Development of efficient transformation and regeneration protocols in elite genotypes of cassava (*Manihot esculenta* Crantz)

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(2010-09-117)

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Certified that this thesis entitled "Development of efficient transformation and regeneration protocols in elite genotypes of cassava (*Manihot esculenta* Crantz)" is a record of research work done independently by Ms. Rini Jose E. (2010-09-117) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

BAP	6- benzyl amino purine
FEC	Friable embryogenic callus
MS	Murashige and Skoog
2,4 D	2,4 dichlorophenyl acetic acid
PEC	Primary embryigenic callus
NaCl	Sodium chloride
NAA	α- naphthaleneacetic acid
GUS	β glucuronidase
AgNo$_3$	Silver Nitrate
TDZ	Thiadiazuron
HgCl$_2$	Mercuric chloride
PVP	Polyvinyl pyrrolidone
SDS	Sodium dodecyl sulphate
%	Per cent
µm	Micro molar

μl	Micro litre
hrs	Hours
°C	Degree Celsius
cm	Centimeter
DAI	Days after inocultaion
$CuSO_4$	Copper sulphate
et al.	And other co workers
Fig.	Figure
g	Gram
M	molar
mg	Milli gram
ml	Milli litre
min	Minutes
CMD	Cassava mosaic disease
KAU	Kerala Agricultural University
MS	Murashige and Skoog
SH	Shenck and Hildebrandt

INTRODUCTION

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz*)* is a tuberous, woody, shrubby perennial plant, of the Euphorbiaceae family characterized by palmately lobed leaves. Cassava is the fourth largest source of food for carbohydrates after rice, wheat and maize and also one of the major staple food in the developing world, providing a basic diet for over 500 million people (Bahekar *et al.,* 2013). It is one of the most drought-tolerant crops, capable of growing on marginal soils. Nigeria is the world's largest producer of cassava and the tuber yield of cassava is influenced by both the quality of planting material used and the agronomic practices employed. All the major cassava growing countries in the Asian continent have higher productivity than the world average. Indonesia, Thailand, Vietnam and India are the major countries growing cassava in Asia. Although it is cultivated in India in 13 states, it is concentrated in the southern peninsular region and to a certain extent in northeast region of the country. In India, cassava is mainly grown in Kerala, Tamil Nadu, Andhra Pradesh, NEH region etc. over an area of 0.28 million ha with a production of 9.62 million t and having the productivity of 34.37 t ha^{-1}, the highest in the world.

Cassava is regarded as a 'famine reserve crop', and is even planted in areas where other crops fail. Cassava starch is also used for the production of biodegradable plastics (Sam *et al.*, 2009). In Brazil alcohol extracted from cassava starch is used as a major component in gasoline. Cassava root is essentially a carbohydrate source. Its composition shows 60–65 percent moisture, 20–31 percent carbohydrate, 1–2 percent crude protein and a comparatively low content of vitamins and minerals. However, the roots are rich in calcium and vitamin C and contain a nutritionally significant quantity of thiamine, riboflavin and nicotinic acid. Cassava starch contains 70 percent amylopectin and 20 percent amylose. Cooked cassava starch has a digestibility of over 75 percent.

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Cassava is one of the main food intakes of many people in different parts of the world. Development of improved varieties through conventional breeding is problematic in cassava which is vegetatively propagated and characterized by strong heterozygosity and inbreeding depression. Breeding programs in cassava consist of crossing elite parents and screening thousands of first-generation progeny for desired characteristics. Conventional breeding leads to inclusion and multiplication of undesired characters along with the desirable traits (Wendy *et al.*, 2015).

Biotechnological tools like tissue culture techniques can play a major role in overcoming the problems associated with those classical modes of propagation and improvement (Santana *et al.*, 2009). *In vitro* culture techniques are mainly used for rapid multiplication of planting materials, meristem culture for virus elimination, for producing synthetic seeds, transgenics, germplasm exchange etc.

As an alternative, in order to overcome the drawbacks of traditional hybridization, transgenic technology opens new vistas of even trans kingdom gene transfer. This powerful tool allows plant breeders to do faster what they have been doing for years in order to generate superior plant varieties. One of the main target areas of transformation is protection against pests, pathogens, and environment stresses while the recent plant engineering programs are aimed at increasing food quality, in particular at increasing nutritional characters of food crops.

The most widely used method for the genetic transformation of plants is based on the conjugation like DNA transfer from the plant pathogenic soil bacterium *Agrobacterium tumefaciens* to wounded plant cells. It is attached to the amino acids, sugars, and organic acids released from the wounds of the plant tissues. *Agrobacterium* is the commonly used plant vector because it satisfies the desirable features of the plant vector (Stanton 2003). *Agrobacterium* mediated plant transformation is a highly complex and evolved process involving genetic determinants of both the bacterium and the host plant cell.

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Development of transgenic crops during 1990s is an important landmark in the history of crop improvement. Transgenic technology can have the biggest impact among the vegetatively propagated crops. Being one of the most important climate resilient crops intense research is being carried out all over the world to develop transgenics in cassava with improved traits *viz.* high starch content, waxiness, high protein content, CMD and brown streak disease resistance, low cyanogen content etc. The limiting factor for the improvement of cassava by genetic engineering, however, has been the lack of a reproducible transformation system to facilitate the introduction of foreign genes into this crop.

Although a number of transformation systems and selection regimes have been developed for cassava, they have only been applied in a limited number of genotypes. This limitation of the applicability of the systems is mainly due to variation in morphological responses of different genotypes to the regeneration and transformation procedures employed, which underscores the need to study all possible parameters for any given cultivar. In India, the development of transgenics has been hampered by the non availability of highly efficient transformation and regeneration protocol in popular varieties. Development of friable embryogenic calli, even though difficult, will improve the transformation efficiency in cassava. Hence the present investigation was undertaken to identify cassava clones with high regeneration efficiency through somatic embryogenesis and to standardize efficient protocol for transformation using different *Agrobacterium* strains.

REVIEW OF

LITERATURE

2. REVIEW OF LITERATURE

Cassava (*Manihot esculenta* Crantz) is a staple food for more than 800 million people which has a great economic importance worldwide. It is one of the most important food crops in tropical and subtropical regions in the world and occupies fifth position in terms of global calorie consumption, just behind wheat, rice, corn, and sorghum (Adeyemo, 2009). According to FAOSTAT 2013, cassava is the second most important staple in the least developed countries after rice both in terms of production and food consumption. It is a major tuber crop cultivated in 13 states of India which is grown in an area of 2.34 mha, with Kerala ranking first with an area of 1.04 mha followed by Tamil Nadu. Cassava is known to be well adapted to drought prone environments and it also grows well in poor soil, where cereals and other crops do not thrive. There are about 100 wild *Manihot* species, which provide an important genetic treasure for cassava breeding.

An estimated 600 million people in tropical and subtropical climates depend on cassava as their source of energy (Bull *et al*. 2011). Also its importance is increasing as animal feed (Nwokoro, *et al*., 2002); as starch source (Srinivas, 2007) in processing and chemical industries; and for paper products textiles, cosmetics and pharmaceuticals (Balagopalan, 2002). Cassava has been used as a source for biofuel production in countries such as Nigeria (Ademiluyi and Mepba, 2013) and Uganda (Nuwamanya, *et al*., 2012). Also the cellulosic waste which is obtained after starch processing was utilized for bio-ethanol production and the insoluble carbohydrate, protein, fibre and residual starch content of the waste was hydrolysed, saccharified and fermented using α-amylase or HCl, amyloglucosidase and *Saccharomyces cerevisiae* respectively. Elias *et al*., (2015) reported that combination of enzymatic and acid hydrolysis recovered much of the starch and cellulose than when either of them was used. Dilute HCl was more helpful in converting the cellulosic materials to

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reducing sugars. A total of 32.4% alcohol (2.7 g ethanol/15 g cellulosic waste) was obtained which indicates that cassava wastes could also be transformed to chemicals for use as fuels, biochemicals, synthetic intermediates etc.

Vegetative propagation of cassava was carried out by using cuttings but low productivity is caused by continuous clonal multiplication and the infestation by diseases transmitted through successive generations. Because of the slow and low propagation rate of cassava, high genetic heterozygosity, low pollen fertility, limited gene availability in the sexually compatible germplasm and the outcrossing nature with strong inbreeding depression of the plants prevent genetic improvement through conventional techniques (Raemakers, *et al.*, 1996). The conventional breeding of cassava is seriously hindered due to the serious trait separation in progeny (Ceballos, *et al.*, 2010, 2012).

Establishment of *in vitro* cultures to initiate micropropagation were carried out by growing meristems, which allows for the production of healthy plantlets which gave high-quality propagating material. And this strategy is ideal for a transgenic approach to crop improvement, because gene segregation through outcrossing is limited. However, clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species (Ribeiro, 2012).

Biotechnology is a powerful tool to overcome the main problems in traditional breeding programe and it can widen the genetic pool for useful genes over species barrier (Raemakers *et. al*, 1997). Genetic engineering is an alternative effective method used to introduce value-adding genes to modify important agronomic traits (Taylor *et al.*, 2004; Liu *et al.*, 2011; Sayre *et al.*, 2011). Transgenic method has become an important tool for cultivar improvement in several crops as well as for studying the gene function in plants.

2.1. Somatic embryogenesis

Somatic embryogenesis forms the basis of all current transformation methods of cassava. Cyclic embryogenesis is the most routinely used method of *de novo* plant regeneration of cassava *in vitro* and provides constant source of material for transformation. Shoot organogenesis from cotyledons of somatic embryos and friable embryogenic callus   are the two most frequently used systems for transformed cassava plants.

The first observations of *in vitro* somatic embryogenesis were made in *Daucus carota* (Reinert, 1958, 1959, Steward *et al.,* 1958). Successful regeneration was reported when explants were grown in induction medium with plant growth regulators or with different growth regulators. Roca (1984) reported that methods for regeneration of cassava cultures, is generally difficult and genotype-dependent. Regeneration through direct somatic embryogenesis has been obtained from cotyledons, embryogenic axes of seeds or from young leaflets (Tilquin *et al.,* 1979, Stamp and Henshaw 1982, 1987, Konan *et al.,* 1994; Guo-Huama and Jun- Yan Guo 1996; Mathews *et al.,* 1993, Mroginski and Scochi 1993, Li *et al.,* 1996). Successful regeneration was reported when explants were grown on an induction medium with plant growth regulators or with different growth regulators. Attempts have been made to increase the efficiency of regeneration and recovery of plants using several methods like 'desiccation 'of the initial embryogenic callus before transfer to the subsequent regeneration medium (Reamakers *et al.,* 1993; Mathews *et al* 1993). Use of suppressants like abscisic acid (Konan *et al.,* 1994), synthetic promoters like picloram and dicamba promoted somatic embryogenesis.

The most amenable transformation technique in cassava plant is *via* regeneration through somatic embryogenesis. Subculturing and recycling of somatic embryos for a lengthy period of time serve as constant sources of explants required for genetic transformation. But recycling of cassava somatic embryos is very difficult

and it reduces the frequency and efficiency of somatic embryogenesis and plant regeneration (Opabode *et al.,* 2014) and it induces genetic alteration in the form of DNA methylation, chromosome rearrangements and point mutations (Nadha *et al.,* 2011). For overcoming these challenges, there is need to establish *in vitro* preservation procedure of cassava somatic embryo to serve as a reliable source of explants for genetic transformation. Mannitol and sorbitol addition to the culture media has been reported to be efficient in reducing growth and increases the storage life of plants (Shibli *et al.,* 2006). Withers in 1979 carried out an experiment to recover the plantlets from somatic embryos which were frozen on semi solid medium. Also, Jayasankar *et al.,* 2005 successfully stored somatic embryos of grapevine for 26 months at 40°C and 80% plantlet were recovered. Fresh cotyledonary stage secondary somatic embryos were carefully removed from solid medium on which they were developed under sterile conditions. Isolated secondary embryos were transferred to fresh BM supplemented with phytohormones supplements. After three weeks embryogenic calli were transferred to BM supplemented with 0.1 mg/l of BAP for maturation under light. To determine the genetic stability of the regenerated plants RAPD technique was used (Opabode *et al.,* 2015).

Auxins and cytokinins are used during somatic embryo maturation and regeneration (Hankoua *et al.,* 2005; 2006; Medina *et al.,* 2007). Fan *et al.,* 2011 demonstrated that NAA auxin regulated organ growth while BA cytokinin facilitated cell division and elongation in cassava tuberisation. He carried out the work to standardize the media for regeneration were the medium M3 comprising 2 mg l^{-1} BAP in combination with 0.01 mg l^{-1} NAA and 1.5 mg l^{-1}GA3, was the best for maturation of somatic embryos. Activated charcoal provides a dark environment in medium, adsorb inhibitory chemical compounds, mainly phenols provides a soil-like conditions (Liu, 1993; Teixeria *et al.,* 1994). Also the release of adsorbed products

such as nutrients and PGRs in culture media, in addition to substances naturally present in activated charcoal that promote plant growth (Thomas et al., 2008).

Friable embryogenic callus (FEC) is considered as the most suitable material for efficient genetic transformation of cassava (Bull *et al.*, 2009; Liu *et al.*, 2011; Xu *et al.*, 2013). Several protocols using FEC as target tissues and particle bombardment or *Agrobacterium*-mediated transformation procedures have been reported (Taylor *et al.,* 2012; Zainuddin *et al.,* 2012; Nyaboga *et al.,* 2013). FEC was produced in GD medium with varying levels of maltose, sucrose, tyrosine, tryptophan, NAA under light and dark conditions (Hellen *et al.*, 2015). FEC was produced through several steps as follows: primary somatic embryo (SE) induction, secondary SE multiplication, fresh FEC (FFEC) induction, and FEC subculturing and multiplication (Zhang, 2000). In cassava, primary and secondary SEs are produced by inoculating explants like immature leaf lobes, axillary buds, petioles in basal media with auxins like picloram or 2,4-D. 8mg/l 2,4-D concentration given best callus formation for the cassava cultivars (Eugene *et al.*, 2011). On inoculation of these secondary embryos in GD medium supplemented with 50μM picloram, produced FEC in the model cultivar TMS60444 (Taylor *et al.*, 1996; Bull *et al.*, 2009). But there are various factors including explant types, basal media, and hormones that affect somatic embryo and FEC induction process (Liu *et al.,* 2011). The studies to optimize the FEC transformation in several farmer preferred cassava cultivars were conducted (Zainuddin *et al.*, 2012; Chetty *et al.,* 2013; Chauhan *et al.*, 2015). In OFEC(old FEC) there was a decrease of sucrose and increase of fructose and glucose were detected. Also there was a significant reduction of genomic DNA methylation in OFEC which indicated altered gene expression *via* chromatin modification. From these results it indicated that the induction and long-term subculture of FEC is a complicated biological process involving changes of genome modification, sub cellular reconstruction, and gene expression. These findings pointed out that

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improving FEC induction and maintenance can be done through cassava cultivars through genetic engineering tools. (Qiuxiang *et al.*, 2015*)*.

2.2. *Agrobacterium tumefaciens*

Agrobacterium tumefaciens naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors The GUS reporter system (*GUS*: betaglucuronidase; *uid*A) is a reporter gene system, particularly useful in plant molecular biology. The GUS assay technique helps for the confirmation step in the successful transformation process (Paula *et al.*, 2014).

2.3 Transgenics in Cassava

Genetic transformation of cassava is being done across the world for reducing cyanogenic content, creating disease and pest resistant varieties, delaying post-harvest physiology deterioration in storage roots, increasing starch production, and for improving the overall quality of cassava (Liu *et al.*, 2011, Sayre *et al.*, 2011).

2.3.1. Reduced cyanogens content

Leaves and roots of cassava contain potentially toxic levels of cyanogenic glucosides like linamarin which is stored in the vacuole. By tissue disruption linamarin is deglycosylated by the apolplastic enzyme, linamarase and produce acetone cyanohydrins which can spontaneously decomposes and enzymatically broken down by hydroxynitrile lyase (HNL) to produce acetone and free cyanide which is then volatilized. According to Narayanan *et al.*, (2011) the over-expression of HNL in cassava roots under the control of a root-specific, patatin promoter would accelerate cyanogenesis during food processing also resulting in a safer food product, but lead to increased root protein levels. There was increase in HNL level in transgenic lines expressing patatin promoter when compared with the wild type. The linamarin levels in intact tissues were reduced by 80% in transgenic cassava roots.

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Siritunga and Sayre, (2003) reported the transgenic approaches to reduce cyanogen in cassava root by suppressing cyanogen synthesis or by accelerating cyanogen breakdown and thus transgenic acyanogenic cassava plants were generated in which the expression of cytochrome P450 genes (CYP79D1 and CYP79D2), which catalyze the linamarin synthesis, was inhibited. There was a reduction in 94% leaf linamarin by inhibition of CYP79D1 and CYP79D2 expression. Also the linamarin content of roots also was reduced by 99% in transgenic plants.

