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Effectiveness of meristem culture and chemotherapy on the production of virus free sweet potato (*Ipomoea batatas* (L.) Lam.)

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By

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List of abbreviations

AAU	Addis Ababa University
AVRDC	Asian Vegetable Research and Development Center
BAP	6-benzylaminopurine
BC	Before Christ
CIP	International Centre for Potato
CMV	Cucumber Mosaic Virus
CMV	Cowpea Mottle Virus
C/R	Cross Reference
DAC	Direct Antigen Coating
DAC- ELISA	Direct Antigen Coating -Enzyme linked immunosorbent assay
DDW	Double Distilled Water
DIBA	Dot Immunoblotting Assay
DNA	Deoxyribonucleic acid
df	degree of freedom
ELISA	Enzyme linked immunosorbent assay
FAOSTAT	Food and Agriculture Organization Statistics
GA_3	Gibberellic acid
IBA	Indole butyric acid
IITA	International Institute of Tropical Agriculture
ISEM	Immunosorbent Electron Microscope
MS	Murashige and Skoog (1962)
NAA	α-naphthalene acetic acid
NCM-ELISA	Nitrocellulose Membrane-Enzyme Linked Immunosorbent Assay

PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulator
P^H	power of hydrogen
PVX	Potato virus x
RDA	Recommended Daily Allowance
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
SD	Standard Deviation
SPCaLV	Sweet Potato Caulimo-like Virus
SPCFV	Sweet Potato Chlorotic Fleck Virus,
SPCSV	Sweet Potato Chlorotic Stunt Virus
SPFMV	Sweet Potato Feathery Mottle Virus
SPLCV	Sweet Potato Leaf Curl Virus
SPLV	Sweet Potato Latent Virus
SPMMV	Sweet Potato Mild Mottle Virus
SPVD	Sweet Potato Virus Diseases
T	Treatment
WHO	World Health Organization
USDE	United States Department of Energy

Abstract

Sweet potato, (*Ipomoea batatas*), is vegetatively propagated crop. Due to the following cycles of propagation, viruses are accumulated, which seriously affects the sweet potato yield and quality. The objective of this work was to produce virus free sweet potato planting materials through meristem culture and chemotherapy. Stem cuttings of four cultivars were collected from Awasa Agricultural Research Center and planted in a greenhouse at Science faculty AAU. These samples were tested for the presence of sweet potato viruses using NCM-ELISA. From the infected plants, meristems were taken and cultured to make them free from viruses. In addition, shoots from all varieties were taken and treated with ribavirin under different concentrations for one month. NCM-ELISA test was carried out to confirm the success of virus elimination. The *in vitro* propagated plants from meristem culture were found to be virus free. Samples treated with 10 mg/l ribavirin, gave positive results for SPFMV. On the other hand, 20 mg/l and 30 mg/l, treated samples gave negative results for the tested viruses.

In this study, micropropagation system in four sweet potato varieties using meristem culture was established. MS medium with 3% sucrose, 0.7% agar and different concentrations and combinations of plant growth regulators adjusted at a pH of 5.8 was used throughout all experiments. Twelve treatments for initiation of shoots from meristems were done. Significant differences in response to initiation existed among the varieties ($p \leq 0.05$). Among all combinations tested, 1 mg/l BAP, 2 mg/l GA3 and 0.01 mg/l NAA gave maximum shoot initiation percentage (90% for Beletech, 70% for Koka-12 and 56.7% for Ogensegen). Shoot initiation for TIS-8250 was not possible in all of the combinations but callusing was highly evident.

Shoot multiplication was dramatically improved by subculturing of the initiated shoots onto different concentrations of BAP enriched medium. The highest significant mean numbers of shoots per shooted explant (8.0 for Beletech and 6.7 for Koka-12) were obtained in 2 mg/l and 1.5 mg/l BAP containing medium respectively. Subsequently, well formed shoots were rooted on growth regulator free and IBA enriched medium. The maximum rooting percentage (100%) and the highest mean number of roots per shoot (4.2 and 3.7) were obtained from growth regulator free and 0.1 mg/l IBA containing medium for Beletech and Koka-12 respectively. Rooted plantlets were acclimatized and 91.4% of acclimatized shoots of Beletech and 73.0% of shoots of Koka-12 were survived and successfully established in the greenhouse.
Key words/Phrases:-Culture initiation, Multiplication, Acclimatization, Chemotherapy, Virus testing, Sweet potato

1. Introduction

Root and tuber crops are the most important food crops after cereals. They have the highest rate of dry matter production per day and are major calorie contributors (Edison *et al.*, 2006). Tuber crops find an important place in the dietary habits of small and marginal farmers especially in the food security of tribal population (Withers, 1991).

The tuber crops are rich in dietary fiber and carotenoids (carotene and anthocyanin) (Zhang *et al*., 1998). Tuber crops not only enrich the diet of the people but also possess medicinal properties to cure many diseases (WHO, 2003). Among the root and tuber crops, sweet potato has become an important dietary source for many countries including Ethiopia.

Sweet potato is a dicotyledonous, perennial plant (Austin, 1987). Botanically, the underground part is classified as a storage root. It ranks as the seventh important staple crop in the world and the fifth in developing countries after rice, wheat, maize and cassava (Loebenstein *et al*., 2003). It is considered to be the second important root crop after cassava in many tropical countries (FAOSTAT, 2006). Its tubers are mostly used as food while the surplus is used as feed and raw materials for industries (Scott *et al*., 2000).

Because of the enormous genetic diversity of sweet potato (Zhang *et al*., 1998), and the accompanying diversity in phenotypic and morphological traits (Woolfe, 1992), the crop has great potential for further development to accommodate specific uses.

Despite of the advantages that the cultivation of sweet potato offers, production is greatly constrained by pests and diseases particularly viral diseases (Tairo *et al*., 2005; Valverde *et al*., 2007; Karyeija *et al*., 1998). Yield loss in sweet potato caused by viral diseases may reach up to 100% depending on cultivar, infecting virus, stage of infection and whether the crop is infected with a single or multiple viruses (Ngeve and Bouwkamp, 1991).

Several viruses have been reported to infect sweet potato in Africa. The most common ones include the sweet potato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV), sweet potato mild mottle virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), sweet potato latent virus (SPLV), sweet potato caulimo-like virus (SPCaLV), cucumber mosaic virus (CMV), sweet potato virus Y, sweet

potato virus G and sweet potato leaf curl virus (SPLCV) (Hahn, 1979; Geddes, 1990; Wambugu, 1991; Mukasa *et al*., 2003; Ateka *et al*., 2004; Tairo *et al*., 2005).

Since sweet potato is cultivated from vine cuttings grown in the field from previous season, percentage of virus infection increases, as a consequence, total yield is dramatically reduced. The most common of these viral diseases known under the name Sweet Potato Virus Diseases (SPVDs), caused by simultaneous infection with Sweet Potato Feathery Mottle Virus (SPFMV) and Sweet Potato Chlorotic Stunt Virus (SPCSV), (Winter *et al*., 1992; Gibson *et al*., 1998a). SPFMV (family Potyviridae, genus potyvirus), is found most commonly in sweet potatoes in different parts of the world (Loebenstein *et al*., 2003; Tairo *et al*., 2005).

In vitro methods in sweet potato were used earlier at several international centers like International Center for Potato, (CIP), Peru, the International Institute of Tropical Agriculture (IITA), Nigeria and Asian Vegetable Research and Development Center (AVRDC), Taiwan for conservation and virus free production of sweet potato germplasm. Besides, several national programmes in USA, Costa Rica and India have been maintaining a large number of *in vitro* collections of sweet potato over the last 10 years (Karyeija *et al*., 2000; Latham and Wilson, 2008).

Meristem cultures (Hahn, 1979) and thermotherapy (Byamukama *et al*., 2004) along with the use of antiviral drugs (Carey *et al*., 1999) have been found effective in virus elimination in sweet potato. Meristem culture technique coupled with indexing by ELISA and grafting on indicator plants was used to establish healthy cultures (Mandal and Chandel, 1996). *In vitro* propagation technique has been refined so as to develop a single medium capable of eliciting desirable response from all varieties and related species (Carey *et al*., 1999).

MS medium supplemented with sucrose and various plant growth regulators has been successfully used to establish various explant cultures of sweet potato cultivars (Schaefers and Terry, 1976; Ishac *et al*., 2000; Carey *et al*., 1999; Njeru *et al*., 2004; Mukasa *et al*., 2006).

Successful establishment of tissue and meristem culture is governed by several factors. This work mainly emphasizes on investigating the effect of growth regulators of culture media interacted with plant variety

for initiation of shoots from meristem explants, multiplication of shoots, rooting and acclimatization of sweet potato varieties. Investigations were conducted on the utilization of meristem culture technology for elimination of sweet potato viruses and exchange and conservation of germplasm for four genotypes of sweet potato. In addition; chemotherapy technique is applied in these four varieties to eliminate viruses.

2. OBJECTIVE OF THE STUDY

2.1 General objectives

The objective of this thesis has been to produce virus free sweet potato planting materials through meristem culture and chemotherapy.

2.2 Specific objectives

- To initiate shoots from meristem culture
- To establish a protocol for shoot multiplication
- To evaluate the effectiveness of meristem culture and chemotherapy in production of virus free varieties
- To acclimatize the virus free varieties in greenhouse and evaluate their rate of survival.

3. LITERATURE REVIEW

3.1 Sweet potato: description, origin and distribution

Despite its name, sweet potato is not related to potato. Potatoes are members of the *Solanaceae* family, which also includes tomatoes, red peppers, and eggplant, while sweet potatoes belong to the morning glory family (*Convolvulaceae*) (Hahn, 1979). Unlike the potato which is a tuber or thickened stem, the sweet potato is a storage root (Geddes, 1990). The cultivated sweet potato (*Ipomoea batatas*) and the wild species closely related to it belong to the order *Solanales*, family *Convolvulaceae*, genus *Ipomoea*, subgenus *Eriospermum* and species *batatas* (Austin, 1987). In addition to *I. batatas*, there exist 13 wild species closely related to sweet potato (FAOSTAT, 2006).

I. batatas is a hexaploid with 2n = 90 (C/R Wheatley and Song, 2000), while most of the wild species are 2x or 4x, although there are some species like *I. trifida* which has 2x, 3x, 4x and possibly 6x cytotypes (Winter *et al*., 1992). Sweet potato varieties exist in many colors of skin and flesh, ranging from white to deep purple, although white and yellow-orange fleshed ones are the most common (Austin, 1987).

Sweet potato is an ancient crop, originated in the north western part of South America (Karyeija *et al*, 2000). Archaeological evidence from Peru showed that domestication of sweet potato dates back to 6000 BC (Hoyer, 1996). The sweet potato was already widely established in the Americas by the time Europeans first arrived there. From there, it spread to the Old World via diverse routes. The crop was introduced into China in the late 16th century (Hollings, 1976).

Because of its hardy nature and broad adaptability, and because its planting material can be rapidly multiplied from very few roots, the sweet potato spread through Asia and Africa during the 17th and 18th centuries (Gibson *et al*., 1998a).

Sweet potatoes are widely eaten in the island nations of the South Pacific, although how they reached there is open to debate (Woolfe, 1992). Some researchers believe European explorers took them there in the wake of the Spanish conquest of Latin America (Karyeija *et al*., 1998); others favor the idea that long before this, the sweet potato moved from island to island across the Pacific, taken there in boats by indigenous people (Karyeija *et al*., 2000).

Linguistic evidence has also shown three lines of dispersal of the sweet potato from America. The **kumara** line is prehistoric and based on lexical parallels between the Quechua name and Polynesian word kumara. This could explain the transfer of sweet potato by Peruvian or Polynesian voyagers from northern South America to eastern Polynesia around AD 400. The **batata** line dates from the first voyage of Columbus in 1492, which resulted in the introduction of West Indian sweet potatoes to Western Europe. Portuguese explorers transferred sweet potatoes grown in western Mediterranean Europe to Africa, India and the East Indies in the 16th century. The **camote** line represents the direct transfer of Mexican sweet potatoes by Spanish trading galleons between Acapulco and Manila, Philippines, in the 16th century (Yen, 1982).

The primary center of diversity of sweet potatoes is located in northwestern South America (Colombia, Ecuador and Peru) and parts of Central America (such as Guatemala) where a great diversity of native sweet potatoes, weeds, and wild *Ipomoea* exists. Secondary centers of sweet potato diversity outside of the Americas are in China, Southeast Asia, New Guinea and East Africa (Austin 1983, 1987; Yen 1982).

Over 95 percent of the global sweet potato crop is produced in developing countries, where it is the fifth most important food crop in terms of fresh weight (Hahn, 1979). Asia is the world's largest sweet potato producing region, with 114 million tons of annual production. China supplies about 80 percent of the world's production, making it the leading supplier of sweet potatoes in the world (FAOSTAT, 2006).

Nearly half of the sweet potatoes produced in Asia are used for animal feed, with the remainder primarily used for human consumption, either as fresh or processed products (Loebenstein *et al*., 2003). In contrast, African farmers produce only about 12 million tons of sweet potatoes annually but most of the crop is cultivated for human consumption (FAOSTAT, 2000). The crop is particularly important in East Africa (Uganda has the largest production in Africa; Tanzania has the second largest area of production but only the seventh largest production due to poor yields) (Cohen and Loebenstein, 1991).

Ethiopia is the second most populous country in Africa. Sweet potato is one of the most important crops for at least 20 million Ethiopians. The total area under sweet potato in Ethiopia is 75,000 ha with an average productivity of 8 t/ha. White fleshed sweet potato is a staple food for 13 million people in the Southern Regional State (Assefa *et al*., 2007).

3.2 Growing conditions

Sweet potatoes grow well in many farming conditions. Because they are sown by vine cuttings rather than seeds sweet potatoes are relatively easiest to plant. In addition, the crop is highly tolerant of weeds, allowing farmers to devote time to other crops (Mukasa *et al*., 2006).

