Norwegian University of Life Sciences
Faculty of Biosciences
Department of Plant Sciences

Philosophiae Doctor (PhD)
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Sweet Potato Virus in Ethiopia - Detection, Characterization, Elimination and Management

Virus i søtpotet i Etiopia – påvisning, karakterisering, rensing og forebygging

Dereje Haile Buko

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Dedication

I grew up in rural family. My parent have never been to school. They raised 4 boys and 2 girls. I am the 5th born to them. They raised and equipped me to fit in all challenges of life. They taught me how to do farming and be a good student. When childhood friends of mine backed from their schooling due to many challenges, my parents overcome all the challenges and were the foundation of the reason who I am today. My father died when I was 11th grade student. After that we relied on our mother, the strongest lady who cared not only me but also my immediate elders and younger brothers to presume our education. Though you both are not here to see the fruit of the seed you saw, I dedicate this piece of work for the efforts you have put in our life today.

Abbreviation

GA_3	Gibberellic Acid
BAP	6-Benzylaminopurine
DAS-ELISA	Double antibody sandwiched- enzyme linked immunosorbent assay
CIP	International Potato Center
CMV	Cucumber mosaic virus
cDNA	Copy deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EIAR	Ethiopian Institute of Agricultural Research
ICTV	International Committee on Taxonomy of Viruses
OFSP	Orange-fleshed sweet potato
PCR	Polymerase chain reaction
PGRs	Plant growth regulators
NGS	Next generation sequencing
NAA	Naphthalene acetic acid () and (BAP)
RT-PCR	Reverse transcription polymerase chain reaction
SNNPR	South Nation Nationalities and Peoples Regions
SPFMV	*Sweet potato Feathery mottle virus*
SPCSV	*Sweet potato chlorotic stunt virus*
SPMMV	*Sweet potato mild mottle virus*
SPVG	*Sweet potato virus G*
SPVC	*Sweet potato viruses C*
SPVD	*Sweet potato viral diseases*
SPV2	*Sweet potato virus 2*
SRDS	Small RNAs deep sequencing
SPSMV	*Sweet potato symptomless mastrevirus*
SPBV	Sweet potato Badnavirus
TAS-ELISA	Triple antibody sandwiched - Enzyme linked immunosorbent assay
VIGS	Virus-induced gene silencing

Acknowledgements

This PhD study has come to an end but has won two main challenges. The first challenge was the struggle to fulfill PhD requirements to end the study and second was the worst political situation in my home country (2014 - 2018) which coincides with the main time of this PhD study. The impact of the later was immense on the moral and psychology of many of us who care human life matter. I appreciated those of you who have asked and prayed for us during that difficult time. Thank God, situation is improving a bit at present, however, challenges always continue, and finally the battle to get the PhD degree will also soon end.

The results presented in this thesis are obtained from experiments conducted in Ethiopia and at the Norwegian University of life Sciences (NMBU), Norway. All my research work and living expenses in Norway was supported by the NORAD funded collaborative project ‘‘Controlling disease in sweet potato and enset in South Sudan and Ethiopia to improve productivity and livelihoods under changing climatic conditions using modern technologies’’ under the NORHED program (agreement no ETH-13/0017, 2013). I am grateful for all the supports provided for the successfully completion my PhD studies. I am grateful to both NMBU and Hawassa University, respectively, for good working environment and granted my study leave during the period of my PhD study.

I would like to thank Prof. Trine Hvoslef-Eide, the main coordinator of the Enset-sweet potato NORHED project and the main supervisor of my PhD work, for allowing me work in this project and exercise academic freedom. Really, I enjoyed the academic freedom under your supervision. More than anything else, thank you for the flexibility in arranging travel during the fieldwork as well as during the distress time I had travel home. I have seen your determination and kindness and allowing students to do their best. Generally, I say thank you for your contribution in my success.

I also thank Dr. Carl Spetz, my co-supervisor. Carl, I know we argued sometimes. The reason I tried to argue with you was you are the expert to guide fresh student like me into a new field of

study. Finally, I found my argument was helpful and learned from your challenges. Therefore, I thank you for great input to my success.

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My special gratitude goes to Astrid and Gry, Silje, the laboratory technicians in the Plant Cell Lab, NMBU and Sissel Haugslien technician in the Virus lab at NIBIO, without them I would not be the one I am today. I learned many tissue culture techniques, mainly meristem cutting from Astrid, and plant managements in green house from Gry. Silje has been helping me in sub culturing my plants in tissue culture. The help received from Sissel during my virus detection in green house and laboratory is highly acknowledged. You were all so cooperative and helpful to me, I say thank you! Beside the hardship of PhD work, you were one among the few individuals who have been eager to hear and sharing troubles we have been in as a country, as nation.

My sincere friends and fellow Ethiopian students at NMBU: the time we had during these PhD studies is not only educational hardship, regardless of our sensitivity to it, we have been in very difficult times due to the bad political situation back home. During this all, our being together and discuss on our issues was very helpful to relieve of pain and stress. Particularly, I appreciate the encouragement I received from some of you, on your contribution in ideas, editing my papers etc.

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Last, but the most important, appreciations goes to my parents. My late father and late mother, you were the one brought me to this world, initiated and invested in my educational journey. You both never went to school, but you were respected hard working farmers, you taught us, that hard work is full of stress, but the fruit is mostly sweet. I thank you because you invested in education of your children and grandsons. I must say it was not God's will for you to see the final fruit of you sons.

However, you are still the reason why I am here today. I cannot get more than this opportunity to thank my late father who died when I was 11th grade student. I never forget your encouragement and determination to send others and me in the family to school during my elementary grades, which motivated me to this day highest academic achievement. My mother: you shouldered our family when our Dad went to rest for ever. You have seen most of the achievement of your sons and daughters, but you were also hurried to follow your husband at the time, before you were able to collect the fruit of your investment in us. I had a plan to fully serve you after my PhD journey, but my plan was too late according to God's plan and you also left our family. I do not have words to expresses your efforts to raise and educate us all under uncomfortable conditions. Therefore, I dedicate my PhD academic success to you both on behalf of all my family.

My Son Bari Dereje and daughter Barite Dereje, my Wife Melesch Tolosa, I know of you were alone at the time when you need my presence utmost. However, you may know that all my effort and success in the future is yours. Melesech, your kindness, trustworthy, and shouldering responsibility is amazing. Though you were a month a bride when I left you, you have never complained during my absence for this PhD work. Thank you for bearing responsibility in raising our beloved children during my absences. My grateful to all families and relatives who have been encouraging me throughout the period of my study and powered me for this success.

Funs during the stress times and coffee break: PhD is not life; Life is not all about PhD.

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Paper IV: Detection of viruses and elimination of Sweet potato feathery mottle virus (SPFMV) in high yielding varieties of sweet potato from Ethiopia (Submitted to Journal of Plant Protection, Elsevier)

Paper V: Next generation sequencing as a method to verify virus elimination using heat treatment and meristem tip culture in the five most widely used sweet potato varieties in Ethiopia.

Summary

The initiative to improve sweet potato production and productivity in Ethiopia began in the 1980s and so far, there are 26 improved sweet potato varieties made available for farmers. Lack of a rigorous quarantine scheme during exchanges of sweet potato germplasm for improvements may have been the main routes for introduction of sweet potato viruses into Ethiopia. Subsequently, the viruses have been disseminated with high infection levels in the country as a result. Viral diseases has been stated as the main reason for the declining productivity of sweet potato in Ethiopia during the last two decades. In spite of this, there have been few efforts to document the damage it causes, little knowledge on farmers' perception of the viruses, no efforts in generating high yielding varieties free from economically important viruses and no plan for such virus tested material for dissemination to end users. Furthermore, the tissue culture protocols to generate virus-free plant are often genotype specific and have not been optimized for a wider diversity of genotypes to use for virus elimination and further large-scale propagation.

This PhD thesis was generated as a subproject under a NORHED project. It has been designed to increase production and productivity of sweet potato in Ethiopia by addressing five specific objectives. The first objective was to review and document previous research findings that have been conducted on virus detections and eliminations in Ethiopia and bring it to further attention (Paper I). Through literature review and personal communication, we found few virus surveys and sweet potato virus elimination trials undertaken in Ethiopia for the last two decades. We found that *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato virus G* (SPVG), *Sweet potato virus* II (SPV2) and *Cucumber mosaic virus* (CMV) have been identified through surveys and that little virus elimination methods have been applied in Ethiopia. We concluded that intervention and actions are needed, and this is recommended in this review. We hope that this review paper will facilitate further actions by the authorities, research centers and extension workers, together with the universities.

The second objective was to investigate farmers' perception of virus (es), virus transmission, insect pests and their management in order to improve prevention and eradication of the

sources for infections in the future (Paper II). The majority of the farmers (64.7%) and a high proportion of the extension workers (41.2%) interviewed had low perception of sweet potato diseases caused by viral infection and its associated symptoms. A majority of the interviewed farmers know that insects feeds on sweet potato plants, but did not know if insects could transmit viruses from an infected plant to a healthy one. We conclude that neither the farmers nor extension workers have received adequate training related to sweet potato diseases and insect pest management. Training farmers and extension workers is highly advisable in order to enhance the management of virus diseases in Ethiopia.

The third objective was to optimize a rapid multiplication method for various many genotypes through tissue culture, as this would facilitate rapid propagation of cleaned stock plants (Paper III). Here we looked at 1) concentrations and combinations of cytokinin (6-benzylamino purine (BAP) and auxin)) and auxin (Naphthalene Acetic Acid (NAA)) for initiation of meristems of four high yielding sweet potato varieties, and 2) varying concentrations of BAP combined with Gibberellic acid (GA_3) for better shoot multiplication from single nodal cuttings of five Ethiopian sweet potato high yielding varieties. Concentrations and combinations of NAA and BAP significantly affected the initiation of shoot from meristems and the weight of callus produced per culture ($p< 0.05$). Moreover, there was a significant genotype x environment interaction. The highest success rate on average over all genotypes was 54% and was obtained with 0.1 mg/L NAA combined with 1 mg/L BAP. The number and height of shoot obtained per nodal cuttings was also significantly affected by the concentrations of BAP, the varieties and the interaction (all at $p< 0.05$). The highest number of shoot per plants was obtained with ½ concentration Murashige and Skoog's (MS) medium salts, supplemented with 2 mg/L BAP. This should be the first medium to try when new varieties are to be taken into tissue culture propagation.

Paper IV has two objectives: 1) detect the viruses infecting five selected high yielding sweet potato varieties from Ethiopia and 2) compare the elimination efficiency of meristem culture alone or meristem cultures combined with thermotherapy. We applied the following virus detection methods: a) infection test using indicator plant, b) enzyme linked immune sorbent assay (ELISA), and c) reverse transcriptase polymerase chain reaction (RT-PCR).

This was combined with virus elimination treatments (meristem culture alone or thermotherapy combined meristem culture) for the chosen five varieties. We found that grafting shoot tips of symptomless test plants on an indicator plant (*Ipomea setosa*) facilitates an easy detection of SPFMV using ELISA. *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV) have been previously reported, and were also detected in this study. We further report of *Sweet potato virus C* (SPVC) for the first time in Ethiopia. Heat-treating donor plants before meristem culture is more efficient for virus elimination than meristem culture alone. This method generated the highest number of plants free from the virus. The efficiency of each of these methods varied with the cultivars tested. A clean stock of five sweet potato varieties have been obtained to be used as further multiplication and use of virus free planting materials.

In the last paper (Paper V), we compared the efficiency of using reverse transcription PCR (RT-PCR) and Next Generation Sequencing (NGS) as a method to verify efficient elimination of viruses and detection of potential novel viruses in plants before and after virus elimination using VirusDetect software. NGS has confirmed the presences of SPFMV, SPVC and SPCSV previously detected in plants before virus elimination treatment by RT-PCR. Moreover, the NGS method detected some of the common viruses in some of plants before virus elimination where these viruses had not been detected using RT-PCR. In addition, NGS confirms the presence of three viruses in Genus badnaviruses all collectively belongs to species known as *Sweet potato pakkakuy virus* (SPPV) and *Sweet potato symptomless mastrevirus-1* (SPSMV-1) previously unknown to be present in Ethiopia in most of plants before virus elimination treatment and in some of plants after the treatment. Therefore, the use of NGS in virus certification schemes is more reliable than RT-PCR and can be used in the developing country like Ethiopia in the future, as the cost is getting lower and lower.

Sammendrag

Det ble tatt flere initiativ på 1980-tallet for å forbedre søtpotetproduksjon og øke avlingene i Etiopia. Så langt har det fremkommet 26 forbedrete sorter av søtpotet, og som er tilgjengelig for bønder. Mangel på strenge karantenebestemmelser ved utveksling av nytt foredlingsmateriale av søtpotet kan være årsaken til spredning av virus på søtpotet i Etiopia. Som en følge av dette, har alle deler av landet som dyrker søtpotet høy grad av virusinfeksjoner. Virussykdommer har oppgitt som hovedårsaken til at avlingene har gått tilbake de siste tyve årene. Likevel, har det vært liten innsats for å dokumentere skadene, liten kunnskap om hvordan bøndene opplever situasjonen i forhold til virusinfeksjoner, ingen innsats for å rense høytytende sorter for virusinfeksjoner og heller ingen plan for å starte rensing med etterfølgende tilbud om virusrenset materiale til bøndene. I tillegg til dette, er de vevskulturoppskriftene som finnes kun utviklet for noen få genotyper, og de er oftest svært genotypespesifikke. Det finnes ingen medier som er brukbare for en større bredde av sorter og med god mulighet for påfølgende masseformering, slik at bøndene kan få tilgang til rensede sorter.

Denne PhD-avhandlingen kom i gang som en følge av et delprosjekt under et NORHED prosjekt (2013-2019). Delprosjektet er laget for å øke produksjonen og avlingene i Etiopia ved å søke svar på fem spesifikke delmål. Det første delmålet var å sammenfatte tidligere vitenskapelige arbeider på virustesting og –eliminering i Etiopia. Dette belyses i **artikkel I**. Ved gjennomgang av litteraturen, samt personlige samtaler, fant vi at det har vært få undersøkelser i Etiopia omkring søtpotet de siste tyve årene. Vi fant at *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato virus G* (SPVG), *Sweet potato virus* II (SPV2) og *Cucumber mosaic virus* (CMV) alle er påvist, men at svært lite virusrensning har funnet sted. Vi anbefaler tiltak mot ytterligere spredning og håper at denne oversiktsartikkelen vil føre til tiltak fra myndigheter, forskningsstasjoner og veiledningstjenesten, sammen med universitetene.

Det andre delmålet var å se på hva bønder og veiledningstjenesten hadde av kunnskap om virus og virusspredning i søtpotet, samt effektive tiltak for å hindre avlingsnedgang i fremtiden

(artikkel II). Et flertall av bøndene (64,7%) og en høy andel av rådgivere (41,2%) som ble intervjuet hadde liten kunnskap om sykdommer i søtpotet og at disse kan være forårsaket av virus. De fleste visste at insekter spiser av bladene, men var uvitende om at disse kunne bidra til smitte fra syke planter til friske planter. Vi konkluderte med at verken bønder eller deres rådgivere i veiledningstjenesten har fått god nok opplæring i forhold til sykdommer i søtpotet og bekjempelse av skadelige insekter. Opplæring av disse to gruppene bør finne sted for å bedre mulighetene for å bekjempe virussykdommer i søtpotet i Etiopia.

Det tredje delmålet var å utvikle en masseformeringsprotokoll gjennom vevskultur som kan brukes til et bredere knippe av søtpotetsorter. Dette er nødvendig for å kunne rense for virus og tilby bøndene renset material (**artikkel III**). Vi utførte forsøk med 1) konsentrasjoner og kombinasjoner av cytokinin (6-benzylamino purine) og auxin (Naphthalene Acetic Acid) for å initiere meristem fra fire høytytende søtpotetsorter, og 2) forskjellige konsentrasjoner av cytokinin (BAP) kombinert med en konsentrasjon av gibberellin (GA_3) for øke skuddanningen fra enkeltskudd hos fem høytytende etiopiske søtpotetsorter. Konsentrasjonen og kombinasjonen av NAA og BAP hadde signifikant effekt på initiering av skudd og kallusvekten ($p< 0.05$). Videre var det et klart signifikant genotype x miljøsamspill. Det beste mediet ga 54,3% av meristemene med skudd; 0,1 mg/L NAA kombinert med 1 mg/L BAP. Antall skudd som ble dannet ved videre oppformering ble også påvirket på en signifikant måte av BAP konsentrasjonen, sortene og samspillet mellom disse ($p< 0.05$). Den høyeste skuddanningen var ved bruk av ½ saltkonsentrasjon av Murashige og Skoogs medium (MS), beriket med 2 mg/L BAP. Dersom man skulle begynne med en ny sort, er dette det første mediet man burde prøve ut.

Artikkel IV har to delmål: 1) undersøke virusinfeksjonen innen fem utvalgte høytytende søtpotetsorter fra Etiopia og 2) sammenligne effektiviteten til to rensestrategier for virusinfiserte planter; meristemkultur alene eller termoterapi etterfulgt av meristemkultur. Vi brukte følgende virusdeteksjonsmetoder: a) synlige infeksjoner i indikatorplanter b) enzyme linked immune sorbent assay (ELISA), og c) reverse transcriptase polymerase chain reaction (RT-PCR). Disse ble kombinert med virusrensing (meristemkultur alene eller termoterapi etterfulgt av meristemkultur) for alle fem sortene.

Poding av skudd fra symptomfrie planter ga utslag på indikatorplanten *Ipomea setosa*, og denne oppformering av virustiter ga muligheten for å påvise SPFMV ved bruk av ELISA.

Sweet potato feathery mottle virus (SPFMV) og *Sweet potato chlorotic stunt virus* (SPCSV) har blitt oppdaget tidligere, og vi fant også disse gjennom vårt arbeid. I tillegg fant vi *Sweet potato virus C* (SPVC) for første gang i Etiopia. Å varmebehandle donorplantene før meristemkultur er mer effektivt for å rense for virus enn meristemkultur alene. Kombinasjonen ga høyest antall virusfrie planter. Effektiviteten varierte med sort. Vi oppnådde å få virusfrie planter av alle fem sortene. Disse danner nå grunnlaget for videre masseformering og bruk av virusrenset material av søtpotet i Etiopia.

I den siste artikkelen (**artikkel V**), sammenlignet vi effektiviteten til reverse transcription PCR (RT-PCR) og Next Generation Sequencing (NGS) som metoder for å verifisere effektiv rensing av virus og påvisning av potensielle nye virus i planter før og etter virusrensing. Vi brukte VirusDetect software til denne analysen. NGS har bekreftet funn av SPFMV, SPVC og SPCSV som var funnet i plantene før virusrensing gjennom bruk av RT-PCR. Videre påviste NGS at det fantes vanlige virus før virusrensing, som ikke var plukket opp ved hjelp av RT-PCR. I tillegg påviste NGS tilstedeværelsen av tre virus innen slekten badnaviruses, som alle tilhører arter er som kjent som *Sweet potato pakkakuy virus* (SPPV) og *Sweet potato symptomless mastrevirus-1* (SPSMV-1). Disse er aldri før påvist i Etiopia og de ble funnet i flesteparten av plantene før virusrensing og i noen etter rensing. Vi kan konkludere med at NGS er mer pålitelig enn RT-PCR og at denne metoden vil kunne brukes i fremtiden av land i den tredje verden, slik som Etiopia, fordi prisen går stadig nedover.

1. General Introduction

1.1 Importance and production status of sweet potato

Sweet potato (*Ipomoea batatas* L. Lam.) is a herbaceous perennial dicotyledonous plant belonging to the *Convolvulaceae* family that is usually cultivated as an annual root crop. It is thought to be indigenous to Central or South America (Purseglove 1968) and widely distributed and grown worldwide (Onwueme 1978). Sweet potato is the world's seventh most important crop, according to (FAO 2017). Africa is ranked second, after Asia, in sweet potato production (FAO 2017, Fig. 1). In Africa, sweet potato, cocoyam and cassava are the most commonly produced root crops and they are mainly used for human consumption (Apata and Babalola 2012). Ethiopia has been ranked fifteenth in the world in terms of sweet potato production (Jones et al 2013). Sweet potato is commonly propagated using stem cuttings (McEwan 2016), with the risk of transmitting diseases to the next generation. Major constraints to sweet potato production in Ethiopia include a lack of quality planting materials, pests and diseases, and underdeveloped markets (Jones et al 2013).

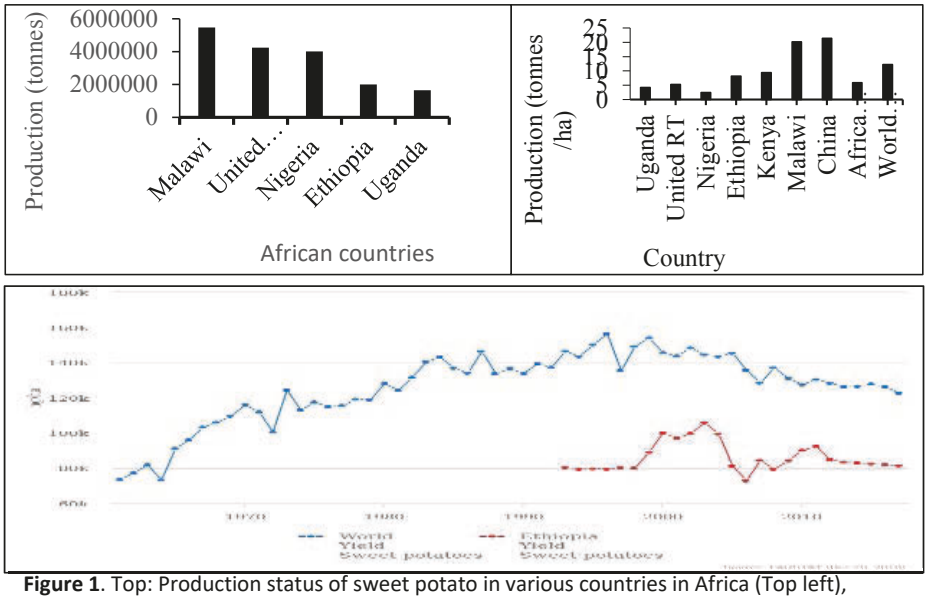

Figure 1. Top: Production status of sweet potato in various countries in Africa (Top left), including Ethiopia and production per ha (Top right). Bottom: The world statistics for sweet potato yields over the last 50 years compared to the yields in Ethiopia (FAO, 2017).

Sweet potato is among the common food crops and has been cultivated in Ethiopia for a long time. It's arrival in Ethiopia is uncertain, but one of the hypothesis is that sweet potatoes are thought to have been introduced into Africa either by the Portuguese to Mozambique and possibly Angola directly from Lisbon in the sixteenth century. An alternative could be as late as the nineteenth century via British influences from India (O'brien 1972). It is mainly cultivated by smallholder farmers and to less of an extent by commercial vine producer farmers. Ethiopia is ranked sixth in the world, by volume, in sweet potato production, having 246,503 ha of total area allocated and 2,008,290.00 tonnes harvested (FAO 2017). However, according to the world atlas, Ethiopia is the fourth largest producer in the world, with an annual production of 2,701,599 metric tons of sweet potato(https://www.worldatlas.com/articles/top-sweet-potato-growing-countries.html).

Whichever is correct, Ethiopia is one of the leading producers of sweet potato in the world and is cultivated in most of the regions of Ethiopia. However, the main producing regions are Southern Nations Nationalities and Peoples Region (SNNPR) and Oromia. These two regions cover over 95% of the country's annual sweet potato production (Central Statistical Agency 2010). More recently, improved varieties of sweet potato are being introduced to the different regions of Ethiopia, in particular to those experiencing frequent shortages of rain (Shiferaw et al. 2014; Aldow 2017). Many national and international organizations are involved in promoting the distribution of sweet potatoes into new locations. For example, the International Potato Center (CIP), Mums for Mums in collaboration with the Ethiopian Ministry of Agriculture and the Ethiopian Institute of Agricultural Research (EIAR), are all working hard to promote the orange-fleshed varieties (Aldow 2017). Consequently, the production area and volume are increasing in Ethiopia, and as well as in Africa [(FAO 2017) Fig. 1)]. However, the national average yield per hectare (8.15 tonnes) is declining and is far behind the world average of 12.26 tonnes (FAO 2017).

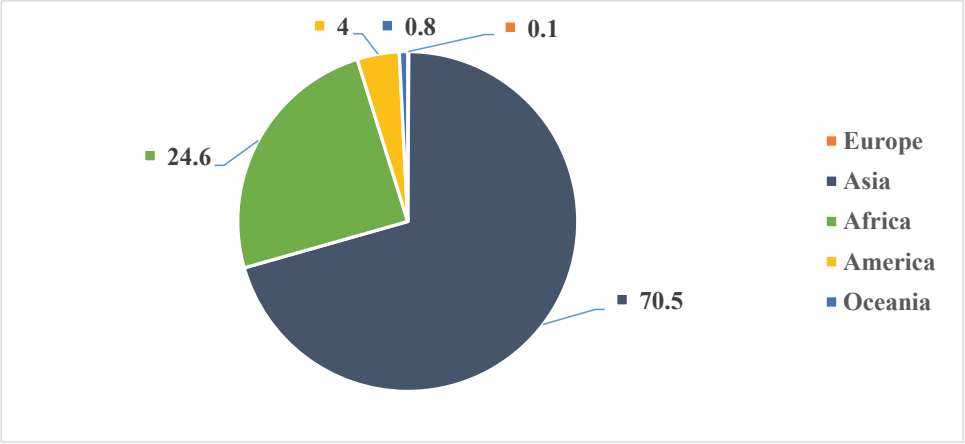

Figure 2. Production share of sweet potatoes by global region (%)

Sweet potato is a multipurpose crop. It is grown primarily for tuberous roots and vine tips for human consumption, as well as vines for planting materials and for animal fodder. Sweet potato is a crop with a high nutritional value and is classified as the most energy-rich tuber. In addition, sweet potato plant provides products for industrial purposes. It provides raw materials for starch and alcohol production. It is also used for the production of amylases, pectins, anthocyanins, carotenoids and the use of root residues as a culture medium (Woolfe 1992).

Due to its different attributes, sweet potato is a widely grown plant. It is grown worldwide because of its easy management, strong adaptability to the environment, high productivity per unit of area and time, nutrient rich, and it serves as a food security crop where other food crops fail to perform (De Vries et al. 1967; Bovell-Benjamin 2007). Furthermore, growers prefer it because it can be harvested for piecemeal as needed, due to its flexible growing season over 3-to 10-months, thus offering a flexible source of food and income to rural households. It is among the candidate crops for bio fortification – to improve vitamin A, iron and zinc deficiencies in many African countries (Asare-Marfo et al. 2013).

1.2 Sweet potato development work in Ethiopia

From the 1980s, research has been performed in Ethiopia to improve sweet potato production. Previous work primarily focused on yield related attributes. To date, around 26 sweet potato varieties have been evaluated and selected for improved yield potential (Kosmowski et al. 2016; Gurmu et al. 2017). Both white and orange-fleshed sweet potato varieties have been released. Most of the sweet potato varieties in sub-Saharan Africa (including Ethiopia) are white-fleshed. The white fleshed cultivars are preferred among growers and consumers in Ethiopia. However, from 2001 to 2003 alone, over 40 orange-fleshed sweet potato (OFSP) cultivars were introduced into Ethiopia and evaluated for yield performances. The five best performing OFSP varieties were approved for use and some of them are under production (Tofu et al. 2007, ARC, 2015). However, the farmers resisted OFSP expansion due to its poor acceptability of dry matter and poor cooking quality. Furthermore, at present four new OFSP varieties with better yielding ability, root quality and beta-carotene content are in the pipeline at the Hawassa Research Center. The grower and consumers are traditionally used to the white fleshed types. Previous reports show that, less research has been performed on cultivar resistance to diseases and insect pests than to other aspects. Four varieties were selected for relative tolerance to general viral diseases, most recently (Gurmu et al 2018). However, the yield loss due to viral diseases and insect pests remain the main challenges for sweet potato production in Ethiopia (Adane 2010; Tesfaye et al. 2013).

1.3 Constraints of production

Several abiotic and biotic factors affect the production of sweet potato and of it reaching its maximum agricultural potential in Ethiopia. Drought stress, limited land availability, shortage of planting materials, shortage of improved varieties, lack of virus-tested materials, post-harvest problems such as storage, and access to market, low market prices, lack of knowledge on processing and preservation are among the abiotic factors limiting the crop's production (Gurmu et al. 2015; Markos and Loha 2016; Fite T 2014). Similar production constraints were reported from Tanzania (Ngailo et al. 2016).

Insects and diseases are the two biotic constraints of sweet potato production (Gurmu et al. 2015). Sweet potato weevils and butterflies are the two most detrimental insect pests of sweet potato in Ethiopia (Azerefegne 1999; Mebrate 2018). Viral, fungal and bacterial diseases are also common in sweet potato. Virial diseases are the most limiting factor of sweet potato production in Ethiopia (Mebrate 2018). Propagation is largely based on vegetative asexual reproduction, through the use of vine cuttings selected from the previous crop (Gaba and Singer 2009). High viral diseases incidences in farmers' fields are mostly attributed to the use of vines from previous seasons, as well as the year round cultivation of the crop (Aritua et al. 1998).

1.4 Plant viruses and damages it causes on crops

Plant-infecting viruses have been studied for over 120 years. *Tobacco mosaic virus* (TMV) was the first virus to be described in the Netherlands (Beijerinck 1898). Thereafter, thousands of viruses infecting different species of plants have been described, characterized and identified in the world [International Committee on Taxonomy of Viruses (ICTV, 2108)]. Plant-infecting viruses are defined as infectious, obligate and intracellular parasites that are only able to replicate inside colonized living cells of its host (Hull 2013).

Viruses are the second most important plant pathogens worldwide after fungus (Vidaver and Lambrecht 2004). Viruses cause economic, social, and environmental impact (Wilson 2014). Viral diseases are the cause of large losses of important crops all over the world which threatens food security (Lal et al. 2015). For example, virus infected plants are small in size and weak that results in reduced quantity and poor quality of the produce as well as reduced market value (Nicaise 2014). Moreover, virus infection predispose the plants to insect pests and other pathogens (Wilson 2014). The damage to a crop by viruses varies depending on the virus type, host plant, type of interaction between the infecting viruses (synergistic or antagonistic), host-virus interactions and environment. It is difficult to estimate losses caused by plant viruses. However, plant diseases cause an estimated 15% loss of overall crop production (Boualem et al. 2016) of which plant viruses account for 47% (Hull, 2013). Annual worldwide economic losses due to plant viruses is estimated at billions of US dollars (Jeong et al. 2014; Sastry and Zitter 2014).

Viruses use the metabolic machinery of the host cells by using host proteins for replication, and interfering with intracellular trafficking systems which all leads to inhibition of plant growth, causing significant economic yield losses (Gergerich and Dolja 2006; Wilson 2014). Although many viruses do not produce disease symptoms, they adversely affect plant metabolism and increase, progressively, over time with repeated vegetative propagation (Wang P and Hu 1980). Virus infected plants are often characterized by stunted growth, reduced vigor, poor product market values and a total loss of yield in the worst cases. Plant viruses in the family of *Potyvirus* are most studied plant-infecting viruses; which also encompass the most common sweet potato infecting viruses.

1.5 Sweet potato viruses identified in Ethiopia and worldwide

Sweet potato plants are vegetatively propagated, usually prone to virus infection and hence, more than 30 viruses infecting sweet potato were identified worldwide (Clark C et al. 2012). However, only some of these viruses cause economic yield losses (Clark C et al. 2012). *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), and *Sweet potato mild mottle virus* (SPMMV), *Sweet potato virus G* (SPVG) and *Sweet potato virus C* (SPVC) are among the most commonly detected pathogenic viruses of sweet potato. The mixed infection of SPCSV with unrelated viruses, such as SPFMV and/ with other common sweet potato infecting *Potyvirus,* has been designated as Sweet potato viral diseases (SPVD), which is the most severe disease of the crop (Karyeija et al. 2000).

Virus infection is the second most limiting factor of sweet potato production in Ethiopia (Fite et al. 2014). Although the first virus was reported in 1986, the high severity and the geographic coverage of sweet potato viruses was recognized in southern Ethiopia during 2006 to 2009 (Shiferaw et al. 2014). SPFMV, SPCSV, SPVG and Sweet potato virus 2 (SPV2) and *Cucumber mosaic virus* (CMV) have previously been identified and reported from Ethiopia (Adane 2010; Tesfaye et al. 2011). These viruses were identified in limited production regions in Ethiopia, mostly in SNNPR (Reviewed in paper I). As a consequence of the limited geographic coverage of the surveys, the countrywide status of infection is unknown. As previously stated, the total land area under sweet potato cultivation is increasing whereas the national yield average is far below

the world average, and is declining. Among possible reasons for this yield decline in Ethiopia are; lack of virus tested planting materials and farmers use of their own infected planting materials over several seasons. Moreover, there is also poor sweet potato disease management in the fields, as farmers are unaware of this threat (Buko et al, manuscript 2). These challenges affect sweet potato production and limit its potential contribution to national food security.

1.6 Importance of virus-free sweet potato planting materials

Using virus-tested sweet potato vines improves root yield and root quality compared to infected vines. Several studies reported that planting virus-free plants increases the root diameter and root weight and gives thicker and more vigorous roots compared to virus-infected ones (Kano and Nagata 1999; Bryan et al. 2003; Alam et al. 2013; Adikini et al. 2016). However, obtaining the yield advantage depends on the cultivars. For instance, compared to farmers' materials, planting virus-free vines increased the yield by 12-49% in Korea depending on the cultivars planted (Yoo and Lee 2013). Furthermore, virus-free plants produce larger above ground biomass (more weight of the leaf and the stem of the plant) and provides better physiological functions compared to virus infected plants (Kano and Nagata 1999). We have also demonstrated that virus-free sweet potato plants yield higher total root and marketable mass than virus infected plants (own unpublished data). As a country, Ethiopia has missed out on the advantages of improved yields and quality from planting clean materials. Better yields and an improved quality of sweet potato can only be obtained by initiating disease-free plant stock. Using "pathogenic virus free" planting materials is economically viable if there is an effective and efficient system for their production, multiplication, and distribution (Carey et al. 1997; Feng et al. 2000). "Pathogenic virus free" plants are obtained by screening healthy crops from virus-infected fields or by eliminating viruses from infected plants of desired traits (preferred genotype).

Plant viruses spread, predominantly, through infected, symptomless planting materials that farmers collect from the previous season's crop and then use for the next cropping cycle (Rukarwa et al. 2010). In Ethiopia, lack of access to certified disease-free sweet potato plants remains the biggest challenge and as a consequence the majority of farmers use their own planting materials over many seasons (Mebrate 2018). Such year-round production of infected plants remains a

source of virus infection and a host for virus vectors (Aritua et al. 2007). These practices assist the widespread dissemination of viruses in southern Ethiopia. The pressure of virus disease can be effectively reduced if the management measures are applied at the initial step of disease development and by planting virus tested planting materials (Jeong et al. 2014). Moreover, virus tested plants are important for the safe import of novel cultivars, germplasm exchanges for breeding purposes as well as preservation of germplasm (Imarhiagbe et al. 2016). The current problems related to the spread of viruses in the country might be reduced through production, multiplication and distribution of certified planting materials to the growers.

