In Vitro Production of Clean Planting Material: Setting the Timelines for an Efficient Seed System for Vegetatively Propagated Crops in Ghana

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Abstract
In vitro propagation provides the required micro-climate critical for growth and development of plants. It is used to rapidly multiply vegetatively propagated crops such as yam, cassava, cocoyam, taro and sweet potato. Conventional vegetative propagation has limitations when large numbers of "clean" disease-free planting materials are required. This is because vegetative propagules usually habour disease pathogens namely fungi, bacteria, and viruses from the previous growing season, and the multiplication rates are low, unlike in grains and cereals. Meristem culture in vitro technique coupled with thermotherapy, chemotherapy and cryotherapy, are techniques routinely used to eliminate such pathogens from plant tissues. This results in the production of "clean" disease-free planting materials. This study used meristem and thermotherapy systems where applicable to produce clean planting materials of sweet potato, cassava and yam. Molecular diagnostics and enzyme-linked immune sorbent assay (ELISA) based methods were used to index cleaned cultures to certify that the germplasm is clean. Results from this study have indicated that it takes up to 12, 18, and 24 months to generate clean planting materials of cassava, sweet potato and yam respectively. Similar systems are being investigated for taro and cocoyam and preliminary results indicate it takes up to six months to generate clean planting material. This paper reports the detailed timelines in the production of clean planting material using tissue culture techniques up to the time of certification. This information generated is very paramount for a sustainable pre-basic seed production of root and tuber crops in West Africa, particularly Ghana.

Key words; Indexing, Meristem, Regeneration, Tissue Cultures, Thermotherapy,

Production in vitro de matériel végétal propre: établir les délais pour un système de semences efficace pour les cultures à multiplication végétative au Ghana

Résumé
La propagation in vitro fournit le micro-climat nécessaire à la croissance et au développement des plantes. Il est utilisé pour multiplier rapidement les cultures multipliées par voie végétative telles que l'igname, le manioc, le taro, le taro et la patate douce. La multiplication végétative conventionnelle a des limites quand un grand nombre de matériel végétal exempt de maladie "propre" est requis. C'est parce que les propagules végétatives habillent habituellement les agents pathogènes de la maladie à savoir les champignons, les bactéries et les virus de la saison de croissance précédente, et les taux de multiplication sont

faibles, contrairement aux céréales et aux céréales. La technique de culture in vitro Meristem couplée à la thermothérapie, la chimiothérapie et la cryothérapie sont des techniques couramment utilisées pour éliminer de tels pathogènes des tissus végétaux. Ceci a pour résultat la production de matériel végétal "propre" sans maladie. Cette étude a utilisé des systèmes de méristème et de thermothérapie, le cas échéant, pour produire des plants propres de patate douce, de manioc et d'igname. Des méthodes basées sur le diagnostic moléculaire et sur le dosage des sorbants immuno-enzymatiques (ELISA) ont été utilisées pour indexer les cultures nettoyées afin de certifier que le germoplasme est propre. Les résultats de cette étude ont indiqué qu'il faut jusqu'à 12, 18 et 24 mois pour produire des plants propres de manioc, de patate douce et d'igname, respectivement. Des systèmes similaires sont étudiés pour le taro et le taro et les résultats préliminaires indiquent qu'il faut jusqu'à six mois pour produire du matériel végétal propre. Cet article rapporte les délais détaillés dans la production de matériel végétal propre en utilisant des techniques de culture tissulaire jusqu'au moment de la certification. Cette information générée est primordiale pour une production semencière pré-fondamentale durable des cultures de racines et de tubercules en Afrique de l'Ouest, en particulier au Ghana.

Mots clés; Indexation, Méristém, Régénération, Cultures Tissulaires, Thermothérapie,

Introduction

In-vitro propagation, is the growth of plants in glass or equivalent vessel (plastic vessels) under sterile conditions, providing all the required growth conditions. Other terms used interchangeably with in vitro propagations are tissue culture and micropropagation. Micropropagation technique is utilized as an integral part of generating planting materials of vegetatively propagated crops.

