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Full Length Research Paper

Use of vegetable oils in the control of *Colletotrichum* sp. in banana fruits

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Anthracnose is a disease caused by the fungus of the genus *Colletotrichum*. It is considered an important post-harvest disease in fruits of banana (*Musa* sp.) which depreciates the commercial value of the fruit. The use of vegetable oils to control fungal growth and disease progression in plants is an important alternative to minimize the deleterious effects of toxic chemicals. Studies were carried out to evaluate the effects of the vegetable oils of murumuru (*Astrocaryum* sp.), andiroba (*Carapa guianensis*) and copaiba (*Copaifera* sp.) *in vitro* on the mycelial growth of *Colletotrichum* sp. The fungus *Colletotrichum* sp. was isolated from banana fruits. The treatments including control (mycelial growth in culture medium without the presence of oil) and vegetable oils (murumuru, andiroba and copaiba) at dosages of 0, 50, 100, 150 and 200 µL.mL^{-1} were applied. The experiment was conducted using a Completely Randomized Design (CRD), in a 3x6 factorial scheme (3 oils x 5 concentrations + 1 control), with five replications. Copaíba oil (*Copaifera* sp.) had a higher mycelial inhibition percentage (MIP) with a dose of 150 µL.mL^{-1} (65.56%). As for murumuru oil, a higher percentage of inhibition was also obtained in the concentration of 150 µL.mL^{-1} (46.44%) and the andiroba oil had a greater inhibitory effect in the concentration of 200 µL.mL-1 (34.89%). The results showed that only the copaiba oil, at all concentrations tested, had an inhibitory effect on *Colletotrichum* sp. Copaiba oil, therefore, is recommended for the control of phytopathogenic diseases caused by *Colletotrichum* sp.

Key words: Alternative control, anthracnose, crop protection, fungal diseases, *Musa* sp.

INTRODUCTION

Brazil is one of the world's largest banana (*Musa* sp.) producers, and this production plays an important role in

local development, since it generates income and provides food resources to the population (Li et al., 2011). Thus, due to the increasing demand and importance of production, the consumer market imposes some requirements for high-quality fruits, and it is suggested that the harvest be carried out in order to minimize losses and with low or no toxicity inputs (Cruz et al., 2010).

It is known that the quality of agroforestry depends on aspects related to the production system and socio-environmental impacts, nutritional value and product flavor, presence of agrochemical residues, micro-organisms and insects on fruits, and visual aspects such as shape, size, color, pattern, stage of maturation and factors related to fruit appearance (Costa et al., 2013). In this scenario, the microbiological factor deserves special attention, since fungi are economically important organisms for medicine, phytopathology and bioindustry, and in addition, act as decomposers in the food chain (Leite et al., 2012).

Diseases such as panama disease, cordana leaf spot, yellow sigatoka, black sigatoka and anthracnose in fruits decrease productivity and depreciate fruits. Anthracnose, the main post-harvest disease in banana (Negreiros et al., 2013), caused by the fungus of the genus *Colletotrichum*, is responsible for large crop losses (Coelho et al., 2010). *Colletotrichum* is considered one of the most important pathogen genera that cause plant diseases (Solino et al., 2012). The symptoms of anthracnose in banana fruits are characterized to dark and depressed lesions, and as the disease progresses they are covered with pink fructification (Coelho et al., 2010).

Thus, the control of postharvest diseases should be performed alternative techniques to the use of fungicides chemicals, in order to avoid the risks of contamination of the agricultural product and the induction of resistance of the pathogen (Oliveira et al., 2019). The application of chemical fungicides in fruits to increase storage time is a major theme in the World Health Organization (WHO) (Dukare et al., 2018) due to the residual effects and risks to consumer health and the environment that these products represent (Ncama et al., 2019).

In this respect, extensive scientific research on the use of antifungal substances may help to control pre and post-harvest diseases (Kamei et al., 2014). In view of possible problems of contamination with the use of chemical substances, has been recommended the use of natural products for the control pf diseases in plants (Araujo et al., 2013). The advantage of post-harvest disease control in fruits with vegetable oils is that they present higher levels of food safety and lower risk of contamination to the environment (Fischer et al., 2018).

The use of murumuru (*Astrocaryum* sp.) oil has grown considerably in recent years, which can be explained by the investments made on discoveries for utilization of these vegetable products. The andiroba (*Carapa guianensis* Aubl.) and copaiba (*Copaifera* sp.) oils have shown a wide range of use in the Amazon region in the control of diseases (Solino et al., 2012). Therefore, the chemical composition of vegetable oils may define their potential type of use and associated biological activity (Funasaki et al., 2016).

Amazon region has a considerable diversity of vegetable species with natural substances that should be investigated for applications with the most diverse purposes, and oils from native plants can be used as alternative sources in the control of plants-infesting pathogens. Thus, this work was conducted to evaluate *in vitro* action of oils from *Astrocaryum* sp*., Carapa guianenses* and *Copaifera* sp. on the mycelial growth of *Colletotrichum* sp*.*, the causative agent of anthracnose in banana fruit, with the intent of suggesting the use of natural products for the control of phytopathogenic agents and, therefore, add value to the utilization of plant products.

MATERIALS AND METHODS

This work was carried out at the Phytopathology Laboratory of the Federal University of Acre, *Campus Floresta*, Multidisciplinary Center – CMULTI, in Cruzeiro do Sul, Acre, Brazil. The vegetable oils were acquired from producers of natural oils in the region of the production of August 2017. The accesses on genetic diversity and associated traditional knowledge present in this work were registered in the database of SisGen - National System of Genetic Heritage and Associated Traditional Knowledge under register AE76FF6.

Isolate used

Sample material with anthracnose symptoms was collected from banana plant fruits obtained from farmers in Cruzeiro do Sul – AC and was taken to the laboratory for removal of fragments containing disease. 20 fruits were examined, of which presented average of 5 infection points, and lesions with a mean diameter of 3 cm. The lesion fragments were washed in running water, subjected to 70% alcohol for one minute and 1% sodium hypochlorite for 30 seconds. Finally, the sample was washed in sterile water for isolation of *Colletotrichum* sp. The infested materials were transferred to 9 cm diameter Petri dishes with 20 mL of Potato Dextrose Agar (PDA) as growth medium. The dishes were then maintained in Biochemical Oxygen Demand (BOD) at 25°C with a photoperiod of 12 hours for seven days. After seven days of fungal culture growth, 5 mm diameter discs containing mycelia of *Colletotrichum* sp. were inoculated in the center of the Petri dishes. The phytopathogenic agent was identified at the genus level by the culture and morphological characteristics of the hyphae with the help of area

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experts and comparisons in the literature.

Bioassay

In this study, the fungitoxic effects of different concentrations (0, 50, 100, 150 and 200 µL.mL^{-1}) of the oils of *Astrocaryum* sp., *Carapa guianensis* and *Copaifera* sp. *in vitro* on the mycelium growth of *Colletotrichum* sp. in PDA medium were analyzed. The control consisted of the fungus isolate cultured only in the PDA medium (Nascimento et al., 2014). The emulsifying agent added to the oils was Tween 80 (1% v/v), which was also used in the control treatment. The effect of different concentrations of vegetable oils on fungal growth was considered after daily measurements of the diameter of the growth area on the two diametrically opposite orthogonal axes of the petri dish. The evaluations were completed when the control mycelial growth completely covered the surface of the culture medium (Ferreira et al., 2012). Based on data of the daily growth of the colony under effect of the abovementioned vegetable oils, the mycelial growth rate was calculated, according to the formula by Dias et al. (2005):

$$MGR = \Sigma \frac{(D - Da)}{N}$$

Where: MGR = mycelial growth rate, D= current mean diameter of the colony, Da= mean diameter of the colony in the previous day, N= number of days after inoculation.

Percentage of growth inhibition (PGI) of the treatments was also calculated, compared to the control, using the following equation:

$$PGI = \frac{(\text{Control diameter} - \text{treatment diameter})}{\text{Control diameter}} \times 100$$

The assay was carried out in a completely randomized design, in 3x6 factorial arrangement (3 oils x 5 concentrations + 1 control). For each oil/concentration treatment five replications were considered. The data obtained in this study were subjected to Shapiro Wilk's test to check for normality of residuals and to the Levene's test for variance homogeneity. Subsequently, to meet the parametric analysis premises, data were subjected to analysis of variance, and the means were compared by the Scott-Knott's test ($p \leq 0.05$). Subsequently, the data were submitted to Multiple Linear Regression Analysis ($p \leq 0.05$) to verify the relationship between the sources of variations (oils and concentrations) on fungal mycelial growth rate. The analyses were carried out in the R statistical program (R Core Team, 2017).

RESULTS

The results obtained for Mycelium Growth Rate (MGR) were significant ($p < 0.05$) for the different oils at the same concentrations tested, but with no significant differences ($p > 0.05$) for different concentrations considering the same vegetable oil (Figure 1). The analysis of the oils effect on mycelial growth indicated that only the copaiba oil, for all concentrations, had an inhibitory effect on the mycelial growth of *Colletotrichum* sp., when compared to the control ($p = 0.02$) (Figures 1 and 2). However, there was no difference among the means of the concentrations used for this oil, indicating that the minimum concentration of copaiba oil to cause an inhibitory effect was 50 µL.mL^{-1}.

Copaiba oil had a higher fungitoxic effect on the MGR of *Colletotrichum* sp. (*F-statistic* = 10.39; *DF* = 58; R-square = 0.52 and $p < 0.01$) with an estimated reduction of the mycelial growth rate of 0.9 cm/day to each microliter (µL) of oil added to the culture medium ($p = 0.02$). In fact, the reduced MGR achieved with the use of *Copaifera* sp. oil, compared to the other oils tested, demonstrates its potential fungitoxic effect, which makes it a promising alternative product to control diseases caused by fungi of the genus *Colletotrichum*.

The mean inhibition percentage of mycelium growth of *Colletotrichum* sp. (Table 1) indicates that all oils used in the study had some percentage of mycelium growth inhibition, but some of them with higher efficiency than the others, indicating promising results especially with the use of copaiba oil.

With respect to the mycelial inhibition percentage (MIP), the copaiba (*Copaifera* sp.) oil exhibited a higher effect with the dose of 150 µL.mL^{-1} (65.56%) (Figure 3). Considering the same concentration value, it was also found greater effects for the murumuru oil (46.44%), and the andiroba oil exhibited a greater inhibitory effect at the concentration of 200 µL.mL^{-1}. Of the oils studied, copaiba (*Copaifera* sp.) oil was the most effective, with over 50% of inhibitory effects for all concentrations tested.

DISCUSSION

In this study, only the copaiba oil showed a fungitoxic effect for all concentrations tested, inhibiting more than 50% of *in vitro* mycelial growth of *Colletotrichum* sp. isolated from banana plant fruits. Our results are in accordance with the work done by Solino et al. (2012), they observed that copaiba oil reduced the mycelial growth of *Colletotrichum gloeosporioides in vitro* and *in vivo* experiment. The application of natural defensive agents based on vegetable oil can be done by the method of spraying on the surface (Mamarabadi et al., 2018) or by immersion of the fruits (Solino et al., 2012).

The chemical composition of the oils has a direct influence on the inhibitory effects on the phytopathogen, since the possible antifungal action of the oils can be attributed to the presence of biologically active chemical substances (Pieri et al., 2009). Some chemical compounds have an excellent fungicidal potential for the control of phytopathogenic fungi, such as alcohol, aldehydes, derivatives of fatty acids, terpenes and phenols can be found in vegetable oils (Ncama et al., 2019). Thus, acting alone or synergistically, these molecules contribute to the biological activity exerted by vegetable oils (Calvo-Garrido et al., 2014).

The chemical composition of the oil can define its potential type of use and indicate possible biological activities. The andiroba and murumuru oils, for example, have a high content of fatty acids (Funasaki et al., 2016), and the lipid profile of andiroba can be represented in

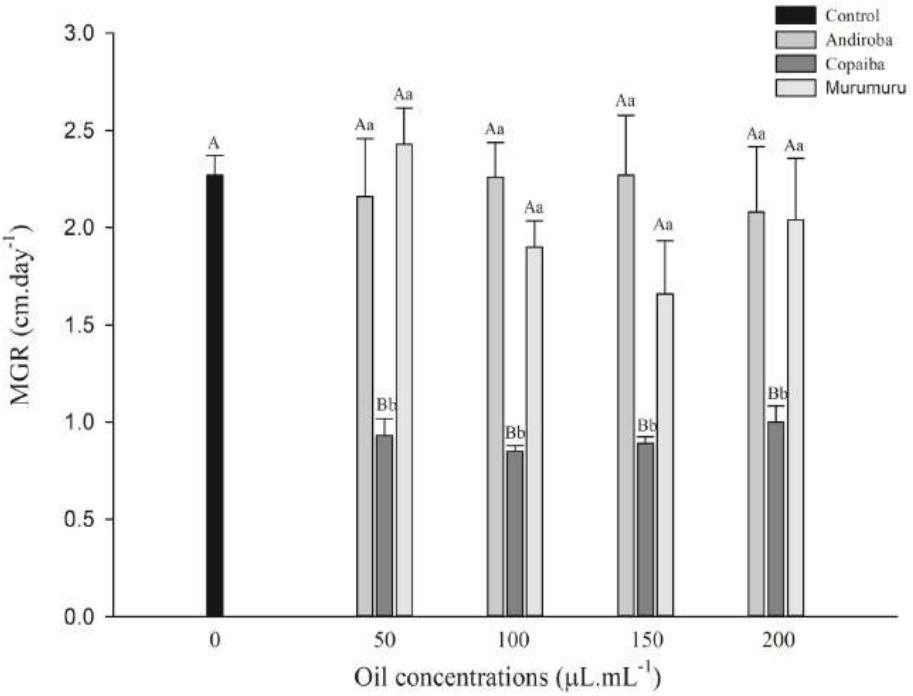

Figure 1. *In vitro* effects of vegetable oils on mycelium growth of *Colletotrichum* sp. * MGR: Micelial Growth Rate. Means followed by same uppercase letter refer to the comparison between different concentrations of the same oil, and lowercase letters for different oils for each concentration at 95% significance level by the Scott-Knott's test.

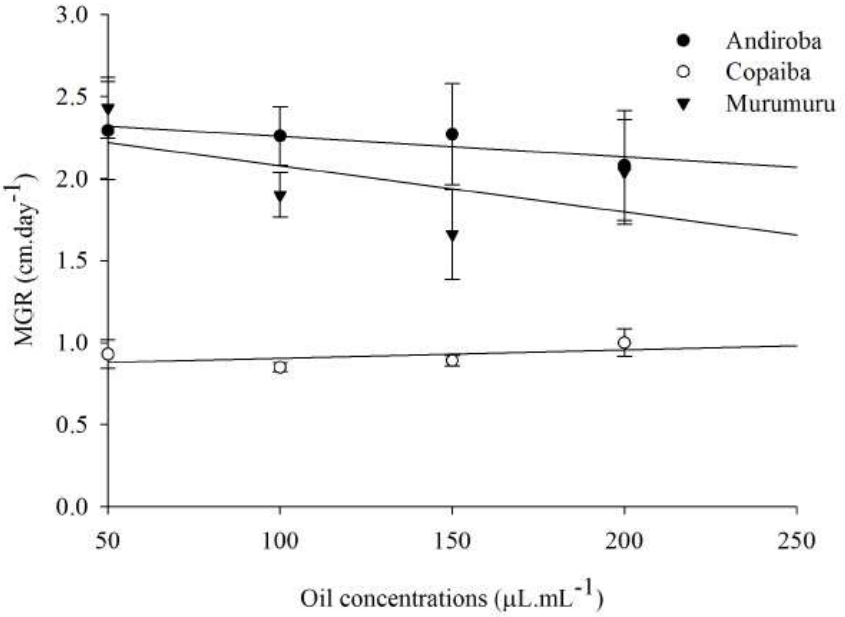

Figure 2. Effect of different concentrations of vegetable oils on the mycelial growth of *Colletotrichum* sp., which causes anthracnose in banana fruits.

more than 97% by oleic, palmitic, stearic, linoleic and arachidonic acids (Milhomem-Paixão et al., 2016). Copaíba oil presents diterpene acids, including copalic acid, kaurenoic acid, alepterolic acid and polyaltic acid in

Table 1. Mean mycelial inhibition percentage (MIP) of *Colletotrichum* sp. with different concentrations of oils from andiroba, copaiba and murumuru. Cruzeiro do Sul, Acre, Brazil. August of 2017.

Concentrations ($\mu L.mL^{-1}$)	Percentage of inhibition of vegetable oils		
	Andiroba	Copaiba	Murumuru
50	17.6±6.5	61.11±6.1	23.56±4.3
100	12.67±6.4	62.89±1.1	39.11±3.9
150	25.5±5.9	65.56±1.1	46.44±6.2
200	34.89±6.3	63.56±1.7	37.78±6.8

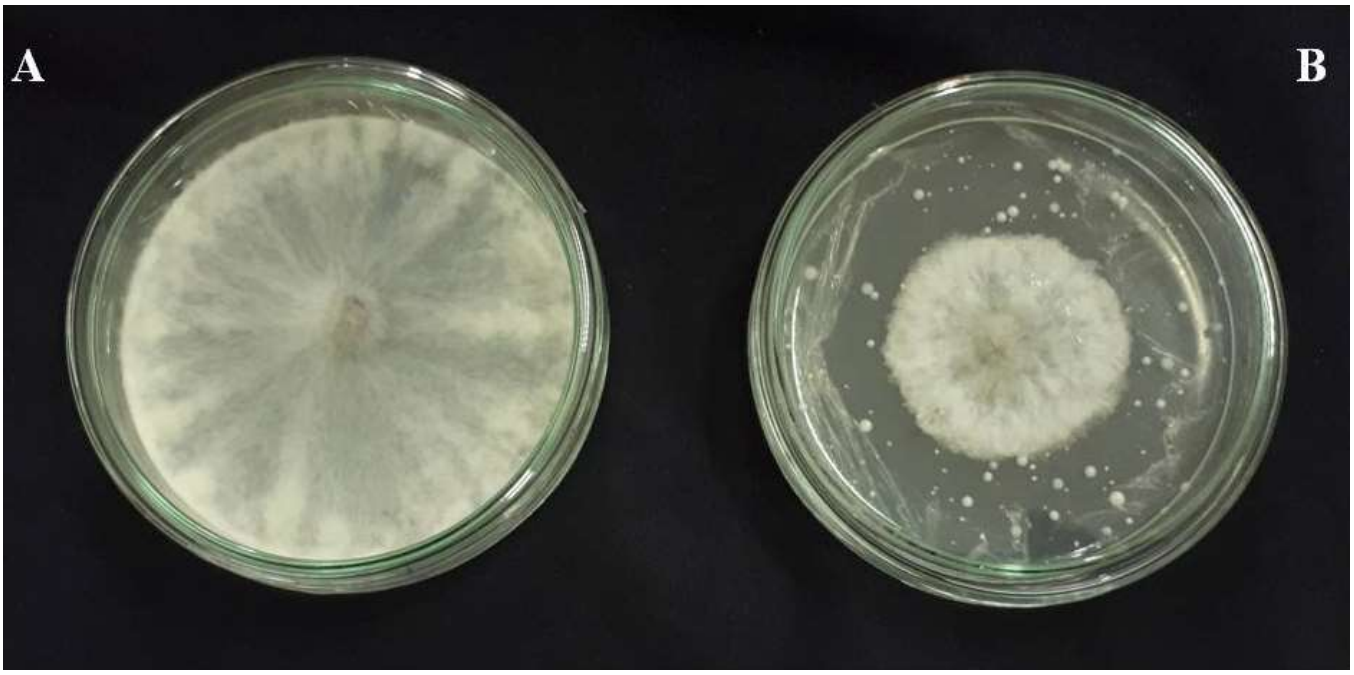

Figure 3. Effect of vegetable oil on the mycelial growth of *Colletotrichum* sp., isolated from banana fruits. A: Control treatment. B: Copaiba oil (150 $\mu L.mL^{-1}$).

its composition and which are considered biologically active (Trindade et al., 2018). With regard to the murumuru oil, no inhibitory effect on *Colletotrichum* sp. growth was found for the concentrations used. However, promising results were obtained in studies with other species of phytopathogenic fungi (Nascimento et al., 2014). The chemical substances present in vegetable oils have potential for the control of phytopathogenic fungi, and in this case the characteristics of the cell wall of the fungi and their interaction with the molecular structure of the applied compounds should also be considered (Avis and Bélanger, 2001). The ability to destabilize fungal structures and interfere with cell wall, plasma and mitochondrial functions, and depends on the specific interaction between fungus and vegetable oil used (Ncama et al., 2019).

The chemical components of plant oils cause morphological changes, increased fluidity in the membranes of fungal cells, alterations in protein conformation or enzymatic activity, followed by destruction of organelles (Knechtle et al., 2014; Shokri, 2016), and changes in production of reactive oxygen species (ROS) (Nazzaro et al., 2017). The andiroba oil did not provide a fungitoxic effect on the mycelial growth of *Colletotrichum* sp. when compared to the control. Other researches with this oil also reported low inhibition of the mycelial growth with fungi of other species (Machado et al., 2013). However, Nascimento et al. (2019), working with the oil of two species of the genus *Carapa*, verified antifungal activity, suggesting that qualitative and quantitative differences in the chemical components of vegetable oils may also indicate their fungitoxic potential.

Investigations on the biological activity of plant oils and extracts along with biological control and induced resistance are technologies with a potential control of

fungi that attack plants. The easy access and low toxicity potential of vegetable oils suggests that they should be recommended as alternative products for the control of these diseases (Fernandes and Bonaldo, 2011). It is worth noting that the mechanisms of action of vegetable oils on the mycelial growth of phytopathogenic fungi are relatively unknown. In this work, we identified inhibition of the mycelial growth of *Colletotrichum* sp. after subjecting it to different concentrations of vegetable oils. However, the mechanism of action still requires further investigations to evidence and substantiate the actual synergetic or isolated action of compounds.

Conclusion

Out of vegetable oils used in this work, copaiba oil showed promising results for the control of mycelial growth of *Colletotrichum* sp. at concentrations of 50 $\mu L.mL^{-1}$ and over and inhibition rates higher than 50%. These results show its potential use in the control of anthracnose in banana plant fruits. Further studies should be conducted in order to substantiate soundly the mechanism of action of the substances contained in these vegetable oils, and also to identify the active ingredient of each chemical component present in oil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Maize-lupine intercrop response to applied nitrogen and phosphorus in North-Western Ethiopia

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Maize (*Zea mays*) is a major staple crop in North-Western Ethiopia. Narrow leaf lupine (*Lupinus angustifolius*) grain is a commercial concentrates for livestock feed. Maize-lupine intercropping is a sustainable and emerging crop production approach for the resource poor smallholder farmers of North-Western Ethiopia; however, there is no recommended fertilizer rate for the intercrop. Therefore, field experiment was undertaken to determine maize-lupine intercrop yield response to applied N and P; to determine N use efficiency of maize; and to establish the basis for determining the most economical N and P rates with varying costs and commodity prices for the intercrop. The experiment was conducted at South Achefer and Mecha areas of North-Western Ethiopia in 2013 crop season. Four levels of each N (0, 64, 128 and 192 kg N ha^{-1}) and P (0, 20, 40 and 60 kg P ha^{-1}) were arranged in factorial combination. Sole crop maize and lupine were included as check treatments. The treatments were laid out in a randomized complete block design with three replications. The results indicated that maize growth parameters and yield components, maize grain yield, and total maize-lupine intercrop yield increased significantly with applied N, P, and N x P interactions. The highest total intercrop yields were obtained on 162/45 kg N/P ha^{-1} at South Achefer and 205/61 kg N/P ha^{-1} at Mecha with yield advantage of 4.14 and 7.05 t ha^{-1} over unfertilized, respectively. The economic optimum rates for maize-narrow leaf lupine intercrop were 130/39 kg N/P ha^{-1} at South Achefer and 177/53 kg N/P ha^{-1} at Mecha with cost price ratio of N cost kg^{-1}/maize grain price kg^{-1} equal to 6 and P cost kg^{-1}/maize grain price kg^{-1} equal to 11. The economic optimum rates decreased as cost price ratio increased, therefore, seasonal price information is vital to adjust the economic optimum rates. Maize N use efficiency declined as N rates increased in the maize-lupine intercrop.

Key words: Intercropping, lupine, maize, optimum rate, yield response.

INTRODUCTION

In Ethiopia, maize ranks second next to *tef* (*Eragrostis tef L*) in area coverage and first in quantity of grain produced (CSA, 2015). Mean maize yield, however, is very low (3.43 t ha^{-1}) (CSA, 2015) as compared to a global mean of 5.0 t ha^{-1} (CIMMYT and IITA, 2011). It is a priority staple food crop in many parts of the country. Narrow leaf lupine is of a recent introduction to Ethiopia and is commercial concentrate feeds for livestock in North-

Western Ethiopia (Likawent et al., 2012). The grain is an alternative to dry bean (*Phaseolus vulgaris*) and soya bean (*Glycine max*) for human consumption, and several modern cultivars have been developed for use as human food (Shahidul et al., 2011).

Maize-legume intercropping systems offer several advantages to small-scale maize farmers such as improved soil fertility, healthier diets, increased productivity, and reduced risk of total crop failure (CCRP, 2009). Seran and Brintha (2010) reported legume intercropping with maize as a way to grow a staple crop while benefiting from the additional crop. Higher productivity of the system as a whole in maize-common bean intercrop compared with maize sole crop has been reported for other parts of Ethiopia (Wortmann et al., 1996; Tamado and Eshetu, 2000).

Nitrogen (N) and phosphorus (P) are important constraints to maize production in Ethiopia (Kebede et al., 1993). Tilahun et al. (2007) recommended application of 128 kg N and 40 kg P ha^{-1} to maximize net returns to fertilizer use with sole crop maize in Dera area of North-Western Ethiopia. However, information is scarce on fertility response of maize-legume intercrop in North-Western Ethiopia. Narrow leaf lupine, hereafter referred to as lupine, is a relatively new crop with grain and forage potential in the region and more information is needed on intercropping it with maize. Optimum nutrient application rates and efficiency for maize-lupine intercrop is required to increase crop yields and profitability while minimizing environmental pollution. Therefore, this study was conducted to: 1) determine maize-lupine intercrop yield response to applied N and P; 2) determine N use efficiency of maize; and 3) establish the basis for determining the most economical N and P rates with varying costs and commodity prices for the intercrop.

MATERIALS AND METHODS

The experiment was conducted on Nitosols at Mecha (11.39° latitude and 37.11° longitude, 1982 meters above sea level) and South Achefer (11.34° latitude and 36.94° longitude, 2021 m above sea level) of North-Western Ethiopia in the 2013 crop growing season (June to October).

The soil analysis result at soil depth of 0 to 40 cm indicated that the sites had clay texture with low values of pH and available P (Table 1). According to Halm (1978), the available P was low (0-15 mg kg^{-1}, Bray) and according to the ratings by Tekalign (1991) the soils for both sites are strongly acid (4.5 to 5.2), organic carbon was moderate (15 to 30 g kg^{-1}), and total N content was high (1.2 to 2.5 g kg^{-1}) at both sites.

Treatments consisted of N (0, 64, 128 and 192 kg N ha^{-1}) and P (0, 20, 40 and 60 kg P ha^{-1}) arranged in factorial arrangement. Sole crop maize at spacing of 75 cm x 30 cm with recommended fertilizer rate of 128/40 N/P kg ha^{-1} and sole crop lupine at spacing of 40 cm x 10 cm with no fertilizer application were included as check treatments. The treatments were laid out in randomized complete block design with three replications. The fertilizer urea (46% N), DAP (18% N and 20% P) and triple super phosphate (TSP) (20% P) were used as sources of N and P. The fertilizers were applied as band application in the maize furrow. All P was applied at planting while one-third of the N was applied at planting and the remaining N was side-dress applied, and covered, at the 8- to 10-leaf stage of maize. The crop varieties used for the experiment were *BH-540* for maize and *Sanabor* for narrow leaf lupine. Both crops were planted in June at the same date in an additive series with 100% of maize plant population, and with lupine planted at 40% of sole crop stands. Maize was planted in paired rows spaced at 50 and 112.5 cm within and between paired rows, respectively. Paired rows of lupine were planted between the paired maize rows with 37.5 cm apart from adjacent maize row and within paired rows lupine (Figure 1). Intra row spacing was 30 and 10 cm for maize and lupine, respectively. Maize leaf area index (LAI) was recorded at silking stage (Dwyer and Stewart, 1986). Plant height, above ground dry biomass yield, ears $plant^{-1}$, kernels ear^{-1} were determined in middle 4 rows of 1.8m length which was the net plot for determination of grain yield of both crops.

Components of N use efficiencies of maize were calculated as described by Cassman et al. (2002).

$$PFP_N = Y_N / F_N ; AE_N = (Y_N - Y_0)/F_N ; RE_N = (U_N - U_0)/F_N$$

;and $$PE_N = (Y_N - Y_0)/(U_N - U_0)$$

Where, PFP_N = Partial factor productivity of applied N (kg grain per kg N applied); Y_N = crop yield (0% moisture) with applied N (kg ha^{-1}); F_N = amount of N applied (kg ha^{-1}); AE_N =Agronomic efficiency of applied N (kg grain increase per kg N applied); Y_0 = crop yield (0% moisture) in a control treatment with no fertilizer (kg ha^{-1}); RE_N = Crop recovery efficiency of applied N (kg increase in N uptake kg^{-1} N applied); U_N = total grain N uptake at maturity (kg ha^{-1}) in a plot that received N; U_0 = total grain N uptake at maturity (kg ha^{-1}) in a plot that received no N; and PE_N = Physiological efficiency of applied N (kg grain increase kg^{-1} increase in N uptake from fertilizer). A 500-g grain sample was taken to measure maize grain protein using Infratec 1241 Grain Analyser (Foss, Hilleroed, Denmark) and N concentration in the dry grain was calculated as protein content divided by 6.25.

Total intercrop yield (measured as equivalent yield of maize, EY_M) was calculated as $EY_M = Y_{ML} + (Y_{LM} x P_L / P_M)$ (Verma and Modgal, 1983), where Y_{ML} = intercrop maize grain yield ha^{-1}; Y_{LM} = intercrop lupine grain yield; P_M = price of maize grain kg^{-1}; P_L = price of lupine grain kg^{-1}. Grain moisture content was measured using a grain moisture tester (Dickey-John Multigrain) and final grain yield was adjusted to the moisture contents of 12.5% for maize and 10% for lupine.

Data were analyzed using the GLM procedure of the SAS 9.4 version (SAS Institute, 2013). Maize grain and total intercrop yield response to N, P, and N x P interaction were tested using single degree of freedom orthogonal contrasts to determine whether the response was linear or not. Mean separation for significant responses were compared using SAS LSMEANS test (probability of difference, PDIFF) at $P < 0.05$. Prediction of the optimal level of N and P for maximum and economic yield of the total intercrop was done using polynomial response equation (Dillon and Andreson, 1991). Economic return from the intercrop was performed at three scenarios of fertilizer cost to grain price ratios (CPR) for each N and

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Table 1. Soil properties of the Mecha and South Achefer experimental sites at the time of planting in the year 2013.

Soil properties	Mecha	South Achefer
Soil texture (%)		
Sand	13	8
Clay	65	65
Silt	22	27
Soil pH (H_2O) 1:2.5	4.6	4.8
Organic C (g kg^{-1})	24.4	20.3
Total N (g kg^{-1})	1.8	1.5
Available P (mg kg^{-1})	7.9	6.7

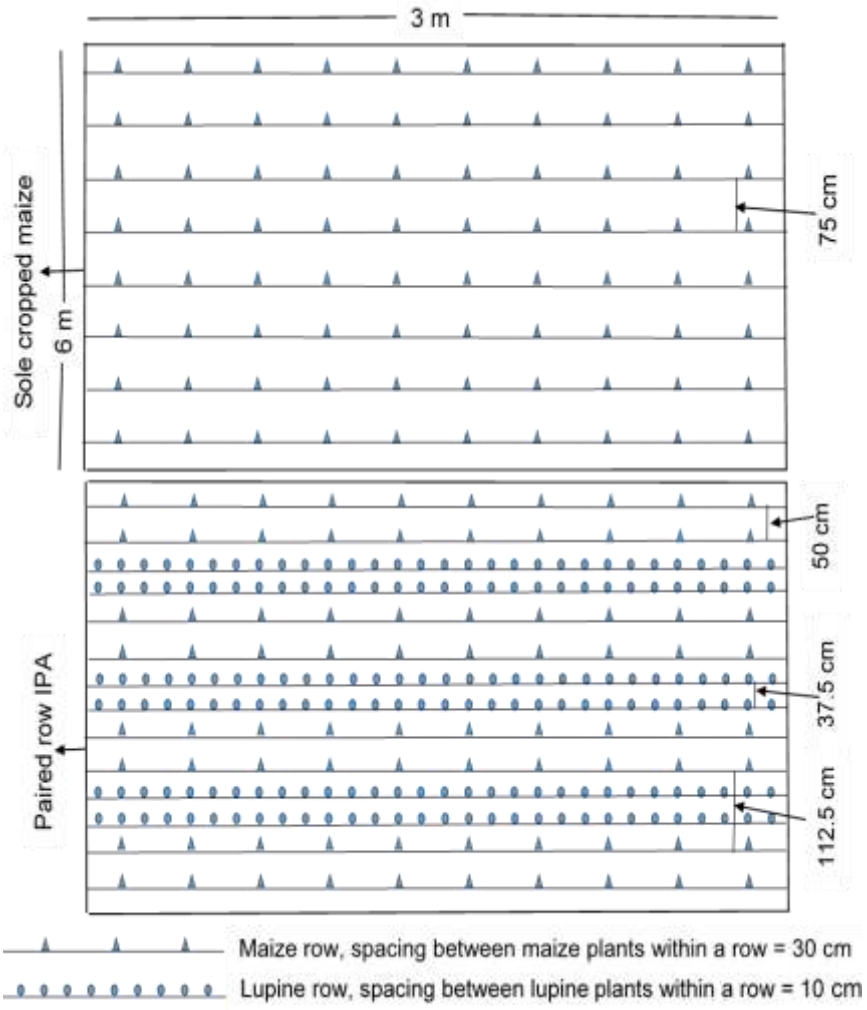

Figure 1. Experimental field layout for paired row intercrop planting arrangement (IPA) in comparison to sole crop maize.

P including ratios of 6, 9 and 13 for N cost kg^{-1} to maize grain price kg^{-1}; and 11, 18 and 25 for P cost kg^{-1} to maize grain price kg^{-1}. Cost of N, Birr 26.5 kg^{-1} (derived from cost of urea, Birr 12.2 kg^{-1}); cost of P, Birr 51 kg^{-1} (derived from cost of DAP, Birr 15 kg^{-1}) of the year 2013; and mean price of the maize grain (Birr 4.5 kg^{-1}) and lupine grain (Birr 3.0 kg^{-1}) from December to February of the year 2013/2014 were used as base for determination of current cost price ratio of 6 for N cost kg^{-1} to maize grain price kg^{-1} and 11 for P cost kg^{-1} to maize grain price kg^{-1}. The other CPR were determined based on the existing trend in increasing cost of production and it

was assumed that cost of N increased to 42 and 58, cost of P to 82 and 112 while maize and lupine grain price kept constant.

RESULTS AND DISCUSSION

The intercrop system was 60% more productive at south Achefer and 100% more productive at Mecha relative to sole crop production as measured from land equivalent ratio (Table 2). Yield advantages of 27% at South Achefer and 61% at Mecha were obtained from intercropped maize relative to sole-cropped maize at 128/40 kg N/P ha^{-1} applied. Increased maize yield when intercropped with lupine relative to sole-cropped maize might be due to a positive effect of lupine to maize crop. Palmason et al. (1992) reported significant N transfer from narrow-leaf lupine to intercropped ryegrass (*Lolium multiflorum*).

Growth and yield components of maize such as LAI, plant height, biomass yield, ear plant^{-1}, kernel ear^{-1}, and TKW were significantly affected by N and P application at both sites. Interaction effect of N and P significantly affected plant height at South Achefer, and LAI, plant height, biomass yield and number of kernels ear^{-1} at Mecha. Maize LAI and plant height increased as N and P rates increased (Tables 3 and 4). The highest LAI and plant height were recorded on the application of 192/60 N/P kg ha^{-1} at both sites (Table 4). Increases in maize plant height and LAI in response to N and P was reported by Onasanya et al. (2009). The effect of N fertilization in enhancing LAI is well documented in maize because of higher photo assimilates that result in more dry matter accumulation (Uhart and Andrade, 1995).

Biomass yield, TKW, number of kernels ear^{-1} and ears plant^{-1} were also increased in response to N and P applied (Table 5). The increase in biomass yield and TKW with N applications was consistent with the findings of Kaleem et al. (2012) who reported significant increase in biomass yield and TKW with increasing N rates as high as 210 kg N ha^{-1}. The response of TKW to N and P rate is due to the fact that N and P deficiency decrease biomass production and partitioning, especially in reproductive organs, resulting in small kernel size (Uhart and Andrade, 1995). The increase in kernels ear^{-1} with N application was also consistent with those of Ali and Raouf (2012) who reported highest maize kernels ear^{-1} at the highest N rate of 225 kg N ha^{-1}. The highest biomass yield (17.07 t ha^{-1}) at Mecha was obtained at the highest fertilizer rates of 192/60 N/P kg ha^{-1} (Table 5).

Maize grain yield and the total intercrop yield, which was measured as equivalent yield of maize, were significantly affected by N and P application at both sites. Maize grain yield and equivalent yield of maize were significantly affected by N x P interaction at Mecha but not at South Achefer. Yield response functions were polynomial for the applied N and P rates at both sites with greater response at Mecha (Figure 2) though basal soil fertility status of the two sites was at the same range as indicated in Table 1. Therefore, the greater responses at Mecha compared to South Achefer suggests the need to repeat the study over seasons and sites to verify the results. Yields increased with nutrient rate to certain level. Averaged over P, total intercrop yield ranged from 3.68 to 6.07 t ha^{-1} at 0 and 162 kg N ha^{-1}, respectively at South Achefer (Figure 2a), and from 1.64 to 6.22 t ha^{-1} at 0 and 165 kg N ha^{-1}, respectively at Mecha (Figure 2b). Yield increased by 65 and 279% at South Achefer and Mecha, respectively, due to N application. Similarly, averaged over N, yield ranged from 4.10 to 5.83 t ha^{-1} at South Achefer (Figure 2c) and from 2.68 to 5.60 t ha^{-1} at Mecha (Figure 2d) at 0 and 45 kg P ha^{-1}, respectively. Yield increased by 42 and 109% at South Achefer and Mecha, respectively, due to P application.

The total intercrop yield (equivalent yield of maize) response functions for N and P applications were:

$$Yield_{Achefer} = 3.68 + 0.0296N - 9.155x10^{-5}N^2;\ R^2 = 0.96$$

$$Yield_{Mecha} = 1.64 + 0.0553N - 1.672x10^{-4}N^2;\ R^2 = 0.96$$

$$Yield_{Achefer} = 4.10 + 0.0765P - 8.438x10^{-4}P^2;\ R^2 = 0.98$$

$$Yield_{Mecha} = 2.68 + 0.131P - 1.47x10^{-3}P^2;\ R^2 = 0.99$$

The increased maize yield in both sites with N and P application has commonly occurred with maize (Ardell and Michael, 2014; Sun et al., 2014). The polynomial response for the highest N and P rates may suggest levels below the highest rates plus the residual nutrients in the soil is sufficient to sustain normal vegetative growth of maize. Depression in grain yield at higher levels of N and P might be also due to nutrient imbalances. Komljenovic et al. (2010) reported significant reduction of zinc status in the maize leaf at higher level of P application. Depression in grain yield of maize at higher N supply was reported (Sun et al., 2014).

The N x P interaction was very important to maize-lupine intercrop yield. Dramatically, yield increased at the lower rates of each nutrient in the presence of the other one (Figure 2) demonstrated the two nutrients are equally important and yield limiting for maize at these sites. Yield responses to N without P application (Figure 2a, b) and to P without N application (Figure 2c, d) were low. This result is in agreement with those of Alzubaidi et al. (1990) who reported significant grain yield response of maize for the interaction effect of N and P compared to application of N and P alone. Masaka (2006) further indicated that this was partly attributed to better plant growth by which N-fertilized plants have larger root systems for the capture of other nutrients. However, the N x P interaction

Table 2. Land equivalent ratio (LER) in maize-lupine intercrop at South Achefer and Mecha in North-Western Ethiopia.

Cropping system	South Achefer		Mecha	
	Grain yield (t ha^{-1})	LER	Grain yield (t ha^{-1})	LER
Intercropped				
Maize with 128/40 N/P kg ha^{-1}	6.75		7.24	
Lupine without N/P application	1.06		0.82	
		1.6		2.0
Sole cropped				
Maize with 128/40 N/P kg ha^{-1}	5.31		4.49	
Lupine without N/P application	3.30		2.08	

Table 3. Main effect of applied N and P on Leaf area index and yield components of maize under maize-lupine intercropping at South Achefer and Mecha in North-Western Ethiopia.