1.7 Testing methods of plants virus

Accurate diagnosis and detection of the pathogenic viruses is essential for prediction of crop losses, obtaining virus-free plants, use of quality seeds and to apply virus management practices (Van der Want and Dijkstra 2006; Aboul-Ata et al. 2011; Wang B et al. 2011). Many methods, from the traditional to the latest next generation sequencing (NGS), have been developed and made available for testing of viruses that infect plants. Standard methods like Enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR) and grafting are the most commonly used techniques for virus detection (Hull 2013). For reliable and accurate detection, two or more detection methods that depend on the different properties of viruses are recommended. Each testing method has advantages and limitations. Some methods determine the shape and the size of the plant virus particles (Loebenstein 2001), others are based on the proteins and nucleic acids sequences of the infecting viruses, whereas some others do not need a template or antibody for detection. Some are highly sensitive, others less so, both rapid and non-rapid techniques are used for diagnosis of plant viral diseases. The different methods are described below in more detail.

Grafting test plant to indicator plant,

Ipomoea setosa Ker Gawl is important for screening sweet potato plants for detection of a viral infection since it is extremely susceptible to many viruses, developing severe symptoms that can

easily be observed. In many parts of the world, grafting a small section (2 to 5 internodes) of sweet potato shoot tip to *I. setosa* is the common method to determine the presence, or absence, of virus infections. Grafting is important as virus-induced symptoms may or may not develop in infected cultivated plants. Grafting helps to separate between healthy and infected plants, based on the presence or absence of symptom development in the indicator plants after certain weeks/months following graft inoculation. However, it involves tedious activities and requires a lot of green house space and some investment. Grafting on to *I. setosa* requires skill in making the cut on the *I. setosa* plant and the sweet potato cutting to be of approximately the same size, to line up the cambiums for joining the vascular tissue of both plants. Unless this is successful, the graft will fail to grow. Using *I. setosa* as a detection method will only provide the information that a virus is present, but it will not be able to determine type of the infecting virus. Nevertheless, grafting is particularly helpful to determine the presence of virus/es infections in symptomless plants. It can also be helpful with boosting latent viral infections, so that other methods can better detect the virus (as described in Paper IV).

Enzyme-linked immunosorbent assay (ELISA)

As a virus detection method developed in 1970s (Clark and Adams 1977), ELISA makes use of labelled antibodies developed against a particular virus, which is coated on the bottoms of a 96-well plate. Through exposure to the virus and a series of controls, the label is developed to form a yellow precipitate where the virus binds to the antibody. The intensity of the yellow color can be read by a colorimetric ELISA-reader and quantified. ELISA is relatively easy to work with, cost effective and can be used for large sample series in 96-well plates. It has been used as a routine diagnostic method to check the phytosanitory status of plants and for the quarantine purposes/virus certification (Torrance and Jones 1981). It needs high quality antisera to correctly identify virus strains. However, ELISA lacks the sensitivity, flexibility and compatibility compared to more modern molecular methods, in particular when many pathogens need to be tested for in the same crop (Boonham et al. 2014). The dependence of ELISA on antibodies for the detection, limits application to detecting only known viruses, where antibodies have been developed for each virus. Moreover, sometimes the detection may not be reliable when the titer concentration is below the

detection limit of ELISA. Therefore, there are many known (and, of course, all unknown) viruses where ELISA cannot be applied for detection.

Molecular detection methods

Molecular techniques were developed in the 1990s and are commonly used in many laboratories to test for plant viruses. Depending on the genome of targeted virus, PCR and RT-PCR have been the most common molecular methods for virus testing (Webster et al. 2004). PCR is a molecular technique that amplifies segments of DNA molecules to increases the amount of genetic material in a sample to a level where it can easily be detected. For the amplification, the DNA samples and all other necessary reagents are first heated to denature the double stranded DNA molecules in to two single stranded ones. Primers bind to specified sequences of DNA where enzymes, called polymerase, bind themselves to synthesize a new strand of DNA from each template strand. This procedure is repeated in many cycles to amplify the specific sequences. Once amplified, the DNA samples can be used in many different laboratory procedures.

RT-PCR works for RNA in a similar way, except that the RNA molecules has to be transcribed into copy DNA (cDNA), which can then be amplified following similar principles as for DNA molecules using regular PCR.

DNA viruses can be directly tested using PCR. Whereas for RNA virus RT-PCR is used. They both have a high accuracy and high sensitivity compared to the ELISA method (Jeong et al. 2014). However, PCR and RT-PCR also have their own limitations. Both require sequence information of the target virus; i.e., PCR and RT-PCR cannot be applied when genetic information of the targeted viruses is not available. Furthermore, sometimes the detection may not be reliable when the titer concentration is low and/or the primer annealing is poor. Fortunately, pathogenic viruses of sweet potato have been detected using RT-PCR in many countries (Clark et al. 2012) making it easier to detect viruses using these techniques.

Small RNAs deep sequencing (SRDS)

Small RNAs deep sequencing techniques have become a common way for detection in recent years. SRDS has been used as a tool for the identification and reconstruction of both known and unknown RNA and DNA viruses, in single and in mixed infections (Pooggin 2018). Several new viruses infecting plants and vertebrates have been discovered using this method. It has become a preferred method in recent years because of its many advantages over the other detection methods discussed previously (sections 1.7.1 to 1.7.3). SRDS increases accuracy and rate of virus discovery (Muhire et al. 2013). In addition, it avoids the cross-linking reactions which can be the case in ELISA, as well as the separate detection of RT-PCR for viruses of different genome types. Moreover, it does not require the prior knowledge of the sequence information of the target viruses (Bi et al. 2012; Kashif et al. 2012). This method is able to recover the viruses from diverse species having combined effects in the plants.

In general, developing a new detection method or improving existing methods is constant as society and technology advances. Therefore, simplicity, speed, specificity, sensitivity, robustness, cost-effectiveness and the information required are the major criteria for selecting optimal detection techniques for any particular study (Gong et al. 2019). In addition to these considerations, a major constraint is often the capital investment required for acquiring the equipment and accessories needed. In reality, not all of the laboratories in Africa are equipped with the latest technologies and may have to settle for the second, or third, best options that fit their circumstances.

1.8 Methods of pathogenic virus elimination in plants

Meristem culture, thermotherapy, chemotherapy and cryotherapy are methods of virus elimination from infected plants (Wang and Valkonen 2008; Dugassa and Feyissa 2009; Hull 2013; Mwangangi 2015). Virus-free plants can be obtained from previously infected plants of a species using one or a combination of virus elimination methods. Some of elimination methods exploit the fact that viruses are unevenly distributed in plants (Holmes 1948; Kassanis 1950; Ng et al. 1992; Panattoni et al. 2013; Mwangangi 2015). Part of a plant, mostly the meristematic tissue, may be free of infections, since diseases are often distributed in the vascular tissue of the plants and meristems have not differentiated vascular tissue. Meristem culture, where only a small portion of the shoot tip is excised, alone or in combination with one or more of these methods, was applied to many plant species. Consequently, virus-free plants have been generated in many cases (Panattoni et al. 2013). It is often a balancing act between taking such a small portion of the plant that ensures no cells with the infections are included, and taking a large enough portion to ensure survival of the meristem and further development into a shoot, potentially free from the disease(s) (Hvoslef-Eide, NMBU lectures in BIO244).

Virus eradication using thermotherapy is the oldest method and has been used since the end of the nineteenth century (Loebenstein et at. 2001). Thermotherapy treatment of plants was initially applied for making cellular environment progressively less suitable for virus vitality or inhibit virus multiplication, as heat often kills microorganisms, or reduce the growth (Baker 1962, Pennazio, 1995). It is applied because thermal sensitivity of plants is usually higher than that of the viruses. An elevated temperature will, therefore, be tolerated by the plant cells, but to a lesser degree by the viruses and the plant will grow, while the virus is staggered. The distance between the vascular tissue with virus and the meristem dome will increase, with a better chance of obtaining a meristem without the virus. Viruses of many different families of plants have been eradicated using thermotherapy followed by meristem culture (Panattoni et al. 2013). In order to eliminate virus using a combination of thermotherapy and meristem cultures, virus infected plants are exposed to a certain temperature range. The temperature to apply depends on the survival limits of the plant species, the thermal sensitivity of the targeted virus and virus-host combination (Spiegel et al. 1993; Laimer and Barba 2011; Panattoni et al. 2013). Thermotherapy reduced the

virus titer contents in infected raspberry shoot tips by degrading products of the viral RNA (Wang et al. 2008). It's effect being different with different virus types as it works best on viruses with an isometric shape than those that are round in shape (Panattoni et al. 2013; Wilson 2014). In contrast, a moderately increased temperature favors high cell division and plant growth increasing the chance of getting virus-free meristematic cells (Laimer and Barba 2011). Virus-induced gene silencing (VIGS) operates ineffectively at low temperature and the host plant's defense immune system improves at a higher temperature (Panattoni et al. 2013). Several studies have confirmed the relation between temperature and VIGS (Szittya et al. 2003; Chellappan et al. 2005; Qu et al. 2005). According to these authors, a rising temperature during heat treatment decreases symptom severity and elevates levels of virus-derived siRNAs. Similar results were recorded from cassava Gemini virus-induced RNA silencing, which also increased with rising temperatures (Chellappan et al., 2005).

High temperatures induces stress to plants (Rao et al. 2006; Wahid et al. 2007), so there is a limit to how high temperatures is beneficial for the purpose of slowing down the virus, but maintaining growth in the plant. It is reported that, thermotherapy is potentially effective in eradicating viral particles present in cells but it is not effective on the new synthesized viral particles (Panattoni et al. 2013), in other words; the thermotherapy has its limitations.

Chemotherapy is initiated and well-studied in clinical medicine (Panattoni et al. 2013). It is described as an application of chemicals to infected tissue/cells, targeting the reduction of virus replication. The most promising antiviral chemicals tested, in chemotherapy of potato, is ribavirin (Landesamt et al. 1953). Virus content in infected plants decreased after chemo-treatment (Yang et al. 2014). It makes for an easy excision by increasing the size of the virus-free meristematic tissues. The concentration of the chemicals, the cultivar sensitivity and the virus species also affects the efficacy of chemotherapy methods (Cordeiro et al. 2003). Chemotherapy is ineffective at lower dosage but also kills/inhibits growth of the host plants at higher dose (Yang et al. 2014). A summary of virus elimination experiments using chemotherapy between 1991-2010 indicates that chemotherapy was more successful on herbaceous plants than to woody plants, with a 66.0% and 34.0% success rate respectively (Panattoni et al. 2013). It has been applied to potato, tobacco, orchid, grapevines, apples and plum. It is applied to viruses in 9 families and un assigned genus

(Panattoni et al. 2013). Mechanisms of action is poorly understood as it has not been well investigated.

Meristem culture consists of culturing a small (0.1–0.5 mm) piece of tissue removed from the meristematic area of shoot tips or root tissue on a nutrient medium. It is used as the routine method and as the standard virus eradication technique (Sastry and Zitter 2014). Apical meristems without leaf primordia is excised to obtain virus-free plantlets (Verma *et al*., 2004). Meristem culture is the standard method for producing virus-free potato, sweet potato and many other crops (Danci et al. 2012). The success in plant virus elimination by meristem culture is mostly influenced by the size of the meristems, the species of plant, and the species of the viruses (Loebenstein 2001). As summarized by Wang et al (2018), the elimination efficiency of common potato viruses is influenced by the size of the meristem. Generally, the smaller the size of the excised meristems, the higher the success of virus elimination, indicating that virus content within smaller meristems to be less. However, there is a limit to how small parts of the meristem that will successfully be developed into a shoot and rescued. It was reported that the duration of the treatment and the type of cultivar affects the successful eradication of potato viruses, rather than the type of virus infecting the plant (Waswa et al. 2017). The pre-treatment of donor plants to enhance virus eradication by thermotherapy and chemotherapy has proven to be quite useful in achieving the maximum number of virus-free plants (Ruiz et al. 1998; Aguilar-Camacho et al. 2016).

To enhance viral elimination and regeneration of virus-free plants, meristem culture in combination with one or more virus elimination therapy methods is recommended. For example, the best results of Potato virus Y (PVY) elimination was obtained from chemotherapy and thermotherapy (Cordeiro et al. 2003). Combined chemo-thermotherapy significantly reduced the damage of the plants and increased the efficiency of virus elimination in potato plants (Antonova et al. 2017). We have obtained a high degree of virus elimination efficiency from combined heat treatment and meristem culture in sweet potato (Paper IV).

1.9 Objectives of the study

This study is part of a larger project funded by Norad (Norwegian Agency for Development Cooperation) through the NORHED Programme 2013-2019. ***This thesis focuses on the research needed to establish a system for virus indexing, virus elimination and clean stock plant propagation for sweet potato in Ethiopia.*** To fulfil these aims, a review of the literature, a survey of the current knowledge of farmers and extension workers, as well as the scientific experiments has been undertaken. The main aim of this study was to provide basic data required for development of a virus management regime for sweet potato production in Ethiopia, and thereby contributing to productivity and improve farmers' income through high-yielding, virus-tested planting materials.

1.9.1 Specific objectives

I. To review the literature on identified sweet potato viral species and the level of incidence and impact of the diseases on sweet potato production in Ethiopia

II. To assess farmers' and extension workers' perception of sweet potato virus disease, it's transmission and management

III. To evaluate the effects of the concentration and the combinations of plant growth regulators on the initiation of meristems and the multiplications of shoots of five sweet potato varieties with the aim of developing a protocol for cleaning sweet potato genotypes from viruses

IV. To identify the most common viruses and their elimination from the most important sweet potato varieties in Ethiopia

V. To evaluate the efficiency of pathogenic virus elimination methods

VI. To compare the efficiency of next generation sequencing and RT-PCR as verification methods of virus-free materials

2. Materials and Methods

2.1 Plant materials and experimental sites

Five high yielding sweet potato varieties (Table 1) obtained from institutions and farmers' field in Ethiopia were used in the virus detection, and virus elimination experiments (**Paper IV and V**) and media optimization experiments (**Paper III**). *I. setosa,* a universal indicator plant was used for virus infection tests (**Paper IV**).

Table 1. Descriptions of Ethiopian sweet potato varieties used in this study

Varieties	Number of plants collected	Source	Yield (qt/ha)	Year released	Root flesh color	Maturity Period
'Berkume'	3	Haramaya University	322	2007	White	3-4 months
'Guntute'	3	HARC*	354	1996	Orange	4-5 months
'Hawassa-83'	3	Farmers field in Wolayta zone	366	1990	White	5-6 months
'Kulfo'	3	HARC	270	2005	Orange	4-5 months
'Tola'	3	BARC**	322	2012	White	3-4 months

*HARC: Hawassa Agricultural Research Center, **BARC: Bako Agricultural Research Center

Most of the experiments were conducted at the Norwegian University of Life Sciences (NMBU), Norway and small parts at Hawassa University (Haw U), Ethiopia. The PhD thesis is the result of data generated from field and laboratory experiments in both countries.

2.2 Methods

In **Paper I**, literature on sweet potato virus surveys, testing and virus elimination activities that have been carried out to date in Ethiopia were reviewed. Information has been collected from the published articles, unpublished research work, field visits and personal communication with farmers. In additions, we added our own experiences on the subject matter after visiting fields. Information from the literature was summarized and presented in tables and figures. Explanation and summary of the relevant literature were made based on the summarized data and personal suggestions, recommendations and conclusions have been made.

For the second objective (**Paper II**), 160 house head farmers and 34 extension workers were interviewed to generate the data. This survey was conducted in 2015, in three districts: Sodo Zuriya and Boloso Sore districts in the Wolayta zone, SNNPR and Tulo district in the west Hararge zone; Oromia region. The districts were selected based on volume of sweet potato production and reports from previous studies (incidences of sweet potato diseases as well as insect pests). The interviews were conducted using a semi-structured questionnaire that was developed in English and later translated into the local language to avoid language barriers. Well-trained interviewers conducted the interviews. The interviews were conducted face to face sitting nearby the respondents' sweet potato fields. Data were collected on various parameters and selected later based on the availability of enough information. The data were analysed using Statistical Packages for Social Sciences (SPSS: Version 25.0).

The third paper (Paper III) was to optimize best media for meristem initiation and shoot multiplication of different sweet potato varieties. Four concentrations of both NAA and BAP were combined into 16 different combinations in a factorial manner (Paper III; Table 1A). Each combination was tested for meristem initiations of four Ethiopian sweet potato varieties (Kulfo, Berkume, Guntute, and Tola). In addition, five different concentrations of BAP combined with one concentration of Gibberellic Acid (GA_3) were optimized for multiplication of shoots of the five varieties: 'Kulfo', 'Berkume', 'Guntute', 'Tola', and 'Hawassa-83' using nodal cuttings (Paper III: Table 1B). All of the experimental procedures, experimental design, and tissue culture

practices and culture conditions conducted were presented in the Materials and Methods section of Paper III. The meristem survival, initiation of shoot from meristem, number of shoots produced per explant and the height of shoots were recorded, and the data were analyzed using Minitab Software V.18. These protocols are a prerequisite for the elimination of viruses performed in Paper IV.

In Paper IV, leaf samples of 15 plants (three plants from each of the five varieties listed in Table 1) were tested for common sweet potato infecting viruses: SPFMV, SPCSV, SPVC, SPV-2, SPVG, and SPMMV before virus elimination treatments. Three different methods were used to test the virus presence and its identification (Paper IV). First, shoot tips from each of the initial genotypes were grafted onto *I. setosa* following detailed procedures as described in Paper IV. Viral disease symptoms development, if any, were checked and recorded for a period of at least six weeks after grafting. Infecting viruses, if any, were identified using double antibody sandwiched - enzyme linked immunosorbent assay (DAS-ELISA) and triple antibody sandwiched - Enzyme linked immunosorbent assay (TAS-ELISA) procedures according to (Clark and Adams (1977) with minor modification detailed in Paper IV. In addition, RT-PCR was used as the main test for SPVG and SPVC, and as a confirmation test method of other viruses previously tested by ELISA. Detailed procedures of RNA isolation and virus testing using the RT-PCR method and NGS were conducted, as described in Paper IV & V. The steps of virus detection in Paper IV are illustrated in Fig 2.

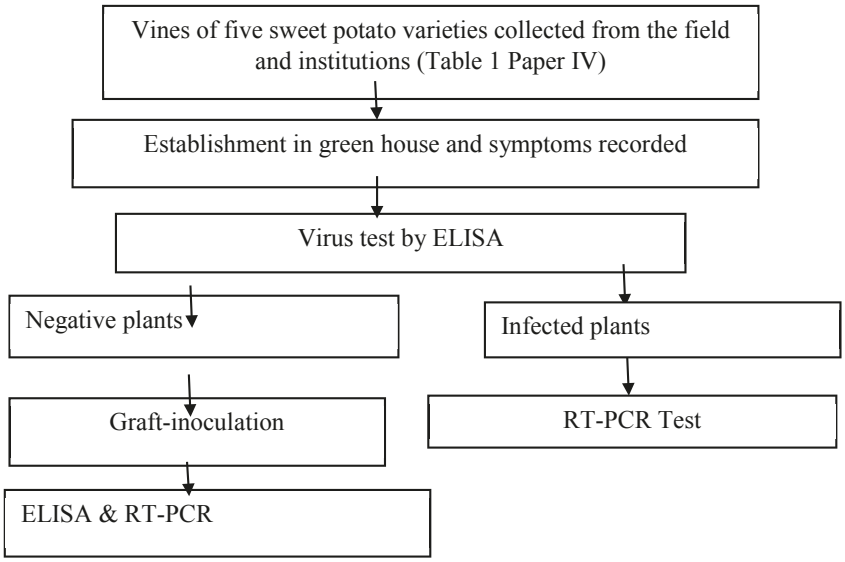

Figure 3: Flow chart diagram depicting steps involved in virus testing of initial sweet potato sample collected from farmers' fields and research stations in Ethiopia, before virus elimination treatment.

In paper V: for the Next Generation Sequencing (NGS) experiment, two sets of potted test plants (five sweet potato varieties) were established using stem cuttings and grown for three months. One set of the potted test plants was transferred into a new growth chamber and heat-treated for 8 weeks at growth conditions described in Paper IV following the detailed procedure of Dennien et al. (2013) but with minor modifications. The other set of plants remained in the same normal growth conditions and used as a control (non-treated). Meristems excised from plants before and after heat-treated were cultured into a media suitable for regeneration of sweet potato (from Paper III). The plants regenerated were tested for the presence of virus infection using RT-PCR as described in Paper IV.

Total RNA was isolated from the six initial test plants and the six plants regenerated after heat treatment followed by meristem culture. The purity and quantities of total RNA samples were checked and sent to Fasteris (Geneva, Switzerland), where small RNA were deep sequenced using

an Illumina genome sequencer. An assembly of the NGS sequences reads into a set of contigs and the mapping of the contigs to the reference virus nucleic acid sequences in databases were performed using VirusDetect software (Zheng et al. 2017), to confirm the identity of the virus(es).

3. Main Results and Discussions

3.1 Review paper (Paper I)

Current status of sweet potato viruses in Ethiopia

A piece of up-to-date information about viruses infecting sweet potato in Ethiopia included in this section is obtained from a review of previous virus surveys and viruses testing conducted in this Ph.D. studies. Our literature review indicated that only a few sweet potato virus surveys have been conducted, mostly in the southern part of Ethiopia (Paper I: Fig. 1). Being one of the dominant sweet potato production regions, southern Ethiopia received more research attention than other regions. On other hand, this indicates the need for more detailed countrywide surveys to document virus infection status of all the other sweet potato production regions in Ethiopia.

List of the five sweet potato infecting viruses that were identified by the previous survey studies, location surveyed in Ethiopia were summarized in Paper I: Table 1. SPFMV is the most commonly detected virus followed by the SPCSV in most of the virus surveys. In addition, mixed infections of both SPFMV and SPCSV were also reported (Adane 2010; Tesfaye et al. 2011). Plant virus has received little research attention in Ethiopia. However, the number of sweet potato virus surveys conducted and types of viruses being identified in Ethiopia has been increasing since 2004, following the virus survey report by Alemu (2004). FAO data show that the productivity of sweet potato has declined over the past two decades (FAO 2017, Fig 1). Declined productivity may be linked party to the increasing spread of virus infections. This has finally received researcher attention to conduct the virus surveys. In addition to confirming the presence of the five viruses previously reported in Ethiopia, we detected new viruses and some viroids in the high yielding sweet potato varieties from Ethiopia (Paper V, Table 1). The new viruses were not found or tested

for in any of the previous surveys in Ethiopia**.** Therefore, it is difficult to say the new viruses and viroids have been recently introduced, or were there for a long time. An increasing number of sweet potato-infecting viruses being identified in the farmers' sweet potato field and research sites in Ethiopia shows the importance of giving more attention for the training of farmers and extension workers on virus management practices. In addition, the necessity to plan and take decision to prevent/reduce the survival, dissemination and transmission of the viruses when distributing planting material is very important. Moreover, it is also helpful to look for an alternative sources of virus-free planting materials. Though it is known that virus infection is common in sweet potato farms in southern Ethiopia, there no restriction of the movement of planting materials between region and location to protect farmers' local cultivars. Indeed, this is the main way by which viruses have been introduced and spread from one area to another in African countries such as Uganda (Karyeija et al. 1998).

Ninety percent of sweet potato production in Ethiopia is from two dominant producing regions. Only one of the two production regions was covered in the previous virus surveys. Other parts of Ethiopia had never been studied, except for one study conducted by Tesfaye et al (2011) in eastern Ethiopia, which detected no viruses. It is possible that farmers often exchange planting materials and further studies are necessary in all production regions to determine the countrywide status of virus infections.

Incidences of SPFMV, SPCSV and mixed infection are most common in Ethiopia

SPFMV is the most common virus detected, in current (Paper IV and V) and previous (Alemu 2004; Tesfaye et al. 2013; Mebrate 2018), studies conducted in Ethiopia. This indicates that this virus has been widely spread in sweet potato fields in Ethiopia. It is not surprise that SPFMV is thought widespread and being detected with high incidences in every sweet potato growing country (Valverde et al. 2007). It has been reported in Africa, China, Europe, Japan, Korea, Peru, Taiwan and USA (Kreuze et al. 2008; Clark et al. 2012). Study have shown that SPFMV infected plants are mostly symptomless when it comes to single infections (Gibson 2009). The frequent detection of this viruses in sweet potato plants may show it can easily disseminate with symptomless plants,

but still spread. SPFMV can cause large yield losses, particularly when it is co-infect with sweet potatoes with SPCSV, which has been reported in all of the previous studies in southern Ethiopia.

Mixed infections of SPFMV and SPCSV know as sweet potato virus disease (SPVD) is only detected in one test plant out of the fifteen samples tested (Paper IV). Such mixed infection were also common infection detected previously in sweet potato samples from farmers' fields (Tesfaye et al. 2011) and in the national sweet potato collection centres (Adane 2010) of Ethiopia. The mixed infection reported explains the severe virus-like symptoms we observed in the sweet potato fields (Paper I: Fig. 2A). This could well be the reason for the decline in sweet potato productivity in Ethiopia over the past two decades (Fig. 1). Infection of SPVD is very common around the world and has resulted in huge yield losses for sweet potatoes everywhere (Gibson et al. 1998). Hence, it requires appropriate virus management practices, including removing sources of infection, controlling vectors, generating and the use of virus-free plants. SPVD causes more severe yield losses than losses induced by any other pathogen of the crop (Gibson et al. 1998). In Uganda, triple infection of SPCSV, SPFMV and SPMMV was reported in both cultivated and wild sweet potato plants (Tugume et al. 2016). When looking at the present situation in Ethiopia, there is a potential of even further yield losses, if the already infected sweet potato fields get co-infected with other viruses causing mixed infections and outbreaks of complex viral diseases.

From the literature review, most of previous studies in Ethiopia have shown that the incidences of SPCSV infection is the second to SPFMV (Adane 2010, Tesfaye et al. 2011). An incidence of SPCSV has been increasing in the consecutives virus surveys that have been conducted in Ethiopia. SPCSV has been responsible for severe infections by breaking the resistance against SPFMV. SPCSV is detected in only one of the 15 plant used as test plants (Paper IV). This doesn't mean the virus is not spread widely, perhaps it means the varieties are resistant.

Sources of virus infections and means of dissemination in Ethiopia

Sweet potato infecting virus has been thought to be introduced into and disseminated within Ethiopia through exchange of germplasm between countries and free exchange of planting materials amongst farmers (Tesfaye et al 2013). However, no clear information exists regarding

the time when viruses were introduced for the first time into the country. Although SPFMV was identified for the first time in Ethiopia in 1986, sweet potato viral diseases only became recognized as a serious threat to sweet potato production starting from 2004 - 2006 onwards, during which additional viruses; SPVG and SPCSV were identified (Alemu 2004). Today farmers' fields and research sites in southern Ethiopia suffer from a high degree of virus infections (own unpublished data; Mebrate 2018).

Information on the main sources of virus infections and the possible means of virus dissemination within the country was collected in the survey. Based on some previous literature, personal observations and our own analysis considering how farmers obtain their planting materials, the exchange of planting materials between productions regions is one of the most likely means of virus dissemination within Ethiopia. Moreover, the lack of virus-tested clean stock and use of planting materials from season to season by farmers, might have contributed to the survival of the viruses and the dissemination in the country.

'If we don't have it, we don't want it' this is the idea to protect the introduction of new pathogens to locations where it does not exist. In Ethiopia, there is a low awareness of the necessity for quarantine schemes for import and exports of plant materials. Moreover, the country's certification scheme for virus-free plants is mainly dependent on visual evaluation in the fields. This study has demonstrated that this does not take into account symptomless infections. Moreover, in most of cases, it does not even involve testing for viruses. Therefore, Ethiopia needs to have a strong quarantine system in place for imports to the country. A system for the certification of planting materials movement between regions is also very important.

3.2 Virus testing methods and viruses detected in present study in Ethiopia (Paper IV, V)

Most virus detection and elimination methods in use elsewhere are rarely applied to sweet potato viral disease management in Ethiopia. Serological techniques has been the most common virus detection method used for testing sweet potato viruses (**Paper I:** Table 2). Moreover, the virus

elimination experiments conducted were also very limited and not efficient in producing virus-free plants of elite cultivars. The virus-free materials was not preserved, there are no virus-free materials of elite cultivars available to the growers. The present study applied three testing methods. The testing efficiency of each method varied. Incorporating grafting as a method of infection test is very important specially for testing viruses in symptomless plants. Using ELISA, RT-PCR and NGS of small RNA, viruses that belongs to different genus were detected in our plants.

New and previously reported viruses detected in elite cultivar from Ethiopia

The detection of new viruses in the present study (paper IV and V) increased the number of sweet potato infecting viruses identified in Ethiopia. SPFMV, SPCSV, SPVG, SPV2 and CMV were the five viruses previously reported in our literature review (**Paper I)**, the present study detected SPVC, *Sweet potato symptomless mastrevirus* (SPSMV-1) and) three sweet potato badnaviruses (SPBV-A, SPBV-B, SPBV-C) collectively called SPPV, and some more viruses that belong to 10 genus and 2 viroids (data not shown) are a new sweet potato infecting viruses detected for the first time in Ethiopia. Although, it needs further experimental confirmation, many viruses and viroids of other plants species were also detected in sweet potato plants by NGS (Appendix 1). Viruses are widely considered to be of great economic importance in sweet potato production (Clark et al., 2012; Gibson & Kreuze, 2014). Sweet potato production in Ethiopia is critical for food security and the increasing number of viruses being identified and insect pests described are threatening its production. Considering the importance of sweet potato plants in Ethiopia and the lack of information about the occurrence and distribution of sweet potato viruses in these plants, the present study contributed new information of infecting viruses that is a prerequisite for the management of viruses. With our holistic approach to the issue in this PhD thesis, we believe there are now good tools in place, as well as the competence in Hawassa U and commercial tissue culture labs, to encounter the challenge in a meaningful way.

Graft-inoculation significantly improves detection of SPFMV by DAS-ELISA and RT-PCR

Most of the test plants (Table 1) collected from the farmers' field and research stations appeared to be symptomless when grown in the greenhouse and observed for symptoms, except 'Guntute' plant B, which showed typical virus-like symptoms (Paper IV: Figure 2). The ELISA method was able to detect SPFMV and SPCSV only in the plant with severe virus-like symptoms. However, ELISA did not detect viruses in the rest of other seemingly symptomless initial plants before grafting. However, SPFMV was detected in 6/15 of the symptomless initial plants when using RT-PCR, showing the improved sensitivity of RT-PCR over ELISA. However, most of the *I setosa* plants developed varied types of virus-induced symptoms (Figure 5) 3 weeks after graft-inoculated with shoot tips of the symptomless plants (Paper IV: Table 1). When viruses were retested in symptomatic leaves of graft inoculated *I. setosa* plant using ELISA, the presence of SPFMV was confirmed in 12/15 of the plants (Table 2). This results was confirmed by RT-PCR. This depicts most of our plants carries virus without any recognizable symptom in the case of latent infection and that *Ipomea setosa* was able to multiply the virus titer to a degree which made it detectable with ELISA.

Grafting of a scion tissue or shot tip from suspected plants into healthy test plant is commonly used for detecting viruses found in low concentrations. A plant free of virus-induced symptoms is not necessarily free of viruses, and we have also exemplified this phenomenon in the present work (Paper IV, Table I). Virus induced symptoms did not develop on any of the indicator plants that were grafted with shoot tips of the symptomless plants of 'Berkume' (Paper IV). Testing of virus in the leaves of both grafted *I. setosa* and leave of 'Berkume' did not give positive tests, which also confirms that the plants of 'Berkume' are at least free of the common viruses we tested for (Figure. 4B). The studies have shown that including biological indexing is necessary to confirm the presence or absence of virus infections when working on symptomless plants. Symptoms observed *I setosa* varied according to the source of the graft (Figure 5). This agrees with the fact that symptoms vary according to the plant variety, the environmental conditions and the species of the viruses involved (Hull 2013).

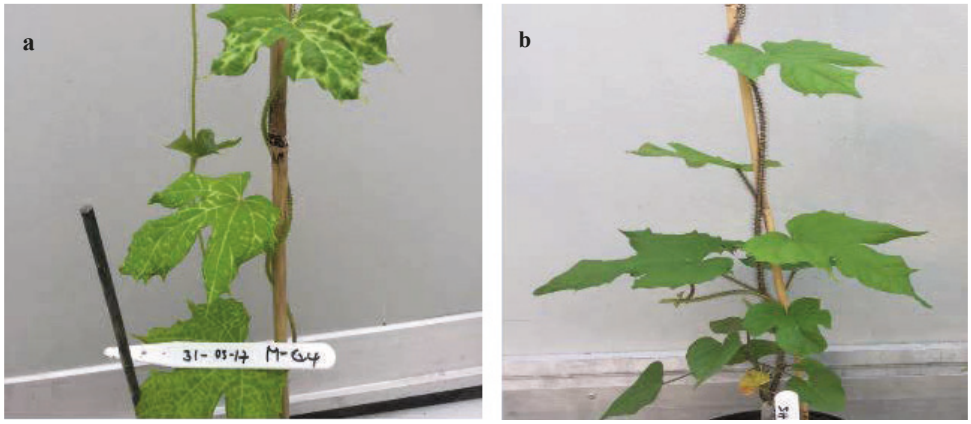

Figure.4 *Ipomea setosa* indicator plants grafted with sweet potato shoot tips; virus symptoms developed on *I. setosa* graft inoculated plants with virus infected shoot tip (left) versus a healthy plant (right).

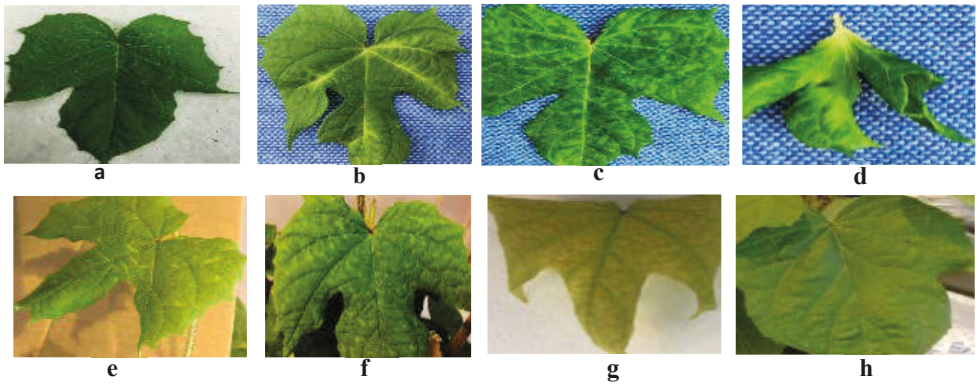

Figure. 5. Virus-induced symptoms on sweet potato graft-inoculated *Ipomea setosa* plant. 3a-f is symptoms induced by SPFMV, showing varying symptoms of chlorotic spots (a), vein clearing (b), feathery (c), leaf curling and leaf deformation (d), (g) is a symptom induced by a shoot from a plant co-infected with SPFMV, SPCSV, and SPVC (severe chlorosis, yellowing) and (h) is a healthy leaf of *I. setosa* plant (control). The causal viruses were tested using DAS-ELISA and TAS-ELISA/ RT-PCR.