In West and Central sub-Saharan Africa, most of the staple starchy food crops are vegetatively propagated and this is facilitated by the production of clonal material to ensure that taste and other qualities of the product are not compromised or lost through the use of traditional seeds. Most vegetatively propagated crops do not produce viable seeds that can be planted as next generation crops. Even when they do, these seeds may be genetically different from the mother plant (Bisognin, 2011). In some varieties, there is no synchronization between the male and female reproductive organs leading to inability of these varieties to produce seeds. Hence, vegetative propagation has been used over several years, and a proper seed system, which ensures the production of clean seed, does not exist. There is the need for seed source to be routinely cleaned off pathogens through the use of tissue culture to have clean planting materials available for propagation. The system of saving vegetative plant parts and using as planting material for the following season has led to endemic pathogens persisting in germplasm over generations. Distribution of vegetatively propagated planting materials across borders both inter and intra nationally comes along with the disbursement of pathogens. Some of these pathogens having co-existed with the crops over several generations currently have their genomic information integrated into the genome of some of these crops as reported in sweetpotato (Kyndt et al., 2015), yam (Seal *et al.,* 2014) and Musa sp (Geering et al., 2005; Haper *et al.,* 1999). It is therefore paramount that planting materials of vegetatively propagated crops be subjected to in-vitro procedures for the elimination of pathogens. This system involves the use of meristem

culture techniques with or without thermotherapy, chemotherapy and cryotherapy (Sastry and Zitter, 2014). The theory associated with the use of apical meristem cultures for the generation of clean planting material is that, meristematic cells are actively dividing and this does not permit the multiplication and persistence of viruses, bacteria and fungi (Walkey, 1978; Pierik, 1989).

Apical meristem-during embryo development, is a dome of actively dividing cells located at the apex of shoots and roots. Plantlets derived from meristem tip culture usually retain the genetic characteristics of mother plants. Virus elimination from selected plants do not differ from mother plants and the high auxin concentration in meristematic cells as well as competition for nutrients and enzymes suppress virus replication in meristematic cells. The ongoing active metabolic process associated with growth regulators (cytokinins) and inhibitors (Phenolamines) are not suitable for virus multiplication, hence the elimination of viruses through the use of apical meristem tissue culture (Ravichandra, 2016). Improved seed systems have proven track record in raising productivity of clonal crops such as sweetpotato and Musa species (Quain and Dzomeku; 2013).

In-vitro propagation of apical meristems is an important part of virus-elimination therapy for improving the health of plant collections. Distribution of tissue culture derived germplasm often assists breeders and nurseries in meeting quarantine regulations, stop the distribution of viruses in propagative plant material, and avoid productivity losses due to viruses. It also contributes to lowering the inoculum potential of viruses within the environment.

The generation of clean planting material via tissue culture is a critical aspect of the development of vegetatively propagated plant varieties for dissemination. Although rapid multiplication in vitro guarantees exponential multiplication rate to generate planting material, coupled with virus elimination, the procedure is initially long, however, once a few clean seedlings are generated, they can be rapidly multiplied to generate the required numbers. This paper focuses on providing the timelines in the generation of clean planting material and serves as a source of reference information in demanding for tissue culture derived clean planting of vegetatively propagated crops. The information is important to be considered during project development. Again, this will facilitate the establishment of a reliable pre-basic seed production system for the vegetatively propagated crops.

Materials and Methods

The research was conducted in the screenhouse and Biotechnology laboratories of the CSIR - Crops Research Institute at Fumesua, Kumasi, Ghana. Vine, treated minisett, nodal cuttings of sweetpotato, yam and cassava respectively, were obtained from the CSIR-CRI breeding programs and planted in sterilized soil under screenhouse conditions (Temperature 285^{o}C and humidity 79%5) as source of mother plants. Plantain suckers, cocoyam and taro corms were obtained from fields of the breeding program as source of mother plant for in-vitro manipulations.