Parameter	South Achefer					Mecha	
N rate (kg ha^{-1})	LAI	BiomassYield (t ha^{-1})	TKW (g)	Ear plant^{-1}	Kernels ear^{-1}	TKW (g)	Ears plant^{-1}
0	1.60^{c†}	6.06^{c}	301^{b}	0.8^{b}	228^{c}	351^{b}	0.8^{b}
64	3.07^{b}	10.87^{b}	334^{a}	0.9^{a}	328^{b}	389^{a}	0.9^{b}
128	3.61^{a}	13.49^{a}	343^{a}	1.0^{a}	377^{a}	397^{a}	1.1^{a}
192	3.83^{a}	13.23^{a}	319^{ab}	1.0^{a}	386^{a}	349^{b}	1.1^{a}
PDIFF	***	***	*	***	***	*	***
P rate (kg ha^{-1})							
0	2.50^{b}	9.21^{b}	295^{c}	0.9^{b}	293^{b}	332^{c}	0.8^{b}
20	3.20^{a}	11.03^{a}	322^{b}	1.0^{a}	340^{a}	354^{bc}	1.0^{a}
40	3.17^{a}	11.80^{a}	349^{a}	0.9^{b}	331^{a}	410^{a}	1.1^{a}
60	3.24^{a}	11.63^{a}	331^{ab}	1.0^{a}	356^{a}	389^{ab}	1.1^{a}
PDIFF	***	***	**	*	**	***	***
CV (%)	11.39	13.82	9.43	12.05	12.96	11.95	14.50

† Numbers followed by different letters on main effect of N and P on the same column indicated significant difference of each other (PDIFF) at P < 0.05. *, ** and *** significant difference at probability level of 0.05, 0.01 and 0.001, respectively.

Table 4. Interaction effect of applied N and P on maize growth parameters under maize-lupine intercropping at South Achefer and Mecha in North-Western Ethiopia.

P rate (kg ha^{-1})	South Achefer				Mecha							
	N rate (kg ha^{-1})				N rate (kg ha^{-1})				N rate (kg ha^{-1})			
	0	64	128	192	0	64	128	192	0	64	128	192
	Plant height (cm)				Plant height (cm)				Leaf area index			
0	133^{i†}	161^{g}	176^{ef}	173^{f}	103^{fg}	129de	142^{d}	123^{def}	1.00^{h}	1.89^{efg}	1.83^{fg}	1.63^{gh}
20	142^{hi}	176^{ef}	200^{ab}	187^{cde}	94^{g}	164^{c}	190^{ab}	172^{bc}	0.95^{h}	2.77^{cd}	3.76^{b}	3.75^{b}
40	147^{h}	184de	197^{abc}	194^{bcd}	116^{ef}	175^{bc}	197^{a}	195^{a}	1.39^{gh}	2.53^{def}	3.95^{ab}	4.12^{ab}
60	140^{hi}	177^{ef}	191^{bcd}	207^{a}	123^{def}	173^{bc}	184^{ab}	199^{a}	1.32^{gh}	2.65^{cde}	3.43^{bc}	4.65^{a}
PDIFF	*				**				**			
CV (%)	3.71				7.87				18.66			

† Numbers followed by different letters indicated significant difference of each other (PDIFF) at P < 0.05. * and ** significant difference at probability level of 0.05 and 0.01, respectively.

is likely to be much less important in cases where N is much more limiting than P deficiency (Dobermann et al., 2011; Kaizzi et al., 2012).

From response of N, P, and the N x P, polynomial response function for the intercrop yields were generated for each of the site.

Table 5. Interaction effect of applied N and P on maize biomass yield and number of kernels ear^{-1} under maize-lupine intercropping at Mecha in North-Western Ethiopia.

P rate (kg ha^{-1})	N rate (kg ha^{-1})				N rate (kg ha^{-1})			
	0	64	128	192	0	64	128	192
	Biomass yield (t ha^{-1})				Kernels ear^{-1}			
0	2.16^{f†}	6.12^{cdef}	7.68^{cde}	5.06^{def}	102^{ef}	219^{cd}	214^{cd}	181^{def}
20	2.57^{f}	8.98^{cd}	13.45^{ab}	13.84^{a}	96^{f}	261^{cd}	462^{a}	414^{a}
40	4.95^{def}	9.67^{bc}	13.52^{ab}	16.98^{a}	181^{def}	195^{cde}	400^{a}	419^{a}
60	3.70^{ef}	8.68^{cd}	13.88^{a}	17.07^{a}	112^{ef}	286^{bc}	372^{ab}	452^{a}
PDIFF			*				**	
CV (%)	26.82				21.57			

† Numbers followed by different letters indicated significant difference of each other (PDIFF) at P < 0.05. * and ** significant difference at probability level of 0.05 and 0.01, respectively.

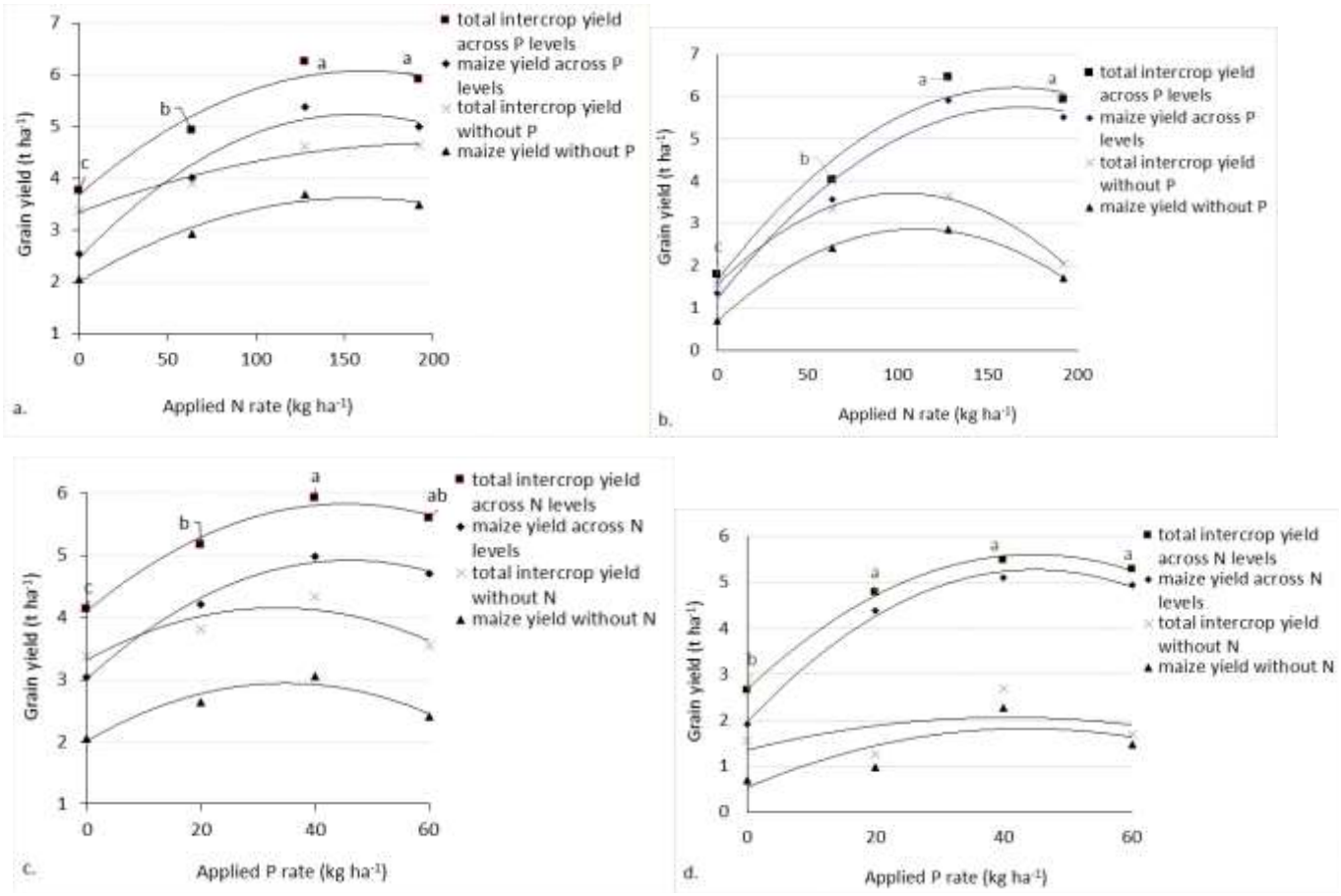

Figure 2. Yield response to applied N and P under maize-lupine intercropping at South Achefer and Mecha in North-Western Ethiopia. **a**) Response to N at South Achefer; b, Response to N Mecha; c, response to P at South Achefer; d) response to P at Mecha (d). Different letters in curve lines indicate significant difference in total intercrop yield (equivalent yield of maize) for N and P levels at P < 0.05.

$$Yield_{Achefer} = 2.56 + 0.0297N + 0.0768P - 9.171x10^{-5}N^2 - 8.484x10^{-4}P^2 \quad R^2 = 0.87 \text{ (Equation 1)}$$

$$Yield_{Mecha} = 1.01 + 0.0424N + 0.0897P - 1.674x10^{-4}N^2 - 1.47x10^{-3}P^2 + 4.32x10^{-4}NP \quad R^2 = 0.91 \text{ (Equation 2)}$$

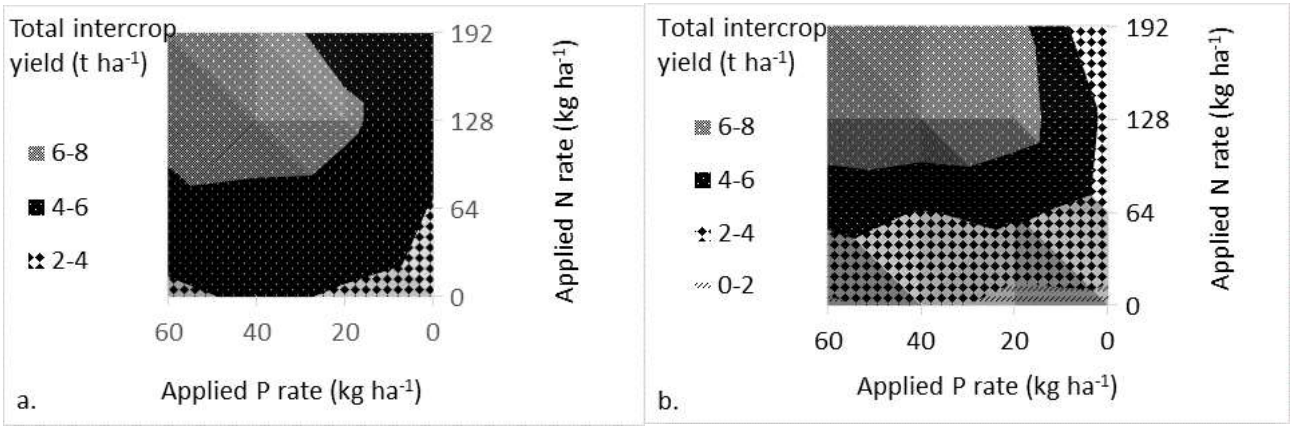

Figure 3. Response surface of N and P interaction effect on maize-lupine total intercrop yield (as measured by equivalent yield of maize) at South Achefer (a) and Mecha (b) in North-Western Ethiopia.

Agronomic optimum N rate (AONR) and P rate (AOPR) at South Achefer were calculated from the following equations which were generated from Equation 1.

$0.0297-1.83x10^{-4}N=0$ and $0.0768-1.7x10^{-3}P=0$

Similarly, AONR and AOPR at Mecha were calculated from the following two simultaneous equations which were generated from Equation 2.

$0.0424-3.348x10^{-4}N+4.32x10^{-4}P=0$ and
$0.0897+4.32x10^{-4}N-0.00294P=0$

The agronomic optimum rates for total intercrop yield were 162/45 kg N/P ha^{-1} at South Achefer and 205/61 kg N/P ha^{-1} at Mecha. Yield ranged from 2.56 at 0/0 N/P to 6.70 t ha^{-1} at 162/45 kg N/P ha^{-1} at South Achefer (Figure 3a), and from 1.01 at 0/0 N/P to 8.06 t ha^{-1} at 205/61 kg N/P ha^{-1} at Mecha (Figure 3b). Yield increased by 4.14 and 7.05 t ha^{-1} at South Achefer and Mecha, respectively relative to the unfertilized.

The cost price ratio affects the economic optimum N rate (EONR) and P rate (EOPR). The economic optimum rates decreased as cost price ratio increased. EONR and EOPR at South Achefer were calculated from equations which were generated from Equation 1 at cost price ratio of N cost kg^{-1} / maize grain price kg^{-1} equals to 6, and P cost kg^{-1} / maize grain price kg^{-1} equals to 11.

$106.97-0.82539N=0$ and $294.465-7.63596P=0$

Similarly, EONR and EOPR at Mecha were calculated from simultaneous equations which were generated from Equation 2.

$164.12-1.50651N+1.943775P=0$ and

$352.83+1.943775N-13.23P=0$

The economic optimum rates were 130/39, 110/35 and 91/31 kg N/P ha^{-1} at South Achefer and 177/53, 160/48 and 143/43 kg N/P ha^{-1} at Mecha at cost price ratios N cost kg^{-1}/maize grain price kg^{-1} and P cost kg^{-1}/maize grain price kg^{-1} of 6 and 11, 9 and 18, 13 and 25, respectively (Figure 4).

Accordingly, the maximum economic net return that can be estimated from maize-lupine intercrop on the above economic optimum rates are 24123, 21110 and 18512 Birr ha^{-1} at South Achefer, and 28338, 24121 and 20314 Birr ha^{-1} at Mecha, respectively. Net return decreased by 23 and 28% at South Achefer and Mecha, respectively, as cost price ratio of N/maize grain and P/maize grain increased from 6 and 11 to 13 and 25. Fertilizer costs and grain prices can fluctuate across years, therefore, profitability of fertilizer use is highly variable. Variations in the profitability of fertilizer use due to variable cost price ratios require that farmers adjust the EONR and EOPR based on current information.

Components of N use efficiencies declined as N rates increased. The decline was significant for most efficiencies except PE_N at South Achefer, and RE_N and AE_N at Mecha (Table 6). At South Achefer, the decline was from 53 to 22, 22 to 12, and 0.32 to 0.19 kg kg^{-1} for PFP_N, AE_N, and RE_N, respectively, as N rates increased from 64 to 192 kg ha^{-1} whereas at Mecha, the decline was from 47 to 24 and from 97 to 69 kg kg^{-1} for PFP_N and PE_N, respectively as N rates increased from 64 to 192 kg ha^{-1}. These declines in N use efficiency might be due to more loss of N in the higher N rate than lower N rate. Decreases in N use efficiencies of maize as N rates increased was due to substantial N losses to the environment and low N use efficiency (Kaizzi et al., 2012 and Wortmann et al., 2011). Ardell and Michael (2014) reported significant decrease in PFP and AE as N rate increased. Sun et al. (2014) and Xueli et al. (2014) also

Table 6. Nitrogen use efficiencies of maize under maize-lupine intercropping as affected by N rates at South Achefer and Mecha in North-Western Ethiopia.

N rate (Kg ha^{-1})	South Achefer			Mecha	
	PFP_N‡	AE_N	RE_N	PFP_N	PE_N
			kg kg^{-1}		
64	53^{a†}	22^{a}	0.32^{a}	47^{a}	97^{a}
128	36^{b}	21^{a}	0.31^{a}	38^{a}	79^{ab}
192	22^{c}	12^{b}	0.19^{b}	24^{b}	69^{b}
PDIFF	***	**	**	**	*
CV (%)	13.89	33.38	31.29	35.64	27.25

† Numbers followed by different letters on the same column indicated significant difference of each other (PDIFF) at $P < 0.05$. ‡ PFP_N,Partial factor productivity of applied N (Kg grain per kg N applied); AE_N, Agronomic efficiency of applied N (kg grain increase per kg N applied); RE_N, Recovery efficiency of applied N (kg increase in N uptake per kg N applied); PE_N, Physiological efficiency of applied N (kg grain increase per kg N uptake increase from applied N); and *, ** and *** significant difference at probability level of 0.05, 0.01 and 0.001, respectively.

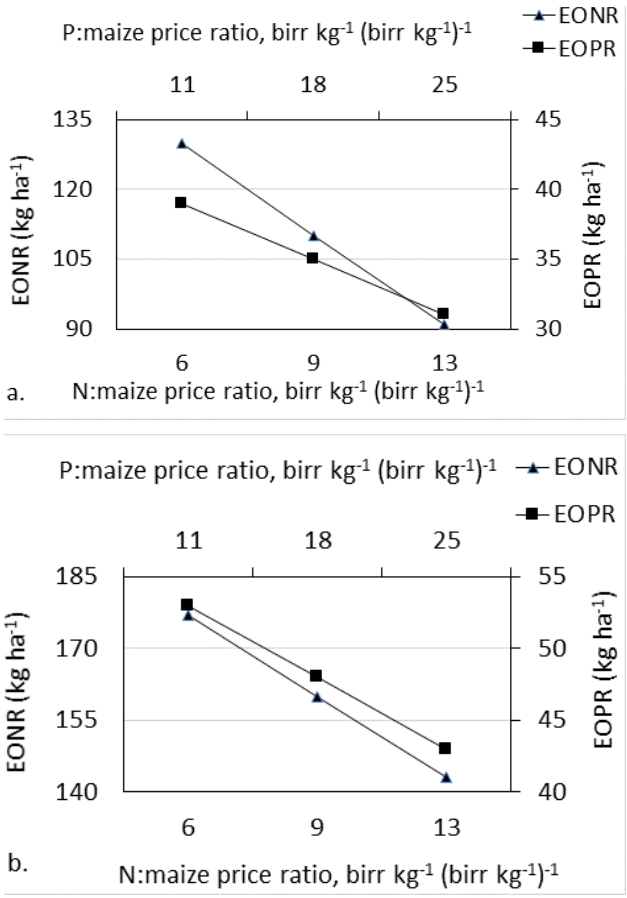

Figure 4. The effect of cost/price ratio on economic optimum N rate and P rate on maize-lupine total intercrop yield at South Achefer (a) and Mecha (b) in North-Western Ethiopia.

reported low N use efficiency at higher N application with increased residual soil nitrate and increased risk of N leaching.

Conclusions

Maize-lupine intercrop responded to N and P application in North-Western Ethiopia with yield increase of 4.14 t ha^{-1} at South Achefer and 7.05 t ha^{-1} at Mecha over unfertilized. Economic optimum rates decreased from 130/39 to 91/31 N/P kg ha^{-1} at South Achefer and from 177/53 to 143/43 kg N/P ha^{-1} at Mecha as cost price ratios of N cost kg^{-1}/maize grain price kg^{-1} increased from 6 to 13 and P cost kg^{-1}/maize grain price kg^{-1} increased from 11 to 25. Therefore, seasonal price information is vital to adjust the economic optimum rates. Nitrogen use efficiencies of maize decreased as high as 58% in maize-lupine intercropping when N applied increased from 64 to 192 kg N ha^{-1}. Further studies need to be conducted over seasons and locations to verify the results.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS:

AEN, agronomic efficiency of applied Nitrogen; **AONR,**

agronomic optimum Nitrogen Rate, **AOPR,** agronomic optimum phosphorus rate; **CPR,** cost price ratio; **EONR,** economic optimum nitrogen rate; **EOPR,** economic optimum phosphorus rate; **EYM,** equivalent Yield of Maize; **LAI,** leaf area index; **PEN,** physiological efficiency of applied nitrogen; **PFPN,** partial factor productivity of applied Nitrogen; **REN,** recovery efficiency of applied Nitrogen; **TKW,** thousand kernel weight.

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Growth of parica seedlings *(Schizolobium amazonicum* Huber ex Ducke*)* cultivated in different organic substrates

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The forest plantations depends on appropriate initial seedling establishment, which occurs when primary factors (water, light, CO_2 and nutrients) are within appropriate ranges. The purpose this study was to evaluate the growth of young parica plants (*Schizolobium amazonicum* Huber ex Ducke) grown in different organic substrates (chicken manure, goat manure and organic matter) in proportions of 5, 10, 15 and 20% in yellow argisol. Among the studied treatments, the best response in plant height growth was observed in T_2 (5% goat manure in yellow argisol). For the mean diameter, it was observed that T_4 (15% goat manure in yellow argisol) allowed greater of growth parica seedlings. In addition, for the quality parameters: ratio of shoot height and root collar diameter (H/RCD), heigh and dry mass air part relation (H/DMAP) and Dickson Quality Index (DQI), the best values were for seedlings cultivated with goat manure, in relation to the other treatments. Therefore, young parica plants grown on substrates with goat and chicken manure showed significant improvements in growth performance, according to fundamentally chemical characteristics of these substrates.

Key words: Chicken manure, goat manure, organic matter, seedling production.

INTRODUCTION

In the Brazilian Amazon, forest cover comprises approximately 321 million hectares, of which about 19% have already been altered by some exploitation activity, be it timber, livestock, agriculture, mining or urbanization (Costa et al., 2014). On the other hand, the counterpart for deforestation actions is technically limited, an

expressive knowledge gap exists concerning the forest plantation (Ferreira et al., 2016), given the diversity of species and variation in ecophysiological traits of the different species, besides the limited knowledge about growth and plant nutrition of tropical tree species (Jaquetti and Gonçalves, 2017).

Stimulating research aimed at the recovery of deforested areas is fundamental, since they can develop technological improvements, mainly aiming at reducing deforestation in order to minimize the ecological and economic threats caused by global warming, due to the emissions of greenhouse effect gases (Tollefson, 2015). Knowledge about nutritional, light and water requirements is diffuse, and in addition, the introduction of silvicultural practices that improve the production of seedlings are also limited.

Currently, several Amazonian tree species, particularly from the botanical family Leguminosae, should receive attention, with an emphasis to their timber and non-timber potential. *Schizolobium amazonicum* Huber ex Ducke (parica tree) stands out due to its silvicultural characteristics, for its rapid growth and high market value (Tavares et al., 2013). Parica is a species with great value for the industrial sector, since its wood is accepted in the plywood industry, making it possible to manufacture sheets with good market acceptance (Galeão et al., 2005). In the field, it is a rustic species and can be used in the recovery of degraded areas, due to its good performance in terms of growth in altered environments, allowing its use in environmental recovery programs (Tavares et al., 2013).

In addition, the demand for good quality wood and the application of stricter environmental legislation in the context of the exploitation of natural forests for selective harvesting has led to a decrease in the supply of this product (Hoffmann et al., 2011). Thus, plantations with native species such as *S. amazonicum*, when well conducted since the production of seedlings, have the capacity to supply part of the growing demand for wood. The production of seedlings should receive greater technical and scientific attention, aiming at the successful installation of forest stands, which in turn depends on the characteristics of the substrate, that are fundamental for the growth and establishment of seedlings after the planting (Sena et al., 2010). The choice of the appropriate substrate for seedling production is important to ensure growth, since it interferes with the structure and functionality of the produced seedlings, and in addition, promotes substantial increases in productivity (Silva and Queiroz, 2014). The use of organic residues from animal and plant remains as a nutrient source for plants in the seedlings production process. It has been constituted as a viable alternative for the certification of agricultural activities and environmental conservation; promoting a significant reduction at application of chemical fertilizers. This is to ensure the minimization of environment contamination with the use of low-cost raw materials (Santos et al., 2010).

Considering these benefits, investigating the chemical characteristics of different organic substrates and their interactions with soils, aiming at the adaptation of growth substrates for tree seedlings production, can contribute to improving the knowledge about techniques in tropical forestry with a strongly applied bias. In this research, the purpose was to verify the response of young parica plants (*S. amazonicum*) to different levels of organic substrates, derived from the use of goat manure, chicken manure and organic matter, as a way of using these compounds in the production of good quality seedlings.

MATERIALS AND METHODS

The experiment was conducted at the forest nursery of the Universidade Federal do Acre (UFAC), Campus Floresta, in Cruzeiro do Sul - Acre state, located at the coordinates 70°36'66" L and 72°40"52" W. The climate of the region is described as Af tropical humid with well distributed rainfall throughout the year and absence of dry season (Alvares et al., 2013).

Parica seeds (*Schizolobium amazonicum* Huber ex Ducke) were obtained from the Fundação de Tecnologia do Estado do Acre - FUNTAC, after being collected in the area of the Colocação São Sebastião, Flona Macauã - Sena Madureira, Acre state. After collection, seeds were stored for a period of 10 months in a cold room, under a temperature of 13ºC. For the beginning of the experiment, seeds were submitted to dormancy breaking using the mechanical scarification method with sandpaper n. 50, opening three striae in the opposite part of the embryo (Rodrigues Filho et al., 2019). The germination process was completed 15 days after sowing.

The organic materials used in this experiment were: chicken manure, goat manure and organic matter mixed in different concentrations with yellow argisol. The chicken and goat manure were tanned outdoors for 60 days. A soil surface layer of 20 cm (organic horizon) was used as organic matter. The substrates were prepared using Becker 500 mL graduated to measure the volumes of the compounds, which were sieved using a 2 cm diameter sieve, thoroughly homogenized and used as treatments in this study. These treatments were as follows: T1: yellow argisol 100%; T2: 5% chicken manure; T3: 10% chicken manure; T4: 15% chicken manure; T5: 20% chicken manure; T6: 5% goat manure; T7: 10% goat manure; T8: 15% goat manure; T9: 20% goat manure; T10: 5% organic matter; T11: 10% organic matter; T12: 15% organic matter and T13: 20% organic matter in yellow argisol. Samples of the different concentrations of the substrates were collected and sent to the Soil Laboratory of the Federal University of Acre, in Rio Branco, for chemical analysis of nutrients (Table 1).

The container used in the experiment to produce parica seedlings were black polyethylene bags with dimensions, 15 cm wide and 20 cm long. The experiment was conducted in a greenhouse with a

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Table 1. Chemical composition of the different substrates.

Treatments	pH	Al	H + Al	C.Org.	P	K	Ca	Mg	Na
	water	cmolc/dm³		g/Kg	mg/dm³		cmolc/dm³		mg/dm³
T_1	4.4	1.55	2.25	0.58	4.7	7.0	0.55	0.10	2.0
T_2	3.8	3.8	6.86	6.52	43	200	1.35	0.8	85
T_3	3.9	1.8	7.0	10.42	128	455	2.5	1.5	16
T_4	3.9	1.2	6.86	10.42	128	559	3.6	1.5	20
T_5	3.8	1.5	7.25	10.51	120	546	3.3	1.4	21
T_6	4.2	4.5	7.35	6.23	10	2.6	0.3	0.55	25
T_7	4.4	2.7	6.61	7.98	26	5.50	0.9	0.85	49
T_8	4.5	0.6	5.48	10.9	59	9.15	1.5	1.25	98
T_9	5.2	0.15	3.92	11.68	128	8.46	1.6	1.3	14
T_{10}	4.0	5.5	8.33	4.01	0.6	38	0.3	0.2	4.0
T_{11}	4.1	5.0	8.57	7.4	0.8	35	0.25	0.65	4.0
T_{12}	4.1	5.2	8.91	10.12	1.4	60	0.3	0.45	6.0
T_{13}	4.2	5.1	8.57	10.51	0.8	36	0.35	0.65	5.0

* Potential of hydrogen (pH), aluminum (Al), potential acidity (H+Al), organic carbon (C. Org.), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sodium (Na).

control system of 50% of the daily irradiance, and the monitoring of seedlings was carried out during 60 days, with daily watering. This period is considered as ideal to take *S. amazonicum* seedlings to the field (Carvalho, 2007). The variables used to estimate the initial growth of the seedlings were: shoot height (H) and root collar diameter (RCD). Measurement of height and diameter were performed weekly, using a millimeter ruler and a digital caliper, respectively. The quality parameters of the seedlings were also determined through ratios among the variables: ratio of shoot height and root collar diameter (H/RCD); heigh and dry mass air part relation (H/DMAP); and the Dickson Quality Index (DQI) (Dickson et al., 1960). The Dickson quality index was calculated from the formula, verifying the conceptual adjustments of the applied concentrations (Equation 1):

$$IQD = \frac{TDM}{\frac{H}{RCD} + \frac{SDM}{RDM}}$$

With: Total dry matter in grams (TDM); Shoot dry matter in grams (SDM); Root dry matter in grams (RDM); Shoot height in cm (H); Root collar diameter in mm (RCD).

In order to quantify the dry mass of the seedlings, five individuals were separated randomly from each treatment (concentration). After collection, roots and shoots were washed in distilled water to remove substrate traces, and later, they were packed in paper bags and taken to a forced air circulation oven at a temperature of 45±1 °C, to dry until constant weight. The used design was a completely randomized one, with a factorial arrangement of 3 (organic substrates) x 4 (concentrations) + control treatment, totaling 13 treatments with 20 replications for each treatment; for the quality parameter of the seedlings, 5 replications were randomly used. The data were submitted to the normality test (Shapiro-Wilk) and homogeneity of variances (Levene) to verify the fulfillment of the premises of the parametric statistic. Subsequently, the data were submitted to the Multivariate Variance Analysis (MANOVA) and analysis of variance (ANOVA) associated with the Scott-Knott test ($p > 0.05$). In addition, the main components were analyzed to verify the relationship between the chemical variables of the substrates together with growth and biomass accumulation results of the seedlings. The statistical programming language R was used for the analysis of this work (R Development Core Team, 2017).

RESULTS AND DISCUSSION

As for the results of the Multivariate Analysis of Variance - MANOVA, considering the growth variables (H, D, RDM, SDM and TDM), it was possible to observe a strong joint contribution of these variables to confirm the statistical difference between the treatments used in this study ($p < 0.0001$; Pillai = 1.4889). Young parica plants presented satisfactory results when cultivated on organic substrates, suggesting the use of agroindustrial residues in the production of quality seedlings. The use of organic substrates may be an alternative for production of quality seedlings promoting a good supply of nutrients to the plants (Mota et al., 2016).

Thus, mean height (H) values of *S. amazonicum* seedlings were observed in an amplitude of 17.00 ± 0.88 at 64.00 ± 4.20 cm, the highest means were verified for seedlings under the influence of the substrate with 15% goat manure – T_8 (Table 2) ($p < 0.0001$), being statistically equal to the treatments: T_5, T_6, T_7 and T_9. For the substrates containing chicken manure, it was possible to observe the lowest values for the height variable. Even this treatment with relatively high nutrient values, when compared to the others, did not provide good results for the height growth variable (Table 1). However, among other factors, under certain conditions the substrate pH is considered as a limiting factor on the availability of nutrients for plants (Ghosh et al., 2016). As for the mean diameter, in this research it was possible to identify values in the range from 3.33 ± 0.08 to 3.90 ± 0.03 mm observing in T_4 (15% chicken manure), the best growth of parica seedlings (Table 2), which in turn did not present

Table 2. Performance of young plants of *S. amazonicum* under the influence of substrates at different concentrations.

T	Conc. (%)	H (cm)		D (mm)		RDM (g)		SDM (g)		TDM (g)	
T_1	0	36.40± 2.62	**bc**	3.33 ± 0.08	**b**	0.21 ± 0.04	**ab**	1.03 ± 0.12	**b**	1.24 ± 0.14	**b**
T_2	5	17.00 ± 0.88	**c**	3.64 ± 0.06	**ab**	0.23 ± 0.02	**ab**	2.03 ± 0.12	**ab**	2.26 ± 0.09	**ab**
T_3	10	22.00 ± 1.26	**c**	3.68 ± 0.10	**ab**	0.18 ± 0.01	**ab**	1.93 ± 0.35	**ab**	2.11 ± 0.35	**ab**
T_4	15	17.90 ± 1.63	**c**	3.90 ± 0.03	**a**	0.14 ± 0.03	**b**	2.16 ± 0.35	**ab**	2.29 ± 0.35	**ab**
T_5	20	48.20 ± 7.00	**ab**	3.79 ± 0.13	**ab**	0.11 ± 0.02	**b**	1.56 ± 0.24	**ab**	1.67 ± 0.24	**ab**
T_6	5	56.60 ± 2.48	**ab**	3.47 ± 0.07	**ab**	0.25 ± 0.02	**ab**	2.16 ± 0.21	**ab**	2.41 ± 0.21	**ab**
T_7	10	57.60 ± 3.36	**ab**	3.58 ± 0.09	**ab**	0.27 ± 0.06	**ab**	1.97 ± 0.23	**ab**	2.24 ± 0.23	**ab**
T_8	15	64.00± 4.20	**a**	3.79 ± 0.07	**ab**	0.34 ± 0.08	**a**	2.57 ± 0.44	**a**	2.91 ± 0.43	**a**
T_9	20	53.40 ± 10.29	**ab**	3.69 ± 0.17	**ab**	0.32 ± 0.03	**a**	2.64 ± 0.17	**a**	2.96 ± 0.17	**a**
T_{10}	5	35.80 ± 2.69	**bc**	3.44 ± 0.14	**ab**	0.34 ± 0.04	**a**	1.01 ± 0.08	**b**	1.35 ± 0.08	**b**
T_{11}	10	37.00 ± 2.61	**bc**	3.69 ± 0.12	**ab**	0.32 ± 0.03	**a**	1.13 ± 0.07	**b**	1.45 ± 0.07	**b**
T_{12}	15	37.80 ± 2.89	**bc**	3.50 ± 0.06	**ab**	0.26 ± 0.03	**ab**	1.74 ± 0.30	**ab**	2.00 ± 0.29	**ab**
T_{13}	20	25.80 ± 5.71	**c**	3.50 ± 0.08	**ab**	0.29 ± 0.04	**ab**	1.37 ± 0.12	**b**	1.66 ± 0.12	**ab**

T: treatments, Conc. (%): Concentration as percentage, H (cm): Height in centimeters, D (mm): Diameter in millimeters, RDM (g): Root Dry Matter in grams, SDM (g) Shoot Dry Matter in grams and TDM (g): Total Dry Matter in grams). * Means followed by the same letters do not present significant difference at the 95% probability level by Scott-Knott's test.

statistical differences when compared to the other treatments. Mendonça et al. (2014) corroborate this work, since, when studying different substrates for the production of *Tamarindus indica* (tamarind) scions, they verified that the best responses as for height were obtained in the treatment containing goat manure (soil + goat manure + cattle manure + humus). The higher the amount of organic compounds, the greater the seedling growth (Gonçalves et al., 2014).

However, it is necessary to consider the chemical composition and the concentration of the substrate to avoid the intoxication of seedlings. In a recent work, the growth of *Ateleia glazioviana* seedlings on substrates containing different concentrations of organic substrates presented, at 120 evaluation days, the best seedling growth as for the height variable in the treatment that contained 30% bovine manure, since the average observed values were went 9.11 to 30.15 cm (Gonçalves et al., 2014).

In addition, it was observed that young parica plants submitted to different concentrations of organic substrates presented different investment strategies in biomass allocation, which is possibly strongly related to the specific nutrient contents of each substrate (Figure 1). Interestingly, there is evidence in this work that parica seedlings grown on substrates with lower phosphorus content presented higher investments in root dry mass production. Phosphorus deficiency is a limiting factor for growth in substrates with high levels of acidity, since it stimulates a greater investment of the plant in the production of roots (Wu et al., 2018).

In general, substrates derived from the concentrations with organic matter allowed higher dry matter productivity in the roots and lower productivity in the shoot (Figure 1). This treatment had the lowest Ca, Ca:Mg ratio, Organic Carbon, K, Na and P contents, while aluminum concentrations and potential acidity presented high levels (Table 1 and Figure 1). However, young parica plants submitted to growth on goat manure substrates had higher values for shoot dry matter (SDM) (2.64 ± 0.17 g), and the highest mean value was observed for the concentration of 20% - $p < 0.0001$ - T_9 (Table 2). As for the total dry matter, the mean superiority of the treatments was confirmed for different concentrations of goat manure, especially T9-20% of chicken manure with 2.96 ± 0.17 g ($p < 0.0001$). On the other hand, for treatments with different organic matter contents and the control treatment (yellow argisol) the lowest average values for total dry matter were identified ($p < 0.0001$) (Table 1 and Figure 1).

The substrates containing different concentrations of chicken manure had the highest concentrations of phosphorus (P) and potassium (K). This nutrient is required for the synthesis of adenosine triphosphate (ATP) and other phosphorylated compounds (Taiz and Zeiger, 2013). As a performance strategy, the greatest root investment occurs in order to increase the uptake of nutrients in substrates where there is low resource availability (Lambers et al., 2008). From a nutritional point of view, this substrate had the highest levels of potassium and phosphorus; the first nutrient is considered an activator of enzymes involved in respiration and photosynthesis, and the latter provides the synthesis of important compounds for plant cells, such as sugars phosphates (Taiz and Zeiger, 2013). Phosphorus is regarded as an important nutrient to stimulate growth and dry matter production in young parica plants (Caione et al., 2012).

In addition, seedlings with low root collar diameter values have difficulties in remaining fixed after planting

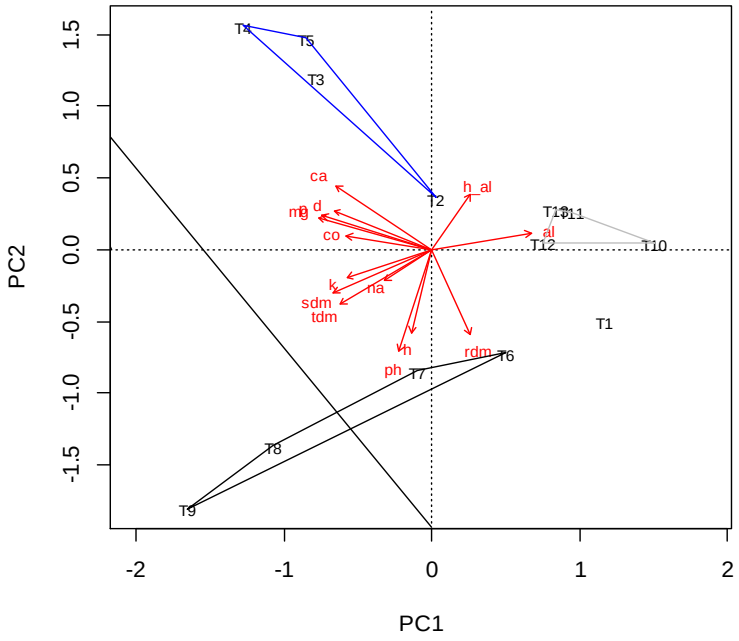

Figure 1. Principal Component Analysis (PCA) considering the chemical variables of the substrates and the growth variables of young plants *S. amazonicum* PC1 = 45% and PC2 = 67%. Blue color polygon (chicken manure treatments); polygon of black color (goat manure treatments) and polygon of gray color (organic matter treatments).

(Cunha et al., 2005). This variable is considered a good indicator of the seedling quality and generally, it is the most appropriate to determine the survival capacity of seedlings in the field (Daniel et al., 1997). In this study, results showed that the use of organic compound provides benefits to the quality of seedlings as for this variable.

In this research, substrates with different levels of goat manure and organic matter presented pH values higher than 4 (Table 1). However, pH values below 4 were found for the chicken manure treatment; this suggests low nutrient availability, resulting in a low increase of young parica individuals under its influence. In addition, the potential of hydrogen (pH) is an important property to consider, since it affects the growth of roots. Root productivity is favoured in substrates with pH values close to 5 and 6, facilitating the availability of K^{+}, Mg^{2+}, Ca^{2+} and Mn^{2+}, also increasing the solubility of carbonates, sulfates and phosphates (Taiz and Zeiger, 2013). According to the H/RCD relation, it was possible to confirm the superiority of the substrate with goat manure in relation to the other treatments, since the control treatment (T1) and the ones with organic matter (OM) were not statistically different ($p < 0.0001$). On the other hand, plants submitted to the treatments using chicken manure (CM) presented the lowest H/RCD value (Figure 2).

According to the analyses obtained from the relation between height and dry mass air part (H/DMAP), the control treatment (T1) and the ones with goat manure (GM) and organic matter (OM), up to 15% concentration, were not statistically differents ($p < 0.0001$). Treatments with chicken manure (CM), goat manure (GM) and organic matter (OM) at the 20% concentration also did not present significant differences, but they differed significantly from the other treatments/concentrations (Figure 3). On the other hand, the control (T1) treatment presented the highest mean value for H/DMAP, when compared to the others. H/DMAP indicates how lignified the seedlings are, suggesting that the lower their value, the more the chances to survive in the field (Welter et al., 2011). Thus, it was possible to identify in this study that the treatment T_2 (5% chicken manure - CM), allowed obtaining the lowest value (8.46) for H/DMAP, suggesting the production of more lignified young parica plants (Figure 4).

