Investigation of physiological and molecular mechanisms for quality assurance in post-harvest management of African nightshade (*Solanum scabrum* Mill.) and cowpea (*Vigna unguiculata* L. Walp)

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PREFACE

This PhD thesis presents research work conducted under field conditions at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya, and greenhouse conditions at Geisenheim University, Germany (January 2015 to June 2018). This research project was a sub-project 4 (SP4) under the HORTINLEA (Horticultural innovation and learning for improved nutrition and livelihood in East Africa) project sponsored by the German Federal Ministry of Education and Research (BMBF) and the Ministry of Economic Cooperation and Development (BMZ). The thesis contains three main chapters: The General introduction (**Chapter 1**) covers a short introduction of African indigenous leafy vegetables (ALVs), their importance in Sub-Saharan Africa (SSA), harvesting, post-harvest storage, losses and technologies applied in SSA. Some parts of introduction are drawn from the review paper and manuscripts in the subsequent chapters. This chapter also contains the objectives of the study.

Chapter 2 contains a review paper with detailed literature of all aspects of the study, and the publications and manuscripts with the relevant research findings; The review paper is titled ″African Leafy Vegetables Pre-harvest and Post-harvest constraints and Technologies for losses reduction along the field to consumer chain″ *African Journal of Horticultural Science* (2017), The second publication covers major findings in cowpeas; ″Development stage, storage temperature and storage duration influence phytonutrient contents in cowpea (*Vigna unguiculata* (L.) Walp.)″ *Heliyon Journal* (2018), Elsevier. The African nightshade research is presented in three manuscripts; (i) "Nutritional composition in African nightshade (*Solanum scabrum* Mill) influenced by harvesting methods, age and storage conditions", *Postharvest biology and technology*, Elsevier, accepted, (ii) "Selection and validation of suitable reference genes for quantitative gene expression analyses in African nightshade (*Solanum scabrum* Mill) during plant development", submitted at *Plant Methods journal*, BioMed Central, and (iii) "Investigation of ethylene biosynthesis, sensitivity and related gene expression in *Solanum scabrum* leaves during development and after harvesting", under preparation.

Chapter 3 &4 contains the general discussion, conclusion and recommendations of the research findings, and the indication of their importance in solving post-harvest losses and quality assurance of the two African crops. Finally, all other important aspects of the research including conference presentations appear in the appendices of the thesis.

ABSTRACT

African indigenous leafy vegetables (ALVs) have a great potential in improving livelihood, offering sustainable food security and solving the malnutrition crisis in Sub-Saharan Africa (SSA). African nightshade (*Solanum scabrum* Mill.) and cowpea (*Vigna unguiculata* L.Walp.) are among the major indigenous leafy vegetables utilized in SSA. Currently, farmers suffer over 50 % qualitative and quantitative losses along the field to consumer chain, due to poor production conditions, unknown maturity indices, poor harvesting methods and post-harvest storage conditions. The research aimed at investigating the harvesting methods in terms of yield and post-harvest nutrient contents, determining development stages with optimal phytonutrients, evaluating the dynamics of phytonutrients at different post-harvest storage conditions, evaluating endogenous ethylene production in African nightshade and their responses to exogenous ethylene application, and finally analyzing ethylene-related gene expression during growth and storage of African nightshade.

African nightshade and cowpea leaves harvested from middle parts of the plants, and from field experiments at Jomo Kenyatta University in Kenya 30, 60, 90 and 120 days after planting (dap), were used for phytonutrient analysis during development and at storage (5 °C and room temperature (RT). Enzymatic and photometric methods were used to quantify glucose, fructose, sucrose (GFS) and starch. The gallic acid equivalents (GAE) phenolic contents were analyzed using the Folin-Ciocalteu method (FC). The aluminum complexation reaction assay was used for quantifying catechin and quercetin equivalent flavonoids, while trolox equivalent antioxidant capacity (TEAC) assays were conducted for antioxidants. Furthermore, African nightshade leaves (2, 4, 6, 8 weeks old) from greenhouse experiments were used in testing ethylene production and sensitivity at RT for 72 hours. Ethylene was quantified with gas chromatography (GC-FID) method and gene expression analysis was studied using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Results indicated that in cowpea the total carbohydrates were highest at 90 dap and at 60 dap in African nightshade. The total phenolics (gallic acid equivalents), catechin equivalent flavonoids, and trolox equivalent antioxidants (TEA) were highest in concentration at 60 dap in cowpea and 90 dap in African nightshade. Chlorophyll content was optimal at 60 dap in both crops. Storage of cowpea and African nightshade leaves at room temperature (RT; 50-55 % relative humidity) led to a strong decline of the analyzed phytonutrients after 4 days, but mostly they remained stable at cold storage (5 °C). The middle leaves aged 4 to 6 weeks (wks) had higher carbohydrate and chlorophyll contents, were less sensitive to exogenous ethylene (5

ppm) application, and emitted less ethylene during storage compared to younger (2 wks) and older (8 wks) leaves. The secondary metabolites were highest in the young leaves (2 wks) compared to other leaves. For RT-qPCR assays, results indicated that the *Adenine phosphoribosyltransferase (APRT)*, *Actin*, and *Glyceraldehyde-3-phosphate dehydrogenase (GAPGH*), were stable and acceptable for use as reference genes in the normalization of gene expression data. Gene expression for ethylene biosynthesis, the *1-aminocyclopropane-1-carboxylic acid oxidase (ACO* 1 and *ACO* 4), and ethylene sensitivity (*ETR;* ethylene receptor) in African nightshade leaves during leaf development, age of the plant and in storage at RT with or without ethylene was investigated. The *ACO* 1 and 4 were upregulated in older leaves and older plants (8 wks and 90 to 120 dap) compared to younger leaves and plants, while the *ETR* was highly expressed in both young leaves and plants (2 wks and 30 dap) than in older leaves.

Results of this study highlight the importance of developmental stages at harvest (60 to 90 dap), storage conditions and duration (low temperatures 5 °C up to 4 days) for optimal availability of phytonutrients in freshly consumed leaves and for post-harvest quality management. Furthermore, considering leaf position and age (4 to 6 wks old) during harvesting of African nightshade would offer optimal quality benefits and reduced sensitivity to ethylene. Adoption of these research findings by the farmers will be a great milestone in solving post-harvest losses and ensuring quality of cowpea and African nightshade.

Keywords. African leafy vegetables, African nightshade, cowpea, ethylene, gene expression, phytonutrients, post-harvest, quality assurance, Sub-Saharan Africa.

ZUSAMMENFASSUNG

Afrikanische einheimische Blattgemüse (ALVs) haben ein großes Potenzial zur Verbesserung der Lebensgrundlage, bieten nachhaltige Ernährungssicherheit und lösen die Unterernährungskrise in Subsahara-Afrika (SSA). Afrikanischer Nachtschatten (*Solanum scabrum* Mill.) und Kuhbohne (*Vigna unguiculata* (L.) Walp.) gehören zu den wichtigsten einheimischen Blattgemüsen, die in SSA verwendet werden. Gegenwärtig erleiden die Landwirte über 50 % qualitative und quantitative Verluste entlang der Kette zwischen Feld und Verbraucher aufgrund von Produktionsbedingungen vor der Ernte, fehlenden Reifeindizes, schlechten Ernteverfahren und unzureichenden Lagerbedingungen nach der Ernte. Ziel der Forschung war es, die Erntemethoden hinsichtlich Ertrag und Nährstoffgehalt nach der Ernte zu untersuchen, Entwicklungsstadien mit optimalen Pflanzeninhaltsstoffgehalten zu bestimmen, die Dynamik der Pflanzeninhaltsstoffe bei verschiedenen Lagerbedingungen nach der Ernte zu bewerten, die endogene Ethylenproduktion im Afrikanischen Nachtschatten und ihre Reaktionen auf die Anwendung von exogenem Ethylen zu bewerten und schließlich die ethylenbezogene Genexpression während des Wachstums und der Lagerung von Blättern des Afrikanischen Nachtschattens zu analysieren.

Afrikanische Nachtschatten- und Kuhbohnenblätter, die nach 30, 60, 90 und 120 Tagen nach der Aussaat (dap) aus dem mittleren Bereich der Pflanzen und aus Feldversuchen an der Jomo Kenyatta University in Kenia geerntet wurden, wurden nach einer Lagerung bei 5 °C oder Raumtemperatur (RT) für die Analyse der primären und sekundären Pflanzeninhaltsstoffe verwendet. Zur Quantifizierung von Glukose, Fruktose, Saccharose (GFS) und Stärke wurden enzymatische und photometrische Methoden eingesetzt. Die Gallussäureäquivalente (GAE) für den Gesamtphenolgehalt (TPC) wurden mit der Folin-Ciocalteu-Methode (FC) analysiert. Der Aluminiumkomplexierungsreaktionstest wurde zur Quantifizierung von Catechin- und Quercetin-Äquivalenten Flavonoiden verwendet, während Trolox-Äquivalent-Antioxidationsmittel (TEAC) Tests für Antioxidantien durchgeführt wurden. Außerdem wurden Afrikanische Nachtschattenblätter (2, 4, 6, 8 Wochen alt) aus Gewächshausversuchen verwendet, um die Ethylenproduktion, die Empfindlichkeit für Ethylen bei der Lagerung (RT) für 72 Stunden zu untersuchen. Das Ethylen wurde mittels Gaschromatographie (GC-FID) quantifiziert und die Genexpressionsanalyse mittels quantitativer Realtime-Polymerase-Kettenreaktion (qRT-PCR) untersucht.

Die Ergebnisse zeigten, dass bei Kuhbohnen die Gesamtkohlenhydrate 90 dap und bei Afrikanischem Nachtschatten 60 dap am höchsten waren. Die Konzentrationen der

Gesamtphenolgehalte (Gallussäureäquivalente), der Catechinäquivalent-Flavonoide, und der Troloxäquivalent-Antioxidantien (TEA) waren 60 dap in Kuhbohnen und 90 dap in Afrikanischem Nachtschatten am höchsten. Der Chlorophyllgehalt war in beiden Kulturen optimal 60 dap. Die Lagerung von Kuhbohnen- und Nachtschattenblättern bei Raumtemperatur (RT; 50-55 % relative Luftfeuchtigkeit) führte nach 4 Tagen zu einem stärkeren Rückgang der analysierten Pflanzeninhaltsstoffe, deren Gehalt blieb aber meist stabil bei Kühllagerung (5 °C). Die mittleren Blätter im Alter von 4 bis 6 Wochen (wks) hatten höhere Kohlenhydrat- und Chlorophyllgehalte, waren weniger empfindlich gegenüber exogenem Ethylen (5 ppm) und emittierten während der Lagerung weniger Ethylen als jüngere und ältere Blätter. Die sekundären Metabolite waren am höchsten in den jungen Blättern (2 Wochen) im Vergleich zu Blättern anderer Alterslassen. Für qRT-PCR-Assays zeigten die Ergebnisse, dass die Gene für *Adenin-Phosphoribosyltransferase* (*APRT*), *Actin* und *Glyceraldehyd-3-phosphat-Dehydrogenase* (*GAPGH*) stabil exprimiert und für die Verwendung als Referenzgene bei der Normalisierung von qRT-PCR-Daten in Genexpressionsstudien akzeptabel waren. Die Expression von Genen für die Ethylenproduktion (*ACO* 1 und 4; *1-Aminocyclopropan-1-carbonsäureoxidase*) und Ethylenempfindlichkeit (*ETR*; Ethylenrezeptor) in Nachtschattenblättern während Blattentwicklung, Alterung der Pflanze und Lagerung bei 5 °C und RT mit oder ohne Ethylen wurden untersucht. Die *ACO* 1 und 4 waren bei älteren Blättern und älteren Pflanzen (8 Wochen und 90 bis 120 dap) im Vergleich zu jüngeren Blättern und Pflanzen hochreguliert, während die *ETR* Expression bei den jungen und älteren Blättern (2 und 8 Wochen) höher war.

Die Ergebnisse dieser Studie zeigen die Bedeutung von Entwicklungsstadien bei der Ernte (60 bis 90 dap) und von Lagerungsbedingungen und -dauer (niedrige Temperaturen 5 °C bis 4 Tage) für die optimale Verfügbarkeit von Phytonährstoffen in frisch verzehrten Blättern und für das Qualitätsmanagement nach der Ernte. Außerdem würde die Berücksichtigung der Position und des Alters (4 bis 6 Wochen alt) der Blätter bei der Ernte des Nachtschattens optimale Qualitätsvorteile und eine geringere Empfindlichkeit gegenüber Ethylen bieten. Die Übernahme solcher Forschungsergebnisse durch die Landwirte kann ein großer Meilenstein bei der Minderung von Nachernteverlusten sein.

Schlüsselwörter. Afrikanisches Blattgemüse, Afrikanischer Nachtschatten, Kuhbohne, Ethylen, Genexpression, pflanzliche Inhaltsstoffe, Erntebedingungen, Nachernte, Qualitätssicherung, Subsahara-Afrika

CONTENTS

ABBREVIATIONS

18S or 28S	Ribosomal units
ACO	*1-aminocyclopropane-1-carboxylic acid oxidase,*
AdoMet	S-adenosyl methionine
AG	Amyloglucosidase
ALVs	African leafy vegetables
APRT	*Adenine phosphoribosyltransferase*
CAE	Catechin equivalent flavonoids
cDNA	Complementary deoxyribonucleic acid
CYC	*Cyclophilin*
Dap	Days after planting
DW	Dry weight
EF	*Elongation factor-1 alpha*
EIN3	*Ethylene-insensitive3*
ETR	Ethylene receptor gene
F-C	Folin-Ciocalteu reagent
FIA	Flow injection analysis
GAE	Gallic acid equivalent phenolics
GAPGH	*Glyceraldehyde-3-phosphate dehydrogenase*
GC	Gas chromatography
GFS	Glucose, fructose and sucrose
HK/G6P-DH	*Hexokinase/glucose 6-phosphate dehydrogenase*
HXK1	Glucose sensor hexokinase
ICP-OES	Inductively coupled plasma optical emission spectrometry
JKUAT	Jomo Kenyatta University of Agriculture and Technology
PGI	Phosphoglucose isomerase
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RH	Relative humidity
RNA	Ribonucleic acid
RT	Room temperature
Rubisco	*Ribulose-1, 5-bisphosphate carboxylase*
SSA	Sub-Saharan Africa
TEA	Trolox equivalent antioxidants
TEAC	Trolox equivalent antioxidants capacity assay
TPC	Total phenolic contents
UBQ	*Ubiquitin*

1 GENERAL INTRODUCTION

1.1 Importance of ALVs in Sub-Saharan Africa

African indigenous leafy vegetables (ALVs) are key to the affordable and sustainable solution to the hunger and malnutrition menace in Sub-Saharan Africa (SSA) (Abukutsa-Onyango *et al.*, 2010; Kamga *et al*., 2013; Grivetti and Ogle, 2017). The ALVs have been used as edible leaves for many centuries and apart from their role in food security, they improve the livelihood of people both in rural and urban/peri-urban areas of SSA (Grivetti and Ogle, 2017). Some of the major indigenous vegetables that are consumed in the African continent include leafy amaranths (*Amaranthus species*), African nightshades (*Solanum* species), Spiderplant (*Cleome gynandra*), African eggplant (*Solanum ethiopicum*), Cowpea (*Vigna unguiculata*), Jute mallow (*Corchorus olitorius*), Slender leaf (*Crotalaria brevidens*), Pumpkin leaves (*Curcurbita muschata*) and African kale (*Brassica carinata*) (Abukutsa-Onyango, 2007; Smith and Eyzaguirre, 2007). The ALVs have several advantages over the exotic vegetables; high nutrient levels, medicinal and antioxidant properties, high seed production rates, short growth periods and withstand both abiotic and biotic stress (Uusiku *et al.*, 2010). One hundred grams of fresh leaves in most of the ALVs contain over 100 % of the recommended daily requirements for an adult in calcium, iron, vitamin A and C, and 40 % for the proteins (Kwenin *et al.*, 2011).

Daily consumption of phytonutrient-rich green vegetables reduces incidences of chronic diseases, cardiovascular diseases, cancer and boosts immunity in humans (Smith and Eyzaguirre, 2007). Currently, secondary metabolites such as flavonoids, antioxidants, phenolics and carotenoids in African vegetables have received a lot of research attention for their wide range of pharmacological and biochemical benefits (Moyo *et al*., 2013; Neugart *et al.*, 2017). The ALVs have high antioxidative capacity to scavenge free radical ions and protect humans against cancer, additionally, the metabolites have anti-inflammatory and antimicrobial properties (Nijveldt *et al.,* 2001). The secondary metabolites also play key roles in the plant defense and protective mechanisms against biotic and abiotic stress (Nijveldt *et al*., 2001; Akula and Ravishankar, 2011). Furthermore, the ALVs can be produced in simple farming systems like intercropping and organic farming (Linguya *et al.*, 2015).

1.1.1 African nightshade and cowpea

Three species of African nightshade are commonly used in Africa*: Solanum villosum*, *Solanum scabrum* and *Solanum nigrum*. *Solanum scabrum* is a rich source of carbohydrates, vitamins, antioxidants, flavonoids, beta-carotene, folic acid, protein and iron, magnesium, calcium,

manganese and zinc (Traoré *et al.,* 2017; Kamga *et al.,* 2013; Kirigia *et al.,* 2018, submitted manuscript). The crop is multipurpose, and although the fruits are not edible due to their alkaloid content, they have been shown to contain high levels of anthocyanins and are used as a natural source of ink or dyes (Kamga *et al.*, 2013; Lehmann *et al.,* 2007). The edible parts of African nightshade include young shoots and leaves which are fried or boiled to improve the organoleptic properties and remove antinutritive compounds. The vegetable mainly accompanies major cereals or starch diets in Africa, e.g. Ugali in Kenya (Abukutsa-Onyango, 2007). The African nightshade has overwhelming pharmacological benefits, and is highly recommended for immune boosting in patients, nursing and pregnant women (Maundu *et al.,* 1999; Mwai & Schippers 2004). Leaf infusions have been used for many centuries to treat eye infections, duodenal ulcers and stomach upsets, swollen glands, wounds and boils, among other ailments (Edmonds and Chweya, 1997).

Cowpea has several agronomic, economic and environmental advantages hence it remains a most suitable and highly cultivated legume crop across Africa, Asia and South America (Linguya *et al*., 2015; Goncalves *et al.*, 2016). Currently, leguminous crops have been identified as affordable and sustainable source of essential nutrients and low-cost proteins for a balanced human diet (Avanza *et al*., 2013; Goncalves *et al.*, 2016). The multipurpose crop is suitable for intensification of a sustainable agri-food system in SSA, with the whole above-ground part, being utilized for its leaves, green pods, green beans, mature beans, or processed into paste or flour. The remaining waste of the crop is used as forage, hay or silage for livestock feeding (Goncalves *et al.,* 2016). Cowpea leaves can be used as fresh green vegetables, fermented or sun-dried (Saidi *et al.,* 2010; Ibrahim *et al.,* 2002; Wafula *et al.,* 2016). The leaves are rich in vitamins, macro and micro minerals, flavonoids, antioxidants, ß-carotene, fatty acids, amino acids, carbohydrates and dietary fibre (Okonya & Maass, 2014; Goncalves *et al.*, 2016). The rich source of phytonutrients in cowpeas makes it one of the most suitable African indigenous crop in provision of a healthy balanced diet and solving malnutrition crisis among the resource-constrained households in SSA (Okonya and Maass, 2014). Daily or frequent inclusion of cowpeas in the diets would therefore not only solve problems with hunger but help in reducing the risk of chronic health conditions.

1.2 Harvesting of ALVs

The quality of vegetables along the field to consumer chain and the subsequent prices are determined by harvesting conditions and post-harvest handling and storage. Physiological

maturity of the plant before harvesting plays a significant role in post-harvest shelf life and nutrient levels of the vegetables (Acedo 2010; Sahou *et al.,* 2014). Small-scale farmers rely on relatively imprecise indicators to determine the start of harvesting the AVLs. These indicators include plant height, leaf size and color, time ordering the product by customers or chain actors, and overall production schedules (Acedo, 2010). Although ALVs can be harvested at different stages of their development, optimization of harvesting maturity is important to ensure best nutritional value, higher yields and post-harvest longevity. The ALVs are harvested mainly by hand in Africa. Machines could be used for large-scale commercial producers where the entire crop needs to be harvested at once (Acedo 2010; Masarirambi *et al.,* 2009). However, harvesting by hand causes less damage to the leaves than using machines.

There are three main methods of harvesting ALVs. In the first method, the young entire plants can be pulled up (uprooted) in 4-6 weeks after sowing when they are about 15 to 30 cm high (Edmonds and Chweya, 1997). In some cases, the first harvest occurs during thinning until the spacing of 15 cm between the plants and 25-50 cm between the rows is achieved. The second method involves topping of the plants 10-15 cm above the ground to encourage lateral growth for successive harvesting. The third method involves continuous harvesting, where plants are harvested at regular intervals, either as single leaves or tender shoots. Harvesting of both African nightshades and cowpeas starts 4-6 weeks after seedling emergence from the soil, and may last for 6 weeks or up to senescence after the start of harvest. Regular harvesting of lateral shoots in African nightshade and disbudding at intervals of 2 weeks encourages production of side shoots (Edmonds and Chweya, 1997). In some cases where seeds are needed, especially for amaranths, spider plants and cowpeas, the plants are left to flower and produce fruits after several successive harvests of the leaves. The seeds are then harvested when the capsules are dry and used for consumption or subsequent planting. The African nightshade berries are picked when they turn completely black (Edmonds and Chweya, 1997). When cowpea is produced mainly for leafy vegetable purposes, the entire seedlings are uprooted when they acquire three to five true leaves in 4-8 weeks after planting, too old leaves become fibrous and unpalatable (Saidi *et al.,* 2010). Cowpeas are also commonly harvested by continuous picking of leaves on weekly intervals during the vegetative stages of the crop until the plants start to be in flower. The pods are then harvested at the end of the season by cutting the heads with the capsules just before they become dry and brittle and are left in a shade to properly dry (Ibrahim *et al.,* 2010; Saidi *et al.,* 2010).

However, the optimal harvesting stages of ALVs remain unclear, and the small-scale farmers in Sub-Saharan Africa (SSA) rely on relatively imprecise indicators to determine the start of harvesting of AVLs. The physiological maturity influences post-harvest quality and storage longevity and marketability of ALVs e.g. the accumulation of phytonutrients varies with development stages (Kirigia *et al.*, 2018). Pre-harvest endogenous carbohydrates have been reported to play a role in post-harvest storability by decreasing ethylene sensitivity hence reducing leaf senescence in *Portulaca grandiflora* (Rapaka *et al.,* 2007). The carbohydrates serve as basic source of energy and initial substrate for the microbes involved in fermentation; a key vegetable preservation method (Wafula *et al., 2*016). Soluble carbohydrates (glucose) have been reported to play a great role in ascorbic acid metabolism during post-harvest storage of spinach. This involves a pathway where glucose is converted to D-glucosone, then to L-sorbosone and finally to ascorbic acid (Toledo *et al.,* 2003). Chlorophyll and carotenoids despite their role as antioxidants and vitamin A precursor, they offer a quick strategy to access storability of vegetables by offering physical characteristics of yellowing during degradation.

Chlorophyll degradation during storage affects market quality, palatability and shelf life of crops (Limantaraa *et al.,* 2015). Phytohormones such as cytokinin contents and ethylene biosynthesis equally vary with development stages and affect storability. Cytokinins play a great role in antagonizing ethylene production and delaying senescence of vegetables (Liu *et al.,* 2013). On the other hand ethylene causes yellowing and decay of the harvested vegetables during storage. Endogenous ethylene production is stimulated by internal and external signals such as development stages, biotic and abiotic stresses, and mechanical damages (Srivastava and Srivastava, 2002; Schaller, 2012). This makes pre-harvest maturity, harvesting methods and post-harvest storage very important in the quality assurance of ALVs.

1.3 Post-harvest handling of ALVs

In most cases, the leaves are used immediately after harvesting as green vegetables. However, for many centuries the post-harvest processing of ALVs has remained unclear, unexamined and unutilized. Small-scale farmers mainly practice some traditional processing and preservation techniques for ALVs. The most common processing methods traditionally used by small-scale farmers include fermentation, sun drying, blanching and solar drying with minimum packaging (Wafula *et al.*, 2016) (Muchoki *et al.*, 2007; Ayua and Omware, 2013; Wafula *et al.*, 2016). The remaining vegetables after daily sales are stored in shades or in houses and water is sprinkled on them frequently to keep them cool (Kitinoja and Kader, 2015). Some farmers use

the on-farm charcoal cooling system to provide cold storage for the vegetables. More details of this section are covered in the review paper (details in section 2.1).

1.4 Constraints facing small-scale farmers in SSA

Despite the importance of ALVs in solving food security crisis in SSA, there are various constraints affecting their production, storability, marketing and consumption. One of the major constraint is post-harvest losses both in quality and quantity along "the field to consumer" chain, whose magnitude is up to or more than 50 % (Fuglie and Nin-Pratt, 2012). These losses are attributed to various factors including inadequate application of fertilizers and water during production, rapid physiological deterioration and microbiological decay of products during transport, storage and marketing (Acedo, 2010; Bartz and Brecht, 2003). Post-harvest losses of vegetables are highly influenced by physiological changes of the harvested organs along the field to consumer chain. These changes include post-harvest hormonal changes, nutrient degradation, chlorophyll and carotenoid degradation and increased transpiration and respiration rates (Bartz and Brecht, 2003). Inadequate harvesting techniques and facilities for storage and transport, insufficient processing and preservation methods, insufficient hygiene conditions in the markets as well as poor infrastructure aggravate the problem. Resource-poor smallholder farmers cannot afford expensive cold storage facilities, use of refrigerated trucks, thus after harvesting ALVs, simple methods such as shading the vegetables are applied (Abukutsa-Onyango and Karimi, 2007). Crop production under stressful conditions with poor soil nutrients, water and salt stress significantly lower the post-harvest life and decrease the nutrient levels of leafy vegetables (Sahou *et al.*, 2014). Moreover, there is a lack of quality control and food safety regulations. Sufficient research has to date not been conducted to incorporate use of modern strategies and techniques for post-harvest losses reduction of African leafy vegetables.

1.5 Research Objectives

The general objective of the study was to investigate physiological and molecular mechanisms for quality assurance in post-harvest management of African nightshade (*Solanum scabrum* Mill.) and cowpeas (*Vigna unguiculata L. Walp.*). The specific research aims were:-

(i) To investigate the effects of harvesting methods on yield and post-harvest nutrient contents in African nightshade.

(ii) To investigate the influence of development stages and postharvest storage conditions on nutrient contents in African nightshade.

(iii) To investigate ethylene biosynthesis, sensitivity and related gene expression in African nightshade during development and after harvesting.

(iv) To investigate the influence of development stages and postharvest storage conditions on nutrient contents in cowpea.

2 PUBLICATIONS AND MANUSCRIPTS

2.1 A review: African Leafy Vegetables Pre-harvest and Post-harvest constrains and Technologies for losses reduction along the field to consumer chain, *African journal of horticultural science*

http://hakenya.net/ajhs/index.php/ajhs/article/view/185

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Highlights

The review paper covers relevant literature of the topic of Ph.D. studies. It highlights on various aspects of ALVs from field to consumer chain, which includes harvesting, post-harvest handling and storage, and the technologies available for post-harvest handling and processing.

African Leafy Vegetables Pre-harvest and Post-harvest constrains and Technologies for losses reduction along the field to consumer chain

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Abstract

African indigenous leafy vegetables (ALVs) play a significant role in food security in Sub Saharan Africa (SSA). Most of the ALVs are rich in vitamins, minerals, dietary fibre, phytochemicals, anti-oxidants, medicinal properties and can withstand both biotic and abiotic stress. Currently, post-harvest losses of AVLs in SSA are more than 50% due to various constrains along "the field to consumer" chain. The constrains influences leaf nutrient value, shelf life and marketability resulting to qualitative and quantitative losses of ALVs. The major pre-harvest factors that leads to losses include; poor production conditions such as unfertile soils, drought stress, unknown mature indices, pest and diseases and poor harvesting techniques. Additionally, post-harvest constrains such as rapid physiological deterioration and microbiological decay, poor infrastructure and poor storage conditions leads to massive losses of ALVs. Post-harvest physiological changes, such as increased respiration and transpiration as well as ethylene biosynthesis highly influences post-harvest quality of vegetables. Ethylene gas is physiologically active at low concentrations and causes significant losses in product shelf life. Despite the importance of ALVs, sufficient research is not yet done to incorporate use of modern strategies and techniques for post-harvest loss reduction of ALVs. However, small scale farmers practice few traditional techniques to preserve and prolong the shelf life of ALVS including, charcoal cooling, fermentation, blanching, solar drying, sun drying and minimum processing and packaging among others. Here we review the available information on pre-harvest and post-harvest constrains of ALVs, as well as strategies and technologies to reduce losses in sub Saharan Africa.

Key words: African leafy vegetable (AVLs), post-harvest losses, Sub Saharan Africa (SSA), physiology, technologies.

Introduction

African indigenous leafy vegetables (ALVs) have been used for many centuries and play a significant role in food security and livelihood in SSA (Grivetti and Ogle, 2000). Some of the major indigenous vegetables that are consumed in the African continent include; leafy amaranths *(Amaranthus species)*, African nightshades (*Solanum species*), Spiderplant *(Cleome gynandra),* African eggplant (*Solanum ethiopicum*), Cowpeas (*Vigna unguiculata*), Jute mallow *(Corchorus olitorius),* Slenderleaf (*Crotalaria brevidens*), Pumpkin leaves (*Curcurbita muschata*) and *A*frican kale *(Brassica carinata)* (Schippers, 2000; Abukutsa-Onyango *et al.*, 2006). The ALVs have several advantages over the exotic vegetables; they have higher nutrient levels, medicinal and antioxidant properties, high seed production rates, short growth period and withstand both abiotic and biotic stresses (Mwai *et al.*, 2007; Odhav *et al.*, 2007; Uusiku *et al.*, 2010). One hundred grams of fresh leaves in most of the ALVs contain over 100 % of the recommended daily requirements for an adult in calcium, iron, vitamin A and C, and 40 % for proteins (Mensah *et al.*, 2008; Uusiku *et al.*, 2010; Kwenin *et al.*, 2011). Some members of *Solanaceae* family have been recognized for their high levels of secondary plant

metabolites, although some like the alkaloids are anti-nutritive (Uusiku *et al.,* 2010).

The AVLs are mostly produced in simple farming systems like intercropping and organic farming (Kimaru *et al.,* 2015). Most of ALVs cultivars grow rapidly and may be harvested from 30 to 55 days after sowing. The ALVs are preferably harvested mainly by hand in SSA during the early hours of the day to reduce water loss (Masarirambi *et al.,* 2010). There are three main methods of harvesting ALVs. In the first method, the young entire plants can be pulled up (uprooted) in 4-6 weeks after sowing when they are about 15 to 30 cm tall (Gockowski *et al.*, 2003). The second method involves topping of the plants 10 cm above the ground to encourage lateral growth for successive harvesting. The third method involves continuous harvesting, where plants are harvested at regular intervals, either as single leaves or tender leafy branches (Onyango and Imungi, 2007). The leafy vegetables are then transported to the packing shed to avoid heat accumulation and water loss after harvesting (Acedo, 2010).

After harvesting small-scale farmers sort the vegetables, bundle and pack them in plastic crates, bamboo baskets, boxes, sacks or heap them together for ease transportation to the market (Fernando, 2006; Chen, 2007; Masarirambi *et al.,* 2010). The farmers transport the vegetables by local means, either by carrying with hands to the market, wheelbarrows or bicycles. The remaining vegetables after daily sales are stored in shades or in houses and water is sprinkled on them frequently to keep them cool (Kitinoja and Kader, 2003; Ndukwu and Manuwa, 2014). The charcoal cooling system is used by some farmers to provide cold storage for the vegetables. In some cases, the vegetables are processed as a strategy for preservation and enhancement of marketability. The most common processing methods traditionally used by small-scale farmers include, fermentation, sun drying, blanching and solar drying and minimum processing and packaging among others. (Muchoki *et al.,* 2007; Ayua and Omware, 2013; Wafula *et al.,* 2016). Production, storability, marketing and consumption of ALVs is affected by various limiting factors.

Effects of Production and Pre-harvest Constraints on African Leafy Vegetables

Production and pre-harvest factors significantly affects the quality and quantity of leafy vegetables. Previous studies have reported effects on shelf life and nutrients value of vegetables as influenced by soil nutrient, cultural practices and water supply during production (Jiang and Pearce, 2005). Crop production under stressful conditions with poor soil nutrients, water and salt stress significantly lowers the post-harvest life and decreases the nutrient levels of leafy vegetables (Jiang and Pearce, 2005; Sahou *et al*., 2014). Use of cattle and goat manure has been reported to increase crude protein and leaf biomass yields in *Cleome gynandra* and *Amaranthus hybridus* (Seeiso and Materechera, 2014). Growing of *Solanum scabrum* under drought stress and solute accumulation led to growth depression and increased electrolyte leakage as a result of membrane damage induced by oxidative stress (Assaha *et al.,* 2016). Use of NPK fertilizers indicated higher antioxidants levels in *Justicia tenella* and higher phenolic contents in *Ceratotheca sesamoïdes* (Sossa-Vihotogbé *et al.,* 2013). Optimum production conditions are essential for assuring quality of ALVs.

The other production or pre-harvest factor that influences quality of ALVS is pest and diseases. Although most of ALVs can withstand both abiotic and biotic factors than the exotic vegetables, pests and diseases still remain a menace to some of the crop species and cultivars. Pests causes mechanical damages and are growth depressants to most of Agricultural crops. Damaged leafy vegetables can act as pathway to infection by disease causing pathogens and spoilage microorganisms. The free beetle is a major pest of spider plant (*Cloeme gynandra*) and causes ≥25% foliage damages (Maina *et al.,* 2015). The damaged leaves affects the

physical quality of ALVs and decreases the marketability hence leads to automatic food loss. Red spider mite (*Acari Tetranychidae*) has been reported to cause severe leaf damages, reduce growth and leaf yield of African nightshade (Murungi *et al.,* 2014). Additionally, diseases such as dumping off, cankers, and various stem and root tots affects growth and yield of Leafy amaranths in wet seasons. *Alternaria* leaf spot disease of amaranths causes serious foliar damage and hence affects the quality and market of amaranths (Das, 2016). Das, 2016 also reported various pests and diseases of leafy Amaranths and their significant effects on yield and quality of the crop.

Physiological maturity of leafy vegetables is another major pre-harvest factor that influences nutrient value and shelf life. Small-scale farmers rely on relatively imprecise indicators to determine the start of harvesting of AVLs. These indicators include plant height, leaf size and color, and the times the product is ordered by customers or other chain actors, and overall production schedules (Barry *et al*., 2009). Nutritional quality of leafy vegetables have been reported to change significantly with plant age and growth conditions. The levels of antioxidants and phenolics have been reported to vary with crop age in *Ceratotheca sesamoïdes*, *Sesamum radiatum* and *Justicia tenella* (Sossa-Vihotogbé *et al.,* 2013). The contents of calcium, iron, and antioxidants has been reported to increase with plant age in leafy amaranths grown in warm temperatures (Mnkeni *et al.,* 2006).

Developing and adopting optimal nutrient and water supply during production can maximize biomass yield, nutritive value, quality and storability of ALVS. The appropriate rates of manure and fertilizer application to each specific leafy vegetable as well as mineral requirements needs to be investigated. Use of leguminous cover crops to improve soil fertility would be cheaper options for resource poor small scale farmer. There is also a need to investigate the agro-ecological areas and environmental conditions suitable for various cultivars of ALVs. Development of integrated pest management strategies as well as good cultural practices such as weeding and crop rotation would lower the infestation rates of most of the pests and diseases. Scanty information exists on effects of plant age on quality and yield of ALVs. Although ALVs can be harvested at different stages of their development, harvesting the vegetables when physiologically mature is important to ensure best nutritional value, higher yields and postharvest longevity. The Hortinlea research project seeks to determine the right physiological age of African leafy vegetables, as well to improve on the harvesting strategies. In addition, experiments to determine optimal fertilizer and water regimes as well as investigation of various pests of ALVs is going on under Hortinlea project. All these strategies aim to improve marketability, quality and to increase the shelf life of ALVs.

Factors that Influence Post-harvest Quality and Losses of African Leafy Vegetables

Quantitative and qualitative losses of leafy vegetables of mainly occur after harvesting, during transportation, processing and in storage (Masarirambi *et al.,* 2010). Post-harvest handling as well as long chains along the field to consumer is very critical in maintaining vegetable quality. A significant decline of mineral content was reported with increase in storage time and handling method in *Pterocarpus soyauxii*, *pterocarpus santalinidies*, *Gongronema latifolium, Corchorus olitorious* and *Amaranthis hybridus* (Nwanekezie and Obiakor-Okeke, 2014). Small scale farmers lack facilities for packaging and transportation of the vegetables to the market, and as well there is poor infrastructure in SSA. Poor post-harvest handling leads to mechanical damages, such as leaf tearing, crushing and other physical injuries. The tissue damages then leads to physiological deterioration due to oxidation of phenolic substances (Bachmann and Earles, 2000). Injuries stimulate ethylene production in vegetables leading to yellowing

and sudden leaf senescence. Additionally, mechanical damage creates openings for microbial infections leading to decay and also increases water loss from the damaged tissue (Bachmann and Earles, 2000). Consumers opt not to buy the damaged vegetables and this leads to automatic losses to the farmer.

The physiological changes of the harvested organs along the field to consumer chain are also influenced by other factors such as;- post-harvest hormonal changes, nutrient degradation, chlorophyll and carotenoids degradation, microbial decay and increased transpiration and respiration rates (Bartz and Brecht, 2003; Acedo, 2010). Resource poor small scale farmers lack cold storage facilities leading to high rates of transpiration and respiration of the ALVs. This is highly influenced by high storage temperatures and relative humidity. The leafy vegetables are reported to wilt just by losing 5-10 % of fresh weight (Kanlayanarat, 2007; Acedo, 2010). Water loss through transpiration induces degradation of nutrients such as vitamin C as well as chlorophyll and carotenoids degradation (Acedo, 2010). Water losses from the harvested plant parts also increases respiration and ethylene production. Cool temperatures extend the shelf life of vegetables by reducing the rate of physiological changes, growth of spoilage microorganisms and prevents nutrient degradation (Kitinoja and Kader, 2003; Acedo, 2010). The rate of respiration and transpiration is low at cold temperatures, this reduces ethylene production and enzymatic activities hence extends the shelf life of ALVs.

