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Full Length Research Paper

Morphological traits associated with anthracnose (*Colletotrichum lindemuthianum*) resistance in selected common bean (*Phaseolus vulgaris* L.) genotypes

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Common bean is among the most important legume crop for protein source in people's diet globally and including Kenya. Anthracnose is a common disease of legumes that causes yield loss of up to 90-100%. The aim of the study is to investigate the morphological traits associated with anthracnose resistance in selected common bean genotypes in Kenya. The study was done in three varied agro-ecological zones; University of Eldoret, Bungoma and Busia. Fifteen genotypes were evaluated on field experiment to ascertain morphological traits associated with anthracnose resistance. Field experiment was done in a random complete block design. Data were collected on morphological traits and subjected to analysis of variance in SAS version 9.1. The genotypes, Ciankui, Tasha, KK15, KK8, Miezi mbili and Chelalang showed morphological traits that were significantly ($P \leq 0.05$) associated with anthracnose resistance, and also with high grain yields of 1.5 to 2.0 t/ha. Morphological traits associated with common bean anthracnose resistance included Leaf width, leaf length, length of fifth internode of the stems, bracteolate size classification and flower colour. It is recommended that management of anthracnose by use of resistant common bean genotype seeds is essential to provide increased bean yields globally and in Kenya.

Key words: Common bean, anthracnose, morphological traits, resistance.

INTRODUCTION

Background information

Common bean (*Phaseolus vulgaris* L.) is one of the most important grain crops grown globally and in Kenya (Wagara and Kimani, 2007). it is considered a major food security crop in Kenya (Mogita et al*.,* 2017). Beans are rich in vitamins (Ekesa et al*.,* 2019) which constitute lysine, tryptophan, methionine, vitamin B, nicotine acid, calcium and iron (Wagara and Kimani, 2007). Beans in Kenya are also valued for their nitrogen-fixing quality in

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Table 1. Selected common bean genotypes.

Genotype	Optional production altitude (mm)	Maturity period (months)	Grain yields (t/ha)	Remarks
KK8	1500-1800	2.5-3.0	1.8-2.0	Tolerant to root rot
KK15	1500-1800	2.5-3.0	1.8-2.0	Tolerant to root rot
Tasha	1000-2000	2.5-3.0	1.5-2.0	Disease and insect pest tolerant
Chelalang	1000-2000	2.5-3.0	1.5-2.0	Disease and insect pest tolerant
Miezi mbili	1000-2000	2.5-3.0	1.2-2.3	Moderately resistant to ALS, Anthracnose, CBB, CBMV
Ciankui	1500-1800	2.5-3.0	1.5-2.0	Disease and insect pest tolerant
Red bean 16	1500-1800	2.5-3.0	1.8-2.0	Moderately susceptible to anthracnose
GLP92-Resistant control	1000-1500	3.0-3.5	1.2-1.7	Wide adaptability, resistant to Anthracnose, HB and Bean Common Mosaic Virus (BCMV)
GLP1127-Resistant control	1000-1500	2.5-3.0	1.0-1.5	Wide adaptability, resistant to CBMV, tolerant to rust
GLP2	1000-2000	2.5-3.0	1.0-1.2	Tolerant to Common Mosaic Virus and Anthracnose
B1-Susceptible control	1000-1500	2.5-3.0	1.0-1.2	Susceptible to anthracnose
B2-Susceptible control	1000-2000	2.5-3.0	1.0-1.5	Susceptible to anthracnose
CAL194	1000-2000	2.5-3.0	1.5-1.8	Susceptible to anthracnose
CAL33	1000-2000	2.5-3.0	1.5-1.8	Susceptible to anthracnose
RED13	1000-2000	2.5-3.0	1.5-1.8	Susceptible to anthracnose

symbiotic relationship with the rhizobium bacteria (Zinga et al*.,* 2017) in the soils.

Anthracnose disease caused by *Colletotrichum lindemuthianum* (Sacc. and Magn.) is a seed-borne fungal disease of the common bean (Leitich et al*.,* 2016). This pathogen is distributed worldwide and also it is found in Kenya. Disease symptoms on bean leaves are evident as dark, linear, and black to brick-red lesions found on the lower surface of the leaf and are mainly seen at primary and trifoliate leaf stage along the veins (Lemessa and Tesfaye, 2005; Manjunath et al., 2012). Field losses may be up to 100% under climatic and soil conditions favourable to the disease (Bassanezi et al., 2001; Lopes and Berger, 2001; Paulert et al., 2009; Tullu et al., 2003). The production and the use of anthracnose resistant seeds is one control and management measure that is effective, safe and cheap in dealing with the disease (Chen et al*.,* 2017).

Food security remains a major challenge in Africa including Kenya. This may be due to biotic and abiotic stresses (Mangeni et al., 2014). Anthracnose is among the destructive disease of common beans globally and including Kenya (Valentini et al., 2017). Most subsistence farmers in cool areas in Kenya grow common beans, which ultimately are destroyed by diseases, including anthracnose (Mangeni et al., 2014).

Quite a number of common bean breeding lines, landraces and varieties used by Kenyan farmers are susceptible to anthracnose or their reaction to the fungus is unknown, thereby limiting common bean genotypes on their improvement for anthracnose resistance (Leitich et al*.,* 2016). Therefore, constant monitoring of the common bean genotypes to be planted in the field is essential to support breeders in the development of resistant genotypes (Pinto et al., 2012). There is, therefore, a need to characterize common bean genotypes grown in Kenya for their tolerance and resistance basing on their morphological trait associated with bean anthracnose infection.

MATERIALS AND METHODS

Description of study areas

Selected common bean genotypes (Table 1) were grown in the fields in different agro-ecological zones; Busia, Bungoma and University of Eldoret.

Busia Agricultural Training Centre experimental field

Busia lies on 00°27’ 48.0’’N, 34°06’ 19.0’’ E (Latitude 0.463333; Longitude 34.105278). It is at an average elevation of 1,227 meters above sea level. Busia has an average annual rainfall of 1691 mm. The average temperature is 22°C. Busia climate is classified as tropical (Jaetzold et al., 2009). The climatic conditions of Busia are favourable for beans. The site is neighbouring Uganda which is a large producer of common beans. The site was therefore chosen for the study to promote growing of common bean genotypes which can be grown in other regions in the country.

Bungoma Agricultural Training Centre experimental field

Bungoma lies at latitude of 0.569525 N and longitude of 34.558376 E. It is located at 0.56° N 34.56° E and has an altitude ranging between 1400-1600 meters above sea level. The mean maximum

temperature is 25°C and relative humidity ranges between 70 and 80% (Jaetzold et al., 2009). The site is neighbouring Kakamega region which is a hot spot for bean growing and therefore the study was done in Bungoma to promote diverse common bean genotypes in the region.

University of Eldoret, Biotechnology field

University of Eldoret lies at latitude 00° 30' N, longitude 35° 15'E and an altitude of 2180 meters above sea level. The area is within Uasin-Gishu plateau, which is the lower highlands (LH3) agro—ecological zone. The site has a maximum temperature of 23°C and relative humidity ranging between 45 and 55% (Okalebo et al., 2007). The site is among major maize growing regions in Kenya. Common bean is among the short season crop which can be cultivated for two seasons in a year. The site was therefore selected to promote common bean growing in the region along with maize and to improve the acidic soils of Uasin-Gishu.

Experimental design and procedures

The field experimental design was a randomized complete block design (RCBD) with three replications. There were 15 blocks and each block had one bean genotype replicated three times; therefore the total blocks in the field experiment were 45. The spacing of bean genotypes was 45 cm between the rows and 15 cm within the rows.

The selected common beans were left for natural infestation of anthracnose disease. The morphological traits were measured at the vegetative (opening of primary leaves and the development of first, second and third trifoliate leaves) and reproductive stage (flowering, pod formation and pod filling) of development (van Schoonhoven, 1987). Characteristics were measured on ten common bean plants chosen at random from the experimental plot. A total of nine morphological traits were evaluated.

Morphological traits

Leaf width

Ten randomly selected common bean plants were sampled in each plot and three center trifoliate leaves were measured across the leaf veins and the midrib to determine the leaf width using 30 cm ruler. The measurements were recorded in centimeters and later converted to means by SAS program. Leaf width was considered a very important trait in morphological characterization of beans (Nassar et al., 2010) as this could associate with genotype resistance to anthracnose disease.

Leaf length

Ten randomly selected plants were sampled in each plot and three center trifoliate leaves were measured at the leaf base to the apex (along the midrib) using 30cm ruler. The measurements were recorded in centimeters and later converted to means by SAS program. Common bean anthracnose leaf symptoms occur as dark, linear, and black to brick-red lesions on the lower surface of the leaf and along the veins at the trifoliate and primary leaf stage (Lemessa and Tesfaye, 2005; Manjunath et al., 2012); therefore the leaf length could determine association of the genotype resistance to anthracnose.

C. lindemuthianum

Length of the fifth internode

Length of the fifth internode on the main stem was measured in centimeters on ten randomly selected plants using a 30cm ruler and the measurements were recorded. Anthracnose (*C. lindemuthianum*) affects the common bean stems; also brown dark eyespot develops on the young seedlings and stems. Anthracnose infections may cause the common bean leaves, pods and stems to rot and die (Masangwa et al., 2013) length of the fifth internode could therefore be an important trait to determine association with anthracnose resistance in common beans.

Bracteole leaf shape

Three leaves from each bean plant were plucked and evaluated on the bracteole leaf shape by visual determination according to earlier reports, and classification was made as; cordate, ovate, lanceolate or triangular following classification developed by Singh et al. (1991). Anthracnose (*C. lindemuthianum*) symptoms may be evident by the presence of enlarged lesions on the lower side of the leaf (Wheeler, 2012). Necrotic lesions may also be observed on the upper leaf surface and on the petioles of the bean plant. Bracteole leaf shape is therefore an important trait that could determine association of bracteole leaf shape trait with anthracnose resistance in the common beans.

Bracteolate size

The three leaves plucked from each bean plant were then measured using a 30-cm ruler to determine bracteolate of the leaves and classified as small, medium and large. Early anthracnose (*C. lindemuthianum*) symptoms are found on leaves, pods and stems of the cotyledon; the growth and development of the bean plant is stunted due to infection and anthracnose diseased areas may girdle the affected areas like leaves, pods and stems and eventually kill the seedlings (Abraham, 2015). Therefore the size of the bracteolate size was considered an important trait in determining the effects of the disease on different genotypes.

Classification of the corolla

The outer base of the corolla was classified using standard classification; striped or smooth. Different common bean genotypes were classified according to their morphology whether striped or smooth (Vazin, 2015).

Pod beak position classification

Pod beak position was classified as either placental or central. By visual look of the shape of the bean pods, their shapes at the bottom of the pod could give true picture of the pod which gave its classification (Duran et al., 2005) and the pod beak position of all the selected genotypes were evaluated on their association with the anthracnose disease. The most common signs of presence of anthracnose (*C. lindemuthianum*) are on the common bean leaves, pods and stems. Small brown-reddish to black blemishes and distinct circulated dish black to brown border with a black-grayish interior (Vazin, 2015).

Table 2. Growth habit classification and description of *Phaseolus vulgaris.*

Growth habit	Description
Type I	Determinate habit; reproductive terminals on main stem and no further node production on main stem after flowering
Type II	Indeterminate habit (vegetative terminal on main stem); further node production on main stem after flowering; erect branches borne on lower nodes; erect plant with extremely variable guide development.
Type IIIa	Indeterminate habit; moderate note production on main stem after flowering ; prostrate canopy with variable number of branches borne on lower nodes; main stem guide development extremely variable but generally showing poor climbing ability.
Type IIIb	Indeterminate habit; considerable node production on main stem after flowering; heavily branched with variable number of facultative climbing branches borne on lower nodes; guide development variable; plants generally show moderate climbing tendency on supports with resulting cone-shaped canopy
Type Iva	Indeterminate habit; heavy node production on main stem after flowering; branches not well developed compared to main stem development; moderate climbing ability on supports, with fruits load carried relatively uniformly along length of the plant.

Source: van Schoonhoven, 1987.

Growth habit

Growth habit was classified using the CIAT 1-to-4 scale where 1= determinate, 2= erect indeterminate, stems and branches prostrate with little or no climbing ability (Table 2). The common bean genotypes were characterized for growth habit because according to earlier reports, there was an indication of plant growth habit and disease development (van Schoonhoven, 1987)

Flower colour

At reproductive stage when the beans started flowering, (the first flower opened until it was fully opened), visual observation was made to identify the flower colour of each common bean genotype (De Ron et al., 2016).

Data collection

Ten plants were selected and pre-tagged from each plot using W-shaped sampling after the plants emerged. Disease epidemic data were collected from pre-tagged plants starting from the onset of the first anthracnose symptoms at vegetative and reproductive stages. At vegetative stage, the data were taken as from 14 days after bean plant emergence when the cotyledon had started appearing at soil level and begun to separate and develop primary leaves which continued to develop into first, second and third trifoliate leaves which opened and the buds on the lower nodes produced branches. At reproductive stage the data were taken from bean plants at flowering when the first flower opened, pod formation when the first pod appeared being more than 2.5 cm long and at pod filling when the first pod begun to fill (seed growth).

Data analysis

Data were collected on morphological traits and subjected to ANOVA in SAS version 9.1. Means were separated using Tukeys' and Pearson correlation analysis was done to estimate interrelationships between the morphological traits association with anthracnose resistance on the genotypes. Variability among the qualitative traits was evaluated on varied percentage rates on the traits. Also, morphological clustering was constructed using UPGMA-based dendrogram depicting Euclidean dissimilarity estimates for morphological traits.

RESULTS AND DISCUSSION

Results from the study showed that incidence and severity of anthracnose (*C. lindemuthianum*) in the three agro-ecological zones varied significantly ($p \leq 0.05$). Analysis of variance revealed that selected common bean genotypes were significantly affected by *C. lindemuthianum* pathogen which contributed to high, moderate and low disease incidence and severity depending on the genotype. This resulted to resistant (R), tolerant (T) and susceptible (S) genotypes. This is in agreement with studies made by Awori et al. (2018) who report pathogen invasion varies significantly in different genotypes. The analysis of variance of mean disease severity and incidence under field conditions revealed highly significant differences ($p \leq 0.001$) among the genotypes. In Busia, some genotypes recorded low incidence and severity and these were; Chelalang, GLP2, GLP1127, Miezi mbili and KK15. The genotypes which had moderate incidence and severity were; KK8, Tasha and Ciankui. The high incidence and severity were realized in; RED13, Redbean16, CAL33, CAL194, GLP92, B2 and B1 genotypes (Table 3).

Table 3. Incidence and Severity of anthracnose (*C. lindemuthianum*) in Busia.

Genotype	DAEI14	DAEI28	DAFI	DAPI	DAES14	DAES28	DAFS	DAPS
RED13- S	70.00^{a}	80.00^{a}	75.00^{a}	83.33^{ab}	7.33^{ab}	8.33^{a}	8.33^{a}	9.00^{a}
Redbean16- S	70.00^{a}	80.00^{a}	73.33^{ab}	88.33^{a}	7.33^{ab}	8.00^{a}	8.33^{a}	9.00^{a}
CAL33- S	73.33^{a}	83.33^{a}	76.66^{a}	88.33^{a}	7.66^{a}	8.66^{a}	8.66^{a}	9.00^{a}
CAL194-S	50.00^{b}	60.00^{ab}	53.33^{bc}	65.00^{bc}	5.00^{bcd}	6.00^{abc}	6.00^{abc}	7.00^{ab}
GLP92-S	50.00^{b}	60.00^{ab}	53.33^{bc}	65.00^{bc}	4.33^{cd}	5.00^{bcd}	5.00^{bcd}	5.66^{bc}
B2- SC	40.00^{bc}	50.00^{b}	43.33^{cd}	56.66^{c}	5.33^{abc}	6.33^{ab}	6.33^{ab}	7.00^{ab}
B1- SC	40.00^{bc}	50.00^{b}	43.33^{cd}	53.33^{c}	4.00^{cde}	5.00^{bcd}	5.00^{bcd}	6.00^{abc}
Ciankui- T	33.33^{bc}	43.33^{bc}	36.66^{cde}	50.00^{cd}	3.33^{cdef}	4.33^{bcde}	4.33^{bcde}	4.66^{bcd}
Tasha- T	26.66^{cd}	36.66^{bc}	31.66^{def}	45.00^{cde}	3.66^{cde}	4.00^{bcde}	4.00^{bcde}	4.33^{bcd}
KK15- R	10.00^{ed}	23.33^{cd}	16.66^{ef}	28.33^{def}	3.00^{cdef}	3.00^{de}	3.00^{de}	3.00^{cd}
KK8- T	10.00^{ed}	23.33^{cd}	16.66^{ef}	26.66^{ef}	2.66^{def}	3.33^{cde}	3.33^{cde}	3.33^{cd}
Miezi mbili- R	10.00^{ed}	20.00^{cd}	15.00^{f}	23.33^{ef}	3.00^{cdef}	3.00^{de}	3.00^{de}	3.00^{cd}
GLP1127- RC	4.00^{e}	11.33^{d}	13.33^{f}	21.66^{f}	1.66^{ef}	2.33^{de}	2.33^{de}	2.33^{d}
GLP2- RC	1.00^{e}	2.00^{d}	11.66^{f}	21.66^{f}	1.00^{f}	2.00^{e}	2.00^{e}	2.00^{d}
Chelalang	1.00^{e}	2.00^{d}	13.33^{f}	20.00^{f}	1.00^{f}	2.00^{e}	2.00^{e}	2.00^{d}
CV%	19.81	18.63	17.58	15.38	21.88	20.19	20.30	20.41
Grand mean	32.62	41.68	38.22	49.11	4.02	4.75	4.77	5.15
Genotype	***	***	***	***	***	***	***	***
MSD	19.56	23.51	20.34	22.87	2.66	2.90	2.93	3.18

Means with same letters are not significantly different. (*, **, ***) and ns is significant at ((P≤0.05, P≤0.01, P≤0.001) and none significant at (P≤0.05) respectively. DAEI14=Incidence at 14 days after emergence; DAEI28=Incidence at 28 days after emergence; DAFI=Incidence at days after flowering; DAPI=Incidence at days after podding; DAES14=Severity at 14 days after emergence; DAES28=Severity at 28 days after emergence;DAFS=Severity at days after flowering; DAPS=Severity at days after podding. S, Susceptible; R, Resistant; T, Tolerance; SC, Susceptible control; RC, Resistant control.

Mean values of incidence and severity among the fifteen genotypes in University of Eldoret site varied significantly (Table 5). The genotypes which recorded high anthracnose incidence and severity were; RED13, Redbean16, CAL33, CAL194, GLP92, and Ciankui while the genotypes which recorded low incidence and severity were; Tasha, KK15, KK8, Miezi mbili and Chelalang (Table 5).

Morphological data were collected on quantitative and qualitative traits. Quantitative traits included; leaf width (LW), leaf length (LL) and length of the fifth internode (Table 6), while qualitative traits included; growth habit (GH) which was realized to be determinate in all the genotypes; bracteole shape (BSH) which was found to be ovate in all genotypes; bracteole size (BSI) which was found to be large and medium; standard corolla (STC) in which all genotypes had smooth and pod beak position (PBP) in which all genotypes were placental (Table 6).

Variability among the qualitative traits

Variability among the qualitative traits was evaluated and results showed varied percentage rates on the traits (Table 7). Flower colour (FC) had three types of colour (variables); white (73%), light purple (20%) and dark purple (7%). Growth habit (GH) had only one type of growth habit (variable); determinate (100%). Bracteolate shape (BSH) had only one type of shape (variable); ovate (100%). Bracteolate size (BSI) had two types (variables); medium (27%) and large (73%). The outer base of the standard of the corolla (STC) had one variable; smooth (100%) and pod beak position (PBP) had one variable (100%).

Morphological clustering

UPGMA-based dendrogram depicting Euclidean dissimilarity estimates for morphological traits was constructed (Figure 1). Both quantitative and qualitative traits were grouped by ascending hierarchical clustering into four groups at 0.88 Euclidian distances. The genotypes which were morphologically related based on their morphological characterization were numbered numerically (1-5). Number 1- indicated that; Ciankui, Tasha, KK8, Miezi mbili, Chelalang, GLP1127 and GLP2 were closely related. Number 2- indicated that; KK15 was more close to number 1. Number 3- indicated that; Redbean16 and RED13 were more close to each other. Number 4 (0.86)- showed that; CAL33 and B1 were closely related to each other. Number 5 (0.84), showed

Table 4. Incidence and Severity of anthracnose (*C. lindemuthianum*) in Bungoma.

Genotype	DAEI14	DAEI28	DAFI	DAPI	DAES14	DAES28	DAFS	DAPS
RED13- S	66.66^{a}	76.66^{a}	71.66^{a}	81.66^{a}	6.66^{a}	8.33^{a}	8.33^{a}	9.00^{a}
Redbean16- S	60.00^{ab}	70.00^{ab}	65.00^{ab}	75.00^{ab}	6.00^{ab}	7.66^{ab}	7.66^{ab}	8.66^{ab}
CAL33- S	56.66^{abc}	66.66^{abc}	61.66^{abc}	71.66^{abc}	5.66^{abc}	7.00^{abc}	7.33^{ab}	8.00^{ab}
CAL194- S	50.00^{abcd}	60.00^{abc}	53.33^{bcd}	65.00^{abc}	5.00^{abcd}	6.00^{bc}	6.33^{ab}	7.33^{ab}
GLP92- S	40.00^{cde}	56.66^{abc}	45.00^{cde}	60.00^{abc}	4.00^{bcde}	5.66^{bcd}	6.33^{ab}	7.33^{ab}
B2- SC	40.00^{cde}	50.00^{bcd}	45.00^{cde}	55.00^{bcd}	4.00^{bcde}	5.00^{cde}	5.33^{bcd}	6.00^{bcd}
B1- SC	46.66^{bcd}	60.00^{abc}	51.66^{bcd}	65.00^{abc}	5.00^{abcd}	6.66^{abc}	7.33^{ab}	8.33^{ab}
Ciankui- T	33.33^{def}	46.66^{cd}	36.66^{def}	50.00^{cde}	3.66^{cdef}	5.00^{cde}	5.66^{bc}	6.33^{abc}
Tasha-T	23.33^{efg}	30.00^{de}	28.33^{efg}	35.00^{def}	2.00^{efg}	3.66^{def}	3.66^{cde}	3.66^{cde}
KK15-T	20.00^{fgh}	30.00^{de}	21.66^{fgh}	33.33^{def}	2.33^{efg}	3.33^{ef}	3.33^{cde}	3.33^{de}
KK8-T	13.33^{ghi}	23.33^{ef}	18.33^{gh}	28.33^{ef}	3.00^{defg}	3.00^{ef}	3.00^{de}	3.00^{e}
Miezi mbili- R	10.00^{ghi}	23.33^{ef}	15.00^{gh}	26.66^{f}	3.00^{defg}	3.00^{ef}	3.00^{de}	3.00^{e}
GLP1127- RC	8.33^{ghi}	16.66^{ef}	13.33^{gh}	18.33^{f}	1.66^{fg}	2.33^{f}	2.33^{e}	2.33^{e}
GLP2- RC	4.00^{hi}	8.00^{f}	13.33^{gh}	20.00^{f}	1.00^{g}	2.00^{f}	2.00^{e}	2.00^{e}
Chelalang- R	1.00^{i}	2.00^{f}	10.00^{h}	20.00^{f}	1.00^{g}	2.33^{f}	2.33^{e}	2.33^{e}
CV%	18.56	17.29	16.48	15.34	18.48	15.35	16.43	16.91
Grand mean	31.55	41.33	36.66	47	3.6	4.73	4.93	5.37
Genotype	***	***	***	***	***	***	***	***
MSD	17.72	21.63	18.29	21.82	2.01	2.20	2.45	2.75

Means with same letters are not significantly different. (*, **, ***) and ns is significant at ((P≤0.05, P≤0.01, P≤0.001) and none significant at (P≤0.05) respectively. DAEI14=Incidence at 14 days after emergence; DAEI28=Incidence at 28 days after emergence; DAFI=Incidence at days after flowering; DAPI=Incidence at days after podding; DAES14=Severity at 14 days after emergence; DAES28=Severity at 28 days after emergence;DAFS=Severity at days after flowering; DAPS=Severity at days after podding. S, Susceptible; R, Resistant; T, Tolerance; SC, Susceptible control; RC, Resistant control.

that; B2 and GLP92 were more closely related to each other than CAL194 which is distantly related.

Some of the common bean genotypes that were grown in different sites of the study had different flower colours. Majority of the genotypes (CAL33, CAL194, GLP92, B2, Ciankui, Tasha, Miezi mbili, KK8, GLP1127, GLP2 and Chelalang) had white flower colour while few of them (RED13, Redbean16, B1 and KK15) had purple flower colour (Figures 2 and 3).

Leaf width

The results of the common bean leaf width in centimeters of the three center trifoliate leaves in the fifteen common bean genotypes revealed significant different measurements. Twelve genotypes; CAL33, RED13, Redbean16, B1, Ciankui, Tasha, Miezi mbili, KK8, KK15, GLP1127, GLP2 and Chelalang had long leaf width above 10 cm while three genotypes; CAL194, GLP92 and B2 had leaf length of below 10 cm. The bean genotypes which had long leaf width of above 10 cm were realized to be resistant and tolerant to anthracnose (*C. lindemuthianum*) while the genotypes which had short leaf width of average 7 to 10 cm showed anthracnose susceptibility on the genotypes. Similarly, Nassar et al. (2010) reported that leaf width may influence disease damage on the leaf. Wide leaf width has large surface area hence the time taken by disease invasion could be long and finally the bean plant may survive through escape mechanism in host plant resistance. Leaf width in the recent research therefore had significant influence on the anthracnose resistance on common bean genotypes.

Leaf length

The results of leaf length proved that, Leaf length was a trait that seems to have contributed to anthracnose resistance and tolerance in most of the common bean genotypes. Among the fifteen genotypes, which were under study, only three (CAL194, GLP92 and B2 were found to have leaf length measuring less than 10 cm and these genotypes recorded high incidence and severities of anthracnose and were considered to be susceptible. The remaining twelve genotypes; CAL33, RED13, Redbean16, B1 Ciankui, Tasha, Miezi mbili, KK8, KK15, GLP1127, GLP2 and Chelalang had leaf length measuring more than 10 cm and these genotypes proved to be anthracnose tolerant. Therefore from the results, leaf length is associated with anthracnose resistance; the longer the leaf the better it could be able to overcome the

Table 5. Incidence and Severity of anthracnose (*C. lindemuthianum*) in University of Eldoret.

Genotype	DAEI14	DAEI28	DAFI	DAPI	DAES14	DAES28	DAFS	DAPS
RED13- S	56.66^{a}	66.66^{ab}	61.66^{a}	71.66^{a}	5.66^{a}	7.66^{a}	7.66^{a}	8.66^{a}
Redbean16- S	56.66^{a}	70.00^{a}	61.66^{a}	75.00^{a}	5.66^{a}	7.66^{a}	7.66^{a}	8.33^{a}
CAL33- S	53.33^{ab}	70.00^{a}	55.00^{ab}	73.33^{a}	5.66^{a}	7.66^{a}	7.66^{a}	8.33^{a}
CAL194- S	50.00^{ab}	63.33^{ab}	51.66^{abc}	63.33^{abc}	5.33^{ab}	7.00^{ab}	7.33^{ab}	8.33^{a}
GLP92- S	50.00^{ab}	60.00^{abc}	55.00^{ab}	65.00^{ab}	5.00^{abc}	7.00^{ab}	7.33^{ab}	8.33^{a}
B2- SC	36.66^{bc}	46.66^{bcd}	38.33^{bcd}	48.33^{bcd}	3.66^{abcd}	4.66^{bc}	5.33^{abc}	6.00^{ab}
B1- SC	30.00^{c}	40.00^{cde}	35.00^{cd}	43.33^{cde}	3.00^{cde}	3.66^{cd}	4.00^{cd}	4.33^{bcd}
Ciankui- T	30.00^{c}	46.66^{bcd}	31.66^{de}	48.33^{bcd}	3.33^{bcd}	4.66^{bc}	5.00^{bc}	5.33^{bc}
Tasha- T	20.00^{cd}	30.00^{de}	25.00def	31.66^{def}	2.66^{de}	3.00^{cd}	3.00^{cd}	3.00^{cd}
KK15- R	10.00^{de}	23.33^{ef}	15.00^{ef}	25.00^{ef}	3.00^{cde}	3.00^{cd}	3.00^{cd}	3.00^{cd}
KK8- T	10.00^{de}	23.33^{ef}	13.33^{f}	25.00^{ef}	3.00^{cde}	3.00^{cd}	3.00^{cd}	3.00^{cd}
Miezi mbili- R	10.00^{de}	23.33^{ef}	13.33^{f}	25.00^{ef}	3.00^{cde}	3.00^{cd}	3.00^{cd}	3.00^{cd}
GLP1127- RC	4.00^{de}	8.00^{fg}	11.66^{f}	18.33^{f}	1.66^{de}	2.33^{cd}	2.33^{d}	2.33^{d}
GLP2- RC	1.00^{e}	2.00^{g}	10.00^{f}	15.00^{f}	1.00^{e}	2.00^{d}	2.00^{d}	2.00^{d}
Chelalang-R	1.00^{e}	2.00^{g}	8.66^{f}	13.33^{f}	1.00^{e}	1.66^{d}	1.66^{d}	2.00^{d}
CV%	20.90	17.38	18.23	16.54	19.42	17.08	18.29	19.16
Grand mean	27.95	38.35	32.46	42.77	3.51	4.53	4.66	5.06
Genotype	***	***	***	***	***	***	***	***
MSD	17.69	20.18	17.91	21.41	2.06	2.34	2.58	2.93

Means with same letters are not significantly different. (*, **, ***) and ns is significant at ((P≤0.05, P≤0.01, P≤0.001) and none significant at (P≤0.05) respectively. DAEI14=Incidence at 14 days after emergence; DAEI28=Incidence at 28 days after emergence; DAFI=Incidence at days after flowering; DAPI=Incidence at days after podding; DAES14=Severity at 14 days after emergence; DAES28=Severity at 28 days after emergence;DAFS=Severity at days after flowering; DAPS=Severity at days after podding. S, Susceptible; R, Resistant; T, Tolerance; SC, Susceptible control; RC, Resistant control.

diseases invasion like anthracnose (*C. lindemuthianum*). Leaf length was not dependent on environment and hence the environment was not significant. Environment and genotype interaction was not significant on leaf length trait but genotypes were highly significant on leaf length trait. This is explained by research of Siahpoosh et al. (2015) which reported leaf length trait as a factor that influenced disease resistance in common bean genotypes. Leaf length therefore in this study had significance in anthracnose resistance.

