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Towards Genetic Engineering in Cocoyam Food Crop: Challenges and Prospects

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Abstract

Cocoyams (Colocasia esculenta and Xanthosoma sagittifolium) are important staple food in many parts of tropics including Africa, Asia and Pacific. Cocoyam is categorised as a neglected food crop and mainly grown for subsistence agriculture. Attempts to improve the crop have been limited due to knowledge gaps in physiological and biological processes affecting breeding against biotic and abiotic stresses. Genetic engineering offers an alternative platform in advancing improvement. This paper highlights the progress made in tissue culture through micropropagation, organogenesis and somatic embryogenesis. The influence of genotypes, explant sources, and culture media is brought into perspective while elucidating system regeneration efficiency using axillary buds, shoot tips, meristem tips and petiole. The efficiency of transformation system using both Agrobacterium tumefaciens and particle bombardment is highlighted while suggestions are made for future research in genetic engineering.

Keywords: Cocoyam; Breeding; Agrobacterium-mediated transformation; Particle bombardment; Genetic engineering

Introduction

Cocoyams (Colocasia esculenta and Xanthosoma sagittifolium) are important staple food in Africa, Asia and the Pacific. The corms and cormels are edible [1] and are usually cooked by boiling, roasted, baked, steamed or fried and used as a starchy vegetable [2] or supplemental food [3]. In addition to sustaining food security in domestic market, it also brings import earnings [4]. They represent an excellent source of carbohydrate, the majority being starch of which 17-28% is amylase, and the remainder amylopectin [5]. Cocoyam starch is one of the most nutritious and 98.8 percent digestible, a quality attributed to its granule size making it ideal for people with digestive difficulties [6]. Cocoyams offer an alternative source of daily calories during periods of food scarcity and economic stress [7].

The removal of cocoyam from the focus of Consultative Group for International Agricultural Research (CGIAR) centres in the past contributed to the limited research investment and knowledge advancement. In addition, many developing countries where cocoyam is grown experience difficulty in sustaining conservation and genetic improvement [8]. Two important diseases that leads to considerable decline in yield include the cocoyam root rot disease (CRRD) caused *Pythium myriotylum* and taro leaf blight (TLB) caused by *Phytophthora colocasiae*. CRRD and TLB have the potential of depleting the diversity in the already narrow genetic base of these crops because of the high susceptibility of most farmers' cultivars which results in their inability to survive in the field [7]. The impacts of these diseases are exacerbated by the fact that cocoyam's vegetative mode of propagation supports transmission of diseases from one generation to the next.

International and regional efforts under the auspices of The International Network for Edible Aroids (INEA), Taro Network for Southeast Asia and Oceania (TANSOA), Taro Genetic Resources: Conservation and Utilization project (TaroGen), Root and Tuber Research Project (ROTREP) in Cameroon, The Cocoyam Rebirth Initiative (TCRI) in Nigeria have sought to develop adaptations to production challenges. Part focus of these initiatives is breeding improvement and international germplasm exchange. The future of cocoyam depends on selection of high yielding, good quality genotypes as well as development of low cost technologies that will enhance its production. A recent report by Onyeka show that available germplasm is highly susceptible to biotic challenges. The main factor limiting classical inter and intra specific hybridization in cocoyam is the irregularity of flowering and the abnormalities of the inflorescence structure. This implies that alternative means of crop improvement including genetic engineering needs to be sought. Genetic engineering offers the potential of disease-resistant, transgenic lines that retain the same genetic composition of the original cultivars but with the addition of a few genes that confer disease resistance [9]. This review presents a timely effort in unfolding present knowledge while highlighting apparent gaps towards traits improvement.