Ethylene induces senescence in plants and prolonged exposure even to very low concentrations causes significant losses on fresh produce (Wills *et al*., 2000). Ethylene not only accelerates aging but also increases susceptibility of fresh produce to decay (Acedo, 2010). Chlorophyll degradation, proteolysis of soluble proteins to free amino acids and deterioration of cell membranes are the major characteristics of senescence induced by ethylene (Ferrante *et al.,* 2004; Hörtensteiner, 2006; Kanlayanarat, 2007). This yellowing of fresh vegetables as a result of chlorophyll degradation is one of postharvest factors affecting shelf life and market value of the vegetables. Production of ethylene gas by vegetables after harvesting is influenced by storage temperatures of above 10°C, endogenous levels of sugars in the plant during harvesting, soil nutrients and water supply during production as well as plant age and cultivars (Kanlayanarat, 2007; Acedo, 2010).

Pathological decay after harvesting is another major factor that affects shelf life. The microorganisms can either be carried from the field, or from contaminated packaging materials, and favorable growth conditions along the field to consumer chain (Kanlayanarat, 2007). These conditions include wetness on the surface of leaves due to water used for washing or harvesting wet vegetables during rainy seasons. The water could be the source of pathogens or creates conducive environment for the microorganisms' growth. Storing vegetables in dirty places or near decaying plant material also leads to microbial infection (Kanlayanarat, 2007). Bacteria, fungi and enzymatic degradation are often responsible for severe losses of nutrients in ALVs due to unfavorable chemical changes. Some microbes like *Aspergillus* produce toxins that are carcinogenic, rendering ALVs and other foods unsuitable for consumption by both animals and human beings (Masarirambi *et al.,* 2010).

Post-harvest handling research on each specific leafy vegetable, including packaging seems to be scanty. Investigations of various postharvest handling and packaging would be a great mile stone in solving post-harvest losses of ALVs. Development and adaptation of ethylene gas depressants and strategies during storage and transportation would also reduce the losses and maintain the quality of ALVs. Farmers could also form unions and cooperatives and with some aid from the government, they can construct cold stores at

various locations. This will increase the shelf life, enhance marketability and assure quality of ALVs. Breeding for improved varieties with prolonged shelf life, higher nutritive value, shipping and processing attributes are the current breeder's efforts on many vegetables and has a potential for enhancing quality (Fonseca, 2004; Acedo, 2010).

Post-harvest Technologies and Strategies to Improve Quality and Reduce Losses of ALVS

All forms of Post-harvest processes and techniques aim to bring benefits in terms of improving produce handling, reducing loss of nutrients, reducing food losses, increasing shelf-life and value addition to the product (Madakadze *et al.,* 2004). Small-scale farmers cannot afford refrigeration equipment's hence cool their vegetables under shades or ventilated stores, and sprinkle water on the leaves (Kitinoja and Kader, 2003; Lyatuu *et al.,* 2009; Ndukwu and Manuwa, 2014). Alternative technologies such as on-farm evaporative coolers or charcoal coolers have been explored for adoption (Lyatuu *et al.,* 2009; Ambuko *et al.,* 2013). The charcoal coolers are simple wooden structures that uses charcoal and water system to provide a cold environment for the vegetables. The charcoal coolers systems is cheap, requires no electricity and uses evaporative cooling principle to keep vegetables fresh for up to 7 days. The limitation of the method is availability of water which also remains a challenge to most rural small scale farmers.

Packaging of vegetables enhances handling and transportation, and reduces water loss from the product (Gast, 1991). Currently, packaging is mainly used for some dried ALVs products, but has limited use in the case of fresh leaf vegetables (Muchoki, 2007). The use of polyethylene and polycarbonate-bags with specific gas permeation has been used on some vegetables and leads to a modified atmosphere (MAP) inside the packages, which reduces respiration of ALVs and thus aid in retaining postharvest freshness and quality (Somjate, 2006; Acedo, 2010; Nyaura *et al.,* 2014). Although MAP packages are locally available in the market, evaluation and commercialization of their use in packaging of ALVs in the local market has not been conducted (Acedo, 2010). Use of ventilated crates and poly sacks have also been reported to reduce postharvest losses and improve quality and safety of vegetables (Adhikari, 2006). Canning technologies have been used as a preservative measure on many vegetables and can also be applied on AVLs. However canning is capital intensive and would require high initial investment, hence remains unaffordable by small scale farmers (Diamante, 2007).

Postharvest treatments such as heat treatments reduce postharvest quality loss, and new emerging postharvest technologies such as use of UV-C irradiation and electrical impulses are current research interests under Hortinlea project (Gogo *et al.,* 2016). Studies have shown the beneficial effects of postharvest treatments such as; control of insect pests, prevention of default fungal rots, and inhibition of undesired acceleration of ripening and senescence and/or promotion of the synthesis of health promoting compounds: such as carotenoids, flavonoids and dietary fibers during storage and marketing (Nicolai *et al*., 2007, Keil *et al*., 2011). These easy-to-apply postharvest treatments can prevent ALVs quality losses effectively and prolong shelf-life, storability and marketability (Gogo *et al.,* 2016).

Traditional preservation and processing technologies such as fermentation and blanching increases storability duration and quality of the vegetables. These technologies also improves food safety and prevents loss of nutrients (Muchoki *et al*., 2007; Wafula *et al.,* 2016). Food fermentation has been used for centuries as a processing technology known to increase storage duration, palatability, aroma and texture and increase the availability of proteins and vitamins. In addition, fermentation enhances food safety by reducing undesired anti-nutritional factors such as phytic acid and glucosinolates

(Habwe *et al.*, 2008; Franz *et al.*, 2014; Ifesan *et al.*, 2014; Wafula *et al.*, 2016). Studies have shown that the fermentation, blanching and drying of ALVs, such as cowpea leaves preserve substantial levels of nutritive compounds like the vitamins and beta-carotene (Muchoki *et al.*, 2007; Wafula *et al.*, 2016).

Traditionally farmers have been practicing natural fermentation, but current studies have involved use of starter cultures in cowpeas (Wafula *et al.*, 2016). Studies have also shown successful fermentation of African kale (*Brassica carinata*) using lactic acid starter strains such as *L. plantarum* BFE 5092 and *L. fermentum* BFE 6620 (Oguntoyinbo *et al* ., 2016).

Drying is one of the food preservation methods that has been used for many centuries and is beneficial in reducing microbial decay, ensuring physiochemical stability, reducing weight and transport costs as well as improving handling and storability (Kumar *et al.*, 2010). Several vegetable drying methods exist including sun drying, oven drying, solar drying, vacuum drying and freeze drying (Fellows, 2009). Leafy vegetables are held into bundles and cut into small pieces with a knife to ensure faster drying. The cut leaves are then Immersed in boiling water (blanching) or steamed for 3 minutes to minimize nutrients loss due to enzyme degradation (Fellows, 2009). Blanching is a pretreatment done on vegetables before most of processing methods or storage to destroy enzymic activity (Fellows, 2009). In addition to inactivating plant enzymes, blanching also reduces some unpleasant secondary metabolites such as oxalates. The chopped blanched leaves are then spread thinly on clean mats or flat open surface directly under the sun, oven dried or solar dried (Fellows, 2009, Masarirambi *et al.*, 2010). The dried vegetables can be stored in pots, tins, or in polythene bags and closed tightly to avoid any moisture re-entry in order to keep the leaf tissues dry (Bencini, 1991, Masarirambi *et al.*, 2010). The dried vegetables are normally soaked in water before cooking.

Although fermentation has been practiced traditionally in Africa, its potential to solve food insecurity and malnutriational crisis has not been fully exploited. Scaling up and development of fermentation, sun drying and solar drying protocols as well as of personnel training would signifantly solve postharvest losses of ALVs. This will also call for developments of proper packaging material to prevent the processed product from moisture and microbial infections to the product. Additionally, adaptation of various packaging, treatments and processing methods on ALVs will ensure quality and minimize losses in SSA. Cheaper storage options like charcoal coolers have been developed, but adaptation and knowledge about them is not widely known. This calls for improved extension services, farmers training and establishments of demonstration farms for all existing technologies and strategies for losses reduction of ALVs.

Future Prospects of ALVS Technologies

The health benefits including medicinal values and high nutrient content of ALVs has led to the global awareness about the vegetables and their utilization in solving food security crisis in SSA. This awareness has led to the awakening of researchers on all aspects of ALVs right from production to consumption. Improvement of production factors such as determining the right rates of fertilizer and water applications, identification of pests of AVLs and their control strategies are some of the current ongoing research interests under HORTINLEA program. Additionally, the HORTINLEA research project seeks to determine the right physiological age of African leafy vegetables and improve on the harvesting strategies. All these strategies aim to improve marketability, quality and to increase the shelf life of ALVs. Therefore, it is expected that more production, harvesting, postharvest handling and preservation techniques and strategies of African leafy vegetables will be revealed to reduce losses and enhance storability and quality of the vegetables.

Conclusion and Recommendations

Although ALVs have been used for many centuries as leafy vegetables, much research to explore techniques that can be used to combat post-harvest losses of the vegetables has not been done. Pre-harvest and post-harvest physiology specifically for each African leafy vegetable need to be investigated as well as the application of modern techniques in post-harvest handling of the vegetables. Adoption of such research findings by the farmers can be a great milestone in solving postharvest losses of the vegetables. Application of molecular biology techniques would also enhance the understanding on the various postharvest responses that lead to post-harvest losses of each specific vegetable crop.

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2.2 Development stage, Storage Temperature and Storage Duration influence phytonutrient content in Cowpea (*Vigna unguiculata L. Walp*.) *Heliyon* journal, 2018, https://doi.org/10.1016/j.heliyon.2018.e00656

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Highlights
The paper highlights on the dynamics of the health promoting phytonutrients and mineral contents in cowpea during growth and under different storage conditions. The pre-harvest and postharvest conditions are key to optimal availability of phytonutrients and post-harvest quality assurance.

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Development stage, storage temperature and storage duration influence phytonutrient content in cowpea (*Vigna unguiculata* L. Walp.)

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Abstract

Cowpea (*Vigna unguiculata*) plays an important role in sustainable food security and livelihood improvement in Sub-Saharan Africa (SSA). The crop is rich in phytonutrients and minerals, which are key in solving malnutrition and hunger crisis, a major challenge in SSA. However, physiological status, storage temperature and duration affect phytonutrient levels and postharvest life of the leafy vegetable. Despite the significant importance of cowpeas, the maturity and postharvest storage effects on quality of the leafy vegetable remains unrevealed. The aim of this study was to analyze the dynamics of phytonutrients in cowpea leaves during development under field conditions in Kenya and in storage. The total carbohydrates (glucose, fructose, sucrose and starch) were highest at 90 d after planting (105.9 ± 2.5 g kg^{-1}) compared to 30, 60 and 120 d. The total Phenolics (Gallic acid equivalents) increased gradually with age up to 12.0 ± 0.2 g kg^{-1} by 120 d. Catechin equivalent flavonoids, trolox equivalent antioxidants (TEA) and chlorophyll were highest in concentrations at 60 d after

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planting with 8.0 ± 0.5 g kg^{-1}, 26.19 ± 0.5 g kg^{-1} and 5.7 ± 0.4 g kg^{-1}, respectively. Quercetin equivalent flavonoids and total carotenoids did not show significant changes with age, while mineral concentration dynamics were specific for each element. Storage of cowpea leaves at room temperature (50–55 % relative humidity) led to a stronger decline of phytonutrients after 4 d, but mostly they remained stable at cold storage (5 °C). Results of this study highlight the importance of developmental stage at harvest, storage conditions and duration for the optimal availability of phytonutrients in freshly consumed leaves and for postharvest management strategies.

Keywords: Nutrition, Food analysis, Food science

1. Introduction

African leafy vegetables (ALVs) hold high potential in solving food insecurity menace and improving livelihoods in Sub-Saharan Africa (SSA) (Grivetti and Ogle, 2017). In addition to lack of enough food (calories), the hidden-hunger crisis due to the deficiency in proteins, minerals and microelements has been a health concern for many centuries in SSA (Okonya and Maass, 2014). This clearly shows the need for a sustainable supply of both quality and quantity of food. Currently, leguminous crops have been identified as the affordable and sustainable source of essential nutrients and low cost proteins for a balanced human diet (Avanza et al., 2013; Goncalves et al., 2016). Cowpea has several agronomic, economic and environmental advantages hence it remains most suitable and highly cultivated legume crop across Africa, Asia and South America (Linguya et al., 2015; Goncalves et al., 2016). The multipurpose crop is suitable for intensification of a sustainable agri-food system in SSA, with the whole above-ground part, being utilized for its leaves, green pods, green beans, mature beans, or processed into paste or flour. The remaining waste of the crop is used as forage, hay or silage for livestock feeding (Goncalves et al., 2016).

Cowpea leaves can be used as fresh green vegetables, fermented or sun-dried (Saidi et al., 2010; Ibrahim et al., 2002; Wafula et al., 2016). The leaves are rich in vitamins, macro and micro minerals, flavonoids, antioxidants, ß-carotene, fatty acids, amino acids, carbohydrates and dietary fibre (Okonya and Maass, 2014; Goncalves et al., 2016). The rich source of phytonutrients in cowpeas makes it one of the most suitable African indigenous crop in the provision of a health balanced diet and solving malnutrition crisis among the resource-constrained households in SSA (Okonya and Maass, 2014). Daily or frequent inclusion of cowpeas in the diets would therefore, not only solve problems with hunger but help in reducing the risk of chronic health conditions. Currently, secondary metabolites such as flavonoids, antioxidants, phenolics and carotenoids in African vegetables have received a lot of research attention for their wide range of pharmacological and

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biochemical benefits (Moyo et al., 2013; Neugart et al., 2017). They have antioxidative capacity, hence scavenges free radical ions and protect humans against cancer illnesses, and as well the metabolites have anti-inflammatory and antimicrobial properties (Nijveldt et al., 2001). The secondary metabolites also play key roles in the plant defense and protective mechanisms against biotic and abiotic stress (Nijveldt et al., 2001; Akula and Ravishankar, 2011).

Phytonutrient contents in vegetables and fruits are influenced by crop age or developmental stage, storage conditions and duration among other factors. Harvesting stages have been shown to influence postharvest behavior, nutritional quality and shelf life in some vegetables such as leaf amaranthus, spinach and lettuce (Lee and Kader, 2000; Modi, 2007; Oloyede et al., 2013; Spinardi et al., 2016). However, there is little information about effects of age on the nutrient content of many indigenous vegetables. Establishment of maturity stages or indices for harvesting would ensure optimum nutrient accumulation in cowpeas for uptake in human diets and/or postharvest processing. Additionally, storage temperature after harvesting of vegetables directly influences their metabolism. Unfavorable storage conditions and prolonged storage duration have been reported to lead to degradation of chlorophyll, carotenoids, ascorbic acid, antioxidants and carbohydrates on vegetables (Acedo, 2010; Spinardi et al., 2016). The changes of phytonutrients in cowpea leaves during development and after harvesting has not been studied so far. Despite the contribution of cowpeas and other traditional vegetables in household food security and poverty eradication, sufficient research has to date not been conducted to explore their full potential. This study aimed at evaluating the effects of plant age, storage conditions and storage duration on phytonutrient content for quality assurance and postharvest losses management of cowpea leaves.

2. Materials and methods

2.1. Experimental set-up

Seeds of the commonly grown Tanzanian variety *V. unguiculata* cv. Tumaini were kindly provided by World Vegetable Center, Arusha, Tanzania. Field experiments were conducted at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja, Kenya (S01°05.9′ E037 °00.8 and 1476 m above the sea level). Three field experiments were set with randomized complete block design (RCBD) in the period from February to May 2015, November 2015 to February 2016 and August to November 2016. Sowing of cowpeas seeds was done at a spacing of 20 × 30 cm between plants and rows, in 6 plots measuring 10 × 15 m wide. Di-ammonium phosphate ($(NH_4)_2HPO_4$) fertilizer was applied at a rate of 2 g per plant (18 g/m^2) and incorporated into the soil by mixing before placing the seed. Drip-irrigation was done to keep the soil moist. Harvesting was done by picking 2−3 leaves at random from the

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middle part of the plants at 30, 60, 90 and 120 d after planting (Fig. 1). Plant from outer rows in each plot were not harvested or used in the experiment.

Harvesting was done after 4 h of sunlight, in 6 biological replicates for each experiment, and each replicate consisted of leaves picked from 3 plants at random and pooled together. All the harvested samples were packed in perforated zip-rock polythene bags before storage. The control samples were immediately frozen at −20 °C after harvesting and represented day zero for the storage duration experiments. The other samples were stored in darkness at 5 °C with 80−85 % relative humidity (in refrigerator) and at 20−22 °C (room temperature (RT)) with 50−55 % relative humidity, for 2, 4, 6 and 8 d. The samples were then frozen at −20 °C shortly before freeze-drying. The freeze-dried samples were ground into fine powder and weighed (20−30 mg) into 2 ml Eppendorf tubes for phytonutrient analysis. The data is presented as g kg^{-1} or mg kg^{-1} dry weight (DW) of the leaf material. The main presented and described data in this study are from the field experiments August to November 2016, while few data comprising of mainly carbohydrates, chlorophyll and carotenoids from field experiments February to May 2015 and November 2015 to February 2016, are presented as additional data.

2.2. Carbohydrates analysis

2.2.1. Glucose, fructose and sucrose (GFS)

Glucose, fructose, sucrose (GFS) and starch were analyzed using enzymatic and photometric methods with slight modification as described by Gomez et al. (2007)

Fig. 1. Images of cowpea plants at different days after planting; (a) 30 d, (b) 60 d, (c) 90 d and (d) 120 d.

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and Zhao et al. (2010). The freeze-dried samples were ground into powder and were weighed into 2 ml Eppendorf tubes (20–30 mg) and used for carbohydrates analysis. Extraction was done with 80 % (v/v) ethanol, incubated in a water bath at 80 °C for 15 min. The ethanol extracts were used for GFS enzymatic assays while the pellet was further processed for starch extraction. For GFS, 20 µl aliquots of the extracts were pipetted into 96 well microplates and placed in an oven at 50 °C for 30 min to evaporate the ethanol. The dried wells were then eluted with an equal volume of double distilled water (ddH_20). The ddH_2O was used as blank and the standards for the calibration curve were made from serial dilutions of 1.0 g L^{-1} glucose. A 96 well microplate was loaded with samples in triplicates, and analysis of GFS was done sequentially starting with glucose, followed by fructose and finally sucrose.

A buffer (100 ml) containing 14 g of triethonalamine hydrochloride (Ref 4804, Roth) and 250 mg of $MgSO_4 \times 7H_2O$ (pH 7.6) (TRA buffer) was prepared. The TRA buffer (10 ml) was then used to dissolve a mixture of 100 g $NaHCO_3$, 100 g ATP (Ref 10519987001, Roche) and 20 g NADP (Ref AE13.2, Roth). The mixture of $NaHCO_3$, ATP and NADP in TRA buffer was then pipetted (100 µl each) into the wells. Ten microliter of hexokinase/glucose 6-phosphate dehydrogenase (HK/G6P-DH) (Roche, 10737275001), was then added to the wells and mixed by shaking gently before incubating for 30 min at 30 °C. Glucose absorbance was read at 340 nm on the Versamax® MP photometric reader (Molecular devices, Canada). The phosphoglucose isomerase (PGI) (Ref 10128139001, Roche) (0.25 U) was then added to the wells (10 µl), mixed by shaking gently, incubated for 30 min at 30 °C and absorbance reading for fructose was taken at 340 nm. For sucrose analysis, Invertase from baker's yeast (960 U, Lot 21K7435, Sigma-Aldrich) was dissolved in citric buffer (citric acid and Tri-sodium citrate, pH 4.6) and 20 µl was added to the wells and mixed gently, then incubated at 30 °C for 45–60 min. The final absorbance reading for sucrose was taken at 340 nm. Calculations of each of the GFS was done as described by Gomez et al. (2007).

2.2.2. Starch

The pellet derived after GFS extraction (Section 2.2.1) was washed with ddH_2O, dried in an oven at 60 °C and used for starch analysis with slight modifications as described by Gomez et al. (2007) and Zhao et al. (2010). Starch was hydrolyzed by adding 500 µl of 0.5 M NaOH and incubated at 60 °C on a shaker for 1 h. The pellet was then cooled and neutralized with 25 µl 1 M acetic acid and 475 µl ddH2O and mixed by vortexing followed by spinning at 4200 × g for 10 min. From the supernatant, 10 µl was transferred to a clear 96-well microplate in triplicates. The Amyloglucosidae (AG) (9 U) was weighed (4 mg) and dissolved in 1.4 ml citric buffer (Section 2.2.1), from which 20 µl was added to the wells, and

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incubated for 30 min at 60 °C. The plate was cooled and 100 µl of TRA buffer (Section 2.2.1) added followed by hexokinase/glucose 6-phosphate dehydrogenase (HK/G6P-DH) (Section 2.2.1) (10 µl), and incubated for 30 min at 30 °C. The absorbance was then recorded at 340 nm and the calculations were based on the calibration curve equation derived from glucose standards as described by Gomez et al. (2007).

2.3. Total phenolics content

Determination of total phenolics content using the Folin-Ciocalteu (F-C) assay was done as described by Ainsworth and Gillespie (2007). Finely ground freeze-dried samples (20–30 mg) were weighed into 2 ml tubes. The total phenolic compounds were extracted with 2 ml 97 % (v/v) methanol for two days at room temperature (RT) in darkness. Ten percent (v/v) of Folin & Ciocalteu's phenol reagent (F-C) (Ref F9252, Sigma- Aldrich) was added to 100 µl of the plant extract, and mixed thoroughly by vortexing. This was followed by the addition of 800 µl 700 mM Na_2CO_3 and incubation for 2 h at RT. Samples were spinned at 15800 × g for 1 min and 200 µl of supernatant was transferred to a clear 96-well microplate in triplicates. The absorbance of each well was measured and recorded at 765 nm using a Tecan microplate reader (Infinite® 200). Gallic acid was used to prepare the calibration curve. The calculated amount of total phenolic content was expressed as gallic acid equivalents per gram of dry weight.

2.4. Flavonoids

The aluminium complexation reaction assay was carried out for catechin and quercetin equivalents as described by Eghdami and Sadeghi (2010). For catechin, 100 µl of the methanol extracts described in Section 2.3 were mixed with 300 µl distilled water and 30 µl 5 % (w/v) $NaNO_2$, and incubated for 5 min. Thirty microliters of 10 % (w/v) aluminum chloride ($AlCl_3$) were added followed by another incubation for 5 min. The reaction mixture was then treated with 200 µl 1M NaOH and 340 µl distilled H_2O to reach a final volume of 1000 µl. Aliquots of 200 µl from the supernatant of each sample were pipetted to a clear 96-well microplate in triplicates. Absorbance at 510 nm was measured with a Tecan microplate reader (Infinite® 200). The calibration curve was done with catechin serial dilutions of 12.5, 25, 50, 100, 250 g L^{-1}. The calculated amount of catechin and its derivatives were then expressed as catechin equivalents in g kg^{-1}. In the quercetin analysis, 100 µl of the extract was mixed with 300 µl of 97 % methanol, plus 20 µl of 10 % $AlCl_3$ and 20 µl of 1M NaOAc. Finally, 560 µl H_2O were added to bring the total volume to 1000 µl. Aliquots of 200 µl from the supernatant from each sample were pipetted to a clear 96-well microplate in triplicates. Absorbance at 415 nm was measured with a Tecan microplate reader (Infinite® 200). The calculated amount of quercetin flavonoids was then expressed as quercetin equivalents.

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2.5. Antioxidants

Antioxidants were analyzed using the Trolox equivalent antioxidants capacity (TEAC) assay against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS• + radical cation) (Ref A1888-1G, Sigma-Aldrich) as described by Miller et al. (1993), and with slight modifications as suggested by Tyrakowska et al. (1999). The principle of the method was based on the ability of the antioxidants to scavenge the ABTS•+ radical cation in comparison to the antioxidative capacity of an artificial and water-soluble vitamin E derivative, Trolox® (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (Ref 1238813-1G, Sigma-Aldrich). The pre-formed blue/green radical of ABTS• + cation was generated by addition of potassium persulfate to a solution of the ABTS diammonium salt, which has an absorption maximum at 734 nm. The ABTS• + radical cation is reduced in presence of hydrogen-donating antioxidants leading to decolorization of the blue/green-colored solution. The decolorization of the ABTS• + radical cation is then a measure of the antioxidant capacity, which is given in Trolox equivalents (Miller et al., 1993). In the TEAC assay, the methanol extracts described in Section 2.3 above, were diluted 1:20 with 50 mM of phosphate buffer (41 mM Na_2HPO_4 and 9 mM NaH_2PO_4, pH 7.2). Sample aliquots of 10 µL were pipetted into 96 well microplates with three replicates each. ABTS stock solution (7 mM) was prepared and incubated overnight at RT in darkness where an intensely blue colored complex developed. An ABTS working solution (about 140 µM) was prepared by diluting with phosphate buffer (1:50 to 1:70) until the extinction at 734 nm was between 0.7 and 0.8. The diluted ABTS solution (190 µl) was then added to the samples by the injector in the Tecan microplate reader, and after 5 min the absorbance was recorded at 734 nm. All analyses were carried out in triplicates. The calibration and regression lines were received from the Trolox® stock solution in a dilution series of 0.025−0.4 mM in phosphate buffer.

2.6. Chlorophyll and carotenoids

The total chlorophyll and carotenoids content were determined in cowpea leaves by UV-VIS spectroscopy, as described by Lichtenthaler (1987). The freeze-dried material was ground into powder, 20−30 mg weighed into Eppendorf tubes with 6 replicates, and 4.5 ml of 90 % (v/v) ethanol extractions were done for the pigments. Samples were then vortexed and spinned at 15800 × g for 1 min and 200 µl of the supernatant was aliquoted to the 96 well plate in triplicates and covered with aluminium foil. The absorbance readings were done at 470 nm, 648 nm and 664 nm (as well as 750 nm to check for impurities) using the Tecan microplate reader (infinite® M200). Calculations for total chlorophyll and carotenoids were done according to the formula described by Lichtenthaler (1987).

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2.7. Mineral analysis

Finely ground plant samples were weighed into 100 ml test tubes (250 mg) in three biological replicates, and each with 3 technical replicates. Digestion of plant material was done with lithium sulfate buffer (0.48 g of selenium powder, 14 g of lithium sulfate monohydrate 99.99 Suprapur® (CAS 10102-25-7, Sigma-Aldrich), 420 ml sulfuric acid 95–97 %, and 330 ml hydrogen peroxide 30 % stabilized). This buffer was prepared by dissolving selenium and lithium sulfate in 3 L of sulfuric acid in a PE beaker while placed in a bowl of cold water. Hydrogen peroxide was then added slowly while stirring, and the liquid became clear after cooling. Ten microlitre of the buffer was then added by tilting pipette to the samples and heated in the Turbotherm apparatus Gerhardt (Gerhardt, analytical systems, Königswinter, Germany). After cooling, the samples were filled with deionized water to 50 ml, mixed on a test tube shaker and filtered through a pleated filter (Munktell 3/N) in PE bottles. The measurement of Ca, K, Mg, Fe, Zn, Mn, P and Cu elements was carried out by inductively coupled plasma optical emission spectrometry (ICP-OES), while nitrates was quantified through flow injection analysis (FIA) method (Jones, 2001; Brabcová et al., 2003).

2.8. Statistical analysis

All the acquired data were subjected to normality test before analysis of variance was conducted using the SPSS (version 20) statistical software. The means were compared using Tukey test at $p < 0.05$. The data were presented as means ± standard errors g kg^{-1} or mg kg^{-1} dry weight.

3. Results

The phytonutrient analysis were done from cowpeas samples harvested at 30, 60, 90 and 120 d after planting (Fig. 1). Cowpeas was at vegetative phase from 30 to 60 d, while at 90 d, the crop was at reproductive phase with over 90 % of the plants having flowers or already developing young pods (Fig. 1). By 120 d, plants already had mature pods and most of the older leaves were already turning yellow as a sign of senescence (Fig. 1). In this study, glucose concentration was significantly lower at 30 d (1.5 ± 0.15 g kg^{-1}) than at 60, 90 and 120 d (9.9 ± 0.5, 11.0 ± 2.7, 10.2 ± 1.7 g kg^{-1}), respectively (Fig. 2). Fructose and sucrose concentrations were highest at 90 d (9.5 ± 0.03 and 48.12 ± 5.2 g kg^{-1}), respectively, while the starch concentration did not show any significant difference between the developmental stages. The total of all the four sugars indicated highest carbohydrate contents at 90 d after planting (Fig. 2). From the field experiments done from February to May 2015 and November 2015 to February 2016, although there were changes with specific sugar quantities with age, the total carbohydrates remained highest at 90 d after planting

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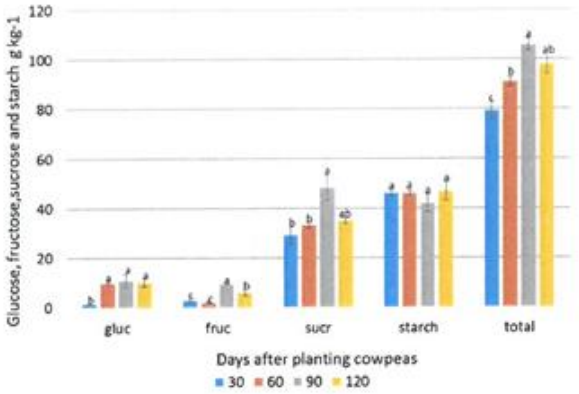

Fig. 2. Glucose (gluc), fructose (fruc), sucrose (sucr) and starch concentrations and their totals in cowpea leaves harvested 30, 60, 90 and 120 d after planting. The data is from field experiment August to November 2016. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

compared to other development stages (Fig. 3). The storage experiments were conducted from 60 d old plants from the field experiments of August to November 2016. During storage at 5 °C and 80–85 % RH, there were no pronounced changes in total sugars and sucrose concentrations, while on the other hand the fructose and glucose increased as the starch content decreased after 6 d of storage (Fig. 4). Storage of cowpea leaves at RT (20–25 °C) at 55–60 % RH led to a significant decrease in concentrations of all three sugars with the steepest reduction being observed after 4 d of storage (Fig. 4). The carbohydrates declined by 81 % if stored under RT as compared to 16 % at cold storage (Fig. 4). The same phenomena of carbohydrates changes was observed during a repeat of storage experiment at 90 d after planting (Fig. 5).

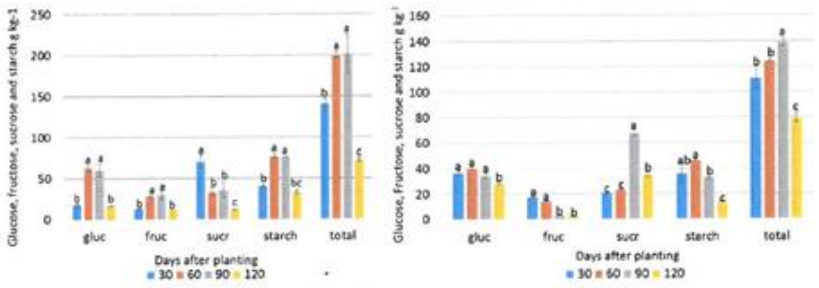

Fig. 3. A = Field experiments February to May 2015 and B = Field experiments November 2015 to February 2016: Glucose (gluc), fructose (fruc), sucrose (sucr) and starch concentrations and their totals in cowpea leaves harvested 30, 60, 90 and 120 d after planting. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

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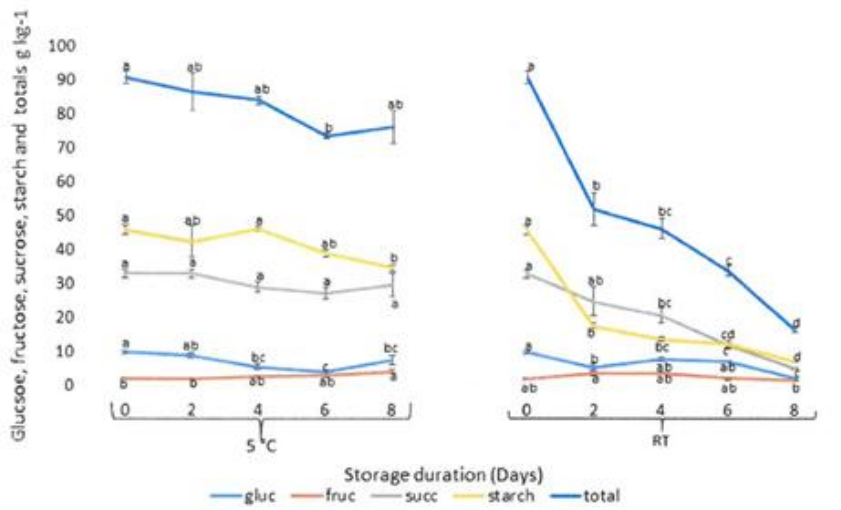

Fig. 4. Glucose (gluc), fructose (fruc), sucrose (sucr) and starch and their totals in cowpea leaves harvested at 60 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

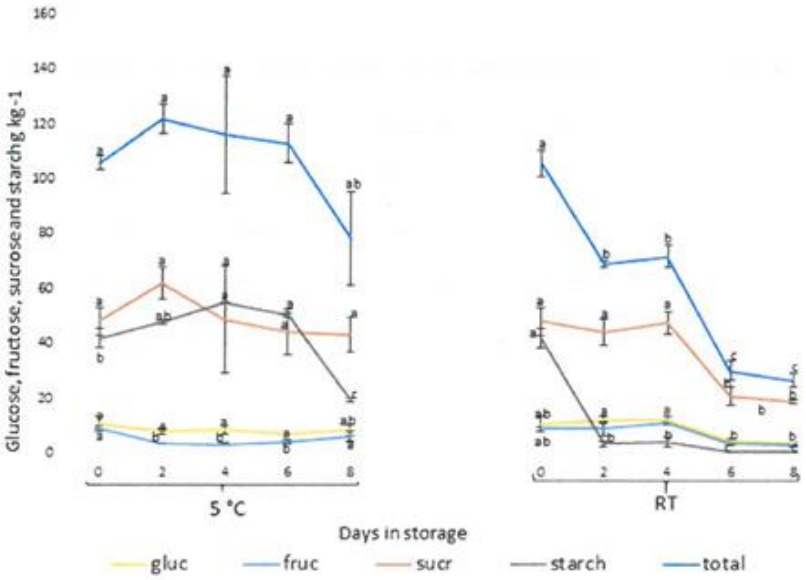

Fig. 5. Glucose (gluc), fructose (fruc), sucrose (sucr) and starch and their totals in cowpea leaves harvested at 90 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

The dynamics of secondary metabolites during development and storage were also investigated. Quercetin equivalent flavonoids were detected in higher concentrations than catechin equivalent flavonoids in cowpea leaves (Fig. 6). For quercetin, no significant differences in concentration were observed among the different plant ages

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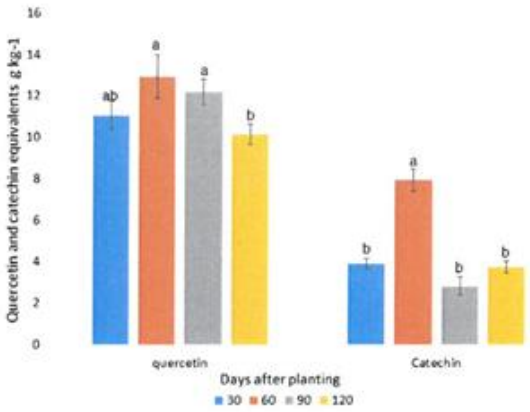

Fig. 6. Flavonoids (quercetin and catechin equivalents) content in cowpea leaves harvested at 30, 60, 90 and 120 d after planting. The values represent means ± standard error. Means labeled with the same letters within a metabolite group were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

(Fig. 6). In contrast, catechin was detected in significantly higher concentrations at 60 d after planting (8.0 g kg^{-1}) compared to 30, 90 and 120 d (3.9 ± 0.3, 2.8 ± 0.4 and 3.7 ± 0.3 g kg^{-1}, respectively) (Fig. 6). In both storage temperatures, there was degradation of flavonoids which was more pronounced for quercetin than for catechin flavonoids (Fig. 7). Between storage conditions, only minor differences were observed during the first 6 d of storage, whereas thereafter, higher storage

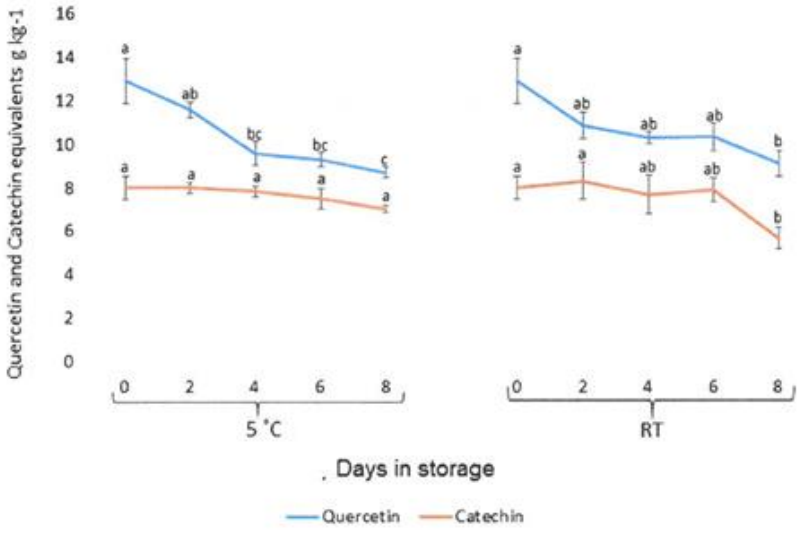

Fig. 7. Flavonoids (quercetin and catechin equivalents) in Cowpeas leaves harvested at 60 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within a metabolite were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

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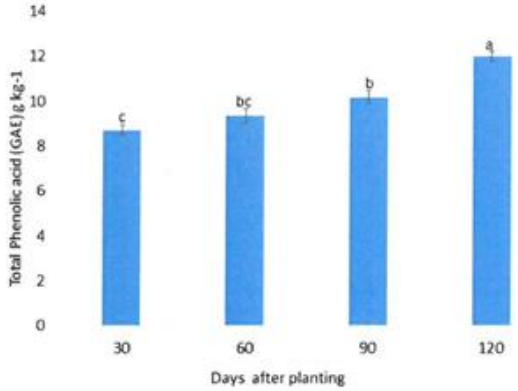

Fig. 8. Total phenolic (Gallic acid equivalents (GAE) content in cowpea leaves harvested 30, 60, 90 and 120 d after planting. The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

temperatures resulted in a steeper decrease in concentration of both groups of flavonoids (Fig. 7).