2.3.2 Pest and disease resistance

The vegetative propagation of cassava through stem cuttings has many advantages, but the pathogens are passed from one generation to the next and thus there was a gradual decrease in cassava production. Cassava mosaic Gemini viruses are the major cause in African and Asian countries. Virus control strategies were examined which includes diagnostics and surveillance, prevention and control of infection using phytosanitation, and control of disease through the breeding and promotion of varieties that inhibit virus replication and/or movement (James *et al.*, 2015). But all these strategies depend on conventional improvement programmes, which have encountered massive limitations (Ceballos *et al.*, 2004). The reports indicated success in generation of disease-free plants, such as transgenic papaya against ringspot virus (Gonsalves, 1998) and CMD-free cassava (Vanderschuren *et al.*, 2007). By genetic engineering tool CMD and CBSD resistance cassava was developed. CMD is another disease caused to cassava by ICMV and SLCMV which reduces the cassava production. Transgenic cassava lines resistant to SLCMV were developed through RNAi vector targeting a conserved 440 bp of 5' end of SLCMV *Rep* (AC1) gene which also overlaps with part of AC4 gene, and functions as a viral RNAi suppressor protein. Co cultivation of cassava embryogenic calli with *Agrobacterium* was done with the developed RNAi constructs using two different

explants namely, immature leaf lobes and somatic cotyledons (Anuradha *et al.*, 2015).

Ntui *et al.*, (2015) carried out a work to make cassava plant resistant to SLCMV by RNA interference (RNAi) in order to increase biomass yield. DsRNA of SLCMV was highly expressed and it did not induce any growth abnormality in the transgenic plants and highly resistant to SLCMV compared to wild plants. Analysis was carried out by PCR amplification and RT-PCR. It shows that the resistance is correlated with post-transcriptional gene silencing.

2.3.3 To delay post harvest deterioration

Post harvest physiological deterioration (PPD) of cassava storage roots is the result of a rapid oxidative burst, which leads to discoloration of the vascular tissues due to the oxidation of phenolic compounds (Xu *et al.*, 2013). By increasing the level of APX and GPX activity, and the total protein contents occurred from 3 to 5 days of storage were correlated with the delay of PPD. Periodic Acid-Schiff (PAS) stain used in cassava samples stained and highlighted the presence of cellulose degradation of starch granules during PPD. Metachromatic reaction toluidine blue method also indicated the increase in acidic polysaccharides which play an important role in PPD delay. Samples were classified according to Principal component analysis (PCA) and the levels of enzymatic activity based on the decision tree model (Uarrota *et al.*, 2016). In 2013, Xu reported that coexpression of the reactive oxygen species (ROS)-scavenging enzymes copper / zinc Superoxide dismutase (MeCu/ZnSOD) and catalase in transgenic cassava was used to explore the intrinsic relationship between ROS scavenging and PPD occurrence. Quantitative reverse transcription-polymerase chain reaction and southern blot analysis were used to study the enzymatic activity and expression both in the leaves and storage roots. Levels of chlorophyll degradation, malondialdehyde, lipid peroxidation, and H_2O_2 accumulation were

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reduced in the transgenic lines compared with the wild type. In transgenic cassava lines PPD response was delayed upto 10 days compared with those of the wild type. (Xu *et al.*, 2013). *Arabidopsis* AOX over expression in transgenic cassava roots showed reduction in ROS accumulation and delayed the PPD for about three weeks after the harvest of the crop (Solomon *et al.*, 2011)

Rapid burst of reactive oxygen species (ROS) accumulation is the earliest biochemical events during the initiation of PPD. ROS accumulation was measured in transgenic low-cyanogen plants with and without cyanide complementation. To reduce cyanide-dependent ROS production in cassava root mitochondria new transgenic plants are generated which express a codon-optimized *Arabidopsis (Arabidopsis thaliana)* mitochondrial alternative oxidase gene (AOX1A) which is cyanide insensitive. Transgenic plants over expressing AOX exhibited over a 10-fold reduction in ROS accumulation compared with wild-type plants. It was observed that there was biomass yield loss in AOX expressing lines (Tawanda *et al.*, 2015)

2.3.4 To increase starch production

Genetic modification of cassava was carried out to express zeolin, a nutritionally balanced storage protein under control of the patatin promoter. Transgenic plants that accumulated zeolin recorded increase in protein levels. Transgenic approaches are therefore an attractive method for the accumulation of nutritionally balanced or industrially valuable protein to these large storage organs (Abhary *et al.*, 2011). Waxy cassava was developed by CIAT, using the antisense RNA technology. The key enzymes in starch biosynthetic pathway are ADP-glucose pyrophosphorylase and starch synthases. The starch synthase enzymes add glucose units to the non reducing end of glucan chains via α1-4 linkages. Waxy starch from cassava has improved paste clarity, low retro-gradation and better freeze thaw stability (Krishna *et al.*, 2014). Xuchu *et al.*, (2016) reported that many enzymes involved in starch and sugar metabolism were upregulated. Among them, three 14-3-

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3 isoforms were induced to be clearly phosphorylated during storage root enlargement. Over expression of a cassava 14-3-3 gene in *Arabidopsis thaliana* confirmed that the older leaves of these transgenic plants contained higher sugar and starch contents than the wild-type leaves.

2.3.5 To improve stress tolerance

Transgenic cassava plants that co-express cytosolic superoxide dismutase (SOD), MeCu/ZnSOD, and ascorbate peroxidase (APX), MeAPX2, were produced and tested for tolerance against oxidative and chilling stresses. The up-regulation was confirmed by the quantitative reverse transcriptase polymerase chain reaction, and enzymatic activity analyses in the leaves of transgenic cassava plant lines with a single-transgene integration site. Xu *et al.*, (2014) suggested that the expression of native cytosolic SOD and APX simultaneously activated the antioxidative defense mechanisms *via* cyclic ROS scavenging, thereby improving its tolerance to cold stress.

2.3.6 To improve nutritional quality

Food insecurity and inadequate caloric intake is a major cause of death and mortility in the world, particularly in developing countries (Fiedler *et al.*, 2009, Pelletier *et al.*, 2005, Zimmermann *et al.*, 2007). Chronic malnutrition, or insufficient intake of essential nutrients and vitamins, affects more than two billion people worldwide, contributing to considerable illness, disability, and mortality (Caulfield *et al.*, 2006). Solution for malnutrition is the development of biofortified crops (Qaim *et al.*, 2007, Stein *et al.*, 2006, White *et al.*, 2005). Ihemere *et al.*, (2006) Siritunga *et al.*, (2003) reported that efficient Class II patatin promoter determined the selectively drive expression of transgenes coding for nutritional traits in storage roots.

Carotenoid content in cassava roots has been increased through biofortification programs to solve the problem of vitamin A deficiency (Aragon *et al.,* 2016). Iron is extremely abundant in the soil, but its uptake in plants is limited due to low solubility in neutral or alkaline soils. By the expression of *AtVIT1* in cassava using patatin promoter showed 3–4 times higher values when compared with wild-type plants. There was an increase in 4 and 16 times higher values of iron concentration in the young stem and stem base tissues of transgenic cassava plants respectively. These results demonstrated vacuolar iron sequestration as a viable transgenic strategy to biofortify crops for solving micronutrient malnutrition in human populations. (Narayanan *et al.,* 2015).   Telengech, 2015 reported that biofortification with pro-vitamin A can help to reduce Vitamin A Deficiency among the undernourished communities. BioCassava Plus project has developed transgenic cassava that expressed beta carotene in roots using root specific patatin promoter. The study aimed at confirming expression of *nptII, crtB* and *DXS* gene. In 2010 Fregene, reported the accumulation of β carotene, a precursor of Vitamin A, in the model cultivar TMS 60444 in Puertorico, Uganda and Kenya . In Puerto Rico it was found out that the biofortified cassava contains up to 40 % increase in beta carotene.

The transgenic cassava was transformed with two transgenes: *crtB* phytoene Synthase gene from *Erwinia herbi*color and 1-deoxy-D-xylulose-5- phosphate synthase gene (*DXS*) from *Arabidopsis thaliana* controlled by patatin promoter. The *crtB* gene controlled beta carotene synthesis through geranylgeranyl-diphosphate (GGDP) in the plastid isoprenoid pathway. *DXS* is intended for increased concentration of GGDP for b-carotene synthesis. (Naumkina *et al.,* 2007). The beta glucoronidase (GUS) gene under the control of class I patatin promoter in the transgenic potato is sufficient to drive tuber specific and sucrose inducible expression of the fused *GUS* gene. (Martin *et al.,* 1997, Hellmann *et al.,* 2000). Expression studies were carried out by RT-PCR and southern blot analysis.

In 2012 Ademola *et al* conducted a study on developing GM super cassava for improved health and food security Recently, multi-national non-governmental organizations have enhanced the provitamin A carotenoid content of cassava, either through traditional plant breeding (Harvest Plus) or bioengineering (BioCassava Plus) (Sayre *et al.*, 2011, Bouis *et al.,* 2011). Their efforts have resulted in yellow-orange-fleshed cassava cultivars with moderately high concentrations of β carotene and other provitamin A carotenoids.

2.4 Transformation and regeneration

Recovery of transgenic 60444 plants was achieved through the production of friable embryogenic callus (FEC) (Taylor *et al.*, 1996, 2001) which is brought into contact with *Agrobacterium*, followed by selection on medium supplemented with an aminoglycoside antibiotic such as paramomycin (Taylor *et al.*, 2001) or hygromycin (Bull *et al.*, 2009) and the somatic tissues were formed *in vitro* using immature leaf lobes and axillary buds, which were in turn converted to the FEC by sequential subculture on medium containing Gresshoff and Doy (GD) basal salts (Gresshoff *et al.*, 1972). By coculturing with *Agrobacterium* strain LBA4404, colonies of putatively transgenic FEC were recovered on antibiotic medium and regenerated to mature cotyledon stage embryos. Whole plants were recovered on MS media (Murashige *et al.*, 1962) supplemented with benzyl amino purine (BAP). Sheela and Nigel 2007 studies the efficiency of different *Agrobacterium* strains *viz.* AGL 8, GV3101, C58C1, LBA 4404,and EH 105 in transformation of cassava. They reported maximum recovery of transgenic tissues when EH 105 was used.

Opabode *et al.*, (2013) investigated the somatic embryogenesis, plant regeneration and genetic stability of regenerants grown from cassava secondary somatic cotyledon preserved at 16^0C on medium containing mannitol or sorbitol alone and their combinations. Irrespective of osmotic agents in the medium, survival

of cotyledon explant, frequency of somatic embryos, shoot induction, number of somatic embryo per explant, shoot elongation and rooting decreased as preservation period increased.

Plants were recovered on media with 6-Benzyl aminopurine (BAP) and GA_3 under a 16 hour light/8 hour darkness photoperiod. High frequencies of callus induction (>98%) for both cultivars, were obtained when 2,4-D and Picloram were used. Similarly, both auxins initiated somatic embryogenesis, with Picloram producing the highest frequency of somatic embryos (>92%) in TMS 60444, using stem explants. Gus assays revealed high frequencies of transformation of >77% in TMS 60444 (Marigi *et al.*, 2016).

Anjum and Shazia 2015 carried out some studies to develop an easy high frequency reproducible method for plant propagation and regeneration through plant tissue culture of cassava (*Manihot esculanta*). Explants were initiated using Murashige and Skoog (Ms) with 3mg/L BAP, 0.5mg/L NAA and 1mg/L IBA. Adenine sulphate and glutamine were also added at the rate of 25 mg/L and 50 mg/L respectively, along with 0.6 g/L charcoal. Highly proficient root length was observed on MS in combination with 0.5 mg/L NAA.

Cassava Mosaic Virus (pCsVMV) 35S promoter from the Cauliflower Mosaic Virus (pCaMV) is a constitutive promoter commonly used in plant genetic transformation. The combination of the two promoters was used to form pOYE153. The method adopted includes the insertion of a *β–glucuronidase* reporter gene (*UidA*) into a promoter cassette comprising the CsVMV promoter. The second construct (pCAMBIA2310) had (pCaMV) used for the selectable marker and gene of interest. This construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404 and then tested for expression of the *UidA* gene in transient assays in cassava somatic embryos. The results showed that the pCsVMV was able to drive high gene

expression of β–glucuronidase reporter gene (UidA) in the transient assays in cassava somatic embryo (Olufemi *et al.*, 2015).

2.4.1 Visual markers in transformation:

Red fluorescent protein (DsRed) from reef coral was evaluated in comparison with green fluorescent protein (GFP) as a reporter gene for cassava transformation. Cassava friable embryogenic callus (FEC) was transformed with ERtargeted versions of DsRed and GFP constructs driven by the 35S cauliflower mosaic virus promoter. High and uniform DsRed expression was observed at the single cell and proliferating callus stages, in somatic embryos. Plants expressing GFP and DsRed were phenotypically normal with regard to growth, vigor, and formation of storage roots. A comparative evaluation of DsRed expression with GFP was carried out to determine the possible application of DsRed as an additional visual marker system in cassava transformation (Ihuoma *et al.*, 2015). The transformation efficiency for cassava variety 60444 used in this study has been extensively optimized as compared to other farmer-preferred cultivars (Taylor *et al.*, 2012).

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MATERIALS
AND METHODS

3. MATERIALS AND METHODS

The study entitled "Development of efficient transformation and regeneration protocols in elite genotypes of cassava (*Manihot esculenta* Crantz) was carried out at the Division of Crop Improvement; ICAR- Central Tuber crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. In this chapter, details pertaining to the experimental materials and procedures used in the study are elaborated.

3.1 Genotypes of cassava

Thirteen genotypes of cassava that include popular released varieties and CMD resistant prerelease lines were used for the study as enlisted in Table 1.

Table 1. Details of the varieties/prebreeding lines used for the study

Sl.No.	Genotype No	Details	Source
1.	9S-127	CMD resistant hybrid	ICAR-CTCRI
2.	9S-174	CMD resistant hybrid	ICAR-CTCRI
3.	Sree Sahya	Released variety	ICAR-CTCRI
4.	Sree Prakash	Released variety	ICAR-CTCRI
5.	CI-273	CMD resistant accession	ICAR-CTCRI
6.	Vellayani Hraswa	Released variety	KAU
7.	IPS2-1	CMD resistant hybrid	ICAR-CTCRI
8.	8W5	CMD resistant hybrid	ICAR-CTCRI
9.	11S4	CMD resistant hybrid	ICAR-CTCRI
10.	Mulluvadi	Released variety	TNAU
11.	Sree Athulya	Released variety	ICAR-CTCRI
12.	Sree Apoorva	Released variety	ICAR-CTCRI
13.	H 226	Released variety	ICAR-CTCRI

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3.2 Medias used for screening the cassava genotypes

The basal media standardised by Murashige & Skoog, 1962 (MS) and Schenk & Hidebrandt, 1972 (SH) were used in the experiments. In different experiments the formulation of the basal media was suitably modified. Different growth regulators (NAA, BAP, 2 4- D, picloram, Thiadizuron) were also included in the media depending on the requirements.

3.2a Media used for initial regeneration

Table 2. Medium used for the *in vitro* establishment of cassava varieties

MEDIUM	COMBINATIONS (1 L)
M 1	4.4 g MS Salt + 30 g Sucrose + 0.5 μM NAA + 2 μM BAP + 2 μM $CuSO_4$ + 8 g Agar
M 2	4.4 g MS Salt + 30 g Sucrose + 0.25 μM NAA + 1 μM BAP + 2 μM $CuSo_4$ + 8 g Agar
M 3	4.4 g MS Salt + 30 g Sucrose + 2 μM $CuSo_4$ + 8 g Agar
M 4	4.4 g MS Salt + 30 g Sucrose + 0.5 μM BAP + 2 μM $CuSo_4$ + 8 g Agar

3.2 b. Standardization of SH and MS medium with varying combinations of TDZ and $AGNO_3$.

Cassava varieties namely 9S 127 and H 226 were inoculated in MS and SH medium with varying combinations of TDZ and $AgNO_3$ to standardize the growth response.

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Table 3 MS medium with varying combinations of TDZ and AgNO$_3$.

Sl No	Medium	Combinations of TDZ and AgNo$_3$
1	A1	4.4 g MS Salt + 30 g Sucrose + 1 µM TDZ + 1 µM AgNO$_3$ + 8 g Agar
2	A2	4.4 g MS Salt + 30 g Sucrose + 1 µM TDZ + 2 µM AgNO$_3$ + 8 g Agar
3	A3	4.4 g MS Salt + 30 g Sucrose + 1 µM TDZ + 5 µM AgNO$_3$ + 8 g Agar
4	A4	4.4 g MS Salt + 30 g Sucrose + 1 µM TDZ + 10 µM AgNO$_3$ + 8 g Agar
5	A5	4.4 g MS Salt + 30 g Sucrose + 2 µM TDZ + 1 µM AgNO$_3$ + 8 g Agar
6	A6	4.4 g MS Salt + 30 g Sucrose + 2 µM TDZ + 2 µM AgNO$_3$ + 8 g Agar
7	A7	4.4 g MS Salt + 30 g Sucrose + 2 µM TDZ + 5 µM AgNO$_3$ + 8 g Agar
8	A8	4.4 g MS Salt + 30 g Sucrose + 2 µM TDZ + 10 µM AgNO$_3$ + 8 g Agar
9	A9	4.4 g MS Salt + 30 g Sucrose + 5 µM TDZ + 1 µM AgNO$_3$ + 8 g Agar
10	A10	4.4 g MS Salt + 30 g Sucrose + 5 µM TDZ + 2 µM AgNO$_3$ + 8 g Agar
11	A11	4.4 g MS Salt + 30 g Sucrose + 5 µM TDZ + 5 µM AgNO$_3$ + 8 g Agar
12	A12	4.4 g MS Salt + 30 g Sucrose + 5 µM TDZ + 10 µM AgNO$_3$ + 8 g Agar

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Table 4. SH medium with varying combinations of TDZ and AgNo$_3$.

