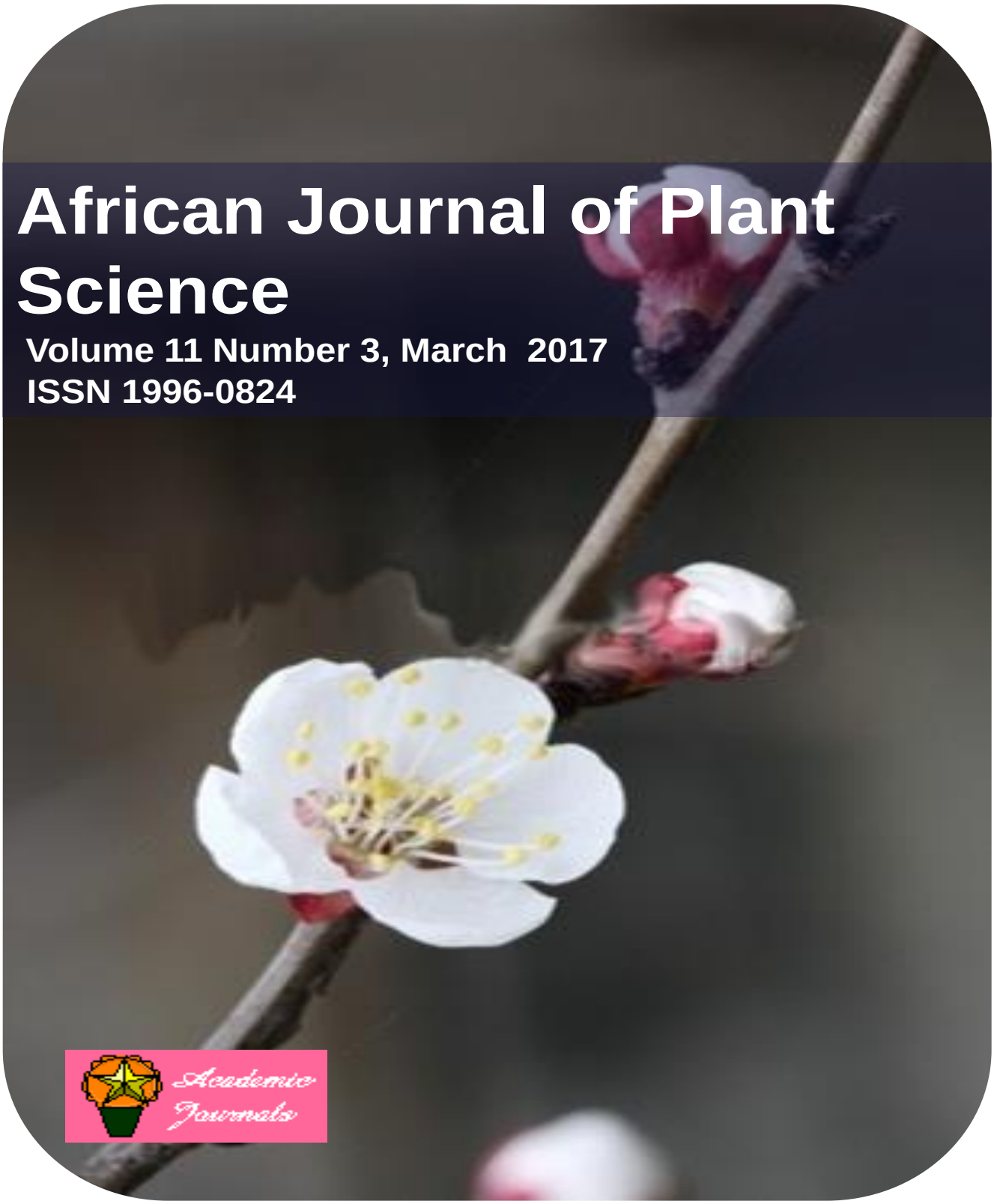

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Full Length Research Paper

Role of endomycorrhizae and *Pseudomonas fluorescens* on the acclimatization of micropropagated *Stevia rebaudiana* Bert. plantlets

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***Stevia rebaudiana* Bert. is a non-caloric sweetener plant of medicinal and commercial values. This study was performed to micropropagate Stevia and improve its ability to withstand the sudden shocks of the environmental changes during acclimatization by using endomycorrhizae and/or *Pseudomonas fluorescens*. *In vitro* establishment of Stevia was superior by culturing shoot tips and nodal segments of mature plants on Murashige and Skoog medium supplemented with 0.5 mg/l 6-benzyl adenine and 1 mg/l β-naphthalene acetic acid, and nodal segments gave better response when compared to shoot tips. While 6-benzyl adenine alone at 1 mg/l was found to be the most promising concentration for shoots multiplication for four successive subculturing. The best rooting response was obtained on half strength Murashige and Skoog medium supplemented with 1 mg/l β-naphthalene acetic acid. Adding 8 ml/l mycorrhizal root extract gave 100% rooting with the highest mean number and length of roots and shoot height. Inoculation of plantlets with endomycorrhizal spores alone in non-sterilized soil increased the survival percentage to 90% and gave the highest mean number and length of both shoots and roots, as well as leaves number. Moreover, they recorded the highest values of total chlorophyll, NPK and relative water contents.**

Key words: Stevia, micropropagation, mycorrhizae, mycorrhization helper bacteria.

INTRODUCTION

Stevia rebaudiana Bert. (Stevia) belongs to the family Asteraceae. It contains natural sweetening compounds that are non-calorific and 230 times sweeter than sucrose (Kinghorn, 1987). It is useful for hypoglycemia and diabetes, as well as nourishes and healing pancreas. Also, It is suitable as a row material for the production of food components, since it is a good source of carbohydrates, protein, fiber materials as well as

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dispensable and indispensable amino acids (Laribi et al., 2012).

Large-scale cultivation of Stevia is restricted because of the low germination percentage of seeds. On the other hand, propagation through seeds produces heterogeneous plants, resulting in variations in sweetening levels. Propagation by cutting is also limited, because of a lower number of individuals that can be obtained simultaneously from a single plant (Dey et al., 2013). Tissue culture techniques hold great promise for micropropagation, and enhancement the natural levels of *in vitro* Stevia plant (Jitendra et al., 2012). Considerable efforts have been directed to optimize the conditions for micropropagation of Stevia, but the acclimatization process of micropropagated plants remains the major hurdle, as it gave low success reports. Desiccation and wilting are the main causes of low survival. Plantlets develop within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in high level of humidity. These contribute a phenotype that cannot survive the environmental conditions when directly transferred in a greenhouse or field (Manjusha and Sathyanarayana, 2010).

Inoculation of endomycorrhizae to micropropagated plants has proved their effectiveness in the resistance of these plants to transplanting stress and improving the growth and mineral nutrient uptake especially phosphorus (Jaizme-Vega et al., 2002), as well as improving physiological parameters, such as increment in photosynthetic rates and stomatal conductance and protecting plant against biotic and abiotic stresses and disease (Arriagada et al., 2012; Bárzana et al., 2014).

Pseudomonas fluorescens inhabiting the rhizosphere of various plants and release auxins as secondary metabolites (Lee et al., 2004). These auxins induces the formation of additional root hair and/or lateral root (Tien et al., 1979), enhancing the ability of plants to take up nutrients from soil and increasing their yield. *Ps. fluorescens* is one of the most important mycorrhization helper bacteria as they affect the symbiotic establishment of mycorrhizal fungi on plant roots in various ways (Garbaye, 1994). The soil-borne *Ps. fluorescens* has received particular attention, because of their capacity to produce a wide range of enzymes and metabolites (Kapoor et al., 2012).

The present study was conducted to investigate the effect of endomycorrhizae and *Ps. fluorescens* on the survival and growth of micropropagated Stevia, either *in vitro* and *ex vitro*.

MATERIALS AND METHODS

Plant material and surface sterilization

Stevia shoots with terminal buds (10 cm in length) were obtained from a nursery in El-Mansurya, Egypt. After defoliating the shoots, they were cut into 2 cm nodal segments and 0.5-1 cm shoot tips. Surface sterilization of explants were done in 95% ethyl alcohol for 10 seconds, then, explants were immersed in sterile distilled water with two drops of Dettol for 10 min, then immersed into 1.05% sodium hypochlorite solution (NaOCl) for 10 min for shoot tips and 1.58% NaOCl solution for 15 min for nodal segments. After each treatment the explants were rinsed 5-6 times with sterile distilled water.

Culture media and growth conditions

Explants were cultured on Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, Netherlands) (Murashige and Skoog, 1962) with 30 g/l sucrose, 100 mg/l myo-inositol and solidified with 2 g/l phytagel (Duchefa, Haarlem, Netherlands). Various concentrations and combinations of plant growth regulators (PGRs, Sigma Cell Culture, min. 90%, St. Louis, USA) were added to the culture medium according to the growth stage. The pH of the media was adjusted to 5.7±0.1 and autoclaved at 121°C at a pressure of 1.1 kg/cm² for 15 min (Harvey Sterilemax autoclave, Thermo Scientific, USA). Cultures were incubated in a temperature of 26±2°C under a photoperiod of 16 hours with a light intensity of 20 µmol/m^2/s (F140t9d/38, Toshiba), and under relative humidity of 60-65%.

Micropropagation

Establishment stage

Shoot tips and nodal segments of Stevia were cultured on MS medium supplemented with 1 mg/l β–naphthalene acetic acid (NAA), individually, or in combination with 6-benzyl adenine (BA) at 0.5, 1, 1.5 and 2 mg/l, and MS medium without plant growth regulators (PGRs) was used as a control. Percentage of growth induction (%), mean number of axillary shoots/explant and mean length of axillary shoots (cm) were recorded after four weeks from culturing.

Multiplication stage

The *in vitro* established shoots were cultured on MS medium supplemented with BA at 0.5, 1, 1.5 and 2 mg/l, individually, or in combination with 0.5 mg/l kinetin (Kn), and MS medium free from cytokinins was used as control. Shoots were subcultured six times on the best medium every four weeks. Mean number and length (cm) of axillary shoots/explant were recorded for each subculture.

Rooting stage

Elongated shoots were excised and cultured on half strength MS medium supplemented with indole-3-butyric acid (IBA) or NAA at 0.1, 0.5, 1 and 1.5 mg/l, in addition to the control treatment of 1/2 MS medium without auxins. Data were recorded in terms of rooting percentage (%), mean number of roots/explant, mean length of roots (cm) and mean shoot height (cm) after four weeks from culturing.

Preparation of endomycorrhizal inoculant

Endomycorrhizal spores were originally extracted from soil around roots of maize grown in the Experimental Field of Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt, using the wet sieving and decanting technique as described by Gerdemann and Nicolson (1963). The concentration of endomycorrhizal spore suspension was about 20 spores /ml, and

250 spores/plantlet was used as a standard inoculum in the experiment.

Estimation of root infection with endomycorrhizae

Inoculated Stevia roots were collected after two months from infection. The percentage of root infection with endomycorrhizae was estimated by the method described by Phillips and Hayman (1970).

Preparation of mycorrhizal infected root extract (MIRE)

Ten gram from fresh roots of Stevia plants grown in the greenhouse and colonized with endomycorrhizae that exhibited 90% mycorrhizal infection, were excised. Mycorrhizal infected root extract (MIRE) was prepared using the method described by Sharma et al. (2005). The MIRE was sterilized by filtration using 0.2 µm filter.

Preparation of *Pseudomonas* free supernatant (PsFS)

Ps. fluorescens was obtained from Unit of Biofertilizers, Microbiology Department, Faculty of Agriculture, Ain Shams University. Microbial inoculant was maintained on King's medium (King et al., 1954), supplemented with 0.01 g/l tryptophan at 25°C for 5-7 days. Culture containing 10^9 CFU/ml was centrifuged at 5500 rpm for 20 min. The *Ps. fluorescens* free supernatant (PsFS) was sterilized by filtration using 0.2 µm filter.

Estimation of PGRs in MIRE and PsFS

The MIRE and PsFS were examined for the presence of gibberellins (GA_3), zeatin and indole-3-acetic acid (IAA) using high-performance liquid chromatography (HPLC) system (Agilent 1100 series, Agilent Technologies, Germany) according to the method described by Tien et al. (1979).

***In vitro* application of MIRE and PsFS**

Two concentrations of MIRE (4 and 8 ml/l) and PsFS (10 and 15 ml/l) were added to Stevia rooting media of 1/2 MS medium with and without 1 mg/l NAA. Media without MIRE and PsFS were served as control. Rooting percentage (%), number of roots/explant, length (cm) of roots and shoot height (cm) were recorded after four weeks from culturing.

Ex vitro* inoculation of mycorrhizae and *Ps. fluorescens

Endomycorrhizal spore suspension and *Ps. fluorescens* maintained in King's medium supplemented with 0.01 g/l tryptophan containing 10^9 CFU/ml were used for the *ex vitro* inoculation of Stevia plantlets. Rooted plantlets were washed from the medium residues and transplanted *ex vitro* into pots containing sterilized and non-sterilized mixture of sand, peat moss and vermiculite (1:1:1 v/v/v). Plantlets were inoculated with 12.5 ml mycorrhizal spore suspension at a concentration of 20 spores/ml and 5 ml *Ps. fluorescens* at a concentration of 10^9 CFU/ml, either individually or in combination. Pots were covered with transparent polyethylene bags to maintain a high relative humidity around shoots, and placed in a greenhouse. Irrigation took place once a week with ½ MS medium. Relative humidity was reduced by gradual removal of the covers within two months. Cultures without mycorrhizal spore suspension and *Ps. fluorescens* were served as control. The survival percentage of plantlets (%), mean number of shoots/plantlet, length of shoots (cm), number of leaves/plantlet, number of roots/plantlet and length of roots (cm) were recorded after six months from transplanting in the greenhouse.

Biochemical analysis of the *in vitro* produced plantlets

Some biochemical analyses were carried out on the greenhouse acclimatized plantlets after six months.

Total chlorophyll

Chlorophyll content was measured in the leaves of acclimatized plantlets using Minolta chlorophyll meter SPAD-502 that expressed as SPAD.