Young vigorously growing nodal cuttings were harvested from mother plants as explants for in vitro establishment. Surface sterilization of trimmed growing point of plantain, taro and cocoyam and nodal cuttings of yam, sweetpotato and cassava were as follows: explants were initially washed under running tap water and then soaked in a solution of fungicide containing 50% Carbendazim at a concentration of 10 g/l

water for 30 minutes. Explants were rinsed with sterilized water and transferred into 70% ethanol for 2 minutes, followed by rinsing and subjection to 20% solution of household bleach with 6% active Sodium Hypochlorite (NaOCL) for 20 minutes, rinsed with sterilized deionized water, trimmed further and transferred into 10% solution of household bleach with 6% active NaClO for 10 minutes. Explants were subsequently rinsed with sterilized water and maintained in the water prior to culturing on medium. Dissection microscope was used to excise apical meristem and cultured on appropriate meristem culture medium. Cultures generated from meristem were maintained in the dark for 2 weeks or till signs of growth and development were observed, prior to transfer to 16hr photoperiod. Composition of media used to culture meristem of the various crops was Murashige and Skoog's (1962) complete medium with vitamins supplemented with 3% sucrose 0.4% phytagel, growth additives as in the Table 1 and pH 5.7±0.2.

Cultures were inspected daily and transferred to the same medium every eight weeks till shoot and root developments were observed. Growing cultures were sub-cultured onto their respective rapid multiplication medium as detailed in Table 1 below. Cassava and yam cultures actively growing in vitro were subjected to molecular based virus indexing to certify they are clean from specified viruses.

Cultures were also subjected to indexing for bacterial to ensure there are no endogenous bacteria. Well-developed shoots of sweetpotato were subcultured onto rooting medium (rooting medium composition was the complete MS medium with 3% sucrose, 0.7% agar and no growth regulators) and established in the screenhouse prior to indexing via I. setosa grafting and NCM-ELISA procedures. NCM-ELISA kit was obtained from the International Potato Center (CIP). Following indexing, where cultures test positive for viruses, 2-3 week old cultures were subjected to thermotherapy treatment for four weeks. Thermotherapy treatment comprises of incubation in growth chamber at temperatures of 34°C for eight hours in darkness and 36°C for 16 hours of light photoperiod daily at relative humidity of 70-80%. Thermotherapy was followed by shoot tip excision and culturing on meristem medium. Cultures were tested again for virus. Cultures that tested negative for viruses were subjected to rapid multiplication to obtain required quantities, subcultures onto rooting medium prior to screenhouse establishment to raise seedlings for the field. Cultures that tested positive for the virus again were taken through thermotherapy treatment once again. The workflow is as presented in the Figure 1.

The various stages in the production of clean planting materials were identified and are as described in Table 2.

Table 1: Composition of initiation medium

Component Conc./L medium	Yam	Sweetpotato	Cassava	Taro & Cocoyam	Plantain
BAP	5 µM	50 µM	0.05 mg/L	5 µM	-
NAA	0.5 µM	-	0.01mg/L	0.5 µM	2 µM
AdSO4	80 mg/L	-	80 mg/L	80 mg/L	-
GA_3	0.225 µM	-	0.05 mg/L	-	-

