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Full Length Research Paper

Towards sustainable stakeholder engagement in smallholder irrigation schemes in Zimbabwe.

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Smallholder irrigation schemes in most developing countries including Zimbabwe have proved to be unsustainable after withdrawal of external assistance. The pre-independence community owned smallholder irrigation schemes had a fairly efficient management framework that, unfortunately, lacked the community ownership and professional execution. The post-independence smallholder irrigation schemes were heavily subsidized and failed to effectively empower the farmers to be managers of their own entities. This study was aimed at examining the stakeholder engagement and the sustainability of smallholder irrigation schemes. Understanding the problems faced in the engagement of these stakeholders will go a long way in enhancing the sustainability of the irrigation schemes. Three smallholder irrigation schemes from the Southern Eastern Low veld of Zimbabwe were purposively selected for the study. A total of 130 farmers were interviewed using questionnaires, 11 interviews of key informants and 3 focus group discussions with farmers in the 3 schemes were conducted. The study revealed that farmers had unsustainable sources of livelihood that were compromising their commitment to schemes. Due to very low levels of literacy, farmers were not participating in training programmes that were aimed at improving the production level. The farmers were struggling to pay the schemes' utility bills and the billing systems were perceived to be unfair. The Government departments involved in the schemes were under resourced and less skilled to leverage sustainable commercial production in the schemes. Private sector participation was very minimal. The stakeholder engagement in the schemes lacked owners and the development agencies were failing to involve the beneficiary farmers on strategic issues about their scheme.

Key words: Sustainability, smallholder irrigation schemes, stakeholder.

INTRODUCTION

Globally, irrigated agriculture plays a very important role in food security and livelihood improvements, especially in Asian farming systems and in most parts of Africa, especially West Africa (Dittoh, 1991; World Bank, 2008). Irrigated agriculture is the most viable means of reducing food crop failure, hunger, and malnutrition in Africa, and

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an effective means for improving the competitiveness of smallholder farming in most parts of Africa (Meinzen–Dick et al., 1993). The pre- and post- independence histories of irrigation schemes of smallholder community in Zimbabwe exposes serious pitfalls in the engagement of relevant stakeholders involved in their establishment, rehabilitation and management, thereby compromising their sustainability.

The history of the development of smallholder community irrigation schemes in Zimbabwe can be traced back from the colonial era, where the Ministry of African Affairs helped in the schemes development. From 1912 to 1927, farmers enjoyed considerable autonomy in the development and management of their schemes, without much interference from central government (Alvord, 1933). Between 1927 and 1945, the Ministry started taking over the management and control of schemes, imposing compulsory crops like beans and wheat, under the name of technical assistance and famine relief (Rukuni, 1984; Manzungu, 1995; Meinzen–Dick, 1993). Smallholder irrigation farmers were made to surrender their dry land plots from 1936, and the government would identify, design and construct new schemes without consulting the intended beneficiaries- the indigenous population (Rukuni, 1988; Rukuni and Makadho, 1994). The regulations obliged farmers to solely depend on irrigation, discouraged them from involvement in rain-fed agriculture or off-farm activities and stipulated the types of commercial crops to be grown, following a prescribed cropping calendar and inputs (Manzungu, 1995). All the smallholder irrigation schemes were under the supervision of the then District Commissioner. With the land apportionment Act in 1948 and 1956, the Department of Native Agriculture took over the development and management of smallholder irrigations (Rukuni, 1988). The Control of Irrigable Areas Regulations of 1970 required farmers to sign annual renewable permits for residence, managing stock and cultivation- which were obligatory in terms of Section 9 (1) of G.N. 69/70). This clause allowed eviction of farmers not complying strictly with payment of fees and cultivation practices- reducing farmers to tenants in the schemes (Manzungu, 1995). There was widespread opposition by farmers to these requirements culminating in the closure of some schemes in the early 1970s (Roder, 1965; Manzungu, 1995). The post-independence smallholder irrigation schemes thrust was on poverty alleviation, to enhance food security, create employment, curb rural urban drift and modernize peasant farming (Manzungu and van der Zaag, 1996; Matsika, 1996; FAO, 1997; Chancellor, 2004; FAO, 2002; Makombe and Sampath, 2010). The schemes were all heavily subsidized by the Government. The Department of Rural Development (DERUDE) retained the development and management of irrigation schemes while the design and planning was transferred to the Department of Agricultural, Technical and Extension Services, (AGRITEX). Before transferring development and management functions to AGRITEX in 1985, DERUDE introduced the concept of Irrigation Management Committees to promote democracy in the running of the schemes (Chidenga, 2003). AGRITEX retained the management responsibility of the schemes until 2001. For the smallholder irrigation farmers-out grower model under the management of the Agricultural and Rural Development Agency (ARDA), the Government designed operate and transfer schemes in which ARDA was expected to gradually transfer management and ownership to the smallholder irrigation farmers (FAO, 2001). Unfortunately, with sudden changes in economic and agricultural policies between 1980 and 1992, the ARDA's management transfer failed to be as gradual as was planned to the detriment of the farmers (Ruigu and Rukuni, 1990; Mombeshora, 2003).

A new Water Act replaced water rights and water permits in the 1990s- introducing Catchment Councils to manage water. This led to the creation of ZINWA (under the Ministry of Water Development) to manage water and the Catchment Councils (Chidenga, 2003; Makombe et al., 2004). In 2000, all new schemes were compelled to directly register themselves as clients of the Zimbabwe Electricity Supply Authority (ZESA) so that electricity bills could be billed directly to the plot holders (Chidenga, 2003). These new developments, in keeping with the Economic Structural Adjustment Programme (ESAP) and the user pay principles, led to the direct rise in production costs for the small holder farmers.

In 1999, 81% of area under irrigation was occupied by large scale commercial farms, 8,5 by government farms, 2% by out grower schemes whiles the smallholder schemes (including small-scale purchase areas) occupied 8.5% (AGRITEX, 1999). From the early 1980s up to the time of ESAP, 100% of capital costs and 89% of recurrent costs for community owned smallholder irrigation schemes were covered by the government (Scoones, 2013). These heavy subsidies provide little incentive for investments into privately owned smallholder irrigation farming by individual farmers. Therefore, individually owned small-scale irrigation farming, has not attracted the attention of as many researchers and development stakeholders as community owned smallholder irrigation schemes.

The agricultural sector suffered considerable neglect since early 2000, due the chaotic Fast Tract Land reform program, bad publicity and Zimbabwe's international isolation, resulting in lack of investment in the agricultural sector (including in research and development) and decreased productivity (Nhundu and Mushunje, 2010; Makadho, 2000). In addition, there was a lack of public–private partnership investment in the rural and agricultural commodity sector—a prerequisite and important catalyst for agricultural development and food production in developing countries (Chidenga, 2003). There was also a reduction in donor funding and foreign direct investment for agriculture since 2001. For example, between 1980 and 2002, multilateral institutions slashed official development assistance on agriculture, to Zimbabwe, from US$3.4 billion to US$500 million, an 85% decline (Poulton et al., 2002; Nhundu and Mushunje, 2010).

All these unfortunate developments culminated in Zimbabwe's worst economic crisis in its history, between 2000 and 2009, characterized by food shortages and record inflation. The annual inflation rate which averaged 12% in the 1980s skyrocketed to a record high of 11.3 million percent as of June 2008 (Nhundu and Mushunje, 2010). The economic melt- down during the 15 years preceding 2009 led to reduced capacity of the relevant Government departments, owing to high staff turn-over (which affected NGOs as well) and poor resources, to give sufficient support to the irrigation farmers (Mutambara and Hungwe, 2011).

The post hyper-inflation era in Zimbabwe attracted a lot of donor support with the focus of interventions shifting from relief programmes to longer term food security and livelihood recovery programs. This phase witnessed an increasing number of NGOs participating in the development, rehabilitation and management of smallholder irrigation schemes. Unfortunately, some community irrigation schemes that were rehabilitated after the hyper- inflation phase became non-functional or malfunctional barely 3 years after rehabilitation. The Zimbabwe Rural Vulnerability Assessment for 2012 revealed that 24% of the wards in Zimbabwe had irrigation schemes and of the wards with irrigation schemes, 38% had functional schemes, whilst 30% had partially functional schemes. Thirty two percent of the wards had non-functional irrigation schemes amongst which were smallholder community irrigation schemes rehabilitated in the post inflation era (ZimVac, 2012). In the year 2013, only 40% of the irrigation schemes were fully functional (ZimVac, 2013). The biggest challenge faced by Zimbabwean smallholder irrigation schemes, as is the case with the whole of Africa, is to ensure that farmers are able to sustain their functionality status (Karugia, 2003; Svendsen et al., 2009; Webb, 1991). Considering the fact that the development and management of smallholder irrigation schemes involve a variety of stakeholders, the process of making them sustainable demands the use of holistic and more informed stakeholder engagement strategies (Koopman et al., 2001; FAO, 2000, 2001; Filcak et al., 2006).

The responsibility to manage and ensure functionality of communal irrigation schemes is split among several independent stakeholders with different interests and competencies, who require deliberate coordination effort to ensure smooth integration and cooperation. It is therefore critical that all the stakeholders are effectively engaged to ensure sustainability of the schemes (Commonwealth of Australia, 2000; Institute of Social and Ethical Accountability, 2005). No known research has explored how the different stakeholders involved in smallholder irrigation schemes in Zimbabwe have been engaged and how the different engagement strategies have been affecting their sustainability. The main objective of the study is to investigate the factors that affect effective engagement of stakeholder in the smallholder irrigation schemes in Zimbabwe with a view to identifying loop holes in the engagement process that can be targeted for intervention and to recommend empirically based solutions to unlock the potential of these schemes.

MATERIALS AND METHODS

Three community-owned smallholder irrigation schemes were purposively selected for the study. Two of them (Tsvovani and Dendere) were operating below capacity while the other one (Mtandahwe) has been operating at full capacity in the 3 years preceding the survey. They were therefore, purposively chosen to be laboratories for the investigation of stakeholder engagement challenges facing community owned smallholder irrigation schemes (Tsovani and Dendere) and a locally designed solution for such challenges based on a successful example (Mtandahwe) close-by and within the same agro-climatic zone. All the 3 irrigation schemes had the same irrigation technology- flooding and had Save River as their source of water.

This research was an exploratory case study in which both quantitative (questionnaire survey) and qualitative (Focus group discussion and key informant interviews) research methods were employed. A semi structured household questionnaire was used to collect both quantitative and qualitative data from the irrigation plot holders in 3 small holder irrigation schemes. Data obtained from the questionnaire survey was augmented by Focus Group Discussions (FGDs), key informant interviews and direct observations targeting the 3 schemes. The combination of different research methods (questionnaire survey, FGDs and key informant interviews) allowed for triangulation of information. The simple random sampling method was used to select 40% of the farmers in the targeted schemes for questionnaire survey. The names of all the farmers were put in hat and 40% were randomly picked for the interviews. Tsovani (300 ha), Dendere (20 ha) and Mtandahwe (23 ha) irrigation schemes had a total membership of 120, 38 and 167 farmers respectively. Therefore, a total of130 farmers were interviewed in the questionnaire survey. The farmer to irrigated surface ration for Tsvovani irrigation scheme was lower than the other two because Tsvovani was formerly designed to be institutionally managed by Agricultural and Rural development Authority (ARDA) in an arrangement in which each farmer, as an out-grower owned 3 ha. Dendere and Mtandahwe were designed to be community managed and each farmer owned an average of 0.1 ha, possibly to avert possible challenges in managing bigger hectarages (Chidenga, 2003). Three FGDs were conducted in the three schemes (one FGD per scheme). FGD participants were selected from the farmers who had not participated in the questionnaire interviews. In order for one to qualify to be a participant in the FGDs, the farmer needed to have been working in the scheme consistently in the 3 years preceding the survey so that they could give meaningful contributions to the discussions. A total of 10 farmers participated in each FGDs, to give a total of 30 participants (50% of which were females). District Heads of institutions from Agricultural Research Technical and Extension Services (AGRITEX), Department of Irrigation (DOI), Zimbabwe Electricity Supply Authority (ZESA), Zimbabwe National Water Authority (ZINWA) and Rural District Council (RDC) from the two Districts (Chipinge and Chiredzi) were interviewed as key informants to the study. Eight key informant interviews were conducted with these institutions to provide institutional perspective on the sustainability of the irrigation schemes. Three key informant interviews were also conducted with the Irrigation Management Committees (IMCs) of the three schemes to give a total of eleven key informant interviews conducted in this study. The data obtained from the questionnaire survey was inputted into SPSS version 16.0 (Statistical Package for Social Scientists). Data was subjected to both descriptive analysis (frequencies and percentages and averages) and advanced statistical analysis in the form of one way ANOVA and Chi-square.

RESULTS AND DISCUSSION

Demographic characteristics of the respondents

The majority of the respondents were between 30 and 69 years of age. Fifty-eight percent of the respondents were females while 42% were males. Seventy-two percent of the respondents were married, 21% were widowed while 6 and 3% were single and divorced, respectively. The average household size for all interviewed households was 7 against 5 at national level (ZimVac, 2012). The sex and age disaggregation of the farmers in the schemes confirmed the report of Muparange (2002) which showed that, in smallholder irrigation schemes**,** the most interested people were females and that the youth were generally not interested in agricultural production. This can impose potential threats to the future sustainability of these schemes since no institutional memory will be left after the current generation of farmers got out of picture.

Sixty-eight percent of the households had children less than 5 years of age with an average of 3 children under 5. Twenty percent had members who were chronically ill, 3% had terminally ill patients. Four percent had at least a member who was disabled or mentally ill and 37% had orphans. These findings are in line with national estimates which revealed that for all the rural households, 30% had orphans, 8% had a chronically ill or a mentally or physically challenged member (ZimVac, 2012). The vulnerability status of the households has a direct negative bearing on the viability of irrigation schemes in that, all the vulnerability categories need to be looked after by women who usually provide labour in the schemes. Parker et al. (2009) argued that shocks to households from diseases like HIV/AIDS can reverse developmental progress threatening economic sustainability of smallholder farming systems.

Educational level of the farmers

The level of illiteracy was on average higher than the national average, with an average of 37% (26% females and 11% males) of the farmers having not attained any level of education against a national average of 18.7%. This was especially true for Dendere and Tsvovani whose illiteracy level was 60 and 65% respectively, while at Mtandahwe, only 12% of farmers had not attained any education at all as shown in Table 1. Less than 2% were educated beyond Ordinary Level against a national average of 3% (ZimVac, 2011).

The differences in the level of education of members in the three irrigation schemes were found to be significant by one way ANOVA at $P < 0.005$, in favour of Mtandahwe irrigation scheme which had the least number of farmers that had not attained any level of education. In Dendere, AGRITEX officers confirmed that due to the very low levels of literacy, farmers were not participating in training programmes that were aimed at improving the production level. The production of high value horticultural crops in irrigation schemes is usually knowledge intensive and the level of education of the farmer can be an important variable in the choice of crop and level of production. In Sub Saharan Africa, low level of education has been blamed for limiting access to information and understanding of commercial farming concepts which are critical to sustaining high production levels in irrigation schemes (Shah et al., 2002).

Irrigation farmers and casual labour

Farmers were also engaged in casual labour, locally termed “magau” which involves weeding, cutting cotton straws, picking cotton and watering gardens for other people in order to supplement their production. Fifty-eight percent of the farmers (45% females and 13% males) 26, 13 and 19% from Tsvovani, Mtandahwe and Dendere respectively, were engaged in casual labour.

The variety of livelihoods activities employed by the farmers in the three irrigation schemes may act as disincentive for serious commitment to the schemes by the farmers. Casual labouring activities (like stumping cotton stocks) had very low wage-rates and were frequently paid for in kind (usually maize and other staple foods). The FGDs revealed that these traded goods were then sold, often at poor or seasonally variable local rates, to generate cash needed for school fees or grinding mill fees. Involvement in casual labour was also blamed for keeping household members away from their own fields when they most needed attention, which could result in the depression of productivity in their own fields, threatening the sustainability of the schemes (Bodibe, 2006). This finding confirms the conclusion of Pocock (2012) that in Africa, casual work is not only poorly paid but leaches commitment to work at the scheme and affects productivity of the critical stakeholder- the farmers.

Irrigation Management Committees

All the schemes had male dominated Irrigation Management Committees (IMCs) (80% male and 20% female) by the time of the survey and all the respondents

Table 1. Highest level of education attained by farmers in the schemes.

Highest level of education attained	Scheme (%)			Total
	Mtandahwe	Dendere	Tsvovani	
None	12	60	65	37
primary	46	27	6	28
ZJC	24	13	10	18
O' level	16	0	17	14
A' level	2	0	2	2
Total	100	100	100	100

concurred on the idea that the role of the IMC was to manage all the aspects of the scheme. Differences were on the perception of the effectiveness of the IMC. All the respondents from Mtandahwe and Dendere felt their IMC was effective while 31% of the Tsvovani respondents felt their IMC was not very effective.

The differences in the perceived effectiveness of the IMCs of the 3 irrigation schemes were found to be significant by one way ANOVA at P<0.007, in favour of Mtandahwe and Dendere irrigation schemes that had 100% of the farmers feeling that their IMC was effective. Those who felt their IMC was effective cited smooth flow of activities (82%), peaceful sharing of water (30%), and transparent and safe keeping of money (60%), compliance of farmers to their orders (70%), limited down times after irrigation pump breakdowns (20%). Those who felt the IMC was not effective cited lack of leadership qualities as the major indicator of their ineffectiveness (30%), lack of transparency on their handling of cash (25%), succumbing to intimidation (15%) and the existence of inter personal conflicts in the scheme (15%). It was noted in one of the FGDs that the IMC in Tsvovani needed to be more transparent on the way they used cash in the scheme. Some farmers no longer had confidence in the IMC as they strongly suspected some of the IMC members were pocketing their money. Consequently, some farmers were resisting payment of contribution towards the running of the scheme. Some blamed the IMC for lacking leadership skills and for being ineffective in containing conflicts. This negative attitude towards the effectiveness of the IMC in Tsvovani possibly explains why the members were failing to pay utility bills which according to the farmers were the major threat to the continued functionality of the scheme. Chidenga (2003) posited that if plot holders are well informed about the financial affairs of the IMC, they will have no choice but to be accountable to the members. This will have a significant positive impact on farmers' willingness to cooperate with the leadership they would have chosen. Transparency also creates an atmosphere in which fraud becomes difficult, increasing the likelihood that the farmers retain control and responsibility for their irrigation schemes, a critical element in sustainability (OECD, 1989; Muparange, 2002; Dzinavatonga, 2008).

The enforcement of the constitution in the schemes was found to be a strong pointer of the effectiveness of the IMC to engage the farmer. All the respondents indicated that they had a constitution in their respective schemes. Ninety-two percent felt their constitutions were being used and only 8% felt it was not being used. Evidence for the utilisation of the constitution includes the punishment of people whose behaviour was not in line with the provisions of the constitution and that all the farmers were contributing towards ZESA bills. Those who indicated that the constitution was not being used cited lack of compliance to the provisions of the constitution as evidence. In Tsvovani, some farmers indicated that if all the farmers had contributed towards the payment of electricity, the ZESA bill could not have reached $40 000. Some farmers were not paying up. Although it was enshrined in their constitution that if someone fails to pay utility bills he/she can be expelled from the scheme, no serious action had been taken against the defaulters. Failure to expel non payers was tantamount to rewarding of bad behaviour and setting wrong precedence in the scheme. The IMC lacked power to operationalise the constitution. One participant in the FGDs said that the IMC were using all tactics to make farmers pay, like preventing one from watering, but when it comes to expelling one from the scheme, it was almost impossible for the IMC. During one of the FGDs in Tsvovani, one farmer said *"Simba racho unenge waripiwa nani rokudzinga munhu. Zvakango nyorwa muconstitution asi hazvitoiti*"-(Where would you get the power to expel someone from the scheme, it is not practical).

The effectiveness of the IMC in Mtandahwe and Dendere was shown by the fact that they had no problem in expelling non paying members from the scheme. In Dendere, the membership of the scheme shrank from 96 to the current level of 38 farmers, due to the non payment of critical contributions by some members. Consequently, Dendere actually had a positive balance of around $500 in electricity bills and utility bills were the least of their worries. One striking thing about Dendere was that they had a reserved fund specifically for the repair of pumps which by the time of the survey was $900, kept in the scheme's bank account. They were all confident that after a pump breaks down, it would never have a downtime of over two days as the reserved money was used to pay for its repair. The farmers, having this culture of group saving, unfortunately were not making group efforts to procure critical inputs like fertilizers and certified seeds to boost their production. Some of the crops were pale due to lack of fertilizer but the very farmers were boasting of having reserved funds waiting for pump break down. Therefore, this level of functional dissonance in the saving pattern of the scheme was counter productive and rendered all their saving efforts unsustainable.

Mtandahwe had no outstanding arrears but had no reserved funds; neither did they have a bank account. The advantage of Mtandahwe was that they procured their inputs in groups which allowed them to have fair uniformity and timely operations in the scheme. They were also involved in group marketing of products to far away markets, especially during times of local market glut. The most striking feature about the IMC in Mtandahwe was the presence of a Marketing Sub-Committee overseeing the marketing dimension of their farming operations. This, according to Mtandahwe farmers who participated in the FGDs, explains why they had fewer problems in marketing their produce.

It was observed that environmental issues were better streamlined in Mtandahwe irrigation scheme than the other 2 schemes. Vegetative/live fence was planted along the perimeter fence of the scheme to ascertain the existence of the fence beyond the fencing poles and the barbed wire. Vetiver grass was also planted in the scheme, around areas highly susceptible to active erosion and gully formation to fortify the soil. Contrary, Dendere although well fenced with diamond mesh had no vegetative fence and the pump's suction point was not protected from the erosive forces of the river, threatening not only the pumps but the pump house as well. In Tsvovani the last piece of barbed wire around the perimeter fence of the scheme was last seen in 1996 before it was stolen. The scheme currently resembles an open communal crop field. Domestic animals pose serious security threats for the crops in the scheme. The fields are guarded day and night, giving more burdens to the already burdened farmers.

Tsvovani was paying 3 pump minders $50 per month each to guard the pump while farmers in Dendere were taking turns to guard the pump at night. In Mtandahwe, the guards were allocated 0.1 ha of land to use as their pay for guarding the pump and the irrigation scheme. The Mtandahwe way of protecting pumps was a fairly sustainable way of payment because cash payment for a scheme that was struggling to pay monthly utility bills like Tsvovani means that one day the farmers may fail to pay the pump guards.

The different level of success of the IMCs was consistent with Chidenga (2003) findings that other schemes have disciplinary control while others were not tight enough as their real power and duties has never been clear. Chidenga (2003) noted that the IMC never got the legal status and administrative authority exercised by the pre-independence irrigation managers and District Commissioners. Consequently, although the IMCs had the potential to effectively manage the scheme, they lacked power to operationalise their constitution and failed to transform the production levels, of the irrigation schemes to enhance their sustainability.

Zimbabwe National Water Authority (ZINWA)

Interviews with the farmers and the AGRITEX officers revealed that ZINWA played no role in the initial development of the scheme and only started to engage the farmer to make them pay water charges after the successful rehabilitation of the 3 schemes in 2009. Farmers in Tsvovani were aware that the ZINWA billing system was as follows; $6.06 per hectare + 40% transmission losses + 25% value added tax. By the time of the survey, the scheme owed ZINWA US$36 000.00 in water charges and had not paid anything to ZINWA since they started receiving the statements. It was not clear how ZINWA was going to react to the non payment although they were speculations that they were going to lock off their pumps to force them to pay, a development that will threaten the functionality of the schemes. Many stakeholders from the RDC, AGRITEX and Department of Irrigation have however questioned the sincerity of ZINWA in its dealing with farmers. When the pumps were under breakdown, ZINWA could not be seen anywhere closer to the farmers to give a hand in fixing them. It is only after the farmers would have won their war in the pump rehabilitation that ZINWA would chip in to bill water they did not help to extract. It was revealed in the discussion with stakeholders that when disconnecting farmers from water supply, ZINWA usually plans it when the crops in the schemes will be at a critical water demand stage as a way of forcing them to pay. This was in line with Mombeshora (2003) finding that ZESA and ZINWA usually disconnect electricity and water from farmers when the crops critically needed water. ZINWA's engagement with farmers lacked materiality (International Association of Public Participation, 2005) and farmers felt that ZINWA wanted to harvest where it did not sow. There is a need for ZINWA to come up with better packages and engagement strategies for farmers to deduce the ethical and economic logic of cooperating with it.

Department of Agricultural, Technical and Extension Services (AGRITEX)

Each irrigation scheme had at least one AGRITEX officer to provide extension services to the farmers. In Tsvovani, there were 4 Agritex officers, one in each block. During the initial development of the scheme, Agritex was responsible for subdividing the plots and guiding the perimeter fencing of the schemes. In Tsvovani, the first Agritex officers were deployed in 2000; 3 years after the withdrawal of ARDA staff. Farmers in Tsvovani felt the Agritex officers were not as technically knowledgeable as ARDA extension officers. One farmer who participated in the FGD said *"Vatinavo ava vanongotaura, havapindi mumunda saka hatizonzwani. Vamwe vacho tinotovakundavo ruzivo"* (Unlike ARDA officers, the Agritex officers we have now just have theoretical knowledge and lack practical knowledge, we are even better than some of them). These shortcomings in the technical knowledge of the extension staff in the schemes was confirmed by the District Agritex officer, Chiredzi who indicated that some of them were trained through the Government's Fast Track training programs and lacked practical skills. They were popularly called "the half backed extensionists". The lack of technical capacity, according to the farmers in Tsvovani was compromising the production capacity of the schemes and restricting the type of crops the farmers could grow.

Conversely, Agritex officers in Dendere and Mtandahwe were highly valued and respected by the farmers. It was well expressed in the FGDs that farmers in the two schemes felt greatly indebted to the service of the Agritex officers that they allocated them a plot of land in their respective schemes. This, in turn was a strong motivational factor for the extension workers. Nevertheless, it was strongly felt in all the interviews with District AGRITEX officers that the extension support from the department was not adequate to leverage commercial production in the schemes. This confirms the finding of Denison and Musona (2007) in the South African smallholder irrigation extension support which they rated inadequate and unreliable to sustain commercial entities.

Department Of Irrigation (DOI)

The farmers felt the Department of Irrigation was almost invisible and were not aware of its roles and responsibilities. They took no part in the rehabilitation of Mtandahwe and Dendere. In Tsvovani, they were seen once when the water pumps were being installed in 2010. The district officers for the department felt the Irrigation Department was the least resourced Government department in the district. Their responsibility in smallholder irrigation scheme was mainly land survey, canal pegging and certification of work done by contractors. They had no vehicle and their visit to irrigation scheme was contingent upon the convergence of interests by some NGOs or other Government departments visiting the scheme in which case the officer would ask for transport assistance. They were largely office bound and did not have up to date information about the smallholder schemes' functionality status and requirement. The Department of Irrigation was largely an uninformed and disempowered stakeholder in the rehabilitation and management of smallholder irrigation schemes.

ARDA

Although ARDA was no longer managing any of the 3 irrigation schemes under investigation, its role in Tsvovani was worth explored. The Farmers in Tsvovani indicated that when ARDA was still managing the schemes, it was doing everything for them on the scheme ranging from the provision of inputs, tillage, planting, weed management, nutritional management, harvesting and marketing. The farmers were at times asked to weed and provide manual labour in their plot and would just be treated like farm workers. For harvesting of maize and wheat, the farmers narrated that ARDA had combine harvesters which were rotating all ARDA estates during harvesting time to harvest maize or wheat. Fertilizer and seeds would come in 30 ton trucks for the farmers and all the cost were deducted from the farmers' cheques after every cropping cycle. ARDA would also arrange loans from AgriBank for the farmers. ARDA owned the engagement process for stakeholders in the input and output supply market, the financial resources and general farm management. Farmers were very happy with the arrangement and would have wanted the arrangement to last for ever as they were now failing to manage the scheme on their own- pushing them into grinding poverty. One farmer said *"…that is the arrangement that bought us the tractors we have but now I am failing to buy diesel for the very tractor to till my land*". The arrangement was good for them but its exit strategy was not well managed as ARDA suddenly withdrew from the scheme without proper handover and takeover of the management of the scheme. Its major weakness was its failure to involve the farmers themselves in the process to preserve institutional memory and for the sustainability of the scheme beyond the management of ARDA. The ARDA management left a dependency syndrome in the farmer, that was not seen in Dendere and Mtandahwe, which is threatening the functionality of the Tsvovani scheme as farmers still expected outside assistance in the payment of utility bills and procurement of inputs. What was probably lacking in the engagement process of ARDA's operate and transfer method was an empowerment element as it was devoid of plans about farmer's future after ARDA's departure. The arrangement was also a victim of unfortunate economic dynamics in the national economy, particularly the aftermath of ESAP.

Zimbabwe Electricity Supply Authority (ZESA)

ZESA confirmed, during key informant interviews, that it

Table 2. Perception on continued functionality without external support.

Name of scheme	Perception on functionality without any external support, in the next 5 years		Total
	Yes	No	Yes
Mtandahwe	46	21	67
Dendere	7	8	15
Tsvovani	15	33	48
Total	68	62	130

was charging commercial rates on the smallholder irrigation schemes and farmers felt ZESA was not fair in its billing system. In Mtandahwe, farmers were collectively paying around $900 per month for electricity and although the farmers were fully paid up, farmers complained that there were no variations in the electricity charges to reflect the different electricity utilisation pattern of the different cropping cycles and watering intervals in the scheme. This was believed to be caused by the use of estimates to bill farmers as ZESA officials rarely visited the scheme to take actual readings. Even if they later discovered that they had overcharged farmers, the rectification of the problem was never done and explanations to it were not convincing to the farmers. Dendere farmers had similar experience with the farmers having about $500 positive balance due to previous overcharge by ZESA which took a long time to rectify. In both schemes farmers reported that ZESA would be very quick to disconnect the supply without verifying the accuracy of their bills.

Tsvovani's future was dangling in the air due to the ever ballooning electricity bill. Like in the other 2 schemes, charges were accumulating during the decade of disrepair. When the scheme was successfully rehabilitated, the farmers had over $10000 outstanding electricity bill. When they commenced production, the farmers were consuming electricity worth around $6000 per month but were only able to pay $1200 per month which was only 20% of their monthly consumption. Consequently, the charges accumulated to around $40000 (from the main pumping unit, 3 sub pumping units at reservoirs and 2 disused borehole pumping units) by the time of the survey for this study. The scheme was once disconnected only to be connected after the intervention of the political leadership after which a contract was reached to extend the grace period for the payment to 6 months.

Approaching the deadline in October 2012, farmers were nowhere closer to half payment of the bill and expect another extension by 6 months. In order to convince ZESA, the farmers had agreed to pay $100 each per month for the month of August and September which could raise them $24000 if every member paid up. Asked why they have not been making such big payments, farmers indicated that they expected to raise enough money to pay the ZESA bill from the sale of cotton but when the cotton price dropped by over 260%, during the 2011-2012 season, farmers resorted to the alternative debt settlement plan. ZESA indicated that disconnecting farmers from the electricity grid was the last option if they prove to be uncooperative and uncommitted to the settlement of their bill. Farmers indicated that they would not be able to pay the electricity bills without external assistance, making it a major threat to the future functionality and sustainability of the scheme as shown in Table 2.

The difference on the sustainability perception of the scheme beyond external assistance in the different schemes was found to be statistically significant at P< 0.000 using one way ANOVA. A Chi square analysis also proved the differences in perceived functionality of the schemes without external support to be significant at P< 0.05. Farmers in Mtandahwe strongly thought their scheme would continue to function beyond external assistance while those in Tsvovani strong felt their scheme will not remain functional. The explanation given by Tsvovani farmers for this perception revolved around the arrears in electricity and water bills amounting to over $60 000 in Tsvovani and farmers felt would not be able pay off and remain functional.

Non Governmental Organisations (NGOs)

It was revealed that NGOs were major players in the establishment of small-scale irrigation schemes and in their rehabilitation. They provided funds for the scheme establishment and in the rehabilitation of the schemes. Mtandahwe and Dendere were established through NGOs, World Vision and Red Barna respectively. After the pegging by Agritex, the NGO would oversee the engagement of the community, consultants, contractors/ service provider and all the relevant Government stakeholders. The meetings, workshops and trainings linked to the establishment and rehabilitation of the schemes were all financed by the NGO. The NGO was also responsible for hiring an engineer who did pump installation at the schemes, procuring the pump and paying for the perimeter fencing of the scheme. For Dendere, the Agritex officers who participated in the perimeter fencing of the scheme were paid travel and subsistence allowances by Red Barna. After successfully establishing the scheme, the Red Barna grew crops for two years providing farmers with all the inputs at zero cost. One farmer said *“we were just taking fertilizers and other input from this warehouse for 2 years”*. After harvest, the farmers would pocket the proceeds. The scheme was handed over to the community in 1997 and Red Barna left.

In Mtandahwe, the NGO that rehabilitated the scheme also constructed a grading shade, a 3 roomed office, a plinth and a summer season pump house to prevent the pump from damage by floods. It also procured all the fencing materials for the 23 ha scheme. The fencing was done by the community under the supervision of Agritex and Mercy Corps. The chairman of the scheme had records on the costs of the rehabilitation cost and the cost of material and labour contributed by the community. The organisation also gave seeds and fertilizer to farmers for the first two cropping cycle after rehabilitation as a way of supporting farmers’ post rehabilitation production. All the ZESA bills and installation costs were covered by the organisation and farmers started on a clean sheet. This helped to unleash the potential of the farmers as production cost at the initial stages were reduced to a minimum and would face actual cost when they have fully recovered. The variety of activities or intervention implemented by Mercy Corps in the rehabilitation of Mtandahwe irrigation scheme confirms (VanSant, 2003) argument that the sustainability of NGO efforts in rural development depend on the program quality and diversification.

This was in sharp contrast to the experiences of Tsvovani and Dendere after rehabilitation in 2010 where they were not assisted with input by the NGO that helped them to rehabilitate the scheme. In Tsvovani it was just the replacement of the pumps, no perimeter fencing and canal rehabilitation was done. Farmers struggled to finance their first cropping cycle without fertilizers and sufficient seeds. The poor yields that ensued set the tone that perpetuated up to date and farmers were never given a chance to unleash their potential. Their ZESA bill that had accumulated over the period of breakdown welcomed the farmers after rehabilitation. They described the bill as the ghost that is haunting the scheme, threatening the sustainability of the scheme. This explains why the Common Wealth of Australia (2003) cautioned that, if donors wish to see benefits sustained, they should, on a case-by-case basis, also consider taking on responsibility for contributing to solving operation and maintenance cost problems in a more direct way. The approach used by the NGOs who rehabilitated the Tsvovani and Dendere schemes lacked materiality and responsiveness as they failed to address the crucial and most important concerns of the farmers they were trying to assist (AccountAbility, 2005).

The unfortunate thing about the NGOs that rehabilitated the 2 schemes (Tsvovani and Dendere) was that they were not disclosing to the farmers information about the cost incurred to establish the scheme. As a result, farmers were not aware of the value of the assets handed over to them by development agencies. The engineers who installed the pumps and the suppliers of the pumps had contracts with the donor and not with the farmer, imposing legal complications when the community attempted to get some restitutions or backup services or follow up on contractual obligations. The farmers did not know where to get new pumps for replacement or where to get spare parts for repairs. Knowledge about suppliers of equipments and item prices is ideally a sustainability measure as people will appreciate the value of assets entrusted to them by outsiders and the amount of care they should give to safeguard them, who will fix it in case of break down and at what cost. Effective engagement depends upon a shared understanding of issues which works best when all participants have access to the same information (AccountAbility, 2005; Crosby, 2000; Perry 1997). This explains why Chandrasekera (2004) opined that lack of information can be a critical sustainability threat. Ideally, stakeholder engagement in the development of smallholder irrigation schemes promotes community ownership of issues and inculcates a sense of responsibility and accountability for both private, Governmental and Non Governmental stakeholders (Commonwealth of Australia, 2000).

Business community participation

The private sector today is increasingly called upon to take significant responsibility for resolving some of the world’s most intractable problems like the sustainability challenges of smallholder irrigation schemes (World Bank, 2008; Stakeholder dialogue, 2012; Dittoh et al., 2010). Unfortunately for the establishment and rehabilitation of the three schemes under investigation, the business community involvement was very limited. Contractors and middlemen who were supplying parts and pumps during the rehabilitation of the irrigation schemes were the major private sector players. The only spectacular private sector engagement was noted in Mtandahwe where Triangle and Hippo valley sugar companies were engaged to provide tillage services as part of their corporate social responsibility. The NGO that assisted in the rehabilitation of Mtandahwe (Mercy Corps) partnered with Hippo valley and Triangle under an arrangement where the two organisations provided the first tillage service after rehabilitation and did land scarping and levelling to allow efficient flow of the flood irrigation water in the beds. This was done at no cost to both Mercy Corps and the farmers. The farmers who participated in the FGDs acknowledged that the service provided by the two sugar giant companies made the irrigation more efficient than it was before the rehabilitation as it ensured that all the part of the scheme accessed water. Farmers in Mtandahwe indicated that there was no way farmers could have approached these two sugar giant companies without the help of Mercy Corps and that this partnership, hitherto, embolden them to confidently interact with the private sector in search of markets and other agricultural synergetic linkages. The Mtandahwe experience confirmed Fowler's (1997) revelation that NGOs skate on a thin ice and what is required to implement effective and sustainable programs under such circumstances are interactive-authentic partnerships for greater impact and reducing dependence on donor funding. Farmers in Tsvovani were linked to National foods as the buyer of their wheat in the late 1980s and Windmill as their supplier of fertilizers on credit payable after harvest. After being weaned from ARDA they never had any meaningful private sector partnership. It was also unfortunate to note that all the 3 schemes were not under any form of contract farming with the private organisations by the time of the survey.

Rural District Councils (RDC)

One missing link in the irrigation scheme management was the RDC. It was revealed in the FGDs with farmers and stakeholders that the major problem with small holder irrigation scheme was lack of owners in the engagement process. Several stakeholders were involved from initial development, rehabilitation and cropping management and marketing. But the question was who will bring these stakeholders together? It was not Agritex because Agritex ended at provision of extension services neither was it the responsibility of the Department of Irrigation, whose scope was restricted to designing of irrigation schemes, pump installation or repair and canal construction. Farmers, ideally, through their leadership (IMC) should be owners of the engagement process, but they had limited powers to stand on an equal footing with other stakeholders like ZESA, NGOs, Government departments and other private companies. The engagement of the stakeholder lacked inclusivity as there was no one to hold them accountable to strategically respond to sustainability concerns of the schemes. There was no one to establish the boundaries of disclosure of the engagement specifying what information should be shared with other stakeholders. Ideally, the RDC as the local Government at district level thought to be the owner of the engagement process for the management of the smallholder irrigation schemes. Unfortunately, the RDC was neither an actor nor a factor in the management of the three irrigation schemes by the time of the survey.