In this study, the values obtained for the Dickson quality index (DQI) presented extreme values from 0.08 to 0.16 (Figure 4). For the DQI, treatments using chicken manure (CM), goat manure (GM) and organic matter (OM) were not statistically different ($p = 0.0038$) (Figure 4b). The control treatment (T1) for this variable was the treatment that provided the lowest values, differing significantly from the others ($p = 0.0038$), and suggesting lower quality seedlings. The Dickson Quality Index (DQI) is considered a good integrated morphological measure, since it encompasses several important characteristics such as height, diameter, shoot dry matter and root dry matter,

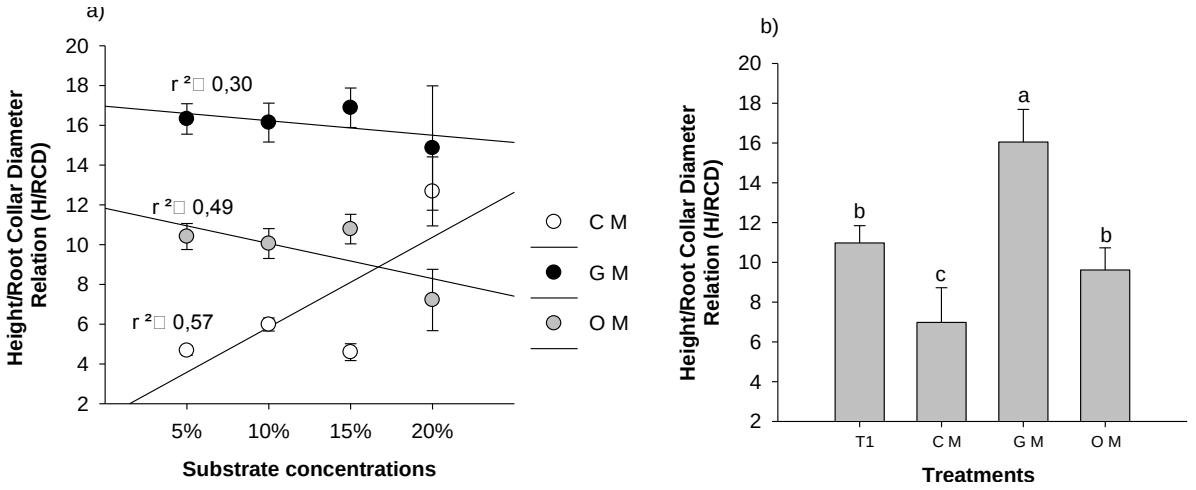

Figure 2. a) Height/Root Collar Diameter relation (H/RCD). (b) Comparison of the values between the different treatments of *S. amazonicum*. T1: control treatment; CM: chicken manure; GM: goat manure and OM: organic matter

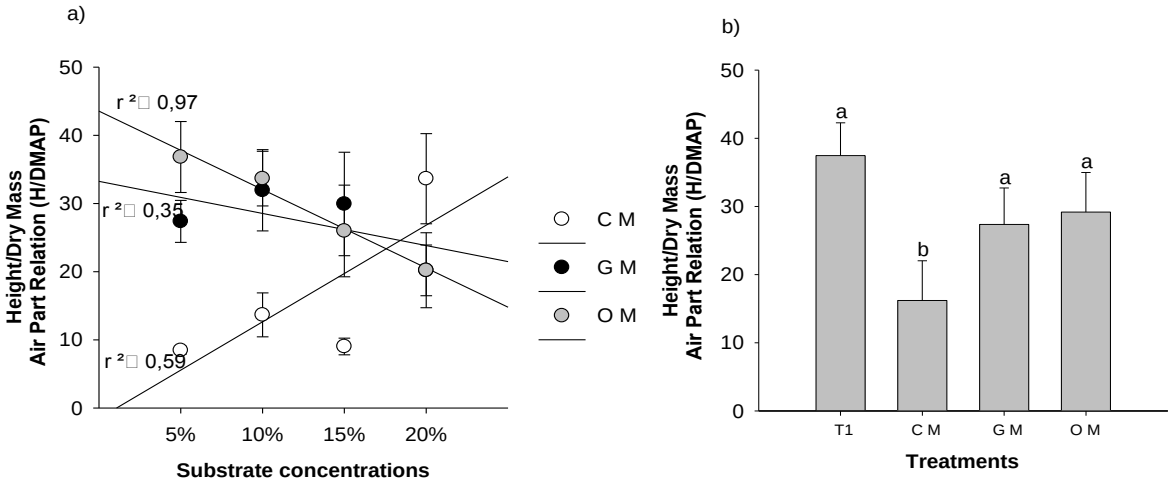

Figure 3. a) Heigh/Dry Mass Air Part relation (H/DMAP) of *S. amazonicum*. b) Comparison of the values between the different treatments. T1: control treatment; CM: chicken manure; GM: goat manure and OM: organic matter.

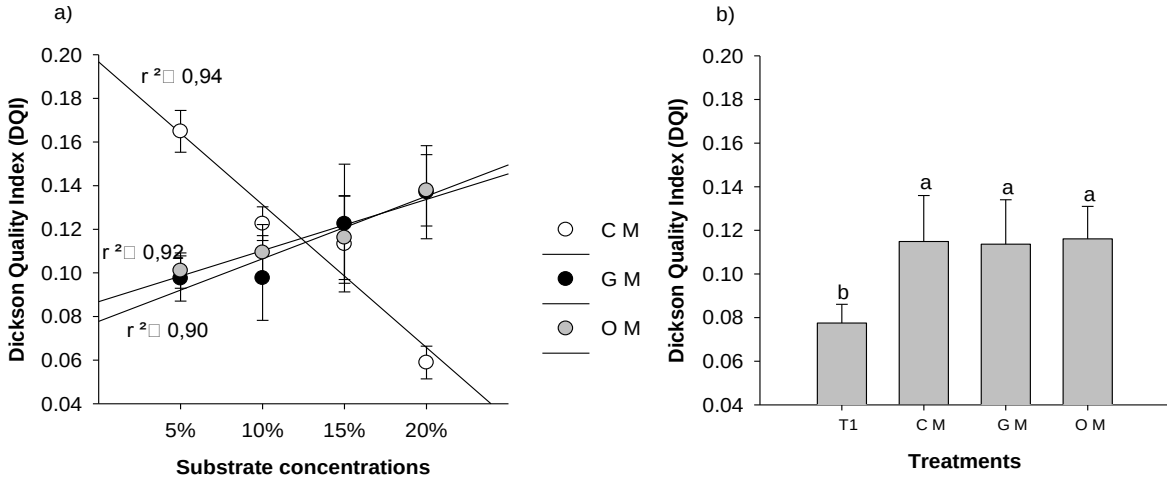

Figure 4. a) Dickson Quality Index (DQI) among the different treatments. b) Comparison of the values between the different treatments. T1: control treatment; CM: chicken manure; GM: goat manure and OM: organic matter.

considering the robustness and balance of mass distribution (Fonseca et al., 2002). Therefore, the success of planting species will depend on the quality of the seedlings, which in turn, depends on the characteristics of the substrate, which are fundamental for the initial growth and establishment of seedlings in the field (Sena et al., 2010).

Conclusion

In terms of initial growth, the use of organic substrates helped the production of *Schizolobium amazonicum* (parica) seedlings. Substrates with 15% goat manure (T4) and 15% chicken manure (T8) were significantly superior to the other treatments, among the used ones, were that provided the highest values, considering the parameters evaluated in this research. The production of good quality seedlings is an important step for excellent silviculture and the use of organic waste supports the supply of nutrients, supplying the initial demands for energy production that will be converted into better performance in the growth and biomass accumulation of seedlings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Virulence and characterization of isolates of potato bacterial wilt caused by *Ralstonia solanacearum* (Smith) in Rwanda

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Bacterial wilt (*Ralstonia solanacearum*) is one of the major potato diseases in Rwanda. An *in vitro* study was carried out to identify and characterize the pathogen isolated from three potato cultivars (Kinigi, Kirundo and Gikungu) in Rwanda. This was achieved by cultural and morphological tests on triphenyl tetrazolium chloride (TTC) and casamino peptone glucose (CPG) agar as well as biochemical tests through Gram staining and biovar test. An *in vivo* experiment was also performed to assess the pathogenicity of those isolates on potatoes. All isolates showed typical morphological traits of virulent *R. solanacearum* on TTC and CPG media. The test isolates were Gram-negative bacteria. Biovar test confirmed that all the isolates belonged to race 1 biovar 3 of the pathogen. Furthermore, the highest disease severity (DS=100%) and disease incidence (DI=100%) were observed in Gikungu isolate followed by Kinigi (DS=97.33% and DI=98.25) and Kirundo (DS=94.67% and DI=92.61%). From this study, all three isolates were typical *R. solanaceraum* belonging to race 1 biovar 3 and were all pathogenic to potato plants. Gikungu and Kinigi isolates were highly virulent than Kirundo isolate. Therefore, Gikungu or Kinigi isolates can be used for further studies in plant protection in management of the disease.

Key words: Biovar test, Gram-negative, Gram-positive, pathogenicity test.

INTRODUCTION

Potato is the world's most widely grown tuber crop (Strange and Scott, 2005; Were et al., 2013) and the fourth major food crop of the world after rice, wheat and maize (Wagura et al., 2011; Guchi, 2015). In Rwanda, potato is among the priority crops, one of the most important food and cash crops for small scale farmers especially in the Northern regions (REMA, 2011; RAB, 2012; Muhinyuza et al., 2014; Uwamahoro et al., 2018). Annual consumption of potato is around 125 kg per person per year and it is the country's second most important source of energy after cassava (REMA, 2011; Muhinyuza et al., 2014). Although Rwanda is the third

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most important potato producer in sub-Saharan African after Malawi and South Africa (FAO, 2014), average yield is estimated at 9 t ha^{-1} which is below the potential yield of 40 to 60 t ha^{-1} (Masengesho et al., 2012; Muhinyuza et al., 2014).

Pests and diseases are among the major limiting factors in potato production worldwide which decrease the production of the crop by 36% (Muhinyuza et al., 2007; Wagura et al., 2011; Yuliar et al., 2015). In Rwanda, brown rot or bacterial wilt caused by *Ralstonia solanacearum* (Smith) (Yabuuchi et al., 1995) is the second most destructive disease in Rwanda after potato late blight caused by *Phytophthora infestans (*Mont. De Bary) (Muhinyuza et al., 2007; REMA, 2011; Uwamahoro et al., 2018). Although late blight is ranked as a priority disease, its management can somehow be achieved by applying fungicides like Dithane M45 or Ridomil in combination with the use of resistant varieties (REMA, 2011; Uwamahoro et al., 2018). However, bacterial wilt should be considered as more problematic than late blight because it cannot be controlled by chemicals or agronomic practices like other main potato diseases (Wagura et al., 2011; Masengesho et al., 2012; RAB, 2012; Huet, 2014; Uwamahoro et al., 2018). This is due to the fact that there is no known chemical that can be used to control it once potato plant is infected because of its lethality, persistence, wide host range and broad geographic distribution (Strange and Scott, 2005; Guchi, 2015; Huet, 2014; Yuliar et al., 2015).

In addition, the application of cultural practices such as crop rotation, use of non-infected seeds, planting in non-infected soils and growing resistant cultivars have various limitations (Huet, 2014; Uwamahoro et al., 2018). For example, crop rotation is not effective because the pathogen can survive in the soil for long periods even in the absence of host plants and it also has a broad host range. Moreover, the disease has been observed even in first planting in newly cleared land. In addition, small farm size is also another challenge for crop rotation program. Furthermore, planting materials are exposed to bacteria attack because potato is mainly propagated vegetatively, and this method favors pathogen spread from mother tubers to the next crop generation. The use of resistant varieties is also limited because they are scarce, some of them are not appreciated by farmers, and they can harbor the latent infection in tubers (Priou et al., 2001; REMA, 2011; Wagura et al., 2011; Huet, 2014; Yuliar et al., 2015; Uwamahoro et al., 2018).

R. solanacearum is a non-spore forming, non-fluorescent species of the genus *Ralstonia*. It is a Gram-negative bacterium, strictly aerobic with round-shaped cells, 0.5 to 1.5 µm in length, with a single, polar flagellum (Hayward, 1991; Priou et al., 2001; Stevenson et al., 2001). The pathogen enters plant through wounds or stomata. Once inside the plant, it moves towards the vascular bundles, and finally colonizes the xylem. The presence of the bacteria inside the xylem is coupled with the production of exopolysaccharides which block the vascular vessels inducing a water shortage throughout the plant (CIP, 1996; Muthoni et al., 2012; Rahman et al., 2012; Guchi, 2015). External disease symptoms of *R. solanacearum* in potato are mainly wilting of the plant and droplets of bacterial ooze from the eyes of the potato tubers. The internal symptoms are brownish discoloration of the vascular ring inside the tuber and grayish white droplets of bacterial cream which come out of them (Priou et al., 2001; Pradhanang et al., 2003; Strange and Scott, 2005; Muthoni et al., 2012; IPDN, 2014; Guchi, 2015).

R. solanacearum is usually closely associated with its living host plants mainly solanaceous crops and temporarily in infected host-plant debris or host weeds. This plant pathogenic bacterium survives for relatively a period of five to eight years in soil or other environment where there are host plants or weeds like *Datura* and *Portulaca* (Hammes, 2013). In the soil, the pathogen may remain there almost indefinitely because it can survive saprophytically and/or it can parasitize a number of very common weeds (REMA, 2011; Uwamahoro et al., 2018). Host debris, latent infected tubers and deeper soil layers were most important factors for its survival (Muthoni et al., 2012; Hammes, 2013). Various environmental factors such as temperature, moisture and rainfall, soil type, inoculum potential, and other soil biological factors such as nematode populations have been reported to correlate with development rate, survival and incidence of pathogen (Hayward, 1991; Uwamahoro et al., 2018).

The isolates of this bacterium can be classified into five different races based on the host range and five biovars on the basis of their ability to utilize the disaccharides (cellobiose, lactose, and maltose) and to oxidize the hexose alcohols (dulcitol, mannitol and sorbitol) (Muthoni et al., 2012; IPDN, 2014). Race 1 of *R. solanacearum* affects a wide range of plant species in the Solanaceae family including potato, tomato and eggplant. It has been observed that *R. solanacearum* race 2 affects some plants of the Musaceae family such as banana and plantains. Race 3 affects mainly potato and tomato and to a less extent to other solanaceous species. Race 4 affects particularly ginger whereas race 5 affects mulberry trees (CIP, 1996; OEPP/EPPO, 2004; Sikirou et al., 2017). In addition, there is also a relationship between biovars and races of *R. solanacearum* and their location. This means that each race contains specific biovars and the races can also adapt to different regions due to the specific requirements in climatic conditions for their survival (Muthoni et al., 2012; Sikirou et al., 2017).

Although Rwanda is one of African countries where bacterial wilt is threatening potato production, the pathogen is poorly studied and its management is getting more difficult due to the challenges mentioned earlier. Identification of the biovars and characterization of the

Figure 1. Symptoms of potato bacterial wilt (*Rasltonia solanacearum*) on potato plants and tubers collected from Kinigi site. Stem/Leaf wilting of Kinigi, Gikungu, and Kirundo cultivars (A, B, and C, respectively). Bacterial ooze and soil clumps on eyes of Kinigi, Gikungu, and Kirundo cultivars (D, E, and F, respectively). Brown ring and ooze from tubers of Kinigi, Gikungu, and Kirundo cultivars (G, H, and I, respectively).

isolates is important for example in management of the disease because there is a correlation between the biovar type, race and ultimately the host range of *R. solanacearum* as well as the climatic conditions it may adapt to (Fock et al., 2001; Strange and Scott, 2005; Popoola et al., 2015). In addition, deep understanding on how different isolates may be virulent on plant hosts can also provide the main key in application of adequate control measures of the pathogen such as creation of potato resistant cultivars. The objectives of this study were, therefore, to identify the biovars of different bacterial isolates and to evaluate their pathogenicity and virulence on potato plants.

MATERIALS AND METHODS

Origin of bacterial isolates

Three isolates of *R. solanacearum* were collected from three infected and most susceptible potato cultivars grown in Rwanda namely Kinigi, Kirundo, and Gikungu with typical bacterial wilt symptoms as shown in Figure 1. The isolates were collected from farmers' fields at Kinigi in November 2017. Kinigi is located in the highland of volcanic soils at an altitude of 2,300 m above sea level (m.a.s.l.), with low temperature (average of 20°C) and high rainfall (1,400 mm to 1,800 mm and well distributed throughout the year) (Birasa et al., 1990; Lepoint and Maraite, 2002; RAB, 2012; MINIRENA, 2013).

Isolation, identification and storage of isolates

All activities for isolation, culturing and identification of bacterial isolates were done in the Plant Pathology Laboratory of Rwanda Agriculture Board (RAB)-Northern zone at Musanze. A vascular flow test and observation of natural slime drop formation after cutting tubers or plants are unique to *R. solanacearum* (Chaudhry and Rashid, 2011; Priou et al., 2001; IPDN, 2014). In this study, diseased tubers from Kinigi, Kirundo and Gikungu potato cultivars collected from grower's fields were used. Tubers were first washed in tap water, then surface sterilized by soaking them in 70% ethanol solution for 5 min and well rinsed three times in distilled water and left to dry. Then, tubers were cut into circular pieces which were suspended for 10 min in sterile distilled water in a glass container to

detect the presence or absence of exudation from the tubers.

Cultural, morphological, and physiological characteristics of single colonies were determined on Petri plates containing specific culture medium. Kelman's Triphenyl Tetrazolium Chloride (TTC) and Casamino peptone glucose (CPG) agar were used as selective media for isolation, identification and biochemical characterization of *R. solanacearum* (Kelman, 1954; IPDN, 2014). Culture of bacteria on both TTC and CPG was achieved by fivefold serial dilution in order to estimate the number of bacteria cells or colony forming units (CFU) in 1 mL of original inocula from three bacterial isolates. Initial bacterial inocula were obtained by collecting suspension of bacterial streaming in sterile distilled water through a vascular flow technique as described earlier. Serial dilution was done from 10^{-1} up to 10^{-5} dilution factor in five tubes each filled of sterile nutrient broth containing peptone, sodium chloride, meat and yeast extracts as described by Marangoni et al. (2001). Each suspension of serially diluted cells were plated on TTC and CPG agar media and incubated at 28°C for 48 h, a period after which bacterial colonies were counted and calculation of CFU mL^{-1} was performed by the following formula (IPDN, 2014):

CFU mL^{-1} = (number of colonies × dilution factor) / volume of culture plate

Counting of the colonies was possible at dilution factor of 1:100,000 (plate labeled as 10^{-5}) on both TTC and CPG media. From the counted colonies, the CFU mL^{-1} in the original bacterial suspension ranged from 4.3×10^7 for Kirundo to 4.8×10^7 for both Gikungu and Kinigi bacterial isolates, respectively on TTC agar and from 4.8×10^7 for Kirundo and Kinigi to 4.9×10^7 for Gikungu isolate on CPG agar.

Purification of bacterial colonies was achieved through culturing two times single colony of virulent *R. solanacearum* isolated from TTC and CPG media on new TTC or CPG growth media. The colonies or cell mass were transferred into a sterile glycerol stock (80% of glycerol mixed with 20% of nutrient sucrose broth) in which a loop full of two days old colonies from TTC or CPG were transferred and kept at -20°C for subsequent uses (IPDN, 2014). A further diagnosis was performed to distinguish this Gram-negative bacterium from Gram-positive bacteria and this was achieved by simple Gram staining as described by Chaudhry and Rashid (2011).

Pathogenicity test

Pathogenicity test for the three bacterial isolates recovered from different potato cultivars (Kinigi, Kirundo and Gikungu) was carried out by soil inoculation. Healthy potato seeds of Kirundo, which is the most susceptible potato cultivar to *R. solanacearum,* were obtained from RAB, Kinigi station. After washing and surface sterilizing the tubers, the later were planted in plastic pots filled with pasteurized mixture of soil, organic matter and sand (2:1:1) and grown under greenhouse conditions (16.7 to 37.4°C temperature and 31 to 75% relative humidity) at RAB, Musanze. Bacterial inoculation was done when seedlings were 30 days old. Soil around plant roots was removed, and then half of the roots of each potato plant were slightly cut. Then 10 ml of bacterial suspension at concentration of 4.8×10^7 CFU mL^{-1} were inoculated around the roots of each pot. Seedlings sprayed with sterile water served as control.

This test was performed using a randomized complete block design (RCBD) in which the pathogenicity of three bacterial isolates was defined as treatments in one potato cultivar. Each treatment was replicated three times (blocks). In each block, five potato seedlings were inoculated with each bacterial isolate or sterile water that served as the control. This means that 20 potato plants per block were used and a total of 60 plants for all the greenhouse experiment were used. Disease incidence (DI %) and disease severity (DS %) in potato plants were evaluated starting from the appearance of symptoms (five days after inoculation) until all the plants inoculated with the most virulent bacterial isolate died. The disease severity (DS %) in plants was evaluated using the scale of Kempe and Sequeira (1983), where 0 = no symptoms; 1 = 1 to 25% leaves wilted; 2 = 26 to 50% leaves wilted; 3 = 51 to 75% leaves wilted; 4 = more than 75% but less than 100% of leaves wilted; 5 = all leaves wilted and plant dead. The severity was calculated using the following formula (Kempe and Sequeira, 1983):

$$DS\ \% = [\Sigma (ni \times vi) \div (V \times N)] \times 100$$

where DS = Disease severity; *ni* = number of plants with the respective disease rating; *vi* = disease rating; *V* = the highest disease rating; and *N* = the number of plants observed.

Disease incidence (DI) was evaluated by the following formula (Kempe and Sequeira, 1983):

$$DI\ \% = \frac{n}{N} \times 100$$

where DI = Disease incidence; n = number of infected leaves per plant; and N = total number of leaves per plant.

From the diseased potato plants, the bacteria were re-isolated and cultured on TZC media and CPG to confirm the presence or absence of the typical colonies of *R. solanacearum* to proof Koch's postulates as well as for further uses.

The analysis of variance (ANOVA) was carried out using SAS software, to determine the difference in wilt incidence and wilt severity due to the three isolates of the pathogen. The treatments means were separated using Tukey's honestly significant difference test at $P \leq 0.05$.

Biovar identification

Biovar determination of the three bacterial isolates was done on both the isolated bacteria strains from Kinigi site and the re-isolated ones from diseased plants during pathogenicity test which was performed under greenhouse conditions. The biovars were determined based on the ability of isolates to oxidize hexose alcohols namely dulcitol, mannitol and sorbitol or to utilize disaccharides like cellobiose, maltose, and lactose. Basal medium as described by Sikirou et al. (2017) was composed of 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1 g of peptone, 0.03 g of bromothymol blue, and 3 g of agar per 1 L of distilled water. The medium was sterilized by autoclaving at 121°C for 15 min. After sterilization, 10% of sugar or alcohol solutions preliminary sterilized by boiling them in water bath for 20 min for three successive days were amended to the basal medium. Then, this mixture was dispensed on 96 well plates by pouring 200 µl of medium in each well. Sterile distilled water served as control. Thereafter, 20 µl of bacterial suspension at concentration of 4.8×10^7 CFU mL^{-1} were added to each well. The plates were incubated at 28°C for seven days.

Utilization of sugars and oxidation of alcohols were shown by a positive (+) reaction which leads to changing in color from green (initial color of medium) to yellow. Otherwise it remains green (-) because the bacterial strains do not utilize the test sugars or oxidize the alcohols (Muthoni et al., 2012; Sikirou et al., 2017). During this experiment, the results of color change of the medium were visually observed from four to seven days. The experimental was set up in completely randomized design (CRD) with three treatments that

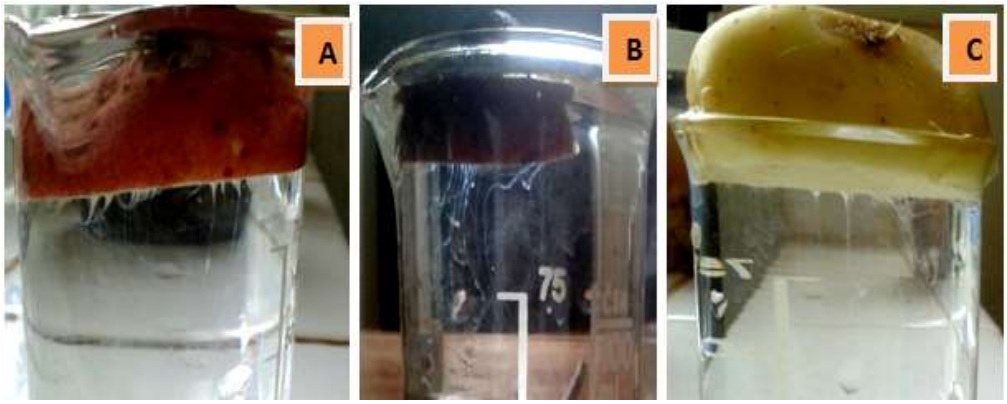

Figure 2. Identification of isolated *R. solanacearum* by vascular flow test: Bacterial streaming characterized by smoke-like milky exudates from infected tubers of Kinigi (A), Gikungu (B), and Kirundo (C) cultivars.

corresponded to each bacterial isolate that was replicated four times.

RESULTS AND DISCUSSION

Bacterial streaming test

In this study, infected potato tubers from Kinigi, Kirundo and Gikungu cultivars were used to identify and confirm the presence of *R. solanacearum* in the samples. A vascular flow test was performed to confirm whether the isolated pathogen from infected potato plant materials was *R. solanacearum*. Other pathogens can also cause wilting symptoms on potato plants such as *Fusarium solani* (Mart. Sacc.), *Verticillium alboatrum* (Reinke and Berth), *Erwinia chrysanthemi* (Burkholder) and *Clavibacter michiganensis* subsp. *Sepedonicus* (Spieckermann and Kotthoff) (Priou et al., 2001; Nadia et al., 2013; El-Habbaa et al., 2016). Through this test, the presence of bacterial wilt in tubers was characterized by smoke-like milky stream that streamed downward from all the cut tubers (Figure 2). Typically, this streaming differentiates *R. solanacearum* from other bacteria which may lead to similar symptoms in potatoes in which these threads are not formed (Priou et al., 2001; Nadia et al., 2013; El-Habbaa et al., 2016). Since the results confirmed the presence of *R. solanacearum*, each isolate was named according to the cultivar that they were isolated from Kinigi, Kirundo and Gikungu respectively.

Morphological features of colonies on growth media

Shape, size and color of colonies on TTC and CPG media are other characteristics which are used to identify the pathogen and to distinguish the virulent and non-vilurent colonies of *R. solanacearum* (Kelman, 1954; Nadia et al., 2013; Sikirou et al., 2017). The colonies which developed on TTC growth medium were fluidal, big, irregularly shaped, and white with pink or red colored center whereas on CPG, they were also big with irregular shape and white color (Figure 3). These features of bacteria on both TTC and CPG agar media confirmed that all the test isolates had typical morphological and cultural characteristics of *R. solanacearum* and were able to infect potato plants and to lead to plant wilting. The same bacterial traits of *R. solanacearum* among other bacteria on culture media were confirmed by Priou et al. (2001), Nadia et al. (2013), IPDN (2014), and Sikirou et al. (2017). These traits are different from non-virulent colonies which are usually smaller, dry, and uniformly dark-red on TTC agar medium and smaller, regularly round and dry on CPG agar (Narasimha and Srinivas, 2012; Muthoni et al., 2012; Nadia et al., 2013; IPDN, 2014; El-Habbaa et al., 2016).

Morphological characteristics of bacterial cells through gram staining

Gram staining was performed to confirm the gram type of *Ralstonia* isolated from Kinigi, Kirundo and Gikungu potato cultivars. From the results of this test, the microscopic observation showed that bacterial cells stained reddish (Figure 4) and this confirmed that the isolated pathogen was a Gram-negative bacterium and distinguished *R. solanacearum* from Gram-positive bacteria which are usually stained purple by this test (Hayward, 1991; Chaudhry and Rashid (2011); Nadia et al., 2013; Popoola et al., 2015). In addition, isolated bacterial cells were rod shaped (Figure 4) and this observation also supported the fact that the isolated pathogen was *R. solanacearum* since its round shape

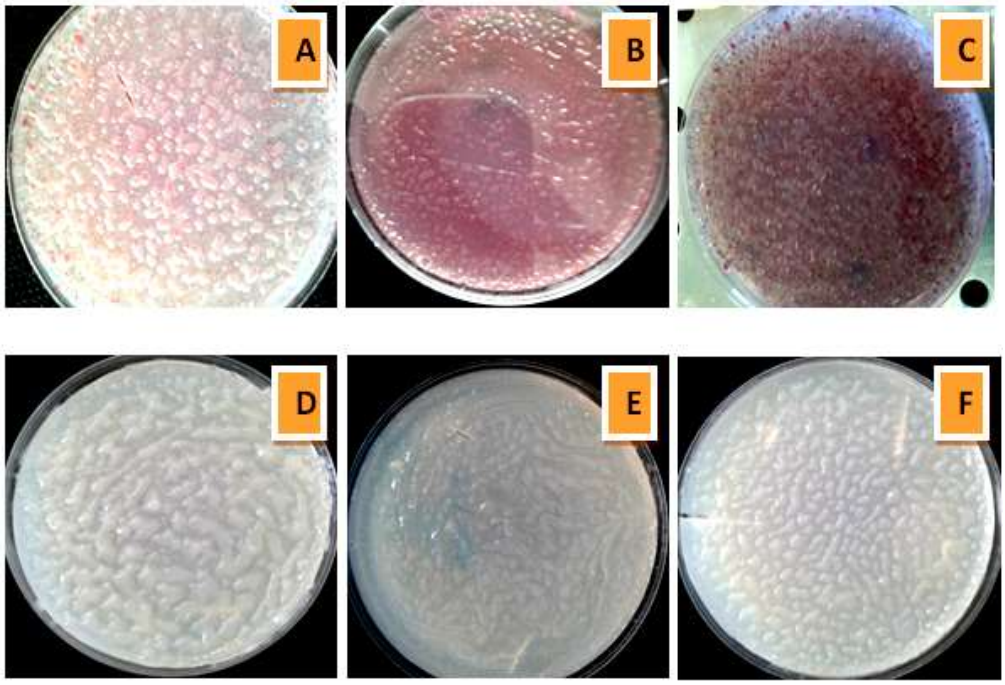

Figure 3. Morphological features of colonies of *R. solanacearum* on TTC and CPG culture media. (A, B, C) Kinigi, Gikungu, and Kirundo isolates respectively on TTC agar at serial dilution 10^{-3}: colonies are mucoid, big with irregular shape, and white with pink colored center. (D, E, F) Kinigi, Gikungu, and Kirundo isolates respectively on CPG agar at serial dilution 10^{-3}: fluidal, big and irregular white shaped colonies.

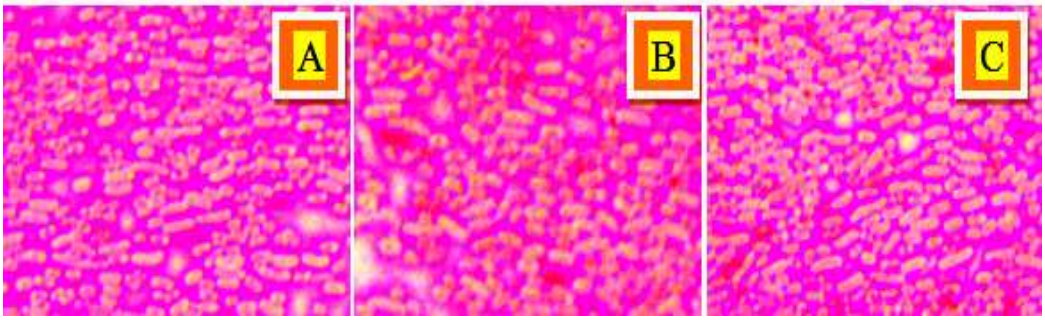

Figure 4. Gram staining pictures from microscopic observation of Kinigi, Gikungu, and Kirundo isolates (A, B, and C, respectively).

was confirmed by Hayward (1991), Stevenson et al. (2001), and Popoola et al. (2015).

Pathogenicity and virulence of the bacterial isolates

Pathogenicity of Kinigi, Kirundo and Gikungu bacterial isolates was tested based on wilting rate they caused to Kirundo potato seedlings under greenhouse conditions. From the wilting rate, disease incidence (DI) and disease severity (DS) were evaluated and calculated from 5 to 20 days after inoculation (5DAI - 20DAI) with an interval of 5 days. The results showed that at 5DAI (Figure 5, 1st row), inoculated plants started to wilt in potatoes treated with Kinigi, Gikungu and Kirundo isolates whereas the control plants did not show any symptom of bacterial wilt. Wilting rate of potato plants increased over time in seedlings inoculated with all test bacterial isolates especially from 10 DAI (Figure 5, 2nd row). Wilting was higher especially in potatoes inoculated with Kinigi and

Figure 5. Wilting of potato seedlings at 5, 10, 15, and 20 days after inoculation (1st, 2nd, 3rd, and 4th row, respectively) with *R. solanacearum* isolates.

Gikungu isolates than in plants inoculated with Kirundo isolate at 10, 15 and 20DAI (Figure 5, 2nd, 3rd, and 4th row respectively). At 20DAI all plants (100%) inoculated with Gikungu isolate died and almost all plants (98.25%) inoculated with Kinigi also died. During the whole experimental period, there was no wilting in plants treated with water which served as the control.

Potatoes treated with sterile water (control) did not show any symptoms of bacterial wilt. Wilting occurred in potatoes inoculated with Kinigi, Gikungu, and Kirundo bacterial isolates, respectively. Wilting of potatoes inoculated with bacterial isolates increased over time either within the isolate or between isolates. At 20DAI all plants inoculated with Gikungu isolate died.

In addition, based on the rates of the potato plant wilting, it was observed that DI as well as DS caused by the three bacterial isolates increased over time. Thus, from 5 to 10 DAI potatoes inoculated with Kinigi, Kirundo and Gikungu bacterial isolates resulted to DI and DS which increased over time but not significantly different between them at $P \leq 0.05$. However, from 15 to 20DAI, there was a significant difference at $P \leq 0.05$ in disease incidence and severity caused by the three bacterial isolates. From 15 up to 20DAI, Kinigi and Gikungu isolates caused higher disease incidence and severity in comparison with Kirundo isolate but with no significant difference between them. Furthermore, at 20DAI inoculation of potatoes with Gikungu isolate caused significant difference level of disease incidence and severity in comparison with Kirundo while Kinigi did not (Table 1). Sterile water inoculation (control) did not cause wilting of potatoes from 5 to 20DAI whereas all three test bacterial isolates were pathogenic to potato plants (Table 1).

Table 1. Disease incidence (DI) and disease severity (DS) over time caused by inoculation of potato seedlings with different isolates of *Ralstonia solanacearum*.

Isolate	DI %				DS (%)			
	5DAI	10DAI	15DAI	20DAI	5DAI	10DAI	15DAI	20DAI
Kinigi	10.03^{a}	27.21^{a}	87.08^{a}	98.25^{ab}	30^{a}	60^{a}	84^{a}	97.33^{ab}
Kirundo	9.51^{a}	16.71^{a}	69.05^{b}	92.61^{b}	23.33 ^{a}	44.7 ^{a}	70.67^{b}	94.67^{b}
Gikungu	1.55^{ab}	22.95^{a}	88.52^{a}	100^{a}	20^{a}	58.33^{a}	84^{a}	100^{a}
Control	0^{b}	0^{b}	0^{c}	0^{c}	0^{a}	0^{b}	0^{c}	0^{c}
P values	0.0107	0.0024	<0.0001	<0.0001	0.3811	0.0159	<0.0001	<0.0001

DAI: Days after inoculation. Values within a column followed by the same letter are not significantly different at p≤0.05.

Pathogenicity of bacteria isolated from tubers of the three potato cultivars (Kinigi, Kirundo and Gikungu) was tested based on wilting severity and disease incidence they caused to Kirundo potato seedlings under greenhouse conditions. From 5 to 10 DAI, all bacterial isolates caused wilting incidence and increased severity in potatoes which were not significantly different between them. However, from 15 to 20 days after inoculation, Gikungu and Kinigi isolates started to cause more wilting as well as an increase in severity to potatoes than Kirundo isolate. All test bacterial isolates were pathogenic to potato seedlings. Moreover, bacteria isolated from Gikungu and Kinigi were more virulent to potatoes than Kirundo isolate. Usually, the difference in virulence may be a result of inoculum from different races and biovars of *R. solanacearum*, different plant hosts, or different plant cultivars due to their genetic base as well as isolate collected from different areas (Hayward, 1991; El-Habbaa et al., 2016; Sikirou et al., 2017). In this study, bacteria were isolated from the same site (Kinigi) and the same plant host (potato) and they were also inoculated in the same host and the same cultivar (Kirundo potato cultivar). In addition, all test isolates belonged to the same biovar (biovar 3 of race 1). These may explain why similar pathogenicity rate of these isolates on one potato cultivar during some periods of the study (5 to 10 DAI) were observed. On the other hand, bacterial cells were isolated from three different potato cultivars (Kinigi, Kirundo and Gikungu). Thus, it is not surprising that the same pathogen isolated from different cultivars resulted to slight difference in the virulence they caused to potatoes during this study especially at 20 DAI.

Biovar identification

From the findings of biovar identification in the present study, all test bacterial isolates acidified all sugars (cellobiose (C), maltose (M), and lactose (L)) and alcohols (dulcitol (D), mannitol (M), and sorbitol (S)) since the medium color changed from green to yellow after four and seven days of plate incubation at 28°C (Figure 6). The ability to utilize both sugars and alcohols characterize biovar 3 of *R. solanacearum* (Lepoint and Maraite, 2002; Muthoni et al., 2012; Sikirou et al., 2017). Therefore, all the three Rwandan collected isolates causing wilting to potato seedlings belonged to *Ralstonia* biovar 3.

Furthermore, it has been reported that there is a relationship between biovars and races of this pathogen and their location (Priou et al., 2001; Muthoni et al., 2012; Sikirou et al., 2017). In this context, biovar 3 generally belongs to race 1 of *R. solanacearum,* a race which usually affects potato and other plant species such as tomato, eggplant, tobacco, chili, peanut, groundnut and several weeds in tropical lowland regions (Fock et al., 2001; Strange and Scott, 2005; Sikirou et al., 2017). However, all the three bacterial isolates were collected from Kinigi site in Musanze District, a region characterized by tropical highland conditions (Birasa et al., 1990; MINIRENA, 2013). Under such conditions, potatoes are mainly affected by *R. solanacearum* biovar 2 race 3, a race which is well adapted to cool temperatures and which affect potatoes and tomatoes (Priou et al., 2001; Muthoni et al., 2012; Popoola et al., 2015). However, the presence of biovar 3 in Kinigi site was previously confirmed by studies conducted by Butare (1987) and Lepoint and Maraite (2002). All these findings confirmed that in Rwanda *R. solanacearum* race 1 biovar 3 can also adapt to tropical highland areas and affect potatoes under these conditions. Therefore, it is concluded that all three isolates from Kinigi site (Rwanda) belonged to race 1 biovar 3 of *R. solanacearum*.

Race 1 biovar 3 is the most widely distributed type of *R. solanacearum* in the world because it has a wide range of plant hosts with potatoes included among the others (Popoola et al., 2015). In addition, potatoes are mainly propagated through vegetative planting materials, a method which favors the dissemination of the bacteria from mother tubers (Hayward, 1991). Therefore, it is not surprising to find race 1 biovar 3 of this pathogen in Musanze since long time ago it is one of the major potato growing areas and the main site of potato seed production in Rwanda (RAB, 2012; Uwamahoro et al., 2018). Thus, the occurrence may be due to the

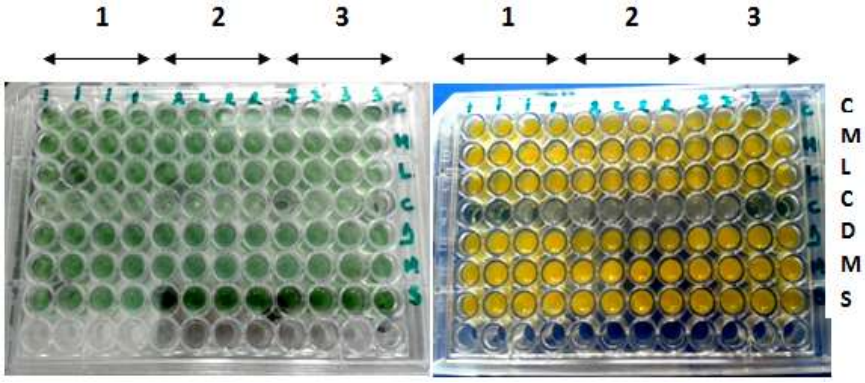

Figure 6. Biovar identification of three isolated of *R. solanacearum* strains. Green color (left) of initial basal medium changed to yellow color (right) at 4 and 7 days of incubation at 28ºC in the wells inoculated with Kinigi (1), Kirundo (2), and Gikungu (3) isolates. C: Cellobiose; M: Maltose; L: Lactose; D: Dulcitol; M: Mannitol; S: Sorbitol, Sterile distilled water (C) in 4th row of plate served as control.

introduction of this strain through potato seeds latently infected by this bacterial strain. To sustain potato production in this region, different potato genotypes should be introduced in order to identify cultivars which are high yielding and adaptable to this region with resistance to the different abiotic and biotic stresses.

Conclusions

This is the first report on a study conducted in Rwanda on pathogenicity of three bacterial wilt isolates on potato host plant. From *in vitro* experiment, all the three (Kinigi, Kirundo and Gikungu) isolates which were collected from Musanze District, Kinigi Sector showed typically cultural, morphological and biochemical traits of virulent *R. solanacearum* on both TTC and CPG agar media. In addition, from biovar test, all these test isolates belonged to *R. solanacearum* biovar 3 which is one of the biovars that belong to race 1 of this pathogen. Furthermore, *in vivo* experiment revealed that all isolates were pathogenic to potato plants. Gikungu and Kinigi isolates are more virulent to potatoes and can cause higher levels of wilting than Kirundo isolate.