Micropropagation, or Ganogenesis and Somatic Embryogenesis

Plant regeneration from tissue and cell culture help in achieving fast clonal multiplication, recovery of pathogen free plants, germplasm preservation and induction of chromosomal and genic variation [10,11]. This is most useful in asexually propagated plants like cocoyam where tubers encourage the pathogen dissemination and subsequent loss of vigor and productivity [12]. It involves the use of defined growth media supplemented with appropriate growth regulators that enable morphogenesis to occur from naturally growing plant parts. This helps in producing a large number of plants from a single individual in short time and in limited space [13]. Table 1 shows different procedures for in vitro culture of various cultivars and target tissue. Cultivars respond differently in respect to callus, shoot and somatic embryogenesis induction [9]. Full-strength nutrients are not conducive for callus formation in C. esculenta var. esculenta [14,15]. Shoots produced with BAP are larger and more normal in appearance than those produced with TDZ, [13]. If the meristem is cultured rather

than the whole bud, it is possible to eliminate viruses, which are particularly problematic in vegetatively propagated crops. Meristem culture of cocoyam perform better in terms of yield than virus-infected in vitro plants [16]. Cormels from meristem-derived plants grow faster and tuberises earlier in comparison with conventionally propagated plants [17]. Meristem-derived plants are also important for a safe exchange of germplasm between countries. In vitro storage of cocoyam under minimal growth conditions [18] and cryopreservation of in vitro shoot tips have been reported as suitable alternatives to field collections [19]. Regeneration of cocoyam plants from protoplasts has been reported [20] due to the establishment of protocols for callus initiation. The major limitation remains that the frequency of regeneration is very low and takes a long time. While de novo regeneration in C. esculenta var. esculenta has been reported [21-24], the initiation of highly regenerable callus is the first step towards an efficient regeneration system and remains a prime requirement for an efficient protoplast culture [20,25]. The protocols developed for *C. esculenta* var. *antiquorum* do not appear to be suitable for cultivars belonging to var. esculenta. To circumvent this limitation, tissue culture system capable of producing highly generative calli as well as a protocol of establishment of in vitro stock culture using shoot tips as explants as well as protocol for inducing calluses and multiple shoots using in vitro shoot tips have been improved [26,27]. A temporary immersion technique for large-scale propagation of *Xanthosoma sagittifolium* using a bioreactor was developed [28]. The authors reported an optimal development with proliferation rate of 68 ± 7% with 20 g l−1 sucrose in the culture medium.

Genetic material/cultivar	Explants	Media/reagents	References
Callus induction			
C. esculenta var. antiquorum	Shoot apex	LS+2, 4, 5 T + K + 0.01, 0.1 and 1 mM S	[48]
C. esculenta var. antiquorum	Axillary buds	MS+ 20 TE + 2NAA + 0.2BA	[14]
C. esculenta var. antiquorum	Axillary buds	Half- strenght MS + 25 TE + 2, 4, 5 T + 200 G	[14]
C. esculenta var. esculenta	Axillary buds	MS+ 1 mg NAA 1(-1) + TE	[14]
C. esculenta var. esculenta	Shoot tips	MS+ 2 mg/L BA + 1 mg/L NAA	[27]
C. esculenta var. esculenta	Shoot tips	LS + 15 p.p.m. IAA or 2 p.p.m. 2,4,5-T	[49]
X. sagittifolium and X. violaceum	Apical meristem	AZ + 2.0 mg 1-1 + 1-NAA	[50]
X. sagittifolium	Shoot tips/petiole	MS + 1.36 µM dicamba+ 0.045 µM TDZ	[51]
C. esculenta var. esculenta	Shoot tips	Nitsch medium + 2, 4-D + 6-BA at 1 mg/liter-1	[25]
C. esculenta var. esculenta	Etiolated stem segments	MS + 30 g/liter-1sucrose + 2 mg/liter-12, 4-D + 2 mg/liter-12 ip	[20]
	Axillary buds	MS + 1 mg L-1 BAP + 2.0 mg L-1 NAA	[12]
Shoot induction			
C. esculenta var. esculenta	Callus	BA (2) + NAA (1)	[9]
C. esculenta var. esculenta	Callus	BA (0.2) + 2,4-D (0.5)	[9]
C. esculenta var. esculenta	Callus	BA (1) + 2,4-D (3)	[9]
C. esculenta var. esculenta	Callus	BA (3) + 2,4-D (3)	[9]
C. esculenta var. esculenta	Callus	Kinetin (1) + NAA (1.5)	[9]
C. esculenta var. esculenta	Primary shoot apices	LS + 5.5 mg 1(-1) NAA +0.2 mg 1-1 K	[52]
C. esculenta var. esculenta	Primary shoot apices	LS + 1.85 mg 1-1 NAA + 2 mg 1-1 K + 10-4 or 10-3 mol•1-1 of polyamine spermine	[52]
C. esculenta var. esculent	Axillary buds	MS + 20 TE	[14]
C. esculenta var. esculent	Axillary buds	1/2 MS + 100 ml coconut water + 25 TE	[14]
C. esculenta var. esculenta	Axillary buds	1/2 MS+ 2.2- 4.4 BA	[53]
C. esculenta var. esculenta	Meristem	MS+ 4.3 µM (1.0 mg L-1) TDZ	[54]
Xanthosoma sagittifolium	Shoot tips	MS + 20 g l−1 sucrose	[55]
Xanthosoma sagittifolium	Shoot tips	1 µM TDZ + 5 µM BAP + 0.05 µM NAA	[56]