Sl No	Medium	Combinations
1	B1	4.4 g SH Salt + 30 g Sucrose + 1 µM TDZ + 1 µM AgNO$_3$+ 8 g Agar
2	B2	4.4 g SH Salt + 30 g Sucrose + 1 µM TDZ + 2 µM AgNO$_3$ + 8 g Agar
3	B3	4.4 g SH Salt + 30 g Sucrose + 1 µM TDZ + 5 µM AgNO$_3$+ 8 g Agar
4	B4	4.4 g SH Salt + 30 g Sucrose + 1 µM TDZ + 10 µM AgNO$_3$ + 8 g Agar
5	B5	4.4 g SH Salt + 30 g Sucrose + 2 µM TDZ + 1 µM AgNO$_3$ + 8 g Agar
6	B6	4.4 g SH Salt + 30 g Sucrose + 2 µM TDZ + 2 µM AgNO$_3$ + 8 g Agar
7	B7	4.4 g SH Salt + 30 g Sucrose + 2 µM TDZ + 5 µM AgNO$_3$ + 8 g Agar
8	B8	4.4 g SH Salt + 30 g Sucrose + 2 µM TDZ + 10 µM AgNO$_3$ + 8 g Agar
9	B9	4.4 g SH Salt + 30 g Sucrose + 5 µM TDZ + 1 µM AgNO$_3$ + 8 g Agar
10	B10	4.4 g SH Salt + 30 g Sucrose + 5 µM TDZ + 2 µM AgNO$_3$ + 8 g Agar
11	B11	4.4 g SH Salt + 30 g Sucrose + 5 µM TDZ + 5 µM AgNO$_3$ + 8 g Agar
12	B12	4.4 g SH Salt + 30 g Sucrose + 5 µM TDZ + 10 µM AgNO$_3$+ 8 g Agar

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3.3. Sterilization of Nutrient Media, Materials, Glasswares and Instruments.

The test tubes containing media were steam sterilized in autoclave for 15min at 121°C. After sterilization, the culture tubes containing media were transferred to baskets and allowed them to get solidified and left at 20°C prior to use under sterile conditions. In between they were screened for microbial contamination visually under tube lights. Glasswares were autoclaved along with media.

3.4. Sample collection

Tender shoot pieces of cassava varieties were collected from the cassava fields of ICAR- CTCRI, Sreekariyam Thiruvananthapuram. The nodes of the cassava plants of different varieties are collected in small plastic covers very carefully and stored in ice. These samples were taken to the lab.

3.5.1. Nodal culture establishment

3.5.1. a. Processing

The shoots collected from the field were taken out and placed on a newspaper. By using a sterile blade the leaves were removed and then cut in to small size by keeping two nodes per explant.

3.5.1. b. Surface sterilization

The small nodes were moved to small bottles and washed in sterile water for 5 min. Then the water was discarded and two drops of Laboline solution was added, shaken well and kept for half an hour. In between it was shaken by rotating the bottle. After that the laboline solution was discarded and washed 2 or 3 times using sterile distilled water until the soap solution was completely removed. Then bavistin was added to the bottle and kept in shaker for 1.5 hours. After that bavistin solution was

discarded and the explants were washed using sterile distilled water until the impurities were completely removed. Then the explants were taken to the laminar air flow chamber (LAF) where sterile condition was maintained. LAF was wiped well and put on UV for 20 min by keeping the necessary instruments like blade and holder, forceps, scissors etc and media (Table 2) for culturing of explants.

The bottle was kept in the laminar air flow and mercuric chloride was added to the bottle very carefully. It was kept for about 5 min and mercuric chloride solution was discarded. The explants were washed well using sterile distilled water until the remnants were removed. Then the explants were moved to the petri plate and all the end portions of the explants were removed as reminants, some chemicals might be present in the open ends of the explants.

3.5.1. c. Inoculation

The tubes that were kept in the LAF was opened and showed to the flame for a few seconds and by using forceps the nodal explants were kept in to the media by touching the first inter node in the media. Then the tube was covered using sterile aluminium foil and labeled it.

3.5.1. d. Culture conditions

All the cultures were kept at 24-26°C under 12 hour of photoperiod and at 70% to 80% relative humidity. Culture tubes were examined daily and data collected.

3.5.2. Callus induction

The explants developed from *in vitro* plants were used for somatic embryogenesis. For standardizing the protocol different explants were inoculated in different media. Immature leaf lobes and axillary buds were carefully taken from *in vitro* plants by using stereo microscope and they were inoculated in 6 types of media.

Table 5. Different combinations of MS medium with picloram and 2,4-D for callus induction (1L)

MEDIUM	COMBINATIONS
C1	4.4g MS salt + 20g Sucrose + 25µM Picloram + 8g Agar
C2	4.4g MS salt + 20g Sucrose + 50µM Picloram + 8g Agar
C3	4.4g MS salt + 30g Sucrose + 25µM Picloram + 8g Agar
C4	4.4g MS salt + 30g Sucrose + 50µM Picloram + 8g Agar
C5	4.4g MS salt + 40g Sucrose + 25µM Picloram + 8g Agar
C6	4.4g MS salt + 40g Sucrose + 50µM Picloram + 8g Agar
C7	4.4g MS salt + 40g Sucrose + 4mgl^{-1} 2,4- D + 8g Agar
C8	4.4g MS salt + 40g Sucrose + 8mgl^{-1} 2,4- D + 8g Agar

3.5.2. a. Culture conditions

All the cultures were kept at 24-26°C under 12 hour of photoperiod and at 70% to 80% relative humidity. Culture tubes were examined daily and data collected.

3.5.3. Cotyledonary explants

The callus formed was inoculated in MS media supplemented with 30g Sucrose + 2µM BAP + 8g Agar or the initiation of cotyledonary explants.

3.6. Equipments used

Autoclave, Vortex mixer, Electronic precision balance, Electrophoresis apparatus, Gel documentation system, Ice machine, Laboratory oven, Microwave oven, Mortor and pestle, Refrigerated centrifuge, Thermal cycler and Water bath were used in the study

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3.7. Molecular analysis

In order to study the genetic fidelity among the cassava genotypes grown under *in vitro* conditions, molecular characterization using ISSR primers was carried out among them. Young leaves of cassava plants grown in field as well as *in vitro* plants of same variety were collected for DNA extraction to study the genetic fidelity under *in vitro* regeneration.

3.7.1. Sample collection for DNA extraction

Fresh tender leaves of cassava genotypes were collected from fields and in vitro plants in pocket size plastic kits and transferred to lab in an ice box.

3.7.2. Extraction of genomic DNA

- DNA was extracted using 1 g of young cassava leaves. The samples were grinded in liquid nitrogen using pestle and mortar.
- To this 15 ml of extraction buffer (0.1 M Tris Hcl, 0.02M EDTA (pH – 8), 2MNaCl, poly vinyl pyrrolidone and 2% beta mercaptoethanol) was added and grinded to form the slurry.
- The slurry was then transferred in to a 30 ml oak ridge tube and incubated at 4°C in ice.
- To this homogenate 1 ml of 20 % SDS was added, mixed well and incubated at 65°C in water bath for 30 min.
- To this 5 ml of 5M potassium acetate was added, mixed well incubated at 4°C for 30 min.
- Then the tubes were centrifuged at 12000 rpm for 20 min at 4°C. the supernatant was collected and transferred to a new tube.
- To this 10 ml of isopropanol was added, mixed well and incubated at 4°C for 1 hour. Then again the tubes were centrifuged at 12000 rpm for 15 min.

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- Then the supernatant was discarded and pellet was dried and it was dissolved in TE or sterile water.

- To this 5 µl of RNase was added and contents were mixed well by inverting the tubes and were incubated at 37°C for 1 hour in dry bath.

- To that equal volume of chloroform: isoamyl alocohol was added and mixed by inversion. The tubes were then again centrifuged at 12000 rpm for 20 min and supernatant were collected and transferred to a new tube.

- To the supernatant 50µl of sodium acetate and double volume of ice cold ethanol was added and incubated at -20°C for two hours.

- The tubes were then centrifuged at 1000 rpm for 10 min at 4°C and the supernatants were discarded by retaining the pellets. These pellets were then washed in 70% ethanol.

- Tubes were air dried and dissolved in 500µl TE buffer or double distilled water and stored at -20°C.

3.7.2. a. Role of various components of DNA protocol is as follows:

Extraction buffer:

This includes SDS as anionic detergent which disrupts the membranes, a reducing reagent such as β mercaptoethanol which helps in denaturing proteins by breaking the disulphide bonds between the cystine residues and for removing the tannins and polyphenols present in the crude extracts, a chelating agent like EDTA which chelates the magnesium ions required for DNase activity, an extraction buffer which is mostly Tris at pH 8 and salt such as sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA.

Phenol chloroform isoamyl alcohol:

Nucleic acid solutions commonly contain undesirable contaminants that are mainly made of proteins. A classic method of purifying is phenol-chloroform extraction by which the nucleic acid solution is extracted by successively washing with a volume of phenols, pH 8, a volume of phenol : chloroform : Isoamyl alcohol in 24:24:1 ratio and chloroform : isoamyl alcohol in 24:1 ratio. Centrifugation is performed intermittently and the upper aqueous layer is transferred to a new tube. The contaminants are denatured and accumulated in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase. Another way of removing proteins is by using the enzymes proteinase K which however again is denatured by phenol chloroform extraction.

Resuspending DNA:

The nucleic acid pellet can be resusupended in TE (10 mM Tris : 1mM EDTA)

Purification of DNA:

DNA is purified by incubating the nucleic acid solution with RNase A (10mg/ml) at 37ºC and reprecipitation following phenol : chloroform extraction to remove RNase.

3.7.3. Quantification of DNA

Nanodrop spectrophotometer analysis was performed to check the quantity of DNA.

Procedure:

Ensured that both top and bottom sample surface of the spectrophotometer was clean. Then 1μl of the blank solution was pipetted on to the lower sample surface. The top arm was lowered and tapped the blank button. The solution was removed from

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sample surface using a clean dry lab wipes. 1μl of the sample solution was pipetted on to the lower sample surface. The top arm was lowered and tapped the measure button. Concentration of DNA was obtained and it was recorded.

3.7.4. Dilution of samples

Samples were diluted using RNase/DNase free water. They are diluted in to 10ng/μl concentrations.

3.7.5. Checking the quality of DNA

Agarose gel electrophoresis was performed to check the quality of DNA. The negatively charged DNA molecules migrate in an electric field from negatively charged cathode to positively charged anode. Smaller molecules migrate faster than bigger one through the pores of the gel. The pore size of the gel matrix can be changed by altering the agarose concentration. After electrophoresis the DNA fragments which had binded with EtBr were visualized under UV.

Materials:
- Gel loading dye
- 1X TBE buffer
- EtBr
- Agarose
- DNA samples.

Procedure:
The edges of a clean, dry glass plate which was supplied with electrophoresis apparatus, were sealed with a tape to make a mould and placed horizontally. 1g Agarose is weighed and put in a conical flask. 100 ml of 1X TBE buffer was added, and the solution was gently boiled in the oven with occasional mixing until all the

particles were completely dissolved. Allowed to cool to 45°C and then 1.2µl EtBr was added. The gel mould was prepared and the comb was placed. Then the cooled gel was poured and kept it till it was solidified. The horizontal electrophoresis was filled with 1X TBE and the comb was removed and placed it in the chamber. The DNA samples were loaded with bromophenol blue dye carefully in to the wells. Then the gel was electrophoreses at 60V for almost 1.5 hrs and the DNA bands were visualized under UV transilluminator.

3.7.6. Sources of primers

The primers were selected from collected literature showing high polymorphism values in cassava plants.

Table 6. ISSR Primers used in the study

Sl No.	Primer Name	Sequence (5' ⟷ 3')
1	UBC 811	GAGAGAGAGAGAGAGAC
2	UBC 825	ACACACACACACACACT
3	UBC 827	ACACACACACACACACG

3.7.7. PCR amplification

The diluted samples were amplified in thermal cycler using different ISSR primers of ISSR at different conditions, such as temperature gradients. Screened the best primers and optimum amplifying conditions were standardized.

Table 7. ISSR- polymerase chain reaction mixture

Components	Stocks conc.	Required conc.	Volume for one reaction (20 μl)
Buffer with 15mM (Finzyme)	10X	1X	2 μl
MgCl (Thermo Scientific)	25mM	1Mm	0.8 μl
dNTP (Gene i)	2mM	2mM each	0.5 μl
Primer (from DOGR)	10μM	0.3μM	0.6 μl
DNA	10ng/μl	20ng	2 μl
Taq DNA polymerase (Finzyme)	2U/μl	1U	0.5 μl
dH$_2$O	-	-	13.6 μl
Total			20 μl

PCR conditions

PCR was carried out in Biorad thermocycler. The program is as follows.

Lid -105°C

94°C– 5 min (initial denaturation)

94°C- 30 sec (denaturation)

56°C- 1 min (annealing) } cycles X 35

72°C- 1 min (extention)

72°C- 10 min (final extention)

4°C– hold

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The amplified products were separated on 2% agarose gel along with 1Kb and 100 bp ladders to identify molecular weight of obtained bands and for polymorphism studies.

3.7.8. Agarose gel electrophoresis

Weighed 2g of agarose in 250 ml conical flask, added 100 ml 1X TBE buffer and gently boiled the solution in a microwave oven with occasional mixing until agarose completely dissolved in buffer. Allowed it to cool for some time and added 1µl ethidium bromide carefully without spilling. Prepared the gel and kept the comb in position. Poured the warm gel to plate and cooled for 20 min. Flled the horizontal electrophoretic tank with 1X TBE buffer (Appendix II). After gel got solidified removed the comb and placed the plate with the gel to the tank. Loaded 7µl sample along with bromophenol blue dye to wells and also 5µl 1Kb and 100 bp ladders for reference. Run the gel at 85 V and 220mA for 1 to 1.5 hour. Visualized the band under UV transilluminator and documented the image on Alpha Imager. The image could be finally scored to detect polymorphism or to identify specific bands that can be linked to a particular trait.

3.8. Transformation

Transformation work was carried out by co cultivating the explants with *Agrobacterium* strains – LBA4404, EHA 105 and AGL-1. Callus and cotyledonary explants were obtained by transferring them to BAP supplements in MS media. For that first *Agrobacterium* competent cells were prepared.

In the present work transformation was done by co cultivation of explants with *Agrobacterium*. Callus and cotyledonary explants which obtained by transferring the generated callus to the medium contained BAP was used for co culture.

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Preparation of competent *Agrobacterium* cells

- Inoculated *Agrobacterium* colony into 2 ml of media and cultured at 28°C on shaker overnight.
- Next day used this as a starter culture for 50 ml of media in 250ml flask
- Shaked at 220 rpm at 28°C until OD at 600nm reaches 0.6-1 (should be 5hrs).
- Chilled cultures for 5 minutes.
- Took 30 ml of the culture and transferred to a centrifuge tube.
- Centrifuged at 7000 rpm for 5 minutes at 4°C.
- Discarded the supernatant and resuspended the pellet into 10 ml ice-cold 0.15M NaCl.
- Incubated on ice water for 5 minutes.
- Centrifuged at 7000 rpm and resuspended the pellet with 1 ml of ice cold 20mM $CaCl_2$
- Placed 100 µl of competent cells in tube and added 1 microgram of plasmid.
- Followed by *Agrobacterium* mediated transformation protocol.

To store the competent cells

- Transferred cells in 100microlitre aliquots to sterile microfuge tubes.
- Freezed these tubes for 5 minutes in liquid nitrogen.
- Stored tubes immediately in -80°C freezer.

Agrobacterium mediated transformation of competent cells

- Thawed on ice 50-100 µl aliquots of competent cells.
- Added 1µg DNA to cells mixing gently with pipette tip.
- Left on ice for 30 minutes.

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- Freezed tubes in liquid nitrogen for 1 minute.

- Thawed the tubes in 37°C water bath /heat block for 1 minute.

- Added 400 µl LB to eppendorf tube and incubate horizontally on shaker at 28°C for atleast 1 hour.

- Plated the cells to LB plates with 1% w/v glucose containing appropriate antibiotics plate 100-200 µl of *Agrobacterium* cells.

- Inverted plates and incubated for 2 days at 28°C.

Cells can be concentrated prior to plating by:

- Spin down cells in microfuge for 1 minute.

- Poured off suspension.

- Resuspended cells in 100 µl LB.

- Plated on to LB with glucose and antibiotics for 2 days.

Transformation

Procedure:
- Leaf tissue of about 3 months of development grown under *in vitro* conditions were cut into small pieces and transferred into petridish having MS liquid media.

- MS media was replaced with *Agrobacterium* solution.

- Care should be taken that all explants were submerged in the Agrobacterium solutions.

- Plates were incubated at room temperature with mild shaking for 30 min.

- The *Agrobacterium* solution was removed and the explants adhered with *Agrobacterium* were transferred to plates having MS + 200µM acetosyringone.