Therefore, from this study, it is recommended to use Gikungu or Kinigi bacterial wilt isolates in future tests that may be conducted on *R. solanacearum*. It is also recommended to carry out other studies on biovar test or pathogenicity of *R. solanacearum* either by using a high number of bacterial wilt isolates or inocula collected from different agro-ecological zones as well as by using different hosts in order to determine whether there are other biovars and races of the pathogen that affect potato plants in Rwanda or if there are other isolates that can be more virulent to potatoes than the ones that were used in this research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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An assessment of adaptation options enhancing smallholder farmers' resilience to climate variability and change: Case of Mbengwi Central Sub-Division, North-West Region of Cameroon

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Farmers' implementation of suitable adaptation measures in the face of climate variability and change (CVC) depends first and foremost on their ability to perceive CVC. This paper analyzes the adaptation measures implemented by smallholder farmers (SHFs) in Mbengwi Central Sub-Division, North-West Region of Cameroon in the face of CVC as well as the determinants of smallholder farmers' choice of adaptation measures. Climate data collected from meteorological stations in the study area and farmers' perceptions obtained through household surveys were analyzed using descriptive statistics (frequency tables, bar charts, histograms and percentage indices) and inferential statistics (Coefficient of Variation and the Multinomial Logistic (MNL) regression model). Farmers' perceptions of CVC were then compared with actual short and long term meteorological data for the study area. Analysis was done on SPSS 17.0, Microsoft Excel 2007, and STATA 7.0. The results revealed that a majority of the respondents perceived an increase in temperature (79.2%) and a drop in the quantity of rainfall (59.2%) which corroborated almost perfectly with the analyzed meteorological data for the study area. From the twelve adaptation measures identified by SHFs, home gardens emerged as the most prevalent adaptation measure in the study area. Following the categorization of the multifarious adaptation measures implemented by SHFs, agroforestry practices emerged as the most prevalent adaptation measure. Results of the MNL regression model revealed that the main determinants of farmers' choice of adaptation measures in the study area were age of household head, number of farms possessed, farm size, and access to weather information ($p<0.05$).

Key words: Climate variability and change, smallholder farmers, adaptation measures, agroforestry practices, Mbengwi Central Sub-division, Cameroon.

INTRODUCTION

Climate variability and change will be the most devastating for developing countries due to their dependence on agriculture especially rain-fed agriculture coupled with their geographical location on one hand and their limited adaptation capacity on the other hand (UNFCCC's COP 21, 2015; WMO, 2016). This is particularly true for Africa which is the most tropical of all the continents in the world (IPCC, 2007; Kreft et al., 2014). Africa is already facing many hazards caused by climate variability and change, and this is expected to aggravate in the coming decades (FAO, 2016). The IPCC (2001) insinuates temperature changes for Africa between

0.2 and 0.5°C per decade. Total agricultural productivity loss in Africa due to climate variability and change is estimated to be between 17 and 28% as compared to 3 and 16% for the world as a whole (Cline, 2007). Documenting and encouraging the implementation of climate-smart and sustainable adaptation options therefore becomes incumbent (United Nations Sustainable Development Summit, 2015).

Sub-Saharan Africa which is a predominantly tropical region will bear the greatest brunt of climate variability and change according to predicted climate scenarios. This is due principally to its tropical nature on the one hand and high rates of poverty, high dependence on rain-fed agriculture, coupled with governmental and institutional failures on the other hand (World Bank, 2013). Following predicted climate scenarios, smallholder farmers in sub-Saharan Africa are expected to experience decreased precipitation and increased temperatures, initiating troubles in production stability for many of these economically bartered farmers (Cooper et al. 2008). According to Cooper (2004), about 89% of cereals cultivated in sub-Saharan Africa are rain-fed which makes them highly vulnerable to the whims of climate variability and change. Climate is a key determinant of food security which explains why its variation poses a serious problem to agriculture dependent economies like those of sub-Saharan Africa (World Bank, 2013; Kreft et al., 2014). Therefore, environmentally benign adaptation measures need to be actively documented and promoted.

Cameroon which constitutes an integral part of sub-Saharan Africa is therefore expected to face the same fate as the other countries found in this region. A large amount of scholarship already shows that in the absence of adaptation, climate variability and change will be a nuisance in Cameroon, especially in catalytic sectors like agriculture and livestock rearing (Molua, 2006, 2008; Molua and Lambi, 2007; Tingem et al., 2008a, b; Cameroun Vision, 2015; Document de Travail, 2009; GESP, 2009; Norrington-Davies, 2011; Somah, 2013; PNACC, 2015). The average temperature in Cameroon is predicted to increase as a result of global warming according to transient General Circulation Models (GCMs) (Tingem et al., 2009; Norrington-Davies, 2011). Based on the HadCM3 model, annual temperatures in Cameroon are expected to rise by 0.7 to 0.8°C by 2020 (Tingem et al., 2007). Agricultural production in Cameroon is already blighted by low levels of input (for example low quality seeds, limited irrigation, limited and inappropriate fertilizer, pesticide and herbicide use) due to farmers' low purchasing power, low levels of government subsidies, and high dependence on rain-fed agriculture which leads to low crop productivity (Tingem et al., 2009; Witt and Waibel, 2009). According to the World Bank (2011), since the year 2000, Cameroon's annual growth rate has been on a downward trajectory with only 2% growth in 2009. Almost 40% of the population continues to live below the national poverty line and Cameroon currently ranks 131st out of 169 nations in the Human Development Index (World Bank, 2011). A greater part of Cameroon's poor live in rural areas and practice agriculture which is the largest sector of the economy contributing about 45% to the annual Gross Domestic Product of the country (World Bank, 2011). All these factors go a long way to increase the vulnerability of smallholder farmers in Cameroon faced with climate variability and change, hence the necessity to document low-cost, climate-smart and sustainable adaptation measures.

The North-West Region of Cameroon like other parts of the country is already facing and will in the coming decades face even greater climate variability and change especially in precipitation and temperature patterns with devastating impacts on smallholder farmers (de Witt, 2011; Sunjo et al., 2012; Kimengsi et al., 2015). Smallholder farmers in the North-West Region of Cameroon in general and Mbengwi Central sub-Division (MCSD) in particular are already experiencing and will in the coming decades face even greater dire effects of climate variability and change owing mainly to their limited adaptive capacity (excruciating poverty and inadequate or no institutional support) as well as other factors like high dependence on rain-fed agriculture (less than 1% of farmers practice irrigation), limited off-farm activities (absolute dependence on agriculture) and few or no best farming practices (Awazi and Tchamba, 2018).

Based on the aforementioned problems facing smallholder farmers across Africa in general and the study area in particular in their struggle to adapt to climate variability and change, this research paper seeks to provide answers to the following research questions: what is the degree of climate variability and change? What are the different adaptation measures implemented by smallholder farmers in the face of climate variability and change? What are the determinants of smallholder farmers' choice of adaptation measures? What is the effectiveness of the most prevalent adaptation measure in reducing vulnerability to climate variability and change? Providing answers to the aforementioned questions helped in the attainment of the objectives of the study which were:

(1) To assess the degree of climate variability and change
(2) To identify the different adaptation measures implemented by smallholder farmers

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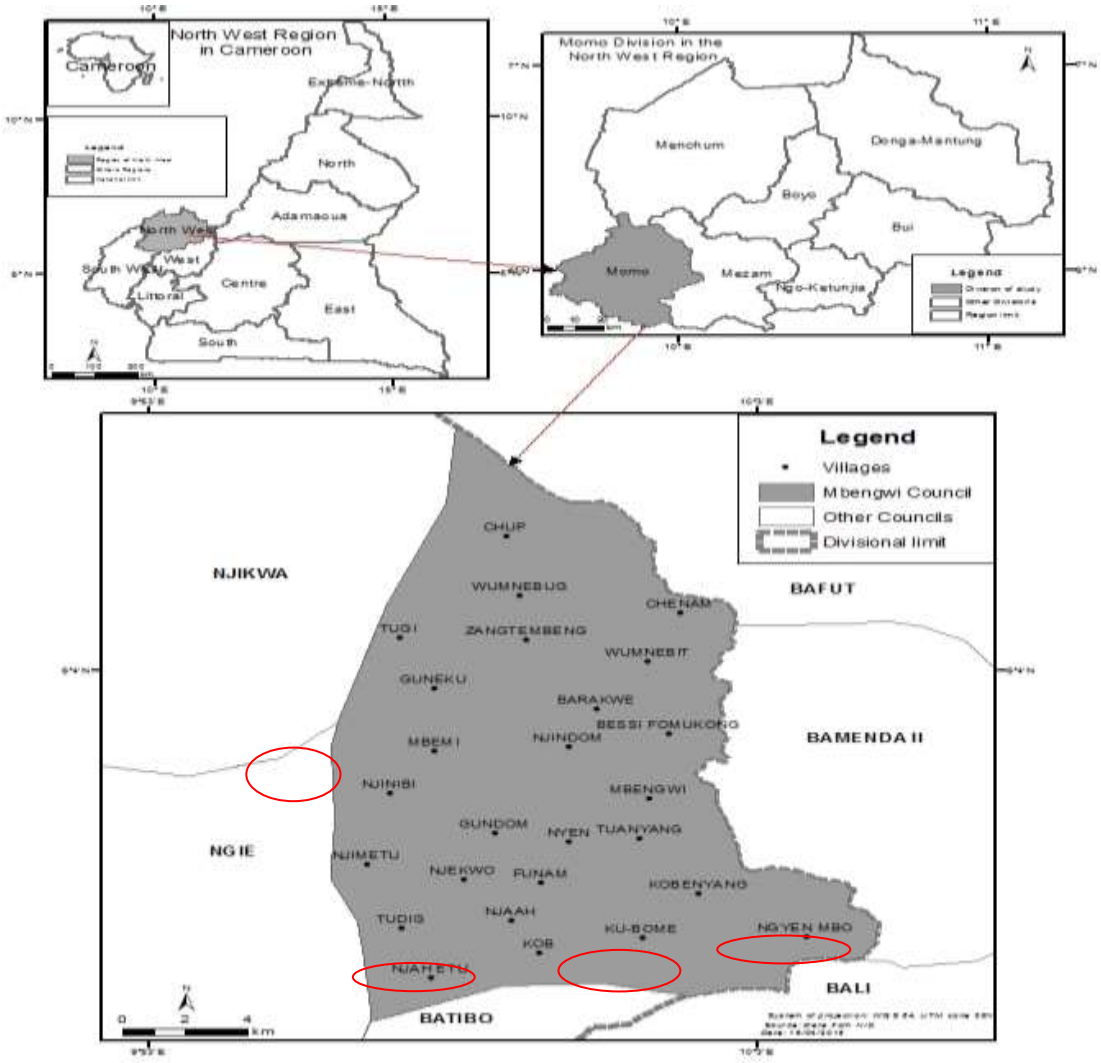

Figure 1. Map showing the study area.

(3) To assess the determinants of smallholder farmers' choice of adaptation measures
(4) To identify the effectiveness of the most prevalent adaptation measure in reducing vulnerability.

MATERIALS AND METHODS

Description of the study site

This study was conducted in Mbengwi Central Sub-Division, North-West Region of Cameroon (Figure 1). It lies between latitude 6°00' and 6°05' North and longitude 10°00' and 10°02' East. It has an essentially tropical climate characterized by two distinct seasons: the dry season and the rainy season. Rainfall mainly occurs during the rainy season which stretches from March to mid-March to mid-October (but this trend has been fluctuating very much in recent decades). The long term annual average temperature is 26°C. The long term annual average rainfall in Mbengwi Central sub-Division is 1450 mm. The sub-division falls within the relief region known as the Western Highlands of Cameroon and is dominated mainly by savannah grasslands. The principal economic activity undertaken by the inhabitants of the study area is agriculture practiced mainly by smallholder farmers who inhabit the rural areas (DDARD and SDARD, 2015).

Sampling procedure

A multiple phase or multi-stage sampling procedure was followed in laying out the survey as used by other scientific studies (Temesgen et al., 2014; Feleke, 2015; Hadgu et al., 2015; Atinkut and Mebrat, 2016).

At the first phase, the study area was purposively selected owing to the high proportion of the population engaged in smallholder agriculture and the high rate of vulnerability of its smallholder farmer population to extreme weather events (due principally to their limited adaptive capacity).

At the second phase, the 29 villages found in the sub-division were grouped into three strata based on their micro agro-ecological-relief characteristics (type of crop grown and livestock raised, vegetation type, and altitude). According to the micro agro-ecological-relief characteristics of MCSD, the three strata are:

Bome zone (60% of the total land surface), Tondig Zone (15% of the total land surface) and Taah zone (25% of the total land surface) (Mbengwi Council Development Plan Report, 2012). Hence, in order to get a representative picture of the entire sub-division, one village was selected from each of these strata with the help of agricultural extension officers. However, owing to the largeness of the Bome zone, an additional village was selected from there, still with the aid of agricultural extension officers. Thus four villages were selected for this survey.

At the third phase, stratified random sampling was conducted. Farmers were stratified based on age into two strata: those with ages below 30 years and those with ages above 30 years. And then 30 smallholder farmer household heads (with ages above 30 years) were randomly sampled in each of the 4 villages (Mainly farmers with ages above 30 were sampled in order to have a better picture of the situation of climate variability and change in the study area). Hence, a total of 120 smallholder farmer household heads were interviewed in the four (04) selected villages giving a sampling rate of 30.38%.

The fourth phase involved Key Informant Interviews (KIIs) with village leaders, chiefs of agricultural posts, delegates and sub-divisional delegates in the Ministries of Agriculture and Rural Development; Environment, Protection of Nature and Sustainable Development; Livestock, Fisheries and Animal Husbandry. KIIs were conducted in order to verify the veracity of the responses given by smallholder farmers during household surveys.

Data collection and analysis

The study made use of both qualitative and quantitative methods to collect primary data for the study area. The methods used were: household surveys, KIIs, and direct observations. Most of these primary data were collected through household survey using structured and semi-structured questionnaires where smallholder farmer household heads (above 30 years) constituted the sampling unit. KIIs with purposely selected resource persons in the study area provided general information to complement the data obtained through household surveys. A semi-structured interview guide approach was employed during KIIs. Direct field observations through transect walks were also frequently undertaken in the study area in order to ascertain the veracity of the responses obtained from household surveys and KIIs. Primary data was collected from four villages selected with the help of agricultural extension agents taking into cognizance the agro-ecological and relief characteristics of the study area. A survey of 120 smallholder farmer households using stratified random sampling of 30 households per village was conducted at a sampling rate of 30.38%.

Secondary data mainly on past temperature and rainfall was collected from the meteorological stations in the study area. Rainfall and temperature data for 11 years (2004 to 2014) was collected from Divisional Delegation of Agriculture and Rural Development (DDARD) based on Mbengwi (the study area), while rainfall and temperature data for 54 years was collected from the Regional Service of Meteorology for the North-West Region (Bamenda-Station) located at some 20 km from the study area.

Statistical analysis of the data obtained from household survey was done on the Statistical Package for Social Sciences (SPSS) 17.0, Microsoft Excel 2007 and STATA version 7.0. Descriptive statistics like percentage indices, frequency tables, bar charts and graphs as well as inferential statistics like Coefficient of Variation (CV) (Equations 1 and 2) and the Multinomial Logistic (MNL) regression model (Equation 3).

CV was used to analyze the short term variability in rainfall and temperature for 11 years. To calculate the CV, the standard deviation is obtained. This is defined as:

σ=Standard Deviation

$$\sigma=\sqrt{\frac{\Sigma\,(x-\bar{x})^2}{n-1}} \quad (1)$$

$$CV = \frac{\sigma}{\bar{x}} \times 100 \quad (2)$$

And as a rule of thumb, when the CV is greater than 10, it is unreliable meaning variability is high but when the CV is less than 10, it is reliable meaning variability is low.

Long term variability and change was analyzed by calculating the rainfall and temperature anomalies for 54 years. This was done by calculating the residual for temperature (annual average - 54 years average) and the residual for rainfall (annual- 54 years mean). As a rule of thumb, variability in climate elements over a period greater than three decades definitely signifies climate change (IPCC, 2007).

The MNL regression model (Equation 3) was used to determine the causal relationship between smallholder farmers' choice of adaptation measures (multinomial dependent variable) with respect to various hypothesized continuous and discontinuous explanatory variables (Table 1). This model has equally been used by other research works on adaptation to climate variability and change (Temesgen et al., 2014).

Following Greene (2003), the MNL model permits the analysis of multiple choice problems. According to Deressa et al. (2009), the MNL model permits the analysis of decisions across more than two categories, enabling the determination of choice probabilities and is equally simple to compute. This model has response probabilities.

$$P(y=j/x) = \frac{\exp(x\beta_j)}{1+\Sigma_{k=1}^{j}\exp(x\beta_k)}, j = 1, \ldots\ldots\ldots J \quad (3)$$

where *y* is a random variable (adaptation options) with the values (1,2,.....J), *j* is a positive integer, *x* is a set of conditioning variables (socio-economic, institutional and environmental factors), and βj is K×1.

The running of the MNL model proper was done on SPSS 17.0. Before running the actual model estimate, Hausman Specification test was run on STATA version 7.0 in order to check the validity of the Independence of Irrelevant Alternatives (IIA) assumption. This test failed to reject the null hypothesis of the independence of the adaptation options under consideration. This implies that the application of the MNL specification was appropriate to model the determinants of adaptation measures.

RESULTS AND DISCUSSION

Degree of climate variability and change

Variations and changes in rainfall and temperature patterns

Analysis of rainfall and temperature data for 11 years showed high levels of variability in these two climate elements (Table 2). In order for greater reliability to exist, there should be less variability. As a rule of thumb, when a CV is greater than 10 as show earlier, it is unreliable meaning variability is high but when the CV is less than 10, it is reliable which means variability is low.

Table 1. Description of hypothesized explanatory variables.

Variable	Description
Household size	Continuous
Sex	Dummy, takes the value of 1 if male and, 0 otherwise
Noticed extreme sunshine	Dummy, takes value of 1 if Yes and 0 otherwise
Age	Continuous
Number of farms	Continuous
Farm size in hectares	Continuous
Noticed high temperatures	Dummy, takes value of 1 if Yes and 0 otherwise
Annual family income	Continuous
Farm experience	Continuous
Access to weather information	Dummy, takes value of 1 if Yes and 0 otherwise
Noticed highly inconsistent rainfall	Dummy, takes value of 1 if Yes and 0 otherwise
Access to extension services	Dummy, takes value of 1 if Yes and 0 otherwise
Education	Dummy, takes value of 0 No education, 1 primary, 2 secondary, 3 tertiary
Access to credit	Dummy, takes value of 1 if Yes and 0 otherwise
Noticed reduced rainfall	Dummy, takes value of 1 if Yes and 0 otherwise
Distance to market	Dummy, takes value of 1 near, 2 moderate, 3 far
Land ownership	Dummy, takes value of 1 if owned, 0 otherwise
Noticed storms	Dummy, takes value of 1 if Yes and 0 otherwise
Membership in farming group	Dummy, takes value of 1 if Yes and, 0 otherwise

Table 2. Annual rainfall and temperature variability in MCSD.

Year	Rainfall (mm) (x)	$(x-\bar{x})$	$(x-\bar{x})^2$	Temperature (°C) (x)	$(x-\bar{x})$	$(x-\bar{x})^2$
2004	2328	-377.73	142680.71	23.42	1.59	2.53
2005	2608	-97.73	9551.35	23.71	1.88	3.53
2006	3355.5	649.77	422199.75	24.01	2.18	4.75
2007	2670	-35.73	1276.70	26.78	4.95	24.50
2008	2567	-138.73	19246.29	23.35	1.52	2.31
2009	2544	-161.73	26156.92	22.42	0.59	0 .35
2010	2244.05	-461.68	213149.34	21.15	-0.68	0.46
2011	3098.60	392.87	154346.05	18.91	-2.92	8.53
2012	3123.09	417.36	174188.53	18.23	-3.60	12.96
2013	2656	-49.73	2473.17	20.93	-0.90	0.81
2014	2568.80	-136.93	18750.10	17.20	-4.63	21.44
Total	29763.04	-	1184018.91	240.11	-	82.17
-	$\bar{x}$ = 2705.73	-	-	$\bar{x}$ = 21.83	-	-

Source: Data courtesy DDARD meteorological station based in Mbengwi.

CV= Coefficient of Variation for rainfall

$$\sigma = \sqrt{\frac{1184018.91}{10}}$$

σ = 344.10

$$CV = \frac{344.10}{2705.731} \times 100$$

CV=12.72%

CV= Coefficient of Variation for temperature

$$\sigma = \sqrt{\frac{82.17}{10}}$$

σ = 2.87

$$CV = \frac{2.87}{21.83} \times 100$$

CV = 13.15%

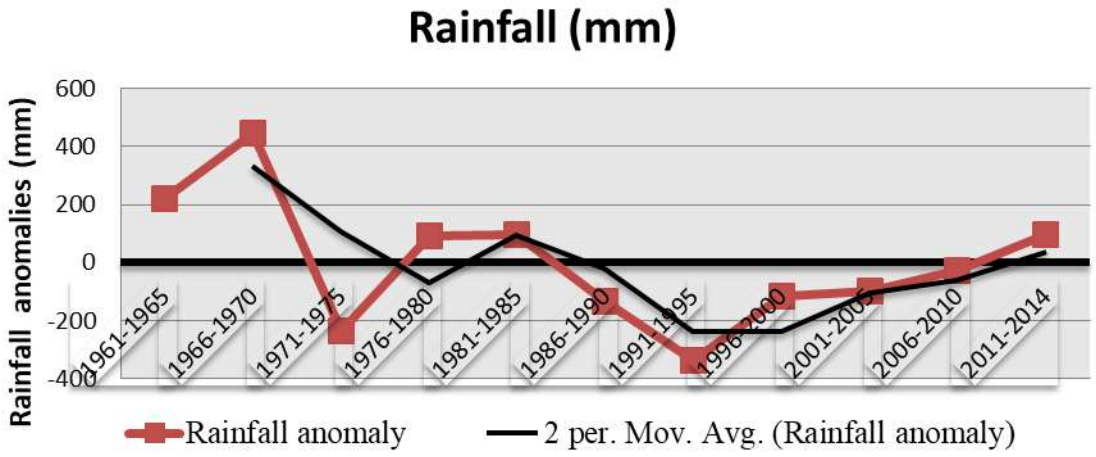

Figure 2. Five year average rainfall residual and moving average for MCSD.

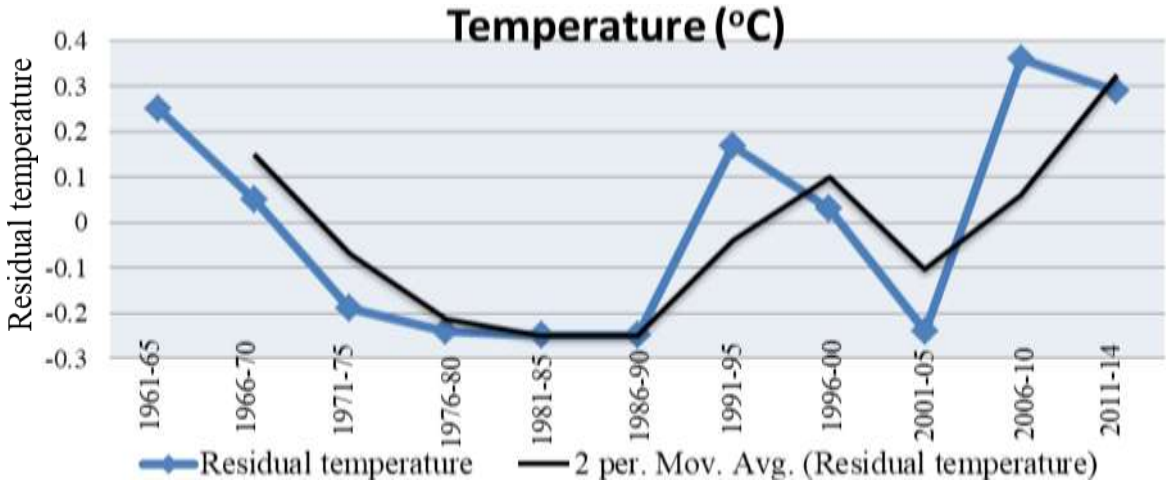

Figure 3. Five year average temperature residual and moving average for MCSD.

From the results, both rainfall and temperature showed high levels of variation, having exceeded the 10% threshold of reliability. The CV for rainfall (12.72%) and that of temperature (13.15%) all exceeded the 10% threshold indicating that there exists significant variability in these climatic elements.

A similar data analysis procedure was followed by Kimengsi and Tosam (2013), in a study conducted to assess the impact of climate variability on cocoa production in Meme Division, South-West Region of Cameroon.

Analysis of long term rainfall data (1961-2014) equally showed high levels of variability and recurrent weather events (especially erratic and inadequate rainfall and relatively high temperatures) (Figures 2, 3 and 4).

Five year average rainfall residuals (quantity of actual average rainfall for five years- 54 years average) and actual average rainfall between 1961 and 2014 showed significant variability (Figure 2). Huge rainfall deficits were observed between 1961 and 2014 with 6 out of the 11 five years intervals experiencing rainfall deficits. Serious rainfall deficits most especially occurred between the periods of 1971-1975, 1986-1990,1991-1995, 1996-2000, 2001-2005, and 2006-2010. The solid line on the residual rainfall graph represents 10 years moving average. Variations in the quantity of rainfall within the five year intervals ranged between 1.16 and 18.80%, with very high variability in the 1960, 1970, 1990 and early 2000s.

Temperature on its part has seen relatively high levels of variability and some relative increase in the past 54 years (Figure 3). Five years temperature residuals (five year average temperature – 54 years average) show some relative degree of variability in temperature especially from the 1980s up to 2014.

The number of rainy days equally fluctuated highly between 1961 and 2014. The number of rainy days

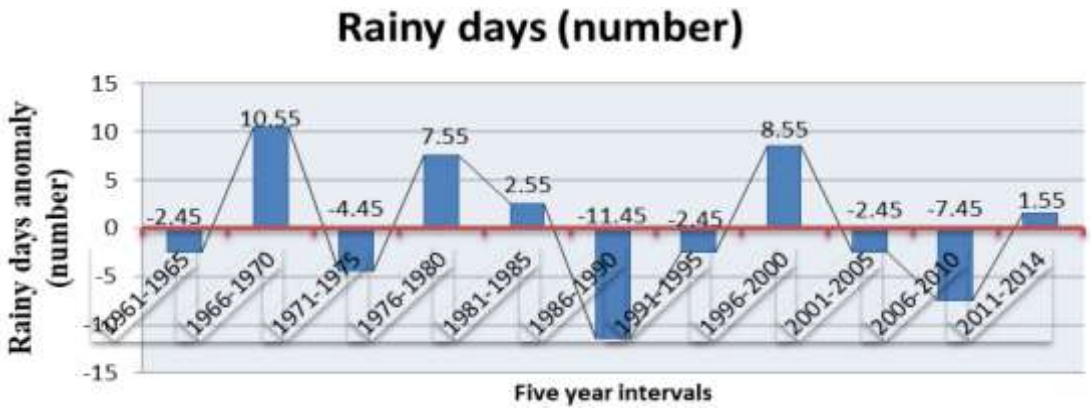

Figure 4. Five year average rainy days anomaly for MCSD.

equally noticed a drastic fall with 6 out of the 11 five year intervals experiencing fewer rainy days (Figure 4). Fewer rainy days most especially occurred between the years 1961-1965, 1971-1975, 1986-1990, 1991-1995, 2001-2005, and 2006-2010.

Smallholder farmers' perception of variations and changes in climate elements

Analysis of household survey showed that all the respondents perceived climate variability and change. But their perceptions varied with respect to the different climate elements of rainfall, temperature, sunshine, wind and storms (Table 3). Pertaining to the total amount of rainfall, the perceptions of most of the respondents was that the total amount of rainfall has been decreasing from decade to decade (1985-2014). 59.2% of the respondents perceived that the total amount of rainfall decreased a lot between 2005 and 2014 as opposed to the 12.5 and 1.7% of respondents who perceived that the rainfall decreased a lot between 1995 and 2004 and 1985 and 1994, respectively.

Pertaining to temperature, 79.2% of the respondents perceived that temperature was much hotter between 2005 and 2014 as opposed to the 21.7 and 0.8% of respondents who perceived much hotter temperature between 1995 and 2004 and 1985 and 1994, respectively.

With regards to rainfall consistency, most of the respondents perceive that rainfall has become increasingly variable as the decades go by. 87.5% of the respondents perceive that rainfall was much more variable between 2005 and 2014 as opposed to the 35 and 1.7% of respondents who perceived that rainfall was much more variable between 1995 and 2004 and 1985 and 1994, respectively.

It must therefore be said that farmers' perceptions of reduction in total quantity of rainfall, rainfall inconsistency and high temperature (Table 3) largely falls in line with meteorological data for the study area. Similar results have been found by other studies across Africa and the tropics (Aggarwal et al., 2015; Sarr et al., 2015) demonstrating that there is increasing unanimity amongst smallholder farmers that climate variability and change is real and characterized by changing weather patterns and extreme climate events.

Smallholder farmers' adaptation measures and determinants of choice of adaptation measures

Smallholder farmers' adaptation measures

In order to counteract the negative impacts of climate variability and change, smallholder farmers implemented a combination of adaptation measures simultaneously (Figure 5). The most recurrent adaptation measures identified by smallholder farmers included staggering of planting dates (64.2%), scattered trees on croplands (53.3%), mono-tree plantations (50.8), home garden (68.3%) and joining community groups (45.8%). Meanwhile the least recurrent adaptation options identified by farmers were irrigation (7.5%), two seasons cropping (15%), use of improved crop and animal species (14.2%). No adaptation equally featured prominently amongst the adaptation options (30%). Several studies conducted on adaptation to climate variability and change have equally found similar results across different parts of Cameroon, Africa and the tropics revealing that a combination of adaptation measures is used simultaneously by smallholder farmers (Molua and lambi, 2007; Atinkut and Mebrat, 2016; FAO, 2016).

Determinants of smallholder farmers' choice of adaptation measures

In order to determine the causal relationship between farmers' choice of adaptation measures with respect to several hypothesized explanatory variables, the MNL

Table 3. Farmer perceived changes in climate elements in three decades.

Total quantity of rainfall	1985-1994		1995-2004		2005-2014	
	Frequency	%	Frequency	%	Frequency	%
Increased a lot	8	6.7	1	0.8	3	2.5
Increased	22	18.3	7	5.8	4	3.3
Stayed the same	74	61.7	32	26.7	6	5.0
Decreased	14	11.7	65	54.2	36	30.0
Decreased a lot	2	1.7	15	12.5	71	59.2
Total	120	100	120	100	120	100
Temperature						
Much hotter	1	0.8	26	21.7	95	79.2
Hotter	7	5.8	59	49.2	18	15.0
Stayed the same	35	29.2	24	20	1	0.8
Cooler	33	27.5	8	6.7	3	2.5
Much cooler	44	36.7	3	2.5	3	2.5
Total	120	100	120	100	120	100
Rainfall consistency						
Much more variable	2	1.7	42	35.0	105	87.5
More variable	6	5.0	45	37.5	10	8.3
Stayed the same	25	20.8	22	18.3	1	0.8
Less variable	33	27.5	10	8.3	1	0.8
Much less variable	54	45.0	1	0.8	3	2.5
Total	120	100	120	100	120	100
Sunshine						
Increased a lot	1	0.8	24	20	102	85
Increased	9	7.5	64	53.3	11	9.2
Stayed the same	64	53.3	24	20	1	0.8
Decreased	27	22.5	6	5	4	3.3
Decreased a lot	19	15.8	2	1.7	2	1.7
Total	120	100	120	100	120	100
Incidence of storms (strong winds, heavy rain and thunder)						
Increased a lot	1	0.8	30	25	98	81.7
Increased	10	8.3	57	47.5	14	11.7
Stayed the same	61	50.8	23	19.2	4	3.3
Decreased	30	25	8	6.7	2	1.7
Decreased a lot	18	15	2	1.7	2	1.7
Total	120	100	120	100	120	100

n = 120; % = percent.
Source: Household Survey Conducted (2015).

model was run (Table 5). In order to run this model, the different adaptation options were categorized (Table 4).

Following the categorization of the different adaptation measures, four major categories emerged: Agroforestry practices, On-farm practices, Off-farm practices and "No adaptation" (Table 4). Agroforestry practices (Combination of home gardens and scattered trees on croplands) were the most recurrent option implemented by smallholder farmers in the study sites (26.7%). This can be attributed to the pro-poor and sustainable nature of agroforestry practices when compared with the other adaptation options. The use of agroforestry practices as an adaptation option in the face of climate variability and change has equally been found useful by other studies

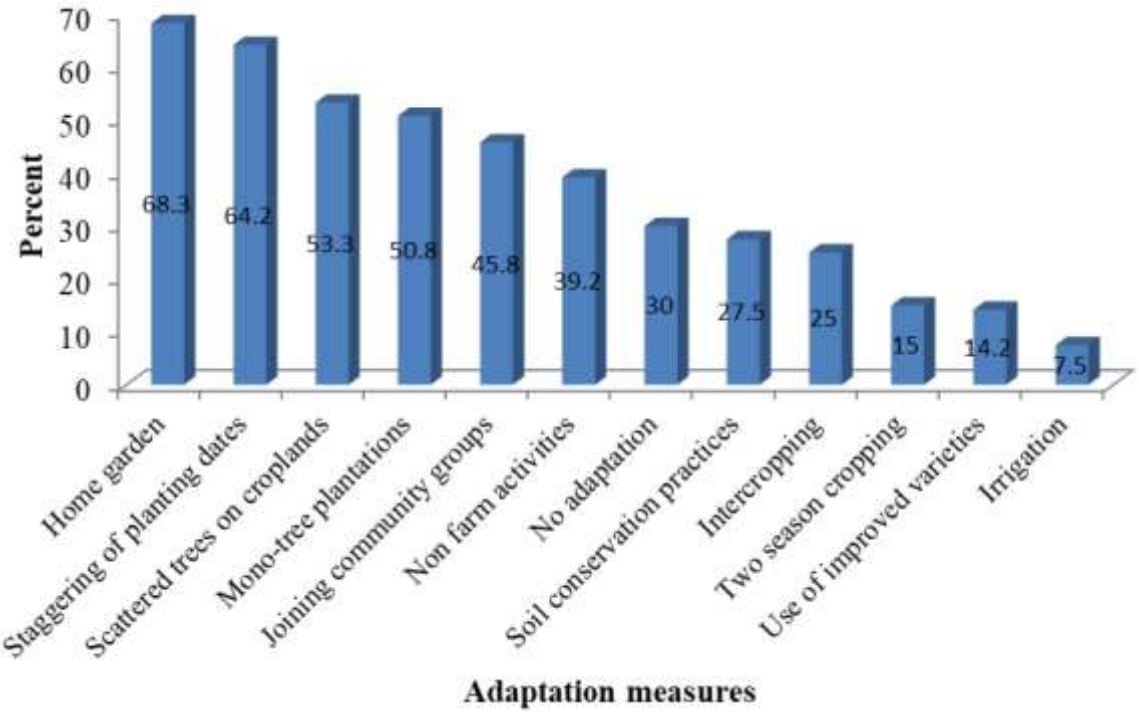

Figure 5. Adaptation measures implemented by smallholder farmers.

Table 4. Categorized adaptation options implemented by smallholder farmers.

Categorized adaptation options	Tugi		Ngyen-Mbo		Ku-Bome		Njah-Etu		Total	
	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%
AFP	11	36.7	11	36.7	3	10	7	23.3	32	26.7
OFP	8	26.7	4	13.3	7	23.3	9	30	28	23.3
OFFP	2	6.7	8	26.7	8	26.7	6	20	24	20
No adaptation	9	30	7	23.3	12	40	8	26.7	36	30
Total	30	100	30	100	30	100	30	100	120	100

Freq. = Frequency; % = Percentage; AFP = Agroforestry Practices; OFP = On-farm practices; OFFP = Off-farm practices.
Source: Own Survey (2015).

(Lin, 2007; Nguyen et al., 2012; Bishaw et al., 2013; Mbow et al., 2013a, b; Kabir et al., 2015; Lasco et al., 2015). These studies all show that agroforestry practices are low cost, climate-smart land-use systems which provide the four ecosystem services described by the Millennium Ecosystem Assessment Report of 2005, provisioning, regulating, cultural, and supporting services. Provisioning services in particular (food, fibre, medicines, wood, and construction materials) provided by agroforestry practices help smallholder farmers to diversify their income sources thereby making them more resilient in the face of climate variability and change (Nguyen et al., 2012; Bishaw et al., 2013; Mbow et al., 2013a; Kabir et al., 2015).

On-farm and off-farm practices were equally adopted by smallholder farmers in their struggle to adapt to climate variability and change. The use of these two practices has equally been identified by other studies conducted in different parts of Africa (Molua, 2006; Tabi et al., 2012).

The “No adaptation option” equally featured prominently with 30% of smallholder farmers taking to it. These smallholder farmers continued their farming activities “business-as-usual”. Several factors explain this state of affairs. The major barriers to adaptation in the study area were: limited or no weather information, shortage of land, low prices of farm products, poverty, lack of good farm inputs, limited or no advice from government and NGO extension agents, and limited irrigation potentials. These barriers to adaptation have

Table 5. Parameter estimates of the MNL model.

Explanatory variable	Agroforestry practices		On-farm practices		Off-farm practices	
	Coefficients	P-level	Coefficients	P level	Coefficients	P-level
Constant	-27.249***	0.001	-31.305***	0.000	-44.622***	0.000
Age of HH head	0.340***	0.006	0.409***	0.001	0.363***	0.003
Number of farms	0.804**	0.039	0.811**	0.039	0.548	0.161
Household size	-0.646	0.157	-0.626	0.175	-0.566	0.220
Ann. family income	0.000	0.141	0.000	0.182	0.000	0.143
Farm size	8.652**	0.024	8.399**	0.029	8.644**	0.024
Access_weather_infos	4.091**	0.035	5.378***	0.008	21.839*	0.075
Base category	No adaptation					
No of observations	120					
-2 Log Likelihood	190.194					
Likelihood Ratio χ^2	139.833***					
Pseudo R-Square	0.735					

*, **, ***Significant at 10, 5 and 1% probability levels, respectively.

equally been found by several studies across Africa (Tabi et al., 2012; Kabir et al., 2015; Mersha and Laerhoven, 2016).

The MNL regression model revealed that age of the household head, number of farms, farm size and access to weather information contributed significantly to influencing smallholder farmers' choice of adaptation measures ($p<0.01$; $p<0.05$; $p<0.10$) meanwhile annual family income and household size did not significantly influence smallholder farmers' choice of adaptation measures ($p>0.10$) (Table 5).

Age of household head ($p<0.01$) significantly influenced the implementation of adaptation measures: agroforestry practices (combination of home gardens and scattered trees on croplands), on-farm practices (combination of staggering of planting dates, soil conservation practices, intercropping, two season cropping, irrigation and improved varieties) and off-farm practices (combination of mono-tree plantations, joining community groups and non-farm activities). This means that the older the farmer, the higher the likelihood to implement adaptation measures like agroforestry practices, on-farm practices and off-farm practices. Several studies undertaken in Africa and the tropics have also found age of the household head to be a major determinant of smallholder farmers' choice of adaptation measures (Deressa et al., 2008; Tabi et al., 2012; Kabir et al., 2015; Atinkut and Mebrat, 2016). This is because age is generally associated with farm experience, and hence the older the farmer, the more experienced they are.

Number of farms ($p<0.05$) positively influenced smallholder farmers' implementation of adaptation measures especially agroforestry practices and on-farm practices. This implies that farmers with more farms are better equipped to adapt in the face of climate variability and change than farmers with few farms. This is probably because farmers with many farms generally cultivate a larger portion of land with a diversity of crops and have more yields making them more food self-sufficient. Farmers with more farms are equally able to sell surplus food making them more financially stable and increasing their ability to buy better farm inputs and tools which goes a long way to enhance adaptation. Tabi et al. (2012) and Taruvinga et al. (2016) equally found that the number of farms positively influences farmers' choice of adaptation measures implying that farmers with more farms adapt better than their counterparts with few farms.

Farm size ($p<0.05$) also positively affected smallholder farmers' choice of adaptation measures. Smallholder farmers with a larger farm size therefore had a higher likelihood to implement adaption measures: agroforestry practices, on-farm practices and off-farm practices than smallholder farmers with smaller farm sizes. This is because farmers with a large farm size are more likely to cultivate more crops and have more yields than their counterparts with a small farm size. With high yields, farmers are more food self-sufficient permitting them to sell surplus food which enables them to buy better farm inputs and tools, thus enhancing adaptation. Farm size has equally been found by other studies to influence farmers' choice of adaptation options (Deressa et al., 2008; Tabi et al., 2012; Kabir et al., 2015; Atinkut and Mebrat, 2016).

Access to weather information ($p<0.05$, $p<0.01$, $p<0.10$) equally influenced smallholder farmers' choice of adaptation measures. Smallholder farmers with better access to weather information were more likely to implement agroforestry practices and on-farm practices in particular. This is because farmers having access to weather information are more exposed to the latest innovations in farming techniques and technologies. They are equally informed in advance of extreme weather

events about to unfold which helps them to take action before the event actually occurs. Several studies conducted in sub-Saharan Africa have equally demonstrated that access to weather information plays a positive role in determining smallholder farmers' choice of adaptation measures (Gbetibouo, 2009; Temesgen et al., 2014; Elia et al., 2015; Atinkut and Mebrat, 2016).