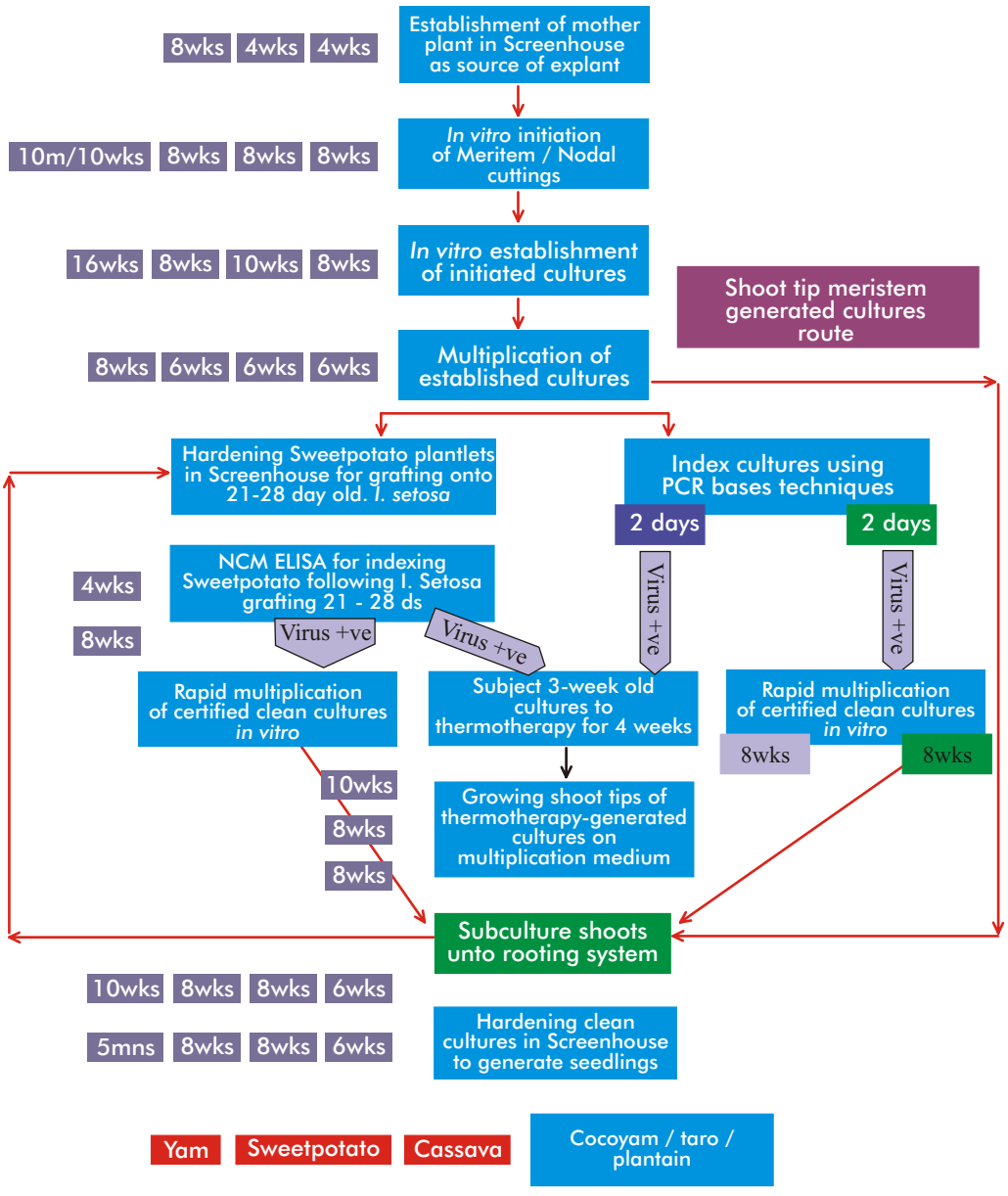

Fig. 1: Flow Chart illustration of production activities

Table 2: Description of Various Production Stages

Production Stage	Description
0	Establish the mother plant in the screenhouse as a routine source of explant for initiation in vitro. However, taro, cocoyam and plantains are normally obtained from the field and established directly in vitro, avoiding the stage 0.
1	In vitro initiation and surface sterilization. Involves initial introduction of explant into sterile environment. Operator has to successfully eliminate pathogens. The stage 1 ends at the period where signs of growth are observed in culture, such as bud sprouting out to form shoot.
2	Culture establishment entails excising the growing part from stage 1 onto fresh medium for further growth and development. May be referred to as rescue stage. Cultures were transferred to fresh medium every 4-5 weeks till further growth and development are observed.
3	Rapid multiplication of Stage 2 established cultures to increase the number of cultures prior to indexing cultures for presence or absence of pathogens.
4	Subculturing onto rooting medium, prior to hardening in screenhouse
5	Disease diagnostics of screenhouse established plantlets
6	Rapid multiplication of certified clean planting material
7	Thermotherapy treatment for cultures that test positive to disease
8	Excision of shoot tips following thermotherapy for growth on fresh medium in vitro
9	Screenhouse establishment of cultures generated from thermotherapy treatment for indexing