The plant does not tolerate frost. It grows best at an average temperature of 24 °C, abundant sunshine and warm nights (Winter *et al*., 1992). Annual rainfalls of 750-1000 mm are considered most suitable, with a minimum of 500 mm in the growing season. The crop is sensitive to drought at the tuber initiation stage 50–60 days after planting and is not tolerant to water-logging, as it may cause tuber rots and reduce growth of storage roots if aeration is poor (Chen *et al*., 2006.).

Sweet potatoes are grown on a variety of soils, but well-drained light and medium textured soils with a pH range of 4.5-7.0 are more favorable for the plant (Woolfe, 1992). They can be grown in poor soils with little fertilizer. However, sweet potatoes are very sensitive to aluminium toxicity and will die about 6 weeks after planting if lime is not applied at planting in this type of soil (Woolfe, 1992).

Fig. 3.1 Sweet potatoes in a field (en.wikipedia.org)

Depending on the cultivar and conditions, tuberous roots mature and can be harvested in 3 to 6 months and are well adapted to warm tropical lowlands (Njeru *et al*., 2004). With care, early maturing cultivars can be grown as an annual summer crop in temperate areas.

Fig. 3.2 Freshly dug sweet potatoes (en.wikipedia.org)

Sweet potatoes rarely flower when the daylight is longer than 11 hours, as is normal outside of the tropics in summer. They are mostly propagated by stem or root cuttings or by adventitious roots called "slips" that grow out from the tuberous roots during storage (Alicai *et al*., 1999).

Fig. 3.3 Sweet potato flower (en.wikipedia.org)

Under optimal conditions of 85 to 90 % relative humidity, at 13 to 16 °C, sweet potatoes can be kept for six months. Colder temperatures injure the roots (Aritua *et al*., 1998a)

3.3 Significance and uses of sweet potato

The versatile sweet potato is ideal for the poor. With the ever-growing interest in health and natural foods, the sweet potato is quickly finding its place in the family weekly diet the year around (Agueguia *et al*., 1994). The sweet potato blends with herbs, spices and flavorings producing delicious dishes of all types. From processed baby foods to the main dishes, salads, breads and desserts, sweet potatoes add valuable, appetizing nutrients and color to any meal (Nutrition Institute, 1995).

In Africa, sweet potato is grown particularly by poorly educated, resource poor farmers, especially women, mostly for family consumption but also for cash (Bashaasha *et al*., 1995). The crop is an important means of poverty alleviation. The crop can grow in relatively marginal soils and its ability to yield quickly with

the onset of rains, even intermittent ones, is invaluable in times of food insecurity (Byamukama *et al*., 2004). It is important in disaster relief in areas of East Africa particularly, Tanzania, Kenya, Uganda and Ethiopia.

As a main dish or prepared as a dessert, the sweet potato is a nutritious and economical food. This nutritious vegetable provides 42% of the Recommended Daily Allowance (RDA) for vitamin C, 6 percent of the RDA for calcium, 10% of the RDA for iron, and 8% of the RDA for thiamine for healthy adults (FAOSTAT, 2006). It is low in sodium and is a good source of fiber and other important vitamins and minerals. It is a complex carbohydrate food source that provides β-carotene which may be a factor in reducing the risk of certain cancers (WHO, 2003; www.nlm.nih.gov).

Sweet potatoes are used for human consumption, as livestock feed, and are in industrial processes to make alcohol and starch, and products such as noodles, candy, desserts, and flour. The green leaves of the plant may also be consumed by humans and animals (FAOSTAT, 2000).

With the growing population pressure on agricultural lands and available foods, escalating cost of food items directly affect the low income population strata which are already deficient in calories. In this context, the sweet potato and other root crops are good sources of energy; the only potential supplementary food crops as they can provide more energy per unit area basis than any other field crop (Wheatley, 2000) and are cheap sources of energy (Austin, 1987). However, in order to fill the anticipated energy gap, production has to be increased. Thus, expansion of cultivation and crop improvement are two important and concurrent prerequisite processes to increase crop production.

Table 3.1 Constituents of sweet potato: values per 100 g edible portion (WHO, 2003).

	Units	Raw sweet potato	Cooked, baked in skin	Cooked, boiled without skin
Water	g	72.84	72.84	72.84
Energy	kcal	105	103	105
Protein	g	1.65	1.72	1.65
Total lipid (fat)	g	0.30	0.11	0.30
Carbohydrate	g	24.28	24.27	24.28
Fiber, total dietary	g	3.0	3.0	1.8
Ash	g	0.95	1.06	0.95
Calcium, Ca	mg	22	28	21
Iron, Fe	mg	0.59	0.45	0.56
Magnesium, Mg	mg	10	20	10
Phosphorous, P	mg	28	55	27
Potassium, K	mg	204	348	184
Sodium, Na	mg	13	10	13
Zinc, Zn	mg	0.28	0.29	0.27
Copper, Cu	mg	0.169	0.208	0.161
Manganese, Mn	mg	0.355	0.560	0.337
Selenium, Se	mg	0.6	0.7	0.7
Vitamin C	mg	22.7	24.6	17.1
Thiamin B_1	mg	0.066	0.073	0.053
Riboflavin B_2	mg	0.147	0.127	0.14
Niacin B_3	mg	0.674	0.604	0.64
Pantothenic acid B_5	mg	0.591	0.646	0.532
Vitamin B_6	mg	0.257	0.241	0.244
Foliate, total	mg	14	23	11
Vitamin B_{12}	mg	0	0	0
Vitamin A	IU	20,063	21,822	17,054
Vitamin A	mg	2,006	2,182	1,705
Vitamin E	mg	0.280	0.280	0.280

3.4 Major problems associated with sweet potato production

Insects

Even when the best available control measures are used, few sweet potato crops entirely escape insect damage (Aritua *et al*., 1998). This damage decreases the quality of sweet potatoes by marring appearance, providing entry points for decay organisms, causing waste when cooked, and sometimes causing objectionable tastes. Roots containing insects or their excrement are usually unfit for human consumption.

According to (Carey *et al*., 1998), at least 18 species of insects feed on sweet potato roots. Among those causing the greatest damage are the sweet potato weevils. These are the most destructive insect pests of sweet potatoes. The adult weevil feeds on all parts of the sweet potato plant but prefers the roots. Feeding scars on tubers consist of tiny shallow holes that usually occur in patches (Chavri *et al.*, 1997). The eggs, laid in vines and roots, hatch in about one week (Clerk, 1960). The legless white grubs with brown heads begin to tunnel through potatoes, causing the potatoes to be too bitter for human consumption (Chavri *et al.*, 1997; Clerk, 1960; Cohen *et al*., 1992). Other insects, such as beetles and caterpillars that chew on the leaves or stems and leafhoppers that withdraw juice from plants also cause damage to sweet potatoes (Cohen *et al*., 1992).

Fungal and Bacterial Diseases

Sweet potatoes are subject to a number of diseases that cause heavy losses in the field and in storage. The most common fungal diseases are scurf, black rot, wilt (stem rot), soft rot and bed blight (Geddes, 1990).

Scurf produces a grayish-brown to black surface discoloration on the roots. These discolored areas can occur in all shapes and sizes with no definite outline. They can be uniform over the surface but are usually worse on the stem end (Gibson *et al*., 1998a). Generally, the sweet potato skin is not broken and the brown discoloration is only skin deep, so it can easily be scraped from the surface (Gibson *et al*., 1998b).

Black rot causes a black lesion to develop on the base of young potato stems before the plants are removed from the bed. The foliage of affected transplants appears yellow and stunted (Gibson *et al*., 1997; Gibson *et al*., 1998a; Gichuki *et al*., 1998). A dark to nearly black spot appears on the potato in the field or in storage. These spots begin as small, round areas, but they enlarge until they cover the entire potato (Gibson *et al*.,

1998). The surface of the diseased spots has a metallic luster, and the tissue just beneath the surface is greenish (Gichuki *et al*., 1998).

Wilt infected vines have a slight off-color of a yellowish tinge, followed by puckering of the foliage and wilting of the plant. The older leaves drop off first, leaving yellow leaves near the vine tip (Hahn, 1979; Hahn *et al*., 1981). On affected young vines, many short stems grow at the center of the hill, giving the vine a rosette appearance. If the skin is peeled away from the diseased stem, the exposed tissue shows a dark discoloration. The discolored tissue extends down the stem into the roots (Hollings *et al*., 1976). Wilt does not cause the stem to rot unless saprophytic organisms enter the diseased vines (Hahn *et al*., 1981).

Bed blight is caused by a soil-borne fungus that attacks the stems of young plants at the soil line, causing a white mold to develop. Infected plants turn yellow and die, creating dead areas in the plant bed (Hahn *et al*., 1981). Soft rot is produced by the same fungus that causes bread mold. It enters the sweet potato through breaks in the skin caused by careless handling. Decay usually begins at one end of the potato and progresses rapidly to the other end (Joubert *et al*., 1979).

Bacterial soft rot can occur in the seed bed, field, or even after harvest (Karyeija *et al*., 1998). Infected plants usually have long black stem lesions, although potatoes on diseased vines can show various degrees of soft rot development. Good sanitation practice is the best method of preventing bacterial soft rot (Karyeija *et al*., 1998; Karyeija *et al*., 2002).

Internal cork forms dark brown to nearly black areas that become hard and gritty when the potato is cooked. Cork symptoms, which are sometimes difficult to see at harvest, are much more visible after two or three months in storage (Kennedy and Moyer, 1982).

Viral diseases

The most harmful diseases in sweet potatoes (*Ipomoea batatas*, family Convolvulaceae) are caused by viruses (Kozai *et al*., 1996; the spread of which is enhanced by the cultivation practices, insects and lack of resistant cultivars (Winter *et al*., 1992). Sweet potato chlorotic stunt crinivirus (SPCSV) breaks down resistance to other viruses in sweet potato cultivars, which results in the severe sweet potato virus disease (SPVD) and high or complete yield loss (Sheffield, 1953).

Sweet potato chlorotic stunt virus (SPCSV, genus *Crinivirus*, family *Closteroviridae*) (Karyeija *et al*., 1998), also known as sweet potato sunken vein virus (Cohen *et al*., 1992) and sweet potato virus disease associated with closterovirus (Winter *et al*., 1992), causes severe symptoms generally referred to as sweet potato virus disease (SPVD) in mixed infection with sweet potato feathery mottle virus (SPFMV) (Gibson *et al*., 1998; Karyeija *et al*., 1998; Karyeija *et al*., 2000). SPVD is the main disease of sweet potato (Geddes, 1990), characterized by small, distorted leaves which are often narrow (strap-like) and crinkled with chlorotic mosaic or vein clearing and stunting (Gibson *et al*., 1998; Karyeija *et al*., 1998; Karyeija *et al*., 2000). The disease has been reported to cause up to 80% yield reduction (Hahn, 1979).

Sweet potato latent virus (SPLV) is more similar morphologically to typical potyviruses than either SPFMV or sweet potato mild mottle virus (SPMMV) (Luo *et al*., 2006) but neither aphids nor whiteflies have been shown to transmit the virus (Liu *et al*., 2001). SPLV has been reported in Taiwan and China and has a wider host range than SPFMV (Karyeija *et al*., 1998). Generally, more than 14 virus diseases of sweet potato have been reported throughout the world (Sheffield, 1953; 1957).

Sweet potato virus disease (SPVD), the most important disease of sweet potato in sub-Saharan Africa (Geddes, 1990), was first reported in 1939 from Kilo Moto Mines in Ituri, in the eastern part of Democratic Republic of Congo. A crop which initially had amounted to 30,000 kg per annum was reduced to 4,000 kg and then abandoned (Sheffield, 1953). SPVD has since been reported from Rwanda, Burundi, Uganda, Kenya, Tanzania, Malawi, South Africa (Sheffield, 1953; 1957), Ghana (Clerk, 1960), Nigeria (Schaefers & Terry, 1976), Cameroon (Ngeve & Boukamp, 1991), Madagascar, Zambia, Zimbabwe (Karyeija *et al*.,1998) Togo, Liberia, Sierra Leone, Sao Tome, Ivory Coast (C/R Thottappilly and Rossel, 1988), Benin, Gabon (Lenne, 1991).

SPVD was shown by Sheffield (1957) to be caused by dual infection with both an aphid-borne virus and a whitefly-borne virus, now known respectively to be sweet potato feathery mottle virus (SPFMV; family *Potyviridae*, genus *Potyvirus*) and sweet potato chlorotic stunt virus (SPCSV; family *Closteroviridae*, genus *Crinivirus*) (Schaefers and Terry, 1976). SPCSV, also called sweet potato sunken vein virus (Cohen *et al.*, 1992) and SPVD-associated closterovirus (Winter *et al.*, 1992), is transmitted by the whitefly *Bemisia tabaci* in the semi-persistent manner (Schaefers & Terry, 1976; Cohen *et al.*, 1992). SPFMV is transmitted by various aphid species in the non-persistent manner (Sheffield, 1957; Stubbs & McLean,

1958; Kennedy & Moyer, 1982). Both SPFMV and SPCSV occur outside Africa and their geographical origin is obscure. Despite this, SPVD has been associated particularly with Africa.

Symptoms, incidence and effects on yield

Symptoms of SPVD differ with sweet potato genotype but are generally severe. Affected plants are very stunted with small distorted leaves which are often narrow (strap-like) and crinkled with a chlorotic mosaic and/or vein-clearing (Lenne, 1991).

Fig. 3.4 Purpling of leaves in plants infected with SPCSV (a), vein clearing in leaves of plants infected with SPFMV (b), chlorotic, small deformed leaves in plants infected with SPFMV and SPCSV (c) and chlorotic spots on leaves of plants infected with SPFMV and SPMMV (d).