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- Plates were incubated at 22°C for 48 hrs of co cultivation.
- After 48 hrs the tissues were transferred sterile into 15 ml tubes having 10 ml sterile water and shaken vigourously inside the LAF
- The tubes were allowed to stand and settle the tissue for about 5 min.
- The liquid pipetted out with a fine tipped pipette
- Then the tissues were washed in the sterile water for 2 or more times.
- Added 10 ml of MS liquid medium containing 500mg/l carbencillin.
- Incubated the tissues in GUS solution at 37°C overnight.

3.9. Statistical Analysis

The observations were recorded and the data relating to each experiment was analyzed by applying the Analysis of Variance (ANOVA) technique (Panse and Sukhatme, 1967).

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RESULTS

4. RESULTS

The results of the study entitled "Development of efficient transformation and regeneration protocols in elite genotypes of cassava (*Manihot esculenta* Crantz) was carried out at the Division of Crop Improvement, ICAR- Central Tuber crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016 and the results obtained are presented in this chapter.

4.1 MEDIA STANDARDIZATION

The popular cassava varieties *viz.* Sree Prakash, Sree Sahya, H 226, Vellayani Hraswa, Mulluvadi, Sree Athulya, Sree Apoorva and the CMD resistant hybrid pre-release varieties *viz.* 9S 127, 9S 174, CI 273, 8W5, 11S4, IPS2-1, were studied for their response to somatic embryogenesis. The shoot tips of the varieties were taken from the field grown plants (Plate 1) and were subjected to sterilization.
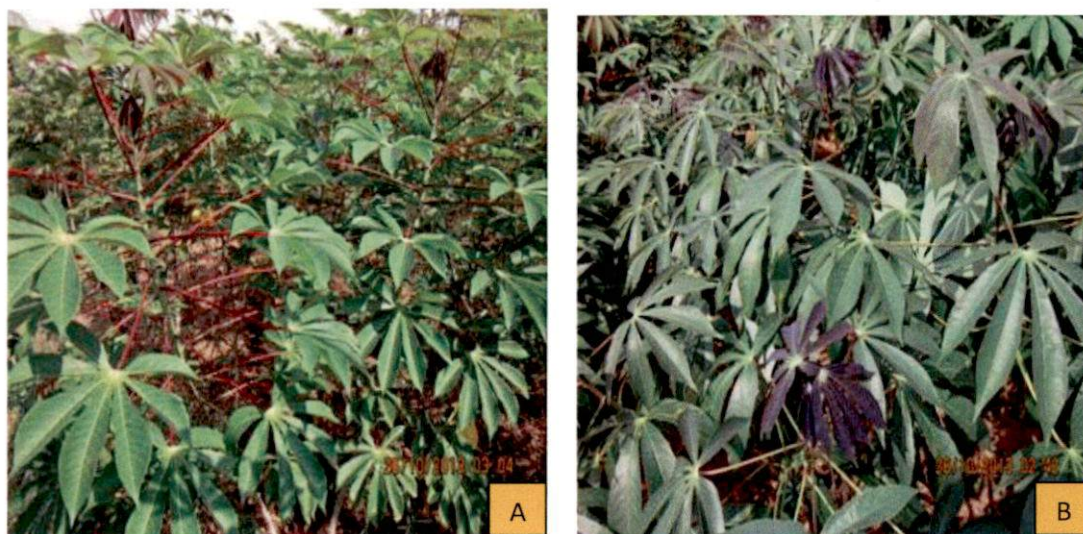

Plate 1: A: Sree Athulya, B: IPS2-1

4.1.a. Media standardization for initiation of *in vitro* culture of cassava varieties

The contamination of the *in vitro* culture of the varieties ranged from 10-40% with Vellayani Hraswa recording the highest contamination under *in vitro* culture (Table 8). The number of days taken for sprouting ranged from 9 (Sree Athulya, H-226, 11S4) to 17 days (Mulluvadi and IPS2-1) after the inoculation of the explants (Fig1). The number of leaves per explants ranged from 1.33 (Sree Sahya) to 4.67 (Sree Prakash). Sree Athulya and 9S 174 also recorded higher number of leaves (>4) at 30 days after inoculation. Shoot length 8W5, H226, Sree Prakash, IPS2-1, CI-273, 11S4, ranged from 0.67 cm (Sree Sahya) to 2.67 cm (9S 127). Better *in vitro* rooting was recorded for most of the varieties while Mulluvadi, Sree Sahya and Vellayani Hraswa recorded poor response.

Four MS medium (M1, M2, M3 and M4) with different concentrations of BAP, NAA and $CuSO_4$ (Table2) were evaluated for *in vitro* shoot and root response.

Nodal explants taken from field grown plants of two varieties *viz.* 9S127 and 8W5 were inoculated in M1, M2, M3 and M4 medium to identify the medium suitable for rapid production of leaves under *in vitro* conditions. In M1 and M2 the initial growth was found to be faster than the other two media. In M3 and M4 the shoot initiation was very slow, it took more than 3 weeks to get sprouting. After 4-5 weeks the growth of the plants in M1 media get good leaves and roots when compared to the M2 media. For 9S 127 the number of leaves per explants was highest (4) in M1 medium on 30 days after inoculation. 8W5 also recorded the same result with maximum no of leaves (3.33) in M1 media (Table 9). Shoot length was also highest in M1 medium for 9S127 (2.66cm) and 8W5 (1.63 cm) (Fig2, Plate 2). The M1 medium also recorded better rooting as compared to other medium combinations. Hence MS media supplemented with 30 g Sucrose + 0.5 μM NAA + 2 μM BAP + 2 μM $CuSO_4$ + 8 g Agar was identified as the best medium for in vitro establishment of the cassava varieties.

56

Table 8. Response of different cassava varieties to *in vitro* culture

Variety	% of contamination	No. of days to sprouting	No. of leaves/explant (30 DAI)	Shoot length (cm) (30 DAI)	Rooting (30 DAI)
8W5	10	14	3.33^{cd}	1.63^{d}	++
H 226	10	9	3.67^{bc}	2.47^{b}	++
Mulluvadi	10	17	2.00^{ef}	1.13^{f}	+
Sree Sahya	20	10	1.33^{f}	0.67^{g}	+
Sree Prakash	20	13	4.67^{a}	1.43^{e}	++
IPS2-1	10	17	3.00^{cd}	2.37^{b}	++
CI 273	20	10	2.00^{ef}	1.67^{d}	++
11S4	30	9	3.67^{bc}	1.63^{d}	++
Vellayani Hraswa	40	12	2.67^{de}	2.07^{c}	+
9S 127	10	12	3.67^{bc}	2.67^{a}	++
9S 174	20	11	4.33^{ab}	2.03^{c}	++
Sree Apoorva	20	11	3.33^{cd}	2.03^{c}	++
Sree Athulya	10	9	4.33^{ab}	2.43^{b}	++
CD (0.969)		22.41	0.97	0.20	
CV (%)			17.87	6.37	

Root length: + (0.5-1 cm), ++:1-2.5 cm

DAI – Days after inoculation

57

Table 9. Effect of MS medium supplemented with different concentrations of growth regulators on no. of leaves, shoot length and rooting in cassava

Varieties	Medium	No. of leaves (30 DAI)	Shoot length (cm) (30 DAI)	Rooting response (30 DAI)
9S 127	M1	4.00	2.66	++
	M2	2.33	2.16	+
	M3	1.00	1.40	+
	M4	1.38	1.30	+
8W5	M1	3.33	1.63	++
	M2	2.33	1.30	+
	M3	1.33	1.00	+
	M4	1.19	1.06	+
CD(0.05)		0.69	0.21	
CV%		18.33	7.60	

Root length: + (0.5-1 cm), ++:1-2.5 cm

DAI- Days after Inoculation

58

Fig 1. Response of different cassava varieties to *in vitro* culture

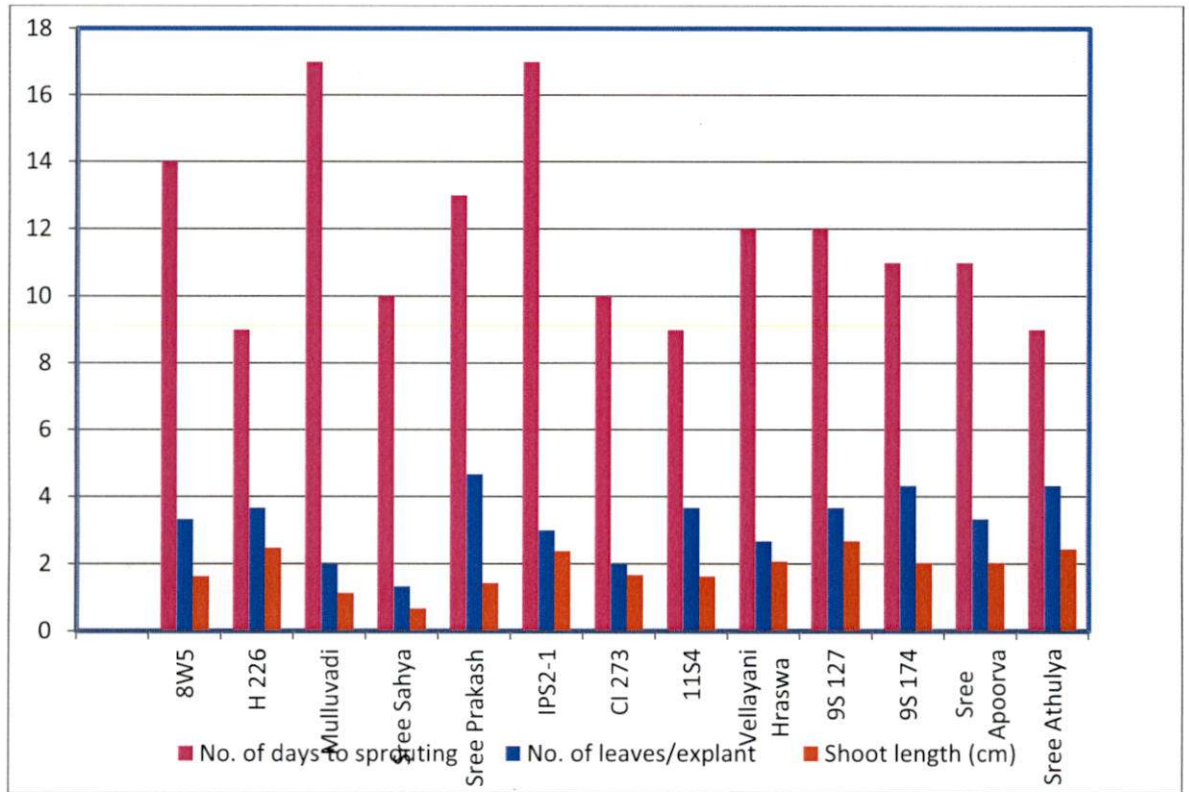Fig 1. Response of different cassava varieties to *in vitro* culture

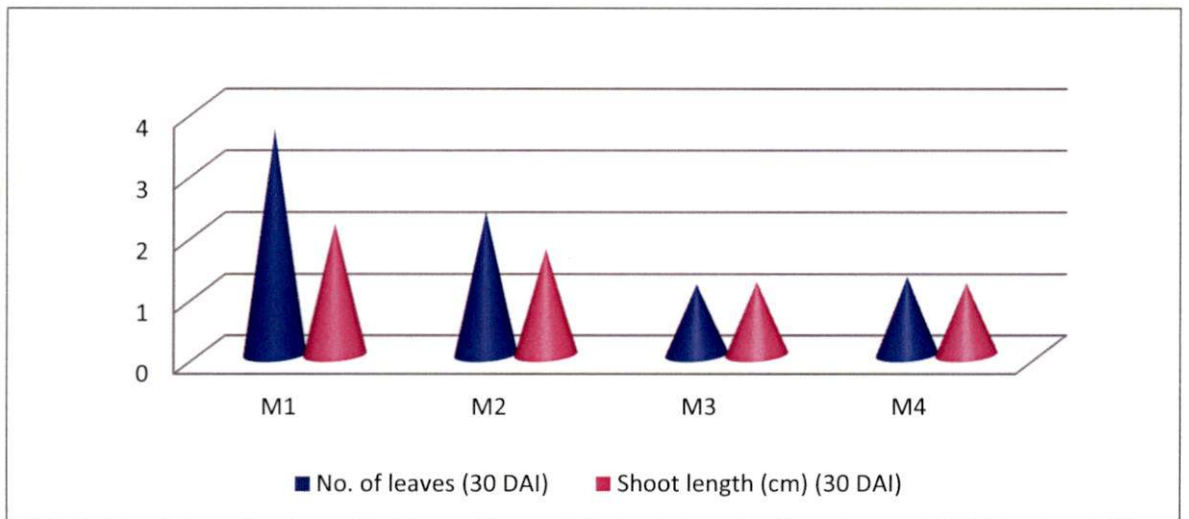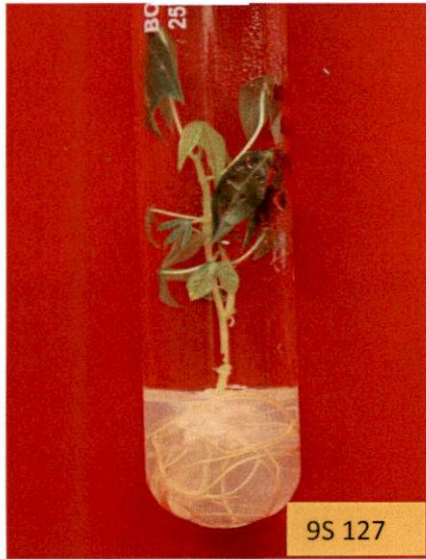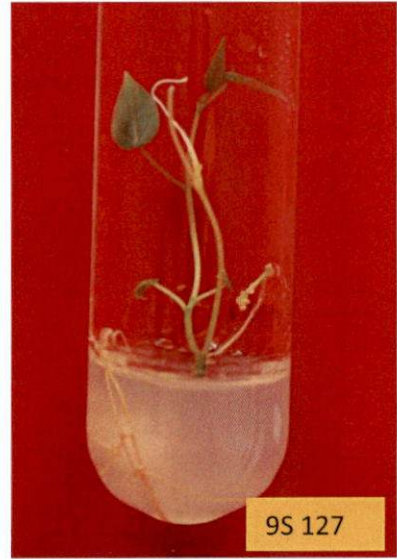# Fig 2. Effect of MS medium on *in vitro* response of cassava varieties

Fig 2. Effect of MS medium on *in vitro* response of cassava varieties

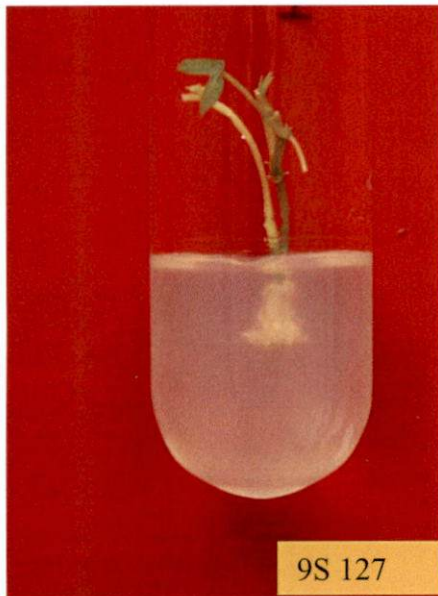**M1**

9S 127
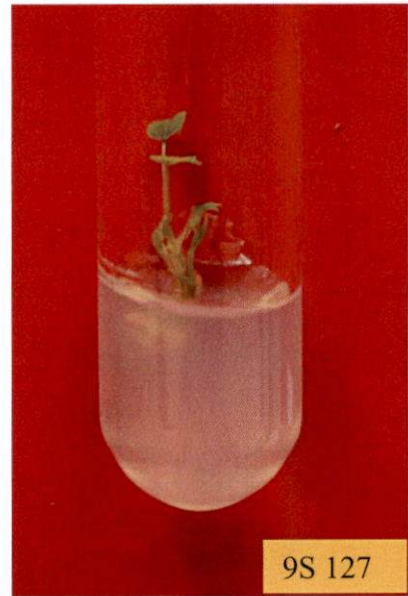
M2

9S 127

M3

9S 127

M 4

9S 127

Plate 2: 9S 127 in M1, M2, M3, M4 medium

60

4.1. b. Media standardization for rapid micropropagation of cassava

For induction of somatic embryogenesis, rapid *in vitro* production of explants is essential. Hence cassava variety 9S127 was subcultured in MS and SH media supplemented with different concentration of silver nitrate and the growth regulator, Thidiazuron (TDZ) to identify the best medium that produce maximum number of leaves during subculturing. The *in vitro* response in 12 combinations of MS media was given in Table10, 11 and 12.

The number of leaves per explant, shoot length (cm) and rooting response were studied at 15 days interval. The A4 medium recorded the highest number of leaves/explants (8) at 15 days after inoculation followed by A3 (5 leaves) and A9 (4 leaves). The highest number of leaves per explants (14) on 45 days after inoculation was also recorded by A4 medium. A3 and A9 medium also recorded production of higher number of leaves per explants (8) followed by A10 (7). The A2, A5, A7 and A12 medium recorded poor response to *in vitro* leaf production. The shoot length was highest in A8 medium (2.3 cm) followed by A4 and A9 that resulted in a shoot length of 2 cm at 15 days after inoculation. The same growth response was also recorded at 30 days after inoculation. The A8 medium recorded the highest shoot length (3.1 cm) at 45 days after inoculation followed by A9 (2.6cm), A1 (2.4 cm), A2 (2.3cm) and A4 (2.1cm). The A8 and A9 media recorded the highest rooting response followed by A10 and A3 medium. The results indicated that the MS medium supplemented with 30 g Sucrose^{-1} + 1 µM TDZ + 10 µM AgNO$_3$ + 8 g Agar was ideal for sub culturing of cassava to facilitate augmented production of leaves/explants.