Table 2. Summary of the virus detected in the sweet potato varieties using ELISA (DAS and TAS) and RT-PCR

Varieties and no. of clones tested		**Virus infection detected**		
Varieties	**genotypes**	**SPFMV**	**SPCSV**	**SPVC***
Hawassa 83	3	+	-	-
Berkume	3	-	-	-
Tola	3	+	-	-
Kulfo	3	+	-	-
Guntute	2	+	-	-
	1	+	+	+

* Detected by RT-PCR

Sensitivity of next generation sequencing (NGS) and RT-PCR for virus detection and screening of virus-free plants (Paper V)

First, we performed RT-PCR to detected known viruses (SPFMV, SPCSV, SPVC, SPVG, *Sweet potato mild mottle virus* (SPMMV) and SPV2) using specific primers for these. Then we did NGS to verify the results from RT-PCR and to detect potential unknown virus(es) in the plants before virus elimination. The RT-PCR revealed that the plants were infected by three viruses: SPFMV, SPCSV, and SPVC. NGS confirms the presences of viruses detected by RT-PCR as well as additional viruses: SPVG, *Sweet potato mastrevirus-1* (SPSMV-1), sweet potato badnaviruses (SPBV-A, SPBV-B, SPBV-C) and some more viruses that belong to another ten genuses and two viroids (Appendix: Table 1). On the other hand, we did not include templates for badinaviruses (SPBV-A, SPBV-B, SPBV-C), SPSMV-1, in the RT-PCR runs due to a lack of primers. So, they were detected for the first time using NGS. This shows the efficiency of NGS technologies as a powerful tool for the analysis of small RNAs sequences in order to simultaneously detect both known and unknown viruses without prior knowledge of the sequences of the infecting pathogen from infected plants. We see this by detecting many other viruses and viroids not specific to sweet potato, but of other plants using NGS. However, this clearly needs further confirmation. One explanation could be that some of the viruses and viroids detected were from a contaminant or from the test plant. Use of

two or more testing methods is recommended for effective detection of viruses (Hull 2013; Wilson 2014). Compared to NGS, RT-PCR did not detect SPVG in any plant, and SPVC in most of the plants, before virus elimination. However, these two viruses were detected later by NGS in many of plants demonstrating the better sensitivity of NGS as a method of virus detection in symptomless plants. It detects most viruses as long as they are in the right concentration, including some of the virus that were not detected by RT-PCR. Detection of virus using RT-PCR influenced by PCR inhibitors like polysaccharides and phenolic compounds which are still a major inhibition to the successful DNA amplification of plant samples (Samarakoon et al. 2013). Besides, as mentioned, NGS is not dependent upon being detected before, have a known sequence for primers (RT-PCR) or antibody made (ELISA).

In addition, this study (Paper V), compared the effectiveness of RT-PCR and NGS as a verification of virus-free materials. RT-PCR and NGS were used again to retest viruses in plants regenerated though meristem culture excised from thermo-treated mother plants. Thus, RT-PCR confirms that SPFMV, SPVC, SPCSV and SPVG were removed after being treated with thermotherapy followed by meristem tip culture from most of plants previously known to be infected by one or more of this viruses before virus elimination treatment (**Paper V, Table 2**). This result demonstrate the virus elimination treatments was successful in all varieties; efficiently eliminating the most common known viruses. NGS also partly confirmed the testing results of RT-PCR for some plants. However, NGS also revealed that none of the viruses found infecting 'Hawassa 83' and SPFMV infecting 'Tola' were eliminated. Moreover, badnaviruses previously detected by NGS are still present in most of the plants even after heat treatment, showing the difficulty to remove these by thermotherapy-meristem culture in most plants. It is suggested that *badinaviruses* are endogenous viruses that are integrated into their host genomes and difficult to remove (Bhat et al. 2016). However, there is a variation in elimination responses as badinaviruses were eliminated from some genotypes and not from others. This may be due to the differences in host-virus interactions as the genotypes used may vary in their integration of the viruses. Paper V was the first to obtain the complete genome sequences of many viruses: SPCSV, SPVC; SPVG, SPBV-A, SPBV-B from Ethiopian sweet potatoes using VirusDetect (Zheng et al., 2017).

NGS detected sweet potato badnaviruses (SPBV-A, SPBV-B, SPBV-C) not know to be economically important for sweet potato in most of elite sweet potato varieties from Ethiopia. What is intriguing is that these viruses were detected in most of the plants both before and after virus elimination treatments (Figure 7). Our results are consistent with several other reports in that most of the symptomatic and symptomless sweet potato plants are infected with SPPV (Kreuze et al. 2017; Nhlapo et al. 2018). Additionally, SPPV were the most commonly detected virus complex in sweet potato in Africa (Mbanzibwa et al. 2014; Kreuze et al. 2017). Most of plant materials infected with sweet potato badnaviruses were obtained from research centers and institutions. Similar results of detection of sweet potato badnavirus infection in symptomatic and symptomless plants that were collected from fields and collection centers were previously reported (Kreuze JF et al. 2009; Kreuze J et al. 2017). This shows that the virus elimination treatments applied has not been able to remove these viruses from most of the plants regenerated from meristem culture (Figure 6).

More than 29 *Mastrevirus* species (including SPSMV-1) have been reported and most of them infect monocot plants (Muhire et al. 2013). This study reports the detection of SPSMV-1 in sweet potato from Ethiopia for the first time. It confirms detection in sweet potato from China and Korea (Kwak et al. 2014; Wang et al. 2015). The detection of SPSMV-1 in both infected and healthy plant shows that heat treatment might not be effective to remove the viruses from tissues. It is true that viruses with no known economic impact and non-pathogenic today to some crops may later become pathogens through genetic change that can occurs though mutation and other factors. So badinaviruses and sweet potato symptomless viruses in our plants need to be monitored for any future economic impact to sweet potato. Moreover, these viruses might be important in other varieties of crops. Although vsiRNAs associated with virus infections is characterized in sweet potato in many countries (Kreuze JF et al. 2009; Kreuze J et al. 2017), this study represents the first report of using NGS to validate the plant are free of viruses by characterizing vsiRNA population in sweet potato plants before and after virus elimination response to high temperature treatment-meristem culture.

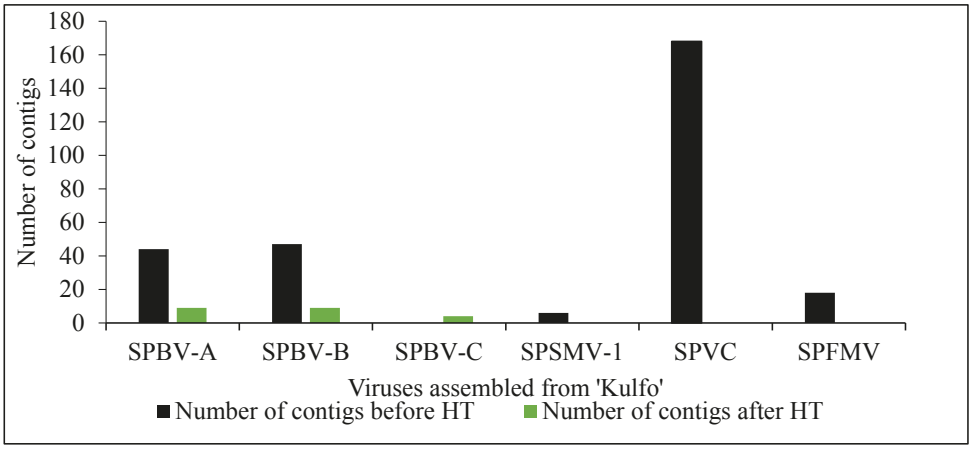

Figure 6. Virus-derived contigs from NGS identified in the non-heat treated and heat-treated samples from sweet potato. Bars indicate the number of viruses derived contigs obtained that covered >50% of the reference virus genome.

3.3 Survey to understand the state of virus awareness amongst farmers and extension workers in regions of Ethiopia (Paper II)

Perception of plant virus diseases, its symptoms and mode of transmission by farmers and extension workers (Paper II)

The survey revealed that farmers had low perception of sweet potato diseases caused by infecting viruses. Over all 64.7% of farmers and 41.2% of extension workers were not aware of infecting virus. Most of the farmers do not know virus-induced symptoms that develop on the infected plants or they link the symptoms to other problems caused by environmental conditions or insects. To increase the knowledge about viruses and virus-induced symptoms is important in the efforts to reduce viral diseases and associated crop losses. Unfortunately, several previous studies in other parts of Eastern Africa have reported the same lack of awareness of viral diseases, associated symptoms and the means of transmission amongst farmers in Kenya, Uganda, Tanzania, Rwanda and Burundi (Rwegasira et al., 2007, Legg et al., 2007, Schreinemachers et al., 2015, Echodu et

al. 2019). Maybe a concerted action with teaching material on viral diseases can be produced for the use of all countries, translated into local languages and training can be done in a comprehensive action in all sweet potato growing regions.

Results of our study farmers' perceptions of parameters related to (Paper II: Table 1) did agree with many of the findings of these earlier studies. Our study show that farmers' awareness varied between the districts studied. For example, more than 54% of farmers in Boloso Sore district had heard about sweet potato virus diseases (Paper II: Table 1). High incidences of SPFMV and SPCSV infections have previously been reported in Wolayta zone in southern Ethiopia where the studied district is located (Tesfaye et al., 2011), which may explain the better awareness of the farmers in Boloso Sore district in our study. Moreover, better awareness of farmers in the district could also be due to its proximity to Areka Research Centre located inside the district, and that has been worked on sweet potato.

The farmers had better awareness of damage caused to sweet potato plant by defoliator insects (Paper II: Table 1). However, a lower percentage knew that viruses can be transmitted from diseased plants to healthy ones by insect vectors. Moreover, the majority of farmers do not perceive that virus transmission can be reduced. Low awareness of virus infections is not limited to the farmers. Similarly to the farmers, extension workers also had a low perception of viruses and their mode of transmission (Paper II: Table 2). The farmers' and the extension workers' low awareness of sweet potato infecting viruses and the low ability to identify general virus-induced symptoms in the fields is an indicator of the fact that both farmers and extension workers have not been well informed about the viral diseases, associated symptoms, and the means of transmission. Moreover, our study reveals that farmers and extension workers received little training on diseases and pest managements. It also suggests that the damage caused by viral disease can be more significant than anticipated, but has not been recognized nor actions taken. Moreover, it clearly demonstrates the need to provide training to the farmers and extensions workers to improve their ability to monitor the onset of disease and disease progression in their fields. In addition, it will enable them to apply cultural practices including rogueing out of the infected plants and other intervention strategies to reduce the infection rate in their fields.

Farmers have, to a small degree, practiced combinations of pest and disease management methods (Paper II: Fig. 6). However, since their knowledge of the diseases and their management is so low, their management strategies must be enforced to target the reduction of the sources of the virus infections, to be effective for virus disease management (Wilson 2014). Sweet potato field observations during this survey indicate the existence of high virus-like symptoms in sweet potato plants which is believed to be a source for further virus infections. Although it does not affect plant viruses directly, farmers practiced the use of locally made non-synthetic insecticide treatments, such as mixture of soap, suspension of plant extracts, application of cow dung and wood ash mixed with water mainly against insect pest and other pathogens. The application of these non-synthetic insecticides may control virus transmitting insect pests including aphids and white fly. In addition, handpicking of worms and caterpillars is a common practice. Similar practices of using non-synthetic insecticides (a mixture of local plants extracts, animal manure and urine) to control insect pest of potato has been reported in the Democratic Republic of Congo (Munyuli, 2016). Even though these are measures against the insects, there is no direct management strategies for virus control. The practices of using such biological insecticides by farmers positively contributes towards the concern about pesticide related environmental problems, increasing demand for healthy food, boosting organic production (Foerster et al. 2001). However, there is a need for more investigatigations on dosage, side effects and mechanism of action. Moreover, they need to be commercialized and made available to farmers at reasonable prices.

Perception of farmers on incidences of and economic damage due to insects and diseases to sweet potato

A majority (58.1%) of the farmers perceived that the damage caused by sweet potato diseases and insect pests was currently decreasing compared to level it had been in the past five years. Farmers had varied perception of the damage to their sweet potato by sweet potato diseases and insect pests. We found a significant difference ($p < 0.05$) between the districts studied. When asked to rank the extent of the damage, 43.8% of the farmers think that sweet potato diseases and insect pests cause an average economic damage to their sweet potato production. The same trend where farmers had different views on the trends of sweet potato diseases and insect was reported from four East African countries (Echodu et al 2019). It is difficult for farmers and extension workers to associate

damages to a given disease causing pathogens, but it is easier for them to give their view of the general impacts due to abiotic and biotic factors.

The majority of the interviewed farmers and extension works claimed that they had no training specific to sweet potato plant disease and insect pest management in the last five-years. Only 19.2% of the farmers and 20.6% of the extension workers received sweet potato related training in the past five years (Paper II: Fig 5b).

In the previous section, virus dissemination within the country through various means was discussed. The conclusion was that unrestricted exchange of planting materials, repeated use of vegetative propagules over seasons, mechanical transmission by agricultural practices and farm tools used during planting materials preparation, cultivation and harvesting are all possible means of virus dissemination. It is quite disappointing to reveal that the majority of the farmers in the locations studied use their own planting materials saved from previous harvests (own unpublished data). Moreover, 72% of the interviewed farmers use non-disinfected machete/sickle to prepare their planting materials at the time of planting, since they have never been told that this could lead to disease and yield loss, or they have not perceived this information and translated the impact on their own farm. All practices of hoeing, weeding and cultivation are carried out using traditional farm tools with a high possibility of making wounds for virus contamination. The farmer need awareness on virus infection sources and this practice may disseminate viruses in infected farms as a preventive measure.

3.3 Media optimizations to ensure efficient regeneration and virus elimination (Paper III)

Variety and PGRs concentration affect initiation and regeneration of meristems

Tissue culture techniques are very important for the production of disease free and genetically uniform plants. Success of plant regeneration in tissue culture depends on media composition and cultivar responses (Feyissa and Dugassa 2011, Addae-Frimpomaah and Amponash 2014; Mbinda et al. 2016). The present study (Paper III) successfully optimized a protocol to initiate shoots from meristems and the subsequent multiplication of shoots from nodal cuttings of selected sweet potato varieties from Ethiopia. This optimized tissue culture media will be used for multiplication of virus-free sweet potato plants using the tissue culture laboratory facilities in Hawassa, as well as the commercial laboratory in Mekelle, Ethiopia to make sure a system for elimination and propagation of these varieties in Ethiopia.

As presented in the analysis of variance summary (Paper III: Table 2), PGRs concentrations, varieties and the interaction of the two had significant effects on the initiation of shoots from meristem culture ($P<0.05$). Moreover, there was a genotype x environment interaction, where the optimal medium PGRs concentration for meristem initiation varied depending on the cultivar studied (Paper III: Fig. 1). However, a concentration of 0.1 mg/L NAA and 1 mg/L BAP initiated around 57% of the cultured meristems on average for all cultivars. Media with low or no PGRs did not initiate regeneration in any cultivar (Paper III: Fig. 2). The effects of concentration and combinations also varied with genotypes (Figure 7)

Previous studies have shown that media optimized for sweet potato cultivar are genotype-specific and if not optimized, results in poor regeneration (Feyissa and Dugassa 2011; Wondimu et al. 2012). Similarly, our results have shown that concentrations and combinations of PGRs had significant interactions also with respect to shoot multiplication. The highest shoot initiations were obtained for 'Kulfo' followed by 'Berkume' when basic MS media was supplemented with 0.5 NAA mg/L and 0.1 mg/L BAP (Paper III; Fig. 1). This agrees with several studies who have

reported varieties of the same species responded differently to media (Abe and Futsuhara 1986; Jan et al. 2001; Wondimu et al. 2012; Mbinda et al. 2016). The varieties we used in our experiment have a high degree of genetic variability, which could explain the variation obtained in their response. Consistent with previous studies, our results have shown that PGRs concentration, genotype and their interaction significantly affects initiations of shoots from meristem and nodal explants (Paper III: Table 2, Fig 1 & 2).

Concentrations of BAP had a significant effect on the number of shoots induced, the height of shoots, and the numbers of internodes produced per single nodal cutting explant($P<0.05$). The varieties tested also had significant differences (Paper III: Table 4 and Fig. 3 and 4). The highest number of shoots were obtained for 'Hawassa 83', 'Kulfo', and 'Berkume' when nodal cuttings were cultured on 2.5 mg/l. On this medium, most of the varieties produced 1.7 to 2.8 shoots from single nodal cuttings within a month. Similar results have been reported by various researchers that medium containing 2 mg/l BAP resulted in highest score of number of shoots per nodal for different varieties of sweet potato such as from Ethiopia (Neja 2009; Dugassa and Feyissa 2011). Similar results have been reported from shoot tips of tea plants cultured in 3 mg/l (Borchetia et al. 2009). Medium with 0 mg/l BAP did not show any multiplication from a nodal sweet potato explant for any of the variety tested. This indicates how important BAP is for multiplication of shoot. On the other hand concentration beyond 2.5 reduced the number and length of shoots indicating its inhibitory effect.

However, half MS media without PGRs induced the longest shoot and larger internode numbers in all the varieties compared to those cultured on media to which PGRs were added. Shoot height declined with an increasing concentration of BAP for most varieties (Paper III: Table 4, Fig. 3 & 4a). According to Nadra et al. (2015) high concentration of cytokinins results in ethylene production that limits the regeneration of shoots and inhibits the elongation of internodes. High concentration of BAP in tissue culture media inhibited adventitious meristem elongation of tea plant (Borchetia et al. 2009). We did get difference in response between the varieties in the number of shoot obtained per nodal cuttings. This suggest the need to optimize multiplication media for different varieties in order improve multiplication rate.

Optimum concentrations and combinations of auxin and cytokinin were reported to be important for cell proliferation and new organ regeneration (Su et al. 2011). The results also show that to obtain shoots, the presence and amount of NAA is more important than the BAP concentration. This study established a rapid and efficient protocol for the initiation and multiplication of five sweet potato varieties released to different location in Ethiopia.

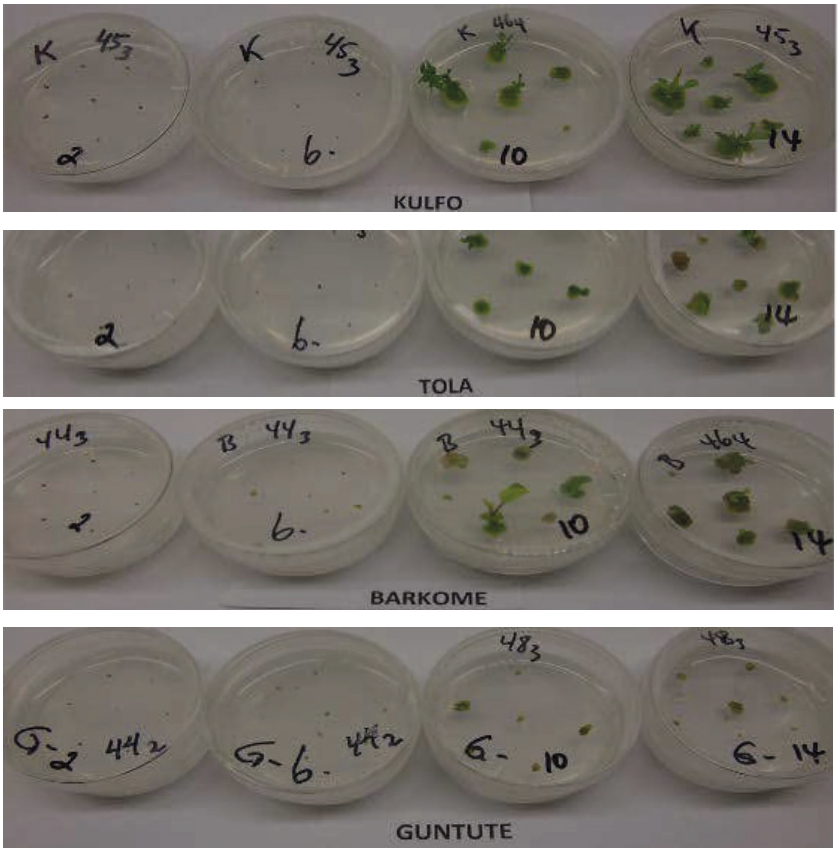

Figure 7. Effect of varying amounts of auxin (NAA) for the initiation of meristems for the four varieties tested; the two Petri dish on right side (2 and 6) represent low concentration of NAA and the two Petri dish on left side (10 and 14) represent a high concentration of NAA.

3.5 Heat treatment increased virus elimination efficiency of meristem culture (Paper IV)

Vegetatively propagated plants are often exposed to the risk of virus accumulation over seasons and need to be freed from viruses. The desire to use quality planting material of clonally propagated plants require an application of efficient therapy techniques to eliminate viruses from elite genotype and the rigorous screening of plants free of viruses (Golino et al. 2017). In Paper IV, we applied meristem culture alone; meristem combined with thermotherapy to plant infected with SPFMV. We did obtained different success rates and percentages of plants free from SPFMV when retested after the treatments (Paper IV: Fig 2).

Using meristem culture after heat treatment of the mother plant increased the likelihood of obtaining a higher number of virus-free plants than meristem culture alone (Paper VI: Fig. 2). The highest and lowest SPFMV elimination efficiency obtained were 93.8% of 'Hawassa 83', and 66.7% of 'Guntute' respectively, using heat treatment of meristem donor plant followed by meristem culture. The success in obtaining virus-free plants by each method was affected by the types of treatments and the plant varieties tested (Panattoni et al. 2013). Higher number of plants that regenerated after heat treatment when grafted to *I. setosa* were confirmed symptomless and free of SPFMV by the ELISA and RT-PCR methods. Samples with an absorbance values of less than double of the positive control were considered virus negative (Figure 8). Results of plants retested using RT-PCR are presented in (Figure 9), where wells with no band show plantlets free of SPFMV after being heat-treated followed by meristem culture.

Our results from the virus indexing of the regenerated plants showed meristem combined thermotherapy is more efficient in eliminating SPFMV than using meristem culture alone. Cutting meristem has two advantages: gives true to type as it develops from differentiated cells and virus elimination. Meristem is microscopic explants excised from the donor plants. If the donor plants have been infected with viruses that may excludes viruses and other pathogenic organisms. Moreover, virus movement within plants through vascular system that is not well developed in the meristem region of plants, increase the chance of getting virus-free plants. Meristem cuttings after

plants did get heat treatment even increased the chance of getting higher percentage of virus-free plants. Heat treatments suggested to inhibit the multiplication of viruses in the infected plants. The observed differences in the efficiency of SPFMV elimination between the genotypes tested might be due to physiological conditions of each genotype (the mother plant) from which meristem was excised and the load of the viruses in each plant tissue (Verma et al. 2004; Sastry and Zitter 2014). Source of explants and shoot tip position influenced success of virus eradication in thermotherapy-based methods (Wang et al. 2018). Our plants may have different concentration of indigenous hormone suggested to affect virus contents in the meristematic tissue. In addition, there were differences in percentage of virus-free materials between meristem donors (varieties), which were previously reported in many crops (Paprstein et al. 2008; Waswa et al. 2017). Differences in virus/viroid elimination efficiency between varieties of the same crop have previously been reported (Paprstein et al. 2008; Waswa et al. 2017) and seems to be mediated by the specific interactions that occur between the virus and the host.

Our study also agreed with previous reports (Ng et al. 1992; Thottappilly and Rossel 1992) where, meristem culture alone could not automatically guarantee total freedom from virus and that its success can be increased by thermotherapy of mother plants before meristem culture. Similar to previous reports, our results have also shown the possibility of obtaining higher number of virus-free plants from meristem excised after heat treatments of mother plants. In general out of all the methods used to eradicate viruses from plants, the combined use of meristem culture and thermotherapy are the most commonly employed methods in many plant species: including nectarine, apple, sweet potato, potato crops (Manganaris et al. 2003; Wang L et al. 2006; Dugassa and Feyissa 2011; Waswa et al. 2017). Thus we also confirm that combined methods is better than using meristem culture alone in eliminating viruses from our donner plants. In line with our results, the review of literature on methods of virus elimination indicated thermotherapy is the most commonly used virus elimination methods (Zhang et al. 2019). Moreover, this study also success fully cleaned viruses from five high yielding varieties from Ethiopia that can be used to for improving food security.

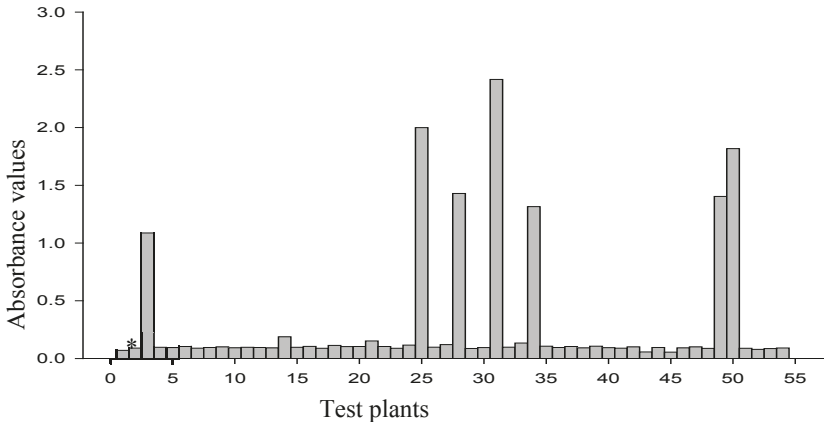

Figure. 8. Absorbance values of ELISA reading using sap extracted from samples collected from plants obtained after heat treatment followed by meristem culture. Absorbance reading: 1, 2 and 3 are blank, negative (*) and positive controls, respectively and the rest (4-55) are samples from test plants. Test plant samples (bars) with the value double of the value of the negative control sample are considered as SPFMV infected.

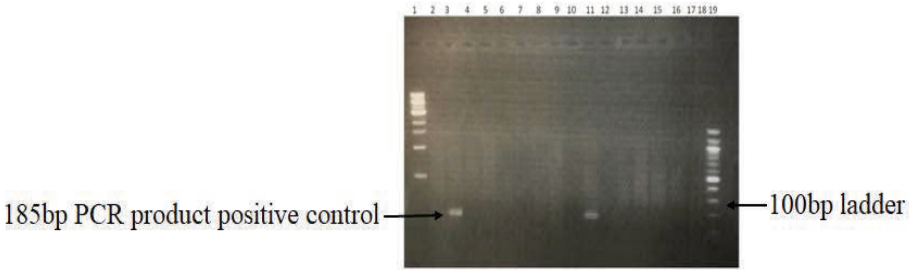

Figure 9. RT-PCR product of SPFMV in samples from tissue culture derived materials after virus elimination treatment of initially SPFMV infected sweet potato. Lane 1 is 1kb ladder; lane 2 is negative control, lane 3 is a positive control, lane 4-17 are tissue culture derived sweet potato clones, lane19, is 100bp ladder.

3.6 The need for virus-free plants in Ethiopia

Sweet potato is an important food security crop of many million Ethiopians. Twenty six improved varieties have been selected for the different production regions of Ethiopia. However, the adoption of the varieties into production is far-behind and it has affected the return from production. In addition, productivity is declining due to a lack of supply of virus-free planting materials, and that farmers continue to use their own planting materials from previous harvests. Results from the previous studies (Alemu, 2004, Adane 2010, Tesfaye et al 2011, Fekadu et al 2015, 2017) and the current studies (Paper I, IV, and V) indicated that sweet potato plants in the farmers' fields are highly infected by viruses and the viruses are being disseminated with infected planting materials. As one of the solution to combat decline in yield and virus spread, the present study successfully eliminated viruses from five high yielding varieties to be used in different production locations of Ethiopia. Healthy plants generated through tissue culture have superior quality and they benefit by providing growers with increased yield opportunities (Wang P and Hu 1980). An average fresh root yields increase by 37.9% has been reported from China (Zhan et al. 2006).

Our plant are with proven natural high yielding potential yet give low yields because of viruses. As using virus-free planting material can increase yields up to 100% when compared to infected plant (Zhan et al. 2006), we recommended that our material reaches the end users (farmers and commercial producers) through the coordinated action of stakeholders. Developing a system that distribute virus-free sweet potato plants to growers not only provide planting material free from viral pathogens but also free from insect pests, bacterial and fungal diseases. The NORHED project has supported establishments of a molecular lab and facilities for virus detection using ELISA. This laboratory facility, if strengthened with consumables at all times, can be used for the future virus testing and virus elimination studies. The Tissue Culture Lab at Hawassa University and Commercial Tissue Culture laboratory at Mekele can be used for multiplication of virus-free stock of the five sweet varieties generated in this study, as well as other genotypes obtained in the future. In addition to the five virus-tested materials we generated in this study (Paper IV), a scheme is needed to clean more elite cultivars being used and to provide farmers with clean planting

materials, which can be renewed at regular intervals. We are in the process of determining (see below) the time between the replacements to maintain high yields in sweet potatoes in Ethiopia.

There was an attempt to clean viruses from few cultivars of Ethiopian sweet potato using meristem-tip culture and thermotherapy (Feyissa and Dugassa 2011; Wondimu et al. 2012). However, virus-free plants that has been obtained were not maintained or supplied to farmers. The reason could be a lack of facilities to preserve a clean a stock or the varieties from which the viruses are cleaned were not of farmers' preferences. Therefore, generating, multiplying and distributing clean planting materials of elite sweet potato genotypes is an important step to avoid infection sources for the build-up of viruses and to realize high root yields that improves the livelihood of communities.

Preliminary result of reinfection test

To see the reinfection rate and yield gain of our virus-free plants, virus-cleaned plants from Norway were multiplied in the tissue culture laboratory in Hawassa, Ethiopia and acclimatized in screen houses there. We then planted them in high virus pressure areas near to sweet potato fields. We also included farmer's varieties as check for the performances of these. Our preliminary results show that the virus-free plant got infected with SPFMV and SPCSV in the first season (data not shown). Field observations show severe virus-like symptoms, especially towards the end of the first growing cycle (Figure 10.). We cannot conclude on the effect of this or the reinfection rate from these preliminary results and observations. However, previous studies have confirmed reinfection in one growing cycle can be as high as 51–84% in Israel (Milgram et al. 1996) and up to 100% in USA (Bryan et al. 2003).

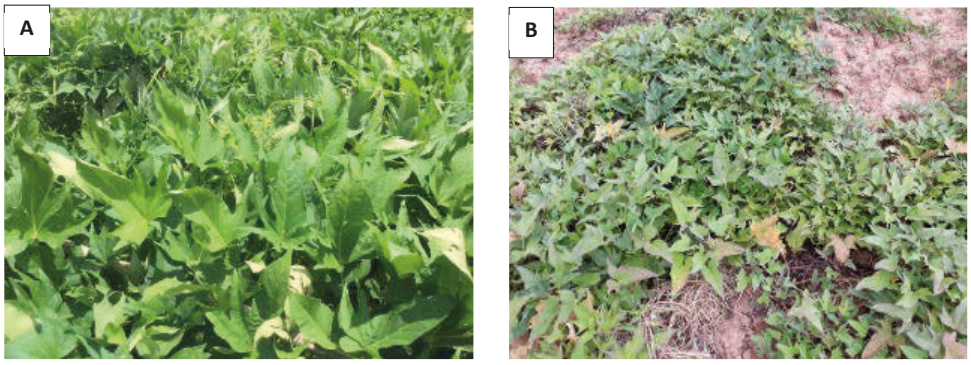

Figure 10. Virus-like symptoms in field grown sweet potatoes observed on plant initially free of common viruses after only one cropping cycle. **A** (left) taken after 4 months planting virus-free plants and **B** (right) after 6 months.

However, many factors determine infection rates. Availability of virus transmitter vectors and their population, presences of infected plants as source of virus in a given location and within range for the vectors affects the time it takes for newly introduced clean planting materials to become infected. Furthermore, it is also influenced by any preventive measures, such as vector management and the use of clean tools when moving from field to field, employed by farmers. Isolation distances from previously infected fields/farms is also a part of this solutions.

Traditionally, sweet potato plants are propagated by vegetative means, which usually carry transmittable disease agents (mainly viruses) to the next generation, accumulating diseases over seasons. Providing clean planting materials at regular intervals, followed by implementation of preventive measures reduces yield reduction due to virus(es). Virus-tested plants, if protected from reinfection, maintain its inherent yield potential. Therefore, training farmers and extension workers in improved agricultural practices is required. A scheme for providing farmers with clean stock materials needs to be in isolated or fully protected locations against reinfection to keep plants for propagation purposes and to prevent deterioration. What remains to be seen, is how long it takes clean sweet potato plants to become re-infected and so heavily burdened with viruses again that reduce yields. These experiments are ongoing, and it is too early to draw any conclusions. If the reinfection rate is too fast and the deterioration is too severe, other approaches will be required to

protect sweet potato production. Modern biotechnology, and perhaps recent advances in gene editing, might provide a better solution in the future, provided that it will be safe to use and that the Ethiopian authorities will permit such use of modern methods for their agriculture.

It is difficult to control viral diseases in practice, despite the fact that significant advancement have been made in the knowledge of plant viral diseases and advanced detection methods. Management of plant viruses is still restricted largely to use of virus- free plant materials after elimination of viruses from infected plant and reducing the population of vectors spreading the viruses from infected plants to healthy plants. As can be seen from the results of the survey of farmers' and extension workers' knowledge on this topic (Paper II), Ethiopia still has a long way to go to reach acceptable management practices.

4. Conclusions and Recommendations

This thesis has included a review of sweet potato viruses in Ethiopia, a survey of farmers to assess their current awareness of viruses, comparisons of virus detection methods, and also surveyed the current situation on the presence of viruses amongst sweet potato farms in Ethiopia. It has also included an exploration for the optimal medium for several elite genotypes, for establishment and multiplication *in vitro;* a prerequisite for being able to use meristem cultures routinely for virus elimination in a future healthy stock sweet potato plant system in Ethiopia. Finally, methods for virus elimination have been compared, and the status of the potentially cleaned plants, both in the lab and currently also in the field (not included in the Thesis).

Farmers and extension worker in the study location had low awareness of sweet potato infecting viruses, their mode of transmission to new plants and dissemination to new locations. The low awareness could hinder application of practices that prevent/reduce virus spread and dissemination. Therefore, appropriate training is needed for both the extension workers and concerned farmers growing sweet potato. Educating farmers through practical, oriented training that targets awareness creation on virus-infection and mechanisms could reduce the spread of the virus and the consequent diseases.

It is true that correct problem identification is a prerequisite for an accurate solution. SPFMV, SPCSV, SPVC, SPVG, SPSMV-1, and SPBV (SPBV-A, SPBV-B, SPBV-C) are viruses detected in sweet potato fields from Ethiopia. This study demonstrates that efficient and reliable detection of SPFMV requires a combination of graft inoculation to *I. setosa* with either ELISA or RT-PCR. The study have confirmed that PCR is a more sensitive method than ELISA; RT-PCR is able to detect viruses at low levels in a symptomless plant. Moreover, the use of NGS method is more reliable and efficient for virus detection than RT-PCR as it facilitates simultaneous detection of known and unknown viruses of different genomes. NGS increases the detection accuracy of viruses and thereby it confirms plants are free from viruses before being used as a clean stock, to be used for established seed systems. However, for the time being, NGS is an expensive method and requires bioinformatics competences which are resources not readily available in Ethiopia at

the moment. Hopefully, NGS can be used commonly in quarantine systems as long as the cost incurred becomes cheaper in the future.