Length of the fifth internode

The length of the common bean stem fifth internode on the main stem measured in the fifteen genotypes showed some discrimination between the resistant, tolerant and susceptible genotypes. The measurements varied among all the genotypes and these results are in agreement with the studies made by Maras et al. (2016) who reported the stem length of fifth internode on the main stem of common bean genotypes to be associated with disease incidence and severity cases. Environment x genotype interaction was not significant but the genotypes were highly significant at $p \leq 0.001$. Length of the fifth internode on the common bean genotypes was significant to anthracnose resistance.

Bracteolate shape classification

The fifteen genotypes were characterized as having ovate bracteole shape leaves. Visual observation made on the fifteen genotypes revealed broadly ovate leaflets with acuminate apices, the petial of the terminal leaflets were longer than those below. Past research made by Buah et al. (2017) and Maras et al. (2016) classified bracteole shape of plant leaves into; cordate, ovate, lanceolate and triangle. Therefore the recent research characterized the fifteen bean genotypes under field study as having one variable (100%), which was ovate leave shape and therefore there was no significance of leaf shapes in anthracnose resistance.

Bracteolate size classification

Bracteole size revealed to be broadly ovate, thin, glabrous to pubescent which measured 4-16 cm long and 2.5-11 cm broad. Bracteolate size varied significantly among the genotypes at $p \leq 0.001$ and this is in accordance with earlier studies made by Buah et al.

Table 6. Morphological traits associated with *C. lindemuthianum* resistance in the selected common beans.

Genotype	LW	LL	LC	FC	GH	BSH	BSI	STC	PBP
CAL33-S	11.00^{a}	11.00^{a}	11.44^{ab}	White	Determinate	Ovate	Medium	Smooth	Placental
RED13-S	10.77^{a}	7.38^{a}	10.11^{d}	Pink	Determinate	Ovate	Large	Smooth	Placental
Redbean16-S	10.94^{a}	10.94^{a}	10.11^{d}	Pink	Determinate	Ovate	Large	Smooth	Placental
CAL194-S	7.38^{b}	6.11^{b}	8.00^{g}	White	Determinate	Ovate	Large	Smooth	Placental
GLP92-S	7.50^{b}	6.00^{b}	8.44^{fg}	White	Determinate	Ovate	Medium	Smooth	Placental
B2-SC	7.44^{b}	6.00^{b}	9.00^{e}	White	Determinate	Ovate	Medium	Smooth	Placental
B1-SC	10.88^{a}	10.88^{a}	11.77^{a}	Pink	Determinate	Ovate	Medium	Smooth	Placental
Ciankui-T	11.05^{a}	11.05^{a}	10.88^{c}	White	Determinate	Ovate	Large	Smooth	Placental
Tasha-T	10.88^{a}	10.88^{a}	11.38^{abc}	White	Determinate	Ovate	Large	Smooth	Placental
Miezi mbili-R	10.66^{a}	7.33^{a}	8.77^{ef}	White	Determinate	Ovate	Large	Smooth	Placental
KK8-T	11.11^{a}	11.11^{a}	10.00^{d}	White	Determinate	Ovate	Large	Smooth	Placental
KK15-R	11.11^{a}	11.11^{a}	11.00^{bc}	Purple	Determinate	Ovate	Large	Smooth	Placental
GLP1127-RC	11.00^{a}	11.00^{a}	11.50^{ab}	White	Determinate	Ovate	Large	Smooth	Placental
GLP2-RC	10.66^{a}	7.33^{a}	11.88^{a}	White	Determinate	Ovate	Large	Smooth	Placental
Chelalang-R	10.77^{a}	10.77^{a}	11.44^{ab}	White	Determinate	Ovate	Large	Smooth	Placental
CV%	4.39	3.09	2.98						
Grand mean	10.21	7.12	10.38						
Environment	ns	ns	ns						
Genotype	**	**	**						
Genotype*Environment	ns	ns	ns						
MSD	0.73	0.36	0.51						

Means with same letters within column are not significantly different. (*, **, ***) and ns is significant at (P≤0.05, P≤0.01, P≤0.001) and not significant at (p≤0.05), respectively. S=Susceptible; R=Resistant; T=Tolerance; SC=Susceptible control and RC=Resistant control. LW=Leaf width; LL=Leaf length; LC=Length of fifth internode; GH=Growth habit; BSH=Bracteole shape; BSI=Bracteole size; STC=Standard corolla; PBP=Pod beak position.

Table 7. Percentages of genotypes showing different qualitative traits.

Variability	Variable	Rate (%)
Flower colour	White	73
	pink	20
	purple	7
Growth habit	Determinate	100
Bracteolate Shape	Ovate	100
Bracteolate Size	Medium	27
	Large	73
Standard Corolla	Smooth	100
Pod Beak Position	Placental	100

FC=Flower colour; GH=Growth habit; BSH=Bracteole shape; BSI=Bracteole size; STC=Standard corolla; PBP=Pod beak position.

(2017) in which the bracteole size of plants was classified to small, medium and large. The fifteen genotypes were therefore characterized into two types; medium and large. Genotypes with large bracteolate size had variability of 73% while genotypes with small bracteolate size had 27%. Therefore larger population of genotypes had large bracteolate size and most of the genotypes having large bracteolate size were those which exhibited anthracnose

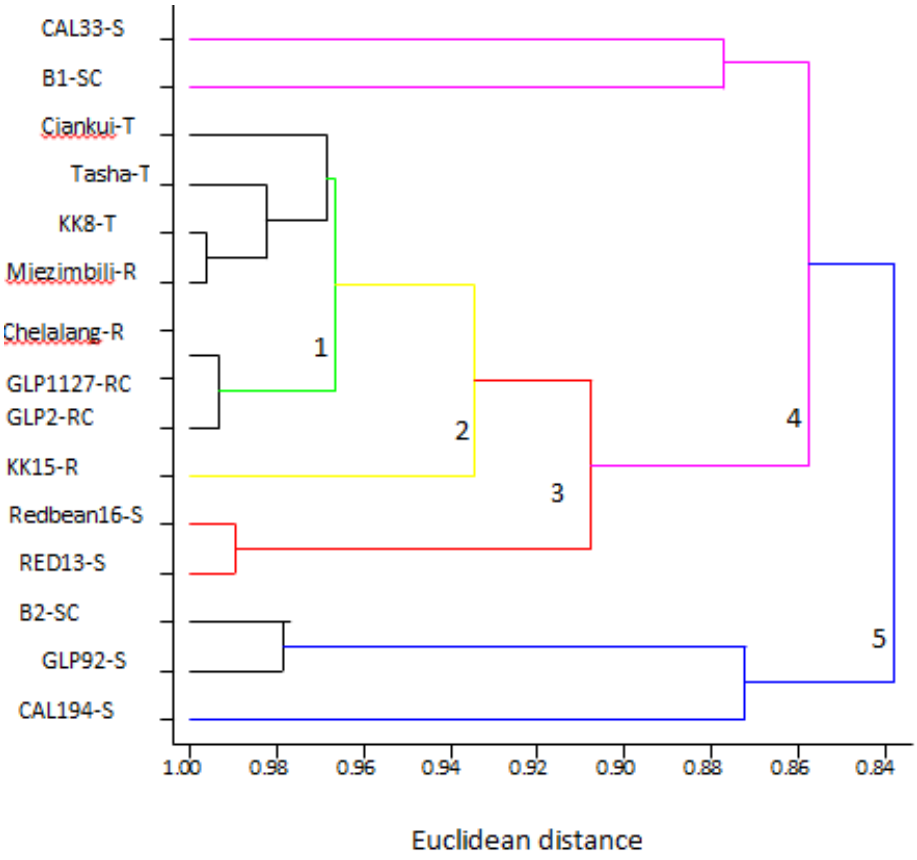

Figure 1. Morphological clustering. S, Susceptible; R, Resistant; T, Tolerant; SC, Susceptible control; RC, Resistant control.

Figure 2. Purple flower colour.

Figure 3**.** White flower colour.

resistance and tolerance. The bracteolate size classification in the study therefore had significance in association with anthracnose resistance.

The outer base of the standard of the corolla of common bean genotypes

The outer base of the standard of the corolla in common beans was all classified as smooth and therefore had one variable (100%), the smooth base. There was no significant difference among the selected common bean genotypes regarding the morphological characteristic of the outer base of the corolla. Therefore the outer base of the corolla did not have any significance in the anthracnose resistance.

The pod beak position of common bean classified as either placental or central

Pod beak positions of the fifteen genotypes studied under field experiment were characterized as having placental pod position only and this was considered as one variable (100%). Similarly, Neupane et al. (2008) reported that most of the common bean genotypes are classified as having either placental or central pod beak position and in relation to anthracnose resistance. The pod beak position is one of the major characters of beans used to identify a particular genotype in association with disease resistance as reported by Neupane et al. (2008). The pod beak position in this study did not show any significance on anthracnose resistance.

Growth habit classification

Growth habit for the genotypes was characterized as determinate type (Type I) where the common bean plant reproductive terminals were on main stem and no further node production on main stem after flowering. Earlier report made by Singh et al. (1996) is able to determine growth habit of common beans into two types; determinate and indeterminate growth habit. The selected common bean genotypes under the recent study demonstrated determinate growth habit only which was considered to be one variable (100%). The determinate growth habit has been exploited for crop breeding to decrease plant biomass and to optimize allocation between vegetative and reproductive growth and this may reduce disease and pests incidence and severities as reported by Sonah et al. (2015). Therefore the growth habit of the genotypes in the study did not demonstrate any significance of anthracnose resistance.

Flower colour

The fifteen genotypes grouped themselves into three groups according to flower colour: those with white and purple, pink and purple flower colour. The flower colour therefore had three variables; white (73%), pink (20%)

and purple (7%). The white flower colour was seen in; CAL33, CAL194, GLP92, B2, Ciankui, Tasha, Miezi mbili, KK8, GLP1127, GLP2 and Chelalang. The genotypes with pink flower colour were; Red13, Red bean16 and B1. The genotype with purple flower colour was KK15 and which was realized to be resistant to anthracnose. The genotypes that had purple flower colour were associated with anthracnose resistance. This is in accordance with research made by Rodiño et al. (2003), where flower colour was associated with disease resistance. Therefore, from the results purple colour was associated with disease resistance because the genotype KK15 which had purple colour were resistant to anthracnose unlike the genotypes which had white flower colour and most of them (CAL33, CAL 194, and B2) were susceptible to the anthracnose disease. Therefore, flower colour trait was significant in this research in associating with anthracnose resistance.

Conclusions and recommendations

Five morphological traits of the selected common bean genotypes which showed significant ($p \leq 0.05$) association with anthracnose resistance in the genotypes were; leaf width, leaf length, length of fifth internode of the stems, bracteole and flower colour. Therefore, morphological traits association with anthracnose resistance was a good indicator for determining potential best genotypes which were resistant and tolerant to anthracnose (*C lindemuthianum*) and of potential use to farmers and plant breeders. The germplasm used represented a valuable source of morphological diversity which could be exploited by plant breeders towards the improvement of the common bean resistance against pest and diseases. Thus, selection of common bean genotypes which are resistant based on morphological traits could certainly lead to genetic improvement in common bean production, hence boost the country's economy through providing income and improved food security.

It is therefore recommended that the five morphological traits which were found to associate with anthracnose resistance (leaf width, leaf length, length of fifth internode of the stems, bracteole and flower colour) can be considered for use by farmers when selecting anthracnose resistant genotypes to plant in their fields.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of the relative feed value of indigenous savanna forage shrub species in Ghana

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Animal performance mainly depends on the quantity and quality of forage available as feed and animal feed represents one of the major costs in animal production. This research was conducted in the Faculty of Natural Resources and Environment Experimental Field at University for Development Studies, Nyankpala Campus to evaluate the relative feed value of four indigenous savanna forage shrubs. These shrubs were cultivated and their leaves harvested at 7, 10 and 13 weeks after establishment. Harvested leaves were pulverized to determine ash, crude fat, crude fiber, crude protein, neutral detergent fibre (NDF) and acid detergent fibre (ADF). Metabolisable energy, dry matter (DM), digestible dry matter (DDM), dry matter intake (DMI) and relative feed values (RFV) were also estimated. Ash, crude fat, crude fibre, CP, ADF and NDF contents ranged from 7.56 to 11.22%, 1.924 to 2.812%, 9.33 to 16.11%, 4.598 to 4.960%, 20.73 to 27.22% and 33.47 to 53.06%, respectively. The DDM, DMI and RFV ranged from 67.70 to 74.84%, 2.27 to 3.66% and 120.2 to 212.6%, respectively. The NDF, ADF contents of *Tephrosia purpurea* was significantly higher than those for *Cajanus cajan* (L), *Stylosanthes mucronata* and *Securinega virosa* ($P < 0.05$). The DDM and RFV of *S. virosa* and *S. mucronata* were significantly higher than those for *C. cajan* and *T. purpurea*. The DMI in *S. virosa* and *S. mucronata* was significantly higher than those for other shrubs. The carbohydrate contents of *S. virosa* were significantly higher than the other shrubs. In conclusion all the species studied offered considerable potential as high quality forage for ruminants during the acute periods of the year when the quantity and quality of forages are limited.

Key words: Relative feed value, indigenous shrubs, plant maturity, dry matter, livestock.

INTRODUCTION

Animal production, particularly ruminants, is one of the most significant socio-economic activities in the savanna zone of Ghana, where irregular rainfall and the seasonality of forage production makes consistent production of animal feed the greatest challenge to ruminant production in the zone (Adam et al., 2010; de Carvalho, 2017).

Animal feed represents one of the major costs in animal production (Khan et al., 2015). The significance of feeding on production and general characteristics of the meat and other products from small ruminants justifies the need for studies investigating the influence of the diet

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on the quality of these animals (Pereira et al., 2016). Animal performance mainly depends on the quantity and quality of forage available to livestock (Lazzarini et al., 2009; Woolley et al., 2009). It is important to determine the nutritional value of forage in livestock nutrition, because effective livestock production is related to the amount of nutrients in the forage (Schut et al., 2010). Indigenous vegetation, broadly, constitutes an important feed reserve for the herds of ruminants in the savanna zone, but in most cases, the native vegetation is not sufficient to meet the nutritional requirements of the animals, which results in low performance rates and low profitability for rural producers (de Carvalho, 2017). Livestock feeds is almost entirely dependent on grazing of natural pastures and rangelands within the Savannah woodlands, unimproved pastures and bush fallow, with extreme seasonal variation in quantity and quality.

In the Northern region of Ghana, the climate favours the production of forages, the use of which usually costs less compared to concentrate ingredients and are always used in high proportions in ruminants' diets (Nicory et al., 2015; Silva et al., 2016).

Savanna forage shrub species, such as *Securinega virosa, Cajanus cajan* and *Tephrosia purpureum* are considered important for the savanna ecological zone because of their abundance and nutritional characteristics, besides their productive and nutritional potential to be used to feed ruminants (de Carvalho, 2017). Adequate quality feed supply is the way to curb this trend and this can be achieved by conducting feed quality test into the forages especially the browse species used by livestock. The animal category to be fed dictates the forage quality that best matches their requirements. Hence, it is very important for livestock producers to be able to assess the feed quality of the forage their animals utilize on the rangeland. This study therefore sought to determine and compare the Relative Feed Values (RFVs) of four savanna forage shrub species.

MATERIALS AND METHODS

Location of study

The study was carried out at the experimental field of the Faculty of Natural Resources and Environment, University for Development Studies, Nyankpala Campus in the Tolon District of the Northern Region of Ghana in 2014 and 2015. The study area is located within the savanna ecosystem on latitude 09° 25' N and longitude 00° 55' W at an altitude of 183 m above sea level. Nyankpala Campus of the University for Development Studies is 16 km away from Tamale, the capital of Northern Region. The area experiences mean annual rainfall range of 1,034 -1,150 mm from April to early November with mean monthly temperature of 22°C. Maximum monthly relative humidity value of 80% can be recorded during the rainy season while a minimum monthly value of 42% during the dry season is observed. The vegetation is Guinea savanna with grasses as the dominant plant species and interspersed with economic but drought resistant trees such as *Vitellaria paradoxa*, *Adansonia digitata*, and *Tamarindus indica*. The soils are well drained with low nitrogen content due to the low organic matter cover (Ziblim et al., 2016).

Experimental design

Randomized Complete Block Design (RCBD) was used in this experiment involving four savanna forage shrub species (*C. cajan, Stylosanthes mucronata, T. purpurea* and *S. virosa*) as the treatments with four replications. Experimental plots of size 4 × 4 m were constructed. Viable and clean seeds of the shrub species were planted at a depth of 1 cm and spaced 1 m between rows and within rows. Each plot contained 16 plants. For the nutritional analysis, the leaves of four randomly selected plants, one from each replicate were hand harvested and this done for each of the shrub species.

Collection and preparation of samples

Green leaves of the various shrub species were hand harvested at 7, 10 and 13 weeks after establishment. The leaves were rinsed in distilled water to remove dust and other impurities. The leaves were then chopped, air-dried, pulverized to pass a 1 mm screen, and neatly bagged in well labeled sample poly-bags for the chemical analysis. All analyses were carried out in triplicate samples.

Determination of chemical composition

The pulverized shrub samples were transported to the Spanish Laboratory at University for Development Studies, Nyankpala Campus for nutrient analysis. Nutritional compositions (Moisture, crude protein (CP), crude fat, crude fiber, carbohydrates and ash) were performed using the AOAC procedures (AOAC, 1995). Nitrogen was determined using the Micro - Kjeldahl method (AOAC, 2000) and crude protein (CP) was calculated as N x 6.25.

Crude fat was determined by extracting a known weight of powdered sample with petroleum ether at 150°C using Soxhlet equipment. The ash contents were determined by incineration of a known weight of powdered sample at 550°C for 4 h. Crude fibre content was examined by hydrolysis with weak acid and base to dissolve organic component, save fibre and was further incinerated at 550°C. Total carbohydrates (Nitrogen Free Extract) were calculated by difference. That is 100 – (% moisture + ash + % fat + % crude fibre + % crude protein). The Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF) were analyzed according to Van Soest et al. (1991). The ADF analysis is used to predict the digestible dry matter (DDM) = (88.9 - (0.779 * % ADF)) and NDF to predict dry matter intake (DMI) = (120 / % NDF). For crude fiber samples were refluxed first with 1.25% H_2SO_4 and subsequently 1.25% NaOH for 30 min each to dissolve acid and alkali soluble components present in them. The residue containing the crude fibre was dried to a constant weight and the dried residue was ignited in muffle furnace, loss of weight on ignition was calculated to express it as crude fibre.

Estimation of relative feed value (RFV)

Relative feed value was calculated from the estimates of Dry Matter Digestibility (DMD) and Dry Matter Intake (DMI) (Rohweder et al., 1978). The calculation was based on the work of Rabah and Fodil (2014).

Data analysis

All data obtained were subjected to analysis of variance (ANOVA) where nutrient levels in the shrubs were compared in relation to period of harvest with the aid of Genstat software (Release 10.3 DE (2011). Significant treatment means were separated using Fisher's

Table 1. Means and standard errors for expressions of proximate composition of indigenous shrubs.

Shrub species	Proximate composition (%)						
	Ash	C. Fat	CP	CHO	CF	NDF	ADF
C. cajan	9.17[b]	2.59[a,d]	4.86[a]	9.73[b]	14.89[b]	42.49[b]	27.22[a]
S. mucronata	10.22[a]	2.33[a,c]	4.96[a]	9.10[b]	9.33[c]	33.59[c]	20.73[b]
T. purpurea	7.56[c]	2.81[a]	4.83[a]	10.28[b]	16.11[a]	53.06[a]	26.54[a]
S. virosa	8.90[b]	1.92[b]	4.60[a]	18.16[a]	9.89[c]	33.47[c]	18.03[b]
SEM	0.21	0.13	0.14	1.37	0.27	1.42	1.59
LSD	0.62	0.37	0.42	4.00	0.79	4.16	4.65

SEM- standard error of means. LSD- least significant difference. Means bearing different superscripts within columns are statistically significant from each other ($p < 0.05$). C. Fat- Crude Fat, Crude protein, CHO- Carbohydrates, CF- Crude Fiber, NDF- Neutral Detergent Fibre, ADF- Acid Detergent Fibre.

Table 2. Effects of time of harvest on proximate composition of indigenous shrubs.

Proximate composition (%)	Period of harvest (WAE)			SEM	LSD
	7	10	13		
Ash	9.75[a]	8.71[b]	8.42[b]	0.18	0.54
Crude fat	1.94[c]	2.33[b]	2.97[a]	0.11	0.32
Crude protein	5.98[b]	6.53[a]	1.92[c]	0.12	0.37
Carbohydrates	12.26[a]	7.75[b]	15.43[a]	1.18	3.47
Crude fibre	12.33[a]	12.83[a]	12.50[a]	0.23	0.69
Neutral detergent fibre	41.10[a]	40.69[a]	40.17[a]	1.23	3.61
Acid detergent fibre	23.45[a]	22.01[a]	23.93[a]	1.37	4.03

SEM- standard error of means. LSD- least significant difference. Means bearing different superscripts within rows are statistically significant from each other ($p < 0.05$).

Least Significant Difference at 5% probability.

RESULTS

Mean proximate composition of the shrubs

Some selected chemical compositions of the shrubs were determined during the study and their mean contents are presented in Table 1. It was observed from the analysis that mean ash content varied significantly ($p < 0.001$) among the shrubs. *S. mucronata* had the highest (10.22%) mean ash content while *T. purpurea* recorded the lowest (7.56%). There was significant ($p < 0.001$) variation of the mean crude fat content among the shrub species. The highest (2.812%) mean crude fat content was recorded in *T. purpurea* and *C. cajan* while *S. virosa* had the lowest (1.924%). Though the shrub species had no significant ($p = 0.357$) effect on CP content, *S. mucronata* had the highest (4.960%) mean CP while *S. virosa* recorded the lowest (4.598%) (Table 1).

Analysis of the mean carbohydrate content showed significant differences ($p < 0.001$) among the shrubs. *S. virosa* had a significantly higher mean carbohydrate content than all other shrub species with *S. mucronata* recording the lowest (Table 1). The crude fibre content of the shrubs showed that shrub species had significant ($p < 0.001$) influence on the mean crude fibre content. Mean crude fiber content was highest in *T. purpurea* but lowest in *S. mucronata* (Table 1). The Neutral Detergent Fibre (NDF) contents varied significantly ($p<0.001$) among the shrubs. *T. purpurea* recorded the highest mean NDF while *S. virosa* had the least. Time of harvest had no significant ($p = 0.866$) effect on NDF. Mean Acid Detergent Fibre (ADF) content varied significantly ($p < 0.001$) among the shrub species. *S. virosa* and *S. mucronata* were significantly lower in ADF (Table 1).

Time of harvest and mean proximate composition

The study showed a highly significant ($p < 0.001$) effects of time of harvest on the mean ash content of the shrubs. It was noted that mean ash content was significantly higher in shrubs harvested at 7 WAE (Table 4). The influence of time of harvest on crude fat, CP and carbohydrate contents was also highly significant ($p < 0.001$). Mean crude fat content was higher in shrubs harvested at 13 WAE and whereas mean CP content was significantly higher at 10 WAE, mean carbohydrate content was lowest for shrubs sampled at 10 WAE (Table 2). However, time of harvest had no significant ($p =$

Table 3. Estimated mean relative feed value of shrubs.

Shrub species	Estimated parameters			
	% DM	% DDM	% DMI	RFV
C. cajan	41.23[a]	67.70[b]	2.84[b]	149.2[b]
S. mucronata	35.94[b]	72.76[a]	3.63[a]	204.8[a]
T. purpurea	41.58[a]	68.23[b]	2.27[c]	120.2[c]
S. virosa	43.46[a]	74.84[a]	3.66[a]	212.6[a]
SEM	1.35	1.24	0.14	9.12
LSD	3.95	3.62	0.42	26.74

Means with similar superscripts within columns are not significantly different.

Table 4. Effects of harvesting time on relative feed values of shrubs.

Estimated parameter	Harvest times (WAE)			SEM	LSD
	7	10	13		
% DM	42.27[a]	38.15[b]	41.25[a,b]	1.17	3.42
% DDM	70.63[a]	71.76[a]	70.27[a]	1.07	3.14
% DMI	3.14[a]	3.05[a]	3.12[a]	0.12	0.37
RFV	173.80[a]	170.10[a]	171.20[a]	7.90	23.16

Means with similar superscripts within rows are not significantly different.

0.325, p = 0.598) influence on the mean crude fibre and mean ADF contents (Table 2).

Estimated DDM, DMI and RFV of the shrubs

The dry matter (DM) yields of the shrub species were determined while digestible dry matter, dry matter intake and relative feed values were estimated. Mean DM yield varied significantly *(p = 0.05)* among the shrub species. Apart from *S. mucronata*, which recorded significantly low DM yield, the other three were not significantly ($p < 0.05$) different from each other (Table 3). There was relative marginal significant effect of time of harvest on the DM yield of the shrubs. Dry matter yield was higher in shrubs harvested at 7 and 13 WAE (Table 4).

Digestible dry matter (DDM) also varied significantly ($p < 0.001$) among the shrub species. Mean DDM was significantly higher in *S. mucronata* and *S. virosa* (Table 3). The shrub species presented significant ($p < 0.001$) influence on the dry matter intake (DMI). *S. mucronata* and *S. virosa* recorded significantly higher mean DMI compared to *C. cajan* and *T. purpurea* (Table 3). Time of harvest had no significant (p = 0.852) influence on the DDM and DMI of the shrub species (Table 4). A significant ($p < 0.001$) variation in mean RFV of the shrub species was observed. The highest mean RFV was recorded in *S. virosa* and *S. mucronata* while *T. purpurea* had the lowest (Table 3). The influence of time of harvest on the mean RFV was not significant (p = 0.943) (Table 4).

DISCUSSION

Mean proximate composition

The variation in mean ash content among the shrubs could be related to the physiological differences. The ash content range of 7.56-10.22% obtained from this study was comparable to that observed by Atiya et al. (2011) (7.19-13.50%) on some top fodder tree and shrub leaves in Pakistan. It was also comparable to the ash content range of 5.02-13.41% reported by Barnes (1996) on some browse species in Ghana and that of Rabah and Fobil (2014) which ranged from 9.09% in *Astragalus armatus* to 10.26% in *Anabasis artculata*. Javed et al. (2008) also reported varied ash contents of fodder tree leaves from 3.5% (*Quercus incana)* to 8.1% (*Aesculus indica*).

Generally, it was observed that the ash content decreased with advancing maturity of the leaves. The decline in ash content in older leaves means that less amount of minerals will be present. Shrubs harvested at 7 WAE were higher in ash content than those harvested at 10 and 13 WAE. This observation was in disagreement with the findings of Kökten et al. (2012) who noted a significant increase in ash content with advancing maturity. It was also in contrast with Tolunay et al. (2009) and Haddi et al. (2003) who observed that ash content increased with maturation in kermes oak and halophyte shrubs, respectively. Parlak et al. (2011) also showed significant increase in ash content with advancing

maturity.

There was significant ($p< 0.05$) variation of crude fat content among the shrub species. Similar variations in chemical composition have been reported for some fodder trees and shrubs of Algerian arid and semi-arid areas (Bouazza et al., 2012). This could partly be due to the physiological and genetic differences of the shrubs and also the bioclimatic conditions. The mean crude fat content varied from one species to another and this variability can be attributed to the bioclimatic and genetics of the species. The range of mean crude fat content established from this study (1.92 to 2.81%) was relatively lower than a range of 4.59 to 6.30% of some leguminous forage shrubs reported by Laamouri et al. (2015). Atiya et al. (2011) also reported a crude fat content range of 1.44 to 6.45% of some top fodder tree leaves and shrubs in Pakistan. It was, however, comparable to crude fat content range of 1.25 to 3.24% of some leguminous herbaceous plants (Laamouri et al., 2015). As livestock feed, fats function much like carbohydrates in that they serve as a source of heat and energy and for the formation of fat due to the larger proportion of carbon and hydrogen. However, fats liberate more heat than carbohydrates when digested, furnishing on oxidation approximately 2.25 times as much heat or energy per pound as compared to carbohydrates. The crude fat content of the species increased with maturity. Crude fat content was higher in plants harvested at 13 WAE and this could be attributed to the physiological and structural changes as the plants mature (Oduntan and Olaleye, 2012).