Household size though not statistically significant (p>0.10), negatively influenced choice of adaptation measures. Many studies conducted across Africa and the tropics show that household size has a positive influence on adaptation implying that the larger the household, the greater the likelihood to adapt in the face of climate variability and change. The reason for this is that a larger household size is attributable to more family labour which permits the cultivation of a larger portion of land thereby increasing yields and in essence the food self-sufficiency of the household (Deressa et al., 2008). However, in this study, it was found that the larger the household, the lesser the degree to adapt. This is probably due to the existence of an essentially dependent population (very young or very old) in the households. Another reason could be that, most household members in the study area are not taking active part in farming activities.

Annual family income though not equally statistically significant (p>0.10), positively influenced choice of adaptation measures. This is because farmers with more income are able to buy better farm inputs and tools which permit them to adapt in the face of extreme weather events. They are also able to buy food in case of food shortages.

Overall, the model was statistically significant, Likelihood Ratio χ^2 (6, n = 120) = 139.83, p<0.001. The likelihood ratio statistics from the MNL model therefore indicated that χ^2 statistics was highly significant (χ^2 = 139.83, p<0.001) showing that the model has a strong explanatory power. The model explained 73.5% (Nagelkerke R^2) of the variance in farmers' choice of adaptation options. Pseudo R^2 (0.735) therefore shows that the weighted combination of predictor variables was jointly significant in explaining smallholder farmers' choice of adaptation options.

However, it must be said that the parameter estimates of the MNL model (Table 5) do not provide the actual magnitude of change. Rather, they only provide the direction of the effect of the explanatory variables on the dependent variable. Therefore, to determine the magnitude of change, the marginal effects from the MNL model need to be run which has not been done in this study. Several studies have come out with the marginal effects of the MNL in order to determine the actual magnitude of the change in the dependent variable that is caused by a change in the explanatory variables (Deressa et al., 2008; Temesgen et al., 2014).

The Kruskal-Wallis test (H-test) which was run to see if there was a significant variation in smallholder farmers' adaptation measures across the four villages studied showed that adaptation measures did not vary significantly across the four villages [χ^2 (3, n=120) = 3.946, p>0.10]. Therefore, smallholder farmer across the four villages implement similar adaptation measures categorized as agroforestry practices, on-farm practices, off-farm practices and "No adaptation".

Effectiveness of the most prevalent adaptation measure implemented by smallholder farmers in reducing vulnerability

Analysis of data collected through household survey revealed that the most prevalent adaptation option in the study area was the home garden which was adopted by 82 out of the 120 smallholder farmers interviewed giving a percentage score of 68.3. Out of the 82 smallholder farmers who take to home garden as an adaptation measure, 44 of these respondents rate home garden to have a very high level of effectiveness in reducing vulnerability to climate variability and change giving a percentage score of 53.7%. Meanwhile 7.3% (6 smallholder farmers) of those who take to home garden rate it to have a high level of effectiveness. Equally, 9.8% (8 farmers) rate home garden to have a moderate effectiveness in reducing vulnerability to extreme climatic events. However, 13.4% (11 respondents) and 15.9% (13 respondents) rate home garden to have a low and very low level of effectiveness respectively (Figure 6).

Home gardens have equally been proven by various studies to be a prevalent adaptation measure amongst smallholder farmers with a high level of effectiveness in reducing smallholder farmers' vulnerability to the nefarious effects of climate variability and change (Bishaw et al., 2013; Mbow et al., 2013a, b; Kabir et al., 2015; Lasco et al., 2015). Following these studies, the immediate area around the homestead generally offers numerous ecosystem services (provisioning, regulating, cultural and support services) like increased availability of water, better soil fertility due to organic waste inputs, and easier protection of the crop against animals thereby leading to increased production of food, fibre, medicines and many other products and services.

CONCLUSION AND RECOMMENDATIONS

This study found out that smallholder farmers have a peculiar way of perceiving climate variability and change (CVC) and adapting to its nefarious effects. A thorough review of literature showed a dearth of information pertaining to low cost, pro-poor and environmentally benign adaptation measures for smallholder farmers. Hence, based on the findings from this study, the following recommendations have been made geared towards increasing smallholder farmers' awareness of CVC and enhancing the implementation of more low-

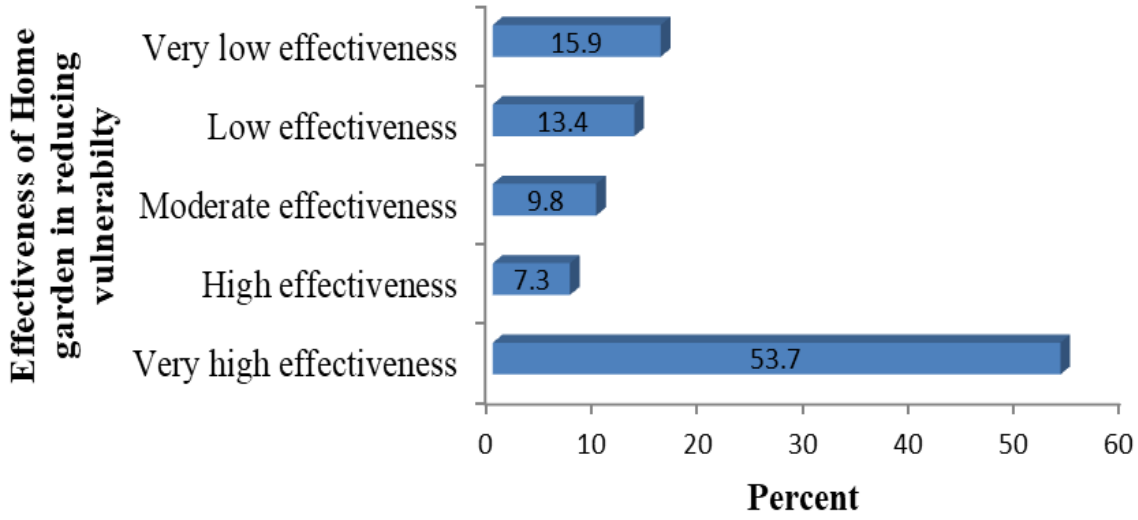

Figure 6. Farmer perceived effectiveness of Home garden in reducing vulnerability.

cost, pro-poor and climate-smart adaptation measures.

First and foremost, the Ministry of Agriculture and Rural Development (MINADER), Ministry of Forestry and Wildlife (MINFOF), Ministry of Environment, Protection of Nature and Sustainable Development (MINEPDED) and their decentralized institutions and delegations need to encourage and support the putting in place of agroforestry practices owing to the pro-poor and environmentally benign nature of this practice when compared with other adaptive measures.

Moreover, the government and NGOs need to provide more institutional support in order to beef-up smallholder farmers' resilience in the face of extreme climate events.

Equally smallholder farmers on their part need to undertake more environmentally friendly agricultural practices (like agroforestry) which are sustainable and at the same time guarantee adequate agricultural productivity.

Last but not the least, local authorities, government agencies, extension officers, Non-Governmental Organizations, policy makers, scientists and research institutions need to accompany smallholder farmers as they strive to adapt to climate variability and change.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison of bioslurry to common nitrogen sources on potato (*Solanum tuberosum* L.) yield and yield components in andisols and oxisols of Northern Rwanda

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This study evaluated the effect of bioslurry from on-farm biogas production units and other sources of nitrogen fertilizer on Irish potato yield and yield components in northern Rwanda. To achieve this, an on-farm experiment was undertaken in the Andisols of Musanze District and the Oxisols of Gicumbi District in 2012. Three farms were selected to host the study in each District. Six treatments were randomly tested in unreplicated field strips by farm, one treatment by strip. The treatments comprised of i_1) a control with no fertilizer (CTL000); i_2) a control supplying 100% of recommended N rate from chemical fertilizers (MIN100); i_3) a treatment supplying 100% of recommended N rate from farm yard manure (FYM100); i_4) a treatment supplying 40% of recommended N amount from bioslurry and 60% from mineral N fertilizer (BIO040); i_5) a treatment supplying 60% of recommended N amount from bioslurry and 40% from mineral N fertilizer (BIO060); and i_6) a treatement supplying 100% of recommended N amount from bioslurry (BIO100). The results indicated that locally produced bioslurry appeared very low-nitrogen concentrated and for some farms, more than 100 m^3 /ha of bioslurry was needed to meet potato nitrogen requirements. Bioslurry appeared as effective as or better than conventional nitrogen sources to improve soil properties such as soil pH and organic matter content, and to sustain potato growth and tuber yield and quality. This was particularly true when bioslurry was combined with mineral fertilizer. In that regard, bioslurry can contribute between 40 and 60% reduction of mineral fertilizers with no subsequent reduction of market or total potato tuber yields.

Key words: Bioslurry, organic fertilizer, mineral fertilizer, organic sources, soil quality, potato yield.

INTRODUCTION

Bioslurry is a biowaste product from biogas generation units that have been promoted in Rwanda to produce energy in replacement of wood charchoal and firewood. Bioslurry offers the potential of both a rich source of nutrients and an amendment for Rwandan soils. It contains appreciable amounts of organic matter (20 to 30%) very much needed for soil quality improvement (Droogers and Bouma, 1996); it is environmentally friendly (Kumar et al., 2015) and has no toxic or harmful effects on the soil (Islam, 2006). Moreover, bioslurry contains

higher concentrations of plant N, P, and K nutrients that are more readily available to crops (Warnars and Oppenoorth, 2014) than farmyard manure and compost. The use of bioslurry could reduce at least 40-50% of chemical fertilizer applications (Islam, 2006) while accelerating organic N mineralization because of its low organic C: N ratio (Warnars and Oppenoorth, 2014).

In Rwanda, long-term land exploitation for agriculture and subsequent water erosion have depleted Rwandan soils in fine particles (Karamage et al., 2016) and organic matter (Mbonigaba et al., 2009). As a result, the application of organic biomass as soil amendments and plant nutrient sources has become a common practice of the crop production intensification program the country has been promoting for adoption by small farmers (Rushemuka et al*.,* 2014). These applications are generally supplemented with mineral sources of plant nutrients.

In terms of efficiency as a source of plant nutrients, several studies reported bioslurry as a good compound fertilizer, which can reduce the application of expensive chemical fertilizers (Haque et al., 2015; Shahbaz et al*.,* 2014). In that regard, bioslury application resulted in yield increases of 30.9, 56.9 and 30.4% for potato tubers, corn, and rice, respectively (Gurung, 1997) while the application of bio-slurry in combination with mineral fertilizers resulted in a 25 to 36% increase of Okra fruit yields in comparison with mineral fertilizer applications alone (Shahbaz et al., 2014). Also, the application of 7.8 tons /ha of bioslurry on carrots increased yields by 8.8% and 23.5% compared to the control over two consecutive seasons (Jeptoo et al*.,* 2013). This high efficacy of bioslurry when combined with mineral fertilizers was also reported in other research findings (Hossain et al. 2014).

Therefore, this study aimed at: i) assessing the efficacy of bioslurry from on-farm biogas production plants as a soil amendment and potato N nutrient source in comparison with farm yard manure and mineral nitrogen fertilizer in two agro-ecological regions of northern Rwanda and, ii) determining the range of bioslurry fraction in its combinations with mineral fertilizer for optimum potato tuber yields in the same regions.

MATERIALS AND METHODS

Description of the study area

This study was carried out in the first rainy season of 2012 in Musanze and Gicumbi Districts respectively located Northwest and Northeast of Northern Province of Rwanda. Musanze is situated in the Northwest of the Northern Province, between 1,850m and 2,500m above sea level (asl) in the agro-ecological region of Birunga or Volcanos (Figure 1, n°4). A moderate and humid climate and abundant rainfalls characterize the region. The annual averages of temperatures and rainfalls are 16^{o}C and 1400 mm, respectively; the maxima annual rain precipitation averages around 1600 mm (Verdoodt and van Ranst, 2003). The soils of Musanze are mainly from volcanic materials and are classified in the Andisols. The experiment was done on three different farms located in Kinigi (2,150 m asl), Nyange (2,111 m asl), and Busogo (2,249 m asl), respectively.

The District of Gicumbi is located in northeast region of the Northern Province covering the natural region of the Buberuka Highlands (Figure 1, n° 6). Gicumbi has a tropical bimodal climate with rain precipitations ranging from 1,200 mm to 1,300 mm and mean temperatures ranging between 13.2 and 20.8°C. It is one of the most environmentally fragile regions of Rwanda characterized by rugged steep slope hills with narrow and wet valleys. The soils of Gicumbi are mainly degraded Oxisols characterized by lateritic materials (Verdoodt and van Ranst, 2003). However, local swamps and lowlands are characterized by rich and deep clay soils. The experiment was conducted on three farms located at Shangasha (2,166 m asl), Rukomo (mean altitude of 2,002 m asl) and Kageyo (2,167 m asl), respectively.

Treatments and application method

Treatments

Six treatments were tested on commonly grown Irish potato varieties, Kinigi variety in Musanze and Mabondo potato variety in Gicumbi. One farm field was selected in each one of the three administrative Sectors by District basing on the similarities of soil chemical characteristics. The selection was also based on the existence of a biogas production unit on the farm, the farmer's willingness to provide the land for the study and the gentle slope (5% slope or lower) of the provided land. The treatments were composed such that each (but the control) supplies potato nitrogen requirement in the amount of 150 kg N /ha (Zebarth et al., 2007) from different sources as follows:

1) Control (CTL000): No fertilizers applied on potato crop;
2) 150 kg N /ha all supplied from mineral fertilizers (MIN100) as follows: 60 kg /ha at planting and 90 kg /ha at hilling; in addition, phosphate and potash were band-applied at planting in the amounts of 15 0kg P_2O_5 /ha and 60 kg K_2O /ha, respectively;
3) 150 kg /ha were entirely supplied from farm yard manure (FYM100);
4) 150 kg N /ha required amount was supplied at 40% from bioslurry (BIO040) and the remaining 60% was supplied from mineral fertilizers;
5) 150 kg N /ha required amount was supplied at 60% from bioslurry (BIO060) and the remaining 40% was supplied from

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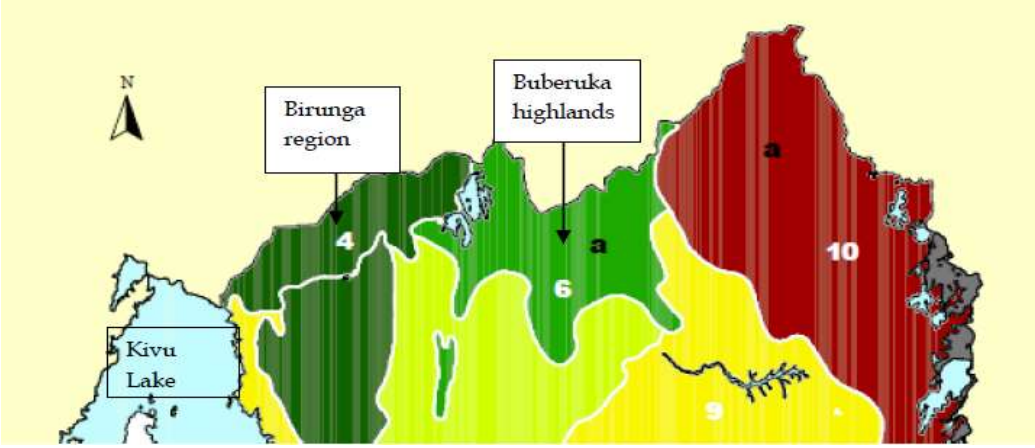

Figure 1. Location of the study area (in zones 4 and 6) in Northern Rwanda, particularly suitable for the potato production (adapted from Verdoodt and van Ranst, 2003).

mineral fertilizers;
6) 150 kg /ha of potato nitrogen fertilizer were all supplied from bio-slurry (BIO100); no mineral phosphate and potash fertilizers were added.

Experimental design and treatments' application

At each site, the six treatments were randomly distributed and tested in un-replicated field strips, a treatment by strip. Each strip was at least 4m wide over the total length of the field. Chemical fertilizers were band-applied in a seed trench just before seeding and slightly covered by the soil to avoid direct contact of seed and fertilizer particles. Bioslurry application rates were calculated based on their actual N nutrient content to supply 150 kg N ha^{-1} to the potato crop. Farmyard manure was applied based on their N content from scientific literature (Sankaranarayanan and Karemangingo, 2012). No mineral P and K fertilizers were added to supplement these organic sources of nitrogen since they were supposed to supply required amounts of other nutrients, notably phosphorus and potassium nutrients. All organic manures were broadcast and incorporated before planting. For bioslurry treatments, which the balance was supplied from mineral N, this application was done at hilling time. Bioslurry application rates were adjusted based on a 50% nitrogen loss (Zebarth et al., 2007).

Potato seeds were sown at the spacing of 80cm between rows and 30 cm within rows. All seeds were medium size grade with a similar sprout status. Weeding operations were done two weeks after emergence and at hilling, 45 days after planting. Pests and diseases were controlled through regular field scounting and weekly applications of Dithane M45 to protect against the mildew. In Rukomo Sector, a combination of bacteria (Ralstonia solanacearum) and a fungii *(*Rhizoctonia *solanii)* caused severe damages to the crop. Harvesting was done about 120 days after planting.

Evaluation of the effects of treatments

Assessing bioslurry quality

Semi-liquid bioslurry samples were collected from the storage lagoons using a locally fabricated aluminum-made can with a spring-operated cup. A 2-m long handle fixed on the can was used to collect liquid samples from different depths and angles of the pit. Samples were collected after thoroughly mixing the sludge with wooden paddles. Composite samples made of single six samples were collected at 10 cm and 1 m depth from each farmer's pit. Each bioslurry sample was stored in 1L Nalgen bottles, kept in a cooler with icebags, taken to the laboratory, and kept frozen until analysis for nutrient contents.

Assessing the effects of N sources on soil quality

Before planting, a composite sample was taken from each farmer's field and tested for initial soil quality. After harvest, a composite sample was also taken from each field strip and tested to evaluate the treatment effect on the soil quality. In fact, soil samples were analyzed for pH in a 1: 2.5 soil-water solution (pH_{water}) using the glass electrode method, organic carbon (OC) using the Walkely and Black method, total nitrogen (total N) using the Kjeldahl method, total phosphorus (total P) using the vanado-molybdate method, available phosphorus using the Mehlich-3 method, potassium using the Na cobaltinitrite method, calcium (Ca) and magnesium (Mg) using the EDTA method, exchangeable aluminum (Al^{3+}) and total exchangeable acidity using titration method, and cation exchange capacity (CEC) using the ammonium acetate method.

Assessing the effects of N sources on potato growth, tuber yield and quality

The effects of treatments on the potato growth, yield and yield quality were performed on three sub-plots randomly selected by strip. Each sub-plot was 1.6m wide x 2.4m long or 3.84 m^2. The crop growth was estimated by measuring the plant length (cm) from the ground surface to the top of the plant using a measuring tape 30, 60, and 90 days after planting. The number of plants and shoots were counted at harvest. The potato tuber quality was assessed using a tuber calibration table which graded potato tubers into small size (< 5 cm circumference), medium size (5 to 10 cm), big size (10 to 15 cm) and very big size (> 15 cm circumference). The total weight obtained by summing up the weights of the potato tubers of all sizes, including rough and rot tubers represented the total potato tuber yield. The market potato tuber yield was the difference between the total potato tuber yield and the weights of small size tubers as well as rough and rot potato tubers.

Data statistical analyses

Data collected were organized using Excel data sheet. The one-way analysis of variance was performed District by District using the 4th Edition of NCSS computer package (Hintze, 2004). Data were eventually log-transformed when non-homogeneity of variance was detected with the Bartlett Chi-square test (Steel and Torie, 1980). Repeated measures analysis of variance was performed for parameters related to the plant growth. In all cases, Duncan's Multiple Range Test (DMRT) was perfomed for comparison of mean effects of treatments. A 5% probability level was used for the significance of all statistical analyses.

RESULTS AND DISCUSSION

On-fam generated bioslurry characteristics

Bioslurry characteristics as tested from different farms in Musanze and Gicumbi are presented in Table 1. These slurries appeared much less concentrated, particularly in N, P, and K nutrients than previously reported (Bonten et al*.,* 2014; Sankaranarayanan and Karemangingo, 2012). Their quality is however variable from farm to farm as highlighted by the coefficients of variation. They are all in the ranges of those reported by Haque (2013) as well as Warnars and Oppenoorth (2014) for N, P, and K nutrients and by Kumar et al. (2015) for nitrogen, only.

This variability can be explained by the high variability of the quality of animal feeds, the method of loading biowaste into the biodigester and, at the end of the gas production process, the method of storing bioslurry. Biowaste loading into the biodigester is done by adding uncontrolled volume of water while, at the end of the process, the farmers reported frequent additions of soil into liquid bioslurry in the earthern lagoon to make it a biosolid easy for transport.

Effects of nitrogen sources on soil quality

Status of soil chemical properties prior to applying treatments

Prior to the application of treatments, the soil characteristics of the different sites are presented in Table 2. These soil properties are quite homogeneous across sites by District for each soil characteristic. They also indicate the differences that exist between Andisols and Oxisols, particularly with regard to soil pH, organic carbon, available phosphore, exchangeable Ca^{2+} cation, and CEC for which the Andisols contained higher values than the Oxisols while the opposite is true for exchangeable Al^{3+} and total acidity. The soils are classified from strongly-acid for Oxisols to weakly-acid for Andisols basing on their actual pH values as per Parent and Gagne (2010).

Consistent with their formation and chemical nature, Andisols are richer in Ca and organic matter than Oxisols (Hengel et al*.,* 2017).

In these specific soils, the organic carbon varies from 1.6% to 2.4% in the Oxisols of Gicumbi and from 2.2% to 3.6% in the Andisols of Musanze, while the exchangeable calcium varies from 5.0 meq to 8.0 meq /100 g soil and from 11.8 meq to 13.8 meq /100 g soil in the two types of soils, respectively.

Status of soil chemical properties after treatments' application

The effects of different nitrogen sources on soil chemical properties as monitored immediately after harvest are here below presented in Table 3 for the parameters only offering significant differences in one or in both of the two soils. In general, the application of organic-based treatments, particularly the bioslurry ones, resulted in significantly higher values of assessed soil characteristics than CTL000 and MIN100 in the two soils. That is particularily true for soil organic matter, available phosphorus and cation exchange capacity.

Otherwise, a comparison between organic sources alone constantly indicates highest values from bioslurry-containing treatments. Therefore, organic-based treatments, particularly bioslurry-based treatments, contributed to significantly improving reported soil characteristics. These results are consistent with many previous findings on the improvement of soil chemical characteristics by organic materials (Kismanyoky and Toth, 2010; Rutunga et al*.,* 1998; Mwanga, 2016).

Effects of nitrogen sources on potato growth, yields and yield components

Effects on the potato plant growth rate

The mean rate of potato plant growth as measured by the plant length 45, 60, 75 and 90 days after planting in Musanze District are ploted on Figure 2 while the results monitored 45 and 60days after planting for Gicumbi are on Figure 3. Optimum plant growth rates varying from 40cm to 60cm were recorded 75days after planting in the Andisols of Musanze. However, repeated measures analyses of variance detected no significant differences among various sources of nitrogen with regard to the plant growth rate in these soils while very significant differences ($P \leq 0.01$) were found between the same N

Table 1. Characteristics of bioslurry materials in Musanze and Gicumbi Districts (on-dry matter basis).

District	Sector	Bioslurry chemical characteristics						
		DM (%)	OC (%)	TKN (%)	P (%)	K (%)	Ca (%)	Mg (%)
Gicumbi	Rukomo	47.08	21.61	0.54	0.22	0.07	0.87	0.37
	Shangasha	45.67	25.77	1.08	0.21	0.02	0.82	0.70
	Kageyo	44.76	32.55	0.63	0.14	0.04	0.81	1.21
Musanze	Busogo	47.52	22.18	0.54	0.13	0.02	0.73	0.17
	Nyange	47.17	26.48	0.97	0.12	0.06	0.91	0.85
	Kinigi	47.51	28.94	1.29	0.13	0.04	0.88	0.76
Mean values		46.62	26.26	0.84	0.16	0.04	0.84	0.68
Coefficients of variation		2.45	15.73	38.02	25.26	48.00	7.17	54.68

DM, Dry matter; OC, Organic carbon; TKN, Total Kjeldhal nitrogen; P, Phosphorus; K, Potassium; Ca, Calcium; Mg, Magnesium.

Table 2. Status of soil chemical properties of the sites prior to the experiment.

Chemical property	Musanze				Gicumbi			
	Sectors			District	Sectors			District
	Busogo	Nyange	Kinigi	All	Rukomo	Kageyo	Shangasha	All
Soil pH_{water}	6.2	6.3	6.2	6.2	5.3	5.3	5.4	5.3
Organic carbon (%)	2.2	3.2	3.6	3.0	1.9	1.6	2.4	1.9
Total nitrogen (%)	0.2	0.3	0.2	0.2	0.2	0.1	0.2	0.2
C :N ratio	14.0	13.0	16.0	14.3	12.0	13.0	12.0	12.3
Available P (ppm)	59.0	54.7	51.0	54.9	40.0	44.3	43.3	42.5
Exchangeable K^+	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Exchangeable Ca^{2+} (meq /100g soil)	13.5	13.8	11.8	13.0	8.0	7.7	5.0	6.9
Exchangeable Mg^{2+} (meq /100g soil)	0.5	1.4	1.4	1.1	1.5	2.4	1.8	1.9
Exchangeable Al^{3+} (meq/100g soil)	0.0	0.0	0.0	0.0	0.8	0.7	0.8	0.7
Total acidity (meq /100g soil)	1.5	1.0	1.1	1.2	2.4	2.4	1.7	2.2
Estimated CEC (meq /100g soil)	15.7	16.2	14.4	15.4	12.1	12.7	8.8	11.2

Table 3. Soil chemical properties as affected by nitrogen sources in the two soils.

Soil characteristics		Nitrogen sources					
		CTL000	MIN100	FYM100	BIO040	BIO060	BIO100
Andisols	Soil pH_{water}	6.00^{a}	6.1^{ab}	6.3^{bc}	6.4^{c}	6.3^{c}	6.3^{bc}
	Organic matter (%)	5.5^{a}	5.8^{ab}	6.1^{ab}	5.8^{ab}	6.4^{b}	6.3^{b}
	Total N (%)	0.25^{c}	0.27^{bc}	0.30^{a}	0.27^{abc}	0.29^{ab}	0.30^{a}
	Available P (ppm)	56.4^{c}	59.6^{bc}	65.2^{ab}	69.3^{a}	66.3^{ab}	69.3^{a}
	Exchangeable Mg^{2+} (meq /100g soil)	1.30^{a}	1.51^{a}	1.62^{a}	1.30^{a}	1.33^{a}	1.67^{a}
	Soil CEC (meq /100g)	17.8^{b}	18.9^{ab}	20.0^{a}	20.4^{a}	19.2^{ab}	20.5^{a}
Oxisols	Soil pH_{water}	5.2^{a}	5.2^{a}	5.5^{ab}	5.6^{b}	5.4^{ab}	5.7^{b}
	Organic matter (%)	3.3^{d}	3.7^{c}	3.8^{bc}	4.1a	3.8^{bc}	4.1^{ab}
	Total N (%)	0.16^{c}	0.19^{b}	0.20a^{b}	0.19a^{b}	0.22^{a}	0.21^{a}
	Available P (ppm)	41.1^{a}	45.8^{ab}	49.7^{b}	49.4^{b}	50.0^{b}	50.6^{b}
	Exchangeable Mg^{2+} (meq /100g soil)	1.89^{b}	2.17^{ab}	1.82b	2.08^{ab}	1.97^{ab}	2.73^{a}
	Soil CEC (meq /100g)	13.9b	14.7ab	14.9ab	15.7a	15.5ab	15.1ab

Different letters in the same row indicate significantly different values.

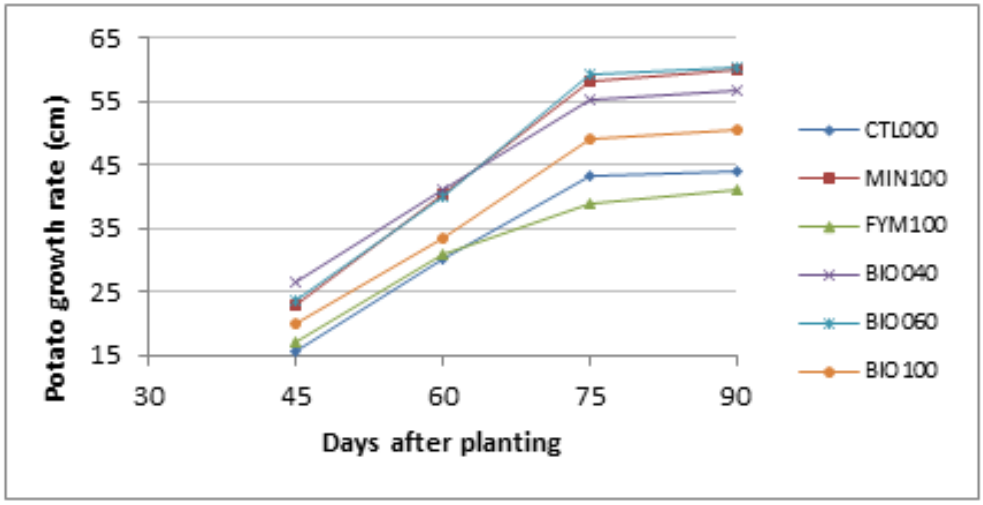

Figure 2. Effects of nitrogen sources on potato growth in Musanze Andisols.

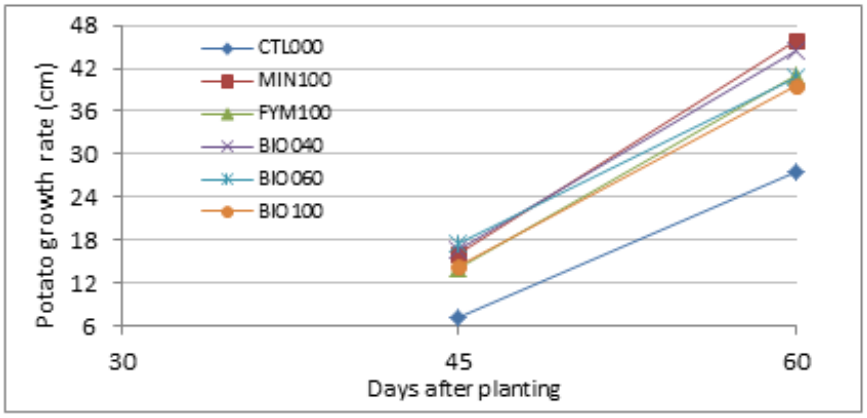

Figure 3. Effects of nitrogen sources on potato growth in Gicumbi Oxisols.

sources in the Oxisols of Gicumbi.

As anticipated, very significant differences ($P \leq 0.001$) exist between the dates of monitoring the plant growth for the two types of soils. The results on the plant growth are consistent with the growth range between 15cm and 100cm previously reported (Haque et al., 2015; Hill, 2002) and with the 60cm average growth length of Irish potato stems reported in Rwanda (MINAGRI, 1988). The plant growth was uniform across all treatments in the Andisols (Figure 1) and significant differences detected in the Oxisols were related to the lower performance of CTL000 than N sources (Figure 2). This can reasonably be related to the low organic matter of these Oxisols which implies a lower mineralization (meaning a lower N supply) and a lower water holding capacity than the Andisols. The results also validate previous studies whereby bioslurry alone (BIO100) or in combination with mineral N (BIO040 and BIO060) was as a good fertilizer as or better than chemical fertilizers on the potato growth rate (Haque et al., 2015; Haque, 2013).

Effects of treatments on the number of shoots and tubers by plant

The average number of shoots per plant as evaluated 45 days after planting ranged between 2.9 and 3.3 in the Andisols and from 2.4 to 2.9 shoots in the Oxisols (Table 4). The analyses of variance of data showed no significant differences between nitrogen sources in none of the two soils with regard to the number of shoots by plant. With regard to the number of tubers by plant, significantly

Table 4. Effects of nitrogen sources on the number of shoots 45 days after planting and the number of potato tubers by plant at harvest time.

Treatments	Musanze Andisols		Gicumbi Oxisols	
	Shoots number by plant	Tubers number by plant	Shoot number by plant	Tubers number by plant
CTL000	2.9[a]	5.5[a]	2.4[a]	7.4[a]
MIN100	3.3[a]	7.6[b]	2.8[a]	12.8[a]
FYM100	3.0[a]	6.0[a]	2.5[a]	10.3[a]
BIO040	3.3[a]	7.9[b]	2.7[a]	9.4[a]
BIO060	3.2[a]	7.5[b]	2.9[a]	10.0[a]
BIO100	3.3[a]	6.7[ab]	2.6[a]	9.0[a]

Numbers sufficed with different letters in the same column indicate significantly different values.

Table 5. Effects of different nitrogen sources on the potato tuber yield and yield quality.

Treatments	Musanze Andisols (metric tons/ha)				Gicumbi Oxisols (metric tons /ha)			
	Small	R&R	Market	Total	Small	R&R	Market	Total
CTL000	1.60[a]	0.03[a]	5.97[a]	7.60[a]	3.13[a]	1.36[a]	4.11[a]	8.60[a]
MIN100	2.87[a]	0.07[a]	11.96[b]	14.90[bc]	4.17[a]	2.78[a]	6.65[b]	13.60[b]
FYM100	2.15[a]	0.02[a]	7.03[a]	9.18[ab]	3.32[a]	1.62[a]	5.97[ab]	10.91[ab]
BIO040	2.38[a]	1.28[a]	11.24[bc]	14.92[bc]	4.35[a]	3.48[a]	6.87[b]	14.70[b]
BIO060	3.33[a]	0.10[a]	13.98[c]	17.41[c]	3.56[a]	2.76[a]	7.19[b]	13.51[b]
BIO100	2.63[a]	0.07[a]	9.21[b]	11.91[b]	3.18[a]	3.02[a]	7.81[b]	14.01[b]

Numbers sufficed with different letters in the same column indicate significantly different values.

different effects ($P \leq 0.05$) between the nitrogen sources existed in the Andisols while no such effects were found in the Oxisols. In the Andisols, bioslurry-based N sources (BIO040, BIO060, and BIO100) statistically provided higher tuber numbers than FYM100, but as equal number of tubers as chemical fertilizer (MIN100).

Potato tuber numbers are consistent with the standards established by Soltner (1985) whereby the number of potato tubers varied from 3 to 20 in normal conditions of cultivation. However, current numbers are consistent in Oxisols and much lower in Andisols than previous findings in Northern Rwanda (Nyiransabimana, 2011).

Over all, the results confirmed the effectiveness of bioslurry whether alone or in combination with mineral nitrogen fertilizer to supply nitrogen and other nutrients to the potato crop as measured by the number of potato tubers. This is also consistent with previous findings (Haque et al., 2015; Warnars, 2014; Nyiransabimana, 2011).

Effects of nitrogen sources on potato tuber yield and yield quality

The mean effects of N sources on potato tuber yields are presented in Table 5 for small size (lower than 5 cm circumference), rough or rot tubers (R&R), marketable, and total potato tuber yields for the two regions. The analysis of variance was performed on log-transformed total yield data in Andisols. Potato market yields were low, particularly in Oxisols because of a severe outbreak of the bacterial blight (Pseumonas solanacearum) during the crop gowth. The disease caused small size and R&R tubers to be much more important in Oxisols than in Andisols. However, such low yields were not exceptional under rainfed conditions as evidenced by previous findings for the region (Turamyenyirijuru, 2013; Nyiransabimana, 2011). Moreover, no significant differences were detected between N sources with regard to small size and R&R tuber yields in either soil. However, very significant differences were found in the Andisols (on log-transformed data at $P \leq 0.01$) and in the Oxisols ($P \leq 0.01$) with regard to market and total potato tuber yields.

Overall, the bioslurry-based N sources were proven as good N fertilizers as mineral N fertilizer in the two regions and better than farmyard manure only in Musanze for market potato yields. Moreover, whether statistically significant or not, combinations of mineral and bioslurry sources constantly resulted in higher yields than other treatments. This fact is a clear evidence of a beneficial symbiosis of combinations of mineral nitrogen and

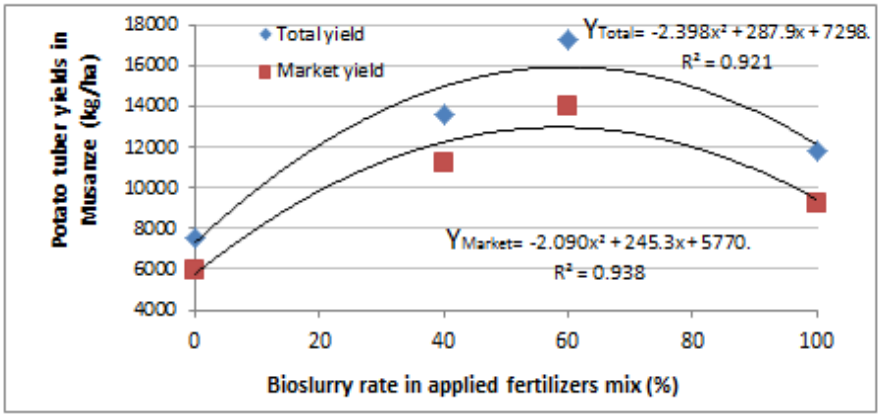

Figure 4. Effect of increasing bioslurry rate on potato yields in Andisols.

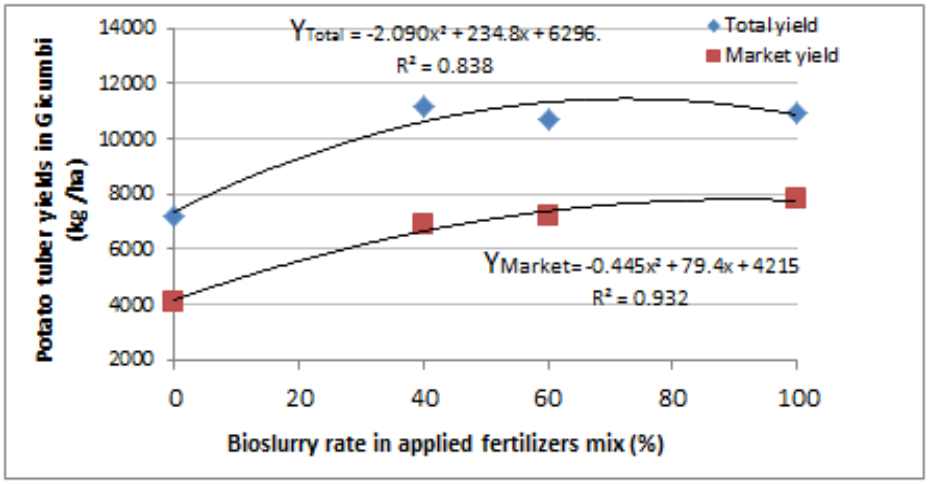

Figure 5. Effect of increasing bioslurry rate on potato yields in Oxisols.

bioslurry to feed the potato plant. Previous findings indicated that bioslurry-containing treatments resulted in as equal as or higher potato yields than mineral fertilizers alone (Haque et al., 2015). The best combinations of the two sources can be determined through the projection of the potato yield as a function of increasing bioslurry rate in the mixes of the two N sources. In this study, the zero solutions of the derivatives of the projection equations determined the best combinations of mineral N and bioslurry N for Musanze (Figure 4) and Gicumbi (Figure 5).

The computed zero-solutions of the projection equations were located at 60 and 58.7% maximum bioslurry rates in potato N fertilizer amounts for Musanze Andisols and at 56.0 and 89.2% for Gicumbi Oxisols for total and market potato tuber yields, respectively.

The high percentage of bioslurry in fertilizer mixes for market potato tuber yields in Oxisols is undoubtfully due to the importance of small size and R&R tuber yields in total tuber yields. Obviously, the interval between 40% and 60% of bioslurry in its combinations with chemical nitrogen fertilizers should contain the optimum rates producing optimum market potato tuber yields in both soils. This would represent between 40 and 60% cost reduction of chemical fertilizers from bioslurry use and a substantial extra-profit from on-farm biogas production plants. Islam (2006) in Bangladesh and Muhmood (2014) in Pakistan reported comparable results on various vegetable crops whereby the use of bioslurry reduced the application of chemical fertilizers up to 50% of N recommended amounts. However, on Kale crops (*Brassica oleracea* L.), the highest yields were harvested under 100% bioslurry when compared to increasing rates of bioslurry in its combinations with mineral fertilizers

(Haile and Ayalew, 2018).