This study optimized PGRs for initiation and multiplication of sweet potato plants *in vitro*. The study demonstrated that varieties of sweet potato respond differently to the shoot initiation and shoot multiplication media with respect to PGRs concentration, in a gentotype x environment interaction. With an optimal PGR combination and concentration for a wider range of genotypes, as demonstrated here, the prerequisite for using meristem cultures for cleaning is in place. A combination of heat therapy and meristem tip culture is the most efficient way to remove sweet potato viruses compared to using meristem tip culture alone. The efficiency of SPFMV elimination also varied with the variety of sweet potato used as test plants. Although virus elimination is costly and time consuming, our study represents a stepping-stone and produced pathogenic plants free from known pathogenic viruses in five high yielding varieties of Ethiopian sweet potato plants. We recommend the maintenance of this virus-free stock, its further multiplication, and incorporation into the seed systems of the country; any of the virus detection methods can be used to rapidly test a large number of plants required for clean planting material schemes, or for screening in the event of a virus outbreak in a production region

5. Future Perspectives

- Organizing and the strengthening of the quarantine systems during importation to the country and certification of planting materials movement between regions is very important.

- Standardize method for large scale virus detection in Ethiopia

- Future virus surveys should address more production regions in the country and use appropriate testing methods

- Virus surveys that cover wider production regions in Ethiopia using appropriate testing methods

- Increase awareness of viruses to farmers and extension workers

- If our field experiments with clean material of sweet potatoes get reinfected and the yields reduced too soon, then a scheme of virus elimination and providing farmers with clean elite stock will be futile and not worth further investments. However, it is still possible to use alternative management methods that effectively reduce sources of infection and transmitter vector.

- An alternative strategy could be to use the newly developed gene editing tools to make elite stock more/fully resistant to the main pathogenic viruses. CRISPR technology has been found to be very effective for virus protection in bacteria and *Archea* and could be an option for the not too distant future. Our efforts to establish a system for virus elimination is not in vain, as this may be the necessary steps to test first, before the authorities allow researchers to try gene editing as to test the potential for the best way forward.

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Appendix 1

Table 1. List of virus and viroids detected in initial plant and plants generated after heat treatment and meristem culture

Cultivar	**Before HT**		**After HT**	
	RT-PCR	NGS		
Berkume	-	SPBV-A, SPBV-B, SPBV-C, CYMV, CaVX, HSVd	-	PVY, GYSVd1, 4 HSVd, CCaV
Guntute B	SPFMV, SPVC, SPCSV	SPFMV, SPVC, SPVG, SPCSV, SPBV-A, SPBV-B, SPBV-C, SPSMV-1	-	MoCV1
Kulfo	SPFMV	SPFMV, SPVC SPBV-A, SPBV-B, SPBV-C, SPSMV-1	-	SPBV-A, SPBV-B, SPBV-C,
Hawassa 83	SPFMV	SPFMV, SPVC, SPVG, SPBV-A, SPBV-B, SPSMV-1	-	SPFMV, SPVC, SPVG, SPBV-A, SPBV-B, SPBV-C, SPSMV-1
Tola	SPFMV	SPFMV, SPVG SPBV-A, SPBV-B, SPBV-C, LYSV	-	SPVG, SPBV-A, SPBV-B, SPBV-C, SPSMV-1

Cactus virus X (CVX); Citrus yellow mosaic virus (CYMV), Grapevine yellow speckle 1(GYSvd1), Leek yellow stripe virus (LYSV), Grapevine fleck virus (GFkV), Magnaporthe oryzae chrysovirus-1(MoCV1), Cymbidium mosaic virus (CMV); Grapevine Syrah virus -1(GSyV-1), Sweet potato chlorotic stunt virus (SPCSV); Sweet potato virus G (SPVG); Sweet potato Badnavirus (SPBV); Sweet potato symptomless Mastrevirus (SPMVS); Sweet potato virus C (SPVC); Sweet potato feathery mottle virus (SPFMV). Grapevine yellow speckle 1 viroid (GYSVd1), Hop stunt viroid (HSVd), Citrus cachexia viroid (CcVd).

Errata list

PhD candidate: Dereje Haile Buko

Thesis: Sweet Potato Virus in Ethiopia - Detection, Characterization, Elimination and Management

Date:24 10 2019

Side	Line	Original text	Corrected text
Thesis Introduction			
Page: i	1	, missed	, added
Page: iii	5	of	to
Page:vii			for ever
Xiii -xv		i - viii (wrong page numbers that are doubled in the second half of the thesis front)	x-xv
Page viii	4 5 6	List of table content changed in thesis front List of paper - ii Summary - iii Sammendrag - vi	 List of paper-ix Summary-xi Sammendrag - xiii
Page 42	9	Equation	solutions
Paper 1			
Page: 11	20	Fig. 1A, 1B, 1C	Fig. 2A, 2B, 2C
Page: 14	1	Tabel 1.	Table 3.
Page:16	4	Tabel 2.	Table 4.

Paper I-V

PAPER I

An update of sweet potato viral disease incidence and spread in Ethiopia

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Abstract: Sweet potato (*Ipomoea batatas* (L.) Lam.) is an important root crop for poor farmers in developing countries. Since the late 1980s, viral diseases have increasingly become a threat to sweet potato production in Ethiopia. This review paper presents the role of sweet potato production for ensuring food security, the level of sweet potato virus research, including the types of viral species identified and their current level of incidences in Ethiopia. *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), Sweet potato virus 2 (SPV2), Sweet potato virus G (SPVG), and *Cucumber mosaic virus* (CMV) were reported in Ethiopia, where the first two are the most common and exist at high incidences. In addition, this paper discusses the virus vectors, virus transmission methods to new farms, factors exacerbating the rate of viral incidence and the methods used to reduce the incidences. Moreover, it highlights methods of sweet potato viruses' detection and cleaning of infected materials in use and the challenges encountered towards the efficient utilization of the methods. Finally, we suggest major intervention techniques that will integrate all key players in managing the impact of the virus on sweet potato production to improve productivity and ensuring food security in Ethiopia. The findings obtained from this review could be an input for the current research on sweet potato improvement (both planting materials and routines) in Ethiopia.

Key words: sweet potato, virus diseases, detection, planting materials

Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam.) is an important root crop in developing countries. It is grown in 115 countries, where China is the leading producer. Amongst the main crops produced in the world, sweet potato is ranked 7th on the base of production volume (FAO 2017). In most developing countries, it ranks fifth in the order of food importance (Som 2007) and is the third main crop after cassava and maize in East Africa (FAO 2014).

There are many reasons why sweet potato is important and preferred by growers. It is a food, feed and an important raw material for the industry (Bovell-Benjamin 2007). Growers choose to grow sweet potato crops because of its ability to tolerate a wide range of growth conditions, it has lower demand for agricultural inputs, high yielding potential per unit area per unit time, ease of cultivation and an effective vegetative propagation method (Woolfe 1992). Moreover, it has a high nutritive value (primarily of carbohydrates and vitamins) and is suitable to grow on marginal lands. Due to all these merits, sweet potato remains a competitive crop for food security in developing countries (Gibson 2009).

Sweet potato improvement researches have been started in the early 1980's in Ethiopia, and so far, 26 improved varieties were released with their appropriate production packages (Shonga et al. 2013). On the other hand, little is known and less attention was given to sweet potato viruses and the associated diseases until very recently. Furthermore, there is no adequately documented information on viral diseases (virus species, incidence, impacts on yield and efforts made so far to reduce its incidence) in Ethiopia. Thus, this research gap has been a problem for researchers in identifying, prioritizing and tackling constraints to sweet potato production, as well as in designing appropriate disease management strategies for Ethiopia.

Therefore, the objectives of this paper are 1) to review the literature on sweet potato viral species identified the level of incidence and impact of the diseases on sweet potato production in Ethiopia

2) to identify research questions and bring to the attention of researchers and stakeholders 3) suggest possible alleviation strategies.

Production status of sweet potato and its role in food security in Ethiopia

Sweet potato production in Ethiopia is currently increasing, hence its role to combat food insecurity. Based on the production volume, Ethiopia is ranked seven sweet potato producer in the world (FAO 2017). Sweet potato stands second, after potato *(Solanum tuberosum)* in area coverage among the root crops grown in the country, but is ranked first in terms of production per hectare (Central Statistical Agency 2015). Sweet potato is cultivated on 130,000 hectares of land in Ethiopia, with an annual total production of 2, 0089, 290 tons (FAO 2017). Over 95% of the sweet potato cultivations are in the densely populated areas in the southern, southwestern and eastern parts of the country (Central Statistical Agency 2010). Oromia Regional State and Southern Nations Nationalities and Peoples Region (SNNPRS) are the two major producers contributing 52.15% and 47.15% respectively to the annual sweet potato production (Central Statistical Agency 2010).

The contribution of sweet potato to poor farmers of Ethiopia has so far been underestimated. Farmers of Ethiopia cultivated sweet potato for several years either as a main or as a supplementary source of food. Farmers produce sweet potato mainly for own consumption and also to some extent as sources of income. Sweet potato is a food security crop for at least 20 million Ethiopians (Tofu et al. 2007). It is highly valued when there is shortage of other crops (Emanna 1990). This is because it withstands drought and performs well on less fertile soil without significant compromises. Sweet potato crop has potential to improve food and nutritional security (especially the orange fleshed with pro vitamin A precursor) for poor farmers (Tsou and Hong 1992). It is amongst the underutilized crops in most Sub-Saharan Africa countries, including Ethiopia, compared to other sweet potato producing countries in Asia. Recently, there are efforts by various NGOs and Government institutions to introduce sweet potato to other regions in the northern, eastern and western parts of the country, to diversify their crop production (Shiferaw et al. 2014;

Aldow 2017). However, sweet potato yields can vary drastically due to viral diseases (Alemu 2004; Tesfaye et al. 2013).

Sweet potato-infecting viruses identified in Ethiopia

There is no clear evidence of when or how sweet potato viruses were introduced into Ethiopia. However, *Sweet potato feathery mottle virus* (SPFMV) was first identified around three decades ago, at a place called Nazret [(Scientific Phytopathological Laboratory 1986)]. There has been no sweet potato virus study in Ethiopia before the study by Alemu (2004). Since then, a number of surveys have been conducted to document the incidences, severities and identities of sweet potato viruses; mostly performed in southern Ethiopia (Alemu 2004; Adane 2010; Tesfaye et al. 2011; Tesfaye et al. 2013). The infecting viruses were tested in samples obtained from sweet potato germplasm collections maintained at the research sites and in farmers' fields, mostly located in southern Ethiopia. The presence of five sweet potato infecting viruses in Ethiopia were confirmed by these survey studies (Table 1), out of the thirty virus species known to infect sweet potato worldwide (Clark et al. 2012). Moreover, the surveys also revealed that SPFMV is the most frequently detected virus in southern Ethiopia, followed by SPCSV. None of the other viruses tested [*Sweet potato mild mottle virus* (SPMMV), *Sweet potato latent virus* (SPLV), *Sweet potato chlorotic fleck virus* (SPCFV), Sweet potato caulimo-like virus (SPCaLV), *Sweet potato mild speckling virus (SPMSV) and C-6 virus*] were detected in these surveys. However, a recent report indicated that all these six viruses have later been detected in germplasm, imported into Ethiopia for the purpose of screening for diseases incidence and other traits (Shiferaw et al. 2017).

SPFMV and SPCSV, their mixed infections: a threat to sweet potato production in Ethiopia

Sweet potato viral diseases are the second most important limiting factor to sweet potato production next to the weevil in Ethiopia (Fite et al. 2014). SPFMV and SPCSV are the most frequently detected viruses in Ethiopia. For example, high infection of these viruses reported in sweet potato germplasm collections in the research fields at the Hawassa and Wendo Genet

Agricultural Research Centers (Adane 2010). The extent of SPFMV and SPCSV incidence and its economic importance has been described previously in many more sweet potato producing locations in the SNNPRS (Alemu 2004; Tesfaye et al. 2011; Tesfaye et al. 2013). SPFMV and SPCSV can occur as single infections or as mixed infections. Single infection of these viruses results in mild symptoms (and many times as symptomless infections). However, when both viruses occur as mixed infection, the symptoms are more severe and results in what is known as Sweet potato viral disease (SPVD). Single and multiple infections of sweet potato plants with SPFMV, SPCSV and SPVG and sweet potato virus II (SPV2) are also not uncommon in Ethiopia (Adane 2010; Dugassa and Feyissa 2011; Tesfaye et al. 2011). Recent studies also confirmed that SPFMV and SPCSV infections and their co-infection have become serious problems in the farmers' and sweet potato multipliers' fields [(Own unpublished data) (Mebrate 2018)].

Summary of virus survey literature review reveals few studies that covered only limited locations were conducted on sweet potato virus diseases in Ethiopia (Fig. 1). Moreover, most of the studies were limited to locations mostly in SNNPRS (Adane 2010; Tesfaye et al. 2011; Tesfaye et al. 2013). Unfortunately, no study has been carried out in other sweet potato growing areas of Ethiopia, except a single study data that was generated from samples collected from Hararge zone, eastern Ethiopia (Tesfaye et al. 2011). Therefore, extensive surveys that cover all the sweet potato production regions are required to determine the current status of sweet potato viral diseases in each location to determine the appropriate preventive measures. Furthermore, it is also important to study virus incidences in wild relatives of sweet potato, since these can act as alternative hosts to viruses infecting cultivated sweet potato.

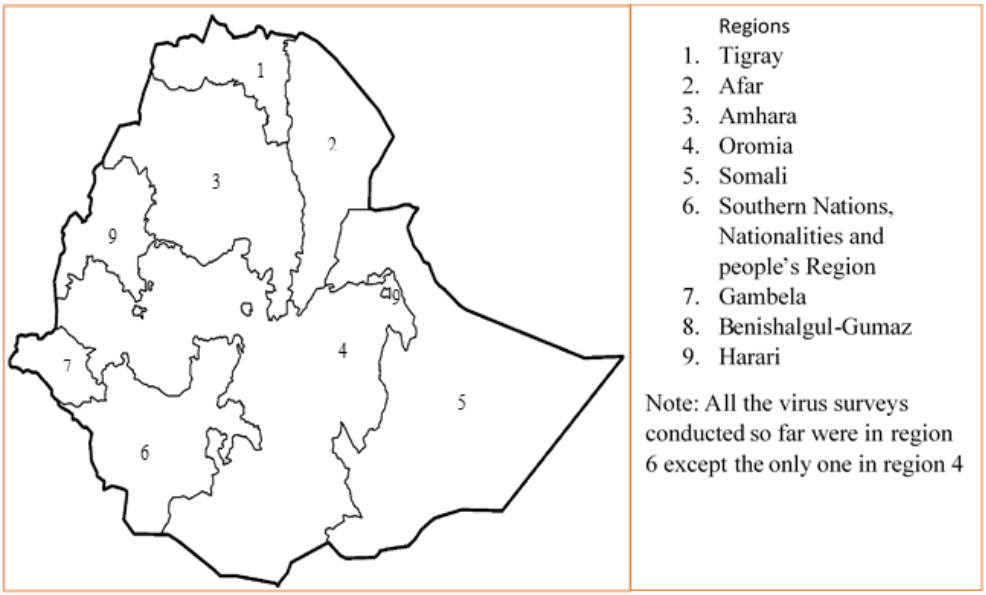

Figure 1. The nine regions in Ethiopia

Disease incidence and yield reduction

The incidences of viruses in sweet potato research sites and in farmer's fields in the southern part of Ethiopia are summarized in Table 1. Sadly, research sites (germplasm collections and experimental stations) are more infected than the farmers' fields. Up to 80% and 100% virus incidences were reported in the samples collected from farmers' fields and germplasm collection sites, respectively (Adane 2010). The author reported one or more viruses detected in those samples. Likewise, Tesfaye et al (2013) reported an incidences of 75% in the samples from farmer's fields and 100% in the experimental stations. The relatively higher viral diseases incidences documented in samples collected from germplasm collections at research stations might be an indicator of the fact that the germplasms imported for adaptation trials were sources of virus infection. Exchange of germplasm between countries for adaptation trials has been a common practice in Ethiopia. However, farmers still grow few improved and mostly local cultivars. Free

exchange of planting materials could also be one of the largest contributors to the spread and distribution of viruses in Ethiopia. Therefore, designing and establishing strong quarantine procedures is required to prevent introduction of infected materials. Moreover, any imported germplasm should be restricted from field planting for propagation and adaptation trials until confirmed free of pathogen and insect pest. The above findings confirmed the status of viral diseases incidence in germplasm in Ethiopia is similar to that of Uganda, which ranges from 86 to 100 % (Aritua et al. 2007). However, it differs from that of Kenya 48% (Ateka et al. 2004) and Tanzania 17 to 33% (Ndunguru and Kapinga 2007). Thus, the incidences and severity of sweet potato viral diseases in East Africa are variable.

SPVD is the main bottleneck of sweet potato production in many parts of the world as reviewed by (Gibson and Kreuze 2015). As sated before, SPFMV and SPCSV incidences are at a high level and SPVD is widely spread in SNNPR, Ethiopia, (Adane 2010; Tesfaye et al. 2011). SNNPR is the main source of germplasm for trials and planting materials for production to all production regions in the country. Hence, virus-infected materials distributed from SNNPR could be an important sweet potato production threat of the country. A general decline in sweet potato productivity per hectare was observed over the decades (FAO 2017). However, no reliable studies have been conducted to estimate the extent of yield loss by virus infections.

As mentioned previously in this report, the incidence of viral diseases in Ethiopia is similar to that of Uganda. If the incidence of viral diseases correlates with the observed yield loss in Uganda, one could expect losses of up to 98% in Ethiopia (Gibson et al. 1998; Karyeija et al. 1998; Mukasa et al. 2003). A recent study that compared infected and healthy planting plants in screen house in Ethiopia showed up to 100% losses yields which depending on varieties and infecting virus(es) infection (Own unpublished). However, yield losses are also dependent on the varieties grown, viral type present and climatic condition during the growth period. For example, the incidence of sweet potato viruses in China can be up to 90 % (Wang et al. 2010), although the average yield loss due to viral diseases ranges between 20 - 30% (Feng et al. 2000). Nevertheless, the high

incidences of viral diseases in Ethiopia, the lack of efficient diagnostic tools, and lack of virus-free planting materials are among the factors that continue to contribute to the dissemination of viruses within the country. Thus, there is a need to set up diagnostic laboratories and reliable detection methods.

Methods of virus detection

Virus testing employs different diagnostic methods. These methods range from the screening of disease symptoms in the fields to the use of more sophisticated molecular detection techniques (Boonham et al. 2014; Jeong et al. 2014). The available technological level, existing laboratory facilities and competent workforce to conduct the work influences the choice of method. An assays based on the biological and serological properties of viruses is the commonly used method in developing countries. However, molecular detection methods are more rapidly emerging this days.

Many sweet potato infecting-viruses induces no or mild symptoms on infected plants. For example, SPFMV infected plants is mostly symptomless. Sweet potato badinaviruses causes no symptoms (Kreuze et al. 2017). In this circumstances, ELISA could not be a good testing methods. Therefore, grafting sweet potato to an indicator plant is very useful, especially when the virus titer concentration in the original host is below the detection limit of serological tests; Enzyme Linked Immunosorbent Assay (ELISA). However, grafting alone cannot decided the type of the infecting virus. Unfortunately, grafting is a lengthy method (at least a month) and needs greenhouse space. In some cases, some viruses induce no symptoms even in the highly susceptible indicator plant *Ipomoea setosa* (Clark et al. 2012). Therefore, identification of viruses solely based on symptom expression on host plants is not recommended and combined with other testing methods.

ELISA is widely used in many laboratories across the globe. This method is very quick and sensitive for detecting plant viruses, provided that antibodies are available. As described before, due to the low virus titer, an initial grafting to *Ipomoea setosa* or other hosts is required to increase

virus titer to detectable levels in many cases. It also requires laboratory equipment, which makes ELISA less accessible, particularly in developing countries. In recent years, a combination of serological and nucleic acid-based assays are common plant virus detection methods. Lack of proper laboratory facilities and technical capabilities, access to reagents have limited many developing countries from establishing these detection methods. The methods used to detect viruses in sweet potato plants grown in Ethiopia are summarized in Table 2.

Table 1. List of areas surveyed, number of sweet potato samples tested and sweet potato specific viruses detected in Ethiopia.

Location of sampling	Total No. of samples tested	Numbers of samples reacted positive to virus and mixed infection*									References
		SPFMV	SPCSV	SPFMV+ SPCSV (SPVD)	SPFMV +SPVG	SPFMV+ SPCSV +SPVG	CMV	SPVG	SPV2		
Southern Ethiopia	318	196	0	0	0	0	-	3	-		(Alemu 2004)
Hawassa ARC	57	22	21	7	0	0	-	0	1		(Adane 2010)
Wondo Genet ARC	127	79	13		0	0	0	0	2		
Hawassa Research Center	6	6	6	0	0	0	0	0	0		(Dugassa and Feyissa 2011)
SNNPRP and eastern Oromia	970	146	125	90	0	0	0	44	0		
Symptomatic samples	235	134	115	88	25	0	0	28	0		(Tesfaye et al. 2011)
Asymptomatic samples	735	13	10	0	7	0	0	15	0		
Hawassa ARC	32	+	+	+	0	+	+	+	0		(Wondimu et al. 2012)
Southern Ethiopia Farmer field	166	ni	ni	83	ni	0	ni	ni	ni		(Tesfaye et al. 2013).
Research stations		ni	ni	+ (46-100%)	ni	0	ni	ni	ni		

*Presence of the viruses were confirmed by test methods listed in table 2.

SPFMV*: Sweet potato feathery mottle virus,* SPCSV*: Sweet potato chlorotic stunt virus,* SPVG*: Sweet potato virus G,* CMV*: Cucumber Mosaic Virus,* SPV-2: Sweet potato virus 2, +: detected but number not indicated**, -**: not tested for, ni: no information if tested or not, and ARC: Agricultural Research Centre.

Sources of virus infectious agents and possible means of dissemination

As previously stated, there is no clear evidence of when or how sweet potato viruses were introduced into Ethiopia. Nevertheless, it is believed that viruses were introduced after the 1980s, when the exchange of planting material for breeding purposes increased between many African countries. Many sweet potato cultivars were introduced between 2001 to 2003 from African (Tofu et al. 2007). Consequently, viruses problem in southern Ethiopia was recognized during 2006 to 2009 (Shiferaw et al. 2014). For example, as presented in this paper, recently six new viruses not previously identified in farmer's fields in Ethiopia were detected in sweet potato germplasm that was introduced from international sources (Shiferaw et al. 2017). This confirms that the exchange of materials within and between places can be one of the main sources for infecting new areas.

Even though breeding programs have been running since the late 1980s and sweet potato is the second most widely grown root crop in Ethiopia, there are no well-developed sweet potato certified seed production systems. Consequently, there is no established mechanism to generate and supply healthy planting materials to the farmers. Farmers obtain planting materials from many different sources none of them go through reliable phytosanitary control. Most farmers save their own sweet potato planting material from the previous year harvest, while others obtain it either through local exchange from neighboring farmers or buy it from nearby local markets (Own unpublished). Such exchange of planting materials is done irrespective of the knowledge if the material is virus-free. For example, picture in Fig. 2A is an example of farmer's fields infected with virus and show virus-like symptoms. Fig. 2B and 2C respectively, show sweet potato cultivars in fields infected with SPFMV and double infection of SPFMV and SPCSV commonly called SPVD. Indeed, this is the main way in which viruses were introduced and spread from one area to another in African countries such as Uganda (Karyeija et al. 1998). In addition, farmers seldom renew their planting materials, but they keep it for many years by vegetative propagation. Therefore it builds up infection within their fields every year. This planting practice, combined with the fact that many sweet potato viruses are transmitted by aphids and whiteflies [widely distributed in Ethiopia,

(Table 3)] increases the risk of more severe infections and the establishment of viral disease in neighboring virus-free fields.

A

B

C

Figure. 2. Sweet potato plants with viral disease symptoms in Wolayta zone, southern Ethiopia. (Fig. 2A): Virus like symptoms in farmers field, (Fig. 2B): Mild symptoms of SPFMV infected sweet potato plant ('Kulfo') in the farmer field, (Fig. 2C): Stunted sweet potato plant due to co-infection by SPFMV and SPCSV initially from fields (Photo: D.H. Buko)

Table 2. Virus detection methods used in Ethiopia.

Methods used for detection	**References**
Based on biological properties of the virus Biological(graft-inoculation)*	(Alemu 2004)
Nucleic acid based (PCR, Sequencing of coat protein)*	(Alemu 2004)
Based on viral proteins NCM-ELISA, DAS and TAS-ELISA	(Alemu 2004; Adane 2010; Dugassa and Feyissa 2011; Tesfaye et al. 2011; Wondimu et al. 2012; Tesfaye et al. 2013)

*Performed in Germany on Ethiopian plant material

NCM-ELISA: Nitrocellulose membrane-Enzyme linked immunosorbent assay, DAS-ELISA: double antibody sandwich enzyme-linked immunosorbent assay, TAS-ELISA: Triple antibody sandwich enzyme-linked immunosorbent assay, PCR: polymerase chain reaction

Table 3. Symptoms of common sweet potato viral diseases and vectors involved in their transmission.

Virus	Symptom observed in sweet potato	Ways of transmission	Geographic distribution
SPFMV	Single infection: no clear observable symptoms when it infects alone or only mild circular spot on the older leaves or light green pattern along veins. Feathery, purple pattern in the leaves (Gibson et al. 1997; Ryu et al. 1998). It could vary based on cultivar infected and growth conditions	Via stylet of several aphid species in a non-persistent manner, (Stubbs and McLean 1958)	Worldwide, Reported in Ethiopia
SPCSV	Single infection: causes slight stunting, purpling of lower leaves, mild chlorotic mottle and yellowing (Gibson et al. 1998; Gibson and Aritua 2002).	Transmitted by whiteflies in Semi-persistently manner (Sheffield 1957; Sim et al. 2000)	Worldwide, reported in Ethiopia
SPFMV + SPCSV (SPVD)	Dual infection: Infected plant became stunted and produce small-distorted edges, narrow crinkled, strap like leaves with chlorotic mosaic or vein clearing, purpling of older leaves, chlorosis along main leaf veins (Schaefers and Terry 1976; Gibson et al. 1998)	See above for individual virus	Worldwide, but severe in Africa Reported in Ethiopia
SPVG	Ranges from symptomless to yellow spotting on the leaves	Aphids	Worldwide, reported in Ethiopia
SPMMV	Symptomless to mild leaf mottling and stunting	May be transmitted by whiteflies to sweet potato (Sheffield 1957; Hollings et al. 1976)	Burundi, Kenya, Tanzania, Uganda, Philippines
SPVG + SPCSV	Symptomless to purple spots and inter-veinal yellow spots	Aphid (SPVG) and Whitefly (SPCSV)	
SPV2	No information for single infection	Transmitted by Aphid (Moyer et al. 1989)	Worldwide, reported in Ethiopia
SPVC	No information for single infection	Transmitted by Aphid (Moyer et al. 1989)	Worldwide, recently identified in Ethiopia (own unpublished)

SPFMV: Sweet potato feathery mottle virus, SPCSV: Sweet potato chlorotic stunt virus, SPVG: Sweet potato virus G, SPMMV*: Sweet potato mild mottle virus, SPVC: Sweet potato virus C.*

Methods of virus elimination and virus-free planting material in Ethiopia.

Different virus elimination methods have been developed and applied to produce disease-free clones of economically important crops around the world. Meristem tip culture and shoot tip culture alone and/or in combination with different therapeutic actions: heat treatment

cryotherapy and chemotherapy have been used to eliminate virus from many crops, including sweet potato infecting virus in many countries (Spiegel et al. 1994; Panta et al. 2006; Wang and Valkonen 2008; Panattoni et al. 2013). These methods have been applied to generate virus-free sweet potato in many countries of the world: Taiwan (Green et al. 1992), United States of America (Clark and Hoy 1999), China (Feng et al. 2000), many countries in Europe (Wang and Valkonen 2008) and Japan (Yamasaki et al. 2009). Virus from different plant species (root crops, ornamental crops, and tree) have been eliminated by heat-treating mother plants followed by meristem tip culture (Hakkaart and Quak 1964).

In general, developing countries in East Africa, including Ethiopia, are seemingly left far behind in the adoption and application of tissue culture techniques for virus elimination. In Ethiopia, few attempts have been accomplished to develop *in vitro* propagation protocols and use of virus elimination techniques for sweet potato. However, meristem culture and heat treatment have been used and were able to eliminate viruses from three varieties of sweet potato in Ethiopia [(Table 4) (Dugassa and Feyissa 2011)]. There varieties cleaned of viruses were not maintained. The efficiency of meristem culture and combined heat treatment have also been evaluated and compared (Dugassa and Feyissa 2011). However, there is no schemes developed and in use to provide virus tested material.

Generating and providing 'virus-free' sweet potato planting materials increases yield per hectare, which improve human food security and livestock fodder. Virus elimination and explant-regeneration requires a good tissue culture protocol. Developing new or adopting and modifying existing protocols previously developed elsewhere in the world important. However, lack of and /or limited laboratory facilities, lack of practically trained workforce, less access to reagents, absence of functional greenhouses and insect proof screen houses are still considered to be the main challenges to adopt and use the existing techniques in Ethiopia.

Still virus infection high level, planting materials available to farmers are unreliable in terms of viral infection. Therefore, urgent attention and action is in need to increase the yield of sweet potato, mainly by providing clean planting material and measure to reduce re-infection rate.

Implementation of integrated strategies that target the prevention of the introduction of virus, their vector, and their distribution into uninfected areas. Such strategies in turn, could enable resource-poor farmers to maintain healthy sweet potato planting materials.

Table 4. List of sweet potato cultivars tested for virus and virus elimination methods used in Ethiopia.

Cultivar/Accessions	Elimination methods	Number of clone tested	Elimination efficiency*	References
'Hawassa 83'	Meristem culture	9	100%	
	Shoot tip thermotherapy	9	88.89%	
'Guntute'	Meristem culture	6	100%	(Dugassa and Feyissa 2011)
'Hawassa local'	Meristem culture	8	100%	
	Shoot tip thermotherapy	6	100%	
'Bellela'	Meristem culture	24	99.96%	
'Temesgen'	Meristem culture	24	100%	(Wondimu et al. 2012)
'LO-323'	Meristem culture	25	100%	
'Zapallo'	Meristem culture	25	100%	

*Efficiency of virus elimination methods were determined based on the percentage of virus-free plantlets obtained by each methods

Production challenge due to sweet potato virus call for intervention in Ethiopia

Strong quarantine restrictions

As presented in Table 1, five sweet potato infecting virus species were detected in southern Ethiopia. To limit further spread of the existing viruses into new production regions, the

different key players need to work in synergy. Before distributing planting materials from virus spot location of southern Ethiopia to a new locations, healthy status must be first confirmed. In this regard, Research Centers must apply rigorous quarantine checks. The technical guidelines for the exchange of pathogen-free sweet potato plant materials should be followed (Moyer et al. 1989). Both exporting and importing bodies ought to abide by these guidelines.

Screening and breeding for resistance

In Ethiopia, cultivar diversity is getting lost due to low yielding and infection of viral diseases. This true particularly in the virus-prone areas of SNNPR where one of the improved variety called Hawassaa-83 dominantly grown. It is possible to screen virus tolerant sweet potato cultivars from local cultivars and use them for resistance breeding. Experts in Agricultural Research Centers in Ethiopia have been trying for a long time to screen and use disease tolerant varieties. The effort to solve the problem appears not yet to be successful, thus, the yielding potential of sweet potato cultivars is declining. Though it needs further effort, Shiferaw et al, (2017) reported of promising accessions for virus resistance. As presented earlier in the paper, the efforts to screen for disease resistance and better yield in southern Ethiopia was not without the risk of introducing viruses along with germplasm. It is advisable to exploit local cultivar gene pools in the country instead of introducing infected material as quarantine restriction of the country is strong enough to screen out.

Therefore, it appears that no adequate and appropriate interventions were made in screening and breeding for disease resistances in sweet potato and it needs a more coordinated effort of all stakeholders. Exploiting resistant genotypes from germplasm pools using both traditional and recent advanced molecular methods would be important.

Training

Sweet potato growing farmers and extension workers in Ethiopia have low perceptions of viral diseases (own, unpublished data). Inabilities to identify a virus-infected plant based on symptoms in the field and lacking basic know-how on its mechanisms of transmission affects

proper selection of healthy looking plating materials. Moreover, it contributes to the continuous use and exchange of infected planting materials from season to season. Therefore, training basic practices on disease identification and management is very important. Moreover, training that enable farmers selecting and using of good planting materials and how to practice sanitation measures would be vital.

Naturally virus resistant/tolerant cultivars, show no symptom depending on many factors. If the farmers do not have access to virus-tested planting materials, and still have to grow it, training would help them to select the best mother plants from the existing symptomless plants (with possible low concentration of virus titers or healthy) in their farm. Farmers' training on removing weeds that may harbor virus-transmitting insects is vital. In additions, weeds may serve as an alternative host for the viruses, must be removed on time. Educating farmers how to identify and rouging out infected plants, proper and timely application of sanitation practices and crop management is very important.

Farmers' closer mentors have high impacts in improving agricultural practices. Study conducted in Ethiopia shows extension workers in the studied areas were less exposed to training on sweet potato diseases identification and management (own unpublished data). It is important to provide problem-solving practical training to those who work closely with farmers. Extension workers should get awareness and training on the sources and choice of good planting materials (diseases free, high yielding), the negative effects of sweet potato viral diseases, practical virus identification in the field and knowhow of appropriate disease management principles. In general, training will greatly contribute to proper virus management that results in the higher chance of reducing the infection of new areas and improves the yield of crops. Training should include practicing sanitation measures in the field, removing infected plants timely to avoid virus spreads within plants and avoiding contamination of pathogen-tested planting materials.

Technical capacity building and laboratory facility

Expertise and basic laboratories are required for diagnosis, identification, and elimination of viruses. Without proper knowledge, it is more difficult to manage virus diseases. There are limited numbers of professionals and poor laboratory facilities in developing countries in general, both for virus diagnosis and elimination. In Ethiopia, there are very limited numbers of experienced plant virologists. Moreover, they have limited access to practically oriented training on identification and elimination of plant viruses, mainly because of a lack of access to properly equipped laboratories and reagents, both at the regional and national levels. This may have limited the applications of the practical knowledge learned and experiences gained during their studies abroad. In Ethiopia, virus elimination from sweet potato using shoot-tip and meristem culture is at an infant stage (Dugassa and Feyissa 2011; Wondimu et al. 2012). Even though virus elimination techniques have been developed and largely utilized across the world, they are less used in Ethiopia. Because of poor facility and technical problem, farmers in Ethiopia have no access to virus-tested planting materials. As a result, farmers continue to use virus-infected vegetatively propagated sweet potato planting materials that could build up over years. Therefore, availability of basic facilities and technically skilled professionals is important to develop/adopt effective methods and establish programs to develop and maintain pathogen tested propagation stocks of farmers preferred root crop cultivars.