The mean CP contents of the shrub species studied were similar. The CP values obtained in this study were very low and inconsistent with those found by Blair (1990) who reported CP values between the range of 12- 32% with an average of 18%. They were also incomparable with the range of 11- 20% (Bayer, 1996; Larbi et al., 1993) and 15.59- 20.99% (Oji et al., 1998; Oji and Isilebo, 2000) reported for browse species in Southern Nigeria. Similarly, higher CP content range of 12 -30% for tropical tree legumes (Norton, 1998) and an average of 12.50% in West African browse species (Le Houerou, 1980) have been documented.

Generally, the CP contents of the shrubs were lower than the minimum requirement of 7 - 8% DM for optimum rumen microbial function in ruminants (Van Soest, 1994; Norton, 1998) and by extension, the function of the rumen microbes is affected when the CP level in a ration is less than 10%. This means that the shrubs have low potential as protein source in ruminant feeding and therefore supplementation with high protein feed will be required. However, the CP contents of shrubs were comparable to report by Lardy et al. (2004) who indicated that CP content of 5% is common in range forage during late fall and winter. They were also comparable with that of Rogosic et al. (2006) who reported CP values of leaves and twigs of some Mediterranean maquis shrubs ranged from 4.9 to 7.8%. The results were also in conformity with the observation by Villalobos et al. (1997) and Patterson et al. (2003) that nutrient deficiencies in a cow are more probable in fall and winter when nutrient contents of the grazed forages are low.

The relatively low CP content of the shrubs could have been influenced by the presence of condensed tannins limiting its availability (Yifei et al., 2009). Rittner and Reed (1992) observed that CT negatively correlated with N degradability and CP for that matter of fodder shrub leaves. The low CP contents of the shrubs could also be attributed to the low N content and by extension to low organic matter content of the soil (Oyinlola and Jinadu, 2012). The slight variation in mean CP content among the shrubs could possibly be due to differences in amount of NDF and ADF. This assertion is consistent with results of others (Haddi et al., 2003; Pecetti et al., 2007). The study observed that mean CP content of the shrubs declined as the shrubs advanced in maturity, which was in concordance with other investigations (Distel et al., 2005; Kökten et al., 2012). This is because as CP content in the plant declined protein synthesis is being inhibited by the weak photosynthesis at the more mature stage (Throop, 2005).

The variation in the carbohydrate levels in the shrubs could be genetic since they were exposed to the same environmental conditions. The range of carbohydrate content obtained from this study was lower than 21.38-47.67% reported by Farrukh and Mufakhirah (2009) in Pakistan. However, Farrukh and Mufakhirah (2009) reported that high carbohydrates content at late phenological stage of plants is less beneficial to livestock due to their low digestibility. The low carbohydrate contents are indicative that the shrubs could be poor sources of energy and the low carbohydrates could be caused by moisture stress since the experiment was carried out during the dry season. There was an increased trend of carbohydrates levels in the shrubs from 7 to 13 WAE. This means that carbohydrate contents in the shrubs increased as they advanced in maturity (Gąsecka et al., 2008). As the plants mature there could be accumulation of soluble sugars resulting in the high carbohydrate content. The reduction in carbohydrate contents at 10 WAE could have been influenced by temperature stress (Jaleel et al., 2009).

The high CF value for *T. purpurea* was in concordance with a 14.62% CF value reported by Mbomi et al. (2012) among other *Tephrosia* species. The range of CF values recorded in this study was lower than that of CF contents of browse trees reported by Ogunbosoye and Otukoya (2014) which ranged from 23.1% in *Leucaena leucocephala* to 38.1% in *Tamarindus indica*. However, it was comparable to CF contents of some browse plants with a range of 15.74% (*Mangifera indica*) to 19.24% (*Daniellia oliveri*) as reported by Ladipo and Akinfemi (2014). The relatively low CF content of the shrubs indicates that their digestibility (enzymatic degradation)

will be high since the two are inversely related. Nutritionally, fibre has both physical and chemical attributes that are related to the mechanical processes of digestion and to enzymatic degradation associated with fermentation. The difference in the CF contents of the shrub species could be attributed to their physiological characteristics and their responses to climatic conditions of the study area. Analysis of the CF contents of the shrubs among the harvesting stages showed no significant difference. This means that period of harvest had little effect on the CF content of the shrubs investigated.

Neutral detergent fibre can be a significant determinant of forage quality and digestibility, which directly may affect intake of dry matter and animal performance (Linn, 2004). Mean NDF value varied significantly among the shrubs, where the highest value (53.06%) was recorded by *T. purpurea* and lowest value (33.47%) by *S. virosa.* According to NRC (2001), 36% NDF is ideal for forage for domestic animals. Greater than 36% NDF increases limits of intake due to rumen fill and less than 36% results in insufficient fibre for rumen scratch factor and proper rumen function. The NDF contents of *C. cajan* and *T. purpurea* were above the ideal NDF of 36% for domestic animals (NRC, 2001) while that of *S. mucronata* and *S. virosa* were less. However, the NDF contents of *S. mucronata* and *S. virosa* were within the range of 25-35% of DM as the optimum requirement which will maximize energy intake of cows in early lactation (NRC, 1989). NDF range of 35-40% has been recommended by El Shaer and Gihad (1994) to be within the normal range of nutritious fodders.

Fibre stimulates the cardinal region of the reticulum to induce regurgitation, rumination and ruminal motility. The high NDF (hemicellulose) content of *C. cajan* and *T. purpurea* could have been caused by genetic factors and also environmental factors such as temperature which might have aided the thickening of the cell walls (Wilson et al., 1991) and enhanced lignin synthesis, both of which lower digestibility (Buxton and Fales, 1994). The high NDF contents of *C. cajan* and *T. purpurea* in this case might be considered as a potential energy source for the rumen microorganisms.

Advance in maturity of the shrubs had no significant effect on the NDF content. This observation was inconsistent with Kökten et al. (2012) who documented a significant increase of NDF contents with age of plant. The observation may primarily be due to inadequate spacing among the periods of harvest used. The mean ADF content recorded in this study ranged from 18.03% (*S. virosa*) to 27.22% (*C. cajan*). The mean ADF contents were lower than that reported by Ogunbosoye and Babayemi (2012) of some browse plants in Southern Nigeria. However, the ADF contents of the shrubs were comparable to the recommended dietary minimum range of 19- 21% for dairy cows (NRC, 1989). The mean ADF contents obtained were also within the range of ADF values reported by Kökten et al. (2012). The mean ADF values were comparable to those reported by Daryl et al. (2006) of foliages of some savanna plants. High fibre content (NDF and ADF) reduces forage digestibility. It was revealed in this study that there was no significant influence of time of harvest on mean ADF values of the shrubs, however, means ADF content was highest in plants harvested at 13 WAE. This observation was consistent with the findings of Kramberger and Klemencic (2003) and Sultan et al. (2007) who reported increased ADF with maturity of grasses.

Estimated DDM, DMI and RFV of the shrubs

The variations in DM content could be related to physiology of the various shrubs and their response to environmental conditions of the study site. The mean DM content obtained from this study was similar to those reported by Yavuz (2007) and Ogunbosoye and Babayemi (2012) of some browse plants. The DM results were also comparable to DM values obtained by Bouazza et al. (2012) on some forages in Algeria. Relatively, the DM content of the shrubs was higher than that obtained by Gonzalez-Garcia et al. (2009) on *Leucaena leucocephala* cultivars in both dry and wet seasons. The shrubs, therefore, would be able to provide enough dry matter as feed for ruminant animals in the study area.

The marginal decrease in DM contents with advancing maturity of the shrub species is in agreement with the findings of Kamalak (2006) and Khazaal et al. (1993) who noted decreased DM contents with the advancing maturity of Lucerne hay. The decrease in DM content may presumably be due to an increase in the number of senesce leaves as the plants aged. The estimated parameters (DDM, DMI, and RFV) varied significantly among the shrub species and could be due to the different physiology of the shrubs and their response to the environmental conditions of the site. The highest DM, DDM, DMI and RFV contents were determined in *S. virosa*. The relative feed values ranged from 120.20 (*T. purpurea*) to 212.60 (*S. virosa*) and the range was within the standards quoted by Hay Market Task Force of American Forage and Grassland Council (Rabah and Fodil, 2014). Based on these standards, *S. mucronata* and *S. virosa* would be considered prime feed ingredients (RFV>151). It was observed that shrubs with higher RFVs had lower NDF and ADF contents indicating the influence of the fibre content on the feeding values of the shrubs. Consequently, as percent NDF and ADF decreased the RFV increased and this observation is in concordance with the findings of Shroeder (1994) and Appiah et al. (2012). Larger RFV of a shrub species could indicate higher feed quality. The estimated parameters of all the shrub species decreased with increased in the age of the leaves (Kökten et al., 2012). The estimated parameters were highest in leaves harvested at 7 WAE.

Conclusion

Maturity (age) of shrub species affected the proximate compositions. Though the protein content was relatively low in the shrubs, the other proximate compositions were within the requirements for the different categories of animals. *S. mucronata* and *S. virosa* were considered prime feed ingredients (RFV>151).

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Expression of *Cocculus hirsutus* trypsin inhibitor promotes endogenous defensive response against *Helicoverpa armigera* and enhanced levels of antioxidants

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Proteinase inhibitors are a group of defense related proteins, natural antagonists of proteinases, induced upon herbivory, play a defensive role against polyphagous insects and phytopathogens. Serine proteinase inhibitor isolated from *Cocculus hirsutus* (L.) *Diels*, *Cocculus hirsutus* trypsin inhibtor (ChTI) was found effective as antifungal, bactericidal and nematicidal agent. Tomato plants expressing ChTI have been developed by agro-infection with almost 27% transformation efficiency. Stable integration and expression of ChTI has been established by polymerase chain reaction (PCR), inhibitory assay and western blot assay. Transgenic plants showed increased fruit yield, antioxidants, phenolics, flavonoids and titratable acidity. Protein extracts of tomato plants inhibited *Helicoverpa armigera* (gut proteinases up to 40%. Transgenic plants MT_2 and JT_2 challenged with 2nd and 4thinstar *H. armigera* (Hubner) larvae, showed delayed larval growth with 100% mortality. The results put together suggest that ChTI is a potential candidate for developing transgenic plant with multiple biotic stress tolerance.

Key words: ChTI, *Helicoverpa armigera*, insect bioassay, transgenic tomato, trypsin inhibitory assay.

INTRODUCTION

Environmental stress is one of the major challenges for plants' growth and productivity. To overcome stress spawned by herbivory, plants up-regulate defense genes encoding for proteins, secondary metabolites, toxic chemicals and repellents (Jamal et al., 2013). In plants, proteinase inhibitors are major defendants, form inhibitory complexes with specific proteinases by irreversible trapping or tight binding interactions (Clemente et al., 2019). Proteinase inhibitor genes are expressed to regulate proteinase activity within the cell (Rustgi et al., 2017), as response to insect damage, mechanical wounding (Tamayo et al., 2000; Haruta et al., 2001; Laluk

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and Mengiste, 2011) possibly through systemin / jasmonic acid mediated signalling cascade (Yang et al., 2015). Plant Protease inhibitors (PIs) are classified based on their specificity into 4 mechanistic classes' cysteine, serine, aspartate and metallocarboxy proteinase inhibitors and data base on plant–PIs is accessible at http://bighost.area.ba.cnr.it/PLANT-PIs (De Leo et al., 2002; Habib and Fazili, 2007) .

Most of the lepidopterans gut proteinases are trypsin/chymotrypsin like enzymes (Giri et al., 2002; Tanpure et al., 2017), hence plants accumulate SPIs in the tissues as one of the defense strategy against herbivores. These inhibitors are widely studied as defense proteins against bacteria (Kim et al., 2009; Bacha et al., 2017), fungi (Quilis et al., 2007; Bhattacharjee et al., 2010; Pariani et al., 2016), insects (Dunse et al., 2010; War et al., 2012), nematodes (Turra et al., 2009, Vieira et al., 2015; Papolu et al., 2016). Many insects combine multiple strategies to circumvent the antinutritional effect of PIs viz. (a) up-regulation of proteinases with different substrate specificity to compensate the loss (Winterer and Bergelson, 2001; Zhu-Salzman et al., 2003), (b) synthesis of proteinases degrading PIs (Wu et al., 1997) and (c) over-consumption of PI expressing tissues to minimize nutritional stress (Cloutier et al., 2000). Further, insects switch to an alternative set of proteinases that allow them to thrive on host plants. Since, insects have the ability to adapt to endogenous PIs as a result of coevolution (Wu et al. 2010, Harsulkar et al. 2002) and transgenic plants expressing these PIs. Identification of novel PIs from non-host plants and their effectiveness against insect gut proteinases and express constitutively could be a promising approach to develop plants resistant to biotic stresses (Stout et al., 1999; Tamayo et al., 2000; Srinivasan et al., 2005; Tamhane et al., 2005). Expression of the proteinase inhibitor gene has been reported to be positive and resulted in increase in seed content, growth rate and biomass (Gutiérrez-Campos et al., 2001; Schluter et al., 2010). Leaf proteome analysis indicates ectopic expression of stress related genes in leaves of transgenic plants (Munger et al., 2012). Transgenic plants with increased tolerance to abiotic stress have also been reported by many groups (Stout et al., 1999; Goulet et al., 2008). These pleiotropic effects have paved way for crop improvement.

Serine proteinase inhibitor belonging to the Kunitz inhibitor family has been characterized from *C. hirsutus* (ChTI) and found effective against mid-gut proteinases of *Helicoverpa armigera* and *Spodoptera litura*. ChTI is a monomeric protein of ~18kDa, with a narrow pH range (7-9) and higher thermo-stability (70°C). *In vitro* feeding of ChTI caused significant mortality of *H. armigera*, *S. litura* (Fabricius) larvae, and exhibited potential antifungal activity (Bhattacharjee et al., 2010). In the present study, we discuss the results of stable integration of ChTI and its constitutive expression at various stages of plant growth, effectiveness against *H. armigera* larval growth, nutritive and morphological traits during growth and fruit development in tomato.

MATERIALS AND METHODS

Plasmid constructs

pChTI (amp^r) and p*CAMBIA* (kan^r, hyg^r) were grown in LB media containing ampicillin and kanamycin at 37°C. *pChTI* was amplified with M13/ChTI specific forward and reverse primers(*ChTI*- Forward- 5' ACCTGCGCCAATCAATGAG 3'; Reverse- 5' GCAGAAGTCACGACCGAC 3' and M13 - Forward- 5' GTAAAACGACGGCAG 3'; Reverse- 5' CAGGAAACAGCTATGAC 3'). *ChTI* amplicons and p*CAMBIA* were digested with *EcoR I* and *Hind III* and separated on 1.0% agarose gel. Fragments were co-eluted by freeze thaw method, washed with phenol:chloroform:isoamylalchohol (25:24:1 v/v) followed by 70% aqueous ethanol. Air dried pellet was dissolved in 5 µl of sterilized water and ligation was carried out using T4 DNA ligase (*pCAM-ChTI*) and transformed into *Escherichia coli* DH5α and *Agrobacterium* strain LBA4404 (Sambrook et al., 1989; Jyothishwaran et al., 2007). Transformed colonies selected on LB plates containing ampicillin and kanamycin, were analyzed for the presence of *ChTI* and *hpt* (Forward- 5'-TAGAAAAAGCCTGAACTCACCG-3' and Reverse- 5'-TATTTCTTTGCCCTCGGACG-3') using gene specific primers.

Development of transgenic tomato

Seeds of cherry tomato line 252 obtained from University of Horticulture Sciences, Bagalkot were rinsed with sterile distilled water, followed by 4% (v/v) sodium hypochlorite solution for 15 min and sterile distilled water. Tomato plants were transformed with *pCAM-ChTI*, as per protocol developed in our group using cotyledons of the germinated seedling as explants (Manamohan et al., 2011; Somayaji et al., 2014). MS media composition used at different stages of transgene development are as follows: 1. MS media without any hormones; 2. MS media for co-cultivation- MS media + 0.1 mg L^{-1} IAA + 4.0 mg L^{-1} BAP + 100 µM Acetosyringone; 3. MS media for proliferation- MS media + 0.1 mg L^{-1} IAA + 4.0 mg L^{-1} BAP; 4. MS media for selection- MS media + 0.1 mg L^{-1} IAA + 4.0 mg L^{-1} BAP + 2.00 mg L^{-1} Hygromycin, and 5. MS media for rooting- MS media + 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 2.00 mg L^{-1} Hygromycin. Plantlets with well-developed roots were carefully washed and transferred to paper cups containing coco-peat and irrigated with sterile water regularly, and half strength MS media once a week, maintained at 25°C with relative humidity of 95% and light intensity 30 lux. After two weeks, plants were transplanted into large earthen pots containing farm yard manure, red soil and sand (1:2:1 w/w).

PCR analysis

Genomic DNA from a total of 56 plants was isolated from 5th leaves from the top (Doyle and Doyle, 1987) and purity of DNA was assessed by absorbance ratio at 260/280 nm. PCR was carried out using *ChTI* and *hpt* primers to confirm the recombinants: - initial denaturation at 94°C for 5 min, with 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 90 s, extension at 72°C for 2 min and final extension of 72°C for 10 min. Positive transformants were forwarded to subsequent generations and stable integration of *ChTI* was assessed by PCR.

Trypsin inhibitory assay

Acetone powder of the leaves from individual plants was ground with extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 25 mM ascorbic acid, and 10 mM β-mercaptoethanol at pH 7.4; 1:5 w/v), centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant having total soluble proteins (TSPs) was incubated at 70°C for 10 min; snap chilled, centrifuged at 12,000 rpm for 20 min at 4°C (Bhattacharjee et al., 2010). Most of the plant serine proteinase inhibitors are thermostable unlike their counter acting proteinases and ensures limited contribution of endogenous proteolytic activity during the assay. Further use of acetone powder during the isolation of ChTI, also avoids the interaction of low molecular weight metabolites interfering with the inhibitory assay. Supernatant (Heat Stable Proteins -HSPs) was used for trypsin inhibitor assay using casein digestion method (Kakade et al., 1969; Bhattacharjee et al., 2010).

Specific activity staining

50 µg of HSP from the leaf extract of control and transgenic plants were separated on 10% anionic PAGE (Sambrook et al., 1989) and gel was incubated with 0.1 M potassium phosphate buffer pH 7.6 for 10 min followed by incubation with trypsin solution (100 µg trypsin/ml in 0.1 M potassium phosphate buffer pH 7.6) for 30 min at 37°C. Gel was washed with distilled water couple of times, and incubated with 10 ml of substrate solution containing 2.5 mg of acetyl-DL-phenylalanine-β-napthylester and 0.55 mg/ml fast blue RR in 0.1 M potassium phosphate buffer, pH 7.6 (Filho et al., 1978; Bhattacharjee et al., 2010), till colour development. The bands containing trypsin inhibitor showed unstained clear zone against intense pink background.

Immunoblot analysis

Laying hens were injected subcutaneously with 100 µg of affinity purified ChTI (Bhattacharjee et al., 2010) in Freund's complete adjuvant followed by 3 doses in Freund's incomplete adjuvant in week interval. IgYs were purified as per method described by (Song et al. 1985). HSPs from transgenic leaf, fruit and shoot were separated on 10% SDS-PAGE and electro-blotted onto PVDF membrane (NEN Life sciences, England), ChTI specific band was detected with ChTI-IgY (1:1000 v/v) and anti-IgY-rabbit antibody conjugated with HRP (1:2000 v/v). Bands were visualised by incubating with TMB/H_2O_2 substrate solution (1:10 v/v). For *in vivo* localization of ChTI, leaf tissue imprint analysis was carried out. Fully expanded leaves were placed on PVDF membrane sandwiched between Whatman filter papers and pressed with even force. Serological reactions were carried out to detect ChTI.

Phenotyping transgenic plants

Leaf shape, plant height, number of fruits per plant, fruit weight, time taken for flowering, fruiting, and ripening were recorded in control and transgene plants. Two transgenic lines from MT_0 and JT_0 were taken for analysis. T_0 lines were forwarded for further generations up to T_2 and 5 plants from each line were used to study.

Biochemical analysis

Fruits from MT_0 and JT_0 and their subsequent generations up to T_2 from randomly selected 5 plants in each line were analyzed for nutritional traits. 10 ripened fruits were harvested and individually analysed for titratable acidity (TA), ascorbate, phenols, flavonoids, lycopene, β-carotene, total antioxidant capacity. Titratable acidity was determined by titration method using citric acid standard and expressed as % acidity (AOAC, 2000). Ascorbate was estimated by 2, 6 dichloro phenol indophenol method expressed as mg ascorbic acid equivalent per 100 g fresh weight (AOAC, 2006). Total phenolic content was measured using Lowry's method expressed as mg gallic acid equivalents per 100 g fresh weight. Total flavonoids were expressed as catechin equivalent per 100 g fresh weight (Singleton and Rossi, 1965). β-carotene and lycopene were expressed as mg of carotene equivalent /100 g fresh weight (Lichtenthaler, 1987). Total antioxidant capacity (Aoc) was measured using FRAP assay (Benzie and Strain, 1996) and expressed as mg ascorbic acid equivalent (AEAC) per 100 g of fresh weight.

Insect bioassay of transgenic plants

Detached leaf assay was carried out using fully expanded leaves of control (WT) and transgenic plants (JT_2) expressing ChTI [3100–3200 TIU/g tissue] were placed on 2% (w/v) agar plates (Giri et al., 2002). Single larva (2^{nd} instar and 4^{th} instar) per leaf was released and larval biomasses were measured at 24 h. For feeding choice assay, two larvae per plate containing control (WT) and transgenic leaves (JT_2) were used. After 24 h amount of leaf left after feeding was recorded. 5 leaves each from each transgenic plants was used per assay.

Effect of ChTI on *H.armigera* gut enzymes

Second instar *H. armigera* larvae were dissected on ice, mid gut was separated and stored at 4°C. Mid gut was suspended in 0.2 M glycine- NaOH buffer pH 10.0 (1:5 w/v), homogenized, and centrifuged for 15 min, 12000 rpm at 4°C. *H. armigera* gut proteinases (HGPs) and inhibitory activity of ChTI (2000 TIU/ml) extracted from transgenic leaves (JT_2) on gut proteinases was assayed by casein digestion method. HGP's were separated on native PAGE at 4°C and were visualized by activity staining by pre-incubation with transgenic (JT_2) and control (WT) leaf extracts.

Statistical analysis

Analysis of variance was carried out to assess the differences, some of the parameters documented in transgenic and control plants. Results were analysed by one way ANOVA program of graph pad prism 5.0. Comparisons of means were done using "Bonferroni's Multiple Comparison Test" ($p<0.05$). Correlation between inhibitory activity and phenols, flavonoids, carotenoids, ascorbate and titratable acidity was made by the Person's procedure ($p<0.001$). Intergeneration regression was carried out using Microsoft excel programme ($p<0.05$).

RESULTS

Transgenic tomato expressing ChTI

pCHTI (amp^r), p*CAMBIA1301* (kan^r) were grown in LB media with appropriate selection markers at 37°C. Since ChTI exhibited bactericidal activity when *E. coli* harbouring *pChTI* was grown for long periods, the plasmid was isolated from 3h grown culture. *pChTI* amplified with M13/ChTI specific forward and reverse

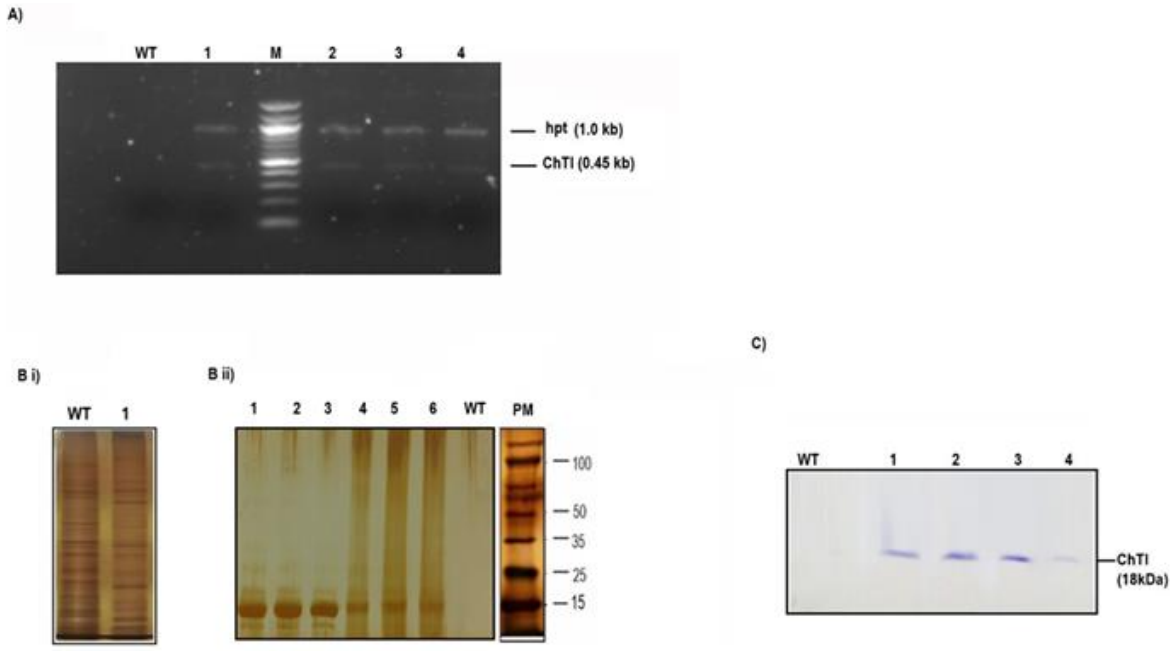

Figure 1. Screening and confirmation of ChTI in transgenic tomato plants. Plants conferring hpt resistance were screened for the presence of ChTI by PCR, and its expression by western blotting. A- PCR analysis of T0 transgenic and WT plants using *ChTI* and *hpt* specific forward and reverse primers. Lane 1-4: PCR amplicons of *ChTI* (0.45kb) and *hpt* (1kb) confirming transgene integration, M: 100bp DNA ladder, WT- non transgenic (control). B- Silver stained SDS-PAGE visualisation at different stages of extraction and purification of ChTI (18kDa) from WT and transgenic leaf extracts. B i) - lane 1- Total soluble fractions isolated from WT extract, Lane 2- TSPs from transgenic extract, B ii) Lane WT - HSPs from WT tissue , Lane 1-3- HSPs from Transgenic tissue , Lane 4-6 - Affinity purified ChTI from transgenic extracts. C -Western blot visualisation of ChTI in T0 plant tissue extracts, in the figures lanes: WT- non transgenic, expression levels of ChTI in transgenic tissue, Lane 1- root, Lane 2- shoot, Lane 3 - fruit, Lane 4- leaf.

primers, yielded 0.7 kb and 0.45 kb amplicons respectively (Supplementary Appendix Figure S1-A). M13 amplicons of ChTI and *pCAMBIA1301* were digested with *EcoRI* and *HindIII* and separated on 1.0% agarose gel. Suitable gel bits were pooled, co-eluted by freeze thaw method, and ligated using T_4 DNA ligase. Recombinant *pCAM-ChTI*, was transformed into *Agrobacterium tumefaciens* LBA4404 *(*rif^r) and selected on LB containing kanamycin. PCR amplification of *pCAM-ChTI* with *ChTI* and *hpt* gene specific primers resulting in 0.45 and 1.0 kb amplicons, confirmed the transformants selected on LB (kan^r, rif^r) media (Supplementary Appendix Figure S1-C). *Agrobacterium* mediated transformation of ChTI resulted in 25.7% efficiency and positive T_0 plants transferred to soil medium, grown under greenhouse conditions.

Screening and expression of ChTI in tomato plants

Leaf genomic DNA amplification with *ChTI* and *hpt* primers yielded amplicons corresponding to 0.45 kb and 1 kb of *ChTI* and *hpt* in transgene plants only, confirms *ChTI* integration (Figure 1A). Further, HSPs fraction was subjected to SDS-PAGE analysis, to ascertain the presence of ChTI specific protein (Bhattacharjee et al., 2010; Figure1Bii). Western blot analysis using ChTI-IgY indicated the presence of 18 kD protein corresponding to ChTI in transgene plants (Figure 1C) and *in gel* staining activity for ChTI confirmed inhibitory activity (Figure 2B). Tissue imprinting of leaves showed the uniform distribution of ChTI (Figure 2C). HSPs exhibited high inhibitory activity ranging from 3000-3500 TIU/ g in aerial tissues (leaf, shoot and fruits) and 2000-2800 TIU/g in roots. However, there was no ChTI activity in the WT (control) plant tissues (Figure 2A). Leaf TSP's, HSPs, and trypsin affinity purified inhibitor (4.5, 2.1 and 0.89 mg/ml) showed 1310, 3100 and 5940 TIU/g tissue, respectively.