Conclusion

The main objective of this study was to assess the efficiency of bio-slurry as a soil amendment and N nutrient source for potato crop in the Andisols and Oxisols of Northern Rwanda. Six treatments including commonly applied farmyard manure and mineral fertilizers were tested against bioslurry alone or in combination with mineral N fertilizer. The results indicated that the application of bio-slurry alone or in combinations with mineral N fertilizers contributed as much as or better than conventional farm yard manure (FYM100) to improving the soil quality (pH, organic matter content, notably). They also indicated that the application of bioslurry alone or in combinations with mineral fertilizer resulted in as good potato plant growth, tuber yield and tuber quality as or better than mineral N fertilizer alone, regardless of the soil type. The highest potato tuber market yields were harvested in MIN100 (11.96T /ha) and BIO060 (13.98T/ha) in Andisols and BIO060 (7.19T /ha) and BIO100 (7.81T /ha) in Oxisols. These yield levels are locally common under rainfed conditions. Overall, the bioslurry fractions varying from 40% to 60% in its combinations with mineral N fertilizers contain both the optimum and maximum market potato tuber yields in these two soils. This would represent an effective cost reduction varying from 40% to 60% of mineral fertilizers. However, local bioslurry appeared low-nitrogen concentrated for two possible reasons: the addition of water to get fresh manure into the biodigester and the subsequent additions of soil in the slurry pit to make it semi-solid for easy transport with domestic tools. Farmers should be better trained to avoid these additions, which reduce the quality of their bioslurry while excessively increasing its application rate.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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Biomass accumulation and potassium concentrations in tissue of Teff (*Eragrostis tef Zucc.* Trotter) at three growth stages in Vertisols and Nitisols of the Central Highlands of Ethiopia

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Understanding the physiology and time-course of above ground biomass (AGBM) and potassium (K) accumulation pattern in plants and removal from soil is essential to simultaneously increase crop yield and synchronize K demand and K supply, thereby predict crop yield. It is also an essential criterion for optimizing fertilizer practices, and may help to enhance soil and crop quality. A pot experiment was conducted using teff (*Eragrostis tef Zucc. Trotte*r) to determine AGBM, K concentration and uptake at three growth stages. Two soil types (Vertisols and Nitisols) and four K levels were used. Soil samples (120) were collected at planting and three stages while plant samples (160) were collected at the three growth stages. Above ground biomass increased as growth stage advances regardless of K levels in both soil types. Maximum AGBM was observed at tillering stage and at 120 kg K ha^{-1} and was higher in Vertisols at all growth stages. The study concluded that sufficient supply of nutrients from soil/fertilizers at early growth stages is required for higher yields. The study suggested that determining the right rate for different soil and crops is required. Repeating the experiment at field condition to draw sound conclusions was also recommended.

Key words: Growth stages, potassium concentration, biomass, teff, Vertisols, Nitisols, Central Highlands.

INTRODUCTION

Teff (*Eragrostis tef Zucc.* Trotter) is a major cereal crop indigenous to Ethiopia (Demissie, 2011; Seyfu, 1997) but is a minor cereal crop worldwide (Schneider and Anderson, 2010; Yigzaw et al., 2001). In other countries like Australia, South Africa, and United States, it is principally used as a forage crop for animal feed. Teff is a warm-season, annual grass that has rapid seed germination and seedling development. It is also well adapted to dry climates. In Ethiopia, teff performs well in 'Weina Dega' agro-ecological zones or medium altitude with ranges of 1,700 to 2,400 m above sea level. Depending on variety and altitude, teff requires 90 to 130

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days for growth (Gebretsadik et al., 2009). Teff has large area coverage annually in Ethiopia and it was first (24%) in area coverage and second in production (17.29%) in 2016/2017 cropping season. An area of 3.02 million ha was cultivated and yielded 5.02 million metric tons (Central Statistics Agency of Ethiopia (CSA), 2017).

Potassium (K) is an essential element for crop growth. It plays a vital role as macronutrient in plant growth and sustainable crop production. Adequate K nutrition enhances the efficiency of photosynthetic apparatus (Wang et al., 2012; Zhao et al., 2001) and promotes plant roots (Zia-Ul-Hassan and Arshad, 2010). However, K has been given less attention than nitrogen (N) and phosphorus (P) with respect to increasing cereal production because the effect of K on increasing cereal production is more gradual compared with N and P, especially in K-enriched soils (Niu et al., 2011). Potassium uptake and availability for plant growth and development vary depending on environmental conditions associated with a particular set of growing conditions. The soil itself contributes greatly to K availability. Availability and uptake of potassium (K) is often complicated by many interacting components. Two factors that have predominating effects are the soil and plant characteristics involved. A third factor is improved fertilizer and management practices, which can be used to modify the inherent characteristics of soils and plants involving K uptake (Mallarino and Murrell, 1998).

Vertisols and Nitisols are the major crop growing soils in Ethiopia. Vertisols cover 12.6 million ha, or about 10% of the country. In addition, there are 2.5 million ha of soils with vertic properties. About 70% of these soils are in the highlands and about 25% (1.93 million ha) of the highland Vertisols are cropped (Debele, 1985). Different reports provide different area estimates of Nitisols in the Ethiopian highlands (Zewdie, 2013). The most recent survey puts the extent of Nitisols to cover about one million ha that account for 31% of the agricultural lands in the Ethiopian highlands (Elias, 2016). The soils are particularly extensive in the south-western and north-central highlands representing 64 and 25% of the agricultural landmass, respectively (Elias, 2017).

Like other cereals, the development of the above ground organs of teff, the dynamics of biomass accumulation, the yield and other traits are all fundamentally influenced by ecological conditions (Fufa et al., 2001), variety traits and the technology used to manage them (Teklu and Tefera, 2005). Of the many factors involved, the most decisive ones are the water supplies, the yield potential and the nutrient supplies (Assefa et al., 2011; Tulema et al., 2005). The amounts, dynamics and within-plant distribution patterns of biomass accumulation and nutrient uptake vary with growth stage of the plant (Karlen and Whitney, 1980; Lal et al., 1978), and are affected by crop species, cultivars and soil-climatic conditions (Gawronska and Nalborczyk, 1989).

In small grains, seed yield is closely related to total biomass production (Reynolds et al., 1999; Vandenboogaard et al., 1995). Grain yield has usually been positively correlated with total DM production and nutrient accumulation in crops (Rhoads and Stanley, 1981). Meanwhile, DM accumulation and nutrient accumulation vary with growth stage of crops (Jones et al., 2011; Miller and Jacobsen, 2004). Cereal crops generally followed a similar pattern of biomass and nutrient accumulation in the growing season, which increased continuously with growing time, maximum biomass accumulation rate and amount usually occurred at late boot stage (46 to 47 Days after emergence (DAE)) and ripening stage (Malhi et al., 2006).

Teff crop was generally fertilized by farmers in Ethiopia using either nitrogen only or nitrogen (N) and phosphorus (P) fertilizers, mainly as urea and Di-ammonium phosphate (DAP) fertilizers. Intensive farming of cereal crops to produce more food often compels the use of additional nutrients, besides N and P, in Ethiopia in recent years. Response to application of nutrients such as K, S, Zn and other micronutrients are reported (Astatke et al., 2004; Haile and Boke, 2011; Mulugeta et al., 2017,2018) and soils are also showing deficiency of these nutrients (Ethiopian Soils Information System (EthioSIS), 2016).

The increased focus on optimizing yield response to nutrient inputs and the need to ensure balanced nutrition has increased demand for information on biomass accumulation, nutrient concentration and nutrient sufficiency levels of crops and on the relationship of biomass accumulation and nutrient uptake to seed yield. However, there is little information available to date, on the accumulation and distribution of biomass and nutrients of teff throughout the growing season in Ethiopia. For whole and seasonal mineral nutrients requirements of crops, fertilizer scheduling and synchronizing nutrient supply with nutrient demand of the crops, it is essential to determine the exact amount of nutrient uptake over the growing season. The availability of K may further be conditioned by the demand of K at different growth stages, the information on which is not available for teff in Ethiopia (Mulugeta et al., 2018).

The objective of this study was therefore to determine the dynamics of biomass accumulation in the plant biomass, changes in nutrient concentration during the growing period, and the nutrient uptake of teff grown in a fertilization experiment in two different soil types collected from central highlands of Ethiopia.

MATERIALS AND METHODS

A pot experiment was conducted using teff (*E. tef*, variety-Kuncho) as test crop in a lath house in the period between October 2015 and February 2016 at the Debre Zeit Agricultural Research Center (08° 46'10.10'' N and 38° 59'56.13'' E) and an altitude of 1889 m.a.s.l. Sixty surface representative soil samples were obtained by composing 10 to 12 random cores from the top 20 cm from major

soil types varying in available K, pH and texture across 20 teff growing districts of Ethiopia to be used as test soils in this experiment. The soils were collected from areas identified as low, medium and high based on their available K status by the national soil fertility status mapping report (Ethiopian Soils Information System (EthioSIS), 2013). The samples were mixed, air-dried, and passed through 1 cm sieve to remove gravel and debris and prepared for pot trials. A portion of the sample was ground and sieved through a 2 mm sieve for physical and chemical analyses. The particle size distribution was done by the HORIBA-Partica (LA-950V2) laser scattering particle size distribution analyzer (Agrawal et al., 1991) and LA-950 software version 7.01 for Windows (Horiba Ltd, NextGen® 2010). Soil pH and electrical conductivity (EC) were measured using 1:2.5 and 1:5 soil: water ratios, respectively. Exchangeable K, Ca, and Mg and available P, Sulfate-S and extractable Zn were extracted following Mehlich-3 (M-3) procedure (Mehlich, 1984). Organic carbon was predicted from mid infrared spectra of soil samples using OPUS version 7.0 software (Bruker® Optic GmbH, 2011) with 32 scans and spectral range of 7400 to 600 cm^{-1} (wave numbers) including part of NIR region.

The pot trial was conducted by using a total of 3 kg of the dried soil sample in a plastic pot having 16 cm top and 14 cm bottom diameters. Four levels of potassium: 0, 60 120 and 180 kg ha^{-1} K, were applied as potassium chloride (60% K_2O) replicated three times in a completely randomized design. There were thus 12 pots per soil and a total of 720 experimental pots. To ensure that K was the only nutrient element limiting teff production, optimum and uniform doses of N, P, S and Zn were applied at the rates of 120, 60, 15 and 3 kg ha^{-1}, respectively, as NPSZn compound fertilizer (12-45-0 + 5S+1Zn). Of the nutrients supplied, 100% of P, K, S and Zn and 30% of N were applied as basal fertilizers before planting while 70% of N was applied as urea 30 days after planting to all pots. All the nutrients were applied as solution. Teff was planted in each pot, managed well and harvested at maturity and the required data were collected and analyzed. Based on the data, the critical limits of potassium in soil was determined graphically by plotting percentage yield against soil available K using the procedure of Cate and Nelson (1971). Out of these soils collected from 60 locations, soils collected from 10 locations, five each for Vertisols and Nitisols, were selected and the soil of each pot was thoroughly mixed and the old roots sieved out and refilled to their respective pots in 5 replications for both soils making a total of 40 pots as experimental unit.

The lath house experiment was conducted between November 2016 and February 2017 at the Debre Zeit Agricultural Research Center. Three kilograms of the dried soil sample was placed into a plastic pot (16 cm top and 14 cm bottom diameter). To ensure that K was the only nutrient element limiting teff production, optimum and uniform dose of N, P, S and Zn (120, 60, 15 and 3 kg ha^{-1}), respectively were applied as NPSZn compound fertilizer (12-45-0+5S+1Zn). In this experiment, similar doze and type of nutrients were applied, except for K. Plants growing in the soil of the previous check pots had to draw further K from the soil while the plants growing in the formerly fertilized soils had largely profited from the residual effect of the previously applied nutrient. Of the nutrients supplied, 100% of P, S and Zn and 30% of N were applied as basal fertilizers before planting while 70% of N was applied as urea 30 days after planting to all pots. All nutrients were applied as nutrient solution. The soil samples collected from each pot were mixed, air-dried and sieved to pass through a 1 cm screen for pot trials. A portion of the sample was ground and sieved through a 2 mm screen for physicochemical analysis. About 30 uniform seeds were sown in each pot. Soil moisture was maintained at nearly 60% field capacity throughout the experiment. Watering and intercultural operations like weeds control and plant protection measures were employed uniformly in each pot whenever required. The plant samples were collected by mowing close to the surface from 1/3 of the pot area in each stage at three separate growth stages. The first sampling occurred at tillering; the second at heading, and the last at the maturity stage for determinations of biomass and K concentration. Similarly, soil samples were collected at planting and the three growth stages. Accordingly, 160 soil and 120 plant samples were collected and analyzed. For the soil samples, exchangeable K, Ca, and Mg and available P, Sulfate-S and extractable Zn were extracted following Mehlich-3(M-3) procedure (Mehlich, 1984).

Plant nutrient concentrations were assessed on the harvested product. The teff plant samples were washed with distilled water to remove the dust and soil particles from the samples. The sun-dried plant samples were kept in paper bags and then dried at 65°C in an oven to constant weight. The dried samples were weighed for their dry matter (DM) yield. The dried plant samples were separately powdered in a warring stainless-steel grinder. Dry powdered plant samples were ashed in a muffle furnace at 500°C and then the ash was extracted in 10 ml of 6N HCl and dried on hot plate for 15 min at 140°C. The ash was dissolved in 10 ml of 1N HCl and K content in filtered digest was analyzed with Inductive Couple Plasma (ICP). Total K uptake was calculated by the following formula as used by Sharma et al. (2012).

K uptake (kg ha^{-1}) = (% K in the plant × Total dry matter (kg ha^{-1}) / 100

Data were analyzed by analysis of variance using SAS software version 9.2 (SAS Institute Inc, 2008) and graphs were developed using Excel. The differences between treatments were tested using the least significant difference (LSD) test at the 0.05 probability level.

RESULTS AND DISCUSSION

Soil properties

The results revealed that soil texture varied from clay loam to clay, with the clay content varying from 29 to 75.0% with a mean clay content of 45% and the sand content varies from 13 to 31% with a mean value of 24% (Table 1). These values show that the clay fraction of the soil sample was higher than silt then much higher than sand in that order.

The soil pH values ranged from 5.0 to 7.8 with a mean value of 6.0 indicating the soils were mostly acidic in reactions. Some of the tested soils had pH < 5.5 (Table 1) showing the characteristics of a highly weathered tropical soil. The pH also shows that these soils are suitable for crop production (Quirine et al., 2005; Redmon and Mcfarland, 2013). In accordance with the ratings of (Ethiopian Soils Information System (EthioSIS), 2016), the available P ranged from very low to medium, exchangeable K from low to high, while available S and extractable Zn were low and medium, respectively (Table 1). The data also showed that the experimental soils were variable not only in their K status but also in other physical and chemical parameters.

The result also showed that the predominant exchangeable cation, which accounts for more than 80% of the exchange complex was Ca^{++} followed by Mg^{++}, K^{+} and Na^{+}. Exchangeable Ca, Mg and K content in the studied soils ranged from 2072.5 to 11548.6 mg kg^{-1} and

Table 1. Physico-chemical properties of the experimental soils (Vertislos and Nitosols) collected from 60 locations in central highland of Ethiopia.

Soil type	Site	Soil parameters									
		Sand	Clay	Textural class	pH	Av. P	Exch. K	Exch. Ca	Exch. Mg	Sulfate S	Extract. Zn
		(%)			(1:2.5)	(mg kg^{-1})- Mehlich-3					
Nitisols	1	31	29	Clay loam	5.8	14.7	175	2651.1	488.1	13.13	7.68
	2	25	38	Clay loam	5.1	6.2	179	2370.0	563.7	11.86	5.21
	3	29	42	Clay	5.9	5.7	186	1419.3	356.4	14.31	3.01
	4	24	44	Clay	5.0	10.5	198	2369.3	482.0	16.93	4.07
	5	26	43	Clay	6.5	7.9	216	2357.7	590.3	12.49	4.77
Vertisols	1	23	47	Clay	5.7	22.1	245	2072.5	473.3	12.12	5.15
	2	26	36	Clay loam	5.1	5.7	252	2268.1	450.9	11.57	5.18
	3	13	75	Clay	7.8	37.5	345	11548.6	874.7	19.14	6.98
	4	19	57	Clay	7.2	40.1	415	10955.2	797.9	17.18	8.19
	5	22	36	Clay loam	5.7	42.1	699	4741.5	1031.6	11.92	4.36

Av= Available; Exch.= exchangeable; Extract = extractable.

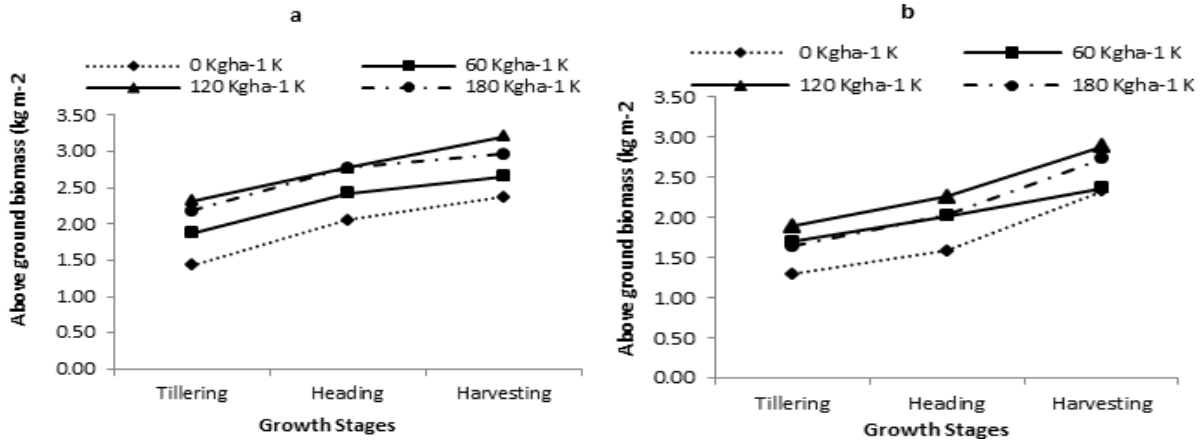

Figure 1. Changes of biomass (kg DM m^{-2}) of teff with growth stage in the pot experiments at Debre Zeit, on a) Vertisols and b) Nitisols.

356.4 to 1031.6 mg kg^{-1}and 175 to 699 respectively. Similarly, available P ranged from 5.7 to 42 .1 mg kg^{-1}. The available P content of vertisols was much higher than nitisols in the test soils. Sulfate S and extractable Zn varied from 11.86 to 19.14 mg kg^{-1} and 3.01 to 8.19 mg kg^{-1}. Vertisols have higher values of most of the parameters except pH than nitisols indicating that vertisols have better inherent soil fertility condition than nitisols (Mamo et al., 2001).

Aboveground biomass

Determination of above ground biomass indicated that biomass accumulation increased with plant age, the maximum being at late growth stage (Figure 1). Similar results were reported by Malhi et al. (2006) in their studies on wheat, barley and oat crops reported that the biomasses of the crops reached their estimated maximum at ripening growth stage. Gawronska and Nalborczyk (1989) also observed similar seasonal biomass accumulation among different winter rye (*Secale cereale* L.) cultivars, despite considerable differences in morphology and absolute values of biomass.

Biomass accumulation increased with increasing K level up to 120 kg K ha^{-1} and decreased afterwards (Figure 1) and was significantly ($p<0.0001$) different from the other K levels (Table 2). Generally, higher biomass

Table 2. Aboveground biomass, and soil and plant K concentration and K uptake of teff at different growth stages in Vertisols and Nitisols of central highlands of Ethiopia.

Parameter	Soil K concentration (mg kg^{-1})	Aboveground biomass K concentration (%)	Aboveground biomass (kg m^{-2})	Aboveground biomass K uptake (kg ha^{-1})
Treatment (T)	***	***	***	***
Soil type (ST)	***	***	***	***
T × ST	NS	NS	NS	NS
Stage (S)	***	***	***	NS
T × S	NS	NS	NS	NS
ST × S	NS	NS	NS	NS
T × ST × S	NS	NS	NS	NS
CV	9.92	12.04	16.04	19.77
LSD	14.47	0.06	0.18	23.08
Treatments				
0 kg ha^{-1} K	262.78^{c}	0.91^{c}	1.84^{c}	155.34^{c}
60 kg ha^{-1} K	273.01^{bc}	1.09^{ab}	2.17^{b}	230.15^{b}
120 kg ha^{-1} K	287.41^{b}	1.14^{a}	2.56^{a}	283.14^{a}
180 kg ha^{-1} K	314.59^{a}	1.05^{b}	2.39^{a}	241.38^{b}
LSD	0.0648	0.1845	23.08	10.235
Soil type				
Vertisols	358.47^{a}	1.10^{a}	2.42^{a}	263.14^{a}
Nitisols	210.42^{b}	0.99^{b}	2.06^{b}	191.87^{b}
LSD	10.24	0.05	0.13	16.32
Stage				
Tillering	268.18^{b}	1.32^{a}	1.79^{c}	238.08^{a}
Heading	292.64^{a}	1.01^{b}	2.24^{b}	227.96^{ab}
Maturity	292.52^{a}	0.81^{b}	2.69^{a}	216.47^{b}
LSD	12.54	0.06	0.16	19.99

Significant at *P ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001; NS: Not significant; LSD- least significant difference; CV- coefficient of variations; Means followed by same letter(s) within a column do not differ at P ≤ 0.05.

was observed in Vertisols than Nitisols and the effect of K application had similar trend for both soil types for pots that received K. In pots that did not receive K, biomass increment was not similar to those that received K, especially in Nitisols. This result indicated that potassium application in Nitiosls has much significant effect than Vertisols. This might be attributed to the low initial soil K level in Nitisols, which ranged from 175 to 216 mg kg^{-1} soil, while the range was from 245 to 699 mg kg^{-1} in Vertisols (Table 1) indicating that soils with low K status respond more to application of the nutrient as compared to those with high K status. The findings were in line with the results of Meena et al. (2015) on sorghum and Zou and Lu (2008) on rapeseed, which showed higher response to K in soils with low K status and low response in high K status soils.

Besides, the higher production of dry matter and absorption of K by the teff plant from K fertilized pots as compared to control indicated that teff has greater potential to produce better grain yield when appropriate rate of K is applied under optimum nutrition of other nutrients for its proper growth and development.

Potassium concentration in the plant

Maximum concentrations of K were attained at the tillering stage in both soils for all treatments and the contents decreased with advanced plant age (Figure 2 and Table 2). Potassium fertilizer application increased K concentrations at all the stages over the control. Average maximum K concentration was obtained with application of 120 kg K ha^{-1} in Vertisols and 60 kg K ha^{-1} in Nitisols. The concentration of potassium was rapidly decreased with plant age (Figure 2) and the values appeared to be affected by K fertilization. This is in line with results of

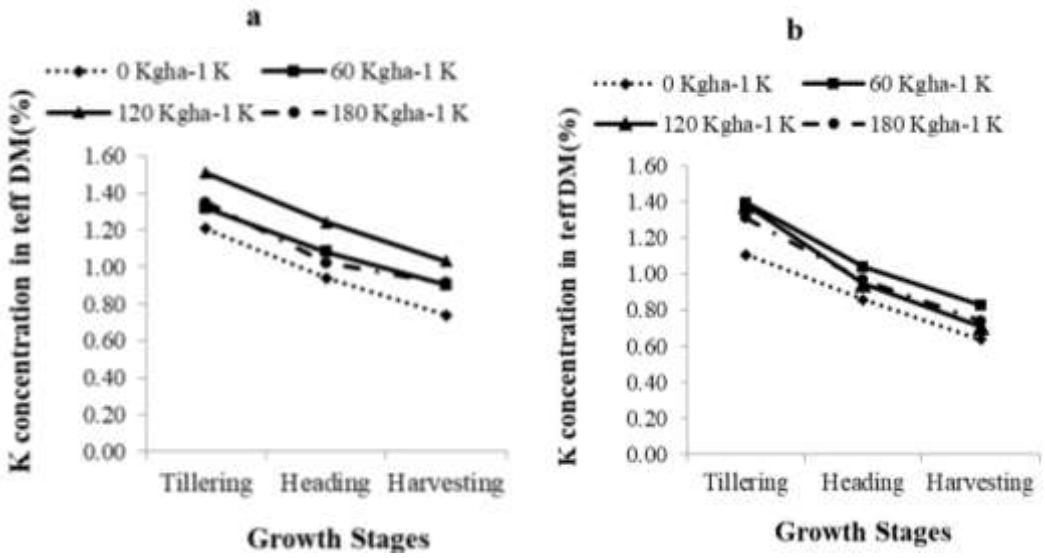

Figure 2. Changes of K concentration in teff biomass (%) at different growth stages in the pot experiments at Debre Zeit, on a) Vertisols and b) Nitisols.

Rhue et al. (1986) and Westermann et al. (1994) which indicated decreases in K concentration as growing season advances.

There are two viewpoints about plant nutrient concentration at different growth stages: (1) decreasing plant nutrient concentrations with increasing DMY may result in a dilution effect and (2) decreasing DMY with increasing plant nutrient concentrations may be due to toxicity effects. Our results showed that DMY increased with application of K because of accelerated plant growth, but K concentration of plant decreased, probably because of the dilution effect. For instance, Izsáki and Kádi (2013) reported a 33% K dilution in 30 days in Jerusalem Artichoke (*Helianthus tuberosus* L.). Besides, the high nutrient concentration in younger tissues is related to water content of the tissue as young tissues are rich in nutrients which are dissolved in water, mainly in the vacuole and in the cytosol (Mengel and Kirkby, 2001).

Potassium uptake by the aboveground biomass

The results indicated that the K uptake by the biomass of teff was significantly ($p<0.0001$) affected by the different treatments (Table 2). Mean potassium uptake was 191.87 kg ha^{-1} in Nitisols and 263.14 kg ha^{-1} in Vertisols. The highest K uptake was observed at the application of 120 kg K ha^{-1}, which was statistically different from other treatments (Table 2). The lowest K uptake was recorded in the control (without K application). Potassium uptake by teff follows a pattern similar to dry weight accumulation, except that dry matter continued to increase until maturity, whereas maximum K accumulation was reached at tillering after which there was a decrease. Similar result was reported by Akporhonor et al. (2005) on maize. Potassium uptake showed different trends across growth stages in the two soils (Figure 2). In Nitisols, K uptake was the highest at tillering stage, decrease at heading and showed a slight increase at maturity (Figure 3b), while in Vertisols the highest K uptake was at tillering and gradually decreased afterwards (Figure 3a).

A comparison of the two soil types showed that the uptake of K was significantly ($p<0.0001$) higher in Vertisols than Nitisols (Table 2). This could be explained by the higher aboveground biomass yield and K concentration of teff in Vertisols as compared to Nitisols. Besides, the higher K uptake in Vertisols could be associated with the soil moisture content, as availability of K is strongly related to soil water content (Olivera et al., 2004).

Potassium concentration in the soil

The soil analytical data showed that the soil K content significantly ($P < 0.0001$) increased with increasing K rates at all growth stages, and the difference between the soil types was also significant. Potassium concentration was higher in Vertisols than Nitisols (Table 2). The two soils vary in their K status a different growth stages may be based upon whether fixation or release dominates, which in turn is dependent upon the types of clays and the amount of weathering they have undergone (Laboski and Carrie, 2006). Besides, soil test levels were higher in fertilized pots than the unfertilized and increased with increasing K rates (Figure 4).

The concentration of soil K showed inverse relationship

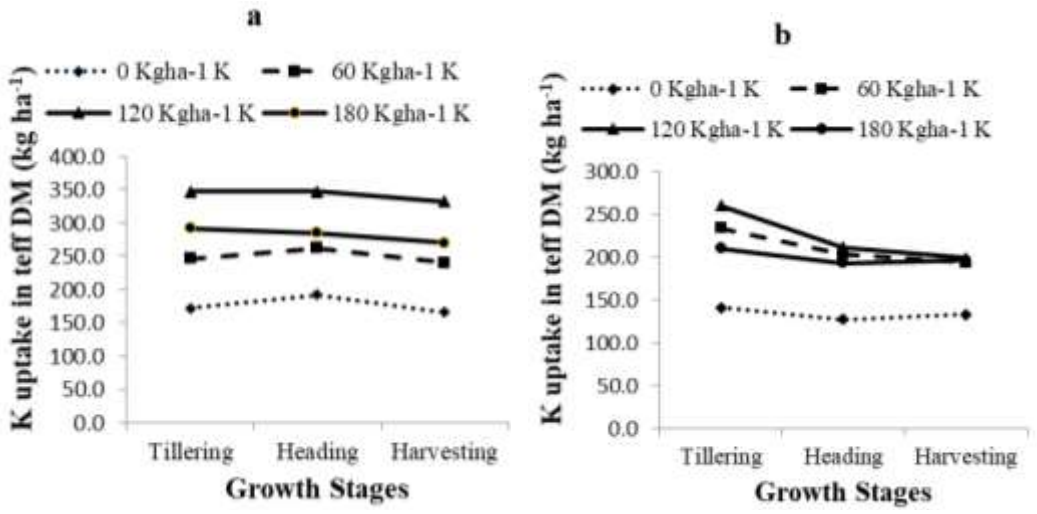

Figure 3. Changes of K uptake in soils (kg ha^{-1}) at different growth stages in the pot experiments at Debre Zeit, on (a) Vertisols and (b) Nitisols

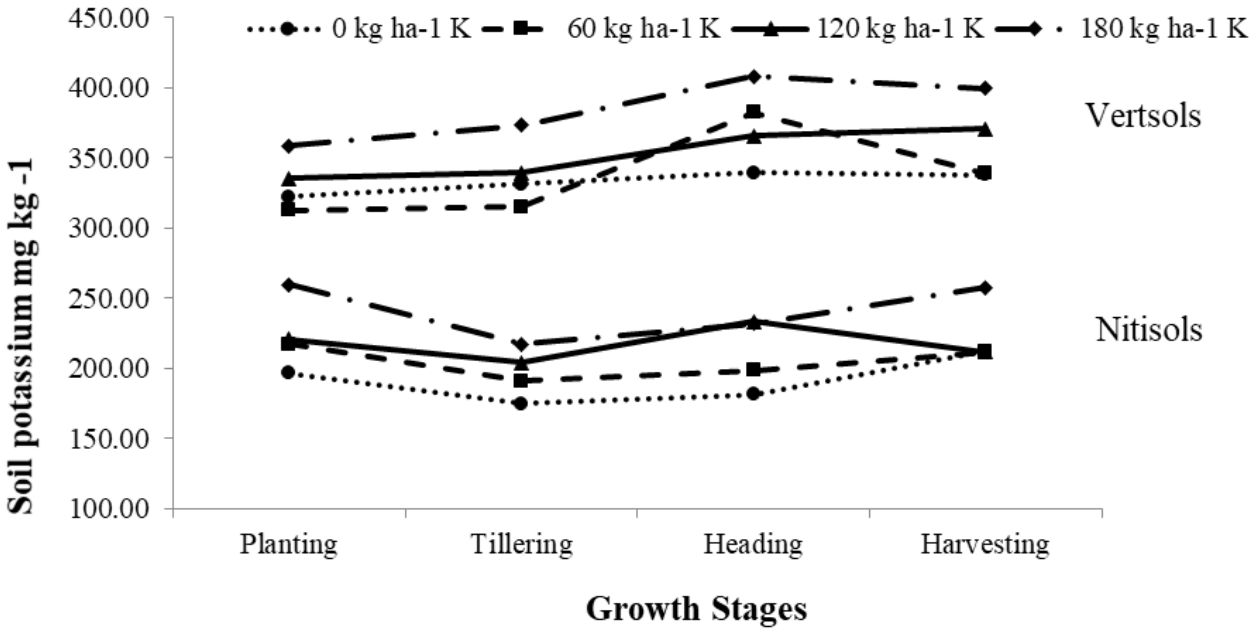

Figure 4. Changes of K concentration in soils (mg kg^{-1}) at different growth stages in the pot experiments at Debre Zeit, on Vertisols and Nitisols.

with plant K concentrations and plant age. As the plant gets older, teff plant K concentration decreased, whereas the soil K level increased. These could be due to moist condition of the soil that allows continuous release of K from the soil during the growing season, as well as the decreased uptake of K with advanced growth stage (Sangakkara et al., 2001).

Usually, soil and plant nutrient concentrations, for most nutrient elements, are positively correlated such that a greater concentration of available nutrient in the soil would be reflected in the plant-tissue nutrient contents. However, one of the reasons why concentrations of some plant nutrients do not mirror soil concentrations is that the plant nutrient concentration reflects not only soil nutrient concentration but also plant age and availability of other nutrients (Mengel and Kirkby, 2001). For short-season crops, there is evidence that a large proportion of the total K uptake occurs during early stages of growth and

the K availability at this stage determines the final yield (Costigan et al., 1983).

Conclusion

Measuring aboveground biomass and nutrient concentrations may help us understand the fertility requirements of teff and lead to better fertilization programs for the crop. The results of our experiment revealed that application of K fertilizer increased the aboveground biomass, plant and soil K concentration and K uptake by teff. The K concentrations in the plants were maximum at tillering in both soil types and decreased with advanced growth stages. Both above ground biomass accumulation and plant K uptake generally followed a similar pattern in the growing season, whereby both increased continuously with growing age, with a much faster increase at early growth stages than at late growth stages. Potassium application significantly affected biomass accumulation, K concentration in the plant and K uptake, the highest being at 120 kg K ha^{-1}. Higher yield was obtained on vertisols than nitisols. On the other hand, plant K concentration was not positively related to the soil K content, which might be due to other factors affecting its availability. The findings suggest that the supply of nutrients from soil and fertilizers must be sufficient at early growth stages to ensure that plants have higher nutrient uptake rate at tillering for optimum development and thereby higher yields. The findings on K fertilization at different stages are new for teff as well as for cereals in Ethiopia. Thus, the present recommendation to apply nutrients at early stage of growth will provide useful information both on the time and levels of potassium application for teff. However, we suggest repeating the experiment under field conditions to draw sound conclusions.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Isotopic signature of the relation between environment and the quality of spatial coffee

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The intrinsic quality of the Bourbon cultivar is well known for a high level of sweetness, intense aroma and pleasant acidity. With the evident relationship between product quality and the environment in mind, the need arises for scientific studies to provide a foundation for discrimination of product origin. Given this context, the aim of this study was to evaluate the use of stable isotopes in discrimination of production environments of Bourbon amarelo coffees from the Serra da Mantiqueira of Minas Gerais by means of statistical modeling. It is believed that upon studying a single variety with high sensory potential, the relation of expression of quality, environment and isotopes may be more evident. Thus, 24 samples of the Bourbon amarelo variety were used for composition of a model through the use of isotopes of $\delta^{18}O$, $\delta^{15}N$, $\delta^{13}C$, %C, %N, δD, and sensory analysis scores. The generated model had a 91.7% accuracy rate for classification of environments, showing in a new way that the use of isotopes may assist understanding of how the Bourbon variety responds to the environmental factors that affect isotope fractionation of C, N, O, and how much the environment collaborates in production of these *terroirs*.

Key words: Isotopic signature, bourbon, quality, geographic origin, coffee.

INTRODUCTION

The quality and complexity of the beverage are the differential that a special coffee can have, making it more valued as it is more rare and exotic. It is grown in various regions of the world. Among these regions with capacity for specialty coffee production, the south of Minas Gerais, Brazil, stands out, and it has been recognized by the Cup of Excellence (COE) as one of the most highly awarded regions in recent years. Part of this success comes from producers in the region returning to planting the Bourbon amarelo cultivar. As one of the purposes in meeting market demand is no longer growing the cultivar for greatest yield but rather the variety which represents quality, the re-emergence of Bourbon amarelo may be observed in production of Brazilian specialty coffees.

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The cultivar manifests early maturity, favoring growing in high altitude locations, leading to the production of chemical compounds that make for sensory quality, resulting in the production of a fine beverage with pleasant nuances for the palate. This notable quality is recognized worldwide and El Salvador is one of the main producer countries. This country is internationally recognized as the great producer of specialty coffee and the Bourbon variety occupies around 70% of the cropped area (Salvadoran Coffee Council, 2009). In this context, designation of origin, which is considered a way of protecting the production location and its products, in addition to adding value, has become a requirement of the international market which has consequently made greater visibility of the product possible. However, by means of measurement of the stable isotope ratios such as $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, information may be obtained about the geographic and botanical origin of many types of food, which makes this one of the most used methodologies in verifying food authenticity and in discrimination of geographic origin (Weckeler et al., 2002). The isotopic composition ($^{18}O/^{16}O$, $^{15}N/^{14}N$, $^{13}C/^{12}C$) of the coffee plant is strongly affected by the environmental conditions of the production location. Rainfall, temperature and relative humidity are characteristic indicating factors of an isotopic signature (Shibuya et al., 2007). In studies on coffee, some authors have shown that the coffee bean has an elemental isotopic composition that varies as a result of the production location, like an isotopic signature or fingerprint (Krivan et al., 1993; Serra et al., 2005; Gonzalvez et al., 2009; Rodrigues et al., 2011). This may be explained by isotope fractionation, which is strongly affected by climate. Thus, fractionation of meteoric water and fractionation of carbon and nitrogen are strongly affected by temperature and by altitude/latitude (Bowen and Revenaugh, 2003), by photosynthetic and respiratory processes, in addition to the strong contribution of gas exchanges in the variation of $^{13}C/^{12}C$ (Ehleringer et al., 2002), and by land use and agricultural practices (Ducatti et al., 2011), respectively. All the studies that cite the use of isotopes as a tool for protected designation of origin for coffees, refer to a continental geographic scale. A recent study performed on the Ilha de Reunião of France (Techer et al., 2011), although on a smaller scale, does not come to work with a regional scale as is proposed in this study. The authors made use of the Sr isotope as a tool of protection of the geographic origin of Bourbon coffees grown in the region of Ilha de Reunião; they related it to the isotopic composition of the rocks, of the meteoric water, of the coffee plants and they compared it in green and roasted coffee beans. Techer et al. (2011) confirmed that the isotopic ratio of $^{87}Sr/^{86}Sr$ found in the rocks and meteoric water are similar to those found in the green and roasted coffee beans, indicating the potential of this tool in geographic discrimination.

However, in light of the aforementioned, it is believed that upon studying a single variety with high potential for quality, the relation of expression of quality, environment and isotopes may be more evident. Thus, the aim of this study was to create a methodology for identifying different production environments of Bourbon amarelo coffees coming from the municipality of Carmo de Minas, a region located in Southeast Brazil.

MATERIALS AND METHODS

Samples and the climate

The coffee beans under study are from the municipality of Carmo de Minas, within the region of the Serra da Mantiqueira of Minas Gerais, which is the second geographic indication for coffee in Brazil. According to the Cup of Excellence, this region has gained worldwide recognition as a producer of high quality coffees (OIC, 2009), which explains the choice of this location to develop this study. The area under study is delimited by the geographic coordinates 22°07'21" Latitude South and 45°07'45" Longitude West (IBGE, 2009). The altitude ranges from a minimum of 864 m to a maximum of 1,634 m. Mean annual temperature is 19.1°C and mean annual rainfall is 1,568 mm (IBGE, 2009).

Design and quality control

Experimental design considered natural processing and hulled/mucilage removed processing, only fruits of the Bourbon amarelo variety, and the three ranges of altitude of below 1,000, 1,000 to 1,200 and above 1,200 m. To ensure the reliability of the samples, each representative lot contained 3 biological replications. All the samples were georeferenced (latitude, longitude and altitude) and collected manually in the ripe cherry stage. They were processed and selected, maintaining the highest quality beans for the purpose of verifying the true effect of the environment.

Quality control of the analyses

Sensory

Sensory analyses were performed only by certified specialty coffee judges, using the methodology proposed by the Specialty Coffee Association of America (SCAA) (LINGLE, 2001). The sensory analysis protocol of the SCAA was used for roasting the coffee, whose coloring must correspond to 58 points on the Agtron scale for the whole bean and 63 points for the ground bean, with tolerance of ±1 point. In each evaluation, five cups of coffee were cupped, representative of the interactions between genotype and environment, performing a session of sensory analysis for each replication, for a total of three repetitions/replications. Each type of processing was evaluated separately. For this study, only the final score of the attributes was considered.

Isotope-ratio mass spectrometry (IRMS)

The green coffee beans were ground in a Retsch mill for 5 min. This was performed three times to achieve a particle size of less than 1 mm. After grinding, the samples were dried in the oven for 12 h at 60°C and placed in tin capsules, folded and weighed again. The weight of the folded capsule was recorded and used to calculate C and N percentage. Elemental analysis was performed in triplicate and the mean and standard deviation was calculated. The certified

Table 1. Summary of the multivariate observations classified according to the linear discriminant model.

Variable		Predicted classification	
		π_1	π_2
True Classification	π_1	$n_{(1,1)}$	$n_{(1,2)}$
	π_2	$n_{(2,1)}$	$n_{(2,2)}$
Total	-	N = total number of observations	

reference material (CRM) for validation of the method was Wheat Flour Standard OAS. The values certified for C and N of the CRM were determined with an elemental analyzer calibrated for acetanilide 141 days of the Stable Isotopes and Instrumental Analysis Facility (SIIAF), Lisbon, Portugal.

Combustion mode (EA-C): The stable isotopes of carbon were determined by a Sira II isotope ratio mass spectrometer coupled to a EuroEA elemental analyzer preparation of the sample for combustion-reduction. The nitrogen isotope ratio was determined in an Isoprime (Micromass, Lisbon); the isotope ratio mass spectrometer was coupled to an elemental analyzer EuroEA. The coupling of the elemental analyzers and of the isotope ratio mass spectrometer was through open-split. The isotope proportion of the samples was adjusted according to international standards (IAEA CH6 and IAEA CH7 for carbon isotope ratio and IAEA N1 for nitrogen isotope ratio). The efficiency of the method was verified by means of insertion of laboratory standards among the samples to check stability and to allow correction of "drift" when necessary. Precision was 0.06‰ for determination of the carbon isotope ratio and 0.08‰ for the nitrogen isotope ratio.