This calls for collective and individual roles of all key-players including the government, Non-Governmental Organisation (NGOs), private sectors, research institutions, Ministry of Agriculture and universities in funding for laboratories and capacity building. Universities and research institutions should be more involved in training extension workers, farmers and other stakeholders. The government should play a major role in allocating funds for laboratory facilities and research activities. Researchers are expected to conduct studies and know the virus species associated with farmers preferred varieties in all the production regions. They should also work to design methods adapted to local conditions and evaluate the best virus elimination and subsequent management methods for the respective viruses.

Provision of virus-tested planting materials

Availability of disease-tested planting materials with desired agronomic traits is key to increase production and thereby improving the life of the farmers. Virus-tested materials can be obtained either through screening naturally existing plant materials or by eliminating viruses from mother plants. Very little progress has been made to identify and eliminate virus from vegetatively propagated materials in Ethiopia. There are no big companies certified to supply virus tested sweet potato planting materials, except for some recent practices of using tissue-cultured plants as a starter. Further multiplication in open fields makes the plants prone to re-infection before reaching the farmers. Moreover, these small-scale multipliers are not getting basic clean starting materials and have no rigorous follow up. In multiplier fields, viruses can also get multiplied and when distributed to farmers, it transmitted to the susceptible host in the nearby field and infect sweet potato landraces on farmer's hands. Therefore, a short-term solution to tackle the problem is to intervene through the provision of vines of pathogen-tested sweet potato plants to the farmers and giving awareness on subsequent management practices to reduce the infestation rate. The use of clean and virus-tested planting materials is economically viable if there is an effective and efficient system for production, multiplication, and distribution of planting materials (Carey et al. 1997; Feng et al. 2000).

What intervention is needed? Providing clean planting materials of root and tuber crops boosts yield and farmer's income. Therefore, all stakeholders (Government, NGOs, Research centers and Universities) are advised to give attention and acknowledge the necessity to provide resources for virus assessment and elimination. The private sector should be encouraged to collaborate with the universities and research centres and invest on tissue culture facilities for commercial production of healthy and quality vines. Initiating new ideas of investment in tissue culture and strengthening existing institutions and farmers' associations to propagate virus-tested plant is a priority. In addition, extension officers should contribute to demonstrate that the use of clean/symptomless planting materials would consistently produce higher storage root yield than the naturally infected farmers' planting materials.

Conclusions and recommendation

Surveys on sweet potato viral diseases in Ethiopia revealed that viral disease incidence and severity is a critical issue for sweet potato production in the southern region of Ethiopia. The Southern Nations Nationalities and Peoples Regional State is the National Center and source for a further introduction of sweet potato plant materials to other parts of the country. The current incidence of sweet potato virus in the southern parts of Ethiopia is a potential threat to sweet potato production in the whole country. As reported before, national germplasm collection and farmers' fields are contaminated with the most common sweet potato viruses; SPFMV, and SPCSV. As a result, the rate of spread and its negative impact on the yield is discouraging farmers who grow and use sweet potato as a main food security crop. Collectively, this demands intervention at all levels (i.e. both at institutions and farmers' levels). Moreover, new viruses are being introduced with germplasm from international sources. Therefore, germplasm introduction should be regulated and new materials should be inspected prior to introduction and multiplication in the open fields. Supplying virus-tested planting materials and establishing a system of distribution would enhance the farmers' ability to increase production and productivity of sweet potato.

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PAPER II

Sweet potato viral diseases and insect pests in Ethiopia: farmers' perception of their importance and management practices

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Abstract

Viral diseases and insect pests have become detrimental to sweet potato production in Ethiopia. This study was conducted to investigate farmers' perception of sweet potato viral diseases, insect pests transmitting the viruses, and farmers' management practices in Wolayta and the west Hararge zones in Ethiopia. We conducted in-depth, face-to-face interviews involving 160 farmers who grow sweet potato and 34 extension workers. The majority of the interviewed farmers (64.7%) had never heard about sweet potato virus diseases and associated symptoms. Their perception is that the virus symptoms they observe on the plants are caused by environmental stress (moisture and sun scorch) during the dry season. Most of the farmers (71.15%) do not know that viruses can be transmitted, and 56% do not know the mode of virus transmissions and the ways to reduce the transmission. However, 77.9% of the respondent farmers were aware that insects feed on sweet potato but did not know if the insects transmit viruses or not. Weevil, butterfly, aphids and whitefly are among insect pest known to infest sweet potato in Ethiopia. Farmers use their own local knowledge and practices and also practices recommended by extension workers to manage insect pests and diseases. The majority of the farmers (85%) and extension workers (80%) reported that they received neither training for the identification of sweet potato diseases and insect pests nor for management methods. The study revealed a lack of awareness amongst farmers and extension workers on sweet potato viruses, associated symptoms and means of transmission. Therefore, we recommend that the government should provide adequate awareness training for all

stakeholders, particularly for the extension workers and farmers, to improve the management of sweet potato viral diseases.

Keywords: Sweet potato, farmers, perception, virus, diseases, cultural practices

Highlights

- Viral diseases and insect pests are the major constraints of sweet potato production in Ethiopia
- Farmers use indigenous knowledge and practice to manage diseases and insects
- Farmers had low awareness of plant infecting viruses, symptoms and modes of transmission and their proper management
- Farmers and extension workers received little training on both sweet potato diseases and insect pest management
- Need to strengthen awareness through different forms of interventions

1. Introduction

The number of different sweet potato infecting viruses that have been identified in the world is increasing and the viral diseases that they cause have become more damaging to sweet potato production. This has resulted in as much as a 50% reduction of yield caused by viral diseases (Loebenstein, 2015). Since an early report on a suspected virus in Eastern Africa (Hansford, 1944), more than 30 different virus types have been identified to infect sweet potato crops worldwide (Clark et al., 2012). Viral diseases and insect pests are major restraining factors of sweet potato production in Ethiopia (Tesfaye et al., 2013). Incidences of two common sweet potato infecting virus types, *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV), have repeatedly been reported to be very high, especially in southern Ethiopia (Adane, 2010, Tesfaye et al., 2011, Tesfaye et al., 2013). SPFMV was the first sweet potato virus reported from Ethiopia in 1986, whilst SPCSV was latterly detected in 2010. Viral disease is recognized as a major problem to sweet potato production in the farming areas of Southern Nation, Nationalities and People's Region (SNNPR) of Ethiopia and has resulted in yield decline.

Several sweet potato insect pests were reported in Ethiopia over the last 3 - 4 decades. Sweet potato weevil (*Cylas puncticollis* Boehman) and Sweet potato butterfly (*Acraea acerata Hew*), are the major sweet potato insects (Azerefegne, 1999, Emanna, 1990). Furthermore, a farmer perception study in SNNPR indicated that insect pests are the major constraint of sweet potato production (Ashebir, 2006). According to Ashebir, two insect pests, sweet potato weevil (63.8%) and sweet potato butterfly (27.6%), were perceived as important insect pest for farmers. In addition, virus transmitters including *Aphis gossypii Glover* (Homoptera: *Aphididae*) and *Bemisia tabaci* (*Gennadius)* (Homoptera: *Aleyrodidae*) can be pests in Ethiopia. A recent study also showed weevils to be a common threat to sweet potato production in eastern Ethiopia (Fite et al., 2017).

Successful management of virus and insect pests at a farm level is extremely difficult without participating farmers in the process participation. Despite the negative impact of viral infection to sweet potato production (Wambugu, 2003), traditional farmers in developing countries have little understanding about plant diseases caused by viruses, bacteria and fungi, and the possible means of their management (Adam et al., 2015). To date, there are no

effective and complete methods to control plant viral diseases in the field, and the best option is to prevent the vectors from sucking sap of crop of interest using different barrier crops (Hooks & Fereres, 2006).

. In a subsistence cropping system, it is difficult to control viral disease (Rukarwa et al., 2010) because farmers cannot afford insecticides for vector control. In addition, there are no effective chemicals available against virus diseases (García-Arenal & McDonald, 2003). However, vector management and the use of cultural practices to reduce the source of infections remain important management tactics. Several other management strategies including phytosanitory measures and deployment of genetic resistance to prevent or reduce infection have been recommended (Maule et al., 2007, Van den Bosch et al., 2007). However, the efficiency of these strategies depends on the farmers' awareness of the plant infecting viruses.

Implementing appropriate disease management practices is very important for profitable crop production, and assurance food security. Farmers often use their indigenous knowledge for the management of insect pests and plant diseases Democratic Congo (Munyuli, 2016). We assume that such basic knowledge and practices to manage disease and insect pests vary depending on the farmers' education and exposure to training and extension services. Research has shown that a farmers' decision to use or not to use chemical pesticides on rice crops varied between those farmers who attended integrated pest management farmers' field school, and those who did not attend (Andriarti et al., 2019). It is difficult for uneducated farmers to identify disease causing agents. However, it was evident that farmers understand the discomfort their crops experience just by observing the symptoms and sign on the plants (Adam et al., 2015). It was also evident that farmers have their own ways of managing sweet potato disease causing agents, when they know the nature of the disease from past experiences (Adam et al., 2015).Studies undertaken to investigate farmers' perception of plant viruses in vegetables and legumes in Asia indicated that farmers had not received any training in this regard (Schreinemachers et al., 2015). In Tanzania, a study revealed that farmers have very little information about economically important sweet potato virus diseases (Rwegasira et al., 2004a) and the researchers recommended the urgent need for research that is aimed directly at the practitioners at the farm level.

Viruses and insect pests are key constraints to sweet potato production in southern Ethiopia (Adane, 2010, Tesfaye et al., 2011, Tesfaye et al., 2013). Food and Agricultural Organization statistical data show that sweet potato productivity per hectare in Ethiopia has declined in the last decade (FAO, 2017). Effective management of plant diseases and insect pests needs the involvement and cooperation of farmers. A farmers' willingness to cooperate depends on the level of understanding that they have on the damage caused by the diseases and insect pests. The farmers' perception and knowledge of sweet potato viral diseases, as well as the identification of vectors of viruses, understanding of symptoms of plant diseases and the means of transmission of diseases have not yet been studied in southern Ethiopia, despite the existence of significant yield losses of sweet potato due to viral diseases. A survey to study farmers' indigenous knowledge on viral diseases and insects, incorporating their knowledge into control solutions, may further improve their knowledge of plant diseases. This particular study was conducted with the objectives of evaluating farmers' perception of sweet potato viral diseases and insect pests, mode of transmission of the viruses, and their management practices.

2. Materials and Methods

2.1 The study sites

The study was conducted in 2015, at two main sweet potato producing zones: Wolayta in SNNPRS and west Hararge in Oromia Regional State of Ethiopia (Fig. 1). The Wolayta zone is located at 037°35′ - 037°58′E and 06°57′ - 07°04′N (Laekemariam et al., 2016) in southern Ethiopia. The West Hararge zone is located in the eastern part of Ethiopia at 8° 39' 59.99" N and 40° 29' 59.99" E. In each zone, two main sweet potato growing districts were purposely selected with the assistance of the extension officers and researchers from the International Potato Centre (CIP) working on sweet potatoes. Sodo Zuriya and Boloso Sore districts are in the Wolayta zone of SNNPR and Tulo district is in the west Hararge zone of Oromia region. The selection of these districts was based on production status, and severity of Sweet potato virus disease (SPVD). In each district, three kebeles (lower administrative units) were selected based on the intensity of sweet potato being grown as recommended by the extension officers in the localities.

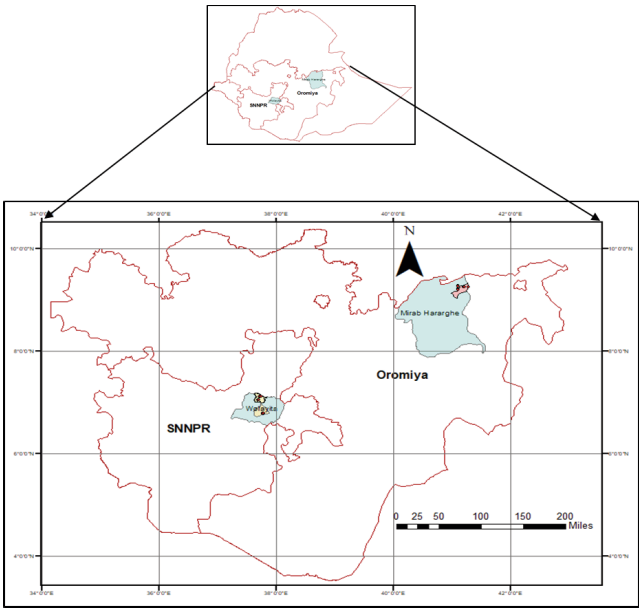


Figure 1. A map of Ethiopia showing the regions where the survey was conducted in southern and eastern Ethiopia.

2.2 Sample size and sampling procedures

Twenty farmers with more than 3-years of sweet potato farming experience were randomly selected from each kebele. A total of 160 household heads (121 from Wotayta zone and 39 from west Hararge zone) and 34 agricultural extension workers, who work at *kebele* and district administrative levels in Wotayta zone, were interviewed. Agricultural extension workers who have a good working relationship with the local respondents in each location accompanied the survey team as a guide to select the households. The survey team obtained the respondents' consent and explained the purpose of the study before starting the interview.

To resolve any language barrier, we selected interviewers in the field of plant sciences who spoke the native language of the respondent farmers. The interviews were conducted face to face, sitting next to sweet potato fields and were based on pre-prepared questionnaires that had been translated to the language of the respondent farmers. The investigator accompanied the survey teams to explain questions when necessary.

Direct field observations and visual aids (A4 laminated colour prints of virus disease symptoms and insect pests) were used to provide a more detailed explanation of the questionnaires to the farmers. These tools were particularly used during the field visits to gather adequate information on the severity of virus disease symptoms and damages caused by insect pests, and the trend of damage due viral diseases. In addition to household's demographics, the following data were collected: farmers' perception and knowledge of sweet potato viruses, vectors of viruses, diseases and insect pest management practices and intervention experiences. The interviews, in general, took between one hour and thirty minutes to two hours to complete, depending on the farmers' understanding of the questions.

2.3 Data Analysis

The primary data collected were manually transferred to a spread sheet and analysed using the Statistical Package for Social Sciences (SPSS, Version 25, SPSS Inc., Chicago, USA). The analysis used descriptive statistical measures, percentage and frequency. Tests for the statistical significance of differences in farmers' responses between districts were performed for some variables using chi-square (χ^2) tests.

3. Results and Discussion

3.1 Respondent's demographic profile

Nearly 82% of the interviewed household heads were males. In a previous study conducted in the same locations with similar methodologies, the majority of the respondents were also male-headed households (Gebru et al., 2017). A similar trend with a male majority was reported from Kenya, where more than 60% of farmers were male (Muthoni et al., 2013). This may represent a bias in the survey with respect to gender, as the women may be the ones actually growing the sweet potato crops, at least as long as sweet potato is not grown as a cash crop. More than half of the respondent farmers (51.3%) had farming experience of more than 20 years, 62.5% had an average family size of 5-8, and 39.4% were between the ages of 31-40 years. Gebru et al. (2107) reported an average family size of four in Sodo Zuria and five in Damote Gale districts, which is located in the same zone as our study. An average family size of more than five was reported in Nigeria (Simonyan & Obiakor, 2012), and as high as seven and a half in Uganda (Okonya et al., 2014a). Large families are common in rural areas to fulfil the demand of labour required for agricultural farming activities (Okoye et al., 2008, Udensi

et al., 2011). Most of the respondents had attended school, but at varying levels: 35.6%, 31.9%, and 22.5% attended primary (1 - 4th), secondary (5 - 8th grade) and high school (9 - 12th grade) education, respectively, and 10% were illiterate. Our results are in line with Gebru et al., (2017), who also reported that only 10% of the farmers were illiterate and that 1.9% were educated beyond high school. There were no significant differences in the education levels of the respondent farmers between the three districts.

3.2 Perception on viruses and virus transmission

Overall, 64.7% of the interviewed farmers and 41.2% of the agricultural extension workers had not heard about sweet potato plant diseases caused by virus infection. This shows that most farmers do not understand the symptoms following infection by viruses or that they might mistakenly link virus symptoms to other problems. The farmers' perception of virus infection varied between districts ($P < 0.001$; Table 1). The farmers in Tulo district were the least aware of virus infecting sweet potato (15.4%). Conversely, 54% of respondents in Boloso Sore district had heard about sweet potato virus diseases. High incidences of SPFMV and SPCSV infections have previously been reported in Wolayta zone in southern Ethiopia (Tesfaye et al., 2011), which might explain the better awareness of the respondent farmers in Boloso Sore district in our study. Moreover, better awareness of respondents in the district could also be due to its proximity to Areka Research Centre that is located inside the district, and has been working on sweet potato. This institution might have disseminated information about the virus problems to nearby farmers. So far there is no report of virus infection of sweet potato and resulting yield reduction in eastern Oromia, which may explain why most of the interviewed farmers from Tulo district were not aware of the sweet potato infecting viruses. In general, farmers in the studied districts of the SNNPR had better awareness (42.2%) about sweet potato virus than those in the western Hararge (15.4%). The farmers from the areas with a higher virus infection rates had relatively better awareness, suggesting virus severity and farmers awareness were related.

Very few farmers knew of the symptoms for virus infections. The majority of the farmers wrongly associated the virus-induced symptoms with the symptoms that are caused by drought and poor soil fertility. Some of the farmers only told of their experience of noting the symptoms on sweet potato fields after they were shown the A4 laminated colour print images of symptoms of virus-infected sweet potato leaves and tuber of the crops. This kind of

perception where only a few farmers knew the virus induced symptoms when shown has also been reported in Tanzania (Rwegasira et al., 2004b). There is study where no farmers in the mentioned viruses though a large proportion of old gardens showed foliar symptoms consistent with viral infection (Gurr et al., 2016). However, another study in Tanzania stated that more than 50% of the farmers identified infected plants based on the symptoms but unable to identify the diseases and its causal agent (Adam et al., 2015). Of the interviewed agricultural extension workers in this study, the majority (57.6%) reported that they knew some of the virus-like symptoms caused by virus infections.

Overall, 78% of the farmers recognized the damage caused by insect pests to sweet potato plants. However, the majority of farmers (71.2%) did not know that insects can transmit viruses from a diseased plant to healthy ones, and 83.3% of the farmers did not know that virus transmission can be reduced to a certain level using management practices (Table 1). Several studies have reported that farmers have a low awareness of viral diseases and the means of transmission. For example, a study conducted in Thailand and Vietnam reported that farmers did not know that insects could transmit plant viruses (Schreinemachers et al., 2015). Another study in East Africa showed that farmers have a low 'knowhow' on virus infection, the resulting SPVD and its possible means of transmission (Legg et al., 2007). Study conducted in four East African country, Kenya, Uganda, Rwanda and Tanzania showed variability in the ability of farmers to correctly identify sweet potato diseases (Echodu et al. 2019). It is reported that a large percentage of farmers in East Africa fail to distinguish symptoms of viral, bacterial and fungal sweet potatoes diseases (Echodu et al. 2019). However, the awarness levels of those farmers who understood symptoms of diseases varied among the country studied (Echodu et al. 2019). Surprisingly, interviewed agricultural extension workers had a similar perception as to that of the farmers on transmission and prevention of viruses. The majority of extension workers (82.4%) and 88.2% respectively, did not know the means of virus transmission and transmission could be reduced (Table 2). From this findings we can may say that farmers and extension workers in the study areas are not well informed about the viral diseases, associated symptoms, and the means of transmission. The lack of knowledge by the farmers of virus transmission shows the need for training and practical based demonstrations. Virus infections can only be reduced by vector management and phytosanitory practices, which cannot be effective without a good awareness of the farming communities and agricultural extension workers.

Table 1. Farmer's perception of sweet potato viruses, mode of transmission and insect pests in study areas in Ethiopia.

Variables on farmers knowledge of virus and its transmission	Possible response	Response in each district (%)					Total households	
		SZ	BS	Tulo	χ^2	P-Value	Frequency	Percent
Heard of sweet potato virus?					16.82	0.000		
	Yes	30.0	54.0	15.4			57	35.3
	No	70.0	46.0	84.6			103	64.7
	Total	100	100	100			160	100
Do insects eat sweet potato?					5.004	0.082		
	Yes	71.6	90.7	87.9			106	77.9
	No	28.4	10.3	12.1			30	22.1
	Total	100	100	100			156	100
Know how on virus transmit from infected to healthy plant?					36.06	0.000		
	Yes	46.4	18.0	5			45	28,84
	No	53.6	82	84.8			111	71.15
	Total	100	100	33			156	100
Can virus transmissions be reduced?					25.45	0.000		
	Yes	25	42.4	15.4			49	31.01
	No	75	35.6	23.1			90	56.96
	I don't know	0	20.3	61.5			18	11.39
	Total	100	100	100			158	100
Are you involved in sweet potato virus protection activities lasting five years?					4.13	0.127		
	Yes	13.3	27.9	15.2			30	19.2
	No	86.7	72.1	84.8			126	80.8
	Total	100	100	100			156	100

Note: SZ: Sodo Zuria, BS: Boloso Sore

Table 2. Extension workers perception of sweet potato viruses, transmission and insect pests in Ethiopia.

Variables	Number of respondent	Percentage
Awareness about sweet potato virus disease		
Yes	20	58.8
No	14	41.2
Total	34	100.0
Do you know virus symptoms on infected plants		
Yes	19	57.6
No	14	42.4
Total	33	100.0
Does sweet potato virus disease cause yield loss		
Yes	30	88.2
No	4	11.8
Total	34	100.0
Knowledge of plant virus transmission ways		
Yes	6	17.6
No	28	82.4
Total	34	100.0
Do you know how to reduce transmission		
Yes	4	11.8
No	30	88.2
Total	34	100.0

3.3 Means of virus distribution

From discussions we had with extension workers and farmers, we identified that the main sources of virus infection are the infected planting materials, contaminated agricultural tools used to prepare and handle during planting. Majority of farmers (72%) use the farm tools machete and cultivation tools though out the field and between fields for preparation of planting, weeding and cultivating the farm without understanding the need for disinfection. The use of such contaminated farm tools could be a source of infection, especially in a field where the virus-infected plants and healthy plants exist together. Pathogen that causes disease and other weed species can spread by human activity and farm machinery when no sanitation is applied. Therefore, awareness needs to be created among the extension workers and sweet potato farmers on the potential sources of virus contamination including contaminated planting materials and farm equipment.

Therefore, the repeated use of the farmers' own sweet potato planting materials over multiple seasons, unrestricted exchange of planting materials, mechanical transmission by agricultural practices could be one possible factor for the distribution, and persistence, of sweet potato virus in southern Ethiopia. The majority of the farmers collect vine from their own field at the time of harvesting of the previous season's crop. Farmers establish collected vines around the home for later use as a planting material (own unpublished data) without knowing if it was virus free. From discussions, we deduced that these practices could be the main means of virus infection and distribution, apart from vectors. In most of the developing countries, 80%-90% of seed systems are informal or traditional (Bishaw & Turner, 2008). This kind of seed system is the most dominant in Ethiopia and is most likely prone to spread infecting viruses particular in vegetative propagated plants. This has been reported to significantly contribute to the persistence of sweet potato viral diseases in vegetatively propagated materials (Opiyo et al., 2010, Bryan et al., 2003). (Bryan et al., 2003). Furthermore, continuous use of such plant materials from the same source (mother stock), over numerous seasons hinders achievement of optimum yields (Adikini et al., 2016). Therefore, farmers should be linked to a supply of plant material tested for economically important viruses. They also need to receive training on the selection of the right planting material, improved ways of plant handling during seed preparation, cultivation, irrigation and pesticide application (to control vectors) which may reduce virus' spread (Wilson, 2014). Farmers should also be encouraged to apply hygiene practices on their farm.

3.4 Current damage trends and incidences of sweet potato diseases and insect pests

None of the farmers able to link symptoms they observe on plant to any pathogen. We did provided the farmers with colour printed pictures of virus-infected sweet potato leaves and tubers with clear virus symptoms and pictures of major insect pests of sweet potato. Later we asked them that if they had seen similar disease symptoms and insect pests in their sweet potato fields in the past two years before they rank the damage. In all the study areas, the majority (58.1%) of the respondent farmers perception was that the damage caused by sweet potato diseases and insect pests was decreasing compared to previous levels. However, there were differences farmers' perception between the districts studied regarding of how much the damage was decreasing ($(p < 0.05)$, Table 3). The highest percentage of decreasing trends (73.78%) was perceived by farmers in Boloso sore district whereas the least (46.2 %) was

perceived in Tulo district. The same trend where farmers had different view on the trends of sweet potato diseases and insect was reported in four East African country (Echodu et al 2019).

There were significant differences in the farmer's perception of incidence of diseases and insect pests among the districts studied ($p < 0.05$). Over all farmers (43.8%) and extension (35.29%) workers in Wolayta zone perceived as average incidences of sweet potato diseases and insect pest, compared with in the last two years (Fig 3). Comparing damage levels caused by diseases and insect pests of sweet potato with disease and insect damage of other field crops they grow, 41.29% of the farmers think that the extent of damage to sweet potato is average (data not included).

We did ask farmers if they recalled any occurrences of outbreaks of disease and insect pests in the past, Majority of the farmers (55.1%) said they had remember its occurrences. The farmers' responses were significantly different between the districts (Table 3). When asked for the reason of outbreak, many the farmers' told us shortage of rain, and a high planting density was the cause. Some farmers didn't know the cause of the outbreak.

Table 3. Perception of farmers' on general status of sweet potato diseases and insect pest, and outbreak in the past two years (2014, 2015).

Study district	Perception on general status of sweet potato diseases and insect pests in the past two years		Incidences of sweet potato insects and disease outbreak you remember in the past		
	Response (%)		Response (%)		
	Increase	Decrease	Yes	No	Don't know
Sodo Zuria	50	50	80	15	5
Boloso Sore	26.22	73.78	47.5	44.3	8.2
Tulo	53.8	46.2	27	73.012	0
Total N=155	41.9	58.1	55.1	39.87	5.0
$\chi 2$= 10.538, P-Value = 0.005			$\chi 2$= 35.51, P-Value = 0.000		

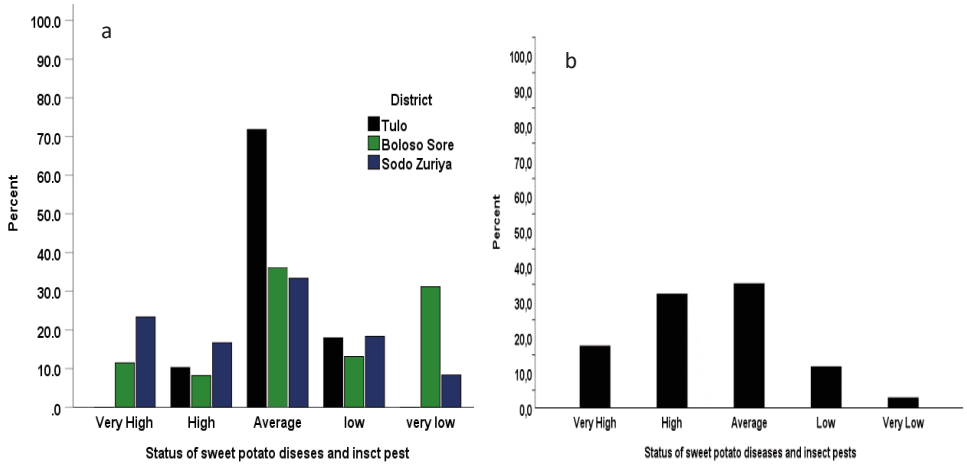


Figure 3. Farmers' and extension workers' perception of the status of general sweet potato diseases and insect pests. a: farmers' perception, b: extension workers in Wolyta zone.

3.5 Sweet potato production constraints

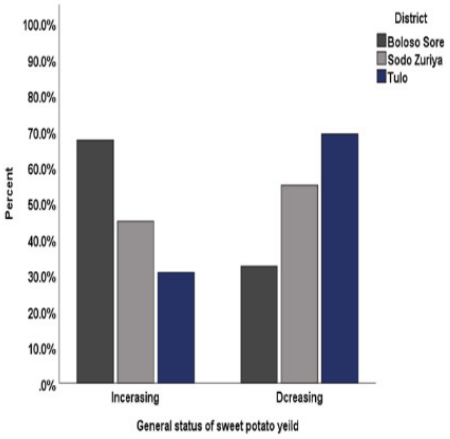
The farmers' perception of the general status economic impact of diseases and insect pests of sweet potato on yield is significantly different among the districts studied ($P < 0.05$) (Fig. 4B). The majority of the farmers (52.5%) perceived that sweet potato yield is declining due to various reasons. Overall, half farmers (48%) and the majority of the extension workers claimed that more than two factors contributed to the reduced production of sweet potato (Table 4). It is not single factor effect that declined sweet potato yield, but it was the added effects of a lack of access to improved varieties, an absence of disease-free planting materials, limited plot of land and climate change. Similar studies conducted in Uganda showed many setbacks to sweet potato production, as perceived by farmers (Okonya & Kroschel, 2016, Shields & Fletcher, 2013). The most important sweet potato production constraints reported from Uganda and Burundi were diseases and insect pests followed by the lack of quality planting materials (Okonya et al., 2019). According to farmer, an absence of disease free materials alone has contributed 5.7% for the current reduction in sweet potato yield. The share seems lower compared to the virus survey results and the actual observation we have seen on the fields. Because of the dry season, farmers have difficulty maintaining vines and obtaining enough planting materials for the next planting season. A survey of document an opinion of sweet potato researchers working in 29 developing countries including Ethiopia have been investigated in 2007. Scientist in Sub-Saharan Africa indicated the need of development of

varietal resistance to virus , quality planting material, cultivars exhibiting high and stable yield, and crop management against viruses, enhancing the availability and quality of sweet potato planting material, weevil control and cultivars with high b-carotene (Fuglie, 2007). Also the effort is ongoing in Ethiopia, it seems much more is needed especially on management of virus diseases

We observed severe virus-like symptoms on the leave of plants and damage of leaves due to insect pest in the farmers' sweet potato fields during the survey **(Fig 4A).** A yellow and purple discoloration in older leaves and stunting of whole plants were among the most common symptoms observed in sweet potato fields that were about to mature. As previously mentioned, when we asked farmers what they thought the cause was, they linked it to sun scorch and other unfavourable climatic conditions such as frost and drought.

Table 4. List of non-disease and insect pest constraints of sweet potato yield reduction.

Reason of yield reduction	Respondent farmers	
	Frequency	Percent
No improved variety	11	15.71
No disease free planting materials	4	5.7
Environmental changes	3	4.3
Limited plot size	9	12.85
No improved variety, No disease free materials and Environmental changes, limited land size	34	48.57
Climate change and land size	4	5.7
Lack of an appropriate farming system	5	7.14



A B

Figure 4. Typical virus-like symptoms in sweet potato fields and farmers' perception on damage to sweet potato yields. A: farmer's sweet potato field showing virus -like symptoms and fed by leave defoliators, Areka district, southern Ethiopia. B: Farmer's perception on status of sweet potato yields

3.6 Main diseases and insect pests of sweet potato in Ethiopia

Having questioned the respondents on their knowledge of pests and diseases in sweet potato, we showed them colour prints of symptoms of diseases, and asked them to respond on diseases limiting sweet potato productions. The farmers then ranked viral diseases as the main sweet potato diseases, followed by fungal diseases. Thirty seven point seven percent of the farmers said virus alone is the main problem (Table 5). However, the majority (61.3 %) indicated that virus and alternaria are the major problems. The majority (86.99%) of the farmers perceived that more than one insect pest accounts for the limitation of sweet potato production. According to farmers, weevil is mentioned by all of the farmers indicating that weevil is the most dominant factor responsible for low production. This agree with previous reports which state that weevils are the number one insect pest of sweet potato in Ethiopia (Ashebir, 2006) and Africa and global (Fuglie, 2007).

Table 5. Farmer's perception on major diseases and insect pests of sweet potato from color printed pictures of diseases symptoms and insects pests.

Diseases	Frequency	Percent
Virus	40	37.7
Virus and Alternaria	65	61.3
Others	1	0.9
Total	106	100
Insect		
Only weevil	12	9,75
Only butter fly	2	1,63
Weevil, butterfly, aphids and whitefly	107	86,99
Total	123	100

3.7 Intervention by training

Despite the negative economic impact due to insect pests and diseases in Ethiopia (Ferdu et al., 2009, Tesfaye et al., 2013), the majority of the farmers and the extension workers were not involved in any kind training to reduce the deleterious economic impact related to viral diseases and insect pest (Fig 5). Only 19.2% of the farmers and 20.6% of the extension workers received training on sweet potato production practices and t management diseases and insects of in the past five years. This explains why farmers and extension workers had limited awareness of the symptoms of diseases associated with viruses, indicating a need for training. Among the extension workers that had received training, only 50% of them gave informal training to the farmers and only 21.2% were involved in at least one kind of intervention to help the farmers. This study demonstrate farmers and the extension workers received a little attention of training to support them practice their indigenous knowledge and improve their skills on diseases and insect pest management. The failure to train farmers did limits farmers' understanding of the technologies and practices that have been generated via scientific knowledge and research. This consequently affects the level of engagement from farmers and extension workers participation towards viruses and insect pest management. Meanwhile, appropriate training relevant to their professions should also be given to the district's development agents and coordinators. As suggested from knowledgeable scientist in the fields, it is important to generate scientific knowledge that involves farmers through research and improve a system to communicate research results to target audiences mainly of the farmers and extension workers in the way they better understand (Fuglie, 2007)

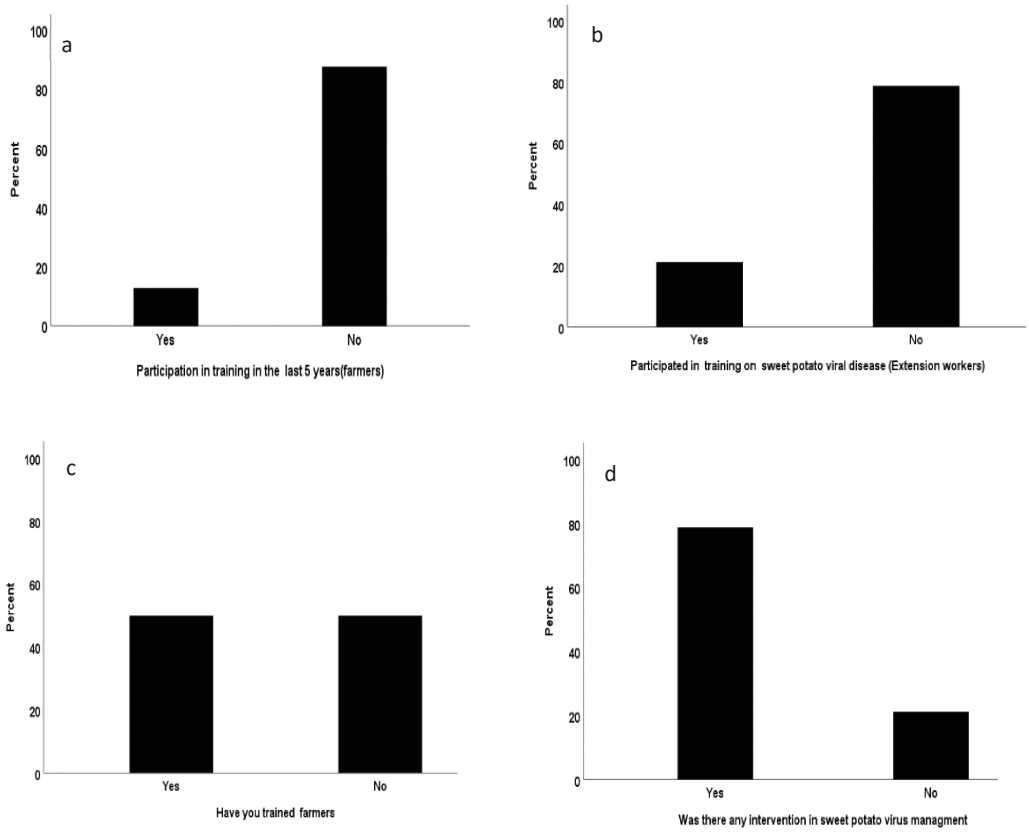


Figure 5. Extension workers' and farmers' response on training and interventions of sweet potato diseases and insect pests. **a** and **b** responses of farmers and extension workers on sweet potato viral diseases management, **c** and **d**: responses of extension workers if they have trained farmers and remember any intervention on sweet potato virus management, respectively.