The total phenolics content gradually increased with plant age in cowpeas, 8.7 ± 0.2, 9.3 ± 0.3, 10.2 ± 0.3 and 12.0 ± 0.2 g kg^{-1} for 30, 60, 90 and 120 d after planting respectively (Fig. 8). During storage, there was 34.5 % decline of the phenolics at RT and 47 % decline (from 10.6 ± 0.6 to 5.6 ± 0.2 g kg^{-1}) was observed after storing at 5 °C for 8 d (Fig. 9). This signifies losses of phenolics in longer storage duration despite the temperature. The antioxidants just like total phenolics increased with plant age and were significantly higher after 60 d (26.2 ± 0.6 g kg^{-1}) than at younger stages, 30 d (22.3 ± 0.5 g kg^{-1}) (Fig. 10). The antioxidants appeared stable up to 6

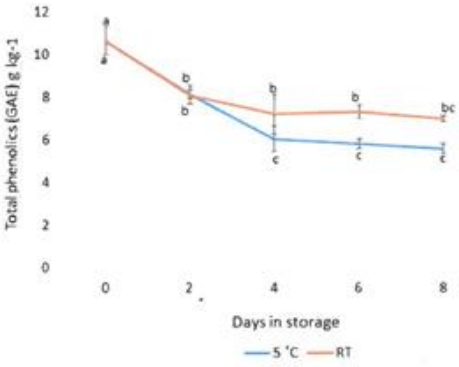

Fig. 9. Total phenolic (GAE) contents in Cowpeas leaves harvested at 60 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within a metabolite were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

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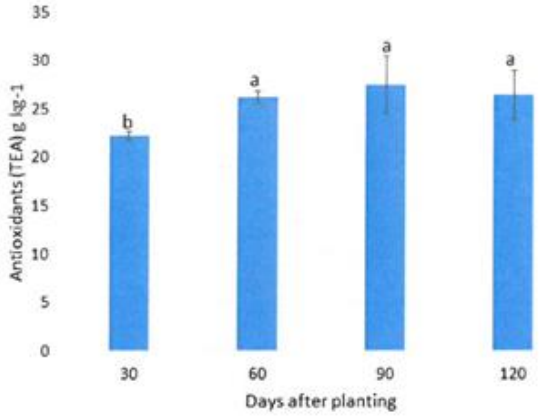

Fig. 10. Trolox equivalents antioxidants (TEA) content in cowpeas leaves harvested 30, 60, 90 and 120 d after planting. The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

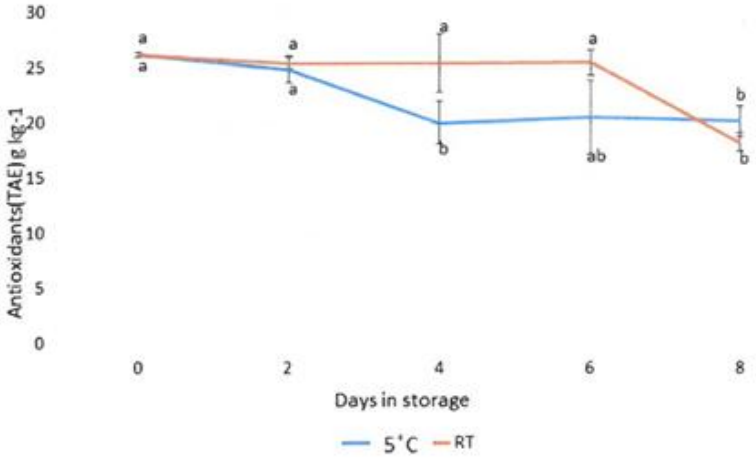

Fig. 11. Antioxidants (TEA) content in cowpeas leaves harvested at 60 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within a metabolite were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

d in storage at RT, but a significant decline was observed by 8 d of storage (Fig. 11). A slight decline was observed during storage at cold temperatures, after 4 d and later remained stable (Fig. 11).

The total chlorophyll content during plant development increased significantly between 30 d (4.3 ± 0.2 g kg^{-1}) and 60 d (5.7 ± 0.4 g kg^{-1}) and remained stable thereafter (Fig. 12; A). Carotenoids did not show any significant difference between the four development stages of cowpeas and the concentration ranged from 0.8 to 0.9 g kg^{-1} (Fig. 12; A). However, the field experiments in February to May 2015 and November 2015 to February 2016, indicated high chlorophyll content 60 d after

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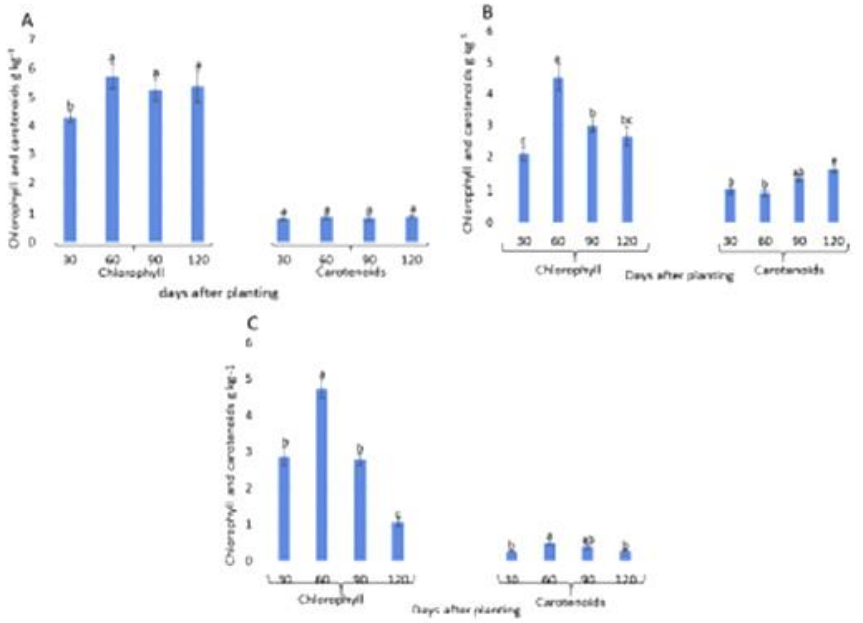

Fig. 12. Chlorophyll and carotenoid concentration in cowpeas leaves harvested 30, 60, 90 and 120 d after planting. A = Field experiments August to November 2016, B = Field experiments February to May 2015, and C = Field experiments November 2015 to February 2016. The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p < 0.05$ using Tukey's test (n = 6).

planting and an increase of carotenoids with age (Fig. 12; B&C). The chlorophyll later decreased in 90−120 d after planting (Fig. 12; B&C). Storage of cowpeas harvested at 60 dap at 5 °C indicated no significant difference in chlorophylls and carotenoids after storing for 0, 2, 4, 6 and 8 d (Fig. 13). On the other hand storage at RT led to a 49 % decline of the total chlorophyll by day eight. The carotenoids quantities were not significantly affected by RT storage at 60 d after planting

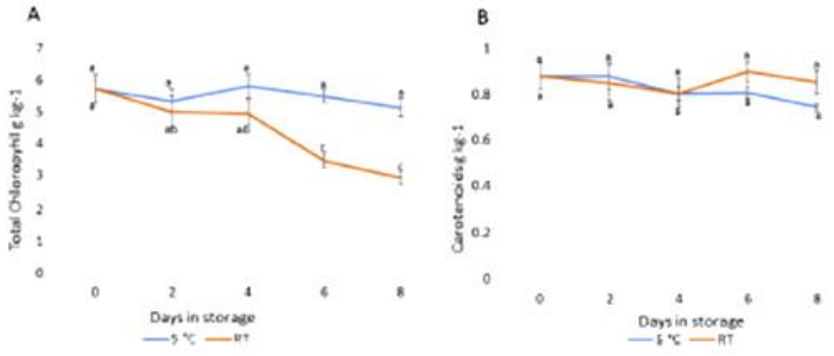

Fig. 13. A = Chlorophyll and B = carotenoid (g kg^{-1}) concentration in cowpea leaves harvested at 60 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within a group and storage condition were not significantly different at $p < 0.05$ using Tukey's test (n = 6).

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(Fig. 13). The same phenomena of chlorophyll and carotenoids changes were observed during a repeat of the storage experiment from cowpea leaves harvested at 30, 90 and 120 d after planting, although slight changes of carotenoids were seen at young leaves at 30 d after planting (Table 1). The greatest percentage losses of chlorophyll was observed at 90 and 120 d after planting (67.9 and 68.5 %), compared to 39.5 % at 30 d after planting (Table 1).

Results in this study indicated that cowpeas is a rich source of both macro and microelements mineral contents. The dynamics of mineral concentrations in cowpea leaves during development was specific for different elements (Table 2). Minerals such as phosphorous, zinc, potassium and magnesium, nitrates and copper were found to be relatively stable at different development stages. Iron and manganese were higher at later development stages (559.9 ± 72 and 327.5 ± 11 mg kg^{-1}), while the calcium concentration was highest at 30 d (19 g kg^{-1}) and indicated a decline thereafter (Table 2). During storage, the minerals did not show significant changes (data is not presented).

Table 1. Chlorophyll and carotenoid (g kg^{-1}) concentration in cowpea leaves harvested at 30, 90 and 120 d after planting and stored at 5 °C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within a group and storage condition were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

Days after planting	Days in storage	Chlorophyll		Carotenoids	
		5 °C	RT	5 °C	RT
30	0	4.3 ± 0.2a	4.3 ± 0.2a	0.8 ± 0.0a	0.8 ± 0.0ab
	2	4.1 ± 0.1a	4.4 ± 0.2a	0.8 ± 0.0a	0.9 ± 0.1a
	4	4.0 ± 0.2a	4.2 ± 0.3a	0.8 ± 0.0a	0.9 ± 0.1a
	6	4.2 ± 0.1a	2.9 ± 0.5ab	0.7 ± 0.0a	0.7 ± 0.0b
	8	4.1 ± 0.2a	2.6 ± 0.8ab	0.8 ± 0.0a	0.7 ± 0.1b
90	0	5.3 ± 0.4a	5.3 ± 0.4a	0.9 ± 0.1a	0.9 ± 0.1a
	2	5.1 ± 0.4a	5.5 ± 0.6a	0.8 ± 0.1a	0.9 ± 0.1a
	4	4.9 ± 0.6a	3.4 ± 0.3b	0.6 ± 0.0a	0.8 ± 0.1ab
	6	4.8 ± 0.3a	1.9 ± 0.3b	0.7 ± 0.0a	0.7 ± 0.0b
	8	4.9 ± 0.2a	1.7 ± 0.2b	0.7 ± 0.0a	0.6 ± 0.0b
120	0	5.4 ± 0.5a	5.4 ± 0.5a	0.9 ± 0.0a	0.9 ± 0.0a
	2	5.0 ± 0.4a	4.6 ± 1.1ab	0.9 ± 0.0a	0.7 ± 0.2b
	4	5.5 ± 0.2a	4.0 ± 0.8ab	0.9 ± 0.1a	0.6 ± 0.1b
	6	5.2 ± 0.3a	2.3 ± 0.5ab	0.8 ± 0.0a	0.7 ± 0.1b
	8	5.3 ± 0.3a	1.7 ± 0.4b	1.1 ± 0.3a	0.7 ± 0.1b

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Table 2. Mineral content in cowpea leaves harvested at 30, 60, 90 and 120 d after planting. The values represent means ± standard error. Means labeled with the same letters within a column were not significantly different at $p < 0.05$ using Tukey's test ($n = 6$).

dap	N g kg^{-1}	P g kg^{-1}	K g kg^{-1}	Ca g kg^{-1}	Mg g kg^{-1}	Fe mg kg^{-1}	Zn mg kg^{-1}	Mn mg kg^{-1}	Cu mg kg^{-1}
30	41.2 ± 0.3b	3.1 ± 0.2a	26.0 ± 1.8a	19.0 ± 0.6a	3.9 ± 0.1a	305.4 ± 4.3b	29.3 ± 3.8a	187.4 ± 10.0b	22.7 ± 3.7a
60	44.3 ± 1.3ab	3.5 ± 0.0a	24.5 ± 0.2a	14.2 ± 0.1b	3.3 ± 0.1b	419.5 ± 16ab	32.2 ± 0.1a	275.1 ± 11.9a	14.9 ± 0.6a
90	41.2 ± 2.2b	2.7 ± 0.3a	20.5 ± 0.3b	12.1 ± 0.8b	2.5 ± 0.2b	371.9 ± 28b	24.6 ± 1.3a	218.4 ± 16.3b	14.7 ± 0.3a
120	47.3 ± 0.7a	3.4 ± 0.2a	23.5 ± 1.0ab	14.2 ± 1.1b	3.0 ± 0.0ab	559.9 ± 71.7a	33.2 ± 0.2a	327.5 ± 10.9a	17.2 ± 1.5a

4. Discussion

4.1. Carbohydrates

Carbohydrates are the major products of photosynthesis and play a central role in driving diverse biological processes from embryogenesis to senescence, by supporting and integrating functions and actions of internal and external regulatory signal. In general, the highest total concentration of all carbohydrates analyzed (105.9 ± 2.5 g kg^{-1}) was detected at 90 d after planting in cowpea leaves (Fig. 2). This high concentration of total sugars at 90 d can be attributed to the well-developed canopy of leaves presenting a high light-exposed surface area and hence a high photosynthetic capacity leading to an accumulation of photosynthetic assimilates. By 120 d, a slight decline in sugar concentration was noticed possibly due to translocation of much sugars from the leaves to the pods, which can be considered as sinks for carbohydrates at this stage (Lemoine et al., 2013). Alternatively, there was probably reduced photosynthetic potential due to aging leaves at 120 d. Previous studies have indicated a cross-link between various metabolites and phytohormones and the regulation of sugar metabolism and transport (Eveland and Jackson, 2012), which could also explain the variation of sugar concentration over development period as observed in this experiment. Sucrose being the major sugar transported from source to sink via the phloem was highest in concentration in the leaves at 90 d, which can be attributed to the development of many fully mature leaves, high chlorophyll content and other signal transductions derived by transition of the crop development phases (Lemoine et al., 2013). The levels of glucose, fructose, sucrose and starch indicated variations and fluctuations during the three experiments and this is due to changes in temperatures, light intensity and possibly slight variations in production conditions. However, in all the cases, carbohydrates contents remained higher 90 d after planting.

During storage at 5 °C and 80–85 % RH, there were no pronounced changes in total sugars and sucrose concentrations, while on the other hand the fructose and glucose increased as starch content decreased after 6 d of storage (Figs. 4 and 5). This can be attributed to the deprivation of simple sugars as a result of metabolic activities still going on in the cells, hence a conversion of starch back to glucose. The degradation

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of starch occurs when there is glucose deficit and helps in maintaining plants metabolism (Smith et al., 2005; Granot et al., 2014). During storage of plant parts at higher temperatures, the respiration rate and most metabolic processes are higher resulting in faster degradation of sugars to provide energy. This explains the sudden decline of starch after two days in storage at RT, which is converted to monosaccharides, presumably causing the stable contents of sugars between 2 and 4 d. The degradation of carbohydrates during storage consequently leads to spoilage of vegetables and loss of good quality attributes.

4.2. Flavonoids, total phenolics and antioxidants

Plant secondary metabolites, such as phenolics, flavonoids and antioxidants play important roles in the human diet and plant development (Wojakowska et al., 2013). Flavonoids and their conjugates have previously been reported to be involved in defense mechanisms against pathogenic attacks in *Fabaceae* species (Akula and Ravishankar, 2011). This study reveals higher concentrations of quercetin equivalent flavonoids in cowpeas leaves than catechin equivalents (Fig. 6). For quercetin, no significant differences in concentration were observed among the different plant ages. The higher levels of catechin flavonoids after 60 d implies that plants synthesize species-specific secondary metabolites after recognition and perception of various signals. These signals can be derived from external biotic and abiotic factors as well as internal factors such as plant development stages (Grotewold, 2005; Veitch, 2007).

The catechin levels are in the same range as those detected in other leafy vegetables such as *A. hybridus, C. olitorius, B. pilosa, C. gynandra, G. parviflora* and *L. sativa* (Chipurura, 2010). Previous studies show that the influence of age on secondary metabolite composition was specific to different crops (Bergquist et al., 2005; Akula and Ravishankar, 2011; Ghasemzadeh et al., 2014). This indicates that different crops respond differently to various factors that either upregulate or downregulate production of secondary metabolites as well as their degradation. Oloyede et al. (2013), reported an increase in antioxidants, flavonoids and phenolic contents at 6 and 5 weeks after planting of *Amaranthus cruentus* compared to 4 and 3 weeks. On the other hand phenolics, flavonoids and antioxidants decreased with age in *Celosia argentea* (Oloyede et al., 2013). However, the three metabolites are lower in cowpeas as indicated in this study compared to those reported in *Amaranthus cruentus* (Oloyede et al., 2013). In both storage temperatures, there was degradation of flavonoids which was more pronounced for quercetin than for catechin flavonoids (Fig. 7). Between the two storage conditions, only minor differences were observed during the first six days of storage, whereas thereafter, higher storage temperatures resulted in a steeper decrease in concentration of both groups of flavonoids. This indicates that the long storage duration, irrespective of temperature, leads to losses of

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secondary metabolites. The stability of flavonoids has been reported on baby spinach during the normal retail storage conditions, and this correlates with our findings up to six days (Bergquist et al., 2005). The researchers however, indicated that the changes during storage is specific for different types of flavonoids (Bergquist et al., 2005).

The total phenolics content in cowpeas gradually increased with the crop maturity (Fig. 8). Increase in total phenolics with age has been reported in various crops such as *Amaranthus cruentus* (Oloyede et al., 2013). Longer storage duration of cowpeas led to a decline of total phenolics although to a higher extent at cold storage than at RT. A decline in phenolic contents at cold storage and an increase during storage at RT has been reported on other leafy vegetables such as *Amaranthus caudatus, Amaranthus viridis, Moringa oleifera* and in baby lettuce among others (Mathiventhan and Sivakanesan, 2013). Therefore, although total phenolics are affected by various biotic and abiotic factors, the stability during storage is variety or species dependent (Mathiventhan and Sivakanesan, 2013). Since the phenolics play roles in plant defense, the stability during storage at RT up to 8 d could be as a result of continuous phenols build up as a physiological response to infection and damage of the leave cells and oxidative stress after harvesting.

The antioxidants just like other secondary metabolites increased with plant age and were significantly higher after 60 d than at younger stages 30 d (Fig. 10). In general, the antioxidative capacity of cowpeas leaves seemed stable up to 6 d despite the storage temperature, and was only affected by prolonged storage duration at RT after 8 d (Fig. 11). Mathiventhan and Sivakanesan, 2013, pointed out an increase of antioxidants in storage both at RT and at 4 °C and in *Amaranthus caudatus, Amaranthus viridis and Moringa oleifera*. These researchers indicated that the increase of both total phenolics and antioxidants during storage could be due to a continuous breakdown of tannins to free phenolics. Therefore this could also explain the stability of this compounds observed in this study.

4.3. Chlorophylls and carotenoids

The physical characteristics of vegetables after harvesting can easily be determined by greenness and freshness, key factors that affect the market. Therefore chlorophyll quantification can be used as a marker to evaluate the status and quality of green leafy vegetables during storage and development. Results in this study indicated higher chlorophyll contents after 60 d than at younger stages (30 d), while carotenoids did not show any significant difference between the four development stages of cowpeas (Fig. 12). However, in the preliminary experiments and increase in carotenoids with age was observed while chlorophyll remained higher 60 d after planting. The differences in the three experiments could be due to different radiation and temperature. Storage of cowpeas at 5 °C indicated no significant difference in chlorophyll and carotenoids after storing for 0, 2, 4, 6 and 8 d (Fig. 13). This shows that

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cold storage preserves the chlorophyll and carotenoids integrity in cowpeas hence quality can be enhanced for a longer duration. On the other hand, storage at RT led to a significant decline of chlorophyll, but carotenoid concentration remained stable, and thus resulted in a significantly lower chlorophyll to carotenoid ratio after 8 d in storage. The low chlorophyll to carotenoid ratio indicates degradation of chlorophyll at higher temperatures and longer storage duration. This is possibly due to break down of various physiological processes and increase in ethylene gas production leading to chlorophyll degradation. Previous reports have highlighted various enzymes such as chlorophyll oxidase, peroxidase-hydrogen peroxide and lipid peroxidation to be involved in chlorophyll degradation in leafy vegetables such as spinach (Yamauchi and Watada, 1991; Limantara et al., 2015). The decline of the chlorophyll content can also be associated with the wilting, yellowing and browning which was observed after 6 d of storage at RT. Low temperatures are known to slow down leaf metabolism hence preserves the color and quality and this explains why degradation of chlorophyll was not observed at low temperature storage.

4.4. Minerals

Mineral content contributes significantly in solving hidden hunger crisis especially among children and women in SSA (Okonya and Maass, 2014). Results revealed that cowpea leaves are a rich source of minerals such as iron, potassium, calcium, magnesium, phosphorus, manganese and zinc (Table 2). The mineral contents in cowpeas in this study was in the same range with some of the green leafy vegetables, including *C. gynandra* (Schönfeldt and Pretorius, 2011; Okonya and Maass, 2014; Kim et al., 2014; Omondi et al., 2017). However fluctuations can occur due to changes in production conditions, temperature and light just as seen in the carbohydrates dynamics. The dynamics of mineral concentrations in cowpea leaves during development was specific for different elements (Table 2). During cowpeas developments, most mineral contents such as zinc, potassium, phosphorous, nitrogen, magenesium and copper were observed to be stable although slight variations were observed. However, calcium decreased with age while Fe and Mn increased (Table 2). Previous studies have reported a wide variability of minerals in different cowpeas cultivars (Kim et al., 2014). However, little information is available about dynamics of mineral elements during the development of leafy vegetables.

5. Conclusion

Results from this study show the dynamics of phytonutrient composition in cowpea leaves based on developmental stages and postharvest storage conditions and duration. This raises the importance of biochemical markers such as chlorophyll, antioxidants, and carbohydrate contents amongst others in determination of harvesting indices for cowpeas, a nutrient-rich crop. Generally, the results indicated that

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60–90 d as the maturity stage with optimal nutritional content, important for the human diet. Therefore, harvesting cowpeas when the nutrients are optimal can offer maximum benefits in terms of nutritional quality and also may compensate for losses incurred under suboptimal storage conditions. An optimal harvesting time would be important for the postharvest processing treatments, such as fermentation, solar drying or sun drying among others, and phytonutrients should also be analyzed after such treatments. Most phytonutrients in cowpeas would be preserved at cold storage at least up to four days. Although cold storage facilities are not available to many resource-poor farmers in SSA, use of on-farm evaporative coolers should be emphasized to preserve most of the beneficial phytonutrients in cowpea leaves and a cooling chain is strongly recommended if cowpea leaves need to be transported to supermarket/marketing centers.

Declarations

Author contribution statement

Dinah Kirigia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Traud Winkelmann, Remmy Kasili: Conceived and designed the experiments; Wrote the paper.

Heiko Mibus: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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2.3 Nutritional composition in African nightshade (*Solanum scabrum* Mill.) influenced by harvesting methods, age and storage conditions: Manuscript - Postharvest Biology and Technology, accepted

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Abstract

African indigenous vegetables have a great potential in improving livelihood, offering sustainable food security and solving the malnutrition crisis in sub-Saharan Africa (SSA). African nightshade (*Solanum scabrum*) is one of such indigenous vegetable, highly appreciated for its nutritive and pharmacological benefits. The vegetable is rich in antioxidative compounds, vitamins, carbohydrates, carotenoids, folic acid, minerals and amino acids. However, the optimal nutritional benefits of nightshade are influenced by pre-harvest physiological maturity, postharvest storage conditions and harvesting methods. We analyzed the dynamics of phytonutrients in nightshade grown under Kenyan field conditions, harvested using different methods and at different developmental stages, 30, 60, 90 and 120 d after planting (dap) and storing them at 5 °C and room temperature (RT). Harvesting by uprooting and cutting offered higher yields, but had the lowest nutrients retained after 4 d storage at RT, while picking single leaves (continuous method) had lower yields but higher nutrients retained. The total carbohydrates (glucose, fructose, sucrose and starch) and chlorophyll were highest in concentration at 60 dap (136.3 ± 2.9 g kg^{-1} and 5.8 ± 0.3 g kg^{-1} dry weight (DW) respectively). Gallic acid equivalent phenolics (GAE), catechin equivalent flavonoids (CAE), trolox equivalent antioxidants (TEA) and total carotenoids were highest in concentrations at 90 dap with 12.6 ± 1.0 g kg^{-1}, 24.1 ± 1.6 g kg^{-1}, 108.8 ± 6.0 g kg^{-1} and 1.01 ± 0.04 g kg^{-1} DW respectively. The mineral elements mainly remained stable during development apart from iron which was highest at 30 dap (1.3 ± 0.5 g kg^{-1}), zinc was highest at 120 dap (62.1 ± 4.4 mg kg^{-1}) and calcium was highest at 90 dap (24.3 ± 0.7 g kg^{-1} DW). A significant decline of phytonutrients was evidenced after 2 d storage at RT. At cold storage (5 °C), there was no significant decline on most of the phytonutrients, at least up to up to 4 d. Results in this study highlight the importance of maturity stages and low storage temperature for optimal nutrients and postharvest quality management of African nightshades.

Keywords: African nightshade; postharvest; physiological maturity; nutrients; storage

1.0 INTRODUCTION

Food insecurity, poor livelihood, and malnutrition crisis have remained a major challenge for many centuries in sub-Saharan Africa (SSA) (FAO, IFAD, UNICEF and WHO, 2017). African indigenous leafy vegetables (ALVs) are key to the affordable and sustainable solution to the

hunger and malnutrition menace in SSA (Abukutsa-Onyango *et al.,* 2010; Kamga *et al*., 2013; Grivetti and Ogle, 2017). The ALVs contain higher nutritional benefits than the exotic ones, and can withstand both biotic and abiotic stresses (Abukutsa-Onyango, 2007). Daily consumption of phytonutrient-rich green vegetables reduces incidences of chronic diseases, cardiovascular diseases, cancer and boosts immunity in humans (Smith and Eyzaguirre, 2007). Some of the major indigenous vegetables that are consumed in the African continent includes;- African nightshades (*Solanum spp*), leafy amaranths (*Amaranthus spp*), spider plant (*Cleome gynandra*), African eggplant (*Solanum ethiopicum*), cowpea (*Vigna unguiculata*), Jute mallow (*Corchorus olitorius*), slender leaf (*Crotalaria brevidens*), pumpkin leaves (*Curcurbita muschata*) and African kale (*Brassica carinata*) (Abukutsa-Onyango, 2007). The African nightshade (*Solanum scabrum*) has been used for many centuries and it's widely distributed and used because of its nutritive and pharmacological benefits. The phytonutrient contents of nightshade include vitamins, antioxidants, flavonoids, beta-carotene, folic acid, protein (39.7 %) and mineral nutrients (Traoré *et al.,* 2017; Kamga *et al.,* 2013). The crop is multipurpose, and although the fruits are not edible due to alkaloid content, they have been shown to have high levels of anthocyanins and are used as a natural source of ink or dyes (Kamga *et al.,* 2013; Lehmann *et al.,* 2007).

The edible parts of African nightshade include leaves and young shoots, which are fried or boiled to improve the organoleptic properties and remove antinutritive compounds. The vegetable mainly accompanies major cereals or starch diets in Africa, e.g. Ugali in Kenya (Abukutsa-Onyango, 2007). Furthermore, simple processing methods like sun drying and fermentation are used by small-scale farmers for food safety and longer utilization of the vegetable (Mensah, 1997). Despite the importance of ALVs in SSA, there still exists wide research gaps and their full potential is not yet exploited. Small-scale farmers to date are facing quantitative and qualitative losses in the field and during post-harvest handling and storage. Physiological maturity and production of the crop under stressful conditions like poor nutrients (soil fertility) and water stress affect the post-harvest life and nutrient levels of leafy vegetables (Modi, 2007; Acedo, 2010). Maturity indicators for most indigenous leafy vegetables to date remain unclear, hence small-scale farmers rely on relatively imprecise indicators such as plant height, leaf size and color to determine the start of harvesting of AVLs.

Storage conditions equally affect the phytonutrient contents of leafy vegetables, but the extent of the changes vary with crop species, leaf age and accumulation of various endogenous contents before harvesting (Bergquist *et al.,* 2006; Hasperué *et al.,* 2011; Park, 2016). The

phytonutrients composition are also influenced by postharvest handling, storage duration and temperature, hence affecting the quality (Acedo, 2010; Spinardi *et al.*, 2016). Despite the high nutritional and pharmacological benefits of ALVs, the dynamics of the phytochemical and phytonutrient contents during development and after harvesting remains unknown. This study aimed at analyzing the effect of harvesting methods, development stages and post-harvest storage conditions on phytonutrients of African nightshade, for optimal nutritional benefits and quality assurance in postharvest management.

2.0 MATERIALS AND METHODS

2.1 Experimental set-up

Seeds of the Tanzanian commonly grown African nightshade (*Solanum scabrum)*, the variety 'Olevolosi' were provided by the World Vegetable Center, Arusha, Tanzania. Field experiments were conducted at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja, Kenya (S01˚05.9' E037 ˚00.8 and 1476 m above the sea level). Three field experiments were done in the periods February to May 2015, November 2015 to February 2016 and August to November 2016. Di-ammonium phosphate ($(NH_4)_2HPO_4$) fertilizer was applied at a rate of 2 g per planting hole (18 g/m^2) and was incorporated into the soil by mixing. Direct sowing of nightshade seeds was done at a spacing of 20 x 30 cm between plants and between rows, in 6 plots measuring 10 x 15 m wide. Drip-irrigation was practiced to keep the soil moist.

The growth characteristics of nightshade were recorded in the two field experiments conducted in February to May 2015 and August to November 2016 at JKUAT, Kenya. The influence of the harvesting methods on yield and nutrient contents of nightshade in terms of dry matter was investigated. Three harvesting methods; Uprooting, cutting/pinching and continuous picking of single leaves at weekly intervals was done and the samples were dried at 60 ℃ for 5 days before weighing. However, roots were cut out from the vegetables harvested by uprooting method, and the entire shoot was weighed. To investigate the influence of the harvesting methods on phytonutrients in nightshade leaves, samples harvested by the three methods were stored for 4 days at RT before freeze-drying and grinded them into fine powder, and were used for nutrient analysis. To determine the position of leaf samples for nutrient contents analysis, top-most young leaves, middle position leaves and old leaves from the bottom position of nightshade plants were harvested at 60 dap in the season February to May 2015. This led to a decision to harvest at the middle position of plants in the subsequent experiments.

To analyze the nutrients at different development stages and storage conditions, 2-3 leaves were harvested the from the middle part (4 to 6 nodes from top) of the plants at 30, 60, 90 and 120 d after planting (dap) (Fig.1). Harvesting was done after 4 h of sunlight, in 6 biological replicates for each experiment, and each replicate consisted 2-3 of leaves picked from 2 different plants at random and pooled together. The control samples were immediately frozen at -20 ˚C after harvesting and represented day zero in storage (0 d). The other samples were stored in zip-lock bags in darkness at 5 ℃ in the fridge with 85-90 % relative humidity (RH) and at 20-22 ˚C (room temperature (RT) at 60-65 % RH, for 2, 4, 6 and 8 d. The samples were then frozen at -20 ˚C before freeze-drying. The freeze-dried samples were ground into powder and weighed into 2 ml Eppendorf tubes for phytonutrient analysis. The data is presented as g kg^{-1} or mg kg^{-1} dry weight (DW) of the leaf material. The data from the field experiment at JKUAT, Juja, Kenya in the period August to November 2016 is presented exemplary as the main data during growth and storage of nightshade, while other relevant replicates from the previous years are mainly in the supplementary document.

2.2 Carbohydrates analysis

2.2.1 Glucose, fructose and sucrose (GFS)

Enzymatic and photometric methods were used for analysis of glucose, fructose, sucrose (GFS) and starch, as described by Gomez *et al.,* (2007) and Zhao *et al., (*2010). The finely ground freeze-dried leaf samples (30 mg) were weighed into 2 ml Eppendorf tubes and used for carbohydrates analysis. Ethanol 80% (v/v) (1.5 ml) was used for extractions followed by incubation in a water bath at 80 ˚C for 15 min. The extraction step was repeated 3 times resulting to 4.5 ml volume of extracts. The ethanol extracts were used for GFS enzymatic and photometric analysis, while the pellet was further processed for starch extraction and analysis. A 96 well microplate was loaded with samples in triplicates, and analysis of GFS was done sequentially starting with glucose, followed by fructose and finally sucrose. The enzymes; hexokinase/glucose 6-phosphate dehydrogenase (HK/G6P-DH) (Roche, 10737275001), phosphoglucose isomerase (PGI) (Ref 10128139001, Roche) (0.25 U) and invertase from baker´s yeast (960 U, Lot 21K7435, Sigma-Aldrich) were used for breakdown of glucose, fructose and sucrose respectively. Absorbance was read at 340 nm on the Versamax® MP photometric reader (Molecular Devices, Canada). The calibration curve was derived from glucose standards and was used for calculations of each of the GFS as described by Gomez *et al.,* (2007).

2.2.2 Starch

The pellet (section 2.2.1) was washed with ddH_2O, dried in an oven at 60 °C for 30 min and used for starch analysis as described by Gomez *et al*., (2007) and Zhao *et al*., (2010). Hydrolysis of starch was done by adding 500 µl of 0.5 M NaOH and incubating at 60 °C on a shaker for 1 h. After cooling the pellet, neutralization was done with 25 µl acetic acid (1 M) followed by an addition of 475 µl double distilled H_2O (ddH_2O). Mixing was done by vortexing and after spinning at 4,200 x g for 10 min, 10 µl of the supernatant was transferred to a clear 96-well microplate. Amyloglucosidase (AG) (9 U) (Ref 11202367001, Roche) was added to the wells and incubated for 30 min at 60 ˚C under continuous shaking. Hexokinase/glucose 6-phosphate dehydrogenase (HK/G6P-DH) (Roche, 10737275001), was then added (10 µl) to break down the hydrolyzed starch to glucose, and was incubated for 30 min at 30 ˚C. Absorbance was recorded at 340 nm. The calibration curve was derived from glucose standards and was used for calculations of starch as described by Gomez *et al.*, (2007).

2.3 Total phenolics

Folin-Ciocalteu (F-C) assay was used for determination of gallic acid equivalent phenolic (GAE) contents as described by Ainsworth & Gillespie (2007). Extractions were done with 2 ml of 97 % (v/v) methanol using 20-30 mg of finely ground freeze-dried material, overnight at RT in dark. The extracts (100 µl) were further treated with 10 % (v/v) Folin & Ciocalteu`s phenol reagent (F-C) (Ref F9252, Sigma- Aldrich) and mixed thoroughly by vortexing. This followed an addition of 800 µl 700 mM Na_2CO_3 and incubation for 2 h at RT. Spinning at 15,800 x g for 1 min were done and 200 µl of supernatant was pipetted to a 96-well microplate. Absorbance at 765 nm was recorded using a Tecan microplate reader (Infinite® 200). Gallic acid was used to prepare standards for the calibration curve, which was used to calculate phenolic contents. The calculated phenolic contents were expressed as gallic equivalents (GAE) in g kg^{-1} dry weight.

2.4 Flavonoids

Catechin and quercetin equivalents flavonoids were analyzed using the aluminium complexation reaction assay as described by Eghdami & Sadeghi (2010). From the methanol extracts (section 2.3), 100 µl was diluted with 300 µl of distilled water and 30 µl 5 % (w/v) $NaNO_2$ was added followed by an incubation for 5 min. Thirty microliters of 10 % (w/v) aluminum chloride ($AlCl_3$) was added followed by another incubation for 5 min. After the incubation, 200 µl 1M NaOH was added to the reaction mixture followed by 340 µl ddH_2O to reach a final volume of 1000 µl. Spinning was done at 15,800 x g and from the supernatant,

aliquots of 200 µl were transferred to a clear 96-well microplate as triplicates. Absorbance at 510 nm was measured with a Tecan microplate reader (Infinite® 200). The calibration curve was done with catechin standards made of serial dilutions of 12.5, 25, 50, 100, 250 g l^{-1}. The calculated amount of flavonoids were expressed as catechin equivalents in g kg^{-1} dry weight. Quercetin analysis was done with 100 µl of the extracts (section 2.3) diluted with 300 µl of 97 % methanol, to which 20 µl of 10 % $AlCl_3$ was added followed by 20 µl of 1M sodium acetate (NaOAc). The mixture was incubated for 30 minutes and finally, 560 µl ddH_2O was added to bring the total reaction volume to 1000 µl. After spinning, aliquots of 200 µl from the supernatant were transferred to the microplate in triplicates. Absorbance at 415 nm was measured with a Tecan microplate reader (Infinite® 200). The calibration curve was done with quercetin serial dilutions of 12.5, 25, 50, 100, 250 g l^{-1}. The calculated flavonoids were expressed as quercetin equivalents g kg^{-1}dry weight

2.5 Antioxidants

The trolox equivalent antioxidants capacity (TEAC) assay against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+ radical cation) (Ref A1888-1G, Sigma-Aldrich) was conducted as described by Miller *et al.,* (1993) and Tyrakowska *et al.,* (1999). The ability of the antioxidants in the sample to scavenge the ABTS•+ in comparison to the antioxidative capacity of an artificial water-soluble vitamin E derivative, Trolox® (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (Ref 1238813-1G, Sigma-Aldrich) were evaluated. ABTS stock solution (7 mM) was prepared and left in dark overnight at RT to form an intense blue colored complex. The ABTS working solution was then prepared by diluting the stock with phosphate buffer (41 mM Na_2HPO_4 and 9 mM NaH_2PO_4, pH 7.2-7.4) to a final concentration of 140 µM and until the extinction at 734 nm was between 0.7 and 0.8. The methanol extracts (section 2.3) were diluted 1:20 with phosphate buffer and aliquots of 10 µl transferred into 96 well microplates. The 140 µM ABTS solution was added (190 µl) to the samples automatically by an injector installed in the Tecan microplate reader machine. After 5 min incubation, absorbance was recorded at 734 nm. The calibration curve was derived from serial dilutions of Trolox® (0.025 to 0.4 mM) and the calculated antioxidants were expressed as trolox equivalent antioxidants (TEA) g kg^{-1} dry weight**.**

2.6 Chlorophyll and carotenoids

UV-VIS spectroscopy was conducted for total chlorophyll and carotenoid content in nightshade leaf extracts, as described by Lichtenthaler, (1987). The freeze-dried leave samples (20-30 mg) were extracted with 1.5 ml (repeated 3 times, giving a final volume of extracts to 4.5 ml) of 90

% ethanol (v/v). Samples were then vortexed and spinned at 15,800 x g for 1 min and 200 µl of the supernatant was transferred into 96 well plate in triplicates and covered with aluminium foil. The absorbance readings were done at 470 nm, 648 nm and 664 nm using the Tecan microplate reader (infinite® M200). Calculations for Total chlorophyll and carotenoids were done according to the formulas described by Lichtenthaler, (1987).