Xanthosoma sagittifolium	Shoot tips	Gamborg's B5 + 0.05 µM 1-NAA + 20 µM BAP and/or 2 µM TDZ	[13]
Xanthosoma sagittifolium	Shoot tip callus	Liquid MS + 1.36 µM dicamba or 1.35 µM 2,4-D + 0.46 µM K or 1.36 µM dicamba + 0.46 µM K	[51]
C. esculenta var. esculenta	Callus	MS + 0.6% agar + NAA + BA at 1 mg/liter^{-1}	[25]
C. esculenta var. esculenta	Protoplast-derived callus	MS + 0.2 mg/liter-1 NAA + 2 mg/liter-1 BA.	[20]
	Meristem	MS + BA (8 mg/l) + IAA(3 mg/l)	[57]
C. esculenta var. esculenta	Petiole	Step 1- MS medium + 2.2 mg/L 2,4-D and 0.44 mg/L TDZ Step 2- culture phase with 1.1 mg/L TDZ	[23]
Somatic embryogenesis			
Unknown	Petiole	MS medium + 1.0-2.0 mg/L TDZ	[22]
Embryo induction			
Maturation and germination	Callus	Half-strength MS + TDZ (0.1 mg/L),+ 2,4-D (0.05 mg/L), glutamine (100 mg/L) + sucrose (50 g/L).	[15]
C. esculenta var. esculenta	Embryo derived from Callus	Half-strength MS + IAA (0.1 mg/L) + BAP (0.05 mg/L).	[15]
Organ differentiation			
X. violaceum	Callus	MS + 5.0 mg 1-1 NAA +100 ml 1-1 coconut-milk + 2.0 mg 1-1 K	[50]
Xanthosoma sagittifolium	Callus	MS + 2.0 mg 1-1 k + 0.2-2.0 mg 1-1 NAA	[50]
C. esculenta var. esculenta	Callus	LS + adenine + N-benzyl-9-(tetrahydro-2H-pyran-2-yl)	[49]
Protoplast culture			
C. esculenta var. esculenta	Protoplast from callus	Half MS + KM + NAA + 2 mg/liter-1 BA + 0.1M glucose + 0.3M mannitol	[20]

MS: Murashige and skoog; KM: organic substances of Kao and Michayluk (1975); LS: Linsmaier and skoog (1965) medium; BA: Benzyladenine; BAP: Benzylaminopurine; NAA: Napthaleneacetic acid; IAA: Indoleacetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; 2, 4, 5 T: 2, 4, 5-trichlorophenoxyacetic ; K: kinetin; G: Glutamine; S: Spermine; TDZ: Thidiazuron; Parentheses contain concentrations in mgL–1.

Table 1: Characteristics of growth media, target cultivar and explant for the induction of callus, shoot, somatic embryogenesis, embryo, organ differentiation and maturation in cocoyam.