3.8 Perception on effects of cropping system and cultivars in tolerances to disease and insect pest pressure

We asked farmers and extension workers if they perceive any differences between sweet potato cultivars and cropping systems in relation to tolerance and/or resistances to diseases and insect pests (Table 6). The majority of farmers (48.4%) perceived different sweet potato cultivars have different tolerances to diseases and insect pests (Table 6). Whereas 35.8% and 15.7% of them respectively said there is no difference or did not know if differences exist or not. There was also a significant differences in the farmer's perception between districts studied ($P< 0.05$). Most of the farmers in Tulo (58.97%) and Sodo Zuria (53.33%) observed cropping systems affects economic impact of diseases and insect pests. The majority think

that that sweet potato grown in mono-cropping systems more affected compared to the one grown under intercropping and crop rotations. A similar response was obtained from extension workers, of which 70.6% perceived sweet potato cultivated in a different cropping system differ in their tolerance to diseases and insect pests. However, 72.7% of interviewed extension workers perceived an absence of any difference between varieties in respect of tolerance to virus infection and insect pest damage. However, some of the extension workers commented that Hawassa-83 (the most commonly grown improved variety) is the least affected when compared to local cultivars. Local cultivar, called "Fesise", is perceived as the most susceptible cultivar.

Table 6. Farmer perception on cropping systems and varietal differences on diseases and insect pressures.

Respondent	Variables	Responses (%)		
Farmers in district	Do cropping systems have an effect on diseases and insect pest pressure	Yes	No	I don't know
	Sodo Z	53.33	46.66	0
	Boloso S	36.66	25	38.33
	Xulo	58.97	35.89	5.13
	Over all	48.4	35.5	15.7
	N = 159, χ^2 =38.56 , P-value = 0.000			
Extension worker		29.4	70.6	0
	Are all varieties equally affected by the viral diseases and insect pests			
Farmers in district	Sodo Z	33	67.67	0
	Boloso S	24	67.2	8.06
	Xulo	41.2	58.8	0
	Over all	29.91	65.81	4.27
	N=117, χ^2 = 6.7, P-value = 0.149			
Extension worker		72.7	27.3	0

3.9 Farmer's management practices of sweet potato diseases and insect pests

Farmers in the study areas manage sweet potato diseases and insect pests in the field using different methods (Fig. 6), how every to less extent. The major methods include insecticide application and single or combinations of more than two different cultural and physical pest-controlling methods listed in Fig. 6a. There is a significant difference ($p < 0.05$) between

farmers in the three districts on chemical application to control insect pests. According to the interviewed farmers, the use of insecticides is the least used option to control insect pests similar to responses of farmers in Uganda (Okonya et al., 2014b). The use of cultural practices such as crop rotation, sanitations, rouging out infected plants and physical methods (hand picking and killing of the insects) are the most commonly used options. Cultural practices, crop rotation and sanitation are strategies to reduce inoculum sources by removing dead and live plants on which pathogen survive. Implementation of management strategies that target a reduce of inoculum sources are highly effective for virus disease management (Wilson, 2014). Farmers respect the importance of using management practices in controlling the diseases and pests, although they do not understand the scientific principles behind them. Gap filling with training is very important for the best efficiency of the management practices applied by the farmers. Training on appropriate sequence of species in crop rotation as to which crop should come first is also important. Crop rotation avoids pest buildup, and interrupts infection cycles in mono-cropping system (Wilson, 2014).

Furthermore, from discussions with the farmers, we noted that they use locally made treatments such as a mixture of soap, suspension of plant extracts, application of cow dung and wood ash mixed with water and the hand-picking of worms and caterpillars. They spray mixture on to the plants to control insects. Similar practices where farmers use non synthetic insecticides such as mixture of local plants extracts, animal manure and urine to control potato pests was reported in Democratic Congo (Munyuli, 2016). Chemicals made from plant extracts can provide insect-repellent activity that discourage insects from entering the field or modify virus vector behaviors that prevent probing. This has been proposed to bean effective deterrent of insects Wilson (2014). Moreover, it is reported that plant extract reduces infectivity of plant viruses in the absences of vectors (Wilson, 2014). Though we did not explore why they preferred to use homemade insecticide treatments instead of commercial insecticides. It could be linked to the higher prices and/or poor accessibility of commercial insecticides relative to the low price or zero cost of locally available plant extract, wood ash, cow dung and urine. The high cost of insecticides is reported to prevent farmers from using it (Okonya et al., 2014).

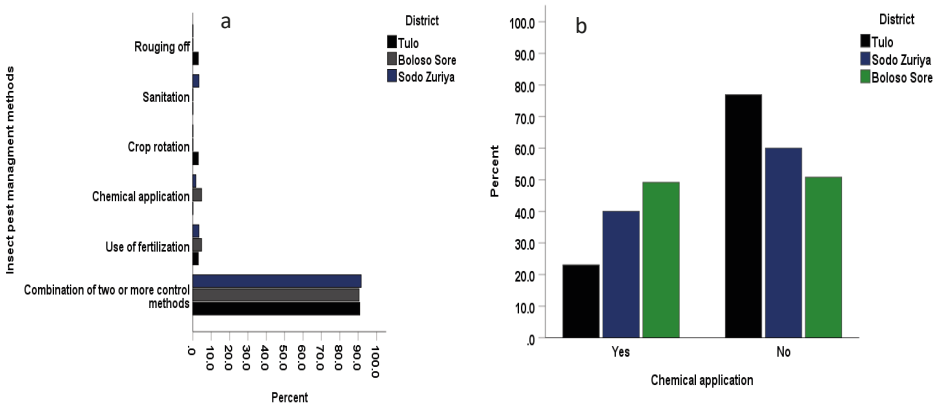


Figure 6. Farmer's methods of sweet potato diseases and insect pest management.

4. Conclusions and Recommendations

This survey investigated farmers perception on different parameters related to sweet potato diseases and insect pests. From the survey, we conclude that the majority of the farmers and government extension workers lacked the awareness of plant viruses and their mode of transmission and symptoms of infected plants. We recommend that educating farmers through practical, oriented training that targets awareness of virus-infections and mechanisms of transmission could reduce the spread of the virus and the consequently diseases. Only through the creation of awareness as well as demonstration and training in the field can farmers detect early symptoms and manage the prevention of spreading the viruses further.

Diseases and insect pests, lack of quality planting materials that are free of viruses major sweet potato production constraints. There is a need to supply healthy planting materials and to educate farmers about the basic principles and practical application of sanitation practices to reduce reinfection rate and to keep only healthy plants in the fields to maximize their yield and income. Encouraging and supporting large scale commercial vine propagators is strongly needed to supply virus free planting materials.

The farmers had better awareness of damage caused to sweet potato plant by defoliator insects. However, only a lower percentage of farmers and extenstion workers knew that viruses can be transmitted from diseased plants to healthy ones by insect vectors. Farmers have, to a small

degree, practiced combinations of pest and disease management methods. More over, there practices of using non synthetic insecticdal by the farmers. Farmers and extention workers recived very litte trainings related to sweet potato plant disease and insect pest management in the last five-years indicating a need to train them on better sytems of dieases and pest management and improve production . |This study provided information on present awarness of farmers and extention workers which can be used as base for any futuree intervetion and action of dieases and insect pest managments.

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PAPER III

Optimization of plant growth regulators for meristem initiation and subsequent multiplication of five virus tested elite sweet potato varieties from Ethiopia

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Abstract

Vegetatively propagated crops are exposed to pests every growing season, providing them with the opportunity to feed on the plants and transmit diseases. Meristem culture is an important method to clean for viruses to restore high yields. A pre-requisite for cleaning is to regenerate shoots *in vitro*, using meristem cultures and subsequent propagation of virus-tested plantlets. Media optimizations with different concentrations and combinations of 6-benzylamino purine (BAP) and Naphthalene Acetic Acid (NAA) were tested for their effects on shoot initiation from meristems. This was followed by regeneration and shoot multiplication from nodal cuttings in five elite varieties of sweet potato performing well in different parts of Ethiopia. There was a highly significant genotype (variety) x environment (media) interaction for all experiments. Concentrations and combinations of BAP and NAA significantly affected the percentage of meristem survival/regeneration and subsequent shoot multiplication. The best combination of the plant growth regulators was NAA (0.1.mg/L) and BAP (1.0 mg/L), which gave the highest overall rate of success. For further propagation, shoot numbers per single nodal cuttings were significantly affected by BAP concentrations ($p < 0.05$) and genotypes: almost three shoots per nodal cutting for 'Berkume' and no multiplication for 'Tola'. The medium which gave shoots was ½ MS media supplemented with BAP (2.5 mg/L) and GA_3 (0.5 mg/L). The

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tallest shoots, with the highest number of internodes, suitable for transfer to soil, was obtained from ½ MS media without any growth regulators for all of the cultivars.

Keywords: Media, meristem, multiplication, regeneration, shoot, sweet potato, variety

Key Message

The study explores media responses for five sweet potato genotypes from different Ethiopian regions to optimize establishment, shoot proliferation and rooting when cleaning genotypes for viruses by meristem culture.

Introduction

Sweet potato (*Ipomoea batatas* L. Lam) ranks as the fifth most important food for most developing countries (FAO 2017). Africa stands second in total production of sweet potato and this production is increasing at the fastest global rate (FAO 2017). Ethiopia is the six largest sweet potato producer in Africa. The crop is critical at both household and national levels for food security (FAO 2017). However, the production of sweet potato suffers from virus infections which results in low national average yields (8.2 tonnes), far below the world average of 12.3 tonnes (FAO 2017).

Tissue culture techniques have been used as a biotechnological tool for virus elimination and mass multiplication of vegetatively propagated crops, including sweet potatoes (International Atomic Energy Agency 2004). Uniform and disease-free planting materials can be multiplied using tissue culture. Tissue culture may improve agriculture by developing quality-planting materials to meet the ever-increasing global demand for food and feeds. In addition, plant tissue culture techniques have a great potential for mass multiplication, conserving elite cultivars and rare cultivars treated to extinction using artificial media (Torres 1989). Tissue-cultured plants often out-perform those propagated conventionally in productivity and also in the quality of produce.

Initiation and regeneration of an explant cultured on artificial nutrient media is influenced by the type and concentration of nutrients, plant growth regulators (PGRs) and their appropriate ratios, genotype and type of explants (Varshney and Anis 2014) in addition to mother plant treatments prior to

propagation *in vitro*. For the initiation of growth and development of an explant in any culture, in addition to the basic MS medium (Murashige and Skoog 1962), the explant requires additional inputs and is highly affected by concerted and cooperative activities of PGRs, particularly auxins and cytokinesis (Gaspar et al. 1996). The most important and most commonly used synthetic auxins in tissue culture are 2, 4-dichlorophenoxyacetic acid (2, 4-D) and naphthalene acetic acid (NAA) whereas that of cytokines are kinetin and BAP (Gaspar et al. 1996). The concentrations and the combination between PGRs determines the morphogenetic responses towards shoot initiation.

Varieties of the same species, often respond differently to artificial media (Abe and Futsuhara 1986; Jan et al. 2001), through genotype x environment interactions. The success of sweet potato shoot initiation and multiplication in tissue culture may vary depending on the genotype used and the type and concentrations of PGRs supplemented (Dugassa and Feyissa 2011; Wondimu et al. 2012; Abubakar et al. 2018). These previous studies show that media optimized for sweet potato cultivar are genotype-specific and if not optimized, may result in poor, or no, regeneration. In Ethiopia, several sweet potato varieties have been evaluated for traits of interest (yield, disease resistance, nutritional content, adaptability to locations) and released for use (Tofu et al. 2007; Gurmu et al. 2017). However, efficient and universal protocols for rapid multiplication of virus-free planting materials has not been optimized, except for a few of these cultivars (Dugassa and Feyissa 2011; Wondimu et al. 2012). For this study, we have used five high yielding varieties of sweet potato released for different regions (southern, eastern and western) of Ethiopia. Three of them are white and the remaining two are orange fleshed sweet potato varieties (containing the valuable β-carotene, the precursor of vitamin A).

Our hypothesis is that these elite genotypes will vary in their responses to the PGRs. It is important to optimize the best media for each genotype. Moreover, we intend to clean the elite varieties from the infecting virus(es) and undertake further mass propagation of these clean genotypes for distribution to the farmers. The present study was carried out to 1) optimize the best combinations of PGRs to obtain a high rate of shoot initiation from meristem explants of four local varieties selected for their strong agronomic performances and 2) to optimize the appropriate concentrations of BAP for the shoot multiplication of five Ethiopian sweet potato varieties using single nodal cuttings. These two aims will, potentially, secure enough clean plant material for Ethiopian famers of these

genotypes, since Ethiopia already has commercial tissue culture facilities in place for propagation of sugar cane with the capacity to take on disease-free sweet potato propagation as well.

2. Materials and Methods

2.1. Plant materials and experimental sites

Five high yielding Ethiopian sweet potato varieties, three of which ('Hwassa-83', 'Guntute' and 'Kulfo') were obtained from the Hawassa Agricultural Research Center, and two from Bako Agricultural Research Center ('Tola') and Haramaya University ('Berkume') were used for this study. The vines of the varieties were imported into Norway with a permit from the Norwegian Food Authority (Mattilsynet) for virus indexing and virus-cleaning. Plants were grown in quarantine rooms and used as stock to provide shoot tips for excising meristems. The studies on PGRs in the two media were conducted in the Plant Cell Laboratory at the Norwegian University of Life Sciences (NMBU), Norway. Meristem tip culture and single nodal cuttings were used as explant for shoot initiation and shoot multiplication experiments, respectively. The nodal cuttings used as explants for the multiplication experiments were from plants in tissue cultures grown on MS media without PGRs, as the last medium before the transfer to soil.

The virus-cleaned and virus-tested cultures were exported back to Ethiopia as tissue cultures where the acclimations were performed at Hawassa University.

2.2. Experimental design and treatment set-up

2.2.1. Medium preparation

The basal medium used throughout was Murashige and Skoog (MS) (1962) from Duchefa Biochemie (M0222.0001) containing 4.4g/L (macronutrients, micronutrients, and vitamins). The MS medium was used in either full (initiation) or half concentration (multiplication). The media were all supplemented with 30 g/L sucrose and 6 g/L agar (Sigma-Aldrich, Spain). Plant growth regulators (PGRs) were supplied according to the two experimental set-ups described below.

2.2.2. Experimental design:

Initiation of meristems

The experiments were set up as complete factorial experiments (4x4x4) with 16 media combinations of two PGRs (NAA and BAP) (Table 1A, media 1-16) for each of the four varieties ('Berkume', 'Guntute', 'Kulfo' and 'Tola').

2.2.3. Regeneration of nodal cuttings

MS medium (1/2 strength) supplemented with five concentrations of BAP (0, 0.5, 1.5, 2.5, and 3.5) mg/L was combined with 0.5 mg/L of the natural gibberellin, GA_3 (Table 1B, media 17-21) and were tested to assess the response differences of the five sweet potato varieties ('Berkume', 'Guntute Kulfo', 'Tola' and 'Hawassa-83'). No PGR was included as a control. Shoot multiplication from each single nodal *in vitro* cutting was recorded for each combination.

Table 1. Plant growth regulator combinations for A: Initiation of meristems using NAA and BAP in 16 combinations (media 1-16) for all four genotypes: 'Guntute', 'Kulfo', 'Berkume' and 'Tola'. B: Regeneration of nodal cuttings of 5 combinations (media 17-21) and 5 genotypes: 'Guntute', 'Kulfo', 'Berkume', 'Tola' and 'Hawassa 83'.

A: 1/1 MS	**BAP mg/L**				
NAA mg/L	**0**	**0.1**	**1.0**	**2.5**	
0	1	2	3	4	
0.01	5	6	7	8	
0.1	9	10	11	12	
0.5	13	14	15	16	
B: ½ MS	**BAP mg/L**				
GA_3 mg/L	**0**	**0.5**	**1.5**	**2.5**	**3.5**
0	17	Not tested (NT)			
0.5	NT	18	19	20	21

2.3. Preparation of explants, surface sterilization and the establishment of a culture

2.3.1. Preparation of meristems

Shoots of about 2 - 3 cm were trimmed for leaves and parts of leaf petioles, the surface was then sterilized in 70% ethanol for about 30 seconds, and thereafter 10 minutes in a solution of 5% (v/v) sodium hypochlorite

(VWR) and 0.02% v/v of Tween 20 (Sigma-Aldrich, St. Louis, USA). Finally, the shoot tips were rinsed three times with double distilled, autoclaved (sterile) water. The apical and the two adjacent axillary meristems from the top, were excised under a dissecting microscope and placed on the various media under sterile conditions. Seven meristems were cultured on each of the 16 different combinations of BAP and NAA levels. Treatments were arranged in a completely randomized design (CRD) and each experiment was replicated three times. In total, 336 meristems per variety were cultured on the different combinations of BAP and NAA.

2.3.2 Regeneration of nodal cuttings for multiplication

In vitro cultures of five sweet potato varieties, which were first kept on hormone-free ½ conc MS medium for 6 weeks, were used as the starting material for this experiment. They were grown under the same growth room conditions as described below and transferred to ½ MS medium, with PGR combinations 17-21 (Table 1B) to evaluate shoot multiplication using single nodal cuttings. Four single nodal cuttings were cultured in each jam jar as a single experimental unit and each combination was replicated three times, i.e. 12 single nodal cuttings of each of the varieties represented each treatment.

2.3.3 Culture conditions

All the *in vitro* cultured explants were grown under the same growth room conditions: temperature of 25 ± 1°C, a light intensity of 28 $\mu molm^{-2}s^{-1}$ and 16/8 h (light/dark) photoperiod provided by white fluorescent lamps (Osram L 58W/840 Lumilux), and a relative humidity of 70%.

2.3.4. Acclimatization

Cleaned, virus tested tissue culture plantlets were returned to Ethiopia from Norway with an issued Health Certificate according to the Convension of Biological Diversity (1993). The cultures were transferred to an insect-proof screen house at Hawassa University, Ethiopia into polyethylene plastic tubes filled with solarized top soil: compost: sand in a ratio of 3:2:1. Plants were covered with a plastic sheet for the first two weeks to maintain plant turgidity. One month after planting the surviving plants were counted and a percentage of survival was calculated.

2.4. Data collections and subculturing

Shoot initiation: Cultures with infection symptoms (if any) were, immediately recorded and discarded. Meristems with a green color after two weeks of culture were counted as survived; whereas those that failed to turn green were considered to be dead. The percentage of meristem survival was

determined by dividing the green meristems that survived by total cultured. After 6 weeks of incubation, the survived meristems were transferred into test tubes, which contained the same fresh media combination, and to allow for shoot regeneration. Twelve weeks later, the number of meristems that induced a shoot size of more than 1cm, the number of meristems that produced callus, the callus weight (mg) induced for each meristem combination was recorded.

For the regeneration of nodal cuttings experiment: culture survival, the number of shoots induced per nodal explant, the length of shoots, the callus weight per nodal explant, and the number of nodes were counted after four weeks.

2.5. Data analysis

All of the data were subjected to ANOVA (General Linear Model) using Minitab Statistical Software version 18 (Minitab Inc., Pennsylvania, U.S.A). Means that were significantly different were separated using a Tukey LSD test at the probability level of 5% ($p \leq 0.05$).

3. Results and Discussion

3.1. Genotype and PGR effects on meristem survival and further shoot development

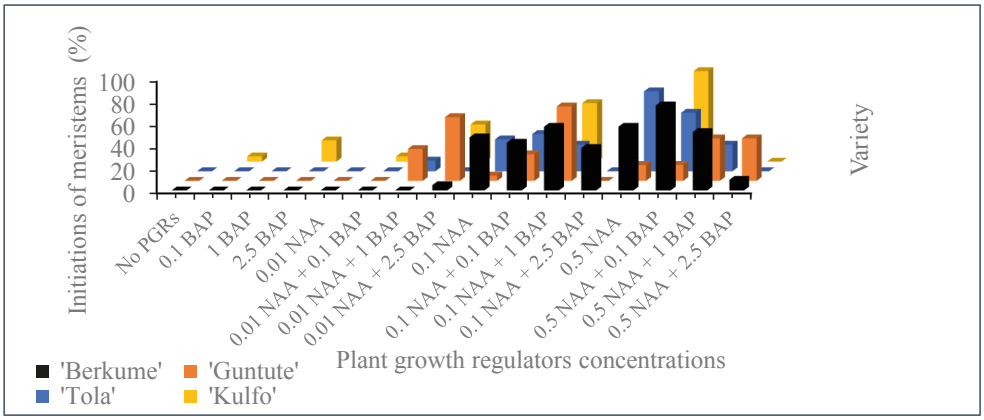
Different concentrations and combinations of NAA and BAP had no significant effects on the survival of the meristems after two weeks; they greened in a similar way for all PGR combinations (data not shown). As time went by (a further 16 weeks), the different concentration and combinations of PGRs gave significantly different survival responses amongst the four varieties tested (Table 2). Many meristems did not develop further into shoots and eventually died, particularly when no NAA was added (Figure 1). We can safely conclude that auxin is needed for meristem survival of sweet potato. This is in accordance with previous findings by Dugassa and Feyissa (2011).

Table 2. Differences in responses of Ethiopian sweet varieties to basic MS media, supplemented with NAA and BAP, calculated after 12 weeks of culture and as an average over all media combinations.

Variety	Mean survival of meristem (%)*	Mean shoot initiation from meristems (%)*	Mean callus weight produced per meristem* (mg)
'Kulfo'	84.3^{ab}	39.1^{a}	261.0^{a}
'Berkume'	83.3^{a}	24.1^{b}	248.6^{a}
'Tola'	74.7^{ab}	15.2^{b}	236.7^{a}
'Guntute'	71.1^{b}	16.1^{b}	147.0^{b}

*Mean values in the same column followed by same superscripts letter are not significantly different at $P < 0.05$ according to Turkey pairwise comparisons

'Kulfo' obtained the highest shoot initiation rate (89%) after a further 16 weeks on the MS media supplemented with 0.01 mg/L NAA and 1.0 mg/L BAP (medium 7) (Figure 1). This is the same optimal medium as 'Berkume', while 'Tola' preferred a medium with only NAA (0.5 mg/L) and 'Guntute' performed best on 0.1 mg/L NAA + 1 mg/L BAP. On average across all of the media, the highest survival rate of 83 % was recorded for 'Kulfo', while the lowest (of the media with growth) was for 'Guntute' (71%) (Table 2). It is reported that two or more hormones (BAP and NAA in this



case) can interact synergistically or antagonistically in many circumstances (George et al. 2008).

Figure 1. The average percentage of initiations shoot from meristem combined for four sweet potato varieties from Ethiopia as affected by 16 different concentrations and combinations of plant growth regulators (NAA and BAP) supplemented to MS medium.

When comparing our study with that of Dugassa and Feyissa (2011), we have included a wider variety of genotypes from several regions of Ethiopia. Our aim was to include the best varieties from different sweet potato producing regions in Ethiopia to be able to clean elite Ethiopian varieties from viruses. Our results are in agreement with the results of Dugassa and Feyissa (2011) for the two varieties that they tested. We have demonstrated the optimal PGR combinations for improved initiation of shoots from shoot tip meristems of three more varieties. In descending order of response: 'Kulfo', 'Berkume', Guntute' (same as Dugassa and Feyissa 2011) and 'Tola' as the least responsive on these media. Dugassa and Feyissa (2011) noted that 'Guntute' had the highest
response rate of the three varieties tested, on their media combinations. However, we have tested a much higher level of NAA, combined with the same levels of BAP, and found that their optimal NAA (0.01 mg/L) had not reached an optimum for any of the varieties that we tested, and 'Guntute' was tested in both studies. Our overall optimal NAA level was x10 higher (0.1 mg/L), combined with 1.0 mg/L BAP. The BAP optimal for all varieties combined (Figure 1), on the other hand, is in accordance with Dugassa and Feyissa's findings (2011). We can conclude that the optimal medium for a new variety would be to try out this combination (NAA 0.1 mg/L + BAP 1.0 mg/L).
The picture is different when looking at each variety separately, since we have a clear genotype x environment interaction (Figure 1). This shows that each variety has it's own requirements, as previous reports have also concluded for both Ethiopian (Dugassa and Feyissa) and west African varieties (Addae-Frimpomaah and Amponash 2014). This kind of factorial experiment to reveal the optimal concentrations of PGRs on meristem development has been confirmed by (Su et al. 2011). The number of weeks to induce shoots varied for the varieties; 'Kulfo', 'Hawassa 83' and 'Berkume' took 5 to 7 weeks, while 'Tola' and 'Guntute' took 3 weeks longer to respond.

The statistical analysis shows that the interaction in the response of meristems, between the varieties and PGRs combinations, was highly significant ($p=0.001$), while the response was only significant, at a level of 5%, when testing the varieties and media independently (Table 3).

Table 3. Analysis of variances for the effect of media, variety (genotype) and media x variety interaction on regeneration of sweet potato shoots *in vitro*.

Source	Var	SE Var	Z-Value	P-Value
Media	259.70	128.82	2.02	0.022
Variety	71.11	85.36	0.83	0.022
Media*Variety	223.69	69.87	3.22	0.001
Error	207.73	27.76	7.48	0.000
Total	762.22			

Figure 1 illustrates the best concentration and combinations of BAP and NAA for shoot initiation media. The details of the statistics behind this figure are presented in Online Resource 1. No, or low, concentration of PGRs did not result in shoot regeneration, as with the initiation meristems. Both 'Kulfo' (81%) and 'Berkume' (76%) had the highest percentage of meristems giving shoots on the same PGR combination: MS with 0.5 mg/L NAA and 0.1 mg/L BAP (medium 14). 'Guntute' performed best on MS medium supplemented with 1 mg/L BAP and 0.1 mg/L NAA (medium 7) with (67 %) of the meristems producing shoots. This is the same result that Dugassa and Feyissa (2011) obtained for 'Guntute', except that they also added 1 mg/L GA_3 in their medium, in addition to BAP and NAA. We can conclude that adding gibberellin was not necessary in our case. The maximum regeneration from meristems in 'Tola' was 72%, on yet another medium: 0.5mg/L NAA without any added BAP (medium13). These three genotypes were not part of Dugassa and Feyissa (2011), so this is the first report of their optimal combinations. Preliminary studies (data not shown) with 'Hawassa-83' was also consistent with Dugassa and Feyissa (2011), so we decided to omit this genotype from the large factorial media experiment, but kept 'Guntute' for reference. Other studies, using other genotypes, have also reported on the effect of variety, the concentrations and combinations of PGRs on the regeneration capacity of sweet potato plants from meristems (González et al. 2008; Sivparsad and Gubba 2012; Alam et al. 2013; Mbinda et al. 2016). So, it is not surprising that the regeneration of sweet potato plants from meristem cells varies depending on the tissue culture media components (PGRs, nutrients, vitamins, sugar, agar), genotypes and the culture conditions (Dugassa and Feyissa 2011; Addae-Frimpomaah and Amponash 2014). Already in 1957, Miller and Skoog revealed the importance of auxin and cytokinin concentrations and their combinations for cell proliferation and new organ regeneration in tobacco. Since then, this kind of factorial experiment, to reveal the optimal concentrations of PGRs, has been repeated by numerous authors for other plants. When optimizing

for a species with genotype x media interactions, we believe it is important to perform such an exercise. It is needed to be sure that we have the best possibility of obtaining surviving meristems when the purpose is to clean particular genotype for viruses, to increase the chance of obtaining a clean plant. From our results for the initiation of shoots from meristems for all varieties, it averaged between a low of 1.2 (%) on 1 mg/L BAP to a high of (57.7%) on 1 mg/L BAP and 0.1 NAA (Figure 2). Moreover, this media combination induced an average low callus weight of 98 mg/meristem, which is desired to reduce the risk of somaclonal variation. Therefore, this combination of 1 mg/L BAP and 0.1 NAA could be the one to use for new cultivars, if a factorial PGR experiment is not feasible.

PGRs combinations and the varieties, when analyzed separately, both had statistically significant effects ($p<0.05$) on the initiations of shoot from meristems (Table 2). When considering overall performances for all tested genotypes, 0.1 mg/L NAA and 1 mg/L BAP (medium 11) was the best concentration and combination tested that regenerated shoots in 58% of the cultures (Fig. 2). Figure 2 reveals that media 9, 15, 13, 14 and 11 are not significantly different at a level of 5%, but do show an increasing level of regeneration when averaging over all genotypes. Significant differences, in respect of days for shoot induction, were also observed. ‘Kulfo’ and ‘Berkume’ took 5 to 7 weeks to regenerate shoots of about 1 cm or more, whereas ‘Tola’ and ‘Guntute’ took between 8 to 10 weeks (data not shown).

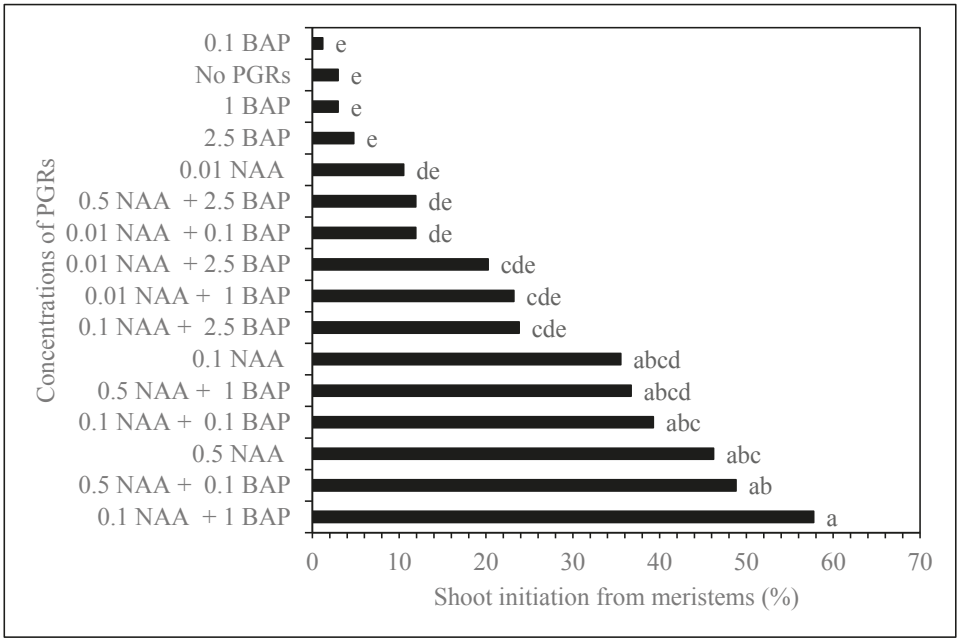


Figure 2. The average initiations of shoot tip meristems of four Ethiopian sweet potato varieties on basic MS media supplemented with 16 different concentrations of NAA and BAP. Mean (represented by the bars) that do not share the same letter are significantly different at 5% level.

3.2. PGRs and genotypes highly affected shoot multiplication and height of nodal explants

The factorial media experiment using nodal explants further emphasized the importance of such media experiments. Table 4 shows the highly significant effects of the five media combinations, the genotypes on shoot multiplication from nodal cuttings, as well as the interaction between them.

Table 4. Analysis of variance for the number of sweet potato shoots obtained from singles nodal cuttings *in vitro*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Media	4	5.578	1.3946	9.56	0.000
Variety	4	8.795	2.1987	15.08	0.000
Media*Variety	16	8.522	0.5326	3.65	0.000
Error	50	7.292	0.1458		
Total	74	30.187			

The sweet potato nodal cuttings responded well to transfer from *in vitro* cultures when propagated on to hormone-free ½ MS medium. As expected, the survival rate was between 95 to 100% in all cases.

Figure 4A shows that the highest mean number of shoots for all varieties were obtained when supplying either 1.5 or 2.5 mg/L of BAP combined with 0.5 mg/L GA_3 (medium 19 or 20). Figure 3 provides more details for the optimal medium for each cultivar. 'Berkume', 'Hawassa' and 'Guntute' produced the maximum mean number of shoots of 2.8, 1.7 and 1.8 correspondingly on ½ basic MS (medium 20) supplemented with 2.5 mg/L of BAP and 0.5 mg/L GA_3 (Figure 3). However, 'Kulfo' produced the highest mean shoot number of 1.6 at a concentration of 3.5 mg/L BAP and 0.5 mg/L GA_3 (medium 21). 'Berkume' produced a significantly higher number of shoots, compared to the other four varieties, on average over all media tested (Figure 4B). 'Tola', essentially, did not multiply on any of these media, as it produced only, or at the very best, 1.1 shoot per nodal cutting (medium 21) in one month (Figure 3).

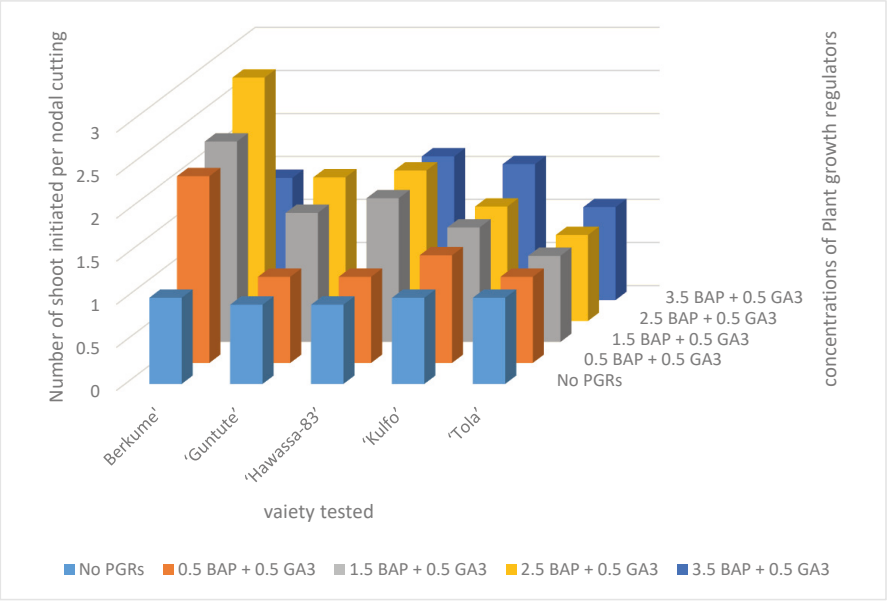


Figure 3. The effect of media combinations and varieties on number of shoots initiated per nodal cutting of five Ethiopian cultivars from different regions.