Morphological attributes

Transgenic plants expressing high levels of ChTI (3100-3300 TIU/g tissue, Table 2) were found taller than controls with increased internode distance and stem diameter (Table 1 and Figure 3A). Difference in number of leaves at inflorescence was not significant. Transgenic plants had larger leaf area, smoother edges with more

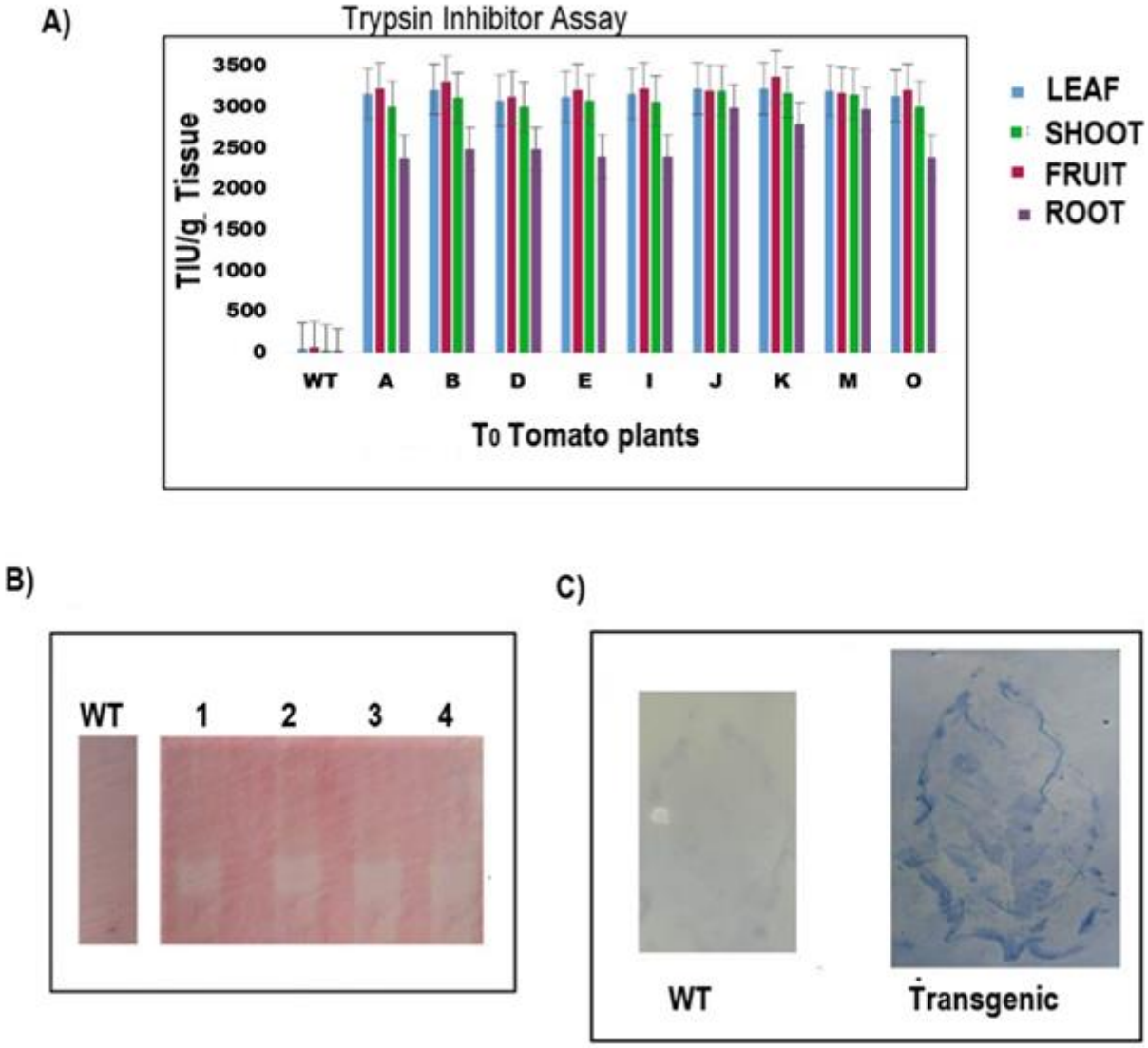

Figure 2. Trypsin inhibitory activity of Transgene Tomato plants expressing ChTI. Positively screened transgenic plant tissues were assayed for inhibitory activity. A- Trypsin inhibitory activity from tissues (leaf, shoot, fruit and root) from control and ChTI expressing T_0 plants. Stable inhibitory activity observed in M and J plant tissue extracts. B- In gel activity of ChTI. HSP of MT_0 and JT_0 leaf extracts were separated on 10% SDS gels and bovine trypsin inhibitory activity was visualised using acetyl-DL-phenylalanine-□-napthylester as substrate. Lane WT- control, Lane 1&2- transgenic leaf extracts from MT0, Lane 3&4- transgenic leaf extracts from JT_0 and C- Tissue print immune-localization of ChTI showing uniform distribution of transgene in transgenic leaf.

heft compared to small, serrated and narrow leaves in WT (Figure 3A). Although, flowering occurred 10 ± 2 days earlier than WT plants, there was hardly any difference in flower morphology (Table 1). WT plants produced more flowers than transgenic plants. Fifteen days' delay in fruit ripening was noticed in transgenic plants (Table 1). Total number of fruits (MT_0-18 ± 2; JT_0-16 ± 2) per transgenic plants was less than WT (30 ± 3), and individual fruit weight in transgenic plants (MT_0-36.46 ± 1.67; JT_0-34.86 ± 2.23) was 10 fold higher than WT (3.85 ± 0.70, Table 1, Figure 3C), resulting in improved net yield.

Biochemical analysis

Titratable acidity (TA) in transgenic fruit (MT_0-0.31 ± 0.07, JT_0-0.34 ±0. 01) was 25% higher than WT fruits (0.27 ± 0.01). Further transgenic fruits had almost 35% higher ascorbic acid (MT_0-16.07 ± 1.06 and JT_0-14.32 ± 0.21 mg per /100 g respectively) than WT (10.53 ± 0.24 mg/100 g, Table 2), resulting in a positive correlation between TA and ascorbate (Table 3). Carotenoids, lycopene and β-carotene were high in transgenic fruits (Lycopene: MT_0-2.65 ± 0.06 and JT_0- 2.24 ± 0.04 mg/100 g fw; β-

Table 1. Variation in the vegetative and reproductive parameters of WT and ChTI expressing tomato plants at T0 generation.

T_0	Internode length (cm)	Stem diameter (cm)	No. of Leaves at first inflorescence	Plant height at first inflorescence (cm)	Onset of flowering[@] (days)	Blossom set to mature green[#] (days)	Mature green to red[$] (days)	Fruit weight (g/ fruit)	No. of fruits / plant
WT	1.49 ± 0.04	0.52 ± 0.02	12.00 ± 1.00	33.00 ± 0.11	33.00 ± 0.11	21.5 ± 0.70	26.50 ± 0.70	3.85 ± 0.70	30.00 ± 3.00
M	1.90 ± 0.24	0.80 ± 0.03	8.00 ± 1.00	42.88 ± 2.60	26.55 ± 1.06	30.0 ± 1.56	40.22 ± 2.61	36.46 ± 1.67	18.00 ± 2.00
J	1.94 ± 0.36	0.81 ± 0.04	7.50 ± 2.00	40.37 ± 2.97	24.25 ± 1.03	32.0 ± 2.61	38.75 ± 1.66	34.86 ± 2.23	16.00 ± 2.00

[@]Onset of flowering corresponds to first 5 flowers appearing, [#]First flower opening up to first fruit breaker, [$]First fruit breaker maturing to red. Data represented as mean ± SD (p values were significant from each other at p value <0.05) corresponds to 2 T_0 generation lines M, J (Transgenic) are compared with WT plant (non transgenic). 10 samplings are taken for each observation.

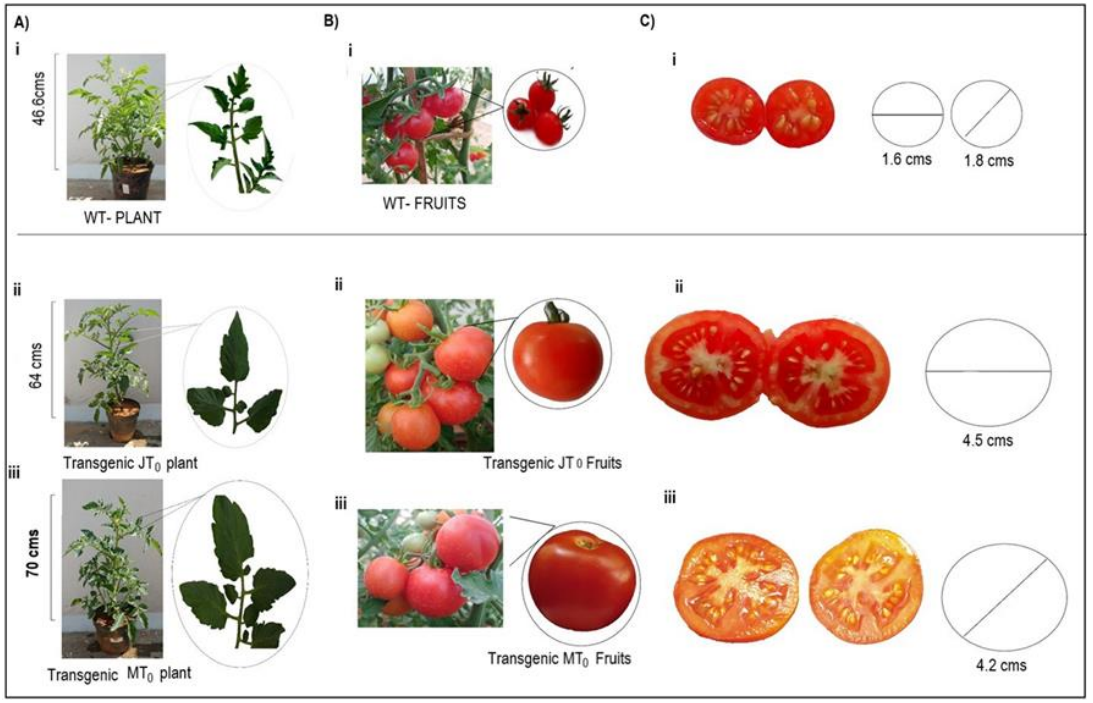

Figure 3. Phenotypes of transgenic tomato plants expressing ChTI. A- Plant height of WT and Transgenic plants (MT_0 and JT_0) at flowering stage, transgenic leaves with larger area, smoother edges and small, serrated and narrow WT leaves. B- WT and Transgenic fruits at harvesting stage. C- Cross section of WT and transgenic fruits. Note: WT - non transgenic plants considered as control. M and J - T_0 generation transgenic plants.

Table 2. Trypsin inhibitory activity and variation in nutrition and morphological parameters among T0 - control and transgenic plants.

T_0	WT	M	J
		Inhibitory activity@	
Leaf	0.00 0.00	3220.0– 3224.0 3221.00 ± 1.51	3197.0 – 3198.0 3198.00 ± 0.63
Fruit	0.00 0.00	3187.0 – 3199.0 3195.00 ± 3.96	3159.0 – 3166.0 3161.00 ± 3.55
Shoot	0.00 0.00	3196.0– 3199.0 3197.00 ± 1.41	3154.0 – 3157.0 3155.00 ± 1.47
Root	0.00 0.00	2975.0 – 2978.0 2976.00 ± 1.16	2972.0 – 2978.0 2976.00 ± 1.16
		Biochemical parameters	
Ascorbate[ß]	10.21 - 11.09 10.53 ± 0.24	15.32 - 17.88 16.07 ± 1.06	13.98 - 14.47 14.32 ± 0.21
Titratable acidity[ơ]	0.23 - 0.28 0.26 ± 0.08	0.34 - 0.36 0.31 ± 0.07	0.31 - 0.32 0.34 ± 0.01
Phenols[đ]	20.11 - 21.99 20.52 ± 0.13	49.76 - 51.14 50.45 ± 0.55	46.07 - 49.47 47.04 ± 1.20
Flavonoids[ε]	9.87 - 10.25 10.22 ± 0.02	15.01 - 15.79 15.22 ± 0.28	14.12 - 15.05 15.58 ± 0.40
Lycopene[ŧ]	1.62 - 01.84 1.82 ± 0.04	2.55 - 2.72 2.65 ± 0.06	2.11 - 2.56 2.24 ± 0.04
β -Carotene[ŧ]	2.11 - 2.13 2.12 ± 0.02	4.24 - 4.31 4.25 ± 0.12	3.25 - 3.89 3.60 ± 0.26
AoC[Ӣ]	7.10 - 7.15 7.14 ± 0.01	24.02 - 25.11 24.84 ± 0.84	22.00 - 24.12 22.67 ± 0.86
		Morphological attributes	
Onset of flowering in days	29.00 - 36.00 33.00 ± 2.00	28.00 - 31.00 29.00 ± 1.00	23.00 - 25.00 24 .50 ± 1.00
Mature green to red in days	21.00 - 22.00 23.66 ± 00.57	32.00 - 38.00 35.00 ± 3.00	37.00 - 39.00 37.80 ± 2.01
Fruit weight[Ï]	3.80 - 04.00 3.90 ± 0.10	25.60 - 32.55 31.05 ± 3.04	29.45 - 34.56 28.99 ± 3.63
Fruits / plant [Ţ]	30.00 - 40.00 35.33 ± 5.03	16.05 - 17.03 16.58 ± 0.76	17.40 - 21.44 19.69 ± 2.20

Data represented as mean ± SD. ($p < 0.05$) of Transgenic lines M T_0 and J T_0 and compared with non-transgenic plants – WT. 10 samplings from each line are taken for each observation. @- Inhibitory activity expressed as TIU/g tissue. Various Biochemical parameters of T_0 fruit values expressed as ß- mg ascorbic acid equivalent /100 g fw, ơ –% acidity, đ –mg gallic acid equivalents / 100 g fw, Ɛ – Flavonoids content expressed as mg catechin equivalents / 100 g fw, ŧ – carotene equivalents/ 100 g fw, Ӣ – AEAC/100 g fw, Ï – weight in grams/fruit , Ţ- fruits/ plant in number.

Table 3. Relationship between inhibitory activity and biochemical constituents of T0 transgenic fruits.

Variable	Inhibitory activity	Phenols	Flavonoids	βCarotene	Lycopene	Titratable acidity	Acsorbate	Total antioxidant capacity
Inhibitory activity	0.00	0.85***	0.54*	0.75**	0.87***	0.75***	0.70***	0.90***
Phenols	0.85***	0.00	0.75**	0.63**	0.82***	0.68**	0.77***	0.93***
Flavonoids	0.54*	0.75**	0.00	0.59*	0.54*	0.74***	0.82***	0.59*
β-Carotene	0.75**	0.77**	0.59*	0.00	0.72**	0.61*	0.83***	0.64*
Lycopene	0.87***	0.74**	0.54*	0.72***	0.00	0.77**	0.69**	0.96***
Titratable acidity	0.75**	0.86***	0.74**	0.61**	0.77**	0.00	0.51*	0.47*
Acsorbate	0.70**	0.82**	0.54*	0.82***	0.69**	0.51*	0.00	0.65**
Total antioxidant capacity	0.90***	0.93***	0.59*	0.64**	0.96***	0.47*	0.65**	0.00

Transgenic plants - T_0 M, T_0 J were considered. n*** , n**, n* indicates values were significant at p-value < 0.001 , < 0.05 ,< 0.01 respectively, n# indicates non-significant.

Table 4. ChTI activity of HSPs from the leaf extracts of tomato expressing ChTI against H.armigera gut protease.

Protein	Trypsin like activity of proteinases (TU/mg)	Trypsin like activity of proteinases incubated with ChTI (TU)	% Inhibition	IC_{50} of ChTI required to inhibit proteinases
H.armigera gut proteinase (HGPs)	144.00 ± 2.87	93.67 ± 0.94	34.5	700 TIU/g tissue

Leaf extracts from transgenic lines - M T2, J T2 were taken for the assay. The data obtained are the means ±SD (P<0.001). TU/mg – Trypsin unit , TIU/g- Trypsin inhibitory units.

carotene: MT_0-4.25 ± 0.12 and JT_0-3.60 ± 0.26 mg/100 gfw, respectively) which was about 30% higher than WT (lycopene: 1.82 ± 0.04 and β-carotene: 2.12 ± 0.02 mg/100 g fw; Table 2). Phenolic content in transgenic fruits was 60% higher than WT. Transgenic fruits had the highest phenol content (MT_0- 50.45 ± 0.55 mg, JT_0-47.04 ± 1.20 mg /100 g fw, respectively) over the WT (20.52 ± 0.13 mg /100 g fw). Flavonoid content was 40% more in transgenics, (MT_0-15.22 ± 0.28, JT_0-15.58 ± 0.40 mg /100 g fw, respectively), relative to WT (10.22 ± 0.02 mg/100 g fw; Table 2). Total Aoc activity in transgenic fruits (MT_0-24.84 ± 0.84 mg, JT_0-22.67 ± 0.86 AEAC/100 g fw, respectively) was higher compared to WT (7.14 ± 0.01mg AEAC/100g fw; Table 2). Positive correlation was observed in transgenic fruits at T_0 generation between biochemical traits and ChTI activity (Tables 3 and 4).

Inheritance ChTI in T_1 and T_2 generations

Based on the performance of T_0 plants and keeping inhibitory activity as the main criteria for selection, plants were forwarded to next generation. Plants from MT_1 and JT_1) were subjected multiplex PCR and inhibitory assay. Twelve plants, confirmed for the presence of *ChTI* and *hpt* by multiplex PCR (Supplementary Appendix Figure S2-A) and those with higher inhibitory activity were forwarded (Supplementary Appendix Figure S3) to next generation. Western blot analysis of plants expressing stable inhibitory activity showed similar immunobloting pattern (Supplementary Appendix Figure S2-C). Parent progeny regression analysis showed positive correlation with respect to their inhibitory activity and morphological/ nutritive traits (Supplementary Appendix Tables S1, S2, S3). Based on the morphological, biochemical, PCR analysis and Trypsin inhibitory activity (TIA), plants were further forwarded to T_2 generation. Random analysis of 10 plants from each, confirmed the presence of ChTI gene (Supplementary Appendix Figure S2-

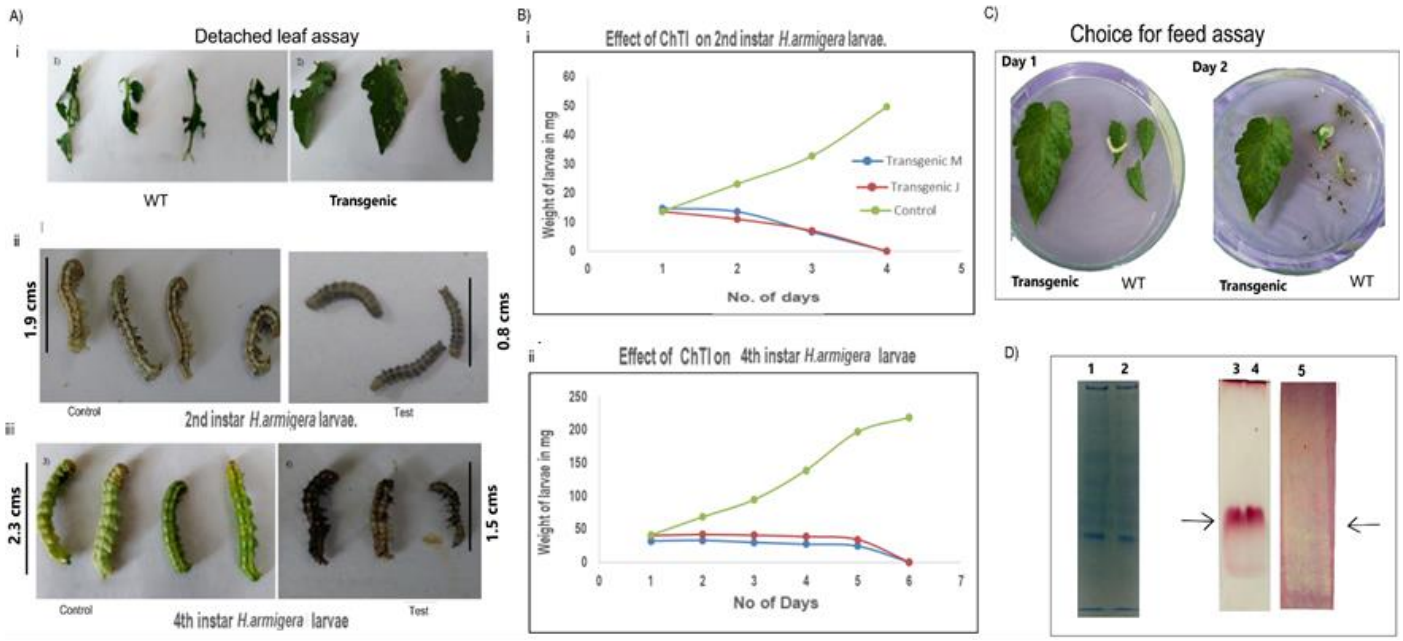

Figure 4. Effect of feeding on transgenic tomato plants expressing ChTI on H. armigera larval growth. A- Detached leaf feeding assay using WT and transgenic leaf damage with 2nd instar larvae. ii, iii) Retardation in growth of 2nd and 4th instar larvae fed on transgenic and WT leaves. B- Rate of H.armigera larval growth of 2nd and 4th instar feeding on WT and transgenic tomato leaves, C- Feeding choice assay using 2nd instar larvae. Larvae feed on WT leaves preferentially over transgenic leaves. D- In-gel assay visualize the effect of ChTI on HGPs. Lane 1, 2: HGPs visualized on SDS- PAGE. Lane 3: HGPs incubated with phosphate buffer, lane 4: HGPs incubated with WT leaf extract showing no inhibition in activity. Lane 5: HGPs incubated with transgenic leaf extract showing inhibition in activity. Note: Leaves and leaf extracts from two individual transgenic plants (MT2 and JT2) and non-transgenic (WT) were taken for the assay. The data obtained are the means ± SD ($p<0.001$) from 15 larvae performed in duplicates.

D) as well its expression (Supplementary Appendix Figure S2-F). Inter-generation regression and correlation analysis (Supplementary Appendix Table S4) showed strong association between individual mean of traits in T_1 and T_2 generation plants. These results put together indicated the inheritance of traits analysed from T_0 to T_2 generation.

Bioassay against *H. armigera*

T_2 generation plants were used to evaluate the effect of ChTI on growth of 2nd and 4th instar *H.armigera* larvae. Larval feeding assay showed significant reduction in mean larval weight and increase in mortality compared to WT (control). LT_{50} for 2nd instar larvae ranged between 3.5 to 4 days, and 4-6 days was for 4th instar larvae (Figure 4B) followed by 100% mortality in both cases at later stages of growth (Figure 4A ii; iii). Leaves of WT plants were severely damaged compared to transgenic plants (Figure 4i). Feeding choice assay showed that *H. armigera* larvae preferred WT over transgenic leaves. Transgenic leaves remained untouched whereas complete feeding on WT leaves was observed (Figure 4C). *In gel* assay showed that ChTI inhibits some of the major HGPs (Figure 4D). HSPs (700 TIU/g) from transgenic leaf extracts inhibited gut caseinolytic activity up to 34.5% (Table 4). These results together suggest that the amount of ChTI constitutively in transgenic plants (~2000 to 3500 TIU per g. of fresh weight), is more than enough to retard the growth and development of *H. armigera* larvae.

DISCUSSION

C. hirsutus possesses serine proteinase inhibitor (ChTI) which has potential insecticidal and antifungal activity (Bhattacharjee et al., 2010). *H. armigera* is a true generalist and agricultural pest that feeds on at least 161 plant species in 49 plant families (Wang et al., 2017). Since *C. hirsutus* is a non-host plant of *H. armigera,* we presumed that the insects are less likely to develop resistance against ChTI compared to similar type of inhibitors from host plants. The study was focussed on the development of transgenic tomato plants expressing ChTI and its *in vivo* evaluation against *H. armigera*. *Agrobacterium* mediated transformation (Manamohan et al., 2011; Somayaji et al., 2014) allowed us to achieve transformation efficiency up to 25.7% in tomato. Selection of plants were made based on the high expression of ChTI in vegetative tissues and fruits, and forwarded to

subsequent generations. SDS-PAGE followed by western blot analysis / *in gel* activity assay suggests that ChTI is 18 kDa protein, expressed constitutively in all these tissues. High level ChTI was expressed in leaves followed by fruits, shoot and roots. Earlier study report that proteinase inhibitor gene expressed under 35S promoter showed more accumulation of inhibitor in mature leaves than in flowers (Thomas et al., 1994). SDS-PAGE / western blot analysis using ChTI-IgYs showed the presence of ~18 kDa protein in partially purified tissue extracts corresponding to ChTI in T_0-T_2 plants. Leaf tissue imprinting analysis showed uniform distribution of ChTI. In earlier study, immuno-analysis in *L. peruvianum* has revealed the presence proteinase inhibitor throughout fruit development (Wingate et al. 2008). Plants expressing ChTI showed 40-45% increased plant height, early flowering by 10 days and increased fruit size. Although, there was delay in fruit ripening, an increase in nutrient and antioxidant levels was observed. Transgenic tobacco plants over-expressing OCI, showed increased plant height, biomass, earlier flowering and decreased life cycle (Gutierrez-Campos et al., 2001). Serine proteinases are likely to be associated in regulating programmed cell death, or associated processes such as senescence and cellular metabolic processes at every stage of plant growth and development (Fluhr et al., 2012; Santamaria et al., 2014; Ghorbani et al., 2016). High level constitutive expression of ChTI in tissues seem to have interfered in signalling mechanisms associated with physiological processes related to plant growth and fruit development.

Digestive process in lepidopteron gut mainly depends upon amylases, proteinases and lipases. Trypsin and chymotrypsin like serine proteinases play major role in providing amino acids pool for the growth and development of insects/pests through hydrolysis of ingested proteins. Large amount of the larval gut proteolytic enzymes are serine proteinases (Johnston et al., 1991). Use of proteinase inhibitors targeting these enzymes is one among the accomplishable crop management strategies against insect/pest control. However, insects under selective pressures have developed multiple mechanisms of adaptation to overcome plant's defense, especially, to proteinase inhibitors of domesticated crops by modifying their digestive physiology (Gatehouse, 2011). Up-regulation of chymotrypsin and other diverged serine proteinases and down regulation of trypsin like enzymes in gut has been reported in *H. armigera* larvae fed with artificial diet containing SkTI (Kuwar et al., 2015). Studies also suggest up-regulation of inhibitor insensitive proteinases in chickpea, pigeon pea, and cotton resulting in 35-55% larval growth. Non host plant PIs from *Pongamia pinnata, Mucuna pruriens*, *Capsicum annuum*, *Nigela sativa* and wild relatives of Chickpea (*Cicer arietinum)* showed maximum inhibitory potential towards HGPs *in vivo*, also exhibited moderate level of inhibition of pro-proteinases, *H. armigera* gut pro-proteinases (HGPPs) (Parde et al., 2010; Golla et al., 2018). Plant proteinase inhibitors from groundnut, potato, winged bean caused 80-100% larval mortality (Harsulkar et al., 2002). Affinity purified ChTI caused significant reduction in 2^{nd} and 3^{rd} instar larval growth (up to 84%), and resulted in 100% mortality in *in vitro* assay (Bhattacharjee et al., 2010). Earlier studies have shown that pro-proteinase levels increased during larval growth, and maximum HGPPs activity was observed in the fifth-instar. Larvae fed on diets with non host plant PIs showed greater inhibition of HGPPs as compared to HGPs. *In vitro* studies on HGPs treated with gut extract of larvae fed on *D. alba* inhibitor showed that out of 10 proteinase isoforms, two were activators of pro-proteinases. Larval growth and development were significantly reduced in the larvae fed on non-host plant PIs, resulting in stunted growth of *H.armigera* larvae. *In vivo* studies indicated that non-host plant PIs were good candidates as inhibitors of the HGPs as well as HGPPs (Parde et al., 2010). Considering the importance of crop protection / improvement, transgenic tomato plants over-expressing ChTI were developed. Leaf feeding bioassay using second and fourth instar *H.armigera* on transgenic tomato plants expressing ChTI showed larval mortality within 2-6 days. Several groups have reported plant protection with development of transgenic plants expressing non host PIs. Transgenic cotton over expressing PI-I and PI-II have reported LT_{50} of 11 days (War et al., 2012). Transgenic tomato over expressing CanPI-17 proved effective against *H. armigera* larvae with $LT_{50}$of 7days (Giri et al., 2010). It is observed 40% larval mortality in *H. armigera* larvae fed with cowpea trypsin inhibitor and 33% mortality of *H. armigera* larvae was observed upon feeding artificial diet impregnated with mung bean (Kansal et al., 2009). The results of our study indicate that 1000 TIU of transgenic leaf extract bring in almost 40% inhibition of HGPs. Constitutive levels of ChTI in leaves is almost 3000TIU, is more toxic leading to severe larval mortality. The reasons for decreased larval mass fed with inhibitors or feeding on transgenic leaves expressing sufficient amount of PIs, could be ascribed to amino acid starvation. Accumulation of proteins and nutrients is very crucial for larvae to progressively switch from one instar to another, disruption of which results in growth retardation, finally resulting in mortality. Feeding choice assay shows preference for WT leaves, but not ChTI expressing leaves, suggest that change in olfactory network. Avoidance of transgenic leaves might be due to release of anti-agents, volatiles gases, which may have mimicked the non host plant volatiles (Wang et al., 2017; Anderson and Anton, 2014). Previous work on *H. armigera* feeding choice assay has indicated that neurons in the medial sensillastyloconica on the maxillary glea contribute to the gustatory discrimination between cotton and pepper leaf saps (Tang et al., 2006). Further, isoforms of gut trypsin like enzymes are expressed in different developmental

stages and also on the basis of diets they were fed with. During the feeding experiments, it has been observed the proteolytic enzymes produced in early instars get inhibited and inhibitor resistant enzymes get expressed. Serine like proteinases expressed in early stages of larval growth is predominantly sensitive to PIs than the once expressed in the later stages of growth. Therefore, the larval growth and mortality rates depend on the stage of the larval growth and the effectiveness of the PIs resulting in delay of mortality rate of *H. armigera* (Bhattacharjee et al., 2010; Lomate et al., 2018; Chikate et al., 2013). These findings indicated that ingestion of transgenic leaves expressing ChTI at early stages of larval growth could control the lepidopteran population effectively.