Nitrogen, phosphorus and potassium (NPK) content

Six months old acclimatized plantlets were oven dried at 70ºC until constant weight was obtained. Dried materials were ground to a fine powder. A constant weight (0.2 g) of oven dried plant samples were digested with concentrated sulfuric acid (98%); as well few drops of H_2O_2 were added. The mixture was placed on a hot plate until being colourless. The aliquot was completed to 50 ml volume with double distilled water (Murphy and Riley, 1962). Nitrogen content (%) was determined according to Bradford (1976) and expressed as mg/g dry weight. Phosphorus and potassium content was determined according to the method described by Murphy and Riley (1962) and expressed as mg/g dry weight.

Moisture content (%)

The moisture content (%) was determined as the difference between dry weight (DW) and fresh weight (FW) and calculated as [(FW - DW)/FW] × 100.

Statistical analysis

Experiments were subjected to completely randomized design. Variance analysis ANOVA was done using Costat software program. Treatments contained at least 10 replicates. The differences among means of treatments were tested for their significance at 5% level by using Duncan (1955) and multiple range tests as modified by Snedecor and Cochran (1990).

RESULTS AND DISCUSSION

Micropropagtion

Establishment stage

Data recorded in Table 1 represent the effect of different concentrations of BA in addition to NAA at 1 mg/l on the *in vitro* establishment of Stevia from shoot tips and nodal segments. The percentage of growth induction reached 100% on all tested treatments for both explants. Shoot tips on MS medium with BA at 0.5 mg/l in combination with 1 mg/l NAA gave the highest significant mean

Table 1. *In vitro* establishment of shoot tip and nodal segment of Stevia on MS medium supplemented 1.0 mg/l NAA and different concentrations of BA. Growth induction reached 100% on all tested treatments.

Treatments		Shoot tip		Nodal segment	
		Mean number of axillary shoots/ explant	Mean length of axillary shoot (cm)	Mean number of axillary shoots/ explant	Mean length of axillary shoot (cm)
Control		4.7^{bc}	1.30^{a}	4.4^{c}	0.66^{c}
BA mg/l	0.0	4.6^{bc}	1.03^{b}	6.0^{bc}	1.03^{b}
	0.5	7.2^{a}	1.10^{b}	14.1^{a}	1.54^{a}
	1.0	5.1^{b}	0.70^{c}	7.8^{b}	0.89^{b}
	1.5	5.2^{b}	0.69^{c}	6.5^{bc}	0.87^{b}
	2.0	3.7^{c}	0.54^{d}	5.8^{bc}	1.50^{a}

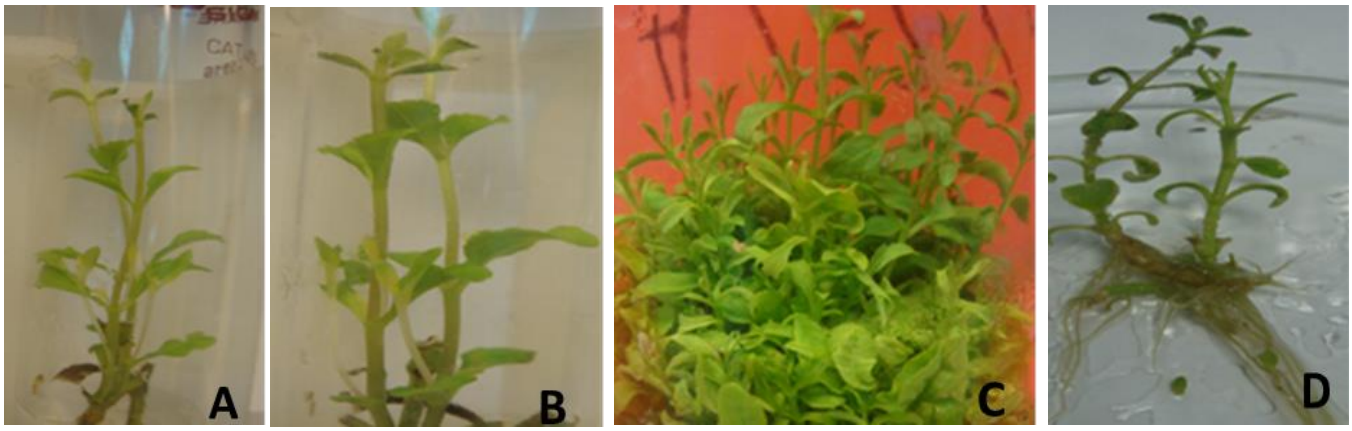

Figure 1. Micropropagation of Stevia. A) Establishment of the shoot tips of Stevia on MS medium supplemented with 0.5 mg/L BA + 1 mg/L NAA B) Establishment of nodal segments of Stevia on MS medium supplemented with 0.5 mg/L BA + 1 mg/L NAA C) Multiplication of Stevia axillary shoots on MS medium supplemented with 1 mg/L BA D) Rooting of Stevia on ½ MS medium supplemented with 1 mg/L NAA.

number of axillary shoots per explant, being 7.2 (Figure 1A), while the highest significant mean length of axillary shoots was recorded within control explant without PGRs, which gave 1.3 cm. This could be contributes to the absence of cytokinin supplementation that caused reduction in the number of axillary shoots and the increase in length as compared to the treatments with BA. Increasing BA concentrations significantly decreased the mean number and length of axillary shoots. The concentration of 2 mg/l BA and 1 mg/l NAA gave the lowest significant value for mean number and length of axillary shoots, which reached 3.7 and 0.54 cm, respectively.

Concerning the *in vitro* establishment of axillary shoots from nodal segments, the results in Table 1 show that MS medium supplemented with 0.5 mg/l BA and 1 mg/l NAA gave the highest significant mean number and length of axillary shoots, being 14.1 and 1.54 cm, respectively (Figure 1B). While MS control medium (free from PGRs) gave the lowest significant mean number and length of axillary shoots, being 4.4 and 0.66 cm, respectively.

These results are supported by Pratibha et al. (2010), who found that the nodal explants of Stevia cultured on 0.5 mg/l BA and 1 mg/l NAA recorded the best shoot establishment and proliferation. Singh and Singh (2005) recorded that cytokinins and auxins stimulate cell division even in non-meristematic tissues, as well as, parenchyma cell. Furthermore, the ratio of cytokinins to auxins controls cell differentiation. When the ratio is in the favor of cytokinins, shoot formation takes place, while for root formation will be in favor of auxins.

The length of axillary shoots has the priority in the establishment stage as the aim of this stage is to obtain a clean growing culture for further stages. Therefore, by comparing the two different explant types of Stevia used in the present study, it could be concluded that the nodal segments gave better response, since it gave higher mean number and length of axillary shoots, when compared to shoot tips.

Multiplication stage

Data recorded in Table 2 show the multiplication of the *in*

Table 2. *In vitro* multiplication of Stevia shoots on MS medium supplemented with BA and Kn.

Cytokinins conc. (mg/l)		Mean number of axillary shoots/ explant	Mean length of axillary shoots (cm)
Kn	BA		
0.0	0.0	15.2^{d}	2.16^{a}
	0.5	17.6^{abc}	1.76^{bc}
	1.0	19.2^{a}	1.54^{de}
	1.5	16.4^{bcd}	1.73^{bc}
	2.0	12.0^{e}	1.75^{bc}
0.5	0.5	15.4^{cd}	1.40^{e}
	1.0	17.8^{ab}	1.86^{b}
	1.5	18.1^{ab}	1.44^{e}
	2.0	15.3^{cd}	1.65^{cd}

Table 3. *In vitro* multiplication of Stevia during six successive subcultures on MS medium containing the best cytokinins for multiple shoots production.

Subculture no.	1 mg/l BA		1.5 mg/l BA + 0.5 mg/l Kn	
	Mean no. of axillary shoots/explant	Mean length of axillary shoots (cm)	Mean no. of axillary shoots/explant	Mean length of axillary shoots (cm)
1st	16.0^{b}	1.41^{ab}	14.0^{b}	1.14^{b}
2nd	18.4^{ab}	1.45^{ab}	17.1^{ab}	1.25^{ab}
3rd	19.5^{a}	1.44^{ab}	18.3^{a}	1.15^{b}
4th	20.7^{a}	0.41^{b}	18.0^{ab}	1.14^{b}
5th	10.9^{c}	1.45^{ab}	8.2^{c}	1.25^{ab}
6th	8.1^{d}	1.54^{a}	7.4^{d}	1.45^{a}

vitro established Stevia shoots using BA, individually, or in combination with Kn. The highest significant mean number of axillary shoots/explant was recorded with 1 mg/l BA alone, being 19.2 (Figure 1C). This result is supported by Ali et al. (2010) who mentioned that MS medium with 1 mg/l BA give maximum shoot multiplication response in Stevia. While, MS medium supplemented with 1.5 mg/l BA and 0.5 mg/l Kn gave 18.1 as mean number of shoots per explant and 1.44 cm as shoot length, which could be considered significantly for production of both. While MS medium without cytokinins gave the significantly highest mean length of axillary shoots, being 2.16 cm, but with lower mean number of axillary shoots per explant. Shoot multiplication increased with increase in concentration of BA and Kn, except for the highest concentration, which could be supra-optimum for the shoots to multiply. Cytokinins have been considered to be one of the most crucial components of plant tissue culture protocol, as optimum culture proliferation and shoot growth is impossible in their absence (Hamide and Mustafa, 2004) and treatments, which stimulated multiple shoot formation hindered shoot elongation. Shoot length is found to be the highest in medium supplemented with no or low dose of cytokinin (Waman and Bohra, 2016).

The six successive subculturing of Stevia shoots were attained on MS medium either supplemented with 1 mg/l BA only or with 1.5 mg/l BA, in combination with 0.5 mg/l Kn (Table 3). In both media the mean number of axillary shoots increased till the 4th subculture, then decreased in the 5th and 6th subcultures. MS medium supplemented with 1 mg/l BA alone found to be the most promising concentration for shoot multiplication of Stevia for four successive subcultures, as it gave 20.7 as mean number of axillary shoots per explant. In this respect, Prakash et al. (2006) successfully used this technique to increase the number of shoot buds in *Ptertocarpus santalinus*. They observed increase in shoot bud multiplication rate up to the 6th subculture stage.

Rooting stage

Rooting of Stevia was studied on ½ MS medium supplemented with different concentrations of IBA and NAA, as represented in Table 4. Data revealed that all the used concentrations gave 100% rooting. However, the concentration of 1 mg/l NAA gave the highest significant mean number (21.5) and length (3.55 cm) of roots with a mean shoot height of 4.8 cm (Figure 1D). It

Table 4. Effect of ½ MS medium containing different concentrations of IBA or NAA on the rooting of Stevia axillary shoots. Rooting percentage reached 100% on all tested treatments.

Auxins conc. (mg/l)		Mean number of roots/ explant	Mean length of roots (cm)	Mean shoot height (cm)
IBA	**NAA**			
0.0		14.6^{bc}	1.5^{cd}	2.4^{h}
0.1		8.1^{d}	0.816^{ef}	2.4^{h}
0.5	**0.0**	13.1^{bc}	1.634^{c}	3.45^{f}
1.0		14.3^{bc}	1.69^{c}	4.1^{d}
1.5		12.7^{bc}	1.143^{de}	5.1^{b}
	0.1	10.1^{cd}	0.685^{f}	2.95^{g}
0.0	**0.5**	15.4^{b}	1.29^{cd}	3.85^{e}
	1.0	21.5^{a}	3.55^{a}	4.8^{c}
	1.5	16.8^{b}	2.25^{b}	5.4^{a}

was also noticed that by increasing the concentration of auxins (either IBA or NAA) the mean number and length of roots increased until the concentration of 1 mg/l. While the mean shoot height significantly increased by the increase of auxin concentration until 1.5 mg/l. These results are in harmony with that obtained by Ali et al. (2010) who found that the best *in vitro* rooting response of Stevia could be obtained on MS medium containing 1 mg/l NAA either from apical or nodal meristem, being 96%.

Roots are mostly induced in the presence of an auxin. IAA, IBA and NAA promoted rhizogenesis in general. However, their response differed according to the plant species and the physiological state of the explant. NAA showed the most positive effect on roots number and length among the different auxins used in this study. Such an effect of NAA was also observed in numerous studies, which have indicated that, among the common auxins, NAA is the most effective auxin for induction of root regeneration (Chae et al., 2016).

PGRs content in the mycorrhizal infected root extract (MIRE) and *Pseudomonas* free supernatant (PsFS)

The PGRs content detected in the form of GA_3, zeatin and IAA recorded 12.391, 64.711 and 2.435 µg/ml in MIRE and 56.5, 205.8 and 14.02 µg/ml in PsFS, respectively. Results show that the concentrations of GA_3, zeatin and IAA were higher in PsFS than MRE.

Effect of mycorrhizal infected root extract (MIRE) and *Pseudomonas* free supernatant (PsFS) on *in vitro* produced plantlets

This experiment was conducted to evaluate the efficiency of MIRE and PsFS on rhizogenesis of Stevia *in vitro* during rooting stage. All the treatments gave 100% rooting, as well as control. However, as recorded in Table 5, MS medium containing MIRE at 8 ml/l with 1 mg/l NAA gave the significantly highest mean number and length of roots, being 32.3 and 5.13 cm, respectively, as well as the significantly highest mean shoot height of 5.85 cm (Figure 2). Followed by MS medium supplemented with 15 g/l PsFS and 1 mg/l NAA, which gave 26.5 and 4.02 cm as mean number and length of roots, respectively, and 5.3 cm as mean shoot height. This result is similar to that obtained by Sharma et al. (2005), who found that endomycorrhizal root extract of *Morus alba* was equivalent to IBA in promoting plant rhizogenesis, root growth and proliferation. These results could be explained as endomycorrhizal fungi are able to produce several growth promoting substances (Hasan, 2002), which are capable to alter plant internal hormone balance (Scagel and Linderman, 1998).