In this study, increasing the concentration of BAP significantly decreased the height of the shoots induced from the nodal cuttings in all of the studied varieties (Figure 4A and Figure 6). The results agreed with the findings of the previous studies conducted that evaluated the effect of BAP concentration on nodal cuttings regenerated using sweet potato from Ghana and Ethiopia (George et al. 2008; Dugassa and Feyissa 2011; Addae-Frimpomaah et al. 2014). Interestingly, the lowest mean number of shoots and the shortest mean shoot height was obtained from 'Tola' among the other varieties that were tested in response of the BAP concentrations. This could agree with the suggestion that the effect of hormones on any developmental process depends on the species (George et al. 2008). However, the highest (6.9 cm) and shortest (1.68 cm) mean shoot height were registered for 'Tola' and 'Berkume' cultured in basic ½ MS media, without PGRs (Table 4; Fig. 5). Furthermore, this study also showed that varieties responded differently to the same concentration of BAP and resulted in varying mean shoot number. This can be explained by the suggested theories that the effects of plant growth regulators varies depending on culture conditions, explant types and the genotype (Gaspar et al. 1996).

Nodal cuttings planted on MS media without BAP generated the highest shoots compared to any other levels of BAP concentration we tested. It appears that nodal cuttings cultured on different BAP levels induced shoots of varying number and length (Dugassa and Feyissa 2011). The higher shoot length can provide more cuttings per plant and can compensate for the number of shoot induced by adding BAP. We suggest that using ½ MS media without PGRs could be good for shoot multiplication of sweet potato varieties as it avoids costs of PGRs and gives longer shoots that can provide more nodal explant for multiplication.

The tallest shoots and highest number of internodes were obtained on ½ basic MS media without BAP nor GA_3 (hormone-free medium 17). Shoot length declined with increasing concentration of BAP for most varieties (Figure Fig. 4A & Figure 6). 'Kulfo' obtained the tallest shoots of all varieties (Figure 4B) on average over all media tested. 'Tola', however, had the tallest shoots of all, 6.90cm on the hormone-free medium 17 (Online Resource 4). Taller shoots secure a better transfer to soil and are, therefore, beneficial.

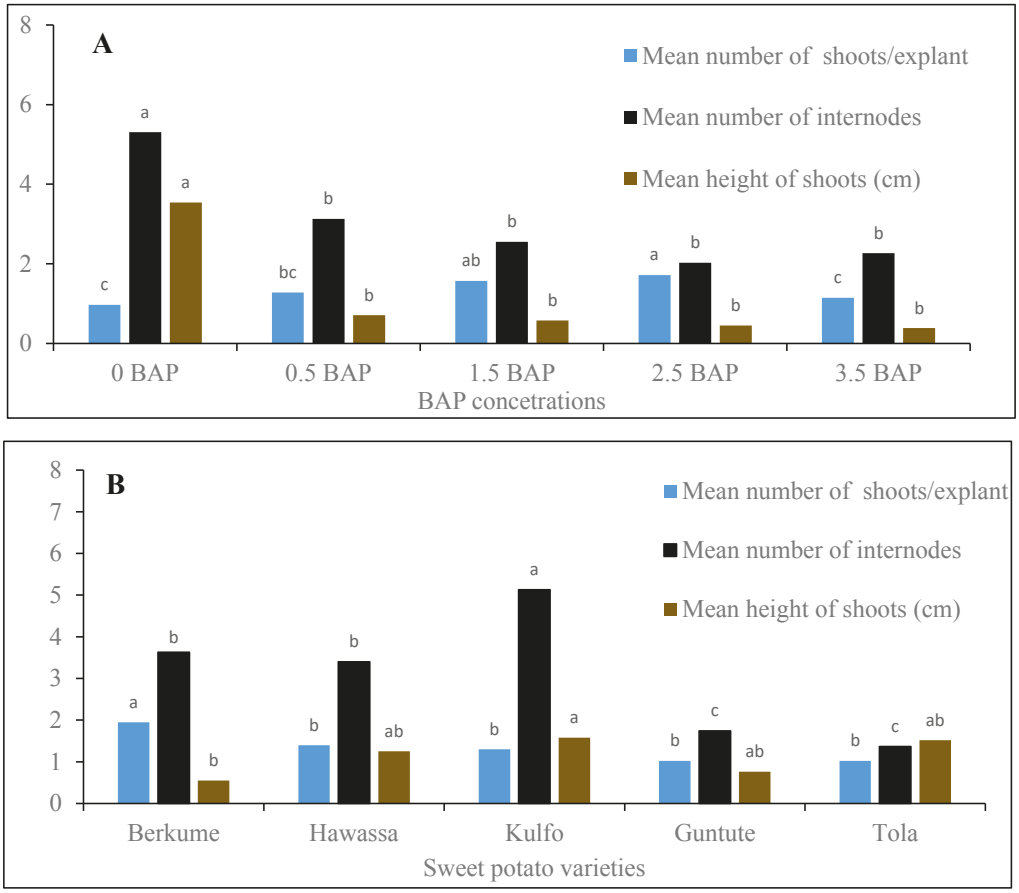


Figure 4. Mean shoot multiplication parameters (shoot number, internode number and shoot height) as affected by varying concentrations of BAP (each combined with GA_3 of 0.5 mg/L) for five elite sweet potato varieties from Ethiopia. **A**: BAP concentrations affected the parameters of shoot multiplications; **B**: varieties affected the shoot multiplication parameters. Bars represent mean values. The same colored bars followed by same superscripts letter are not significantly different at $P < 0.05$ according to Turkey pairwise comparisons.

Figure 5. The shoot height of sweet potato *in vitro*, from the start (left), decreases with increasing concentrations of BAP.

3.3. PGR and genotype affect internode number

The BAP concentrations (Fig. 4A) as well as varieties (genotypes) tested (Fig. 4B) significantly affected the number of internodes per single initial nodal explant. The number of internodes gives an indication of the potential of mass propagation since there will normally be at least one axillary bud for each node. Basic ½ MS media without BAP nor GA_3 (medium 17) gave the highest average (over all genotypes) internode number of 5.3, whereas the minimum (2.0) was obtained with a concentration of 2.5 mg/L BAP and 0.5 mg/L GA_3 (Figure 4A). The highest average internode number obtained from the various genotypes was 5.1 from 'Kulfo', while the lowest was 'Tola' with 1.4 internodes from each initial nodal cutting (Figure. 4B).

No significant differences between varieties were recorded on the establishment rate (survival) when the tissue culture of sweet potato plants were planted in screen house. In all of the varieties, more than 90% of the planted plants survived the acclimation and successfully established in to mature plants within one month after planting (Fig. 6).

Figure 6. Establishment of tissue culture plants in soil. **A**: Shoot of 4 - 5 internodes from tissue culture used for planting. **B:** Examples of plants that survived were acclimatized and became established.

3.4. PGR x genotype interactions for callus growth

We did not set out to produce callus but we recorded it, as a way of describing the responses to the media combinations. Also, callus has a potential use for the development of other regeneration methods, such as somatic embryogenesis protocols. In our case, this is an undesired feature, as we aim for shoots, not callus. Regeneration of shoots from callus is more prone to somaclonal variation than direct shoot organogenesis (Krishna et al. 2016), while the differentiation into somatic embryos probably requires an intact genetic constitution to facilitate the complex differentiation into somatic embryos. This hypothesis is supported by the work in poinsettia (Geier et al. 1992) who documented a higher variation in callus than in the subsequent somatic embryos.

The mean weight of callus induced per meristem was significantly affected by the concentrations and the combination of NAA and BAP in the varieties studied. The interaction of PGRs and varieties was significant at $p < 0.05$ for callus weight. Overall, on average across all varieties, the highest average callus weight (654 mg) was obtained from meristems cultured on basic MS medium (medium 16) supplemented with combinations of 0.5 mg/L NAA and 2.5 mg/L BAP (Fig. 4). Medium 16 produced the highest callus weight for all varieties; 'Berkume' obtained a maximum mean weight (864 mg) of callus per culture, while 'Kulfo' obtained of 643 mg, 'Tola' 642 mg, while 'Guntute' had only 465 mg (Figure 7, with statistics in Online Resource 2).

Meristems cultured on a basic MS media, supplemented with all concentrations of BAP but without NAA did not induce callus in any of the varieties (Fig. 7 & 8, Online Resource 2). 'Berkume' was the culture with the highest callus mass that quickly outgrew the shoot bud.

The differences in weight of the callus measured between the varieties that we tested on the specific media concentrations and combinations, could be explained by the differences in the amount of endogenous auxins in each variety (Onwubiko et al. 2015). Therefore, in order to improve the growth of the cultures, these authors suggested PGRs that supplemented the basic MS media and should be in line with the concentration of endogenously PGRs in tissue. While this may be the explanation for the genotype variation, we would recommend to test a variety of PGR combinations systematically, rather than the lengthy process of determining the endogenous hormone content.

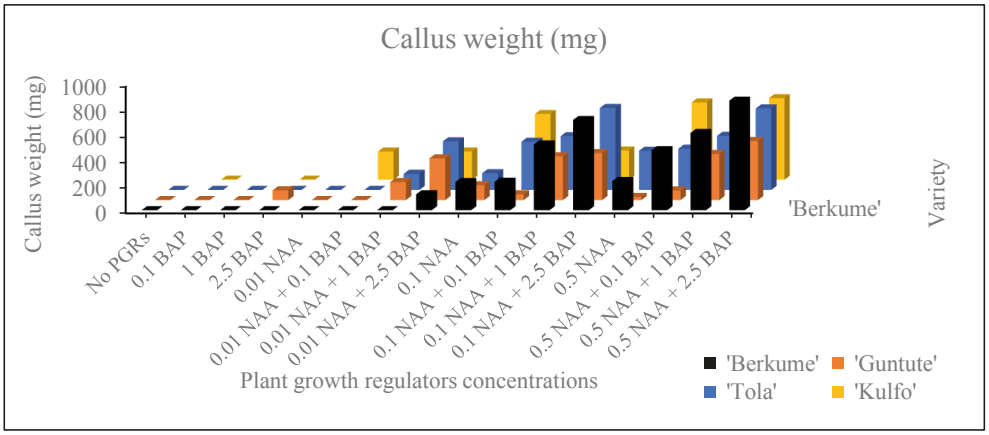


Figure 7. Effects of 16 combinations of NAA and BAP on the weight of callus induced from meristem explants of four sweet potato varieties from Ethiopia, after 12 weeks.

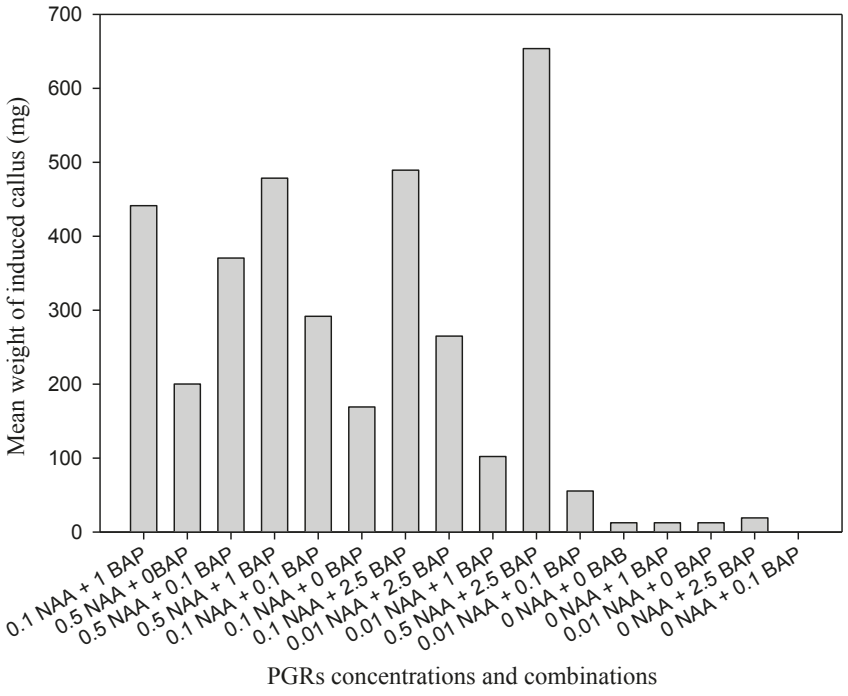


Figure 8. Mean weight of callus (mg) for four Ethiopian sweet potato varieties induced on 16 combinations of PGR (NAA and BAP) concentrations in MS media.

Conclusion and recommendations

This study demonstrated that different varieties of sweet potato respond very differently to the shoot initiation and shoot multiplication media, with respect to PGRs. Sweet potato have a clear requirement for auxin (NAA) for initiation, without this the addition of cytokinin (BAP) at any concentration has no effect. Then, the combinations of auxin and cytokinin is vital for meristem initiation and development into shoots. For the establishment of a novel genotype that did not form part of these experiments, the best chance of success would be to use MS medium supplemented with 0.1-0.5 mg/L NAA and 1.0 mg/L BAP.

For further multiplication *in vitro* of virus-free material , the best medium for 'Berkume', 'Guntute' and 'Hawassa-83' , would be 2.5 mg/L BAP combined with 0.5 mg/L GA_3 and *no auxin*, would be the medium to try for new genotypes. However, 'Kulfo' preferred higher amounts of BAP (3.5 mg/L and 0.5 mg/L GA_3) to reach it's highest potential. Since this is the highest amount of BAP used in this study, the optimum may be even higher. The same is probably true for 'Tola', which failed to multiply under our conditions, but still had it's highest score on the same medium.

For all five of the tested varieties, the longest shoots with a higher number of internodes was obtained when single nodal cuttings cultured on basic ½ MS media without any PGRs. These shoots are suitable for transfer to soil. We, therefore, recommend to use hormone-free medium as the last medium prior to transfer to soil, to produce longer shoots with a greater chance of survival.

With this study, we have provided initiation medium, multiplication medium and elongation medium for elite sweet potato genotypes from Ethiopia, which can be used for multiplication of elite virus-free sweet potato varieties, and then distributed to the farmers or private multipliers.

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Figure legends

Figure 1. The average percentage of initiations shoot from meristem combined for four sweet potato varieties from Ethiopia as affected by 16 different concentrations and combinations of plant growth regulators (NAA and BAP) supplemented to MS medium.

Figure 2. The average initiations of shoot tip meristems of four Ethiopian sweet potato varieties on basic MS media supplemented with 16 different concentrations of NAA and BAP. Mean (represented by the bars) that do not share the same letter are significantly different at 5% level.

Figure 3. The effect of media combinations and varieties on number of shoots initiated per nodal cutting of five Ethiopian cultivars from different regions.

Figure 4. Mean shoot multiplication parameters (shoot number, internode number and shoot height) as affected by varying concentrations of BAP (each combined with GA_3 of 0.5 mg/L) for five elite sweet potato varieties from Ethiopia. **A**: BAP concentrations affected the parameters of shoot multiplications; **B**: varieties affected the shoot multiplication parameters. Bars represent mean values. The same colored bars followed by same superscripts letter are not significantly different at $P < 0.05$ according to Turkey pairwise comparisons.

Figure 5. The shoot height of sweet potato *in vitro*, from the start (left), decreases with increasing concentrations of BAP.

Figure 6. Establishment of tissue culture plants in soil. **A**: Shoot of 4 - 5 internodes from tissue culture used for planting. **B:** Examples of plants that survived, were acclimatized and became established.

Figure 7. Effects of 16 combinations of NAA and BAP on the weight of callus induced from meristem explants of four sweet potato varieties from Ethiopia, after 12 weeks.

Figure 8. Mean weight of callus (mg) for four Ethiopian sweet potato varieties induced on 16 combinations of PGR (NAA and BAP) concentrations in MS media.

Online Resource 1. The effects of basal MS media supplemented with plant growth regulators on the percentage of shoot regeneration of four sweet potato varieties from Ethiopia. Results were recorded at 12 weeks. T: treatment number

PGRs concentrations (mg/l)			Shoot regeneration (%) Variety			
T	**NAA**	**BAP**	**'Berkume'**	**'Guntute'**	**'Tola'**	**'Kulfo'**
1	0	0	0^{d}	0^{d}	0^{d}	
2		0.1	0^{d}	0^{d}	0^{d}	4.77 ± 4.8^{c}
3		1	0^{d}	0^{d}	0^{d}	
4		2.5	0^{d}	0^{d}	0^{d}	19.05 ± 1.9^{bc}
5	0.01	0	0^{d}	0^{d}	0^{d}	
6		0.1	0^{d}	0^{d}	0^{d}	4.76 ± 4.8^{c}
7		1	0^{d}	28.57 ± 8.3^{bcd}	9.52 ± 4.8^{c}	
8		2.5	4.76 ± 4.8^{cd}	57.14 ± 8.3^{ab}	0^{d}	33.33 ± 4.8^{bc}
9	0.1	0	47.62 ± 4.8^{abcd}	4.76 ± 4.8^{cd}	28.57 ± 0.0^{bc}	
10		0.1	42.86 ± 21.8^{abcd}	23.81 ± 4.8^{bcd}	33.33 ± 12.6^{bc}	52.38 ± 14.3^{ab}
11		1	57.14 ± 14.0^{ab}	66.67 ± 4.8 a	23.81 ± 12.6^{bc}	
12		2.5	38.10 ± 7.2^{abcd}	0^{d}	0^{d}	0^{c}
13	0.5	0	57.10 ± 6.5^{ab}	14.29 ± 8.3^{cd}	71.43 ± 14.30^{a}	
14		0.1	76.20 ± 12.6^{a}	14.29 ± 8.3^{cd}	52.40 ± 4.8^{ab}	80.95 ± 76.0^{a}
15		1	52.38 ± 4.8^{abc}	38.10 ± 7.2^{abc}	23.81 ± 12.6^{bc}	
16		2.5	9.52 ± 4.8^{bcd}	38.10 ± 4.8^{abc}	0^{d}	0^{c}

Values represent means ± SE. values in a column that share the same letter are not significantly different at $p < 0.05$ based on Tukey's LSD test.

Online Resource 2. Effects of NAA and BAP on the weight of callus induced from each meristem explant of five sweet potato varieties from Ethiopia at 12 weeks after culture.

PGRs concentrations (mg/l)			Callus weight/meristem (mg)			
			Variety			
T	**NAA**	**BAP**	**'Berkume'**	**'Guntute'**	**'Tola'**	**'Kulfo'**
1	0	0	0^{e}	0^{d}	0^{e}	
2		0.1	0^{e}	0^{d}	0^{e}	0^{e}
3		1	0^{e}	0^{d}	0^{e}	
4		2.5	0^{e}	76.7 ± 63.0^{bcd}	0^{e}	0^{e}
5	0.01	0	0^{e}	0^{d}	0^{e}	
6		0.1	0^{e}	0^{d}	0^{e}	222.38 ± 64.5^{bc}
7		1	0^{e}	141.91 ± 28.6^{abcd}	126.67 ± 63.8^{de}	
8		2.5	125.71 ± 34.2^{de}	329.10 ± 92.8^{abcd}	382.40 ± 65.7^{abc}	222.40 ± 64.5^{bc}
9	0.1	0	222.40 ± 64.5^{abc}	116.19 ± 36.7^{bcd}	130.95 ± 65.3^{de}	
10		0.1	224.29 ± 29.0^{abc}	45.24 ± 32.0^{bcd}	379.05 ± 43.9^{bcd}	518.57 ± 134^{ab}
11		1	518.57 ± 34.0^{abc}	344.76 ± 55.8^{abc}	422.86 ± 55.3^{abc}	
12		2.5	711.00 ± 26.6^{ab}	370.00 ± 37.0^{ab}	645.70 ± 62.4^{a}	230.19 ± 76.0^{abc}
13	0.5	0	230.19 ± 76.0^{cde}	24.62 ± 15.4^{cd}	308.10 ± 37.7^{cd}	
14		0.1	472.86 ± 87.3^{bcd}	76.19 ± 41.2^{bcd}	323.81 ± 29.3^{cd}	609.50 ± 37.2^{ab}
15		1	609.52 ± 37.2^{ab}	362.86 ± 118.0^{ab}	425.24 ± 82.8^{ab}	
16		2.5	863.81 ± 46.2^{a}	465.14 ± 182.0^{a}	642.38 ± 3.3^{ab}	643.33 ± 107.0^{a}

Values represent means ± SE. values in a column that share the same letter are not significantly different at $p<0.05$ based on Tukey's LSD test.

Online Resource 3. Multiplication rate of shoots induced from single nodal cutting explants of five elite varieties of sweet potato from Ethiopia cultured on ½ basic MS media containing five different combinations BAP and GA_3. 1 or less than 1 means that some cultures have no multiplication or reduced number of shoots (some die).

Concentration of PGRs (mg/L)		Mean number of shoot induced from nodal cuttings				
		Variety				
BAP	**GA_3**	**'Berkume'**	**'Guntute'**	**'Hawassa-83'**	**'Kulfo'**	**'Tola'**
0	0	1.00 ± 0.0	0.92 ± 0.1	0.92 ± 0.1	1.00 ± 0.0	1.00 ± 0.0
0.5	0.5	2.17 ± 0.2	1.00 ± 0.1	1.00 ± 0.0	1.25 ± 0.0	1.00 ± 0.0
1.5	0.5	2.33 ± 0.3	1.50 ± 0.1	1.67 ± 0.2	1.33 ± 0.2	1.00 ± 0.0
2.5	0.5	2.83 ± 0.8	1.67 ± 0.2	1.75 ± 0.1	1.33 ± 0.1	1.00 ± 0.0
3.5	0.5	1.42 ± 0.4	-	1.67 ± 0.2	1.58 ± 0.2	1.08 ± 0.1

Online Resource 4. Mean height of the shoots regenerated from single nodal cuttings of the five elite varieties of sweet potato from Ethiopia cultured on ½ basic MS media containing five different combinations BAP and GA_3.

Concentration of PGRs(mg/L)		Mean height of shoot induced (cm)				
		Variety				
BAP	**GA_3**	**'Berkume'**	**'Guntute'**	**'Hawassa-83'**	**'Kulfo'**	**'Tola'**
0	0	1.68 ± 0.0	2.58 ± 0.5	3.50 ± 1.2	3.00 ± 1.00	6.90 ± 0.4
0.5	0.5	0.28 ± 0.1	0.40 ± 0.0	0.80 ± 0.1	1.83 ± 0.2	0.25 ± 0.1
1.5	0.5	0.27 ± 0.1	0.40 ± 0.1	0.55 ± 0.1	1.50 ± 0.3	0.17 ± 0.0
2.5	0.5	0.27 ± 0.0	0.40 ± 0.1	0.73 ± 0.3	0.71 ± 0.2	0.13 ± 0.0
3.5	0.5	0.25 ± 0.0	-	0.68 ± 0.3	0.88 ± 0.2	0.12 ± 0.0

PAPER IV

Detection of viruses and elimination of *Sweet potato feathery mottle virus* (SPFMV*)* in high yielding varieties of sweet potato from Ethiopia

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Highlights

- Viral diseases have become the main threat to sweet potato production in Ethiopia.
- Obtaining and multiplying virus-free planting materials have been difficult for growers.
- Five common viruses were tested in five sweet potato varieties prior to heat treatment.
- This study reports detection of *Sweet potato virus C* (SPVC) for the first time in Ethiopian sweet potato variety.
- Meristem culture from heat-treated plants further increases the percentage of virus-free plantlets compared to meristem culture in none treated plants.
- Five virus-tested sweet potato planting materials were generated.

Abstract

In recent years, viral diseases have become the main threat to sweet potato (*Ipomoea batatas* L. Lam.) production which is the main and supplementary source of food for millions of Ethiopians. Studies carried out from 1986 to 2011 reported the detection of five sweet potato infecting viruses.

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Consequently, obtaining and multiplying virus-free planting materials have been difficult for farmers and commercial multipliers. This study was conducted to:

i) detect the virus/es that are infecting the five sweet potato varieties preferred by the farmers

ii) compare the virus elimination efficiency between meristem culture from none-treated and heat treated mother plants and

iii) produce virus-free sweet potato planting materials.

Seven common viruses were tested using Grafting, Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) before and after elimination procedures as a screening and confirmatory methods. SPFMV elimination efficiency of meristem culture from none treated (grown at 25 ± 1 °C) and heat-treated (grown at 39 ± 1 °C) potted plants of four sweet potato varieties infected by SPFMV were evaluated and compared. This study reports detection of *Sweet potato virus C* (SPVC) for the first time in sweet potato plants in Ethiopia. *Sweet potato feathery mottle virus* (SPFMV) was detected in 12 of the 15 plants tested. A triple infection of SPFMV, *Sweet potato chlorotic stunt virus* (SPCSV) and SPVC was detected in only one of the plants. Cuttings meristems from heat-treated plant was found to further increase the percentage of virus-free plantlets by 10% to 16%, depending on the varieties. Elimination efficiency seems also varied with varieties: highest in 'Tola' and least in 'Guntute'. The present study generated five virus-free genotypes of sweet potato planting materials for future use by the farmers in Ethiopia.

Keywords: Sweet potato, virus elimination, *Sweet potato feathery mottle virus*, grafting, Ethiopia

1. Introduction

Sweet potato (*Ipomoea batatas*) is expanding faster than any other major food crop, in terms of area under production, in Sub-Saharan Africa (Low et al. 2009). Sweet potato is the second most important root crop grown by small-scale farmers in two regions of Ethiopia; Southern Nation, Nationalities and People's Region (SNNPR) and Oromia Region. Sweet potato is used as a source of food and feed in Ethiopia (Belehu 2003). It is a key crop to combat food insecurity and to enhance nutritional balance due to it's high drought resistance and higher yield per unit area. Therefore, non-governmental organizations (NGOs), including the International Potato Center (CIP) and "Better Potato for a Better Life", are currently promoting the distribution of orange fleshed sweet potato (high in pro-vitamin A) to new areas in the northern part of the country (Aldow 2017). Nevertheless, virus diseases are still the main factor limiting the production of this crop.

Viral diseases have become the main threat to sweet potato production in Ethiopia. To date, thirty sweet potato-infecting viruses have been identified worldwide (Clark et al. 2012). Of these, only the following five have been reported in Ethiopia during studies carried out from 1986 to 2011: *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato virus 2* (SPV-2), and *Sweet potato virus G* (SPVG), *Cucumber mosaic virus* (CMV) (Alemu 2004; Adane 2010; Tesfaye et al. 2011; Wondimu et al. 2012). SPFMV and SPCSV are the most frequently detected viruses with high incidence levels in both farmers' fields and research stations in SNNPR of Ethiopia (Adane 2010; Tesfaye et al. 2011). These viruses tend to occur as single infections and mixed infections (Adane 2010; Tesfaye et al. 2011). These viruses are still infecting and being distributed with planting materials because of a lack of a supply of virus-free materials in Ethiopia.

In Ethiopia, the farmers use their own planting materials from previous harvests and it is normal to exchange it with their neighbors. There is no dependable certification scheme to verify that the planting material is free of viruses. Viruses accumulate, perpetuate, and transmit over seasons in vegatatively propagated materials. These could be the main reasons why viruses are distributed in SNNPR of Ethiopia. The use of virus-infected planting materials not only reduce the yield and quality (Clark and Moyer 1988) but also significantly contributes to the persistent and continued

spread of viral diseases (Opiyo et al. 2010). Furthermore, research institutions, where virus infections were reported very high in their sweet potato collection (Adane 2010), are the only source of planting materials for commercial propagators. The propagators sell planting material to national and international none Governmental organizations (NGOs) and Government organizations who distribute to farmers in drought-prone areas (own, unpublished data).Therefore, there is an obvious need for a more rigorous certification scheme in Ethiopia which should also encompass a scheme for detection and virus elimination to provide clean mother stocks.

Virus detection and elimination is an important virus management practice. Detection of pathogens (including viruses) is key to their management, prediction of disease occurrences and prevention of subsequent crop loss due to infection (Van der Want and Dijkstra 2006). Therefore, detection and elimination of possible viruses from preferred high yielding varieties is essential before distribution of the planting materials to the grower. The application of efficient therapy methodologies to eliminate viruses and rigorous screening of elite accessions for viruses are required in clonally propagated plant (Golino et al. 2017) and it determines the success of securing quality propagating material. Therefore, the use of more than one technique, depending on the different properties of virus, is essential for correct detection. Different therapeutic actions, in combination with meristem culture and/or shoot tip culture, have been used to eliminate viruses from many crops, including sweet potato (Spiegel et al. 1994; Wang and Valkonen 2008; Panattoni et al. 2013). Virus elimination by heat therapy is achieved by exposing plants to temperatures of between 35^0C -54 ^{0}C (Panattoni et al. 2013) for different periods, depending on plant type. Heat therapy is the oldest, most effective and most commonly used virus elimination method (Nyland and Goheen 1969). However, combinations of one or more of these methods could further enhance the elimination efficiencies of the methods.

Attempts were made to clean viruses from a few cultivars of Ethiopian sweet potato using meristem-tip culture and thermotherapy (Dugassa and Feyissa 2011; Wondimu et al. 2012). However, the cleaned stock wasn't maintained for further multiplication and distribution to farmers and multipliers. This study was conducted to i) detect the virus(es) infecting five sweet potato varieties preferred by farmers in Ethiopia ii) to compare the virus elimination efficiency of

meristem culture and heat therapy combined with meristem culture and iii) to produce virus-free sweet potato planting materials.

2. Materials and Methods

2.1 Sampling, plant materials and greenhouse conditions

This study was conducted in the Plant Cell Laboratory at the Norwegian University of Life Sciences (NMBU) and the Virus Laboratory at the Norwegian Institute of Bioeconomy Research (NIBIO), both laboratories are located in Ås, Norway.

Five high yielding sweet potato varieties: Hawassa-83, Berkume, Kulfo, Guntute and Tola were used for this study. Vines from three plants of each variety, were collected from farmers' fields, university research sites and research institutions in the beginning of August 2014, in Ethiopia. These vines were then established in a screen house at Hawassa University, Ethiopia.

Vine cuttings from the three plants of each variety (designated as plant A, B, and C) were transported to Norway, established in the quarantine rooms at NMBU and grown at 25 °C, with a light intensity of 150 $\mu molm^{-2}s^{-1}$ and RH of 70%. These plants were used for the virus testing and virus elimination experiments.

2.2 Virus testing

Three standard testing methods were used: Enzyme-Linked-Immuno-Sorbent-Assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR) and graft-inoculation using *Ipomea setosa* as the indicator plant.

2.2.1 ELISA

The ELISA test was applied to leaf samples of the initial sweet potato plants (before grafting) and leaf samples of *Ipomoea setosa* grafted with initial plants (after grafting) before virus elimination. In addition, the leaf samples of *Ipomoea setosa* graft-inoculated with plantlets regenerated after virus elimination experiments were tested. This was done to determine the initial status of the

plants prior to subjecting them to procedures for virus elimination and to evaluate if the virus elimination procedures were effective.

The initial plants were inspected and virus symptoms observed (if any) were recorded during the active growth stage of the plants for a period of up to three months. Five common sweet potato infecting viruses SPFMV, SPMMV, SPCSV, SPV2 and CMV were tested using modifications of double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) procedures (Clark MF and Adams 1977). These were modified as follows: 0.5g fresh leave samples and 4.5 ml of sample extraction buffer were added into extraction bags (BioReba). The sap was extracted using homogenizer (semi-automated HOMEX 6, BioReba). Polyclonal antibodies and positive controls of the target viruses obtained from the Leibniz institutes DMSZ-German collection of microorganism and cell culture (Germany) were used. Dilution of antibodies was undertaken as advised by the manufactures. However, we used 100µl of the antibodies-coating solution into each microtiter plates instead of 200 µl. 100µl of unpurified sample extracts were dispensed into microtiter plates. All buffers used were prepared, based on the recommendation of (Clark MF and Adams 1977). All incubation and washing steps were carried out according to Adam and Clark, (1977). Positive and negative controls were incorporated to verify ELISA procedures. Results were measured two hours after addition and incubation of the colorless substrate-solution made from P-Nitrophenyl Phosphate Disodium 20mg*Ta (Sigma) commercial tablet dissolved in 40 ml of substrate buffer. Virus-infected samples were determined based on visual assessments of the yellow color that developed, and the absorbency value (AV) measured at 405 nm using SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Califonia USA). Samples that developed yellow color and had a mean absorbency value greater than twice the mean of the absorbance of the negative control sample were considered as virus-infected samples.

2.2.2 RT-PCR

RT-PCR was used to verify the results obtained with ELISA and as a main testing method for SPVC and SPVG. Briefly, total RNA was extracted from 0.1g fresh leaf samples. These samples were grounded to a fine powder in liquid nitrogen using mortar and pestle. RNA extraction was performed using Trizol reagents (Invitrogen, Life Technologies) following the manufacturer's

procedures. Total RNA was quantified and the quality verified using Nanodrop (Thermo Fisher Scientific, USA), Agilent 2100 Bio-analyzer (Agilent Technologies, USA) and with a 1% agarose gel electrophoresis stained with ethidium bromide.

First strand cDNA was synthesized using 2.5µg total RNA template, random primer (Invitrogen) and Super Script™ II Reverse Transcriptase (Invitrogen) and all other reaction components and reaction conditions were according to the manufacturer's recommendations (Invitrogen, California ©2010 Life Technologies).

PCR reactions were carried out using the first strand cDNA as a template, virus-specific primers to SPVC, SPVG, SPV2 (Li et al. 2012) and SPFMV, SPCSV, and SPMMV (Kathurima et al. 2011) and TIF DNA polymerase (Invitrogen) following the procedures of the manufacturers and the appropriate controls. Amplification was performed under the reaction conditions of 94 ℃ for 2 minutes, 35 cycles of 94 ℃ for 20 seconds, 52 ℃ (for SPVC, SPVG, SPMMV and SPCSV) or 53 ℃ (for SPFMV) for 20 seconds, and 72 ℃ for 30 seconds. A final elongation was carried out at 72 ℃ for 5 minutes.

2.2.3 Grafting

Shoot tips of the initial sweet potato plants and *in vitro* plantlets regenerated after heat treatments were grafted onto the indicator plant *Ipomoea setosa* before and after virus elimination treatments respectively. Previous studies have shown (Aritua et al. 2007) that some viruses occur in very low titters in sweet potato, which might be below the detection threshold of ELISA. Therefore, all initial plants which tested negative by ELISA were grafted to *Ipomoea setosa* (a highly susceptible indictor host) to increase the virus concentration for a second round of testing. In all cases, grafting was carried out as follows: a three week old *Ipomoea setosa* was graft-inoculated with a 3cm long shoot tip, with 3-4 internodes (side grafting) taken from test plants. Three *Ipomoea setosa* were grafted with shoot tips of the test plants. Two shoot tips were grafted on each *Ipomoea setosa* to maximize the inoculum. The graft union was tightly wrapped with Para-film and covered with a plastic bag for a week, and was cultivated under greenhouse conditions consisting of: day/night temperature 21/19 ℃, RH of 70% and light intensity of 150 $\mu molm^{-2}s^{-1}$. Grafted *Ipomoea setosa*

plants were checked daily and the presence of systemic symptoms were recorded for up to 45 days after grafting. However, *Ipomoea setosa* leaf samples were collected one month after graft-inoculation and stored at -80 ℃ for virus testing using ELISA and RT-PCR, as described previously.