Before independence, the RDCs through the district Commissioners were critical players in the enforcement of by laws, management of pumps and collection of tax revenues from the schemes. Some stakeholders who were interviewed during the survey weighed the options of adapting the pre-independence model of running the smallholder irrigation schemes to enhance the sustainability of the scheme in the modern day Zimbabwe. However, some stakeholders felt that giving the RDC the responsibility to oversee schemes can open a can of worms for the farmers as they alleged that most RDCs were corrupt and mismanaged. The RDCs played no role in the rehabilitation and management of scheme other than having issues discussed in the full council meetings; allocate schemes for rehabilitation to donors or referring pressing issues about schemes to the relevant Government ministry. It was strongly felt that even if the RDCs might lack money to finance some scheme requirements; its oversight responsibility could go a long way in trying to ensure that the schemes were not allowed to deteriorate. It was widely believed that the Central Government would have interests in having the RDCs oversee these smallholders considering that the Government have invested a lot of money in the schemes and their criticality to the communal subsistence farmers. It was concluded from interviews with the Chipinge and Chiredzi RDCs that as a potential owner of the engagement process in the management of community smallholder irrigation schemes, the RDCs were strategically positioned to determine the level(s) and method(s) of engaging with stakeholders. Considering that some of the critical stakeholders like the department of irrigation and Agritex are poorly resourced and disempowered, RDCs as owners of the engagement would identify where capacity to engage needs to be built and respond appropriately to these needs. This would enable effective engagement to prevent them from participation fatigue and disengagement.

This is in line with sustainability recommendations made for rural development project in India where the local government institutions were tasked with the responsibility to establish a collaborative partnership in developing a local vision and strategy; and designing/planning, allocating resources, implementing and monitoring/evaluating of development projects (Chandrasekera, 2004).

Conclusion

Multiple stakeholders were involved in the smallholder irrigation schemes and the farmer was one of the critical stakeholders in the scheme. The sex and age disaggregation of the farmers in the schemes show that females dominated the schemes and only a few youth participated in the schemes. The absence of the youth had the potential to impose threats to the future sustainability of these schemes as no institutional memory will be left after the current generation of farmers got out of picture (Shah et al., 2002). Over thirty percent of the household had member who were orphans, chronically and young children who were below 5 years of age. Such a vulnerability status of the households had a direct negative bearing on the viability of irrigation schemes in that, all the vulnerability categories need to be looked after by women who usually provide labour in the schemes (Parker et al., 2009). The illiteracy level of the farmers in the 3 schemes were 18.3% higher than the national average and considering that high value horticultural crops grown in the scheme are usually knowledge intensive and their level of education could not leverage high level of productivity in the schemes. The involvement of farmers in lowly paying casual labour as a source of livelihood was not only eroding their commitment to their irrigation schemes but was also trapping them in a poverty circle.

Some of the IMC were perceived to be ineffective owing to lack of leadership qualities, lack of transparency and the power to operationalize their constitution. Such perceptions determined the farmers' level of willingness to cooperate with the IMC especially on making contributions towards the operations of the scheme. For example, transparency creates an atmosphere in which fraud becomes difficult, increasing the likelihood that the farmers retain control and responsibility for their irrigation schemes- a critical element in sustainability (OECD, 1989; Muparange, 2002; Dzinavatonga, 2008). Although some of the schemes, like Dendere, displayed a remarkable level of cohesion among the farmers, their IMCs displayed functional dissonance as they were failing to take advantage of the farmers' cohesiveness to do group procurement of inputs and group marketing of their agricultural products, for the schemes to sustain high levels of production. The IMCs were generally failing to transform the schemes into commercial production entities to enhance their sustainability. It was also shown that the scheme that had a Marketing Sub Committee had fewer problems in marketing their agricultural produce than those that did not have such a committee.

It was revealed that ZINWA's engagement with farmers lacked materiality. Farmers and other stakeholders felt ZINWA was not fair in its dealings with farmers as they could not understand why this organisation was charging farmers for water they were taking from the river without giving them any help in the water extraction. Also, as a way of forcing farmers to pay their water bills, ZINWA would also disconnect farmers from water supply, when the crops in the schemes will be at a critical water demand. There is, therefore, need for ZINWA to align its operational strategies to the needs of the farmers. This would potentially elicit the needed cooperation and mutual understanding for sustainable engagement. Farmers had the same perception with ZESA whose electricity bill was usually based on inflated estimates and not on actual meter readings. ZESA was charging commercial rates on the electricity for the schemes and was quick to disconnect farmers from the power grid in case of any outstanding bills , at times without verifying the accuracy of their bills. Consequently, water and electricity bills were the major operational costs threatening the sustainability of the schemes.

Some of the AGRITEX officers lacked the requisite qualification and experience to leverage commercial production in the schemes. Evidence from Dendere and Mtandahwe suggest that farmers were able to motivate their extension workers by respecting them and making them plot holders in the scheme. On the other hand, the Department of irrigation was one of the poorly resourced depart in the district and this prevented from rendering any meaningful support to the smallholder irrigation schemes. ARDA failed to effect a gradual and strategic handover of the Tsvovani farm and its management to farmers, in keeping with the Operate and Transfer model for the scheme. This left a dependency syndrome amongst the farmers as ARDA never empowered them to be independent.

The NGOs that rehabilitated some of the schemes failed to involve farmers on critical strategic issues like the hiring and contracting of service providers. They were also not transparent enough to disclose to farmers, information about the costs incurred during the establishment or rehabilitation of their scheme neither were they also telling the farmers the costs and suppliers of the equipment critical for the rehabilitation of the scheme. This made farmers passive recipients of external assistance which does not only discourage ownership but sustainability of the scheme maintenance. The involvement of the business community in the establishment, rehabilitation and operations of the scheme was very marginal although they had the potential to be strategic partners in different spheres of the scheme.

It was revealed that the engagement process for the multiple stakeholders involved in different aspects of the smallholder irrigation schemes lacked ownership. The responsibility to oversee the sustainability of the schemes is split amongst different stakeholders and there was no one with the responsibility to bring the stakeholder together to enhance cohesiveness, responsibility and accountability in their service to the smallholder irrigation scheme. Although the Rural District Councils were strategically positioned to coordinate the stakeholders, as was the case during the pre-independence era, they were neither a player nor an actor in the smallholder schemes. Consequently, there was no standard way engaging farmers by the multiple stakeholders and the stakeholders lacked supervision.

RECOMMENDATIONS

1) The farmers need to be trained in agronomic practices, farming as a business and marketing of agricultural produce for them to transform smallholder irrigation schemes into commercial production entities.

2) The IMCs of the respective schemes need to be trained in group dynamics and transformational leadership to enhance their effectiveness and transparency in leading farmers. There is need for the IMC to be guided by the relevant policies to make the provisions of the constitutions guiding operations in the schemes enforceable.
3) The development agencies rehabilitating and establishing the irrigation schemes should involve farmers at all the critical stages and should aim at seeing the farmers through instead of piecemeal interventions. They should also inform the farmer about the costs involved in either rehabilitation or establishing the schemes as well as the suppliers of the critical equipment needed for the scheme to enhance ownership and sustainability of the scheme.
4) ZINWA and ZESA need to realign their operational strategies to the needs of the farmers to ensure that their billing systems are pro-poor and justifiable. This will elicit the farmers' cooperation and mutual understanding needed for the sustainability of the schemes.
5) Government department like AGRITEX and Department of irrigation need to be adequately trained and resourced to effectively render the necessary support to lead irrigation schemes into commercial production entities. In order to be effective, these two government department need a supervisory board that hold them accountable.
6) It is, therefore, recommended that at district level, there be an adequately resourced government department in the form of RDC, responsible for coordinating the all the affairs of smallholder irrigation schemes. This would be responsible for supervision and holding accountable the multiple stakeholders involved in different aspects of smallholder farmers in a holistic sense. This will not only give an institutional frontage to the farmer when dealing with other organisations but will also ensure that the vulnerable farmers are not exploited.
7) Further research is needed to compare the performance of individually owned smallholder irrigation entities with community owned smallholder irrigation schemes in a view to draw some best practices from both types to inform policies.

Conflict of Interest

There are no conflicts of interests regarding this publication.

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Full Length Research Paper

Growth of ungrafted and grafted citrus rootstocks under saline water irrigation

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Growth of citrus genotypes under salinity during the plant formation was assessed. The experimental design consisted of a randomized block design with three replications in a greenhouse. Five salinity levels (0.8; 1.6; 2.4; 3.2; and 4.0 dS m^{-1}) of irrigation water were applied to 12 genotypes (citrus varieties and hybrids from crossings involving *Citrus* and *Poncirus*) of citrus rootstocks, ungrafted and grafted with 'Tahiti' lime and 'Star Ruby' grapefruit. The irrigation with saline water was started at 60 days after sowing. Plants were initially grown in polyethylene tubes of 288 ml, and were transplanted to plastic bags, filled with commercial substrate after five months. The stem diameter, stem height and number of leaves on ungrafted rootstocks and scion-rootstock combinations were evaluated every 30 days. Data were assessed by analysis of variance by the 'F' test. Regression analyses were performed for quantitative variables (salinity) and means were compared at 5% of probability for qualitative factors (rootstocks and scions combinations) by Scott-Knott and Tukey tests. Citrus growth was reduced by salinity. The hybrid between Sunki of Florida mandarin (TSKFL) and citrange C25 (CTC25) - 010 and 'Troyer' citrange had greater growth compared to ungrafted. 'Rangpur' lime under 'Tahiti' lime is the most indicated combination for irrigation with saline water.

Key words: *Citrus* spp., salt stress, rootstocks, varieties and hybrids, water quality, salinity, seedlings.

INTRODUCTION

High salt concentrations in soil reduce the growth and productivity of crops. Salinity effects are most pronounced in arid and semiarid regions due to droughts and high evaporation during the year (Ayers and Westcot, 1999; Tester and Davenport, 2003).

However, some crops produce economically viable

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yields even at high levels of soil salinity, due to the higher capacity of osmotic adaptation of these species, which allows the absorption of sufficient water even under saline conditions (Ayers and Westcot, 1999). Such adaptability is very useful and allows the selection of tolerant genotypes when it is not possible to keep soil salinity at low levels (Tester and Davenport, 2003).

Salinity tolerance varies among species and within species; its effects vary between stages of development. In general, the tolerance has been identified as specific for some development stages, that is, a genotype may be more tolerant during one stage and more sensitive during other stage (Foolad et al., 1998; Silva et al., 2014). Irrigated fruit production is considered salt sensitive and requires attention to prevent salinization, particularly when the water source has high salt concentrations.

Brazil is the third largest fruit producer in the world (FAO, 2013), therefore, it is essential to generate knowledge that enables the use of saline waters and ensures sustainability of production. Among fruit crops, citrus stand out as having the highest national importance and the sweet orange (*Citrus sinensis* (L) Osbeck) as having the highest production and exportation (FAO, 2013). Citrus fruits also stand out in northeastern Brazil for undoubted social and economic importance. A practice that may enable the use of low quality water and salt affected soils is the use of genotypes with high tolerance to this abiotic factor.

However, the development of cultivars tolerant to salinity by breeding programs requires the study of the effects of salinity on plant physiology to identify processes responsible for any tolerance mechanisms (Silva et al., 2014). In fruit crops propagated by grafting, such as citrus, salt tolerance should be assessed considering the scion-rootstock plant. Careful selection of both scion and rootstock is critical to the success of citrus crop, given that the main effect of salinity on plants are the suspension of growth, leaf injury symptoms and yield reduction (Syvertsen and García-Sanchez, 2014).

According to Wei et al. (2013), the investigation of possible salt tolerance mechanisms in various cultivars may obtain more insight into the role of scion in citrus to tolerate salinity. Brito (2007), while evaluating 18 citrus genotypes, highlighted some varieties as being tolerant and moderately tolerant to salinity during the period of rootstock formation and indicated individuals with potential for production of citrus seedlings under salt stress. These genotypes, however, were not studied after grafting. Therefore, in this study, the growth of different combinations of citrus scion-rootstock under saline water application by irrigation during the formation of seedlings (rootstocks ungrafted and grafted) was assessed.

MATERIALS AND METHODS

Study site

Experiments were conducted under greenhouse conditions at the Center of Technology and Natural Resources - CTRN of Federal University of Campina Grande - UFCG, located in Campina Grande, state of Paraiba, Brazil, in the geographic coordinates 7°15'18"S and 35°52'28" W with an altitude of 550 m.

Treatments and experimental design

Five levels of salinity of irrigation water were applied: S1 - water with electrical conductivity (ECw) of 0.8 dS m^{-1}; S2 - ECw of 1.6 dS m^{-1}; S3 - ECw of 2.4 dS m^{-1}; S4 - ECw of 3.2 dS m^{-1}; and S5 - ECw of 4.0 dS m^{-1}. The threshold salinity of 1.7 dS m^{-1} described in Ayers and Westcot (1999) for 'Pera' sweet orange was used to define these levels, having two levels below and three above this limit. The levels of electrical conductivity of water (ECw) were applied in five genotypes tolerant to salinity and six moderately tolerant genotypes that were selected by Brito (2007) in an experiment that comprised only the seedling stage (probably plants of nucelar origin), rootstock production, and used 'Rangpur' lime (moderately sensitive) as control (Table 1). Seeds of genotypes were provided by the Citrus Breeding Program of Embrapa Cassava and Fruits - Embrapa CBP. After the formation of rootstocks, genotypes were grafted with two scion varieties: Tahiti lime [*C. latifolia* (Yu. Tanaka) Tanaka], and Star Ruby grapefruit (*C. paradisi* Macfad.), whose buds were also provided by Embrapa CBP. Combining all factors, we had 12 rootstocks x 5 salinity levels x 2 scions, totaling 120 treatments. The experimental design was a randomized block with three replications, and each plot consisted of four plants.

Sowing and management practices

The seeds were properly selected and treated with fungicide (4 g of Thiran per kg of seeds) and sown at the rate of three per tube with a capacity of 288 ml, in commercial substrate containing a combination of vermiculite, pine bark and humus, in a proportion of 1:1:1, keeping only one seedling in each container (Schäfer et al., 2005).

Plants grown in tubes were transplanted after germination and early growth (five months), to plastic bags 35 cm high, 22 cm upper diameter and 20 cm bottom diameter. Grafting was performed after transplantation and establishment of rootstocks in the bags. Irrigation was applied in same volume, depending on the evapotranspiration in the control treatment, which was obtained by weighing bags of different blocks, adding a leaching fraction equivalent to 20% (LF = 0.2). The control of weeds and prevention of insects, as well as fertilization, were carried out as usually recommended for production of citrus (Rozane et al., 2007).

Growth measurements

Every 30 days, from the beginning of treatment until grafting [240 days after sowing (DAS)], the number of leaves (NL), plant height (PH, cm) and stem diameter (SD, mm) from rootstocks were recorded. After grafting until 330 DAS, the number of leaves in the scion (NLScion), the length of the scion's stem (cm) measured from grafting point to stem's apex of scion, the diameter of rootstock's stem (mm), measured near the substrate by using a digital caliper rule, and the scion's stem diameter (mm) measured at 2 cm above the grafting point were obtained.

Statistical analysis

The factor "irrigation water salinity" was evaluated by analysis of variance using 'F' test, and polynomial regression (linear and quadratic). The Scott-Knott test was used to evaluate the factor

Table 1. Citrus genotypes[1] analyzed in the study.

S/N	Genotype (Classification of tolerance[2])
01	'Rangpur Santa Cruz' lime (MS)
02	TSKC x CTSW - 064 (T)
03	TSKFL x CTC25 - 010 (T)
04	TSKFL x CTC13 - 005 (MT)
05	HTR - 069 (T)
06	'Troyer' citrange (MT)
07	LRF x (LCR x TR) - 005 (MT)
08	TSKC x CTSW - 031 (T)
09	'Volkamer' lemon LVK (T)
10	TSKC x (LCR x TR) - 029 (MT)
11	TSKC x CTARG - 015 (MT)
12	TSKFL x CTTR - 013 (MT)

[1] 'Rangpur Santa Cruz' lime (*Citrus limonia* Osbeck), TSKC: 'Sunki' mandarin [*C. sunki* (Hayata) hort. ex Tanaka] common selection, CTSW: 'Swingle' citrumelo [*C. Paradisi* Macfad x *Poncirus trifoliata* (L.) Raf.], TSKFL: 'Sunki' mandarin Florida's selection, CTC25: C25 citrange [*C. sinensis* (L.) Osbeck x *P .trifoliata*], CTC13: C13 citrange, HTR: trifoliate hybrid, CTTR: 'Troyer' citrange, LRF: 'Rough' lemon (*C. jambhiri* Lush.) Florida's selection, TR: *Poncirus trifoliata*, LCR: 'Rangpur' lime, LVK: 'Volkamer' lemon (*C. volkameriana* V. Ten. & Pasq.), CTARG: 'Argentina' citrange. [2]MS: Moderately sensitive genotype; MT: Moderately tolerant genotype; T: Tolerant genotype.

"rootstock", and the Tukey test was used to verify differences between scions. All tests were performed at 95% of probability (Ferreira, 2011).

RESULTS AND DISCUSSION

Seedlings growth

According to the Scott-Knott test, seedlings with the largest number of leaves were relatives to the hybrids TSKFL x CTC25 - 010, and had about 21 leaves at 180 DAS (Figure 1A). More than four groups of genotypes were obtained, with emphasis to TSKFL x CTC13 - 005, which appears in the group with lower mean (about 10 leaves). However, it should be noted that greater or lesser number of leaves may be related to genetic material and not just due to the potential effects of salinity. This can be verified when comparing different genotypes for this characteristic, taking as base the average salinity and the lack of significant effect on interaction, which indicates that behavior was similar among salinities.

Fochesato et al. (2007), when studying growth of citrus rootstocks on different substrates, found a higher number of leaves on 'Rangpur' lime and a lower number in citrange 'C13'. This last genotype is composed of the hybrid TSKFL x CTC13 - 005, which confirms the results shown in this study.

Salinity reduced the number of leaves in 8.7% at 180 DAS (Figure 2A) with unit increase in salinity of water (dS m^{-1}), based on the lowest salinity level.

Levy and Syvertsen (2004) reported in their reviews that the absorbed chloride accumulates in leaves of citrus causing abscission and/or reducing growth. This effect may have happened in our research, which led to a reduction in the formation of leaves with increased salinity, because we did not consider the fall of leaves.

With regard to plant height, the TSKFL x CTC25 - 010 and 'Troyer' citrange stood out in the group of genotypes with the highest averages (Scott-Knott test $p < 0.05$; Figure 1B). The lowest average was observed in the group of the hybrid TSKFL x CTC13 - 005. Simpson et al. (2014), studying the growth response of grafted and ungrafted citrus plants under saline irrigation, did show a difference among three genotypes evaluated by them, with the best result for C146 and C22, and smaller in the sour orange (*C. aurantium* L.) from dates of relative growth rate in ungrafted plants, such as found in the data of this study. This can mean that the effect of salinity is variable among genotypes and these results reinforce the theory about the need to choose the best genotype for use under saline water conditions.

The height of rootstocks decreased linearly by increasing water salinity (dS m^{-1}) (Figure 2A) to 6.4% at 240 DAS. Presence of genotypes with reduced growth under salinity conditions is indicative of materials potentials, as identified by Fernandes et al. (2011) and Silva et al. (2014). However, these low values during citrus plants in the ungrafted stage can be relative to phase, such as observed by Simpson et al. (2014); these authors did show that plants during the ungrafted stage are more tolerant than in the grafted stage. But keeping in view that stress was applied in the substrate without salinity, in other words, the increase in substrate salinity was gradual, the plants may have hardened and became resistant to saline stress, as cited by Syvertsen and García-Sanchez (2014) in their reviews about multiple stress on citrus under salinity.

The values of stem diameters at 240 DAS ranged from 6.0 to 8.5 mm. 'Troyer' citrange stood out as having the highest average values (Figure 1C). Stem diameter is one of the most important variables in study of citrus rootstocks because it indicates the plant's potential for grafting. Genotypes with larger diameter have more success when grafted with buds. Based on the lowest level of salinity, the increase of one unit in the salinity of water also reduced the diameter by 5.6% at 240 DAS (Figure 2C). These results were similar to those obtained by Soares et al. (2006), who studied three citrus rootstocks in saline waters, in a nursery, in São Paulo, Brazil. According to Syvertsen and García-Sanchez (2014) salinity may have caused nutritional disturbances, reduced accumulation of biomass and plant growth. Moreover, salinity may cause toxic disturbances by the action of specific ions or drought stress by reducing the water potential in soil.

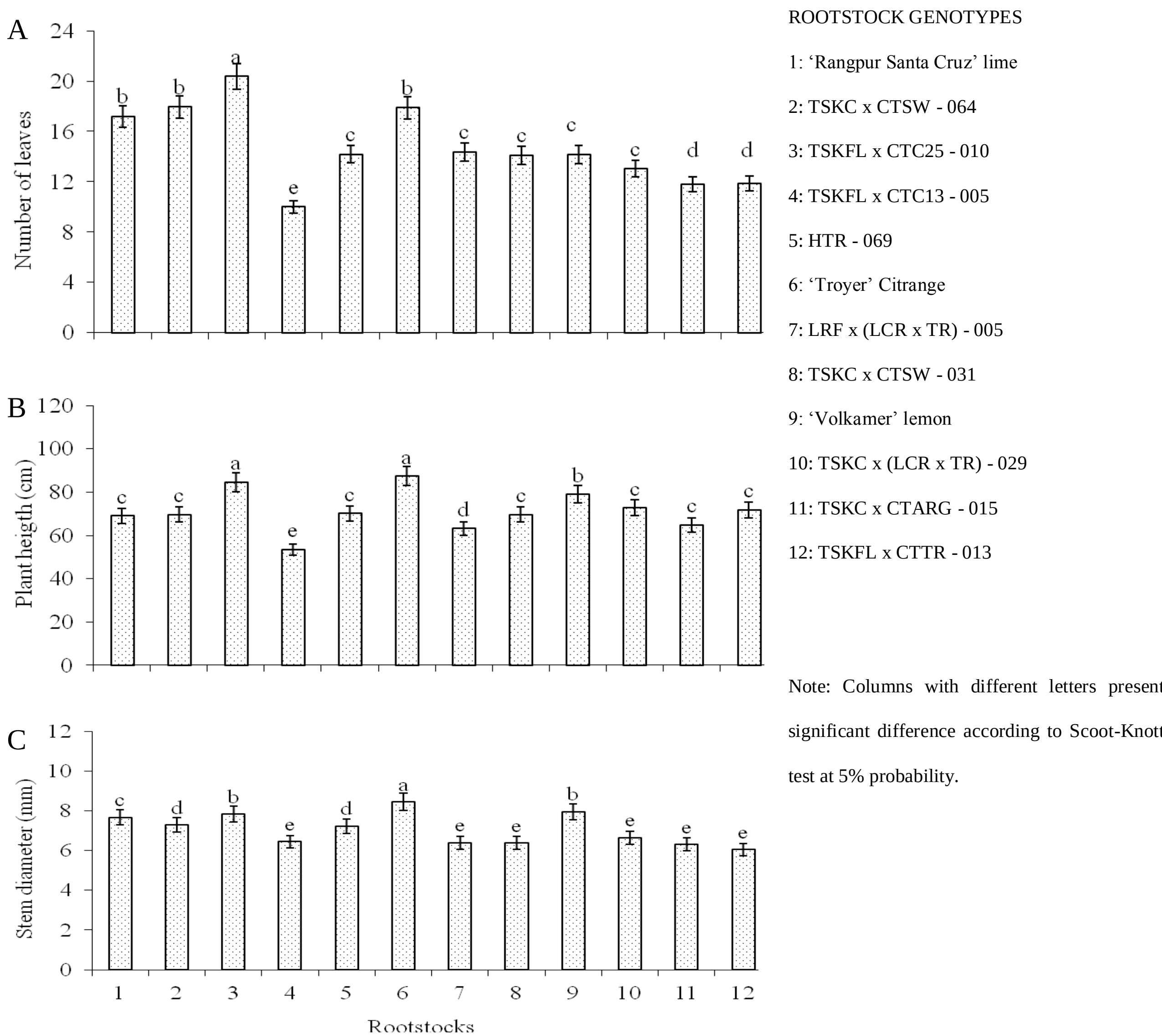

Figure 1. Number of leaves at 180 days after sowing (DAS) (A), plant height (B) and stem diameter at 240 DAS (C) of 12 citrus rootstocks.

Plant growth after grafting

The increase of rootstock diameter after grafting may be an evidence of adaptation of scion to rootstock; in this relationship, the rootstock provides support and input of water and nutrients absorbed from soil, while the scion produces organic compounds that are transported to all organs, including roots. The best adaptation was found in 'Rangpur' lime under high salt concentration. Average values of 'Rangpur' lime ranged from 9.6 mm at the lowest level of salinity to 8.5 mm at the highest level, while in the last assessment before grafting 7.8 mm in diameter was obtained (Figure 1C). The 'Rangpur Santa Cruz' lime, TSKFL x CTC25 - 010, HTR - 069, 'Troyer' citrange and 'Volkamer' lemon, which had higher estimated averages, showed linear decreases in stem diameter of 3.3, 3.4, 5.7, 4.4 and 6.0%, respectively, with 1 dS m^{-1} increase in EC of water with respect to the lowest level of salinity (Figure 3).

According to Fageria and Gheyi (1997), when comparing yields of material (genotype) under salt stress to the same material without stress, and the difference is less than 20%, this material is considered tolerant. Therefore, taking the rootstock's stem diameter as a criterion for selection of genotypes, with respect of tolerance to salinity, it can be concluded that all are tolerant to salt stress. However, it is not appropriate to take only the stem diameter of rootstock as criterion

The behavior of rootstocks on each scion genotype relative to the number of leaves is observed in Table 2

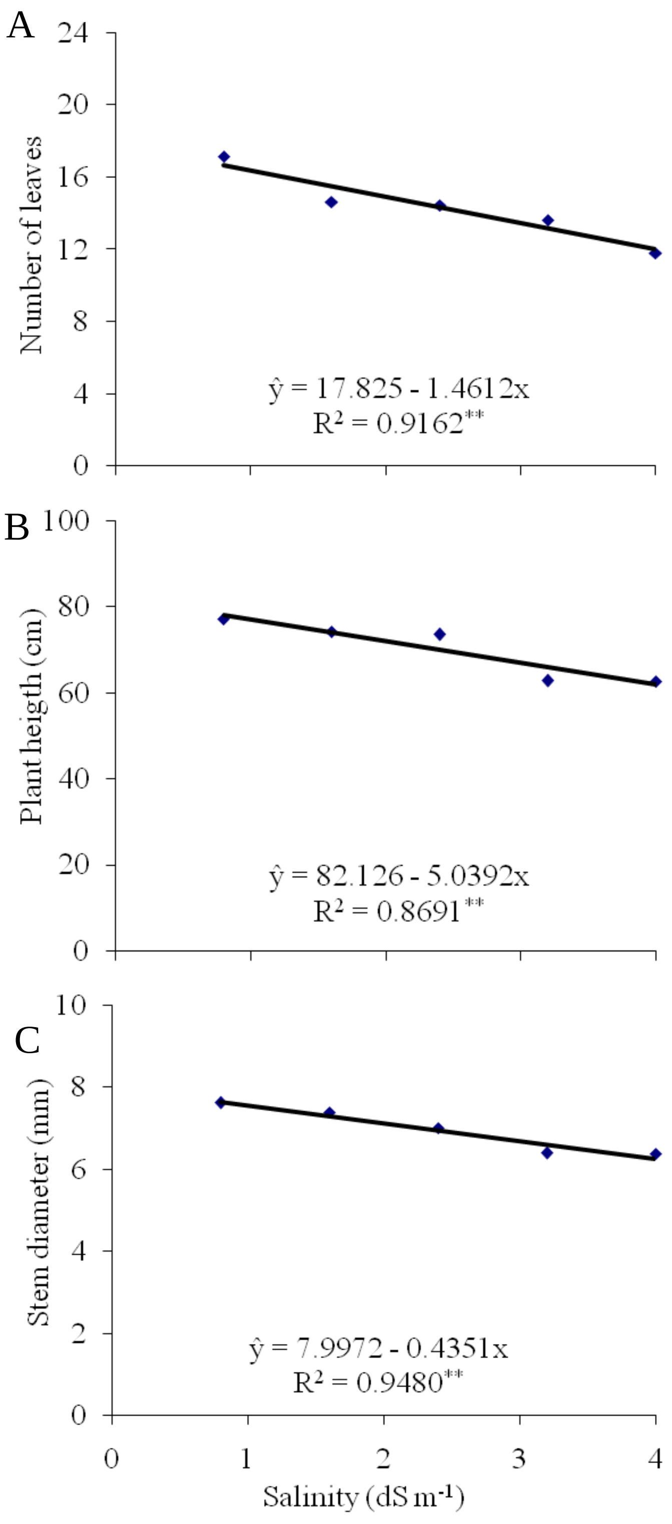

Figure 2. Effect of water salinity on numberof leaves(A) at 180 days after sowing, plant height(B) (cm) and stem diameter(C) (mm) at 240 (DAS) of 12citrus genotypes.

with linear and quadratic equations. 'Rangpur Santa Cruz' lime grafted under 'Tahiti' lime stood out with highest means under the lowest level of salinity (0.8 dS m^{-1}), showing a gradual reduction and quadratic trend, with the increase in electrical conductivity of irrigation water. On the other hand, when ‘Rangpur’ is grafted with 'Star Ruby' grapefruit, a linear decrease with increase in water salinity can be noted, with reduction of about 24% between 0.8 and 4.0 dS m^{-1} ECw. Also, in this scion (‘Star Ruby’), other genotypes with potential can be noted, highlighting the HTR - 069 and ‘Troyer’ citrange with a reduction of 39 and 35% in the number of leaves when salinity was increased from 0.8 to 4.0 dS m^{-1}. Thus, this may mean that it is better to choose the combination scion/rootstock to be successful in a citrus crop under salinity conditions.

Quadratic behavior was also observed in the TSKFL x CTC25 - 010, TSKFL x CTC13 - 005, LRF x (LCR x TR) - 005 and TSKC x CTSW - 031 grafted with 'Tahiti' lime and 'Star Ruby' grapefruit, and in TSKC x (LCR x TR) - 029 grafted with 'Star Ruby' (Table 2). Remaining combinations decreased the number of leaves with increasing salinity. Even in the quadratic model showing the best adjustment, salinity decreased the number of leaves. This may be related to toxic effects of ions in the water, which compete in nutrition and osmotic adjustment. Thus, the plants use more energy to maintain their photosynthetic apparatus (Taiz and Zeiger, 2009).

In Table 3, the behavior of scion/rootstock combinations for length of scion stems is shown, with a significant triple interaction (salinity x rootstock x scion). Linear decrease was observed when the rootstocks TSKC x CTSW - 064, TSKFL x CTC25 - 010, 'Troyer' citrange and TSKFL x CTTR - 013 were grafted with 'Tahiti' or 'Star Ruby'. A similar situation was observed when ‘Rangpur’ lime and the hybrid TSKFL x CTC13 - 005 were combined with 'Star Ruby', and when the hybrid LRF x (LCR x TR) - 005 was grafted with 'Tahiti'. There was a quadratic behavior for the remaining genotypes, except for the hybrid TSKC x (LCR x TR) - 029. The effect of salt promoted reduction both in number of leaves and plant height. Soares et al. (2006) and Fernandes et al. (2011) also identified a decrease in height of citrus under salt stress. These authors emphasized that growth occurs by cell division and expansion and this latter occurs by increase in cell turgor pressure by storage of water in vacuoles (Taiz and Zeiger, 2009).

Thus, salt stress reduces water uptake and the plant may have difficulties to carry out such processes, which results in growth limitations. Relative to variable stem diameter of scion, the behavior of scion-rootstock combinations under water salinity is shown in Table 4. The highest values for this parameter were found in combinations of 'Rangpur Santa Cruz' lime with 'Tahiti' and 'Star Ruby', however, this reduces with increase in salinity of irrigation water (dS m^{-1}). Besides these combinations, the hybrid TSKFL x CTC25 - 010 grafted with 'Tahiti', as well as the hybrid TSKFL x CTC13 - 005 and ‘troyer’ citrange grafted with 'star ruby' had a

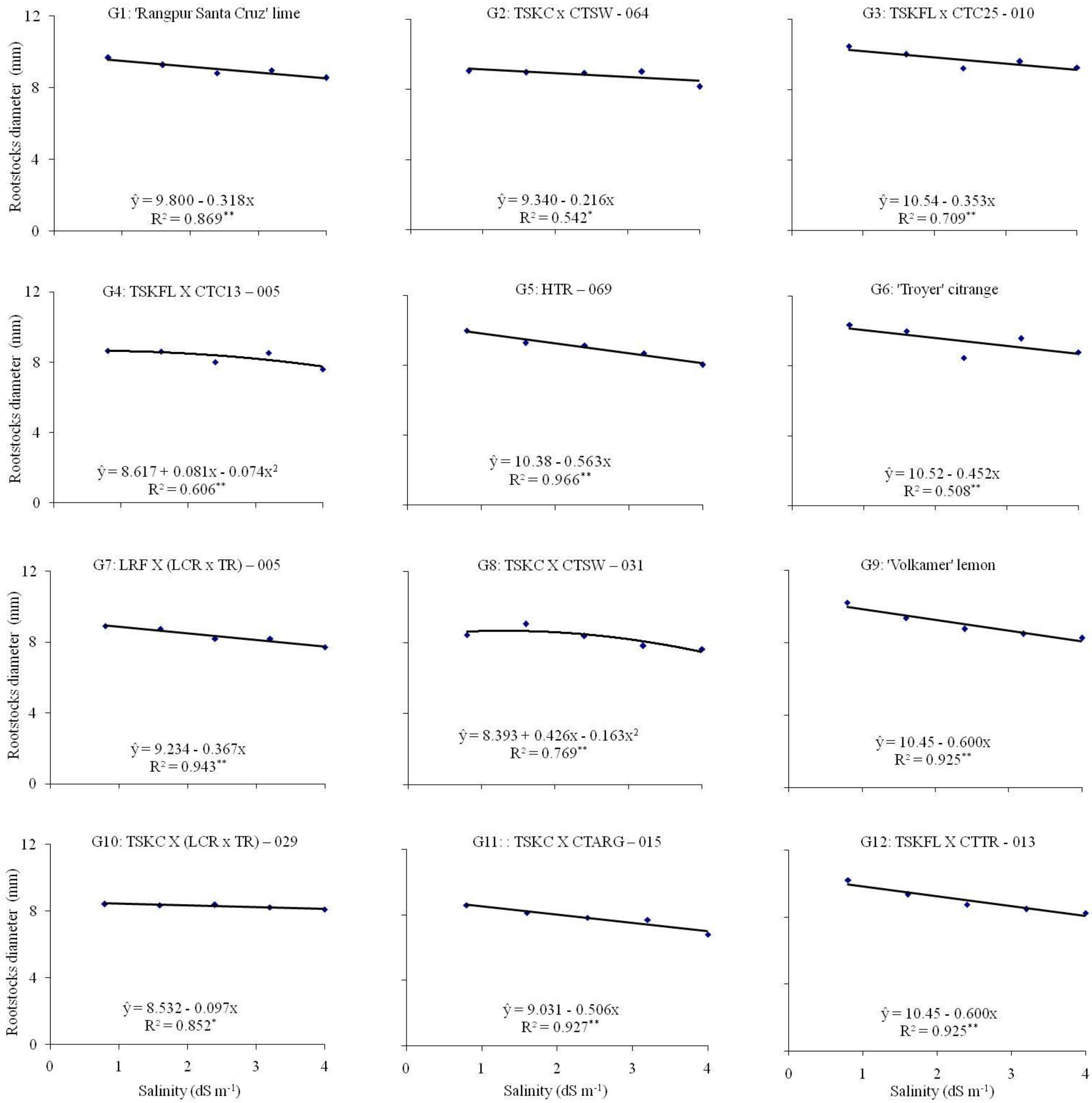

Figure 3. Effect of water salinity on diameter of rootstock (RS) (mm) at 330 days after sowing (DAS) in 12 genotype of citrus rootstocks.

decrease in stem diameter of the scion until the plants died with increasing salinity.

The remaining genotypes best fitted to the quadratic model, however, had a gradual decrease with increasing salinity. Behavior similar to ‘Rangpur’ lime combined with the scion varieties was observed in 'Volkamer' lemon, with greater stem diameter of scion in the scion-rootstocks combination "Star Ruby/Volkamer" in some salinity levels.

Another interesting combination between hybrid trifoliate (HTR - 069) as rootstock and scion of grapefruit was that except for the first salinity level (S1, 0.8 dS m^{-1}), the estimated averages were not close to those of "Star Ruby-Rangpur" and "Star Ruby-Volkamer"; moreover, in

Table 2. Regression equations for number of leaves according to water salinity (dS m^{-1}) at 330 days after sowing (DAS) for 24 scion-rootstock citrus combinations.

Rootstock	Scion	
	'Tahiti' lime	'Star Ruby' grapefruit
01. 'Rangpur Santa Cruz' lime	$y = -1.5956x^2+3.9438x+18.112$ $R^2 = 0.9693$**	$y = -1.1728x+16.365$ $R^2 = 0.5075$**
02. TSKC x CTSW - 064	y = 4.5 ns	y = 7.9 ns
03. TSKFL x CTC25 - 010	$y = -0.7175x^2+0.0058x+10.348$ $R^2 = 0.8286$**	$y = -1.3213x^2+3.0389x+7.5315$ $R^2 = 0.7792$**
04. TSKFL x CTC13 - 005	$y = -0.0728x^2-4.8606x+19.463$ $R^2 = 0.9044$**	$y = -0.8758x^2+0.3318x+12.55$ $R^2 = 0.9163$**
05. HTR - 069	$y = -1.338x^2+1.6196x+17.443$ $R^2 = 0.8427$**	$y = -0.5168x^2+0.6357x+13.962$ $R^2 = 0.6599$**
06. 'Troyer' citrange	$y = -5.4093Ln(x)+9.1946$ $R^2 = 0.6934$**	$y = -1.3194x+13.033$ $R^2 = 0.7333$**
07. LRF x (LCR x TR) - 005	$y = -0.3162x^2-2.4196x+15.25$ $R^2 = 0.7331$**	$y = -0.5673x^2-1.3539x+14.67$ $R^2 = 0.8406$**
08. TSKC x CTSW - 031	$y = -0.8309x^2+3.6825x+4.9333$ $R^2 = 0.3482$**	$y = -0.0577x^2-1.4128x+12.227$ $R^2 = 0.9652$**
09. 'Volkamer' lemon	$y = -2.5882x+17.092$ $R^2 = 0.7231$**	$y = -3.6181x+19.471$ $R^2 = 0.8269$**
10. TSKC x (LCR x TR) - 029	$y = -1.0805x+6.5044$ $R^2 = 0.3018$**	$y = -1.4475x^2+5.0247x+3.9333$ $R^2 = 0.8724$**
11. TSKC x CTARG - 015	$y = 5.4^{ns}$	$y = -1.0278x+9.3944$; $R^2 = 0.5098$**
12. TSKFL x CTTR - 013	$y = -1.3681x+9.3289$ $R^2 = 0.4711$**	$y = -1.8264x+13.206$ $R^2 = 0.8592$**

this combination no significant reduction occurred when water up to ECw = 2.4 dS m^{-1} was used. Thus, for a wider range of materials to choose from, HTR-069 under scions of 'Star Ruby' may be added to the list of best combinations, and it can use water with EC equal to 2.4 dS m^{-1}. It also shows that using 'Star Ruby' grapefruit as scion can improve tolerance to salinity.