2.7 Mineral elements

Finely ground leave samples (250 mg) were weighed into 100 ml test tubes each with 3 technical replicates. Digestion was done with lithium sulfate buffer (0.48 g of selenium powder, 14 g of Lithium sulfate monohydrate 99.99 Suprapur® (CAS 10102-25-7, Sigma-Aldrich), 420 ml sulfuric acid 95-97 %, and 330 ml hydrogen peroxide 30 % stabilized). The buffer 10 µl was then added to the samples and heated in the Turbotherm apparatus Gerhardt (Gerhardt Analytical Systems, Königswinter, Germany). Samples were left to cool, filled with 50 ml of deionized water and mixed on a test tube shaker before filtering through a pleated filter (Munktell 3/N,) in PE bottles. The measurement of mineral elements (N, Ca^{2+}, K^{+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, P and Cu^{2+}) was carried out by inductively coupled plasma optical emission spectrometry (ICP-OES), and the flow injection analysis (FIA) method was used for nitrate quantification (Jones, 2001; Brabcová *et al*., 2003).

2.8 Statistical analysis

All the acquired data were subjected to normality test before analysis of variance was conducted using the SPSS (version 20) statistical software. The means were compared using Tukey test at $p<0.05$. The data were presented as g kg^{-1} or mg kg^{-1} dry weight with means ± standard errors.

3.0 RESULTS

3.1 Nightshade development characteristics

Nightshade was growing vegetatively at 30 to 60 dap (Fig. 1). The onset of flowering was 75 dap, and by 90 dap almost 95 % of the plants had flowers (Fig. 1). Taking the values from August to November 2016, the plants had 12.1 ± 1.9 and 25.1 ± 6.2 cm in height, with 6.8 ± 1 and 18.9 ± 3.8 leaves per plant 30 to 60 dap, respectively (supplementary Table. A1). Plants were 56.3 ± 9.6 cm in height with 45.4 ± 8.8 leaves at 90 dap. Subsequently, at 120 dap, the plants had formed fruits and were 100.5 ± 11.7 cm in height with 87.6 ± 12.7 leaves (supplementary Table. A1). The growth characteristics were in the same range with the observations made in February to May 2015 (supplementary Table. A1). Results indicated

higher carbohydrate contents on the young and middle leaves than in the older leaves (91.9 ± 5.8, 121.4 ± 7.6 and 56.2 ± 7.5 respectively (Supplementary Fig. A1). Therefore, leaves samples for all nutrient analysis in the following experiments were picked from the middle part of the plants, approximately 4 to 6 nodes from the tips of the apical shoots.

Fig. 1: Images of nightshade plants at different days after planting; a=30 d, b= 60 d, c= 90 d and d= 120 dap

3.2 Harvesting methods influence on yield and post-harvest nutrient contents

Plants harvested with the three methods and stored in dark at RT for 4 d indicated significant differences in the amount of carbohydrates, total phenolics, and flavonoids retained in the leaves (Fig. 2). Harvesting by continuous picking of leaves at weekly intervals (continuous method) gave the lowest yield of 166.4 g/m^2 dry mass (Supplementary Fig. A2). On the other hand, harvesting by cutting of young shoots resulted in high yields (274.7 g/m^2) because the variety grew vigorously forming many healthy shoots after harvesting. The shoots were ready for harvesting every 2 weeks. Uprooting plants 4-5 weeks after planting and re-planting also gave higher yields in a period of 4 months compared to continuous picking of leaves (306.6 g/m^2) (Supplementary Fig. A2). Furthermore, the harvesting methods also influenced the nutrient contents in nightshade leaves. Plants harvested with the three methods and stored in dark at RT for 4 d indicated significant differences in the amount of carbohydrates, total phenolics, and flavonoids retained in the leaves (Fig. 2). The total carbohydrates, phenolics, and flavonoid contents remained higher in leaves harvested by picking of single leaves (continuous method) than with uprooting and cutting method (Fig. 2). Sucrose levels indicated the greatest differences with 3.2 ± 0.3, 18.4 ± 3.2 and 11.5 ± 2.7 g kg^{-1} DW for cutting, continuous and uprooting harvesting methods respectively.

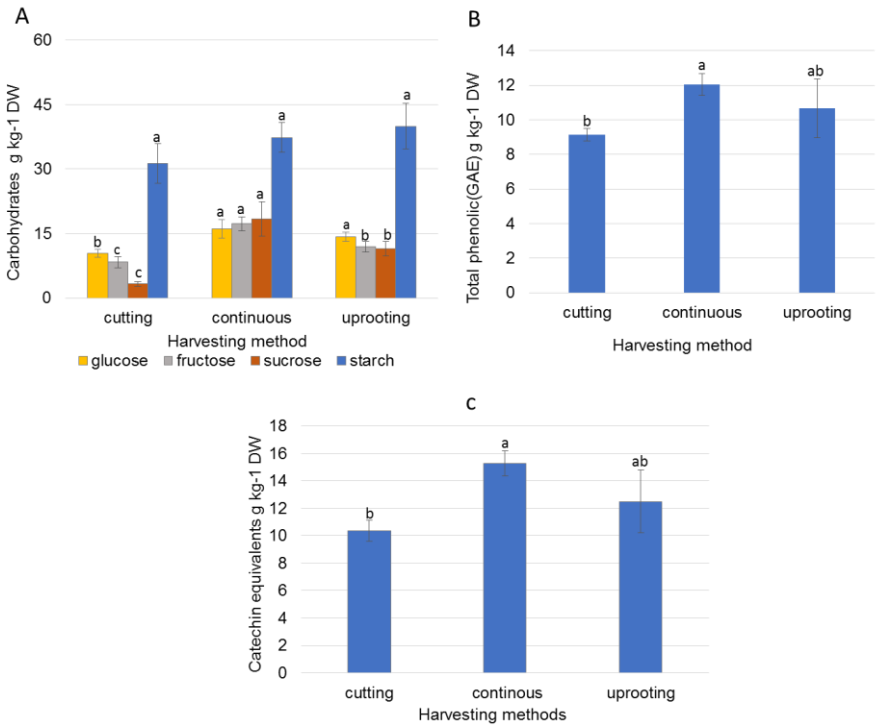

Fig. 2: Phytonutrients in nightshade as influenced by harvesting method; A = carbohydrates (glucose, fructose, sucrose and starch), B = gallic acid equivalent (GAE) phenolics, C = flavonoid's (catechin equivalents) in nightshade harvested by the three different methods. The values represent means ± standard error. Means labeled with the same letters within each content were not significantly different at $p < 0.05$ using Tukey´s test (n=6).

3.2 Development stages and post-harvest storage influence on phytonutrients

3.2.1 Carbohydrates

Carbohydrates contents displayed significant changes with plant age. The overall sugars combined gave the highest values at 60 dap (136 ± 3.0 g kg^{-1}) than at 30, 90 and 120 dap (Fig. 3). Glucose levels had an inconsistent pattern with 5.2 ± 0.5 g kg^{-1}, 31.1 ± 0.8 g kg^{-1}, and 4.9 ± 0.2 g $kg^{-1}$and 10.1 ± 0.4 g kg^{-1} at 30, 60, 90 and 120 dap respectively (Fig. 3). Similarly, fructose indicated the same pattern like the glucose with the highest concentration at 60 dap (Fig. 3). Although the total carbohydrates remained higher at 60 dap in the three field experiments, there was an inconsistent pattern of glucose, fructose and sucrose (Supplementary Table. A2). Sucrose was higher at 60 dap in two field experiments (36.2 ± 0.8 g kg^{-1}) (Fig 3) and (43.0 ± 3.2 g kg^{-1}) (Supplementary Table. A2; A) and at 90 dap in one field experiment (51.7 ± 5.8 g kg^{-1} DW) (Supplementary Table. A2; B). Starch prevailed in high quantities and did not indicate changes at 30, 60 and 90 dap but had a sharp significant decline at 120 dap (43.5 ± 1.5, 48.4 ± 2.9, 40.1 ± 8.9, and 19.5 ± 0.5 g kg^{-1} DW respectively) (Fig. 3). Low starch quantities were also evidenced at 120 dap (4.7 ± 0.5 g kg^{-1}) in the field experiment November 2015 to February 2016 (Supplementary Table. A2).

The quantity of the sugars at 60 d after planting were significantly influenced by days in storage and the storage temperature ($F_{4, 20}$=16.1, 21.9, 5.1, 13.7, 13.4), with p values < 0.005 for glucose, fructose, sucrose, starch and total sugars respectively (Fig. 4). Storage of nightshade leaves at cold temperature (5 °C) and RT resulted in significant changes in carbohydrates, with a sharper decline observed for starch, glucose and fructose (Fig. 4). Concentrations of all the four sugars highly declined after storing at RT compared to cold temperatures. Glucose showed instability at cold storage, with a decline observed at 4 d followed by a subsequent increase, while starch declined from 6 to 8 d (Fig. 4). At RT glucose and starch declined significantly within 2 d in storage. Sucrose concentrations indicated a decline in 8 d storage at 5 °C, while at RT the decline was seen by 6 d in storage. Fructose concentration had significantly reduced 4 to 8 d in storage in both temperatures (Fig. 4). The same dynamics of sugars were noticed in the repeat of storage experiment with leaves harvested at 30, 90 and 120 dap (Supplementary Table A3, A4 and A5). Three way Anova indicated an interaction between plant age (d after planting), storage duration (days) and the storage temperature (Supplementary Tables. A3, A4 and A5). This was with values of $F_{12, 80}$= of 8.5, 6.3, 4.4, 4.3 and 7.8 for glucose, fructose, sucrose, starch and their total, all with a p value < 0.001. In all the sugars, there was a significance difference as a result of age (d after planting) and storage duration and storage temperature. Considering the total carbohydrates at 2 d in storage at (RT), 30 d after planting indicated the highest percentage decline (68.7 ± 12.5) compared to 16.7 ± 4.3, 55.4 ± 6.8 and 43.9 ± 1.6 at 60, 90 and 120 d after planting respectively (Fig.4, and Supplementary Tables. A3, A4 and A5).

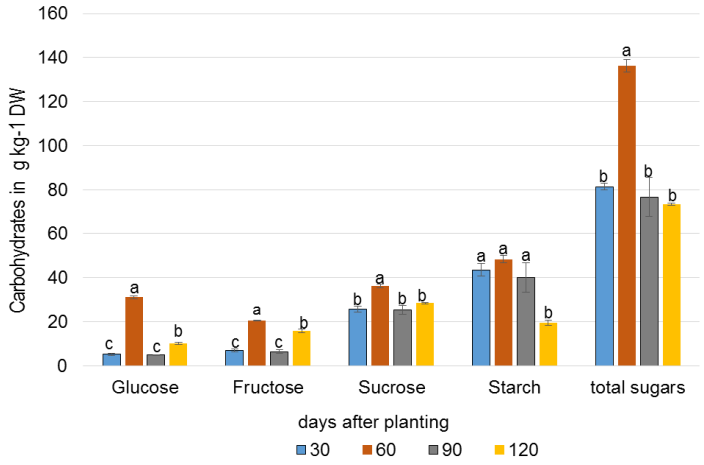

Fig. 3: Glucose, fructose, sucrose and starch concentrations and their totals in nightshade leaves harvested 30, 60, 90 and 120 dap. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at p<0.05 using Tukey´s test (n=6).

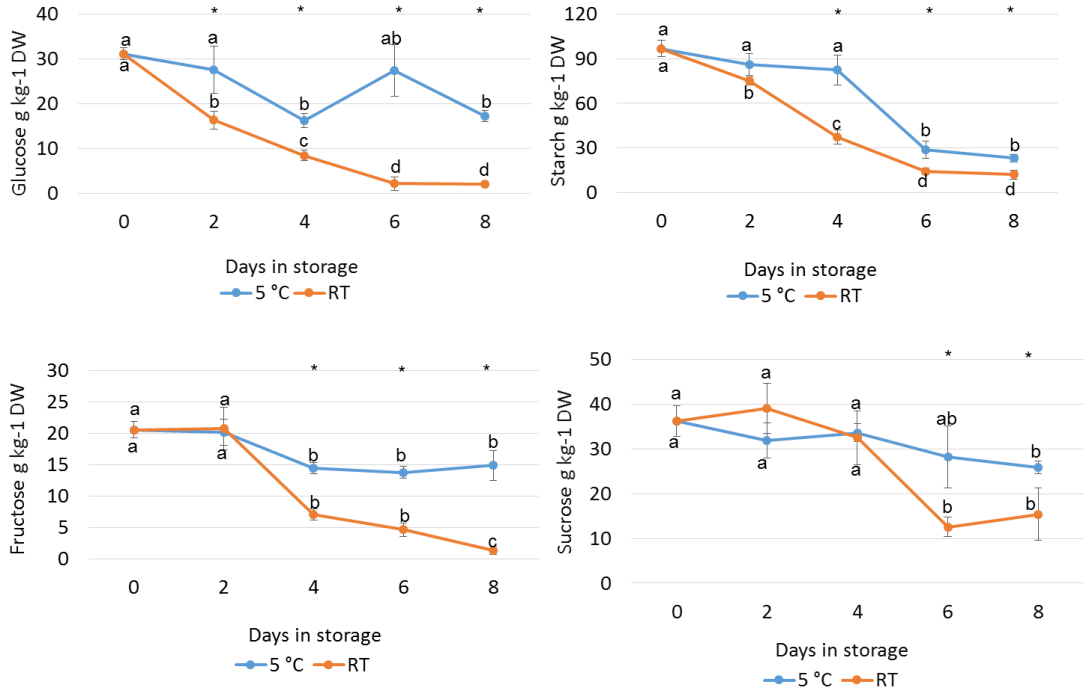

Fig. 4: Glucose, fructose, sucrose and starch and their totals in nightshade leaves harvested at 60 dap and stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage were not significantly different, while * indicates a significantly different between the two storage temperatures, at $p<0.05$ using Tukey´s test (n=6).

3.2.2 Total phenolic content

Nightshade leaves had significant levels of phenolics which indicated an increase with plant age (8.3 ± 0.3, 10.15 ± 0.2, 12.6 ± 1.0 and 11.8 ±1.5 g kg^{-1} in leaves harvested at 30, 60, 90 and 120 dap respectively (Fig. 5). During storage there was a significant interaction between plant age (d after planting), storage duration and storage temperature on the phenolic contents ($F_{12, 80}=8.6$, $p<0.001$. Storage of nightshade leaves after harvesting indicated stability of phenolic contents up to 4 d at 5 °C (Fig. 6). A significant degradation occurred by 6 and 8 d at 5 °C with percentage losses of 23-41 % for leaves harvested at 30 to 120 dap (Fig. 6). At RT, a significant decline was seen after 2 d in storage, with 62 to 91 % decline of total phenolics by 8 d in storage (Fig. 6). Plant development stages had no influence on post-harvest losses, with results indicating a decline of phenolics at all development stages (Fig. 6).

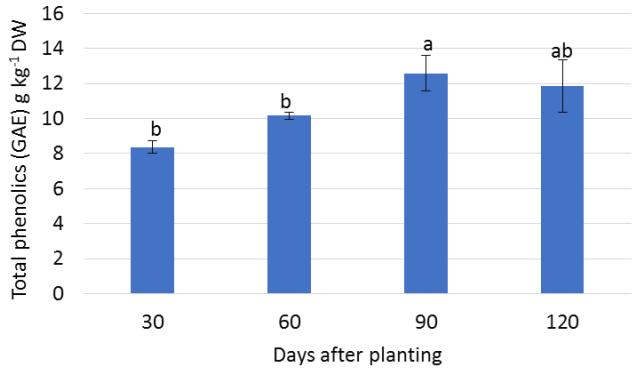

Fig. 5: Total phenolic (gallic acid equivalents (GAE) contents in nightshade leaves harvested 30, 60, 90 and 120 dap. The values represent means ± standard error. Means labeled with the same letters were not significantly different at p<0.05 using Tukey´s test (n=6).

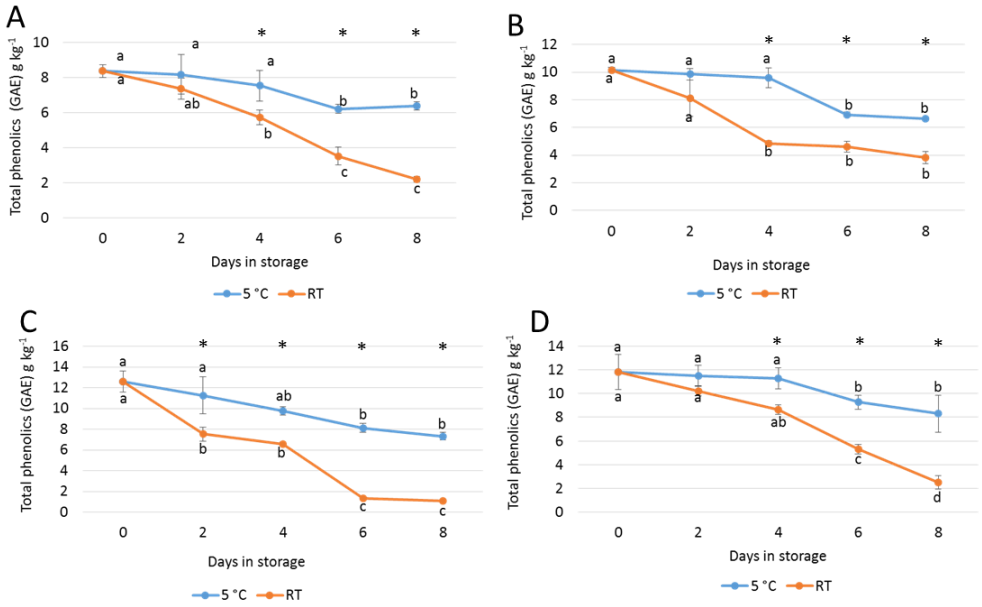

Fig. 6: Total phenolic (gallic acid equivalents (GAE)) contents in nightshades leaves harvested at; A) 30 dap, B) 60 dap, C) 90 dap and D) 120 dap, stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage were not significantly different, while * shows a significant difference between the two storage temperatures, at p<0.05 using Tukey´s test (n=6).

3.2.3 Flavonoid's

Nightshade leaves contained significant amounts of flavonoids (catechin and quercetin equivalents). Catechin equivalents in nightshade leaves were found to be higher in concentration than the quercetin equivalents (Fig. 7). Catechin increased with plant age and was highest at 90 dap (24.1 ± 1.6 g kg^{-1}) and lowest at 30 dap (15.5 ± 2.1 g kg^{-1} (Fig. 7). A sharp decline of catechin was seen at 120 dap. On the other hand, quercetin indicated no significant changes with plant age in nightshade and the amount ranged from 10.2 ± 0.4 g kg^{-1} at 30 dap to 11.5 ± 0.9 g kg^{-1} by 120 dap (Fig. 7). During the storage experiment, flavonoids

were significantly influenced by crop age (d after planting), storage duration (days) and storage temperature with $F_{12, 80}$=5.3, p<0.001 for catechin and $F_{12, 80}$=6.4, p<0.001 for quercetin (Fig. 8 and Fig. 9). Storage of nightshade at cold temperature (5 °C) indicated a higher stability of both flavonoids while a significant decline was observed during storage at RT with steepest losses occurring by 6 and 8 d in storage (Fig. 8 and Fig. 9). However, steeper declines occurred in catechin (Fig. 8), while quercetin appeared more stable during storage at RT (Fig. 9).

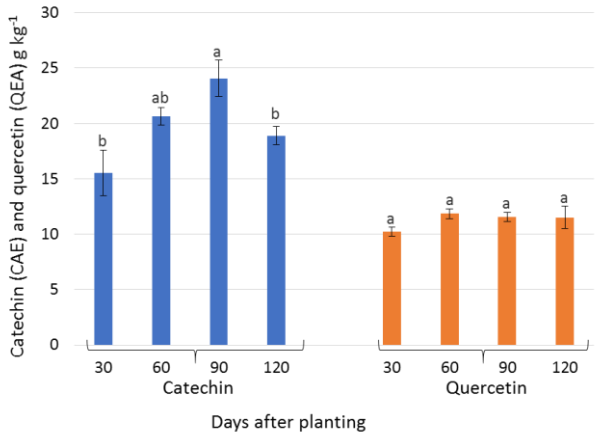

Fig. 7: Flavonoids (quercetin and catechin equivalents) content in nightshade leaves harvested at 30, 60, 90 and 120 dap. The values represent means ± standard error. Means labeled with the same letters within each flavonoid group were not significantly different at p<0.05 using Tukey´s test (n=6).

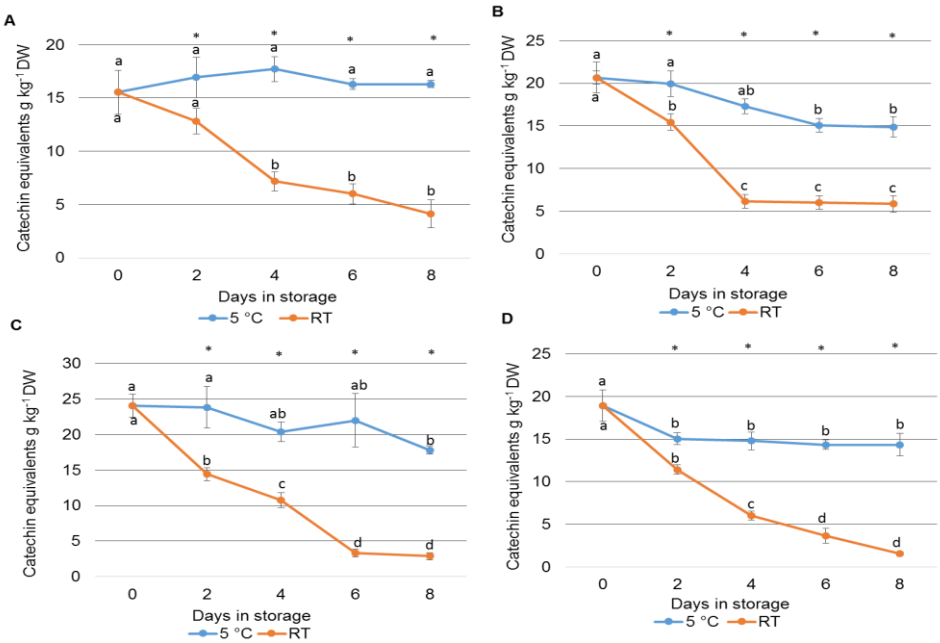

Fig. 8: Flavonoids (catechin equivalents) in nightshades leaves harvested at; A) 30 dap, B) 60 dap, C) 90 dap and D) 120 dap, and stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage each temperature were not significantly different, while * indicates a significant difference between the two temperatures at p<0.05 using Tukey´s test (n=6).

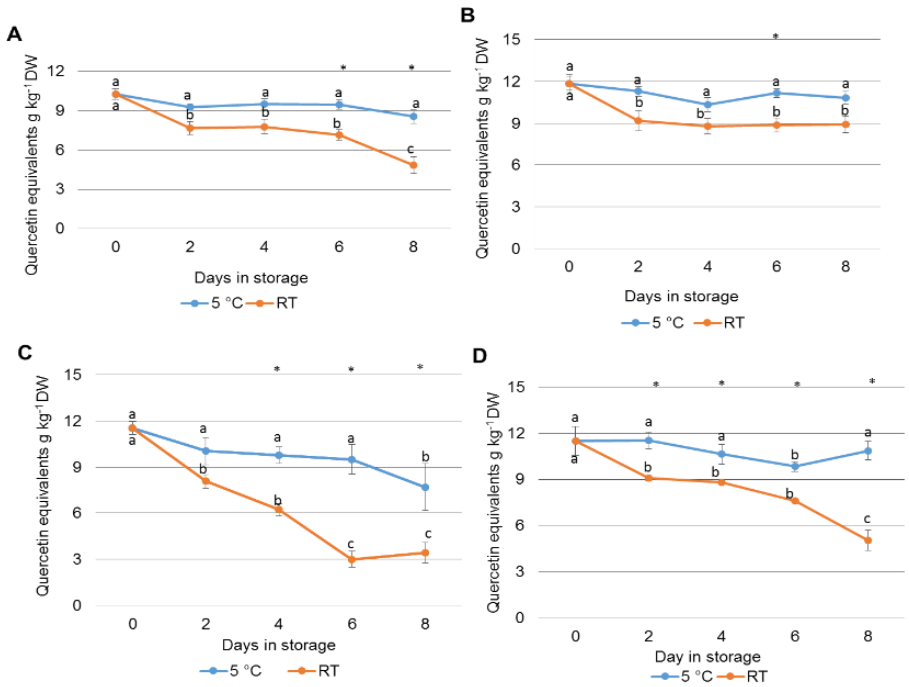

Fig. 9: Flavonoids (quercetin equivalents) in nightshades leaves harvested at; A) 30 dap, B) 60 dap, C) 90 dap and D) 120 dap, and stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage each temperature were not significantly different, while * indicates a significant difference between the two temperatures at $p<0.05$ using Tukey´s test (n=6).

3.2.4 Antioxidants

Results indicated high levels of antioxidants measured as trolox equivalents (TEA) in nightshade leaves with 27.2 ± 1.1, 42.9 ± 1.5, 80.8 ± 5.9, 36.4 ± 4.4 g kg^{-1} DW at 30, 60, 90 and 120 dap respectively. The total antioxidants indicated a sharp increase at 90 dap followed by a decline by 120 dap (Fig.10). There was a significant interaction between plant age (d after planting), days in storage and storage temperature $F_{12, 80}=9.4$, $p<0.001$ on antioxidants during storage of nightshade leaves. Antioxidants showed strong stability during storage at cold temperatures while RT conditions led to a significant decline by 4 d. The percentage loss after 8 d in storage were 72, 57, 73, and 74 % for 30, 60, 90 and 120 dap. (Fig. 11).

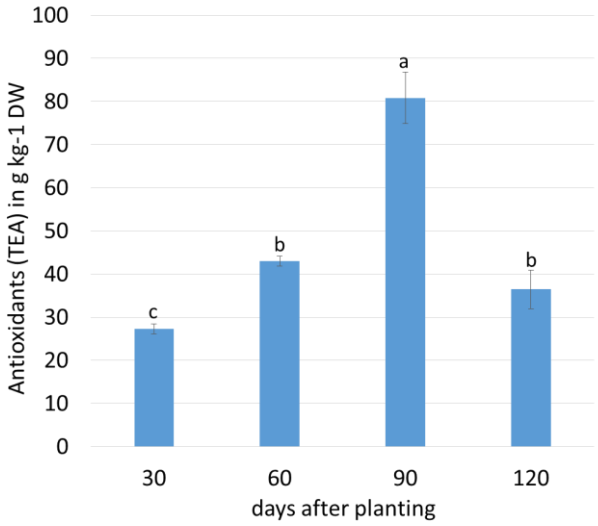

Fig. 10: Antioxidants (trolox equivalents) (TEA) content in nightshades leaves harvested 30, 60, 90 and 120 dap. The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=6).

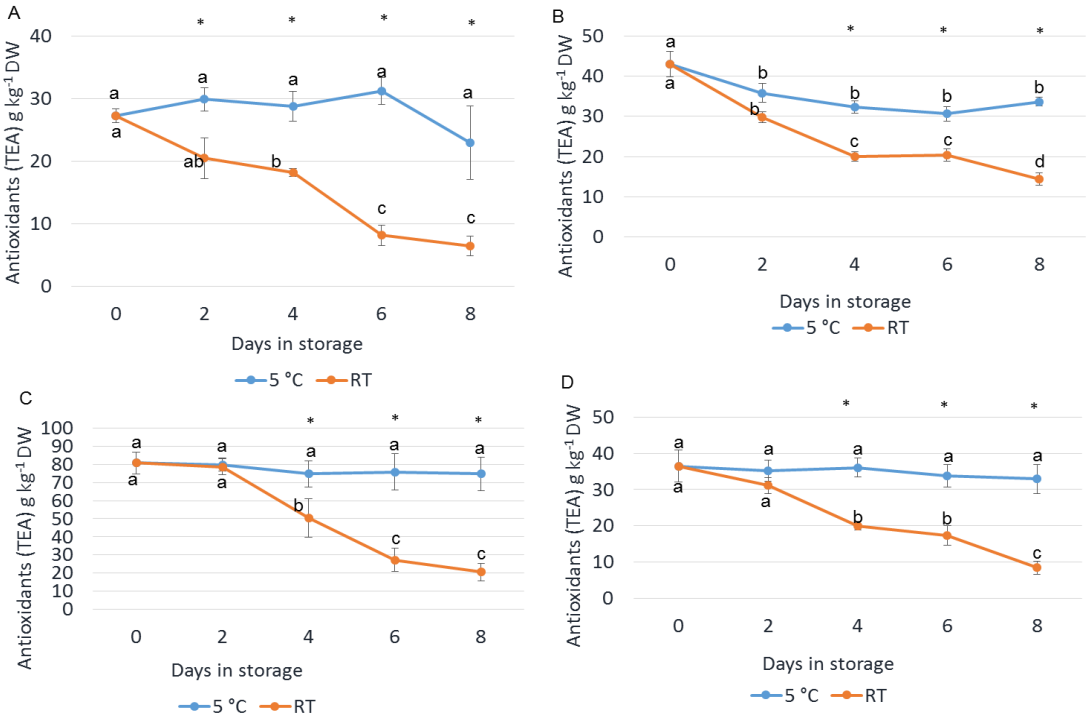

Fig. 11: Antioxidants (Trolox equivalents) (TEA) content in nightshades leaves harvested at; A) 30 dap, B) 60 dap, C) 90 dap, and D) 120 dap, stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage each temperature were not significantly different, while * indicates a significant difference between the two temperatures at $p<0.05$ using Tukey´s test (n=6).

3.2.5 Chlorophyll and carotenoids

Leaves contained highest chlorophyll concentrations 60-90 dap in nightshade grown under field conditions in Kenya (Fig.12). High chlorophyll contents were observed at 60 dap in the two other repetitions of the field experiments in Kenya (Supplementary Table. A2). The total

carotenoids were highest at 90 dap (Fig.12). This was also observed in two other repeated field experiments (Supplementary Table. A2). The chlorophyll contents during storage was significantly influenced by plant age (d after planting), storage duration and storage temperature ($F_{12, 80}$=3.2, p=0.003) (Fig. 13). However, there was no interaction between d after planting and storage duration ($F_{12, 80}$=1.5, p=0.151) during storage of nightshade (Fig. 13). At 5 °C storage, chlorophyll remained significantly stable, while at RT more than 50 % degradation of chlorophyll occurred by 6 to 8 d in storage (Fig. 13). The carotenoid on the other hand, were not significantly influenced by plant age (d after planting), storage duration and storage temperature ($F_{12, 80}$=1.3, p=0.236). However, 2 way Anova showed that plant age and storage duration had a significant interaction ($F_{12, 80}$=2.0, p=0.04), hence had an influence on carotenoids. Carotenoids showed high stability during storage at RT for leaves harvested at 30 and 60 dap, apart from at 90 dap where a significant decline was observed after 4 d in storage. A slight decline of carotenoids was also noticed at 8 d storage at RT for samples harvested from 120 dap.

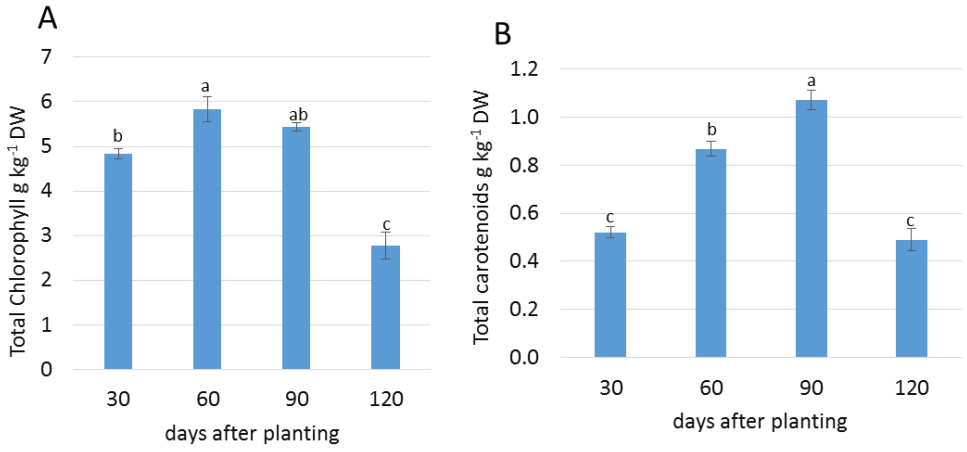

Fig. 12: A= total chlorophyll and B=total carotenoid contents in nightshades leaves harvested 30, 60, 90 and 120 dap. The values represent means ± standard error. Means labeled with the same letters were not significantly different at p< 0.05 using Tukey´s test (n=6).

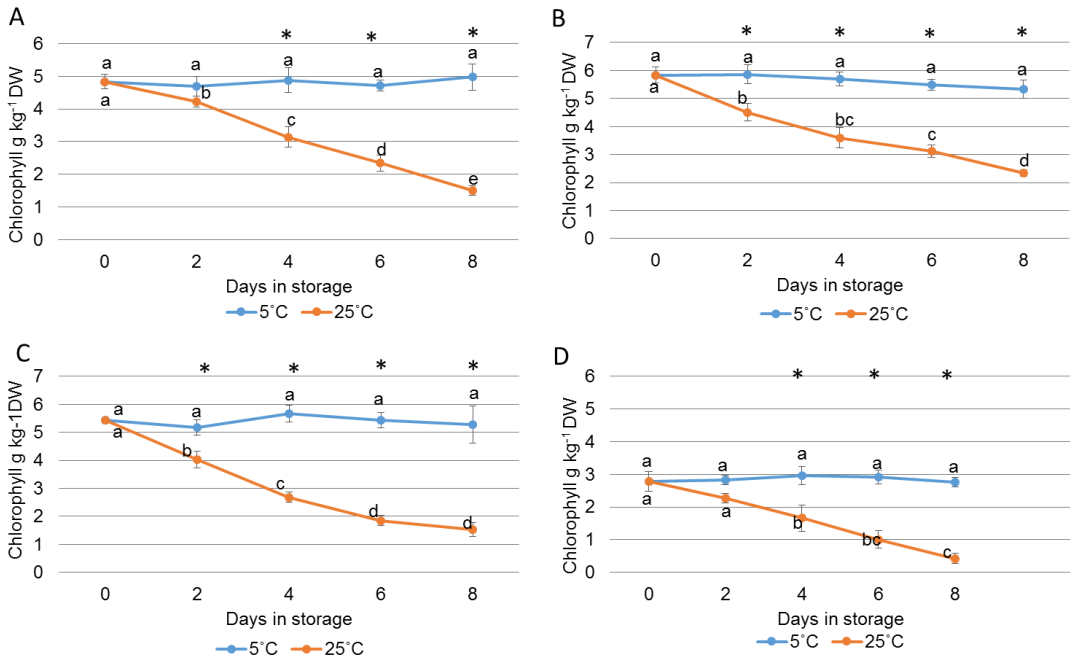

Fig. 13: Total chlorophyll content in nightshade leaves harvested at 30, 60, 90 and 120 dap and stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage each temperature were not significantly different, while * indicates a significant difference between the two temperatures at $p<0.05$ using Tukey´s test (n=6).

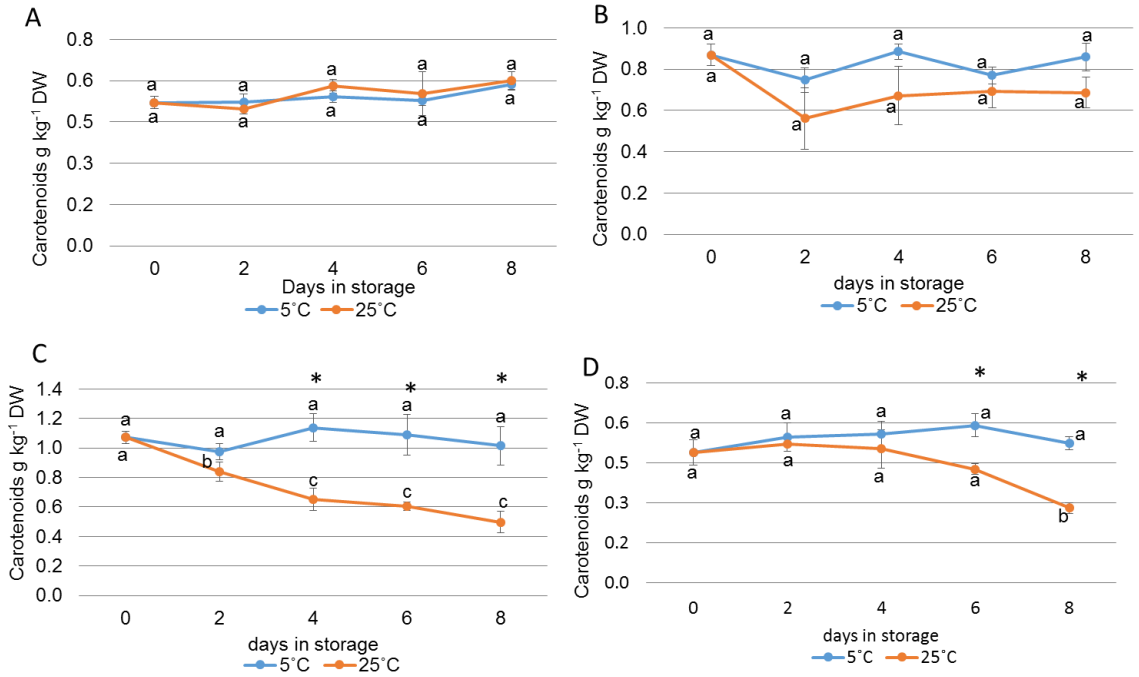

Fig. 14: Carotenoid content in nightshade leaves harvested at 30, 60, 90 and 120 dap and stored at 5 ˚C and RT for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within days in storage each temperature were not significantly different, while * indicates a significant difference between the two temperatures at $p<0.05$ using Tukey´s test (n=6).

3.2.6 Mineral contents

Results detected high mineral nutrient content in nightshade leaves. Potassium, nitrogen and calcium were highest compared to other minerals (Table. 1). The highest values were 45.8 ± 2.7, 24.3 ± 0.7, and 58.6 ± 14.4 for potassium, calcium and nitrogen respectively (Table. 1). The vegetable also has high amounts of iron, phosphorous. Magnesium, zinc and manganese (Table. 1). The mineral contents showed no significant changes during development apart from calcium, iron and zinc (Table. 1). The mineral elements mainly remained stable during development apart from iron which was higher at 30 dap (1.3 ± 0.5 g kg^{-1}), zinc was highest at 120 dap (62.1 ± 4.4 mg kg^{-1}) and calcium was highest at 90 dap (24.3 ± 0.7 g kg^{-1}) (Table. 1).