The media standardization of SH medium with varying combinations of TDZ and AgNO$_3$ was also carried out and the results are presented in Table 13, 14 and 15. The highest number of leaves per explants (10) was recorded for B10 followed by B1 (7). The shoot length ranged from 1.5 cm (B4) to 4.2cm (B10).

41

Table 10. No. of leaves in MS medium with 2 weeks of time interval

MEDIUM	15 DAI	30 DAI	45 DAI
A1	2	4	6
A2	0	0	0
A3	5	6	8
A4	8	12	14
A5	0	0	0
A6	0	0	1
A7	1	0	0
A8	3	5	6
A9	4	6	8
A10	3	4	7
A11	0	0	1
A12	1	0	0
SD	2.49	3.80	4.60
CV(5)	110.75	123.27	107.65

DAI – Days after inoculation

62

Table 11. Shooting (cm) response in MS medium at 2 weeks of time interval.

MEDIUM	15 DAI	30 DAI	45 DAI
A1	1.5	2.0	2.4
A2	1.0	1.7	2.3
A3	1.3	1.6	2.0
A4	2.0	2.0	2.1
A5	1.0	1.2	1.5
A6	1.0	1.3	1.8
A7	1.0	1.3	1.9
A8	2.3	2.8	3.1
A9	2.0	2.1	2.6
A10	1.0	1.3	1.8
A11	1.0	1.1	1.5
A12	1.0	1.2	1.7
SD	0.47	0.49	0.45
CV(5)	34.89	29.82	22.04

DAI – Days after inoculation

Table 12. Rooting response in MS medium with 2 weeks of time interval.

MEDIUM	15 DAI	30 DAI	45 DAI
A1	+	+	+
A2	+	+	+
A3	++	++	++
A4	+	+	+
A5	+	+	+
A6	+	+	+
A7	+	+	+
A8	++	+++	++++
A9	++	+++	++++
A10	++	++	++
A11	+	+	+
A12	+	+	+

Root length : + (0.5-1 cm), ++:1-2.5 cm; +++:>2.6 cm

DAI – Days after inoculation

64

Table 13. No. of leaves in SH medium with 2 weeks of time interval.

MEDIUM	15 DAI	30 DAI	45 DAI
B1	2	4	7
B2	3	4	5
B3	2	4	6
B4	2	3	5
B5	2	3	6
B6	2	3	5
B7	4	5	6
B8	2	3	5
B9	3	4	6
B10	6	8	10
B11	3	4	5
B12	2	3	5
SD	1.21	1.41	1.44
CV(5)	44.20	35.20	24.40

The observation was taken after 2 weeks with continuous intervals, the B7 and B10 media combinations given a good response for leaf multiplication at the time of last observation.

*DAI – Days after inoculation

Table 14. Shooting (cm) response to SH medium with 2 weeks of time interval.

MEDIUM	15 DAI	30 DAI	45 DAI
B1	1.3	1.6	1.7
B2	2.3	2.5	2.6
B3	3.0	3.2	3.3
B4	1.0	1.3	1.5
B5	2.3	2.5	2.7
B6	2.0	2.4	2.5
B7	2.0	2.3	2.5
B8	1.8	2.0	2.3
B9	2.3	2.6	2.8
B10	3.2	4.0	4.2
B11	2.7	3.0	3.2
B12	2.6	3.0	3.3
SD	0.61	0.69	0.70
CV (5)	27.94	27.41	25.85

The observation was taken after 2 weeks intervals, the B3 and B10 media combinations given a good response for shooting at the time of last observation.

* **DAI** – Days after inoculation

Table 15. Rooting response in SH medium with 2 weeks of time interval.

| MEDIUM	15 DAI	30 DAI	45 DAI
B1	+	++	+++
B2	+	++	++
B3	+	++	++
B4	+	++	+++
B5	+	++	++
B6	+	++	++
B7	+	+++	++++
B8	+	++	++
B9	+	+++	++++
B10	+	+++	++++
B11	+	++	++
B12	+	+++	++++

Root length: + (0.5-1 cm), ++:1-2.5 cm

*DAI – Days after inoculation

The observation was taken after 2 weeks intervals, the B7, B9, B10 and B12 media combinations given a good response for rooting at the time of last observation.

Twenty four media were tested for their efficiency in the production of leaves per explant and shoot length and the result is depicted in Fig: 3. The study revealed the highest response in A4 medium ie. the MS medium supplemented with 30 g Sucrose^{-1} + 1 μM TDZ + 10 μM AgNO3 + 8 g Agar followed by B10 medium (SH Salt + 30 g Sucrose + 5 μM TDZ + 2 μM AgNO$_3$ + 8 g Agar).
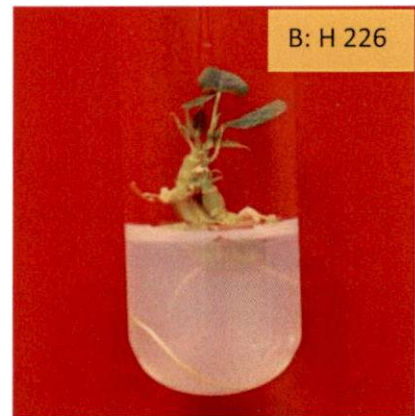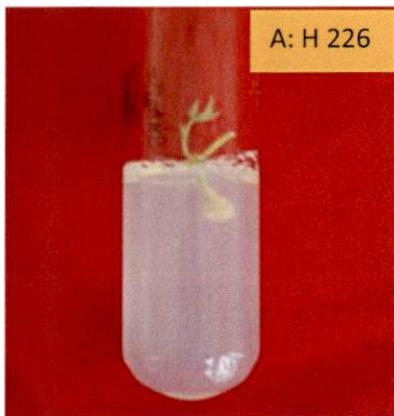
47

Fig.3 Effect of media on no. of leaves per explant and shoot length at 45 days after inoculation.

68
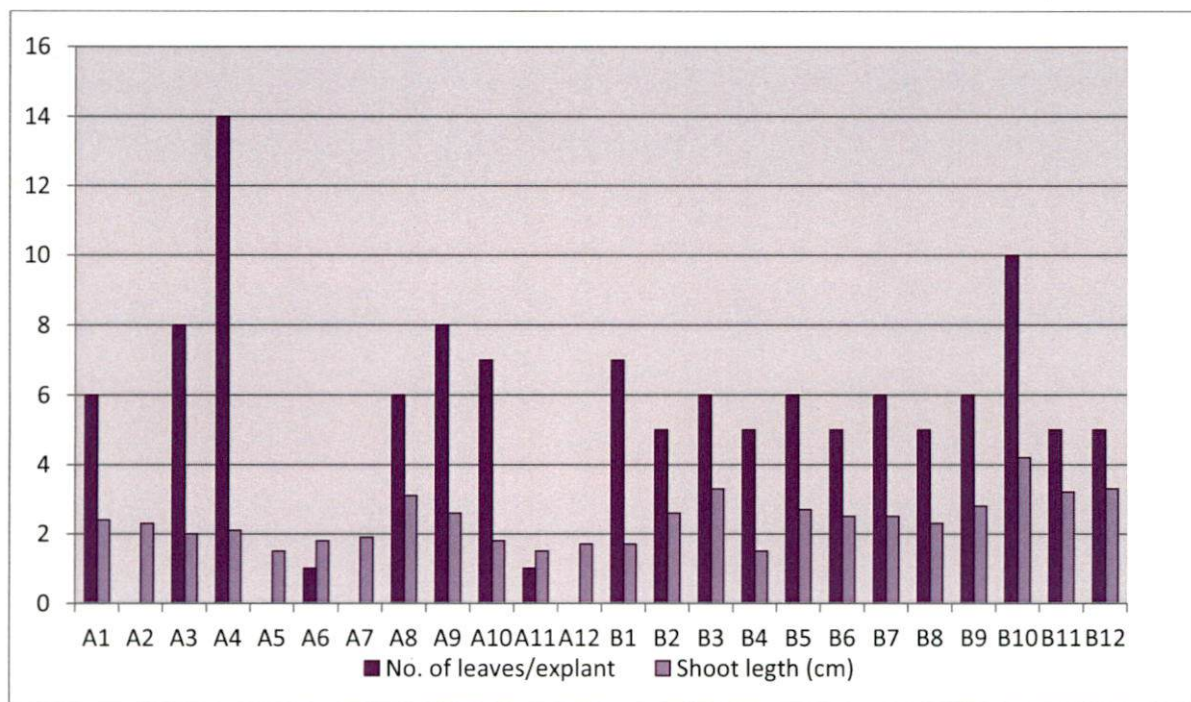
In vitro propagation of cassava varieties in different stages
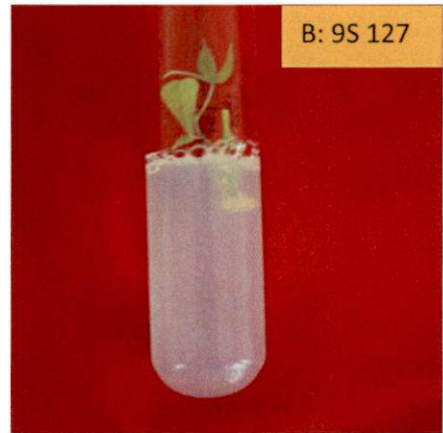
Plate 3: Growth of 9S 127 in MS medium A: observed after 2 weeks
B: observed after 4 weeks

Plate 4. Growth of H226 in SH medium A: observed after 2 weeks,
B: observed after 4 weeks

69

Plate 5. A: Mulluvadi variety inoculated in MS medium

      B: Mulluvadi variety observed after 11 days

      C: CI 273 variety observed after 23 days

      D: Sree Prakash variety observed after 33 days

      E: IPS2-1 variety observed after 50 days

174108
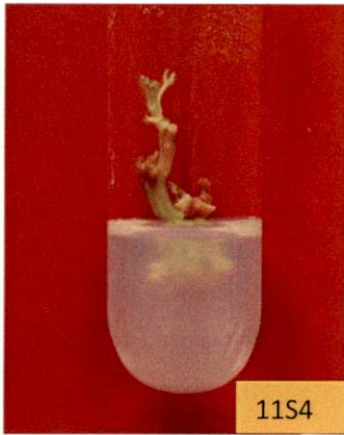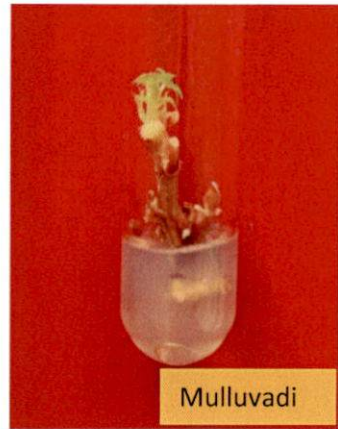
Plate No 6: Initial establishment of cassava genotypes 11S4, CI 273, Mulluvadi, Sree Prakash, 8W5, Sree Sahya
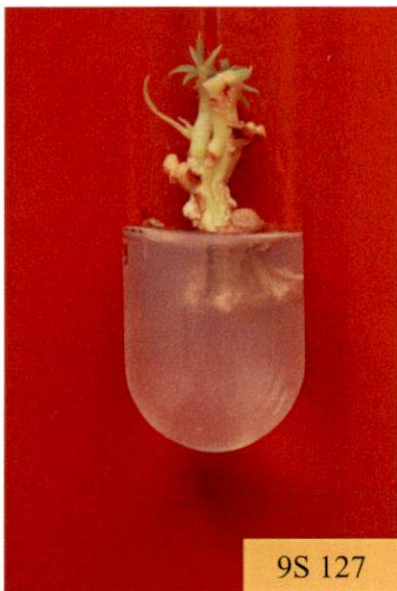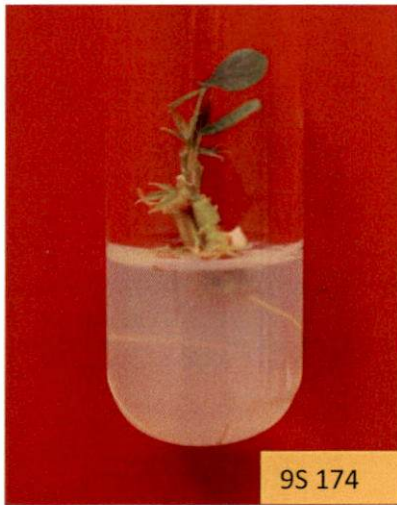
Plate No7: Initial establishment of cassava genotypes   9S 174, IPS2-1, 9S 127, Vellayani Hraswa

72
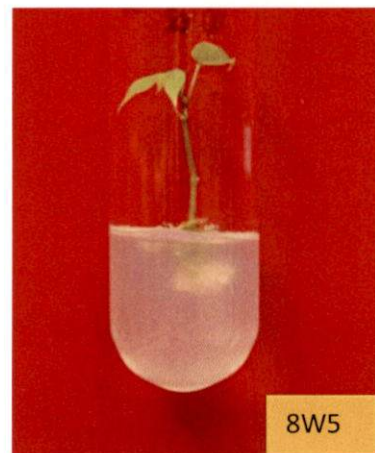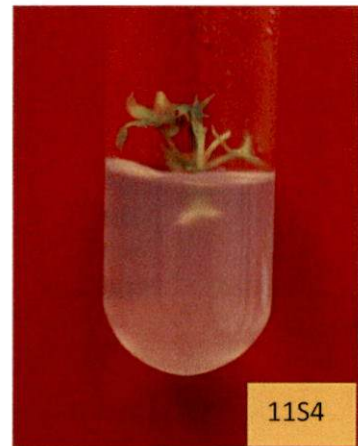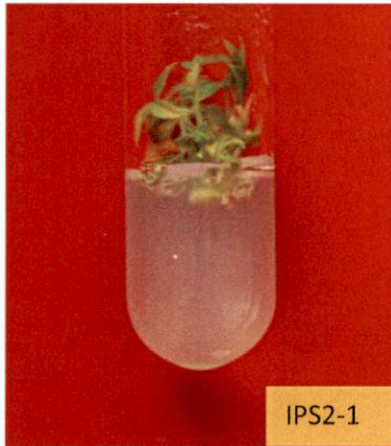
Plate No 8. Initial establishment of cassava genotypes IPS2-1, 11S4, CI 273, 8W5

73
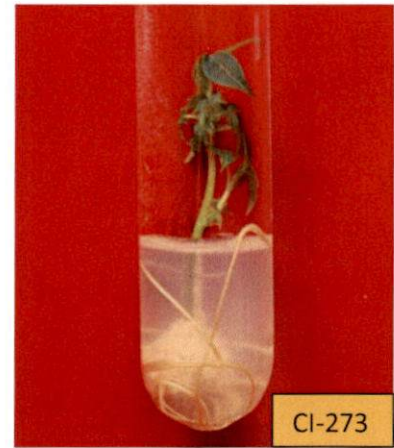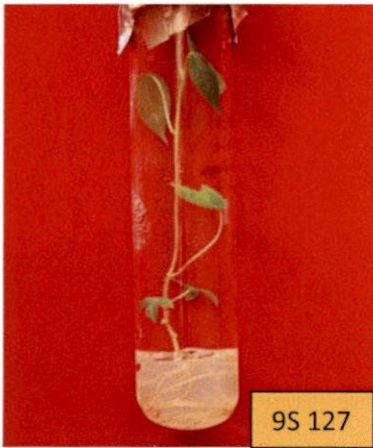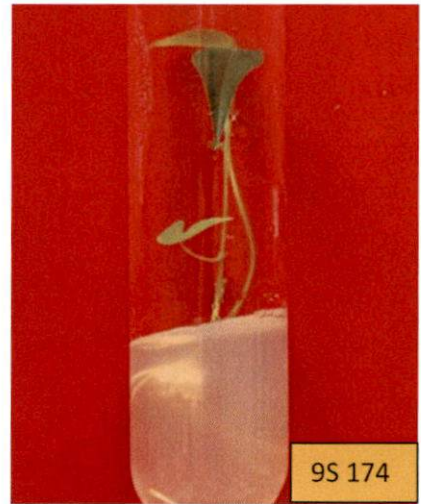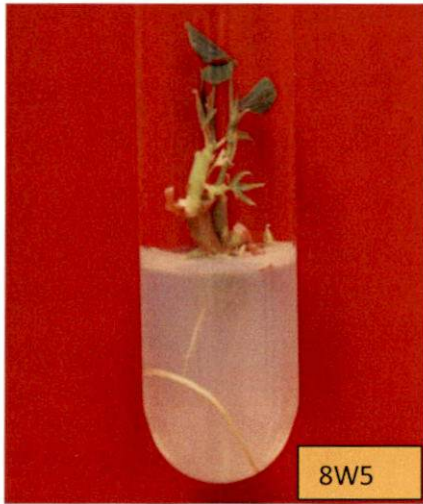
Plate No. 9. Initial establishment of cassava genotypes 8W5, 9S174, 9S 127, CI-273

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4.1. c. Hardening

Cassava genotypes raised through micro propagation were taken for hardening. The plants were grown in M1 media for about 2 months with continuous subculturing in an interval of 20 days. Plants with good roots were selected for hardening.