Also, shoots treated with MIRE gave the best results in all growth parameters when compared to PsFS, which may be due to the presence of GA_3, zeatin and IAA in the PsFS in supra-optimal level for plants, that cause some inhibition in plant growth parameters (Kunkel and Chen, 2006; Zulfiqar et al., 2009). On the other hand, Peyvandi et al. (2010) found that length and numbers of adventitious and lateral roots of olive micro-shoots increased *in vitro* by using the supernatant of *Ps. fluorescens* maintained on King's medium that supplemented with L-tryptophan.

Effect of the endomycorrhizae and *Ps. fluorescens* on the acclimatized Stevia plantlets

Data recorded in Table 6, after six months from culturing Stevia plantlets in the greenhouse, show that inoculation of Stevia plantlets with endomycorrhizal spores and/or *Ps. fluorescens* in non-sterilized soil gave higher significant values on all parameters when compared within same treatments in sterilized soil. In this respect,

Table 5. Effect of MIRE and PsFS on the rooting of Stevia axillary shoots.

Treatments			No. of roots/ explant	Roots length (cm)	Shoot height (cm)
1.0 mg/l NAA	Control		20.5^{f}	3.55^{d}	4.80^{bc}
	MIRE (ml/l)	4.0	25.6^{c}	3.99^{bc}	5.05^{bc}
		8.0	32.3^{a}	5.13^{a}	5.85^{a}
	PsFS (ml/l)	10.0	22.9^{de}	3.81^{c}	4.81^{bc}
		15.0	26.5^{b}	4.02^{b}	5.30^{b}
0.0 mg/l NAA	Control		21.8^{ef}	3.560^{d}	4.64^{c}
	MIRE (ml/l)	4.0	22.6^{e}	3.63^{cd}	4.85^{bc}
		8.0	23.7^{d}	3.90^{bc}	4.84^{bc}
	PsFS (ml/l)	10.0	21.8^{ef}	3.56^{d}	4.64^{c}
		15.0	21.9^{ef}	3.63^{cd}	4.85^{bc}

Figure 2. *In vitro* rooting of Stevia on ½ MS medium supplemented with **(A)** 1 mg/l NAA only and **(B)** 1 mg/l NAA in addition to 8 mg/l MIRE.

Smith and Read (2008) showed that plantlets cultivated in non-sterile soils could develop endomycorrhizae and grew better than plantlets cultivated in sterilized soils, as they mentioned that sterilization by heat may be responsible for the production of toxic compounds, which are harmful for plant development. Also, Martins (2008) mentioned that endomycorrhizae formed in non-sterile soil are responsible for the increased performances of the plants, which may be explained by the presence of some soil microbiota that enhance the germination of endomycorrhizal spores.

As shown in Table 6, inoculation of non-sterilized soil with endomycorrhizal spores alone recorded the significantly highest values of survival percentage (90%), mean number and length of shoots (5.8 and 38.95 cm), mean number of leaves (34), mean number and length of roots (35 and 21.83 cm), total chlorophyll (33.83 SPAD), NPK (1.16%, 0.72 and 43.02 mg/g dry weight) and relative water content (84.43%), when compared to all tested treatments (Figure 3). Followed by those inoculated by endomycorrhizal spores in combination with *Ps. fluorescens* in non-sterilized soil that gave 90% survival percentage, 4.6 and 30.50 cm as mean number and length of shoots, respectively, 28.2 as mean number of leaves, 31.30 and 18.30 cm as mean number and length of roots, respectively, 29.813 SPAD as relative chlorophyll content, 0.98%, 0.61 and 41.40 mg/g dry weight as NPK content, respectively, and 78.76% as relative water content. These results are in agreement with those reported by Estrada-Luna et al. (2000), who showed that mycorrhizal Guava plantlets had greater shoot length, leaf area, leaf, stem, root dry mass, mineral levels of P, Mg, Cu, and Mo. While, Earanna (2007) mentioned that inoculation with arbuscular mycorrhizae and plant growth promoting rhizobacteria improve the growth and biomass of several plants by supplementing plant with nutrients and producing growth hormones.

Arbuscular mycorrhizal fungi can also benefit plants by stimulating the production of growth regulating substances, increasing photosynthesis, improving osmotic adjustment under drought and salinity stresses as well as enhance root enzymes activity, shoot nutrient and pigments content (chlorophyll and carotenoid), and water use efficiency (Wang et al., 2008; Manaf and Zayed, 2015). Also, Estrada-Luna and Davies (2003) mentioned that arbuscular mycorrhizal fungi help plantlets to recover rapidly during acclimatization and gave great growth during post-acclimatization. Also, in this respect, Gray and Smith (2005), Glick et al. (2007) and Manaf and Zayed (2015) mentioned that *Ps. fluorescens* have substantial effects on plant growth, particularly under stress conditions, and play an important role in plant physiology by secretion of PGRs (auxins, cytokinins, and gibberellins), which enhance various stages of plant growth or synthesize enzymes that modulate plant growth and development as well as improving nutrients uptake, and enhancement of stress resistance.

As mentioned in the present study, inoculation

Table 6. Effect of mycorrhizal spores and *Ps. fluorescens* on acclimatization and total chlorophyll, NPK and relative water contents in Stevia plantlets acclimatized in sterilized and non-sterilized soil, after 6 months of culture.

Treatments		Survival %	No. of shoots/ explant	Length of shoots (cm)	No. of leaves/ explant	No. of roots/ explant	Length of roots (cm)	Total chlorophyll content (SPAD)	N content (%)	P content (mg/g)	K content (mg/g)	Relative water content (%)
Non-sterilized soil	Control	20^{c}	0.3^{d}	3.00^{e}	3.8^{e}	13.67^{e}	9.83^{de}	22.090^{b}	0.78^{g}	0.41^{c}	24.18^{g}	50.89^{g}
	Endomycorrhizal spores	90^{a}	5.8^{a}	38.95^{a}	34.0^{a}	35.00^{a}	21.83^{a}	33.830^{a}	1.16^{a}	0.72^{a}	43.02^{a}	84.43^{a}
	Ps. fluorescens	50^{abc}	1.7^{cd}	13.85^{cde}	14.2^{cde}	20.70^{d}	13.17^{c}	26.850^{ab}	0.86^{e}	0.47^{bc}	29.55^{e}	61.83^{e}
	Endomycorrhizal spores + *Ps. fluorescens*	90^{a}	4.6^{ab}	30.50^{ab}	28.2^{ab}	31.30^{b}	18.30^{b}	29.813^{ab}	0.98^{b}	0.61^{ab}	41.40^{b}	78.76^{b}
Sterilized soil	Control	10^{c}	0.2^{d}	1.50^{e}	2.2^{e}	11.70^{e}	7.67^{e}	21.088^{b}	0.72^{h}	0.40^{c}	22.20^{h}	45.52^{h}
	Endomycorrhizal spores	70^{a}	4.4^{ab}	25.00^{bc}	22.6^{abc}	27.67^{c}	17.50^{b}	28.413^{ab}	0.95^{c}	0.60^{ab}	39.12^{c}	75.47^{c}
	Ps. fluorescens	30^{bc}	1.0^{d}	8.55^{de}	6.8^{de}	17.67^{d}	10.83^{d}	24.713^{b}	0.84^{f}	0.43^{bc}	25.95^{f}	58.93^{f}
	Endomycorrhizal spores + *Ps. fluorescens*	70^{ab}	3.1^{bc}	21.95^{bcd}	18.0^{bcd}	20.67^{d}	16.30^{b}	27.813^{ab}	0.91^{d}	0.53^{b}	38.20^{d}	70.86^{d}

Figure 3. Stevia plantlets, in the greenhouse, **(A)** inoculated with mycorrhizal spores in non-sterilized soil. **(B)** inoculated with mycorrhizal spores + *Ps. fluorescent* in non-sterilized, and **(C)** in soil without biofertilizers (control treatment).

of Stevia with mycorrhizal fungi alone gave the best results than inoculation with mycorrhizal fungi in combination with *Ps. fluorescent*, which was discussed by Bisht et al. (2009), who found that the mycorrhizal infection was lower when the plant growth promoting rhizobacteria was applied in combination with arbuscular mycorrhizal fungi as well as lower photosynthetic and transpiration rates. Also, Ravanskov et al. (1999) reported the

negative effect of mycorrhizae (*Glomus intraradices*) on the population size of *Ps. fluorescent* in rhizosphere.

Conclusion

The present study clearly demonstrated a successful and efficient micropropagation protocol for Stevia plant and solved the main difficulty that limits the commercial production of the plant by presenting the benefits of using arbuscular mycorrhizal fungi for enhancing the growth and survival of micropropagated Stevia plantlets. Mycorrhization can be combined by micropropagation to provide a sustainable tool to facilitate difficult propagating plants adaptation to *ex vitro* conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

BA, 6-benzyl adenine; **GA_3**, gibberellic acid, **HPLC,** high-performance liquid chromatography; **IBA,** indole-3-butyric acid; Kn, kinetin; **MIRE,** mycorrhizal infected root extract; **MS,** Murashige and Skoog; **NAA,** β-naphthalene acetic acid; **NaOCl,** sodium hypochlorite solution; **PGRs,** plant growth regulators; **PsFS,** *Ps. fluorescens* free supernatant.

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Full Length Research Paper

Participatory varietal selection of intermediate altitude sorghum (*Sorghum bicolor* (L.) Moench) genotypes in Western Part of Ethiopia

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Seven sorghum genotypes obtained from Melkassa Agricultural Research Center and local check collected from Assosa district were evaluated in randomized complete block design with three replications under farmers' participatory selection scheme in western part of Ethiopia during 2013 main cropping season. The objectives of this experiment were to select superior intermediate sorghum varieties in the study area and to identify farmers' preference and selection criteria. Farmers' set; grain yield, early maturity and bird damage resistance as selection criteria at maturity stage of the crop. The results of analysis of variance indicated highly significant differences among genotypes for all traits tested at 1% probability level. The highest mean grain yield was obtained from the genotype Adukara (4017 Kg ha^{-1}) whereas the lowest from the variety Geremew (1050 Kg ha^{-1}). Likewise, local-check (8667 Kg ha^{-1}) and Adukara (7651 Kg ha^{-1}) had given comparatively the highest above ground biomass yield which will be used as a good source of feed for animals in the study area. The maximum percentage of bird damage was recorded from early matured genotypes Geremew (33.34%) and IS-9302 (31.67%) whereas the least obtained from late matured genotypes Adukara (5%), Baji (8%) and local-check (10%). Farmers' evaluation of direct matrix ranking showed Adukara (score 27) and RAYA (score 25) were the most preferred genotypes and IS-9302 (18) the least one. Likewise, pair-wise ranking revealed that Adukara and RAYA were chosen equally, six times by farmers to be the most important ones. Therefore, the genotypes Adukara and RAYA were chosen for their performance in the experimental field and also acceptable from farmers' participatory evaluation. Thus, based on the results of this study and previous works done on sorghum in Assosa Agricultural Research Center the selected varieties need to be multiplied and distributed to farmers.

Key words: Bird damage, direct-matrix, genotype, pair-wise ranking, participatory evaluation, selection scheme.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important cereal crop grown in arid and semi-arid parts of the world. It is the fifth leading cereal grain worldwide after wheat, rice, maize and barley with average productivity of 1.4 tons ha^{-1} and area coverage of about 40 million hectare and total production of 61.1 million ton (FAO, 2008). It is a staple food for millions of the poorest and most food insecure people in the semi-arid tropics (SAT) of Africa and Asia. It is one among the few resilient crops that can adapt well to future climate

change conditions, particularly the increasing drought, soil salinity and high temperatures. Over 60% of the total area devoted to sorghum in the world is in developing countries. According to FAO (2008), the area under sorghum production in Africa is about 24.23 million ha and total production and average yield being 21.9 million tons and 0.9 ton ha^{-1}, respectively.

Ethiopia is third largest sorghum producer in Africa next to Nigeria and Sudan (FAO, 2008), where the crop is one of the major food cereals like: Tef, wheat, maize and barley (CSA, 2013). It ranks third in area cultivated and in total production among cereals. It is grown in 12 of the 18 major agro-ecological zones including the lowland, mid and high altitude areas of the country (MoARD, 1998), which covers a wide range of ecological habitats, in the range of 400 to 3000 m.a.s.l. (Teshome et al., 1997). The current sorghum production in Ethiopia is estimated to be 3,604,262 tons on an area of 1,711,485 ha of land giving the national average grain yield of 2.11 tons per hectare (CSA, 2013).

In Benishangul-Gumuz Region cereals covers over 77% of grain crops cultivated land mainly sorghum, millet and maize. Out of the area cultivated for grains, sorghum, maize and finger millet took up an area of 27, 20.30 and 12%, and yielding 30, 33 and 8% of the regional grain production, respectively. The area covered, total production and average yield of sorghum was estimated to be 65,933.36 ha, 130, 995 tons and 1.99 tons ha^{-1}, respectively (CSA, 2013). Therefore, it is the most dominant crop grown as staple food in the region. Despite the economic importance of the crop in attaining food security and food self-sufficiency in the study area, very little have been done to change the livelihood of sorghum producing farmers through generation, adaptation and dissemination of sorghum technologies. So the contribution of improved varieties of sorghum is almost negligible mainly due to poor participation of farmers in the selection process, poor intervention of improved agricultural technologies (absence of improved varieties), birds damage to early maturing varieties, diseases (grain mold, head smut, anthracnose) and insect pests (shoot fly and stalk borer) (AsARC, 2011). Thus it is crucial to evaluate the sorghum genotypes in their agro-ecology using the participatory varietal selection (PVS) approach so as to provide choices of varieties to the farmers for increasing production in their diversity of socioeconomic and agro-ecological conditions. PVS also helps to disseminate the adoption of released varieties in larger areas; allow varietal selection in targeted areas at cost-effectiveness and also in less time and as a consequence help seed production and scaling-up at community level.