The rootstock has a critical role in the growth of the scion genotype. The appropriate combination of rootstock and scion genotypes is essential for plant development and success of crops, especially under conditions of irrigation with saline water. This fact is corroborated in studies carried out by Singh et al. (2003) and Syvertsen and Garcia-Sanchez (2014). Another observation in this study is that the salinity effect is more in ungrafted rootstocks, because the growth reduction was highest in the grafted phase, and death of plants was noted with irrigation water of ECw = 4.0 dS m^{-1}, as observed by Simpson et al. (2014).

In most combinations growth was reduced due to increased salinity, but not up to zero, which allows to affirm that salinity provokes different effects among genotypes and among phases of development. The combination scion/rootstock plays a critical role in this process. Several approaches of plant adaptation to salt stress are present in literature (Syvertsen and Garcia-Sanchez, 2014). It is believed that non-tolerant plants do their adjustment by partitioning salts in vacuoles of old leaves, as described by Taiz and Zeiger (2009). Thus, when the canopy was cut from rootstock, salts began to accumulate in the graft tissue, which become thin and, thus, fail to grow. This fact is associated to the combinations of TSKFL x CTC25 - 010 under 'Tahiti' and 'Star Ruby' and TSKFL x CTC13 - 005 under 'Star Ruby'.

Table 3. Regression equations for length of stem scion and water salinity (dS m^{-1}) 330 days after sowing (DAS) in 24 different scion-rootstock citrus combinations.

Rootstock	Scion	
	'Tahiti' lime	'Star Ruby' grapefruit
01. 'Rangpur Santa Cruz' lime	$y = -0.8885x^2 – 2.6067x + 36.95$ $R^2 = 0.7132^{**}$	$y = -5.3707x + 34.583$ $R^2 = 0.9368^{**}$
02. TSKC x CTSW - 064	$y = -1.6617x+10.976$ $R^2 = 0.8713^{**}$	$y = -1.7722x + 12.534$ $R^2 = 0.7049^{**}$
03. TSKFL x CTC25 - 010	$y = -4.6038x + 17.239$ $R^2 = 0.8087^{**}$	$y = -3.9606x + 17.216$ $R^2 = 0.7239^{**}$
04. TSKFL x CTC13 - 005	$y = 1.7903x^2-13.864x+33.168$ $R^2 = 0.9216^{**}$	$y = -5.3201x + 25.442$ $R^2 = 0.9601^{**}$
05. HTR - 069	$y = -1.37x^2 + 1.3029x + 26.483$ $R^2 = 0.8495^{**}$	$y = -0.9031x^2 + 0.0756x + 22.446$ $R^2 = 0.8318^{**}$
06. 'Troyer' citrange	$y = -3.4561x + 14.536$ $R^2 = 0.5137^{**}$	$y = -1.5778x + 11.641$ $R^2 = 0.7988^{**}$
07. LRF x (LCR x TR) - 005	$y = -3.1517x + 17.98$ $R^2 = 0.8301^{**}$	$y = -0.7856x^2 - 0.1913x + 17.428$ $R^2 = 0.8413^{**}$
08. TSKC x CTSW - 031	$y = -2.4516x^2+10.014x+1.8733$ $R^2 = 0.8324^{**}$	$y = -0.3614x^2 - 1.2887x + 14.092$ $R^2 = 0.7707^{**}$
09. 'Volkamer' lemon	$y = 3.6855x^2-24.917x + 51.211$ $R^2 = 0.9889^{**}$	$y = 2.1187x^2-15.143x+33.355$ $R^2 = 0.9808^{**}$
10. TSKC x (LCR x TR) - 029	$y = 4.91^{ns}$	$y = -1.4878x^2 + 5.7463x + 2.9844$ $R^2 = 0.5622^{**}$
11. TSKC x CTARG - 015	$y = 5.92^{ns}$	$y = 7{,}28^{ns}$
12. TSKFL x CTTR - 013	$y = 7.90^{ns}$	$y = -1.4409x + 13.086$ $R^2 = 0.8827^{**}$

Although some genotypes have not been prominent among the ones with the highest estimated means, TSKC x CTSW - 064, LRF x (TR x LCR) - 005 and TSKFL x CTTR - 013 have the potential to be used in genetic breeding programs in order to obtain plants with tolerance to salinity, since by analysing averages of these genotypes in the highest and the lowest levels of salinity, the difference is less than 10% (Table 4). Compatibility between individuals (scion and rootstock) should be verified during the formation of citrus plants (scion-rootstock combination), especially because these are new materials. Thereby, according to the results obtained in this study there was no "elephant foot" phenomenon. However, as the materials are young, and the demand for scions is still small, definitive conclusions in this regard must be obtained from field studies.

Conclusion

This study showed that: (1) Salinity reduces the growth of young plants of citrus and the scion-rootstock combination should be analysed to determine the best combination. (2) Evaluation of scion or rootstock separately is vague, since the interaction between these two individuals is predominant in the production systems of citrus plants. (3) When irrigating with saline water, the hybrid TSKFL x CTC25 - 010 and 'Troyer' citrange show higher growth when ungrafted, but die after being grafted and irrigated with water of ECw of 4.0 dS m^{-1}. (4) The combination 'Rangpur Santa Cruz' lime with 'Tahiti' lime is adequate for citrus production with saline water. (5) The 'Volkamer' lemon and the trifoliate hybrid HTR - 069 may compose production systems of citrus seedlings irrigated

Table 4. Regression equations relating stem diameter of scion (mm) and water salinity (dS m^{-1}) 330 days after sowing (DAS) in 24 scion-rootstock citrus combinations.

Rootstock	Scion	
	'Tahiti'	'Star Ruby'
01. 'Rangpur Santa Cruz' lime	$y = -0.4577x + 5.3973$ $R^2 = 0.8450^{**}$	$y = -0.7198x + 5.3811$ $R^2 = 0.8409^{**}$
02. TSKC x CTSW - 064	$y = 2.64^{ns}$	$y = 2.67^{ns}$
03. TSKFL x CTC25 - 010	$y = -1.1125x + 4.4022$ $R^2 = 0.759^{**}$	$y = 2.12^{ns}$
04. TSKFL x CTC13 - 005	$y = -0.4501x^2 + 1.4915x + 2.1489$ $R^2 = 0.8979^{**}$	$y = -0.7668x + 3.9617$ $R^2 = 0.6471^{**}$
05. HTR - 069	$y = -0.3981x^2 + 1.6149x + 2.4679$ $R^2 = 0.8737^{**}$	$y = -0.1304x^2 + 0.1884x + 3.924$ $R^2 = 0.8669^{**}$
06. 'Troyer' citrange	$y = 2.31^{ns}$	$y = -0.2669x + 3.3577$ $R^2 = 0.9115^{**}$
07. LRF x (LCR x TR) - 005	$y = 2.82^{ns}$	$y = -0.1452x^2 + 0.3783x + 2.6614$ $R^2 = 0.7755^{**}$
08. TSKC x CTSW - 031	$y = -0.22x^2 + 0.8712x + 2.2827$ $R^2 = 0.8135^{**}$	$y = -0.0435x^2 + 0.0058x + 3.1151$ $R^2 = 0.8696^{**}$
09. 'Volkamer' lemon	$y = 0.3549x^2 - 2.2425x + 6.8855$ $R^2 = 0.9122^{**}$	$y = 0.2148x^2 - 1.4301x + 5.8009$ $R^2 = 0.9384^{**}$
10. TSKC x (LCR x TR) - 029	$y = 1.94^{ns}$	$y = -0.6904x^2 + 3.4207x - 0.9992$ $R^2 = 0.9103^{**}$
11. TSKC x CTARG - 015	$y = -0.2468x^2 + 0.8759x + 2.1002$ $R^2 = 0.6145^{*}$	$y = 2.46^{ns}$
12. TSKFL x CTTR - 013	$y = 2.35^{ns}$	$y = 2.76^{ns}$

with saline water until 2.4 dS m^{-1}, when grafted with 'Star Ruby' grapefruit.

Conflict of Interest

The authors declared no conflict of interest.

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Water and salt stress in germinating seeds of pitaya genotypes (*Hylocereus* spp.)

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Lack of water and salinity are commonly encountered problems in many regions worldwide. For this reason, certain robust cactus species may represent promising crops. Because it is necessary to assess the ability of cactus species to survive and adapt under conditions of natural stress, the present study aimed to evaluate the effect of water and salt stress on the vigor and viability of seeds of pitaya genotypes using different osmotic potential gradients and different osmotically active agents. The experiment had a completely randomized design with a 3 × 6 × 4 factorial scheme corresponding to three pitaya genotypes (white, hybrid I, and hybrid II), six osmotic potential gradients (0.0, -0.2, -0.4, -0.6, -0.8, and -1.0 MPa), and four osmotically active agents (PEG 6000, KCl, NaCl, and $MgCl_2$), with four replicates. The following variables were analyzed: germination percentage, germination speed index (GSI), and mean germination time (MGT). Statistical analyses were performed for each pitaya genotype. The data pertaining to germination were fitted to a binomial model; the data pertaining to GSI and MGT were fitted to regression models. The germination, GSI, and MGT values for all three pitaya genotypes were optimal with the osmotically active agents KCl and NaCl, regardless of the osmotic potential gradient. At osmotic potential gradients lower than -0.2 MPa, the PEG 6000 polymer was detrimental to pitaya seed vigor and viability. The pitaya hybrid I seeds were more resistant to the adverse conditions, exhibiting higher rates of germination and GSI than those of the other genotypes. The osmotic effect negatively influenced the vigor and viability of seeds of the three pitaya genotypes to a greater extent than the salt effect.

Key words: Cactaceae, *Hylocereus undatus*, *Hylocereus costaricensis*, polyethylene glycol, vigor.

INTRODUCTION

With climate change occurs worldwide and water scarcity becoming increasingly pronounced in many areas, the species of family Cactaceae, which can be produced under conditions of limited water resources, have become promising for the future of mankind. The pitaya can adapt to different environmental conditions and has therefore been introduced into countries with different edaphoclimatic conditions; because of Its robustness, this, crop may, represent a cultivation option in unfavorable areas (Mizrahi et al., 2002; Tel-Zur et al., 2004).

The organoleptic characteristics and nutraceutical

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properties of the pitaya have made this crop attractive to the consumers; in addition, certain pitaya species are rich in antioxidants, vitamins, and fiber and are sources of vitamin A, phosphorus, calcium, potassium, and sodium (Crane and Balerdi, 2005; Wu et al., 2006; Esquivel et al., 2007).

Because the pitaya culture can be propagated by seeds, it becomes necessary to evaluate the germination process these in different conditions in order to know the possible factors influencing it; since the germination percentage is dependent on external and internal factors regarding the seed, for example, water, salinity, oxygen, temperature, light, the substrate, health, etc.

Water is one of the most important environmental factors influencing the seed germination process, because is the matrix where most of the biochemical and physiological processes occur; with reactivation of metabolism; participates in enzymatic reactions, in solubilization, and in the transport of metabolites, besides being a reagent in the hydrolytic digestion of proteins, carbohydrates and lipids of seed reserve tissues (Marcos Filho, 2005; Virgens et al., 2012).

Imbibition depends on the osmotic potential gradient (water tension) that exists between the seed and the external environment (Ávila et al., 2007). When the osmotic potential of the solution is lower than the potential within the embryonic cells, absorption of the water necessary for the seed to germinate becomes difficult, affecting the uniformity, speed, and percentage of seed germination, which, in turn, affects cell elongation, cell wall synthesis, and seedling formation (Marcos Filho, 2005; Machado Neto et al., 2006).

Thus, for the germination process to occur, it is essential that the moisture content, which is dependent on seed chemical composition and testa permeability, be minimal (critical point). For each species, there is a critical osmotic potential value below which germination does not occur (Carvalho and Nakagawa, 2012).

Salinity is an important environmental issue that can adversely affect crops worldwide, especially in arid and semi-arid regions. The sensitivity of species to salinity during the germination phase can affect the establishment of a crop and may affect the crop productivity. Salts act on the osmotic potential of the substrate, reducing the potential gradient between the substrate and the seed surface and thus restricting water uptake by the seed (Oliveira et al., 2011).

The reduced osmotic potential associated with salt toxicity not only prevents seeds from absorbing water but also affects seed germination, cell division and elongation, reserve mobilization, and the development of many species in different regions (Lima et al., 2005; Marcos Filho, 2005; Nogueira et al., 2005). It is known that high total salt concentrations in cells can inactivate enzymes and inhibit protein synthesis (Taiz and Zeiger, 2013). However, because adaptation to stressful conditions results in integrated events that occur at several levels, the mechanisms by which plants tolerate high levels of salinity remain unclear due to involve morphological, anatomical, cellular, biochemical, physiological, and molecular changes (Zhu, 2002; Abreu et al., 2008). These changes vary with plant species, the development stage of the plant, and the type, duration, and intensity of the stress (Larcher, 2000).

The ability of plants to tolerate water and salt stress has been extensively studied, with the goal of finding species that are more resistant to these conditions. In these studies, solutions with different osmotic potentials are used to moisten substrates (usually paper towels), and seeds are placed to germinate on these substrates in an attempt to simulate conditions of water and salt stress; the point of tolerance of different species to drought and salinity is then identified. Certain authors have observed that saline solutions have the strongest effect on seed germination, whereas other authors have observed that polyethylene glycol (PEG) solutions more strongly affect germination; some authors found that both solutions had equal effects on germination (Moraes and Menezes, 2003; Duan et al., 2004; Sosa et al., 2005; Zhang et al., 2010).

Water restriction is usually induced by adding osmotically active solutes such as PEG, potassium chloride (KCl), sodium chloride (NaCl), and magnesium chloride ($MgCl_2$). However, each osmotic agent has chemical differences that may affect seed germination differently, even in the presence of similar osmotic potentials.

PEG, which is not easily metabolized by living organisms, not absorbed by cells because of its high molecular weight (>4000), and is chemically inert, stable, and non-toxic to seeds, has been extensively used in seed germination studies (Mexal et al., 1975; Santos et al., 2008). Studies of the germination response of seeds subjected to conditions of artificial stress are important ecophysiological tools that can be used to understand the ability of species to survive and adapt to conditions of natural stress (such as drought and saline soils, which are commonly encountered in agricultural regions). These tools are also used to evaluate the sensitivity of these species and their ability to adapt aggressiveness and dominance strategies when subjected to adverse and/or new environments (Larcher, 2000; Rosa et al., 2005; Pereira et al., 2012).

In light of the diversity of the family Cactaceae, basic information on the optimal germination conditions of this plant group is lacking. Thus, the present study aimed to evaluate the effect of water and salt stress on the vigor and viability of seeds of pitaya genotypes using different osmotic potential gradients and different osmotically active agents.

MATERIALS AND METHODS

The study was conducted in May 2012 at the Seed Production and Technology Laboratory of Londrina State University (Universidade

Estadual de Londrina - UEL) located in Londrina, Paraná state, Brazil. The seeds were collected from ripe fruit from mother plants of three pitaya genotypes - *Hylocereus undatus* (white pitaya); *H. undatus* x *H. costaricensis* (pitaya hybrid I), and *H. costaricensis* x *H. undatus* (pitaya hybrid II) – grown at the experimental site of the UEL Department of Agronomy located at 23°23' S and 51°11' W at a mean altitude of 566 m. The approximately 10-year-old pitaya plants were grown on an area of soil classified as eutrophic, latosolic red nitosol (Embrapa, 2013). The plants were spaced 2.0 × 3.0 m apart and trellised with 2.5 m tall stands, with two plants per stand.

The pulp was manually extracted from the fruit with a spoon and placed in a beaker containing a solution of water (1 L) and sucrose (25 g L^{-1}); the mixture was left for 48 h at room temperature to promote the fermentation process and facilitate seed extraction. Subsequently, the solution was sieved under running water to eliminate the pulp residues and retain the seeds. The seeds were then placed on paper and shade-dried at room temperature for 48 h.

The following were used for the study: three pitaya genotypes (*Hylocereus undatus* (white pitaya); *H. undatus* x *H. costaricensis* (pitaya hybrid I), and *H. costaricensis* x *H. undatus* (pitaya hybrid II)), six osmotic potential gradients (0.0, -0.2, -0.4, -0.6, -0.8, and -1.0 MPa), and four osmotically active agents (PEG 6000 polymer and potassium chloride (KCl), sodium chloride (NaCl), and magnesium chloride ($MgCl_2$) salts) in a 3 x 6 x 4 factorial scheme. The experimental design was completely randomized with four replicates.

The osmotic potential gradients were obtained according to the methods described by Braccini et al. (1998). The physiological quality of the seeds was evaluated by conducting a germination test in which 50 seeds per replicate were arranged in crystal polystyrene boxes (Gerbox®) lined with blotting paper moistened with the respective solutions for each treatment (using two and a half times the dry weight of the substrate). The experiment was conducted in a germinator with a 24-hour photoperiod regulated with a fluorescent lamp and kept at a constant temperature of 25°C.

The seeds were evaluated daily for 23 days (that is, at which point the germination process was stabilized), and seeds were considered germinated when they exhibited root extension greater than or equal to 2 mm. The following variables were analyzed: germination percentage (G); germination speed index (GSI), calculated according to the method described by Maguire (1962); and mean germination time (MGT), in days, determined according to the method described by Lima et al. (2006).

The statistical analyses were performed using the software R (R DEVELOPMENT CORE TEAM, 2014). To test the germination potential of the three pitaya genotypes exposed to the different osmotic potential gradients and osmotically active agents, the data were fitted to three binomial models (one for each pitaya genotype), with the percentage of germinated seeds as the response variable. The variability of the observed responses was greater than the variability that the binomial model is able to accommodate, that is, the data exhibited over dispersion. To accommodate this extra variability, the variances of the binomial model parameters were multiplied by the heterogeneity factor (Mascarin et al., 2010). For the variables GSI and MGT, the data were fitted to regression models in which the explanatory variables were the osmotic potential gradients and the osmotically active agents. To test the fit of the models, semi-normal probability plots were constructed with simulation envelopes; the model provides a good fit if most of the points are within the envelope (Urbano et al., 2013).

RESULTS

Figure 1 shows the germination percentages of the white pitaya (A), pitaya hybrid I (B), and pitaya hybrid II (C) genotypes with the different osmotically active agents (KCl, $MgCl_2$, NaCl, and PEG) at each osmotic potential gradient (MPa).

Regardless of the osmotic potential gradient of the KCl and NaCl salts, the germination percentages of the white pitaya (Figure 1A) did not significantly differ (approximately 82% and 87%, respectively). For the $MgCl_2$ salt, lower osmotic potential gradients were associated with lower germination percentages (decreasing from 96% at 0.0 MPa to 42% at -1.0 MPa).

The PEG 6000 polymer was the osmotically active agent that most negatively affected white pitaya seed germination; reduced germination percentages were observed as the osmotic potential gradient decreased (from 97% at 0.0 MPa to 0% at -0.8 and -1.0 MPa). In other words, the white pitaya genotype did not germinate at osmotic potential gradients lower than -0.6 MPa.

For pitaya hybrids I and II (Figure 1B and C), the germination percentages (approximately 90% and 83%, respectively) did not significantly differ when different KCl, $MgCl_2$, and NaCl salt concentrations were used, that is, germination is statistically equal regardless of the osmotic potential gradient.

With PEG, the seed germination of both genotypes decreased as the osmotic potential gradient decreased. The germination of pitaya hybrid I was 97% at 0.0 MPa and 0% at -1.0 MPa; the germination of pitaya hybrid II decreased from 93% at 0.0 MPa to 0% at osmotic potential gradients lower than -0.6 MPa. Osmotic potential gradients lower than -0.2 MPa obtained with PEG were unfavorable for the germination of the seeds of all three genotypes.

It is noteworthy that for all of the osmotically active agents and for all osmotic potential gradients, the pitaya hybrid I achieved better seed germination than the other two genotypes, indicating that the pitaya hybrid I seeds are more resistant to the adverse conditions to which they were exposed.

The germination speed index (GSI) of all three pitaya genotypes (Figure 2A, B and C) did not significantly differ at the different osmotic potential gradients achieved with KCl and NaCl. However, for all three genotypes, the GSI decreased with decreasing osmotic potential gradient (MPa) for the osmotically active agents $MgCl_2$ and PEG.

PEG led to a more pronounced decrease in GSI for all of the pitaya genotypes: for the white pitaya, hybrid I, and hybrid II, the GSI decreased from 21.62, 22.30, and 17.91 at 0.0 MPa to less than 4.96 at -0.6, -0.8, and -0.6 MPa, respectively. The GSI was not calculated at potentials below -1.0 MPa for any of the genotypes, as no white pitaya or pitaya hybrid II seeds germinated at osmotic potentials lower than -0.6 MPa and no pitaya hybrid I seeds germinated at osmotic potentials lower than -0.8 MPa.

The GSI of pitaya hybrid I seeds (Figure 2) was higher than that of the other two genotypes under all conditions,

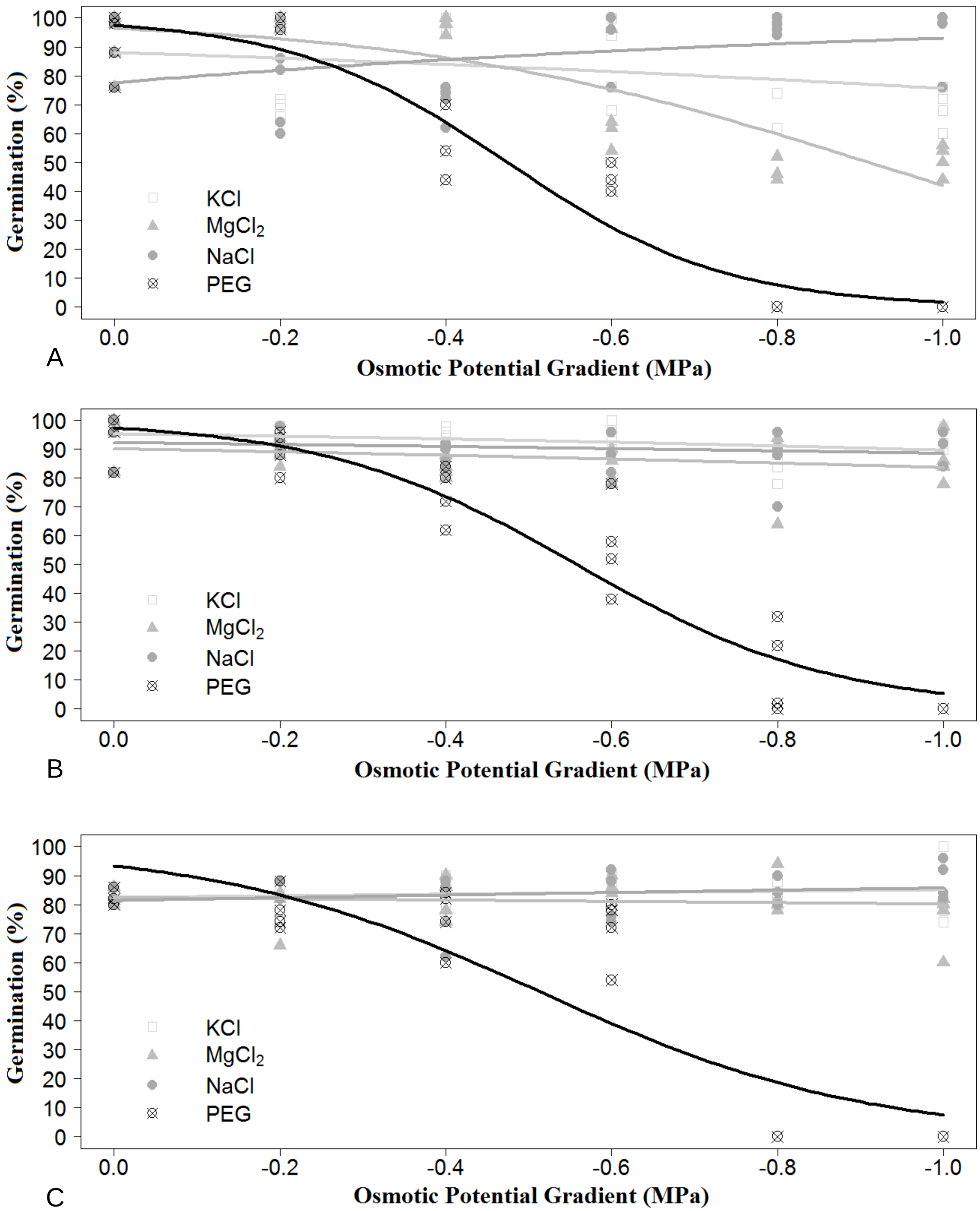

Figure 1. Germination (%) of white pitaya (A), pitaya hybrid I (B), and pitaya hybrid II (C) seeds exposed to different osmotically active agents (KCl, $MgCl_2$, NaCl, and PEG) and osmotic potential gradients (MPa) with fitted curves for the models.

indicating that this genotype develops to a greater extent under adverse conditions than the other genotypes.

For all three pitaya genotypes, the mean germination time (MGT - days) did not significantly differ by osmotic potential gradient (MPa) with the KCl and NaCl salts (Figure 3); the average MGT was 4.98 days for the white

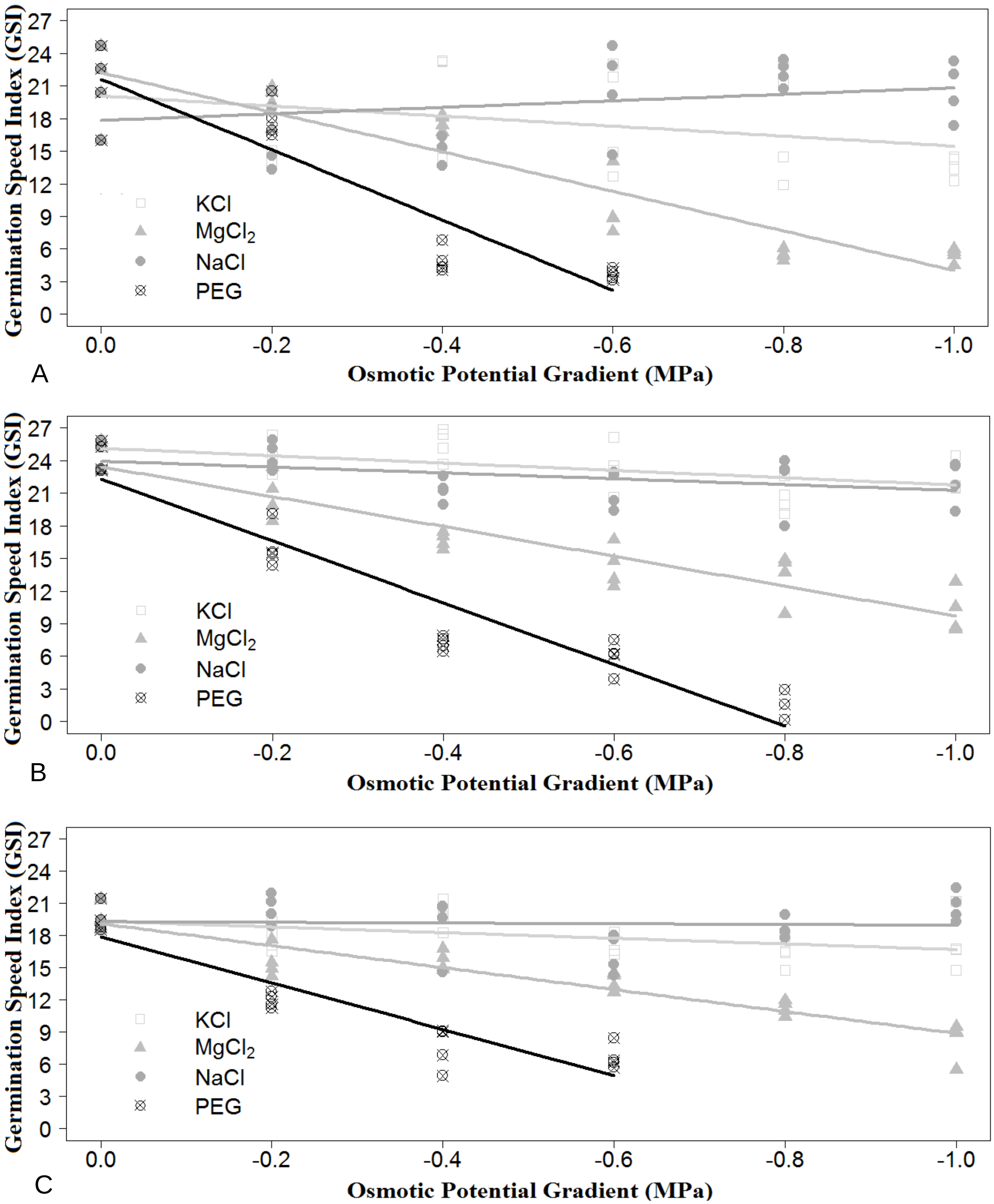

Figure 2. Germination speed index (GSI) of white pitaya (A), pitaya hybrid I (B), and pitaya hybrid II (C) seeds with different osmotically active agents (KCl, $MgCl_2$, NaCl, and PEG) and osmotic potential gradients (MPa) with fitted curves for the models.

pitaya (Figure 3A), 4.16 days for pitaya hybrid I (Figure 3B), and 4.83 days for pitaya hybrid II (Figure 3C).

For seeds exposed to $MgCl_2$, the MGT increased with decreasing osmotic potential gradient, indicating that osmotic potential gradients lower than -0.4 MPa increase the duration of the germination process. In the field, such

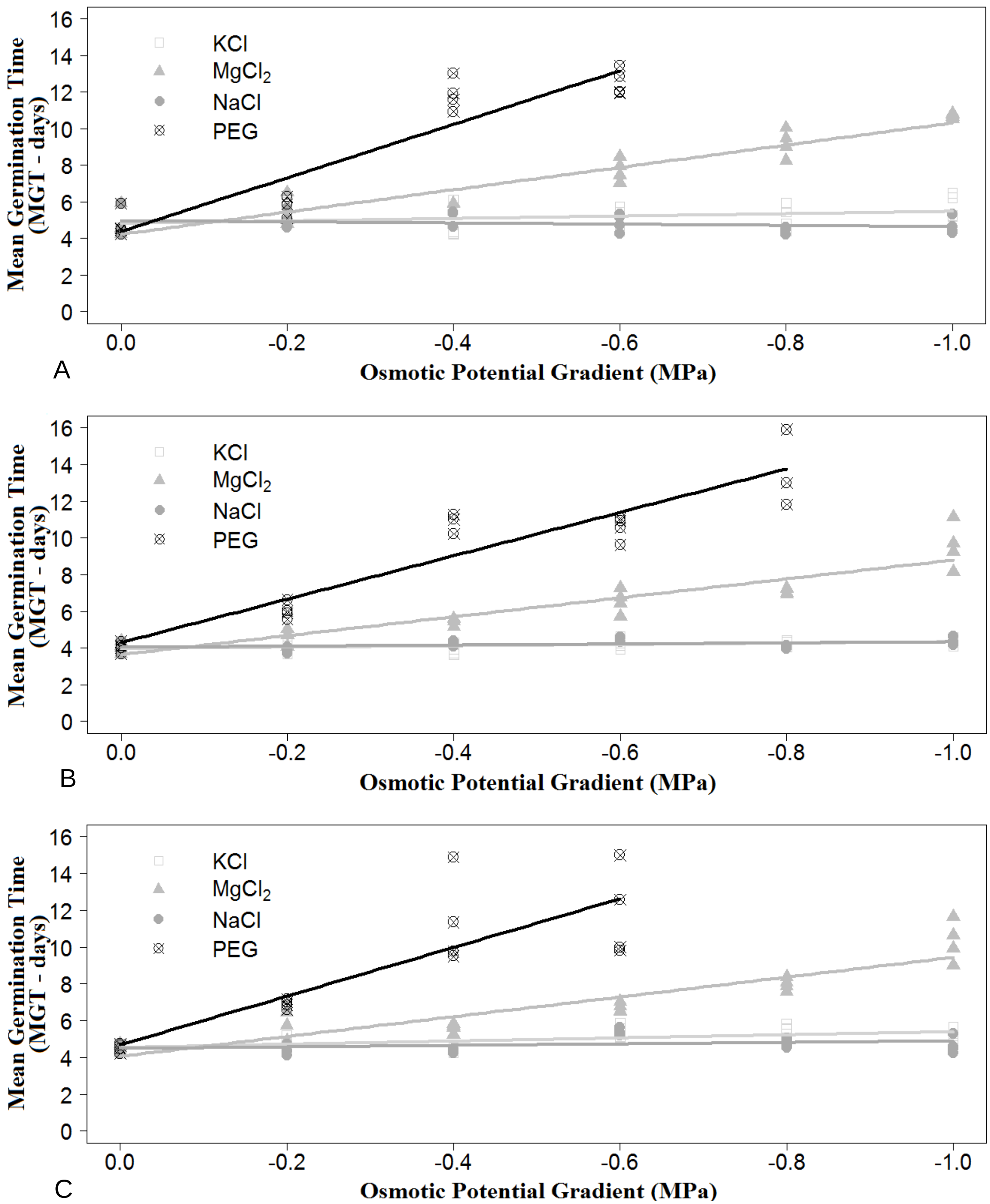

Figure 3. Mean germination time (MGT - days) of white pitaya (A), pitaya hybrid I (B), and pitaya hybrid II (C) seeds exposed to different osmotically active agents (KCl, MgCl2, NaCl, and PEG) and osmotic potential gradients (MPa) with fitted curves for the models.

an increased germination time would be detrimental to the crop, as the seeds would be exposed to unfavorable edaphoclimatic conditions for longer periods of time.

For the three pitaya genotypes, the MGT of seeds exposed to substrates moistened with PEG varied with the osmotic potential gradient; more specifically, lower gradients resulted in higher MGTs. The MGT was 4.39 days at 0.0 MPa and 13.17 days at -0.6 MPa for the white pitaya; the MGT increased from 4.30 and 4.69 days at 0.0 MPa to 13.77 and 12.64 days at -0.8 and -0.6 MPa for pitaya hybrids I and II, respectively. When the gradients were lower than -0.6 MPa for the white pitaya and pitaya hybrid II and lower than -0.8 MPa for pitaya hybrid I, no seeds germinated and it was impossible to calculate the MGT. It is noteworthy that PEG and osmotic potential gradients lower than -0.2 MPa were detrimental to the MGT for all of the pitaya genotypes.

DISCUSSION

According to Mizrahi et al. (1997), cacti in general are drought-tolerant and salt stress-susceptible. However, to confirm the tolerance and/or susceptibility of a species, its developmental stage should be considered. Strogonov (1964) studied plant physiological responses and concluded that the occurrence of salinity-related damage depends on the vegetative phase of the plant.

The three pitaya genotypes evaluated in this study were water stress-susceptible during the germination-emergence stage at osmotic potential gradients lower than -0.2 MPa obtained with the PEG 6000 polymer; the germination, GSI, and MGT were all lower at these gradients than at 0.0 MPa (distilled water). Similar results were obtained by Moraes and Menezes (2003) who found that PEG 6000 produces more stressful effects on the performance of *Glycine max* seeds than KCl, $MgCl_2$, and NaCl salts when the osmotic potential is reduced to -0.8 MPa. This most likely occurs because the PEG solutions exhibit high viscosity, thus limiting the amount of O_2 available to the seeds and consequently reducing their germination potential (Yoon et al., 1997).

According to Moraes and Menezes (2003), osmotic potentials of -0.8 MPa induced by PEG 6000 and $MgCl_2$ prevent germination and reduce the vigor of *G. max* seeds. In the present study, the -0.6 MPa potential was able to prevent germination in the white pitaya seeds and pitaya hybrid II seeds and the -0.8 MPa potential produced the same effect in pitaya hybrid I. However, in solutions of $MgCl_2$, the germination process remained uninhibited for all of the genotypes evaluated until the potential reached a value of -1.0 MPa.

The results pertaining to the salt effect were inconsistent with those obtained by Mizrahi et al. (1997); in this study, none of the variables differed significantly when the seeds were exposed to substrates moistened with distilled water or with KCl and NaCl regardless of the osmotic potential gradient, indicating that all of the genotypes were salt-stress tolerant. Corroborating the results obtained in this study, Ungar (1978) reported that mannitol and PEG have a more pronounced inhibitory effect on several halophytes than inorganic ions, indicating that the seeds are affected by osmotic stress rather than by the toxicity of specific ions.

Salinity affects seed germination via osmotic effects (Bliss et al., 1986), ion toxicity (Hampson and Simpson, 1990), or both (Huang and Redmann, 1995). Zehra et al. (2013) support the claim that different salts produce both osmotic and ionic effects on seed germination and vigor; so only one salt is not applicable to field conditions, which is a mixture of different salts. For this reason, different osmotically active agents (KCl, $MgCl_2$, NaCl, and PEG) were used in this study to determine the specific toxicity of ions in different pitaya genotypes.

It is known that the osmotic and toxic effects of salts are exerted simultaneously on plants: toxicity directly affects plant physiological and metabolic processes, whereas the osmotic factor acts indirectly by reducing osmotic pressure and consequently limiting the absorption of water and nutrients (Hu and Schmidhalter, 2005). According to Zhang et al. (2010), ionic effects can be distinguished from osmotic effects by comparing the effects of saline solutions and iso-osmotic solutions with those of an inert osmotic agent such as PEG (which cannot penetrate the cell wall). Germination inhibition in PEG-treated seeds is attributed to osmotic effects only, and any difference in the germination of salt-treated seeds and PEG-treated seeds is usually attributed to ionic effects (Dodd and Donovan, 1999).

Thus, in this study, the osmotic effect was more detrimental to seed vigor and viability in the three pitaya genotypes than the toxic effect of the salts; indeed, reduced germination, GSI, and MGT occurred in the seeds exposed to the PEG solution (which is not a salt), suggesting that the osmotic effect was responsible.