Significant increase in the fruit size was observed in T_0-T_2 tomato plants expressing ChTI. Earlier studies in breeding crops for higher yield has been invariably associated with compromise on nutritional traits. Micronutrient malnutrition is a major threat in the present scenario, related to quantity and quality of food produced using modern agricultural technologies. Antioxidants are more stable in acidic pH. Increase in TA (68%) with increase in fruit size shows positive correlation of ascorbate (R^2= 0.511). Tittonell et al. (2001) report that higher TA (lower pH) provides stability of ascorbate and related antioxidants. Besides, intensity of light and amount of foliage is of particular interest contributing to the level of ascorbate in fruits (Ntagkas et al., 2019). Transgenic tomato plants expressing ChTI are taller, with low foliage levels compared to shorter control plants with heavy foliage. Burge et al. (1975) report that the higher fruit ascorbate levels in plants with less foliage (23 mg /100 g fw) and reduced level (18 mg/100 g fw) in plants with heavy foliage. Development of horticulture crops with increased Aoc is becoming increasingly relevant in accomplishing nutritional security in addition to increased production. Ascorbic acid, lycopene, flavonoids and phenols contribute to Aoc of the fruits (Toor and Savage, 2005). High Aoc activity in ChTI expressing tomato fruits showed strong positive correlation with TA, ascorbate, lycopene, phenols and carotenoids. Regression and correlation analysis between T_0-T_2 showed improvement in almost every trait documented in our study, thus suggesting successful inheritance of the traits along with ChTI expression for resistance to *H. armigera*.

Conclusion

The results of our study suggest that tomato plants incorporated with ChTI shows very high constitutive expression in all the vegetative tissues and fruits. ChTI effectively inhibits HGPs and larval growth of *H. armigera* effectively. The data reveals no compromise on the phytonutrients content viz. titratable acidity, antioxidant content, phenolics, flavonoids, ascorbate and lycopene, which was significantly higher in transgene fruits. PIs from the non-host plants have the potential to be expressed in genetically engineered plants to confer resistance to *H. armigera*. However, insect herbivores develop multiple mechanism of adaptation to overcome the defensive effects due to selection pressure. Future prospects for using proteinase inhibitor genes to enhance insect resistance in transgenic crops will require assessment of their mechanisms of action like their role in cell signalling, PCD other metabolic processes at various stages of plant growth and development.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

Aoc, Antioxidant capacity; **ChTI,** *C. hirsutus* Trypsin Inhibitor; **EDTA,** Ethylene di-amine tetra-acetic acid; **HGP,** *H. armigera* gut proteinase; **HGPP,** *H. armigera* gut pro-proteinase; **Hpt,** Hygromycin phosphotransferase; **HRP,** Horse radish peroxidase; **HSPs,** Heat stable proteins; **LD,** Lethal dose; **MS,** Murashige and Skoog; **PCD,**Programmed cell death; **PIs,** Proteinase Inhibitors; **SPIs,** Serine proteinase inhibitors; **TA,** Titratable acidity; **TIA,** Trypsin Inhibitory activity; **TIU,** Trypsin inhibitory unit; **TMP,** 3,3',5,5'-Tetramethylbenzidine; **TSPs,** Total soluble proteins.

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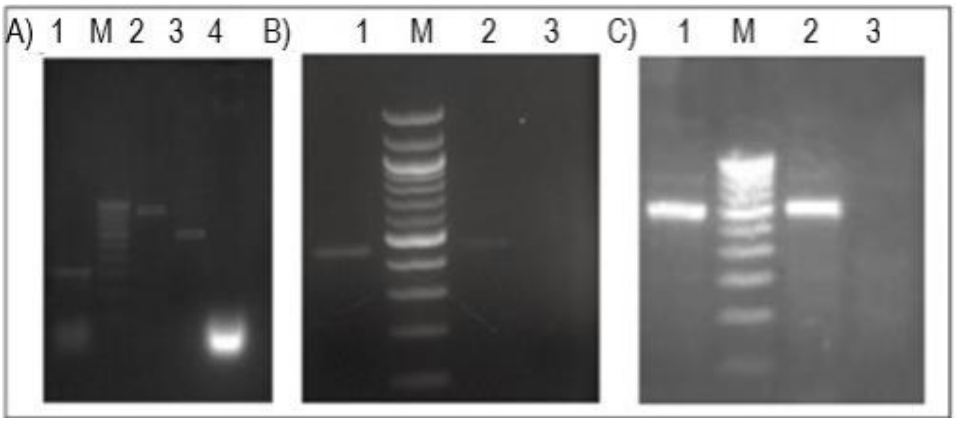
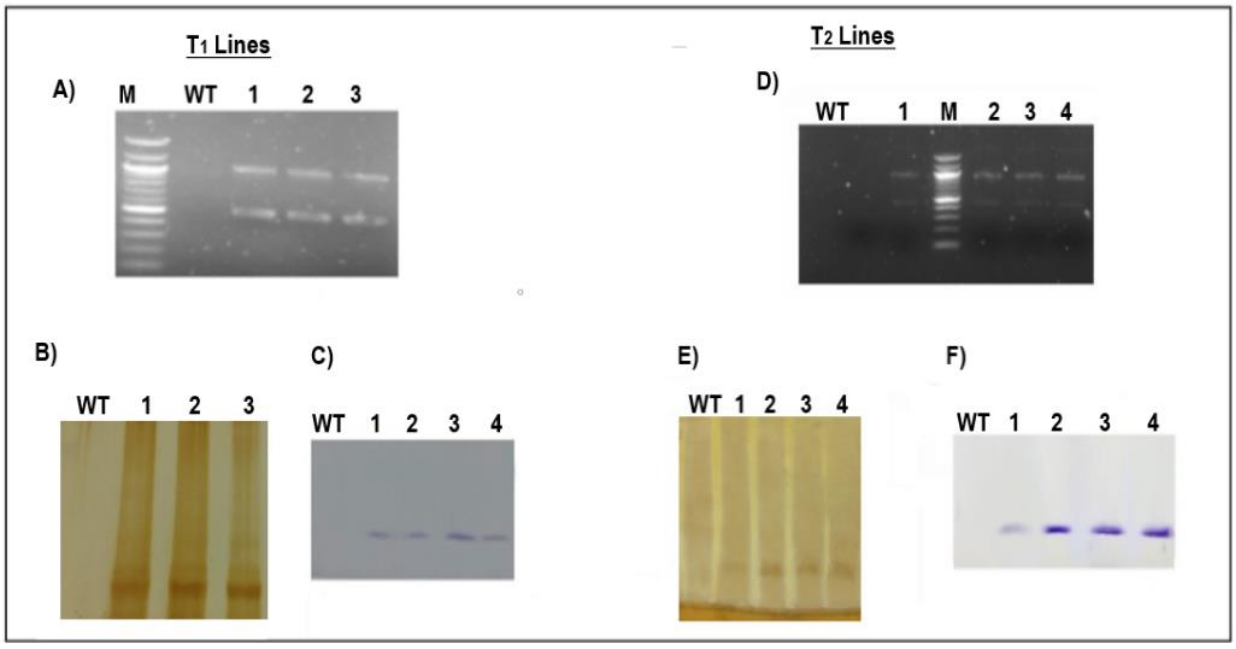
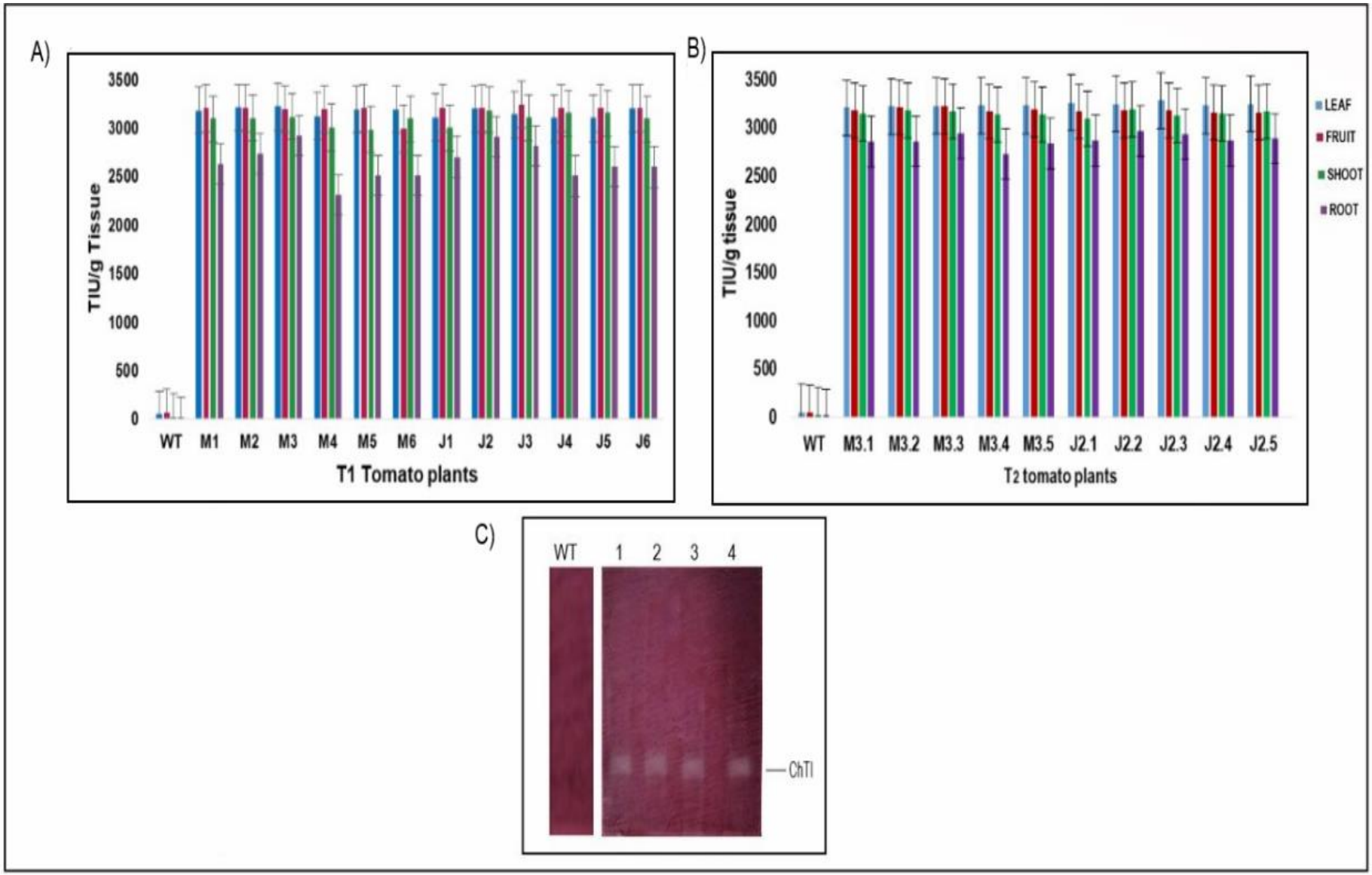
SUPPLEMENTARY MATERIAL



Supplementary Figure 1 (S1). Construction of recombinant vector pCAM-ChTI. ChTI amplicons amplified with M13 forward and reverse primers as well as pCAMBIA 1301 were digested with EcoR I and Hind III to provide cohesive termini for ligation. Fragments separated on 2% agarose gel were co-eluted and ligated. Construction of pCAM-ChTI was confirmed by PCR analysis. a) M-100 bp DNA ladder, lane2 - ChTI amplicons amplified with M13 forward and reverse primers, lane3 - ChTI amplicons amplified with M13 forward and reverse primers digested with EcoR I and HindIII, and lane4 - pCAMBIA digested with EcoR I and HindIII; b) M-100 bp DNA ladder, lane 1 and 2 - pCAM-ChTI amplified with ChTI specific primers, showing 450bp amplicons of ChTI, lane3 - pCAMBIA 1301 amplified with ChTI specific forward and reverse primers, showing the absence of 450bp amplicons of ChTI; c) Confirmation of transformation in pCAM-ChTI in Agrobacterium strain LB4404 by PCR using ChTI specific primers. Lane1 and 2 - pCAM-ChTI isolated from Agrobacterium strain LB4404, lane3 - pCAMBIA 1301 isolated from Agrobacterium strain LB4404.



Supplementary Figure 2 (S2). Confirmation of ChTI stability in transgenic tomato plants. Plants exhibiting trypsin inhibitory activity were screened for the stability of ChTI in T1 and T2generation. Plants conferring hpt resistance were screened for the presence of ChTI by PCR, and its expression by western blotting. A, D- PCR analysis of T1 and T2 plants using ChTI and hpt specific primers. Lane 1-3: PCR amplicons of ChTI (0.45kb) and hpt (1kb), M- 100bp DNA ladder, WT- non transgenic (control). B, E- Silver stained SDS-PAGE showing ChTI (18kDa) purified via Trypsin affinity column from WT, T1 and T2 plants, in the figures lane WT: control tissue extract, lane 1-4: transgenic tissue extract. C, F- Western blot visualisation of ChTI in T1 and T2 plant tissue extracts, in the figures lanes: WT- non transgenic, expression levels of ChTI from lane 1- root, lane 2- shoot, lane 3 - fruit, lane 4- leaf.



Supplementary Figure 3 (S3). Trypsin inhibitory activity of Transgene expressing T1 and T2 - tomato plants. Positively screened transgenic plant tissues were assayed for inhibitory activity. A, B - Trypsin inhibitory activity from tissues (leaf, shoot, fruit and root) from WT and ChTI expressingT1 and T2 tomato plants. C - In gel activity of ChTI. HSP fraction of leaf extracts were separated on 10% SDS gels and bovine trypsin inhibitory activity was visualised using acetyl-DL-phenylalanine-□-napthyl ester as substrate. Lane WT: control, lane 1-4: transgenic leaf extracts from two independent T1 and T2 plants.

Supplementary Table 1 (S1). Variation in the vegetative and reproductive parameters of WT and ChTI expressing tomato plants at T1 and T2 generation.

T_1	Internode length (cm)	Stem diameter (cm)	No. of leaves at first inflorescence	Plant height at first inflorescence (cm)	Onset of flowering[@] (days)	Blossom set to mature green[#] (days)	Mature green to red[$] (days)	Fruit weight (g/ fruit)	No. of fruits / plant
WT	1.45 ± 0.06	0.56 ± 0.08	12.00 ± 1.00	38.00 ± 0.28	31.00 ± 0.05	21.0 ± 0.56	24.50 ± 0.68	3.72 ± 0.81	32.00 ± 2.00
M	2.10 ± 0.15	0.78 ± 0.06	8.00 ± 2.00	48. 39 ± 1.80	23.95 ± 1.01	28.0 ± 1.90	39.18 ± 3.80	36.00 ± 1.55	14.00 ± 3.00
J	1.90 ± 0.26	0.80 ± 0.03	7.00 ± 1.00	46.41 ± 2.00	23.21 ± 1.09	28.0 ± 1.91	38.61 ± 1.66	36.06 ± 1.03	19.00 ± 1.00
T_2									
WT	1.39 ± 0.10	0.58 ± 0.13	11.00 ± 2.00	37.00 ± 0.10	30.00 ± 0.10	22.0 ± 0.60	27.50 ± 0.11	3.90 ± 0.11	25.00 ± 5.00
M	2.08 ± 0.12	0.81 ± 0.10	9.00 ± 1.00	49. 19 ± 2.10	24.81 ± 2.00	29.0 ± 1.00	39.10± 2.00	34.00 ± 1.00	15.00 ± 3.00
J	2.00 ± 0.11	0.71 ± 0.30	7.00 ± 1.00	49.70 ± 1.92	22.05 ± 1.00	30.0 ± 1.00	40.9 ± 0.05	36.13 ± 0.09	18.00 ± 2.00

[@]Onset of flowering corresponds to first 5 flowers appearing, [#]First flower opening up to first fruit breaker, [$]First fruit breaker maturing to red. Data represented as mean ± SD (p values were significant from each other at p value < 0.05) corresponds to 2 independent T_1 and T_2 generation lines - M, J (Transgenic), and 5 progeny plants from each lines (T_1 M and T_1J) are compared with WT plants. (non transgenic). 10 samplings are taken for each observation.

Supplementary Table 2 (S2). Trypsin inhibitory activity and variation in nutrition and morphological parameters among T1, T2- control and transgenic plants.

	T_1			T_2		
	WT	**M**	**J**	**WT**	**M**	**J**
			Inhibitory activity[@]			
Leaf	0.00	3200.0 – 3216.0	3209.0 – 3248.0	0.00	3021.0– 3232.0	3169.0– 3189.0
	0.00	3208.00 ± 6.34	3216.00 ± 15.38	0.00	3190.00 ± 83.45	3176.00 ± 11.76
Fruit	0.00	3125.0 – 3224.0	3108.0 – 3201.0	0.00	3170.0 – 3221.0	3175.0 – 3189.0
	0.00	3190.00 ± 35.19	3148.00 ± 46.31	0.00	3195.00 ± 19.75	3174 ± 11.86
Shoot	0.00	3154.0 – 3157.0	3005.0 – 3190.0	0.00	3140.0– 3185.0	3100.0 – 3198.0
	0.00	3155.00 ± 01.41	3120.00 ± 65.91	0.00	3156.00 ± 18.53	3143.00 ± 37.36
Root	0.00	2712.0– 2931.0	2510.0 – 2917.0	0.00	2512.0– 2931.0	2873.0– 2970.00
	0.00	2823.00 ± 89.16	2727.0 ± 217.10	0.00	2606.00± 213.89	2904.00 ± 38.96
Biochemical parameters						
Ascorbate[ß]	9.57 - 11.89	14.51 - 17.35	13.66 - 17.03	10.97 - 11.08	15.14 - 18.13	14.35 - 17.85
	11.32 ± 1.53	16.28 ± 1.28	13.88 ± 1.59	10.83 ± 0.40	16.98 ± 1.10	16.01 ± 1.23
Titratable acidity[ơ]	0.20 - 0.22	0.38 - 0.43	0.31 - 0.40	0.20 - 0.21	0.35 - 0.43	0.31 - 0.36
	0.21 ± 0.01	0.39 ± 0.02	0.33 ± 0.04	0.20 ± 0.05	0.40 ± 0.04	0.33 ± 0.01
	21.10 - 22.44	49.98 - 60. 81	45.19 - 56.99	20.01 - 21.06	46.19 - 60.13	44.57 - 56.13
Phenolsđ	22.11 ± 0.86	55.36 ± 4.28	51.19 ± 5.82	20.78 ± 0.51	54.31 ± 5.46	48.81 ± 4.22

Supplementary Table 2 (S2). Contd

Flavanoidsε	8.34 - 10.23 9.43 ± 0.84	14.83 - 16.33 15.92 ± 0.64	14.69 - 16.35 15.32 ± 0.69	10.29 - 10.03 10.09 ± 0.13	15.68 - 17.54 16.17 ± 0.93	14.55 - 15.56 15.08 ± 0.37
Lycopenef	1.02 - 1.13 1.08 ± 0.06	3.68 - 4.12 3.96 ± 0.15	3.14 - 4.02 3.57 ± 0.46	1.09 - 2.07 1.80 ± 0.47	2.34 - 3.53 3.26 ± 0.47	2.98 - 3.44 2.83 ± 0.87
β-Carotenef	1.96 - 2.03 2.00 ± 0.03	3.47 - 4.23 3.94 ± 0.35	3.14 - 4.02 3.50 ± 0.46	2.08 - 02.28 2.13 ± 0.09	3.27 - 4.23 3.94 ± 0.95	3.04 - 3.91 3.50 ± 0.37
AoCИ	10.23 - 12.93 10.87 ± 0.96	22.88 - 27.65 25.28 ± 1.70	22.06 - 24.11 23.12 ± 0.89	10.23 - 11.08 10.83 ± 0.40	22.88 - 26.19 25.28 ± 1.70	23.14 - 24.11 23.12 ± 0.89
Morphological attributes						
Onset of flowering in days	30.00 -31.00 31.00 ± 1.00	27.00 - 30.00 28.00 ± 2.00	24.00 - 28.00 25.00 ± 3.00	29.00 - 33 .00 31.00 ± 2.00	24.00 - 26.00 25.00 ± 1.00	25.00 - 28.00 27.00 ± 1.00
Mature green to red in days	21.00 - 23.00 22.00 ± 1.00	34.00 - 37.00 35.00 ± 1.00	31.00 - 40.00 35.00 ± 5.00	21.00 -23.00 22.00 ± 01.00	34.00 - 42.00 39.00 ± 2.00	36.00 - 42.00 39.00 ± 2.00
Fruit weightΪ	3.20 - 3.80 3.40 ± 0.32	31.00 - 38.00 33.80 ± 2.50	34.00 - 37.00 35.25 ± 1.50	3.50 - 4.20 3.90 ± 0.11	28.86 - 34.93 32.17 ± 2.50	27.89 - 35.90 31.44 ± 3.38
Fruits / plantΓ	30.00 - 36.00 33.00 ± 3.00	15.00 - 17.00 16.20 ± 0.83	14.00 - 16.00 15.05 ± 1.29	30.00 - 40.00 36.00 ± 03.00	16.04 - 20.01 17.95 ± 01.45	17.50 - 23.00 20.45 ± 02.00

Data represented as mean ± SD. ($p< 0.05$) of M T_1 and J T_1, M T_2 and J T_2 - Transgenic plants, WT- Non transgenic plants. 5 plants from each T_1 ,T_2 was taken for study. With 10 samplings per plant. The results are compared with non-transgenic plant (WT). Note : @- Inhibitory activity expressed as TIU/g tissue. Various Biochemical parameters of T_0 fruit values expressed as ß- mg ascorbic acid equivalent /100g fw, ơ –% acidity, đ –mg gallic acid equivalents / 100 g fw, ε – Flavanoids content expressed as mg catechin equivalents / 100g fw, f – carotene equivalents/ 100g fw, И – AEAC/100g fw, Ï – weight in grams, Γ- fruits/ plant in number.

Supplementary Table 3 (S3). Relationship between inhibitory activity and biochemical constituents of T1, T2 transgenic fruits.

	Inhibitory activity		Phenols		Flavanoids		βCarotene		Lycopene		Titratable acidity		Ascorbate		Total antioxidant capacity	
	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Inhibitory activity	0.00	0.00	0.9^{***}	0.9^{***}	0.93^{***}	0.93^{***}	0.88^{***}	0.81^{***}	0.8^{***}	0.83^{***}	0.77^{***}	0.77^{***}	0.67^{**}	0.40^{*}	0.72^{**}	0.81^{***}
Phenols	0.9^{***}	0.9^{***}	0.00	0.00	0.73^{**}	0.76^{**}	0.63^{**}	0.77^{**}	0.68^{**}	0.78^{***}	0.64^{**}	0.61^{**}	0.76^{***}	0.72^{***}	0.69^{*}	0.89^{***}
Flavanoids	0.93^{***}	0.93^{***}	0.73^{**}	0.76^{**}	0.00	0.00	0.62^{**}	0.70^{**}	0.78^{***}	0.59^{**}	0.76^{***}	0.8^{***}	0.89^{***}	0.63^{*}	0.87^{***}	0.87^{***}
β-Carotene	0.88^{***}	0.81^{*}	0.63^{*}	0.77^{**}	0.62^{**}	0.7^{***}	0.00	0.00	0.79^{**}	0.89^{***}	0.57^{*}	0.45^{*}	0.67^{*}	0.80^{***}	0.56^{*}	0.78^{**}
Lycopene	0.8^{***}	0.33^{*}	0.68^{*}	0.78^{***}	0.78^{***}	0.59^{***}	0.79^{**}	0.89^{***}	0.00	0.00	0.67^{*}	0.31^{*}	0.75^{**}	0.77^{**}	0.57^{**}	0.74^{**}
Titratable acidity	0.77^{**}	0.77^{**}	0.79^{*}	0.61^{**}	0.76^{***}	0.8^{***}	0.57^{*}	0.45^{*}	0.67^{**}	0.31^{*}	0.00	0.00	0.55^{*}	0.42^{*}	0.63^{**}	0.72^{**}
Acsorbate	0.67^{*}	0.40^{*}	0.76^{**}	0.72^{**}	0.89^{***}	0.63^{*}	0.67^{***}	0.80^{***}	0.75^{***}	0.77^{***}	0.55^{*}	0.42^{*}	0.00	0.00	0.75^{**}	0.51^{**}
Total antioxidant capacity	0.72^{**}	0.81^{**}	0.69^{**}	0.89^{***}	0.87^{***}	0.87^{***}	0.56^{*}	0.78^{*}	0.57^{**}	0.74^{**}	0.75^{*}	0.72^{**}	0.75^{**}	0.51^{**}	0.00	0.00

Five $T_1$and T_2- Transgenic plants M and J with 10 samplings each were considered. n*** , n**, n* indicates values were significant at p-value <0.001 , < 0.05 ,< 0.01 respectively, $n^{\#}$ indicates non-significant.

Supplementary Table 4 (S4). Intergeneration correlation and regression analysis from T1-T2 generation.

Characters	$T_1 - T_0$				$T_2 - T_1$			
	M		J		M		J	
	Correlation	Regression	Correlation	Regression	Correlation	Regression	Correlation	Regression
Inhibitory activity								
Leaf	0.81**	0.65**	0.78**	0.61*	0.98***	0.97***	0.93***	0.86**
Shoot	0.93**	0.86**	0.84***	0.71**	0.76**	0.57*	0.95***	0.9***
Fruit	0.93***	0.87***	0.88**	0.78**	0.93***	0.87**	0.9***	0.81**
Root	0.65*	0.42*	0.79**	0.63**	0.94***	0.89**	0.88***	0.78**
Biochemical attributes								
Phenols	0.85**	0.72**	0.85**	0.73**	0.9***	0.82**	0.94***	0.89***
Flavanoids	0.8**	0.64**	0.87**	0.75**	0.95***	0.9***	0.94***	0.89***
Lycopene	0.89**	0.8**	0.75**	0.56*	0.83***	0.69*	0.76**	0.59*
β- Carotene	0.96***	0.92***	0.92***	0.84**	0.98***	0.97***	0.93***	0.87**
Ascorbate	0.93***	0.86**	0.83**	0.7**	0.94***	0.89**	0.78**	0.61*
Acidity	0.85**	0.73**	0.84**	0.7**	0.86***	0.74**	0.78**	0.61*
Total antioxidant capacity	0.78**	0.62*	0.86**	0.72**	0.84**	0.72**	0.87**	0.75**
Morphological attributes								
Onset of flowering	0.92***	0.85**	0.63*	0.39#	0.8**	0.65*	0.86**	0.74**

Supplementary Table 4 (S4). Contd

Blossom set to mature green	0.81**	0.66**	0.63*	0.4 #	0.88***	0.77**	0.92***	0.85**
Mature green to red	0.72**	0.52*	0.59*	0.35 #	0.93***	0.86**	0.71**	0.5*
Fruit weight	0.93***	0.86***	0.9***	0.81**	0.78**	0.61**	0.9***	0.81***
Fruits / plant	0.78**	0.61*	0.7**	0.41#	0.83**	0.69**	0.87**	0.65**

T_0, T_1, T_2 - Transgenic plants M and J were considered. n*** , n**, n* indicates values were significant at p-value < 0.001 , < 0.05 ,< 0.01 respectively, $n^{\#}$ indicates non-significant.

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Effects of severity of apical shoot harvest on growth and tuber yield of two sweet potatoes varieties

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Leaf harvesting of sweet potato during vegetative stage is common in most parts of Liberia. There is little information on the effects of severity of apical shoot harvesting on tuber yield of sweet potato. Experiments were conducted in 2017 at the Federal University of Agriculture, Abeokuta to determine the effects of severity of apical shoots harvest on growth and tuber yields. Experiment consisted of two varieties (SHABA and SPK-004) and three levels of cutting severity; no cutting, cutting of 15 and 30 cm long apical shoots at 4-weeks. Treatments were arranged in split plot with variety as the main plot and cutting severity as sub-plot arranged in (RCBD) with three replications. Data were collected on vine length, number of leaves per plant, number of branches per plant, leaf area, and leaf area index, fresh and dry apical shoots weight, tuber, unmarketable yield, marketable yield and total number of tuber. Data collected on growth, yield parameters were subjected to analysis of variance and mean values separated using standard error at ($p<0.05$). In cutting severity, vegetative growth and tuber yield of SHABA were significantly higher than those of SPK-004. Cutting at 15 cm long apical shoots gave higher total tuber yields in SHABA than SPK-004. Cutting at 30 cm long apical shoots increased fresh apical shoot weigh in SPK-004 than in SHABA. For SHABA and SPK-004 had more tuber weight than 30 cm long apical shoots. Therefore, sweet potatoes whose apical shoot was cut at 15cm long for 4 weeks are recommended.

Key words: Bacterial blight, disease development, grain yield, planting densities, percentage severity index.