The plants were taken carefully from the tubes and they were kept in bavistin solution for 20 min. Then the plants were washed well with clean water and transferred to a tray having potting mixture and coir pith with 2:1 ratio. After 2 weeks they were transferred to small pots and kept in cage under glass house. The pots were then moved to net house when the plants are grown.

Plate 10. A:Plants transferred to tray with potting mixture and coir pith.

B: Plants transferred to cups and kept in cage

C: Plants in cups transferred to net house.

1. H226
2. 9S 127
3. Sree Apoorva

4.2. Induction of somatic embryogenesis

The *in vitro* propagated plants were used to take the explants *viz.* immature leaf lobes and axillary buds to produce the somatic embryos. For the large scale production of explants and for easy handling, the *in vitro* plants were cultured in big petriplates of 120mm size, where the plants grew very thickly and quickly to fill the plates in media identified previously for rapid propagation.
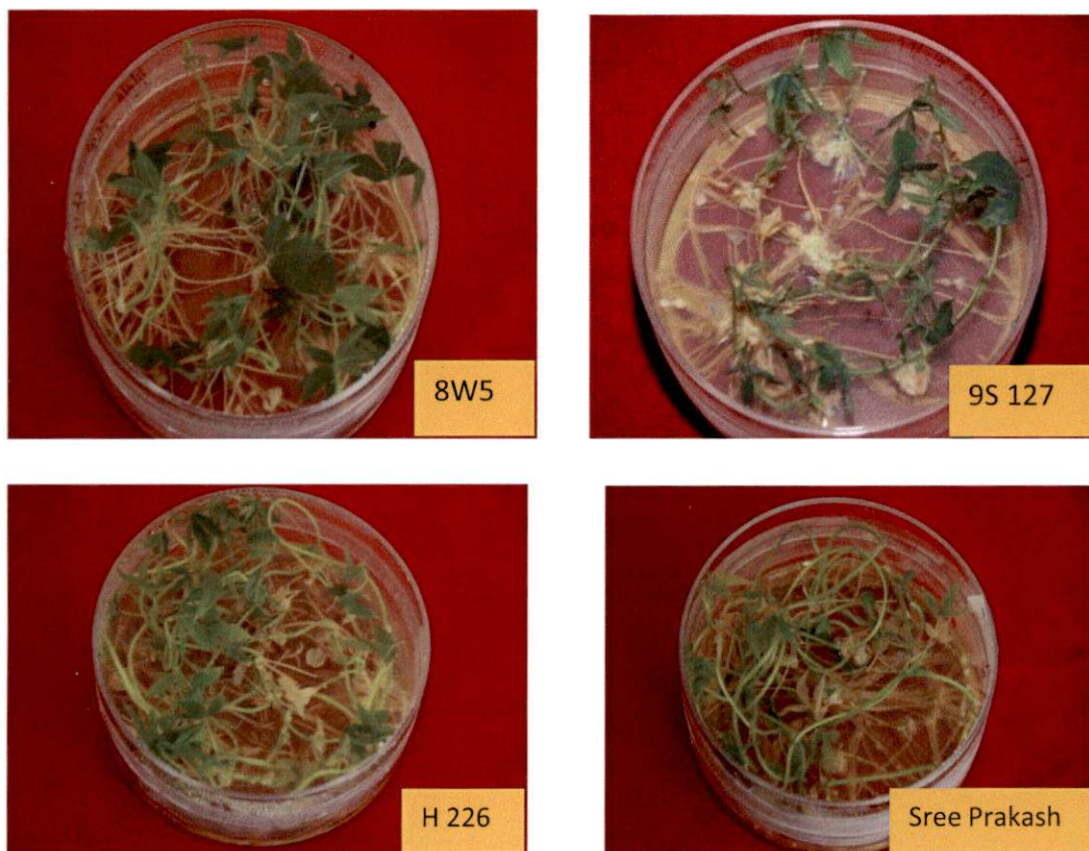
Plate 11. Raising of cassava varieties for explants production

4.2.1. Effect of explants, media and genotypes on induction of somatic embryogenesis in cassava plants

The immature leaf lobes and axillary buds were taken from four to six weeks old *in vitro* plants raised in petridishes (Plate 11). The immature leaflobes were excised from mother plants using fine forceps and a scalpel and placed with the abaxial surface in contact with the media. Eight media combinations (Table 5) of MS media with varying concentrations of sucrose and growth regulators viz. Picloram and 2,4-D were evaluated for the induction of somatic embryogenesis in ten cassava varieties selected for the study.

Eight combinations of MS medium (Table 5.) with varying concentrations of sucrose (20, 30, 40), picloram (25 μm, 50 μm) and 2, 4 D (4 mgl^{-1}, 8mgl^{-1}) were evaluated for the identification of best media for induction of somatic embryogenesis in cassava varieties. The effect of sucrose in induction of somatic embryos is given in Table 16. Sucrose @ 30 % resulted in higher no of somatic embryo per explant (10.5) followed by 20% (5.25) and 40 %(4.75). Higher % of sucrose was found to have inhibitory effect on somatic embryo production. Significant variety sucrose interaction was also recorded as is given in Table 17.

The mean no of somatic embryos per explant produced in MS media with picloram and 2,4-D ranged from 4.25 to 13.25 and is given in Table 18. The highest no of explants (13.25) was obtained in MS medium supplemented with 2, 4 D 8mgl^{-1} followed by picloram @ 50μm (11.0). The lower concentration of hormones resulted in less no of somatic embryos per explant. Interaction effect of variety sucrose is given in Table 19. The highest no of somatic embryos (18) was recorded for Sree Sahya in C8 media.

Somatic embryogenesis from axillary buds and immature leaf lobes was recorded in all the cassava varieties selected for the study (Table 18, Fig.4). When axillary buds were used as explants the callusing percentage ranged from 0-100%. Sree Sahya

and Sree Athulya recorded 100 % callus induction followed by H 226 (60%), Sree Apoorva (50%) and IPS2-1 (40%). Other cassava varieties did not produce any calli. Embryogenic callus was also produced from these cassava varieties when axillary buds were used as explants and it ranged from 0-30%. Sree Sahya recorded the highest percentage of embryogenic callus (30%) followed by Sree Athulya (12 %), H 226 (12 %) and H 165 (6%). Other cassava varieties did not produce any embryogenic callus.

When immature leaf lobes were used as explants the callusing percentage ranged from 40-100%. All the cassava varieties recorded 100 % callusing except Vellayani Hraswa (80%) and 11S4 (40%). Embryogenic calli were produced from these cassava varieties when immature leaf lobes were used as explants and the percentage of embryogenic callus induction varied from 0-98% (Plate11 and 12). Among the cassava varieties, Sree Sahya recorded the highest percentage of embryogenic callus (98%) followed by Sree Athulya (92 %), Sree Apoorva (90%), H226 (80%) and H-165 (50%). Other cassava varieties produced very less embryogenic calli when immature leaf lobes were used as explants.

The results indicated higher induction of embryogenic calli when immature leaf lobes were used as explants as compared to axillary buds (Fig4.). Among the varieties evaluated Sree Sahya, Sree Athulya Sree Apoorva and H-226 has high embryogenic potential (>80%) followed by H-226 with medium embryogenic efficiency (50%).

4.2.1.1. Regeneration of somatic embryos

The somatic embryos were regenerated (Plate 12) in MS medium supplemented with sucrose (30g l^{-1}) and BAP (2μM).

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Table 16. Mean number of embryos produced due to different levels of Sucrose in MS medium

Cassava genotype	Sucrose 20 %	Sucrose 30 %	Sucrose 40 %
Sree Sahya	6*	14*	7*
Sree Apoorva	6*	11*	5*
Sree Athulya	5*	9*	4*
H 226	4*	8*	3
Mean	5.25	10.50	4.75
STDEV	0.95	2.65	1.70
CV (%)	18.30	25.20	35.90

Table 17. ANOVA for the variety X sucrose interaction on somatic embryogenesis in cassava

Source	Df	Mean Square	F	CD(0.05)
Variety	3	32.52	5.95	0.26
Sucrose	2	173.52	31.75	0.45
VarietyX Sucrose	6	3.19	0.58	0.18
Error	36	5.47		

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Table 18: Mean number of embryos produced in MS medium with different levels of Picloram and 2,4-D

Cassava genotypea	Picloram 25 μM	Picloram 50 μM	2,4-D 4mg l⁻¹	2,4-D 8 mg l⁻¹
Sree Sahya	7*	15*	5*	18*
Sree Apoorva	6*	12*	4	15*
Sree Athulya	5*	9*	4	11*
H 226	4	8*	4	9*
Mean	5.5	11.0	4.25	13.25
STDEV	1.29	3.16	0.5	4.03
CV (%)	23.47	28.74	11.76	30.42

Table 19. ANOVA for the variety X growth regulators interaction on somatic embryogenesis in cassava

Source	df	Mean Square	F	CD(0.05)
Variety	3	73.04	14.46	0.29
Growth regulators	3	295.17	58.43	0.36
VarietyX Growth regulators	9	13.00	2.40	0.12
Error	48	5.04		

Table 20. Somatic embryogenesis from the immature leaf lobes and axillary buds of cassava varieties.

Genotype	Axillary buds		Immature leaf lobes	
	% callus	% Embryogenic callus	% callus	% Embryogenic callus
Sree Apoorva	50	8	100	90
9S- 127	0	0	100	2
8W5	0	0	100	2
Sree Athulya	100	12	100	92
CI 273	0	0	100	1
Sree Prakash	0	0	100	5
H- 165	0	6	100	50
H- 226	60	12	100	80
Sree Sahya	100	30	100	98
9S174	0	0	100	3
Vellayani Hraswa	0	0	80	0
11S4	0	0	40	0
IPS2-1	40	0	100	0

8)

Fig.4. Callus induction and somatic embryogenesis from the immature leaf lobes and axillary buds of cassava varieties.

82
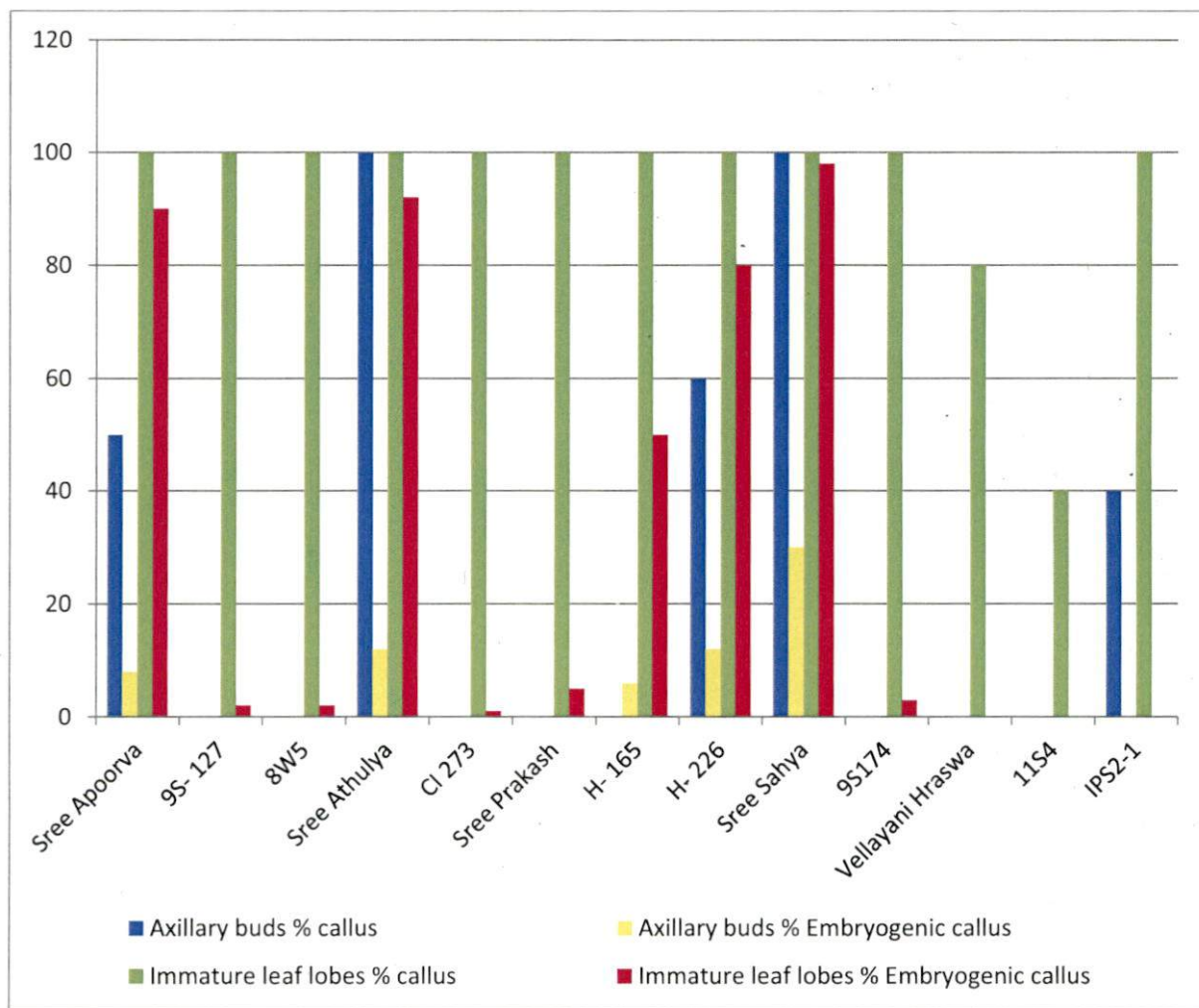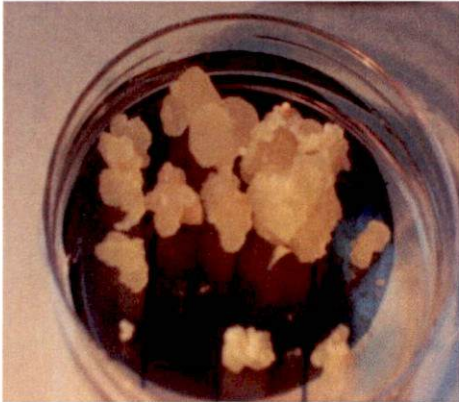
| H-226: Non embryogenic callus | H-226: embryogenic callus |

| Sree Sahya | Sree Athulya | Sree Apoorva |

Plate 12: Somatic embryos were observed in H 226, Sree Athulya, Sree Sahya
Sree Apoorva.

83
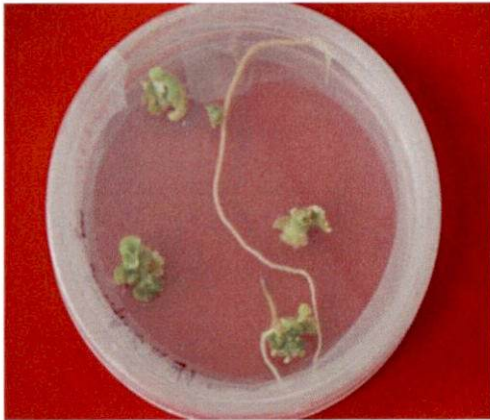
SREE APOORVA

H-226

SREE SAHYA

SREE ATHULYA

Plate 13: Cotyledonary explants observed from somatic embryos from *invitro* cassava genotypes of Sree Apoorva, H 165, Sree Sahya Sree Athulya,

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4.3. *Agrobacterium* mediated transformation

The *Agrobacterium* mediated transformation was carried out following the method described (3.8) using the strains *viz.* LBA4404, EHA105 and AGL-1 containing PBI121 vector with a cocultivation duration of 12hrs, 24 hrs and 48 hrs. Transformation was obtained only when LBA4404 was used with a co-cultivation time of 48 hrs. The callus and cotyledon explants of the variety Sree Sahya were co-cultivated with *Agrobacterium-* LBA 4404 which possessed PBI121 vector and examined through X- Gluc assay. Transformed callus and cotyledon explants were appeared in blue color after co-cultivation for 48 hours. In transformed one, β-glucuronidase enzyme came into function and cleaved X-Gluc and produced 5, 5'-dibromo-4, 4' – dichloro indigo which appeared in blue color. As compared to callus the cotyledonary explants recorded higher transformation efficiency as depicted in plate 14.