According to Gulzar and Khan (2001), the salinity threshold for a significant decrease in germination varies among species. Moraes and Menezes (2003) suggest that the negative effects caused by salt stress may be related to the type of salt used. According to Sosa et al. (2005), the germination of *Prosopis strombulifera* seeds was not only affected by salt concentrations (or osmotic potential) but also by the nature of the ions in the saline solutions and their interactions. These authors found that the osmotic agent KCl inhibited germination to a greater extent than NaCl; indeed, at an osmotic potential of -0.8 MPa, the germination percentage was inhibited by a KCl solution but not by a NaCl solution. High intracellular concentrations of Na^+ and Cl^- can inhibit cell division and expansion, slowing seed germination and even leading to seed death (Neumann, 1997; Zhang et al., 2010).

For Duan et al. (2004), the germination of *C. glaucum* seeds decreased with increasing salinity, and germination was inhibited to a greater extent by $MgCl_2$ than by NaCl. Zehra et al. (2013) found that $MgCl_2$ had more pronounced toxic effects on *Phragmites karka* seeds than KCl, which, in turn, was more toxic than NaCl.

In the present study, $MgCl_2$ was the only salt that reduced the GSI and increased the MGT for all three pitaya genotypes and delayed germination in the white pitaya. At osmotic potential gradients lower than -0.4 Mpa, the toxic effect of this salt was intensified.

Ungar (1978) reported that the toxic effects of specific ions have less influence on seed germination than the osmotic potential. This phenomenon was observed in the present study, as PEG (i.e., the osmotic effect) reduced germination, the GSI, and the MGT more than the saline solutions (ion toxicity).

Corroborating the results obtained in the present study for the pitaya genotypes, Zhang et al. (2010) assessed the germination and GSI of *Hordeum vulgare* seeds exposed to PEG or NaCl and found that both variables were higher in the saline solution and that germination occurred faster and at lower osmotic potentials in seeds exposed to NaCl. Thus, these authors suggested that seeds incubated in NaCl were less negatively affected by osmotic potential or better able to adapt to decreasing osmotic potentials than seeds incubated in PEG. According to these authors, *H. vulgare* seeds absorb sodium, which results in an additional osmotic potential, higher water absorption, and faster germination, even in environments with lower osmotic potential.

In contrast, for Sosa et al. (2005), the germination of *P. strombulifera* seeds was greater in treatments with PEG than in all of the salt treatments at osmotic potential gradients less than or equal to -0.8 MPa. Additionally, Katembe et al. (1998) demonstrated that high NaCl concentrations were more inhibitory to water absorption, germination, and seedling root length in *Atriplex prostrata* than PEG. In opposition to, Duan et al. (2004) found that similar concentrations of NaCl and PEG have similar effects on *C. glaucum* germination.

Because sodium chloride is commonly encountered in soils (Khan and Gul, 2006) and causes salinization, it has been extensively used in studies of germination. Thus, it is not surprising that plants have developed mechanisms to regulate the accumulation of sodium chloride (Munns and Tester, 2008). However, other chloride-, sulfate-, and carbonate-based salts and their interactions can also affect seed germination (Khan, 2002).

Zehra et al. (2013) found that the germination of *P. karka* seeds decreases with increasing salinity and attributed this decrease to ion toxicity and variable osmotic stress due to composition of salts; more specifically, the K^+ cation was usually the most toxic, followed by Mg^{2+} and Na^+. However, the salts did not affect seed viability, which suggests that the seeds went into dormancy. Shaikh et al. (2007) found that the germination of *Urochondra setulosa* seeds was inhibited by increasing concentrations of salts (NaCl, Na_2SO_4, $MgSO_4$, and KCl).

Luders and McMahon (2006) found that the pitaya does not tolerate saline environments. However, Bárcenas-Abogado et al. (2002), in a comparative study of different *Hylocereus*, found that the number of shoots and the root dry matter yield did not decrease with increasing salinity. These findings confirm the results of the present study that found that the salt effect was not detrimental to the plants at the development stage. In contrast, Cavalcante et al. (2008) found that salinity resulted in reduced plant height, stem diameter, root length, number of additional stems, and dry root and shoot weight in *H. undatus;* these authors also found that the stem tissues collapsed under conditions of high salinity and concluded that pitaya roots are as sensitive to salt effects as the shoots.

Information on pitaya salt tolerance remains limited. However, the fact that this species displays cross fertilization and can consequently experience high genetic variability may explain the different saline classifications recorded in the scientific literature (Cavalcante et al., 2008). The pitaya hybrid I seeds were more resistant to the adverse conditions of water restriction and salinity, as they exhibited higher germination and GSI values than the other genotypes. This result may be explained by heterosis. Coimbra et al. (2006) also concluded that achieving heterosis in hybrid rice cultivars is one of the most important technical applications of genetics in agriculture and can produce more vigorous cultivars with higher production.

For species such as the pitaya (*H. undatus*), the critical level of saline solution has not yet been determined. Thus, studies involving salt tolerance are valuable, especially in regions with arid and semi-arid climates where these conditions are a major environmental problem (Cavalcante et al., 2008).

Conclusion

The germination, germination speed index, and mean germination time were more optimal for all three genotypes in the presence of the osmotically active agents KCl and NaCl, regardless of the osmotic potential gradient.

At osmotic potential gradients lower than -0.2 MPa, the PEG 6000 polymer was detrimental to pitaya seed vigor and viability.

The pitaya hybrid I seeds exhibited higher germination percentages and GSI values than the other genotypes and were thus more resistant to the adverse conditions to which they were exposed.

The osmotic effect negatively influences the vigor and viability of the three pitaya genotypes to a greater extent than the salt effect.

Conflict of Interest

The authors have not declared any conflict of interest.

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Agronomic biofortification of maize, soybean and groundnut with selenium in intercropping and sole cropping systems

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Agronomic biofortification of staple food crops with micronutrients important for human nutrition, such as selenium (Se), is currently being advocated to address widespread deficiencies in the diets of populations in sub-Saharan Africa. Previous research has shown that there is likely to be widespread dietary Se deficiency in Malawi due to low concentration of Se in edible parts of the staple crops, such as maize (*Zea mays* L), on low-pH soils, but that this could be addressed through agronomic biofortification using Se-enriched fertilisers. Farmers often intercrop maize with legumes such as groundnut (*Arachis hypogaea*) and soybean (*Glycine max*). Therefore, a field study during the 2012/2013 cropping season examined the effect of foliar application of Se on its concentration in grains and stover of maize, soybean and groundnut grown as intercrops or sole crops at three sites in Malawi. Mean Se concentrations were highest in soybean seed, followed by groundnut seed and maize grain, both in plots with added Se and without. Application of 10 g ha-1 of Se increased Se concentration in maize grain by 8-fold, in groundnut seed by 9-fold and in soybean seed by 18-fold; thus universal adoption could increase estimated average dietary Se supply in Malawi from between 21 and 31 µg cap-1 d-1 to between 68 and 78 µg cap^{-1} d^{-1}.

Key words: Selenium, intercropping, food security, hidden hunger, biofortification, fertilizers, mineral micronutrient deficiencies.

INTRODUCTION

SeleniumThe element selenium (Se) is an essential element in the nutrition ofnutrient for humans and livestock. A total of 25 selenoproteins have been identified in humans including iodothyronine deiodinases, thioredoxin reductases, glutathione peroxidases and a range of other selenoproteins, with critical roles in thyroid functioning, cell proliferation, antioxidant defence and the immune response (Fairweather-Tait et al., 2011). Chronic and extremely low levels of Se intake leading to concentrations in blood plasma levels of <20-40 µg L^{-1}

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are thought to be a major underlying cause of Keshan disease (a cardiomyopathy) and Kashin-Beck disease (an osteoarthropathy). Moderate deficiencies (blood plasma <100 µg L^{-1}) resulting from low dietary intakes or malabsorption and high losses of Se (e.g. due to infection such as HIV) can cause immune dysfunction and increased viral pathogenicity and low Se intakes have been associated with certain cardiovascular disorders and cancers (Beck, 2007; Fairweather-Tait et al., 2011).

Suboptimal dietary Se intake is likely to be widespread in Malawi due to limited phytoavailability of Se in the predominant low-pH soils, and narrow food choices including limited animal-source products (Eick et al., 2009; Chilimba et al., 2011; Gibson et al., 2011). Through analysis of composite diet samples, Hurst et al. (2013) found median intake of Se for adult women in rural Malawi of just 6.8 µg cap-1 d-1 (median absolute deviation, MAD=2.9, range=1.1-62.3, n=56) in an area of low-pH soils but 55.3 µg cap^{-1} d^{-1} (MAD=25.9, range=5.8-192, n=58) in an area of calcareous soils with pH >6.5. This compares to the adult female estimated average requirement (EAR) of 45 µg d^{-1} (IOM, 2000). The EAR is the average daily nutrient intake that meets the requirements of 50 percent of apparently healthy individuals in a particular age and sex group.

Chilimba et al. (2012) reported a linear response in maize grain and stover Se concentration to selenium application using a variety of application methods and rates (R^2 > 0.90). For each g ha^{-1} of Se applied, the Se concentration increased by 11-29 and 3-21 µg kg^{-1} in grain and stover, respectively. Annual application of 5 g ha^{-1} of Se to maize crops grown on low-pH soils in Malawi would raise average dietary Se supply by 26-37 µg $person^{-1}$ d^{-1}, greatly reducing risks of dietary Se deficiency. Agronomic biofortification via Se-enriched fertilisers might therefore be a cost-effective way to address widespread Se deficiency in Malawi, and could follow the policy-precedent of Finland which has successfully increased dietary Se intake through Se biofortification of major crops since 1984 (Eurola et al., 2004; Eurola, 2005; Lyons et al., 2005; Broadley et al., 2006; White and Broadley, 2009; Broadley et al., 2010; Alfthan, 2013).

Current knowledge suggests that Se recovery by crops is inefficient and applied Se is likely to be rapidly leached as soluble selenate (SeVIO42-), adsorbed as selenite ($Se^{IV}O_3^{2-}$; pK_2 = 7.3) or immobilised into organic forms (Mayland et al., 1991; Fordyce, 2013; Gabos et al., 2014). For example, Sager and Hoesch (2006) reported that between 0.7 and 4.7% of applied Se was transferred to barley grain. Chilimba et al., (2012) observed recovery rates in maize grain of 6.5 and 10.8% after applying 10 g Se ha^{-1} at two contrasting sites in Malawi, but in the subsequent residual year < 0.1% of the original Se application was recovered in the crop. These studies were conducted using sole crops, whereas many smallholder farmers in Malawi intercrop maize with other species including legumes and root and tuber crops. It is likely that Se recovery by different crops will differ and suspected that intercropping systems may also influence Se recovery by individual species. The objectives of this study were (i) to determine Se concentration in grains and stover of crops grown in sole and intercropping systems with, and without, application of Se-enriched fertiliser, and (ii) to evaluate the effectiveness of different cropping systems in recovering applied Se.

MATERIALS AND METHODS

Experiments were conducted during the 2012/13 growing season at three research stations of the Malawi Ministry of Agriculture and Food Security: Chitedze Research Station, Zombwe Extension Planning Area and Lunyangwa Research Station (Table 1). The experimental treatments consisted of five cropping systems: monocrop maize, monocrop groundnuts, monocrop soybean, intercrop maize/groundnuts and intercrop maize/soybean. There were two application rates of Se: 0 and 10 g ha^{-1}. The experimental plots were laid out in a randomised complete block design with three replicates. The gross plots contained four ridges of 30 cm height, spaced 75 cm apart and 5 m long. The net plot data were collected comprised 4 m lengths of the two central ridges.

Maize (variety SC627) was used with four seeds per planting station at 75 cm intervals along the ridges, thinned to three plants per planting station after emergence. Soybean (variety Ocepara-4) was used with one seed per planting station at 2.5 cm intervals along the ridges. Rhizobia inoculation was achieved by preparing a mixture of 200 ml of 5% sugar solution and one 50 g sachet of rhizobium inoculant to form a slurry which was poured over the soybean seed and mixed until all seeds were evenly covered; planting of the seed was undertaken on the same day. Groundnut (variety CG7) was used with one seed per planting station at 15 cm intervals along the ridges. Planting spacing for intercrops was identical to the corresponding sole crop. Seeds of the intercropped species were planted between the maize planting stations.

The Malawi national fertiliser recommendation was used with base application of N, P2O5 and K2O (46, 20 and 10 kg ha^{-1}) soon after seed emergence using a 23:10:5 +3S fertiliser and a top dressing of urea at 46kg N ha^{-1} applied two weeks after basal dressing (Table 1). Sodium selenate ($Na_2SeO_{4(aq)}$) solution containing 15.0 mg L^{-1} of Se was applied to the gross plot area at early stem extension stage (~'knee high'). To ensure even application to the crop, the $Na_2SeO_{4(aq)}$ was applied as a high-volume drench (667 ha-1 of water) using a knapsack sprayer, with the operator wearing personal protective equipment of overalls, boots, face-shield and nitrile gloves (Broadley et al., 2010). A 16 L Berthoud Vermorel 2000Pro knapsack tank (Exel GSA, Villefanche-sur-Saône, France) was connected to a 1 m boom, housing three Lurmark 110°, flat-fan spray nozzles (Hypro EU Ltd, Longstanton, Cambridge, UK), spaced equally, with a spray-swath of 1.5 m. A coarse nozzle type "08 white" was used (1180 ml $nozzle^{-1}$ min^{-1}; British Crop Protection Council, 2001) to minimise potential for aerosol drift. Ergonomically acceptable drench rates were calibrated to treat four replicate plots from a single tank at appropriate walking speed with two passes.

Maize and legumes were harvested and weighted to determine biomass and grain yield when the crop was mature and had dried in the field to a moisture content of approximately 15%. Sub-samples of biomass and grain were collected and dried in drying ovens at Lunyangwa and Chitedze research stations at 65 °C for 24 h in preparation for elemental analyses including Se. grainWhole grains and stover were milled in a kitchen blender before shipping to the UK. Samples (~0.4 g dry weight, DW) were digested with microwave heating for 45 min at a controlled pressure of 2 MPa in 3.0 ml of 70% trace analysis grade (TAG) HNO_3, 2.0 ml H_2O_2 and 3.0 ml Milli-Q water (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). The microwave system comprised a Multiwave 3000 platform with a 48-vessel 48MF50 rotor (Anton Paar GmbH, Graz, Austria). Samples were digested in vessels comprising perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). Digested samples were diluted to 20 ml (30% HNO_3) with Milli-Q water (18.2 MΩ cm) and stored at room temperature pending elemental analysis. Immediately prior to analysis, samples were diluted 1-in-10 with Milli-Q water. Selenium (^{78}Se) analysis was undertaken by ICP-MS (X-SeriesII, Thermo Fisher Scientific Inc., Waltham, MA, USA) using a hydrogen reaction cell. Samples were introduced from an autosampler (Cetac ASX-520, Omaha, NE, USA) with 4 × 60-place sample racks, at 1 ml min^{-1} through a concentric glass venturi nebuliser and Peltier-cooled (3°C) spray chamber (Thermo Fisher Scientific Inc.). Internal standards introduced to the sample stream via a T-piece included Ge and Rh (10 µg L^{-1}) in 2% TAG HNO_3 and 2% methanol to enhance Se ionization in the plasma. An external wheat flour standard (NIST 1567a; National Institute of Standards and Technology, Gaithersburg, MD, USA) was used as reference material. Each digestion batch (n=48) included two blank digestions and two certified reference samples; final Se concentrations were converted to mg kg^{-1} DW.

Statistical analysis was conducted in GenStat (V.16.1.10916, VSN International, Hemel Hempstead, UK). ANOVA was performed to determine the influence of Se application, crop-type, site and intercropping versus monocropping on Se concentrations in plant tissues. Data were not transformed prior to ANOVA on the basis of visual inspection and a Shapiro-Wilk test for normality. The efficiency of recovery (R_{Se}, %) of exogenous Se for each crop component was calculated as:

$$R_{Se} = \frac{100 \times Y \times (1\text{-}M) \times (C_{Se} - C_o)}{F_{Se}}$$

Where: Y = fresh-weight yield of the crop component (kg ha^{-1}); M = moisture content (proportion) of the crop component;

C_{Se} = Se concentration in crop component (mg kg^{-1} DW) with exogenous Se application; C_0 = Se concentration in the crop component (mg kg^{-1} DW) without exogenous Se application; F_{Se} = rate of exogenous Se application (mg ha^{-1}).

Recovery efficiency of plots was calculated as the sum of crop components. For calculation of rates of recovery, maize grain moisture content was assumed to be 15%, the national standard for moisture level at harvest. USDA moisture content data (USDA-ARS, 2013) was used for groundnut (6.91%, “Peanuts, virginia, raw”) and soybean (8.54%, “Soybeans, mature seeds, raw”). Moisture content of 15% was assumed for stover of all crop types.

RESULTS AND DISCUSSION

Some samples were excluded from Se analysis due to low yield; termite damage contributed to missing samples of soybean biomass. Yield data was combined across sites to form average yields for each crop. Three samples (two soybean seed and one groundnut seed from plots without Se application) were identified as outliers, defined as Se concentration in excess of three standard deviations from the mean. These samples were presumed to be contaminated, either in the field or post-harvest, so were excluded from statistical analysis. The influence of exogenous Se application, crop-type, site and intercropping versus monocropping was tested using ANOVA (unbalanced design; Table 1).

Application of Se at 10 g ha^{-1} significantly increased Se concentration in crops p<0.001; Tables 2 and 3). There were also significant differences in Se concentration between crop types (p<0.001 but not between sites (p=0.242; Tables 2 and 3). The ANOVA detected a significant influence of intercropping on Se concentration at the 95% level (p=0.038), with crops from intercropped plots having higher mean Se concentration. This might have been due to the denser and more varied canopy cover of intercrop versus monocrop stands which could have spread the arrival of exogenous Se to the root zone, effectively slowing the rate of Se and deposition to the soil surface and thereby increasing opportunity for root-uptake prior to Se being leached, immobilised into organic forms or strongly adsorbed as selenite following reduction in the soil. Greater foliar interception may also have increased the amount of foliar absorption through the leaf epidermis. However, further investigation for each crop-type at both Se application rates using two - sample t-tests did not find significant differences at the 95% level between samples that were intercropped versus those that were monocropped. Sample sizes may have been insufficient.

Mean Se concentrations at 0 g ha^{-1} Se application rate were highest in groundnut stover (0.0648 mg kg^{-1}, n=6, SD=0.0221; Table 3 and Figure 1) followed by soybean seed (0.0453 mg kg^{-1}, SD=0.0167, n=17), groundnut seed (0.0437 mg kg^{-1}, SD=0.0200, n=17), maize stover (0.0234 mg kg^{-1}, SD=0.0125, n=12) and maize grain (0.0135 mg kg^{-1}, SD=0.00505, n=32). With Se application of 10 g ha^{-1}, mean Se concentration increased 5-fold in groundnut stover (0.347 mg kg^{-1}, SD=0.130, n=6; Table 3 and Figure 1), 18-fold in soybean seed (0.813 mg kg^{-1}, SD=0.364, n=18), 9-fold in groundnut seed (0.415 mg kg^{-1}, SD=0.210, n=18), 3-fold in maize stover (0.0791 mg kg^{-1}, SD=0.0547, n=12) and 8-fold in maize grain (0.113 mg kg^{-1}, SD=0.0678, n=36). Thus, Se application of 10 g ha^{-1} raised Se concentration in maize grain and stover by 0.0995 and 0.0557 mg kg^{-1}, respectively. This is consistent with the study of Chilimba et al. (2012) who found that each g ha−1 of Se applied as Na2SeO4 (aq) increased Se concentration in maize grain and stover by 0.011 to 0.029 and 0.003 to 0.021 mg kg^{-1} respectively.

Application of Se did not significantly affect yield of any crop. However, mean maize stover yield in intercropped plots (2,640 kg ha^{-1}, SD=1550, n=18) was less than sole-cropped maize (4,210 kg ha^{-1}, SD=1730, n=26) (t(24)=2.30, p=0.030), mean maize grain yield in intercropped plots (1,940 kg ha^{-1}, SD=1050, n=18) was less than that in sole-cropped maize (3,120 kg ha^{-1}, SD=1260, n=24) (t(22)=2.14, p=0.044), and mean groundnut seed yield in intercropped plots (551 kg ha^{-1}, SD=89.5, n=6) was less than that in sole-cropped groundnuts (908 kg ha^{-1}, SD=242, n=11) (t(9)=3.63,

Table 1. Site location and soil characteristics.

Site	Location (°) (Lat., Long.)	Soil type[a]	Texture class[b]	pH[c]	OM (%)[d]	Soil Se concentration (mg kg^{-1})[e]	Fertiliser applied (kg ha^{-1})[f]		
							N	P_2O_5	K_2O
Chitedze	−13.98, 33.63	Chromic luvisol	scl	5.2	2.03	0.350	92	20	10
Zombwe	-11.32, 33.83	Lixisol	scl	5.8	n.d.	0.200	92	20	10
Lunyangwa	-11.43, 34.05	Ferralsol	scl	5.0	n.d.	0.100	92	20	10

[a]FAO classification (Green and Nanthambwe, 1992); [b]scl = sandy clay loam; [c]Measured in water; [d]Loss on ignition. n.d. = no data; [e]Total soil Se measured by XRF; [f]NPK (base, 23:10:5 + 3S) and N (top, urea)

Table 2. ANOVA to test the influence of Se application, crop-type, site and intercropping versus monocropping on crop Se concentration.

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ Se_rate	1	3.74	3.74	303	<0.001
+ Crop_type	4	3.81	0.95	77.0	<0.001
+ Site	2	0.04	0.02	1.43	0.242
+ Intercrop_yes/no	1	0.05	0.05	4.38	0.038
+ Se_rate. Crop_type	4	3.20	0.80	64.8	<0.001
+ Se_rate.Site	2	0.12	0.06	5.01	0.008
+ Crop_type.Site	7	0.72	0.10	8.31	<0.001
+ Se_rate.Intercrop_ yes/no	1	0.01	0.01	0.86	0.355
+ Crop type.Intercrop_ yes/no	4	0.02	0.00	0.38	0.821
+ Site.Intercrop_y/n	2	0.00	0.00	0.05	0.952
+ Se_rate. Crop_type.Site	3	0.34	0.12	9.29	<0.001
+ Se_rate. Crop_type.Intercrop_ yes/no	4	0.02453	0.00613	0.50	0.738
+ Se_rate.Site.Intercrop_ yes/no	2	0.00016	0.00008	0.01	0.993
+ Crop_type.Site.Intercrop_ yes/no	5	0.14434	0.02887	2.34	0.045
Residual	131	1.61888	0.01236		
Total	173	13.84570	0.08003		

Table 3. Selenium (Se) concentrations (dry-weight, DW) in crop components at Se application rates 0 and 10 g ha^{-1}.

Se application (g ha^{-1})	Crop component	n	Se concentration (mg kg^{-1} DW)			
			Mean	SD	Min	Max
0	Maize grain	32	0.0135	0.00505	0.00415	0.0262
0	Maize stover	12	0.0234	0.0125	0.0107	0.0504
0	Groundnut seed	17	0.0437	0.0200	0.0216	0.0930
0	Groundnut stover	6	0.0648	0.0221	0.0438	0.106
0	Soybean seed	17	0.0454	0.0167	0.0262	0.0947
10	Maize grain	36	0.113	0.0678	0.00669	0.301
10	Maize stover	12	0.0791	0.0547	0.0418	0.241
10	Groundnut seed	18	0.415	0.210	0.0295	0.837
10	Groundnut stover	6	0.347	0.130	0.208	0.563
10	Soybean seed	18	0.813	0.364	0.264	1.55

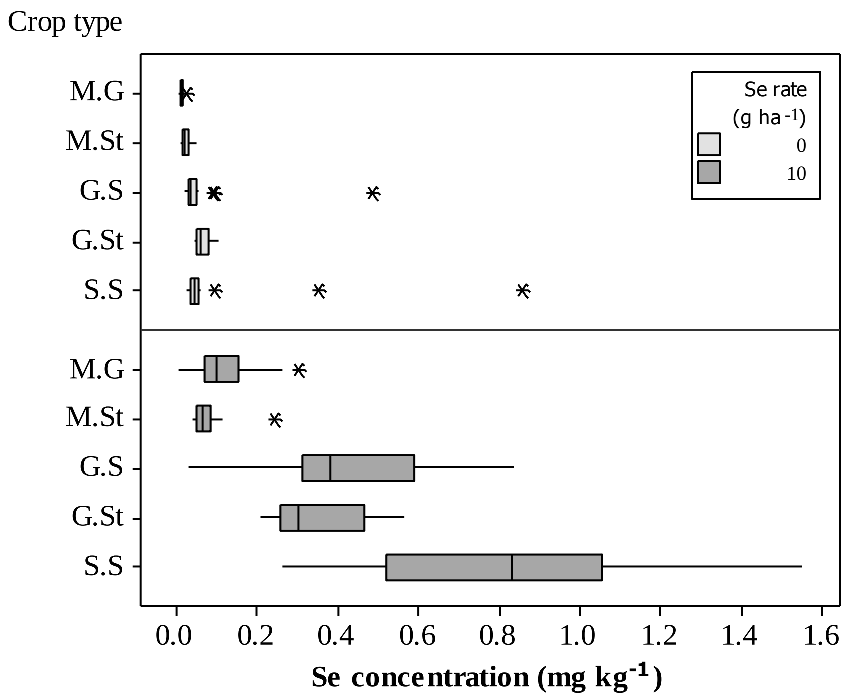

Figure 1. Selenium (Se) concentration in dried maize grain (M.G), maize stover (M.St), groundnut seed (G.S), groundnut stover (G.St) and soybean seed (S.S) at Se application rates of 0 and 10 g ha-1. Boxes represent Q1, Q2 and Q3; whiskers represent 10th and 90th percentiles.

Table 4. Proportional recovery (R_{Se}, %) of Se following application of 10 g ha^{-1} in maize, groundnut, soybean and pigeon pea under contrasting cropping systems.

Cropping system	Constituent component						
	Maize grain	Maize stover	G'nut seed	G'nut stover	Soybean seed	Soybean stover	TOTAL
	R_{se} (%)						
Maize sole	2.64	1.99					4.63
G'nut sole			3.14	11.55			14.69
Soybean sole					7.41	n.d.	n.d.
Maize/g'nut	1.64	1.25	1.91	7.86			12.66
Maize/soybean	1.64	1.25			10.54	n.d.	n.d.

n.d. = no data.

p=0.006). There were no significant differences in yields of soybean seed or groundnut stover due to intercropping. Soybean stover yield could not be tested due to insufficient samples. Negative yield effects of intercropping are likely to be due to competition for water and nutrients and shading effects (Natarajan and Willey, 1986; Yunusa, 1989). However, when the edible components of intercrop plots were combined, mean yields (2,630 kg ha^{-1}, SD=974, n=18) were greater than those of sole crops (1,850 kg ha^{-1}, SD=1500, n=16), although the difference was only marginally significant (t(32)=-1.81, p=0.080).

The cropping system with the highest proportional recovery (R_{Se}) of exogenous Se was sole groundnut (14.7%; Table 4), followed by maize/groundnut intercrop (12.7%) and maize monocrop (4.6%). Soybean seed recovered 7.4% of exogenous Se when monocropped and 10.5% when intercropped with maize. Due to missing data for soybean stover it was not possible to calculate Se recovery in sole soybean or soybean/maize intercrop treatments. Uptake of added Se is generally greater in legumes than cereals (Bisbjerg and Gissel-Nielsen, 1969) and this might be related to protein content as the most common form of Se in plants is generally Se-methionine (Tapiero et al., 2003). With Se fertiliser applied, the cropping system that yielded the most Se in the edible parts (i.e. seed fraction) of the crop was maize/soybean intercrop (1,300 mg ha^{-1}), followed by soybean sole crop (785 mg ha^{-1}), maize/groundnut intercrop (400 mg ha^{-1}), groundnut sole crop (351 mg ha^{-1}) and finally maize sole crop (300 mg ha^{-1}).

The average amounts of maize, groundnut and soybean (fresh-weight) available for consumption in Malawi are 365, 14 and 7 g cap^{-1} d^{-1} according to food balance sheets from the United Nations Food and Agriculture Organization (FAO, 2012). Using composition data for crops at 0 g ha^{-1} Se application, the estimated supply of Se from maize, groundnuts and soybean (that is, 4.2, 0.6 and 0.2903 µg cap-1 d-1, respectively) appears to contribute little towards dietary Se requirements considering an EAR for adult women of 45 µg cap^{-1} d^{-1} (IOM, 2000). With universal coverage of Se biofortification at 10 g ha^{-1}, maize, groundnuts and soybean would supply an average of 35.1, 5.4 and 5.2 µg cap^{-1} d^{-1} of Se, respectively. Average dietary supply of Se from sources other than maize, groundnut and soybean is likely to range between 15 and 25 µg cap^{-1} d^{-1} in Malawi (Donovan et al., 1992; Eick et al., 2009). Average dietary Se intake would therefore range between 61 and 71 µg cap^{-1} d^{-1} which is likely to be optimum for most people (Fairweather-Tait et al., 2011), and provides minimal risk of overdose based on a current safe upper limit of 400 µg Se $person^{-1}$ d^{-1} (IOM, 2000). There may be further benefits of Se biofortification on livestock health since cattle and goats are commonly fed on maize stover residue during the dry season. For example, >6 mg d^{-1} of supplemental Se is required to optimise serum Se status of dairy cattle (Gerloff, 1992). Feed containing >0.1 mg kg^{-1} Se will protect against Se deficiency disorders (Girling, 1984); this compares to concentrations of Se in maize stover of 0.079 and 0.023 mg kg^{-1} with and without Se application, respectively.

Risks of negative environmental or health impacts due to Se toxicity are minimal with agronomic biofortification at 10 g ha^{-1} yr^{-1} of Se. Selenium toxicity can occur over some sedimentary rocks, e.g. the black shale and sandstone deposits of the Great Plains in the USA, where concentrations of total Se in the soil are high (1-10 mg kg^{-1}) and the soil environment alkaline (Muth and Allaway, 1963). However, Oldfield (1999) report that soils with up to 20 mg kg^{-1} total Se did not cause problems to vegetation and livestock in humid lateritic soils in Hawaii. Application of Se at 10 g ha^{-1} yr^{-1} is equivalent to 0.0036 mg kg^{-1} topsoil, assuming 2.8 t ha^{-1} topsoil, and it would take >5 k yr to reach a concentration of 20 mg kg^{-1} Se in the topsoil.

Conclusion

Dietary Se deficiency appears to be widespread in Malawi, on the basis of crop, soil, diet composite, blood and urine surveys (Eick et al., 2009; Chilimba et al., 2011; Gibson et al., 2011; Hurst et al., 2013). Effective biofortification of the staple grain maize with Se through sodium selenate application has been demonstrated previously (Chilimba et al., 2012). This study measured the effect of sodium selenate application on Se concentration in maize, groundnut and soybean in sole- and intercrop systems at three sites with low-pH soils. Foliar application of 10 g ha^{-1} of Se in the form of a sodium selenate liquid drench was effective in increasing Se concentration in maize grain by 8-fold, groundnut seed by 9-fold and soybean seed by 18-fold. Considering all grain and stover components combined, recovery efficiency of exogenous Se was greater in groundnuts as sole crop and maize/groundnut intercrop compared to maize sole-crop. Considering only the edible portion of grain, maize-soybean intercrop provided the highest yield of Se (1,310 mg ha^{-1}). Universal adoption of Se-enriched fertiliser would lead to a 150-225% increase in estimated average dietary supply of Se, from between 21 and 31 µg cap^{-1} d^{-1} to between 68 and 78 µg cap^{-1} d^{-1}. Further research is now required to validate this estimate at wide scales and to monitor impact on human health and nutrition.

Conflict of Interest

The authors have not declared any conflict of interest.

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Study on occurrence and importance of faba bean diseases with special consideration to the newly emerging "faba bean gall" in Tigray, Ethiopia

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Faba bean is one of the most important crops in Ethiopia. The production of the crop is, however, constrained by several disease infections including fungal diseases. A survey was conducted in 2013 to assess the occurrence and importance diseases affecting faba bean in south Tigray. The observed diseases were gall, chocolate spot, ascochyta blight, alternaria leaf spot, black root rot and rust in order of their prevalence. The overall mean incidences of 66, 45.5, 45.9, 28.9, 5.7, 1.1, and 0.4% and with severities of 64.4, 47.5, 15.3, 7.7, 11.8 and 0.2%, respectively. Faba bean gall was the most devastating newly identified disease in Tigray. The severity of the disease ranged from 30% in Emba-Alaje to 100% in Ofla, Enda-Mekoni and Raya-Alamata districts indicating the seriousness of the disease in the area. In addition, improved varieties were relatively tolerant to most identified diseases as compared to the local cultivars except for the new "gall" disease wipes-out fields without any tolerance. Hence, it is very important to use integrated management tactics and risk forecasting that operate on different aspects of the disease etiology.

Key words: Disease, faba bean, gall.

INTRODUCTION

Faba bean is botanically known as *Vicia faba* L.; with the common names including broad bean, horse bean, tic bean and field bean, is one of the earliest domesticated food legumes in the world, probably in the late Neolithic period (Metayer, 2004). Faba bean is used as an important human food in developing countries and as an animal feed in industrialized countries. Feeding value of faba bean is high and this legume has been considered as a meat extender or substitute due to its high protein content (20 to 41%) (Crépona et al., 2010). It has been produced for centuries in Ethiopia and provide the much needed protein supplement to the diet of rural households, which otherwise includes mainly cereals or root crops. From the economic standpoint, faba bean is a source of cash to the farmers and foreign currency to the country. Ethiopian farmers are also cognizant of the role of legumes in general and faba bean in particular in improving soil health by fixing atmospheric nitrogen, and widely use them in rotation with cereals (Sahile et al., 2008). It takes the largest share of the area and production of the pulses grown in Ethiopia including Tigray region. It occupies close to 574, 060 ha of land

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with annual production about 943, 964.2 tones. In Tigray region, it covers an area of 18, 580 ha and production of 32, 175.2 tons annually (CSA, 2013).

Even though Ethiopia is the world's second largest producer of faba bean after China, its share is only 6.96% of world production and 40.5% within Africa (Chopra et al., 1989). The average yield of faba bean under small-holder farmers is not more than 1.6 t ha^{-1} (CSA, 2013). The low productivity of the crop is attributed to susceptibility to biotic and abiotic stresses (Mussa et al., 2008; Sahile et al., 2008). Of the biotic category, diseases are important factors limiting the production of food-legume crops as a whole and faba bean specifically in Ethiopia (Berhanu et al., 2003; Nigussie et al., 2008). More diseases are affecting faba bean, but only a few of them have either major or intermediate economic significance. Among them, fungi are the largest and perhaps the most important groups affecting all parts of the plant at all growth stages. Diseases such as chocolate spot (*Botrytis fabae* Sard.), rust (*Uromyces vicia-fabae*), black root rot (*Fusarium solani*), and foot rot (*F. avenaceum*) are among fungal groups that contributes to the low productivity of the crop (Berhanu et al., 2003; Nigussie et al., 2008).

Furthermore, a newly emerging disease known as "faba bean gall" incited by the pathogen *Olpidium viciae* Kusano causing up to complete crop failure over wide areas within short period of time and aggravates the diminution of yield to maximum nationwide (Dereje et al., 2012). Therefore, disease monitoring and surveillance are of paramount significant for sustainable faba bean production and tackle food insecurity. The assessment of diseases involves the measurement and quantification of plant diseases and is therefore of fundamental importance in the study and analysis of plant disease epidemics. Hence, the paper presents the major faba bean diseases with special focus on the newly emerging "faba bean gall" with respect to the distribution and economic importance.

MATERIALS AND METHODS

Study area description

The survey was conducted in south Tigray, Ethiopia. South Tigray is one of the seven administrative zones in Tigray National Regional State. It is bounded by Afar region in the East, Eastern zone of Tigray in the North, Amhara region in the South and South West. It lies 12°57'37''.19 North latitude and 39°31'41''.91 East longitude. The zone includes five districts namely, Raya-Alamata, Ofla, Enda-Mekoni, Emba-Alaje and Raya-Azebo. The former four districts are the major faba bean growing areas of the zone and the region as a whole.

Survey of faba bean diseases

The study was conducted in 2013 main cropping season (July to September) to determine the spatial and temporal occurrences of faba bean affecting diseases. Faba bean farmers' sown fields in the four districts and research experimental sites were included in the assessment. The survey trips was made following the main roads and accessible routes in each survey district, and stops were made randomly at every 5 to10 km intervals based on vehicles odometers. Five stops or samples were made in each faba bean field by moving in 'W' fashion of the fields using 1 m^2 quadrants and data were collected from individual quadrants. Data included were the number of affected and non affected plants per quadrant, the percent severity of each disease, the variety grown, the response of varieties to each disease and other pertinent data. The collected samples that is, five samples per field were used as one site after averaged. Hence, a total of 50 faba bean fields were surveyed from flowering up to maturity growth stage of the crop.

The assessment of each disease was based on the disease incidence, the number of diseased plants compared to the total number of assessed plants expressed as a percentage, and on the disease severity, as the infected area of tissue to the total area of tissue expressed as a percentage. The severity of the disease was examined visually on the whole plants within the quadrants and recorded as the percentage of plant part (tissue) affected, using respective scoring scale of each disease. Faba bean gall, chocolate spot, ascochyta blight and alternaria leaf spot severities were recorded based on both the percentage of infected leaves/leaf and/or stem (for gall) area damaged and the extent of defoliation when scoring infection level symptoms on the foliage using a 0–9 scale (Bernier et al., 1985; Ding et al., 1993).

The following infection levels on the scale were used: 0, no visible infection on leaves; 1, a few dot-like accounting for less than 5% of total leaf area; 3-4, discrete spots/galls less than 2 mm in diameter, accounting for 6 to 25% of leaf area; 5, numerous scattered spots/galls with a few linkages, diameter 3 to 5 mm, on 26 to 50% of leaf area with a little defoliation; 6, confluent spot lesions/galls accounting for 51 to 75% of leaf/stem area, mild sporulation, half the leaves dead or defoliated; 7, complete destruction of the larger leaves, spot lesions/galls covering more than 76% of leaf area, abundant sporulation,; 8, 80% of the defoliated and plants darkened and dead; 9, disease covering more than 80% of the foliar tissue heavy defoliation and plants darkened and dead. The severity of black root rot was determined according to (Abdou et al., 2001) rating scale of 0 to 5 on the basis of root discoloration or leaf yellowing as follows, 0 = neither root discoloration nor leaf yellowing, 1= 1 to 25% root discoloration or one leaf yellowed, 2= 26 to 50% root discoloration or more than one leaf yellowed, 3= 51- 75% root discoloration plus one leaf wilted, 4= up to 76% root discoloration or more than one leaf wilted, and 5= completely dead plants. Scale (1 to 9) was used for the evaluation of the reaction of faba bean plants to rust under field conditions (Van Schoonhoven and Pastor-Corrales, 1987). For the simplicity purpose, the severities of the identified diseases were expressed in percentage (Zadoks and Schein, 1979). The prevalence of the disease was computed by using the number of fields affected divided by total number of fields assessed and expressed in percentage.