Pyrolysis mode (EA-P): The oxygen isotope ratio was determined by an Isoprime, isotope ratio mass spectrometer coupled to a "EuroEA" elemental analyzer for pyrolysis. Pyrolysis occurred at 1,300°C in a glassy carbon reactor with glassy carbon chips and carbon nickel as catalyzers, mounted in a coaxial manner over a ceramic tube. Coupling of the elemental analyzer to the isotope ratio mass spectrometer was through open-split. The isotope ratio of the samples was adjusted by international standards (IAEA 601 and IAEA 602). Analytic performance was verified by means of insertion of laboratory standards among samples to verify stability and to allow correction of deviation when necessary. Precision was 0.14‰.

Data statistics

Models were established by Fisher discriminant analysis, mentioned by Johnson and Wichern (2007) for discrimination of sampled geographic locations. The discriminant function is responsible for explaining the differences among the classification variables (altitude). Classification determines the functions of the variables observed, which allows new objects to be classified in one of the "g" populations. The model created follows the proposed sampling design. The predictive factors tested for the model were: the final sensory analysis score of the coffee samples, delta nitrogen ($\delta^{15}N$), carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$) of the coffee bean, oxygen ($\delta^{18}O$), deuterium (δD), and percentages of carbon (%C) and nitrogen (%N).

Classification of the model

After obtaining the discriminant model and for its validation, a frequency table was obtained, as shown in Table 1, in which each cell represented the total number of observations classified within the following situations: $n_{(1,1)}$ equal to the number of observations belonging to π_1 which were classified in π1; $n_{(1,2)}$ equal to the number of observations belonging to π_1 which were classified in π2; $n_{(2,1)}$ equal to the number of observations belonging to π_2 which were classified in π1 and, finally, $n_{(2,2)}$ represented the number of observations belonging to π_2 which were classified in π_2.

From the results obtained by means of construction of Table 1, it was possible to compute the accuracy rate, which was used to evaluate the quality of classification resulting from Fisher's discriminant linear function. Thus, this rate was obtained according to expression 1:

$$T = \frac{n_{(1,1)} + n_{(2,2)}}{N} \quad (1)$$

In working with situations that involved more than two classification variables, as is the case of classification by ranges of altitude, a similar procedure was adopted, making due adaptations in discriminant analysis so that the Fisher's discriminant function and the estimate of the cutoff point were adapted to three classifications.

RESULTS AND DISCUSSION

Classification model-altitude

The model generated by means of the linear response method was able to classify 22 of the 24 samples studied, with an accuracy rate of 91.7%. The relations between the classification variables and the FITS1 are represented in Figure 1.

The relation between the altitude and the $\delta^{15}N$ is represented in Figure 1A. As may be observed, there is a decline in the concentration of the isotopes with increasing altitude. This same observation may be made for $^{18}O/^{16}O$ and for %N (Figure 1C and E, respectively), however, with less evidence of this relation with altitude. Contrary to what was observed for $\delta^{15}N$, $\delta^{18}O$ and %N, the sensory score and the percentage of carbon (Figure 1F and D) manifested a tendency to increase the rates and values as a result of an increase in altitude.

Altitudes below 1000 m had ‰ values for $\delta^{15}N$ (as shown in Table 2) greater than those found at higher altitudes (5.86 ± 3‰). These data show a trend toward lower isotopic abundance of $\delta^{15}N$ in production environments at high altitudes. The same observation

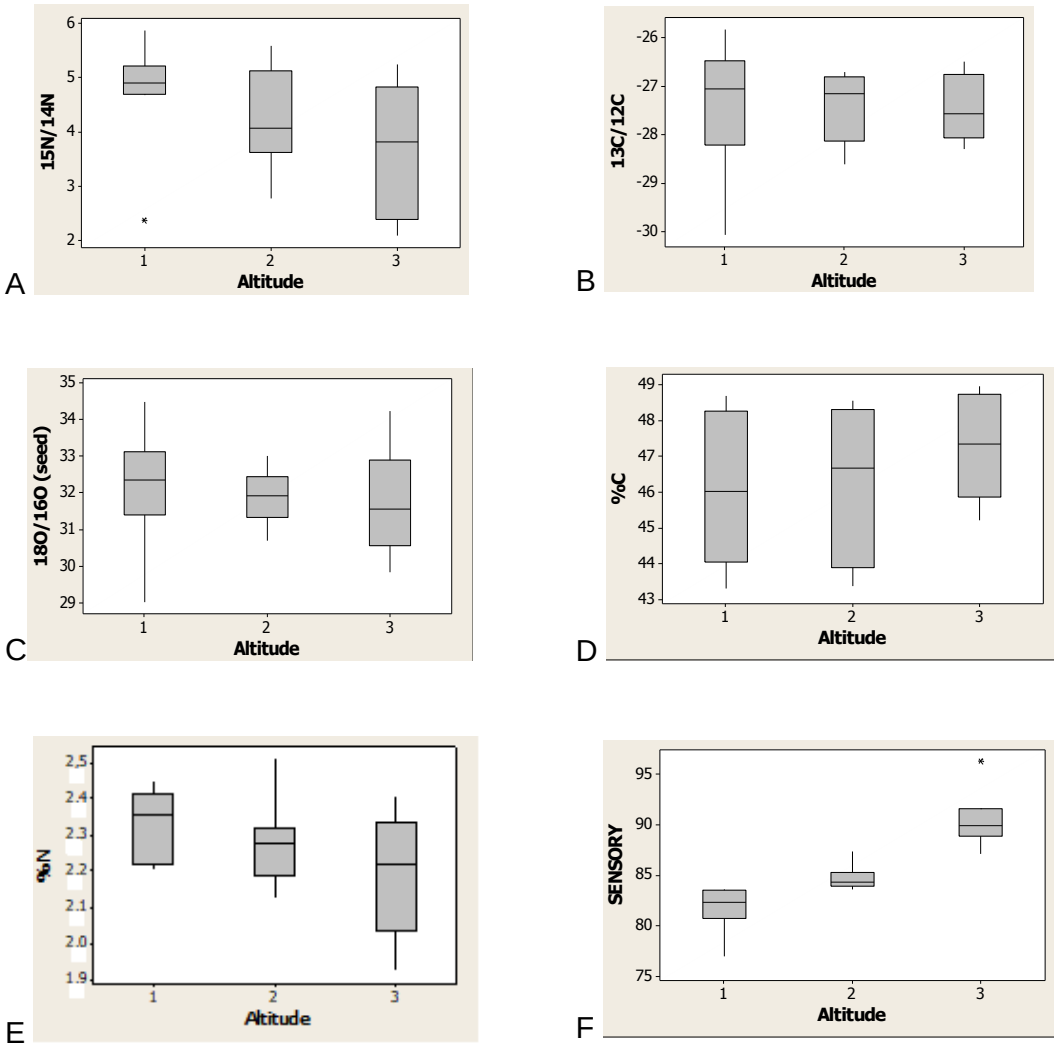

Figure 1. Boxplot of the classification variables (A= $\delta^{15}N$; B=$\delta^{13}C$; C= $\delta^{18}O$; D=%C, E= %N F= final sensory score) of the *Altitude* model. The numbers 1, 2 and 3 correspond to the altitude ranges of below 1000 m, 1000-1200 m and above 1200 m, respectively.

may be made for $^{18}O/^{16}O$ (29.0±34.5‰) in relation to the altitude classifications. A supposition that may explain the result exhibited by $\delta^{15}N$ is isotope fractionation of nitrogen, which is strongly affected by N_2 cycling. Some authors relate this phenomenon to the effects of agricultural practices or even to a result of leaching caused by excess rainfall (Borbemisza, 1982). Nevertheless, greater studies are necessary to determine the cause of this decline in the values found for the coffee beans since there are no reports regarding this fractionation in seeds or in coffee beans. For the same purpose, on a larger geographic scale, some authors studied the composition of a selected element and stable isotope ratio for food products from some European countries (Gonzalvez et al., 2009). Gonzalvez et al. (2009) observed that differences found in $^{15}N/^{14}N$ and $^{13}C/^{12}C$ are related to agricultural practices. Other factors such as rainfall and differences in soil type are reported as having a strong influence on variations of nitrogen cycling patterns and, therefore, on nitrogen isotope composition in plants (Martinelli et al., 1999). As for $\delta^{18}O$, the values found varied for altitude(s) <1000 m (32±34.5‰) and for altitudes >1200 m (29±33‰). However, the differences among the $\delta^{18}O$ values in relation to the altitude classifications are not very evident. It is worth noting that the data presented here are being reported for the first time on a small geographic scale. Therefore, the isotope values found cannot be compared to those found in the literature in reference to a global geographic scale. Nevertheless, it is worth pointing out some results of studies performed in similar geographic areas, such as the study performed by Rodrigues et al. (2011). The authors studied coffees from different islands in Hawaii and were able to discriminate the different

Table 2. Mean values, standard deviation and range of values of C%, N%, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ (seed) and final sensory analysis score of green coffee beans for the three altitude classifications (<1000 m; 1000 to 1200 m; >1200 m).

Altitude	Mean	StanDev	Ranges	Mean	StanDev	Ranges
	$\delta^{18}O$ Natural (seed)			$\delta^{18}O$ Mucilage removed (seed)		
1000	33.12	1.19	32.2 - 34.5	31.61	1.63	29.0 - 33.3
1000-1200	31.47	0.65	30.7 - 32.2	32.31	0.57	31.7 - 33
1200	31.54	1.38	29.8 - 33.2	31.94	1.65	30.3 - 34.2
	%C Natural			%C Mucilage removed		
1000	45.90	2.48	43.80 - 48.64	46.15	2.11	43.30 - 48.69
1000-1200	45.80	2.22	43.78 - 48.56	46.59	2.26	43.37 - 48.50
1200	47.58	1.5	45.47 - 48.83	47.09	1.53	45.21 - 48.96
	$\delta^{15}N$ Natural			$\delta^{15}N$ Mucilage removed		
1000	4.9	0.25	4.68 - 5.18	4.6	1.32	2.38 - 5.86
1000-1200	4.2	0.9	3.58 - 5.59	4.18	1.07	2.78 - 5.25
1200	3.5	1.28	2.3 - 4.99	3.7	1.3	2.09 - 5.24
	%N Natural			%N Mucilage removed		
1000	2.27	0.12	2.20 - 2.41	2.37	0.06	2.28 - 2.44
1000-1200	2.26	0.07	2.16 - 2.31	2.29	0.15	2.12 - 2.51
1200	2.18	0.12	2.01 - 2.29	2.19	0.22	1.92 - 2.41
	$\delta^{13}C$ Natural			$\delta^{13}C$ Mucilage removed		
1000	-28.63	1.37	-27.31 to -30.05	-26.58	0.52	-25.82 to -27.27
1000-1200	-27.22	0.58	-26.69 to -27.96	-27.61	0.91	-26.82 to -28.60
1200	-27.65	0.72	-26.73 to -28.29	-27.18	0.64	-26.47 to -27.78
	Sensory Natural			Sensory Mucilage removed		
1000	81.45	0.97	80.37 - 82.25	81.97	2.82	77 - 83.62
1000-1200	84.37	0.27	84.12 - 84.75	85.09	1.73	83.62 - 87.37
1200	89.78	1.23	88.87 - 91.5	91.25	3.8	87.12 - 96.25

environments by means of the isotopic signature of oxygen $^{18}O/^{16}O$. They observed that the isotope ratio of $\delta^{18}O$ decreases with an increase in altitude; there is a reduction of this element in the chemical composition of the coffee beans. These observations made by the authors validate what was analyzed in this study for the values of $\delta^{18}O$ and $\delta^{15}N$. Nevertheless, it may be seen that in regard to the Bourbon amarelo variety, of high sensory potential, the relationship of quality expression, environment and isotopes is more evident, as shown in Figure 2A, B and C.

A contrary behavior of the $\delta^{15}N$ and $\delta^{18}O$ isotopes may be observed when compared to the sensory score in relation to quality. In other words, in this plot it is possible to visualize that with the increase in altitude classifications there is an increase in the sensory score and a reduction in the respective isotopes. Avelino et al. (2005) performed studies on the quality of Costa Rican coffees and observed a positive relation of the effect of altitude on quality. Although this phenomenon is not very well understood, it was also reported by Barbosa et al. (2012). According to the authors that performed studies on Brazilian coffees, in addition to the quality vs. altitude relation, there is also a relation with latitude as a result of rainfall distribution and temperature. According to Rodrigues et al. (2011), a more refined interpretation of the isotopic abundance of the coffee bean becomes difficult due to the combination of diverse environmental, climatic and physiological processes. In the face of these environmental effects, it has been reported in the literature that isotopic fractionation of oxygen occurs in the plant leaves (Yakir and Sternberg, 2000); however, there are no reports in relation to fractionation in seeds. Taking into consideration that the products formed by photosynthesis are divided up among the locations of greatest demand in the plant (Larcher, 2006) and considering that these products will be deposited and built up during formation and ripening of the coffee fruits,

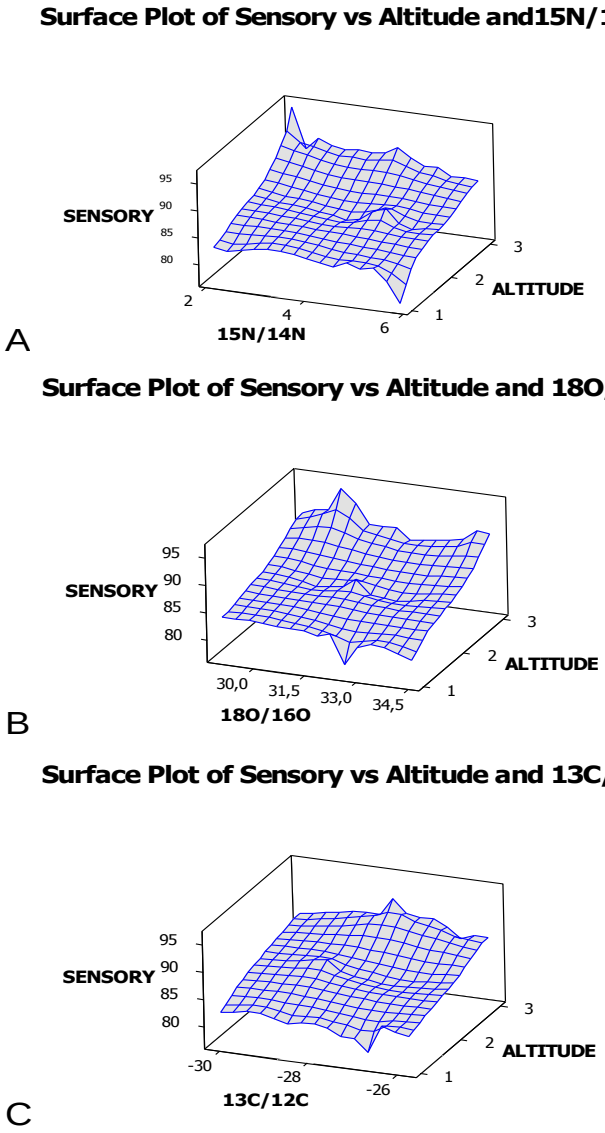

Figure 2. (A) 3D surface plot composed of the variables: stable isotope of nitrogen ($\delta^{15}N$), final sensory score (Sensory) and the altitude classifications (1 = <1000 m, 2 = 1000-1200 m and 3 = >1200 m) represented by the X, Y and Z axes, respectively; (B) 3D surface plot composed of the variables: stable isotope of oxygen ($\delta^{18}O$), final sensory score (Sensory) and the altitude classifications; and (C) 3D surface plot composed of the variables: stable isotope of carbon ($\delta^{13}C$), final sensory score (Sensory) and the altitude classifications.

these events contribute to the distribution of the isotopes studied in the coffee beans. In relation to the isotopes contained in the meteoric water that falls on the soil, authors report that it may reflect the same isotopic abundance found in the rain (Yakir and Sternberg, 2000). Another consideration that may be taken into account is the relation of the water balance in which, by means of transpiration, plants take up the water contained in the soil through the roots and transport it to the transpiration surfaces (Larcher, 2006). Thus, the organic compounds recently synthesized by the plant may contain $\delta^{18}O$, but this may be dependent on fractionation and also on enzymatic, regulatory and synthesis processes. Barbour et al. (2000) report that the isotopic composition of the organic matter of plants is known for reflecting the water absorbed in evapotranspiration conditions at the time the organic matter is formed. From this, it is plausible to infer that the isotopic composition of the $\delta^{18}O$ found in the

coffee beans will be reflected in the physiological performance of the coffee plant.

Conclusions

The results presented show that when a single variety of high sensory potential is the point of focus, the relation of quality expression, environment and isotopes is more evident. With the unprecedented nature of the study on such a small geographic scale in mind, greater information in regard to the matter is necessary to clarify such events that create the mystique of the prestigious “terroirs”.

Since the international market requires excellent standards of quality, placing value on products with country of origin labeling, the need is seen for creation and application of methodologies that add value to fine products such as coffees from the Serra da Mantiqueira of Minas Gerais, thus providing for a positive perception of the coffees produced in Brazil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Osmotic stress in *Chenopodium quinoa* Willd.: Variations in osmoprotectants at different phenological stages

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***Chenopodium quinoa* Willd. is an edible crop plant adapted to the climatic conditions of the South American Andes, where it thrives under extreme environmental conditions such as saline soils, drought, high UV radiation and broad temperature fluctuations. The prolonged exposure of this crop to high salinity and low relative humidity has promoted the development of efficient mechanisms to retain water within the intracellular compartments and avoid desiccation, including accumulation of osmoprotectants. In this study, the effect of osmotic stress (250 mM KCl) was evaluated on osmolyte accumulation at different phenological stages of quinoa growth (branched, panicle, flowering) compared to control (0 mM KCl). The osmotic stimulus increased the concentrations of proline, glycine betaine, sucrose, fructose, glucose, and trehalose two- to seven-fold compared to a low salinity conditions. This is the first report to show significant increase in trehalose in response to osmotic disturbance at three different phenological stages of quinoa growth, opening a new avenue to explore the protective role of trehalose against osmotic-induced damage in this crop.**

Key words: Compatible solutes, osmotic stress, quinoa, phenology.

INTRODUCTION

In the face of climate change, soil salinization, and a rising world population; the research and development of effective approaches to produce stress tolerant food crops of high nutritional quality is of increasing importance (Delorge et al., 2014). Quinoa (*Chenopodium quinoa* Willd., Amaranthaceae) is a seed crop high in nutritional value that has achieved rising global demand in recent years and holds promise as an alternative crop for the promotion of food security (Vega-Galvez et al., 2010; Lutz et al., 2013; Graf et al., 2016;). Native of the Andean Altiplano of South America, quinoa is adapted to thrive in regions characterized by intense abiotic stresses, including saline soils, low precipitation, frequent drought, temperature fluctuations from -4 to 27°C, and

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strong winds (Arenas and Lanino, 2008). Agronomists have recently begun to expand quinoa production to other regions with unique abiotic stress patterns throughout Africa, Asia, and North America (Graf et al., 2016).

Soil salinity is one of the major abiotic stresses affecting crop growth and yield (Wang et al., 2003). About 830 million hectares of land have been identified as salt-affected soils around the world (Rengasamy, 2006). Salt stress impedes crop growth, development, and yield by inducing an osmotic effect and ion toxicity (Delatorre-Herrera and Pinto, 2009; Adolf et al., 2013), each of which differentially affect plant physiology (Delatorre-Herrera and Pinto, 2009). Salt-tolerant plants employ complex defense mechanisms, including osmoregulation, ion homeostasis, activation of antioxidant systems (Hasegawa et al., 2000), and accumulation of soluble sugars such as glucose, fructose, sucrose and trehalose (Ancillotti et al., 2015).

Quinoa is a facultative halophytic plant that has adapted physiological mechanisms to provide osmoregulation in highly saline environments (300 mM NaCl or more) including the production of compatible solutes, also known as osmoprotectants (Morales et al., 2011; Adolf et al., 2013). Compatible solutes, such as soluble sugars, proline, and glycine betaine, adjust the osmotic potential of plant tissue to cope with the high accumulation of salt without disrupting essential physiological processes, enzyme functions, or cell membrane integrity (Morales et al., 2011). Previous studies have shown that quinoa seedlings or young plants demonstrated increased sucrose, glucose, fructose, proline, and glycine betaine content under salt stress (NaCl levels 450 mM) compared to normal conditions (Rose et al., 2009; Morales et al., 2011). The accumulation of these compatible solutes has also been observed in many other plant species, including sugar beet, rice, potato (Ghoulam et al., 2002; Boriboonkaset et al., 2013).

However, quinoa is unique to most other crop plants in that this species has recently been shown to produce detectable levels of trehalose under salt stress (450 mM NaCl) (Morales et al., 2011). Trehalose is a thermostable, non-reducing disaccharide composed of two glucose units linked by an α,α -1,1,-glycosidic bond (Higashiyama, 2002) that was previously thought to be restricted to only a few higher plants, including the salt-tolerant resurrection plants *Myrothamnus flabellifolius* and *Selaginella lepidophylla* (Fernandez et al., 2010). Trehalose functions in these plants to protect and stabilize cytoplasmic and membrane proteins by establishing hydrogen bonds that replace water during dehydration, and by crystallizing in a glassy state to prevent the denaturation of biomolecules (Elbein et al., 2003; Fernandez et al., 2010). Trehalose has also been well-documented to confer osmoregulation and stress resistance in bacteria, fungi, and invertebrates. (For example, *Escherichia coli* synthesizes large amounts of trehalose when placed in a high osmolarity environment; *Saccharomyces cerevisiae* accumulates trehalose during exposure to hydrogen peroxide; the nematode *Aphelenchus avenae* converts up to 20% of its dry weight to trehalose during dehydration (Elbein et al., 2003).

Though the trehalose biosynthetic genes have been documented in higher plants for which genomes have been sequenced, detectable levels of trehalose have not been observed in most higher plants (Goddijn and van Dun, 1999; Avonce et al., 2005). Given the role of trehalose as an effective osmoprotectant, edible plants which naturally produce trehalose are of increasing interest as alternative crops in extreme environments.

Trehalose accumulation has been rarely studied as a stress tolerant mechanism in plants (Fernandez et al., 2010; Tsai and Gazzarrini, 2014). It has been suggested that the genome of *C. quinoa* encodes functional genes associated with trehalose metabolism, because trehalose content increases under high saline conditions (Morales et al., 2011). However, the accumulation of trehalose and other carbohydrates has not been investigated in different stages of quinoa growth under osmotic stress. It was hypothesized that trehalose levels increase in response to osmotic stimulus during all quinoa phenological growth stages.

This study measured the accumulation of trehalose and other osmolytes (sucrose, glucose, fructose, glycine, betaine and proline) in quinoa plants grown under KCl-induced osmotic stimulus compared to non-stimulated plants at three different phenological growth stages: branched, the stage when the quinoa plant first produces axillary branches; panicle, the stage when the quinoa plant produces flower buds, and flowering, the stage when flowers buds within the panicle open.

MATERIALS AND METHODS

Plant

The seed used in this study was a white variety of quinoa (accession no. 28) initially obtained from Cariquima in the Tarapacá province of the northern Chilean Altiplano and stored at Arturo Prat University, Iquique, Chile. This seed variety was selected for the study because it is well adapted to the sandy, saline soils of Cariquima, a region located at 3,800 m above sea level with less than 300 mm annual rainfall (Becares and Bazile, 2009).

Experimental design

KCl was used to avoid the effect of ionic stress and the nutritional imbalance caused by sodium and to induce only an osmotic stress (Munns et al., 1995; Delatorre-Herrera and Pinto, 2009). A randomized block design was implemented with two treatment groups: KCl treatment (250 mM) and a control group (0 mM KCl). Each treatment group comprised four blocks each containing eight quinoa plants (32 plants per treatment). Quinoa plants were grown in a hydroponic perlite system, according to the method described elsewhere (Delatorre-Herrera and Pinto, 2009). Quinoa seeds were

sown directly in perlite plastic containers (4 L volume) and initially irrigated with a nutrient solution using a cotton wick (Delatorre-Herrera and Pinto, 2009). The nutrient solution, formulated as described previously (Schlick and Bubenheim, 1993), was changed once a week. The temperature of the greenhouse was set to 27 to 2°C during cultivation, and the aeration flow of the culture system was continuous throughout the test. The photoperiod applied was environmental, that is between 12 and 13 h of light. The treatments were applied at 2 weeks when the plants had 2 true leaves and were prolonged until the end of the trial (approximately 120 days). The plants in the KCl treatment group were irrigated with the same nutrient solution as the controls with the addition of 250 mM KCl. KCl was used to reduce osmotic potential of the nutrient solution to -0.8 MPa, as described elsewhere (Delatorre-Herrera and Pinto, 2009; Panuccio et al., 2014;), whereas the control group exhibited an osmotic potential of -0.1 MPa. KCl was chosen for salt treatment, instead of NaCl, because KCl can reduce osmotic potential and thereby induce osmotic stress without leading to sodium-induced plant toxicity. Osmotic potential of the nutrient solution was monitored with an osmometer (Advanced Instrument, INC, Mod. 3320), throughout the study to maintain osmotic stress treatment.

Plant growth was monitored every two days to verify the phenological stage throughout the experiment. Plant samples were collected at three different phenological growth stages (branched, panicle and flowering) as described elsewhere (Delatorre-Herrera and Pinto, 2009; Panuccio et al., 2014). During the branched stage, apical buds from three or four plants (500 mg dry weight) were randomly collected for analysis of osmotic potential, leaf water potential, proline, glycine betaine, and sugar concentrations.

Analysis of osmotic potential and leaf water potential

The leaf osmotic potential (Ψs) was determined from fresh leaf samples (0.1 g) that were ground with a mortar and pestle in 1.5 mL of ultrapure water. The mixture was centrifuged at 1,000 ×g for 10 min. The supernatant was analyzed with an osmometer (Advanced Instrument, INC, Mod. 3320). The data was transformed to osmotic potential according to the van´t Hoff equation (Ghneim-Herrera et al., 2006).

The leaf water potential (LW) was measured in a pressure chamber as described elsewhere (Scholander et al., 1965). The pressure of neutral gas required to induce an exudate from the leaf was measured and expressed in negative Mega Pascal units (-MPa).

Analysis of proline and glycine betaine content

Proline content was measured as previously described (Bates et al., 1973). Briefly, 50 mg of lyophilized leaf tissue was smashed and mixed with sulfosalicylic acid (3% w/v). The mixture was centrifuged at 10,000 ×g at 4°C and the supernatant was mixed with acetic acid and ninhydrin. The solution was heated at 90°C for 1 h and transferred to ice for 30 min. This solution was vigorously mixed with toluene in a ratio of 5:1 for 30 s. The toluene phase was analyzed using a spectrophotometer (Mecasys MOD: OptizenPOP) at 520 nm. A proline standard (Merck) curve was performed with concentration of 10, 20, 50, 100 and 200 mg/mL.

Glycine betaine content was determined as described previously (Grieve and Grattan, 1983) with minor adaptations (Delatorre-Herrera and Pinto, 2009). Briefly, 0.1 g dry matter was stirred in 4 mL of distilled water for 24 h at 25°C. Then, the mixture was filtered and stored at -4°C. For determining quaternary complex, extract was thawed and 50 µL was mixed with 50 µL of 2 N sulfuric acid. Then, the mixture was chilled to 0°C, and 40 µL of KI-I2 (15.7 g iodine and 20 g of potassium iodide in 100 mL of distilled water) was added with gentle shaking on a vortex. This solution was stored at -4°C for 16 h, and centrifuged at 10,000 ×g for 15 min. The supernatant was carefully aspirated and the pellet was dissolved in 1.6 mL of 1,2-dichloroethane, stirred vigorously with a vortex and then allowed to stand for 2.5 h. Samples were read in a spectrophotometer at 365 nm. The calibration curve was performed with pure betaine standard (Merck) at concentrations of 10, 20, 50, 100 and 200 mg/mL diluted in 1,2-dichloroethane.

Sugar analysis

The concentration of individual sugars was measured by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI) as described previously (Singh et al., 1994). Briefly, lyophilized leaf tissue (100 mg dry weight) was ground with mortar and pestle. The powder was homogenized with ethanol 80% (v/v) and the mixture heated to 90°C for 10 min. The mixture was centrifuged at 2,500 ×g for 10 min, and the supernatant was dried via rotary evaporation and resuspended in 1.5 mL of acetonitrile:water (78:22 v/v). The solution was analyzed by HPLC using a Librocart 250-4 column linked to a pre-column Librocart 4-4 at a flow of 1.5 mL/min under isocratic conditions. The mobile phase was acetonitrile:water (78:22 v/v). The detector was a Merck Hitachi Refractive Index Detector LaChrom 7490. The retention times (tR) of standards were 4.22 min (fructose), 4.62 min (glucose), 6.17 min (sucrose), and 8.13 min (trehalose). Calibration curves for each standard (Merck) were used to determine the concentration of each sugar within the samples.

Data analysis

Tukey's multiple comparison one-way analysis of variance (ANOVA) was performed using GraphPad Prism 6.0 (La Jolla, CA, USA). $P < 0.05$ was considered significant. Student t-test was performed using Infostat V 2016.

RESULTS

Osmotic potential and leaf water potential

At all phenological growth stages, the osmotic potential of quinoa leaves was significantly lower in KCl treated plants versus control by 81 to 163% (Table 1). This corresponded with a significant 79 to 114% decrease in leaf water potential in KCl treated plants versus control among the three different phenological growth stages (Table 1). Osmotic potentials of the leaves at the branched and flowering stages: -2.74 and -2.51, respectively; were statistically similar, whereas osmotic potential at the panicle stage was significantly higher (-2.10 MPa). Leaf water potentials at the branched, panicle, and flowering stages significantly differed, and values incrementally decreased through successivel stages of plant growth: -1.70 > -1.82 > -1.84 MPa.

Proline and glycine betaine levels

Compared to control plants, proline content significantly increased by 202, 166, and 99% in KCl-treated plants at the branched, panicle, and flowering stages, respectively

Table 1. Leaf water potential and osmotic potential in *C. quinoa* leaves plant under osmotic stress compared to control.

Phenological stage	Leaf water potential (MPa)		Osmotic potential (MPa)	
	Control	KCl treatment	Control	KCl treatment
Branched	-0.95± 0.0011	-1.70 ± 0.0028*	-1.04 ± 0.0046	-2.74 ± 0.0003*
Panicle	-0.93 ± 0.0036	-1.82 ± 0.01*	-1.16 ± 0.00063	-2.10± 0.02*
Flowering	-0.86 ± 0.00024	-1.74 ± 0.00064*	-1.23 ± 0.0017	-2.51± 0.05*

*Significant difference between the treatment and control at each phenological stage ($P \leq 0.05$), according to Student's t-test.

Table 2. Total content of the osmolytes proline and betaine (mg g^{-1} dry matter) in *C. quinoa* leaves under osmotic stress and control conditions during three phenological stages of plant growth.

Osmolyte	Treatment	Phenological stage		
		Branched	Panicle	Flowering
Proline	Control	4.63 ± 0.58	5.45 ± 0.23	5.61 ± 0.81
	KCl	13.97 ± 2.03*	14.5 ± 2.99*	11.17 ± 3.89*
Betaine	KCl	1.17 ± 0.13	1.00 ± 0.06	1.17 ± 0.08
	Control	0.46 ± 0.09*	0.44 ± 0.2*	0.66 ± 0.2*

*Significant difference among the treatments at each phenological stage ($P \leq 0.05$), according to two-way ANOVA test.

(Table 2). Glycine betaine content significantly increased by 154, 127, and 77% at the same phenological growth stages. Proline and glycine betaine levels were statistically similar at each phenological growth stage in control and KCl-treated plants, respectively.

Sugar concentrations

Among the four sugars analyzed in quinoa leaves grown in control conditions, fructose generally showed the highest levels among all phenological growth stages (7.51 - 14.85 mg g^{-1} dry weight), followed by glucose (5.96 - 7.64 mg g^{-1} dry weight), sucrose (3.24 - 4.72 mg g^{-1} dry weight), and trehalose (0.96 - 1.13 mg g^{-1} dry weight) (Figure 1). However, under KCl treatment, glucose showed the highest levels among all phenological growth stages (22.49 - 43.37 mg g^{-1} dry weight), followed by fructose (16.41 - 24.10 mg g^{-1} dry weight), sucrose (7.98 - 10.65 mg g^{-1} dry weight), and trehalose (2.79 - 3.63 mg g^{-1} dry weight).

The levels of individual sugars were statistically similar to the levels of the corresponding sugar at each phenological growth stage, except in the case of glucose, in which glucose levels were significantly higher in KCl-treated plants at the branched and flowering stages (40.07 - 43.37 mg g^{-1} dry weight) than at the panicle stage (22.49 mg g^{-1} dry weight).

Plants exposed to KCl treatment showed significantly higher levels of all sugars (fructose, glucose, sucrose, and trehalose) compared to those of the control group at all phenological stages. Glucose showed the largest increase, with 628, 240, and 424% higher levels at the branched, panicle, and flowering stages, respectively. Trehalose showed the second greatest increase, with 150, 172, and 280% higher levels at the same phenological growth stages. Sucrose showed the third greatest increase, with 126, 149, and 146% levels at each growth stage, respectively. Among the sugars measured, fructose showed the smallest increases, with 99, 118, and 24% increases at each growth stage.

DISCUSSION

According to our hypothesis, the results showed that 250 mM KCl treatment reduced both the water potential and the osmotic potential of the quinoa leaves, showing that the concentration applied causes a stress condition in the treated plants. The KCl effect on the osmotic potential could be part of the osmotic adjustment of quinoa, by which the plant maintains the gradient of water potential and turgor pressure (Smirnoff, 1998). This osmotic adjustment in quinoa has been widely demonstrated by several authors (Hariadi et al., 2011; Orsini et al., 2011; Ruiz et al., 2015) and has also been described in other plant species, such as sugar beet, rice and corn (Hasegawa et al., 2000; Ghoulam et al., 2002; de Sousa et al., 2016,).

The reduction in osmotic potential and leaf water

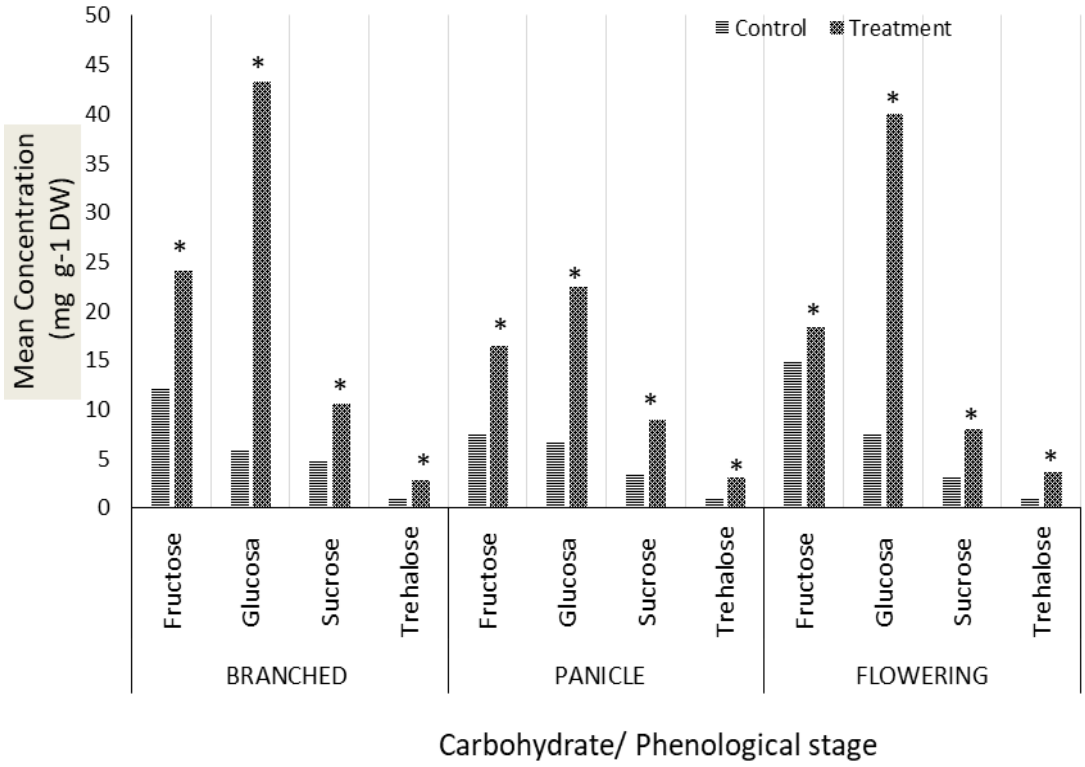

Figure 1. Glucose, fructose, sucrose, and trehalose concentrations in C. quinoa leaves grown under KCl treatment compared to control at three different stages of phenological growth**.** Data are the mean (n=3). Asterisk denote significant differences across treatments and phenological stages for an individual sugar ($P < 0.05$), according to two-way ANOVA test.

potential induced by KCl treatment was correlated with a significant increase in concentrations of compatible solutes in quinoa leaves, including glycine betaine, proline, glucose, fructose, and sucrose (Table 2 and Figure 1). Furthermore, these increases were consistently observed for all compatible solutes across all three phenological stages (branched, panicle, and flowering) (Figure 1). The observed accumulation of these molecules is consistent with literature that reports that proline, glycine betaine, and soluble sugars play roles as osmolytes and salt defense mediators, especially in halophyte species (Gupta and Kaur, 2005; Morales et al., 2011; Boriboonkaset et al., 2013; Ruiz et al., 2015). However, further studies are needed to understand the relative contribution of these osmolytes to the multicomponent response of quinoa to osmotic stress, which also involves the use of salt bladders, ion sequestration, and chaperon protein synthesis (Adolf et al., 2013; Raney et al., 2014).

The results show that there are changes in the concentration of three of the sugars (sucrose, glucose, fructose), in the phenological stages (branched, panicle, and flowering) (Figure 1). Trehalose is the only sugar that does not change in the phenological stages, maintaining a similar concentration in all of the stress conditions.

Sucrose increased 2 to 3 times its concentration in the plants with KCl (osmotic stress) compared to the control treatment (without KCl), the bibliography in general coincides with these results, showing an increase ranging from 2 times, in *Atriplex halimus* (Hassine and Lutts, 2010), reaching up to 9 times in *Vernonia herbaceous* (Garcia et al., 2011). Overall, demonstrating that this sugar has a direct participation in the mechanism that plants have to overcome periods of water deficit, since it not only acts as an osmolyte to maintain the osmotic potential of the cells, but also acts to protect the proteins from denaturation (Da Silva and Arrabaca, 2004; Murata et al., 2012). Likewise, a decrease in the concentration of sucrose in the expansion and maturation of cassava leaves (*Manihot esculenta*) has also been found, this decrease being 60% in maturation and 33% in expansion (Alves and Setter, 2004); in our results it was observed that there was a slight decrease in the concentration of this sugar as the phenological phases arose, showing the lowest concentration in the beginning of flowering stage, which suggests that sucrose could be used as a source of energy for glycolysis by degrading it into glucose and fructose.

Also, the period during which the plant is exposed to stress conditions strongly influences the plant response.

In the present study, the period of exposure was constant and prolonged by four months, which produced high concentrations in glucose and fructose, in the three phenological stages. These results agree with that obtained by García (2004), who observed in tomatoes, that more time of exposure to stress increases concentration of these sugars. Similar results were obtained by Márques and Celeste (2004) who observed an increase of up to ten times in the concentration of these two sugars. Nio et al. (2011), who subjected *Triticum aestivum* to a prolonged period of water stress, reported an increase in glucose and fructose, but a decrease in sucrose in leaves.

These results are similar to those found in the present study; in each of the phenological stages, sucrose always showed the lowest concentration with respect to glucose and fructose. This could be explained by the low use of fructose for the synthesis of sucroses (Trevanion, 2002). Fructose forms polymers and oligomers with a low percentage of glucose in their structure called fructans, which are the main reserve carbohydrates in some plants and microorganisms (Mancilla and Lopez, 2006). The ability of some plant species to synthesize fructans has been associated with their survival when growing in cold and dry climates, which seems to indicate that they play an important role in the response to stress conditions (Ritsema and Smeeken, 2003). It has been suggested that fructans can stabilize membranes by direct H-bonding to the phosphate and choline groups of membrane lipids, resulting in a reduced water outflow from the dry membranes (Valluru and Van den Ende, 2008). In the present case, it can be seen that the branched stage, when the fructose content increases, this produced the biggest difference between the control and the application of KCl, followed by panicle (Figure 1), these results could indicate the need for the plant to adjust its growth as a consequence of the higher energy requirement for the formation of new organs such as the panicle, a situation that is affected by the stress to which the plants are subjected with KCl application.

On the other hand, the high content of trehalose observed in quinoa plants stressed by salt, with respect to the control, is consistent with the effect observed in *C. quinoa* ,in the eight-leaf stage by Morales et al. (2011) and in other plants, under similar stress conditions (Da Silva and Arrabaca, 2004; Iordachescu and Imai, 2008; Delorge et al., 2014). However, this phenomenon was further described by studying the effect of KCl across other phenological stages of quinoa growth.

The present experiments showed that, regardless of the phenological stage of *C. quinoa*, trehalose levels increased in response to the osmotic stimulus induced by KCl (from 1.07 to 3.16 mg g^{-1} dry weight). Interestingly, the maximum concentrations of trehalose in KCl-treated plants were significantly lower than those of other sugars, such as sucrose and glucose, suggesting that trehalose may function as a signaling molecule, rather than an osmoprotectant (Hasegawa et al., 2000; Avonce et al., 2005). Additionally, trehalose can also act as a structural component when incorporated into glycolipids, in order to stabilize membranes (Elbein and Mitchell, 1973; Elbein, 1974). However, the most important feature of trehalose is the property it possesses to act as a protein stabilizer, when certain organisms are exposed to stress conditions. Trehalose has the ability to associate with membrane proteins and replace the function of water as a protein stabilizer, especially under dehydration conditions (Donnamaria et al., 1994).