Table. 1: Mineral content in nightshade leaves harvested at 30, 60, 90 and 120 dap. The values represent means ± standard error dry weight (DW). Means labeled with the same letters within a column were not significantly different at $p<0.05$ using Tukey´s test (n=6)

dap	N g kg^{-1}	P g kg^{-1}	K^{2+} g kg^{-1}	Ca^{2+} g kg^{-1}	Mg^{2+} g kg^{-1}	Fe^{2+} g kg^{-1}	Zn^{2+} mg kg^{-1}	Mn^{2+} mg kg^{-1}	Cu^{2+} mg kg^{-1}
30	43.8 ± 4.9a	2.7 ± 0.3a	41.1 ± 1.4a	14.6 ± 1.3b	3.8 ± 0.4a	1.3 ± 0.5a	39.8 ± 1.9b	257.6 ± 44.9a	42.2 ± 12.7a
60	40.2 ± 3.1a	2.8 ± 0.1a	45.3 ± 2.3a	20.2 ± 1.1a	3.3 ± 0.3a	1.0 ± 0.0a	42.2 ± 3.7b	276.3 ± 20.4a	26 ± 2.2a
90	38.8 ± 1.6a	2.3 ± 0.1a	45.8 ± 2.7a	24.3 ± 0.7a	4.1 ± 0.2a	1.0 ± 0.0a	46.7 ± 2.4b	289.3 ± 16.2a	30 ± 1.6a
120	58.6 ± 14.4a	3.2 ± 0.4a	40.5 ± 3.1a	21.6 ± 3.9a	3.7 ± 0.5a	0.7 ± 0.1b	62.1 ± 4.4a	287.5 ± 18.4a	29.7 ± 3.8a

4.0 Discussion

4.1 Carbohydrates

The study of developmental changes in phytonutrients was undertaken since the physiological status of the plant is influenced by age, especially during the transition from vegetative to reproductive phase. Moreover, in *Phaseolus*, the carbohydrates can differ between the position of an individual leaf of a plant (Araya *et al.*, 2006). Therefore, this led to the studies to identify the carbohydrates changes based on leaf positions in nightshade. On the other hand, harvesting method influenced retention of nutrients in the nightshade leaves (Fig. 2). Harvesting by continuous picking of middle leaves led to higher retention of carbohydrate, phenolic and flavonoid contents, whereas harvesting by cutting method resulted in lower contents in the leaves after 4 days storage at RT. This is possibly due to continuous translocation of nutrients to the stem at least for minutes or few hours after harvesting by cutting or uprooting the entire plants (Lemoine *et al.*, 2013). On the other hand, continuous sap flow immediately after cutting might lead to oozing out of solutes through the wound which could also reduce the assimilates in the leaves. Harvesting through cutting also may create bigger wounds which could hence

lead to various healing reactions either at the injury site or systematically, which includes translocation of various phytochemicals as a defense mechanism (León *et al.,* 2001).

Carbohydrates play important roles not only in the human diet but also in driving various biological processes in plants during development, in stress responses, and as fuel energy for metabolic processes (Rolland *et al.,* 2006). Sugars are signaling molecules and are involved in modulating expression of the various gene in plants (Koch, 1996; Araya *et al.,* 2006; Rolland *et al.,* 2006). Results in this study indicated dynamics of carbohydrates with plant age. The high levels of total sugars at 60 dap can be attributed to well-developed leaves with higher chlorophyll content and a surface area for optimal photosynthetic activities compared to younger plants. On the other hand, accumulation of carbohydrates at 60 dap could be a signal response to produce and accumulate photosynthetic assimilates ready for initiation of reproductive phase. However, total carbohydrates in the leaves at 90 and 120 dap were lower possibly due to higher translocation from source (leaves) to sink (flowers and fruits) organs because at these stages the plants were in the reproductive phase (Lemoine *et al.,* 2013). The low level at 120 dap could be due to hydrolysis of starch to glucose in response to the increased demand to the sink sites (maturing fruits), or due to reduced photosynthetic capacity on the aging plants leaves (Granot *et al*., 2014). The repetitions of the field experiments in Nov. 2015 to Feb. 2016 and in Jan. to March 2017 indicate that the carbohydrate contents in nightshade leaves are consistently higher at 60 dap compared to other development stages. The slight fluctuations noticed with glucose, fructose, sucrose and starch with different years can be due to differences in temperature, light intensity and slight variations in production conditions (Spinardi *et al.,* 2016).

Storage of nightshade leaves at 5 °C and RT revealed significant changes of the carbohydrates in both conditions. At cold storage, less degradation of sugars especially glucose, fructose and sucrose at least up to 4 d was observed, while starch had a steep decline after 6 d in storage (Fig. 4). The levels of glucose were fluctuating most likely due to the continuous breakdown of starch to maintain a glucose balance that is needed for continued metabolic processes in the leaf cells (Smith *et al*., 2005; Granot *et al.*, 2014). Storage of the leaves at RT led to a significant decline of glucose within 2 d, which can be attributed to increased respiratory rates and metabolic processes. Soluble carbohydrates (glucose) have been reported to play a great role in ascorbic acid metabolism during post-harvest storage of spinach. This involves a pathway where glucose is converted to D-glucosone, then to L-sorbosone and finally to ascorbic acid (Toledo *et al.,* 2003). The results imply that storage of nightshade leaves at higher temperatures

would lead to a quick loss of quality within two days while at cold storage carbohydrates would remain unchanged at least up to four days.

4.2 Total phenolics, Flavonoids, and antioxidants

Phenolic compounds play various roles in plants during development and contribute to antioxidative properties in the human diet as well. The total phenolics increased with plant age irrespective of the growing environment with highest contents seen at 90-120 dap (Fig. 5). Also, a previous study in *Amaranthus cruentus* reported an increase in phenolic contents with plant age (Oloyede *et al.,* 2013). However, the production of phenolics and dynamics with age and during storage is species dependent (Mathiventhan and Sivakanesan, 2013). During storage of nightshade leaves, there was a significant reduction of phenolic contents by day 4 in storage at RT. The total phenolics indicated a decrease with increased storage duration irrespective of temperature. The gradual decrease with prolonged storage at RT could be due to reaction with other chemical compounds, oxidation or exhaustion during the plant defense activities. By 6 d in storage at RT other contents like sugars indicated decline and there could be a closer link between the carbohydrates and phenolics (Arnold *et al.,* 2004).

The catechin equivalent flavonoids prevailed in nightshade leaves compared to quercetin derivatives. Although under field conditions quercetin did not indicate changes with plant age, it increased with age under greenhouse conditions (data not shown). This shows the quantities and dynamics of some specific secondary metabolites are highly influenced not only by age but also production and environmental conditions. The dynamics of flavonoids is also specific for different species of plants, and although they increased with age in nightshade, previous studies have indicated higher quantities at younger stages in *Spinacia oleracea L.* and *Celosia argentea* (Bergqust *et al.*, 2005; Oloyede *et al.,* 2013). This implies that different plant species respond differently to signals that either upregulate or downregulate production of secondary metabolites (Akula and Ravishankar, 2011). Storage conditions equally influenced changes in flavonoids after harvesting. The stability of flavonoids under cold storage conditions and the decline during storage at RT in Kenya could be due to oxidative stress. Degradation of flavonoids, phenolic compounds and antioxidants during storage has previously been observed in other leafy vegetables including spinach (Bergqust *et al.,* 2005). During production and storage, other unknown factors can influence the dynamics of flavonoids.

The trolox equivalent antioxidants were in high amount in nightshade hence signifying the pharmacological attributes and the importance of the crop for human health. The increasing

health concern and cancer incidences in the world have led to a growing demand for natural dietary sources containing free radical scavenging potential, and African nightshade offers such benefit. The sudden increase of antioxidants at 90 dap under field conditions indicate that some signaling mechanisms in the plant lead to such dynamics. Various chemical interactions in plants due to various environmental changes or storage conditions may lead to variation in amounts of antioxidants and other secondary metabolites in the same plant species or variety (Telascrea *et al.,* 2007). During storage, there was a continuous decline of antioxidants at RT which could be attributed increased oxidative stress under such condition. Plants defense system against oxidative stress consists of enzymatic and non-enzymatic mechanisms, explaining possible causes of such variations in nightshade (Ahmad *et al.,* 2010).

4.3 Chlorophyll and carotenoids

Chlorophyll plays a great role in the marketability of leafy vegetables. Some small-scale farmers use the greenness of vegetables as maturity indicators to start harvesting. In the market, the greenness and freshness are among the major physical characteristics that influence the acceptability of vegetables. Results indicated higher chlorophyll content 60 to 90 dap in nightshade leaves. This can also be correlated to the increase photosynthetic assimilates especially the sugars at this growth phase. The lower chlorophyll content at 120 dap could be due to the onset of senescence. During storage, chlorophyll contents were highly maintained at 5 °C up to 8 d, while at RT, a significant decline due to chlorophyll degradation was observed by 2 d. Physiological changes during storage at RT include ethylene biosynthesis and the activation of chlorophyll degradation enzymes such as peroxidase-hydrogen peroxide and chlorophyll oxidase among others (Yamauchi and Watada, 1991; Limantaraa *et al.,* 2015). Therefore, chlorophyll can also be used as a phytochemical marker to evaluate quality and status of green leafy vegetables during storage. The carotenoids, on the other hand, play a role as food colorants and are important in preventing diseases such as cancer, cardiovascular illnesses and in improving eye health. Results in this study showed an accumulation of carotenoids with plant age up to 90 dap and a high stability during storage, although a slight decline was seen in samples harvested at 90 dap and stored at RT. Greater stability of carotenoids, unlike chlorophyll, has been reported previously (Ferrante *et al.,* 2004).

4.4 Mineral contents

Results indicated high mineral nutrients in nightshade with very high contents of potassium, calcium, iron, magnesium, zinc, manganese and phosphorous. This proved that nightshade is a

great source of essential minerals that are required in solving the mineral deficiency menace in SSA (Okonya and Maass, 2014). The mineral contents were in the same range with previous reports on other ALVs including spider plant (*Cleome gynandra),* jute mallow (*Corchorus olitorius*), and amaranthus (*Amaranthus cruentus*) (Kamga *et al.*, 2013; Omondi *et al.*, 2017). Results showed no significant changes in mineral contents in nightshade leaves during development apart from the decrease of iron at 120 dap, while calcium and zinc increased with age. However, production conditions including soil nutrients highly influence the mineral contents in the leaves of vegetables. This indicates that the mineral composition in the nightshade leaves may not be influenced by age, but mainly by the soil nutrient conditions. Storage of nightshade leaves up to 8 d did not result in significant changes in mineral concentration, data not shown.

5.0 Conclusion and recommendations

Nightshade is a rich source of health-promoting phytonutrients and mineral elements. However, the phytonutrient contents have been shown to be highly influenced by the physiological maturity of the crop, harvesting methods and storage conditions. Harvesting by continuous picking of single leaves at the middle part of the plant at 1 to 2 weeks intervals offers higher nutrients retention during post-harvest storage. Most of the phytonutrients and phytochemical compounds including carbohydrates, chlorophyll and carotenoids, total phenolics, antioxidants and flavonoids were high 60 to 90 dap. This suggests that harvesting the vegetables 60 to 90 dap would offer higher nutritional benefits and quality of nightshade. Most phytonutrients were stable 4 to 6 d in storage at 5 °C while at RT a high portion of most nutrients was lost within 2 d in storage. Although results did not clearly indicate if age could influence postharvest losses of nutrients, harvesting the crop when the nutrients are optimal would compensate for losses during post-harvest storage. High nutrients could also be retained in the leaves if stored at cold temperatures at least up to 4 days while on the other hand RT storage would not be recommended. A cool chain would equally be recommended for commercial farmers to facilitate cold temperatures during transport and marketing of nightshade.

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2.3.1 Supplementary data (Manuscript; 2.3)

Nutritional composition in African nightshade (*Solanum scabrum*) influenced by harvesting methods, age and storage conditions

Dinah Kirigia[1, 2], Traud Winkelmann[1], Remmy Kasili [3], and Heiko Mibus[2]

1.0 Nightshade characteristics during development

Table A1: Leaf number and plant height in nightshade at 30, 60, 90 and 120 d after planting (dap) grown in two seasons under field conditions in Kenya. The values represent means ± standard error (n=10).

	Height (cm)		Leaf number	
dap	2015	2016	2015	2016
30	11.8 ± 3.1	12.1 ± 1.9	5.9 ± 1.0	6.8 ± 1.0
60	28.2 ± 6.6	25.1 ± 6.2	14.2 ± 2.4	18.9 ± 3.8
90	52.0 ± 11.2	56.3 ± 9.6	40.5 ± 4.6	45.4 ± 8.8
120	91.4 ± 7.7	100.6 ± 11.7	77.3 ± 4.8	87.6 ± 12.7

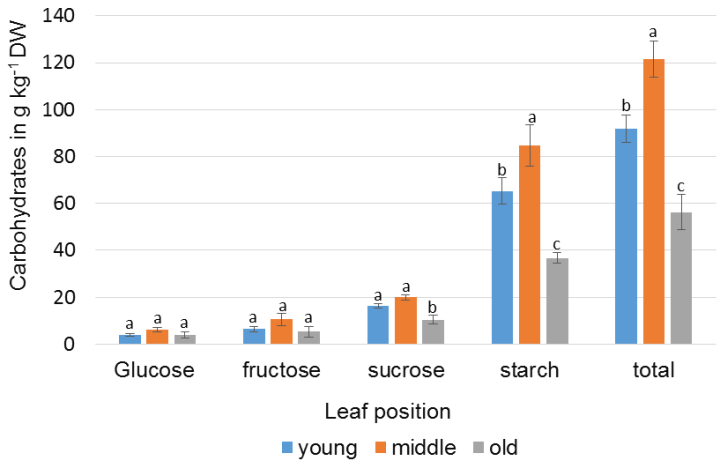

Fig. A1: Carbohydrate contents in nightshade based on leaf positions (young topmost, middle, and bottom leaves) (data Feb to May 2015). The values represent means ± standard error. Means labeled with the same letters within each sugar content were not significantly different at $p < 0.05$ using Tukey´s test (n=6).

2.0 Harvesting methods effects on yield

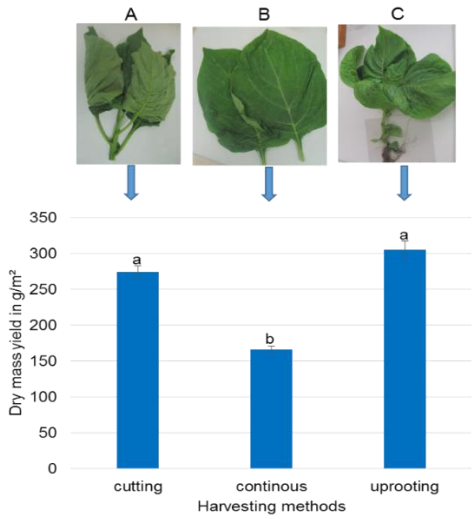

Fig. A2: Nightshade yield from three harvesting methods; A=cutting, B= continuous (single leaves), C= uprooting. The values represent means ± standard error. Means labeled with the same letters were not significantly different at p< 0.05 using Tukey´s test (n=10).

3.0 Phytonutrients changes during development

Table. A2: Carbohydrates, chlorophyll and carotenoids in nightshade leaves harvested 30, 60, 90 and 120 d after planting (dap). A=Field experiment at JKUAT (Feb to May 2015), and B= Field experiment at JKUAT (Nov 2015 to Feb 2016). The values represent means ± standard error. Means labeled with the same letters within each content were not significantly different at p<0.05 using Tukey´s test (n=6).

A

Feb to May 2015 (Field experiment; JKUAT)							
dap	glucose	fructose	sucrose	starch	Total carbohydrates	Total chlorophyll	Total carotenoids
30	11.2 ± 0.6a	6.5 ± 0.1a	16.8 ±0.7b	29.9 ± 0.4a	64.4 ± 1.2b	3.3 ± 0.3c	1.2 ± 0.6b
60	3.8 ± 0.4b	2.8 ± 0.1b	43.0 ±3.2a	29.4 ± 0.4a	77.1 ± 3.7a	5.6 ± 0.7a	1.2 ± 0.6b
90	9.6 ± 0.5a	1.9 ±0.1c	11.9 ±0.1c	27.9 ± 0.1ab	51.2 ± 0.5c	4.1 ± 0.4 b	1.8 ± 0.1a
120	3.8 ± 0.4 b	1.4 ± 0.0c	12.5 ± 0.4c	30.5 ± 0.6a	47.2 ± 1.4c	3.0 ± 0.3c	1.2 ± 0.1b

B

Nov 2015 to Feb 2016 (Field experiment; JKUAT)							
dap	glucose	fructose	sucrose	starch	Total Carbohydrates	Total Chlorophyll	Total carotenoids
30	35.5 ± 2.3b	20.3 ± 1.2ab	13.1 ± 0.6c	50.9 ± 0.7a	119.8 ± 5.3b	3.2 ± 0.0b	0.8 ± 0.0c
60	56.7 ± 11.8a	22.9 ± 3.5a	33.2 ± 2.6b	47.1 ± 1.6a	159.8 ± 15.0a	4.1 ± 0.1a	1.0 ± 0.1b
90	37.0 ± 7.3b	17.0 ± 0.8b	51.7 ± 5.8a	33.2 ± 1.0b	138.8 ± 14.8ab	3.0 ± 0.1b	1.4 ± 0.1a
120	24.6 ± 3.5b	14.7 ± 1.2bc	13.7 ± 1.3c	4.7 ± 0.5c	57.7 ± 5.7c	1.2 ± 0.2c	0.6 ± 0.3bc

4.0 Influence of storage temperature and duration on carbohydrates

Table. A3: Glucose, fructose, sucrose and starch and their totals in nightshade leaves grown in Field at JKUAT (August to Nov 2016), harvested at 30 dap and stored at 5 ˚C (**A**) and RT (**B**) for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at p<0.05 using Tukey´s test (n=6).

A

days in storage	temp (˚C)	glucose	fructose	sucrose	starch	total Sugars	decline in total sugars (%)
0	-20 ctrl	5.2± 0.5^{c}	6.9± 0.7^{c}	25.7± 1.4^{a}	43.5± 2.9^{a}	81.3± 1.5^{a}	-- -
2	5	12.1± 1.7^{b}	15.2± 2.4^{b}	29.4± 3.2^{a}	10.4± 5.9^{b}	67.2± 4.8^{b}	17.4± 5.9^{a}
4	5	16.8± 1.3^{b}	20.5± 1.0^{b}	39.0± 7.7^{a}	9.6± 5.4^{b}	85.9± 13.7^{a}	5.7± 6.8^{a}
6	5	20.3± 1.2^{a}	29.1± 1.7^{a}	29.3± 3.3^{a}	2.4± 0.2^{c}	81.1± 1.6^{a}	0.3± 2.0^{a}
8	5	17.0± 2.8^{a}	30.1± 2.8^{a}	30.7± 0.9^{a}	2.9± 0.4^{c}	80.7± 7.0^{a}	0.7± 8.6^{a}

B

days in storage	temp (˚C)	glucose	fructose	sucrose	starch	total Sugars	decline in total sugars (%)
0	-20 ctrl	5.2± 0.5^{a}	6.9± 0.7^{a}	25.7± 1.4^{a}	43.5± 2.9^{a}	81.3± 1.5^{a}	-- -
2	25	5.7± 2.3^{a}	7.1± 2.5^{a}	10.5± 4.9^{b}	2.2± 0.7^{b}	25.5± 10.2^{b}	68.7± 12.5^{a}
4	25	3.2± 0.5^{b}	2.2± 0.5^{b}	5.7± 2.1^{c}	1.2± 0.3^{c}	12.2± 2.7^{b}	85.0± 3.3^{a}
6	25	3.9± 0.5^{b}	3.3± b^{b}	2.8± 0.7^{c}	2.4± 0.1^{b}	12.3± 1.8^{b}	84.9± 2.2^{a}
8	25	1.9± 0.1^{c}	1.6± 0.1^{c}	1.4± 0.3^{d}	1.6± 0.2^{c}	6.5± 0.5^{c}	92.0± 0.6^{a}

Table. A4: Glucose, fructose, sucrose and starch and their totals in nightshade leaves grown in Field at JKUAT (August to Nov 2016), harvested at 90 dap and stored at 5 ˚C (**A**) and RT (**B**) for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at p<0.05 using Tukey´s test (n=6).

A

days in storage	temp (˚C)	glucose	fructose	sucrose	starch	total Sugars	decline in total sugars (%)
0	-20 ctrl	4.9± 0.2^{b}	6.4± 0.7^{b}	25.2± 2.0^{a}	40.1± 6.6^{a}	76.7± 8.9^{a}	-- -
2	5	18.0± 7.8^{a}	23.1± 9.5^{a}	31.3± 6.8^{a}	8.3± 4.6^{b}	80.7±18.6^{a}	-5.3± 24.2^{a}
4	5	18.3± 4.8^{a}	27.2± 6.0^{a}	34.0± 2.8^{a}	2.5± 1.1^{c}	81.9±12.5^{a}	-6.9± 16.3^{a}
6	5	12.8± 1.4^{a}	23.5± 1.7^{a}	25.8± 2.3^{a}	0.3± 0.0^{d}	62.5± 0.8^{b}	18.5± 1.1^{a}
8	5	12.3± 2.5^{a}	23.0± 7.2^{a}	27.7± 1.6^{a}	0.6± 0.0^{d}	63.6± 8.0^{ab}	17.0± 10.5^{a}

B

days in storage	temp (˚C)	glucose	fructose	sucrose	starch	total Sugars	decline in total sugars (%)
0	-20 ctrl	4.9± 0.2^{b}	6.4± 0.7^{b}	25.2± 2.0^{a}	40.1± 6.6^{a}	76.7± 8.9^{a}	-- -
2	25	7.6± 1.3^{a}	18.1± 4.0^{a}	9.8± 0.8^{b}	1.4± 0.2^{b}	34.1± 5.2^{b}	55.4± 6.8^{b}
4	25	1.9± 0.2^{c}	2.7± 0.7^{c}	2.4± 0.1^{c}	0.7± 0.4^{b}	6.3± 1.3^{c}	91.8± 1.6^{a}
6	25	1.9± 0.3^{c}	1.7± 0.3^{c}	1.7± 0.2^{c}	1.7± 0.5^{b}	3.6± 0.4^{c}	95.3± 0.6^{a}
8	25	2.1± 0.1^{bc}	1.8± 0.0^{c}	1.3± 0.1^{c}	0.2± 0.1^{c}	5.4± 0.1^{c}	93.0± 0.1^{a}

Table. A5: Glucose, fructose, sucrose and starch and their totals in nightshade leaves grown in Field at JKUAT (August to Nov 2016), harvested at 120 dap and stored at 5 ˚C (**A**) and RT (**B**) for 0, 2, 4, 6 and 8 d. The values represent means ± standard error. Means labeled with the same letters within each sugar were not significantly different at $p<0.05$ using Tukey´s test (n=6).

A

days in storage	temp (˚C)	glucose	fructose	sucrose	starch	total Sugars	decline in total sugars (%)
0	-20 ctrl	10.1± 0.3 b	15.8± 0.9b	28.5± 0.4a	19.5± 1.8 a	73.5± 3.1a	-- -
2	5	14.8± 0.1a	25.8± 0.2 a	30.9± 0.2a	12.6± 0.3b	84.1± 0.2a	13.0± 0.2 c
4	5	10.1± 0.1 b	21.2± 0.6a	23.7± 1.7ab	2.1± 0.0 d	57.1± 2.4b	18.6± 2.5 b
6	5	7.7± 1.7bc	15.3± 3.0 b	29.6± 3.7a	4.9± 1.2c	57.5± 9.5b	18.6± 9.8 b
8	5	5.6± 0.7c	9.3± 1.4 c	19.6± 1.3b	6.0± 1.4c	40.5± 3.8c	42.2± 3.9 a

B

days in storage	temp (˚C)	glucose	fructose	sucrose	starch	total Sugars	decline in total sugars (%)
0	-20 ctrl	10.1± 0.3a	15.8± 0.9a	28.5± 0.4 a	19.5± 1.8a	73.5± 3.1 a	-- -
2	25	6.6± 0.4c	12.5± 1.1ab	16.7± 1.2 b	3.5± 0.3b	39.3± 1.5 b	43.9± 1.6c
4	25	8.4± 0.1b	13.6± 0.7ab	10.8± 1.0 c	5.0± 0.8b	37.8± 1.5 b	46.1± 1.5c
6	25	6.7± 0.3c	10.1± 0.7b	5.6± 0.7d	5.5± 0.6b	27.9± 1.0 c	60.1± 1.1b
8	25	2.3± 0.1d	2.5± 0.1 c	3.0± 0.3 d	5.5± 1.5b	13.4± 1.1 d	81.1± 1.2a

2.4 Selection and validation of suitable reference genes for quantitative gene expression analyses in African Nightshade (*Solanum scabrum*) during plant development (Manuscript, submitted, plants methods journal).

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Abstract

Background: Quantitative real-time RT-PCR is the current powerful technique for gene expression analysis in plants. The accuracy and reliability of the analysis is determined by the stability of the housekeeping genes used as the references for normalization of the expression data. African indigenous vegetables such as African nightshade (*Solanum scabrum* and other *Solanum* species*)* have recently gained popularity because of their nutritional and pharmacological benefits. This has led to increased research interests in all aspects of the crops, including molecular studies. Gene expression studies for the crops requires evaluation and determination of the stable reference genes which to date have not been determined. The commonly used housekeeping genes include; *Cyclophilin (CYC), Elongation factors (EF), Adenine phosphoribosyltransferase (APRT), Actin, Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP)* and *Glyceraldehyde-3-phosphate dehydrogenase (GAPGH*), *ribosomal units (18S or 28S*) and *Ubiquitin (UBQ)* among others.

Results: This study evaluated six housekeeping genes (*Actin, APRT, EF, GAPDH, RuBP and CYC)* for their expression stability in African nightshade during development under field and greenhouse conditions. The bestkeeper and geNorm algorithms were used for the analysis of the housekeeping gene stability. The genes *APRT, GAPDH* and *Actin* were found to be the most stable across all the examined samples. CYC was also stable in certain sample subsets, but could require tests across experimental samples before using it as a reference gene.

Conclusion: The genes *APRT, GAPDH* and *ACTIN* are recommended for the use as reference genes in the normalization of qPCR data during gene expression studies in African nightshade.

Keywords: Gene expression analyses, quantitative RT-PCR, reference genes, *Solanum scabrum*

1. Introduction

Gene expression in plants is regulated by various factors that involve both internal and external signal transductions. These include changes in environmental factors, developmental stages, tissue or cell types, physiological and phytochemical changes (Marquez *et al.*, 2012). Functional genomics is among the current approaches for evaluating molecular mechanisms responsible for phytochemical changes and physiological responses in plants. Gene expression analyses make use of different methodological approaches such as Northern blot, Real-time PCR (qPCR), *in situ*-hybridization, ribonuclease protection assay and reverse transcription (RT)-PCR (Fryer *et al.*, 2002). The quantification through qPCR remains the most reliable and simplest for high throughput gene expression analyses (Gantasala et al., 2013).

The accuracy of gene expression through the qPCR approach requires stable housekeeping genes for use as references in normalization of the data (Pfaffl *et al.*, 2004; Huggett *et al*., 2005). The selected reference genes are expected to be stably expressed across various environmental conditions, tissues and development stages among other treatments *(Bustin et al.*, 2009). The errors that could occur during genes expression analyses as a result of quality of mRNA, efficiency of cDNA synthesis, differences in initial sample quantity or during qPCR assays are corrected by normalization of the data using stable reference genes (Huggett *et al.*, 2005; Expósito-Rodríguez *et al.*, 2008). Use of more than one reference gene would offer more accurate analysis and conclusions of the qPCR results (Pfaffl *et al.*, 2004; Bustin *et al.*, 2009). According to the MIQE guideline for qPCR analysis, normalization against a single reference gene is not acceptable unless with clear evidence showing its invariant expression under the experimental conditions described (Bustin *et al.*, 2009).

In general, housekeeping genes are often used as references, but their expression may vary depending on the plant species, environmental and growth conditions, or developmental stages (Schmittgen and Zakrajsek, 2000). This requires that the genes are investigated for stability across a variety of cDNA samples derived from various mRNA samples of interest. Frequently used housekeeping genes in qPCR assays include; *Cyclophilin (CYC), Elongation factors (EF), Adenine phosphoribosyltransferase (APRT), Actin, micro-globulins, Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP)* and *Glyceraldehyde-3-phosphate dehydrogenase (GAPGH*), ribosomal units (18S or 28S) and *Ubiquitin (*UBQ) among others (Pfaffl *et al.*, 2004; Gantasala *et al.*, 2013). The Beekeeper, GeNorm and Normfinder programs are among the main

programs used to analyze and rank the suitability and acceptability of a housekeeping gene for use as reference genes in the qPCR assays (Vandesompele *et al.,* 2002; Pfaffl *et al.,* 2004).

Although the African nightshade (members of the species *Solanum scabrum* and *S. villosum*) has been used for many centuries as a leafy vegetable in sub-Saharan Africa, there still exist wide research gaps regarding molecular genetic approaches to understand various mechanisms involved in physiological changes and responses under different conditions. Gene expression analysis within the crop species will allow in-depth insights into molecular responses depending on the research aspects under investigation. Since suitable reference genes for gene-expression analyses in African nightshade have not been reported up to now, this study aimed at evaluating expression of a series of housekeeping genes in this crop and at determining the most stable and suitable genes for normalization of qPCR data during upcoming gene expression studies.

2. Materials and methods

Seeds of the Tanzanian commonly grown African nightshade (*Solanum scabrum)* variety 'Olevolosi' were provided by the World Vegetable Center, Arusha, Tanzania. Greenhouse experiments were conducted at Hochschule Geisenheim University, in the period September to December 2017. Field experiments were done at (JKUAT), Juja, Kenya under conditions described by Kirigia *et al.,* (2018). The evaluation was done with nightshade leaf tissues derived from middle height positions of the plants at different developmental stages (30, 60 and 120 day after planting (dap) grown under field conditions in Kenya and greenhouse conditions in Germany. Three biological replicates were used at each development stage. The numbers 30F, 60F and 120F represent samples from the field, while 30G, 60G and 120G represent samples from the greenhouse experiments at the respective age.

2.1 Tissues collection and RNA extraction

Nightshade leaf samples were collected in 2 ml Eppendorf tubes with 3 biological replicates, and immediately frozen in liquid nitrogen. The tissues were then homogenized in liquid nitrogen into fine powder using a mixer mill (Retsch, MM301). Approximately 100 mg of ground tissue was used for total RNA extraction following the instructions of the InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular GmbH, Ref no 1064100X0, Berlin, Germany). Cell lysis was done by adding 900 µl of the RP buffer to the ground tissue and incubation for 15-30 min before spinning for 2 min at 14,000 x g was done. The total RNA was subjected to an on-column DNase treatment by adding 10 µl of the DNase1 mix (Thermo Fisher scientific, Ref no EN0521) directly to the RNA binding column membrane, and incubation at 37 °C for

10 min. To elute the RNA, 30-35 µl of RNase-free elution buffer provided in the kit were added directly to the column membrane, followed by an incubation for 2 min and spinning for 1 minute at 11000 x g. The eluted RNA was immediately placed on ice and stored at -80 °C until cDNA synthesis.

2.2 cDNA synthesis

The total RNA extracted in section 2.2.1 was quantified using a spectrophotometer (Nanodrop 1000, PeQlab Biotechnologie GmbH, Germany) and checked for integrity by 1.2 % agarose gel electrophoresis. The total RNA (1 µg) was then reverse transcribed to cDNA using superscript® III first-strand synthesis kit (Invitrogen, Cat 18080-051, Germany) following the instructions of the manufacturer. The first strand cDNA synthesis reactions were primed using 1 µl of the provided random hexamers (10 mM), mixed with 1 µl of 10 mM dNTP and 1 µg of the total RNA plus RNase-free water in a volume of 10 µl. The mixture was then incubated for 5 min at 65 °C and chilled on ice for at least 1 minute. The second master mix was prepared by adding 2 µl 10x reaction buffer, 4 µl $MgCl_2$ (25 mM), 2 µl 0.1 M DTT, 1 µl RNaseOUT™ (40 U/µl) and 1µl Superscript III RT (200 U/µl) to a total volume of 10 µl. The second master mix (10 µl) was added to the 10 µl of the first mixture, and mixed gently before incubating as follows; 10 min at 25 °C, 50 min at 50 °C, and 5 min at 85 °C, then cooled at 4 °C. The synthesized cDNA was spinned briefly and 1 µl *E. coli* RNase H (2 U/µL) was added to each tube and incubated at 37 °C for 20 min. The synthesized first strand cDNA was stored at -20 °C to await quantitative PCR.

2.3 Selection of housekeeping genes and qRT-PCR

The expression stability of six candidate reference genes was evaluated; *CYC, EF (1-alpha), APRT, Actin, RuBP* and *GAPGH*. Primers for the housekeeping genes were designed based on the existing gene sequences of other members of the Solanaceae in the NCBI database and previous studies on *Solanum melongena L.* (Gantasala *et al.*, 2013). The primers were derived from accession numbers CK270447, U17005, AF126551, X55749, FS083183 and AJ536671 for *APRT, GAPDH, CYC, Actin, RuBP* and *EF* respectively (Table 1). Primers for *CYC, APRT, GAPDH* and *EF* were designed using Primer 3 (Untergasser *et al.*, 2012). The *Actin* and *RuBP* primers were used directly from the report on *Solanum melongena* (Gantasala *et al.*, 2013). The primers were designed to have an amplified fragment size of 140-200 bp, a melting temperature T_M of 60 ˚C and GC contents of 45-60 %. To confirm that the right gene fractions were amplified, the PCR product was run in 1.5 % agarose gel electrophoresis, the corresponding

band excised, purified using the Geneaid purification kit (GenepHlow™ Gel/PCR kit, Ref DFH100, Taipei, Taiwan) and sent for Sanger sequencing (StarSeq, Germany). The absence of gDNA in all cDNA samples was further evaluated by conducting a PCR using the *GADPH* primer, which amplified bands of approximately 600 bp for genomic DNA and 190 bp for cDNA. Multiple alignments of the sequences was done using Clustal Muscle (version 3.8), with sequences of other members of Solanaceae to confirm their identity. The SYBR green qPCR assay was done using the CFX96 Maestro real-time system from Bio-Rad with the powerUp™ SYBR Green master mix (Cat no A25776, Applied Biosystems).

To test primer efficiencies, the cDNA across all the biological samples were picked (2 µL), pulled together, and were used as initial concentration for the standard curve dilutions. Using nuclease free water, serial dilutions consisting of four points, with a dilution factor of 5 were made and used as standards. All the cDNA samples for the assay were diluted with nuclease free water in the ratio 1:20 before setting up the PCR. The RT-PCR amplification mixture consisted of cDNA template 2 µl, 0.5 µl each for the forward and reverse primer (10 µM) , 10 µL of the PowerUp™ SYBR Green master mix (Cat no A25776, Applied Biosystems) and 7 µL of distilled H_2O to a total volume of 20 µl. The Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Cat no MSB1001, Bio-Rad) were set so as to contain samples with primers for the gene of study, standards, and negative control (NTC). Each sample contained 3 technical replicates. The plates were sealed with an optical adhesive Microseal® 'B' PCR plate sealing film (Cat no: MSB1001, Bio-Rad), spinned briefly and run in the C1000 touch thermocycler (CFX Optics Module, Bio-Rad, Singapore).

The thermocycler was installed with CFX96 Maestro real-time software, which was used to generate the data. The PCR cycling consisted initial polymerase activation at 50 °C for 2 min and 90 °C dual- lock DNA polymerase (hot start) for 2 min, followed by 40 cycles of 95 °C for 2 min and 60 °C for 1min. The dissociation curve (melting curve) was included after the 40 cycles to check for specificity, at 65-95 °C with 0.5 °C increment per second. The gene expression data was then generated and analyzed with Bio-Rad CFX maestro software, version 4.1. The mRNA expression levels for each housekeeping gene across all the samples were measured and were indicated as threshold cycles (Ct) (Livak and Schmittgen, 2001). The housekeeping genes expression stability were analyzed using GeNorm and Bestkeeper algorithms (Vandesompele *et al.*, 2002; Pfaffl *et al.*, 2004*)*. The geNorm algorithms were

incorporated in the CFX96 Maestro software with color code rankings; Green for ideal genes, yellow for acceptable genes and red for most unstable genes. For the analysis, samples were first divided into two groups based on the growth conditions (field and greenhouse). The geNorm stability analysis (M) algorithms were expressed as log-transformed inverse of M values (Ln (1/Avg M)) and were used to rank the genes for their stability (Vandesompele et al., 2002). Additionally, the expression quantity (Δct) was done to compare relative mRNA changes across all samples for each of the genes (Pfaffl et al., 2004).

Table.1: Primer details of the candidate reference genes

Housekeeping gene	Accession number	Forward primer 5`-3`	Reverse primer 5`-3`	Length (bp) cDNA	Primer Efficiency (%)	Tm (°C)
CYC	AF126551	ATGGCTAATGCTGGACCTGG	CAACAGCTCTCGGCCTTCTT	140	92	60
EF	AJ536671	ACATCCATTGCTTGCTTTCACC	CCTTCAAAACCAGAGATGGGGA	189	100	60
APRT	DQ284483	GTCCCCATGAGGAAACCCAA	AGTTAGGGTCCCTCCAGTCG	162	98.9	60
Actin	X55749	TGTGTTGGACTCTGGTGATGGTGT	TCACATCCCTGACGATTTCTCGCT	200	88.7	60
RuBP	FS083182	TCGAGACTGAGCACGGATTTGTGT	TGCACTCTCCGACCTCATTCAACA	141	89.4	60
GAPGH	U17005	GGCATTGTGGAGGGTCTTATGA	CCATTCCAGTCAATTTGCCGTT	190	99.3	60

3. Results

Images from the confirmed that the right gene fractions were amplified (Fig 1). The band sizes were accorded to the expectations with 200 bp, 190 bp, 162 bp, 140 bp, 141 bp, and 189 bp for *Actin, GAPDH, APRT, CYC, RuBP* and *EF* respectively (Fig 1). The *GAPDH* primer had and amplification of approximately 600 bp for the genomic DNA, and as well 300 bp with the *EF* primers (Fig 1). The agarose gel electrophoresis did not indicate primers dimers for the candidate genes of study (Fig 1). The sequence pairwise analysis using Blast algorithms indicated 85 to 100 % similarity with other *Solanaceae* plants especially *S. tuberosum, S.lycopersicum* and *S. melongena* (Fig.2). This equally confirmed that the right genes were amplified. The target sites were mostly on the conserved regions indicated by the clustlal Muscle multiple alignment of the *Solanaceae* plants (Fig.2). The qPCR amplification specificity using housekeeping gene-specific primers were proven by evaluating the dissociation curves (Fig. 3). Results from the melting curve analysis indicated the amplification of single fragments with 82, 80, 81, 83.5, 83 and 83.5 °C melting temperatures for *CYC, EF,*

APRT, *Actin*, *RuBP* and *GAPDH*, respectively (Fig. 3). The amplification of the housekeeping gene fragments from the field and greenhouse samples indicated a variability of the mRNA transcripts (shown by Ct values) specific for each gene across all the tissues at 30, 60 and 120 days after planting (Fig. 4). The *RuBP* housekeeping gene showed highest expression levels with the earliest signal detected but also had a big variability across all the different developmental stages from the field experiments (Fig. 4). The EF gene had unstable expressions across all samples under field and green house conditions. The *APRT* gene showed the highest Ct value and was observed to be the most stable. Pearson's correlation of coefficient indicated a p-value of 0.119, 0.068, 0.463, 0.593, 0.221 and 0.808 for *CYC*, *EF*, *APRT*, *Actin*, *RuBP* and *GAPDH* respectively across all samples.