The approach of participatory varietal selection in Ethiopia has been done on many crops including common bean (Firew, 1997; Assefa et al., 2005; Asrat and Fitsum, 2008; Mekonen et al., 2010), sorghum (Eshetu and Ketema, 2001), maize (Eshetu and Habtamu, 2002), tef (Belay et al., 2006), barley (Abay et al., 2008; Yetsedaw et al., 2010) and wheat (Alebachew, 2012). So far the involvement of farmers in decision making process has been observed in the region on some rice varieties on different approach, that is, through Farmers' Research Group (FRG) by Assosa agricultural research center (AsARC) (Personal communication). Otherwise PVS for mid-altitude genotypes of sorghum was not done in the study area. Hence, it was found imperative to evaluate the introduced new sorghum genotypes for their performance and farmers' preferences for achieving good quality, high yielding and farmers' preferred varieties thereby enhancing sorghum production and productivity in the region.

Therefore, this study was conducted to evaluate and select the best performing mid-altitude sorghum genotypes and to identify farmers' preference and selection criteria for sorghum varieties in the study site.

MATERIALS AND METHODS

Description of the study area

The study was conducted in western part of Ethiopia at Assosa district, which is located at an attitude of 1547 m.a.s.l.. The area is situated in the East of Assosa town and West of Addis Ababa at about 4 and 660 km distance, respectively.

Assosa district has uni-modal rainfall pattern, which starts at the end of April and extends to mid-November, with maximum rainfall received during June to October. The total annual average (2000-2012) rainfall of Assosa is 1276.22 mm. The mean annual air temperature is 23°C from 2000 to 2012. The major soil types found in Assosa area is Nitisols.

Experimental design and procedures

A total of eight sorghum genotypes (Birmashi, Baji, Geremew, IS-9302, BARC-acc-18, RAYA and Adukara) including a local check collected from Assosa district were used for this study. The experiments were laid in randomized complete block design (RCBD) with three replications from June to December 2013. During planting, the seeds were manually drilled at a rate of 8 kg/ha into five meters long four row plot spaced 0.75 m apart. At approximately 21 days after planting the seedlings were thinned to 0.20 m distance between plants. Nitrogen and phosphorus fertilizer were applied in the form of urea (46% N) and Diammonium phosphate (DAP) (18% N and 46% P_2O_5) at the rate of 50 kg ha^{-1} urea and 100 kg ha^{-1} of DAP, respectively. DAP fertilizer was applied at the time of planting (as basal application), whereas urea was applied in the form of split application, half of it together with DAP during planting and the rest as top dressing before heading. Hand weeding was practiced as frequently as needed. Birds were

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Table 1. ANOVA of growth, yield and yield components of intermediate altitude sorghum genotypes tested at Assosa district during 2013 main cropping season.

Sources of variance	Degree of freedom	DTF (no.)	DPM (no.)	PLH (cm)	HWPPt (g)	NKPH (numbers)	NHPPL (numbers)	TKW (g)	GY(kg/ha)	AGBM (kg/ha)	HI (%)	BIRD Dam (%)
Rep	2	18.48^{ns}	53.59^{ns}	356^{ns}	193^{ns}	109312^{ns}	179^{ns}	1.59^{ns}	393867^{ns}	253867.8^{ns}	36.91^{ns}	25.00^{ns}
Tret	7	1527**	1068**	15668**	4968**	970203**	356**	39.93**	3215856**	19527592**	164.32**	295.83**
Erorr	14	19.77	21.34	632.32	92.63	213245.30	68.23	1.59	12818287	382340.6	24.84	12.50**
Mean		105.26	157.74	191.19	76.91	1990.47	45.26	22.81	2425.9	5852.47	42.78	17.20
C.V. (%)		4.22	2.93	13.15	12.51	23.19	18.25	5.53	14.76	10.57	11.65	20.53

**Highly significant at 1% probability level ; ns, non-significant at 5% probability leve. DTF, Days to 50% flowering; DPM, Days to physiological maturity; PLH, Plant height; HWPPt, Head weight per plant; NKPH, Number of kernels per head and NHPPL, Number of heads per Plot; TKW , Thousand kernel weight; GY , Grain yield; AGBM , Above ground biomass; HI, Harvest index; BIRD Dam (%), Bird damage estimation in %.

found to be the most important biotic stresses that occurred in the study site. Even though it was very difficult to manage and monitor bird injury, it was tried to be protected by manual guards and data were recorded by estimating bird damage (%).

Data collection

Data were collected on plant and plot basis for different agronomic traits. For data collection on plant basis, ten plants were randomly taken from the two middle rows of each plot excluding the two rows on both sides of each plotas borders and the mean value of those ten plants was calculated and used as plot data for analysis. Plant height (cm), head weight per plant (g), number of kernels per head, thousand kernel weight (g) and percentage of bird damage were recorded on plant basis; whereas days to 50% flowering, days to physiological maturity, number of heads per plot, grain yield (kg ha^{-1}), above ground biomass (kg ha^{-1}) and harvest index (%) were recorded on plot basis.

Participatory varietal selection procedures

Qualitative data on mid-altitude sorghum genotypes including local check were collected using participatory approach of direct matrix and pair wise ranking methods. Farmers' were selected randomly based primarily on their experience for growing sorghum, considering gender and their willingness to participate in the research. At first focus group discussion (FGD) were held to determine challenges and constraints of sorghum production in the study area. Then farmers were allowed to prioritize the challenges and set their demands to combat the aforementioned constraints. Direct matrix ranking gave more detailed evaluations in to the merits and demerits related to the evaluated genotypes. Pair wise ranking was a useful tool whenever it is important to explore and discuss the criteria for decision making between and among alternatives.

The genotypes were evaluated using farmers' selection criteria. A total of twenty farmers of both sexes (male=13, female=7) participated in the study. Farmers were allowed to set their own selection criteria and then both male and female participants prioritized and jointly agreed on preferred characters. All of them were tabulated in a matrix scoring table, and each selection criterion was compared with another in a pair wise fashion. The rank assignments were determined from the number of times each selection criterion was preferred by the group. A direct matrix table was prepared for the mid-altitude sorghum genotypes listed in the row and characteristics preferred by farmers in the column. Scores were given to each variety based on the selection criteria (5 = very good, 4 = good, 3 = average, 2 = poor, and 1 = very poor). During direct matrix ranking farmers have given rating of importance (a relative weight) of a selection criterion ranked from 1 to 3 (3 = very important, 2 = important and 1 = less important) and rating of performance of a variety for each traits of interest (selection criteria) was given based on their level of importance on the basis of common agreement of evaluators'. The score of each variety was multiplied by the relative weight of a given character to get the final result and then added with the results of other characters to determine the total score of a given variety. Scoring and ranking were done on consensus, and differences were resolved by discussion as indicated by de Boef and Thijssen (2006).

Statistical analysis

Microsoft Excel software programmes were used in the calculations of treatment parameters means and summary of tables presented wherever required. The data were analyzed using PROC ANOVA in SAS software version 9.00 with treatment and replications as the class variables and the response variables were the traits on which data were collected. Mean separation was carried out using Duncan's Multiple Range Test (DMRT) at 5% probability level.

RESULTS AND DISCUSSION

Performance evaluation of mid-altitude sorghum genotypes

The results of the analysis of variance (ANOVA) showed highly significant ($P < 0.01$) differences among the mid-altitude sorghum varieties for all characters measured (Table 1). This result confirmed the results of previous studies done by Abdella (1991), Girma (2006) and Mihret (2012). They found that cultivars of grain sorghum were significantly affected by phonological growth as well as yield and yield related parameters.

Table 2. Mean values of growth, yield and yield components of intermediate altitude sorghum varieties at Assosa district.

Varieties	Mean values										
	DTF (no.)	DPM (no.)	PLH (cm)	HWPPt (g)	NKPH (numbers)	NHPPL (numbers)	TKW (g)	GY (kg/ha)	AGBM (kg/ha)	HI (%)	BIRD Dam (%)
IS-9302	92.00^{d}	138.67^{ef}	150.33^{c}	47.63^{de}	1529.30^{cd}	44.33^{abc}	22.00^{cd}	1116.70^{de}	2605.60^{de}	43.13^{bcd}	31.67^{a}
Birmash	86.33^{de}	149.67^{d}	163.00^{c}	50.67^{de}	1797.10^{bcd}	41.33^{bcd}	23.00^{bc}	1983.30^{c}	5633.30^{c}	34.69^{de}	18.33^{b}
Baji	88.00^{de}	144.67 ^{de}	160.83^{c}	42.63^{de}	1920.50^{bcd}	59.67^{a}	23.00^{bc}	2750.00^{b}	6700.00^{bc}	40.87^{cd}	8.33^{de}
BARC-acc-18	125.00^{b}	173.67^{b}	281.33^{a}	117.30^{b}	2462.10^{ab}	52.33^{ab}	19.67^{ef}	2933.30^{b}	7516.70^{ab}	39.27^{de}	16.67^{bc}
Geremew	88.67^{de}	151.00^{c}	131.17^{c}	54.10^{d}	1333.30^{d}	28.00^{d}	20.33^{de}	1050.00^{e}	2066.70^{e}	50.67^{ab}	33.33^{a}
RAYA	82.00^{e}	136.00^{f}	124.00^{c}	32.93^{e}	1391.90^{cd}	38.33^{bcd}	18.00^{f}	1716.70^{cd}	3433.30^{d}	49.69^{ab}	20.00^{b}
Adukara	137.33^{a}	191.00^{a}	155.50^{c}	124.83^{ab}	2142.40^{bcd}	58.33^{a}	30.67^{a}	4016.70^{a}	7550.08^{ab}	53.43^{a}	5.00^{e}
Local	136.33^{a}	177.00^{b}	222.17^{b}	141.17^{a}	2235.10^{bc}	34.00^{cd}	24.00^{bc}	2700.00^{b}	8666.70^{a}	31.26^{e}	10.00^{de}
Mean	105.26	157.70	191.19	76.91	1990.47	45.26	22.81	2425.93	5852.47	42.78	17.22
C.V. (%)	4.22	2.93	13.15	12.52	23.19	18.25	5.53	14.76	10.57	11.65	20.53

Means in the same column followed by the same letters are not significantly different at 5% level of significance according to DMRT where; DTF, Days to 50% flowering; DPM, Days to physiological maturity; PLH, Plant height; HWPPt, Head weight per plant; NKPH, Number of kernels per head and NHPPL, Number of heads per Plot; TKW , Thousand kernelweight; GY , Grain yield; AGBM , Above ground biomass; HI, Harvest index; BIRD Dam (%), Bird damage estimation in %.

Mean values of tested genotypes for the characters studied in Assosa district are given in Table 1. Among the tested varieties; mean days to flowering ranged from 82.00 for RAYA to 137.33 for Adukara. Earliness is a desirable attribute for sorghum production in dry land areas since early maturing varieties can escape drought conditions that set in during the latter growth stages and allow farmers as source of food and cash. As to days to physiological maturity, the earliest varieties were RAYA and IS-9302 with 136.00 and 138 days, respectively. The latest were local check and genotype Adukara with 177 and 191.00 days, respectively.

The mean plant height recorded was 191.19 cm with a range of 124.00 to 281.33 cm. The shortest plant height was recorded from the variety RAYA while the tallest plant height was recorded from the variety BARC-acc-18. There were significant differences among sorghum genotypes tested for above ground biomass ($p < 0.01$). The varieties Geremew (2066.70 kg ha^{-1}) and IS-9302 (2605.60 kg ha^{-1}) had the least above ground biomass production whereas the highest above ground biomass was recorded from local check (8667 Kg ha^{-1}) followed by Adukara (7551.00 kg ha^{-1}) and BARC-acc-18 (7517 kg ha^{-1}).

Table 2 shows the mean values for yield and yield components. The highest head weight per plant was obtained from the local check (141.71 g) and Adukara (124.83 g) whereas the lowest head weight was recorded from the variety RAYA (32.93 g). Among tested varieties BARC-accc-18 (2462.60), local (2235.10) and Adukara (2142.40) had the maximum number of kernels per head than the rest of the varieties, while, the minimum number of kernels per head was recorded by Geremew (1333.3) and RAYA (1391.90).

Among the tested varieties, Baji, Adukara, and BARC-acc-18 had high average number of heads per plot than the rest of the tested varieties. The maximum and minimum number of heads per plot was 59.67 for Baji and 34.00 for local cultivar respectively. The genotypes differed significantly for thousand kernels weight. The values ranged between 18.00 and 30.67 g with a mean of 22.81 g. The highest thousand kernel weight was exhibited by Adukara, whereas the lowest weight was recorded by RAYA. The mean harvest index ranged from 31.26% for local cultivar to 53.43% for Adukara (Table 1). Mean grain yield among tested varieties ranged from4016.70 Kg ha^{-1} for the genotype Adukara to 1050.00 Kg ha^{-1}for the variety Geremew; with over all mean value of 2425.93 Kg ha^{-1}. The grain yields obtained from the varieties BARC-acc-18"(2933.30 kg/ha) and Baji (2750.99 Kg ha^{-1}) revealed a significantly ($P \leq 0.01$) higher grain yield than others and the local variety. The lowest yield was recorded for the variety Geremew, which could be due high bird damage recorded in the experimental field (Table 2). With regard to mean percentage of bird damage, Adukara (5.00%) and Baji (8.33%) were

Table 3. Direct matrix ranking evaluation of intermediate altitude sorghum genotypes by group of farmers' at Assosa district in 2013 main cropping season.

Selection criteria	Grain yield	Bird damage resistance	Early maturity	Total	Rank
Relative weight	3	2	2		
IS-9302	6(2)	2(1)	10(5)	18	8
Birmash	9(3)	4(2)	8(4)	21	5
Baji	12(4)	6(3)	6(3)	24	3
BARC-acc-18	12(4)	6(3)	6(3)	24	3
Geremew	9(3)	2(1)	10(5)	21	5
RAYA	9(3)	6(3)	10(5)	25	2
Adukara	15(5)	10(5)	2(1)	27	1
Local check	12(4)	6(3)	2(1)	20	7

Number of participants = 20 (M = 13 and F=7), numbers in parenthesis indicated the performance rating value of each variety given from 1-5 (5=excellent, 4=very good, 3=good, 2=poor and 1=very poor) and numbers written in the bold indicate total score of a variety as per each selection criteria, which was obtained by multiplying the relative weight of each selection criteria with that of the performance rating number in the parenthesis.

Table 4. Farmers pairwise ranking of intermediate altitude sorghum varieties at Assosa district during main cropping season 2013.