The present results show a relationship between the various sugars in the phenological stages. Cortina and Culiañez-Macià (2005) show that in genetically modified (GM) tomatoes to synthesize trehalose, an increase in the concentration of starch is produced in addition to trehalose. The fructose values in the leaves increased from 3.5 mg g^{-1} for the wild, to 4.9 mg g^{-1} for the GM, while the glucose concentration was 5.5 mg g^{-1} in wild plants and 10.5 mg g^{-1} GM plants.

On the other hand, the concentration of sucrose increased slightly in both wild and modified plants (from 2.9 to 3.1 mg g^{-1}), results similar to those found inthe present study. In yeast, Trehalose-6-phosphate (T-6-P) affects glycolysis and sugar signaling through its interaction with hexokinase, which is a putative sensor (Hohmann and Thevelein, 1995; Paul et al., 2001). Although it remains to be determined whether T-6-P in plants interacts with hexokinase as it does in yeast, T-6-P appears to be important in sugar signalling in plants (Paul et al., 2001). In *Arabidopsis thaliana*, exogenously applied trehalose induces the ADP-Glucose pyrophosphorylase gene *ApL3*, and starch synthesis (Wingler et al., 2000). Also, transgenic TPS1 tomato plants accumulate over 60% more starch in their leaves than the tomato control plants. Thus, trehalose biosynthesis appears to affect starch accumulation by inducing directly the components of its starch biosynthetic pathway (Wingler et al., 2000). The synthesis of sucrose is also influenced by the synthesis of trehalose, since the trehalose-6-phosphate, directly affects the levels of sugars in plants (Wingler, 2000; Avonce et al., 2004; Avonce et al., 2005). Fernandez et al. (2010) state that future studies are necessary to establish whether trehalose is increased in specific organs, cells or organelles of quinoa plants, where it may function as a signaling molecule through the trehalose-6-phosphate pathway. Although the present results show only the increase in the phenological stages measured in quinoa, it is possible to associate the results with the appearance of new organs. For example, in the branched stage, there are only branches; however, in the panicle stage reproductive structures are formed and during flowering they add flowers. In this regard we can see that from branches to panicle and flowering, fructose decreases its concentration (Figure 1), which shows the use of fructose as a source of reserve for the growth of other organs.

Regarding trehalose, the concentration in each phenological stage is not increased, so it can be deduced that in this case it does not play a role as a reserve carbohydrate, but probably acts as a signaling or protective molecule.

Conclusions

Osmotic stress increases proline, glycine betaine, glucose, fructose, sucrose and trehalose leaf contents in *C. quinoa*.

All the measured sugars (sucrose, fructose and glucose) increase their concentration with an osmotic stress in the different phenological stages.

For each phenological stage, the concentration of trehalose is higher in plants with KCl, than in control plants. Given the low concentration present, trehalose likely acts only as a signaling or protective molecule.

Glucose is the sugar that is present in higher concentrations relative to the control across all three stages of growth, and it particularly increased relative to the control in the stages of branching and flowering. Fructose is the second sugar in relative concentration to the control, and presents greater concentration in branches and panicles.

Trehalose, which has rarely been identified in agricultural crop plants, can be a useful biomarker for saline-resistant quinoa ecotypes. Crops that are adapted to grow in salt-stressed environments are very valuable in the context of global climate change and soil desertification. These molecules may function as osmoprotectants and signaling molecules. Trehalose, which has been rarely identified in agricultural crop plants, may be useful biomarker for salt resistant ecotypes of quinoa.

CONFLICT OF INTERESTS

The authors have no declared any conflict of interest.

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Full Length Research Paper

Different dry matters content used for the conservation of annual ryegrass (*Lolium multiflorum* Lam.) in anaerobic environment

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Annual ryegrass (*Lolium multiflorum* Lam.) is widely used for feeding ruminants, and the conservation of this material as haylage can be an alternative to farms. The aim of this work is to study the nutritional and microbiological value of annual ryegrass (*L. multiflorum* Lam.) pre-dried and stored with different dry matter contents (250, 350, 450, 550, 650 and 750 g kg^{-1}) in anaerobic environment. The experimental design was completely randomized with six treatments and four replications. The pH presented at the time of opening of the silos showed a linear increase of 0.003 pH units for each 1 g kg^{-1} of dry matter increase in ryegrass, as the materials with contents above 550 g kg^{-1} of dry matter showed a pH above the desired pH. Materials containing 650 and 750 g kg^{-1} dry matter showed the lowest concentrations of NH_3-N. This fact indicates that haylages with higher levels of dry matter present lower protein losses after aerobic exposure. The increase in dry matter contents of the treatments provided higher crude protein contents to the materials (being 112.91 g kg^{-1} of dry matter) in the treatment with 750 g kg^{-1} of dry matter. The best results for the proliferation of LABs and efficiency in pH decrease were in the treatment with 450 g kg^{-1} of dry matter.

Key words: Haylage, chemical composition, *Lolium multiflorum,* silage, microorganism.

INTRODUCTION

Annual ryegrass (*Lolium multiflorum* Lam.) is widely cultivated in the world for various purposes (Choi et al., 2017), especially in animal feed. Dairy farms depend heavily on ryegrass for high-quality winter food to cows (Han et al., 2014). In this food, the conserved forms used with silage, hay and haylage are keys to herd feeding in shortage periods or during the year (Meinerz et al., 2015). Using this temperate cereal for conservation can be a viable option given their shorter cycle than maize; they can adapt to decreased rainy seasons and frost

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resistant as well as make better use of water by providing early forage or a silage cut for dry season (Celis-Alvarez et al., 2016).

Corn silage is of great importance for dairy cattle feeding systems, demonstrating high rates of mass and energy production at a relatively low cost (Hernandez-Ortega et al., 2011). However, maize silage has low protein content compared to ryegrass. Thus, ryegrass conservation can be a promising option for dairy farms, especially in regions favorable to the production of this forage.

In the conservation of fodder, conservation is sought by the reduction of available water in the hays, anaerobic fermentation in the silages, and both preservation tools in the haylage. The principle of forage conservation through haylage and silage is based on anaerobic fermentation, aiming to provide sufficient amounts of lactic acid to promote a drop in pH (Soundharrajan et al., 2017) and inhibit undesirable microorganisms (Nath et al., 2018). After harvester, silos are used in silage storage, while in the haylage, the bales are individual with plastic wrapped (Han et al., 2014).

In haylage of ryegrass, the pre-drying after the cut is an excellent technological alternative for improving the fermentation pattern of haylages (Nath et al., 2018) and to preserve the nutritional characteristics of original forage. However, the adequate DM content for pre-drying is not known to retain the original nutritional characteristics of ryegrass and to prevent the proliferation of undesirable microorganisms.

Thus, the objective of this work is to evaluate the nutritional and microbiological value of pre-dried annual ryegrass (*L. multiflorum* Lam.) stored with different dry matter contents in anaerobic environment.

MATERIALS AND METHODS

The experiment was conducted at the Animal Nutrition Laboratory of Unipampa - Uruguaiana Campus, located at Latitude 29° 45 '17 "S and Longitude: 57° 05' 18" W, at an altitude of 66 m, Rio Grande do Sul, Brazil. The design was completely randomized with six treatments and four replications. The treatments studied were different dry matter (DM) contents (250, 350, 450, 550, 650 and 750 g kg^{-1}) of annual ryegrass stored in anaerobic environment.

The ryegrass implantation was done in the month of May and its harvest was done with a tractor harvester placed 5 cm in the soil, during the phenological stage of pre-flowering. The cut material was ground in a hammer mill for conditioning and was exposed to the sun for natural dehydration. In dehydration, the material was placed on a plastic canvas with a layer of 10 cm where it remained for a period of time sufficient to reach the desired DM content for ensiling according to the treatments. In the monitoring of DM contents, 100 g samples at 15 points were collected every 2 h for instantaneous measurement (Lacerda et al., 2009).

After reaching the desired MS content, the forage was stored in experimental silos made with polyvinyl chloride (PVC) pipes of 50 cm high and 10 cm diameter. The silos were sealed with caps equipped with Bunsen type valves for the free escape of the gases fixed with the aid of adhesive tape. For the drainage of the effluent produced, 0.5 kg of dried and autoclaved sand, insulated by a cotton cloth, was conditioned at the bottom of each silo. Quantities of 2.210, 2.100, 2.090, 1.240 and 1.000 kg for the DM content of 250, 350, 450, 550, 650 and 750 g kg^{-1} were conditioned. Thus, the stocking densities of forage were obtained: 563, 535, 532, 420, 316 and 255 kg m^{-3} and forage DM of 141, 187, 240, 231, 205 and 191 kg m^{-3}.

In the silos unloaded after 60 days, the upper and lower portions of each silo were discarded (5 cm) with posterior homogenization and sampling of the remaining silage to study the microbiological and bromatological profile of the silages. The fermentative characteristics were studied by determination of pH and ammoniacal nitrogen (AN), being the previous item determined in relation to the total nitrogen (NH_3-N/TN) in the 60 days of fermentation. The pre-drying was determined in samples of 350 g, by drying in oven with forced circulation of air under a temperature of 55°C for 72 h. The pH and NH_3-N were determined in the independent samples according to Silva and Queiroz (2009) and Bolsen et al. (1992), respectively.

The bromatological profile was determined in the samples after obtaining the DM and milling in mill of Willy type knives with stainless steel chamber and sieve, using 1 mm mesh. The dry matter correction at 105°C and contents of organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin, cellulose and hemicellulose were determined (Van Soest et al., 1991). Nitrogen bound to neutral detergent fiber (NIDN) and nitrogen bound to acid detergent fiber (NIDA) were estimated in relation to total nitrogen (TN). Fractions of carbohydrates were estimated according to Sniffen et al. (1992). Total digestible nutrients (TDN) were estimated according to Bolsen et al. (1996) and the relative value of forage.

The microbiological characteristics were studied through the determination of the microbial populations according to Silva et al. (2007). After collection of samples these were homogenized and diluted in the proportion of 10 g to 90 mL of peptone water, obtaining a dilution of 10^1 until 10^8. Afterwards, the samples were inoculated in selective culture media. For the growth and counting of filamentous fungi and yeasts, the Potato Dextrose Agar media was used, maintaining the plates at room temperature for 5 to 7 days. For developing the Acid lactic bacteria (LAB), Lactobacillus MRS Broth media were used in the oven at 35°C for 72 h; for developing Enterobacteria, the Violet Red Bile Agar (Oxford) media were used and maintained at 35°C for 48 h; for developing Clostridia, the Reinforced Clostridial Agar media were used and maintained at 35°C for 48 h in anaerobic chamber. After the incubation period, colony forming units (CFU) between 30 and 300 CFU per Petri dish were counted, and the results expressed in $\log_{10}$ CFU g^{-1} of DM (McDonald et al., 1991).

For statistical analysis of the data, they were submitted to analysis of variance and the means were analyzed by the regression analysis (linear and quadratic models tested). The chosen models were based on determination coefficients (R^2), and significance level (to a 5% level) of the regression coefficients. All the analyses were carried out on Sisvar Statistic Program (Ferreira, 2011).

RESULTS AND DISCUSSION

NH_3-N showed a decrease with increasing DM content of forage stored in 0.02 g kg^{-1} of total N for each gram plus DM (Table 1). The NH_3-N quantification in silages is relevant, since it points out the protein that was degraded during the fermentation phase, being one of the main indicators of the quality of the fermentation process (Santos et al., 2010). Its reduction implies better nutritional quality silage, due to the lower loss of nitrogenous compounds. This is used for the development of undesirable microorganisms and is explained by the decrease in CP and TDN (Table 1), with increase of NH_3-N in the forage (250 g kg^{-1} DM), where these microorganisms are greater among the treatments studied (Table 1).

According to McDonald et al. (1991), the values of NH_3-

N above 100 g kg^{-1} in silages indicate poor preservation of ensiled mass; this ammonia arises from the catabolism of amino acids during the fermentation process. At the lower DM content studied, the percentage of NH_3-N was higher in relation to the others and exceeded the limit suggested by McDonald et al. (1991) for good quality silages. High amounts of NH_3-N decrease the amount of N available for ruminal metabolism due to its high volatilization rate after silo opening.

The dehydration process in the field provided an increase in DM content of ryegrass; however, this was accompanied by an increase in pH values of 0.0107 pH units for each 1 g kg^{-1} increase in DM content (Table 1). This increase makes it difficult to lower the pH after ensilage for adequate food preservation, favoring the proliferation of undesirable microorganisms inside the silo. The pH presented at the time of opening of the silos also showed a linear increase of 0.003 pH units for each 1 g kg^{-1} of DM increase in ryegrass, being that the materials with contents above 550 g kg^{-1} of DM showed a pH above the desired pH (Table 1).

The pH increased with increasing DM content of the ensiled fodder. This increase is due to the higher percentage of oxygen contained in the stored fodder, since the increase in DM also entails greater compaction difficulties, and the presence of residual oxygen delays the establishment of lactic acid bacteria. To limit the growth and proliferation of undesirable microorganisms and to guarantee an adequate fermentation inside the silo, it is necessary that the pH of the silage is less than 4.0 to promote the conservation of nutrients for long periods (Arriola et al., 2011). However, the verification of the pH of the ensiled material alone is not a definitive parameter for silages with high MS content, as opposed to materials with lower concentrations, which is still considered a good standard of evaluation of the fermentative quality of silages (Cherney and Cherney, 2003).

A linear increase of 0.333 g kg^{-1} in DM losses for each 1 g kg^{-1} of increase in DM content of ryegrass was observed (Table 1). These losses are due to the continuity of the respiratory processes inside the silos. This is due to compaction of forage, which is difficult during the ensiling process of materials with high DM contents. The increasing losses of DM in silages occur mainly in grasses that contain reduced levels of fermentable carbohydrates. With the increase of the DM content of the forage to be ensiled, a reduction in the density of the silage fodder also occurred, allowing the maintenance of a greater amount of air inside the silos, favoring the prolongation of the aerobic phase of the ensilage and the continuity of the respiratory processes. During the cellular respiration inside the silos, part of the fermentable substrates is used by the aerobic microorganisms for energy production, causing the consumption of DM and the production of CO_2 and water.

Around 75-90% of the total nitrogen is in the form of protein. During ensiling, proteolysis occurs, causing 40-60% of the nitrogen to be solubilized in non-protein nitrogen compounds (McDonald et al., 2010). CP increased with increasing DM contents (Table 1). This result is due to the lower proteolytic activity and lower loss of nitrogen compounds during the fermentation, since the extent of proteolysis decreases with increasing MS contents. According to Özelçam et al. (2015), determining CP of ryegrass silages and hay presented lower results than those found in this study, being 8.91 g kg^{-1} of DM for silage and 6.35 g $kg^{-1}$of DM for hay, which indicates that the pre-drying of the materials contributed to the increase of CP.

The levels of nitrogen bound to neutral detergent fiber (NIDN) and nitrogen bound to acid detergent fiber (NIDA) decreased with increasing DM content of the ensiled material, indicating a reduction of the proteolysis inside the silos. This results in a lower loss of nitrogen compounds. Elevation of DM content inhibits the development of microorganisms' population of the *genus* Clostridia and proteolytic potentials in silages.

Large swings were observed in the materials (Figure 1). In most of the samples, the silos temperature was lower than the ambient temperature, evidencing that there was no excessive heating of the ensiled material regardless of the DM content adopted. Materials stored immediately with adequate compaction tend not to obtain temperatures above 5 to 8°C at room temperature (Kung et al., 2018), which can be observed at the materials with lower DM contents, and remained less exposed during pre-drying to the field, being ensiladed of faster form.

According to McDonald et al. (1991), in the first days after ensiling, until the end of the aerobic phase, it is common to observe the heating of the ensilage material which can last from 48 to 144 h. After this period, when there is anaerobic fermentation inside the silo accompanied by the drop in pH, the temperature decreases and tends to equal the temperature of the environment. This fact can be observed after the 144 h of fermentation (Figure 1), where the temperature inside the silo was lower than the ambient temperature, extending up to the fifteen days of anaerobic fermentation. In materials with DM contents above 400 g kg^{-1} by the slow fermentation, it is common to observe high temperatures, when compared to silage forages with lower DM content; however the results obtained in the treatments with higher DM values were not affected by high temperatures.

Excessive heating of the ensiled material is undesirable. This condition favors the occurrence of Maillard Reaction which is characterized by the non-enzymatic chemical polymerization of soluble sugars and hemicellulose with amino acids of food when the silage temperature rises above 55°C (McDonald et al., 2010). Silages that go through this process will have reduced protein digestibility. If there is high temperature prolongation above 45-50°C, it will result in protein denaturation with

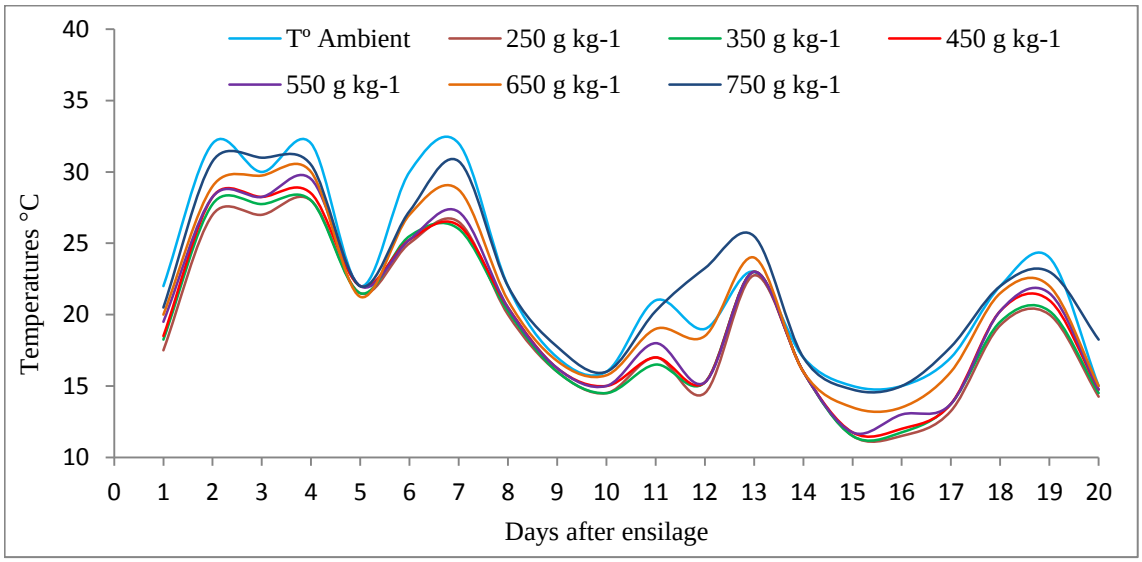

Figure 1. Oscillations at temperatures of stored ryegrass with different levels of dry matter.

consequent increase in NIDA in the ensiled material (Kung et al., 2018). In addition to temperature monitoring, the NIDA contents of the forage before and after ensilage act as indicative of the occurrence or not of this reaction; also, the higher the NIDA content, the greater the losses and the lower the protein available for the ruminal microorganisms.

Materials with high DM contents and prolongation of the aerobic phase due to delay in silo loading and sealing failures are the main factors that predispose Maillard Reaction in silages. In the present study, temperatures above 35°C (Figure 1) were not observed, even in treatments with higher dry matter content, suggesting that ryegrass can be conserved by means of anaerobic fermentation with higher dry matter contents and pre-drying.

No statistical difference was observed between the EE contents of the different treatments (Table 1). Its measurement in animal feed is fundamental because EE provides 2.25 times more energy when compared with carbohydrates and proteins (Garcez Neto et al., 2018). In a study of oat silage, Garcez Neto et al. (2018) obtained a mean result of 29.70 g kg^{-1} of DM of EE after 20 days of storage of the material, lower than the minimum value obtained in this study, which was 31.38 g kg^{-1} of DM (Table 1); also, it is within the range used for conventional ruminant diets. This demonstrates that ryegrass haylage and silages have great potential to provide energy to ruminants when related to their EE.

One of the main sources of energy for the microorganisms present in the rumen are carbohydrates, just as they are needed for food storage in the anaerobic environment. NFCs are the main substrates used for the production of lactic acid to achieve stability inside the silo. The TC contents presented a linear reduction of 0.0590 g for each 1 g kg^{-1} of DM (Table 1). This result is related to the pre-drying of the materials before storage, which during the time that it exposed to the field may have led to volatilization and leaching, consequently causing nutrient losses (Singh et al., 2018). This fact can also be explained by the NFC values, which also obtained a linear reduction, but more marked when compared to the reduction observed in TC; in counterpoint, the contents of FC increased to 0.1398 g in each 1 g kg^{-1} in DM increase, where the pre-drying of the food increased the less volatile fractions, such as FC and as a result decreasing the NFC contents of the forage.

Pre-drying contributed to getting silages with better NDF contents. The data presented a quadratic behavior, with the reduction up to the material with 0.450 g kg^{-1} of DM and subsequent increase (Table 1), but without any result that would impair the nutritional quality of the materials with higher DM content. All treatments generated satisfactory results, being below the 55-60% limit of NDF in the diet of ruminants (Van Soest, 1994).

The ADF contents did not present significant differences among the treatments studied (Table 1). However, it is possible to observe that in the treatment with content of 350 g kg^{-1} DM that obtained high quantification of undesirable microorganisms. The ADF content also increased when compared to the other treatments (Table 2), because these microorganisms consume non-fibrous carbohydrates. When comparing the bromatological composition and microbiological profile of silages of different grasses, Gentu et al. (2018) obtained NDF and ADF higher than those found in this study, but found that the NDF and ADF contents decreased with ensilage of the material and attributed the possibility of bacteria producing enzymes capable of degrading fibers, thus reducing the NDF and ADF content

Table 1. Chemical-bromatology composition of silage and haylage ryegrass with different contents of dry matter.

Variable	Dry matter content g kg^{-1}										Mse (EPM)
	250	350	450	550	650	750	L	Q	Equation	R^2	
NH_3-N	12.96	9.43	7.34	5.18	3.35	2.61	0.000	0.000	Y=17.1196-0.0206x	0.96	0.2283
pH E	6.202	6.213	6.217	6.221	6.228	6.248	0.000	0.115	Y=6.1901+0.0107x	0.92	0.0298
pH	3.84	4.00	4.07	4.66	5.25	5.44	0.000	0.000	Y= 2.7819+0.0035x	0.93	0.0335
DML	4.72	6.52	9.77	16.45	23.13	17.18	0.006	0.142	Y=4.0660+0.3330x	0.80	0.6224
CP	98.16	102.70	103.38	104.37	108.10	112.91	0.021	0.735	Y=91.9396+0.0259	0.93	4.2973
NIDN	11.73	10.86	9.78	9.68	9.58	9.55	0.008	0.153	Y=12.3208-0.0042x	0.78	0.5937
NIDA	6.52	6.03	5.85	5.71	5.32	5.08	0.007	0.909	Y=7.1038-0.0027x	0.97	0.3748
EE	31.57	31.38	31.60	31.50	31.41	31.63	0.968	0.920	-	-	0.8550
TC	793.25	788.90	783.25	774.61	768.47	765.93	0.000	0.887	Y=808.5674-0.0590x	0.98	5.4060
NFC	364.60	346.60	333.38	327.64	299.41	296.22	0.000	0.796	Y=397.8605-0.1398x	0.98	5.4061
FC	423.65	427.30	432.37	441.81	451.26	456.80	0.000	0.625	Y=397.8605+0.1398x	0.96	5.8270
NDF	473.77	467.41	454.99	464.43	478.87	494.42	0.000	0.004	Y=536.8684-0.3454x+0.0003x^2	0.94	6.4502
ADF	302.67	307.29	299.99	301.80	303.62	305.02	0.008	0.000	-	-	3.5448
CEL	265.67	263.15	253.05	251.30	249.55	246.43	0.000	0.001	Y=274.6867-0.0396x	0.91	5.5555
LIG	57.00	49.13	46.94	50.50	49.07	46.10	0.001	0.022	Y=67.936-0.0637+0.00005x²	0.68	1.7856
HEM	176.18	152.63	145.00	162.63	180.26	196.89	0.000	0.000	Y=258.8521-0.4809+0.0054x²	0.92	4.6779
TDN	594.87	622.20	632.25	631.34	622.92	609.93	0.508	0.000	Y=499.3604+0.5137-0.0005x²	0.96	4.3852
Frac C	170.80	152.43	147.82	146.80	153.28	157.00	0.028	0.000	Y=225.3774-0.2957X+0.0003x²	0.92	3.3754
Frac B2	430.81	427.00	417.33	428.86	450.39	473.67	0.012	0.038	Y=498.3348-0.3800+0.0005x²	0.96	12.6718
A+B1	378.39	410.57	449.85	396.33	391.33	381.83	0.429	0.075	-	-	13.9057

NH_3-N: ammoniacal nitrogen (% total N); pH E: hydrogen potential in the ensilage; pH: hydrogen potential; DML: dry matter loss (%); CP: crude protein; NIDN: nitrogen bound to neutral detergent fiber; NIDA: nitrogen bound to acid detergent fiber; EE: ether extract; TC: total carbohydrates; NFC: non-fibrous carbohydrates; FC: fibrous carbohydrates; NDF: neutral detergent fiber; ADF: acid detergent fiber; CEL: cellulose; LIG: lignin; HEM: hemicellulose; TDN: total digestible nutrient; Frac C: C fraction of carbohydrates; Frac B: B fraction of carbohydrates; A+B1: A+B1 fraction of carbohydrates.

of silages. This same behavior can also be observed in the present study.

Horst et al. (2017), comparing the bromatological composition of pre-dried silages of different oat cultivars, obtained a mean of 85.70 g kg^{-1} of DM for lignin. It is higher than that found in this study (Table 1) which is a mean of 49.70 g kg^{-1} of DM. The forage that obtained the highest result was the treatment of 250 g kg^{-1} of DM (Table 1); however it remained within the standards for good quality fodder. The lignin represents the completely indigestible portion of the plant, being negatively related with the capacity to take advantage of the ingested fodder. The results of hemicellulose showed a quadratic regression model, with a reduction up to the DM content of 450 g kg^{-1}, and a subsequent increase (Table 1). The increase of this carbohydrate in forage DM can be considered as a reflection of the reduction in the other constituents of the plant. The hemicellulose has constituents like xylan, which occupies a large part of its fraction and requires specialized systems such as xylanolítico to make its degradation (Saratale et al., 2012).

The cellulose fraction was set to linear regression with a reduction of 0.0396 g for each 1 g kg^{-1} of DM. Cellulose is a fraction of the cell wall that is formed basically by long linear chains of high molecular weight and a high degree of polymerization (Giger and Reverdin, 1995). These

Table 2. Estimates of consumption, energy, relative value of forage and microbiological profile of haylage ryegrass after opening.

Variable	Dry matter content g kg^{-1}										
	250	350	450	550	650	750	L	Q	Equation	R²	Mse (EPM)
CDMRLW	2.54	2.57	2.64	2.58	2.51	2.43	0.000	0.004	Y=2.2032+0.0018x-0.000002x^2	0.94	0.0343
DMD	653.22	649.62	655.31	653.90	652.48	651.39	0.000	0.932	-	-	2.7616
GE	4801.75	4840.45	4853.56	4955.61	5064.41	5109.68	0.000	0.652	Y=4607.0330+0.6610x	0.97	10.6925
DE	2855.57	2833.63	2912.61	2955.72	3002.12	3014.49	0.000	0.000	Y=2737.1398+0.3837x	0.92	36.0055
RVF	128.37	129.49	134.00	130.98	126.75	122.62	0.000	0.004	Y=110.2749+0.0978x-0.0001x^2	0.90	1.8016
FF	5.45	5.31	4.99	4.95	4.86	4.18	0.000	0.178	Y=6.0596-0.0022x	0.86	0.1394
LACT	5.27	5.30	4.93	4.87	4.81	4.60	0.000	0.980	Y=5.6568-0.0013x	0.91	0.1181
ENT	4.62	4.53	4.44	4.44	4.47	4.59	0.654	0.058	-	-	0.0835
CLOST	5.17	5.10	4.69	4.54	4.39	4.08	0.000	0.868	Y=5.7707-0.0022x	0.97	0.1058

CDMRLW: consumption of dry matter in relation to live weight (%); DMD: dry matter digestibility (g kg^{-1}); GE: gross energy (kcal kg^{-1} of DM); DE: digestible energy (kcal kg^{-1} of DM); RVF: relative value of forage; FF: filamentous fungi; LACT: Acid lactic bacteria; ENT: Enterobacteria; CLOST: Clostridia.

characteristics make it difficult to break during fermentative processes inside silos and in the rumen. High levels of this carbohydrate are negatively correlated with forage quality (Van Soest, 1994). The results obtained in this study for constituents of the plant cell wall were similar to those found by Horst et al. (2017), reaffirming that the pre-drying of forages is able to reduce the content of lignin and cellulose proportionally; consequently increasing the content of hemicellulose present in the forage and thus contributing to the nutritional quality of silages. The losses of energy in the silages are generally more associated to losses of DM due to aerobic deterioration than to losses due to fermentation processes, being one of the facts that the treatment with 250 g kg^{-1} of DM presented the lowest levels of TDN. According to Sniffen et al. (1992), the carbohydrates present in the plants can be classified according to the rate of degradation.

The fraction A that is rapidly degradable is composed of soluble sugars; the intermediate degradation fraction B_1 is composed of starch and pectin; B_2 presents slow degradation, containing the available cell wall; and the fraction C that does not present degradation is composed of the cellular wall that is unavailable mainly by the lignin. Frac C presented quadratic regression, with 492 g kg^{-1} reduction of DM, and a subsequent increase (Table 1). This result is related to the LIG content present in fodder, which also set up the quadratic regression model. This fraction is of low nutritional quality and has a very low degradation in the digestive system of ruminants. It needs specified systems of fungi so that other microorganisms may have some access to this phenolic compound, being of low or no rumen degradability and detrimental when present in high concentrations. The highest value was observed in the treatment with 250 g kg^{-1} of DM. The quadratic regression also explains the data of Frac B_2, with reduction up to 380 g kg^{-1} of DM and subsequent increase. The Frac B_2 fate will depend on digestion and passage rates as its fermentation occurs mainly in the rumen and part of the large intestine (Sniffen et al., 1992). The behavior observed in this fraction is related to the HEM and CEL, which are potentially digestible structural carbohydrates of the plant cells and are available for the nutritional exploitation of the ruminants at the digestive level. The fraction A + B_1 is composed of soluble sugars and starch, respectively. The junction of these fractions is accomplished by the difficulty of quantifying these fractions separately. The A + B_1 fraction did not fit the regression models tested (Table 2).

The results of CDMRLW presented quadratic behavior (Table 2) and are directly related to NDF results, where forages that present higher NDF contents, consequently will have a limitation and decrease of CDMRLW. The contents of this variable increased until the use of the 450 g kg^{-1} of DM, with subsequent reduction (Table 2). The treatments of 650 and 750 g kg^{-1} of DM presented the lower results for CDMRLW (Table 2).

When the chemical composition is very variable between crops, both in fresh and preserved fodder, this is reflected in the digestibility of the

Table 3. Main components and indicatives of material quality after aerobic stability (9 days of aerobic exposure).

Variable	Dry matter content g kg^{-1}										
	250	350	450	550	650	750	L	Q	Equation	R²	Mse (EPM)
NH_3-N	14.00	16.42	17.89	11.28	4.66	3.01	0.233	0.000	$Y=4.2551+0.6646x-0.0094x^2$	0.88	0.9022
pH	5.82	4.80	4.44	4.98	5.52	5.62	0.000	0.000	$Y=8.5809-0.01595x+0.0016x^2$	0.73	0.2760
CP	91.71	97.94	86.29	95.05	103.81	99.81	0.000	0.135	-	-	50.971
TDN	651.99	642.62	655.33	657.60	659.88	666.44	0.000	0.725	$Y=652.5373-0.3157x+0.0067x^2$	0.76	53.502
CDMRLW	2.13	2.32	2.60	2.57	2.56	2.45	0.000	0.000	$Y=1.0643+0.0540x-0.0004x^2$	0.95	0.0673
DMD	637.03	626.61	640.76	643.29	645.82	653.12	0.000	0.725	$Y=637.64440-0.3511x+0,0075x^2$	0.76	59.532

NH_3-N: ammoniacal nitrogen (% total N); pH: hydrogen potential; CP: crude protein; TDN: total digestible nutrient; CDMRLW: consumption of dry matter in relation to live weight (%); DMD: dry matter digestibility (g kg^{-1}).

silages and haylages, mainly due to the fiber content of the food that affects the digestibility in quality and quantity (McDonald et al., 2010). However, the estimated digestibility levels (Tables 2 and 3) suggest that ryegrass was harvested with a favorable contribution of non-structural carbohydrates; however, it did not fit the regression models tested (Table 2). After the stability period, DMD levels decreased until the treatment with 350 g kg^{-1} of DM and subsequent increase (Table 3).

The maturation stage of the plants influences the conservation of the forage and its nutritive value (Wilkinson and Davies, 2012); thereafter, it was possible to verify the interference of the quality indicators from the energy estimates after the storage of the materials. The estimate of GE is directly related to CP and pH levels of the food. This variable showed a positive linear regression and an increase of 0.6610 kcal kg^{-1} of DM was identified for each gram of DM in treatments (Table 2). The highest result was in the treatment with 750 g kg^{-1} of DM, which coincides directly with the results of CP (Table 1). The DE is not only directly related to the GE but also correlates with the concentrations of ADF present in OM, and lower levels of ADF in OM are indicators of higher levels of ED. For the estimates of ED, a positive linear regression with increase of 0.3837 kcal kg^{-1} of DM for each gram of augmented MS was also observed (Table 2).

Forage RVF is an indicator of quality when referring to concentrations of plants' cell wall constituents. The higher the contents of cellulose, hemicellulose and lignin the lower the RVF of the food, indicating materials of lower or higher quality. The values found in the present study made it possible to generate a quadratic equation that indicates an increase in RVF up to the level of 450 g kg^{-1} of DM with a subsequent decrease in this variable (Table 2). This result corroborates with those presented in Table 1 regarding the NDF contents of the treatments, being the treatment of 750 g kg^{-1} of DM that presented higher levels of NDF and consequently lower levels of RVF.

The presence of anaerobic microorganisms, such as lactic acid bacteria (LAB) during the forage fermentation process is fundamental. The LABs are the principals responsible for using substrate carbohydrates to produce lactic acid, consequently reducing the pH of the ensiled material after the silo closes. However, it is also possible to observe the growth of other agents, such as enterobacteria and Clostridia (McDonald et al., 2010).

In this study, it was observed that, as DM content increased, there was a reduction of 0.0022 $\log_{10}$ CFU g^{-1} of filamentous fungi for each 1 g kg^{-1} DM of ryegrass haylage (Table 2). This result is explained by the low humidity of the materials, inhibiting the growth of these microorganisms, which develop mainly in materials with higher moisture content and the presence of oxygen. These microorganisms are present mainly in silage with less than 300 g kg^{-1} of DM, as can be observed in this study, where the material with DM content of 250 g kg^{-1} obtained the highest result. Fungi are more common in the silo surface layers, either by access to the air due to storage failures, after opening, during anaerobic removal or deterioration. When present in high amounts they may be malefic, many of them being mycotoxin producers, which decrease the quality of silage in terms of their sanity (Kononenko and Burkin, 2014).

To obtain silages of nutritional and sanitary quality, it is necessary to immediately inhibit the cellular respiration of the plant, inhibiting the growth of undesirable aerobic microorganisms,

which consequently will also decrease the proteolysis performed by these agents and thus conditions favorable to the growth of LAB. LAB provides improvement in the fermentation of pre-dried and silage, and when in greater quantity, controls the growth of undesirable micro-organisms (Soundharrajan et al., 2017). This can be noticed in this study, where the number of colonies of LAB prevailed higher compared to enterobacteria and Clostridia (Table 2).

The highest growth of LAB was observed in the ensiled material with a DM content of 350 g kg^{-1}, and a decrease of 0.0013 log_{10} CFU g^{-1} at each 1 g kg^{-1} of DM (Table 2). This was due to the pH of the ensiled mass, which was above the limit of 4.2 in the treatments with higher DM contents. It is common for materials with MS above 400 g kg^{-1} to increase pH (Kung et al., 2018), which can also be noted in this study. This is because as the content of DM increases it limits the availability of metabolic water for the growth of LAB (Whiter and Kung, 2001).

The values obtained in the quantification of enterobacteria varied between the treatments, and the lowest results were in the treatments of 450 and 550 g kg^{-1} DM (Table 2). The growth of these microorganisms is more evident in the initial stages of fermentation, when the pH of the silage is close to neutrality. This is the environment necessary for its development, because the very enterobacteria themselves have buffer capacity and CO_2 production (Collins et al., 2017). Although in less quantity, the enterobacteria also produce lactic acid, but they damage the sanitary profile of conserved foods. These microorganisms compete for water-soluble carbohydrates with LAB, but the product with the highest concentration at the end of this process is acetic acid, which is detrimental to proper fermentation of the material (McDonald et al., 2010). In this experiment, the different contents of DM and pre-drying contributed to the production of silages with less proliferation of enterobacteria in relation to LAB (Table 2), improving the health profile of silage.

As DM levels increased, there was a decrease of 0.0022 log_{10} CFU g^{-1} for each 1 g kg^{-1} DM of the Clostridia populations (Table 2), evidencing that the higher DM content of the haylage fodder helps in the control of these agents. This fact is related to the pre-drying of the materials, which through the wilting of the ryegrass to the field caused a reduction of humidity and consequently a reduction of the presence of these microorganisms. Gentu et al. (2018), ensiling grasses with low moisture content (<54%), observed the reduction of the populations of these microorganisms as well as the results obtained in this study (Table 2), in which the materials with higher MS content presented lower quantification of populations of this bacterium. This is due to the sensitivity that microorganisms of the genus Clostridia present in relation to water scarcity. The treatment with 250 g kg^{-1} of DM obtained a higher concentration of Clostridia (Table 2), because it contains more than 70% humidity, which is considered an environment conducive to its proliferation (Queiroz et al., 2018). Although the pH of this material remained acidic (3.84) (Table 1), in the low DM, even a pH below 4.00 is not enough to inhibit them (McDonald et al., 2010). It can be observed in the haylages with 650 and 750 g kg^{-1} DM (Table 2) that even with a pH above 5.00, a lower presence of Clostridia was obtained, emphasizing that the higher contents of DM contribute to the decrease of these agents independent of their pH. According to Weirich et al. (2018), good quality haylages are those in which LABs are in higher concentration than the other microorganisms, as can be seen in forage with 750 g kg^{-1} DM (Table 2). After the aerobic stability period, the NH_3-N contents presented a quadratic behavior, increasing until the treatment of 450 g kg^{-1} of DM and subsequent reduction in the other treatments (Table 3). These results are related to the higher presence of undesirable microorganisms in the materials with a higher concentration of NH_3-N, especially in filamentous fungi (Table 2), which are mainly responsible for the aerobic deterioration of silages. Materials containing 650 and 750 g kg^{-1} DM showed the lowest concentrations of NH_3-N. This fact indicates that haylages with higher levels of DM present lower protein losses after aerobic exposure. The pH of the studied forages increased with aerobiosis. This suggests the possible development of aerobic microorganisms that cause material deterioration during exposure to air, which degrade the lactic acid produced by the LAB during the anaerobic process, thus raising the pH of the silages (McDonald et al., 1991).

Although all materials analyzed showed increased pH, when compared to the opening of the silos (Table 1) and after opening in aerobiosis (Table 3), it is possible to observe that in materials with 250 and 350 g kg^{-1} of DM the pH increase was accentuated (1.98 and 0.80 respectively). However, in the treatments with 550, 650 and 750 g kg^{-1} of DM, the increase was 0.32, 0.27 and 0.18 (Tables 1 and 3), respectively.

CP values oscillated between treatments. The highest concentration of this nutrient was obtained in the treatments with 650 and 750 g kg^{-1} of DM. This correlates with the result obtained for NH_3-N, with lower CP losses, and a higher concentration (Table 3). The inverse was observed in the treatment of 450 g kg^{-1} of DM, where it presented the highest concentration of NH_3-N and consequently the lowest value for CP (Table 3), because it has greater protein loss during aerobic exposure. TDN data after the aerobic stability period presented a quadratic behavior, with reduction up to the treatment of 350 g kg^{-1} of DM, followed by a subsequent increase (Table 3). These results are possibly related to the higher CP concentrations in treatments with high DM content.

Conclusions

The increase in DM contents of the treatments provided higher CP contents to the materials (being 112.91 g kg^{-1}

of DM) in the treatment with 750 g kg^{-1} of DM. Higher DM levels contributed to the decrease of NH_3-N contents at the time of opening and during the aerobic exposure period. The temperature of the stored forage remained stable, not exceeding 35°C. This shows the reduction of undesirable microorganisms with higher DM levels in the treatments.

The best results for the proliferation of LAB and efficiency in pH decrease was up to treatment with 450 g kg^{-1} of DM. The different contents of MD tested for the conservation of annual ryegrass in an anaerobic environment can be used; however, different nutritional levels were found.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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