Gene Mapping and Functional Annotation of Genes

The first *C. esculenta* genetic map containing 161 amplified fragment length polymorphisms (AFLPs) and 8 simple- sequence repeats (SSRs) in 2004 [29]. The authors identified several quantitative trait loci (QTLs) for corm yield and corm dimensions. QTL detection was performed on data from one clonal generation for traits like number of suckers, corm dimensions, and dry matter content. Although heritabilities were higher for number of suckers and dry matter content, only six putative QTLs were found for yield and related components. Genes have been characterized, isolated, expressed and validated as soluble starch synthase I and II genes [30,31]. Researchers have equally isolated genes for tarin, a family of storage proteins that accounts for approximately 40% of the total soluble protein in taro corms [32,33]. Gene that encodes a tarin isoform was isolated and characterized in taro and shares a homology with lectins that are thought to play a role in defence [33,34].

Genetic Transformation

Genetic engineering can help in the development of disease-resistant, transgenic cocoyams that retain the same genetic composition of the original cultivars but with the addition of a few genes that confer disease resistance [9,27]. Table 2 shows past transformation efforts in cocoyam as well as adopted strategies. The first transformation *in C. esculenta var.* antiquorum callus was obtained from the apical meristem and maintained in liquid culture via high-velocity particle bombardment [35]. This was done with the insertion of the reporter gene β-glucuronidase (*gus*) into triploid cv. Eguimo (major cultivars grown in Japan). This resulted into two putative transgenics that was confirmed by amplification of the GUS gene and Southern hybridization. Transformation in *Colocasia esculenta var. esculenta* via microprojectile bombardment was subsequently reported [9,36]. The authors showed a successful particle bombardment transformation method in inserting a disease resistance gene, rice chitinase gene Chi 11 with a low efficiency of less than 0.5%. In their Southern blot analysis, a high-copy insertion of the transgene was observed thus indicating a high risk of transgene silencing and rearrangement. The particle bombardment that was adopted in both

studies resulted in low efficiency with only one stably transformed plant generated. Agrobacterium-mediated transformation approach was used to transform *C. esculenta var.* esculenta cv. Bun Long (major commercial variety in Hawaii and the Dominican Republic) [26,37,38]. This approach used two plant disease resistance genes: a rice chitinase gene (RICCHI11) and a wheat oxalate oxidase gene (gf2.8). Two hundred calli were infected with the supervirulent A. tumefaciens strain EHA105 harboring the plant transformation plasmid pBI121:RICCHI11. In all studied lines, only six contained the rice chitinase gene after confirmation with PCR and Southern blot analyses. Transformation was equally done with vector EHA105:pBI121:gf2.8 that contains the wheat oxalate oxidase gene gf2.8. Two independent lines were positive for the specific gf2.8 gene fragment upon confirmation with PCR, Southern blot and RT-PCR analyses. Following a preliminary bioassay, the six transgenic taro lines with the rice chitinase gene showed significant tolerance to the fungal pathogen Sclerotium rolfsii while one transgenic taro line with the wheat oxalate oxidase gene exhibited significant tolerance to both the fungal pathogen Sclerotium rolfsii (causal agent for Southern Blight) and oomycete pathogen Phytophthora colocasiae (the causal agent of TLB). Based on these studies, Agrobacterium tumefaciens-mediated transformation of taro has been shown to be more efficient than the particle bombardment transformation method [27].

Material transformed	Gene transformed	Strategy	Transformation efficiency (%)	Bioassay	Reference
Embryogenic suspension cultures- var. esculenta	gfp reporter gene	PB, AMT	-	-	[24]
Embryogenic calli C. esculenta var. antiquorum	gus reporter gene, hpt selection gene	PB	0.1	Resistance to Sclerotium rolfsii	[35]
Regenerative calli Eguimo (triploid)	gus reporter gene, nptII selection gene, rice chinitase gene chi 11, wheat oxalate oxidase gene gf2.8	PB, AMT	0.05-3	Significant resistance to Sclerotium rolfsii	[26]
Regenerative calli- C. esculenta	gus reporter gene, nptII selection gene, rice chinitase gene chi 11	AMT	3	Significant resistance to Sclerotium rolfsii	[37]
Regenerative calli- C. esculenta	gus reporter gene, nptII selection gene, rice chinitase gene chi 11	PB	0.5	Significant resistance to Sclerotium rolfsii	[9]
Regenerative calli- C. esculenta	wheat oxalate oxidase gene gf2.8	AMT	-	Significant tolerance to Sclerotium rolfsii and Phytophthora colocasia	[38]
Regenerative calli- C. esculenta	rice chitinase gene chi 11 or a wheat oxalate oxidase gene gf2.8	AMT	-	e -	[27]

Table 2: List of genetic material, genes transformed, adopted strategy, transformation efficiency and resistance bioassay in cocoyam.