Cotyledon pieces

Calli

Plate 14: Transformed tissues showing blue color during X-GLUC
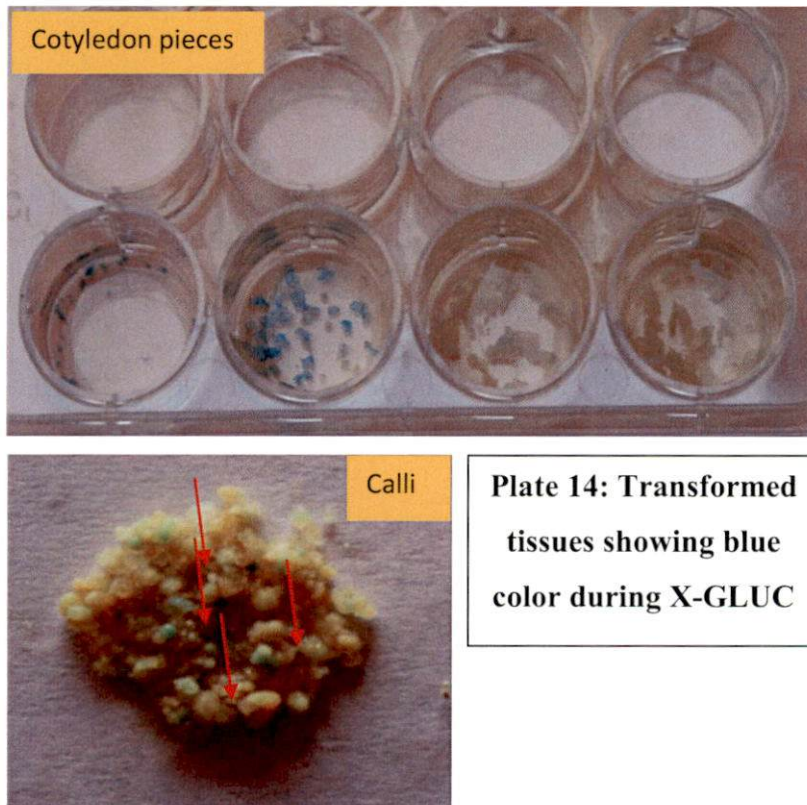
4.4. Molecular Analysis

The DNA was isolated from the leaves of the cassava varieties grown in field and also raised through tissue culture to study the stability of cassava varieties during *in vitro* culture. The quality of DNA was checked using Nanodrop spectrophotometer and the concentration of DNA is given in Table 21.

Table 21: Absorbance value and concentration of DNA used for study

Sample Name	Absorbance (A_{260nm})	Absorbance (A_{280nm})	A_{260nm}/A_{280nm}	Concentration (ng/µl)
Sree Prakash	0.078	0.038	2.07	392
Mulluvadi	0.081	0.040	2.05	405
9S-174	0.132	0.065	2.03	659
CI-273	0.171	0.084	2.03	857
IPS2-1	0.156	0.075	2.07	778
11S4	0.543	0.265	2.05	2715
Vellayani Hraswa	0.120	0.057	2.11	600
9S-127	0.047	0.023	2.06	235
Sree Sahya	0.194	0.095	2.05	971
Sree Prakash(T)	0.045	0.022	2.04	227
Mulluvadi(T)	0.115	0.058	1.98	573
9S-174(T)	0.028	0.014	2.01	142
CI-273(T)	0.037	0.019	2.02	187
IPS2-1(T)	0.159	0.074	2.15	795
Sree Sahya(T)	0.078	0.038	2.07	392
Vellayani Hraswa(T)	0.078	0.038	2.07	392
9S-127(T)	0.081	0.040	2.05	405

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4.4.1. Primer screening

Six ISSR primers were selected for primer screening to identify polymorphic primers and from that 3 were selected for further experiments.
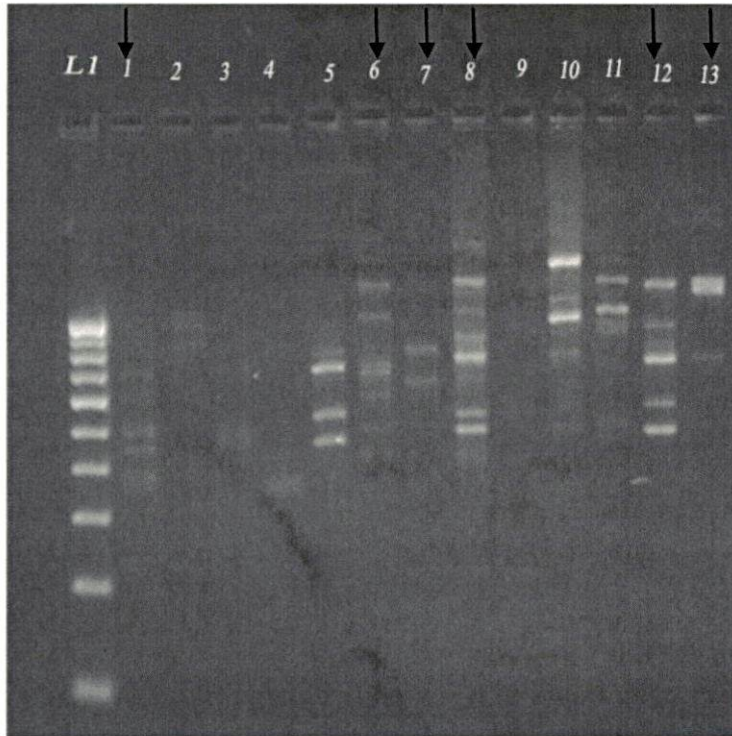
1 – 100 bp

2 - Sree Prakash with primer A

3 - Sree Prakash with primer B

4 - Sree Prakash with primer C

5 - Sree Prakash with primer D

6 - Sree Prakash with primer E

7 - Sree Prakash with primer F

8- 9S 174 with primer A

9 - 9S 174 with primer B

10 - 9S 174 with primer C

11 - 9S 174 with primer D

12 - 9S 174 with primer E

13 - 9S 174 with primer F

Plate. 15 Primer screening in Sree Prakash and 9S 174 with 6 primers.

Based on polymorphism the three ISSR primers (PRIMER A - UBC 811, PRIMER A - UBC 825, PRIMER A - UBC 827) were selected for studying the stability of cassava varieties during *in vitro* culture.

4.4.2. ISSR Analysis of Cassava genotypes

The amplicons obtained after the ISSR analysis of eight field grown plants and eight *in vitro* plants using selected primers were initially resolved in 2% agarose gel (Plates 14 to 16). These three primers *viz.* UBC 811, UBC 825, UBC 827 were used for PCR amplification and agarose gel electrophoresis was carried out and the analysis of these amplicons showed that UBC 811 primer showed difference in banding pattern of the genotypes *viz.* 9S 174, CI 273, IPS2-1 from field and *in vitro* raised plants. UBC 825 primer showed difference in banding patterns of 9S 174, CI 273 and UBC 827 primer showed difference in banding patterns of 9S 174 from field and *in vitro* raised plants. Other cassava accessions did not showed any change in banding patterns indicating their stability during *in vitro* culture.

GEL ANALYSIS OF FIELD AND *IN VITRO* SAMPLES OF SAME VARIETIES WHICH WAS AMPLIFIED BY ISSR PRIMER UBC 811:
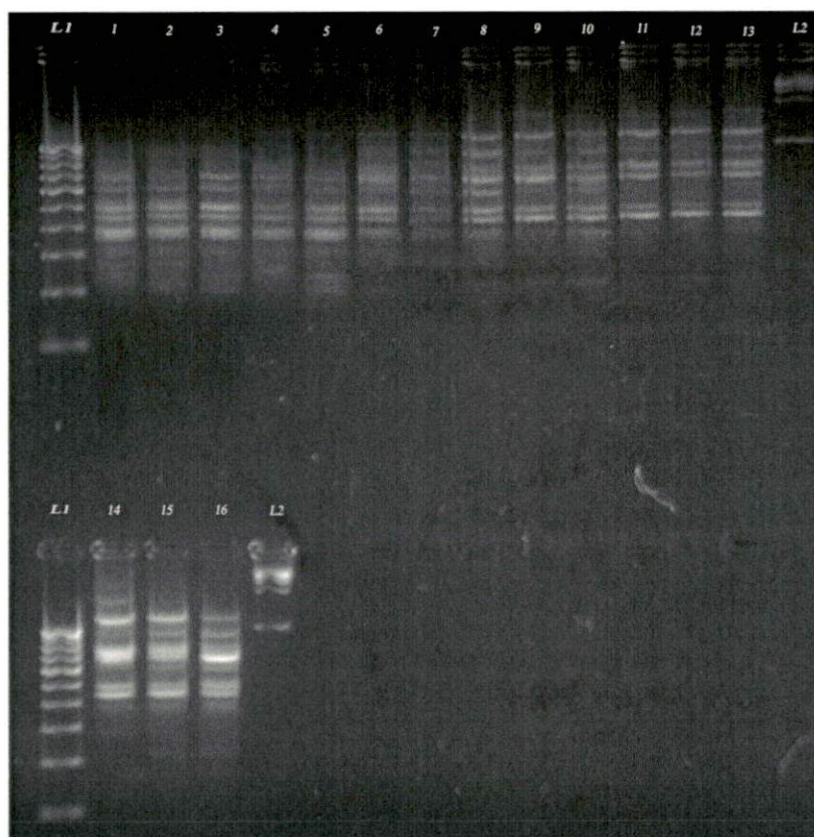
L1. 100 bp ladder

1. Sree Prakash (F)
2. Sree Prakash (I)
3. Mulluvadi(F)
4. Mulluvadi(I)
5. 9S 174(F)
6. 9S 174(I)
7. CI 273(F)
8. CI 273(I)
9. IPS2-1(F)
10. IPS2-1(I)
11. Sree Sahya(F)
12. Sree Sahya(I)
13. Vellayani Hraswa(F)
14. Vellayani Hraswa(I)
15. 9S 127(F)

Plate 16: Agarose gel (2%) showing genetic fidelity of cassava varieties with ISSR primer UBC 811

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GEL ANALYSIS OF FIELD AND *IN VITRO* SAMPLES OF SAME VARIETIES WHICH WAS AMPLIFIED BY ISSR PRIMER UBC 825:
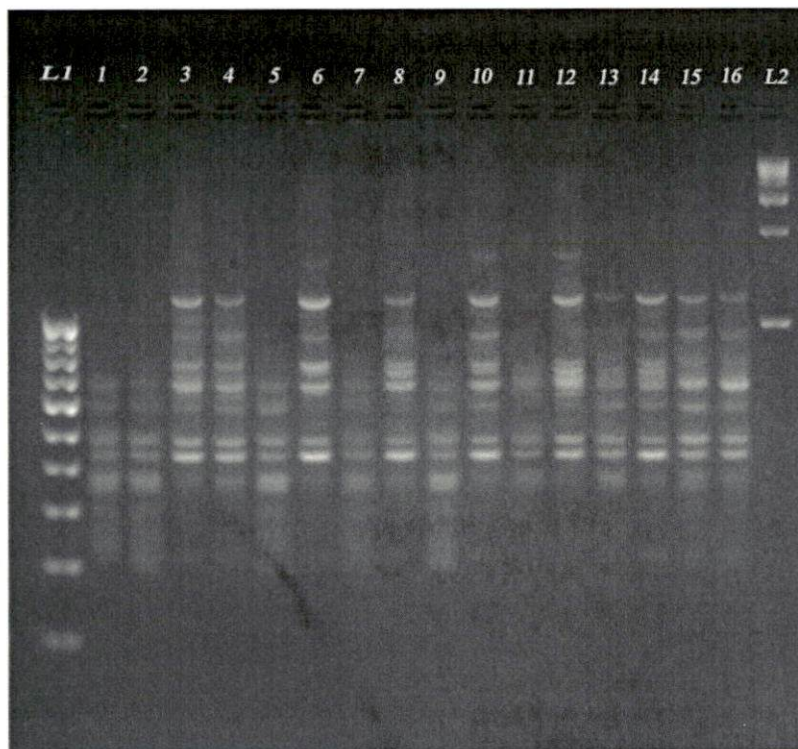
L1. 100 bp ladder
1. Sree Prakash (F)
2. Sree Prakash (I)
3. Mulluvadi(F)
4. Mulluvadi(I)
5. 9S 174(F)
6. 9S 174(I)
7. CI 273(F)
8. CI 273(I)
9. IPS2-1(F)
10. IPS2-1(I)
11. Sree Sahya(F)
12. Sree Sahya(I)
13. Vellayani Hraswa(F)
14. Vellayani Hraswa(I)
15. 9S 127(F)
16. 9S 127(I)
L2. 1KB ladder

Plate 17: Agarose gel (2%) showing genetic fidelity of cassava varieties with ISSR primer UBC 825

GEL ANALYSIS OF FIELD AND *IN VITRO* SAMPLES OF SAME VARIETIES WHICH WAS AMPLIFIED BY ISSR PRIMER UBC 827:

L1. 100 bp ladder
1. Sree Prakash (F)
2. Sree Prakash (I)
3. Mulluvadi(F)
4. Mulluvadi(I)
5. 9S 174(F)
6. 9S 174(I)
7. CI 273(F)
8. CI 273(I)
9. IPS2-1(F)
10. IPS2-1(I)
11. Sree Sahya(F)
12. Sree Sahya(I)
13. Vellayani Hraswa(F)
14. Vellayani Hraswa(I)
15. 9S 127(F)
16. 9S 127(I)
L2. 1KB ladder

Plate 18: Agarose gel (2%) showing genetic fidelity of cassava varieties with ISSR primer UBC 827

DISCUSSION

5. DISCUSSION

The cassava (*Manihot esculenta* Crantz) is a woody shrub comes in Euphorbiaceae family. It has edible starchy tuberous root, and used as an important dietary staple in many countries which is cultivated mainly in the tropic and sub-tropic regions of the world over a wide range of environmental and soil conditions. It is used as food, feed, fuel and industrial crop.

Cassava breeding through conventional approaches is often hampered with limitations like sparse flowering, poor seed set and high heterozygosity which make genetic engineering an attractive and efficient tool to complement traditional breeding in addressing major cassava production constraints. Genetic transformation in cassava was attempted for improving different attributes *viz.* reducing cyanogen content, delaying post harvest deterioration, improved nutritional quality and producing disease and pest resistant variety.

But the main bottleneck for the transformation process is the lack of regeneration system based on somatic embryogenesis. Due to non availability of popular varieties with high regeneration potential, cassava transformation is mainly carried out using the variety, TMS 60444, throughout the world. So there is a need to standardize transformation and regeneration protocols in location specific popular cassava genotypes. Hence the present investigation was carried out to standardize the protocols for regeneration through somatic embryogenesis and transformation and also to identify varieties with high embryogenic potential for the development of transgenic cassava in future.

5.1 Media standardization

5.1.1. *In vitro* culture

In vitro establishment of 13 elite genotypes of cassava *viz.* Sree Sahya, Sree Athulya, Sree Apoorva, Mulluvadi, Vellayani Hraswa, Sree Prakash, 9S 127, 9S 174, 11S4, CI 273, H 226, H 165 and IPS2-1 was carried out in different media combinations supplemented with varying hormone concentration. Initial *in vitro* establishment of the cassava varieties was carried out with varying combination of NAA, BAP and $CuSO_4$. Nodal explants taken from field grown plants of two varieties *viz.* 9S127 and 8W5 were inoculated in M1, M2, M3 and M4 media to identify the medium suitable for rapid production of leaves under *in vitro* conditions. In M1 and M2 the initial growth was found to be faster than the other two media. The better growth of the cassava varieties was obtained in M1 medium with higher number of leaves per explant. The M1 medium also recorded better rooting as compared to other media combinations. Hence M1 medium *ie* MS medium supplemented with Sucrose@ 30 g l^{-1} + 0.5 µM NAA + 2 µM BAP + 2 µM $CuSO_4$ + Agar @8 g l^{-1} was identified as the best medium for *in vitro* establishment of the cassava varieties. The present result was in conformity with the findings of Schopke *et al* 1993. However Anjum and Shazia, 2015 reported that *in vitro* culture was initiated in cassava using MS medium with BAP @3mgl^{-1}, NAA @ 0.5mg l^{-1} and IBA@ 1mg l^{-1}.

In order to produce large number of explants from *in vitro* plants, rapid production of immature leaves is very much essential. Hence media standardization was done for sub culturing of *in vitro* plants with the MS and SH media supplemented with TDZ and $AgNO_3$ in varying concentrations. The A4 medium recorded the highest number of leaves/ explants (8) at 15 days after inoculation followed by A3 (5 leaves) and A9 (4 leaves). The highest number of leaves per explants (14) at 45 days after inoculation was also recorded in A4 medium. The shoot length was highest in A8 medium (2.3

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cm) followed by A4 and A9 that resulted in a shoot length of 2 cm at 15 days after inoculation. The standardization of SH medium with varying combinations of TDZ and $AgNO_3$ was also carried out. The highest number of leaves per explant (10) was recorded for B10 followed by B1 (7). The shoot length ranged from 1.5 cm (B4) to 4.2cm (B10). Among the twenty four media tested for their efficiency in the production of leaves per explant and shoot length, the highest response was obtained in A4 medium *ie* the MS medium supplemented with Sucrose@ 30 g l^{-1} + 1 µM TDZ + 10 µM AgNO3 + Agar @8 g l^{-1} followed by B10 medium (SH Salt + Sucrose@ 30 g l^{-1} + 5 µM TDZ + 2 µM $AgNO_3$ + 8 Agar @8 g l^{-1}). In the present study, thidiazuron and silver nitrate favoured the growth of the cassava *in vitro* cultures. The effect of TDZ on regeneration varied with genotypes. TDZ (N-phenyl-N'-1, 2, 3-thidiazol-5-ylurea) is a substitutional phenyl urea compound that is known for its ability to induce a diverse array of cultural responses ranging from induction of callus to formation of somatic embryos. The TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants, although structurally it is different from either auxins or purine based cytokinins. Helen *et al* (2015) also reported the favorable effect of TDZ on multiple shoot formation in cassava in conformity with the present finding. However they got response with higher concentration of TDZ (10 mgl^{-1}).