Results and Discussions

The experiment investigated the duration required to generate clean planting materials for yam, sweetpotato, cassava, taro, cocoyam and plantain. The various stages involved in the production of clean planting materials were outlined as the stages detailed in table 2. A total of ten major different stages of production were identified. Labeling of cultures were detailed to enable each explant to be tracked at every stage of development, as an original individual. The first stage was, however, not required for taro, cocoyam and plantains, as normally explants obtained from the field and established directly in vitro, avoiding the stage 0. During stage 1, operator has to deal with getting the tissues to survive sterilization to eliminate pathogens and some explants may be lost to over-sterilization or persistent pathogens that result in contamina-

tion and ultimate loss of culture. The stage 1 ends at the period where signs of growth are observed in culture, such as bud sprouting out to form shoot. Results obtained revealed that explants obtained from nodal cuttings may easily be lost to contamination due to persistence of pathogens. Where nodal cuttings were used as the starting explant for initiation, cultures that got contaminated was in the range of 30 to 100%. However, explants isolated from apical meristems had very low rate of contamination ranging from 0 to 30%. During stage 2 cultures were transferred to fresh medium every 4-5 weeks till further development such as more leaf development, shoot increase in height and root development are observed. Established cultures were rapidly multiplied (Stage 3) to increase the number of cultures prior to indexing cultures for presence or absence of pathogens.

Currently, the laboratory has not established indexing and certification processes for taro, cocoyam, and plantain; hence cultures are always generated from apical meristem to assume that seedlings generated are apparently clean. This is due to the fact that initiating cultures at stage 1 using meristem cultures improve the chances of having clean planting cultures by stage 3. Initiated cultures of taro, cocoyam and plantain therefore reach stage 3 within 7 months.

Initiation from meristems is lengthy, our results (Fig. 2) indicated that yam cultures need up to 10 months unlike sweetpotato and cassava which take 2 to 3 months to have a whole plant developing from apical meristem. Following indexing for bacteria and viruses (stage 5), cultures which test negative to pathogens are advanced to rapid multiplication (stage 6) to generate the required quantities. However, cultures that tested positive to pathogens were subjected to thermotherapy (stage 7) followed by excision of the shoot tip onto multiplication medium, (stage 8), establishment in the screenhouse (stage 9) and then subject to indexing again. Our research has indicated that, in vitro manipulations via meristem cultures are able to efficiently eliminate viruses. Where pathogens are persistent, inclusion of thermotherapy process has successfully eliminated pathogens.

Data generated as presented in Figure 2. indicated that the system requires up to 15 months to generate certified clean planting materials of yam using nodal cuttings as initial explants and more than 24 months where apical meristems are used as explants. Sweetpotato, cassava, and taro group (taro, cocoyam and plantain) requires up to 13.8, 11.3 and 7 months respectively. It is therefore critical that as part of breeding programs, production of certified clean planting materials is conducted to make them available for dissemination when required. Once clean planting materials are generated they can be maintained in vitro and rapidly multiplied when required. The advantage of the Tissue culture system is that if the procedure generates just a single clean culture, it can be rapidly multiplied overtime to generate the needed clonal materials and production is all-year-round (Tegen and Mohammed, 2016).