INTRODUCTION

The herbaceous dicot sweet potato plant (*Ipomoea batatas* Lam.) is a native of tropical and subtropical region of America and belongs to the Convolvulaceae family. Many parts of the plant are edible, including leaves, roots, and vines, and varieties exist with a wide range of skin and flesh colour, from white to yellow-orange and deep purple (CIP,1999). In Sub-Saharan Africa, sweet potato is the third most important root (tuber) crop after *cassava (Manihot esculenta)* and yam (*Dioscorea* spp) (Ewell and Mutuura, 1994). This crop plays an important role in household food security and income generation among farmers and supplies substantial amount of nutritional diets that can greatly reduce risk of heart disease, stroke, and even cancer (Carey et al., 1999; Helen, 2012). It yields about 60% industrial starch in Japan and also used as a sweetener in local drinks in Nigeria (Collins, 1993; Agbo and Ene, 1994). In some countries such as Ghana and Liberia, vine tops are used as vegetables and dry forage during scarce grazing periods (Abindin, 2004), Leaf harvesting has been reported to have some detrimental effect on tuberous root yield of sweet potato. Dahniya

(1980) compared the effects of harvesting shoots of two varieties of sweet potato TIS 2154 and TIS 2328. Harvesting the crop for shoots led to a reduction in tuber yield. The reduction was 48% in variety TIS 2328 and 31% in variety TIS 2154. Harvesting the shoots at base led to a reduction of 62% for variety TIS 2328 and 50% for variety 2154. Similarly, Gonzales et al. (1977) reported that topping the sweet potato plants reduced tuberous root yield. Highest tuber yield was obtained where no topping was done.

MATERIALS AND METHODS

Experimental site condition

The experiment was conducted in April, 2017 at the Federal University of Agriculture Abeokuta. The study site lies between Latitude 7°14"N and Longitude 3°26"E and is located within a forest Savannah transition zone (Salako et al., 2007); it has two distinct seasons: wet season, which extends from March to October, and the dry season, which is usually from November to February. The rainfall is bimodal in distribution- usually from March to July and from September to October, with a characteristic of August break. Its temperature is between 32.4 and 33.47°C, relative humidity is 77.38% in April but decreases to 63.24% in November.

Source of planting materials

Sweet potato vines were sourced from the International Institute of Tropical Agriculture (IITA) in Ibadan. The vines obtained were cut into 20 cm length by using a sterilized sharp knife.

Land preparation

The field was ploughed and harrowed mechanically. Ridges were constructed manually with traditional hoes in the two experiments, and a walkway of 1m was left in-between plots.

Pre cropping and soil analysis

Soil sample (0-20 cm) was randomly taken before planting and bulked to form a composite sample. This sample was air dried pass through 2 mm sieve and laboratory for both physical and chemical analysis to determine soil texture and soil fertility

Experimental design and planting methods

The entire plot was measured as 28 m^2 length × 19 m^2 widght giving an area of 532 m^2 and the plots size was 5 m × 3.5 m each. There was walk way of 1 m each between two plots; 1m walk way was also maintained around the perimeter of the entire plot. Five ridges were constructed in each plot with dimension of 35 cm ridge. Planting was done on the ridges in each plot at an inter-row spacing of 0.5 m and intra-row spacing of 1 m. Thus, there were 7 plants on each ridge and 35 plants per plot. This gave a plant population of 630 plants (equivalent to 20,000 plants per hectare). There were two sweet potatoes varieties (SHABA and SPK-004) and three levels of cutting severity, (no cutting, cutting 15 and 30 cm long apical shoots at 4 weeks interval); the experiment was arranged in split-plot with variety as the main plot and cutting severity as sub-plot,in romdonmized complete block designed (RCBD). Weeding was done manually with hoe to minimize weeds infestation to the sweet potato plants. Four weedings were done at 4, 8, 12, 16 (WAP). Earthling up was done on all ridges to establish a desirable soil bulk for root expansion and moisture conservation.

Data collection

Data were randomly collected from 5 plants in the three mid- rows on the following parameters.

Vine length (cm)

Vines length was determined in centimeter using rope to tread from the base of the plant to the tip of five selected plants from the three middle inter rows at 5, 9, 13, 17 (WAP).

Number of leaves per plants

The total number of leaves per plant was counted from 5, 9, 13, 17 (WAP) and recorded as sample from the field.

Number of main branches per plant

The number of main branches per plant was counted and recorded at 5, 9, 13, 17 WAP.

Fresh apical shoot weight

The vine fresh apical shoots were weighed on an electronic balance scale and recorded in gram.

Dry apical shoots weight per plant in gram

The total weight obtained from the apical shoot was oven-dried in the laboratory at 70°C and expressed in gram.

Leaf area per plant (LA) (cm_2)

Leaf area was obtained by using meter rule to measure the length and breadth of the leaf. 90 samples of leaves of different sizes which were traced on graph sheet. The length and breadth measured were regressed on the leaf area as derived by Olasantan and Salau (2008). From the experiments, leaf area was derived for SHABA in equation I and for SPK-004 in equation II. The linear equation ise as follows:

$Y= 8.2988x +11.981 \quad r^2 = 0.4904$ (1)

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Table 1. Weather data, temperature relative humidity and sunshine hour's in 2017.

Months	Rainfall (mm)	Temperature (°C)		Relative humidity (%)	Sunshine hours
		Maximum	Minimum		
January	15.9	35.29	22.39	58.34	4.39
February	0	36.36	23.95	55.31	4.19
March	34.3	35.86	24.07	60.28	5.96
April	112.8	33.47	23.75	63.24	5.64
May	146	32.4	23.16	69.05	5.46
June	111	31.43	31.05	73.83	4.33
July	156.1	29.16	22.8	74.5	2.11
August	90.3	28.18	22.45	77.38	1.28
September	50	30.02	22.12	69.1	2.11
October	92.2	31.94	27.62	72.78	4.16
November	85.4	30.2	24.75	65.54	0.21

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Y= 4.279x-56.393 r^2 = 0.6314 (2)

Where Y = leaf area, X = leaf breadth.

The Leaf Area Index (LAI) was calculated as leaf areas of all plants divided by number of plants/ plot size.

$$LAI = \frac{\text{Leaf areas all of plants (cm}^2\text{) /plot}}{\text{Plot size (cm}^2\text{)}}$$

Number of tuber per plant

The number of tuber harvested per plant was counted and recorded.

Fresh tubers weight per plant during harvest

The fresh tubers' weight was recorded and measured in kilogram and expressed in metric tons per hectare

Numbers of marketable tubers per plant

The number of marketable fresh tuber weight per plant was sampled and sorted for tuber sizes; tuber above 1.5 cm was considered as marketable tuber; disease free tuber, and non-rotten tubers were considered and recorded

Number of unmarketable tubers

The numbers of unmarketable tubers, fresh weight per plant were sorted out; signs of being damaged by disease tubers, rough skin tubers and those eaten by rats and below 1.5 cm were recorded as unmarketable yield and expressed in tons per hectare. The total tuber yield per plot was measured on the field in kilogram and expressed in ton per hectare.

Tuber dry weight per hectare

The tuber weighed were oven dried to constant weight of 70°C and recorded.

Statistical analysis

All sweet potato plants were harvested at 6-7 months and number of tubers and their weight, and fresh tuber yield per hectare were recorded. Statistical analyses were conducted using the analyses of variance procedure according to spilt-plot design of statistical analyses system institute (1990). Treatment means were presented with the associated standard error of the means (S.E.) at 5% probability.

RESULTS

Weather data during the study in 2017 at Alabata Road in Abeokuta

Total rainfall at the Federal University of Agriculture, Abeokuta was 894 mm in 2017. The total rainfall during the period of the experiment (April-November) was 843.3 mm in 2017 (Table 1). Higher rainfall was recorded in July (156.1 mm) while the lowest rainfall was recorded in September (50 mm). Minimum temperature was between 12.12 and 22.8°C, from April to November 2017, while maximum temperature was between 32.4 and 33.47°C, relative humidity was 77.38 in April but decreased to 63.24% in November. Higher sunshine rate per hour during the period of the experiment was 5.64 h recorded in April 2017 and the lowest was 1.28 h which was recorded in August.

Experimental site of soil analysis

The soil used for the experiment was sandy loam, slightly acidic (pH 5.6). The soil was moderate in nitrogen content (0.15 %) but very high in Phosphorus and Potassium contents (40.36 respectively) (Table 2).

Table 2. Soil elements of experimental site.

Parameter	Content
pH	5.6
Clay (g/kg^{-1})	4.4
Sand (g/kg^{-1})	80.6
Silt (g/kg^{-1}	15.0
K-(cmol/kg	0.462
Ca_2 (emol/kg)	0.201
Mg^2-(emol/kg)	0.236
Organic Carbon (%)	2.476
Na Cmol (%)	0.435
AV.P (Mg/kg)	40.36
Nitrogen (%)	0.177
Exchangeable Ac (Cmol/kg)	2.1

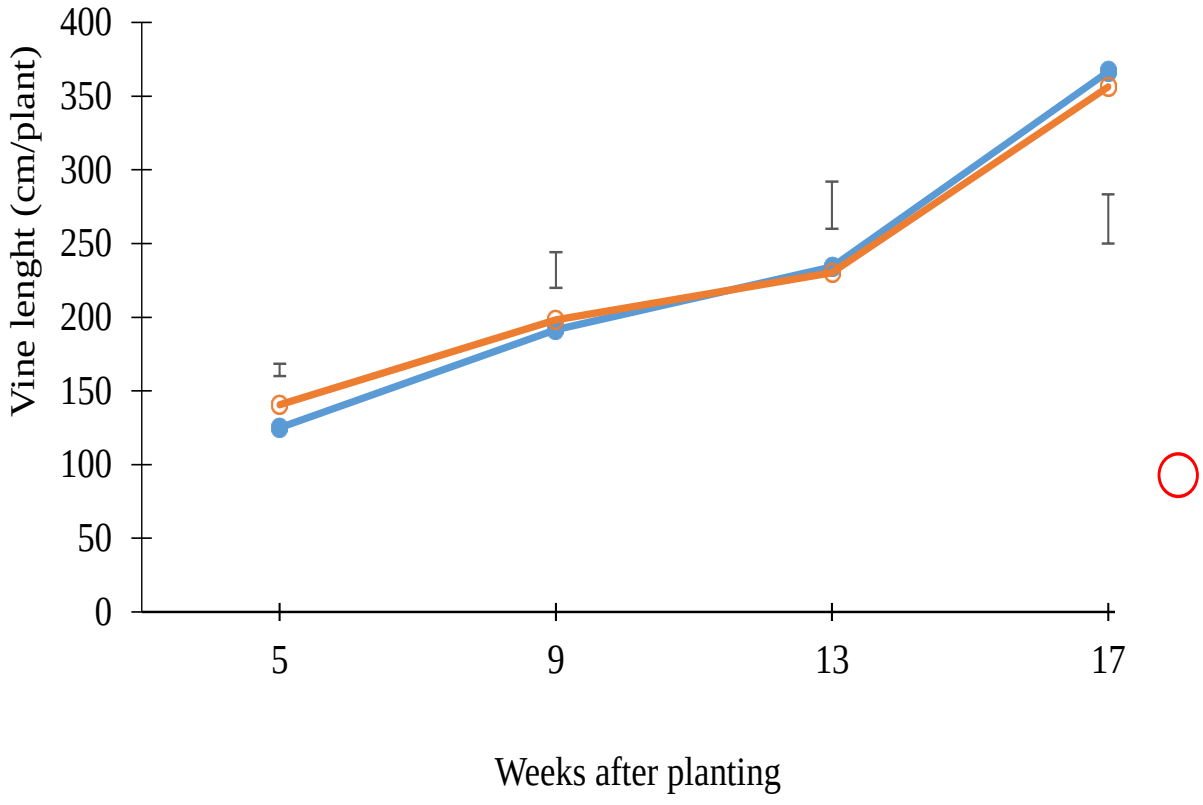

Figure 1. Vine length of two sweet potato varieties. SHABA (●), SPK-004 (○). Bars are SE at p≤0.05.

Vine length of sweet potato as affected by variety and cutting severity

The vines of SPK-004 was longer than that of SHABA at 9 WAP, while from 9-17 WAP, the two vines were similar (Figure 1). The control plant was significantly (P ≤0.05) longer than those plants whose apical shoots were cut at 15 and 30 cm long throughout the period of the experiment (Figure 2). Vine length of plant whose apical shoots were cut at 30 cm long were similar to plants whose apical shoot were cut at 15 cm long at 5 and 9 WAP. At 13 and 17 WAP, however sweet potato plants whose apical shoot was removed at 30 cm long was longer than sweet potato plant whose apical shoot was cut at 15 cm long.

The vine of SPK-004 control plant was significantly

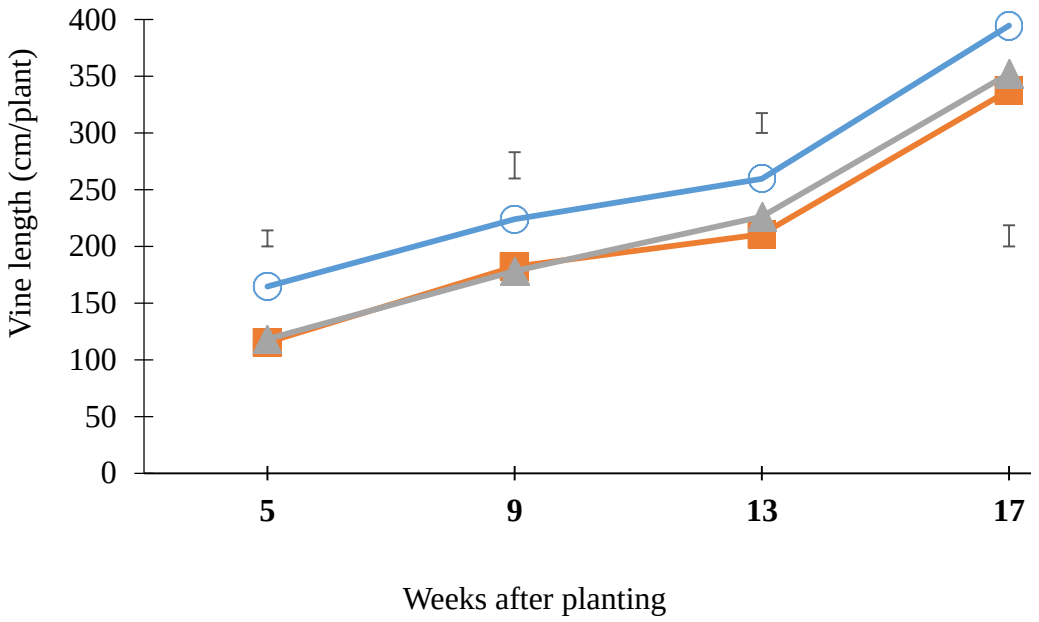

Figure 2. Vine length of two sweet potato Varieties as affected by cutting severity. Control, 15 cm and 30 cm. Bars are SE at p≤0.05.

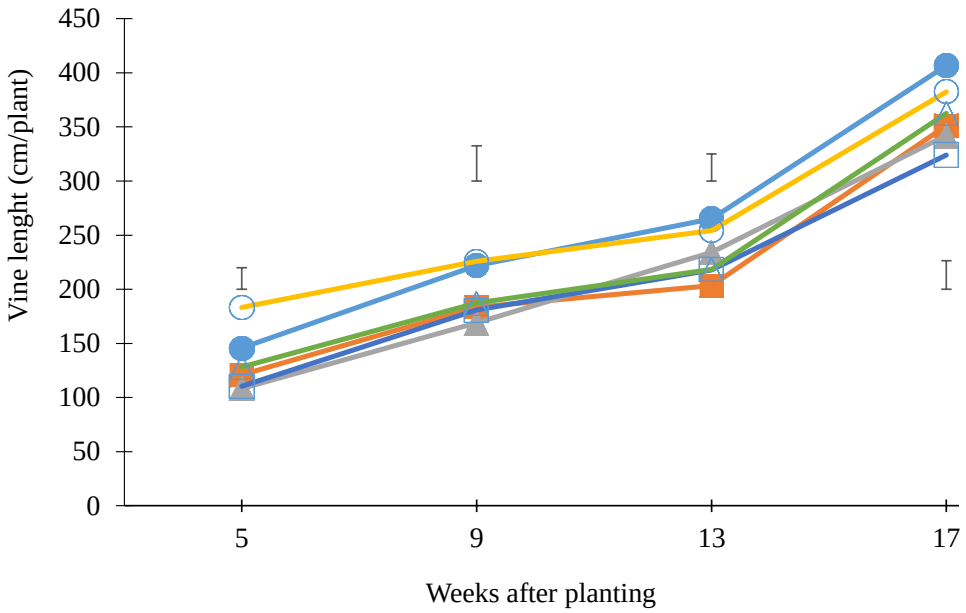

Figure 3. Interaction between variety and cutting severity on vine length of two sweet potato varieties. SHABA x Control, SHABA x 15 cm, SHABA x 30 cm, SPK-004 x Control, SPKK-004 x 15 cm and SPK-004 x 30 cm. Bars are SE at p≤0.05.

(P≤0.05) longer than SPK-004 whose apical shoot was removed at 15 cm long and in (Figure 3). At 5 and 17 WAP, the vine length of SPK-004 control plant was significantly (P≤0.05) longer than sweet potato plant whose apical shoot was cut 30 cm long at 5-9 WAP. However at 13 and 17 WAP SPK-004 control plant whose apical shoot was removed at 30 cm long was similar. At 5 WAP SHABA control plant whose apical shoots were

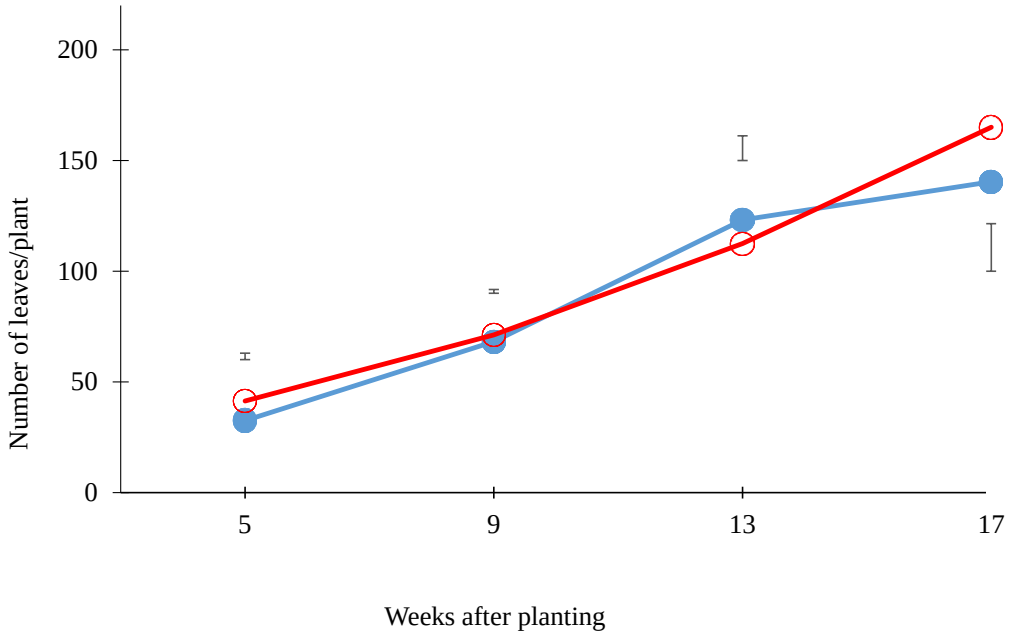

Figure 4. Number of leaves of two sweet potato varieties SHABA (●), SPK-004 (○). Bars are SE at p≤0.05.

removed at 15 and 30 cm long were at similar, while at 9 and 17 WAP SHABA control plant was significantly (P ≤ 0.05) longer than sweet potato whose apical shoot was removed at 30 cm long; but at 13 WAP control plant and SHABA whose apical shoot was cut at 15 cm long have similar vine length. At 5 to 17 WAP SHABA plant whose apical shoots were removed at 15 and 30 cm long produced similar vines (Figure 3).

Number of leaves of two sweet potato varieties (SHABA and SPK-004) as affected by cutting severity

The result in Figure 4 shows the varietal effect on number of leaves of SHABA and SPK-004. At 5-17 WAP the number of leaves on SPK-004 plant was significantly (P≤0.05) highest than the number of leaves on SHABA; however both varieties have similar number of leaves at 9 WAP, whereas at 13 WAP SHABA produced higher number of leaves than SPK-004. There was a gradual increase in the production of leaves of two sweet potato with respect to time. At 5 to 17 WAP, number of leaves of control plant was significantly (p ≤ 0.05) higher than sweet potato plant whose apical shoots were cut at both 15 and 30cm long. However the number of leaves on sweet potato plant whose apical shoots were removed at 15 and 30cm long were also similar at 5- 9 WAP; although at 13 - 17 WAP, the number of leaves on sweet potato plant whose apical shoot were removed at 15cm long were more than sweet potato plant whose apical shoots were removed at 30 cm long (Figure 5). At 5-17 (WAP), leaves produced by SHABA control plant were more than those produced by plant whose apical shoots were removed at both 15 and 30 cm long. However, for SPK-004 number of leaves produced by control plant was similar with that of plant whose apical shoots were removed at both 15 and 30 cm long at 5 WAP; at 13 WAP control sweet potato plant and plant whose apical shoot was removed at 15 cm long had more number of leaves than sweet potato whose apical shoot was removed at 30 cm long. However, control plant, cutting at 15 and 30cm long have similar vine length at 17 WAP .

Varietal effects on number of branches of two sweet potato variety

SPK-004 produced more branches than SHABA from 5-13 WAP, but from 15-17 there was significant (P≤ 0.05) increase in number of branches produced by SHABA as compared to SPK-004 (Figure 6). At 5 WAP, control plant produced higher number of branches than sweet potato plant whose apical shoots were removed at 15 and 30 cm long (Figure 7); however both sweet potato plant whose apical shoots were cut at 15 and 30 cm long were similar, whereas at 9 - 17 WAP control plant had significantly (P≤ 0.05) highest number of branches than sweet potato plant whose apical shoots were removed at 30 cm long. At 9 WAP control plant had significantly (P≤ 0.05) higher number of branches than sweet potato plant whose apical shoot was cut at 15 cm long, whereas at 13 -17 WAP control plant produced more number of branches

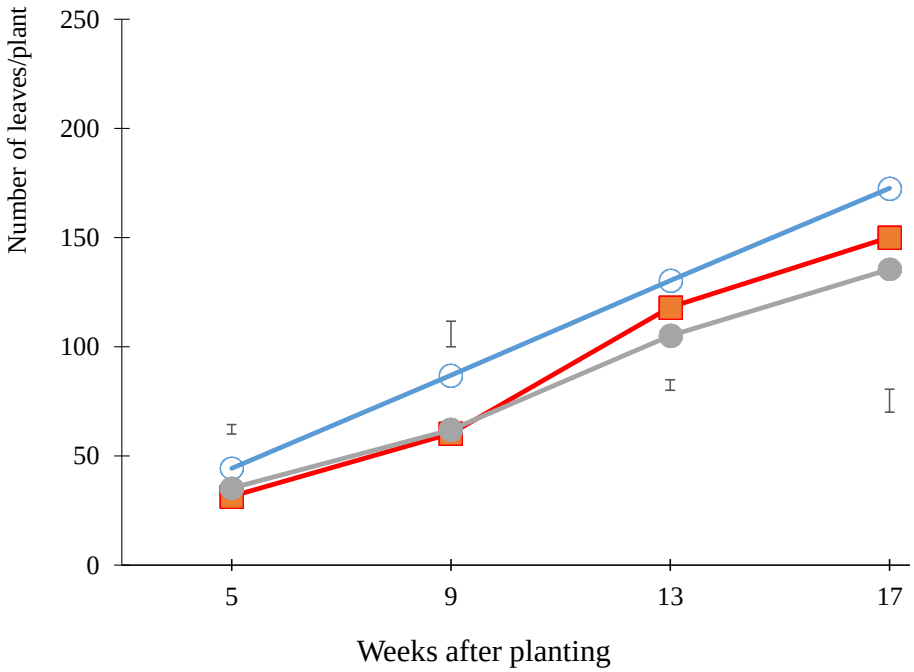

Figure 5. Number of leaves of two sweet potato varieties as affected by cutting severity Control ( ), 15 cm ( ) and 30 cm ( ). Bars are SE at p≤0.05.

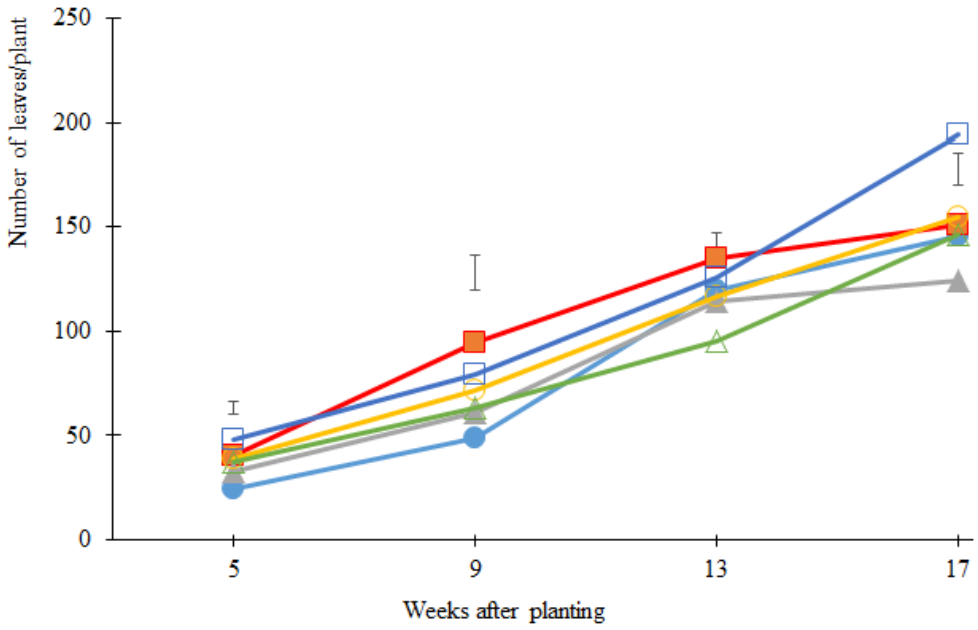

Figure 6. Interaction between variety and cutting severity on number of leaves of two sweet potato varieties. SHABA x Control ( ), SHABA x 15 cm ( ), SHABA x 30 cm ( ), SPK-004 x Control ( ), SPKK-004 x 15 cm ( ) and SPK-004 x 30 cm ( ). Bars are SE at p≤0.05.

than sweet potato plant whose apical shoot was removed at 15 cm long, although at 17 WAP sweet potato plant whose apical shoot was removed at 15cm long produced higher number of branches than sweet potato plant whose apical shoot was removed at 30 cm long. (Figure 8).

At 5-13 WAP SHABA control plant and cutting severity at 15cm long had similar number of branches, however at

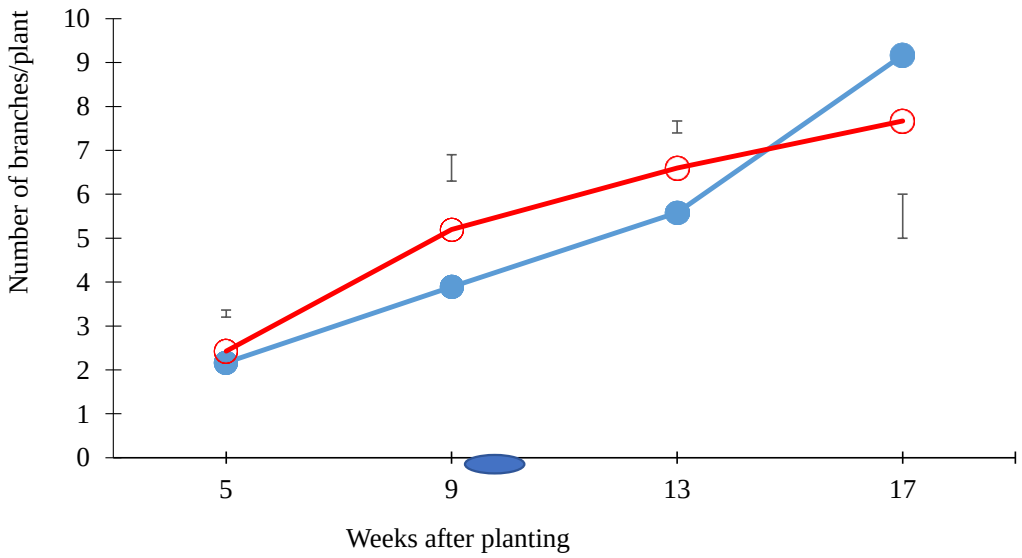

Figure 7. Number of branches of two sweet potato SHABA and SPK-004 of two sweet potato varieties SHABA, SPK-004. Bars are SE at p≤0.05.

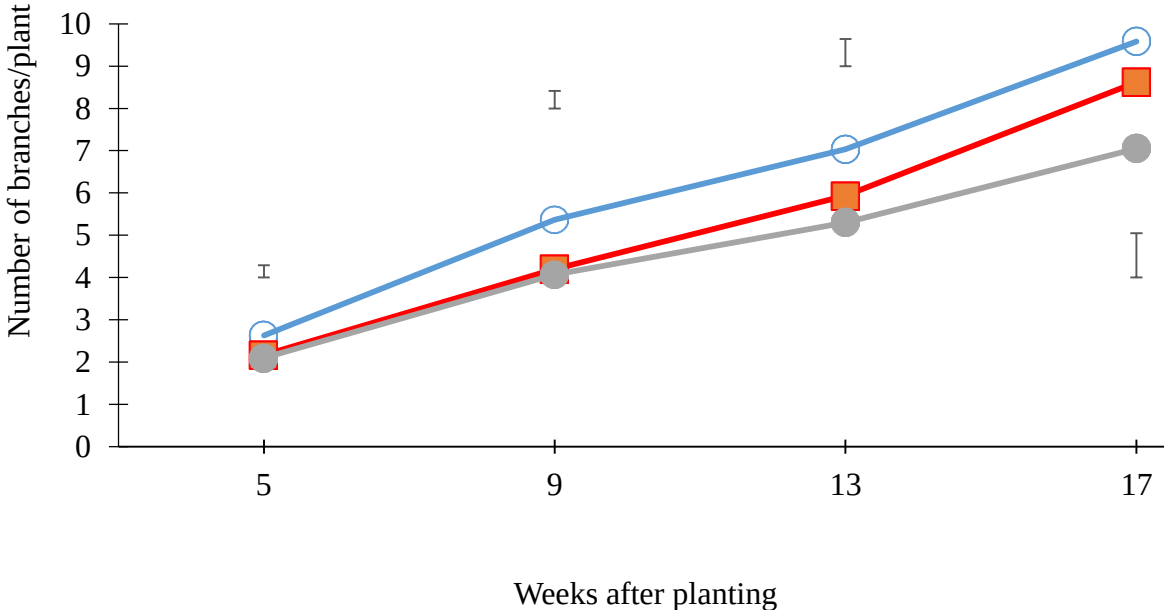

Figure 8. Number of branches of two sweet potato varieties as affected by cutting severity. Control (), 15 cm () and 30 cm (). Bars are SE at p≤0.05.