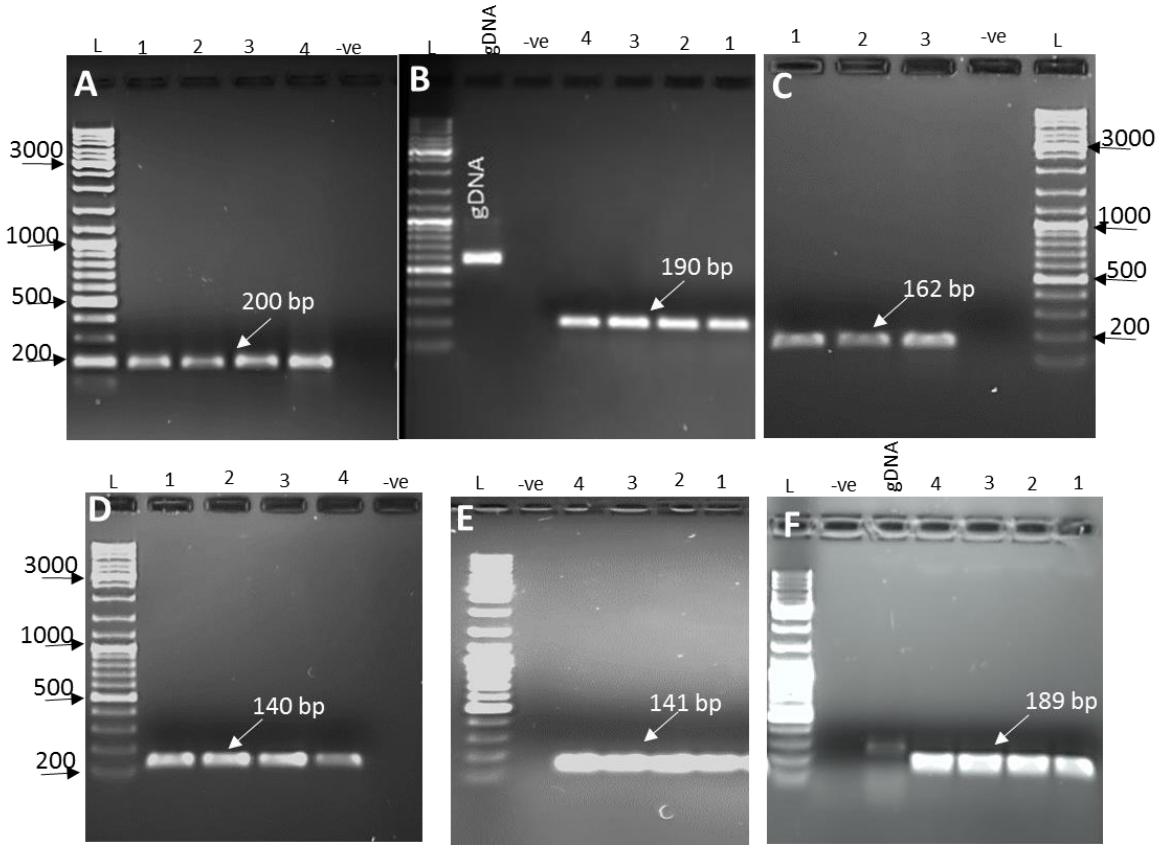

Fig 1: Amplification of cDNA using housekeeping gene specific primers designed for qPCR assays; A=*Actin*, B=*GAPDH*, C=*APRT*, D=*CYC*, E=*RuBP* and F=*EF*. 1-4 cDNA samples, -ve (NTC) and L is the DNA marker (Gene Ruler, Invitrogen)

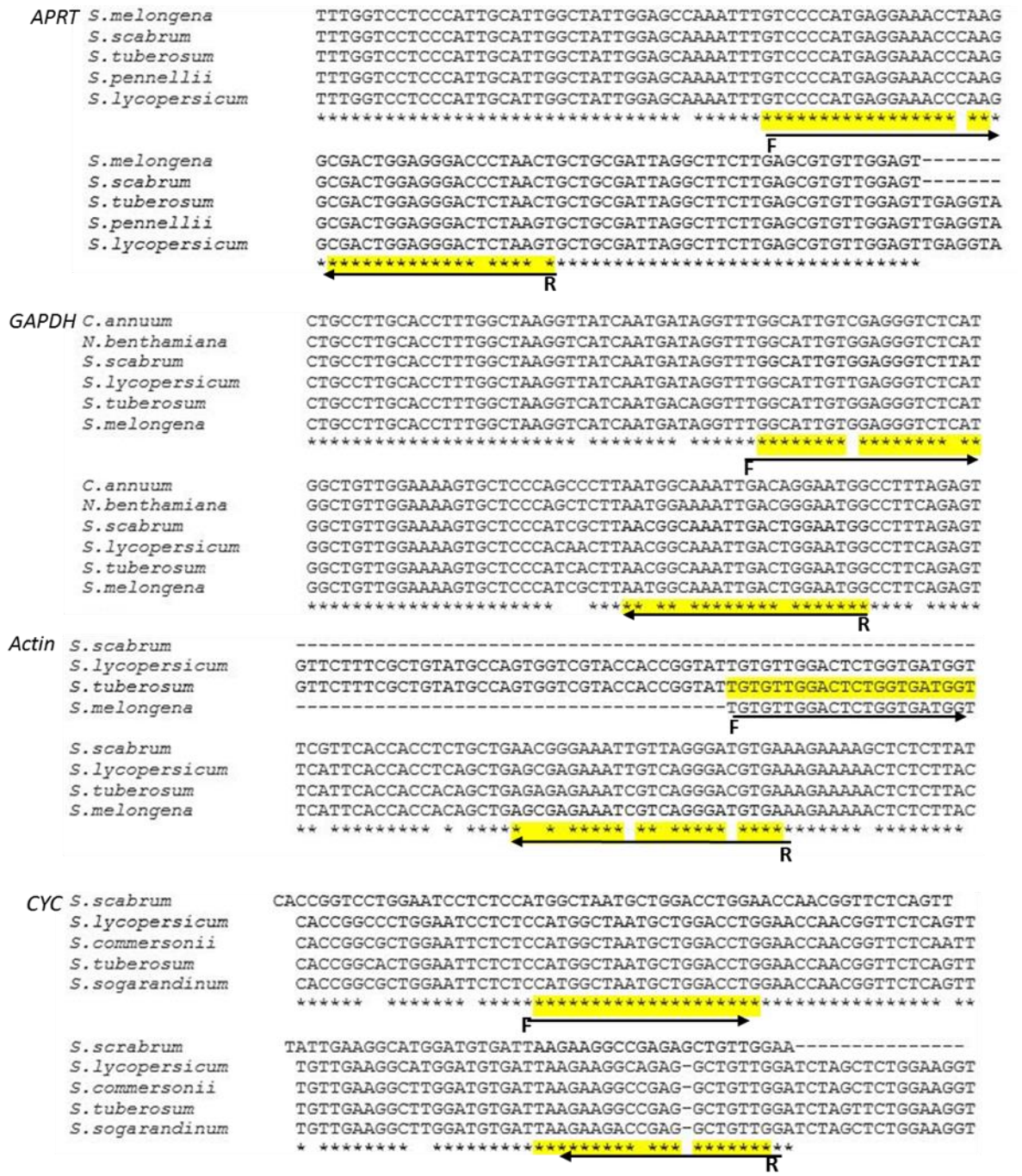

Fig 2: Multiple sequence alignments comparing the *S. scabrum* housekeeping gene sequences with those of other *Solanaceae* plants using Clustal MUSCLE software (version 3.8) and showing positions of forward (F) and reverse (R) primer binding sites for each gene.

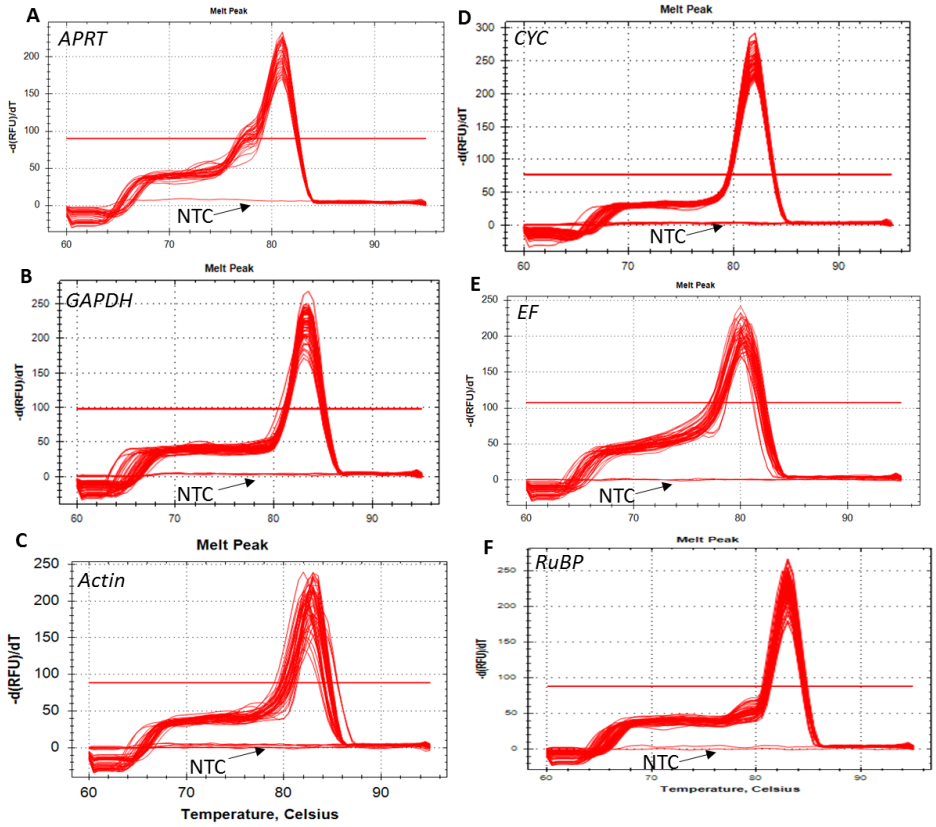

Fig 3: Melting curve analysis for qRT-PCR amplification specificity in the evaluation of the candidate reference genes from different cDNA samples (3 developmental stages x 2 growing environments x 3 biological replicates 3technical replicates = 54)

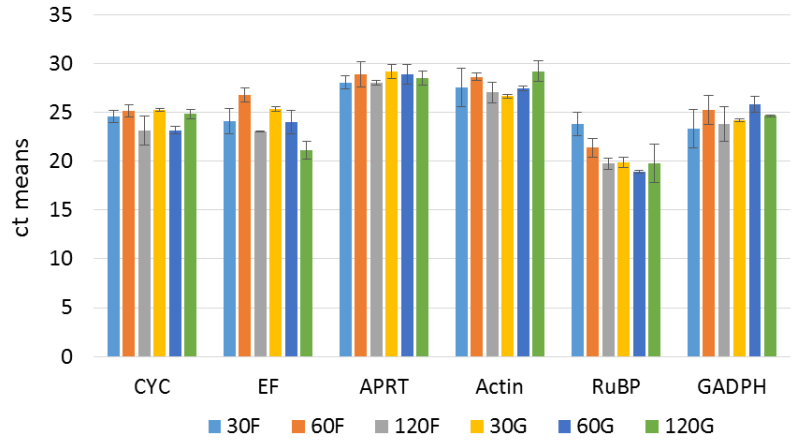

Fig 4: Mean Ct values for the housekeeping genes across samples harvested from field (F) and greenhouse (G) experiments at 30, 60 and 120 days after planting nightshade (n=3±SD).

Stability analyses was supported by two recommended programs, the geNorm and the Bestkeeker software. First, the housekeeping stability analysis was done using Bestkeeper algorithms which uses the raw Ct values. The variation based on the Best keeper's calculated

SD and CV (% Ct) of the Ct values were compared for the six housekeeping genes (Table 2). Base on best keeper´s analysis, genes with SD values of higher than 1 are considered unqualified for being used as reference genes. For the samples derived from field experiments the highest stability was recorded for the *APRT* gene with the least SD of 0.4 Ct (Table 2). The *EF* and *RuBP* were found to be highly unstable with and SD of 1.4 Ct and high CV values (red; Table 2). Similarly the analysis of greenhouse samples indicated highest stability of the *APRT* gene expression with an SD of 0.2 Ct while the *EF* was the most unstable gene with a value higher than 1 (Table 2).

Table 2: Bestkeeper stability analysis of the expression of six housekeeping genes using cDNA samples derived from field and greenhouse experiment.

Bestkeeper analysis	Field experiments						Greenhouse experiments					
	CYC	*EF*	*APRT*	*Actin*	*RuBP*	*GAPDH*	*CYC*	*Ef*	*APRT*	*Actin*	*RuBP*	*GADPH*
geo Mean [Ct]	24.3	24.6	28.3	27.7	21.6	24.1	24.4	23.4	28.9	27.7	19.5	24.9
std dev [± Ct]	0.8	1.4	0.4	0.6	1.4	0.7	0.8	1.6	0.2	0.9	0.4	0.6
CV [% Ct]	3.2	5.8	1.4	2.1	6.6	3.0	3.4	6.8	0.8	3.4	2.0	2.6
coeff. of corr. [r]	1.0	0.9	0.8	0.9	0.5	0.6	0.6	0.8	0.9	0.8	0.5	0.6

Secondly, analysis was done using GeNorm algorithms which shows the statistical measure of stability for each housekeeping gene (M value). The CFX96 Maestro plotted the log transformed inverse values of M, and the higher the bar the higher the stability. The graphical presentation of candidate reference gene stability indicated *APRT, Actin* and *GAPDH* as ideal genes for use as references for q RT-PCR data normalization under field conditions (Fig. 5A). On the other hand, for the samples from the greenhouse experiment showed *CYC, RuBP* and *APRT* to be the most stable reference genes for expression normalization (Fig. 5B). Unexpectedly, samples harvested from the greenhouse conditions indicated a wider variability in gene expression patterns.

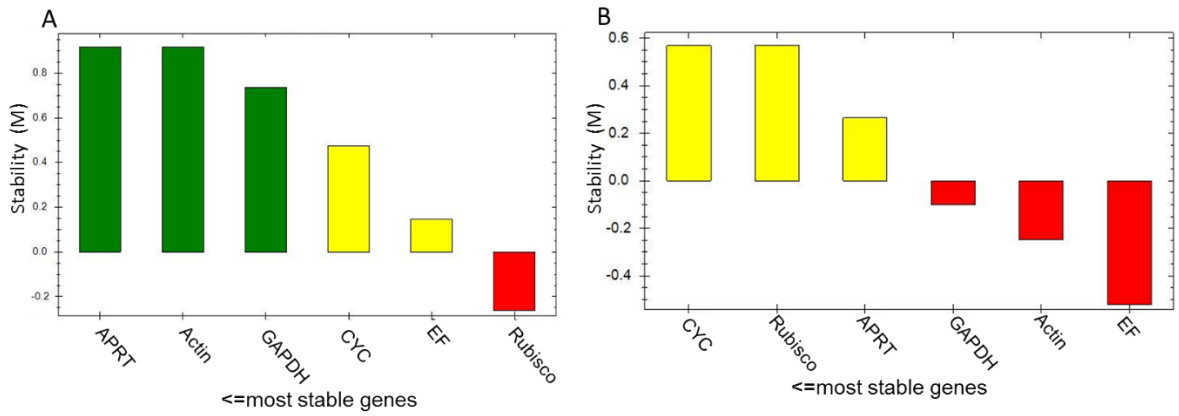

Fig 5: Candidate reference genes using GeNorm stability plots; A= Expression stability analysis using samples from field experiments A= Expression stability analysis using samples from greenhouse experiments.

Furthermore, analysis was done for the final selection of the housekeeping genes stably expression under the two growing conditions. This was done by first disqualifying the least stable genes (*EF and RuBP*) according to bestkeeper analysis (SD (± Ct)) / CV (% Ct)). The final stability of selected housekeeping genes were further evaluated for their validity as reference genes across all samples at 30, 60 and 120 days after planting, both from the field and greenhouse conditions. The geNorm stability algorithms (M) finally ranked *APRT*, *GAPDH* and *Actin* as the most stable housekeeping genes across all samples under different development stages and growth conditions (Fig. 6). In agreement with this, the Bestkeeper algorithms finally ranked the candidate reference genes; APRT*, GAPDH, Actin* and *CYC* at positions 1, 2, 3 and 4, respectively (Table 3).

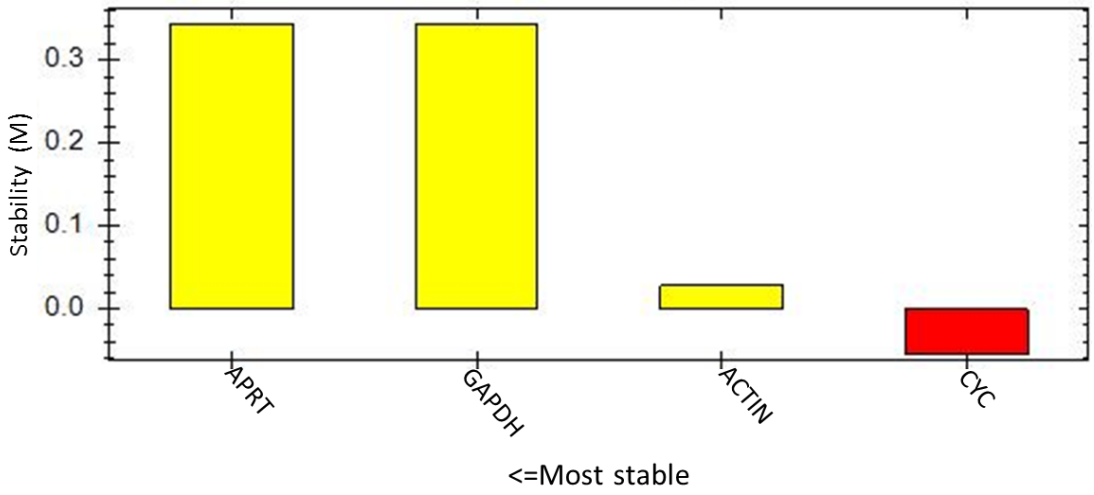

Fig. 6: geNorm´s relative stability plot ranking the four selected housekeeping genes across samples at different developments stages under field and greenhouse conditions, n=6.

Table 3: Bestkeeper ranking of the four selected housekeeping genes across samples at different developments stages under field and greenhouse conditions, n=6.

	geo Mean [Ct]	std dev [± Ct]	CV [% Ct]	coeff. of corr. [r]	p-value	Rank No
CYC	24.33	0.80	3.30	0.629	0.181	4
APRT	28.59	0.41	1.42	0.660	0.153	1
Actin	27.73	0.77	2.78	0.716	0.110	3
GADPH	24.49	0.73	2.97	0.627	0.183	2

Validation of the four selected candidate reference genes for expression normalization was done across all samples at the different developmental stages under field and greenhouse conditions. Results were expressed as the relative expression quantity (ΔCt) of the four genes across the set of six biological samples (Fig. 7). There was high stability across all the samples again with the *APRT* gene being the most stable on under the different growth conditions (Fig. 7; A). The EF and the RuBP clearly indicated strongest expression variations across all the samples and hence cannot be used as reference genes (Fig 7; E &F).

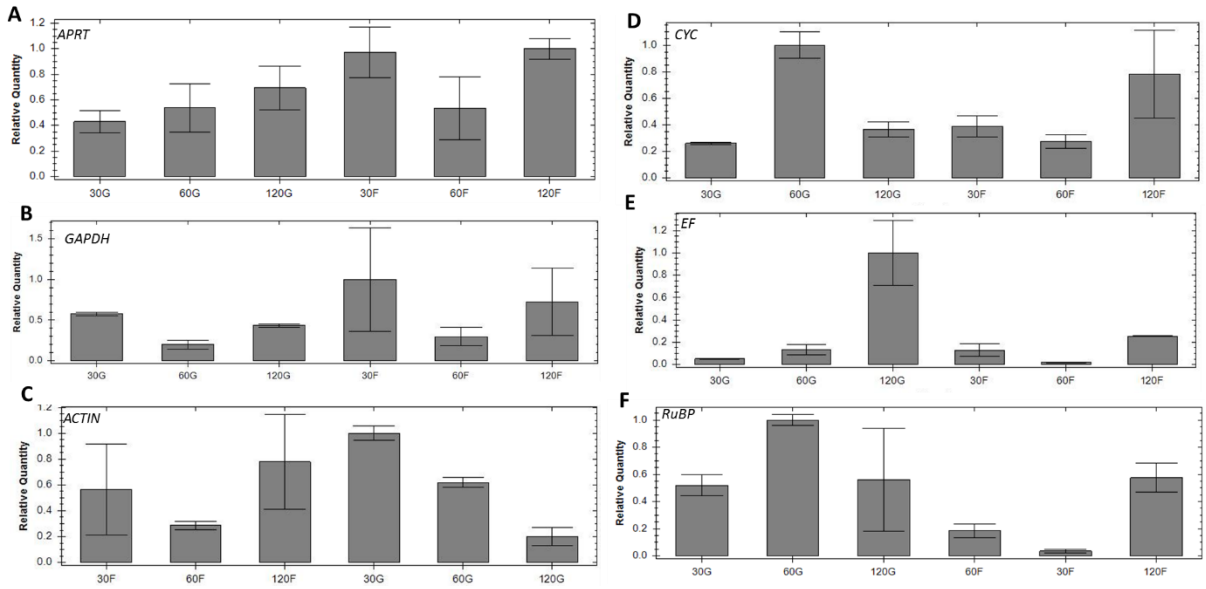

Fig 7: Expression quantitity of the candidate reference to validate their utility in the normalization of qRT-PCR assays during development under greenhouse and field conditions, n=6 ± standard error.

4.0 Discussion

Primer optimization, efficiency and stability are very important in the qRT-PCR assays (Bustin et al., 2009). The designed primers indicated high specificity, with efficiencies in the acceptable range 88.7 -100%, and amplified fragments less than 200 bp. This was also confirmed by the melt curve analysis and hence help to give unbiased qRT-PCR analysis (Fig 1, 2 & 3).Biological processes that lead to physiological and phytochemical changes can be best understood through gene expression approaches (Lu *et al.*, 2012). This would be important in improving the African indigenous crops for their better palatability, storability, yields and tolerance to biotic and abiotic stress. Accurate gene expression assays via qPCR can only be reliable by use of stable reference genes for normalization of the data (Pfaffl *et al.*, 2004; Gantasala *et al.*, 2013). Identifying stable housekeeping genes is not a trivial task, and is a necessary and sometimes neglected step before starting any gene expression experiments (Vandesompele et al., 2002). The selected reference genes should be stably expressed across all samples in the experiment regardless of the experimental treatments. The housekeeping genes analysed in this study were evidenced to vary in expression under greenhouse and field conditions, as well as at different developmental stages (Fig 4). This confirms the previous report that the housekeeping genes may have unstable expression patterns under different conditions, hence it is important to test them for qRT-PCR assays (Schmittgen and Zakrajsek, 2000).

The Bestkeeper and the geNorm algorithms are recommended for evaluating the stability of housekeeping genes before their use as reference genes in qRT-PCR assays (Vandesompele *et al.*, 2002; Pfaffl *et al.*, 2004*).* The identification of the best reference genes is automated and categorized by selection tools which test each housekeeping gene based on its stability across samples of experimental interest (Vandesompele *et al*., 2002; Pfaffl *et al.*, 2004). Bestkeeker results algorithms ranks the most stable housekeeping genes by comparing the (SD (± Ct)) / CV (% Ct) values (Pfaffl et al., 2004). The Pearson's coefficient of correlation (r) is not reliable for gene ranking because of the heterogeneity in expression levels among the tested group of samples (Pfaffl et al., 2004). In this study, the high standard deviations across all samples indicated *EF* and *RuBP* to be unstable and unsuitable for being used as reference genes based on the Bestkeeper analysis (Table 2). Although *RuBP* was highly expressed, it varied significantly across all samples, especially the field samples. *RuBP* gene plays great role in photosynthesis and photorespiration and its regulation to vary with environmental conditions, especially light intensity (Parry *et al.*, 2008). This kind of variation of the RuBP was evidenced under field conditions where days light intensity could have been different during the sampling at different plant development stages. This made RuBP inappropriate for use as reference gene under field experimental conditions, and use of such gene can always require clear testing and evidence of its stability on the test samples. The *APRT* and *GAPDH* and *Actin* were the highly ranked reference gene followed by *CYC* (Table 3).

In geNorm analysis the housekeeping gene with the least Ct variations is not necessarily the most stable gene. This is because samples may vary in the amount of amplifiable cDNA, even after normalizing the starting mRNA inputs using a spectrophotometer. Therefore a geNorm statistical measure of stability for each housekeeping gene (M value), normalizes the Ct of each gene against the Ct`s of all other candidate genes across all the samples in the experimental setup (Vandesompele *et al.*, 2002). The lower the M value the higher the stability, hence value 0 to 0.5 present highly stable and ideal genes (green), 0.5 to 1 are acceptable (yellow) while values greater than 1 present unstable and unacceptable (red) for use as reference genes (Vandesompele *et al*., 2002). However results in this study were presented as log-transformed inverse of M values (Ln (1/Avg M)) (Fig. 5 & 6), and here higher bars stand for higher stability across the samples tested. The geNorm algorithms identified *APRT, ACTIN* and *GAPDH* to be the most stable and ideal genes under field conditions while *RuBP* again was classified as unstable and unqualified for gene expression normalization. For the samples from the greenhouse experiment, *CYC, APRT* and *RuBP* were in the acceptable range of reference genes

according to geNorm analyses. Exclusion of the *EF* and *RuBP* resulted to the same ranking by both the Bestkeeper and geNorm analysis with *APRT, GAPDH, Actin, CYC* ranking as 1, 2, 3 and 4, respectively. This indicates the use of more than one selection tool to get the reliable and most stable reference genes across different set of biological samples. *APRT* proved to be stable under the two growth conditions and developments stages, while the choice of *GAPDH, Actin* and *CYC*, although stable could require test across all sets of experimental samples to choose the highly stable among them. Validation of the genes were seen in the relative quantification analysis across all samples (Fig 7). *APRT* indicated relatively equal expression pattern while *EF* and *RuBP* proves their unstable expression, with a wide variation across all samples. Previous findings in *Solanum melongena*, also showed *APRT* and *CYC* to be stably expressed across different tissues of the plant (Gantasala *et al.*, 2013).

5. Conclusion

The GeNorm stability M-value and the Bestkeeper (SD of Ct values/ CV (% Ct) values showed that *APRT, GAPGH* and *Actin* and *CYC* are stably expressed housekeeping genes in *Solanum scabrum*. The genes can therefore be recommended for use as references for the normalization of gene expression data in quantitative real-time RT-PCR assays. The *APRT* was ranked as the most stable housekeeping gene with the highest stability across samples of all development stages and growth conditions. The elongation factor (*EF*) and *RuBP* were the least stable housekeeping genes with high SD and CV values and hence are unsuitable for use as reference genes in African nightshade

Competing interest

The authors declare no compteting interests

Acknowledgement

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2.5 Investigation of ethylene biosynthesis, sensitivity and related gene expression in African nightshade (*Solanum scabrum* Mill.) leaves during development and after harvesting: manuscript under preparation

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Abstract

African nightshade *(Solanum scabrum)* are among the key indigenous vegetables with high potential for solving malnutrition and food insecurity menace in Sub Saharan Africa (SSA). The vegetable is rich in nutritional and pharmacological benefits. To date, small-scale farmers suffer 50 % post-harvest losses, but still wide research gaps exist, in terms of postharvest physiology and the underlying molecular mechanisms for these vegetables. We aimed at analyzing phytonutrients, the endogenous production of ethylene and the sensitivity to exogenous ethylene in nightshade leaves based on plant and leaf age, and storage conditions. Furthermore, we analyzed the expression of ethylene synthesis genes *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO1* and *ACO4*) and the ethylene receptor gene *ETR1* in nightshade leaves during growth and in post-harvest storage. Leaves aged 4 to 6 wks had higher carbohydrates and chlorophyll contents and were less sensitive to exogenous ethylene. Furthermore, they emitted less ethylene during storage compared to younger and older leaves (2 and 8 wks). Expression of *ACO1* and *ACO4* was upregulated in older leaves and older plants (8 wks and 90 to 120 days after planting), while *ETR*1 was higher expressed in the young and older leaves (2 and 8 wks). Ethylene treatment during storage of young leaves (2 wks) led to a suppression of the investigated *ACO* genes and to an upregulation of the *ETR*1. Results highlight the importance of considering leaf age and plants developmental stages during harvesting of nightshade, with the 4 to 6 wks old middle leaves at 60 days after planting nightshade offering optimal quality benefits and low sensitivity to ethylene.

Keywords: African nightshade, post-harvest, ethylene, gene expression, leaf age, phytonutrients

1. INTRODUCTION

African nightshade plays a great role in human nutrition and food security of Sub-Saharan Africa (SSA) (Abukutsa-Onyango *et al.*, 2010; Kamga *et al*., 2013; Grivetti and Oge, 2017). Although African leafy vegetables contain high nutritional benefits and have pharmacological attributes, there still exist wide research gaps in terms of post-harvest losses. Small-scale

farmers are to date faced with >50 % quantitative and qualitative losses along the field to consumer chain. The post-harvest losses are caused by various factors such as poor post-harvest handling, mechanical damages, unknown maturity indices, inappropriate harvesting methods, exposure to ethylene and poor storage conditions (Acedo, 2010). The plant and leaf age, post-harvest damages and high temperatures are among the factors that lead to ethylene production consequently causing spoilage of vegetables. Endogenous production and exposure to exogenous ethylene lead to post-harvest physiological changes which negatively affect the quality of several vegetables (Saltveit, 1999). These physiological changes include yellowing as a result of chlorophyll degradation, changes in aroma, texture and taste of plant products as well as other colour changes such as browning. Ethylene plays major regulatory roles in plants growth and development, ripening of fruits, senescence and abscission of leaves and flowers (Schaller, 2012; Iqbal *et al.*, 2017). Additionally ethylene elevates the respiration rates during storage leading to faster decay and tissue deterioration (Saltveit, 1999; Fugate *et al.*, 2010). Endogenous ethylene production is stimulated by internal and external signals such as development stages, and biotic and abiotic stresses (Srivastava and Srivastava, 2002; Schaller, 2012).

The biosynthesis and the sensitivity to ethylene are initiated at certain developmental stages depending on the crop species, and are as well activated by various biotic and abiotic stresses. In ethylene sensitive plants, concentrations as low as 0.1 ppm cause significant detrimental effects in the plant tissues (Srivastava and Srivastava, 2002). Ethylene biosynthesis from methionine via S-adenosyl methionine (AdoMet) requires the synthesis of the biochemical precursor, 1-aminocyclopropane-1-carboxylic acid *(ACC)* by the synthase *(ACS)* enzyme and the conversion to ethylene by the oxidase *(ACO)*(Chang and Stadler, 2001). The two enzymes *ACS* and *ACO* are activated under different physiological conditions (Chang and Stadler, 2001; Schaller, 2012). The ethylene receptors *ETR* are known to negatively regulate ethylene signaling (Hua and Meyerowitz, 1998). Quantification of expression of the key genes in ethylene biosynthesis (*ACO*) and perception *(ETR1*) is important to understand the functional role of ethylene in physiological and developmental processes.

There exists only scare to hardly any information on effects of ethylene on post-harvest losses of African leafy vegetables. Therefore, understanding the levels of ethylene production and responses as well as senescence-related genes expression patterns in nightshade will enhance our understanding of the physiological maturity that is optimal for harvesting, and help to

develop post-harvest storage management and transportation of the leafy vegetable. This study aimed at analyzing the endogenous production of ethylene in African nightshade depending on plant and leaf age, evaluating the sensitivity of leaves to exogenous ethylene and analyzing ethylene-related gene expression in leaves during growth and in post-harvest storage. Furthermore, we evaluated dynamics of phytonutrients, i.e. carbohydrates, chlorophyll and secondary metabolites at different leaf developmental stages. The ultimate goal is post-harvest and quality management of African nightshade.

2. MATERIALS AND METHODS

2.1 Measurement of ethylene and CO_2, and investigation of ethylene sensitivity in nightshade leaves

Seeds of the African nightshade (*Solanum scabrum)* variety 'Olevolosi' that is commonly grown in Tanzania were provided by the World Vegetable Center, Arusha, Tanzania. Greenhouse experiments were conducted at Hochschule Geisenheim University, in pots (10 L) with peat substrate (Torf substrat ED 73 Ref 814509, Meyer, Germany**)** in the period March to May 2018. Plants were fertigated with 1% boron and N.P.K (16.6.16) once a week, with daily drip irrigation to keep the media moist. The greenhouse temperature ranged 18-24 °C during the growth of nightshade plants. Field experiments were done at (JKUAT), Juja, Kenya under conditions described by Kirigia *et al*, (2018). Samples from the field experiments were only used for the analysis of ethylene related gene expression in leaves at different developmental stages (30, 60, 90 and 120 days after planting (dap). Greenhouse samples comprises the main samples for collection of data in this study and were used for all the investigations described below.

The Nightshade leaves were marked by tagging once a week. Quantification of endogenous production of ethylene in nightshade leaves was conducted by harvesting leaf samples from 60 days old plants in the greenhouse which were 1-8 wks old. Samples were taken in 4 biological replicates each from different plants. Each replicate composed of 4 leaves (7.9 ± 1.3 g FW) for 1 wks old leaves, 3 leaves (8.3 ± 1.5 g FW) for 2 wks old leaves, 2 leaves (11.2 ± 1.0 g FW) for 3 wks old leaves, and 1 leaf for 4, 5, 6 and 8 wks old leaves, with 11.4 ± 0.5, 17.8 ± 2.9, 16.4 ± 2.8 and 11.0 ± 1.4 g FW, respectively. The leaves were weighed and placed in gas-tight bottles (600 ml) (Schott, Germany) covered with duran screw cups lids that were fixed with silicone seals septa GL 45 (292461002 Duran™, Germany). The gas samples were taken from the bottle (25 ml) at 12 h, 24 h, 48 h and 72 h and injected into vials with septum, and ethylene was

quantified using the GC-FID (Bassi and Spencer, 1989). The leaf area was measured using an area meter (LI-3100C, LI-COR)

The response of nightshade leaves to external ethylene was tested by placing the leaf samples in the gas-tight containers with septum lids and injecting pure ethylene to a final concentration of 5 ppm. The samples were then stored in darkness at room temperature (RT) and after 72 h, observations were made in comparison to untreated controls. Quantification of carbohydrates, chlorophyll and secondary metabolites were done for the ethylene treated samples in comparison to the controls. Furthermore expression of ethylene related genes was done on leaves aged 2, 4, 6 and 8 wks, leaves treated with ethylene and their respective controls and stored at RT for 72 h, leaves stored at 5 °C or at RT for 2 and 4 days and samples derived from field experiments at 30, 60, 90 and 120 dap.

2.2 Phytonutrient analysis in African nightshade leaves of different age and from different storage conditions

The phytonutrient contents in leaves aged 1-8 wks, and after 72 h storage with and without exogenous ethylene treatments (5 ppm) were evaluated. The carbohydrates (glucose, fructose, sucrose and starch) were measured using enzymatic and photometric methods, gallic acid equivalent phenolics (GAE) using Folin-Ciocalteu's (F-C) assay, and catechin and quercetin equivalent flavonoids were analyzed using the aluminum complexation reaction assay. The Trolox equivalent antioxidants capacity (TEAC) assay against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+ radical cation) was conducted for antioxidants. Total chlorophyll was quantified using a non-destructive chlorophyll-meter with a sensor that measures real-time flavonols, anthocyanin and chlorophyll indices (Force-A Dualex Scientific+™). The methodological details of phytonutrients analyses were as described in Kirigia *et al.* (2018).

2.3 Expression of ethylene related genes in different developed and stored African nightshade leaves

2.3.1 Tissues collection and RNA extraction

Nightshade leaf samples were collected in 2 ml Eppendorf tubes with 3 biological replicates, and immediately frozen, and homogenized in liquid nitrogen into fine powder using a mixer mill (Retsch, MM301). Approximately 100 mg of ground tissue was used for total RNA extraction as per the instructions of the InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular

GmbH, Ref no 1064100X0, Berlin, Germany). To remove the genomic DNA, on column *DNase*1 (1 U) treatment was done according to the manufacturer's instructions (Thermo Fisher scientific, Ref no EN0521). The eluted RNA was immediately placed on ice and stored at -80 °C to await cDNA synthesis.

2.3.2 cDNA synthesis

The total RNA extracted in section 2.3.1 was quantified using a spectrophotometer (Nanodrop 1000, PeQlab Biotechnologie GmbH, Germany) and quality rechecked in 1.2 % agarose gels stained with Roti®-gel stain (Carl Roth GmbH, Germany). The total RNA was reverse transcribed to cDNA using superscript® III first-strand synthesis kit (Invitrogen, Cat 18080-051, Germany). The synthesized cDNA was spinned briefly and 1 µl of *E. coli* RNase H (2 U/µL) was added to each tube followed by an incubation at 37 °C for 20 min. The quality of cDNA was tested for remnant genomic DNA by conducting a PCR using *glyceraldehyde-3-phosphate dehydrogenase (GAPGH*) gene-specific primers (Table 1), which amplified bands of 600 bp for genomic DNA and 190 bp for cDNA.