Varieties	IS-9302	Birmash	Baji	BARC-acc-18	Geremew	RAYA	Adukara	Local	Total score	Rank
IS-9302	x								0	8
Birmash	Birmash	x							1	7
Baji	Baji	Baji	x						4	4
BARC-acc-18	BARC-acc-18	BARC-acc-18	BARC-acc-18	x					5	3
Geremew	Geremew	Geremew	Geremew	BARC-acc-18	x				4	4
RAYA	RAYA	RAYA	Baji	RAYA	RAYA	x			6	1
Adukara	Adukara	Adukara	Adukara	Adukara	Adukara	RAYA	x		6	1
Local	Local	Local	Baji	BARC-acc-18	Geremew	RAYA	Adukara	x	2	6

found to be significantly more resistant for bird damage among the tested genotypes. The highest bird damage was recorded from the varieties Geremew (33.33%) and IS-9302 (31.67%) being early to mature.

Farmers' evaluation of mid-altitude sorghum genotypes

Selection criteria of farmers in the study area were based on a wide discussion and consensus and farmers set grain yield, early maturity and bird damage resistance as selection criteria during maturity stage of the crop. Direct-matrix was made with the criteria in the columns and tested varieties in the rows. PVS was done in the study site not only that farmers' cultivars were old but also none of the tested genotypes except local check were grown previously by farmers. Thus, some of the criteria farmers used may not coincide with the merits of improved varieties as to weed *striga* (*Striga hermantica*) resistance and some nutritional quality aspects. In other words the criteria farmers used in identifying the suitable varieties depend on the existing constraints and opportunities farmers faced in their micro environments.

Based on farmers' pair wise ranking results, grain yield, bird damage resistance and earliness were proposed as the three most important criteria in descending order. Buah et al. (2010) in participatory evaluation of drought tolerant varieties of maize and Yetsedaw et al. (2010) on participatory evaluation of malt barley reported similar selection criteria set by farmers. In addition, Alebachew (2012) found also the same selection criteria while farmers' evaluated wheat varieties in Tigray region. Direct matrix ranking evaluation of intermediate attitude sorghum genotypes indicated the total score ranged from 27 to 18 as indicated in Table 3. The highest score was given to the best genotype Adukara (score 27) followed by the variety RAYA (score 25). Out of the eight genotypes tested, IS-9302 (score 18) and local check (score 20) scored the minimum value to be the least preferred. Despite the advantage of earliness, the varieties Geremew and IS-9302 were susceptible to bird damage while the local check were late maturing and reasonably resistant to bird damage. Likewise, pair-wise

ranking revealed that Adukara and RAYA were chosen by farmers to be the most important ones (Table 4). Generally farmers' responded positively to the new genotypes evaluated. Farmers' evaluation indicated that there was alignment with researchers' criteria particularly for grain yield and bird damage resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Assessment of genotype x environment interaction and pod yield evaluation of groundnut (*Arachis hypogaea* L.) genotypes in Zimbabwe

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Groundnut is an important component of the diet of both rural and urban populations in the Sub-Saharan Africa. The national average pod yield is 0.25 t/ha which is far less than the global average. The diverse environmental conditions of Zimbabwe make selection and release of stable groundnut genotypes a challenge, mainly due to genotype x environment interaction (GEI). Twenty-five groundnut genotypes were evaluated to examine the level and type of GEI on pod yield. The genotypes were evaluated under multi-environmental yield trial conducted in (2013/14 summer season) at five environments. The objectives of the experiment were to determine the presence of GEI on pod yield stability of groundnut genotypes, to identify genotypes that are specifically or widely adapted. General combined analysis of variance (GenStat Version 14) at 5% significance level indicated that genotypes (G) were not significant (p = 0.153), environments (E) and genotype x environment interactions (GEI) were highly significant (P < 0.05) on pod yield. The environment influenced yield of the groundnut genotypes. Environment and genotype explained 58.8 and 6.1% respectively of the total treatment variance, whilst the genotype by environment interaction accounted for 35.1%, indicating that environment influenced a lot on the performance of the genotypes. High significant level of GEI indicates that some genotypes may be released for specific environments. Basing on the mean pod yield value from the combined (ANOVA) analysis of variance results, groundnut genotype G24 (3.34 t/ha, check variety) was the highest yielder, followed by G7 (3.31t/ha) and then G2, G14 and G11 (3.29, 3.25 and 3.02 t/ha respectively). The results indicate that the experimental genotypes have great potential to be released and grown on large scale production. Stability analysis based on one multivariate or various uni-variate parameters to extract more information on the GEI on pod yield stability of groundnut is recommended.

Key words: Groundnut, genotypes, pod-yield, multi-environmental trial, experimental, genotype x environment interaction.

INTRODUCTION

Zimbabwean environment is so diverse and so sophisticated (Nyamapfene, 1991; Rukuni et al., 2006) and that leads to very high levels of genotype x environment interactions (GEI). The heterogeneous

nature of the Zimbabwean environments (agro-ecological regions) results in the performance of different groundnut genotypes to differ both within and across environments. Higher genotype x environment interaction is usually expected to be as a result of large environmental differences as in Zimbabwe. In most cases, this kind of interaction may lead one genotype in having the highest yield in some environments and may be lowest in others, whilst the second genotype may excels in other environments in which the first one might have failed (Gauch and Zobel, 1996). For that reason, it is important to know and understand the level of the interactions in the selection of genotypes across several environments rather than only calculating the average performance of the genotypes under evaluation (Fehr, 1991; Gauch and Zobel, 1997).

It has been noted that genotypes tested in different locations or years often have significant fluctuation in yield due to the response of genotypes to environmental factors such as climate, soil fertility, pests and disease pathogens (Kang, 2004). These variations in yield are the ones that are usually referred to as genotype x environment interaction (GEI) and they are so frequent whenever experiments are conducted. Genotype x environment interactions has been studied in many crops by many different researchers. One of the major complications in all breeding programs is genotype x environment (G x E) interaction. It has been noted that a proper understanding of the environmental and genetic factors that causes the interaction as well as an assessment of their importance is likely to have a great impact on the development, evaluation and selection of superior germplasm (Magari and Kang, 1993; Basford and Cooper, 1998).

The phenotypic expression of an organism (plants/crops included) is due to its genotype (G), the surrounding environment (E) as well as the interaction of the two (G x E). The presence of significant GxE interactions complicates the process of selecting only genotypes with higher performance (high yielding) since the genotype is going to yield differently in different environments (this leads to change in rank order). For that reason, multi-environment trials are now broadly used to assess the suitability of genotypes for target environments (DeLacy et al., 1996).

When experiments are conducted under varying environments, genotypes that always give high average yields with minimum G x E interaction have been gaining importance over increased yields (Ceccarelli, 1989; Gauch and Zobel; 1997, Kang, 1998). The analysis of G x E interaction is closely related with the quantitative estimation of phenotypic stability of genotypes over different environments (Kang, 1998). When significant G x E interaction is observed, the effects of genotypes and environments are statistically non-additive; this implies that the differences between genotypes are due to the environment and not genotypes themselves. G x E interactions may, but not all the time, lead to different rank orders of genotypes in different environments. The presence of G x E interaction in multi environment trials leads to a need for the analysis of genotype stability (usually yield stability). Many authors have described yield stability in many different ways over the years and there have also been different concepts of stability tests (Lin et al., 1986). According to Becker and Leon (1988), many researchers use the terms adaptation, phenotypic stability and yield stability in different ways. Chahal and Gosal (2002) noted that stability indicates consistency in performance that would mean minimum variation among environments for a particular genotype.

The prime reason for researchers to perform multi-environmental genotype evaluation is to estimate and or predict how the genotype is likely to perform in future years and future environments, basing on the performance data of the past, and to develop or recommend superior ones. In almost all multi-location trials, there exists interaction and noise (Purchase, 1997). Selecting for high pod yielding and genotypes that have wide adaptation is the ideal situation that breeders would want to concentrate on rather than concentrating on genotypes that might give the highest pod yield in only one environment. Alternatively, in the case that there are no ideal genotypes, then selecting for specifically adapted genotypes would be the next option. That means the genotypes will be released and recommended for specific areas and not for the growing areas. This chapter focuses on the level and nature of G x E interaction based on the general combined analysis of variance techniques as a primary tool to differentiate these groundnut genotypes according to their pod yield performance.

This study was designed to (i) examine the level and type of Genotype x Environment Interaction for pod yield (ii) identify groundnut genotypes with high yielding varieties, and (iii) determine the need of doing pod yield stability analysis.

MATERIALS AND METHODS

A total of 25 genotypes (4 commercially released varieties and 21 intermediate experimental lines) were tested in 2013/14 summer season. All the check varieties and the intermediated experimental lines were obtained from Crop Breeding Institute (C.B.I). Ilanda and Tern are the highest yielding short season groundnut varieties and for that reason they were included as check varieties. More details on genotypes and the information on their breeding status are highlighted in Table 1.

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Table 1. Pedigree information and source of the planting materials.

Variety/line code	Pedigree	Breeding Status	Origin
G1	297/7/29	Intermediate line	C.B.I
G2	302A/6/2	Intermediate line	C.B.I
G3	401/92/14	Intermediate line	C.B.I
G4	262/4/3	Intermediate line	C.B.I
G5	AB/5/11	Intermediate line	C.B.I
G6	321/5/15	Intermediate line	C.B.I
G7	9607/5/14	Intermediate line	C.B.I
G8	9503/6/11	Intermediate line	C.B.I
G9	267/6/13	Intermediate line	C.B.I
G10	9607/5/10	Intermediate line	C.B.I
G11	9607/5/22	Intermediate line	C.B.I
G12	294/5/16	Intermediate line	C.B.I
G13	9503/6/5	Intermediate line	C.B.I
G14	294/5/16	Intermediate line	C.B.I
G15	374/92/16	Intermediate line	C.B.I
G16	9607/5/11	Intermediate line	C.B.I
G17	296/5/4	Intermediate line	C.B.I
G18	295/5/8	Intermediate line	C.B.I
G19	H97/3F7/1	Intermediate line	C.B.I
G20	H97/14F7/1	Intermediate line	C.B.I
G21	267/6/6	Intermediate line	C.B.I
G22	Falcon	Released	C.B.I
G23	Tern	Released	C.B.I
G24	Jesa	Released	C.B.I
G25	Ilanda	Released	C.B.I

Table 2. Description for the sites used on the multi-environmental groundnut yield trials in 2014.

Code	Location	Soil properties	Latitude	Longitude	Altitude masl	Rainfall data (mm)
E1	Harare	Clay	17^{o} 48 S	31° 03 E	1506	660
E2	Gwebi VTC	MG/SCL	17^{o} 41 S	30° 32 E	1448	880
E3	Kadoma	Clay	18^{o} 19 S	29° 53 E	1149	818
E4	Panmure	MG/SCL	17^{o} 16 S	31° 47 E	881	796
E5	Save Valley	Sandy-loam	20^{o} 48 S	33° E	450	500

Study site

The project was conducted at five locations Harare Research Station (HRS), Panmure Experimental Station (PES), Gwebi Variety Testing Centre (GVTC), Save Valley Experimental Station (SVES) and Kadoma Research Station (KRS). Two of the locations belong to high veld (Harare Research Station and Gwebi VTC, the other two to middle veld (Kadoma Research Station and Panmure Experimental Station) and one belongs to the low veld (Save Valley Experimental Station). More details on the testing sites and the agro-ecological characteristics for all the locations used are shown in Table 2.

Management

The seeding rate that was used is 100 kg/ha for all environments. Compound D was applied at planting at a general recommendedrate of 300 kg/ha. Gypsum was also applied during first flowering (7 to 8 weeks after planting) at a general recommended rate of 300 kg/ha. Harvesting was done manually, were 2.4 m (0.3 m from either sides of the row) of the 3 m rows were harvested as net plot by way of hand pulling as well as hand plucking. Pod yield was then recorded after drying the groundnut pods to 12.5% moisture content by exposing the pods to the sun and moisture content was measured using the moisture meter. All other recommended groundnut production practices such as weed, pest and disease management were followed and practiced.

Experimental design

The trials were laid in a Complete Randomized Block Design

Figure 1. Field showing part of the trial under study at Gwebi VTC.

Figure 2. Field showing part of the trial under study at Harare.

(CRBD) at all the sites (Figures 1, 2 and 3). Each of the twenty-five treatments with 3 replicates and that translated to seventy-five plots in total. The plot sizes were 5.4 m^2 with 5 rows of 3 m long with spacing of 0.45 m between rows. The net plot size was 2.16 m^2, 1 row from both sides and 0.3 m from either side was discarded.

Records taken

Records that were taken include, days to flowering, days to maturity, diseases scores, insect pest scores, pod size, seed size, shelling percentage and pod yield. For the sake of this study, only pod yield was considered for statistical analysis. Pod yield was recorded on the net plot basis. After drying and cleaning, the weights of the pods per plot were recorded and converted to t/ha using a formula.

Analysis of variance

General combined analysis of variance (ANOVA) for pod yield data was conducted using GenStat 14th Edition software to determine

Figure 3. Field showing part of the trial under study at Kadoma.

Table 3. General combined analysis of variance for pod yield (t/ha) of twenty-five groundnut genotypes evaluated across five locations over a season.

Source	DF	SS	MS	Percentage total sum of squares
Rep stratum	2	0.8646	0.4323	
Genotype	24	29.2839	1.2202	4.1
Environment	4	281.8406	70.4602***	39.7
Genotype.Environment	96	168.4375	1.7546***	23.7
Residual	248	229.8636	0.9269	
Total	374	710.2903		

Coefficient of variation (%CV) = 18.1%.

the G, E and GEI effects. The effects of the genotypes, environments as well as their interaction were determined from ANOVA analysis.