The direct yield effects of the disease may be underestimated if, as is likely, the controls were not wholly virus free when planted or became infected during the trial (Mukiibi, 1977). On the other hand, yield measurements for single plants or plots solely planted with SPVD affected cuttings may overestimate crop losses (Michelmore, 1995). In farmers' crops, plants generally become diseased during growth (rather than from the outset) and diseased plants grow mixed with unaffected ones, the sprawling, indeterminate growth of most sweet potato varieties providing opportunity for unaffected plants to benefit from reduced competition by diseased plants (Joubert *et al*., 1979).

However, the greatest (but largely unappreciated) economic effect of SPVD is probably that it prevents farmers growing susceptible but high-yielding varieties. Amongst six local Ugandan sweet potato varieties, the more susceptible ones yielded over twice as much as the more resistant ones (Aritua *et al*., 1998a), consistent with most farmers' opinion that local resistant landraces are poor and late yielding. Furthermore, some exotic clones of sweet potato yield much more than even high-yielding local landraces (Gichuki *et al*., 1998) but cannot be grown widely because they are too susceptible to SPVD

Epidemiology

Both SPFMV and SPCSV can be perpetuated by normal vegetative propagation. However, farmers avoid taking cuttings from plants with the severe SPVD: plants infected with SPCSV alone also have discernible though mild symptoms. Plants with SPFMV are generally symptomless but, despite this, planting material is often not infected, probably because SPFMV is only partially systemic in many African varieties of sweet potato (Gibson *et al*., 1997). SPFMV is also at a low titre in sweet potato when infecting alone, only SPVD-affected plants having a high titre of SPFMV and providing an effective source from which aphids can acquire SPFMV (Aritua *et al.*, 1998b).

Aphids are seldom direct pests of sweet potato, and it seems likely that SPFMV is spread mostly by non-colonizing alate aphids making brief probes into plants during host seeking (Wambugu, 1991). In contrast, whiteflies may be seasonally very abundant on sweet potato, and the incidence of SPVD is closely related to their numbers (Aritua *et al*., 1998b). Both SPFMV and SPCSV can infect wild *Ipomoea* spp. (Karyeija *et al.*, 1999) but the sweet potato crop seems now to be the main host of the viruses in Africa.

Control

African farmers largely control SPVD by phytosanitation and the use of resistant varieties. Most farmers select cuttings for planting from symptomless plants (Bashaasha *et al.*, 1995; Kapinga *et al.*, 1995) and some may also rogue out diseased plants when the crop is young. Despite this, farmers seldom seem to remove infected material from mature crops, destroy the infected haulms of harvested crops, or purposely isolate new plantings from affected crops (Agueguia *et al*., 1994). Consequently, improved phytosanitation offers unknown but potentially considerable benefits for SPVD control. Landraces of sweet potato vary in resistance to SPVD but African farmers often have to strike a balance between a good yield and some resistance (Aritua *et al.*, 1998a).

Scientists have attempted to control sweet potato viruses by the use of virus-free planting material and by breeding resistant varieties. The first approach has been used primarily in southern Africa (Joubert *et al*., 1979) whereas resistance has been used in West (Hahn *et al*., 1981) and East (Mwanga *et al*., 1995) Africa. Resistance is to the viruses; sweet potato varieties differing in susceptibility to SPVD have similarly-sized whitefly populations (Aritua *et al.*, 1998b). SPCSV synergises SPFMV to produce SPVD (Karyeija *et al.*, 2000) so resistance to SPVD seems to depend on resistance to SPCSV. SPCSV can be divided into two

serological strains, one of which occurs in West Africa and the other in East and southern Africa (Hoyer *et al.,* 1996: Gibson *et al.,* 1998b). Smaller differences have also been identified within the latter strain by both nucleotide sequencing and monoclonal antibodies (Alicai *et al.,* 1999).

Consistent with the differences in SPCSV strains in Africa, genotypes resistant to SPVD in Nigeria (Hahn *et al.,* 1981) are generally susceptible to SPVD when grown in East and southern Africa. The several small differences identified between isolates of SPCSV in East Africa are also consistent with plant breeders identifying only polygenic sources of resistance to SPVD (Aldrich, 1963). Nucleotide sequencing has revealed little variability in SPFMV in East Africa: more is present in West Africa (Kreuze *et al.,* 2000) whilst SPFMV in Zimbabwe appears to be quite unusual (Chavri *et al.,* 1997).

3.5 Methods of Virus Elimination from sweet potato

3.5.1 Meristem Culture

The yields of tissue cultured crops has been found to be higher in vegetatively propagated crops like sweet Potato (1.8 tons per hectare) than traditional varieties (0.5 tons per hectare) (Mutandwa, 2008; Sihachakr *et al*., 1997). No effective chemicals are available for controlling viruses from infected plants. Tissue culture is one of the biotechnology tools that are applied to crop plants in the production of disease free germplasm. Different methods are applied to obtain initial virus free plant materials. Most commonly, meristem culture is used to eliminate viruses. Meristem culture alone is preferred in that it reduces the time and the space required for thermotherapy (Dunbar, 1993; Valverde *et al*., 1998). However, some times, meristem culture fails to result in virus free plants, even combined with chemotherapy or thermotherapy as anti-viral treatments. For this reason, plants derived from meristems should be detected for pathogenic viruses before field releasing for cultivation (Seal and Coater, 1998).

The basis of virus elimination is that the terminal region of the shoot meristem, above the zone of vascular differentiation is unlikely to contain pathogenic particles. This is because virus distribution is uneven in a plant and viruses cannot travel quickly enough through plasmodesmata to keep up with actively growing tip. It is also assumed that auxin production at the meristematic tissue inhibits viruses and virus replication at meristem (International Potato Center, 1999).

If a sufficiently small explant can be taken from an infected donor plant and cultured *in vitro,* then there is a real possibility of the derived culture being pathogen free (Dunbar, 1993). Such cultures, once screened and certified, can form the basis of a guaranteed disease free stock for further propagation.

Whatever variants of technique are employed for virus eradication, the key to success is undoubtedly the size of the meristem. The smallest explants are those that typically, will be the least successful during *in vitro* culture, but will produce the highest proportion of virus free material (Valverde *et al*., 1998).

The essence of meristem culture is the excision of the organized apex (0.2-1 mm) of the shoot from a selected donor plant for subsequent *in vitro* culture. The conditions of culture are regulated to allow only for organized outgrowth of the apex directly into a shoot, (Murashige, 1978).

The excised meristem is typically small (often less than 1 mm in length) and is removed by sterile dissection under the dissecting microscope (Kapinga *et al*., 1995). The explant comprises the apical dome and a limited number of the youngest leaf primordia, and excludes any differentiated provascular or vascular tissues. A major advantage of working with such a small explant is the potential that it holds for excluding pathogenic organisms that may have been present in the donor plant particularly plant viruses (Mutandwa, 2008).

3.5.2 Chemotherapy

Viral chemotherapy is centered on purine and pyrimidine analogs, with the presumption that the synthesis of the nucleic acid of the virus could be inhibited by such molecules (Mukiibi, 1977). These chemicals are filter sterilized (by millipore filter of 0.22μm) and supplemented into meristem culture media or autoclaved with the medium depending on the nature of the chemical (Zaitlin and Palukaitis, 2000).

The chemicals act as competitive inhibitors, and could interfere either with the incorporation of nucleotides into nucleic acids or with the formation of the bases from their precursors. Unfortunately, although some of the compounds have shown some viral retarding properties, they also affect the nucleic acid metabolism of the host and often injure the host plant (Mangal *et al*., 2004).

Chemotherapy involves the use of chemicals like antibiotics, plant growth regulators, amino acids, purine and pyrimidine analogues to inactivate viruses or inhibit replication or movement of viruses in tissues (Seal and Coater, 1998). These chemicals can either be sprayed on growing plants prior to excision of meristems or incorporated into tissue culture media. As early as in 1954, eradication of SPVX from sweet potato tissue cultures by malachite green and thiouracil treatments was reported. Of all the chemicals tested for plant virus elimination, synthetic nucleotide analogues like ribavirin (1-D-ribofuranosyl-1, 2, 4-triazole-3-3carboxamide) and DHT (5-dihydroazauracil) have been particularly effective in inhibiting different plant viruses.

In vitro chemotherapy of meristematic explants with antiviral chemical ribavirin has been found to be most promising for elimination of major sweet potato viruses. Though the exact mode of action of ribavirin on plant viruses is not understood, following possibilities have been suggested:

- Ribavirin triphosphate, a major derivative of ribavirin, inhibits viral RNA polymerase synthesis.
- Ribavirin-5-phosphate, a derivative of ribavirin, inhibits IMP-dehydrogenase, and thereby decreases the GTP pool and nucleic acid synthesis.
- Ribavirin interferes with capping at the 5' end of viral mRNA leading to inefficient translation.

Other antiviral chemicals such as 8-azaguanine, 5-fluorouracil, 2-thiouracil and Para-fluorophenylalanine have also been tested for virus elimination in sweet potato. The concentrations of many antiviral chemicals required during chemotherapy to inhibit virus multiplication are very close to the toxic concentration for the host plant. In addition, there is always a possibility of mutations when the plants are exposed to antiviral chemical. Therefore *in vitro* ribavirin therapy at low concentrations combined with thermo therapy has been used to eradicate viruses from infected potato cultivars. In such cases simply culturing the shoot cuttings can eliminate some viruses like SPVY (Mangal *et al*., 2004).

Chemotherapy is applied during nodal or shoot culture. The nodal segments or shoot tips from virus infected plants, after proper sterilization, are inoculated on to medium along with various concentrations of antiviral chemicals (Sharma *et al.*, 2007). The efficiency of antiviral chemicals in the elimination of plant viruses depend on concentrations, type of host plant genetic material, and type of virus (Paunovic *et al*., 2007).

3.5.3 Thermotherapy

Thermotherapy (heat treatment) at 35 ± 2 ºC for 4 weeks or to 40 ± 2 ºC for 3 weeks has found to free meristems or shoots of axillary buds cultured from carnation plants (Mangal *et al*., 2004). Incubating explants at 37 ± 2ºC, with a 16 hour photoperiod, and light intensity of 110 μmol m^{-2} s^{-1} for 30 days has found to free node culture of potato (Nascimento *et al*., 2003). As reported by International Potato Center (1999), most commonly *in vitro* potato plantlets (one month older) are kept at temperatures of 36 ºC for 8 hours and 30 ºC for 16 hours daily for 30 days under a high intensity continued light are found to be optimum thermotherapy conditions (Ngeve and Boukamp, 1991).

Growing host plants at higher temperatures significantly reduces replication of many plant viruses by disrupting viral ssRNA and dsRNA synthesis. Higher temperatures (35-37°C) cause disruption in the production and/or activity of virus-encoded movement proteins (MPs) and coat proteins (CPs). MPs are involved in cell-to-cell movement of viruses through plasmodesmata and plant vascular system, while CPs play a role in the reconstitution of virus particles from replicated viral nucleic acids. Therefore, thermotherapy of infected plants improves virus freedom even from relatively large size explants. Reduction in virus titer is higher, if the infected plants are exposed to elevated temperature for longer periods. Current virus elimination programmes involve either growing of whole plants or *in vitro* cultures at temperatures close to the threshold of normal plant growth. The exact temperature and length of treatment vary with the virus and the heat tolerance of the host plant.

3.5.4 Meristem Culture with Thermotherapy and/or Chemotherapy

Meristem culture is very efficient in eliminating viruses when used with high temperature (thermotherapy) and/or chemicals that inhibit virus replication (chemotherapy) (Kassanis, 1975). Meristem culture combined with thermotherapy is widely used for virus elimination in sweet potato. The source plants infected with viruses are incubated in a growth chamber under light intensity of 30-50 μmol m^{-2} s^{-1} at 35-37°C for 2-6 weeks. After respective periods of thermotherapy the meristems are excised and cultured on nutrient medium.

Cold therapy followed by apical meristem culture has also been shown to successfully eliminate several viruses from infected plants. Viroids, some of which are quite resistant to elevated temperatures, have been effectively eliminated by cold therapy. Low temperature therapy (4-7°C) followed by meristem excision and initiation has been used to eliminate virorids from infected sweet potato plants.

3.5.5 Electrotherapy

Electrotherapy of explants of infected potato plants has recently been reported to be an effective means for virus elimination. Potato stems infected with PVX were exposed to 5, 10 or 15 mA for 5-10 minutes followed by immediate culturing of the shoot tips *in vitro*. The highest efficiency was obtained at 15 mA for 5 min, and about 60-100% of the regenerated plantlets tested negative against PVX. Electrotherapy technique is yet to be tested against other potato viruses (Latham and Wilson, 2008).

3.6 Detection (Indexing) of plant Viruses

Detection deals with establishing the presence of a particular target organism within a sample. Progress in molecular biology, biochemistry and immunology has led to the development of many new, accurate, rapid and less labor intensive methods of virus detection. The advent of antibody based detection, in particular monoclonal antibodies and enzyme-linked immunosorbent assay (ELISA), were the two major breaks through for the molecular detection of plant viruses (Falk and Purcifull, 1983).

Precipitation or agglutination tests, ELISA, Immunosorbent Electron Microscopy (ISEM), fluorescent antibody test and dot immuno blotting assay (DIBA) are protein based assays (Ngeve and Boukamp, 1991). Where as, dot blot hybridization, polymerase chain reaction (PCR) and nucleic acid hybridization with radio labeled and non radio labeled DNA/RNA probes are viral nucleic acid based techniques (Yongwei *et al*., 2002).

ELISA is used to detect coat proteins and viral genome is detected by PCR. Virus genome hybridization, PCR (using total DNA extract), fixing of infected and non infected mesophyll tissues for electron microscopy studies and grafting to an indicator plant are commonly used to detect viruses as indicated by Val Verde *et al*. (1998). Though not reliable, electro microscopic searching for viral particles and use of indicator plant are routinely used. Gene probes are most sensitive and accurate methods for detecting specific viruses in tissue culture operations (Yongwei *et al*., 2002).