RESULTS

The occurrences and intensities of faba bean diseases during 2013 are presented in Tables 1 and 2. The survey results indicated that six diseases were found to be important throughout the inspected routes. Among them, faba bean leaf and stem gall disease was the most frequently occurring and devastating disease during the survey (Table 1).

It is for the first time that the disease was identified in Tigray region in 2013. The percentage of distribution or

Table 1. Prevalence and intensities of faba bean leaf and stem gall in south Tigray, in 2013.

District	Altitude range (m.a.s.l)	Total fields	Prevalence (%)	Incidence (%)		Severity (%)	
				Range	Mean	Range	Mean
Ofla	2125-2793	20	80	0-100	77.5	0-100	71.9
Enda-Mekoni	2423-2938	14	78.6	0-100	43.9	0-100	63.8
Emba-Alaje	2463-2767	11	36.4	0-25	9.1	0-30	22.5
Raya-Alamata	2200-2517	5	40	0-5	2	0-100	85
Total/mean	2125-2938	50	66	0-100	45.5	0-100	64.4

Table 2. The percentage distribution and intensities of the main faba bean diseases in South Tigray in 2013.

District	Altitude range (m.a.s.l)	Total field	Chocolate spot			Ascochyta blight			Alternaria leaf spot			Root rot			Rust		
			Pre	Inc	Sev	Pre	Inc	Sev	Pre	Inc	Sev	Pre	Inc	Sev	Pre	Inc	Sev
Ofla	2125-2793	20	65	55.8	61.6	40	8.5	8.8	10	0.3	3	5	40	20	0	0	0
Enda-Mekoni	2423-2938	14	57.1	34.6	45.1	64.3	34.6	25.6	7.1	2.1	10	9.1	2	4.5	0	0	0
Emba-Alaje	2463-2767	11	63.6	33.3	13.6	81.8	40	9	72.7	22.7	7.8	7.1	5	10	18.2	1.8	0.9
Raya-Alamata	2200-2517	5	80	66	20	80	70	18	60	5.2	10	40	6	12.5	0	0	0
Total/mean	2125-2938	50	64	45.9	47.5	60	28.9	15.3	28	5.7	7.7	10	1.1	11.8	4	0.4	0.2

Pre: Prevalence; Inc: Incidence and Sev: Severity.

prevalence of the disease was of 66%. Faba bean leaf and stem gall was highly distributed in all surveyed districts namely: Ofla, Enda-Mekoni, Raya-Alamata and Emba-Alaje with prevalence values of 80, 78.6, 40 and 36.4%, respectively. The incidence range of the disease varied from 5% in Raya-Alamata to as high as 100% in Ofla and Enda-Mekoni districts. The mean incidence of gall was 77.5 and 43.9% in Ofla and Enda-Mekoni districts, respectively. The severity of the disease ranged from 30% in Emba-Alaje to 100% in the rest of three districts. The mean severity value of the disease in Raya-Alamata, Ofla, Enda-Mekoni and Emba-Alaje were 85, 71.9, 63.8 and 22.5%, respectively. The intensity of the disease escalated as the increase of elevation.

The prevalence of chocolate spot in all districts was higher than 57%. It was highly distributed in Raya-Alamata (80%), Ofla (65%) and Emba-Alaje (63.5%). The overall incidence of chocolate spot was reached 45.9%. The severity of the disease was higher in Ofla and Enda-Mekoni with mean values of 61.6 and 45.1%, in that order (Table 2). In similar way, ascochyta blight was also among the diseases identified during the year. The prevalence of this disease was greater than 60% in Enda-Mekoni, Raya-Alamata and Emba-Alaje. The intensity (incidence and severity) of ascochyta blight was also higher as that of its prevalence (Table 2). In addition, alternaria leaf spot and black root rot were also among the diseases found throughout the surveyed routes. Alternaria leaf spot was dominantly present in Emba-Alaje (72.7%) and Raya- Alamata (60%). The incidence of disease was 22.7% in Emba-Alaje, while, lower than 6% in the rest of districts. Black root rot was found in all surveyed districts with less than 10% mean incidence except in Ofla district reached 40%. Faba bean rust was rarely found only in few localities of Emba-Alaje District (Table 2).

Most of the varieties grown by the farmers were affected by one and/or more of the identified diseases. Among the cultivated faba bean varieties, improved varieties (Walki, Moti, CS20DK, and Gebelcho) were relatively tolerant to most diseases except faba bean gall as the compared to local cultivars. The percentage

Table 3. Response of faba bean varieties to different diseases (%).

Diseases	Improved released varieties			Local land races		
	Prevalence (%)	Incidence (%)	Severity (%)	Prevalence (%)	Incidence (%)	Severity (%)
Faba bean gall	37.5	20.04	38.5	92.3	59.1	69.2
Chocolate spot	58.3	34.8	23.8	73.1	58.7	54.8
Ascochyta blight	50	24.4	8	69.2	22.3	19.7
Alternaria leaf spot	12.5	11.7	7.7	42.3	1.2	7.8
Root rot	4.2	3.5	11.3	7.8	23	12.3
Rust	8.3	0.8	0.4	0	0	0

distribution of faba bean gall, chocolate spot and ascochyta blight on the local cultivar were 92.3, 73.1 and 69.2%, respectively (Table 3). The two most destructive diseases namely faba bean leaf and stem gall and chocolate spot were highly important and scored more than 50% mean incidence and severity on local cultivars. Likewise, the mean severity of gall and chocolate spot were 38.5 and 23.8% on the improved varieties, in that order, while other diseases were below 12%. All diseases affected both improved and local faba bean genotypes except for rust that was absent in the local (Table 3).

DISCUSSION

Diseases are the most devastating agents from an economic standpoint and the most difficult to protection efforts. This is mainly associated with complexity of pathogens and unavailability of fungicides to small scale farmers or due to the fact that their use in low input systems is not economically justifiable. Furthermore the importance and distribution of diseases varied as a result of climatic change and other bio-physical phenomenon. According to this study, six diseases; faba bean leaf and stem gall, chocolate spot, ascochyta blight, alternaria leaf spot, black root rot and rust in order of percent distribution. Among them, the newly emerging devastating disease "faba bean leaf and stem gall" was the most dominant both in terms of occurrence and intensity. This disease was characterized by the formation of chlorotic gall and then progressively enlarges to become light brown, circular or elliptical rough spots on both sides of the leaves and finally tissues decay (Li-juan et al., 1993). The industry of faba bean has been further complicated by addition of new disease in the region and the country as a whole. Though, the mechanism of introduction of faba bean gall to Tigray region is questionable; the disease was first reported in the country starting from 2010 at few localities (Dereje et al., 2012). Currently, the occurrence of the disease increased to an epidemic level in almost all fields for no crops to recover and any seed harvest (Dereje et al., 2012). Xing (1984) first identified the pathogen of the disease as *Olpidium viciae* by means of microscopic examination, inoculation, symptom and host range determinations. It was reported as new specie in 1912 in Japan. In 1936, S. Kusano confirmed that the small galls in Japan were caused by the same pathogen which had a wide host range, including faba bean and pea (Li-juan et al., 1993). In Tigray region, the production of faba bean in the area is now very much checked and farmers are frustrated by the nature of the disease. The distribution pattern of the disease was at escalating speed like fire-wood within short period of time. Furthermore, the occurrence of the disease at early growth stage of the plant aggravates the diminution of crop yield to maximum and wipes-out fields without any tolerance. The seriousness of the disease was linearly associated with the increases of elevation. According to Li-juan et al. (1993), the disease was more important at higher elevation between 2500 and 3400 meter above sea level. More recently, Dereje et al. (2012), reported that wider distribution of the disease at higher elevation (2500-3000 meter above sea level). The importance of disease has been getting more serious, because of the fact that most of the local available fungicides were ineffective to manage the disease.

Chocolate spot was among the widely distributed and importance diseases. According to Nigussie et al. (2008) and Teshome and Tagegn (2013) reports, chocolate spot was among the priority important diseases in Ethiopia. It is causing yield loss up to 61% in susceptible genotypes and 34% for tolerant genotypes

(Berhanu et al., 2003) and 62% yield loss in Tigray region (MRC, 1994). Ascochyta blight was also widely distributed disease in the area. However, lower disease severity was recorded with a peak value of 25.6% in Enda-Mekoni District. This is in line with the previous report that disease was categorized as minor in Ethiopia (Nigussie et al., 2008). In addition, alternaria leaf spot and black root rot were among the diseases identified during the survey. Nonetheless, the severity of alternaria leaf spot was not more than 10%. This is in line with the previous report that the disease was less important (Nigussie et al., 2008). Black root rot was found in all surveyed districts with less than 10% mean incidence except in Ofla reached 40%. However, according to the previous report black root rot was the second most important disease of faba bean causing up to complete loss in severe infection condition and when favorable conditions prevail (PPRC, 1996). This could be due to the environmental disparity and variety grown in a specific area. Faba bean rust incited by the pathogen *Uromyces vicia-fabae* was rarely occurred during the year. This could be due to the cool environmental conditions as most fields were surveyed at higher elevation (>2100 m). According to Nigussie et al. (2008) report that faba bean rust has no significant effect in the highland areas of Ethiopia, but, up to 2-15 and 14 to 21% yield loss has been recorded in lowland and midland areas, respectively. During the survey some improved varieties such as CS20DK, Walki, Moti, Gebelcho were showed relative tolerant to the identified diseases than the local cultivar. However, faba bean gall with an explosive character wipes-out most faba bean fields without any tolerance. Previous reports indicated that most improved varieties were moderately resistant to moderately susceptible for most faba bean fungal diseases (Nigussie et al., 2008), except for gall (Dereje et al., 2012). Hence, it is very important to use integrated management tactics and risk forecasting that operate on different aspects of the disease etiology, such that they complement each other and can be applied together in farmers' fields collectively to provide farmers with maximum economic return.

Conflict of Interest

The authors have not declared any conflict of interest.

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A comparative study to improve rooting of English lavender stems cuttings

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An experiment was conducted to compare the ability of two bacteria strains from *Agrobacterium rhizogenes* (DSM30200 and A4), yeast extract (0.5, 1 and 2 g/L) and indole butyric acid (IBA) (50, 100 and 150 mg/L) on rooting of the terminal cuttings of *Lavandula angustifolia* L plant. *A. rhizogenes* strains were more effective than most of the other treatments in increasing rooting percentage, roots number and increase of cuttings length except using IBA at 100 and 150 mg/L, as no significant differences were found between them. In contrast, the controls cuttings and cuttings treated with yeast at higher concentrations produced the lowest rooting percentage. Same trend treatments which showed higher rooting characteristics also recorded higher percentage of total carbohydrates, phenol and N percentage. On commercial scale use of two examined strains of *A. rhizogenes* for rooting of terminal cuttings of *L. angustifolia* can reduce the use of synthetic auxins like IBA.

Key words: *Agrobacterium*, yeast extract, IBA, lavender, stem cutting.

INTRODUCTION

Rooting of stem cutting is an important horticultural tool for the propagation of ornamental, medicinal and aromatic plants. Factors affecting the rooting of cuttings can include application of auxins, seasonal timing at which cuttings are collected, type of cuttings, wounding, physiological and histological factors as well as an imbalance of endogenous phytohormones. Synthetic auxins are known to stimulate adventitious root formation on cuttings, but the world was going to reduce the use of synthetic chemical compounds as a result for its risks to the environment. Indole butyric acid (IBA) is the most widely used among the root promoting auxinic compounds because it is non-toxic and effective over a wide range of species (Couvillon, 1988). *Lavandula angustifolia* L. “Munstead” (English lavender) family Lamiaceae is one of the medicinal and aromatic plants which can commercially cultivate on Egypt especially in the calcareous soils in the north coast of Egypt and other soils. It is a hardy perennial shrub rich in aromatic essential oils and there is an increase in demand for this plant to be used in pharmaceutical preparations, culinary herbs, cosmetics industry, etc.

Also, it is a very beautiful ornamental pot plant and has ability for cut and formation. Lavender plants are commercially propagating by stem cuttings, but have a poor rooting ability (Andrade et al., 1999). The seeds have a poor germination percentage (Takano et al., 1990). As well, *Agrobacterium rhizogenes* is one of the

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plant growths promoting rhizobacteria which have recently been used to stimulate root growth. *A. rhizogenes*, the causative agent of hairy root syndrome is a common soil organism capable of entering a plant through a wound and causing proliferation of root (McAfee et al., 1993). Also, the importance of yeast in some physiological properties may be due to that it contains after the composition of a wide group of amino acids and vitamins. In addition, yeast is a natural source of many growth substances as a protective agent, and most of nutritional elements (Na, Ca, Fe, K, P, S, Mg, Zn and Si) and contained cytokinin as well as some organic compounds (Nagodawithana, 1991). It was also investigated for improving growth and flowering of some crops (Ahmed, 1998; El-Mogy et al., 1998; Fathy et al., 2000).

The aim of this study was to investigate the effect of using two strains from *A. rhizogenes*, yeast extracts and evaluate and compare both of them with different concentrations from IBA to establishment of a commercial propagation protocol for this plant as well as minimizing the use of synthetic auxins.

MATERIALS AND METHODS

Experimental design

Terminal cuttings of *L. angustifolia* L. "Munstead" (English lavender) (15 cm long) were obtained from the Experimental Station Of Ornamental Plants, Faculty of Agriculture, Mansoura University, Egypt in January. Cuttings basal part of 0.5 mm each (fresh wounded) was dipped in 0.5 ml bacterial suspension for 24 h in complete darkness (Damiano et al., 1995) and planted in a plastic black pots 5 cm in diameter at a depth of 3 to 5 cm approximately each contained peat moss:vermiculite 1:1 (v/v). Planted pots were placed on the soil surface in a greenhouse with mist irrigation unit.

Bacterial strains and cultivation

Two strains of virulent *A. rhizogenes* (DSM30200 and A4) were obtained from Cairo Mircen Laboratory, Egypt. The bacterial cells were aerobically cultured on Luria Bertani (LB) medium supplemented with 10.0 g/L tryptone, 5.0 g/L yeast extract, 10.0 g/L NaCl and 15.0 g/L agar at Ph 7 and left for 3 days at 27 ± 1°C. From the previous cultured bacteria transformation was done to inoculate 5 ml of LB liquid medium and incubated overnight at 27 ± 1°C under continuous shaking at 120 rpm. One milliliter (1 ml) of overnight grown *Agrobacterium* culture was used to inoculate 25 ml of LB medium shaken till an optical density reading of 0.6 at 600 nm wave lengths, followed by centrifugation under cooling at 4200 rpm for 20 min (Draper et al., 1988). The supernatant was discarded and *Agrobacterium* cells precipitate pallet was suspended in 25 ml of Murashige and Skoog (1962) (MS) liquid basal medium.

Yeast extract and indole butyric acid (IBA) treatments

Yeast extract was used at concentrations of 0.5, 1 and 2 g/L. Long with IBA at 50, 100 and 150 mg/L plus a control treatment (cuttings dipped on distilled water).

Statistical analysis

Simple experiment in randomize complete block design was used. All experiments were repeated twice and similar trends of data were obtained. The number of terminal cuttings in each of the different treatments was 15. Data of percentages were either converted by arcsin transformation prior to analysis of variance (ANOVA) ($p < 0.05$). Multiple comparison test was performed using Duncan's multiple range test (a = 0.05) following ANOVA. All statistical analyses were performed using Costat version 6.303 (copyright 1998 - 2004 CoHort software, Monterey, CA).

RESULTS AND DISCUSSION

Adventitious root formation and rooting parameters

Adventitious roots induced on the treated terminal cuttings were evaluated through the measurements of four physical root parameters including cuttings rooting percentage, average roots number, roots length and increase in cuttings length. It was clear from data in Table 1 and Figure 1 that the highest rooting percentage of 88.89% was obtained when lavender cuttings were treated with the two bacteria strains (DSM30200 and A4). This extensive root formation is caused by *A. rhizogenes* which induces formation of adventitious roots at sites of infection, resulting in a hairy-root phenotype and expression of *A. rhizogenes* genes encoded by transfer-DNA (T-DNA), a fragment of DNA originating from a root-inducing plasmid (Ri) (Moore et al., 1979). Also, this bacterium transfers its T-DNA which is a portion of the large plasmid called the root-inducing plasmid (pRi) to susceptible plant cells where the T-DNA, if integrated into the nuclear genome of the plant cell, will encode genes that direct the synthesis of auxin (indole-3-acetic acid) and/or increase the sensitivity of the transformed plant cells to auxin plus to the endogenous production of auxin and/or an increase in auxin sensitivity can lead to the formation of hairy roots (Hatta et al., 1996). But, it was observed that there were no significant differences between cuttings treated with bacteria strains, yeast extract at 0.5 and IBA at all its concentrations as they ranged from 44.44 to 55.56%.

On the other hand, the lowest rooting percentage of 22.23% was tabulated for cuttings treated with 2 g/L yeast extract. Data in the same Table 2 and Figure 2 showed that the DSM30200 bacteria strain produced the highest roots number of 6.44 roots when compared with most of the other treatments. Followed by 4.33, 4.45 and 4.56 roots when A4 bacteria and IBA at 100 and 150 mg/L were used, respectively, no significant differences were shown between them. Exogenous IBA may induce changes in enzyme activities [peroxidase and indole acetic acid (IAA) oxidase] and in their effectors contents (phenolic) allowing the establishment of the favorable endogenous hormone balance. It is well known that applied auxins induce modifications in their own metabolism, mostly by conjugation, and in other

Table 1. Effect of Agrobacterium strains, yeast extract and indole butyric acid (IBA) concentrations on rooting parameters of English lavender stem cuttings.

Rooting substance		Rooting percentage	Roots number ± SD	Roots length ± SD	Increase in cuttings length (cm) ± SD
Treatment	Concentrations				
Control	0	33.34 ± 0.00[b]	0.56 ± 0.88[bc]	0.72 ± 1.15[b]	3.17 ± 1.41[cd]
Bacteria strains	DSM30200	88.89 ± 6.24[a]	6.44 ± 3.5[a]	3.73 ± 2.46[a]	3.94 ± 1.42[abc]
	A4	88.89 ± 6.22[a]	4.33 ± 5[abc]	3.17 ± 3.28[ab]	4.39 ± 2.62[abc]
Yeast (g/L)	0.5	44.45 ± 12.91[ab]	0.33 ± 0.71[c]	0.67 ± 1.66[b]	3.5 ± 1[bcd]
	1	33.33 ± 0.00[b]	0.89 ± 1.45[bc]	0.83 ± 1.69[b]	3.5 ± 1.22[bcd]
	2	22.23 ±12.83[b]	1.11 ± 1.70[bc]	1 ± 1.85[ab]	2.83 ± 1.66[cd]
IBA (mg/L)	50	55.55 ± 12.24[ab]	1 ± 1.32[bc]	0.87 ± 1.06[b]	1.94 ± 1.13[d]
	100	55.56 ± 12.91[ab]	4.45 ± 5.98[abc]	2.89 ± 4.34[ab]	5.61 ± 2.42[a]
	150	44.44 ± 6.24[ab]	4.56 ± 7.23[ab]	2.56 ± 3.91[ab]	5.39 ± 2.57[ab]

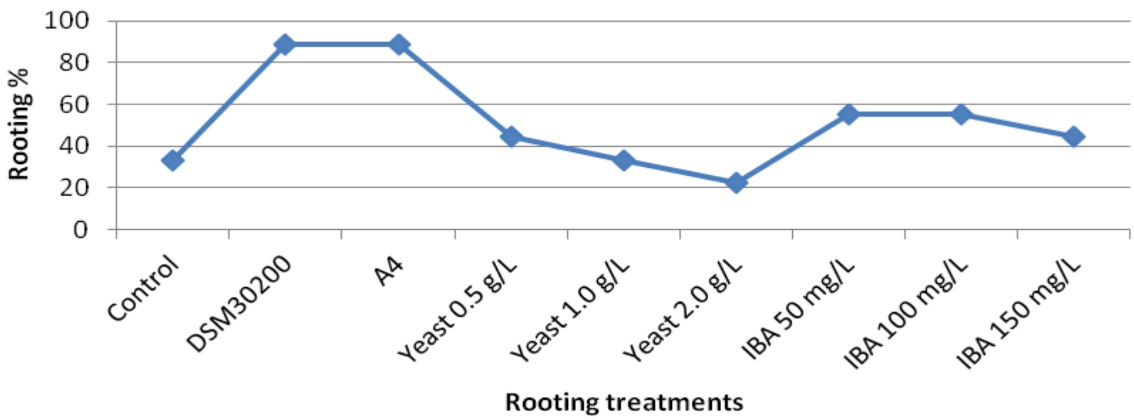

Figure 1. Effects of *Agrobacterium* strains, yeast extract and indole butyric acid (IBA) concentrations on rooting percentage of English lavender stem cuttings.

hormones such as cytokinins (Ribnicky et al., 1996; Gaspar et al., 1997).

It was obvious that cuttings treated with 0.5 g/L yeast gave the lowest roots number and length of 0.33 roots and 0.67 cm. No significant differences were recorded between the previous result and cuttings treated with 2 g/L yeast, 100 and 150 mg/L IBA, as they were 1, 2.89 and 2.56 cm, respectively. Also, the two bacteria strains and IBA at 100 and 150 mg/L, recorded 3.94, 4.39, 5.61 and 5.39 cm increase in cuttings length, respectively and no significant differences were shown between them. The control, yeast at all its concentrations and 50 mg/L IBA gave the lowest increase in cuttings length.

Changes in chemical constituents involved in formed adventitious roots

As shown in Table 2, the adventitious roots formation process and root parameters on cuttings of lavender under study were activated corresponding with the effect of tested treatments. The treatments have the ability to make an increasing effect on nitrogen contents, carbohydrates and phenols succeeded to form adventitious roots of higher qualities. This was in agreement with Samaan et al. (2014). But, it was extremely clear that *A. rhizogenes* strain DSM30200 is significantly still higher than all of the other treatments on these parameters, especially in carbohydrates percentage as shown in Figure 3, followed by using IBA at the higher concentration of 150 mg/L in N percentage, as no significant difference was found between them. The positive relationship between changes in chemical constituents in basal part of the treated cuttings and root parameters using *A. rhizogenes* could be attributed to certain evidences supported by the effective role of this biochemical compound to exert an influence on the rooting of cuttings. Carbohydrates in cutting provide

Table 2. Effect of *Agrobacterium* strains, yeast extract and indole butyric acid (IBA) concentrations on chemical constituents involved in formed adventitious roots of English lavender stem cuttings.

Rooting substance		N % ± SD	Carbohydrates % ± SD	Total phenol % ± SD
Treatment	**Concentrations**			
Control	0	1.91 ± 0.03^{e}	33.23 ± 0.25^{f}	0.25 ± 0.003^{h}
Bacteria strain	DSM30200	2.35 ± 0.05^{a}	36.56 ± 0.42^{a}	0.37 ± 0.01^{a}
	A4	2.13 ± 0.01^{c}	35.53 ± 0.21^{c}	0.34 ± 0.004^{c}
Yeast (g/L)	0.5	1.81 ± 0.04^{f}	31.23 ± 0.16^{i}	0.29 ± 0.002^{f}
	1	1.98 ± 0.03^{d}	31.97 ± 0.15^{h}	0.24 ±0.005^{i}
	2	2.22 ± 0.03^{b}	34.3 ± 0.01^{e}	0.27 ±0.007^{g}
IBA (mg/L)	50	2.03 ± 0.02^{d}	32.7 ± 0.2^{g}	0.31 ± 0.001^{e}
	100	2.09 ± 0.09^{c}	34.90 ± 0.03^{d}	0.33 ± 0.003^{d}
	150	2.31 ± 0.02^{a}	36.04 ± 0.06^{b}	0.36 ± 0.005^{b}

Figure 2. Effect of *Agrobacterium* strains, yeast extract and indole butyric acid (IBA) concentrations on rooting parameters of English lavender stem cuttings.

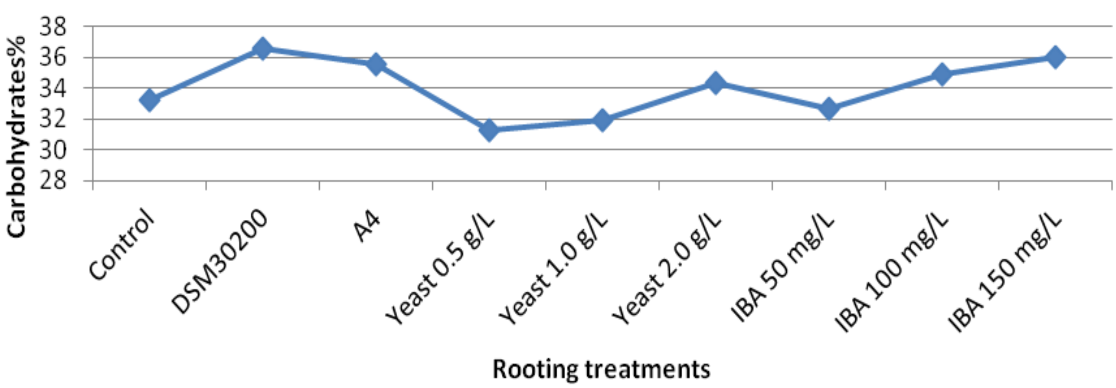

Figure 3. Effects of *Agrobacterium* strains, yeast extract and indole butyric acid (IBA) concentrations on carbohydrates percentage of English lavender stem cuttings.

sufficient amount of food for roots formation (Bleasdale, 1984). In the same point, the former authors reported that the rooting capacity of many cuttings has been correlated with carbohydrates content. Tahir et al. (1998) confirmed the important of carbohydrates accumulation in guava stem cuttings and other hardwood cuttings to produce number of roots and shoots. Sufficient of both carbohydrates and nitrogenous basis works with others in synthesis of the building blocks for nucleic acids (DNA and RNA). These biochemical compounds are the main source in synthesis of proteins as well as carbohydrates and fats metabolism which they all are necessary for normal cell division (Cannon et al., 2002). In that respect, Galle (1965) previously worked on leaf cuttings of cauliflowers (*Brassica oleraca*) and found a marked increase in the RNA content in tissues of cuttings basal part occurred during the period in which root primordia were formed. If the nitrogen level in cuttings decreased below a certain level, root formation was decreased in spite of a high level of carbohydrates. A similar result was cleared in the studies of Hambrick et al. (1985) with *Rosa multiflora* cuttings. He showed also that effect of non-rooting was related to carbohydrates content in cuttings as for C/N ratio, similar relationship of carbohydrates and C/N ratio with root qualities was found. High C/N ratio in hard wood stem cuttings is favorable to good rooting (Mahros, 2000; Hussein, 2008). They added that C/N ratio maybe an important factor influencing the root ability and their levels were positively related to rooting percentage on cuttings under study.

In testing, the biological activity of compounds structurally related to rooting co-factors, since early times Hess (1962) found that the phenol compounds catechol reacts synergistically with IAA in root production in the mung bean bioassay. Bouillenne and Bouillene (1955) suggested that auxidation of an orthodihydroxy phenol is one of the first step leading to root initiation tests using ultraviolet (UV) spectrum analysis and infrared spectroscopy indicated that the rooting factor is a complex structure of high molecular weight (an indole-phenol complex). They reported also that this factor may react at the base of the cuttings with specific enzyme initiating cell division leading to adventitious root formation (Hartmann and Kester, 1968; Fadle and Hartmann, 1967).

Generally, it could be concluded that English lavender terminal stem cuttings infected the basal part with the two strains of *A. rhizogenes* resulting in the best rooting percentage, average roots number and roots length. Moreover, these treatments also increased cuttings length.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Chemico-technological parameters and maturation curves of sweet sorghum genotypes for bioethanol production

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The sweet sorghum has high potential for bioethanol production, especially for using the same industrial production complex and for being processed during sugarcane off-season. The objective of this study was to evaluate the chemico-technological characteristics of the following sorghum genotypes: CVSW80007, CVWS80147, and BRS610, which were grown in Jaboticabal – São Paulo, State, Brasil. The experimental design used was a completely randomized block in a split-split-plot desing. The main treatments were three genotypes, two stalk management systems (stalks with and without leaves and panicles), and the tertiary treatment were six harvesting times (100, 105, 110, 118, 135, and 160 days after sowing) with three replications. The soluble solids content (Brix), total reducing sugars (TRS), pH, total acidity, total phenolic compounds, and starch content of the juice extracted were evaluated. The results indicated that given the chemico-technological characteristics of the genotypes CVWS80147 and CVSW80007, they can be used as raw material for bioethanol production, with useful-period-of-industrialization (UPI) about 40 days, starting at 110 days after sowing. The better results with chemical-technological parameters is find at 118 to135 days after sowing.

Key words: *Sorghum bicolor*, technological quality, productivity, biometrics, bioethanol.

INTRODUCTION

The growing global concern about environmental pollution resulting from the use of non-renewable energy and the release of greenhouse gases has stimulated the search for alternative fuels. These new energy sources may lead to increased and improved energy efficiency besides increasing energy availability and reducing costs

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thus ensuring sustainability and less environmental impact. The use of plant biomass as renewable energy sources, although it will not replace petroleum in its entirety, should help reduce dependence on fossil fuel and, consequently, reduce negative impacts on the environment. This possibility highlights the importance of such change making the production of energy from modern biomass an alternative strategy for all nations.

According to Nassif et al. (2012), sugarcane provides economic, social and environmental contributions, especially as raw material, to Brazil supporting the bioethanol production process. Similarly, other countries have successfully adopted the national ethanol production model, using sugarcane, resulting in a paradigm shift in fuel production in these modern days.

According to Jank (2011), the growing demand for ethanol in the domestic and international market is an issue of major concern, especially considering the predicted increase in industry demand for raw materials in the coming years. By 2020, it will be necessary to process 1.2 billion tons of sugarcane for the production of 51 million tons of sugar, in addition to 69 billion liters of ethanol (anhydrous and hydrated).

Under this scenario, one can deduce that the processing of sugarcane alone will not be sufficient to meet such high demand. Therefore, sweet sorghum has been identified as a high potential crop for biofuel production (Almodares and Hadi, 2009), as it has a short life cycle fully mechanized planting and harvesting and stalks rich in fermentable sugars; besides, its bagasse can be used as forage, for the co-generation of power (Cutz et al., 2013), or for producing second generation ethanol (Heredia-Olea et al*.,* 2013). Data available in the literature also demonstrate the feasibility of its use during the sugarcane off-season in the center-south region of Brazil, allowing the sugarcane mills to anticipate and extend the period of grinding (Teixeira et al., 1997).

Nevertheless, the inherent agronomic characteristics of the deployment of industrial large-scale production, such as planting, management, and harvesting systems and the fermentation process conduction are still liable for characterization. In order to produce high levels of industrial production, it is essential that the raw materials to be processed have high levels of fermentable sugars. Thus, the knowledge of the chemico-technological characteristics of sweet sorghum genotypes and their suitability for the fermentation process are of utmost importance. However, there are few studies that address this topic and little information available in the literature. The objective of the present study was to investigate the behavior of three sorghum genotypes during harvest in terms of their chemico-technological characteristics to evaluate the possibility of using them as raw materials for bioethanol production, in the sugarcane pre harvest season.

MATERIALS AND METHODS

Experimental conditions

The experiment was carried out in an experimental area of the Department of Crop Production at UNESP/FCAV, 21°14'05''S and 48°17'09''W, during the 2011/2012 harvest season. Sowing took place on12/14/2011 using 90 x 70 cm row spacing. A surplus of seeds was used, and 15 days after sowing (DAS) the plants were thinned to 10 plants.m^{-1} in order to obtain a population of 100,000 plants.ha^{-1}. N-P_2O_5-K_2O fertilizer was added at the rate of 20-100-100 kg. ha^{-1}. The weeds were removed manually, and thiamethoxam plus lambda-cyhalothrin was applied in the grooves at the rate of 20+15 g.ha^{-1} for pest control. The area was sprayed at 30 and 45 DAS at the same rate using the same pesticide for fall armyworm (*Spodoptera frugiperda*) control. N-P_2O_5-K_2O fertilizer was added at 30 DAS at the rate of 40-10-40 kg.ha^{-1}.

Experimental design

A split-split-plot design in a completely randomized block with three replications was used. Each plot consisted of 10 rows of 10 m length. The main treatments consisted of the three genotypes (CVSW80007, CVWS80147, and BRS610); the secondary treatments consisted of the two stalk harvesting systems (stalks with and without leaves and panicles), and the tertiary treatment consisted of the six harvesting times (100, 105, 110, 118, 135, and 160 DAS).

Sweet sorghum harvest and juice extraction

For every genotype at each harvesting time, 25 whole stalks (with leaves and panicles) and 25 clean stalks (without leaves and panicles) were harvested. They were sent to the Laboratory of Sugar and Ethanol Technology and Fermentation Microbiology, UNESP/FCAV, where they were weighed and their juice was extracted using a laboratory hand mill. The juice obtained was used in the chemico-technological analyses.

Chemical-technologycal evaluation

The chemico-technological characteristics evaluated were: Brix, determined according to Scheneider (1979); pH, measured using a DMPH-2 pH meter (Digimed) with temperature compensation; Total Reducing Sugars (TRS), determined by the Lane and Eynon (1934) volumetric method; phenolic compounds, quantified according to Folin and Ciocalteau (1927); and total acidity and starch content, determined according to Icumsa (The International Commission for Uniform Methods of Sugar Analysis) (2013). Whereas the sweet sorghum is used as raw material at season early, we compared its characteristics as sugarcane available in region at same time. We used the cultivars RB966928 and RB855156, classified as early maturing (crop in April and May). However, we did not statistical analysis.

Statistics

The results were submitted to analysis of variance (F test), test of multiple comparison of mean (Tukey 5%), and polynomial regression when this was significantly analysed, using the Barbosa and Maldonado (2011) method.

Table 1. Soluble solids (Brix), pH, total acidity, total reducing sugars (TRS), phenolic compounds, and starch content of the juice extracted from sorghum and mean values of sugarcane (Jaboticabal, 2012).

Treatment	Brix (%)	pH	Total Acidity ($g.L^{-1}$ H_2SO_4/L)	TRS (%)	Phenolic compounds (ug/ml)	Starch (ug/ml)
Genotypes (G)	43.32^{**}	0.85^{ns}	2.22^{ns}	89.51^{**}	0.09^{ns}	21.90^{**}
CVSW80007	$16.12^{A\#}$	$4.94^{A\#}$	$1.54^{A\#}$	$12.03^{A\#}$	$705.02^{A\#}$	$385.55^{B\#}$
CVWS80147	$15.89^{A\#}$	$4.96^{A\#}$	$1.76^{A\#}$	$11.70^{A\#}$	$726.42^{A\#}$	$496.86^{A\#}$
BRS610	11.97B	$4.92^{A\#}$	$1.72^{A\#}$	$9.47^{B\#}$	$690.16^{A\#}$	$346.75^{B\#}$
LSD	1.78	0.09	0.40	0.74	303.27	83.91
CV	14.48	2.34	29.03	7.97	51.04	24.38
Harvesting system (HS)	1.31^{ns}	15.20^{**}	5.94^{ns}	1.07^{ns}	1.13^{ns}	6.42^{*}
Without leaves	$14.75^{A\#}$	$4.90^{A\#}$	$1.58^{A\#}$	$11.17^{A\#}$	$730.14^{A\#}$	$447.12^{A\#}$
With leaves	$14.57^{A\#}$	$4.98^{A\#}$	$1.77^{A\#}$	$10.96^{A\#}$	$684.26^{A\#}$	372.32B
LSD	0.36	0.04	0.18	0.50	105.72	72.22
CV	5.32	2.05	23.54	9.70	31.74	37.43
Harvesting time (HT)	38.51^{**}	14.84^{**}	5.36^{**}	12.28^{**}	15.09^{**}	22.23^{**}
100	$11.93^{C\#}$	$5.09^{A\#}$	$1.34^{B\#}$	$9.03^{D\#}$	$450.08^{B\#}$	$205.66^{D\#}$
105	$13.63^{B\#}$	$5.03^{A\#}$	$1.59^{B\#}$	$9.70^{CD\#}$	$316.62^{B\#}$	$303.91^{CD\#}$
110	$14.77^{B\#}$	$4.91^{B\#}$	$1.48^{B\#}$	$11.84^{AB\#}$	$440.37^{B\#}$	$342.21^{CD\#}$
180	$16.28^{A\#}$	$4.90^{BC\#}$	$1.63^{B\#}$	$11.52^{AB\#}$	$1499.12^{A\#}$	$439.98^{BC\#}$
135	$16.68^{A\#}$	$4.91^{BC\#}$	$2.27^{A\#}$	$13.19^{A\#}$	$508.88^{B\#}$	$555.10^{AB\#}$
160	$14.67^{B\#}$	$4.80^{C\#}$	$1.74^{AB\#}$	$11.12^{BC\#}$	$1028.14^{A\#}$	$611.45^{A\#}$
LSD	1.17	0.11	0.57	1.78	493.32	136.87
CV	8.13	2.29	35.00	16.47	71.08	34.04
$F_{Gx HS}$ Test	1.68^{ns}	3.05^{ns}	0.15^{ns}	3.10^{ns}	3.92^{ns}	0.33^{ns}
F_{GxHT} Test	2.98^{**}	1.73^{ns}	0.95^{ns}	0.51^{ns}	0.99^{ns}	1.85^{ns}
F_{HSxHT} Test	0.24^{ns}	1.16^{ns}	0.81^{ns}	0.71^{ns}	1.75^{ns}	2.73^{*}
$F_{GxHSxHT}$ Test	1.12^{ns}	1.57^{ns}	0.39^{ns}	1.45^{ns}	0.61^{ns}	1.20^{ns}
Sugarcane	(Mean obtained at same dates of sweet sorghum harvest)					
RB966928	21.18	5.25	0.99	17.80	505.38	244
RB855156	18.00	5.00	1.72	10.32	655.00	450

**Significant at 1 % probability ($p < 0.01$), *significant at 5% probability ($p<0.05$), and ^{ns} - not significant ($p>0.05$). $^{\#}$ Mean following with same capital letter, in vertical orientation, inside fator analysed (genotypes or harvesting system or harvesting time), did not differ significantly.