RESULTS AND DISCUSSION

General combined ANOVA and mean yield performance

General analysis of variance at 5% significance level indicated that genotypes (G) were not significant ($p = 0.153$), but environments (E) and genotype x environment interactions (GEI) were highly significant both ($P < 0.001$) on pod yield of twenty-five groundnut genotypes and accounted for 4.12, 39.68 and 23.72% of the total sum of squares, respectively (Table 3). This indicates that the environment influenced the yielding ability of the groundnut genotypes. In this research, environment and genotype explained 58.8 and 6.1% of the total treatment variance, whilst the genotype by environment interaction accounted for 35.1%, this indicates that the environment had a lot of influence on the performance of the genotypes. Similar results that confirm that environment contributes a more genotype and environment interaction to the total treatment variance were obtained on wheat, were the genotypes, environments and their interactions were significant, with the environment contributing much of the variation (Gauch, 2006). In their research, the effects of environment and genotype explained 83.78 and 2.71% of total treatment variance respectively, whereas the interaction explained 10.08% of the total treatment variance. In Table 3 it is shown that the (large) total variance for environments was 76.02% indicating higher heterogeneity in the environmental conditions among the five locations used in the study, hence the groundnut genotype pod yield was largely influenced by the environments. This was also consistent with findings

Table 4. Mean pod yield (t/ha) of 25 groundnut genotypes (G1 - G25) evaluated across 5 environments (E1 - E5) in Zimbabwe in summer 2014.

Genotype	Environment					Genotype mean yield (t/ha)
	E1	E2	E3	E4	E5	
G1	3.083	3.929	1.548	**4.254**	2.243	3.0114
G10	2.747	3.758	1.628	3.141	2.329	2.7206
G11	3.657	6.059	**1.887**	1.495	2.015	3.0226
G12	3.086	2.458	1.937	2.811	2.586	2.5756
G13	3.853	2.572	1.363	2.789	2.015	2.5184
G14	3.768	5.53	1.42	3.837	1.7	3.251
G15	3.715	3.472	1.657	2.638	2.143	2.725
G16	3.476	2.486	1.397	2.155	2.515	2.4058
G17	3.546	3.215	1.313	3.134	1.672	2.576
G18	3.684	3.429	1.185	3.502	2.286	2.8172
G19	2.855	3.244	1.412	2.75	2.443	2.5408
G2	4.32	**6.501**	1.336	1.782	2.486	3.285
G20	4.198	3.658	1.753	3.231	1.8	2.928
G21	4.413	3.129	1.486	3.61	2.072	2.942
G22	4.85	2.901	1.545	3.025	1.772	2.8186
G23	4.488	4.572	1.239	2.167	2.115	2.9162
G24	3.652	5.53	1.792	3.228	2.515	3.3434
G25	3.526	2.472	1.983	2.911	2.286	2.6356
G3	3.77	2.1	1.486	1.818	2.372	2.3092
G4	3.635	4.187	0.775	2.927	2.615	2.8278
G5	3.691	2.958	1.181	**4.335**	1.86	2.805
G6	2.84	2.301	1.677	3.806	1.815	2.4878
G7	**6.032**	4.101	1.193	2.582	**2.658**	3.3132
G8	4.183	3.129	1.711	3.092	2.058	2.8346
G9	4.106	1.858	1.218	3.532	2.415	2.6258
Site mean yield (t/ha)	3.80696	3.58196	1.48488	2.98208	2.19144	2.8091

(Yan and Kang, 2003) which showed that environment is the dominant source of variation, while G and GE are relatively small in yield trials across locations. According to Zerihun (2011), in most cases under normal multi-environment yield trials, environment (E) accounts for 80% or higher of the total yield variation, while genotype (G) and genotype x environment interaction (GEI) each account for about 10%. The magnitude of genotype by environment interaction sum of squares (1.89%) was larger than of genotypes (1.32%), indicating that there were substantial differences in genotypic responses across environments (Table 3). The analysis of genotype by environment interaction pattern is highly important for scientists such as plant breeders, because it enables them to design the correct strategies (selecting for wide/general or specific adaptation) for new genotypes to be released for commercial production.

Genotype x environment interaction

There were inconsistencies in pod yield rankings of genotypes across environments as shown in Table 4. This gives rise to cross over type of GEI indicating that there was inconsistent genotype pod yield performance across environments. Table 4 indicates that the following genotypes had highest pod yield at different environments; G7 in E1 (Harare); G11 in E3 (Kadoma); G1 in E4 (Pamure); G7 in E5 (Save Valley); G2 in E2 (Gwebi VTC). The presence of cross over GEI shows the existence of different mega environments in which different winning genotypes can be selected (Crossa et al., 1991). The mean pod yields in Table 4 indicate that there were differences in rankings of pod yield performance among genotypes across environments (cross over GEI). This is shown by some genotypes which attained maximum yields in more than one environment, for instance, G7 in E1 (Harare) and E5 (Save Valley); (Table 4). According to (Crossa et al., 1991), it is common for a multi environment yield trial to constitute a mixture of cross over and non-cross over types of GEI. Crossover interactions result due to the G x E interaction, in which case there will be non-parallel response curves of genotypes (intersecting each other)

with interaction. In other words this is when different genotypes are winning in different environments. It means that the performance of genotypes leads to change in relative rank orders of genotypes in different environments.

The implication crossover interaction to this study is that the selection of superior genotypes is not possible, since different genotypes were performing differently. As a result, researcher will then focus on two sets of genotypes to recommend for release: (i) Choose the genotypes that have wide adaptation, and (ii) Choose genotypes which are specifically adapted to high potential environments. These will then be identified when the data has been subjected to adaptability and stability analysis.

Conclusion

The results shows evidence that there was genotype x environment interaction in this study as well as significance of genotypic performance that was due to the environments. The presence of significant GxE interactions complicates the process of selecting only genotypes with higher performance (high yielding or superior genotypes) since the genotype were yielding differently in different environments (leading to changes in rank orders). For this reason, multi-environment trials are recommended. The results indicate that the environmental main effect influenced the performance of the groundnut genotypes more than the genotypic main effect and the interaction of genotype and the environment. The results indicate that there are some experimental genotypes that have great potential to be released and recommended for commercial production in all areas suitable for groundnut production. The following genotypes would be recommended for release and commercial production; G7 (9607/5/14 an experimental genotype, mean pod yield = 3.31 t/ha) and then G2, G14 and G11 (302A/6/2, 294/5/6 and 9607/5/22 experimental lines, mean pod yields = 3.29, 3.25 and 3.02t/ha respectively). Due to the negative impact of GEI on genotype selection, the researcher recommends that the data must be exposed to analysis of pod yield stability using on one multivariate or various uni-variate parameters to extract more information on the GEI and pod yield stability of groundnut genotypes in Zimbabwe.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of thermotherapy duration, virus type and cultivar interactions on elimination of potato viruses X and S in infected seed stocks

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Virus infection in potato reduces yield through seed degeneration. This can be reduced by use of virus-free seed tubers. However, novel approaches are required to ensure availability of virus-free stocks, especially in developing countries where the seed potato system are evolving. Consequently, a laboratory experiment was conducted at Kachwekano ZARDI in Uganda to evaluate and determine appropriate procedures for cleaning valuable potato cultivars infected with the most prevalent potato viruses; potato virus X (PVX) and potato virus S (PVS) which are frequent in the potato farming system. Thus, 20 *in-vitro* potato plantlets from cultivars Victoria, Kinigi and Rwangume each batch infected with either PVX or PVS in three replicates were grown in a thermotherapy chamber for two, three and four weeks at 37 to 40°C in 16 h of light and 30 to 34°C for 8 h of darkness per day. An equal number plantlets and replicates of the same cultivars and virus infection combinations were grown in a standard tissue culture (TC) growth room at 16 to 18°C with 16 h of light without thermotherapy as controls. Results indicated that plantlet survival after *in-vitro* thermotherapy decreased with increasing duration of heat treatment. Virus elimination efficiency significantly ($P \leq 0.05$) differed between heat-treated plantlets and the controls but not between the duration of thermotherapy treatment. However, the highest proportion of virus-free plantlets was obtained after three weeks of thermotherapy. Significant ($P \leq 0.05$) interactions were observed between thermotherapy duration and virus type on virus elimination efficiency where significantly ($P \leq 0.05$) more PVS-free than PVX-free plantlets were obtained at the same thermotherapy duration. Three weeks of thermotherapy of virus-infected *in-vitro* plantlets, particularly for PVS, offered an equilibrium duration for adequate plantlet survival and maximum meri-clone regeneration to obtain the highest proportion of virus-free plantlets. The use of thermotherapy to obtain a high proportion of PVX-free plantlets from infected potato seed stock showed no clear trend and needs further investigation.

Key words: Latent virus infection, meristem tip culture, plant virus heat therapy, recalcitrant viruses.

INTRODUCTION

Virus infection in crops is known to reduce yield and quality in global production (Salazar, 1996). Viral infections are especially problematic in vegetatively propagated crops in which they are transmitted through successive generations of infected planting material (Agrios, 2005). Therefore, production and maintenance of

virus-free planting stock of vegetatively propagated crops is crucial for controlling the spread of viruses, slowing seed degeneration and maintaining variety yield potential (Muthoni et al., 2013; Wagoire et al., 2005). Potato virus X (PVX) and Potato virus S (PVS) are the major viruses infecting potato that occur at high frequencies in most areas and potato cultivars in Uganda (Kakuhenzire et al., 2000). The two viruses are easily transmitted mechanically requiring regular monitoring and removal in infected seed if the yield potential of the cultivars is to be maintained. Potato virus X belongs to the *Potexvirus* genus and can potentially reduce yield in potato up to 10% when 100% of tubers are infected (Struik and Wiersema, 1999). This virus is inactivated at 68 to 76°C (De Bokx, 1972). Potato virus S belongs to the genus *Carlavirus,* occurs globally and may cause 10% yield loss in potato crops where 100% of seed tubers are infected (Struik and Wiersema, 1999). The thermal inactivation temperature of PVS ranges from 55 to 60°C (De Bokx, 1972). In multiple viral infections however, yield loss is enhanced that having seed free from the two viral infections becomes imperative. The common occurrence of PVS and PVX in Uganda may be attributed to the ease of virus transmission (De Bokx, 1972; Salazaar, 1996), the repeated use of the same seed stock for seed bulking with minimal renewal over time among most smallholder farmers (Tindimubona et al., 2000), the limited seed certification and finally to the lack of affordable and effective locally adapted techniques for virus detection and elimination in infected stocks. This leads to virus accumulation in potato in locally produced planting materials in potato farming systems (Kaguongo et al., 2008). Therefore, there is a need to develop and adapt appropriate techniques for cleaning and returning to the seed system existing and valuable cultivars that have either been abandoned or are being widely grown, but have very low yields due to viral infections. Recovery and restoration of valuable virus-infected cultivars can be achieved through virus cleaning or elimination in infected cultivars and maintaining them in pathogen free and reinfection-proof environment such as *in vitro* tissue culture.

Virus elimination methods can be cultivar and virus sensitive (Panattoni et al., 2013). Several methods of virus elimination have been used in potato to clean viral-infected stocks (Mahmoud et al., 2009; Dhital et al., 2008; Wang et al., 2006). Thermotherapy combined with meristem tip culture has been commonly used in several crop species for eliminating many viruses for its apparent affordability, safety and ease of generating virus-free plants (Panattoni et al., 2013). However, for a given crop such as potato, optimizing the duration high temperature exposure is required to enhance the efficiency and effectiveness of virus elimination in infected stock where positive selection is not possible to recover clean propagules. Success in plant virus elimination by meristem culture is often influenced by size of the excised meristems, crop cultivar, plant and virus species (Loebenstein et al., 2001). This procedure, however, is difficult and alone often results in a lower virus elimination efficiency (Wang and Valkonen, 2008). *In vitro* thermotherapy followed by meristem culture markedly enhances virus elimination by allowing a few specialized cells without or carrying a minimum infective virions to be excised. Excision of a few specialized cells in the apical meristem increases the frequency of meri-clone regeneration with a high proportion of virus-free plantlets (Nascimento et al., 2003; Rukarwa et al., 2010; Wasswa et al., 2010; Wang and Valkonen, 2008). Plant thermotherapy for enhancing virus elimination in infected stock involves exposing infected explants to 35 to 40°C for 14 to 28 days (Agrios, 2005). Longer thermotherapy exposure would be more effective in virus elimination, however, it compromises survival and regeneration of treated plantlets (Tan et al., 2010).

The aim of this study was to determine the effect of thermotherapy duration on potato plantlet survival and virus elimination efficiency in selected important potato cultivars in Uganda. The efficiency of virus elimination was evaluated based on plantlet survival, success of meri-clone regeneration and ratio of virus-free plantlets using different combinations of cultivar, virus type and thermotherapy duration in a laboratory conditions at Kachwekano ZARDI, Uganda.

MATERIALS AND METHODS

Plant and virus stocks

Virus infected stocks of potato cultivars Kinigi, Rwangume and Victoria were used. The stocks were infected either by Potato virus X (PVX) or Potato virus S (PVS). The virus inoculum was maintained in stocks of the three cultivars used in this study.