Serological methods based on the protein components of viruses have been commonly used in plant virus detection (Matthews, 1991). Recently a real time quantitative PCR assay was developed for the detection and quantification of plant viruses. The Reverse Transcriptase PCR (RT-PCR) method has been found to

be more sensitive than direct antigen coating ELISA (DAC- ELISA) for instance in detecting cowpea mottle virus (CMV) (Ateka *et al*., 2004).

The RT-PCR method gives no false positive reaction as is sometimes seen with ELISA. Until now ELISA and its modified forms have been extensively used, because these are quick. However PCR has been widely used with the varying degree of modification for detection of viral genomes in infected plant (Akinjogunla and Taiwo, 2008).

ELISA lacks the sensitivity required for detection of the viruses, which occur in low concentrations in plant tissues. The detection is carried out by measuring the absorbent value to visualize a particular color (Falk and Purcifull, 1983). RT-PCR and nucleic acid hybridization are more sensitive than ELISA as they could detect in some of the ELISA negative plantlets (Sharma *et al*., 2007).

Even after taking all precautions to excise small meristem tips and subjecting them to various treatments favoring virus elimination, ultimately very few virus free mericlones are obtained. Therefore, meristem-derived plants must be tested for virus freedom before using them as mother plants in micro propagation. Accurate, sensitive and rapid detection of potato viruses is critical for identifying virus free mother plants and their integration into seed production programme.

3.7 Tissue culture for micropropagation of plants

Plant tissue culture is the science (or art) of growing plant cells, tissues or organs isolated from the mother plant, on artificial media (George, 1993). It includes techniques and methods appropriate to research into many botanical disciplines and has several practical objectives.

Many plants can now be cultured *in vitro*. These cultures have commercial uses such as production of virus free plants, *in vitro* propagation and sterile culture techniques for producing different seed types (Kumar, 2002).

Several workers cultured stem tips, roots, leaves, floral parts, immature fruits, meristems and embryos of some plants. Plant cultures are commenced by placing one or more explants in to a pre-sterilized container of sterile nutrient medium. Some explants may fail to grow, or may die, due to microbial contamination

(Chawla, 2002). To ensure the survival of an adequate number, it is important to initiate several cultures at the same time, each being started from an identical organ or piece of tissue (C/R Wheatley and Bofu, 2000).

Developmental stages of micropropagation can be classified into culture initiation, multiplication, rooting and acclimatization. Culture initiation needs the selection of the explant and the time at which the explant is taken according to the objective of the micropropagation. Explants taken from stock plants at different times of the year, may not give reproducible results in tissue culture. This may be due to variation in the level of external contaminants or because of several changes in endogenous (internal) growth substance levels in the stock plant (George, 1993).

Plant material will only grow *in vitro* when provided with specialized medium. The most widely used medium is MS medium which is developed by Murashige and Skoog (1962) that gives best result. A medium usually consists of a solution of salts supplying the major and minor elements necessary for the growth of whole plants, together with various vitamins, amino acids carbon and energy source usually sucrose (Kumar, 2002). Growth and development of plant cultures depend on media composition, growth regulators combination and concentration, genotype and type and age of the explant (Gonzalez *et al*., 1999).

4. MATERIALS AND METHODS

4.1 Plant material

Sweet potato vines of four varieties namely Beletech, Koka-12, Ogensegen and TIS-8250 were obtained from the sweet potato research farm at the Awassa Agricultural Research Center, Hawassa, 275 Km south of Addis Ababa. To protect the loss of water and drying during travel, the samples were packed with plastic bags. The collected sweet potato vines were planted in the labeled pots, four vines per pot and eight pots per variety, in the greenhouse and watered regularly.

Plant materials required for the whole meristem culture and shoot culture experiments were obtained from greenhouse grown plants at the Science Faculty of AAU. Sweet potato meristems were excised every 10 - 15 days from four varieties, given in table 4.1.

Table 4.1 Variety name, nature and maturity of the plant materials used.

No.	Name of the variety	Nature	Maturity
1	Beletech	White fleshed	Medium
2	Koka-12	White fleshed	Medium
3	Ogensegen	Orange fleshed	Medium
4	TIS-8250	Orange fleshed	Medium

Planting containers used were plastic pots purchased in a local market and drilled to have holes at the bottom for drainage using a driller. Red soil was filled into each of the pots and watered adequately. Prepared pots were kept in a greenhouse at the Science Faculty of Addis Ababa University (AAU). A total of 32 pots were prepared and used for the whole stock plant retaining up to the end of the research. After 15 days of planting, samples were established and used as a stock plant for the subsequent laboratory work.

4.2 Media preparation

Preparation of MS stock solution

The nutrient media which were used contained inorganic salts and organic compounds according to Murashige and Skoog (1962). Stock solutions for macronutrients, micronutrients and vitamins were

prepared and stored for subsequent culture media preparations. Each compound was measured using either electronic or sensitive balance as given in table 4.2.

For each case, measured constituents were mixed in 1 liter volumetric flask with double distilled water (DDW). The mixture was continuously stirred using magnetic stirrer until all the mixtures were thoroughly dissolved and mixed. The solution is poured into 100 ml plastic bottles (macro), 50 ml plastic bottles (micro) and 10 ml test tubes (vitamin) and kept at -20 ^{o}C. The method of preparation of MS macro, micro and vitamin is the same. In most media preparation cases, macronutrients were measured as gram per liter and micronutrients and vitamins were measured as milligram per 500 ml.

Table 4.2 Macronutrients, micronutrients and vitamins in MS stock solution (Murashige and Skoog, 1962)

Micronutrients mg/500ml		Macronutrients gm/l		Vitamins mg/500ml	
Fe-Na-EDTA	2000	KNO_3	19	Myoinositol	5000
$ZnSO_4.7H_2O$	430	NH_4NO_3	16.5	Glycine	100
H_3BO_3	310	$CaCl_2.2H_2O$	4.4	Nicotinic acid (NaOH)	25
$MnSO_4.4H_2O$	1115	$MgSO_4$	1.8	Pyridoxine (B6)	25
$CuSO_4.5H_2O$	1.25	KH_2PO_4	1.7	Thiamin (B1)	5
KI	41.5				
$Na_2MoO_4.2H_2O$	12.5				
$CoCl_2.6H_2O$	1.25				

Preparation of Plant Growth Regulators (PGRs)

Most commonly, PGRs were prepared as 1 mg/ml. The required PGR (100 mg) was measured using sensitive balance and dissolved by 1N NaOH and thoroughly mixed in 100 ml of DDW with the aid of magnetic stirrer. It was labeled and stored at +4 ^{o}C for subsequent uses until one month. All of the PGRs used were prepared in the same way.

Media preparation for shoot initiation from meristem

In 1liter volumetric flask that contained DDW, 30 g sucrose was dissolved using magnetic stirrer. From the stock solutions prepared, 100 ml of MS macro, 10 ml of micro and 10 ml of vitamin were measured using

measuring cylinder and added to it while stirring. The plant growth regulators, auxin (Naphthalene Acetic Acid/NAA), cytokinin (6-Benzyl Amino Purine/BAP) and gibberellin (Gibberellic acid/GA3) were also added to the medium in different concentrations and combinations (Table 5.1).

After the constituents dissolved and fully mixed, it was transferred to one liter beaker and its pH was adjusted to 5.8 with either 1 N HCl or 1 M NaOH. It was then transferred to 1liter bottle and 7 g agar was added and autoclaved at 121 ^{o}C for 15 minutes. The medium was then poured into autoclaved Petri dishes on air flow cabinet bench. After it is solidified, it is either used immediately or kept at +4 ^{o}C for latter use.

Media preparation for shoot multiplication and rooting

Shoot multiplication and rooting media preparation was the same as stated above. The only difference was, the agar was added in the beaker while heating and stirring after the pH was adjusted at 5.8. When the agar was fully dissolved and mixed, 60 ml of the medium was dispensed into each Magenta culture vessel and autoclaved in the same way.

4.3 Meristem culture

About 1-2 cm long shoots were cut from the young stem apex, and taken to the laboratory in a bottle with tap water. Explants were washed three times in tap water and quickly rinsed in 70% alcohol for 1 minute in a sterilized bottle. They were then sterilized with 10% (w/v) calcium hypochlorite with 3-4 drops of Tween-20, for 15 minutes and rinsed 4 times with sterile DDW.

Disinfected shoots were placed in a sterile Petri dish under a dissecting microscope. Using the forceps and scalpels, the young leaves and leaf primordia were removed. The removal was accomplished by scraping the leaf primordia off with the cutting edge of the scalpel blade. Sterile syringe needle was used to excise meristem and the meristem was cultured on Petri dishes containing 25 ml of the shoot initiation medium. To minimize the risk of contamination, one syringe for one meristem was used and forceps and scalpels were sterilized after each meristem dissection.

Five meristems were cultured per Petri dish and six Petri dishes (30 meristems) were used per treatment. The Petri dishes were sealed with perforated plaster or parafilm and labeled. All operations were carried out on a laminar air flow cabinet bench and all the tools required for dissection were previously sterilized.

The cultures were placed on the bench with average photoperiod of 12 hours using white florescent light (40 μmol m^{-2} s^{-1}) at a temperature of 24 ± 2 ^{o}C. Every change in growth was observed and recorded continuously.

After 2-3 weeks, initiated meristem cultures were transferred to the same fresh medium until shoots were emerged. The shoots were then transferred to shoot multiplication medium on the second subculture. In this case, each shoot which is derived from single meristem, was independently cultured and labeled as an independent clone.

4.4 Shoot multiplication

MS medium with different concentrations of BAP (Table 5.4) was used for shoot multiplication. About fifteen shoots, five explants in each Magenta culture vessel, were used per treatment. After 3 to 4 weeks the number of shoots obtained per explant in each treatment was counted and the data were recorded.

4.5 Chemotherapy

Before applying the chemotherapy technique, the shoot culture condition was assessed. MS medium with 3% sucrose, 0.7% agar and 1 mg/l BAP was mixed and 40 ml of the medium was poured in to 100 ml baby food jars and autoclaved. Shoots (1-2 cm) from the four varieties of the stock plants were taken and sterilized in the same way as culture initiation from meristems. Sterilized shoots were cultured vertically on the baby food jars containing 40 ml of the medium (3 shoots per baby food jar and 5 baby food jars per variety). The cultures were maintained in the same temperature and light conditions as stated above. After the shoots were fully established and grown (5 weeks), the same medium containing ribavirin with different concentrations (10 mg/l, 20 mg/l and 30 mg/l) was prepared. The shoots were transferred and subcultured in the chemical treated medium (15 shoots per treatment). Equal number of shoots was cultured in ribavirin free medium as a control.

4.6 Rooting and acclimatization

Shoots, 1-2 cm long, were cultured in MS medium supplemented with different concentrations of IBA (Table 5.5). The rooted shoots were taken out from the culture vessels and were washed under running tap water to remove the agar from the roots. The plantlets were then transferred to small pots containing red

soil, sand and compost in the ratio of 1:2:1. Pots were covered with transparent plastic bags to retain moisture with random holes for air circulation and under side of the pots were drilled for drainage. Then, they were transferred to the greenhouse and watered every one or two days. Plastic covers were removed partially after a week and completely after two weeks.

4.7 Virus testing

Leaf portions (1 cm in diameter) were taken from upper, middle and lower leaf parts of a meristem origin, chemical treated shoots, untreated shoots (control) and the stock plant. The technique used was serological testing of sweet potato viruses with nitrocellulose membranes enzyme-linked immunosorbent assays (NCM-ELISA) as described by Gibb and Padovan (1993). Virus specific polyclonal antibodies to sweet potato viruses (Appendix 5) as well as NCM strips spotted with sap from virus-positive plants were used. Visual assessment of the development of a purple colour on the sample spots of the nitrocellulose membrane was used to identify virus positive samples (Guitierrez *et al*, 2003).

4.8 Statistical analysis

Statistical analysis of quantitative data was carried out by using SAS/JMP version 8.01 software. Differences among means were analyzed by Tukey's HSD test and a difference at probability level of $p \leq 0.05$ was considered significant for all analysis.

5. RESULT

5.1 Shoot initiation

Expansion of cells to form shoots or calli was observed after 3 weeks of culture. But not all the samples showed this property. Some of the samples were black in appearance and lacked new growth. The sequences of events observed in initiation of shoots from meristems were swelling of the meristem, callus production and shoot development (Fig. 5.1).

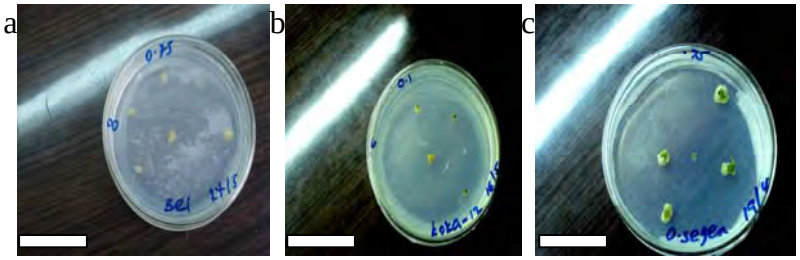


Fig. 5.1 Sequential growth changes in initiation of meristem cultures (a-c). Bars = 0.5cm.

The percentage of shoots induced for each varied depending on the concentration and combinations of growth regulators applied. Significant variations were observed in shoot initiation among treatments in different sweet potato varieties used (Table 5.1).

Effects of PGRs on shoot initiation

As presented in Table 5.1, all the treatments induced the formation of either shoots or calli or both while the explants cultured on growth -regulator-free medium died without producing any callus or shoot.