In vitro manipulation is a reliable method for the mass propagation of true to type planting material of premium quality which would ensure high yields, and several researchers including Bachraz (1998), Mtui (2011) and Quain and Dzomeku (2013), have emphasized the vital role of the technique. Some research programs may consider avoiding the use of tissue culture methods due to the cost involved (Tegen and Mohammed 2016) as well as the duration, however, this is the main tool in horticulture that has contributed to meeting production demands as well as providing jobs (Tengen and Mohammed, 2016). The information provided by this study

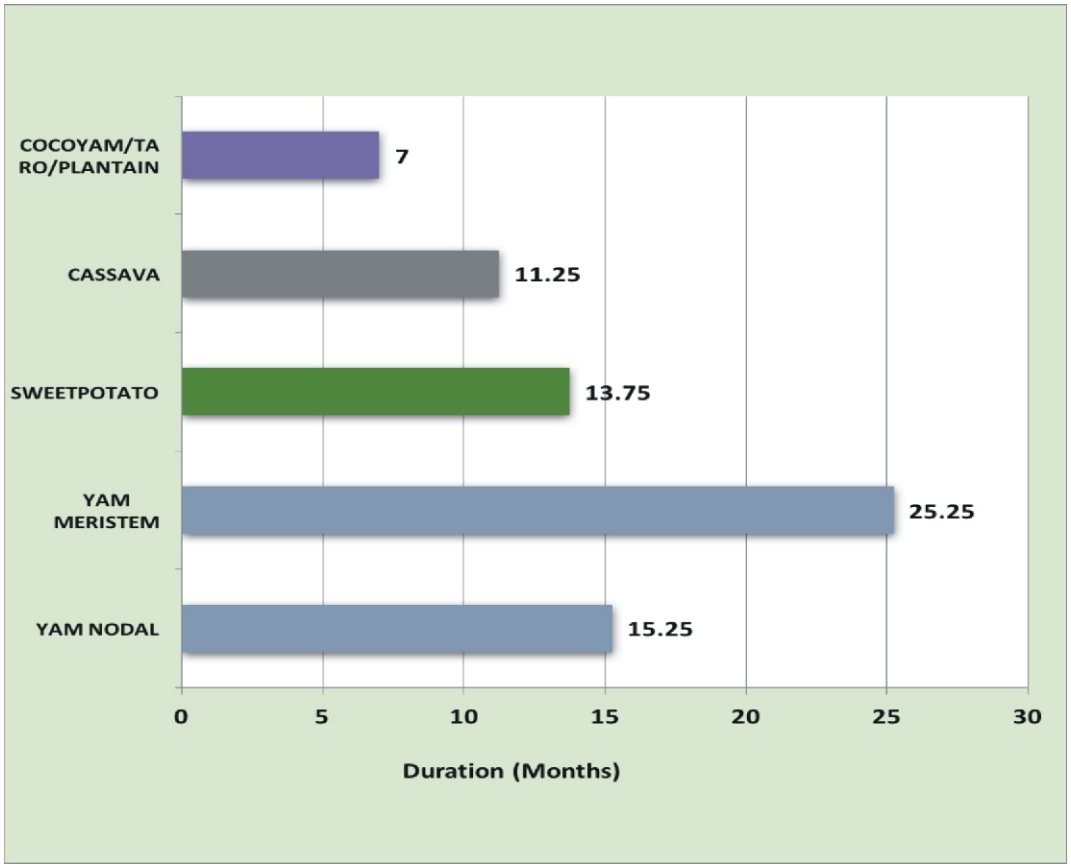

Figure 2: Graphic presentation indicating duration (in months) required for the production of Yam, Sweetpotato, Cassava, Taro, Cocoyam and Plantain Clean planting materials

is vital for researchers and policy makers in their efforts to ensure the safe dissemination of quality declared planting materials.

Conclusion

Plant tissue culture techniques have great potentials for the mass rapid propagation of clonal material. The technique ensures the distribution of clean planting materials. Stakeholders are frequently challenged with the actual duration required to generated a clean planting material once a mother plant is obtained from the field. To the best of our knowledge this is the first publication of a schematic outline of production stages, and duration for vegetatively propagated crops vital for the Sub-Saharan African regions especially Ghana. These timelines should the required information for the effective inclusion of tissue culture techniques in crop production.

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