5.1.2. Somatic embryogenesis

Friable embryogenic callus (FEC) is considered as the most suitable material for efficient genetic transformation of cassava as reported by Bull *et al.*, 2009; Liu *et al.*, 2011 and Xu *et al.*, 2013. For the production of FEC, embryogenic callus induction is the first step and in cassava, primary and secondary somatic embryos were produced by inoculating explants like immature leaf lobes, axillary buds, petioles in basal media supplemented with auxin like herbicides, picloram or 2,4-D (Taylor *et al.*, 1996). For the large scale production of explants and for easy handling, the *in vitro* plants were cultured in petriplates of 120mm size, where the plants grew very thickly

and quickly to fill the plates in media identified previously for rapid propagation. Eight MS with varying concentrations of sucrose (20 g l^{-1}, 30 g l^{-1}, 40 g l^{-1}), picloram (25 µM, 50 µM) and 2, 4 D (4 mgl^{-1}, 8mgl^{-1}) were evaluated for the identification of best media for induction of embryogenesis in cassava varieties. Sucrose @ 30 g l^{-1} resulted in higher no of somatic embryo per explant (10.5) followed by 20 g l^{-1} (5.25) and 40 g l^{-1} (4.75). Higher % of sucrose was found to have inhibitory effect on somatic embryo production. Konan *et al* (1994) also reported the inhibitory effect of higher concentration of sucrose on the development of somatic embryo in cassava in conformity with the present finding. According to them, high sucrose concentration (6%, w/v) inhibited the induction of somatic embryos, while 6% sucrose was optimal concentration for the development of somatic embryos after an induction treatment using 2% sucrose.

The mean no of somatic embryos per explant produced in MS media with hormones ranged from 4.25 to 13.25. The highest no of explants (13.25) was obtained in MS medium supplemented with 2,4 D@ 8mg l^{-1} followed by picloram @ 50µM (11.0). Sarria *et al.*, 2000 also reported good results while using 8mg/l 2,4-D in agreement with the present finding. Regeneration of plants from somatic embryos induced on cotyledons of zygotic embryos, immature leaves or primary somatic embryos had been reported as the only reproducible method of *de novo* regeneration of cassava *in vitro* (Stamp and Henshaw, 1982, 1987; Szabados *et al* .,1987; Mathews *et al.*, 1993 and Raemakers *et al.* 1993 a,b).

Somatic embryogenesis from axillary buds and immature leaf lobes was recorded in all the cassava varieties selected for the study. When axillary buds were used as explants the callusing percentage ranged from 0-100%. Sree Sahya and Sree Athulya recorded 100% callus induction followed by H 226 (60%), Sree Apoorva (50%) and IPS2-1 (40%). Other cassava varieties did not produce any calli. Embryogenic callus was also produced from these cassava varieties when axillary buds were used as explants and it ranged from 0-30%. Eugene *et al* (2011) in a study on the

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callogenesis expression also reported that the leaf explants were superior in callus formation in all the Ugandan cassava varieties studied by them in agreement with the present finding on Indian cassava varieties.

Among the varieties evaluated for embryogenic efficiency, Sree Sahya recorded the highest percentage of embryogenic callus (30%) followed by Sree Athulya (12 %), H 226 (12%) and H 165 (6%). Other cassava varieties did not produce any embryogenic callus.

Somatic embryogenesis is now the most commonly used regeneration method of cassava. Successful regeneration of cassava has been reported, when explants were grown on an induction medium with plant growth regulators and later transferred to regeneration medium with or without growth regulators. In the present study, somatic embryos were inoculated in MS medium supplemented with BAP@2µM and Sucrose@30g l^{-1} and Agar@8 g l^{-1} for regenerating plantlets.

5.2 Transformation

The *Agrobacterium* mediated transformation was carried out using the strains *viz.* LBA4404, EHA105 and AGL-1 with a cocultivation duration of 12 hrs, 24 hrs and 48 hrs. Transformation was obtained only when LBA4404 was used with a co-cultivation time of 48 hrs. The callus of the variety Sree Sahya co-cultivated with *Agrobacterium*- LBA 4404 which possessed PBI121 vector was examined through X-Gluc assay. Transformed callus were appeared in blue color after co-cultivation for 48 hours. In transformed one β- glucuronidase enzyme came in function and cleaved X-Gluc and produced 5,5'-dibromo-4,4' – dichloro indigo which appeared in blue color. As compared to callus the cotyledonary explants recorded higher transformation efficiency. The present finding was in conformity with the reports of Sarria *et al* 2000. Sheela and Nigel, 2007 studied the efficiency of different *Agrobacterium* strains viz. AgL8, GV3101, C58C1, LBA4404 and EH1205 in transforming cassava. They reported the maximum recovery of transgenic tissues

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when EH 105 was used whereas in the present investigation LBA4404 resulted in higher transformation in cassava.

5.3. Molecular Analysis

Genetic fidelity of the cassava genotypes was checked between somatic embryo raised plants and field plants. Primer screening was carried out using 6 ISSR primers and 3 were selected which give better results. Vidal *et al.*, 2015 used ISSR markers to check the genetic fidelity of cassava genotypes. The ISSR primers *viz.* UBC 811, UBC 825, UBC 827 were selected for PCR amplification. The gel analysis of amplicons with UBC 811 showed dissimilarity in 2 cassava genotypes *viz.* 9S 174, CI -273. When UBC 825 was used, three cassava varieties showed dissimilarity *viz.* 9S 174, CI -273 and IPS2-1 while when UBC 827 was used, only one cassava variety showed dissimilarity *viz.* 9S 174.

SUMMARY

6. SUMMARY

The cassava (*Manihot esculenta* Crantz) is an important dietary staple in many countries which is cultivated mainly in the tropic and sub-tropical regions of the world over a wide range of environmental and soil conditions. Cassava breeding through conventional approaches is hampered with limitations like sparse flowering, poor seed set and high heterozygosity which make genetic engineering an attractive and efficient tool to complement traditional breeding in combating major cassava production constraints. But the main bottleneck for the efficient production of transgenics is the lack of regeneration system in popular farmer preferred varieties. Friable embryogenic callus is the most efficient explant for transformation of cassava. Although a number of transformation systems and selection regimes have been developed for cassava, they have only been applied in a limited number of genotypes. Due to nonavailability of popular varieties with high regeneration potential, cassava transformation is mainly carried out using the variety, TMS 60444, throughout the world. So it is essential to standardize transformation and regeneration protocols in location specific popular cassava genotypes. Hence the present investigation was carried out to standardize the protocols for regeneration through somatic embryogenesis and transformation and also to identify varieties with high embryogenic potential for the development of transgenic cassava in future.

In vitro establishment of 13 elite genotypes of cassava *viz.* Sree Sahya, Sree Athulya, Sree Apoorva, Mulluvadi, Vellayani Hraswa, Sree Prakash, 9S 127,9S 174, 11S4, CI 273, H 226, H 165 and IPS2-1 was carried out in different media combinations supplemented with NAA, BAP and $CuSO_4$. The M1 medium *ie* MS medium supplemented with Sucrose @30 g l^{-1} + 0.5 μM NAA + 2 μM BAP + 2 μM $CuSO_4$ + Agar @8 g l^{-1} was identified as the best medium for *in vitro* establishment of the cassava varieties.

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In order to produce large number of explants from *in vitro* plants, rapid production of immature leaves is very much essential. Hence media standardization was done for sub culturing of *in vitro* plants with the MS and SH media supplemented with TDZ and $AgNO_3$ in varying concentrations. The highest number of leaves per explants (14) at 45 days after inoculation was also recorded in A4 medium. Among the twenty four media tested for their efficiency in the production of leaves per explant and shoot length, the highest response was obtained in A4 medium *ie* the MS medium supplemented with Sucrose @30 g l^{-1} + 1 μM TDZ + 10 μM AgNO3 + Agar @ 8g l^{-1} followed by B10 medium (SH Salt + Sucrose @30 g l^{-1} + 5 μM TDZ + 2 μM $AgNO_3$ + Agar@ 8 g l^{-1}).

Eight MS with varying concentrations of sucrose (20 g l^{-1}, 30 g l^{-1}, 40 g l^{-1}), picloram (25 μM, 50 μM) and 2, 4 D (4 mgl^{-1}, 8mgl^{-1}) were evaluated for the identification of best media for induction of embryogenesis in cassava varieties. Sucrose @ 30 g l^{-1} resulted in higher no of somatic embryo per explant (10.5) followed by 20 g l^{-1} (5.25) and 40 g l^{-1} (4.75). Higher percentage of sucrose was found to have inhibitory effect on somatic embryo production.

The highest no of explants (13.25) was obtained in MS medium supplemented with 2, 4 D@ 8mg l^{-1} followed by picloram @ 50μM (11.0). Somatic embryogenesis from axillary buds and immature leaf lobes was recorded in all the cassava varieties selected for the study. When axillary buds were used as explants the callusing percentage ranged from 0-100%. Sree Sahya and Sree Athulya recorded 100 % callus induction followed by H 226 (60%), Sree Apoorva (50%) and IPS2-1 (40%). Other cassava varieties did not produce any calli.

Among the varieties evaluated for embryogenic efficiency, Sree Sahya recorded the highest percentage of embryogenic callus (30%) followed by Sree Athulya (12 %), H 226 (12 %) and H 165 (6%). Other cassava varieties did not produce any embryogenic callus. The somatic embryos were inoculated in MS medium

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supplemented with BAP@2μM and Sucrose@30g l^{-1} and Agar@8 g l^{-1} for regenerating plantlets.

The *Agrobacterium* mediated transformation was carried out using the strains *viz.* LBA4404, EHA105 and AGL-1 with a cocultivation duration of 12hrs, 24 hrs and 48 hrs. Transformation was obtained only when LBA4404 was used with a co-cultivation time of 48 hrs. As compared to callus, the cotyledonary explants recorded higher transformation efficiency.

Genetic fidelity of the cassava genotypes was checked between somatic embryo raised plants and field plants. Primer screening was carried out using 6 ISSR primers and UBC 811, UBC 825, UBC 827 were selected for PCR amplification. The gel analysis of amplicons with UBC 811 showed dissimilarity in two cassava genotypes *viz.* 9S 174, CI -273. When UBC 825 was used, three cassava varieties showed dissimilarity *viz.* 9S 174, CI -273 and IPS2-1 while when UBC 827 was used, only one cassava variety showed dissimilarity *viz* 9S 174. Out of the 13 varieties, ten varieties showed genetic stability during regeneration using the protocol standardized during present investigation.

The regeneration protocol standardized during the present study can be used in developing transgenics in future especially in cassava varieties *viz.* Sree Sahya, Sree Athulya, Sree Apoorva and H-226. However further studies need to be carried out in developing high quality friable embryogenic calli in these popular varieties.

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APPENDICES

MS media:

MS media – 4.4g

Sucrose – 30g

Agar – 8 g

Distilled water – 1000 ml

pH – 5.7 – 5.8

4.4 g of MS salt is dissolved in 800 ml of distilled water and 30 g of sucrose is added to it. Then hormones are added in to the media and the pH is checked and it is adjusted using NaOH and Hcl. Then the media is made up to 1000 ml. Then agar is added and it is melted and poured in test tubes.

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- EXTRACTION BUFFER (15 ml)

1M Tris- HCl   : 1.5ml

0.5M EDTA   : 1.5ml

5M Nacl   : 1.8ml

PVP   : 0.4g

0.02% Mercaptoethanol   : 10µl

Make up to 15ml

Appendix III

- 10X TBE BUFFER (pH: 8.25)

Tris   : 107 g

Boric acid   : 55g

EDTA (0.5M)   : 40ml

Make upto 1000ml with double distilled water.

- 1X TBE BUFFER

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10ml of 10X TBE and 90ml double distilled water.

Appendix IV

- TE BUFFER (pH : 8)

1M Tris base	: 1ml
0.5 M EDTA	: 200µl

Make upto 100 ml and adjust the pH to 8.

Appendix V

- GEL LOADING DYE

Glycerol	: 3.5ml
10X TBE	: 0.25ml
0.5M EDTA	: 0.20ml
20% SDS	: 50µl
10% Bromophenol blue	: 300µl
Sterile Water	: 750µl

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Appendix VI

- ETHIDIUM BROMIDE

  10ng/ml

- RNase A:

  Dissolve 10 mg of RNase in 1ml TE buffer on 1ml double distilled water.

  Preheat the mixture to 80°c for 10 min to activate RNases.

Appendix VII

- AGAROSE GEL (1%)

  Agarose   : 1g

  Doube distilled water   : 100ml

  Gently boil the solution in microwave oven with occasional mixing until all particles are completely dissolved.

Appendix VIII

GUS ASSAY

- Tris – NaCl Buffer (1L)

  Tris Cl (50mM)   : 6.05g

  NaCl (5M)   : 8.76g

  Distilled water   : 800 ml

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Adjusted the pH to 7.5

Make up to 1000ml and stored at 4°c

- Gus Assay solution (1ml)

  Tris – NaCl Buffer   : 89 ml

  X- gluc (10mg/ml   : 10ml

  10% Triton X 100   : 1ml

ABSTRACT

Development of efficient transformation and regeneration protocols in elite genotypes of cassava (*Manihot esculenta* Crantz)

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Abstract of the thesis

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9. ABSTRACT

Cassava (*Manihot esculenta* Crantz) is an important dietary carbohydrate source for approximately 800 million people in the tropics. It is used as a food, feed and industrial crop. It has also been projected as a potential biofuel crop. Cassava breeding through conventional approaches is often hampered with limitations like sparse flowering, poor seed set and high heterozygosity which make genetic engineering an attractive and efficient tool to complement traditional breeding in addressing major cassava production constraints. The main bottleneck for the development of transgenic cassava with desirable traits is the lack of efficient regeneration system based on somatic embryogenesis in popular cassava varieties grown in India. Therefore the present investigation was undertaken with the objective of optimization of efficient regeneration and transformation protocol in elite genotypes of cassava.

The present study was carried out using 13 cassava genotypes including seven released varieties and six CMD resistant pre-release clones. Four media for the initial *in vitro* establishment of these varieties were evaluated and M1 media *ie* MS medium supplemented with Sucrose @ 30 g l^{-1} + 0.5 µM NAA + 2 µM BAP + 2 µM $CuSO_4$ + Agar @ 8 g l^{-1} was identified as the best medium that resulted in faster establishment and growth of the cultures.

In order to produce large number of explants from *in vitro* plants, rapid production of immature leaves is very much essential. Hence media standardization was done for the sub culturing of *in vitro* plants in MS and SH media supplemented with TDZ and $AgNO_3$ in varying concentrations. Among the twenty four media tested for their efficiency in the production of leaves per explant and shoot length, the highest response was obtained in A4 medium *ie* the MS medium supplemented with Sucrose

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@30 g l^{-1} + 1 µM TDZ + 10 µM AgNO$_3$ + Agar @ 8 g l^{-1} followed by B10 medium (SH Salt + Sucrose @30 g l^{-1} + 5 µM TDZ + 2 µM AgNO$_3$ + Agar @ 8 g l^{-1}).

Eight MS with varying concentrations of sucrose (20 g l^{-1}, 30 g l^{-1}, 40 g l^{-1}), Picloram (25 µM, 50 µM) and 2, 4 D (4 mgl^{-1}, 8mgl^{-1}) were evaluated for the identification of best media for induction of embryogenesis in cassava varieties. Sucrose @ 30 g l^{-1} resulted in higher no of somatic embryo per explant (10.5) followed by 20 g l^{-1} (5.25) and 40 g l^{-1} (4.75). The mean no of somatic embryos per explant produced in MS media ranged from 4.25 to 13.25. The highest no of explants (13.25) was obtained in MS medium supplemented with 2,4 D@ 8mgl^{-1} followed by picloram @ 50µM (11.0). Unopened leaf lobes were found to be the best explants for induction of somatic embryogenesis as against axillary buds. Among the varieties evaluated for embryogenic efficiency, Sree Sahya recorded the highest percentage of embryogenic callus (30%) followed by Sree Athulya (12 %), H 226 (12 %) and H 165 (6%). Other cassava varieties did not produce any embryogenic callus. The somatic embryos were inoculated in MS medium supplemented with BAP @2µM and Sucrose @30g l^{-1} and Agar @8 g l^{-1} for regenerating plantlets.

The *Agrobacterium* mediated transformation was carried out using the strains *viz.* LBA4404, EHA105 and AGL-1 with co-cultivation for 12hrs, 24 hrs and 48 hrs. Transformation was obtained only when LBA4404 was used with a co-cultivation time of 48 hrs. Also the cotyledon explants of the variety Sree Sahya recorded higher transformation efficiency as compared to calli revealed by X-gluc assay.

Genetic fidelity of the cassava genotypes was checked between somatic embryo raised plants and field plants. Primer screening was carried out using 6 ISSR primers and UBC 811, UBC 825, UBC 827 were selected for PCR amplification. The gel analysis of amplicons showed genetic stability in ten varieties during regeneration using the protocol standardized during present investigation.

The regeneration protocol standardized during the present study can be used in developing transgenics in future especially in cassava varieties *viz.* Sree Sahya, Sree Athulya, Sree Apoorva and H-226. However further studies need to be carried out in developing high quality friable embryogenic calli in these popular varieties.

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