When the growth regulators supplements were considered, 1mg/l BAP, 2mg/l GA3 and 0.01mg/l NAA induced the highest percentage of shoots 90, 70 and 56.7 for Beletech, Koka-12 and Ogensegen respectively. This was the best growth regulators combination among the treatments to produce shoots. Higher concentrations of NAA (0.05 mg/l) and BAP (1.5-2.5 mg/l) induced callus formation than shoot initiation.

Table 5.1 Percentage growth of meristem cultures of three sweet potato varieties at different growth regulators concentrations and combinations.

	Treatments			Shoot initiation (%)		
Code	BAP	GA3	NAA	Beletech	Koka-12	Ogensegen
1	1.00	1.00	0.01	80.0^{ab}	46.7^{ab}	50.0^{ab}
2	1.00	2.00	0.01	90.0^{a}	70.0^{a}	56.7^{a}
3	1.00	2.00	0.05	16.7^{bc}	0.0^{c}	0.0^{c}
4	2.50	1.00	0.01	3.3^{c}	3.3^{c}	0.0^{c}
5	2.50	2.00	0.01	3.3^{c}	0.0^{c}	0.0^{c}
6	2.50	1.00	0.05	3.3^{c}	0.0^{c}	0.0^{c}
7	1.00	3.00	0.01	0.0^{c}	6.7^{bc}	0.0^{c}
8	1.50	1.00	0.05	6.7^{bc}	6.7^{bc}	0.0^{c}
9	0.75	1.00	0.01	33.3^{abc}	0.0^{c}	26.7^{abc}
10	0.10	1.00	0.01	10.0^{bc}	10.0^{bc}	20.0^{bc}
11	0.25	1.00	0.01	20.0^{bc}	10.0^{bc}	33.3^{abc}
12	0.50	1.00	0.01	26.7^{abc}	3.3^{c}	0.0^{c}
Control	0.00	0.00	0.00	0.0^{c}	0.0^{c}	0.0^{c}

Means within each column connected by the same superscript (a-c) are not significantly different at 5% probability level.

Effects of genotype on shoot initiation

There were significant differences among the four genotypes at all concentrations of growth regulators. Beletech performed best followed by Ogensegen and then Koka-12 (Table 5.2). The highest percentage of shoot initiation (90%) was obtained from Beletech at a growth regulator combination of 1 mg/l BAP, 2 mg/l GA3 and 0.01 mg/l NAA. Generally, performance of TIS – 8250 was the poorest. In all treatments tested, meristems taken from variety TIS-8250, shoots were not initiated. Out of 390 meristems cultured in all treatments from TIS-8250, 285 (73%) meristems formed calli and 105 (27%) were died (Appendix 1).

Table 5.2 Effects of genotype on shoot initiation from meristem

Varieties	Mean number of shoots initiated
Beletech	6.770 [a]
Ogensegen	4.307 [ab]
Koka-12	3.610 [ab]
TIS-8250	0.000 [c]

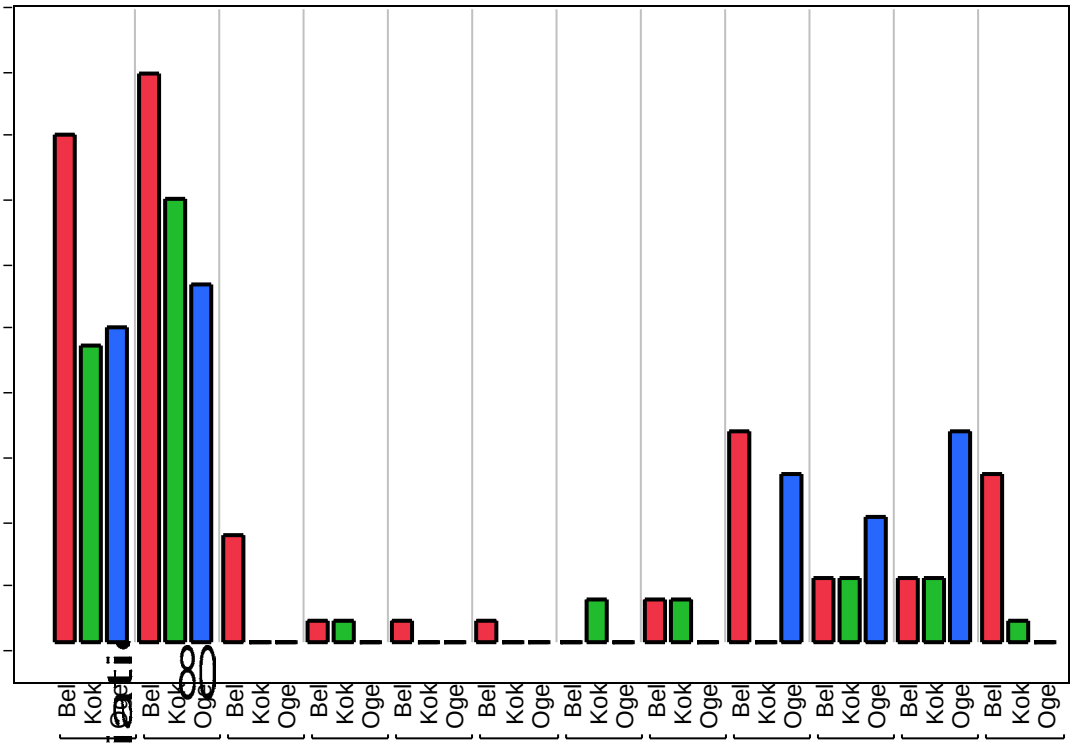
Means connected by the same superscript (a-c) are not significantly different at 5% probability level.

Table 5.3 ANOVA for comparison of the treatment and variety effects on shoot initiation from meristem

ANOVA/ Dependent variables		Initiation (%)	
Source of variation	*df*	Mean square	*F*
VAR	3	1146.55	2.5568*
BAP	1	812.188	1.6814*
GA3	1	96.495	0.194
NAA	1	1077.48	2.2554*
VAR X BAP	7	656.948	1.4193
VAR x GA3	7	512.178	1.0541
VAR x NAA	7	696.769	1.5262*
BAP x GA3	3	660.59	1.3797
BAP x NAA	3	512.997	1.0511
GA3 x NAA	3	461.098	0.9386
VAR x BAP x GA3	15	417.901	0.8047
VAR x BAP x NAA	15	377.049	0.703
BAP x NAA xGA3	7	547.601	1.1402
VAR x BAP x GA3 x NAA	31	289.489	0.3621
Error	566	72.372	
Total	714		

*5% significance level (F test)

Significant variation in initiation was clearly observed in different varieties of sweet potato used as it was observed from the ANOVA table. Among the plant growth regulators used, auxin (NAA) showed higher variation followed by cytokinin (BAP) and then GA3.



Key: Bel- Beletech Kok- Koka-12 Oge- Ogensegen VAR- Variety 1-13- Treatment codes

Fig. 5.2 Treatment effects of PGRs on initiation of shoots from meristems of four sweet potato varieties.

A little difference was seen among sweet potato varieties at shoot initiation stage. Early appearance of shoots was observed in Beletech followed by Koka-12 and Ogensegen sequentially. After six to seven weeks, well formed shoots were observed (Fig. 5.3).

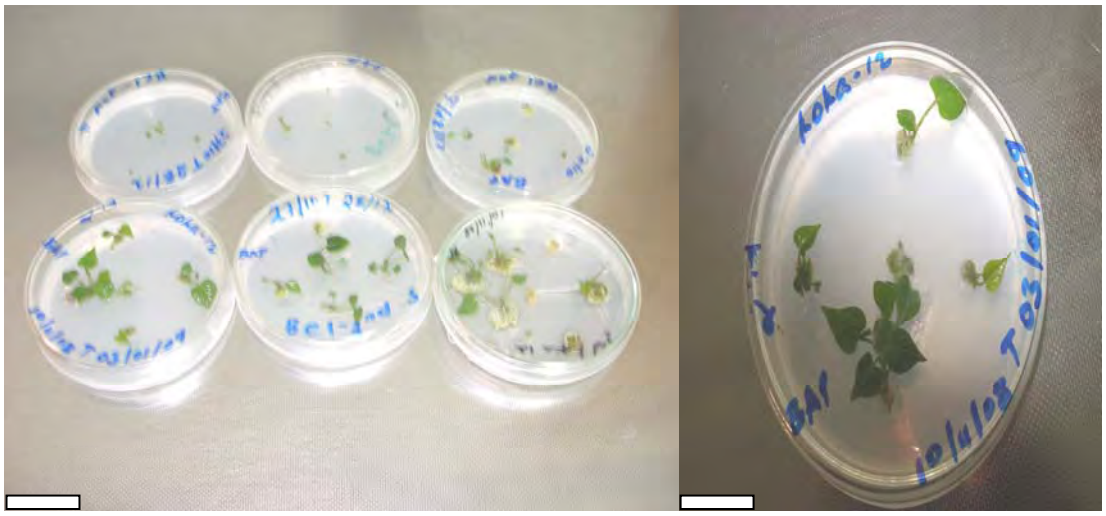


Fig. 5.3 Well formed shoots from culture initiation after seven weeks of culture. Bars = 1cm.

5.2 Subculturing and shoot multiplication

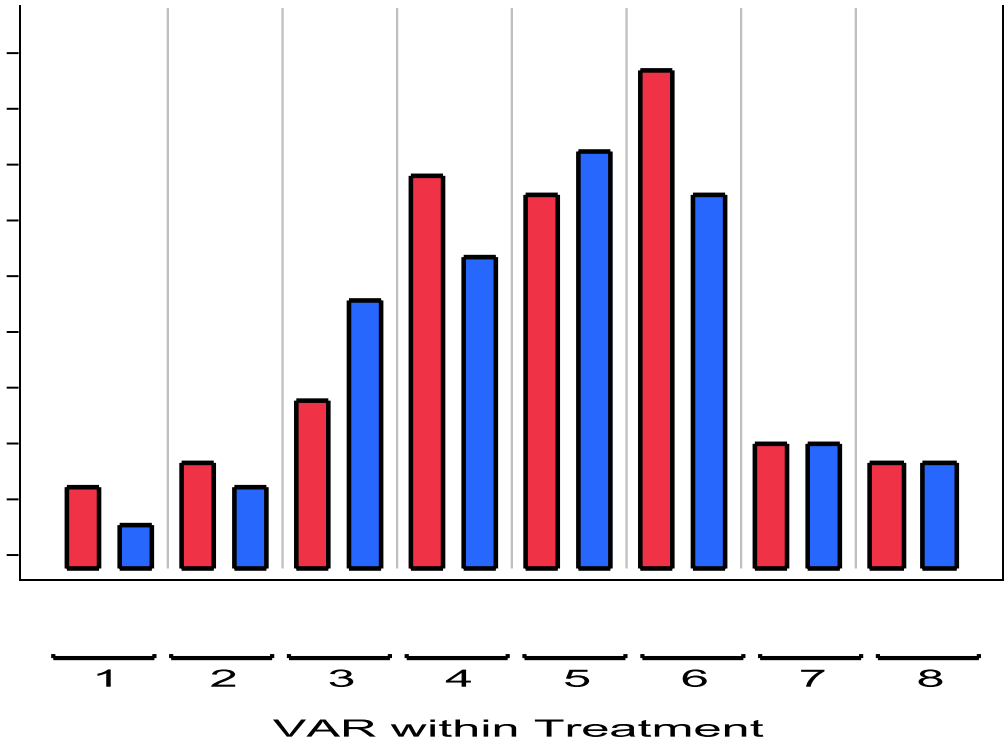
Since the emergence of the first leaves showed the establishment of the culture, it was transferred to a fresh medium with 1or 2 mg/l BAP. After two months of initiation, well formed shoots which were cut into small pieces, each of which containing one or two nodes and placed in MS medium with 1mg/l BAP, showed similar results as the shoots cultured in the same medium.

The highest mean number of shoots per explants (8.0 and 6.7) was obtained on MS medium with 2 mg/l and 1.5 mg/l BAP from Beletech and Koka-12 respectively. As the concentration of BAP increased, it induced callus formation and number of shoots per explant was highly reduced in both varieties. Lower concentrations (0.0 and 0.1 mg/l) and higher concentration (3.0 mg/l) of BAP were not the best concentrations to multiply shoots in both varieties (Table 5.4). The minimum mean number of shoots per explant (1.3 from Beletech and 0.7 from Koka-12) were obtained from growth regulator free medium.

Table 5.4 Number of shoots produced per explant in different concentrations of BAP on MS medium. Data given as means ± SD

	Treatment	Number of shoots per explant	
Code	BAP mg/l	Beletech	Koka-12
1	0.0	1.3 ± 1.2^{c}	1.1 ± 0.4^{c}
2	0.1	1.7 ± 1.1^{c}	1.3 ± 0.5^{c}
3	0.5	2.7 ± 2.0^{abc}	4.3 ± 2.5^{ab}
4	1.0	6.3 ± 4.5^{ab}	5.0 ± 3.6^{ab}
5	1.5	6.5 ± 4.5^{a}	6.7 ± 4.2^{a}
6	2.0	8.0 ± 6.2^{a}	6.0 ± 3.6^{a}
7	2.5	2.0 ± 1.7^{bc}	2.0 ± 1.7^{bc}
8	3.0	1.7 ± 1.3^{c}	1.7 ± 1.1^{c}

Means within each column connected by the same superscript (a-c) are not significantly different at 5% probability level.



Key: BEL- Beletech KOK- Koka-12 VAR- Variety 1-8- Treatments

Fig. 5.4 Effect of variety and treatment on shoot multiplication of Beletech and Koka-12

5.3 Rooting & acclimatization

Rooting in sweet potato varieties was easy even in initiation medium and growth regulator free medium. The longest roots, 11.25 cm for Beletech and 11 cm for Koka-12 were observed in growth regulator free medium (Figures. 5.5 and 5.6). Use of IBA in different concentrations showed different results for the two varieties. When 0.01 mg/l IBA was applied in MS medium, good mean number of roots (3.1) per explants and abundant root hairs were obtained for Beletech while, 0.1 mg/l IBA gave better mean number of roots (3.7) and abundant root hairs for Koka-12 (Table 5.5).