RESULTS AND DISCUSSION

The results obtained for the chemico-technological characteristics of the sweet sorghum juice are shown in Table 1. It was found that the genotype BRS610 had the lowest Brix and total reducing sugars (TRS) in comparison to those of CVSW80007 and CVWS80147, which were approximately the same. This result was expected since BRS610 is classified as a forage type of high biomass productivity, which is generally associated with low sugar. It was observed that the genotypes CVSW80007 and CVWS80147 showed significant increase in Brix values and TRS up to 135 DAS (Table 1, Figures 1 and 2), followed by a significant reduction up to the final harvest at 160 DAS. The harvesting systems evaluated did not significantly influence the raw material quality, where as Ribeiro Filho et al. (2008) found differences verifying Brix values of 12.4 and 11.6 and TRS of 9.66% and 9.81% in stalks without and with leaves, respectively.

According to Schaffert (2012) in order to achieve economic and sustainable production of ethanol from sweet sorghum, it is necessary to obtain a minimum of 12.5% of TRS, which is used to establish the useful-period-of-industrialization (UPI). The results obtained in this study indicate that CVSW80007 and CVWS80147

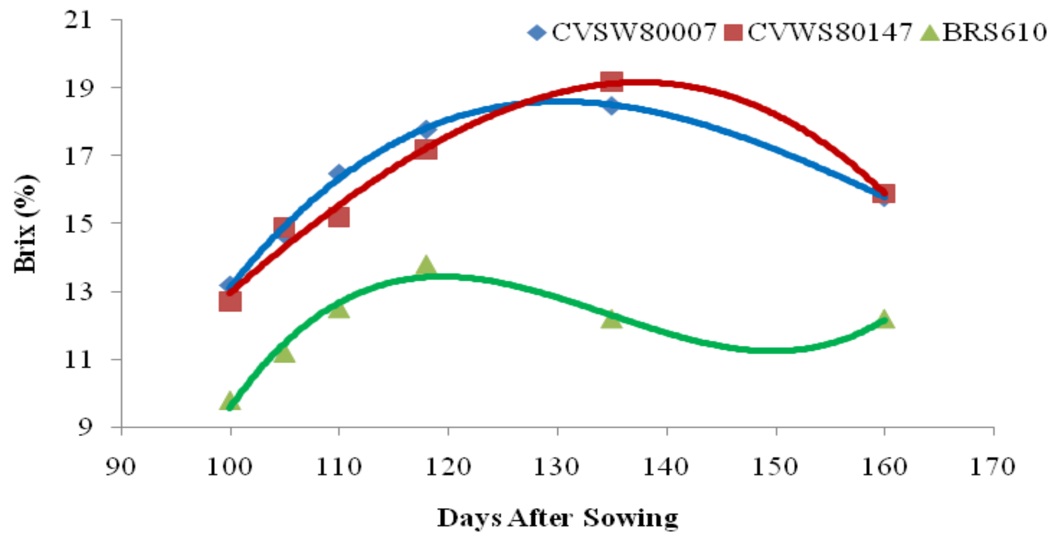

Figure 1. Polynomial regression of soluble solids (Brix) of the genotypes CVSW80007, CVWS80147 and BRS610 as a function of the harvesting time. Jaboticabal-São Paulo, State- Brazil, 2011/2012 harvest season.

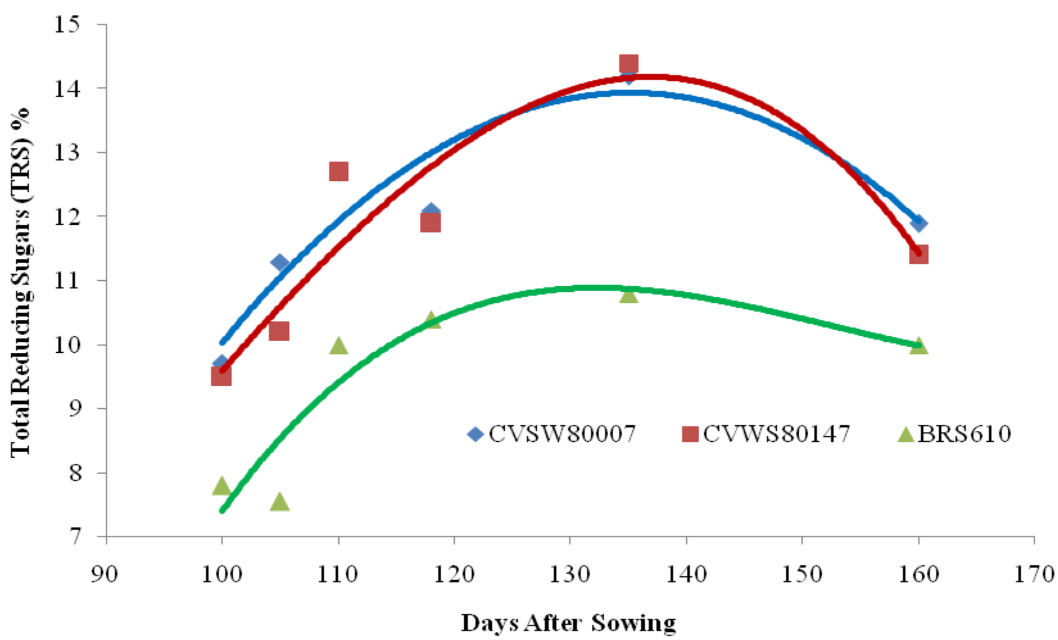

Figure 2. Polynomial regression of Total Reducing Sugars (TRS) for the genotypes CVSW80007, CVWS80147 and BRS610 as a function of the harvesting time. Jaboticabal- São Paulo, State- Brazil, 2011/2012 harvest season.

had a UPI of 40 days, which started around 110 days after sowing (Figure 2). The mean values of TRS obtained ranged from 11.7 to 12.0. These values are in accordance with those found by Teetor et al. (2011), who evaluated 24 different genotypes and found that only 10 had TRS levels between 10 and 11, and only 3 had TRS higher than 12. However, when the mean values of the genotypes of sorghum were compared with those of the sugarcane cultivars RB966928 and RB855156, harvested in April in Jaboticabal-SP, it was found that the Brix values of the juice extracted were lower than those found for the sugarcane cultivars (Table 1). Though the Brix were higher to sugarcane, it should be noted that this raw material was immature, once the acidity was higher than 0.8 g.L^{-1} H_2SO_4 (Ripoli and Ripoli, 2009). In this way, the sugarcane could stay in the field more time.

The levels of TRS in the sorghum juice were lower than those recommended by Amorim (2005), who considers values greater than 15% as optimal when sugarcane juice is used as raw material. The TRS values in the BRS610 genotype were significantly lower than those of CVSW80007 and CVWS80147. However, when compared to those of the sugarcane juice analyzed, it was found that the TRS levels in the RB855156 genotype was lower than the mentioned values, while for the RB966928, they were much higher. Considering the data gathered from the sugarcane producers in the state of São Paulo in the month of April during the 2010/2011, 2011/12, and 2012/2013 harvest seasons (UNIÃO DA INDÚSTRIA DE CANA-DE-AÇÚCAR (Unica), 2013), it can be seen that the TRS values in the juice obtained from the raw materials were in the range between 13.10 and 15.45, and the highest values were found at the end of the month. Thus, it appears that the sorghum materials evaluated (Figure 2) had better quality in the first half of April (135 DAS). For the parameter pH we observed there was no significant difference between the genotypes and the harvesting system (Table 1). The pH determined during the harvesting period from 110 to 135 DAS varied, but the mean values were significantly higher at 100 and 105 DAS. In a study on sorghum juice characterization, Ribeiro Filho et al. (2008) found pH values higher (5.31 for clean stalks and 5.46 for stalk with leaves) than those obtained in the present study. Considering the sugarcanes pH, it was observed values for the cultivar RB966928 higher than those of the sorghum genotypes (Table 1), which indicates it reached a mature stage of development. In general, the values obtained for the sorghum juice extracted are suitable for fermentation process, with optimal pH range for yeast growth from 4.5 to 6.5 (Amorim, 2005).The sorghum harvesting systems and genotypes did not influence the total acidity and phenolic compounds levels of the extracted juice (Table 1).

The mean levels of the phenolic compounds were significantly higher in the fourth (118 DAS) and the sixth period of time evaluated (160 DAS). This behavior may be due to the physiological response of the culture to the environmental conditions prevailing in these periods of time, namely low rainfall, low levels of water storage in the soil, and large water deficit combined with high average temperatures. Under these conditions, the sweet sorghum culture after reaching total metabolic activity started to allocate some photosynthates to produce defense biomolecules such as phenolic compounds (Taiz and Zeiger, 2004). According Dicko et al. (2006), sorghum has a high content of phenolic compounds, reaching up to 6% in some varieties, and the genetic characteristics and environmental conditions in which they are grown are determining factors for the production of these compounds by the plant. The phenolic compound values obtained for RB966928 and RB855156 were similar to those reported for the sorghum genotypes (Table 1). Taking into consideration the quality standard according to Amorim (2005), who recommends phenolic compounds levels in the juice below 500 ppm, it can be said that sorghum was suitable for industrial processing for production ethanol at 135 DAS.

Starch, a biomolecule characteristic of sorghum, is stored mainly in the panicle grains, but it can also be found in the stalk. According to Guiying et al. (2000), the level of starch in the plant depends on the stage of maturity and its genotype; the higher the stage of maturity, the larger the amount of starch stored in the grain. Nan et al. (1994) found contents of starch in the juice ranging from 300 to 9900 ppm and most of them were around 2000 ppm. The genotype CVWS80147 showed significantly higher levels of starch in the juice, about 25 to 30% more than that of CVSW80007 and BRS610, respectively, whose values did not differ significantly (Table 1). According to Ripoli and Ripoli (2009), these values are considered adequate. The cultivar RB855156 showed values similar to those, while the values of RB966928 were around 40% lower than those of BRS610, once it was immature.

The harvesting systems evaluated during the period of time studied indicated that the juice of the stalks without leaves and panicles showed the highest mean values of starch content at 105, 110, 118, and 160 DAS, and no significant differences between the two treatments were observed at 135 DAS (Figure 3). This behavior may be due to leaves, that contain high levels of moisture, and this water could dilute the phenolic compounds.It was found that the starch content increased significantly with time (Figure 4); Guiying et al. (2000) reported similar results, while Nan et al. (1994) found lower values with the growth and development of sorghum. Considering the behavior of the genotypes, starting at 118 DAS, it can be observed that CVSW80147 showed the highest starch content, followed by CVSW80007 and BRS610; the latter showed the lowest content, similar to those of RB855156.

We conclude that the genotypes CVSW80007 and CVWS80147 have the best chemico-technological characteristics. Harvesting the stalks with and without leaves does not influence the quality of the raw material, except for the starch content. In this way, the stalks harvested with leaves could be more economical to industry, and this leaves could be used to burn and generate power. The best technological quality of the raw material was found between 118 and 135 DAS, when

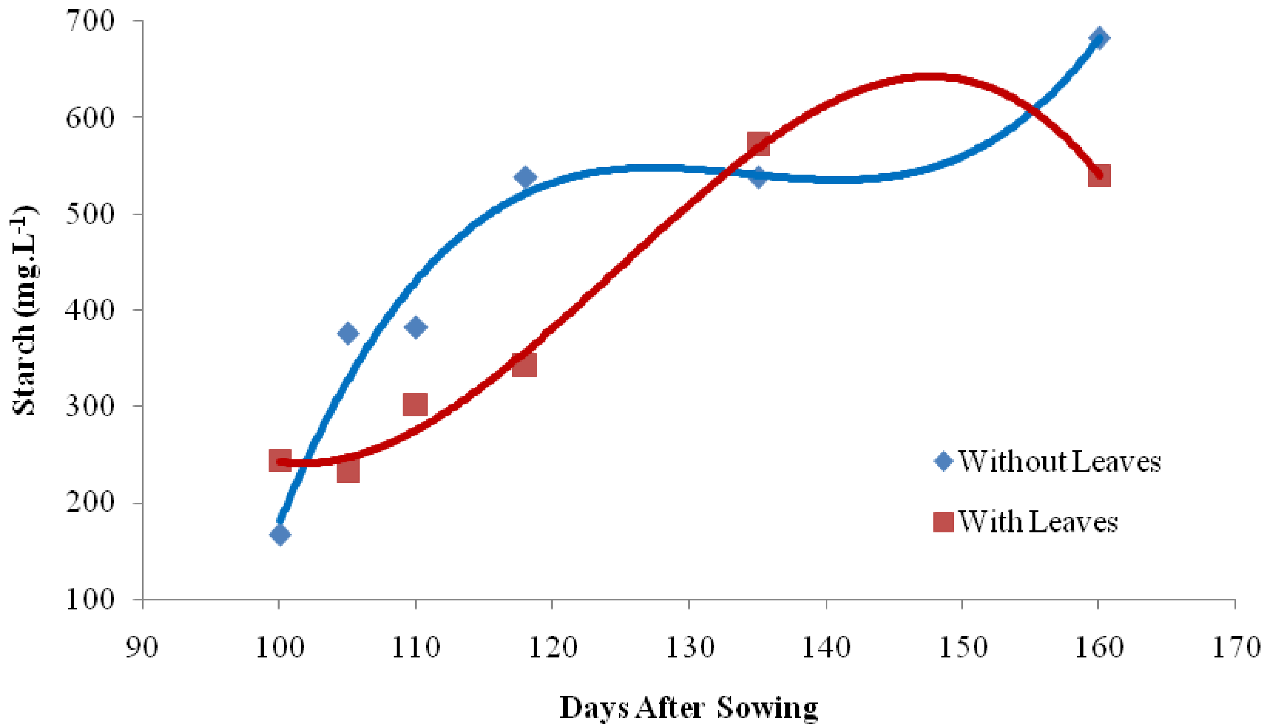

Figure 3. Polynomial regression of the starch content of the juice obtained in the harvest systems studied as a function of the harvesting time. Jaboticabal- São Paulo, State- Brazil, 2011/2012 harvest season.

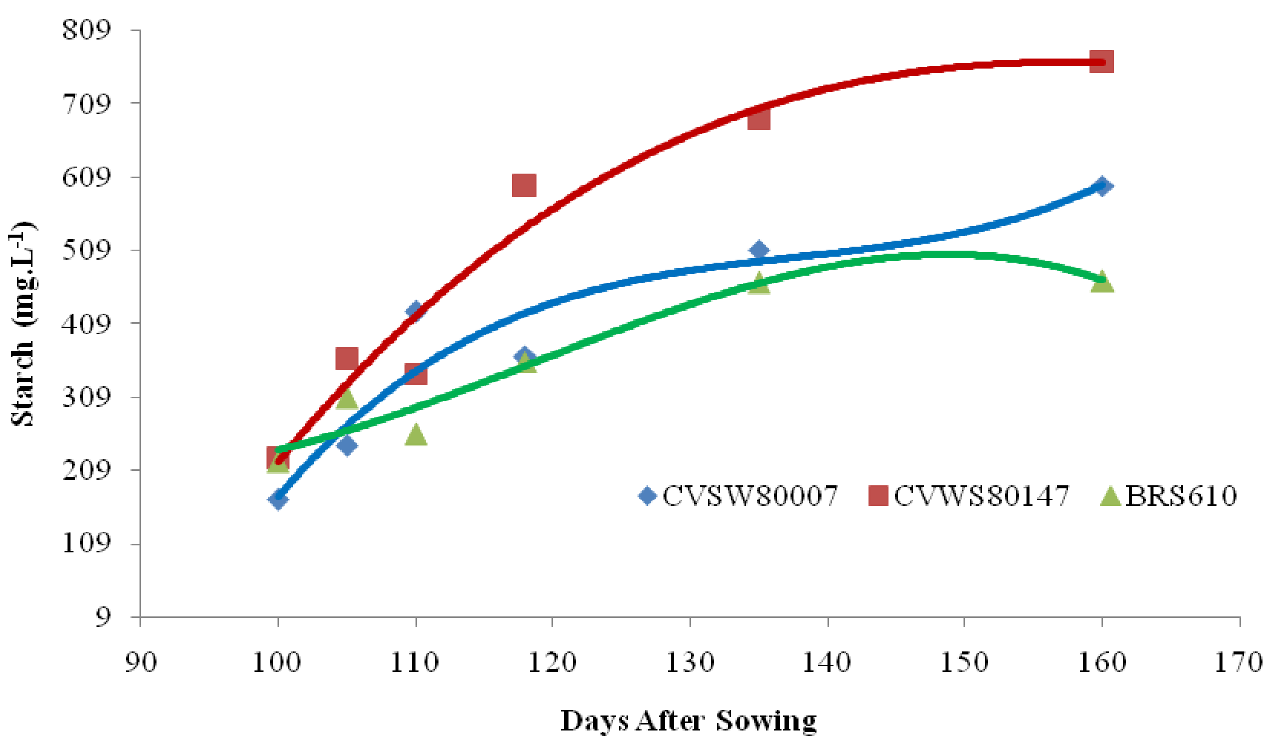

Figure 4. Polynomial regression of starch content of the juice in the genotypes CVSW80007, CVWS80147 and BRS610as a function of the harvesting time. Jaboticabal- São Paulo, State- Brazil, 2011/2012 harvest season.

these cultivars showed high levels of Brix and TRS, and low levels of Phenolic Compounds Total.

Conflict of Interest

The authors have not declared any conflict of interest.

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Gypsum and phosphorus in the development of upland rice under a no-tillage system

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The use of gypsum in a no-tillage system may be a feasible alternative for cultivating upland rice because of its ability to move some nutrients to greater depth in the soil and thereby stimulate root growth. Additionally, phosphorus is one of the nutrients that most limits crop production in the Brazilian Cerrado. Thus, the objective of this work was to study the effect of combining gypsum (applied to the soil surface without tillage) and phosphorus at sowing on soil attributes, plant height, number of panicle m^{-1}, seed mass, and grain yield in a no-tillage cultivation system. The experiment was conducted using a randomized complete block experimental design with four replicates in a factorial scheme of gypsum doses (0, 1000, 2000 and 3000 kg ha^{-1}), phosphorus doses in the furrow (0, 50, 100 and 150 kg ha^{-1}) and growing seasons (2011/2012 and 2012/2013). Gypsum applications provided incremental increases in soil calcium and increased potassium levels in the deeper soil layers, but it did not affect plant height, number of panicle m^{-1}, or grain yield of upland rice cultivated under a no-tillage system. Increasing doses of phosphorus applied at sowing resulted in a significant increase in the plant height, number of panicles m^{-1} and grain yield.

Key words: *Oryza sativa* L., fertilization, Cerrado, leaching.

INTRODUCTION

Rice is a food that is part of the diet of half the world's population (Kumar and Ladha, 2011), and most of this grain is grown in Asia using a controlled-flooding irrigation system (Farooq et al., 2009; Prasad, 2011). However, the reduced availability of water resources for irrigation of crops due to increasing industrial and human consumption has generated a demand for alternatives in the form of water-saving rice cultivation systems (Feng et al., 2007). As alternatives, rice could be cultivated in upland ecosystems, which can be sprinkler irrigated or not irrigated depending on rainfall (Bouman et al., 2007; Crusciol et al., 2013; Nascente et al., 2013). As a component of these alternative cultivation methods, the no-tillage system (NTS), due to its characteristic of

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maintaining a covering of straw over the soil, could bring advancements to rice production as a result of greater retention of water in the soil.

In a NTS, adequately managing soil fertility at depth layers is important to ensuring the system's sustainability (Silva and Lemos, 2008). To this, gypsum ($CaSO_4.2H_2O$) could be used for improving the root environment in deep soil layers (Santos et al., 2006). This product is a soil conditioner and his moderately soluble (2.5 g L^{-1}). It can provide Ca^{2+} and SO_4^{2-} ions in solution, which can be leached, thereby enriching subsoil layers with both nutrients and reducing the saturation of Al^{3+} at depth layers (Alcordo and Rechcigl, 1993). Thus, gypsum use could allow the development of roots in deep layers of the soil, thereby increasing the volume of explored soil and, consequently, the tolerance of plants to drought (Sousa et al., 2005). The successful use of gypsum to enhance the root environment has been implemented extensively in the Brazilian Cerrado region, where approximately 80% of the area has some type of subsoil acidity and a high incidence of dry periods, especially in the months of January and February, which are critical for the development of summer crops (Ramos et al., 2006; Caires et al., 2008). Moreover, gypsum can be found in many parts of the world and often has a low commercial cost for farmers (Melo et al., 2008).

Among the primary macronutrients, phosphorus (P) is the nutrient least required by rice crops; however, it is highly exported in harvested grains crops (Soratto et al., 2010; Fageria et al., 2011) and is less abundant in most tropical Brazilian soils. Its deficiency in Cerrado soils is due to a low natural content and a high fixation capacity (Fageria et al., 2011). This nutrient also has a significant effect on root growth and grain yield of rice (Crusciol et al., 2005).

For upland rice, data on the combined application of gypsum and P are still scarce with regard to soil management carried out in an NTS. Thus, the objective of this work was to determine the effect of the combination of gypsum applied to the soil surface without tillage and P applied in the sowing furrow on soil attributes, plant height, number of panicles m^{-1}, seed mass, and grain yield of upland rice.

MATERIALS AND METHODS

The experiments were conducted at Fazenda Capivara at Embrapa Rice and Beans, which is located in Santo Antônio de Goiás, GO, Brazil, at 16º28'00" S and 49º17'00" W and an altitude of 823 m. The climate is tropical savanna and is considered Aw according to the Köppen classification. There are two well-defined seasons, one normally dry season from May to September (autumn/winter) and one rainy season from October to April (spring/summer). The average annual rainfall is between 1500 to 1700 mm, and the average annual temperature is 22.7°C, ranging annually from 14.2 to 34.8°C. During the two growing seasons in the present study, there was no problem with dry periods.

The soil is classified as a clay loam (kaolinitic, thermic Typic Haplorthox) acidic soil (Embrapa, 2006). Prior to the experiment, in 2010, chemical analysis was performed in the depth ranges 0 to 10, 10 to 20 and 20 to 40 cm for the characterization of the experimental area (Table 1). The chemical analyses were performed according to the methodology proposed by Claessen (1997). The soil pH was determined in water. Exchangeable Ca, Mg, and Al were extracted with neutral 1 mol L^{-1} KCl in a 1:10 soil:solution ratio and determined by titration with a 0.025 mol L^{-1} NaOH solution. Phosphorus and exchangeable K were extracted with a Mehlich 1 extracting solution (0.05 M HCl in 0.0125 M H_2SO_4). The extracts were colorimetrically analyzed for P, and flame photometry was used to analyze K. The base saturation values were calculated using the results from exchangeable bases and total acidity at pH 7.0 (H + Al). Organic matter was determined by wet combustion with external heat.

The experimental area had been cultivated in crop-livestock rotation using a no-tillage system (NTS) for seven consecutive years. The rotation program included soybean (*Glycine max*) (summer), followed by rice (*Oryza sativa*) (summer) and common bean (*Phaseolus vulgaris*) (winter), followed by corn (*Zea mays*) + *Urochloa brizantha* (summer) and two years of grazing pasture. Installation of the current experiments in both years was conducted in plots where upland rice was the crop to be grown as part of the established program of crop rotation.

The experimental design was a randomized complete block in a 4 × 4 × 2 factorial scheme with four replications. The treatments consisted of combinations of gypsum (0, 1000, 2000 and 3000 kg ha^{-1} applied in both years), P in the sowing furrow (0, 50, 100 and 150 kg of P_2O_5 ha^{-1} applied in both years) and growing seasons (2011/2012 and 2012/2013). The plots consisted of 10 five-meter-long rows, spaced 0.35 m apart. The useful area of each plot was formed by the nine central m of the six central rows.

Approximately 15 days before sowing, the experimental area was cleared of weeds with glyphosate + 2,4D. The gypsum application (17% S and 22% Ca) was broadcast on the surface of one day before rice sowing. Based on soil analysis, the recommended P dose for the rice crop was estimated to be 100 kg ha^{-1} (Sousa and Lobato, 2004). The base fertilization applied in the sowing furrows was calculated according to the soil chemical characteristics and the recommendations of Sousa and Lobato (2004). The fertilizer consisted of 40 kg ha^{-1} of N as urea and 50 kg ha^{-1} of K_2O as KCl and was applied together with P_2O_5 as triple superphosphate at sowing. Additionally, topdressing fertilization was performed 21 days after crop emergence using 40 kg ha^{-1} of N as urea.

The sowing was performed mechanically using 80 kg ha^{-1} of rice seeds from a mutant line 07SEQCL441 CL that was derived from a Primavera variety and was resistant to Imazapyr + Imazapic herbicide. The seed was sown on December 15th, 2011 and November 1st, 2012 in the first and second growing seasons, respectively. Before sowing, seeds were treated with Carboxin + Tiram (250 mL 100 kg of seeds^{-1}) + Fipronil (100 mL 100 kg of seeds^{-1}). Weed control was accomplished using Imazapyr + Imazapic herbicide applied at 16 (100 g ha^{-1}) and 26 (50 g ha^{-1}) days after crop emergence. Other cultural practices were performed according to standard recommendations for a rice crop to keep the area free of diseases and insects.

The soil strata were sampled four months after gypsum application in the first season (14/03/2012), in 0-0.10, 0.10-0.20 and 0.20-0.40 m soil layers. Fifteen simple disturbed samples were collected using a screw auger, randomized in each plot and for each depth (five in the sowing row and 10 in the inter-rows), to constitute the work samples. The work samples were air dried, sieved (2 mm mesh), and subsequently analyzed for pH (water) and for exchangeable Al, Ca, Mg and K as described previously.

In addition, the plots were evaluated with regard to: plant height (m), which was determined by measuring 10 plants per plot at the time when the crop was at the phonological stage of pasty grains and recording the distance between the soil surface and the top end of the highest panicle; number of panicles m^{-1} which was

Table 1. Chemical characteristics of the soil in the total experimental area prior to the experiment, in the growing season 2010/2011.

Depth	pH	SOM¹	P	Al	H+Al	K	Ca	Mg	CEC²	V³
cm	Water	g dm^{-3}	mg dm^{-3}	----------------------mmol$_c$ dm^{-3}--------------------						%
0-10	5.7	27	15	0.0	40	2.0	17	12	71.0	44
10-20	5.8	26	16	0.0	37	0.6	18	13	68.9	46
20-40	5.9	23	8	0.0	34	0.6	12	9	55.6	39

¹SOM, soil organic matter; ²CEC, Cation exchange capacity; ³V = (K+Mg+Ca/ H+Al+K+Mg+Ca) * 100.

determined by counting the number of panicles within 1.0 linear m of one of the rows in the useful area of each plot; mass of 1000 grains which was evaluated randomly by collecting and weighing two samples of 100 grains from each plot, corrected to 13% of water content; and grain yield which was determined by weighing the harvested grain of each plot, corrected to 13% of water content and converted to kg ha^{-1}.

For statistical analysis, SAS Statistical Software, SAS Institute, Cary, NC, USA (SAS, 1999) was used. Data were subjected to an analysis of variance and, when necessary, compared by a Tukey test at $p<0.05$. When rates of gypsum and P were significant, the results were submitted to regression analysis at $p<0.05$.

RESULTS AND DISCUSSION

Gypsum applications did not result in statistical significant changes in pH, Mg, Al or P attributes (Table 2). However, it caused significant changes in the Ca content of the 0 to 0.10 and 0.10 to 0.20 m layers, and in the K content of the 0 to 0.10, 0.10 to 0.20 and 0.20 to 0.40 m layers. Based on these results, the Ca data were fitted with quadratic equations (Figure 1). According to Caires et al. (2004), the application of gypsum can provide an increase in the exchangeable Ca content of the soil. Rocha et al. (2008) reported that a 30-day interval after gypsum application was enough to increase the exchangeable Ca content of the deepest soil layer (0.80 m), even when gypsum was broadcast without incorporation in an Oxisol. Soratto and Crusciol (2008) also reported increases in Ca at depth 12 m after gypsum application in an Oxisol.

Through the results obtained in this work, it was observed that there was a reduction in K levels in all layers evaluated. Based on this observation, it was possible to infer that the leaching of K to deeper layers of the soil (> 0.40 m) was caused by the application of higher doses of gypsum (Figure 2). Similar results have been reported by other authors (Caires et al., 1998). According to Caires et al. (2004) and Santos et al. (2013), gypsum application may result in movement of nutrients to deeper soil layers and may also be responsible for nutrient losses, especially a loss of Mg and K, because of the interaction with SO_4^-.

Plant height measurements showed no effects of gypsum application (Table 3). Similar results were obtained by Oliveira et al. (2009) when studying the effect of gypsum on the development of *Brachiaria humidicola*. The authors explained the results as being due to the low Ca requirement of that forage which is similar to that of a rice crops (Fageria et al., 2011). In contrast, P application positively affected plant height (Table 2), and thus, the data were fitted to a quadratic equation (Figure 3). Likewise, Tonello et al. (2012) observed a significant increase in plant height due to the application of increasing P. Garcia et al. (2009) reported significant and positive effects of increasing P on seedling growth in upland rice. According to Fageria et al. (2011), P is the nutrient that most limits crop development, especially in soils of low fertility, such as those of the Brazilian Cerrado, and supplying P, results in improvements in plant growth.

For yield components and grain yield, it was observed that gypsum application did not affect any of the evaluated parameters (Table 3). Santos et al. (2013) also did not observe responses in grain yield when analyzing the effects of gypsum application on two *Pennisetum purpureum* varieties. Similarly, Gomes et al. (2000) found no effect of gypsum applications of up to 20 Mg ha^{-1} on rice. Additionally, Soratto et al. (2010) did not observe increases in upland rice grain yield as a function of gypsum dose. The results of the present study is supported by the findings of previous studies indicating that rice is undemanding with regard to Ca as reported by Fageria et al. (2011).

The results of the present study which were repeated for two consecutive years could reflect this soil does not need Ca or SO_4^-. According to Sousa et al. (2005), using soil analysis results from 0.20 to 0.40 m layer, if Al saturation exceeds 20% or if Ca amount is less than 5 mmol$_c$ dm^{-3}, it is possible to see a response to gypsum application. The experimental area used in this work, in both years, had low Al saturation, and Ca values were higher than 5 mmol$_c$ dm^{-3} at 0.20 to 0.40 m (Table 1). Under these conditions, the absence or low response to gypsum application in the development of agricultural crops could be expected, especially for rice crop which shows low demand for Ca. In addition, according to Sousa et al. (2005), in places with dry periods during the growing season, it is possible to identify effects of gypsum application on growing plants. We did not have a problem with dry periods which could be another reason and we did not observe any effects from gypsum.

Based on the results, one could question whether the

Table 2. Chemical characteristics of the soil cultivated with upland rice in a no-tillage system with regard to phosphorus dose, gypsum doses and sampling depth in the growing season 2011/2012.

0-0.10 m depth*						
Treatments	**pH**	**Ca**	**Mg**	**Al**	**P**	**K**
Gypsum doses (kg ha^{-1})		---------------mmol$_c$ dm^{-3}-----------------			---------mg dm^{-3}--------	
0	6.37	34.94	17.10	0.14	15.79	78.10
1000	6.26	38.53	17.04	0.00	12.57	68.53
2000	6.41	39.00	14.98	0.79	15.68	67.00
3000	6.15	41.09	14.31	0.07	11.20	65.93
Phosphorus doses (kg ha^{-1})						
0	6.35	37.40	16.50	0.05	14.74	76.23
50	6.36	37.71	15.38	0.86	15.02	65.71
100	6.25	39.31	15.87	0.00	13.33	73.85
150	6.25	35.79	15.87	0.13	12.56	68.53
Factors			**ANOVA – Probability of F test**			
Gypsum (G)	0.1291	0.0403	0.3817	0.8378	0.5741	0.0490
Phosphorus (P)	0.4835	0.6672	0.4392	0.4274	0.8356	0.3756
G × P	0.9503	0.9438	0.8174	0.1519	0.87989	0.4367
0.10-0.20 m depth						
Gypsum doses (kg ha^{-1})						
0	6.22	24.43	11.58	0.10	9.76	84.33
1000	6.12	26.97	10.84	0.00	8.79	76.47
2000	6.33	27.39	12.32	0.00	8.32	71.93
3000	6.11	28.51	10.95	0.07	6.06	66.14
Phosphorus doses (kg ha^{-1})						
0	6.19	27.30	11.41	0.09	11.39	82.18
50	6.21	25.91	11.22	0.07	5.76	76.21
100	6.18	27.89	11.20	0.00	7.10	77.85
150	6.19	25.40	11.55	0.00	7.30	73.60
Factors			**ANOVA – Probability of F test**			
Gypsum (G)	0.1912	0.0434	0.3381	0.6384	0.4177	0.0441
Phosphorus (P)	0.6080	0.8629	0.9325	0.5240	0.9386	0.4567
G × P	0.9160	0.9849	0.3617	0.6151	0.7999	0.3166
0.20-0.40 m depth						
Gypsum doses (kg ha^{-1})						
0	6.18	18.93	7.75	0.05	3.95	89.57
1000	6.08	15.97	7.08	0.00	2.23	89.13
2000	6.27	20.30	8.59	0.00	12.84	76.57
3000	6.02	17.98	7.30	0.23	1.83	68.54
Phosphorus doses (kg ha^{-1})						
0	6.15	18.41	7.76	0.14	10.40	87.95
50	6.09	17.46	7.51	0.00	2.15	82.07
100	6.17	20.39	7.81	0.00	2.42	82.77
150	6.16	17.47	7.85	0.07	2.13	81.93
Factors			**ANOVA – Probability of F test**			
Gypsum (G)	0.1110	0.3564	0.2407	0.1497	0.1791	0.0325
Phosphorus (P)	0.7937	0.6629	0.6747	0.2128	0.1698	0.9965
G × P	0.8432	0.7252	0.6812	0.7297	0.2241	0.1589

*Samples were collected 120 days after phosphorus and gypsum application.

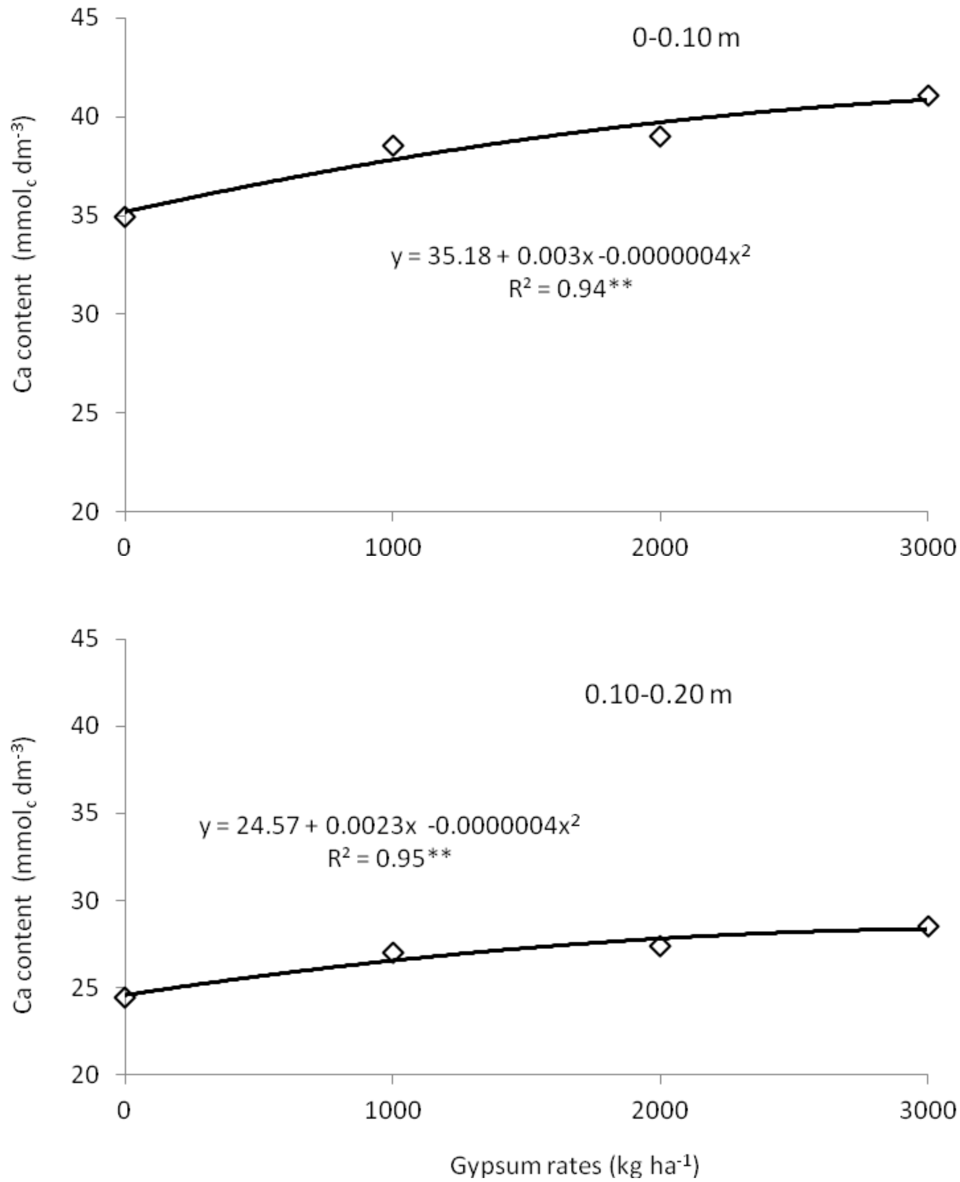

Figure 1. Calcium levels in the soil at depths of 0-0.10 and 0.10-0.20 m in relation to gypsum dose. 2011/2012 growing season.

experimental area was the right choice. However, it appears that this area is representative of those used for growing maize and soybeans in the Cerrado soil, which are naturally acid with low levels of bases (K, Ca and Mg) but after many years using liming and fertilizers by farmers provided high base saturation and fertility to the soil (Oliveira-Júnior et al., 2011; Montezano et al., 2006). Thus, these results are an important guide for farmers; in corrected soils with low subsurface Al and adequate Ca levels (>5 mmol dm^{-3}) gypsum application is not necessary.