2.3.3 qRT-PCR

The stability of housekeeping genes had been confirmed previously for their suitability as reference genes in qRT-PCR gene expression studies (Kirigia *et al.*, submitted manuscript). Therein, we identified *cyclophilin (CYC),* and *adenine phosphoribosyltransferase (APRT), glyceraldehyde-3-phosphate dehydrogenase (GAPGH*) and *actin* to be stably expressed across samples under field conditions and greenhouse conditions at different developmental stages. The *APRT* and *CYC* gene-specific primers (Table 1) were then chosen for normalization in the qRT-PCR assay. Ethylene-related gene expression analysis was conducted on leaf samples at different weeks (2, 4, 6 and 8), different plant developmental stages (30, 60, 90 and 120 days after planting (dap), post-harvest storage for 2, 4 and 6 days at room temperature (RT), and after ethylene (5 ppm) treatment. The studied ethylene-related genes were *1-aminocyclopropane-1-carboxylic acid oxidases ACO1* and *ACO*4, and the *Ethylene receptor* gene *ETR1*. Sequences of other members of the *Solanaceae* (*S. tuberosum, S. esculentum* and *N.tabacum*) at the NCBI data bases were used as templates for the putative candidate genes for identifying suitable primers. Pairwise analysis of the sequences was done using Blastn algorithms at the NCBI database (Altschul *et al.,* 1990). Primer 3 software was used for gene-specific primer design for qRT-PCR analysis (Untergasser *et al.*, 2012), from the conserved regions of the putative sequences (Table 1).

The CFX96 real-time system (Bio-Rad, Germany) was used for nightshade gene expression studies. Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Cat no MSB1001, Bio-Rad) were set to contain samples with primers for the gene of study, standards, reference genes and no-template control. The plate wells were loaded with cDNA template 25ng, forward and reverse primer 500 nM each, 10 µL of the PowerUp™ SYBR Green master mix (2x) (Cat no A25776, Applied Biosystems) and 7 µL of distilled H_2O to a total volume of 20 µl. The plates were sealed with an optical adhesive Microseal® 'B' PCR plate sealing film (Cat no: MSB1001, Bio-Rad), spinned briefly and run in the C1000 touch thermocycler (CFX Optics Module, Bio-Rad, Singapore). The thermocycler was installed with CFX96 Maestro real-time software, which was used to generate the data. The PCR cycling consisted initial polymerase activation at 50 °C for 2 min and 90 °C dual- lock DNA polymerase (hot start) for 2 min, followed by 40 cycles of 95 °C for 2 min and 60 °C for 1min. The dissociation curve (melting curve) was included after the 40 cycles to check for specificity, at 65-95 °C with 0.5 °C increment per second. The gene expression data was then generated and analyzed with Bio-Rad CFX maestro software, version 4.1.

Table 1: Primer sequences used for the qRT-PCR analysis for the reference and target gene expression studies * NCBI data base identity number.

Target gene	Accession number*	Forward primer 5`-3`	Reverse primer 5`-3`	Length of cDNA template [bp]	Annealing Temperature [°C]
APRT	DQ284483	GTCCCCATGAGGAAACCCAA	AGTTAGGGTCCCTCCAGTCG	162	60
CYC	AF126551	ATGGCTAATGCTGGACCTGG	CAACAGCTCTCGGCCTTCTT	140	60
ACO1	AF384820	AGATGAGAGAGCCAACACCATG	ACCTCTGTTCCATGCACTTCTT	152	60
ACO4	XM_006357919	ATCGATGTTCCTCCCATGCG	TTGCTTGCTTTCCTCTGCCT	210	60
ETR	AY300040	AGCGGCTTCTTGTGGATTCA	TGGCAAGACAAGCAGCTTCT	159	60

2.4 Statistical analysis

All the acquired data were subjected to normality test before analysis of variance was conducted using the SPSS (version 20) statistical software. The means were compared using Tukey test and T-test both at $P<0.05$.

3. RESULTS

3.1 Ethylene and CO_2 production of African nightshade leaves

The physical characteristics of nightshade leaves during development was recorded and a significant increase in leaf size (area) and fresh weight was observed (Table. 2). A gradual increase of the two parameters was seen in wks 1 to 6 while old bottom leaves (8 wks) with an average leaf area of 143.5 cm² were smaller compared to those being 4-6 wks old (264.5-368 cm²).

Table 2: Leaf fresh mass and area in African nightshade during development in the greenhouse (n=12). The values represent means ± standard deviation. Means labeled with the same letters within each group were not significantly different at $p<0.05$ using Tukey´s test (n=4).

Leaf age (weeks)	Fresh mass per leaf [g]	Area per leaf [cm²]
1	1.7 ± 0.2d	50.9 ± 3.4d
2	3.0 ± 1.0cd	86.7 ± 8.1d
3	5.55 ± 0.5c	159.4 ± 13.0c
4	11.2 ± 0.6b	264.5 ± 22.4b
5	17.1 ± 2.9a	353 ± 26.4a
6	15 ± 2.5a	368 ± 27.6a
8	11.1 ± 1.3b	143.5 ± 30.7c

The produced ethylene was below detectable limits after 12-24 h in the leaves 1- 4 wks old, while low amounts were detected in the older leaves (6 and 8 wks old, Fig. 1). At 48 and 72 h higher amounts of about 0.06 µl g^{-1} FW ethylene were measured in young leaves (1 and 2 wks) while 4 wks old leaves produced significantly less ethylene than all other age stages. The respiration activity during storage was seen from the measurements of the produced CO_2. A similar trend was seen with the CO_2 emission, with younger leaves (1-3 wks) producing higher amounts of the gas than older ones (4 to 8 wks). The accumulation of the two gases increased with time with the CO_2 getting to very high quantities by 48 to 72 h (Fig. 1).

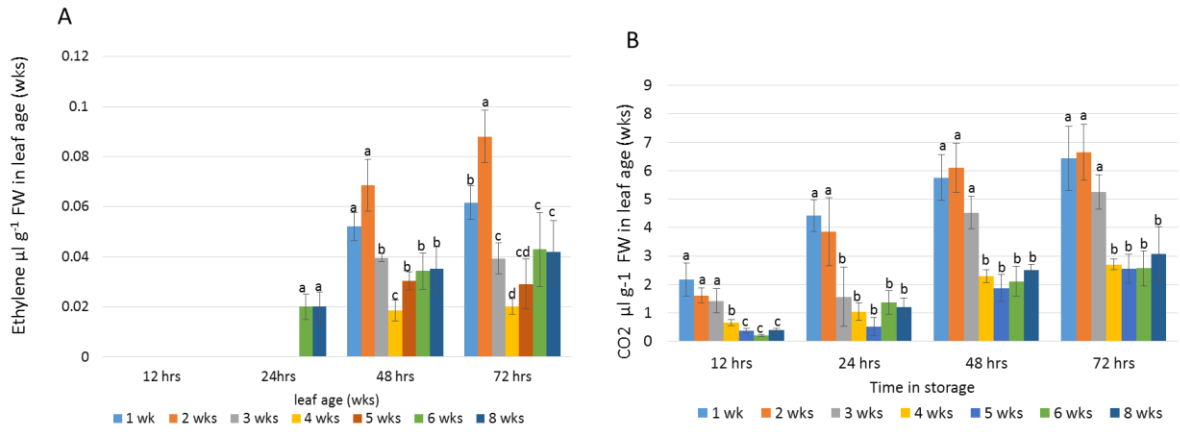

Figure 1: Quantification of ethylene (A) and CO_2 (B) produced by nightshade leaves aged 1 to 8 wks, during storage at room temperature (RT) in darkness. The values represent means ± standard deviation. Means labeled with the same letters within each storage duration (hrs) were not significantly different at $p<0.05$ using Tukey´s test (n=4).

3.2 Ethylene sensitivity and the chlorophyll content of African nightshade leaves

Treatment of leaf samples with 5 ppm ethylene indicated a higher ethylene sensitivity in the young leaves (1-2 wks) and some slight yellowing in old leaves (8 wks), while the 4 wks old leaves hardly showed any senescence symptoms (Fig. 2). By repeating the treatment 3 times, 100 % of the treated leaves aged 1-2 wks indicated yellowing after 72 h of ethylene treatment, while only 58 % of the 8 wks old leaves became yellow (Fig. 2). The 4 to 6 wks old leaves had no visible yellowing symptoms up to 72 h. Some slight yellowing was also observed in the untreated controls of young leaves (Fig. 2).

Figure 2: Response of African nightshade leaves of different ages (2, 4 and 8 wks) after 72 h exposure to 5 ppm ethylene (treated) or ambient air (control) at RT in darkness.

The total chlorophyll at harvest increased with leaf age being highest in 4-6 wks old leaves and lowest in young leaves aged 1-2 wks and in older leaves aged 8 wks (Fig. 3; A). Treatment of leaf samples with ethylene during storage resulted in significant decreases in chlorophyll

contents in leaves of 1, 2, 3 and 8 wks age with a higher magnitude observed in younger leaves (Fig 3; B). The slight degradation of chlorophyll in 4 to 6 wks old leaves was not significant.

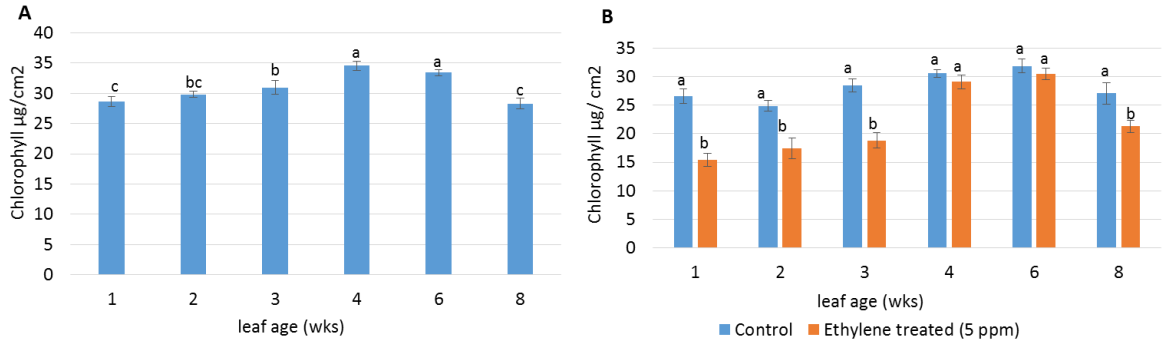

Figure 3: A= Total chlorophyll content in nightshade leaves of different ages from greenhouse culture at the time of harvest and B=Total chlorophyll content of leaf samples 1-8 wks aged after 72 h of storage in darkness at ambient air or treated with 5 ppm ethylene. The values represent means ± standard error. In graph A, means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4), while in graph B, comparison of means through paired sample t-test (n=4) was done and the same letter indicate no significant difference between control and treated leaves at $p< 0.05$.

3.2 Phytonutrient influenced by leaf age and ethylene treatment

3.2.1 Carbohydrates

Results indicated effects of leaf age on the phytonutrient content in African nightshade. The carbohydrates were higher in the leaves 4-6 wks old compared to younger leaves of 2 wks and older ones of 8 wks age (Fig. 4). The total carbohydrates were significantly higher in 4 wks old leaves with 157.6 ± 4.9 g kg^{-1} compared to all other leaf ages, with the oldest leaves (8 wks old) having the lowest amounts with 109.08 ± 8.4 g kg^{-1}. Glucose and fructose did not indicate any change with leaf age, but the sucrose concentration was highest in 4 to 6 old wks old leaves (Fig. 4). The starch concentration was lowest in the old leaves (8 wks) (Fig. 4).

Treatment of the leaf samples with 5 ppm ethylene resulted to significantly lower starch and increased glucose levels in the treated leaf samples compared to the untreated leaves (0 ppm) (Table 3). Fructose levels increased in the treated leaves only at 4 and 6 wks and remained the same in 2 and 8 weeks. The sucrose levels increased in the ethylene treated samples at 2 and 8 weeks but remained the same 4 to 6 wks (Table 3). The total carbohydrates indicated slight decline on the ethylene treated samples but only to a significant level in young leaves aged 2 wks (Table 3).

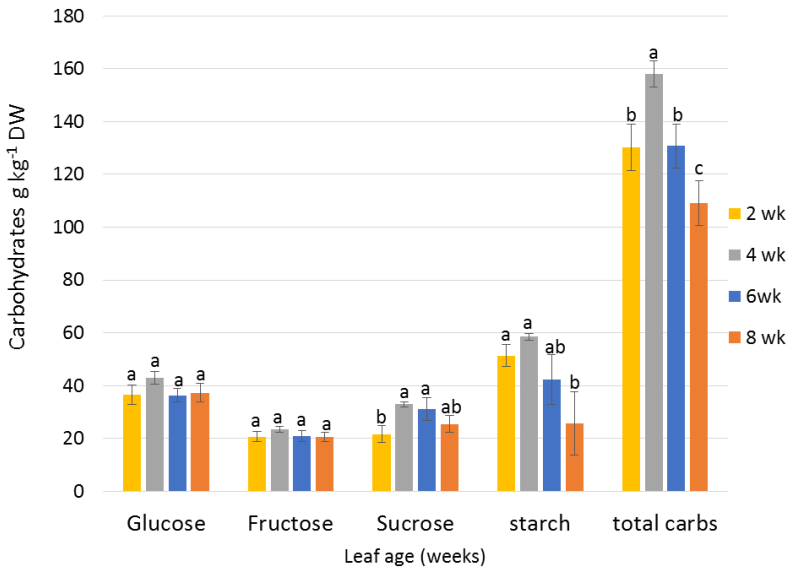

Figure 4: Carbohydrate concentrations in African nightshade leaves of different age (2, 4, 6 and 8 wks old). The values represent means ± standard error. Means labeled with the same letters within each carbohydrate were not significantly different at $p<0.05$ using Tukey´s test (n=4).

Table 3: Carbohydrate concentrations (g $kg^{-1)}$ in African nightshade leaves harvested at different ages of 2, 4, 6 and 8 weeks and treated during storage for 72 h at RT in darkness with 5 ppm ethylene or ambient air (controls). The values represent means ± standard error. Means labeled with * indicate a significant difference between the ethylene treated and control samples at each leaf age, $p < 0.05$ using paired sample t-test (n=4).

leaf age [weeks]	Treatment (5 ppm ethylene vs control)	Glucose	Fructose	Sucrose	Starch	Total
2	Ethylene	25.7 ± 0.3 *	14.9 ± 0.2	1.8 ± 0.3	34.5 ± 2.2	76.9 ± 2.2
	Control	17.2 ± 1.1	12.3 ± 1.2	3.2 ± 0.9*	53.7 ± 1.0*	86.4 ± 3.3*
4	ethylene	37.1 ± 0.4*	20.6 ± 0.2*	12.4 ± 0.5	43.2 ± 0.6	113.3 ± 4.8
	control	24.3 ± 1.7	14.1 ± 0.9	14.8 ± 0.4	54.2 ± 5.2*	107.4 ± 2.1
6	ethylene	56.4 ± 1.5*	30.2 ± 0.8*	15.1 ± 1.0	13.4 ± 2.7	115.1 ± 4.0
	control	47.9 ± 2.9	25.2 ± 2.2	15.2 ± 0.8	19.3 ± 0.7*	107.7 ± 3.7
8	ethylene	51.4 ± 1.2*	27.7± 0.6	8.0± 2.1	2.6 ± 0.2	89.7± 0.5
	control	43.9 ± 1.9	20.9 ± 1.9	17.7 ± 1.7*	7.8 ± 2.2*	90.4 ± 3.4

3.2.2 Secondary metabolites

The secondary metabolites, total phenolics, flavonoids (catechin and quercetin) and trolox equivalent antioxidants indicated the same pattern of changes across leaves of different ages. The total phenolics (gallic acid equivalents) were highest in 2 wks old leaves (14.8 ± 0.6 g kg^{-1} DW) and gradual decreased up to 6 wks followed by an increase thereafter in 8 wks old leaves (Fig 5A). Treatment of the leaf samples with 5 ppm ethylene during storage at RT for 72 h indicated a steep decline of phenolics (GAE) in young 2 wks old leaves, while there was no difference between treated and untreated 4 to 8 wks old leaves (Fig. 5B). The trolox equivalent antioxidants (TEA) were significantly higher in the youngest leaves (2 wks) with 62.0 ± 2.9 g kg^{-1} DW compared to constant and lower contents in older leaves (Fig. 6A). The antioxidants indicated no significant decline in young leaves 2 wks old after ethylene treatment during storage, while a sharp decline was seen in the treated samples of 4 to 8 wks old leaves (Fig. 6B). Catechin and quercetin equivalent flavonoids were both significantly higher in the young 2 wks old leaves (13.0 ± 1.1, 9.4 ± 0.5 g kg^{-1} DW, respectively) than in the older leaves (Fig 7A and C). Treatments of the leaves with 5 ppm ethylene indicated the same trend like described for the phenolics, with a sharp decline seen in the young 2 wks old leaves compared to 4 to 8 wks old leaves (Fig. 7B and D).

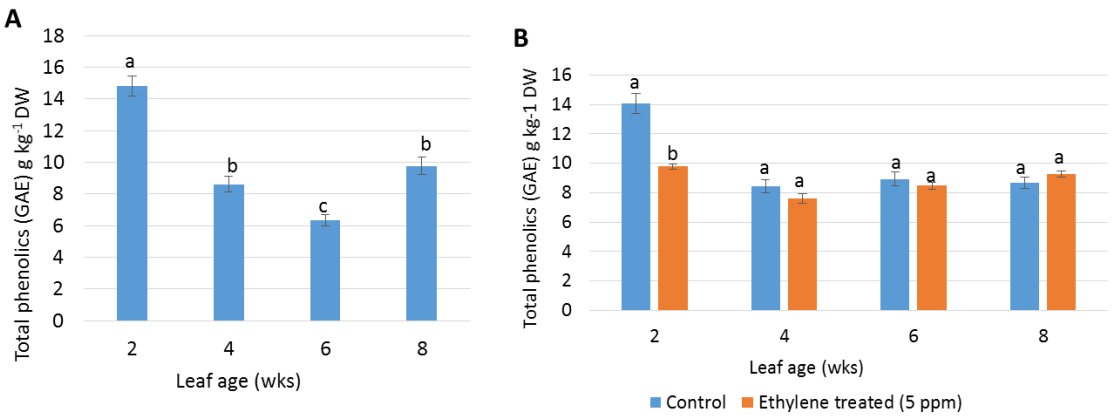

Figure 5: Total phenolics (gallic acid equivalents (GAE)) contents in African nightshade leaves of different ages from greenhouse culture at the time of harvest and B=GAE content of leaf samples 2-8 wks aged after 72 h of storage in darkness at ambient air or treated with 5 ppm ethylene. The values represent means ± standard error. In graph A, means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4), while in graph B, comparison of means through paired sample t-test (n=4) was done and the same letter indicate no significant difference between control and treated leaves at $p< 0.05$.

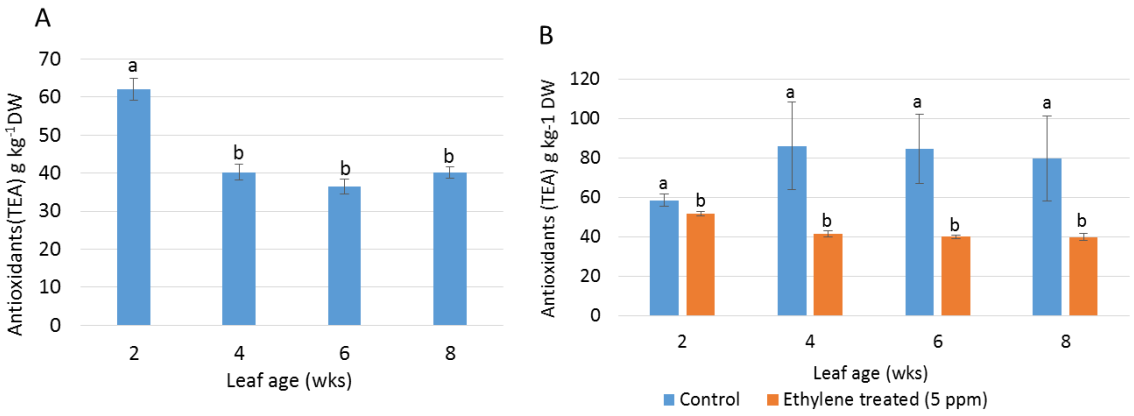

Figure 6: Trolox equivalent antioxidants (TEA) contents in African nightshade leaves of different ages from greenhouse culture at the time of harvest and B=TEA content of leaf samples 1-8 wks aged after 72 h of storage in darkness at ambient air or treated with 5 ppm ethylene. The values represent means ± standard error. In graph A, means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4), while in graph B, comparison of means through paired sample t-test (n=4) was done and the same letter indicate no significant difference between control and treated leaves at $p< 0.05$.

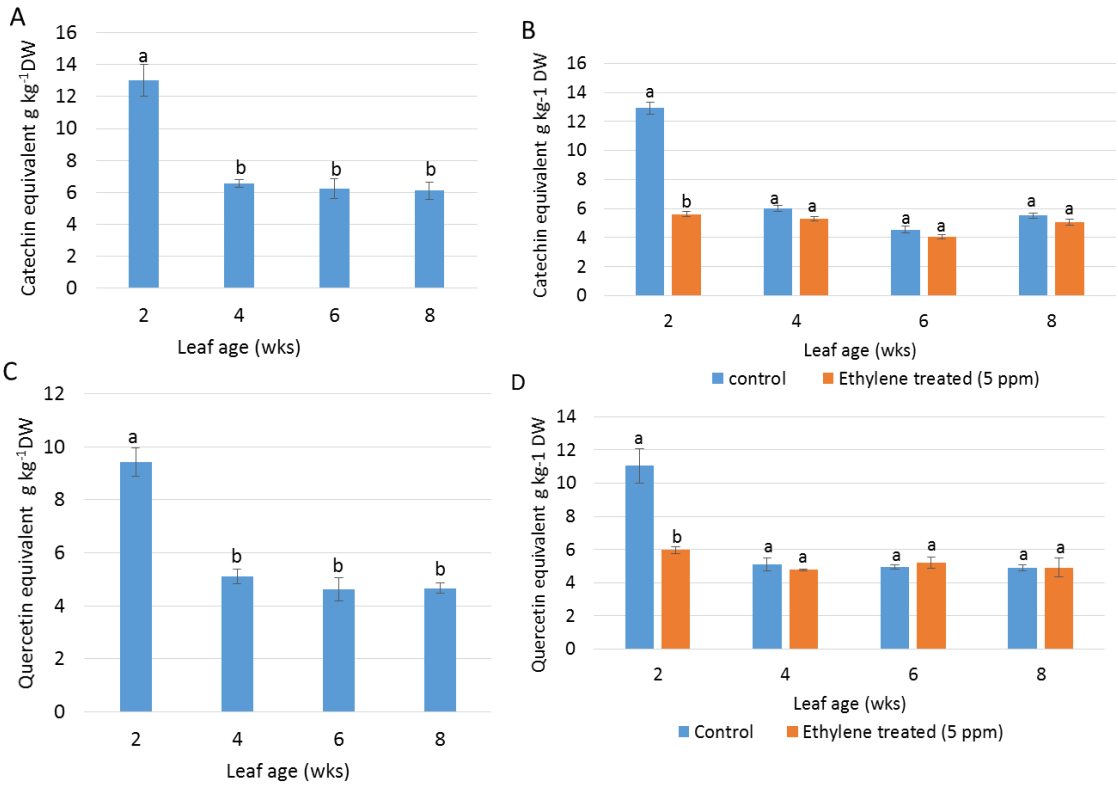

Figure 7: Flavonoids (catechin and quercetin) contents in nightshade of leaf samples 2-8 wks aged after 72 h of storage in darkness at ambient air or treated with 5 ppm ethylene. The values represent means ± standard error. In graph A and C, means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4), while in graph B and D, comparison of means through paired sample t-test (n=4) was done and the same letter indicate no significant difference between control and treated leaves at $p< 0.05$.

3.3 Expression of ethylene-related genes

Diff**e**rence in ethylene-related gene expression levels between leaf ages, plant developmental stages and storage conditions were observed. The *1-aminocyclopropane-1- carboxylic oxidase* (*ACO*1) expression increased with leaf age, with fold changes of 4.2, 4.4 and 21 for 4, 6 and 8 wks old leaves, respectively, compared to the expression at 2 wks (Fig. 8; A). During development under field conditions in Kenya, the leaves harvested from the middle part of the plants at 30, 60, 90 and 120 days after planting (dap) indicated an upregulation of *ACO*1 in the older developmental stages (90 and 120 dap) with fold changes of 2.5 and 2.2, respectively, in comparison to expression at 30 dap (Fig. 8; B). Storage of leaves at RT in darkness led to upregulation of *ACO1* compared to the control leaves directly after harvest, (4 wks ctrl) (Fig. 8; C). Ethylene treatment during this storage reduced this upregulation of *ACO1* in 2 and 4 wks old leaves compared the non-ethylene treated leaves (Fig. 8; C). This indicates that the gene is highly expressed during storage at RT, but suppressed in the presence of exogenous ethylene. In storage experiments involving different temperatures (RT and 5 °C), the *ACO*1 gene was equally expressed in samples either stored for 2 or 4 days regardless of the temperatures (Fig. 8; D).

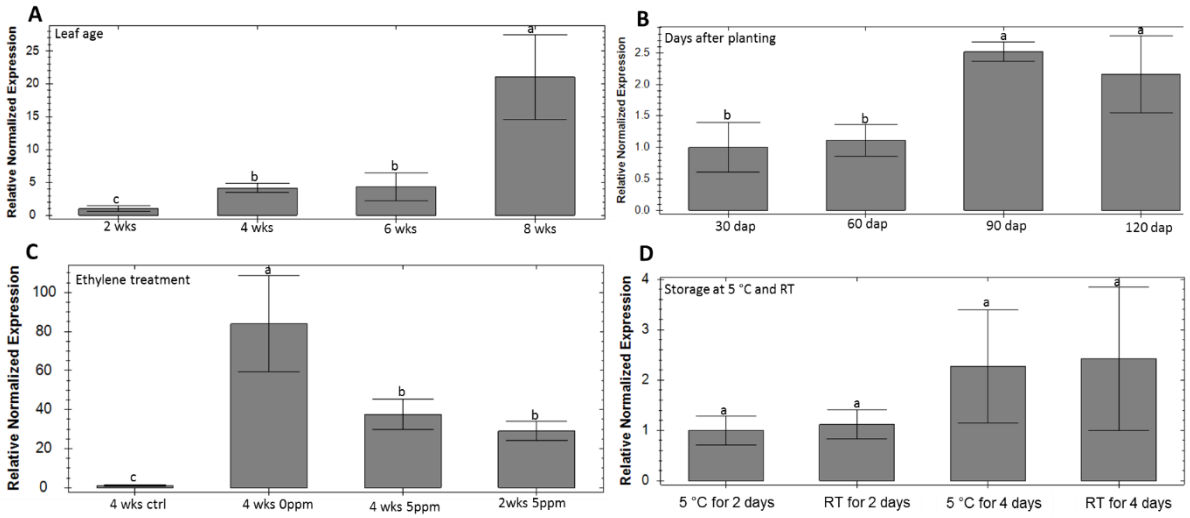

Figure 8: Relative normalized expression of *ACO*1 in nightshade leaves; A = leaves of different ages (2, 4, 6 and 8 wks old), B = leaves harvested 30, 60, 90 and 120 dap from the field experiment in Kenya, C = 4 wks old leaves directly after harvesting (4 wks ctrl) and 4wks and 2 wks old leaves stored for 72 h at RT in darkness treated with 5 ppm ethylene and without treatment (0 ppm); and D= leaves from plants grown in the field and harvested 60 dap plants stored at 5 °C and RT in darkness for 2 and 4 days. The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4).

The *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO*4) gene was highly expressed in the older leaves (8 wks) with an 8.5 fold change compared to the 2 wks old leaves (Fig. 9A). The fold changes were 2.6 and 1.5 for the 6 and 4 wks old leaves compared to the youngest leaves, respectively (Fig. 9A). When leaves were harvested from field grown plants at different stages, 30, 60, 90 and 120 dap, the expression of *ACO*4 increased in leaves of older developmental stages with fold changes of 2.2 and 2.9 for 90 and 120 dap compared to 30 dap, respectively (Fig. 9B). Storage induced an upregulation of *ACO4* especially in the untreated (0 ppm) leaves compared to the freshly harvested control leaves (4 wks ctrl) (Fig. 9C). Furthermore, again no effect of the storage temperature on *ACO*4 expression was found, for a storage time of 2 d, but an upregulation after 4d at RT was seen (Fig. 9D).

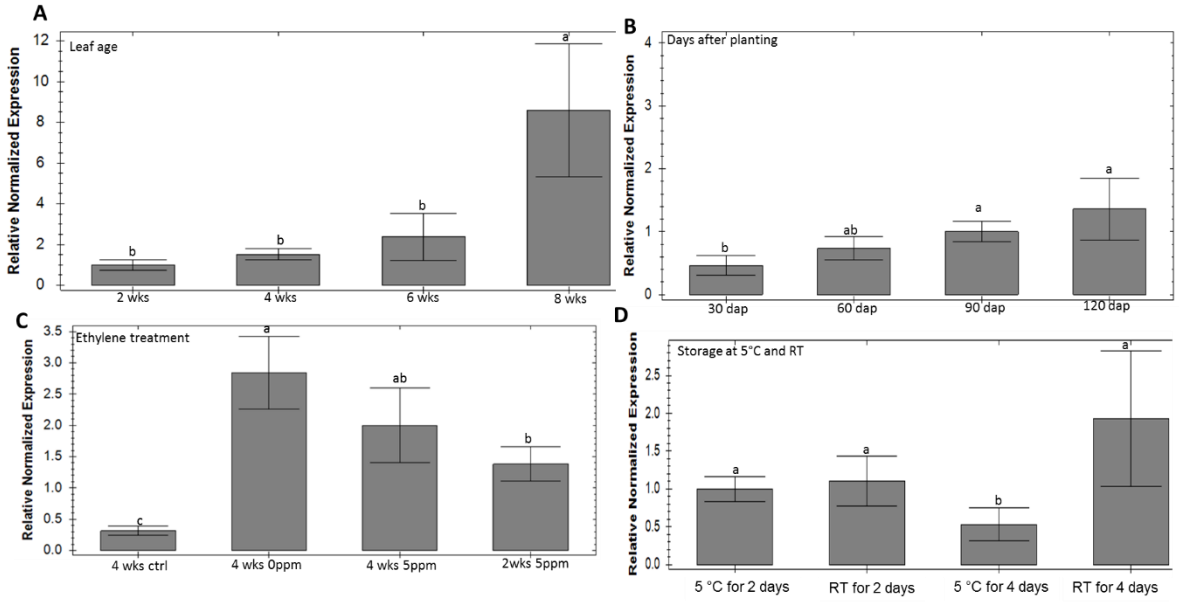

Figure 9: Relative normalized expression of *ACO*4 gene in nightshade leaves; A = leaves aged 2, 4, 6 and 8 wks old, B = 30, 60, 90 and 120 dap nightshade (from the field experiment, kenya), C = 4 wks old leaves direct after harvesting(4 wks ctrl), and 4wks and 2 wks leaves stored for 72 hrs at RT in darkness treated with 5 ppm ethylene and without treatment (0ppm);, and D=middle leaves from 60 dap plants stored at 5 °C and RT in darkness for 2 and 4 days. The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4).

The ethylene receptor gene *ETR*1 was expressed highest in young (2 wks) and older leaves (8 wks), while in 4 and 6 wks old leaves it was found to be less expressed (Fig. 10A). The expression of the *ETR*1 was high in young plants at 30 dap and significantly lower in the older plants grown under field conditions with fold changes -4.2, -7.7 and -5.2 for plants at 60, 90

and 120 dap, respectively (Fig. 10B). Treatment of nightshade leaves with ethylene resulted in a very high (up to 200 fold) upregulation of the *ETR*1 expression compared to freshly harvested leaves (Fig. 10C). Nevertheless, the expression of the *ETR* gene was also 40 fold upregulated in stored leaves that were not treated with ethylene (0 ppm) (Fig 10C).

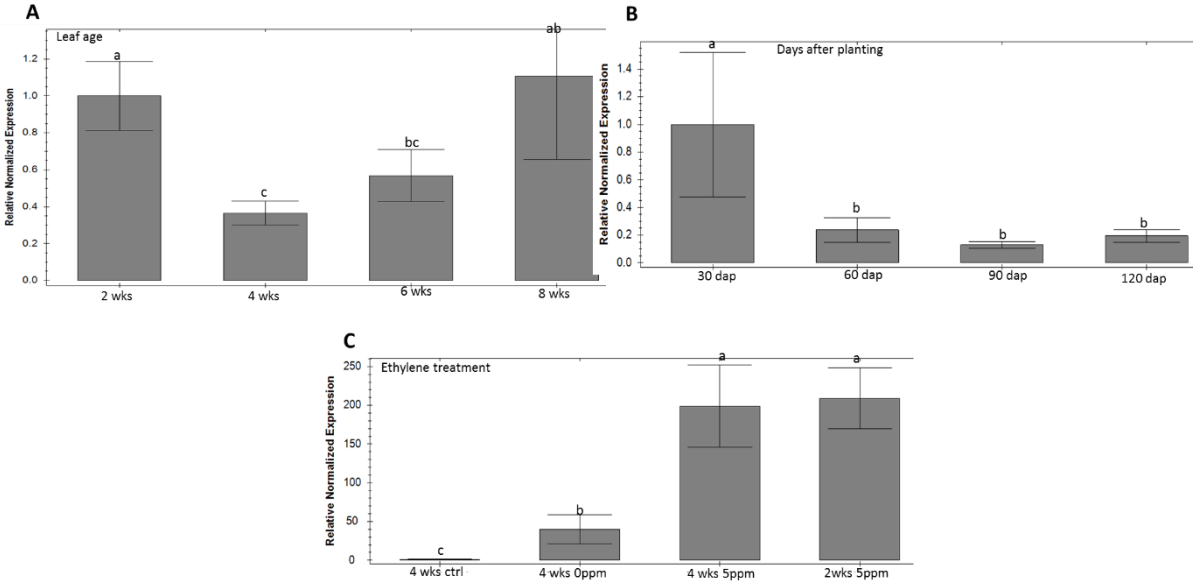

Figure 10: Relative normalized expression of *ETR*1 gene in nightshade leaves; A = leaves aged 2, 4, 6 and 8 wks old, B = 30, 60, 90 and 120 dap nightshade (from the field experiment, kenya), C = 4 wks old leaves direct after harvesting (4 wks ctrl), and 4wks and 2 wks leaves stored for 72 hrs at RT in darkness treated with 5 ppm ethylene and without treatment (0ppm). The values represent means ± standard error. Means labeled with the same letters were not significantly different at $p<0.05$ using Tukey´s test (n=4).

4. Discussion

4.1 CO_2 and ethylene production, and ethylene sensitivity in African nightshade

Ethylene is one of the major phytohormones that plays regulatory roles in plants growth and development, ripening of fruits, senescence and abscission of leaves and flowers (Schaller, 2012; Iqbal *et al.*, 2017). Since ethylene is involved in cell division and expansion, this could be the reason for ethylene production of the young leaves compared to the older leaves (Fig. 1) (Schaller, 2012). The production of ethylene is known to be higher in young developing leaves, it goes down in mature leaves and starts getting high later in the old leaves (Iqbal et al., 2017). This could also explain the variation in ethylene production observed in African nightshade leaves in our study. Ethylene is known to act as aging hormone explaining the increasing production in older leaves, but it has also been associated in stimulating the transition from juvenile to adult phase in plant leaves (Lumba *et al.*, 2012).

Young leaves (1-3 wks old) were observed to be actively increasing in size (leaf area) and fresh mass (Table 2). However, the amount of leaves placed in the bottle during the ethylene production experiments varied with leaf age (section 2.1). Four to 2 small leaves aged 1-3 wks were put into one bottle and the lower leaf area could have had led to higher O_2 availability of the younger leaves, hence increasing respiration rates. Besides, the young leaves contains more and active cell activities. This could have resulting in the higher CO_2 production by the younger leaves compared to older leaves (4 to 8 wks) which had bigger leaf areas. However, since the leaf weight and leaf area were factored out, other mechanisms could be responsible for such observations. Increased respiration rates have been linked to elevated production for ethylene in plants (Fugate *et al.,* 2010). This also could give a hint that big leaves might have quickly depleted O_2 and thus had lower respiration rates as evidenced by lower CO_2 production. However, measuring oxygen concentrations would be necessary to support this hypothesis.

Ethylene promotes chlorosis through chlorophyll degradation in leaves resulting in yellowing effects. During the sensitivity test, the younger leaves indicated much more pronounced responses with clearly visible yellowing after 72 h of ethylene application and storage at RT (Fig. 2). This kind of reaction could be due to thinner leaves and membranes in the young leaves increasing the permeability of the ethylene compared to older leaves (Voesenek and Van Der Veen, 1994). This kind of ethylene sensitivity has been reported seedlings of other Solanaceae plants like solanum melongena, compared to mature plants (Edelman and Jones, 2014). The 8 wks old leaves equally indicated some yellowing after ethylene application (Fig. 2). This could be due to the already programmed aging of the leaves, reduced assimilates and the onset of senescence-related genes including those of ethylene perception and responses (Voesenek and Van Der Veen, 1994; Edelman and Jones, 2014). Interestingly the 4 to 6 wks old leaves did not indicate any yellowing after application of 5 ppm ethylene. The visual impression was supported by the data on chlorophyll contents (Fig. 3).

4.2 Phytonutrients in African nightshade influenced by leaf age and postharvest treatment

Chlorophyll content increased with leaf age but later decreased in the older leaves possibly due to senescence onset which is also correlated to the low carbohydrate contents of 8 wks old leaves (Fig. 4). Chlorophyll declined significantly in young leaves (2wks) and old leaves (8wks), but not 4-6 wks old leaves after 72 h of 5 ppm ethylene treatment and storage at RT (Fig. 2 and 3). The high levels of carbohydrates in the 4 to 6 wks old leaves, together with other unknown factors including stress defense mechanisms could explain the low responses to ethylene at this stage (Rapaka *et al.,* 2007). A close interaction between glucose and ethylene

signaling pathways has been previously reported (Yanagisawa, 2003) (Ramon *et al.*, 2008). Glucose signaling downregulates some ethylene signaling pathways (Zhou *et al.*, 1998) and enhances degradation of the key transcriptional regulator in ethylene signaling (ETHYLENE-INSENSITIVE3 (EIN3), through the plant glucose sensor hexokinase (HXK1)(Yanagisawa, 2003). In contrast, ethylene enhances the stability of EIN3 leading to various responses (Yanagisawa, 2003). All these factors could explain the less pronounced ethylene responses seen observed for 4 to 6 wks old leaves, hence maintaining the chlorophyll after ethylene treatments.