Table 5.5 Percentage of rooting and number of roots per explant of Beletech and Koka-12 at different concentrations of IBA. Mean values are shown as ± SD

IBA mg/l	No. of roots per explant		Root length (cm)		Rooting percentage	
	Beletech	Koka-12	Beletech	Koka-12	Beletech	Koka-12
0.00	4.2±2.0^{a}	2.3±1.5^{ab}	11.25^{a}	11.00^{a}	100^{a}	80.0^{ab}
0.01	3.1±1.6^{ab}	2.8±1.2^{ab}	6.25^{c}	2.75^{bc}	100^{a}	93.3^{a}
0.10	1.9±0.9^{ab}	3.7±1.7^{a}	8.20^{ab}	2.00^{c}	93.3^{a}	100^{a}

Means within each column connected by the same superscript (a-c) are not significantly different at 5% probability level.

When variety effects were considered, no significant differences were observed either in percentage of rooting or number of roots produced per explant, among the two varieties. Different concentrations of IBA showed significant differences in both percentage of rooting and the number of roots per explant. As concentration of IBA increases, mean number of roots per explant and percentage of rooting was decreased for Beletech but the reverse was true for Koka-12.

All of the explants under treatment were rooted at growth regulator free and 0.01 mg/l IBA enriched media for Beletech and 0.1 mg/l IBA enriched medium for Koka-12, producing 4.2 and 3.7 roots per shoot respectively. When root hairs were considered, except IBA free medium for Koka-12 and 0.1 mg/l IBA enriched medium for Beletech which gave few root hairs, other concentrations produce abundant root hairs for both varieties.

Fig. 5.5 Rooted shoots of Beletech, 0.00 mg/l IBA (a) 0.01 mg/l IBA (b) and 0.1 mg/l IBA (c). Bars = 8 cm.

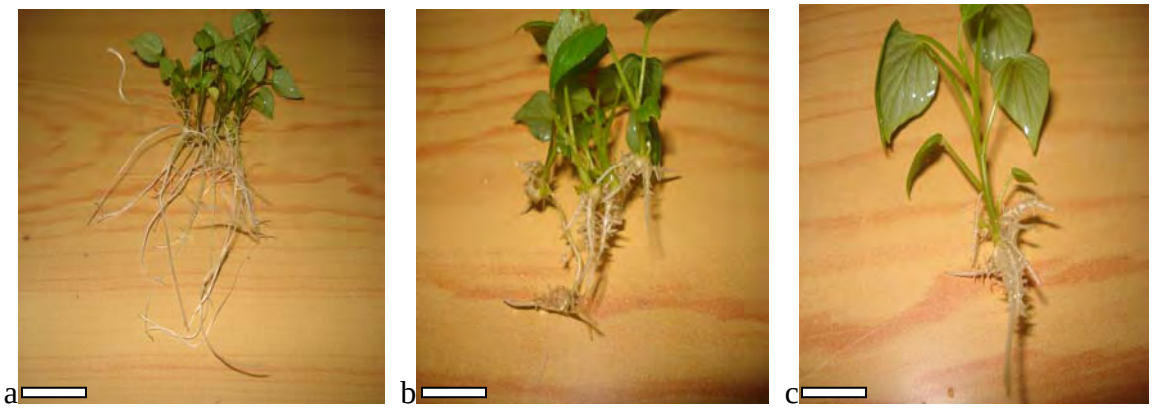


Fig. 5.6 Rooted shoots of Koka-12, 0.00 mg/l IBA (a) 0.01 mg/l IBA (b) and 0.1 mg/l IBA (c). Bars = 8 cm.

Acclimatization in both varieties was done and 91.4% of acclimatized plantlets of Beletech and 73.0% of Koka-12 were survived (Fig. 5.8).

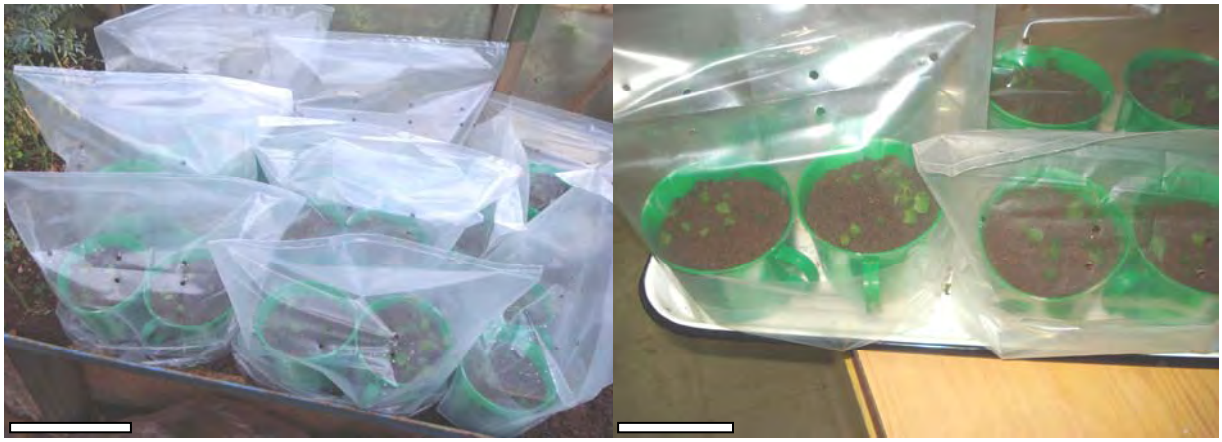


Fig. 5.7 Acclimatized plantlets covered with plastic bags in a greenhouse. Bars = 11.25 cm.

Fig. 5.8 Plantlets after 20 days of acclimatization in a greenhouse. Bar = 3.5.

5.4. Virus indexing results

Samples from stock plants

Out of 22 stock plant samples tested for 10 different viruses (listed in the Appendix), all of the samples were infected with Sweet Potato Feathery Mottle virus (SPFMV). In addition, Sweet Potato Chlorotic Stant Virus (SPCSV) was found only in Ogensegen but other varieties did not give positive result. Slight color change was observed in Ogensegen for Virus G. All the rest of the viruses did not show any reaction in our serologic test in all of the varieties tested when compared to the positive control for each virus (Fig. 5.9).

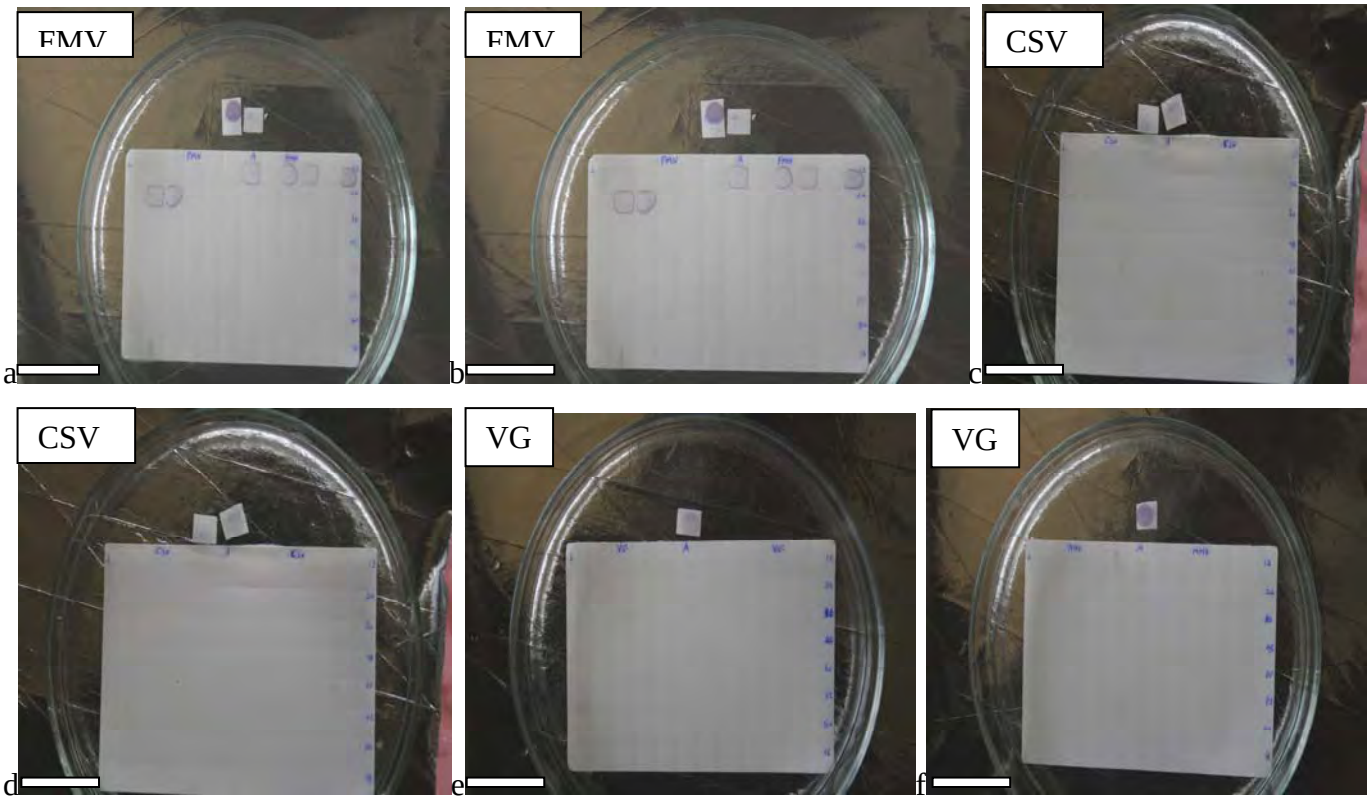


Fig. 5. 9 NCM-ELISA test results for sweet potato viruses. Bars = 7.25 cm.

In visual observation, all of the varieties used were symptomatic for viral infection as the leaf colors were changed to purple in Ogensegen and yellow in others (Fig. 5.10).

Fig. 5.10 Symptoms of virus infection in Ogensegen (a), Beletech (b) and Koka-12 (c). Bars = 9.75 cm.

Samples from meristem derived plants

All of the 9 clones tested for Beletech and 7 clones for Koka-12, were found to be negative for all tested viruses, while the stock or the mother plants were all infected.

Samples from ribavirin treated shoots

Shoots cultured in MS medium with 1 mg/l BAP and different concentrations of ribavirin showed variable responses in survival and length of shoots (Table 5.6). After a month of culture, significant percentage (63.3, 100 and 33.3) of shoots cultured on 30 mg/l ribavirin treated medium were died from Koka-12, Ogensegen and Beletech respectively. In the control and 10 mg/l ribavirin treated medium, survival of cultured shoots was better than shoots cultured on 20 and 30 mg/l ribavirin treated medium. In addition, as the concentration of ribavirin increased, the number of shoots survived and length of shoots were significantly reduced.

Because of degeneration of leaves in ribavirin treated shoots especially in Beletech and TIS-8250, samples were taken only from Koka-12 in all treatments and Ogensegen treated with 20 mg/l ribavirin. Among these samples, only Koka-12 treated with 10 mg/l gave positive result for SPFMV. All samples taken from the control showed positive result for SPFMV.

Table 5.6 Effect of ribavirin on shoot cultures of sweet potato varieties. Shoot lengths are shown as mean values

Rib mg/l	Koka-12		Ogensegen		Beletech		TIS-8250	
	SS %	SL	SS %	SL	SS %	SL	SS %	SL
0	100[a]	5.6 [a]	76.7[a]	3.00 [a]	96.7[a]	4.0 [a]	All callus	-
10	90.0[a]	5.2 [ab]	40.0[bc]	2.75 [a]	76.7[ab]	2.0 [ab]	All callus	-
20	80.0[ab]	5.2 [ab]	26.7[c]	1.60 [ab]	66.7[ab]	1.5 [c]	All callus	-
30	36.7[c]	3.7 [c]	0.0[c]	0.00 [c]	66.7[ab]	1.5 [c]	All callus	-

Means within each column connected by the same superscript (a-c) are not significantly different at 5% probability level.

Where: SS- Shoots Survived SL- Shoot length Rib- Ribavirin

At 30 mg/l ribavirin, more callus formations were observed in all the four varieties (Fig. 5.12).

Fig. 5.11 Leaf degeneration and callus induction in 30 mg/l ribavirin treated medium. Beletech (a), Ogensegen (b), Koka-12 (c) and TIS-8250 (d). Bars = 6.5 cm.

Koka-12 performed best in 10 and 20 mg/l ribavirin followed by Ogensegen (Fig. 5.13).

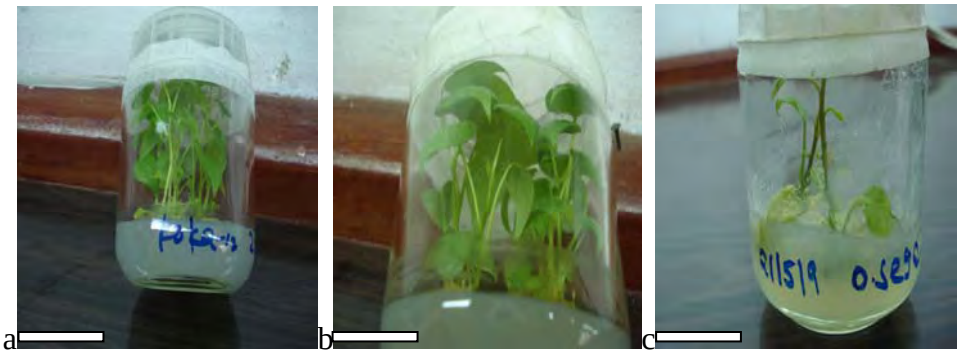


Fig. 5.12 Shoot growth in 10 mg/l (a) and 20 mg/l (b and c) ribavirin treated medium in Koka-12 (a and b) and Ogensegen (c). Bars = 8.25 cm.