5-17 WAP SHABA control plant significantly (P≤ 0.05) produced higher number of branches than sweet potato plant whose apical shoot was removed at 30cm long (Figure 9); at 9 WAP control plant produced higher number of branches than sweet potato plant whose apical shoot was removed at 15 cm long. At 13 WAP cutting severity at 15 cm long had higher number of branches than sweet potato plant whose apical shoot was removed at 30 cm long and control plant. At 5-9 WAP SPK-004 control plant had similar number of branches with sweet potato plant whose apical shoots were cut at 15 and 30 cm long and at 13 WAP SPK-004 control plant produced higher number of branches than sweet potato plant whose apical shoots were removed at

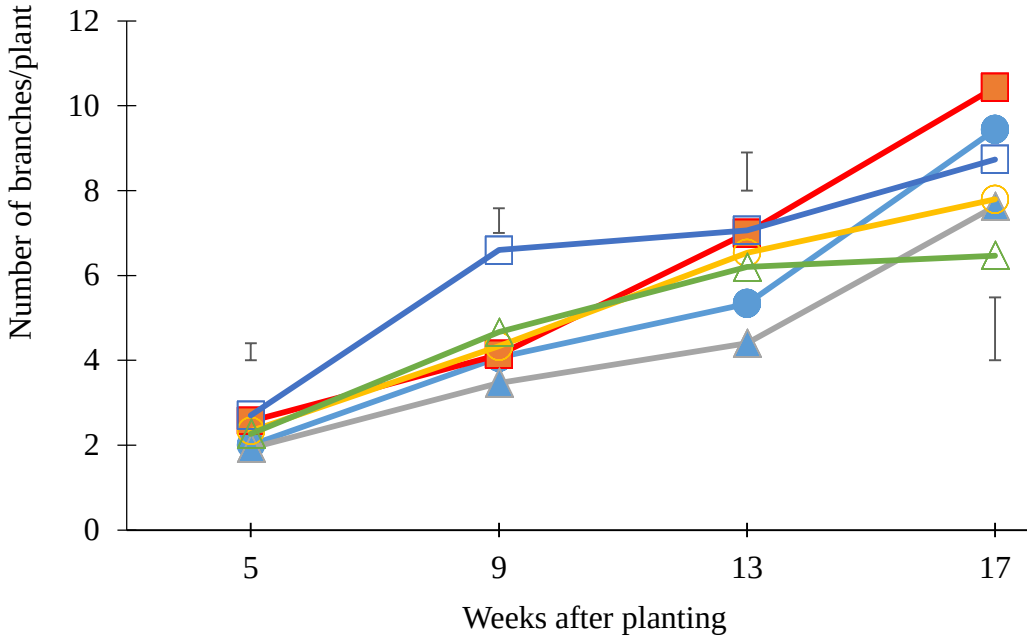

Figure 9. Interaction between variety and cutting severity on number of branches of two sweet potato varieties, SHABA x Control ( ), SHABA x 15 cm ( ), SHABA x 30 cm ( ), SPK-004 x Control ( ) SPKK-004 x 15 cm ( ) and SPK-004 x 30 cm ( ). Bars are SE at p≤0.05.

30 and 15 cm long; although at 17 WAP sweet potato plant whose apical was cut at 15cm long significantly (P≤ 0.95) produced higher number of branches than sweet potato plant whose apical shoot was removed at 30 cm long and control plant (Figure 9).

Leaf area index of two sweet potato as affected by variety and cutting severity

The leaf area index of SHABA had more leaf index cover than SPK-004 at 5 WAP; however at 9 -17 WAP SHABA plant produced significantly (P≤ 0.05) more leaf area index cover than SPK-004 plant (Figure 10). The leaf area index of two sweet potato as affected by cutting severity (Figure 11). Between 9 and 17 WAP, the leaf area index of sweet potato with control plant significantly (P≤ 0.05) produced higher leaves area index cover than that of plant whose apical shoots were removed at 15 and 30 cm long. However, there was no significant difference between sweet potato plants whose apical shoots were removed at 15 and 30 cm long at 5-17 WAP (Figure 11). Between 5-17 WAP, the leaf area index of SHABA control plant produced higher leaf area index cover than SHABA plant whose apical shoots were removed at 15 and 30 cm long (Figure 12). At 5-13 WAP, sweet potato plants whose apical shoot was removed at 30 cm long produced more leaf area index cover than SHABA control plant, whereas at 17 WAP SHABA control plant produced higher leaf area index cover than that of sweet potato plant whose apical shoots were cut at 30 cm long. At 5-9 WAP control plant and sweet potato plant whose apical shoots were removed at 15 and 30 cm long have similar leaf area index cover and at 13 WAP SPK-004 control plant and sweet potato plant whose apical shoots were cut at 15 cm long were similar. But both control plant and sweet potato plant whose apical shoot was cut at 15 cm long produced more leaf area index cover than SPK-004 whose apical shoot was removed at 30 cm. However at 17 WAP sweet potato whose apical shoot was cut at 15 cm long produced more leaf area index cover than control plant and sweet potato whose apical shoot was cut at 30 cm long (Figure 1).

The fresh apical shoot weight as affected by cutting severity

Fresh apical weight of SHABA was higher than that of SPK-004 at 9 WAP, but at 17 WAP, SPK-004, fresh apical shoots decreased at 21 WAP (Figure 13). Between 5 to 9 WAP, the fresh apical shoots weight of sweet potato plant whose apical shoots were removed at 15 - 30cm long was similar except at 13 to 21 WAP (Figure 14). Sweet potato plants whose apical shoot was removed at 30cm long produced significantly (P≤ 0.05) higher fresh shoots weight than sweet potato plant whose apical shoot was removed at 15 cm long (Figure 14). Between 5- 19 WAP, SHABA plant whose apical shoots were removed at 15 cm long produced significantly (P≤

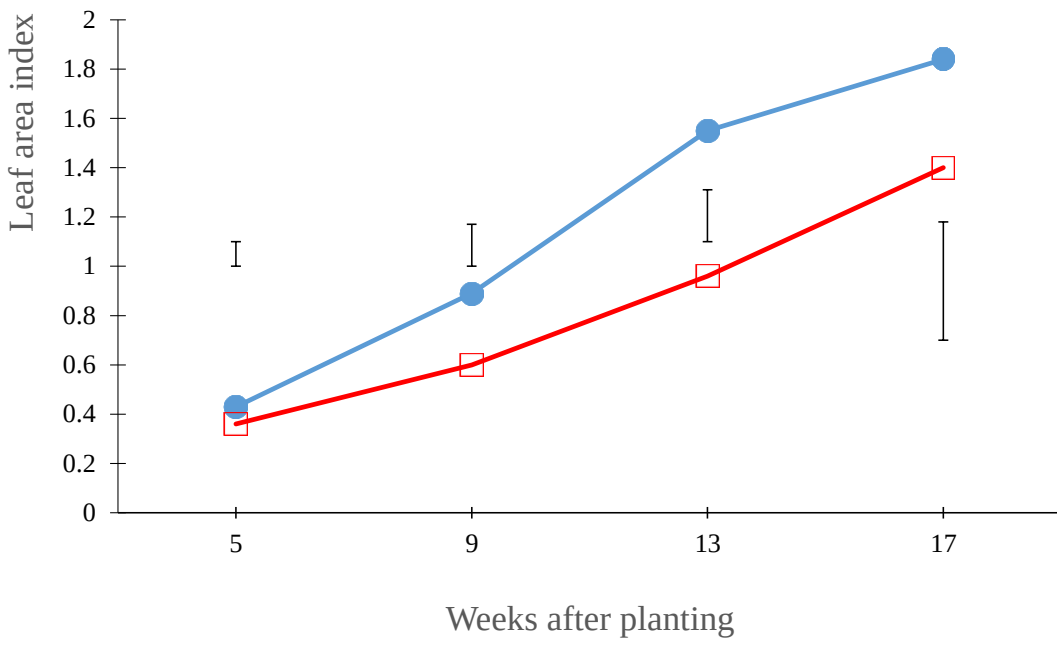

Figure 10. Varietal effect on leaf area index of two sweet potato varieties. SHABA ( ), SPK-004 ( ). Bars are SE at p≤0.05.

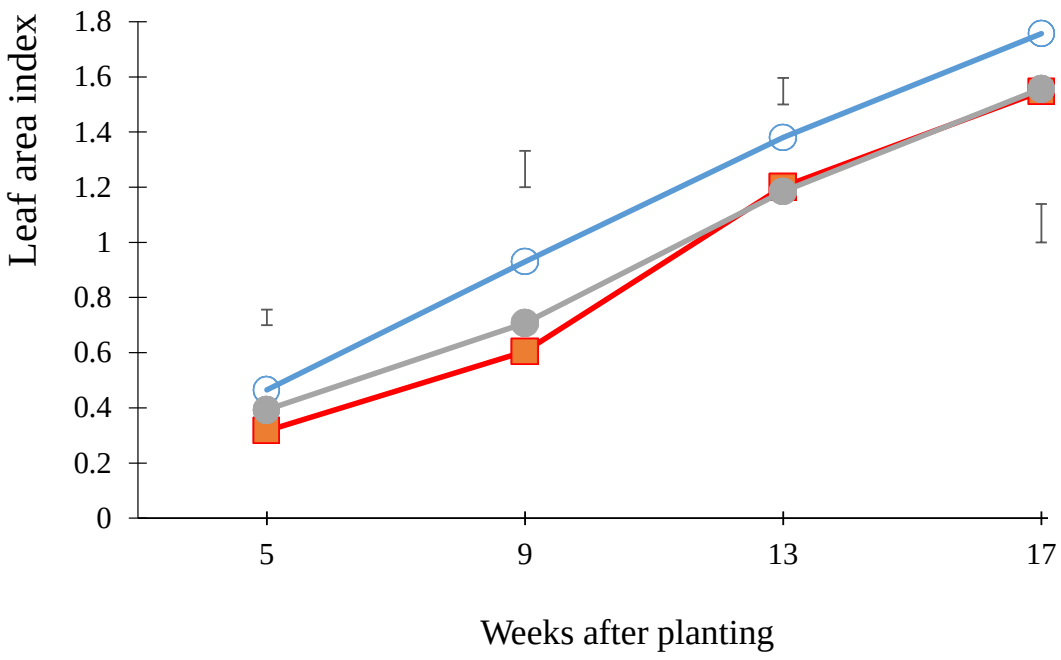

Figure 11. Leaf area index of two sweet potato varieties as affected by cutting severity. Control ( ), 15 cm ( ) and 30 cm ( ). Bars are SE at p≤0.05.

0.05) higher fresh apical shoot weight than SHABA whose apical shoots were removed at 15 cm long (Figure 15). At 9, 13 and 19 WAP, SPK-004 plant whose apical shoots were removed at 30 cm long produced significantly more fresh apical shoot weight than SPK-004 plant whose apical shoot was cut at 15cm long. However, at 5 and 9 WAP, SPK-004 plant whose apical shoot was cut at 15 and 30 cm long produced similar fresh apical

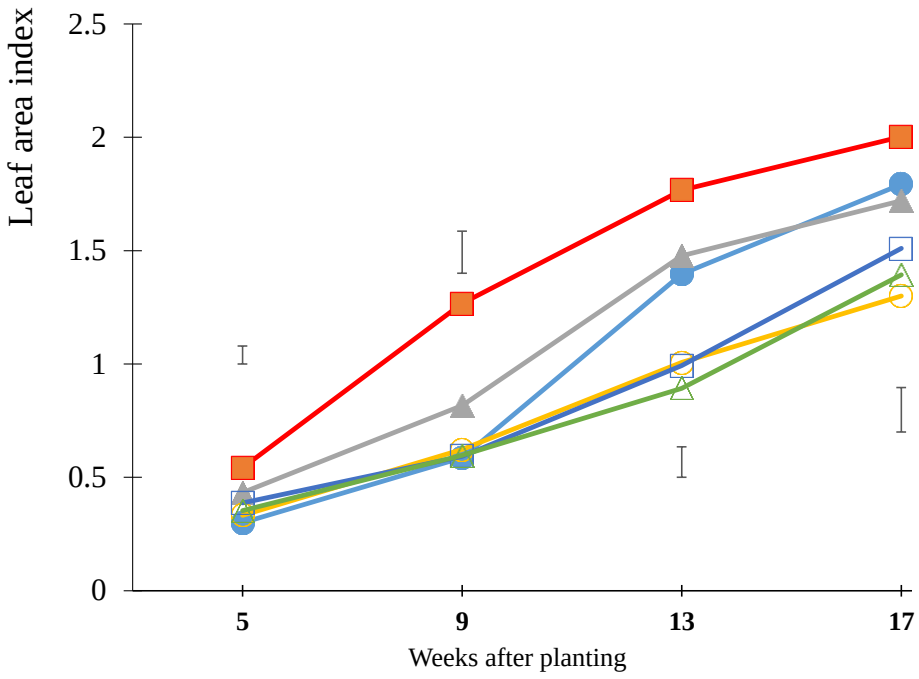

Figure 12. Interaction between variety and cutting severity on leaf area index of two sweet potato varieties. SHABA x Control ( ), SHABA x 15 cm ( ), SHABAx 30 cm ( ), SPK-004 x Control ( ), SPKK-004 x 15 cm ( ) and SPK-004 x 30 cm ( ). Bars are SE at p≤0.05.

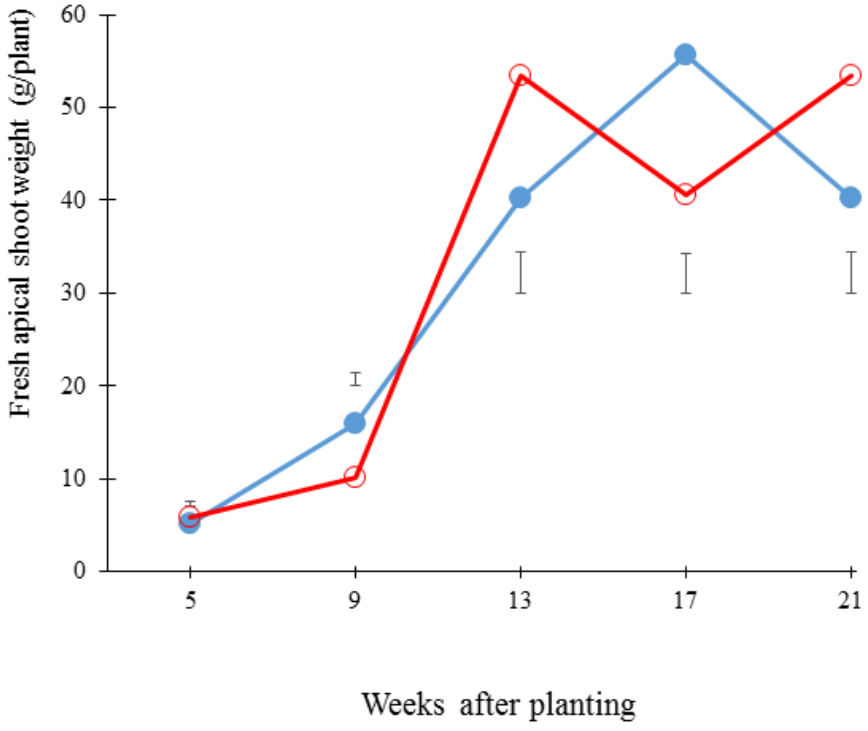

Figure 13. Fresh apical shoots weight of two sweet potato varieties. SHABA ( ) SPK-004 ( ). Bars are SE at p≤0.05.

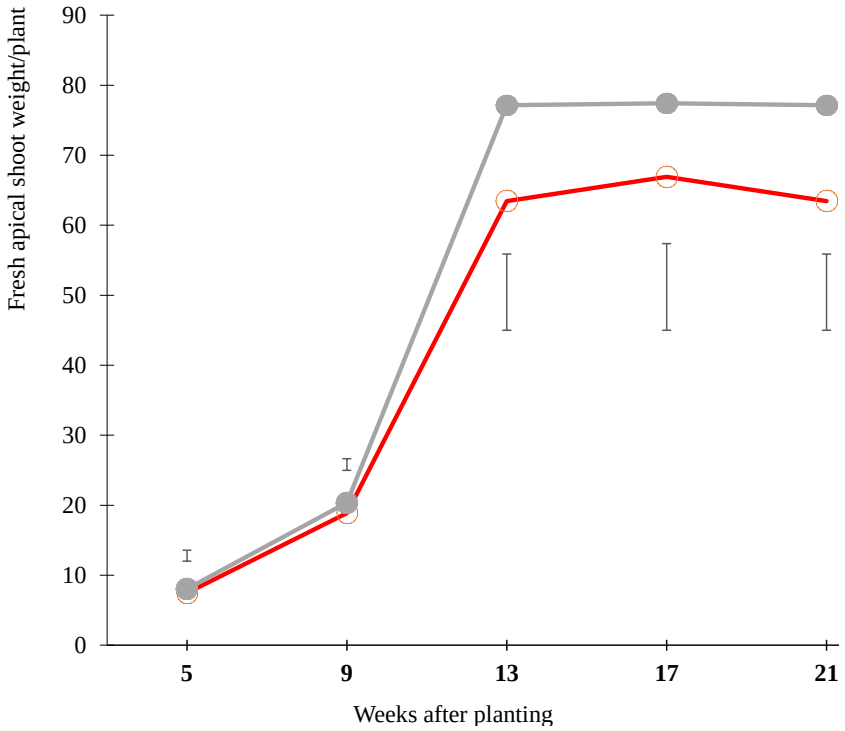

Figure 14. Fresh apical shoots weight of two sweet potato varieties as affected by cutting severity. 15 cm ( ) and 30 cm ( ). Bars are SE at p≤0.05.

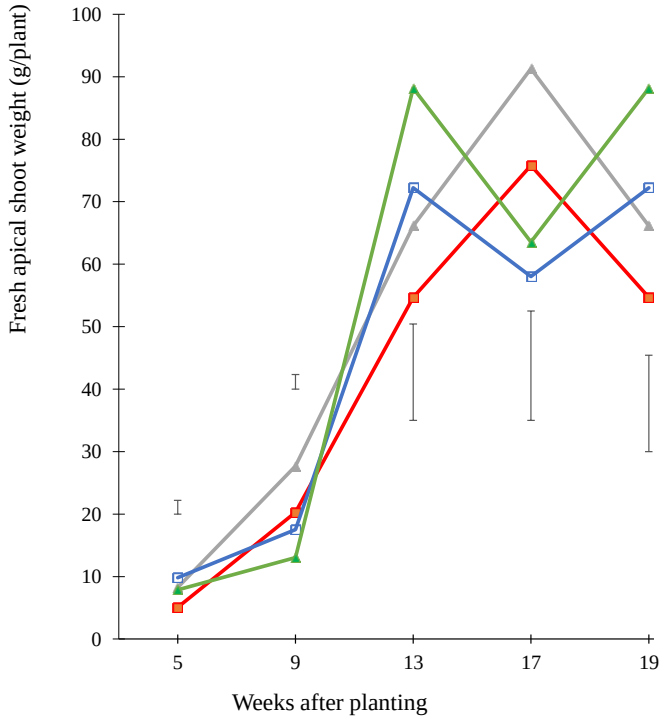

Figure 15. Interaction between variety and cutting severity on fresh apical shoot weight of two sweet potato, SHABA x 15 cm ( ), SHABA x 30 cm ( ), SPKK-004 x 15 cm ( ) and SPK-004 x 30 cm ( ). Bars are SE at p≤0.05.

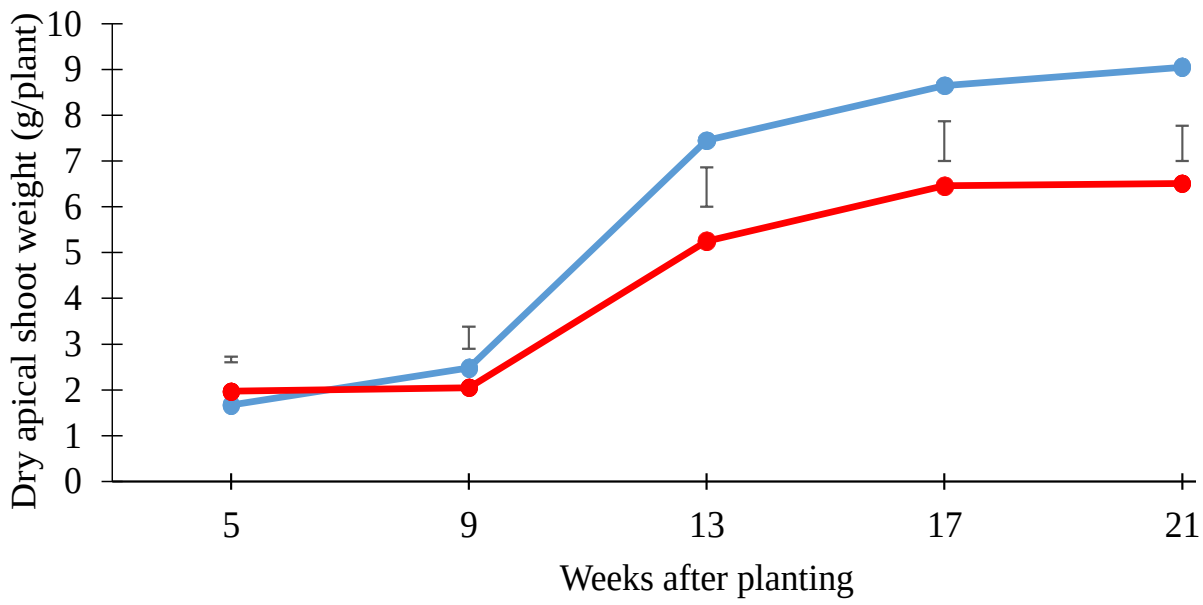

Figure 16. Dry apical shoot weight of two sweet potato varieties SHABA ( ), SPK-004 ( ). Bars are SE at p≤0.05.

shoot weight. Whereas at 13, 17 and 19 WAP, SPK-004 plant whose apical shoot was removed at 30cm long produced more apical shoot weight than SPK-004 plant whose apical shoot was removed at 1m long (Figure 15).

Dry apical shoot weight of two sweet potato as affected by variety and cutting severity

At 5 WAP, SPK-004 produced higher dry apical shoots weight than SHABA; however at 9 WAP SHABA and SPK-004 produced similar dry apical weight. While at 13-21 WAP SHABA plant produced higher dry apical weight than SPK-004 plant (Figure 16). At 5, 9 and 21 WAP, sweet potato whose apical shoot was removed at 30 cm long produced more dry apical shoot weight than sweet potato plant whose apical shoot was cut at 15 cm long; however at 13 - 17 WAP sweet potato plant whose apical shoot was cut at 30 cm long produced higher apical shoot weight than sweet potato plant whose apical shoot was removed at 15 cm long (Figure 17). At 5, 9 and 21 WAP SHABA plant whose apical shoots was cut at 30cm long produced similar dry apical shoot weight, whereas, at 13 and 17 WAP, SHABA plant whose apical shoot was cut at 30cm long produced significantly (P≤ 0.05) more apical shoot than SHANA plant whose apical shoot was removed at 15 cm long (Figure 18). At 5-9 WAP SPK-004 whose apical shoots were cut at 15 and 30cm long produced similar dry apical shoots. However at 13, 17 and 21 WAP SPK-004 plant whose apical shoot was cut at 30cm long produced drier apical shoots weight than 15 cm long (Figure 18) .

Total fresh and dry apical shoot weight of sweet potato as affected by variety and cutting severity

The interaction between variety and cutting severity showed that SHABA whose apical shoots were cut at 30 cm long had significantly (P≤0.05) higher total fresh weight than SHABA cut at 15cm long.SPK-004 whose apical shoots were cut at 30cm had significantly (P≤0.05) higher total fresh shoots weight than SHABA cut at 15cm long (Table 3).

SHABA whose apical shoots was removed at 30cm long had significantly (P≤0.05) higher total dry apical shoot weight than SHABA removed at 15 cm. However, SPK-004 whose apical shoots were cut at 15 and 30 cm long was similar with respect to total dry apical shoot weight. Total dry apical shoot weight of SHABA was significantly (P≤0.05) higher than that of SPK-004. Total fresh and dry apical shoot weight of sweet potato plant whose apical shoots was cut at 30 cm long was significantly higher than those cut at 15 cm long.

Unmarketable, marketable and total tuber weight of sweet potato as affected by variety and cutting severity

The interaction between variety and cutting severity significantly (P≤ 0.05) affected unmarketable, marketable and total tuber weight of sweet potato (Table 4). SHABA plant cut at 15 and 30 cm long produced similar unmarketable and marketable tuber weight. However, SHABA plant whose apical shoot was harvested at 15 cm

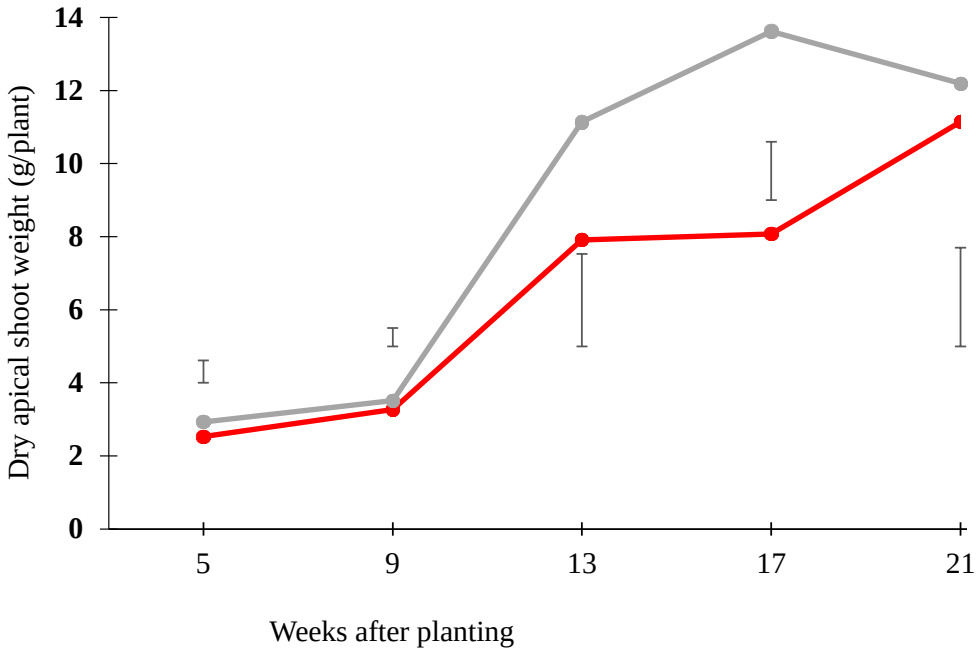

Figure 17. Weight of dry shoot of two sweet potato varieties as affected by cutting severity. 15 cm ( ) and 30 cm ( ). Bars are SE at p≤0.05.

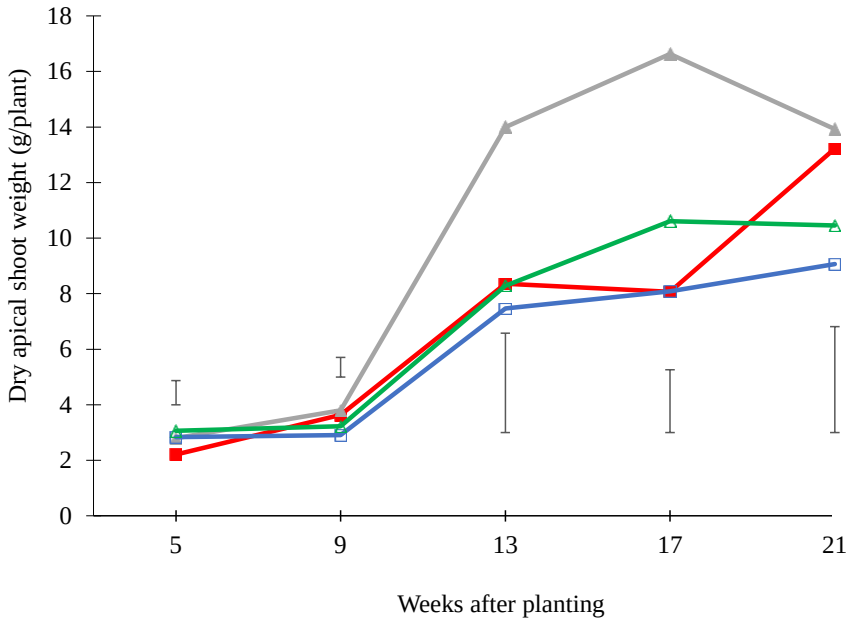

Figure 18. Interaction between variety and cutting severity on dry apical shoot weight of two sweet potato variety. SHABA x 15 cm ( ), SHABA x 30 cm ( ), SPKK-004 x 15 cm ( ) and SPK-004 x 30 cm ( ). Bars are SE at p≤0.05.

long produced significanly (P ≤0.5) highest total tuber weight, followed by SHABA control plant and the least was recorded in SHABA plant whose apical shoot was removed at 30 cm long.