Considering the result obtained, what could be expected in relation to gypsum application and that upland rice is considered a high-risk crop because it is a high-water-demand crop (Crusciol et al., 2013), there are some concerns about the cultivation of upland rice. The use of gypsum could help to reduce the subsurface Al concentration and increase Ca levels to stimulate root development at depth (Ritchey et al., 1982). In a NTS, due to the concentration of nutrients, organic matter and moisture in the first few centimeters of the soil, the rice root system tends to concentrate superficially (Nascente et al., 2013). Thus, the rice plant which has a higher demand for water than other crops, such as soybeans and maize, and has a less developed root system, becomes more subject to water stress and absorbs less

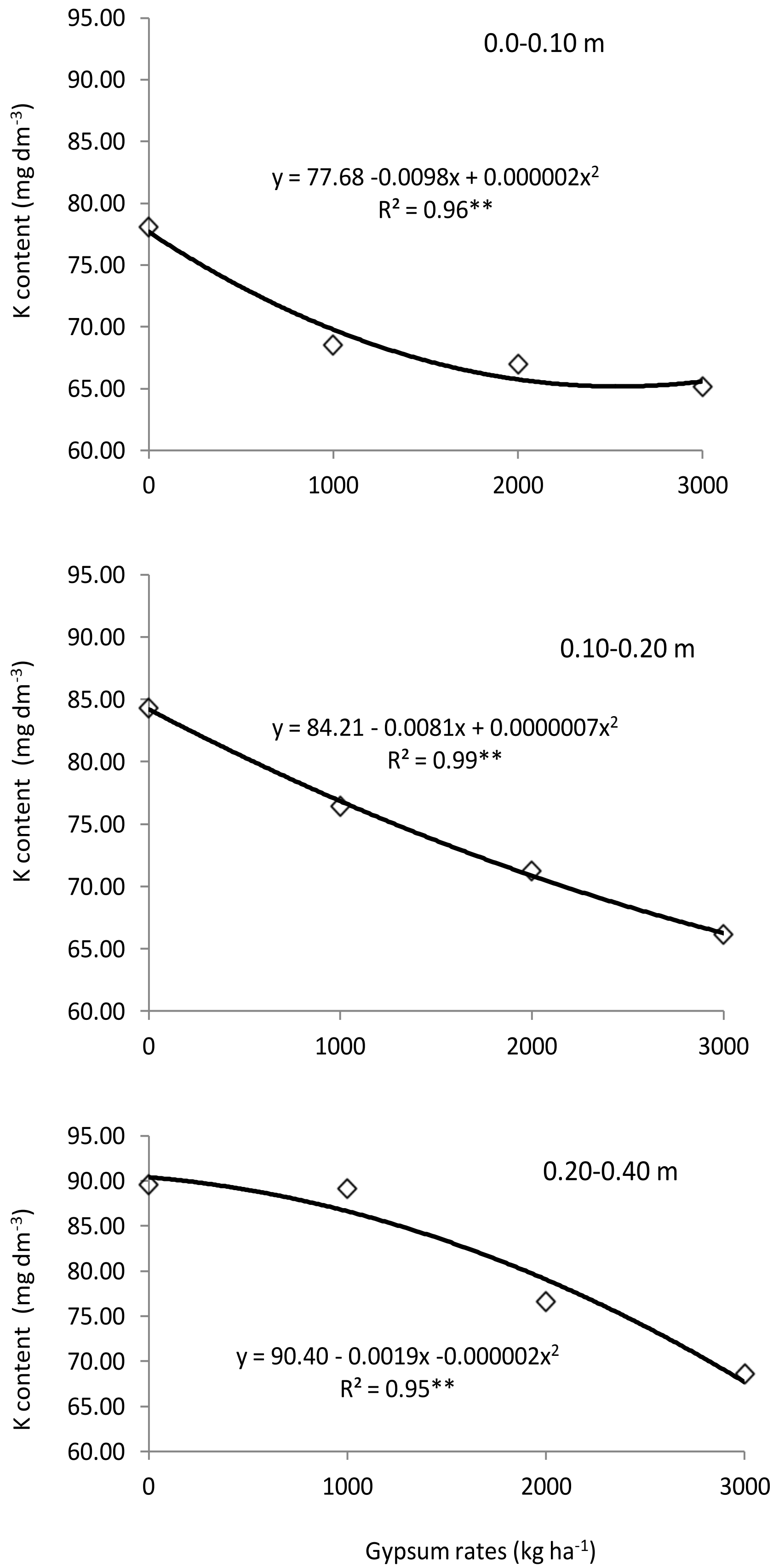

Figure 2. Potassium levels in the soil at depths of 0-0.10, 0.10-0.20 and 0.20-0.40 m as a function of gypsum rates 2011/2012 growing season.

Table 3. Plant height, number of panicles m-[1], mass of 1000 grains and grain yield for upland rice cultivated in a no-tillage system in relation to phosphorus dose, gypsum dose and growing season (2011/2012 and 2012/2013).

Factors of Gypsum doses (kg ha^{-1})	**Plant height (cm)**	**Number of panicles (m^{-1}) Number**	**Mass of 1000 grains (Grams)**	**Grain yield (kg ha^{-1})**
0	104.5	101.91	25.25	5092
1000	105.0	101.00	25.21	4993
2000	102.8	103.30	24.90	4860
3000	108.0	102.31	25.19	5133
Phosphorus doses (kg ha^{-1})				
0	103.8	101.64	25.26	4735
50	104.4	103.13	24.98	4970
100	105.0	103.36	25.09	5103
150	107.4	103.81	24.93	5207
Growing seasons				
2011/2012	105.4a[1]	90.1b	25.17a	4157.7b
2012/2013	104.8a	115.9a	24.97a	5849.7a
Factors		**ANOVA – Probability of F test**		
Growing seasons (GS)	0.4828	<0.001	0.1772	<0.001
Phosphorus (P)	0.0388	0.0155	0.3594	0.0386
Gypsum (G)	0.1003	0.7551	0.1943	0.7606
GS x P	0.9030	0.6866	0.4303	0.7613
GS x G	0.9873	0.4000	0.9219	0.6564
P x G	0.0521	0.6440	0.5551	0.8766
G x P x GS	0.9980	0.5359	0.5231	0.9049

[1]Means followed by the same letter do not differ by the Tukey test for $p<0.05$.

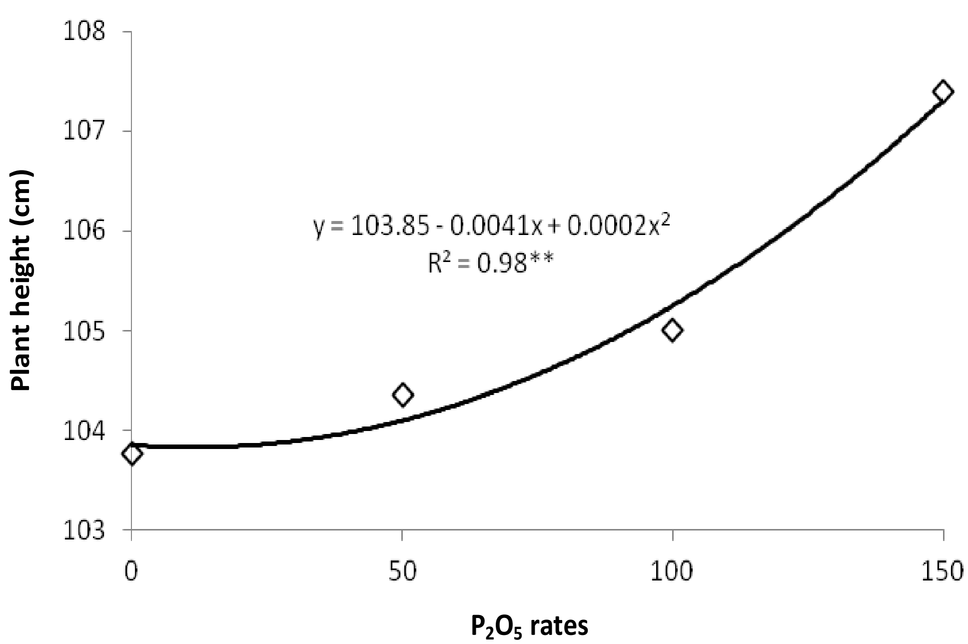

Figure 3. Rice plant height as a function of phosphorus rates applied in the sowing furrow. Average of two growing seasons.

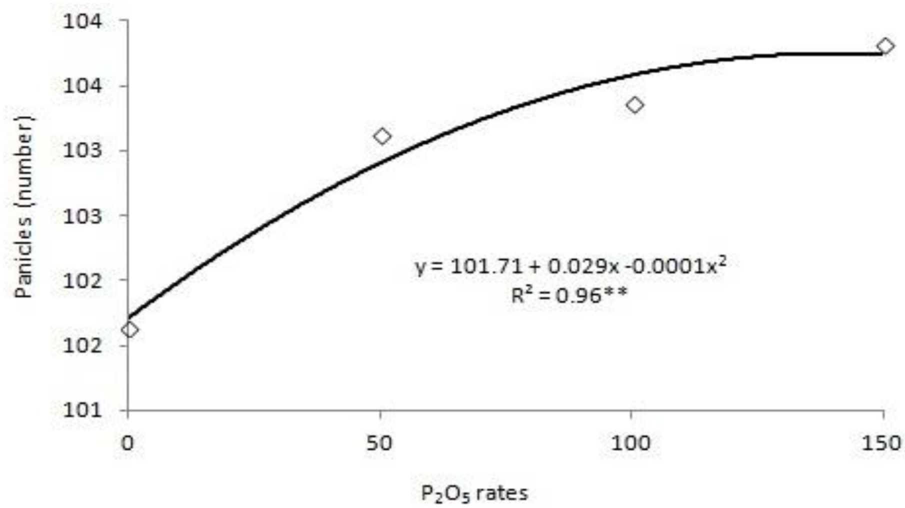

Figure 4. Rice number of panicles m^{-1} as a function of phosphorus rates applied in the sowing furrow. Average of two growing seasons.

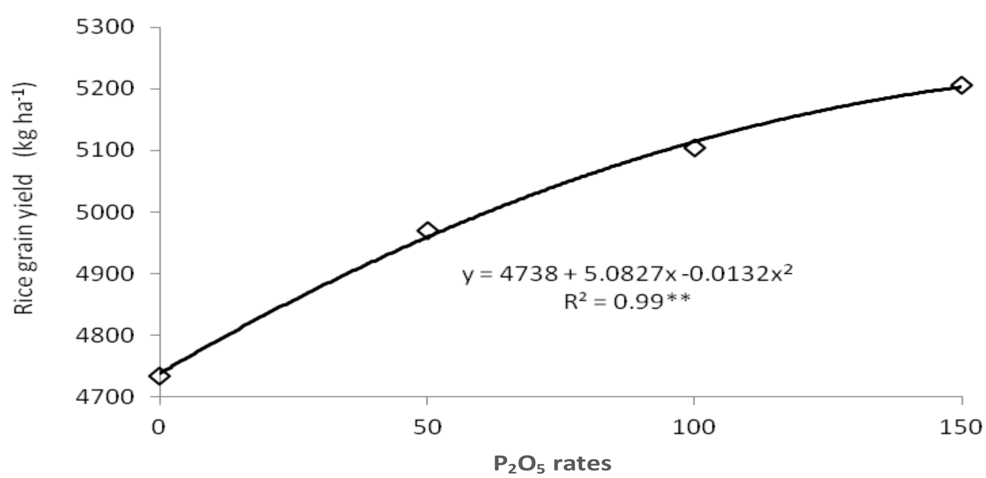

Figure 5. Rice grain yield as a function of phosphorus rates applied in the sowing furrow. Average of two growing seasons.

nutrients, which could cause grain yield reductions (Kluthcouski et al., 2000; Guimarães et al., 2006). As no effect of gypsum was observed in this study, it is possible that the rice root system had not developed in depth, thus, other strategies must be studied with regard to deepening rice root systems and, consequently, increasing the plant's drought resistance.

With respect to P levels, there were significant effects on the number of panicles m^{-1} and grain yield (Table 3). Thus, the data were fitted to quadratic equations for these two parameters (Figures 4 and 5). Crusciol et al. (2005) evaluated four upland rice varieties and also observed significant increases in crop production due to increased P availability. It is important to highlight that grain yield results were close to 5000 kg ha^{-1} in all treatments, even in the treatment with no P, and this is considered a high yield for upland rice. This result is probably due to the high level of P in the soil (Table 1), which had a significant effect on the rice grain yield. According to Fageria et al. (2011), P in the soil directly affects tillering, root development, number of panicles m^{-1} and the grain yield of rice crops.

Regarding growing seasons when evaluating grain yield and its component results in the second year, a higher number of panicles m^{-1} was observed than in the previous growing season (Table 3). Similarly, as rice grain yield is determined by the yield components, including the number of panicles m^{-1} (Yoshida, 1981), higher grain yields were also observed in 2012/2013 growing season.

The results of this experiment allowed us to infer that for the evaluated conditions (absence of Al toxicity in depth), P was more important for the development of the crop than gypsum. In Cerrado soils, which exhibit low natural fertility, it is common to find nutrient deficiencies, although with the development of agriculture in the region, corrected and high fertility soils can be found. Therefore, it is important to evaluate the initial fertility of each area with the objective of determining the real need for crop fertilization. Phosphorus, however, due to its high fixation in Cerrado soils, still has a high probability of response, and its doses should be adjusted to yield expectations to make its use profitable.

Conclusion

The application of gypsum provided incremental increases in Ca levels in addition to increased levels of K in deeper soil layers and did not affect plant height, yield components or grain yield of upland rice cultivated in a no-tillage system. By contrast, the application of increasing doses of P at sowing gave a significant increase in plant height, number of panicles m^{-1} and grain yield of upland rice cultivated in a no-tillage system.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Effect of application rates of selected natural pesticides on soil biochemical parameters and litter decomposition

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An experiment was carried out at the cocoa plantation of the Obafemi Awolowo University Teaching and Research Farm to study the comparative effects of siam weed and neem leaf extracts (as natural pesticides) and a synthetic pesticide- Endocel® (Endosulfan) on soil microorganism and litter decomposition in cocoa plantation to determine a safe application rate on soil microbial population and some soil chemical properties. Soil samples were collected on monthly basis for five months after the application of aqueous extracts of fresh leaves of siam weed and neem plants. Samples were analyzed for total heterotrophic bacteria (THB) and exchangeable cations using standard methods. Results showed that application of neem extract at 40,000 mg/kg (w/v) gave the highest microbial load and aided microbial activity more than siam at 40,000 mg/kg (w/v) with the least microbial load. Hence, neem plant was found to be less toxic than siam weed.

Key words: Agriculture, pesticides, cocoa, soil, microorganisms, exchangeable cations.

INTRODUCTION

World Cocoa bean production, harvested from the cacao tree (*Theobroma Cacao*) is estimated to be 3.5 million tones, 90% of which is grown in Cote d'ivoire, Ghana, Indonesia, Nigeria, Cameroon, Brazil, Ecuador and Malaysia where millions of smallholder farmers depend on the revenue (Norgrove, 2007).

Cocoa export is an important source of foreign exchange earnings for Nigeria. However, maximal yield of the product is not achieved as a result of the incidence of diseases and pests (Wessel, 1966; Opeke, 1982; Egbe et al., 1989). In order to improve product yield, large scale spraying of the cocoa pods with pesticides is usually carried out. This has led to the soils in some cocoa plantations in Nigeria being reportedly contaminated with organochlorine pesticide residues. Inspite of the upsurge in the use of pesticides in Nigerian agriculture, there has been little or no awareness among the users of the effects on the environment (Okoya et al., 2013).

Trees in forests absorb nutrients from the soil to

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support their growth. At the same time, some part of the nutrient taken up is returned to the forest ground via litter fall. This is justified since decomposition processes play an important role in soil fertility in terms of nutrient cycling and formation of soil organic matter (Bargali et al., 1993). Many studies have extensively investigated the effects of climate, litter quality and microorganisms on litter decomposition and their interactions in many ecosystems using a large number of plant species (Berg et al., 1993; Berg and Meetmeyer, 2002). Sariyildiz (2008) reported that a single tree canopy can significantly alter litter decomposition rates of a given tree species, due to changes in environmental conditions under the tree canopy, especially forest floor moisture content and pH Litter, therefore, plays a major role in the transfer of energy and nutrients within a woodland ecosystem. Microflora populations are responsible for the chemical transformation and degradation of complex organic molecules into simpler compounds during decomposition (Crossley and Witkamp, 1964).

The activities of soil microorganisms are beneficial but some may be detrimental to the environment, plant and man. Decomposition of organic materials is one of the most important activities carried out by soil microorganisms as it results in soil organic matter formation and the release of plant nutrients (Edwards et al., 1973; Wardle et al., 2003). Garcia-Pausas et al. (2004) reported that soil microclimate and/or N availability appear to be more important controlling litter decomposition than the distribution of fauna in Mediterranean forest soils. Also, the effect of litter N content on decomposition rates is site dependent. The soil fauna consumes partially degraded litter, resulting in very low faunal activity during the first stages of decomposition.

In modern agriculture, pesticides are frequently used in the field to increase crop production. Besides combating pest, pesticides also affect the population and activity of beneficial microbial communities in the soil (Pandey and Singh, 2004). Although, some soil biota can utilize and degrade pesticides, these pesticides have been found to adversely affect microbial populations, diversity and biochemical activities such as ammonification, nitrification, denitrification and urea hydrolysis (Greaves and Malkomes, 1980; Roslycky, 1986; Taiwo and Oso, 1997). They also may accelerate the development of the pest biotypes resistant to specific pesticidal chemicals (Ivbijaro, 1990). The widespread use of synthetic pesticides poses a potential threat to these decomposition processes and serious danger to human and environmental health. The intensive use of Organo chlorine and Lindane based insecticides for mirid control in Nigeria (Okoya et al., 2013) resulted to the development of resistance by the mirids, thereby rendering the insecticides ineffective (Booker, 1969; Youdeowei, 1971).

Turning to alternative strategies, plant extracts have been widely investigated for pest control. Natural pesticides are active ingredients derived from plants for the management of human and animal pest organisms, or it can be said to be biologically active ingredients principally derived from plants for the management of human and animal pest organisms (Ivbijiaro, 1990). Botanicals are easily biodegradable and their use in crop protection is a practically sustainable alternative (Devlin and Zettel, 1999). It maintains biological diversity of predators (Grange and Ahmed, 1988) and reduces environmental contamination and human health hazards. With the growing global demand for environmentally sound pest management strategies; there is a need to develop alternative pesticides with minimal or non-ecological hazards (Devlin and Zettel, 1999). One widely studied plant in this context is the neem tree, *Azadirachta indica.* Many research works have been carried out to examine the pesticidal activities of various preparations from neem such as the dry leaf powder, seed powder, seed cake, and ethanol extracts (Ofuya et al., 1992; Egwunyenga et al., 1998), aqueous extracts (Jackai and Oyediran, 1999; Padi et al., 2003). Neem leaf extract was also found to have nematicidal properties (Egunjobi and Afolami, 1976; Fatoki and Fawole, 2000).

Earlier studies in Nigeria showed that Siam weed (*Chromolaena odorata*) has some potential to control plant-parasitic nematodes in laboratory trials (Fatoki and Fawole, 1999; Adekunle and Fawole, 2003). The active ingredients in *C. odorata* are Alkaloids and Flavonoids.

This study seeks to determine the effect of the extracts from the leaves of neem plant and siam weed on soil microbial load and litter decomposition in a cocoa plantation. It is also aimed at determining the effective application rates of the two plant extracts.

MATERIALS AND METHODS

Study area

The study site was located on the cocoa (*T. cacao* Linn.) plantation at Obafemi Awolowo University Teaching and Research Farm, Ile-Ife, in southwestern Nigeria. Ile-Ife lies within latitudes 7° 30′ to 7° 35’ and longitude 4° 30’ to 4° 35.6’. The vegetation of Ile-Ife has been described as lowland forest zone (Keay, 1965), semi deciduous moist forests (Charter, 1970) and Guinea-Congolian forest drier type (White, 1983). There are two prominent seasons in Ile-Ife area, the rainy and dry seasons. The dry season is short, usually lasting five months from November to March and longer rainy season prevails during the remaining months. The annual rainfall at Ile-Ife has a mean of 1,272.44 mm in a 5-year (2009 to 2014) survey with a maximum value of 1452.62 mm and a minimum value of 1090.10 mm (Atmospheric Research Unit, Department of Physics, Obafemi Awolowo University, Nigeria, 2014).

The mean annual temperature ranges from 22.5 to 31.4°C. The relative humidity in the early morning is generally high, usually over 90% throughout the year. At midday, it is rather lower, around 80% in the wet season, than and as low as 50 and 60% in the dry season. The area lies within the basement complex and the underlying rock consists of granites, gneisses, and undifferentiated schists (Smyth and Montgomery , 1962). The soil is a low base status forest soil derived from coarse-grained gneiss and granite parent rocks and is classified as an Utisol (Harpstead, 1973; Okusami and Oyediran, 1985; Amusan and Ashaye, 1991). The soils of the study site are of two series (Iwo and Gambari). Ojanuga (1975) and Okoya et al. (2010) described soils of Iwo series as comprising well structured, strong brown to reddish soils with gravelly sand, clay loam to sandy clay texture within the upper horizon and generally slightly more clayey in the subsoil.

Field study

A field study was conducted with aqueous extracts from fresh leaves of neem (*A. indica*) and fresh leaves of siam weed (*C. odorata*) which were prepared using the method of Olabiyi et al. (1992). Ten – grams each of fresh siam weed and neem leaves were weighed and placed into two different reagent bottles. 100 ml of distilled water was added into each reagent bottle which were then placed in a water-bath and heated at 100°C for 1 h. The extract obtained was allowed to cool and filtered through Whatman No. 1 filter paper. The filtrate obtained was 100,000 mg/kg (stock extract). Serial dilutions were prepared to produce 40,000 and 20,000 mg/kg extracts (Adekunle and Fawole, 2003).

Experimental design and layout

A randomized complete block design with four blocks (A, B, C and D) each divided into 5 plots were established. The trees in the plots were tagged T_{1x} – T_{5x} respectively, where x represents the block. The plots were; plot 1 (neem extract application at 40,000 mg/kg), plot 2 (neem extract application at 20,000 mg/kg), plot 3 (siam weed extract application at 40,000 mg/kg), plot 4 (siam weed extract application at 20,000 mg/kg), plot 5 (control). The trees were treated five times with the different application rates of siam and neem extracts at a weekly interval. 30 days after the fifth application, soil samples were collected at 0 to 5 cm soil depth on monthly basis for five months. Soil samples were collected before the application of pesticides as well. The soil samples were analysed for the load of total heterotrophic bacteria and exchangeable cations.

Nutrient agar which has been reported to support the growth of most heterotrophic bacteria was used for bacteria isolation and incubation was at ambient temperature for two days. Microbial enumeration and isolation were carried out as described by Seeley and Vandenmark (1981). Representative bacteria colonies were identified. Some of the soil samples collected were air-dried, crushed, sieved and analysed for exchangeable cations. The exchangeable cations were extracted by leaching 10 g soil with 100 ml of 1N ammonium acetate solution. The filtrate was then taken to the flame analyzer for reading. Na^+, K^+ and Ca^{2+} were measured by the flame emission spectroscopy while Mg^{2+} was measured by the atomic absorption spectroscopy (Anderson and Ingram, 1993).

Data analysis

The data collected were analysed using SPSS for windows, version 16.0. Analysis of variance (ANOVA) was used to determine statistical differences in the data. Mean values of four replicates were used in all the analysis.

RESULTS AND DISCUSSION

At the beginning of the study, soil samples were collected in August from all the plots and analyzed for THB before the application of synthetic pesticide (Endocel) and the two plant extracts (Neem and Siam). Statistical analysis carried out on the data obtained (Table 1) showed that there were no significant differences along the column in the microbial load in soil samples. After the application of pesticides (Endocel, Neem and Siam extracts), however, the microbial load in the control plots (that is, no pesticide application) varied between $7.84 \times 10^6 \pm 4.95 \times 10^6$ and $4.58 \times 10^7 \pm 2.14 \times 10^7$ cfu/g (Table 1). In the soil treated with neem at 20,000 mg/kg (w/v), the load values were from $2.86 \times 10^6 \pm 1.57 \times 10^6$ to $1.18 \times 10^8 \pm 1.11 \times 10^8$ cfu/g while those treated at 40,000 mg/kg (w/v) varied from $1.70 \times 10^6 \pm 5.60 \times 10^5$ to $2.57 \times 10^9 \pm 2.54 \times 10^9$ cfu/g. The microbial load in siam treated soil varied from $1.50 \times 10^7 \pm 6.16 \times 10^6$ to $4.50 \times 10^7 \pm 2.81 \times 10^7$ cfu/g at the application rate of 40,000 mg/kg (w/v) and $4.14 \times 10^6 \pm 2.46 \times 10^6$ to $5.20 \times 10^7 \pm 2.51 \times 10^7$ cfu/g at the application rate of 20,000 mg/kg (w/v). The microbial load in soil treated with Endocel (0.05 L/ha) varied from $1.56 \times 10^6 \pm 9.92 \times 10^5$ to $5.16 \times 10^9 \pm 3.45 \times 10^9$ cfu/g while with 0.1 L/ha Endocel treatment of soil, the load was between $3.51 \times 10^6 \pm 1.75 \times 10^6$ and $6.17 \times 10^7 \pm 2.54 \times 10^7$ cfu/g. The load of THB in the soil samples was not significantly different for the synthetic and plant extracts used compared to control as shown along the columns in each month (Table 1). However, the differences were only statistically significant in March when the load of THB increased considerably with Endocel at 0.05 L/ha. No significant difference was detected between the treated and untreated soils.

In the second month after the application of treatment (December), the highest microbial load was observed in the soil treated with neem at 40,000 mg/kg (w/v) and the lowest was in the soil treated with neem at 20,000 mg/kg (w/v) when compared with the Siam extract only, However, among the three treatments, the lowest microbial load is in the soil treated with Endocel (0.05 L/ha) when comparing synthetic data was with the plant extracts data (Table 1). This is contrary to what was observed in the first month of sampling after treatment application (November). Siam treatment at 40,000 mg/kg (w/v) was observed to favour microbial activity throughout the experimental period except in the second month where the soil treated with siam at 20,000 mg/kg (w/v) had the higher microbial load between the two application rates. Statistical analysis indicated no significant difference between the treatments along the columns. However, to assess if there are significant differences in the microbial load values in different seasons, the trends across the months are presented in Figure 1.

The exchangeable cations likewise show varying results. Exchangeable Na^+ decreased consistently from August to December but slightly increased in January (Tables 2, 3, 4 and 5). This was observed for all the treatments. This same trend was also observed for exchangeable K^+ and Ca^{2+} in all the treatments for all the experimental months except in Siam at 20,000 mg/kg

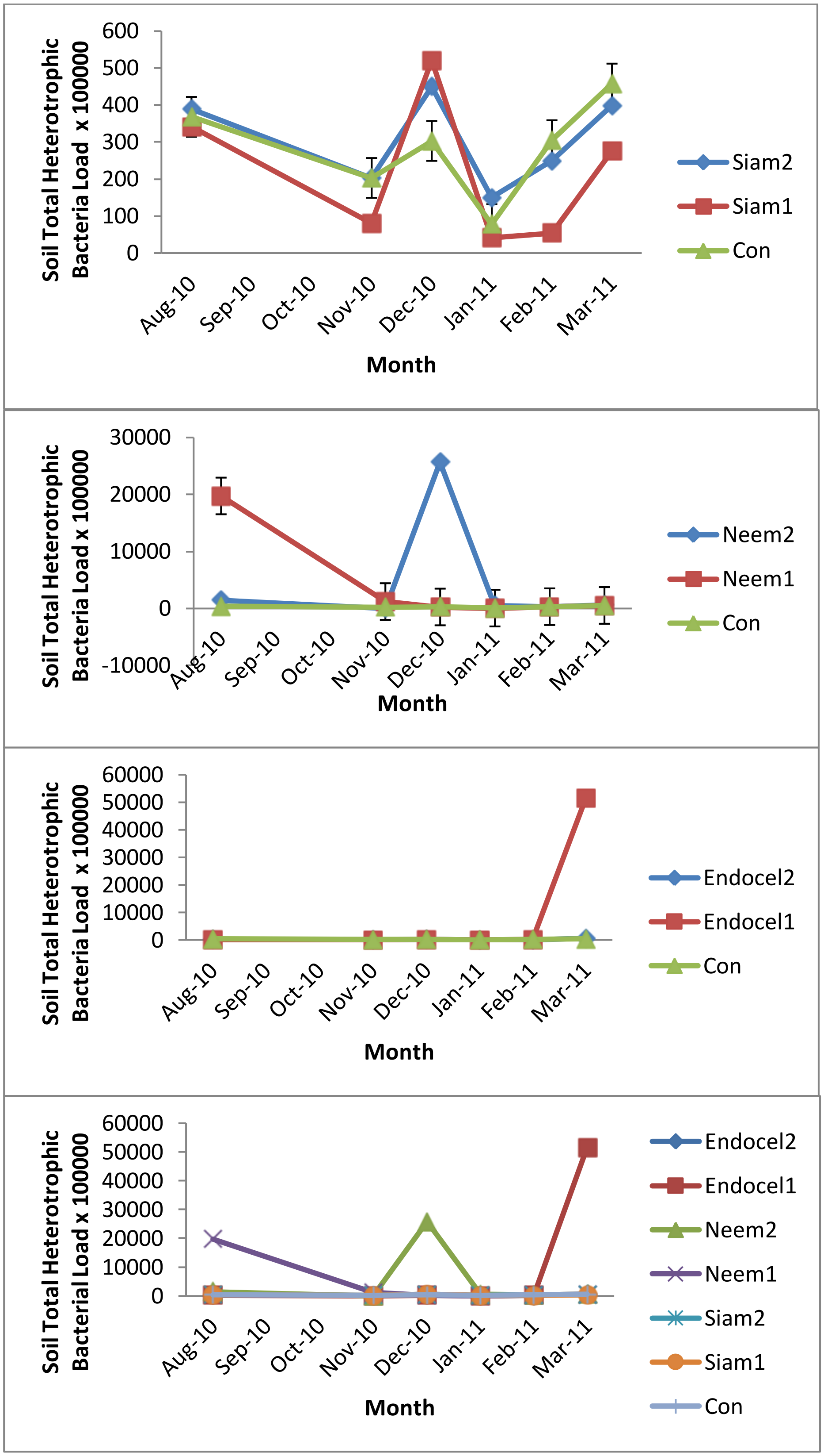

Figure 1. Effects of endocel, aqueous extracts of Neem leaves and Siam weed leaves on soil total heterotrophic bacteria load across the months of study. Note: Jan. – January, Feb. – February, Mar. – March, Aug. – August, Sep. – September, Oct. – October, Nov. – November and Dec. – December.

Table 1. Effects of endocel, aqueous extracts of Neem leaves and Siam weed leaves on soil total heterotrophic bacteria load in the studied cocoa plantation.

Treatment		Microbial concentration (cfu/g) ± S.D.				
	Aug. 2010*	Nov. 2010	Dec. 2010	Jan. 2011	Feb. 2011	Mar. 2011
Endocel at 0.1 L/ha	$3.00 \times 10^7 \pm 6.95 \times 10^{6(a)}$	$1.08 \times 10^7 \pm 4.07 \times 10^{6(a)}$	$3.00 \times 10^7 \pm 1.07 \times 10^{7(a)}$	$3.51 \times 10^6 \pm 1.75 \times 10^{6(a)}$	$1.11 \times 10^7 \pm 4.32 \times 10^{6(a)}$	$6.17 \times 10^7 \pm 2.54 \times 10^{7(a)}$
Endocel at 0.05 L/ha	$1.44 \times 10^7 \pm 3.90 \times 10^{6(a)}$	$3.41 \times 10^6 \pm 1.14 \times 10^{6(a)}$	$1.54 \times 10^7 \pm 5.40 \times 10^{6(a)}$	$1.56 \times 10^6 \pm 9.92 \times 10^{5(a)}$	$2.28 \times 10^7 \pm 1.55 \times 10^{7(a)}$	$5.16 \times 10^9 \pm 3.45 \times 10^{9(b)}$
Neem at 40,000 mg/kg	$1.45 \times 10^8 \pm 2.68 \times 10^{6(a)}$	$1.70 \times 10^6 \pm 5.60 \times 10^{5(a)}$	$2.57 \times 10^9 \pm 2.54 \times 10^{9(a)}$	$5.04 \times 10^7 \pm 4.99 \times 10^{7(a)}$	$2.60 \times 10^7 \pm 1.51 \times 10^{7(a)}$	$6.48 \times 10^7 \pm 5.39 \times 10^{7(a)}$
Neem at 20,000 mg/kg	$1.97 \times 10^9 \pm 1.94 \times 10^{9(a)}$	$1.18 \times 10^8 \pm 1.11 \times 10^{8(a)}$	$2.29 \times 10^7 \pm 1.01 \times 10^{7(a)}$	$2.89 \times 10^6 \pm 1.57 \times 10^{6(a)}$	$2.78 \times 10^7 \pm 1.30 \times 10^{7(a)}$	$4.99 \times 10^7 \pm 1.24 \times 10^{7(a)}$
Siam at 40,000 mg/kg	$3.90 \times 10^7 \pm 2.10 \times 10^{7(a)}$	$2.03 \times 10^7 \pm 1.51 \times 10^{7(a)}$	$4.50 \times 10^7 \pm 2.81 \times 10^{7(a)}$	$1.50 \times 10^7 \pm 6.16 \times 10^{6(a)}$	$2.50 \times 10^7 \pm 8.50 \times 10^{6(a)}$	$3.98 \times 10^7 \pm 1.58 \times 10^{7(a)}$
Siam at 20,000 mg/kg	$3.41 \times 10^7 \pm 5.55 \times 10^{6(a)}$	$8.00 \times 10^6 \pm 1.66 \times 10^{6(a)}$	$5.20 \times 10^7 \pm 2.51 \times 10^{7(a)}$	$4.14 \times 10^6 \pm 2.46 \times 10^{6(a)}$	$5.41 \times 10^6 \pm 1.62 \times 10^{6(a)}$	$2.76 \times 10^7 \pm 4.93 \times 10^{6(a)}$
Control (No pesticide)	$3.68 \times 10^7 \pm 7.97 \times 10^{6(a)}$	$2.03 \times 10^7 \pm 9.00 \times 10^{6(a)}$	$3.03 \times 10^7 \pm 1.32 \times 10^{7(a)}$	$7.84 \times 10^6 \pm 4.95 \times 10^{6(a)}$	$3.05 \times 10^7 \pm 1.03 \times 10^{7(a)}$	$4.58 \times 10^7 \pm 2.14 \times 10^{7(a)}$

Each value is a mean of four replicates. Values having the same alphabet(s) in bracket along the same column are not statistically different (P<0.05). *Values before the application of pesticides. Note: Aug. – August, Nov. – November, Dec. – December, Jan. – January, Feb. – February and Mar. – March.

Table 2. Exchangeable cations of soils in the studied cocoa plantation in August 2010 before application of treatments.

No treatment with	Exchangeable cations (cmol/kg)			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
Endocel at 0.1 L/ha	$0.24 \pm 0.01^{(a)}$	$0.66 \pm 0.11^{(a)}$	$11.79 \pm 0.64^{(a)}$	$6.92 \pm 2.52^{(a)}$
Endocel at 0.05 L/ha	$0.30 \pm 0.03^{(a)}$	$0.59 \pm 0.07^{(a)}$	$11.38 \pm 0.63^{(a)}$	$3.78 \pm 0.17^{(ab)}$
Neem at 40,000 mg/kg	$0.26 \pm 0.04^{(a)}$	$0.46 \pm 0.04^{(a)}$	$10.57 \pm 0.27^{(a)}$	$3.90 \pm 0.27^{(ab)}$
Neem at 20,000 mg/kg	$0.26 \pm 0.00^{(a)}$	$0.60 \pm 0.13^{(a)}$	$11.38 \pm 0.55^{(a)}$	$3.90 \pm 0.25^{(ab)}$
Siam at 40,000 mg/kg	$0.28 \pm 0.02^{(a)}$	$0.44 \pm 0.07^{(a)}$	$10.88 \pm 0.85^{(a)}$	$3.62 \pm 0.29^{(b)}$
Siam at 20,000 mg/kg	$0.27 \pm 0.02^{(a)}$	$0.47 \pm 0.05^{(a)}$	$11.07 \pm 1.38^{(a)}$	$3.97 \pm 0.51^{(ab)}$
Control (No treatment)	$0.23 \pm 0.00^{(a)}$	$0.42 \pm 0.09^{(a)}$	$9.85 \pm 0.47^{(a)}$	$3.54 \pm 0.23^{(b)}$

Mean value ± S.D. with the same superscript along the same column are not significantly different. Each value is a mean of four replicates.

where constant decrease was observed from August to January in both cations. Although, the changes observed in exchangeable Na^+ and Ca^{2+} did not show any significant difference, exchangeable K^+ increased significantly ($P < 0.05$). Exchangeable Mg^{2+} decreased consistently throughout the experimental period for all the treatments except Endocel at 0.1 L/ha, Endocel at 0.05 L/ha and Siam at 40,000 mg/kg where increment was observed in January. However, the values were not significantly different ($P < 0.05$). For all the treatments used, Endocel at 0.05 L/ha had the highest concentration of exchangeable cations within the range (0.16 ± 0.01 to 10.79 ± 0.61), followed by Endocel at 0.1 L/ha (0.18 ± 0.01 to 9.91 ± 0.92). Comparing the two plant extracts, exchangeable cations obtained in Neem treatment is higher than Siam treatment, where the highest value was observed in Neem at 20,000 mg/kg (0.16 ± 0.02 to 9.91 ± 2.10) although closely followed by Neem at 40,000 mg/kg (0.14 ± 0.01 to 9.44 ± 0.70).

Generally, soil microbial load reduced with all the treatments from November to February (Table 1). It is reasonable to deduce that this may have resulted from, at least in part, the extremely dry conditions experienced in these months due to

Table 3. Effects of endocel, aqueous extracts of Neem leaves and Siam weed leaves on soil exchangeable cations of the studied cocoa plantation in November 2010.

Treatment	Exchangeable cations (cmol/kg)			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
Endocel at 0.1 L/ha	0.18 ± 0.01$^{(ab)}$	0.38 ± 0.06$^{(a)}$	9.35 ± 1.01$^{(a)}$	4.57 ± 1.44$^{(a)}$
Endocel at 0.05 L/ha	0.21 ± 0.01$^{(a)}$	0.36 ± 0.05$^{(a)}$	10.79 ± 0.61$^{(a)}$	3.51 ± 0.21$^{(a)}$
Neem at 40,000 mg/kg	0.18 ± 0.00$^{(ab)}$	0.33 ± 0.03$^{(a)}$	9.44 ± 0.70$^{(a)}$	3.19 ± 0.17$^{(a)}$
Neem at 20,000 mg/kg	0.20 ± 0.01$^{(ab)}$	0.38 ± 0.08$^{(a)}$	10.03 ± 0.91$^{(a)}$	3.65 ± 0.31$^{(a)}$
Siam at 40,000 mg/kg	0.19 ± 0.01$^{(ab)}$	0.29 ± 0.07$^{(a)}$	9.19 ± 1.19$^{(a)}$	3.27 ± 0.72$^{(a)}$
Siam at 20,000 mg/kg	0.18 ± 0.01$^{(ab)}$	0.33 ± 0.04$^{(a)}$	9.07 ± 1.26$^{(a)}$	3.06 ± 0.23$^{(a)}$
Control (No treatment)	0.18 ± 0.01$^{(b)}$	0.23 ± 0.02$^{(a)}$	7.91 ± 0.41$^{(a)}$	3.44 ± 0.93$^{(a)}$

Mean value ± S.D. with the same superscript along the same column are not significantly different. Each value is a mean of four replicates.