6. DISCUSSION

In this study, meristem culture and chemotherapy technology has been utilized to produce four virus free sweet potato varieties locally. The essence of meristem culture is the excision of the organized apex of the shoot from a selected donor plant for subsequent *in vitro* culture. The conditions of culture are regulated to allow only for organized outgrowth of the apex directly into a shoot, without the intervention of any adventitious organs (Debergh and Zimmerman, 1991).

The excised meristem tip is typically small (often less than 1 mm) and is removed by sterile dissection under the microscope. A major advantage of working with such a small explant is the potential that it holds for excluding pathogenic organisms that may have been present in the donor plants from the *in vitro* culture. The second advantage is the genetic stability inherent in the technique, since plantlet production is from an actively dividing apical meristem (Ancora *et al*., 1981). Shoot development directly from the meristem avoids adventitious organogenesis, ensuring that genetic instability and somaclonal variations are minimized.

Chemotherapy techniques drew much attention to get virus free varieties of sweet potato. The technique employs the application of different antiretroviral analogs in tissue cultures to retard or hinder viral replications in explants used.

Shoot initiation from meristem explants depends on many factors. The main factors that affected shoot initiation in our study were the genotype and the concentrations of growth regulators. It is found that not only the genotype but also the medium is very significant for growth of shoots from meristem explants.

Thirteen different growth regulators concentrations and combinations on MS medium were tested to get shoots from meristem. Explants were placed on MS medium containing 3% sucrose and three different types of growth regulators in different concentrations and combinations. The first two combinations gave relatively good number of shoots for the 3 varieties (Beletech, Ogensegen & Koka -12). In both treatments combination, high number of callus was produced for TIS-8250. It could be suggested that sensitivity to growth hormones addition is probably affected by the endogenous levels of hormones in cells and it is reasonable to assume that differences in response *via* different sweet potato cultivars, resulted from the genetic differences among genotypes as it was also stated by Otani and Shimada (1996).

Dhir *et al.* (1998), Perez *et al*. (1987), Lazzeri *et al*. (1987) and Chee *et al*. (1990) obtained similar results and they differentiated the genotypes as appropriate for meristem initiation and callus regeneration. According to them, genotypes which form callus from meristem explants found to be appropriate for callus regeneration. This finding might be appropriate for our variety, TIS-8250 to be regenerated from callus since it forms callus in all of the treatments tested. On the other hand, they found out that the responses of meristem initiation to plant growth regulators have varied with the type, concentration and combination of growth regulators, age and type of explant used, species and genotype within the same species.

Results obtained showed that sensitivity to growth regulators addition is probably affected by the endogenous hormonal balance in cells, which control many circumstances expressed by the genetic makeup of the cells. The type of auxin used in initiation medium was found significant for meristem initiation and specific to the individual cultivars (Al-Mazrooei *et al.,* 1997). Gonzalez *et al.* (1999) found that influence of auxin enriched initiation medium was observed to be greater than that with cytokinin enriched medium to induce shoot formation from meristems.

In the light of the results obtained in this study, each of the growth regulators used had different effect on shoot initiation from meristems of sweet potato varieties. As it was reported by Gong *et al.,* (2001), shoot initiation from meristem requires specific concentration and combination of plant growth regulators. Interaction effects between varieties (Beletech, Koka-12 and Ogensegen) and growth regulators (BAP, NAA and GA3) were significant at 5% probability level for meristem initiation.

Chee *et al.* (1990), Newell *et al.* (1995), Dhir *et al.* (1998), and Gong *et al.* (2001) maintained sweet potato explants onto auxin cytokinin enriched medium as an initiation medium. Although, Otani and Shimada (1996), Al-Mazrooei *et al.* (1997) and Triqui *et al.,* (2007) used initiation medium supplemented with NAA as an auxin source, BAP as a cytokinin and GA3 as Gibberellins to induce shoots from sweet potato meristem explants and stated that these were critical for meristem initiation of sweet potato varieties. On the other hand, Oggema *et al.* (2007) synchronized with our results, pointed out that the lower concentrations of NAA and BAP in initiation medium resulted higher shoot initiation percentage.

When NAA concentration is increased from 0.01 to 0.05 mg/l, it sharply decreases the number of shoot production from 27 shoots to 5 shoots in Beletech; 21 shoots to 0 in Koka-12 and 17 shoots to 0 in Ogensegen and enhances callus formation. Compact callus formation with increase in concentration of NAA was reported by Robbins (1972). Increased concentrations of BAP gave more callus than shoots in all of the varieties. Same is the case with increasing the concentration of GA3.

This study showed that BAP concentration of 0.1 mg/l with other growth regulator compositions influenced the formation of shoots from meristems, in that large number of explants died from all of the varieties. This result agrees with the result obtained from the plant growth regulator free initiation medium.

Therefore, according to the results obtained and discussions supported with references, 1mg/l BAP, 2mg/l GA3 and 0.01 mg/l NAA was found to be optimum in meristem initiation of Beletech, Ogensegen and Koka-12 (Table 5.1). This combination was repeated once and similar results were recorded. In all of the combinations, shoot initiation was not possible for TIS-8250 but callus formation was highly evident.

Shoots of 1-2 cm in length originated from meristem culture were subjected to different concentrations of BAP since cytokinins play a predominant role in multiple shoot formation (Jayasree *et al*., 2001). However, correct concentration of BAP is necessary because unfavorable concentration may inhibit the growth of cellular mass as reported by Green (1987).

Shoot multiplication response per explant was increased tremendously when BAP (2 mg/l and 1.5 mg/l) was used for Beletech and Koka-12 respectively (Table 5.4). However, further increase in concentration of BAP resulted in sharp decrease in shoot multiplication response for both varieties .This is possibly due to the reason that normal development of somatic tissue required a fine temporal and spatial regulation of cell division, enlargement and differentiation that could be achieved by correct concentration of cytokinins as also reported by Ammirato (1987).

The results indicated that IBA is important factor on root length, root number and abundance of root hair formation but it was not significant in percentage of rooting as the IBA treated medium and the control gave similar results for both sweet potato varieties used. Since sweet potato is vegetatively propagated plant, rooting is not influenced by the exogenous growth regulators. Similar result was reported by Newell

et al. (1995). The mean rooting percentage in IBA treated and the control (non – treated) medium was numerically different but not significantly.

Significant differences (P = 0.05) were found between the two sweet potato varieties in number of roots and root length. The highest mean number of roots (4.2) in the variety Beletech was obtained in the IBA free medium. This was different from results obtained by Lity and Conooer (1978) who reported that IBA significantly increased the number of roots produced in sweet potato varieties. On the other hand, the highest mean number of roots (3.7) in Koka-12 was obtained in 0.1 mg/l treated IBA medium (Table 5.5). These differences may indicate that the two varieties are genetically different.

The number of roots produced per shoot increased when IBA was used in minimal concentration for Beletech and relatively high concentration for Koka -12. Growth regulator free medium gave longest root with root hairs in both genotypes. Rooting was also common in initiation medium as also reported by Oggema *et al.* (2007).

Plantlets are ready to be established in the soil when they have two or three leaves and at least one strong root. As indicated in the result 1:2:1 (soil: sand: compost) mixture is the best planting medium (Fig. 5.8). Soil mixtures with high peat content often lead to slower growth and a higher death rate (Kuo *et al*., 1985).

Since sweet potato is vegetatively propagated by the use of vine cuttings and storage roots, almost all germplasm is known to be infected with viruses (Green, 1987). Viral infections can have a dramatic effect on yield and marketable quality of a crop and also can affect adversely international distribution of sweet potato germplasm (Ngeve and Boukamp, 1991). Some viruses like sweet potato is feathery mottle virus can alone decrease the yield by 40% and in combination with other viruses, the loss reaches 90% (Njeru *et al*., 2004). The most common virus reported from sweet potato is feathery mottle virus (SPFMV) which occurs world-wide. This was also confirmed in our study that all of the mother plants tested positive for SPFMV.

The best control method for potato viruses is production of healthy plants from meristem culture (Guitierrez *et al*, 2003). These workers showed that for producing healthy plants, small meristems (0.11 - 0.25 mm diameter) had better efficiency than big meristems.

In this study, the two main virus elimination methods, meristem culture and chemotherapy were tested for their use in virus elimination. Serological method, such as NCM-ELISA, was used to test for the presence of viruses in the stock plants and meristem derived and chemical treated plantlets. Meristem culture was found to be very effective in eliminating viruses. Its virus elimination efficiency reached 100% in our study (with a possibility of re-infection) and 75-80% elimination efficiency was reported by Kuo *et al*. (1985). In another study, Mellor and Smith (1977) cultured apical meristems of four potato cultivars in modified MS solid medium with BAP, NAA and GA3 and they obtained 68.1 - 86.6% virus free plantlets.

In the stock plants tested, Ogensegen showed very deep purple color showing that it is severely attacked by viruses. Symptoms in the leaves of this plant also are indicative of infection by viruses. NCM-ELISA confirmed the synergistic effect of the three viruses (SPFMV, SPCSV and SPVG) in this variety. Beletech and Koka-12 showed light purple color showing that they are infected only by single virus, SPFMV. This result is also indirect proof for the absence of whiteflies in our greenhouse. Had these flies been in the greenhouse, the other stock samples, Beletech and Koka-12, would have been infected with SPCSV since it is transmitted by whitefly.

Antiviral chemicals can be used as additives in the culture medium (Quak, 1977), and one of the most widely used is ribavirin, (1-β-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) also known as virazole. This compound is a guanosine analog with broad-spectrum activity against animal viruses and appears also to be active against plant virus replication in whole plants (Gong *et al*., 2005) Increasing concentrations of ribavirin and increasing length of culture incubation in the presence of the compound typically increase the effectiveness of virus elimination (Mellor and Smith, 1977), but slowed growth and phytotoxicity may be evident at high concentrations (Lin *et al*., 1990).

The efficiency of ribavirin in the elimination of plant viruses is subject to ribavirin concentrations, type of host plant genetic material, and type of virus. Mangal *et al*. (2004) reported that by chemotherapy *in vitro*, with application of 20 mg/l of ribavirin, 55.5% virus-free sweet potato plants were obtained but in our varieties, Koka-12 and Ogensegen, 100% of the shoots treated with 20 mg/l ribavirin were free from viruses. Nevertheless, lower concentration (10 mg/l) failed to produce similar results in all treated shoots, which is similar with the result obtained by Lin *et al*. (1990). As the same result obtained by Lin *et al*.

(1990) and Al-Mazrooei *et al.* (1997), 30 mg/l ribavirin treated shoots were 100% free from viruses with sever phytotoxicity effect.

In our study, shoots cultured on media containing the antiviral agent ribavirin at concentrations ranging from 10-30 mg/1 had both virus elimination and phytotoxicity effects. In 30 mg/l ribavirin treated samples, significant number of shoots of Koka-12 followed by Beletech and all shoots of Ogensegen were died. All shoots of TIS-8250 cultured in all concentrations of ribavirin turned to calli. This result may indicate that there is a difference in genetic constituents of the four varieties

All shoots cultured on the control (ribavirin free medium) tested positive for viruses present in their mother plants showing that shoots, 1-2 cm in length also contains enough amount of viruses and culturing them without treatment can not eliminate viruses.

The development of pale green pigmentation in the early storage of meristems could be an indication of survival of meristem tips. If the meristem cultures become white and translucent it could be an indication of degeneration. Growth initiation and organ differentiation of sweet potato meristems require a combination of cytokinin and auxin at low concentration, since higher concentrations promote callusing (Lity and Conooer, 1978). The ability of sweet potatoes to establish under the culture conditions specified varies widely between genotypes. Some of the common problems experienced in this study were the following. Shoot initiation in variety Ogensegen was easy but not a single shoot survived to maturity. When the first initiated shoots were transferred to the same fresh medium or 1 or 2 mg/l BAP, all of them were died. This happened frequently during the subsequent cultures.

Several authors drew the attention to the effect of initiation medium on stimulation of plantlet survival for sweet potato as Al-Mazrooei *et al.* (1997) whom pointed out that the carry over effect of the initiation medium on subsequent growth of plantlets especially in recalcitrant varieties. According to them, the effect of auxin initiation medium is critical and specific to the individual cultivars.

One of the most important approaches for overcoming *in vitro* recalcitrance problems is the optimization of plant growth regulators; however, cultures are under the control of both endogenous and exogenous plant growth regulation, therefore, achieving the correct exogenous balance of the key growth regulators auxin

and cytokinin can be critical, also, their appropriate application can effectively overcome certain recalcitrance problems (Erica, 2000).

Finally, callusing in the initiation medium was frequently observed in all varieties especially in TIS-8250 in which no shoot initiation was possible in all of the treatments used.

7. CONCLUSION AND RECOMMENDATIONS

In this study, initiation of shoots was successful onto auxin, cytokinin and Gibberellin enriched medium. Growth regulator free initiation medium showed significant difference than plant growth regulator enriched initiation medium. Cytokinin enriched medium scored the highest significant increase in number of shoots per explant. Shoots rooted well when subcultured on growth regulator free rooting medium and IBA enriched medium. Rooted plantlets were then transferred into pots and successfully established in the greenhouse.

This protocol is significant for future tissue culture and other researches by giving basic information on optimization of media and production of virus free stock on sweet potato varieties used. The significant differences among genotypes in response to initiation also help in studying the genetic factors responsible for initiation, shoot multiplication and rooting.

Out of ten different viruses tested, SPCSV and SPFMV are found in our varieties. Elimination of these viruses from our plant material became successful by using meristem culture technology and chemotherapy.

Additionally, research on phytosanitary methods of virus control might identify methods which would allow susceptible but highly productive varieties to be grown successfully. This research became successful in production of virus free varieties of sweet potato. This might be a significant contribution to the virus free variety storage and to combat the problem of sweet potato yield and quality reduction in Ethiopia.