The SPK-004 plant without cutting produced highest unmarketable tuber weight than SHABA whose apical

Table 3. Total fresh and dry apical shoot weight per plant of sweet potato as affected by variety and cutting severity.

Treatments		Total fresh shoot weight g/plant	Total dry shoot weight g/plant
Variety	**Severity**		
SHABA	Control	--	--
	15 cm	212.7	35.5
	30 cm	259.3	51.2
SPK-004	Control	-	--
	15 cm	229.8	30.4
	30 cm	260.8	35.6
	SE(8 D.F.)	20.4	7 .9
	Variety		
	SHABA	157.3	29.3
	SPK-004	163.5	22.2
	SE(8 D.F.)	3.9	2.4
	Severity		
	Control	-	-
	15 cm	221.3	32.9
	30 cm	260.0	43.4
	SE(8 D.F.)	14.4	5.6

shoots were removed at 15 and 30 cm long. SPK-004 plant without cutting and those plants whose apical shoots were removed at 15 and 30 cm long produced similar marketable and total tuber weight. The effects of variety on unmarketable, marketable and total tuber weight of sweet potato was not significant at ($P \leq t0.05$).

However, both SHABA and SPK-004 plant produced similar unmarketable, marketable and total tuber weight. The weight of unmarketable tuber in sweet potato as affected by cutting severity was significant. Table 4 shows that the unmarketable tuber weight of the sweet potato control plant was significantly ($P \leq 0.05$) more than those plant whose apical shoot was removed at 30 cm, while the weight of marketable tuber and total tuber weight were similar in control plant, at 15 and 30 cm long (Table 4)

Unmarketable, marketable and total tuber number of sweet potato as affected by cutting severity

There was no significant difference between SHABA without cutting and those whose apical shoots were removed at 15 and 30 cm long with respect to unmarketable, marketable and total tuber number (Table 5). Similarly SPK-004 without cutting and those whose apical shoots were cut at 15 and 30 cm long had similar unmarketable, marketable and total tuber number. SHABA produced significantly ($P \leq 0.05$) similar number of unmarketable and marketable tubers than SPK-004. But total tuber, number of SHABA was more than that of SPK-004 (Table 5). The number of unmarketable, marketable and total tuber of sweet potato as affected by cutting severity is shown in Table 5. The number of unmarketable and total marketable tubers were similar in sweet potato without cutting and those whose apical shoots were cut at 15 and 30 cm long.

Unmarketable, marketable and total tuber yield of sweet potato as affected by cutting severity

The interaction between variety and cutting severity as it affects unmarketable, marketable and total tuber yield of sweet potato is shown in Table 5. Yield of unmarketable and marketable tuber of SHABA without cutting and those whose apical shoots were removed at 15 and 30 cm long were similar. However, SHABA whose apical shoots were removed at 15cm long produed more tuber yield than SHABA without cutting and SHABA whose apical shoots were removed at 30cm long. SPK-004 without cutting had significantly highest unmarketable tuber yield than SPK-004 plant whose apical shoots were removed at 15 and 30 cm long. SPK-004 plant without cutting and those whose apical shoots were removed at 15 and 30 cm long were similar in their marketable and total tuber yield.

Variety did not significantly influence unmarketable, marketable and total tuber yield sweet potato yield of unmarketable, marketable and total tuber yield of SHABA

Table 4. Unmarketable, marketable and total tuber weight per plant of two sweet potato as affected by cutting severity.

Treatment		Unmarketable tuber weight (g/plant)	Marketable tuber weight	Total tuber weight (g/plant)
Variety	**Severity**			
SHABA	Control	77.0	251.8	328.5
	15 cm	102.0	299.9	401.9
	30 cm	53.7	229.0	282.7
SPK-004	Control	115.7	201.0	316.4
	15 cm	74.7	191.9	266.9
	30 cm	64.3	238.4	302.6
	SE(8 D.F.)	27.5	71.5	92.0
	Variety			
	SHABA	260.3	337.7	597.7
	SPK-004	210.4	295.3	505.7
	SE(8 D.F.)	74.1	86.5	160.6
	Severity			
	Control	96.3	226.4	322.4
	15 cm	88.3	245.9	334.4
	30 cm	59.0	233.7	292.6
	SE(8 D.F.)	19.4	50.6	65.1

Table 5. Unmarketable, marketable and total tuber number per plant of two sweet potato as affected by variety and cutting severity.

Treatment		Number of unmarketable tuber/plant	Number of marketable tuber/plant	Total number of tuber/plant
Variety	**Severity**			
Shaba	Control	3.00	2.67	5.67
	15 cm	3.00	3.00	5.67
	30 cm	2.33	2.33	4.67
SPK-004	Control	2.33	3.00	5.33
	15 cm	1.67	2.00	3.33
	30 cm	2.00	2.33	4.33
	SE(8 D.F.)	0.77	0.96	1.33
	Variety			
	SHABA	2.78	2.67	5.33
	SPK-004	2.00	2.44	4.33
	SE(8 D.F.)	0.68	0.29	0.38
	Severity			
	Control	2.67	2.83	5.50
	15 cm	2.33	2.50	4.50
	30 cm	2.17	2.33	4.50
	SE(8 D.F.)	0.54	0.68	0.94

was similar compared to that of SPK-004. The yield of unmarketable, marketable and total tuber of sweet potato as affected by cutting severity is shown in Table 6. The control plant produced more marketable tuber compared to plant whose apical shoots were removed at both 15 cm and 30 cm long; however, unmarketable tuber yield was

Table 6. Unmarketable, marketable and total tuber yield of affected by variety and cutting severity.

Treatment		Unmarketable tuber yield t/ha	Marketable tuber yield t/ha	Total tuber yield t/ha
Variety	**Severity**			
Shaba	Control	3.06	10.07	13.14
	15 cm	4.08	12.00	16.07
	30 cm	2.15	9.16	11.31
SPK-004	Control	4.61	8.04	12.66
	15 cm	3.00	7.68	10.67
	30 cm	2.57	9.53	12.11
	SE(8 D.F.)	1.10	2.86	3.68
	Variety			
	SHABA	3.09	10.41	13.51
	SPK-004	3.39	8.42	11.81
	SE(8 D.F.)	0.81	2.96	3.46
	Severity			
	Control	3.84	9.06	12.90
	15 cm	3.54	9.84	13.37
	30 cm	2.36	9.35	11.71
	SE(8 D.F.)	0.78	2.02	2.60

Table 7. Correlations between yield parameter.

Variable	Severity of cutting			
	Total tuber weight	Total unmarketable weight	Total dry vine weight	Total fresh vine weight
Total marketable weight	0.929**	0.459	0.013	-0.095
Total tuber weight		0.672**	0.005	-0.079
Total unmarketable weight			-0.096	-0.077
Total dry vine weight				0.681**

more in apical shoot cut at 15 cm long than apical shoot cut at 30 cm long. There was no significant difference between control plant and sweet potato whose apical shoots were cut at 15 and 30 cm long in marketable and total tuber yield.

Correlations between yield parameter

Total marketable weight was significantly correlated with total tuber weight; total tuber weight positively correlated with total unmarketable weight and total dry apical shoot weight was significantly correlated with total fresh apical shoot weight under both treatments of cutting severity and frequency of cutting (Table 7).

DISCUSSION

There was high rainfall in May which was maximum in July, while high amount was also recorded in October after a period of low rainfall in August and September. This indicates tri-modal pattern of rainfall. This was against the bi-modal pattern of rainfall reported by Adejuwon and Odekunle (2006). Sweet potato crop grows on negligible soils with partial inputs. It has the capability to tolerate harsh soil and climatic conditions and yet give satisfactory yield. It grows well in fertile and high organic matter, well-drained, light, and medium textured soils. The relatively low fertility status of the soil of the study location is a peculiar characteristic of most soil in South-western Nigeria. This low fertility status could be attributed to the degraded state of most tropical soil Agboola (1973) wrote about some of the farmers in the south who have refused to apply fertilizer to any farmland used in yam production because they have noticed that using fertilizer to grow white yam changes the colour of the yam to brown during pounding. Also this could be as a result of soil erosion and nutrient mining as a result of continuous cropping.

The results obtained in this experiment showed that variety does not influence vine length and number of leaves of sweet potato. However, SHABA variety produced significant higher (P ≤ 0.05%) leaf area index. Severity of cutting affected the growth of sweet potato. Sweet potato without cutting had longer vine length, number of leaves, branches and leaf area and leaf area index. This influence of cutting severity on sweet potato shows that harvesting of sweet potato leaves affect growth. This was in line with result by Olorunnisomo (2007) who reported that leaf harvest intensity influences the branching intensity in sweet potato crop. Better growth performance of SHABA variety cut at 15cm could be as a result of the better ability of the variety response to cutting severity . The dry matter yield and total yield of sweet potato was enhanced by variety. Better performance obtained in the SHABA variety could be attributed to the efficiency of the variety in utilization of photosynthates and soil nutrients. Cutting 15cm long apical shoot generally gave higher total tuber yield than cutting at 30cm long apical shoot at 4 weeks. However, better performance was recorded in SHABA variety cut at 15 cm than SPK=004. This indicated that minimal vine cutting in sweet potato does not adversely affect yield of the variety.

Higher nutrient content was recorded in the SPK-004 than SHABA. The higher nutrient content in the less vigorous variety could be as a result of less dilution effect with respect to moisture accumulation by the vigorous variety. Cutting severity at 30 cm had higher nutrient content and the response of each variety to severity of cutting indicated both varieties cut at 30 cm had higher nutrient content. Harvesting of forage at regular intervals is a potent agronomic tool used in maintaining a balance between yield and quality in forage species (Hong et al., 2003). The result obtained in this study on effect of variety on growth of sweet potato shows that variety affects vine length, number of leaves, number of branches and leaf area index. SHABA variety was more vigorous than SPK-004 vine length and Leaf area index.

Conclusion

Severity of apical shoot harvest had effects on the growth of the sweet potato varieties with the best cutting severity being the control with respect to vine length and number of leaves while cutting at 15 cm was the best for number of branches and leaf area.However, cutting at 30 cm increased shoot yield while cutting at 15 cm increased root yield and nutritional value. Furthermore, cutting severity had effect on the growth performance of the sweet potato varieties; SHABA had the best growth performance with respect to vine length, number of branches and leaf area while variety SPK-004 had the best growth performance with respect to the number of leaves. In the study, variety SPK-004 performed better than SHABA with respect to fresh shoot yield while SHABA performed better than SPK-004 with respect to total tuber yield.

Recommendation

Cutting sweet potato apical shoots at 30 cm is recommended for cultivation intended for optimum shoot production while cutting at 15 cm is recommended if the root yield is of interest. Variety SHABA is recommended for production intended for optimum tuber yield while variety SPK-004 is recommended as shoot yield of interest. A repeat of this study is recommended for the purpose of validation, especially in regions where both sweet potato shoot and root production are of significant economic importance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Modulation in growth and development of potato (*Solanum tuberosum* L.) microtubers by different concentration of 6-benzyl aminopurine

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Two potato varieties namely, *kufri bahar* and *kufri surya* were tested for *in vitro* tuberization response to a same culture medium supplemented with three levels of 6-benzly aminopurine (0.75, 1.5 and 2.25 mg/L) in a completely randomized block design. The study was conducted in the Central Potato Research Institute (CPRI), Modipuram, Meerut Campus, and India during the period of 2012 to 2013 and 2013-2014. The objective was to determine optimum concentration of 6-benzly aminopurine for tuberization. Both varieties, exhibited a better response when the culture medium supplemented with 2.25 mg/L of 6-benzyl aminopurine than the other concentrations and control for mean values of number of days for tuber initiation microtuber number, fresh weight of microtubers and number of eyes per microtuber was found optimum and it may be useful to enhance tuber quality as well as crop growth under *in vitro* conditions at farmer and industrial levels.

Key words: *In vitro* plantlets, potato cultivars, 6- benzyl aminopurine, *in vitro* plants.

INTRODUCTION

In India, potato is an important commercial crop (As per the final estimates of 2017-2018, horticulture production stood at record 311.7 MT, which is 3.7% higher than the previous year and 10% higher than the past five years average production area (https://m.economictimes.com/news). India is second largest producer of potatoes in the world after China. India showed tremendous growth in potato production during last one and half decade (Rana and Anwer, 2018). The crop is damaged by many pests and diseases (like late blight, Bacterial bilt etc), so to provides and cultivate diseases-free plants and enhances genetic manipulation to improve the existing cultivars and to generate of novel plants a good procedure is require. Approximately 15% of the total area under potato cultivation around the world is used for the production of tuber seeds (Amina et al., 2006). However, tubers formed through these conventional conditions are susceptible to pathogen infections, thereby resulting in poor quality and yield and are difficult to transport and store due to their large size (Nhut et al., 2006). Tissue culture is a reliable technique can eliminate viruses in during tuber seed production programs and microtuber is one of the strategies in this perspective (Wang and Hu, 1982). The work on potato

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Figure 1. Macronutrients Solution.

Figure 2. Micronutrients Solution.

plant has been conducted as *in vitro* growth of potato plant (*in vitro* tuberization) with the main purpose of initiation of potato plant culture *in vitro*, micro propagation of potato plant, production of microtubers and hardening and transfer of plant to the field (soil) (Joseph et al., 2015). Microtubers are very small tubers produced by allowing micro plants to grow under tuber inducing conditions.

Micotubers have some advantages over *in vitro* micro plants. They are very convenient and easy to transport and store. Production of microtubers is possible throughout the year. They have the same health status as *in vitro* micro plants, and unlike micro plants they do not require hardening (Struik and Wiersema, 1999). The quality *in vitro* micro plants and micro tubers can be affected by a combination of growth regulators and environmental conditions (Salimi et al., 2010; Yeasmin et al., 2011).

Growth regulators are commonly used in in vitro multiplication. Several compounds, including gibberellic acid (GA3), cytokinin (CK), jasmonic acid, auxin, abscisic acid and sucrose have been reported to participate in the regulation of tuber formation (Rodriguez-Falcon et al., 2006). Kinetin, 6- benzylaminopurine (BAP) and Chloro choline chloride (CCC) have extensively been used in tissue culture medium to promote the micro tuberization (Hussey and Stacey, 1981). BAP at concentration (below 8 mg/L) used for microtuber production average number, weight and eyes, while with the increasing concentration of BAP (up to 10 mg/L) inhibits the average number, weight and eyes number of micro tubers (Badoni and Chauhan, 2010). Since the effect of BAP on potato micro tuberization has been established, in this experiment, influence of three concentrations (0.75, 1.5 and 2.25 mg/L) of 6-benzyl aminopurine on physical characteristics of microtubers such as microtuber initiation, number of microtubers, weight of microtubers and eyes of microtuber of potato were investigated.

MATERIALS AND METHODS

The study was conducted in the Central Potato Research Institute (CPRI), Modipuram, Meerut Campus, and India during the period of 2012-2013 and 2013-2014. Virus free certified micro plants of potato (*Solanum tuberosum* L.) were collected from (CPRI) Modipuram, Meerut. Two varieties namely *kufri bahar* and *kufri surya* were selected for tissue culture studies under lab conditions. For plantlets multiplication, nodal cuttings of potato both varieties were cultured in test tubes (25×150 mm) containing 15 ml of solidified (0.8% agar) Murashige and Skooge MS (Murashige and Skoog, 1962)

Macronutrients amount in g/L

KNO_3-38.00 g/L, NH4NO_3-33.00 g/L, MgSO4.7H_2O-7.4 g/L, KH_2PO_4-3.4 g/L and $CaCl_2$.2H_2O- 8.8 g/l. KNO_3, NH_4NO_3, $MgSO_4$.7H_2O and KH_2PO_4 were dissolved in 1000 ml double distilled (DD) water. $CaCl_2$.2H_2O was dissolved in 500 ml double distilled (DD) water. After that both the solutions were mixed together and make volume to 2000 ml with DD water. The prepared solution was stored at 4°C (Figure 1 and Table 1).

Micronutrients amount in mg/L

$MnSO_4$.4H_2O- 2.230 mg/L, $ZnSO_4$.7H_2O-860 mg/L, H_3BO_3 – 620 mg/L, KI-83 mg/L, $Na_2MOO4.2H_2O$-25 mg/L, $CuSO_4$.5H_2O-2.5 mg/L and $COCl_2$.6H_2O-2.5 mg/L. All the micronutrients were dissolved in DD water and make volume to 1000 ml and were stored at 4°C (Figure 2 and Table 2).

Table 1. Macronutrients amount in g/l

Macronutrients	Amount in g/L
KNO_3	38
NH_4NO_3	33
$MgSO_4.7H_2O$	7.4
KH_2PO_4	3.4
$CaCl_2.2H_2O$	8.8

Table 2. Micronutrient amount in mg/L

Micronutrient	Amount in mg/L
$MnSO_4.4H_2O$	2.23
$ZnSO_4.7H_2O$	860
H_3BO_3	620
KI	83
$Na_2MOO_4.2H_2O$	25
$CuSO_4.5H_2O$	2.5
$COCl_2.6H_2O$	2.5

Figure 3. Vitamins Solution.

Vitamins amount of mg/L

Myo-inositol- 10,000 mg/L, Thiamine-HCl- 10 mg/l, Nicotinic acid-50 mg/L, Pyridoxine-HCl-50 mg/L and Glycine- 200 mg/L. All the vitamins were dissolved in DD water and make volume to 1000 ml Store below 0°C (Figure 3 and Table 3).

Table 3. Vitamins amount in mg/L.

Vitamin	Amount in mg/L
Myo-inositol	10,000
Thiamine-HCl	10
Nicotinic acid	50
Pyridoxine-HCl	50
Glycine	200

Figure 4. Iron solution.

Table 4. Iron source amount in g/L.

Iron source	Amount in g/L
$Na_2EDTA.2H_2O$	3.73
$FeSO_4.7H_2O$	2.78

Iron source Amount of g/L

$Na_2EDTA.2H_2O$-3.73 g/L and $FeSO_4.7H_2O$-2.78 g/L $FeSO_4.7H_2O$ and $Na_2EDTA.2H_2O$ were dissolved separately in 400 ml/L of double distilled water. $Na_2EDTA.2H_2O$ was heated until it dissolved completely. $FeSO_4.7H_2O$ was added to the warm Na_2 EDTA solution with continuous stirring. The solution was slight yellowish tinge. The volume was then adjusted to 1000 ml with double distilled water. This stock was stored in coloured bottle at 4°C. The pH of the medium was adjusted to 5.8 with freshly prepared 1 N HCl or 1 N KOH (Figure 4 and Table 4).

100 ml of macronutrients and 10 ml each of solution micronutrients, vitamins and iron source were taken and mixed together to prepare Murashige and Skoog medium (1962). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C

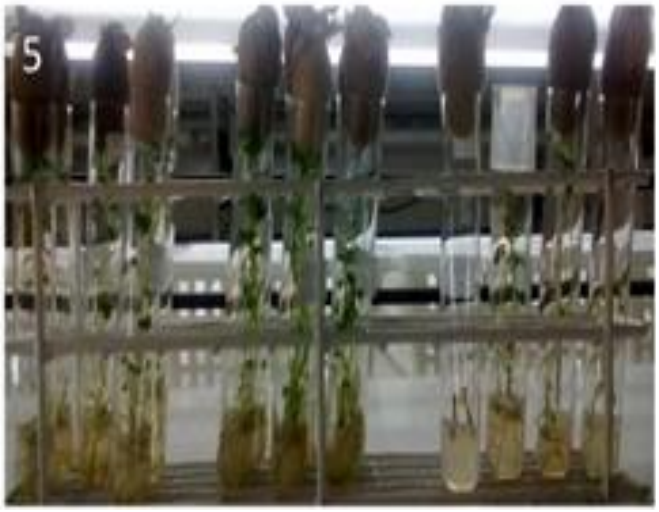

Figure 5. Microplants of Kufri Bahar

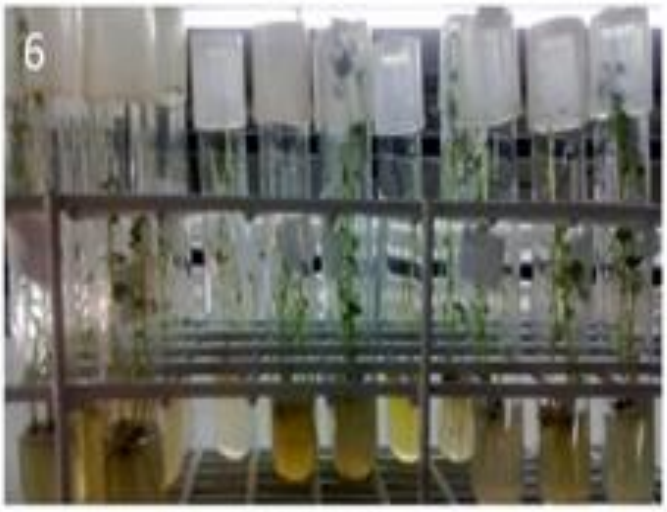

Figure 6. Microplants of Kufri Surya.

for 20 min. Cultures were incubated at 25 1°C for 16 h photoperiod (fluorescent light of 100 µmole/m /s). After 21 days when sufficient numbers of plantlets were formed on solid medium in test tube, they were cut with three node cutting and inoculated in 20 ml of liquid media in 250 ml Erlenmeyer flask. Composition of the liquid medium was the same as that of solid medium except agar which was not added (Figures 5 and 6). For micro tuberization, after 20 days the liquid propagation medium was replaced by tuber induction medium containing MS salt, vitamins, sucrose (8%) and supplemented with different BAP concentrations (0.75, 1.5 and 2.25 mg/L). These flasks were then incubated at 18 ± 2°C under complete dark condition. After 75 days microtuber were harvested and physical (Number of microtuber and fresh weight of microtubers) and growth parameters (Number of eyes and Days to microtuber initiation) analyzed. The pool data obtained were statistically analyzed using completely randomized design with the software IRRISTAT (IRRI, 1999).

RESULTS AND DISCUSSION

In vitro tuberization in potato was studied in *kufri bahar* and *kufri surya* varieties. To evaluate the physical characteristics such as number of microtubers, weight of mircotubers and growth rate including number of eyes and tuber initiations were selected as indicators.

Microtuber initiation

MS media supplemented with different concentration of 6- benzyl aminopurine were used for micro tuber initiation. The data presented in Table 5 revealed that there was significant variation over control with respect to microtuber initiation. MS media supplemented with different concentration of 6- benzyl aminopurine were used for microtuber initiation. Maximum days 20.0 (±1.53) days were recorded for microtuber initiation for the control (no BAP added) *kufri bahar*.

However, there were no significant differences. In *kufri surya* the maximum days for microtubers initiation 20.4 (±1.06) days were recorded for microtubers initiation with the control. However, it was at par with 2.25 and 1.5 mg/L in *kufri surya*. Significantly minimum days for microtuber initiation were recorded in 2.25 mg/L. The findings are in agreement with the studies made by Yong et al. (1996) who observed that 6- benzyl aminopurine promoted initiation and growth of microtubers.

Number of microtubers

Effects of 6- benzyl aminopurine on physiological and growth parameter were studied. The number of microtubers increased with increasing 6- benzyl aminopurine concentration in both the varieties during both the years of study (Table 5). Significant maximum number of microtubers 8.24 ± 0.33 was recorded with 2.25 mg /L of BAP in *kufri bahar*. It was significantly at par with 1.5 and 0.75 mg /L and control. In *kufri surya* maximum number of microtubers 8.83 ± 0.44 was recorded with 2.25 mg /L and it was at par with 1.5 and 0.75 mg/L and control. Significantly minimum number of microtubers (5.60 and 5.30) was recorded in control. These results agree with Naqvi et al. (2019) who found that the higher mean number of microtubers was achieved by the effect of BAP which promotes *in vitro* microtuberization (Figures 7 and 8).

Weight of microtubers

Significant variation was found between concentration of BAP for the fresh weight of microtubers per flask in both varieties during the two years of study (Table 6). Significantly maximum microtubers mean fresh weight (3.13 g) was recorded with 2.25 mg /L in *kufri bahar*.

Table 5. Effect of BAP on microtuber initiation and number of microtubes per flask of varieties.

Treatment	Microtuber initiation (days)				Mean		Number of microtubers per flask				Mean	
	1st year		2nd year				1st year		2nd year			
BAP	KB (A1)	KS (B1)	KB (A2)	KS (B2)	KB (A1, A2)	KS (B1, B2)	KB (A1)	KS (B1)	KB (B1)	KS (B2)	KB (A1, A2)	KS (B1, B2)
0.75 mg/L	17.4	17.2	17.5	17.4	17.4	17.3	6.32	6.66	6.44	6.60	6.38	6.63
1.5 mg/L	15.6	15.5	15.5	15.6	15.5	15.5	6.66	6.78	6.58	6.80	6.62	6.79
2.25 mg/L	12.9	12.4	**12.7**	12.6	12.8	12.5	8.22	8.82	8.26	8.84	8.24	8.83
Control	19.8	20.2	**20.2**	20.6	20.0	20.4	5.40	5.20	5.80	5.40	5.60	5.30
S Em ±	1.82	1.77	2.16	1.79	1.70	1.55	0.71	0.50	0.48	0.57	0.33	0.44
CD or LSD(P=0.05%)	NS	5.32	NS	5.37	NS	4.67	NS	1.51	1.44	1.73	1.00	1.34
CV	24.8	24.3	29.3	24.3	23.1	21.2	24.1	16.5	15.9	18.8	11.2	14.6

kufri bahar (KB) and *kufri surya* (KS) *in vitro* condition.KB- *kufri bahar*, KS- *kufri surya*, NS-non significant .

Figure 7. Microtubers of Kufri Bahar

Figure 8. Micrutubers of Kufri Surya

However, it was at par with 1.5 and 0.75 mg/L and control. In *kufri surya* significantly maximum mean fresh weight of microtubers (3.04 g) was also observed with 2.25 mg /L and the result was at par with 1.5 and 0.75 mg /L and the control. Minimum mean fresh weight of microtubers (0.47 and 0.51 g) was recorded for the control. The findings are in agreement with the studies carried by Hossain et al. (2015) who observed that at [5 mg/L of BAP] and 9% sucrose, increase in the number and fresh weight of microtubers. Aryakia and Hamidoghi

Table 6. Effect of BAP on fresh weight of microtubers per flask and number of eyes per microtuber of potato varieties *kufri bahar* (KB) and *Kufri Surya* (KS) *in vitro* condition.

Treatment	Fresh weight of microtuber per flask (g)				Mean		Number of eyes per microtuber				Mean	
BAP	1st year		2nd year				1st year		2nd year			
	KB	KS	KB	KS	KB	KS	KB	KS	KB	KS	KB	KS
0.75 mg/l	0.55	0.58	0.58	0.57	0.56	0.57	3.38	3.12	3.30	3.00	3.34	3.06
1.5 mg/l	0.95	0.90	0.96	0.92	0.95	0.91	4.90	5.00	4.98	5.04	4.94	5.02
2.25 mg/l	**3.12**	**3.02**	**3.14**	**3.06**	**3.13**	**3.04**	**5.06**	**5.32**	**5.14**	**5.34**	**5.10**	**5.33**
Control	**0.47**	0.51	0.48	**0.52**	0.47	0.51	**1.80**	**2.00**	**1.96**	**1.86**	**1.88**	**1.93**
S Em ±	0.35	0.29	0.31	0.26	0.12	0.13	0.24	0.34	0.21	0.42	0.16	0.32
CD or LSD(P=0.05%)	1.07	0.89	0.94	0.80	0.38	0.41	0.74	1.04	0.62	1.27	0.49	0.96
CV	62.9	53.1	54.4	47.1	22.4	24.7	14.6	20.3	12.2	24.9	9.7	18.8

KB- *kufri bahar*, KS- *kufri surya,* NS-non significant.

(2010) also observed, at high concentrations (0.75 and 1 mg/L) of BAP showed incremental effect on weight and size of microtubers.

Number of eyes

Different concentrations of BAP markedly influenced the number of eyes per microtuber. BAP at 2.25 mg/L gave significant maximum number of eyes per microtuber (5.10) which was at par with 0.75 mg /L and the control whereas it was significantly minimum (1.88) in control in *kufri bahar* (Table 6). Similarly, maximum number of eyes per microtuber (5.33) was also noted with 2.25 mg/L in *kufri surya*. However, it was at par with 0.75 mg/L and the control. Significant mean number of eyes (1.93) per plant was lowest in control in *kufri surya* (Table 6). The findings are in agreement with the studies carried by Badoni and Chauhan (2010) who observed that the lower concentration of BAP (below 8 mg/L) increases the number of eyes per microtuber, while increased concentration of BAP (up to 10 mg/L) inhibits the production of eyes by micro tubers. So that economically and qualitatively both varieties of potato are be useful in enhancing tuber quality as well as crop growth.

Conclusion

The BAP 2.25 mg/L should be useful to enhance tuber quality as well as crop growth under *in vitro* conditions at farmer and industrial levels. The results suggested the need of developing genotype specific protocols to maximize *in vitro* performance for microtuberization and *in vivo* minitubers performance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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