Table 4. Effects of Endocel, aqueous extracts of Neem leaves and Siam weed leaves on soil exchangeable cations of the studied cocoa plantation in December 2010.

Treatment	Exchangeable cations (cmol/kg)			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
Endocel at 0.1 L/ha	0.18 ± 0.01$^{(a)}$	0.35 ± 0.03$^{(ab)}$	7.92 ± 1.13$^{(a)}$	1.03 ± 0.24$^{(a)}$
Endocel at 0.05 L/ha	0.16 ± 0.01$^{(a)}$	0.25 ± 0.04$^{(b)}$	7.60 ± 0.94$^{(a)}$	0.75 ± 0.24$^{(a)}$
Neem at 40,000 mg/kg	0.14 ± 0.01$^{(a)}$	0.26 ± 0.03$^{(ab)}$	6.76 ± 0.33$^{(a)}$	1.27 ± 0.24$^{(a)}$
Neem at 20,000 mg/kg	0.16 ± 0.02$^{(a)}$	0.26 ± 0.03$^{(ab)}$	8.19 ± 1.12$^{(a)}$	0.91 ± 0.31$^{(a)}$
Siam at 40,000 mg/kg	0.17 ± 0.02$^{(a)}$	0.23 ± 0.02$^{(ab)}$	7.85 ± 1.21$^{(a)}$	1.09 ± 0.26$^{(a)}$
Siam at 20,000 mg/kg	0.15 ± 0.01$^{(a)}$	0.27 ± 0.04$^{(a)}$	7.03 ± 1.04$^{(a)}$	1.41 ± 0.50$^{(a)}$
Control (No treatment)	0.14 ± 0.01$^{(a)}$	0.27 ± 0.05$^{(ab)}$	6.07 ± 0.67$^{(a)}$	1.41 ± 0.69$^{(a)}$

Mean value ± S.D. with the same superscript along the same column are not significantly different. Each value is a mean of four replicates.

Table 5. Effects of Endocel, aqueous extracts of Neem leaves and Siam weed leaves on soil exchangeable cations of the studied cocoa plantation in January 2011.

Treatment	Exchangeable cations (cmol/kg)			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
Endocel at 0.1 L/ha	0.18 ± 0.01$^{(a)}$	0.39 ± 0.02$^{(a)}$	9.91 ± 0.92$^{(a)}$	3.11 ± 2.80$^{(a)}$
Endocel at 0.05 L/ha	0.18 ± 0.02$^{(a)}$	0.39 ± 0.09$^{(a)}$	10.48 ± 2.03$^{(a)}$	4.46 ± 4.26$^{(a)}$
Neem at 40,000 mg/kg	0.17 ± 0.02$^{(a)}$	0.31 ± 0.05$^{(ab)}$	8.94 ± 0.77$^{(a)}$	0.41 ± 0.11$^{(a)}$
Neem at 20,000 mg/kg	0.18 ± 0.02$^{(a)}$	0.35 ± 0.05$^{(ab)}$	9.91 ± 2.10$^{(a)}$	0.40 ± 0.11$^{(a)}$
Siam at 40,000 mg/kg	0.17 ± 0.01$^{(a)}$	0.27 ± 0.03$^{(ab)}$	7.97 ± 0.67$^{(a)}$	1.32 ± 0.87$^{(a)}$
Siam at 20,000 mg/kg	0.16 ± 0.01$^{(a)}$	0.16 ± 0.01$^{(b)}$	6.88 ± 0.84$^{(a)}$	0.69 ± 0.34$^{(a)}$
Control (No treatment)	0.17 ± 0.01$^{(a)}$	0.41 ± 0.11$^{(a)}$	8.57 ± 0.84$^{(a)}$	0.76 ± 0.20$^{(a)}$

Mean value ± S.D. with the same superscript along the same column are not significantly different. Each value is a mean of four replicates.

low rainfall and high temperature. The rain in February was just a light shower that could not have had a significant effect on the soil microbial load. Aerts (1997) had reported that temperature and moisture affect the population of microorganisms during decomposition. Rheinheimer (1997) and Knacker et al. (2003) have also noted that microbial populations were generally lower during the dry season. Under the dry spell, the least microbial load was found in soil samples treated with Siam at 40,000 mg/kg while Endocel at 0.05 L/ha aided more microbial population. In March when there was high rainfall, microbial activity thrived because there was increase in microbial load for all the treatments (Table 1). Jordan (1989) reported that water affected decomposition directly through leaching and indirectly through its effect on microbial decomposers. It would appear that moisture and temperature conditions were most conducive during the wet season when microbial population flourished, thus suggesting, that drought affects the population of microbial organisms (Hendrix et al., 1986). The increase in the population of the microorganisms probably indicates a faster decomposition at this period. The application of the synthetic and biological pesticides had no significant effect on the soil micro-organisms except Endocel at 0.05 L/ha that increased the population in March assessment.

Comparing the two plant extracts used, Neem aided litter decomposition better than Siam because the highest microbial load was found in Neem at 40,000 mg/kg followed by Neem at 20,000 mg/kg while the lowest load was found in Siam at 40,000 mg/kg. Foerster (2000) had earlier reported that Neem plant as being toxicologically safe. At the first two months of sampling after the application of pesticides, a consistent reduction was observed in exchangeable Na^+, K^+ and Ca^{2+} concentrations (Tables 3 and 4). This was probably due to the slower rate of leaf litter breakdown in these months as a result of the reduction in soil micro-organisms responsible for litter decomposition which slowed down the release of exchangeable cations. However, Cocoa growing soils have the ability to replenish the lost potassium due to cropping as reported by Ahenkorah et al. (1974).

Conclusion

In this study, the decomposer organisms responsible for litter decomposition were generally not affected by both synthetic pesticide and plant extracts used, although Endocel at low concentration aided more microbial population and nutrient release. This indicates that neem leaves, siam weed extracts and Endocel used at manufacturer's recommended rate did not have adverse effect on soil microorganisms thus aiding litter decomposition and nutrient release when other environmental factors such as moisture, temperature, etc are adequate. Although, both neem and siam weed leaf extracts aided microbial activity, more microorganisms were found in neem treated plots. It is therefore reasonable to conclude that neem leaf extract is less toxic and aids microbial growth better than siam extract.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Biochemical responses of maize (*Zea mays* L.) cultivars subjected to nitrate and glutamine fertilizers

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Maize (*Zea mays* L.) possesses high productive potential, excellent chemical composition and nutritive value, is one of the most important cereals in the world. This work aimed to evaluate the biochemical changes in two cultivars of maize, BRS Planalto and BR5202 Pampa, exposed to different amounts of nitrate and glutamine. The seeds were sown in polyethylene pots acclimated in a greenhouse, where began treatments with nitrate and glutamine at different doses (0.00, 0.10, and 10.00 mM) in five plots a completely randomized statistical design with six replicates. At 30 days, a single collection of material was performed and evaluated: chlorophylls a, b and total, carotenoids, total soluble carbohydrates, starch, sugars reducers and non-reducers, soluble protein and activity of nitrate reductase in leaves and roots. The content of photosynthetic pigments showed differences only between the doses tested. In general, an increase in nitrate concentration and a decrease in starch content, total soluble sugars, reducers and non-reducers for both cultivars. Comparing the effects of different sources and levels of N, the cv. BRS Planalto presented the greatest levels of total soluble proteins in leaves compared to cv. BR5202 Pampa. There was a decline in the activity of RN in both genotypes in response to high doses of N. Overall, there was no significant difference between N sources used. Nitrate reductase activity is greater than the 0.1 mM of nitrogen source, and is greater in leaves in relation to the roots, and glutamine does not inhibit its activity.

Key words: *Zea mays* L., nitrate reductase, nitrogen fonts.

INTRODUCTION

Maize (*Zea mays* L.) is a monocot of the Poaceae family (formerly Gramineae) and is part of a group of plants that perform C4 photosynthetic metabolism (Marenco and Lopes, 2009). Due to high productive potential, excellent

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chemical composition and nutritive value, maize is one of the most important cereals grown and consumed in the world, and in many countries of Africa, Latin America and Asia, maize the food base of the population (Castro et al., 2009).

The culture of corn requires large amounts of nitrogen and usually requires the use of nitrogen fertilizer (organic and/or chemical) in coverage to complement the amount provided by the soil. However, the efficiency in the use of available nitrogen in the fertilizer is generally low which seems to depend on, among other factors, the concentration of the mineral in the soil and losses by leaching process (Figueiredo et al., 2005).

Studies reveal different responses regarding the time of application and nitrogen sources (Lara Cabezas et al., 2005). However, there is a high demand for nitrogen in the early stages of development (Silva et al., 2005). The importance of nitrogen known for duties in the metabolism of plants, participating as a constituent of proteins, cytochromes and chlorophylls, among others, in addition to being considered one of the most relevant factors for the increase in production by influencing the rate of emergence and expansion of foliar area (Taiz and Zeiger, 2009).

According to Marenco and Lopes (2009), nitrogen is preferentially absorbed by the roots of plants in the form of nitrate and ammonia. Nitrate can originate from organic matter mineralization that contains amino acid nitrogen which undergoes several biochemical transformations, but can also be of fertilizers containing such salt. Once absorbed by the cell, nitrate is reduced to nitrite by nitrate reductase (NR) and then to ammonium chloride by nitrite reductase (NIR). The ammonium chloride is immediately assimilated through the joint action of the enzymes glutamine synthetase (GS) and glutamate synthetase (GOGAT). The process of reduction and assimilation of N can occur on leaves and/or roots, simultaneously or not, between these organs, according to the species (Pate, 1980) and with environmental conditions (Costa, 1986).

The first step of this process is the reduction of nitrate to nitrite in the cytosol by the enzyme nitrate reductase (Taiz and Zeiger, 2009). Thus, NR is of fundamental importance in providing nitrogen to the plant. This enzyme has several factors that regulate its activity. The presence of nitrate itself induces its activity in various tissues. Light stimulates the protein levels of NR (Li and Oaks, 1994), as well as the presence needed in large quantity of reducing agents.

Glutamine synthetase is another important enzyme in the process of incorporation of nitrogen because it catalyses the step key of inorganic nitrogen assimilation: incorporation of ammonium chloride to glutamate which results in glutamine. Thus, the synthesis of glutamine by GS is considered a key process for plants to express their maximum productive potential (Unno et al., 2006). Glucose and starch products of the Calvin cycle which comes from the photosynthetic activity also stimulate the accumulation of NR (Lillo, 2004). However, Touraine et al. (2001) found that amino acids repress nitrate absorption.

Based on this context, this work aimed to evaluate the biochemical changes in plants of two cultivars of maize (*Z. mays* L.) exposed to different amounts of nitrate and glutamine in order to increase the knowledge about the influence of these sources of nitrogen for vegetable growth and to test its viability for modern agriculture.

MATERIALS AND METHODS

Study area

The experiment was performed in the greenhouse of the Department of Botany, the Institute of Biology - IB of Universidade Federal de Pelotas - UFPel, Rio Grande do Sul, Brasil located at 31°46'19"S, 52°20'34"W with a height of about 7 m above sea level. The weather according to Köppen classification is the CFA type, that is, moist temperate weather with a warm summer and average rainfall of 1200 mm per year. The average temperature in the warmest month is 23.3°C and in the coldest 12.2°C. The average relative humidity is 78%.

Material and preparation

For this experiment, seeds of corn (*Z. mays* L.) cv. BRS Planalto and cv. BR5202 Pampa were used, provided by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Clima Temperado, Pelotas, Rio Grande do Sul). These varieties were selected for being precocious and both possess tolerance to cold weather in southern Brazil and have been developed for the practice of family agriculture which is quite common in this part of the country.

The seeds (3 seeds/pot) were sown in polyethylene pots with a capacity of 1 L containing washed sand as substrate. At 3 days after emergence (DAE), scraping was carried out so that only one plant remained per pot. Plants remained in the greenhouse with an average daily temperature of 30°C ± 2°C. The plants were irrigated with water whenever required until 10 DAE.

The experiment constituted six treatments, three being different concentrations of nitrate and three different concentrations of glutamine (0.00, 0.10 and 10.00 mM), divided into five instalments, applied with 20 mL of each solution at intervals of 3 days in each treatment. Potassium nitrate was used as a source of nitrate.

Among the applications of the solutions, nitrogenized was provided a nutritive solution without nitrogen at DAE, a single material collection was carried out for the evaluations by biochemical analyses.

Biochemical analysis

For the biochemical analyses, samples of ~100 mg of the tissues of roots and leaves were collected, weighed and conditioned in containers of suitable solutions according to the extractions and maintained at temperatures of -20°C subsequent for processing. Analyses were carried out of photosynthetic pigment content (chlorophylls *a*, *b* and total, and carotenoids), starch, total soluble sugars, reducing and non-reducing sugars, total protein and activity of NR.

Chlorophylls (Chl*a* and Chl*b, total)* and carotenoids (Car)

The procedure began with the maceration of the samples in the dark with addition of 80% acetone until a uniform extract was obtained. The extract centrifuged at 3000 rpm for 5 min and then the supernatant was collected and its volume was made up to 25 mL with 80% acetone. Absorbance of the samples was read in a spectrophotometer at 470, 645 and 663 nm according to Arnon (1949). The contents of chlorophyll *a*, *b* and total, and carotenoids were calculated according Lichtenthäler (1987).

Starch

Starch content determinations were carried out with centrifuged residuals after extraction of soluble carbohydrates according to the method described by Mc Credy et al. (1950).

Total soluble carbohydrates, reducing sugars and non-reducing sugars

The levels of total soluble carbohydrates were determined by means of reactions with Antrona according to Clegg (1956) and reducing sugars by the method of Somogy-Nelson (Nelson, 1944; Somogy, 1952). The non-reducing sugars were estimated by the differences between the concentrations of total soluble and reducing sugars. All results are expressed in mg.g MF^{-1}.

Total soluble proteins

The samples was macerated with NaOH to 0.1 M and then centrifuged at 3000 rpm for 5 min. The supernatant was collected and the final volume was measured. From these, we collected a sample of 100 µL and added 5 µL Coomassie Blue. This mixture was stirred in the vortex for 2 min. The absorbance was measured in the spectrophotometer at 595 nm and the content is expressed in mg.g MF^{-1}.

Nitrate reductase activity - NR

To quantify the *in vivo* activity of nitrate reductase, tissue samples from leaves and roots were used with modifications according to Cataldo et al. (1975). Samples were weighed and incubated in 0.1 M buffer phosphate-K (pH 7.5), 0.02 M KNO_3, 2% propanol and 5% Triton X100. Subsequently, the material was filtered in vacuum desiccator in the dark for 5 min followed by incubation in a water bath at 30°C for 30 min in the dark to avoid the formation of reduced ferredoxin and to measure the nitrite formed before its reduction to ammonium. 2.0 mL of samples, 0.3 mL of 1% sulfanilamide in 3 M HCl and 0.3 mL of 0.02% N-naphthalene diamine hydrochloride was added. The total volume was made up to 4 mL with deionized water. The solution remained at rest for 10 min and was read in a spectrophotometer at 540 nm (Kenis et al., 1992). The activity is expressed in μmol NO_2^- g^{-1} MF h^{-1}.

Statistical analyses

The experimental design utilized was completely randomized with six replications. The experimental unit constituted of a pot containing a plant. The effects of nitrogen levels were analysed by the Tukey test at 5% of probability using the program ASSISTAT (Silva, 1996; Silva and Azevedo, 2002, 2006, 2009).

RESULTS AND DISCUSSION

In this present study, no significant differences were found in the contents of pigments between cultivars BRS Planalto and BR5202 Pampa. However, significant differences in the contents of *a, b* and total chlorophylls were obtained in treatments with GLN and nitrate at a concentration of 10 mM compared to the control and the lowest dose (0.1 mM) which did not differ among themselves (Table 1) in both cultivars.

The carotenoid content of cv. BRS Planalto was significant in relation to the other treatments only at a dose of 10 mM. On the other hand, the levels of carotenoids in cv. BR5202 Pampa were significantly higher than in treatments with 0.1 mM nitrate and 10 mM GLN. Corn plants subjected to higher doses have generally higher content of pigment in relation to the lower doses. Majerowicz et al. (2002) also observed an increase in the contents of pigments in seven varieties of corn in response to an increase in N rates (15). The two genotypes used showed a 100% increase in chlorophyll levels *a* and *b* when the total dose of GLN increased from 0.1 to 10 mM (100-fold).

The content of photosynthetic pigments was sufficient to indicate the difference of the nutritional status of the plants and the efficiency of N rates utilized but not to differentiate between the behaviour of each cultivar. In the same way, the contents of chlorophyll showed the rates effect of N without producing differences between the maize genotypes tested (Majerowicz et al., 2002).

The concentration of starch in cv. BRS Planalto was significantly higher than the control only at a dose of 0.1 mM nitrate (Table 2). In cv. BR5202 Pampa, the starch content did not differ between the doses of 0.1 mM nitrate and GLN but at 10 mM both of nitrate and GLN were lower than the control.

Possibly this fact occurred due to large amount of GLN acting as your enzyme inhibitor preventing its action. Oliveira et al. (2013) stated that the glutamine synthetase is important in the absorption of N, and Hirel et al. (2007) claimed that the presence of N is crucial to grain filling which results in higher levels of starch and sugar.

For total soluble sugars, there was a difference for 0.1 mM nitrates, 0.1 mM and 10 mM GLN relative to the control for BRS Planalto. However, BR5202 Pampa did not show the same differences in soluble sugar levels compared with the control. The availability of N has affect the levels of soluble sugars from the roots, what happens in function of N is required (Table 3), to produce enzymes responsible for the carbohydrate synthesis which may be affected by the availability of N (Alfoldi et al., 1992).

In the cv. BRS Planalto, the content of reducing sugars was significantly different at doses of 0.1 mM of nitrates and 10 mM of GLN as compared to control. In the cv. BR5202 Pampa, there was a decrease in the level of reducing sugars with an increase in the two sources utilized. In both cultivars, the non-reducing sugar content was significantly higher than the control only for the treatments with GLN (Table 2).

In general, we observed that with an increase in the nitrate concentration, there was a decrease in the starch

Table 1. Level (mg g^{-1} MF) of chlorophyll *a*, *b*, total and carotenoids corn plants cv. BRS Planalto and cv. BR5202 Pampa according to different sources and levels of nitrogen.

Treatment (mM)	Chlorophyll a		Chlorophyll b		Total chlorophyll		Carotenoids	
	Planalto	Pampa	Planalto	Pampa	Planalto	Pampa	Planalto	Pampa
Control	0.41^{Ba}	0.35^{Ba}	0.20^{Ba}	0.16^{Ba}	0.61^{Ba}	0.52^{Ba}	0.17^{Ba}	0.14^{Ca}
NO_3^- 0.1	0.43^{Ba}	0.51^{Ba}	0.20^{Ba}	0.21^{Ba}	0.63^{Ba}	0.73^{Ba}	0.15^{Bb}	0.20^{Aa}
NO_3^- 10	0.65^{Aa}	0.66^{Aa}	0.33^{Aa}	0.32^{Aa}	0.98^{Aa}	0.99^{Aa}	0.18^{Ba}	0.18^{Ba}
GLN 0.1	0.38^{Ba}	0.40^{Ba}	0.16^{Ba}	0.17^{Ba}	0.54^{Ba}	0.57^{Ba}	0.17^{Ba}	0.17^{Ba}
GLN 10	0.82^{Aa}	0.84^{Aa}	0.36^{Aa}	0.38^{Aa}	1.19^{Aa}	1.22^{Aa}	0.23^{Aa}	0.22^{Aa}

Means followed by the same letter are not different at 5% probability by Tukey test.

Table 2. Level (mg g MF^{-1}) starch, soluble sugars, and reducing and non-reducing sugars in corn plants cv. and open pollinated cv. BR5202 Pampa according to different sources and levels of nitrogen.

Treatment (mM)	Starch		Total soluble sugars		Reducing sugars		Not reducing sugars	
	Planalto	Pampa	Planalto	Pampa	Planalto	Pampa	Planalto	Pampa
Control	1.60^{Ba}	1.30^{Aa}	3.21^{Ba}	3.02^{Aa}	1.48^{Ba}	1.74^{Aa}	1.72^{Ba}	1.29^{Ba}
NO_3^- 0.1	2.12^{Aa}	1.30^{Ab}	3.91^{Aa}	2.82^{Ab}	1.68^{Aa}	1.54^{Aa}	2.24^{Ba}	1.28^{Bb}
NO_3^- 10	1.17^{Ba}	1.05^{Ba}	1.97^{Ba}	2.19^{Ba}	0.98^{Bb}	1.19^{Ba}	0.99^{Ca}	1.00^{Ba}
GLN 0.1	1.49^{Ba}	1.48^{Aa}	4.22^{Aa}	3.39^{Ab}	1.56^{Ba}	1.68^{Aa}	2.66^{Aa}	1.71^{Ab}
GLN 10	1.20^{Ba}	1.06^{Ba}	4.51^{Aa}	2.71^{Ab}	1.92^{Aa}	1.29^{Bb}	2.59^{Aa}	1.42^{Ab}

Means followed by the same letter are not different at 5% probability by Tukey test.

Table 3. Relationship between carbohydrates and proteins in corn plants cv. BRS Planalto and cv. BR 5202 Pampa according to different sources and levels of nitrogen.

Treatment	BRS Planalto			BR5202 Pampa		
	Leaf	Root	Total	Leaf	Root	Total
Control	2.72^b	2.53^b	2.68^b	4.8^b	2.94^a	4.03^a
NO_3^- 0.1 mM	2.95^b	3.8^a	3.15^b	5.88^a	3.04^a	4.62^a
NO_3^- 10 mM	2.56^b	2.95^b	2.66^b	3.68^b	1.5^b	2.67^b
GLN 0.1 mM	5.28^a	2.32^b	4.26^a	6.12^a	1.95^b	4.13^a
GLN 10 mM	5.94^a	2.33^b	4.7^a	3.62^b	1.36^b	2.4^b

Means followed by the same letter are not different at 5% probability by Tukey test.

content, total soluble sugars, reducing and non- reducing sugars for both cultivars (Table 2). Probably, the increase in nitrate concentration affected the production of these organic compounds in the development stage analysed. Touraine et al. (2001) stated that there should be availability of carbohydrates in roots so that activity in the transporters of nitrate can occur. In this way, it appears that 0.1 mM nitrates can be considered sufficient for the genotypes and stage analysed.

For both genotypes analysed, starch content decreased with the increased availability of N. This shows that the starch can be a source of carbonic skeletons for incorporation of the N, when available in larger concentrations. The synthesis of starch is altered by N availability in plants (Medici, 2003). Carbon which is required during nitrate assimilation in many cases is obtained by slowing the rate of accumulation of starch or by means of starch remobilization (Scheible et al., 1997).

The ratio between carbohydrates and proteins in the leaves of cv. BRS Planalto showed the highest values when GLN was used as a source of N. There were differences between the sources of N and between GLN and control, when the ratio increased (Table 3). This may be due to the decrease in protein (Figure 1) or an increase in the level of total soluble carbohydrates (Table 2).

This demonstrates that the plant manages to make available the N absorbed as GLN and can incorporate it into protein molecules. The source of N available for the production of biomolecules is important for the growth of

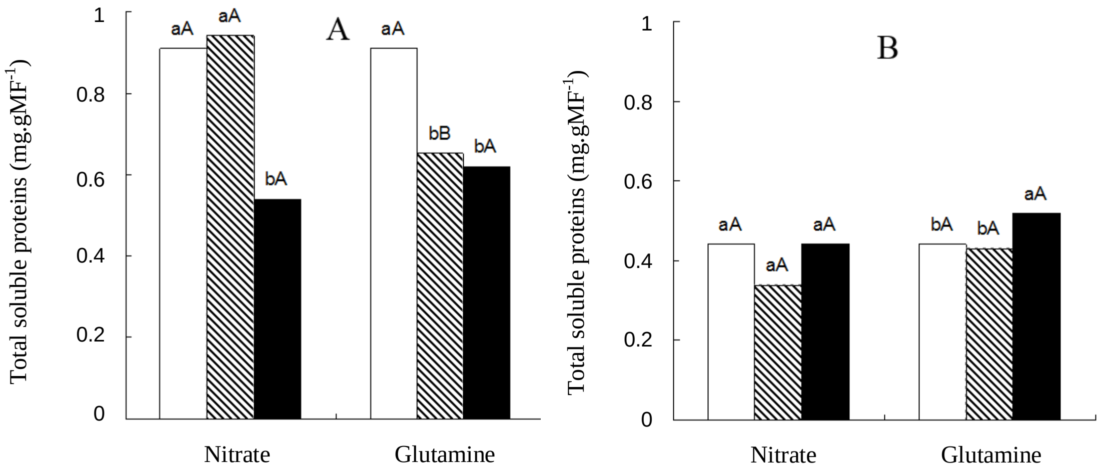

Figure 1. Content of total soluble protein in leaves of corn plants BRS Planalto (A) and BR5202 Pampa (B) grown with 0 mM (□), 0.1 mM (▨) and 10 mM (■) N. Means followed by the same in lowercase letter and uppercase between each source and the sources used do not differ by Tukey test at 5% probability.

Table 4. Ratio of dry matter between shoot and root of corn plants cv. BRS Planalto and cv. BR 5202 Pampa according to different sources and levels of nitrogen.

Treatment	BRS Planalto	BR5202 Pampa
Control	0.70^b	0.71^b
NO_3^- 0.1 mM	0.81^b	0.88^b
NO_3^- 10 mM	1.22^a	1.33^a
GLN 0.1 mM	0.76^b	0.81^b
GLN 10 mM	0.91^b	0.75^b

Means followed by the same letter are not different at 5% probability by Tukey test.

plants and can be distinguished by patterns in the metabolism of nitrogen and carbon (Britto and Kronzucker, 2002).

The cv. BR5202 Pampa had significant differences in the relationship between carbohydrates and proteins of leaves at 0.1 mM nitrate and GLN relative to the control and 10 mM. Meanwhile, in the roots of cv. BR5202 Pampa, there was no difference between the control and 0.1 mM nitrate. While the ratio of the total was similar, between the control and 0.1 mM of nitrate and GLN, there was a decrease in the concentration of leaves and roots.

The relationship between the shoot dry matter aerial part (DMA) and the root dry matter (DMR) was similar for both cultivars presenting a significant difference only for 10 mM nitrate and GLN (Table 4). This demonstrates that there were differences between the biomass partitioning in leaves and roots when different sources and doses of N were used.

The soluble protein contents of the leaves did not demonstrate a direct correlation with increased levels of N in the cv. BRS Planalto being that the increase in the nitrate availability had as a response decrease in soluble protein content probably due to the effect of high concentrations of nitrate (Figure 1).

Concerning the source of N in form of GLN, there was no significant difference between the levels of 0.1 and 10 mM. This may have occurred to treat yourself to a source organic N, that is produced endogenously. Comparing the two nitrogen sources, there were differences between only at 0.1 mM nitrate compared to the same level of GLN in the cv. BRS Planalto (Figure 1). Meanwhile, in the cv. BR5202 Pampa, the same behaviour was not observed since the dose of 0.1 mM nitrate did not differ from GLN (Figure 1). For cv. BR5202 Pampa, there was no difference in the treatments with nitrate and a significant difference was observed only for 10 mM GLN compared to 0.1mM and the control (Figure 1).

In the roots of cv. BRS Planalto, we observed higher protein content at 0.1 mM nitrate in relation to 10 mM. With GLN, there were no significant differences between treatments (Figure 2). The cultivar cv. BR5202 Pampa presented a higher content of protein with increased doses of nitrate or GLN. Shankar and Srivastava (1998)

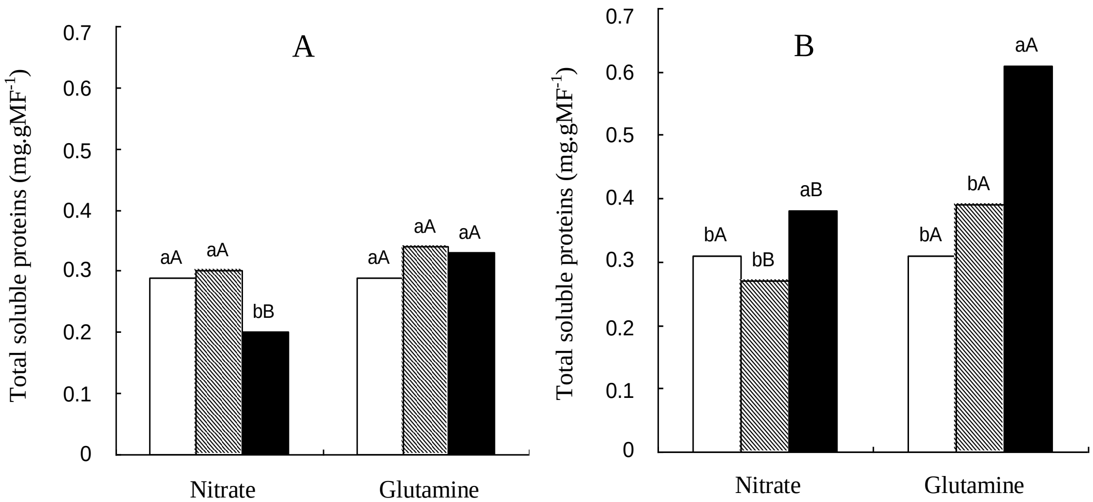

Figure 2. Content of total soluble proteins in roots of corn plants BRS Planalto (A) and BRS5202 Pampa (B) grown with 0 mM (□), 0.1 mM (▨) and 10 mM (■) N. Means followed by the same letter are not different at 5% probability by Tukey test.

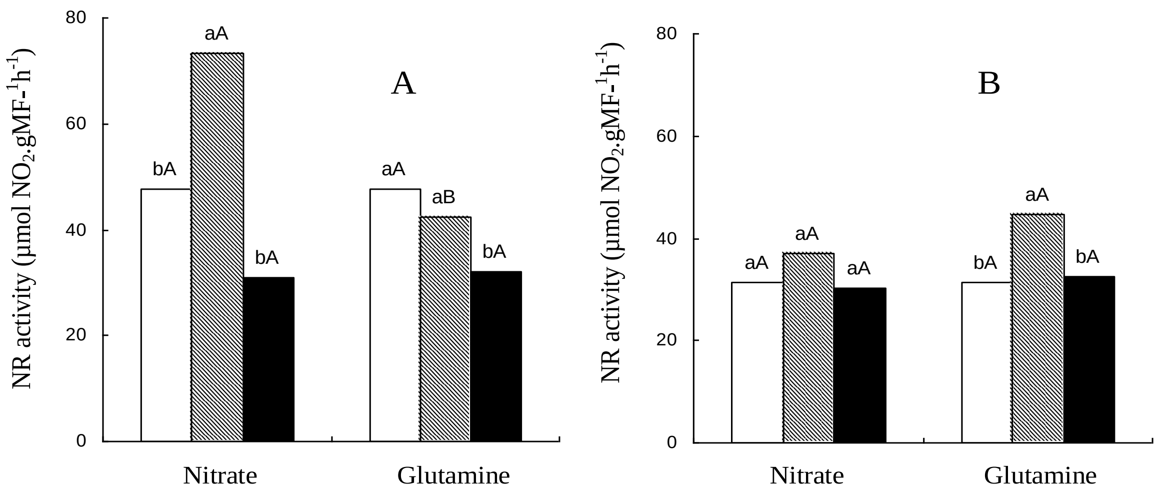

Figure 3. Activity of nitrate reductase (NR) in leaves of corn plants BRS Planalto (A) and BRS5202 Pampa (B) grown with 0 mM (□), 0.1 mM (▨) and 10 mM (■) N. Means followed by the same letter are not different at 5% probability by Tukey test.

obtained an increment in the protein content in roots of corn seedlings subjected to Hoagland solution with 5 mM nitrate and after 1 and 5 mM of GLN.

The increase in protein content most likely occurs due to the incorporation of N of GLN in the other amino acids and eventually in proteins because in the roots, high concentrations of amides cause toxic effects in many processes related to protein metabolism.

In the comparison between cultivars, the two sources were similar for treatments with 0.1 mM compared with 10 mM, where plants cultivated with GLN accumulated higher contents of soluble protein in the roots. In the leaves of cv. BRS Planalto, NR activity was decreased with increased doses of both sources (Figure 3). The cv. BR5202 Pampa also presented a decrease in activity of NR with increased dose of either source. These data are confirmed by the work of Oliveira et al. (2013) who tested the efficiency and use of N and enzyme activity in maize genotypes where it was concluded that the higher the concentration of NR, the lower the efficiency of N and its compounds in the plant.

The behaviours were similar among cultivars with the exception of genotype BRS Planalto that presented the highest NR activity in relation to BR5202 Pampa in the

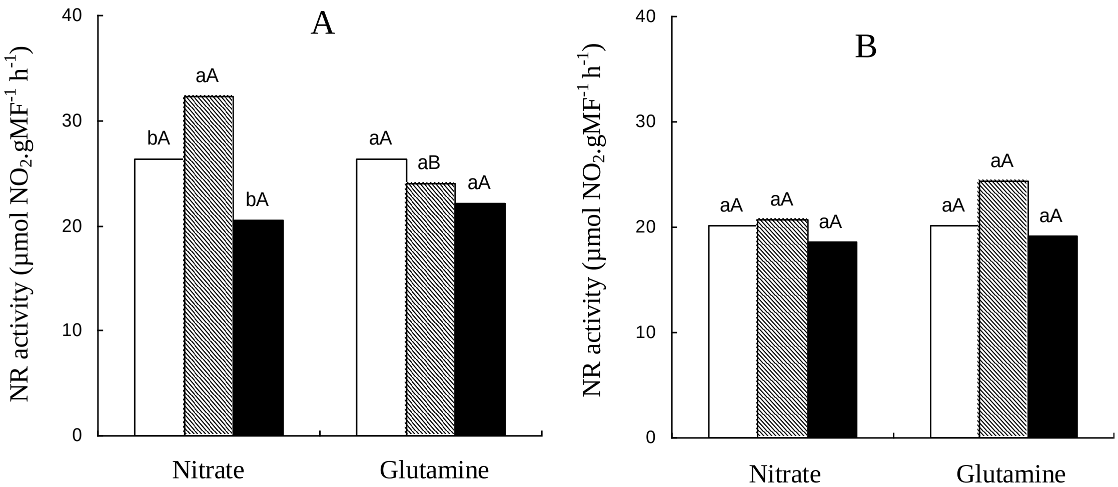

Figure 4. Activity of nitrate reductase (NR) in roots of corn plants BRS Planalto (A) and BRS5202 Pampa (B) grown with 0 mM (□), 0.1 mM (▨) and 10 mM (■) N. Means followed by the same letter are not different at 5% probability by Tukey test.

treatment with 0.1 mM nitrate. The NR activity differed between blocks and N levels but did not show differences between maize genotypes utilized (Majerowicz et al., 2002). The NR activity was similar at 1.6 mM or 16 mM of N among 14 maize genotypes (Purcino et al., 1998). According to Miflin and Habash (2002), the action of glutamine and its enzyme is a key point for the growth and productivity of plants. For the cv. BRS Planalto, there was a decline in the foliar activity of NR when the level of nitrate increased from 0.1 to 10 mM (Figure 4).

Despite that there were no significant differences; there was even a turn slightly to decrease the enzyme activity considering the GLN as a source of N both in the leaves as in roots (Figure 4). Silva et al. (2011), studying the effects of NR activity under fertilization of N and K reported that the higher the concentration of N, the smaller the NR enzyme activity. Shankar and Srivastava (1998) obtained a decline in the activity of NR with the increased concentrations of GLN in the roots of maize seedlings subjected to Hoagland solution with 5 mM KNO_3 and thereafter to 1, 5, 10 and 20 mM of GLN. In contrast, Aslam et al. (2001) showed an increase in the NR activity in response to increased concentrations of GLN from 0.1 for 10 mM in barley plants.

The cv. BR5202 Pampa had a greater activity of NR in the roots compared with the BRS Planalto at the dose 0.1 mM nitrate. Normally, the enzyme NR does not present a high activity when evaluated in conditions of low levels of N, that is, N ≤ 300 kg/ha (Machado et al., 1992). In general, a decline of NR activity occurred in both genotypes in response to increased doses of N.

Purcino et al. (1998) evaluating seven of the cultivars utilized in his experiment presented a decline of NR activity with an increase of 1.6 to 16 mM nitrate. However, Majerowicz et al. (2002) obtained increased from 3.8 to 12.7 ± mol NO_2^- g^{-1} MF h^{-1} in the NR activity when the dose increased from 1 to 15 mM of N. The NR activity was always higher in the leaves in relation to the roots in both cultivars.

According to Shankar and Srivastava (1998), an increase in the protein content in corn seedlings (*Z. mays* L.) occurs after the addition of glutamine. However, Li et al. (1995) claimed that corn sprouts (*Z. mays* L.) subjected to glutamine demonstrated little or no effect on the activity of the NR.

The enzymes naturally presents a linear initial response as a function of substrate availability, then present a saturation to reach their maximum capacity of converting the substrate into their respective products, so it is of no use to increase the concentration of substrate when the enzyme has reached its maximum capacity of metabolism. In the specific case of NR, the form of nitrogen available in the middle is one of the factors that influence its activity. The nitrate is an inducer of enzyme activity, once an enzyme induced by substrate and not constitutive of the middle. However, in higher concentrations and depending on the age of the plant, this may not be beneficial and can cause a decrease in the activity of the enzyme as nitrite and ammonium chloride. Camargos, (2007) suggested that the action of the substrate and enzyme of NR is one of the greatest substrate-induced enzymes in higher plants.

In general, there was no significant difference between the solutions of N utilized. Likewise, Purcino et al. (1998) observed similarities among 14 corn genotypes in NR activity which was similar at 1.6 or 16 mM N. A large number of genes that work together or individually depending on the carbon and nitrogen available (Scheible et al., 1997) command the efficiency of nitrogen use. For the increased availability of nitrogen, a decrease in protein content can be occurred by excess resulting in accumulation of toxic forms of nitrogen and causing inhibition of enzymes involved in nitrogen metabolism. This may explain the fact of cultivars, possibly have different answers, the availability of nitrogen in the soil and by extension in the own plant. In this way, it is possible to find variation phenotypic and genetic in order to understand the genetic basis of efficiency of the use of nitrogen as a key component to the productivity of corn (Hirel et al., 2001).

Conclusions

The content of photosynthetic pigments showed no differences between the analysed genotypes, only between the doses tested. The soluble protein content was higher in leaves of BRS Planalto. The activity of nitrate reductase was greater at dose than 0.1 mM independent of the nitrogen source and organ involved. The activity of nitrate reductase is higher in leaves compared with roots, and glutamine does not inhibit its activity. Further studies are needed with other dosages and cultivars to clarify the action of these sources for the culture of corn and utilize such sources in practice in the camp.

Conflict of Interest

The authors have not declared any conflict of interest.

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