Based on the results obtained in this research, the following recommendations are stated.

The sweet potato varieties found in Ethiopia are infected with viruses but there is no quantitative information available on the economic impacts of these viruses in productivity of the crop. Therefore, it is mandatory to asses the economic impacts and to give attention to optimize for each variety for *in vitro* propagation and hence use meristem culture technology to produce virus free cultivars. In addition, nutrient contents of the soil in which sweet potato varieties are planted should be properly evaluated because disease resistance depends on the nutrient availability of the soil.

Meristem culture technology is very efficient to eliminate viruses from sweet potato than the other methods. Chemicals used for virus treatment have phytotoxicity on the plants, so that using meristem culture is very important and it is cost effective relative to chemotherapy. But there should be very efficient and fully equipped laboratories in universities or other research organizations.

After virus free varieties are produced, there should be well protected screen house to protect viral contaminations and controlled mechanism of distribution is important. Virus free varieties produced in this study should be handled properly and stored as a stock for distribution to farmers.

Germplasm storage systems are very important to save virus free varieties for further research and to save sweet potatoes of Ethiopia from complete loss.

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Appendix I. Raw data collected in shoot initiation from meristem explants in different plant growth regulator concentrations and combinations

	Treatments (mg/L)			Beletech			Koka-12			Ogensegen			TIS-8250		
Co	BAP	GA3	NAA	S	C	D	S	C	D	S	C	D	S	C	D
1	1.00	1	0.01	24	3	3	14	12	4	15	10	5	0	27	3
2	1.00	2	0.01	27	3	0	21	5	4	17	12	1	0	25	5
3	1.00	2	0.05	5	18	7	0	24	6	0	27	3	0	15	15
4	2.50	1	0.01	1	20	9	1	24	5	0	25	5	0	15	15
5	2.50	2	0.01	1	18	11	0	23	7	0	27	3	0	30	0
6	2.50	1	0.05	1	16	13	0	27	3	0	27	3	0	30	0
7	1.00	2	0.01	0	15	15	2	22	6	0	27	3	0	29	1
8	1.50	1	0.05	2	26	2	2	26	2	0	25	5	0	27	3
9	0.75	1	0.01	10	10	0	0	23	7	8	7	15	0	30	0
10	0.10	1	0.01	3	6	3	3	13	14	6	2	22	0	14	16
11	0.25	1	0.01	6	9	3	3	8	19	10	4	16	0	20	10
12	0.50	1	0.01	8	12	1	1	29	0	0	30	0	0	23	7
Con	0.00	0	0.00	0	0	30	0	0	30	0	0	30	0	0	30

Key: - Co- Code S- Shoots C- Callus D- Dead Con- Control

Appendix II Raw data collected from shoot multiplication

Variety	Number of shoots per explant at different BAP concentrations in mg/l							
Beletech	**0.0**	**0.1**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
	1	2	5	11	12	15	3	2
	1	2	5	4	3	12	3	2
	1	2	2	5	4	5	2	2
	1	1	3	6	4	6	2	2
	1	1	4	4	7	10	2	2
	2	1	1	8	8	11	1	1
	1	3	2	9	10	6	2	1
	2	3	3	4	11	5	1	2
	1	3	4	5	8	7	2	1
	1	2	2	10	4	8	1	1
	1	1	2	4	3	6	2	2
	1	1	1	5	5	5	2	2
	2	1	3	6	6	10	3	2
	2	1	13	7	4	4	2	2
	2	2	3	7	3	10	2	2

Appendix II (Cont'd)

Variety	Number of shoots per explant at different BAP concentrations in mg/l							
Koka-12	**0.0**	**0.1**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
	1	1	5	11	12	11	3	2
	1	2	5	4	5	4	3	2
	1	2	8	5	4	5	2	2
	1	1	3	6	4	6	2	2
	1	1	4	4	7	4	2	2
	1	1	1	8	8	8	1	1
	2	2	2	7	10	7	2	1
	2	1	3	4	11	4	1	2
	1	2	4	5	8	5	2	1
	1	2	4	10	4	10	1	1
	1	1	2	4	3	4	2	2
	1	1	10	5	5	5	2	2
	1	1	6	6	6	6	3	2
	1	1	13	7	4	7	2	2
	2	1	8	4	3	4	2	2

Appendix III. Raw data collected from rooting at different IBA concentrations

Variety	Number of roots per explant at different IBA conc. mg/l		
Beletech	0.00	0.01	0.10
	4	4	3
	4	3	2
	2	4	2
	2	7	4
	7	8	3
	5	3	3
	5	2	2
	3	5	2
	1	8	1
	1	5	1
	3	3	1
	2	2	2
	2	2	1
	3	3	2
	3	4	0
Koka-12	4	0	6
	0	3	8
	0	4	4
	0	2	2
	2	2	4
	2	4	3
	4	3	3
	3	3	4
	3	4	4
	4	4	2
	4	2	3
	2	3	1
	3	1	3
	1	3	4
	3	4	5

Appendix IV. NCM-ELISA test results for 10 different sweet potato viruses in three sweet potato varieties.

No.	Source	Variety	Symp	FMV	MMV	LV	CFV	MSV	C-6V	CSV	CaLV	VG	CMV
1	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
2	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
3	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
4	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
5	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
6	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
7	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
8	Stock	Beletech	Symp	+	_	_	_	_	_	_	_	_	_
9	M_1	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
10	M_2	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
11	M_3	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
12	M_4	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
13	M_5	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
14	M_6	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
15	M_7	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
16	M_8	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
17	M_9	Beletech	Asym	_	_	_	_	_	_	_	_	_	_
18	SC_1	Beletech	Asym	+	_	_	_	_	_	_	_	_	_
19	SC_2	Beletech	Asym	+	_	_	_	_	_	_	_	_	_
20	Stock	O.segen	Symp	+	_	_	_	_	_	+	_	+	_
21	Stock	O.segen	Symp	+	_	_	_	_	_	+	_	+	_
22	Stock	O.segen	Symp	+	_	_	_	_	_	+	_	+	_
23	Stock	O.segen	Symp	+	_	_	_	_	_	+	_	+	_
24	Stock	O.segen	Symp	+	_	_	_	_	_	+	_	+	_
25	Stock	O.segen	Symp	+	_	_	_	_	_	+	_	+	_
26	SC_1	O.segen	Asym	+	_	_	_	_	_	+	_	+	_
27	SC_2	O.segen	Asym	+	_	_	_	_	_	+	_	+	_
28	R_{20}	O.segen	Asym	_	_	_	_	_	_	_	_	_	_
29	R_{20}	O.segen	Asym	_	_	_	_	_	_	_	_	_	_
30	Stock	Koka-12	Symp	+	_	_	_	_	_	_	_	_	_
31	Stock	Koka-12	Symp	+	_	_	_	_	_	_	_	_	_
32	Stock	Koka-12	Symp	+	_	_	_	_	_	_	_	_	_
33	Stock	Koka-12	Symp	+	_	_	_	_	_	_	_	_	_
34	Stock	Koka-12	Symp	+	_	_	_	_	_	_	_	_	_
35	Stock	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
36	M_1	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
37	M_2	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
38	M_3	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
39	M_4	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
40	M_5	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
41	M_6	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_

42	M_7	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
43	SC_1	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
44	SC_2	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
45	R_{10}	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
46	R_{10}	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
47	R_{10}	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
48	R_{10}	Koka-12	Asym	+	_	_	_	_	_	_	_	_	_
49	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
50	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
51	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
52	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
53	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
54	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
55	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
56	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
57	R_{20}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
58	R_{30}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
59	R_{30}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_
60	R_{30}	Koka-12	Asym	_	_	_	_	_	_	_	_	_	_

Key: - Symp- Symptomatic

Asym- Asymptomatic

M- Meristem

SC- Shoots cultured without ribavirin (control)

R- Ribavirin

Numbers, 1-9- Clone number

10, 20 and 30- Ribavirin concentrations in mg/l

Appendix V REAGENTS AND MATERIALS INCLUDED IN THE NCM-ELISA KIT

Code	NAME	Chemical composition	Quantity	Physical state	Remarks
1	TBS	0.02M Tris=4.84g 0.50M NaCl=58.44g	5 packets	White powder	Stable at 4°C for 1 month
2	HCl	HCl(18.5%)=10ml	3 bottles	Transp.Liquid	Avoid contact with skin
3	Ext. buffer	Sodium sulfite=1.0g	2 packets	White powder	Stable at 4°C for 6-8 months
4	Blo. buffer	2%Powdered cow milk=12.0g	2 packets	White powder	Prepare fresh, for each test
5	Triton X-100	2% Triton X-100=12ml	1 bottle	Transp.Liquid	Mix thoroughly. Stable at room temperature
6	Con. buffer	2% Powdered cow milk=6.0g	2 packets	White powder	Prepare fresh, for each test
7	T-TBS	Tween-20=1.5ml	2 vials	Yellow liquid	Mix thoroughly. Stable at 4 C
8	SB	0.1 Tris =3.03g 0.1M NaCl=1.45g 5mM MgCl2.6H2O=0.25g	2 packets 2 eppendorf tubes	White powder	Stable at 4 C for 1 month
9	Antibody	SPFMV =0.25 ml	1 vial	Transp.Liquid	Stable at 4°C for 6-8 months
		SPMMV=0.25 ml	1 vial	Transp. Liquid	Stable at 4°C for 6-8 months
		SPLV =0.25 ml	1 vial	Transp.Liquid	Stable at 4°C for 6-8 months
		SPCFV =0.25 ml	1 vial	Transp. Liquid	Stable at 4oC for 6-8 months
		SPMSV =0.25ml	1 vial	Transp.Liquid	Stable at 4oC for 6-8 months
		C-6 virus=0.25ml	1 vial	Transp. Liquid	Stable at 4oC for 6-8 months
		SPCSV =0.25 ml	1 vial	Transp.Liquid	Stable at 4oC for 6-8 months
		SPCaLV=0.25 ml	1 vial	Transp. Liquid	Stable at 4oC for 6-8 months
		SPVG =0.25ml	1 vial	Transp.Liquid	Stable at 4oC for 6-8 months
		CMV =0.25ml	1 vial	Transp. Liquid	Stable at 4oC for 6-8 months
10	Conjugate	GAR=1.1ml	2 vial	Transp.Liquid	Stable at 4°C for 6-8 months
11	Substrate	NBT= 25mg	2 vials	Yellow solid	Stable at 4oC for 6-8 months
		BCIP= 12.5mg	2 vials	White solid	Stable at 4oC for 6-8 months
12	Solvent	DMF(70%)to NBT =2.0 ml	1 vial	Transp.Liquid	Stable at room temperature very toxic, skin absorbed
		DMF(100%)to BCIP=2.0 ml	1 vial		
13	Membranes	Nitrocellulose	20 sheets	White solid	very fragile, always use gloves when handling
14	Filters	Whatman # 4 filter papers	60 sheets	White solid	For blotting and storing. Nitrocellulose membranes

Other materials included in the NCM-ELISA kit:

* Positive and negative controls (20 nitrocellulose membranes strips)

* Instruction manual (1) and sample sheet (1)

* Gloves (1 pair)

* Small tubes of 1 cm in diameter (2)

* Samples plastic bags (10)

Appendix VI Sweet potato viruses (adapted from Valverde *et al*., 2007)

Virus	Family/Genus	Vector
Cucumber mosaic virus (CMV)	*Bromoviridae / Cucumovirus*	Aphids
Ipomoea yellow vein virus (IYVV)	*Geminiviridae / Begomovirus*	Whiteflies
Sweet potato chlorotic stunt virus (SPCSV)	*Closteroviridae / Crinivirus*	Whiteflies
Sweet potato feathery mottle virus (SPFMV)	*Potyviridae / Potyvirus*	Aphids
Sweet potato latent virus (SPLV)	*Potyviridae / Potyvirus*	Aphids
Sweet potato virus G (SPVG)	*Potyviridae / Potyvirus*	Aphids
Sweet potato leaf curl virus (SPLCV)	*Geminiviridae / Begomovirus*	Whiteflies
Sweet potato leaf curl Georgia Virus (SPLCGV)	*Geminiviridae / Begomovirus*	Whiteflies
Sweet potato leaf speckling virus (SPLSV)	*Luteoviridae / Enamovirus*	Aphids
Sweet potato mild mottle virus (SPMMV)	*Potyviridae / Ipomovirus*	?
Sweet potato mild speckling (SPMSV)	*Potyviridae / Potyvirus*	Aphids
Tomato spotted wilt virus (TSWV)	*Bunyaviridae / Tospovirus*	Thrips?

Tentative species	Family / Putative genus	
Sweet potato C-6 virus	?	?
Sweet potato caulimo- like virus	*Caulimoviridae*	?
Sweet potato chlorotic fleck virus (SPCFV)	*Flexiviridae / Carlavirus*	?
Ipomoea crinkle leaf curl virus (ICLCV)	*Geminiviridae / Begomovirus*	?
Sweet potato ringspot virus	*Comoviridae / (epovirus*	?
Sweet potato vein mosaic virus	*Potyviridae*	Aphids
Sweet potato virus 2 (SPV2)	*Potyviridae / Potyvirus*	Aphids?
Sweet potato yellow dwarf virus (SPYDV)	*Potyviridae / Ipomovirus*	?

Appendix VII Units

ha	hectare
t/ha	tone per hectare
mm	millimeter
ºC	degree Centigrade
g	gram
Kcal	Kilocalorie
mg	milligram
Kg	Kilogram
µm	micrometer
Km	Kilometer
ml	milliliter
ml/l	milliliter per liter
N	normal
mg/l	milligram per liter
cm	centimeter
mA	milli Ampere

Declaration

I the undersigned candidate declare that this thesis is my original work. It has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been duly acknowledged.

Candidate Name	Signature	Date
Neja Jemal	_____	_____