Production of virus-infected *in vitro* plantlets

Fully sprouted tubers of cvs Kinigi, Victoria and Rwangume obtained from field-grown, suspected virus-infected potato plants were indexed for PVX, PVS, PLRV, PVY, PVM and PVA using direct double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977). Single-tuber samples per variety obtained from plants singly infected with both PVX and PVS were planted in plastic pots in a greenhouse for viral inoculum maintenance and for obtaining viral infected ex-plants. After full germination at four leaf stage, the plants were re-indexed to ensure that only mother plants infected with either PVX or PVS per cultivar were kept and used. Thereafter, the terminal buds were excised from mother plants to promote axillary bud growth and

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Table 1. Summary of mean squares for the effect of duration of heat thermotherapy, cultivar and virus type on virus elimination efficiency in potato in Uganda in 2012

Source of variation	d.f.	Percent plantlet survival after heat treatment	Meristem survival (%) at 30 days after initiation on media	Meri-clone regeneration (%) at 45 days	Virus-free meri-clones (%)
Cultivar	2	1169.1*	73.9	4088.1*	6105.0*
Virus	1	528.1*	4644.2*	12811.7	10833*
Heat duration	3	11304.1*	276.1	5451.9*	4149*
Cultivar × Virus	2	1071.9*	840.8	1527.9	800
Cultivar × Heat duration	6	171.4*	628.2	772.7	1569
Virus × Heat duration	3	287.4*	397.8	1323.2	3665*
Cultivar × Virus × Heat duration	6	135.3*	932.2*	660.5	654
Residual	48	49.3	325.4	862.6	1067
CV (%)	-	10.5	43.2	55.0	80.5

*Represents significance levels at P≤0.05. Mean squares without asterisk were not significant at any of the levels above.

introduced in *in vitro*. The excised explants were surface-sterilized by soaking in water with liquid detergent for 30 min and then immersed in 70% ethanol for 5 s and later in a solution of 14% sodium hypochlorite and 2 drops of tween-20 for 3 min (Lizaragga et al., 1989). The disinfected explants were rinsed three times in sterile distilled water and introduced *in vitro* for plantlet regeneration. With fully developed virus-infected potato *in vitro* plantlets, single-node explants were initiated in standard tissue culture media containing 6 g agar, 100 mg ascorbic acid, 100 ml macro nutrient stock solution containing 12 ml/l $MgS0_4$, 50 ml/l calcium chloride, NH_4NO_3 (35 g/200 ml), KNO_3 (40 g/200 ml) and KH_2PO_4 (3.5 g/200 ml), 10 ml micro nutrient stock solution containing KI (0.02 g/200 ml), H_3BO_3 (0.1 g/200 ml), $MnSO_4.H_20$ (0.5 g/200 ml), $ZnS0_4.7H_20$ (0.2 g/200 ml), $Na_2MoO_4.2H_2O$ (0.005 g/200 ml), $CuSO_4.5H_2O$ (0.005 g/10 ml), $CoCl_2.6H_2O$ (0.005 g/10 ml), 5 ml $FeSO4.7H_2O$, 100 mg myoinosotol, 10 ml vitamins, , 1 ml folic acid, 4 ml L-Arginine and 30 g sucrose in 1000 ml of distilled water; pH 5.8. The regenerated plantlets were sub-cultured 3 times every 4 weeks to obtain adequate numbers of plantlets per cultivar and viral infection type for the thermotherapy experiment.

Experimental treatments

Freshly excised single-node potato stem cuttings from the virus-infected plantlets described earlier were initiated in a standard *in vitro* plant growth medium in standard tissue culture laboratory conditions (Lizaragga et al., 1989) for 2 weeks to obtain completely regenerated plantlets before exposure to thermotherapy. The fully developed, plantlets were subjected to thermotherapy at 37 to 40°C with 16 h of light (high intensity of over 10,000 lux) and at 30-34°C with eight 8 h of darkness for two, three or four weeks in a thermotherapy chamber. Twenty plantlets per cultivar, virus-infection type and thermotherapy duration treatment combinations were used. The experiment was repeated three times. The same number of virus infected plantlets per cultivar grown in standard tissue culture conditions were used as controls.

At the end of each thermotherapy period (1, 2 or 3 weeks), plantlets that had survived were recorded and apical meristem (~0.2 to 0.5 mm) were excised from each using hypodermic needles under a stereo-microscope in sterile conditions. The excised meristems were cultured on the same media for initiation in tissue culture with addition of 1.0 mg/L gibberellic acid and 0.4 mg/L benzylaminopurine to ensure better growth and regeneration of meri-clones in standard tissue culture growth room conditions (Wang et al., 2006). After one month of growth, the regenerated meristems were transferred to fresh media of the same composition for regrowth to obtain meri-clones (Wang et al., 2006). The meri-clones obtained from both heat-treated and control plantlets were sub-cultured on standard MS media devoid of growth regulators (Wang et al., 2006) to obtain fully developed plantlets that were tested to determine their virus-infection status.

Data collection and analysis

The number of plantlets per thermotherapy treatment period that survived per thermotherapy period were counted and recorded and survival percentage was computed. The regeneration efficiency of meristems was computed as a ratio of mericlones that were recoverd after meristem initiation in standard meristem tip culture medium and expressed as a percentage. Virus elimination efficiency (%) was computed as the ratio of the number of virus free meri-clones to the total number of regenerated meri-clones after full plantlet recovery and expressed as a percentage. The significance of treatment factors and their interactions on plantlet survival, meristem regeneration and virus elimination efficiency were tested using analysis of variance (ANOVA) in GENSTAT 14th Edition statistical software (VSN International, Hemel Hempstead, UK). Means of significant (P≤0.05) main effect and interactions were compared using Fishers' Protected Least Significant Difference (LSD) test at 5% probability.

RESULTS

Effect of heat treatment on potato plantlet survival and meri-clone regeneration

The survival of potato plantlets after heat treatment was significantly (P≤0.05) affected by main effects of cultivar, thermotherapy duration and type of virus infection (Table 1). First and second order interactions among the main effects significantly (P≤0.05) affected plantlet survival after heat treatment (Table 1). The success of meri-clone regeneration was significantly (P≤0.05) influenced by the cultivar and duration of heat treatment but not interaction between the two main effects (Table 1). The efficiency of

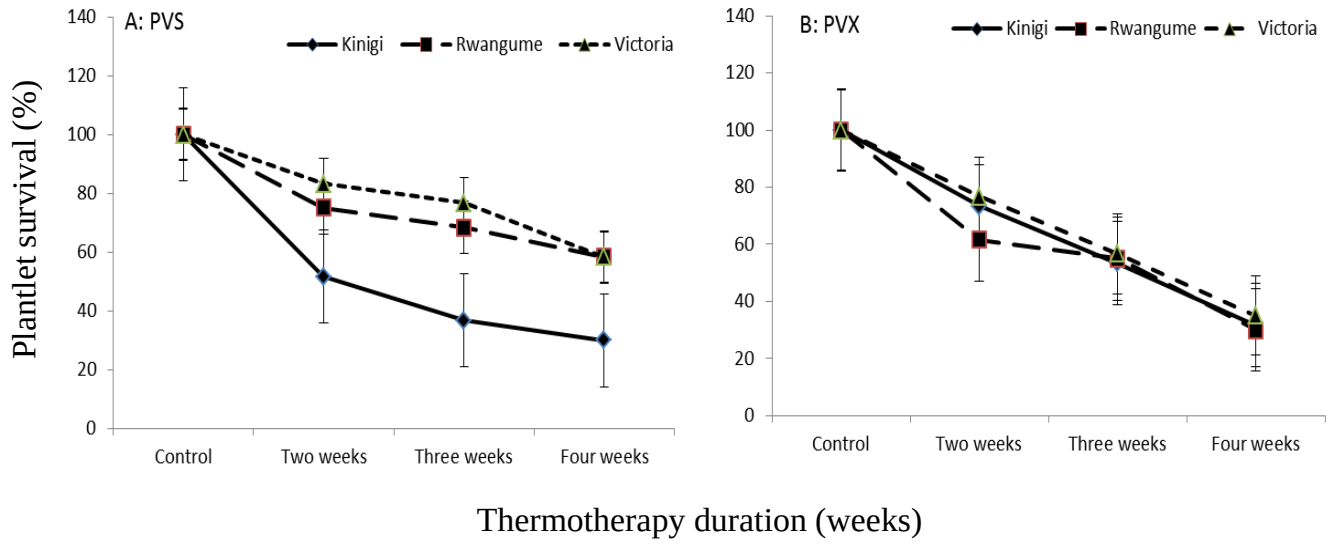

Figure 1. Percentage survival of potato plantlets infected with PVS or PVX for different duration of thermotherapy exposure.

Table 2. Survival as influenced by the two viruses.

Virus	% Survival
PVS	69.86
PVX	64.44
Mean	67.15
LSD	5.763

Table 3. Virus × cultivar interactions on potato plantlet survival after thermotherapy.

Virus	Cultivar			
	Kinigi	Rwangume	Victoria	LSD*
PVS	54.58	75.42	79.58	5.684
PVX	64.58	61.67	67.08	5.684
Mean	59.58	68.54	73.33	-
LSD	4.204	4.204	4.204	-

LSD* for Virus × Cultivar interactions.

obtaining virus-free plantlets after heat treatment was significantly (P≤0.05) influenced by virus infection type, potato cultivar, heat exposure duration and interaction between virus infection type and duration of thermotherapy (Table 1).

Effect of high temperature treatment on plantlet survival

Potato plantlet survival decreased for the three cultivars with the increase in thermotherapy duration (Figure 1) with significant difference between PVS and PVX infected stocks (Table 2). The survival % of cultivar Kinigi was significantly (P≤ 0.05) lower than that of cultivars Victoria and Rwangume which did not in turn significantly (P≤ 0.05) differ from each other when infected with PVS (Figure 1 and Table 3). There were no significant (P≤ 0.05) differences in plantlet survival after thermotherapy between cultivars from PVX-infected stocks (Table 3).

Meristem regeneration 30 days after excision and inoculation into *in-vitro* medium

The duration of thermotherapy on meristem survival at 30

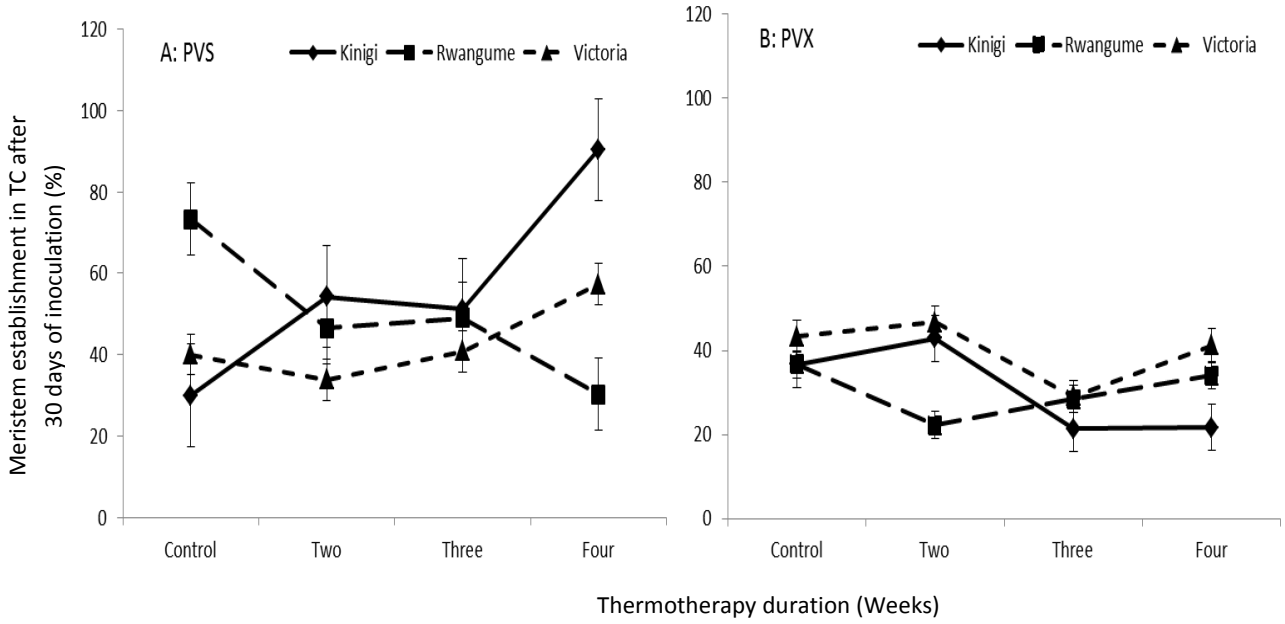

Figure 2. Effect of thermotherapy treatment duration on meristem establishment in tissue culture at 30 days after inoculation.

Table 4. Cultivar differences with respect to mericlone regeneration.

Cultivar	% Regeneration
Kinigi	54.3
Rwangume	65.3
Victoria	39.3
LSD	16.12

Table 5. Effect of thermotherapy duration on mericlone regeneration.

Thermotherapy duration (Weeks)	% Regeneration
0	27.4
2	60.7
3	66.2
4	57.5
LSD	18.62

days after excision and inoculation in meristem tip culture medium under normal tissue culture growth room conditions had no significant impact on meristem survival (Table 1). However, meristem establishment was more influenced by the type of virus present in *in vitro* plantlets (Figure 2) and affected by interaction between cultivar, virus and duration of thermotherapy (Table 1).

Effect of thermotherapy duration on potato meristem regeneration into mericlones

Cultivar Rwangume had the highest meristem regeneration efficiency to mericlones, although it did not significantly (P≤0.05) differ from cultivar Kinigi (Table 4 and Figure 3), while cultivar Victoria had the lowest meristem regeneration efficiency (Table 4 and Figure 3). Amongst the three cultivars however, the three thermotherapy durations did not significantly (P≤0.05) differ from each other, although all the heat treatment duration had higher regeneration success than the control (Table 5).

Effect of virus infection type and thermotherapy duration on virus elimination efficiency

Correlation analysis did not reveal strong relationship among measured or derived variables as factors that would influence the success of plantlet regeneration or virus elimination even when some correlation coefficients between meristem survival and virus-free mericlones was significant (P ≤ 0.05) (Table 8). Therefore, the proportion of regenerated *in-vitro* plantlets that were apparently free from viral infection after successful thermotherapy and meristem tip culture was not significantly (P ≤ 0.05) influenced by the proportion of plantlets that survived after thermotherapy and meri-clone regeneration. Cultivars Rwangume and Victoria had significantly (P≤0.05) higher rate of virus elimination than cv. Kinigi, while cv. Rwangume and cv. Victoria did not significantly (P ≤ 0.05) differ from each other (Table 6 and Figure 4). Thermotherapy exposure for two and three weeks had a significantly (P≤0.05) higher proportion of plantlets that were free from virus infection than the controls and

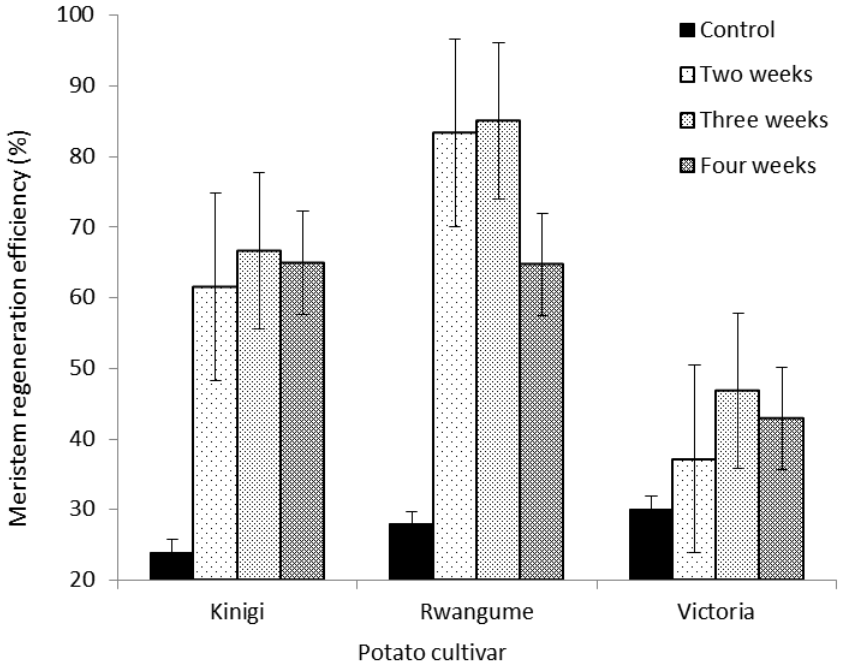

Figure 3. Effect of thermotherapy duration on potato meri-clone regeneration.