In vitro plantlets regenerated after undergoing virus elimination treatments were also graft-inoculated to *Ipomoea setosa* as mentioned above. Graftings were performed twice; at 5 months (after five subcultures) and 18 months (after nine subcultures) after meristem cultures. *In vitro* plantlets were randomly selected and shoot tips of about 3cm long were grafted at each screening time following the procedure described earlier.

2.3 Heat therapy, meristem culture, and plant regeneration

Out of the five sweet potato varieties tested in this study, four were shown to be infected with only SPFMV and free from the other common sweet potato viruses tested (SPMMV, SPCSV, SPV2 and SPVC, SPVG and CMV). Consequently, these four varieties were used in the virus elimination experiment. Heat-therapy treatment, followed by meristem tip culture was applied according to the method described for sweet potato (Dennien et al. 2013), with some modifications described as follows. Six vine cuttings from each variety were potted and grown in a growth chamber at a daily temperature cycle of 25/18 °C under a 12/12 hours photoperiod with a light intensity of 150 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). After four weeks, the potted plants were divided in to two sets each consisting of three potted plants of each variety. One set continued to be grown under same conditions and used as a meristem donor for meristem culture alone (non-heat treated control). The other set was transferred to a different growth room where it was subjected to heat therapy for 8 weeks at daily temperatures cycle of 39 ± 1°C / 25 ± 1 °C day/night, 12/12 hours of photoperiod and light intensity of 150 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). Meristems were excised from both experimental conditions (non-heat treated and the heat-treated donor plants) and cultured *in-vitro* on nutrient media optimized for sweet potatoes. MS medium (Murashige and Skoog 1962) supplemented with vitamins, plant growth regulators in mg/l (0.3 6-Benzylaminopurine, 0.03 1-Naphthaleneacetic acid, and 0.5 Gibberellin A3), iron 48mg/l of Fe and 2% Parts per million (PPm) was used for initiate regeneration. The cultures were placed in the light (18h)/dark(8h) for a week at 25 ± 1 °C and then moved to a growth condition of more intensity

of light (28 μmolm^{-2}s^{-1}) provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). After five weeks, surviving meristems were counted and sub-cultured into test tubes containing a fresh medium of the same composition, for further shoot initiations. After initiation, plantlets were elongated and multiplied on plant growth regulators-free 1/2 basal MS media for subsequent virus indexing using combinations of methods described in section 2.2 of this paper. The virus elimination efficiency of meristem culture alone and combined heat therapy were compared based on the percentages of plantlets that were cured of infecting viruses by each method.

3. Results and Discussion

3.1 Graft-inoculation drastically improves detection of SPFMV by DAS-ELISA and RT-PCR

From the 15 plants collected from the field and research stations and grown in the greenhouse, only one plant (variety 'Guntute', plant B) showed typical virus-like symptoms. The plant presented a general chlorosis, vein clearing, leaf distortion, mosaic, yellowing, stunting of the whole plant, reduced leaf size, and vein chlorosis (Fig. 1b). The rest of the plants showed no visible symptoms (Fig. 1c). DAS-ELSA and TAS-ELISA assay to detected SPFMV, SPMMV, SPCSV, SPV2 and CMV revealed the presence of SPFMV and SPCSV in only one plant ('Guntute' B), which correlated with being the only plant that showed typical virus-like symptoms. No other virus was detected in the other 14 plants according to DAS-ELISA and TAS-ELISA (Table 1). RT-PCR results confirmed the results obtained by ELISA (Table 1). However, RT-PCR results also revealed a higher number of plants (6 out of 15) positive for SPFMV, of which 5 were negative according to ELISA (Table 1).

Subsequently, scions from the 15 plants were used to graft-inoculate *Ipomoea setosa*. From each initial plant, 6 *Ipomoea setosa* plants were graft inoculated. All *Ipomoea setosa* plants inoculated with scions belonging to plants of the varieties Hawassa 83, Tola, Kulfo, and Guntute showed virus like symptoms after 3 weeks post grafting (Table 1). Furthermore, all of these plants were positive for SPFMV by DAS-ELISA and RT-PCR (Table 1). Thus, the number of SPFMV infected plants detected after graft inoculation increased from 6 to 12. Nevertheless, none of the plants graft

inoculated with scions belonging to plants of the variety Berkume developed any virus-like symptoms, nor tested positive for any of the viruses tested for by ELISA or RT-PCR.

As described above, ELISA assays were not able to detect SPFMV in 11 out of 12 plants prior to graft inoculation. On the other hand, RT-PCR assays were able to detect SPFMV in 6 of the 12 plants prior to graft inoculation. Thus, RT-PCR is a more sensitive and reliable method to detect SPFMV from sweet potato plants. Nevertheless, RT-PCR assays efficiently detected the virus in only 50% of the cases. Our findings are in line with previous studies reporting the challenges to detect SPFMV by ELISA (Green et al. 1988; Moyer and Salazar 1989; Aritua et al. 2007). Consequently, we recommend (as previous authors have also recommended) that graft inoculation into *Ipomoea setosa* followed by ELISA and/or RT-PCR obtains the most reliable results.

Previous studies indicate that symptomless infection of sweet potato plants by SPFMV is not uncommon (Gibson et al. 1997; Tugume et al. 2008). These findings are also exemplified in our study where 11 out of the 12 plants found to be infected with SPFMV were symptomless. In Ethiopia, there is a very weak plant virus certification scheme, since it relies on only visual evaluation in the fields which does not consider symptomless infections. Using virus-infected planting materials not only reduces yield and quality (Clark and Moyer 1988) but also spreads the virus, since viruses accumulate and perpetuate over seasons in vegetatively propagated materials.

Table 1. Induced viral symptoms and viruses detected in sweet potato varieties from Ethiopia before and after grafting *Ipomoea setosa.*

Variety	Before grafting			After grafting		
	Symptoms	Viruses detected by ELISA	Viruses detected by RT-PCR	Symptoms	Viruses detected by ELISA	Viruses detected by RT-PCR
'Hawassa-83' A	SL	ND	SPFMV	CRS, LD, FM, LC, VC	SPFMV	SPFMV
'Hawassa-83' B	SL	ND	SPFMV	CRS, LD, F, LC, VC	SPFMV	SPFMV
'Hawassa-83' C	SL	ND	ND	CRS, LD, FM, LC VC	SPFMV	SPFMV
'Berkume' A	SL	ND	ND	SL	-	-
'Berkume' B	SL	ND	ND	SL	-	-
'Berkume' C	SL	ND	ND	SL	-	-
'Tola' A	SL	ND	ND	CRS	SPFMV	SPFMV
'Tola' B	SL	ND	SPFMV	CRS	SPFMV	SPFMV
'Tola' C	SL	ND	ND	CRS	SPFMV	SPFMV
'Kulfo' A	SL	ND	ND	CRS	SPFMV	SPFMV
'Kulfo' B	SL	ND	SPFMV	CRS	SPFMV	SPFMV
'Kulfo' C	SL	ND	SPFMV	CRS	SPFMV	SPFMV
'Guntute' A	SL	ND	ND	RCS, VC, LC FM	SPFMV	SPFMV
'Guntute' B	CRS, S VC	SPFMV &SPCSV	SPFMV &SPCSV	CRP, CY, LD, VC	SPFMV, SPCSV	SPFMV, SPCSV & SPVC
'Guntute' C	SL	ND	ND	CRP, VC, LC, FM	SPFMV	SPFMV

CRP: Chlorotic ring spots, LD: Leaf deformation, FM: Feathery mottle, LC: leaf curling, CY: yellowing and chlorosis, D: defoliation of older leaf, S: Stunting of plants, VC: Vein clearings, SL: Symptomless, ND: Not detected

3.2 First report of SPVC in Ethiopia

In this study SPVC was detected in one plant of sweet potato variety Guntute, which was obtained from Hawassa Research Center, Ethiopia. Detection of this virus was achieved by RT-PCR, where a PCR product with a fragment size of 836 bp was amplified (Fig. 1a). The identity of the virus was verified by next generation sequencing (H. buko, *manuscript in preparation*). The infected plant appeared severely stunted and showed vein yellowing and chlorotic blotches (Fig. 1b). These symptoms differed from all the other plants tested in this study and it was later revealed that this plant suffered from a mixed infection of SPVC, SPFMV and SPCSV (Fig. 1b). None of the other plants and varieties tested were found to be infected by SPVC. It is our understanding that this study represents the first report of SPVC in Ethiopia. The previous surveys carried out in Ethiopia (Alemu 2004; Adane 2010; Tesfaye et al. 2011) did not include SPVC as a target virus. Therefore, it cannot be established if SPVC has been present in the country for a long time or if it has recently been introduced by imported plant material.

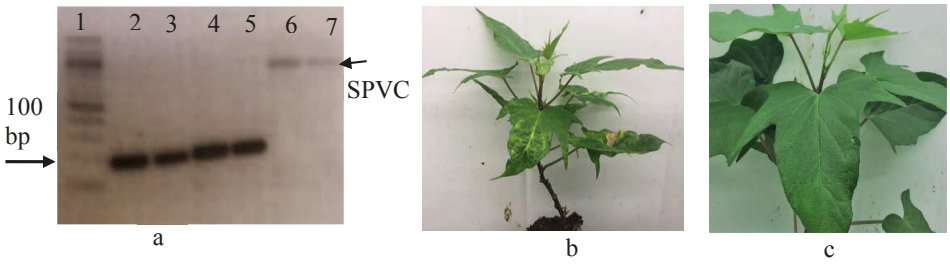

Fig 1. Triple infection of sweet potato plant. 1a: PCR products of triple infection of virus in samples of variety Guntute. Lane 1 is 100bp lader, 2 and 3 are +ve control and PCR product of SPFMV, 4 & 5 are +ve control and PCR product of SPCSV and 6 & 7 are +ve control and PCR product of SPVC. 1b: is stunted plants infected with the three viruses; SPFMV, SPCSV, and SPVC. 1c: Symptomless plant infeced with SPFMV.

3. 3 Heat therapy followed by meristem tip culture increased the efficiency of SPFMV removal

Heat treatment of meristem donor plants before meristem excision resulted in a higher number of virus-free plants as compared to meristem excision without heat treatment (Fig. 2). Samples from shoot tips/plants that did not induce virus symptoms in *Ipomoea setosa* after grafting were SPFMV negative by the ELISA and RT-PCR. On the other hand, grafted plants which showed virus-like symptoms were positive to SPFMV as confirmed by ELISA and RT-PCR.

Heat treatment of 'Hawassa-83' meristem donor plant followed by meristem culture eliminated SPFMV from 93.8% of the plantlets; whereas only 83.3% of 'Hawassa-83' plants obtained from the non-heat treated ones were SPFMV free. In 'Kulfo' 84.7% and 70.6% SPFMV free plantlets were obtained from heat-treated and non-treated meristem donor plants, respectively. Only a small number of plants were regenerated from the variety 'Tola'. We believe that this is due to a poor regeneration media for this genotype; the optimum medium has not been found. Nevertheless, the plants derived from both sources (heat treated and non-heat treated) were 100% virus-free. In the case of 'Guntute', only 66.7% and 40.0 % plants derived from the heat-treated and non-treated meristem donor plants, respectively, were found to be free of SPFMV.

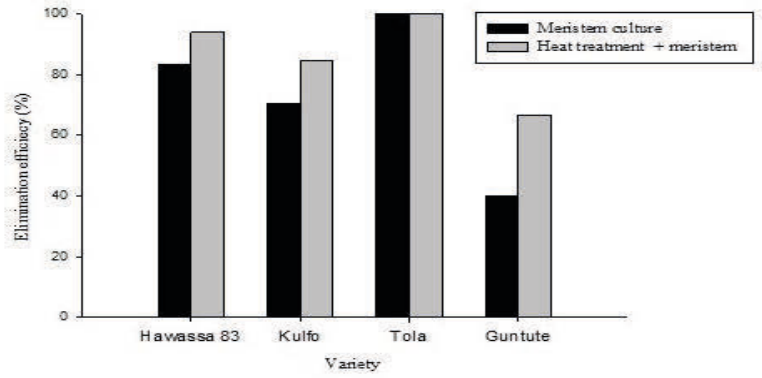

Fig. 2. Efficiency of virus elimination methods; meristem culture alone and combined with heat treatment for 4 virus-infected genotypes of sweet potato

Differences in response to virus elimination treatment was prominent in the varieties tested. The highest (100%) SPFMV elimination efficiency was obtained in 'Tola' followed by 'Hawassa-83' (93.8%) and 'Kulfo' (84.7%). The variety with the lowest efficiency (66.7%) was 'Guntute'.

Differences in virus/viroid elimination efficiency between varieties of the same crop have previously been reported (Paprstein et al. 2008; Waswa et al. 2017) and seems to be mediated by the specific interactions that occur between the virus and the host. For example, in the case *Argyranthemum* plants infected by *Chrysanthemum stunt viroid* (CSVd), the accumulation of Callose (β-1, 3-glucan) around the plasmodesmata of shoot apical meristems differs between varieties and correlates with the feasibility of eliminating the viroid by meristem tip culture. Varieties which accumulate high amounts of callose are less likely to become viroid free after meristem tip culture. Studies have shown that callose accumulation in the plasmodesmata is associated with a decrease in the plasmodesmata opening (Bilska and Sowiński 2010) whereas virus/viroid movement from cell-to-cell is accomplished through the plasmodesmata (Hull 2013). Thus, it has been suggested that callose deposition at the plasmodesmata limits cell-to-cell movement of CSVd and is most likely responsible for the different frequencies of obtaining viroid-free material among *Argyranthemum* varieties (Zhang et al. 2015). All of the sweet potato varieties

in our study have a different genetic background (Kosmowski et al. 2018). Thus, it is conceivable that the different frequencies observed in response to virus elimination treatment are due to specific virus-host interactions that vary among the sweet potato varieties tested.

Meristem tip culture alone and in combination with the other virus elimination methods; heat therapy, chemotherapy and cryotherapy has been sucessfuly used as a virus elimination method in sweet potato (Wang Q et al. 2009). Previous studies have shown that combining heat treatment with meristem culture in sweet potato and potato have a better elimination efficiency than meristem culture alone (Dodds and NG 1988; El Far and Ashoub 2009; Waswa et al. 2017). Thus, our study confirms that heat-treating meristem donor plants increases the efficiency of virus elimination over using meristem culture alone.

4. Conclusions

In this study, we have successfully removed SPFMV from four of the five most widely used sweet potato varieties in Ethiopia. Our data shows that a combination of heat therapy and meristem tip culture is more efficient that meristem tip culture on its own. We have also shown that efficient and reliable detection of SPFMV requires a combination of graft inoculation to *Ipomoea setosa* with either ELISA or RT-PCR. Moreover, our study is the first to report SPVC in Ethiopia. Our study represents a stepping stone on the road towards an efficient virus-free production of sweet potato in Ethiopia. We envision that the experimental procedures presented in this paper will be used towards the development of a virus-free sweet potato production scheme in Ethiopia.

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PAPER V

Next generation sequencing as a method to verify virus elimination using heat treatment and meristem tip culture in the 5 most widely used sweet potato varieties in Ethiopia

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Abstract

Sweet potato (*Ipomoea batatas* L. Lam) has become one of the staple crops in Africa during the last 20 years. In Ethiopia, sweet potato is the second most widely grown root crop and is the first regarding the production per hectare. Worldwide, Ethiopia is ranked the sixth largest producer of sweet potato. Thus, there is a great demand of planting material throughout the country. Currently, planting material is usually obtained from own previous season harvest, local markets or from the neighboring fields since no certified clean planting material production scheme has been established in Ethiopia yet. Unfortunately, this practice has contributed to the spread of viral diseases throughout the country. Production of clean planting material requires the availability of virus-free plants, which can be used as the mother stock source. Elimination of viruses from infected plants is a tedious job, which requires efficient methods to eliminate the virus and also to verify that the plants are indeed virus-free. In the case of sweet potato, we have experience that heat treatment, combined with meristem tip culture is an efficient method for virus elimination. Our previous findings indicate that reverse transcription (RT) PCR is more efficient than ELISA to verify the efficiency of virus elimination. In this study, we explored the use of next generation

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sequencing (NGS) as a verification method and compare it to RT-PCR. Our results show that NGS seems to be more efficient than RT-PCR, although also prone to inconclusive results.

Key words: Viruses, NGS, Sweet potato, RT-PCR

Introduction

Sweet potato [(*Ipomoea batatas* L. Lam)] is the seventh most economically important crops in the world with total annual production of 12.3 million tones (FAO 2017). China leads the production followed by Nigeria, Tanzania, and Uganda and Ethiopia. Sweet potato is grown and consumed mainly by small-scale farmers in Ethiopia. Nevertheless, sweet potato demand, as a food source and as planting material has increased substantially over the last 10 years. In Ethiopia, the Southern Nations Nationalities Peoples Region (SNNPR) is the dominant sweet potato production area. Unfortunately, this area is also where sweet potato viral diseases cause severe losses of crop yields (Tesfaye et al. 2011; Tesfaye et al. 2013).

In order to increase the production of sweet potato, it is necessary to devise a strategy to produce clean planting material that can supply the market. An initial step for the establishment of a certified clean planting material production scheme is to identify the most widely used varieties in the country, identify the virus that are infecting these varieties, and device methods by which these varieties can be "cleaned" from the infecting viruses. During the last five years, we have accomplished all of these goals (Dereje et al submitted). Nevertheless, true development also requires the incorporation of novel techniques, which would contribute to the scientific development of the country.

During the last 10 years, next generation sequencing (NGS) has proven to be a reliable a robust method for screening for viruses in plants (Kreuze et al. 2009; Kashif et al. 2012; Jones et al. 2017). This method allows the detection of viruses infecting a given host, without prior knowledge on the existence of the virus. NGS has been used to determine the nucleotide sequences of RNA/DNA molecules and detected many viruses in sweet potato (Kashif et al. 2012; Mbanzibwa

et al. 2014; Kreuze et al. 2017; Nhlapo et al. 2018), in vineyard (Czotter et al. 2018) and many other crops. The methods was also used for detection of plant viroids. NGS has not been previously used in Ethiopian varieties of sweet potato. Moreover, NGS provides sequences data that can be used to classify the viruses in to taxonomical categories. NGS more sensitive than RT-PCR in detecting viruses in symptomless plants. In addition, NGS has not been used as a verification method for the efficiency of virus elimination by heat treatment-meristem tip culture in sweet potato varieties. Thus, in this paper we compare the efficiency of using reverse transcription PCR (RT-PCR) and NGS as a method to verify efficient elimination of viruses utilizing heat treatment combined with meristem tip culture in the five most widely used sweet potato varieties in Ethiopia. Our results show that NGS seems to be more reliable than RT-PCR, although it can also lead to unexpected results. In addition, by using NGS as a detection method, we have also identified the occurrence of two sweet potato viruses, previously not known to be present in Ethiopia.

Materials and Methods

Plant materials: Five sweet potato varieties ('Hawassa-83', 'Berkume', 'Tola', 'Kulfo' and 'Guntute B') were obtained from research and academic institutions in Ethiopia. Four varieties ('Hawassa-83', 'Berkume', 'Tola', and 'Kulfo') were symptomless, whereas one ('Guntute B') showed severe virus symptoms. Shoot tips of each variety were planted in two sets. One set was subjected to heat treatment followed by meristem culture for virus elimination (see below) and the other set was used as the control initial plants. Following heat treatment, the *in vitro* plants were regenerated from meristem cultures.

Heat therapy

Heat-therapy treatment, followed by meristem tip culture was applied according to the method described for sweet potato (Dennien et al. 2013), with some modifications described as follows. Five vine cuttings from each variety were potted and grown in a growth chamber at a daily temperature cycle of 25/18 °C under a 12/12 hours photoperiod with a light intensity of 150 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). After four weeks, the potted plants were divided in to two sets each consisting of three potted plants of each

variety. One set continued to be grown under same conditions and used as a meristem donor for meristem culture alone (non-heat treated control). The other set was transferred to a different growth room where it was subjected to heat therapy for 8 weeks at daily temperatures cycle of 39 ± 1°C / 25 ± 1 °C day/night, 12/12 hours of photoperiod and light intensity of 150 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux).

Meristem culture and plant regeneration

Meristems were excised from both experimental conditions (non-heat treated and the heat-treated donor plants) and cultured *in-vitro* on nutrient media optimized for the sweet potatoes (*Dereje and Trine 2019, in preparation*). The cultures were placed in the darker for a week at 25 ± 1 °C and then moved to a growth condition of more intensity of light (28 $\mu molm^{-2}s^{-1}$) provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). After five weeks, surviving meristems were counted and sub-cultured into test tubes containing a fresh medium of the same composition, for further shoot initiations. After initiation, plantlets were elongated and multiplied on plant growth regulators-free 1/2 basal MS media for subsequent virus indexing using combinations of methods described in (Dereje *et al., submitted*). The virus elimination efficiency of meristem culture alone and combined heat therapy were compared based on the percentages of plantlets that were cured of infecting viruses by each method.

Virus testing

All plants were tested by RT-PCR and NGS before and after heat treatment-meristem tip culture. RT-PCR was used to test for SPVC, SPVG, SPV2, SPFMV, SPCSV, and SPMMV as previously described (Dereje et al., submitted). For NGS, total RNA was extracted from 100 mg leaf tissue using Trizol (Invitrogen, CA, USA). Extracted RNA samples were purified using PureLink RNA Mini purification kits and DNase treated using the TURBO DNA-free kit (Invitrogen by thermofisher scientific) according to the manufacturer's instructions. The RNA samples quantity and purity were evaluated using a Nanodrop (NanoDropTechnologies, Wilmington, DE, USA). Furthermore, the RNA integrity was confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Finally Total RNA (3µg) kept on dry ice was sent to Fasteris genome sequencing services center (Geneva, Switzerland). There, small RNAs less than 30 nucleotides was separated from the total RNA by electrophoresis in acrylamide gel. The small RNAs were ligated with single stranded 3' adaptor and a bar coded with 5' adaptor. The small RNA library construction were made using Illumina TrueSeq small RNA library preparation kit. The library was sequenced using the Illumina HiSeq instrument (1 × 50+8 for sRNA runs) following the manufacturers' protocol. Small RNA read from the 12 samples were generated and kindly provided. The total numbers sRNA reads generated, percentage of sRNAs reads of 18-26 bp were calculated and presented in the table.

Data analysis

Raw RNA sequence data sets obtained from Fasteris were downloaded and analyzed using the VirusDetect software (Zheng et al., 2016). For the VirusDetect analysis, reads in the size range of 10 to 50 nucleotides were used.

Results and Discussion

Next generation sequencing

Small RNAs reads were generated using small RNA (sRNA) molecules of less than 30 nucleotides in size. sRNA deep sequenced data of the 5 varieties before and after virus elimination treatments were analyzed by denovo assembly, using the VirusDetect software package. Between 8.26 and 10.35 million sRNAs (1–50 bp) reads were generated per sample (Table 1). On average, 9.35 million reads were generated per sample and 79.4% of the sRNAs reads generated are between the size ranges of 18-26 bp (Table 1). The size distribution of the sRNAs read of the 10 samples libraries had slight differences. For all the libraries, reads with size of 21 bp and 24 bp accounted for the majority of sRNAs reads.

Table 1. Small RNA reads generated per samples before virus elimination treatment (A) and after virus elimination (B).

Samples	Varieties	Total # of small RNA reads per sample (million)	# of small RNA reads (size;18-26 bp)	% of small RNAs reads size (18-26 bp)
A. Before heat treatment-meristem tip culture				
1	Berkume I	9.13	6.28	68.74
2	Guntute B I	9.16	7.78	84.78
3	Kulfo I	10.03	8.91	88.85
4	Hawassa-83 I	9.44	7.30	77.36
5	Tola I	10.02	7.52	75.06
B. After heat treatment-meristem tip culture				
1	Berkume T	10.35	8.24	79.61
2	Guntute B T	9.97	7.72	77.43
3	Kulfo T	8.36	7.42	88.81
4	Hawassa-83 T	9.07	8.12	89.44
5	Tola T	8.26	6.92	83.69
Average		**9.35**	**7.42**	**79.40**

NGS reveals the existence of sweet potato viruses previously unknown to be present in Ethiopia.

Table 1 shows viruses known to infect sweet potato detected in the samples before and after heat treatment-meristem tip culture. The following sweet potato viruses were detected: SPFMV, SPVC, SPCSV, SPVG, *Sweet potato symptomless mastrevirus-1* (SPSMV-1) and three sweet potato badnaviruses (SPBV-A, SPBV-B, SPBV-C) collective called *Sweet potato papakuy virus* (SPPV). Among these SPFMV, SPVC, SPCSV and SPVG have previously been detected in Ethiopia. However, and to our knowledge this is the first report of SPSMV-1 and SPPV in Ethiopia.

SPPV (family *Caulimoviridae*; genus *Badinavirus*) is the most commonly detected virus in sweet potato (Mbanzibwa et al. 2014; Kreuze et al. 2017) and has been reported to occur in many accession belonging to the sweet collection at International Center for Potato (CIP) (Kreuze et al. 2017). SPPV was initially identified in sweet potato plants showing no visible symptoms (Kreuze et al. 2009). In our study, SPPV was detected in 5/5 of the initial plants samples and 3/5 of plants regenerated after heat-treated and meristem culture. Thus, heat therapy and meristem tip culture

have 50 % chance of eliminating the virus. Whether SPPV is endemic to Ethiopia, or was introduced into the country through infected material used for breeding purposes, is unknown. Perhaps the wide use of CIP accessions in breeding programs has contributed to the spread of this virus in Ethiopia.

Viruses, which occur in in apparently healthy-looking plants have a higher chance of dissemination between farms when planting materials, are exchanged. Although Badinaviruses do not cause visible damage in sweet potato, they are serious pathogens of tropical horticultural crops: banana, black pepper, cocoa, citrus, sugarcane, taro, and yam (Bhat et al. 2016; Bömer et al. 2018). Thus, NGS can be a potential detection tool in rigorous certification schemes in nations where susceptible crops to these viruses exist.

SPSMV-1 (family *Geminiviridae*, genus *Mastrevirus*) has previously been reported to occur in sweet potato plants in Peru, Tanzania, Central America, China and Korea (Kreuze et al. 2009; Kwak et al. 2014; Mbanzibwa et al. 2014; Wang et al. 2015; Kreuze et al. 2017). The virus usually occurs as a symptomless infection and does not result in a severe disease. SPSMV-1 was detected in three varieties ('Guntute B', 'Kulfo', and 'Hawassa-83') before virus removal treatement. Based on the NGS results from the treated plants, SPSMV-1 was efficiently removed from 'Guntute B' and 'Kulfo', but not from 'Hawassa-83'. However, and to our suprise, SPSMV-1 was also detected in 'Tola' (which was negative to SPSMV-1 before tratment).

Table 2. List of commonly found viruses infecting sweet potato detected by RT-PCR and NGS before (A) and after (B) heat treatment combined with meristem tip culture.

	A. Before heat treatment-meristem tip culture		B. After heat treatment-meristem tip culture	
Variety	**RT-PCR**	**NGS***	**RT-PCR**	**NGS***
Berkume	-	SPPV	-	-
Guntute B	SPFMV, SPVC, SPCSV	SPFMV, SPVC, SPVG, SPCSV, SPPV, SPSMV-1	-	-
Kulfo	SPFMV	SPFMV, SPVC, SPPV, SPSMV-1	-	SPPV
Hawassa 83	SPFMV	SPFMV, SPVC, SPVG, SPPV, SPSMV-1	-	SPFMV, SPVC, SPVG, SPPV, SPSMV-1
Tola	SPFMV	SPFMV, SPVG, SPPV	-	SPVG, SPPV, SPSMV-1

*Viruses which are underlined were only detected by NGS since no primers were available for RT-PCR detection.

Next generation sequencing (NGS) screening is more sensitive than RT-PCR

Throughout this study, we have employed two independent methods (NGS and RT-PCR) to test viruses in our plant material. All plant material was tested by RT-PCR and NGS before and after heat treatment-meristem tip culture. Thus, by using these two independent methods we believe that our results are more reliable.

RT-PCR was initially used to test for SPVC, SPVG, SPV2, SPFMV, SPCSV, and SPMMV in all plants before undergoing virus elimination treatment. Of the six viruses tested only SPFMV, SPVC and SPCSV were detected. The results showed that 4 out of the 5 varieties were infected with SPFMV, and these results were verified by NGS. Furthermore, RT-PCR results indicated that the only one plant ('Guntute B') was infected with SPCSV, and these results were verified by NGS. However, RT-PCR results also indicated that only one plant ('Guntute B') was infected by SPVC whereas NGS indicated that this virus also infected two other varieties 'Kulfo' and 'Hawassa-83'. Moreover, RT-PCR did not detect SPVG in any of the plants, whereas NGS revealed that three varieties ('Guntute B', 'Hawassa 83' and 'Tola') were infected with SPVG.

RT-PCR was also used to test for SPVC, SPVG, SPV2, SPFMV, SPCSV, and SPMMV in all plants after undergoing virus elimination treatment. None of the viruses tested for were detected in any of the plants. Thus, based only on RT-PCR results, the virus elimination treatment was successful in all varieties; efficiently eliminating the viruses (SPFMV, SPVC, SPCSV and SPVG) previously found infecting the untreated material. However, NGS results revealed that none of the viruses found infecting 'Hawassa-83' (SPFMV, SPVC and SPVG) were eliminated, whereas only SPFMV had been eliminated from 'Tola', which was still infected with SPVG. Surprisingly, NGS results also revealed that 'Tola' was also infected with SPSMV-1, even though this virus had not been detected in the plant before virus elimination treatment. More experiments are required to verify that the initial material was indeed free of SPSMV-1 or that the viral concentrations are below the detection threshold of NGS.

Abundances of homology based assembled vsiRNAs contigs differs between non-treated and treated plants and correlated with virus elimination treatments.

Viral-derived siRNA (vsiRNAs) contigs are contigs which are assembled from siRNA molecules that belong to viral sequences. Thus, in theory, in the absence of viral infection, no vsiRNA contigs should be obtained. In this study, the number of viral-derived siRNA (vsiRNAs) contigs assembled from non-treated plants was higher in most of the plants (with the exception of one plant) than that of the treated plants. Heat treatment followed by meristem tip culture is an efficient method for eliminating viruses. Thus, a lower number of vsiRNAs derived contigs in the plants after undergoing heat treatment-meristem tip culture, might be due to the lower viral population present in the plant after the treatment. Nevertheless and to our surprise, one variety ('Hawassa-83') showed a higher number vsiRNA derived contigs in the plant that had gone through heat treatment-meristem tip culture. The initial, non-treated "Hawassa-83" plant was infected with SMFMV, SPVC, SPPV, SPVG and SPSV-1 as determined by NGS. However, none of these viruses where eliminated from the plant by heat-treatment followed by meristem tip culture. Among the viruses found infecting 'Hawassa-83', SPPV belong to the genus *Badnavirus*.

Within the genus *Badnavirus*, *Banana streak virus* (BSV) occurs in two stages (endogenous and episomal). BSV which is present as an integrated sequence in the host's (banana) genome is named endogenous eBSV. On the other hand, when the virus is present as an episomal agent, capable of producing viral particle, it is named BSV. The functional episomal stage of BSV is triggered when the plant goes through some kind of stress, such as meristem tip culture (Gayral et al. 2010). Indeed, it is widely known that meristem tip culture in banana results in an increase of BSV in this host. Therefore, it is tempting to suggest that higher number of vsiRNA-derived contigs found in 'Hawassa-83' after the treatment corresponds to an increase of SPPV caused by the activation of the episomal stage of the virus due to the stress induce by procedure of meristem tip culture. Nevertheless, SPPV was also found in varieties 'Kulfo' and 'Tola' after heat-treatment followed by meristem tip culture. In both of these varieties the number of vsiRNAs derived contig was lower than that of the untreated plants. Thus, further experiments are required to determine if these results are indeed correct or an experimental mistake.

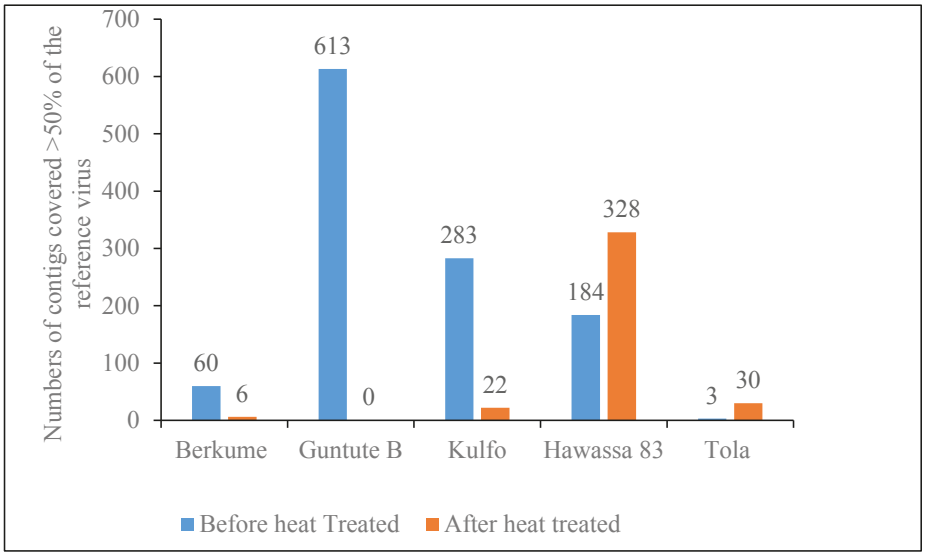

Figure 1. VsiRNAs contigs assembled in plants before and after virus elimination treatments.

Conclusions

In this, study the inclusion of NGS as means to screen viruses in plants before and after virus elimination treatment (heat treatment and meristem culture) helped us verify the efficiency of this method. Our study is the first to use deep sequencing to characterize siRNAs derived from viruses infecting sweet potato from Ethiopia. Using NGS we confirmed the presences of previously reported viruses: SPCSV, SPFMV, SPVC, and SPVG in sweet potato plants from Ethiopia. Moreover, this study reports new, but less important sweet potato viruses: SPPV and SPSMV-1 for the first time. This study demonstrated using the more recent molecular methods (NGS) enables the detection of previously unidentified viruses, without the need of utilizing virus-specific primers or antibodies. Moreover, this study shows that NGS is more sensitive than RT-PCR, although it can also give unpredictable results. Thus, standardizing the method is required before it is used in large scale or in rigorous certification schemes. Although the cost for NGS are still very expensive for routine testing in developing countries (such as Ethiopia), we envision that in the near future NGS will be cheap enough to become the standard testing utilized in certification schemes.

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