The sugars in plants are key signaling molecules involved in modulating expression of various genes, metabolism as well as coordinating internal and external factors that govern growth and development (Araya *et al.*, 2006; Rolland *et al.*, 2006; Ramon *et al.*, 2008). The dynamics of carbohydrates with African nightshade leaf age was revealed in this study (Fig. 4). Higher carbohydrate contents in 4 to 6 wks old leaves compared to 2 and 8 wks old ones can be attributed to the well-developed and expanded leaves with higher chlorophyll content and surface area hence increased photosynthetic potential. Our previous studies have indicated an increase of sugars with plant age followed by a decline in the older stages (Kirigia *et al.*, 2018b, submitted manuscript). The 8 wks old leaves had lowest total carbohydrates with lower levels of sucrose and starch, possibly due to decreased photosynthetic potential, translocation for the source (old leaves) to the sink (young leaves), as well as an onset of senescence signals. Glucose and fructose increased while starch decreased in the ethylene treated samples compared to the controls (Table 3). There was an indication of increased respiration rates corresponding to the elevated CO_2 levels, leading to breakdown of starch and increased monosaccharides in the ethylene treated samples. The increase of respiration rates has previously been associated with the presence of ethylene (Prasath *et al.*, 2018). This could, therefore, explain the lower carbohydrates levels in the ethylene treated samples, especially in the young leaves. Additionally, plants endogenous carbohydrates have been reported to play a role in post-harvest storability by decreasing ethylene sensitivity in leaves (Rapaka *et al.,* 2007; Smeekens, 2000).

The secondary metabolites play important roles in the plant development and stress defense mechanisms (Akula and Ravishankar, 2011; Wojakowska *et al.*, 2013). The total phenolics (GAE), antioxidants (trolox equivalents) and flavonoids (catechin and quercetin) indicated higher quantities in the young leaves, and were lower and stable in 4 to 8 wks old leaves (Fig. 5, 6 & 7). The younger leaves had higher secondary metabolites possibly as a defense mechanism to the young tissues against any type of biotic and abiotic stresses. Ethylene

treatment caused a sharper decline of phenolics and flavonoids only in young leaves, but not in 4 to 8 wks old leaves. The high respiration rates indicated by high CO_2 emission by the young leaves could have accelerated the degradation of the secondary metabolites. However, the antioxidants on the contrary were more reduced in 4 to 8 wks leaves but were stable in the younger leaves (2 wks). The stress induced by high storage temperature and ethylene treatment could have resulted to in a kind of consumption of antioxidants, leading to the lower levels as indicated in the results.

4.2 Ethylene related gene expression depend on leaf age and storage conditions

The two major enzymes of the ethylene synthesis *ACS* and *ACO* are upregulated and activated under different physiological conditions, including age and developments stages of plants and tissues, stress (Chang and Stadler, 2001; Schaller, 2012). In this study, the *ACO*1 gene expression increased with leaf age with highest expression fold changes of 21 in the old leaves at 8 wks relative to the expression at 2 wks (Fig. 8). The high expression of the *ACO*1 gene in the old leaves and at later developmental stages of nightshade plants (120 dap) signifiy the onset of senescence and negatively correlates with the low chlorophyll and carbohydrates in the leaves. Upregulation of *ACO*1 has also been reported in senescing petals, tomato leaves and in during post-harvest degradation of broccoli (John *et al.,* 1995; Alexander and Grierson, 2002). As stated above, in the young leaves, the higher endogenous ethylene levels could be playing roles in the leaf development and the transition from juvenile to mature leaves (Lumba *et al.,* 2012; Schaller, 2012). The *ACO*1 gene expression correlated with the other investigated *ACO* isoform, the *ACO*4 which was also highly expressed in the older leaves (Fig. 9). Storage of the nightshade samples resulted in higher expression of the *ACO* genes irrespective of the leaf-age, temperature and duration compared to the controls (RNA extracted immediately after harvesting) (Fig 8D and 9D). However, untreated samples (0 ppm) stored at RT for 72 h expressed the *ACO* genes significantly higher than those samples treated with ethylene (5 ppm), indicating repression of the expression by ethylene. Despite this upregulation of ethylene biosynthesis the 4 to 6 wks old leaves did not phenotypically indicate yellowing after up to 72 h of ethylene treatment.

ETR1 is involved in the ethylene signal transduction pathway and contains three putative membrane-spanning subdomains at its amino-terminal hydrophobic region, which forms the ethylene binding site (Rodriguez *et al.,* 1999; Chang and Stadler, 2001). The carboxyl terminus of the ETR1 contains both a histidine protein kinase domain and a receiver domain and has been reported to play a role in repression of ethylene responses and is consequently a negative

regulator (Hua and Meyerowitz, 1998). In this study, the ethylene receptor gene *ETR*1 was upregulated in youngest leaves (2 wks) and oldest leaves (8 wks) while during plant development (field experiment), the expression was significantly higher leaves of young plants 30 dap compared to other developmental stages (Fig. 10). Since ETR1 is known to negatively regulate ethylene signaling, the expression in the younger leaves could be a defense mechanism against the negative effects of the endogenous ethylene biosynthesis (Hua and Meyerowitz, 1998). The higher ethylene production in the younger leaves can also be correlated with the higher expression of the *ETR1*. The upregulation of the *ETR1* gene up to 200 fold changes was seen in the ethylene treated samples. This could be a response in attempt to reduce the ethylene sensitivity by increasing the expression of receptors (Agarwal *et al.*, 2012). The 4-6 weeks old leaves would have been expected to have higher *ETR1* expression levels based on the sensitivity tests, which indicated no yellowing, thus, other factors than this receptor might have contributed to the observed response.

5.0 Conclusion

The phytochemical components, ethylene-related gene expression, endogenous production of ethylene and sensitivity to exogenous ethylene application varies with leaf age, plant developmental stage and storage conditions of African nightshade leaves. The outcome of this study allows to identify optimal harvesting stages and effects of post-harvest storage conditions. Thereby, optimal nutritional benefits, marketability, storability and post-harvest processing of African nightshade can be obtained. Leaves of an age of 4 to 6 wks found at a middle height position of stems of plants grown for 8 weeks, had optimal carbohydrate and chlorophyll contents and as well were less sensitive to exogenous ethylene application. They also emitted less ethylene during storage compared to younger and older leaves. This leads to the conclusion that 4 to 6 wks old leaves are best to harvested for African nightshade grown in the greenhouse and could offer optimal quality benefits. Similar experiments under field conditions should follow in order to confirm this before being finally extended to the farmers in SSA.

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3 GENERAL DISCUSSION

The dynamics of phytonutrient composition in African nightshade and cowpea leaves from field experiments in Kenya was influenced by harvesting methods, development stages and storage conditions (publication and manuscript; 2.2 and 2.3). The investigation of harvesting methods from field experiments showed an influence on the yields and nutrients in African nightshade. Harvesting by uprooting and cutting resulted to higher yields than continuous picking of single leaves on weekly basis (Manuscript; 2.3). This was due to vigorous production of side shoots after harvesting compared to the continuous method. However, more nutrients could be retained in the leaves that were harvested by picking of single leaves than if cut or uprooted. The phytonutrient contents in the nightshade and cowpea were optimal at 60 to 90 dap, this proved that the physiological maturity before harvesting plays a great role in ALVs quality. Carbohydrates are the major products of photosynthesis and play a central role in driving diverse biological processes from embryogenesis to senescence, by supporting and integrating functions and actions of internal and external regulatory signals (Lemoine *et al.,* 2013). The fully developed leaves at the middle positions of the plant in nightshade and cowpeas had highest carbohydrate and chlorophyll contents at 60 to 90 dap due to the highly light-exposed surface area, hence a high photosynthetic capacity leading to an accumulation of photosynthetic assimilates (publication and manuscript; 2.2 and 2.3). However, total carbohydrate in the leaves at 120 dap were lower, possibly due to higher translocation from source (leaves) to sink (flowers and fruits) organs, because at these stages the plants were in the reproductive phase (Lemoine *et al.,* 2013). The low level at 120 dap could be due to hydrolysis of starch to glucose in response to the increased demand at the sink sites, or due to reduced photosynthetic capacity on the aging plants leaves, which was also evidenced with reduced chlorophyll contents at this stage (Granot *et al.,* 2014). During storage at RT, carbohydrates and chlorophyll were reduced significantly, due to increased respiration, ethylene accumulation and/or enzymatic breakdown of the contents.

The secondary metabolite (total phenolics, antioxidants and flavonoids) quantities varied with development stages and storage conditions in both nightshade and cowpeas. Plant secondary metabolites, such as phenolics, flavonoids and antioxidants play important roles in human diet and in plant defense mechanisms (Wojakowska *et al.,* 2013). The dynamics of the metabolites in both crops during growth and after harvesting were specific to each crop as reported by previous researchers (Akula and Ravishankar, 2011; Mathiventhan and Sivakanesan, 2013).

This indicates that different crops respond differently to various factors that either upregulate or downregulate production of different types of secondary metabolites as well as their degradation (Veitch, 2007). Previous studies have indicated a cross link between various metabolites, phytohormones and the regulation of sugar metabolism and transport (Eveland and Jackson, 2012), which could also explain the variation of the secondary metabolites over the development period as observed in the experiments (publication and manuscript; 2.2 and 2.3).

Results revealed that cowpea and African nightshade leaves are a rich source of minerals such as iron (Fe), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), manganese (Mn) and zinc (Zn) (publication and manuscript; 2.2 and 2.3). However, the mineral contents were observed to be stable during development of African nightshade and no significant changes occurred at 30 to 120 dap apart from Ca, Zn and Fe. The Fe content decreased with age while Zn increased. These kind of changes in Fe and Zn at 120 dap could be a result of remobilizations of such minerals which has been reported in various crops during reproduction phase and senescence (Kutman, *et al.*, 2011; Maillard *et al.*, 2015). The dynamics of mineral concentrations in cowpea leaves during development was specific for different elements, with Fe and Mn being higher at later development stages, while the Ca concentration was highest at 30 dap and indicated a decline thereafter. In both crops mineral contents were not significantly affected by storage conditions and duration. The irrigation regimes and soil fertility during production should be investigated for their influence on mineral concentrations.

From the greenhouse experiments at Geisenheim Germany, the phytochemical components, ethylene-related gene expression, endogenous production of ethylene and sensitivity to exogenous ethylene application varied with leaf age, African nightshade developmental stages and storage conditions (Manuscript; 2.5). The variation of phytonutrients with leaf maturity or positioning in nightshade were also noted in the field experiments (Supplementary data; 2.3.1). Leaves aged 4 to 6 weeks that can be found at a middle height position of stems of plants grown for 60 days, had optimal carbohydrate and chlorophyll contents and as well were less sensitive to exogenous ethylene application. They also emitted less ethylene during storage compared to younger and older leaves. This leads to the conclusion that 4 to 6 week old leaves are best to be harvested for African nightshade grown in the greenhouse and could offer optimal quality benefits.

4 CONCLUSION AND RECOMMENDATIONS

The outcome of this study allows us to identify optimal harvesting stages and effects of post-harvest storage conditions in African nightshade and cowpea. Thereby, optimal nutritional benefits, marketability, storability and post-harvest processing of both ALVs can be obtained. Harvesting by continuous picking of single leaves at the middle part of the plant at 1 to 2 week intervals would offer higher nutrient retention during post-harvest storage of nightshade. Most of the phytonutrient and phytochemical compounds including carbohydrates, chlorophyll and carotenoids, total phenolics, antioxidants and flavonoids were high at 60 to 90 dap in both crops. This suggests that harvesting the vegetables at 60 to 90 dap would offer higher nutritional benefits and better post-harvest quality. However, the phytonutrients also varied with leave maturity and positioning in African nightshade, hence high nutrients can be achieved if leaves aged 4 to 6 weeks are harvested. Establishment of maturity stages or indices for harvesting of each ALV crop species, will ensure optimum nutrient accumulation for uptake in human diets and/or post-harvest processing and may compensate for losses incurred under suboptimal storage conditions. An optimal harvesting time would be important for the post-harvest processing treatments, such as fermentation, solar drying or sun drying among others, although the phytonutrients should also be analyzed after such treatments.

Most phytonutrients were stable after 4 to 6 days in storage at 5 °C while at RT a high portion of most nutrients were lost within 2 days in storage. The phytonutrients in African nightshade and cowpeas would be preserved at cold storage at least up to four days. Although cold storage facilities are not available to many resource poor farmers in SSA, the use of on-farm evaporative coolers should be emphasized to preserve most of the beneficial phytonutrients in leaves and a cooling chain is strongly recommended if leaves need to be transported to supermarket/marketing centers. Furthermore, the influence of water and fertilizers to African nightshade and cowpeas on quality, quantity, and post-harvest shelf life should be investigated. Additionally, the antinutritive aspects of the ALVs as influenced by developmental stages and storage conditions would further confirm the optimal harvesting stages, and could be investigated after post-harvest processing. Adoption of these research findings by the small-scale farmers will be a great milestone in solving post-harvest losses and assuring quality of African nightshade and cowpea.

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APPENDICES

Poster presentation

Effects of Age, Storage Temperature and Duration on Total Phenolics, Flavonoids and Antioxidants in African Nightshade

Dinah Kirigia, Traud Winkelmann, Remmy Kasili, and Heiko Mibus-Schoppe

Introduction

African nightshade (*Solanum scabrum*) is one of the major indigenous leafy vegetables in Sub-Saharan Africa that plays a great role in food security and livelihood improvement. The vegetable is highly nutritious and contains antioxidants valuable in human diet. These secondary metabolites also play important roles in plant defense mechanism against various biotic and abiotic stresses.

Despite the importance of African nightshade, there are various constraints that affect their quality and quantity along the field to consumer chains. Physiological maturity and storage duration and conditions are some of the major factors affecting quality and quantity. Maturity indices at which phytonutrients are optimal remain unrevealed. The dynamics of various nutrients during development and storage at various conditions is one of our major research interest. The study's ultimate goal is to identify the appropriate harvesting stages, storage duration and conditions for postharvest quality assurance of African nightshade.

Materials and Methods

Nightshade (*Solanum scabrum* accession Olevolosi) from World Vegetable Center, Tanzania was planted at JKUAT, Nairobi, Kenya.

The leaf samples (2-3) were picked from the middle part of the plant after 30, 60, 90 and 120 days. Storage of the samples was done at 5°C with (80-85 % relative humidity (RH) and at room temperature (RT) (20-25°C) with 55-60 % RH, for a duration of 0 (control), 2, 4, 6 and 8 days. After storage, the samples were freeze-dried, ground into fine powder, and methanol extractions were done on 20-30 mg for quantification of the three groups of metabolites.

Total phenolics contents (TPC) were analyzed using the Folin-Ciocalteu method and were expressed as Gallic acid equivalents. Catechin and quercetin flavonoids were analyzed with a calorimetric method and spectrophotometric absorbance was recorded at 510 nm and 415 nm. Trolox equivalent antioxidants capacity (TEAC) assay against ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cations was conducted for antioxidants.

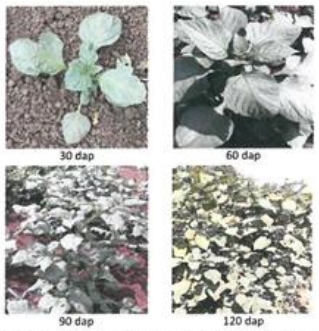

Fig 1: Nightshade images at 30, 60, 90 and 120 days after planting

Literature

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HORTINLEA framework

This study was part of SP4 within the Project **HORTINLEA** (Horticultural Innovation and Learning for Improved Nutrition and Livelihood in East Africa)

HORTINLEA
DIVERSIFYING FOOD SYSTEMS

TO IMPROVE THE LIVELIHOOD AND NUTRITIONAL SITUATION OF THE URBAN AND RURAL POOR

Results

Total phenolics

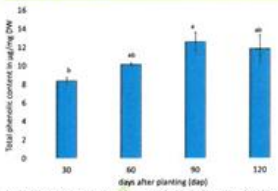

Fig 2: Total phenolics contents in nightshade leaves at 30, 60, 90 and 120 dap. Values represent means ± standard error (SE). Means with different letters indicate significant differences at p<0.05; Tukey test (n=6).

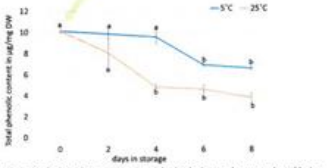

Fig 3: Total phenolics content in nightshade leaves harvested at 60 dap and stored at 5°C and RT for 0, 2, 4, 6 and 8 days. Values represent means ± SE. Within one storage temperature means with different letters indicate significant differences at p<0.05; Tukey test (n=6).

Antioxidants

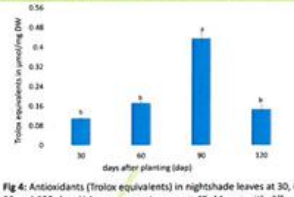

Fig 4: Antioxidants (Trolox equivalents) in nightshade leaves at 30, 60, 90 and 120 dap. Values represent means ± SE. Means with different letters indicate significant differences at p<0.05; Tukey test (n=6).

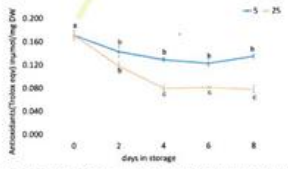

Fig 5: Antioxidants (Trolox equivalents) in nightshade leaves harvested at 60 dap and stored at 5°C and RT for 0, 2, 4, 6 and 8 days. Values represent means ± SE. Within one storage temperature different letters indicate significant differences at p<0.05; Tukey test (n=6).

Flavonoids

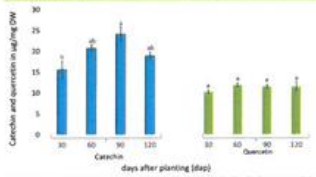

Fig 6: Flavonoids (catechin and quercetin) in nightshade leaves at 30, 60, 90 and 120 dap. Values represent means ± SE. Means with different letters indicate significant differences at p<0.05; Tukey test (n=6).

Table 1: Flavonoids (catechin and quercetin in µg/mgDW) in nightshade leaves harvested at 60 dap and stored at 5°C and RT for 0, 2, 4, 6 and 8 days. Values represent means ± SE. Within one storage temperature means with different letters indicate significant differences at p<0.05; Tukey test (n=6).

Days in storage	Catechin		Quercetin	
	5°C	25°C	5°C	25°C
0	20.7 ± 0.8 ^{a}	20.7 ± 0.8 ^{a}	11.9 ± 0.4 ^{a}	11.9 ± 0.4 ^{a}
2	20.0 ± 0.5 ^{a}	15.4 ± 1.0 ^{b}	11.3 ± 0.4 ^{a}	9.2 ± 0.2 ^{b}
4	17.3 ± 0.6 ^{b}	6.2 ± 0.2 ^{c}	10.3 ± 0.5 ^{a}	8.8 ± 0.1 ^{b}
6	15.1 ± 0.6 ^{b}	6.0 ± 0.3 ^{c}	11.2 ± 0.3 ^{a}	8.9 ± 0.5 ^{b}
8	14.9 ± 0.2 ^{b}	5.8 ± 0.3 ^{c}	10.8 ± 0.5 ^{a}	8.9 ± 0.1 ^{b}

Discussion and Conclusion

- Production of secondary metabolites is influenced by various physiological, biotic and abiotic factors (Akula and Ravishankar, 2011). Total phenolics and catechin concentrations were highest after 60 dap, while antioxidants were highest at 90 dap. However, quercetin concentration did not show any significant difference with age. Thus, accumulation of secondary metabolites in nightshade was influenced by age. Storage temperature and duration also influenced the stability of the metabolites after harvesting. Higher decline percentage (55, 63, 72 and 25 %) for antioxidants, phenolics, catechin and quercetin, respectively, were observed after 4 days in storage at RT, compared to storage at 5°C (22-35 %). This signifies the importance of age, storage conditions and duration for optimal availability of beneficial nutrients and antioxidants. It is, therefore, recommendable to harvest nightshade between 60-90 dap and store at low temperatures, and more importantly only up to 4 days, to avoid losses of antioxidative properties.

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Abstracts: Conference presentation

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"Future Agriculture:
Socio-ecological transitions and bio-cultural shifts"

Effects of Age, Storage Temperature and Duration on Total Phenolics, Flavonoids and Antioxidants in African Nightshade

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Abstract

African nightshade (*Solanum scabrum*), a leafy vegetable plays a great role in food security and livelihood in sub-Saharan Africa. The vegetable is highly nutritious and contains phenolics and flavonoids, major sources of antioxidants highly valuable in human diet. The phenolics and flavonoids have other important properties such as, anti-inflammatory, antimicrobial, anti-allergenic and anti-thrombotic. The study aimed at analysing the secondary metabolites during development, storage at 5°C and 80–85 % relative humidity (RH) and room temperature (RT) (20–25°C) at 55–60 % RH, for a duration of 0, 2, 4, 6 and 8 days. The total phenolic contents (TPC) were analysed using Folin-Ciocalteu method and were expressed as Gallic acid equivalents. The calorimetric method was done for flavonoids followed by spectrophotometric absorbance at 510 nm and 415 nm, the results were expressed as catechin and quercetin equivalents. TEAC assays were conducted for antioxidants. The TPC, flavonoids and antioxidants were higher at 90 days after planting (dap) ($12.6 \pm 1.7 \mu g\, mg^{-1}$, $24.1 \pm 2.8 \mu g\, mg^{-1}$ and $0.45 \mu g\, mg^{-1}$ of dry weight respectively). There was a significant decline of these three after 4, 6 and 8 days storage at RT. After 8 days of storage at RT, the TPC and flavonoids declined to $1.1 \pm 0.1\ \mu g\, mg^{-1}$ and $2.9 \pm 0.9 \mu g\, mg^{-1}$ respectively. There was no significant decline at 5°C storage up to 8 days. Plant age, storage duration and temperature affects TPC and flavonoids and it would be recommendable to store nightshade at low temperatures to avoid losses. Further experiments are going on to determine development stages with optimal secondary metabolites and storage longevity.

Keywords: African nightshade, days after planting, flavonoids, phenolics, temperature

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Tropentag, September 16-18, 2015, Berlin, Germany

"Management of land use systems for enhanced food security: conflicts, controversies and resolutions"

Carbohydrates Content at Different Maturity Stages of African Leafy Vegetables

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Abstract

African indigenous leafy vegetables (ALVs) play a significant role in food security and poverty eradication in sub-Saharan Africa (SSA). Compared to exotic vegetables, ALVs have higher contents of vitamins, minerals, dietary fiber, phytochemicals, anti-oxidants and medicinal properties. Despite the high values of AVLs, post-harvest losses remains > 50 % due to various pre-harvest and post-harvest constrains leading to massive losses along "the field to consumer" chain. Physiological maturity is one of the major factors that highly influences shelf life, phytohormones and nutrients such as carbohydrates and carotenoids contents. Photometric and enzymatic measurement of carbohydrates (glucose, fructose, sucrose and starch) was conducted at 30, 75, and 90 days after planting (d.a.p) in nightshade (*Solanum scabrum*), spider plant (*Cleome gynandra*), amaranthus (*Amaranths cruentus*) and cowpeas (*Vigna unguiculata*). Results indicated the level of carbohydrates to be higher in 30 d.a.p, (170 μg/mg dry weight of the nightshade leaves) than in 90 d.a.p (60 μg/mg dry weight of the nightshade leaves). Higher levels of carbohydrates were observed in Nightshade and cowpeas compared to those of Spider plants and amaranth's. Furthermore, carbohydrates levels were higher in younger leaves (180 μg/mg dry weight in nightshade) than in the older lower leaves (80 μg/mg dry weight) of the same plant in 75 d.a.p. However, storage of vegetables in higher temperatures (24°C) resulted to low starch and high sucrose levels indicating the effects of temperatures on carbohydrates breakdown. Carbohydrates are important in post-harvest processes such fermentation. Determining the right development stages with optimal nutrients levels and longer storability will be a great milestone in solving problems of postharvest losses. Further experiments are going on in Kenya, as well as cytokine measurements, and the results are expected to guide us to determine optimal development stages for harvesting ALVs.

Keywords: African leafy vegetable, carbohydrates, carotenoids, post-harvest

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Book chapter contribution (Hortinlea; submitted)

Optimization of harvesting

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Introduction

Harvesting methods, crop age, storage conditions and duration among other factors influences phytonutrient contents of African leafy vegetables (AVLs). Small-scale farmers in Sub-Saharan Africa (SSA) rely on relatively imprecise indicators to determine the start of harvesting of AVLs. Although ALVs can be harvested at different development stages, physiological maturity is important to ensure best nutritional value, higher yields and postharvest longevity (Acedo, 2010). Physiological maturity in other leafy vegetables such as leaf amaranthus, spinach and lettuce have been shown to influence post-harvest behavior, nutritional quality and shelf life (Spinardi *et al.,* 2012; Modi, 2007). Despite the contribution of ALVs and other traditional crops in household food security and poverty eradication, sufficient research has to date not been conducted to explore their full potential. The present study aimed at evaluating the effect of plant age, harvesting methods, storage conditions and storage duration on phytonutrient contents of ALVs for quality assurance and postharvest loss management. In this study, the commonly grown Tanzanian varieties (*Vigna unguiculata* cv. *Tumaini* and *Solanum scabrum cv. Olevolosi*) were grown at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya (S01˚05.9' E037 ˚00.8 and 1476 m above sea level). Harvesting was done at 30, 60, 90 and 120 days after planting (dap), after 4 h of sunlight, in 6 biological replicates. The freeze-dried samples were ground into powder and used for phytonutrient analysis.

Harvesting methods influence yield and nutrient contents

Harvesting methods indicated significant differences in both yield and nutrient content of ALVS. Taking the case study of nightshade within a period of 120 dap, the yields were 274.7 g $m^{-2,}$ 166.4 g m^{-2} and 306.6 g m^{-2} for cutting, continuous and uprooting harvesting methods (Kirigia *et al.,* 2018, unpublished results). Cutting or pinching resulted to vigorous growth of new side shoots hence the high yield compared to continuously picking of single leaves. To investigate the influence of harvesting methods on phytonutrients, the enzymatic and photometric assays for carbohydrates were analyzed as described by Zhao *et al.* (2010). Total phenolics content,

gallic acid equivalents (GAE) were determined using the Folin-Ciocalteu (F-C) assay as described by Ainsworth and Gillespie (2007). Although continuous method had the lowest yields, it had most carbohydrates and total phenolics retained in the leaves after 4 days storage at room temperature (RT) compared to other two harvesting methods in nightshade (Fig. 1). Cutting and uprooting methods resulted to more nutrient losses in the leaves within 4 days at RT, possibly due to continuous translocation of the phytonutrients compounds to the stem or roots shortly after harvesting. This was more evidenced by a decline of sucrose, the main translocation sugar. The translocation of carbohydrates in plants has previously been linked to phenolics contents explaining their same pattern of variations with harvesting methods (Fig. 1) (Arnold *et al.,* 2004). Harvesting through cutting also may create bigger wounds which could hence lead to various healing reactions either at the injury site or systematically, which includes translocation of various phytochemicals as a defense mechanism (León, Rojo and Sánchez-Serrano, 2001). Therefore harvesting by picking of single leaves would have more nutrients retained up to 4 days in storage than cutting or uprooting the entire plant.

Fig. 1: **A**=Carbohydrates in African nightshade 30 dap, **B**=Total phenolic contents (gallic acid equivalent), in nightshade leaves harvested by cutting, continuous, and uprooting methods. The values represent means ± standard error, g kg^{-1} dry weight (DW). Means indicated with the same letters within each nutrient content were not significantly different at $p<0.05$ using Tukey´s test (n=6).

Physiological maturity influences phytonutrients

The highest levels of phytonutrients in nightshade were seen 60 to 90 dap (Tab. 3). The highest levels were 136.3 ± 2.9 g kg^{-1} in carbohydrates (glucose, fructose, sucrose and starch) and 12.6 ± 1.0 g kg^{-1} total phenolics. The Trolox equivalent antioxidants capacity (TEAC) assay against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS• + radical cation) was conducted as described by (Miller et al., 1993). The antioxidants were highest at 90 dap (108.8 ± 6.0 g kg^{-1}) (Tab. 3). The aluminum complexation reaction assay was carried out for catechin and quercetin equivalent flavonoids as described by Eghdami and Sadeghi (2010). The catechin equivalents flavonoids were 24.1 ± 1.6 g kg^{-1} at 90 dap while the quercetin equivalent antioxidants remained stable throughout the growth period (Tab. 3). The UV-VIS spectroscopy

chlorophyll and carotenoids assays indicated 5.84 ± 0.3 g kg^{-1} chlorophyll at 60 dap and 1.01 ± 0.04 g kg^{-1} carotenoids at 90 dap (Tab. 4) Lichtenthaler (1987). In cowpeas, the total carbohydrates were highest at 90 dap (105.9 ± 2.5 g kg^{-1}) compared to 30, 60 and 120 dap (Tab. 4) (Kirigia *et al.*, 2018). The GAE increased gradually with age up to 12.0 ± 0.2 g kg^{-1} by 120 dap (Tab. 4). Catechin equivalent flavonoids, antioxidants and chlorophyll were at highest concentrations 60 dap with 8.0 ± 0.5 g kg^{-1}, 26.19 ± 0.5 g kg^{-1} and 5.7 ± 0.4 g kg^{-1} respectively (Tab. 4) (Kirigia *et al.*, 2018). Quercetin equivalent flavonoids and total carotenoids did not show significant changes with age in cowpeas (Tab. 4).

Tab. 3. Carbohydrates, total phenolic contents (gallic acid equivalent), trolox equivalent antioxidants (TEA), catechin and quercetin equivalent flavonoids, total chlorophyll and carotenoids in **nightshade** leaves harvested 30, 60, 90 and 120 dap. The values represent means ± standard error, **g kg^{-1} dry weight** (DW). Means indicated with the same letters within each nutrient content were not significantly different at p<0.05 using Tukey´s test (n=6).

dap	Carbohydrates	Phenolics (GAE)	Antioxidants (TE)	Catechin	Quercetin	Total chlorophyll	Carotenoids
30	81.3 ± 1.5b	8.4 ± 0.4b	27.3 ± 1.1b	15.5 ± 2.1b	10.2 ± 0.4a	4.8 ± 0.1b	0.9 ± 0.03b
60	136.3 ± 2.9a	10.2 ± 0.2ab	43.0 ± 1.2b	20.7 ± 0.8a	11.9 ± 0.4a	5.8 ± 0.3a	0.5 ± 0.02c
90	76.7 ± 8.9b	12.6 ± 1.0a	108.8 ± 6.0a	24.1 ± 1.6a	11.5 ± 0.4a	5.4 ± 0.1ab	1.1 ± 0.04a
120	73.5± 0.5b	11.8 ± 1.5a	36.5 ± 4.4b	18.9 ± 0.8ab	11.5 ± 1.0a	2.8 ± 0.3c	0.5 ± 0.05c

Tab. 4. Carbohydrates, total phenolic contents gallic acid equivalent, trolox equivalent antioxidants(TEA), catechin and quercetin equivalent flavonoids, total chlorophyll and carotenoids in **cowpeas** leaves harvested 30, 60, 90 and 120 dap The values represent means ± standard error, **g kg^{-1} dry weight** (DW). Means indicated with the same letters within each nutrient content were not significantly different at p<0.05 using Tukey´s test (n=6).

dap	Carbohydrates	Phenolics (GAE)	Antioxidants (TEA)	Quercetin	Catechin	Total Chlorophyll	Carotenoids
30	79.3 ± 3.3c	8.7 ± 0.2c	22.2 ± 0.5b	11.1 ± 0.7b	3.9 ± 0.3b	4.3 ± 0.2b	0.8 ± 0.0a
60	90.9 ± 1.9bc	9.3 ± 0.3bc	26.2 ± 0.2a	12.9 ± 1.0ab	8.0 ± 0.5a	5.7 ± 0.4a	0.9 ± 0.1a
90	105.9 ± 2.5a	10.2 ± 0.1b	27.4 ± 2.9a	12.2 ± 0.6b	2.8 ± 0.4b	5.3 ± 0.4a	0.9 ± 0.1a
120	97.8 ± 3.9ab	12 ± 0.2a	28.4 ± 1.5a	10.2 ± 0.5b	3.7 ± 0.3b	5.4 ± 0.5a	0.9 ± 0.0a

Carbohydrates are the major products of photosynthesis and play a central role in driving diverse biological processes from embryogenesis to senescence, by supporting and integrating functions and actions of internal and external regulatory signal. At 60 to 90 dap,

total sugars were high possibly due to the well-developed canopy of leaves presenting a high light-exposed surface area and hence a high photosynthetic capacity leading to an accumulation of photosynthetic assimilates (Kirigia *et al.*, 2018). By 120 dap, the decline in sugar concentration was noticed possibly due to translocation of much sugars from the leaves to the fruits, which can be considered as sinks for carbohydrates at this stage (Lemoine *et a*l., 2013). Alternatively, there was probably reduced photosynthetic potential due to aging leaves at 120 dap. The cross-link between various metabolites, phytohormones, regulation of sugar metabolism and transport, could also explain the variation of sugar concentration observed in this experiment (Eveland and Jackson, 2012).

Plant secondary metabolites, such as phenolics, flavonoids and antioxidants play important roles in human diet and in plant defense mechanisms (Wojakowska et al., 2013). The dynamics of the metabolites in both crops during growth and after harvesting were specific to each crop as reported by previous researchers (Mathiventhan and Sivakanesan, 2013; Akula and Ravishankar 2011). This indicates that different crops respond differently to various factors that either upregulate or downregulate production of secondary metabolites as well as their degradation. The higher levels after 60 dap implies that plants synthesize species-specific secondary metabolites after recognition and perception of various signals. These signals are derived from external biotic and abiotic factors as well as internal factors such as plant development stages (Veitch, 2007).

The physical characteristics of vegetables after harvested can easily be determined by greenness and freshness, key factors that affects market. Therefore, chlorophyll quantification can be used as a biochemical marker to evaluate the status and quality of green leafy vegetables during storage and development. Results in this study indicated higher chlorophyll contents 60 dap than at younger stages (30 dap), which can also be correlated with carbohydrates contents at this stage. The carotenoids did not show any significant difference between the four development stages of cowpeas but were higher at 90 dap in nightshade (Kirigia *et al.*, 2018).

Mineral nutrients

The measurement of Ca, K, Mg, Fe, Zn, Mn, P and Cu elements was carried out by inductively coupled plasma optical emission spectrometry (ICP-OES), while nitrates was quantified through flow injection analysis (FIA) method. Results revealed that cowpea and nightshade leaves are a rich source of minerals such iron, potassium, calcium, magnesium, phosphorus, manganese and zinc (Tab. 5 and 6). However, the mineral contents were observed to be stable during development of nightshade and no significant changes occurred 30 to 120 dap apart from Ca, Zn and Fe (Tab. 5). The Fe contents decreased with age while Zn increased (Tab.

5). These kind of changes in Fe and Zn at 120 dap could be as a result of remobilizations of such minerals which has been reported in various crops during reproduction phase and senescence (Kutman, *et al.,* 2011; Maillard *et al.*, 2015). The dynamics of mineral concentrations in cowpea leaves during development was specific for different elements (Tab. 6). Minerals such as phosphorous, zinc, potassium and magnesium, nitrates and copper were found to be relatively stable at different development stages (Tab. 6). Iron and manganese were higher at later development stages (559.9 ± 72 and 327.5 ± 11 mg kg^{-1}), while the calcium concentration was highest at 30 dap 19 g kg-1 and indicated a decline thereafter (Tab. 6). The minerals content were in the same range with some green leafy vegetables, including *C. gynandra* (Okonya and Maass, 2014). Although mineral nutrients contributes significantly in solving hidden hunger crisis in SSA, there exists scanty information about their dynamics during development of leafy vegetables.

Tab. 5. Mineral content in nightshade leaves (dry weight) harvested at 30, 60, 90 and 120 dap. The values represent means ± standard error. Means indicated with the same letters within a column were not significantly different at p<0.05 using Tukey´s test (n=6).

dap	N g kg^{-1}	P g kg^{-1}	K g kg^{-1}	Ca g kg^{-1}	Mg g kg^{-1}	Fe g kg^{-1}	Zn mg kg^{-1}	Mn mg kg^{-1}	Cu mg kg^{-1}
30	43.8 ± 4.9a	2.7 ± 0.3a	41.1 ± 1.4a	14.6 ± 1.3b	3.8 ± 0.4a	1.3 ± 0.5a	39.8 ± 1.9b	257.6 ± 44.9a	42.2 ± 12.7a
60	40.2 ± 3.1a	2.8 ± 0.1a	45.3 ± 2.3a	20.2 ± 1.1a	3.3 ± 0.3a	1.0 ± 0.0a	42.2 ± 3.7b	276.3 ± 20.4a	26 ± 2.2a
90	38.8 ± 1.6a	2.3 ± 0.1a	45.8 ± 2.7a	24.3 ± 0.7a	4.1 ± 0.2a	1.0 ± 0.0a	46.7 ± 2.4b	289.3 ± 16.2a	30 ± 1.6a
120	58.6 ± 14.4a	3.2 ± 0.4a	40.5 ± 3.1a	21.6 ± 3.9a	3.7 ± 0.5a	0.7 ± 0.1b	62.1 ± 4.4a	287.5 ± 18.4a	29.7 ± 3.8a

Tab. 6. Mineral content in cowpea leaves (dry weight) harvested at 30, 60, 90 and 120 dap. The values represent means ± standard error. Means indicated with the same letters within a column were not significantly different at p<0.05 using Tukey´s test (n=6).

dap	N g kg^{-1}	P g kg^{-1}	K g kg^{-1}	Ca g kg^{-1}	Mg g kg^{-1}	Fe mg kg^{-1}	Zn mg kg^{-1}	Mn mg kg^{-1}	Cu mg kg^{-1}
30	41.2 ± 0.3b	3.1 ± 0.2a	26.0 ± 1.8a	19.0 ± 0.6a	3.9 ± 0.1a	305.4 ± 4.3b	29.3 ± 3.8a	187.4 ± 10.0b	22.7 ± 3.7a
60	44.3 ± 1.3ab	3.5 ± 0.0a	24.5 ± 0.2ab	14.2 ± 0.1b	3.3 ± 0.1b	419.5 ± 16ab	32.2 ± 0.1a	275.1 ± 11.9a	14.9 ± 0.6a
90	41.2 ± 2.2b	2.7 ± 0.3a	20.5 ± 0.3b	12.1 ± 0.8b	2.5 ± 0.2b	371.9 ± 28b	24.6 ± 1.3a	218.4 ± 16.3b	14.7 ± 0.3a
120	47.3 ± 0.7a	3.4 ± 0.2a	23.5 ± 1.0ab	14.2 ± 1.1b	3.0 ± 0.0ab	559.9 ± 71.7a	33.2 ± 0.2a	327.5 ± 10.9a	17.2 ± 1.5a

Conclusion

The dynamics of phytonutrients composition in nightshade and cowpea leaves were influenced by harvesting methods and development stages. Harvesting method equally influenced the yields in nightshade. Harvesting by uprooting and cutting would offer higher yields than

continuous method. However, more nutrients can be retained in the leaves if harvested by picking of single leaves than if cut or uprooted. Phytonutrient contents in the two crops were optimal at 60 to 90 dap, which therefore is the recommended development stage for harvesting. Establishment of maturity stages or indices for harvesting of each ALV crop species, would ensure optimum nutrient accumulation for uptake in human diets and/or postharvest processing and may compensate for losses incurred under suboptimal storage conditions.

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DEDICATION

This thesis is dedicated to my Dad William Kirigia (RIP) and my son Victor Koome

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