Table 6. Percent Virus elimination from each of the cultivars.

Cultivar	% Virus elimination
Kinigi	22.8
Rwangume	53.7
Victoria	45.2
LSD	19.63

plantlets that were heat-treated for four weeks (Table 7 and Figure 4).

Analysis of variance indicated significant (P≤0.05) interaction between thermotherapy duration and type of virus infection in a seed stock (Table 1). Mean comparison among main effects and interactions showed that plantlets regenerated from PVS infected seed stocks had a significantly (P≤0.05) higher virus elimination success than the ones regenerated from PVX infected mother plants (Table 9). Similarly, plantlets that were not subjected to thermotherapy had significantly (P≤0.05) lower proportion of virus-free plantlets than those that were heat treated (Table 9). However, examination of trends in success of recovering virus-free plantlets along each virus infection with increasing duration of heat treatment revealed that longer heat exposure periods increased the success of recovering virus-free plants from stock originally infected with PVS. Recovery of PVX-free plantlets after thermotherapy revealed an increasing trend in virus elimination success with thermotherapy duration up to three weeks, after which the trends in elimination decreased at the fourth week (Table 9). Nevertheless, the equilibrium of attaining a high plantlet survival after thermotherapy, high regeneration rate with high proportion of virus-free meri-clones was achieved when plantlets were exposed to heat treatment for 2 to 3 weeks (Figure 5). Thermotherapy exceeding three weeks reduced plantlet survival, meri-clone regeneration and success of recovering virus-free plantlets (Figure 5).

DISCUSSION

This study was designed to determine the effect of thermotherapy on potato plantlet survival and success of eliminating PVX and PVS infection among selected potato cultivars by varying the duration of high temperature exposure. Plantlet survival decreased with the increase in duration of thermotherapy and was significantly (P≤0.05) influenced by potato cultivar, type of viral infection and their interactions. Cultivar Victoria tolerated longer periods of thermotherapy than the other two cultivars indicating different levels of tolerance to extended high temperature exposure by different potato cultivars. However, this is likely to compromise the success of virus elimination since it reduced plantlet survival. The low survival rates for Kinigi and Rwangume even at low thermotherapy exposure periods implied that these cultivars have low tolerance to high temperature (Ali et al., 2013). The higher survival rate of cultivar Victoria after four weeks of heat treatment than the other

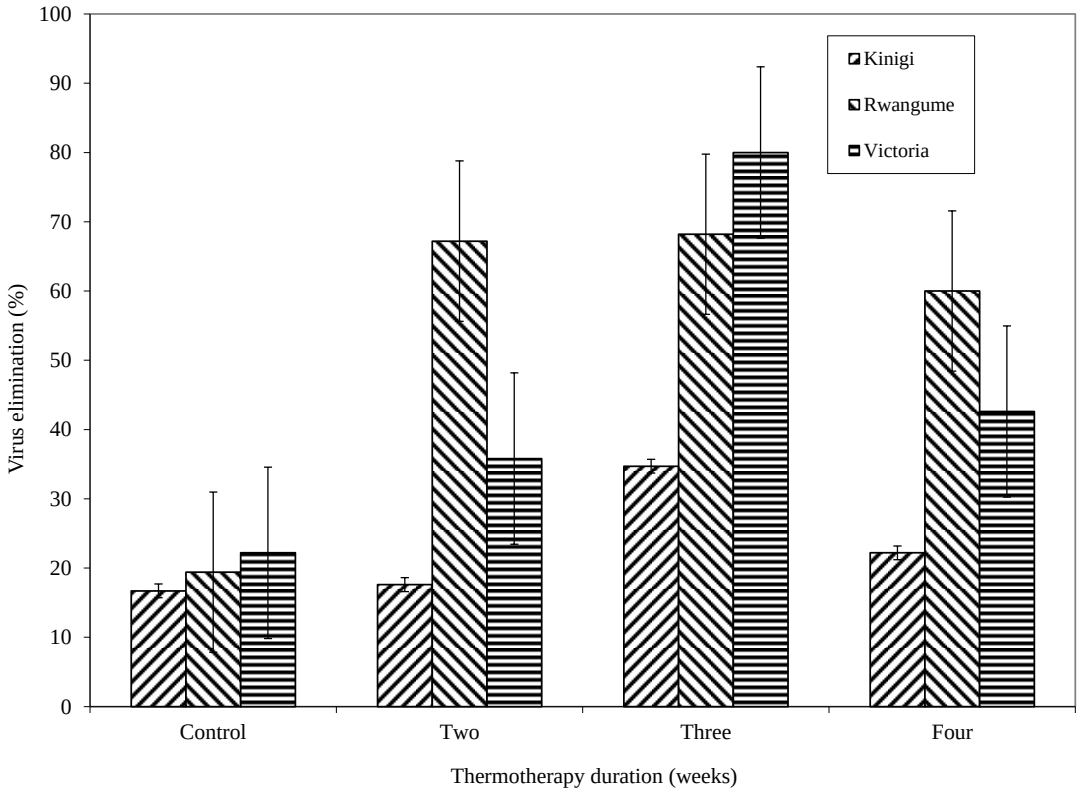

Figure 4. Effect of thermotherapy duration of virus elimination success for different potato cultivars.

Table 7. Percent Virus elimination under each of the Thermotherapy exposure durations.

Thermotherapy duration	% Virus elimination
0	19.4
2	45.9
3	55.3
4	41.6
LSD	22.67

Table 8. Correlation coefficient among plantlet survival after heat treatment (%), meristems survival (%) in tissue culture at 30 days after excision, meri-clone regeneration after 45 days (%) and virus free meri-clones (%).

Variable	Survival (%) after heat treatment	Meristems survival (%) in TC at 30 days after excision	Meri-clone regeneration after 45 days (%)	Virus free meri-clones (%)
Plantlet survival after heat treatment (%)	0			
Meristems survival (%) in TC at 30 days after excision	-0.017	0		
Meri-clone regeneration after 45 days (%)	-0.229	0.155	0	
Virus free meri-clones (%)	-0.034	0.089	0.488***	0

***Implies significant correlation.

cultivars was probably due to its ability to thrive in diverse environments in nature in contrast to cvs. Kinigi and Rwangume which are adapted to cooler agro-ecologies. Nevertheless, cultivars Rwangume and Kinigi had higher

Table 9. Effect of virus infection type and thermotherapy duration on virus elimination efficiency in infected stocks.

Type of virus	Thermotherapy duration				Mean
	0	2	3	4	
PVS	16.7	61.4	61.1	72.1	52.825
PVX	22.2	30.4	49.4	11.1	28.275
Mean	19.45	45.9	55.25	41.6	40.55
LSD	22.67	22.67	22.67	22.67	18.31

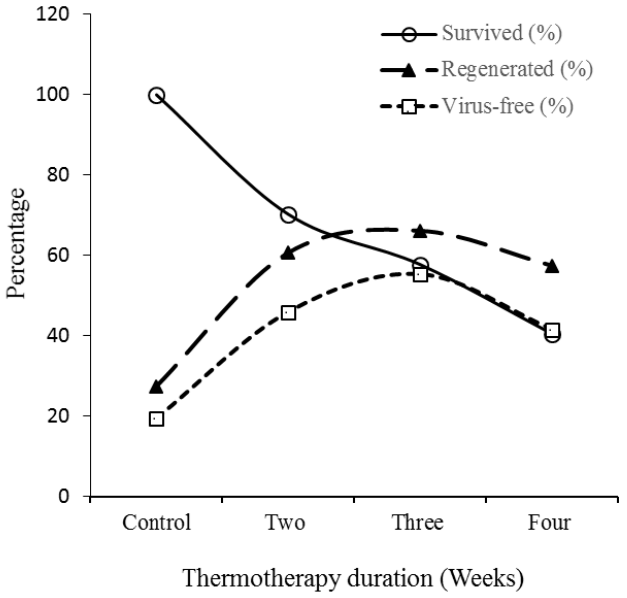

Figure 5. Equilibrium between plantlet survival after thermotherapy and mer-iclone regeneration with virus-elimination success.

meristem regeneration efficiencies implying differences in cultivars for meristem culture and meri-clone regeneration (Cheong et al., 2014; Danci et al., 2012; Biniam and Tadesse, 2008).

Virus-free meri-clones were obtained in both heat treated and controls plantlets, however, the virus-free success rate was higher in the former than the latter. The high virus elimination success in heat treated *in-vitro* plantlets may be due degradation of virions at extended high temperature exposure that resulted in unfavorable conditions for virus replication due to competition among the rapidly diving host cells and replacing virus particles (Hull, 2002). The low virus elimination efficiency of plantlets that were in thermotherapy for four weeks may be attributed to the difficulty in excising the meristems from weakened plantlets. This could have been largely contributed by picking a large portion of already differentiated cells possibly containing infectious virus particles (Biniam and Tadesse, 2008).

In this study, virus elimination efficiency was influenced by the main effects of cultivar, thermotherapy duration, virus type and interaction between thermotherapy duration and type of virus. Among the tested cultivars, Rwangume had the highest overall rate of virus elimination. Based on the data obtained in this study, the absence of significant interaction between potato cultivar and thermotherapy duration indicated that the success of virus elimination at any heat exposure duration was independent of the potato cultivar but influenced by the duration of thermotherapy and the type of virus infection involved. The success of virus elimination generally increased with the duration of thermotherapy up to the third week. The decrease of virus-free plants due to fourth week of thermotherapy could not be explained by this data considering that virus elimination success was not related to plantlet survival or meri-clone regeneration. However, interaction between heat treatment duration and virus type was significant where a high proportion of formerly PVS-positive plantlets were virus-free by the three weeks of heat treatment than stocks that were previously infected with PVX. This implies that the success of virus elimination depends on the virus species possibly due to the existence of complex interactions between the host plant cultivar and the virus species in the infected stock (Panattoni and Triolo, 2010; Panattoni et al., 2013).

The success of virus elimination was the highest for three weeks of thermotherapy especially for stocks that were previously infected with PVS, although there was no significant ($P \leq 0.05$) difference in virus elimination success among heat treatment durations except the controls. However, the impact of high temperature exposure is clearly depicted by the significant difference between heat treated plants and the controls in both PVX and PVS infected stocks. The lower success in obtaining virus-free plants from the stocks that were previously infected with PVX may be related to differences in virus inactivation temperature which is higher for PVX than PVS (Fernow et al., 1962; De Bokx, 1972; Hull, 2002). Thus, accumulated heat-units over the four week heat treatment period were probably not adequate to result in effective destruction or retardation of PVX replication to obtain more PVX-free meri-clones (Lizarraga et al., 1989).

The proportion of PVX-free plantlets after heat treatment could increase by extending the thermotherapy period. However, this may negatively affect plantlet survival, making it difficult to extract a virus-free apical dome and thus reducing the ability of meri-stems to regenerate in tissue culture. Alternatively, the low success of PVX elimination by thermotherapy and meristem tip culture could be attributed to the smaller size of PVX (470-480 nm long and 13 nm wide) than PVS (610-700 nm long and 12 to 15 nm wide) (Hull, 2002). This means that PVX can easily move through cells via the plasmodesmata up to the meristematic tip rendering PVX more recalcitrant for removal in infected potato stem

tissue than PVS (Hsu et al., 2000; Loebenstein et al., 2001).

CONCLUSION AND RECOMMENDATIONS

This study demonstrated that the success of cleaning potato stock previously infected with PVX or PVS using thermotherapy is significantly influenced by the potato cultivar, type of virus and duration of heat treatment. Data further showed that there was no significant interaction between potato cultivar and thermotherapy duration indicating that cultivars used in this study do not need adjustments to heat treatment duration in order to obtain equivalent success in virus elimination. The study additionally showed that plantlet survival decreased with increase in duration of thermotherapy and PVX-infected stock possibly requires a longer thermotherapy exposure period than PVS infected material. However, extended high temperature exposure led to reduced plantlet survival, low meri-clone regeneration and, in consequence, low virus elimination success. Virus-infected plantlets subjected to meristem tip-culture without thermotherapy also generated some virus-free meri-clones after meristem tip culture but frequencies were lower. In heat-treated virus-infected stock, the proportion of virus-free plantlets increased with longer thermotherapy periods (up to three weeks) particularly for PVS. The efficiency of virus elimination was influenced by virus type and potato cultivar on one hand and thermotherapy duration with virus type interactions on the other. However, interaction between thermotherapy and type of virus present was significant as indicated by the low success of removing PVX as compared to PVS. It is therefore imperative to conduct further studies to determine more appropriate duration of thermotherapy beyond the highest exposure period that was tested in this study for maximum success in obtaining PVX-free plants. However, this may negatively impact plantlet survival, success of meri-stem excision and meri-clone regeneration and consequent recovery of PVX-free plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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