There has been effort to develop effective transformation system in the esculenta variety using both Agrobacterium tumefaciens and microprojectile bombardment of regenerable embryogenic suspension cultures [24]. Putative stably transformed embryos expressing the gfp reporter gene with an efficiency of ~ 200 and ~ 17 per mL SCV for microprojectile bombardment and Agrobacterium tumefaciens respectively. The major limitation of this study remains that molecular characterization of the resulting embryos was not done. It has to be noted that the study approach of Deo differs from that of He et al. The former used embryogenic suspension cultures while the later used regenerable callus as the target tissue for transformation. The low frequency recorded by the use of regenerable callus might has resulted to the low frequency observed in the transformation. The advantages of embryogenic suspension cultures include easy proliferation of target tissue, maximal exposure to the transforming agent thus facilitating the identification of independent transformation events within the dispersed cell clusters [39].

Future Perspective

Collection of germplasm representing the genetic diversity is a prerequisite for its effective conservation and utilization. The use of complementary conservation strategies, especially in vitro techniques for efficient conservation and utilization is thus necessary. Plant tissue culture technique help in the production of plants with superior quality. Unfortunately developing countries in Africa, Asia and Pacific who grow cocoyam as staple food crop have not exploited this technology. Micropropagation is more expensive than the traditional methods of plant propagation. It requires trained and enhanced competence while the cost per plant is high. Low cost alternatives are most desirable to reduce production cost of tissue-cultured plants. Such options include the use of analytical grade sucrose on culture growth [40]. Commercial grade chemicals of lower purity than the analytical grades are quite suitable for commercial micropropagation unless deleterious effects are observed. Low cost media have been developed for the multiplication of orchids [41]. Distilled water is an expensive pre-requisite for plant tissue culture. Due to the absence of electrical distillation system in poor economies that grow cocoyam, tap water that is devoid of heavy metals and contaminants can be used as a low cost alternative. This has been successfully demonstrated in the in vitro propagation of banana [42]. Reduction in the cost of energy is essential to lower the production cost of micropropagated plants. Large amount of electrical energy is used up in autoclaving, lighting of the growth room, air filtration in laminar-flow cabinets and air conditioning. In addition to the cost there is evidence of erratic energy supply and fluctuating voltage in most developing countries where cocoyam is a staple food. In vitro cultures can make use of natural lights. To avoid heat buildup this might generate in the tropics,

thermostat-controlled exhaust fans can be installed. Another realistic alternative is the use of low cost solar tube system which can be recharged with natural sun.

Tissue-cultured propagules have small juvenile leaves with reduced photosynthetic capacity. Plants in their natural environment are influenced by microorganisms including bacteria, fungi, and mycorrhizae. Priming for rooting, shooting, and improved photosynthesis under in vitro environment can be achieved with adjustments in growth regulators and growing conditions that affect the post-transplanting performance of the propagules. Biopriming has the potential of improving plant performance under natural field condition. Micropropagation in bioreactors has been advanced as a possible way of reducing propagation cost [43]. The optimal plant production depends upon better understanding of physiological and biochemical responses of the plant to the signals of culture microenvironment and an optimization of specific physical and chemical culture conditions to control the morphogenesis in culture systems [44]. Micropropagation, in combination with radiation-induced mutations can speed up the development and multiplication of improved varieties.

Genetic transformation has not made considerable advancement partly because of difficulties in the development of highly efficient regeneration system. The transformation process thus requires a method of transferring genes into plant cells, a gene conferring the useful new trait together with a promoter directing the appropriate level and pattern of expression, an effective selective agent to suppress the growth of non-transformed cells and the ability to regenerate plants from single transformed cells [37]. The relative activity and tissue specificity of promoters needed to control transgene expression is yet another critical factor. Transient activity of maize polyubiquitin-1 (Ubi -1) promoter, cauliflower mosaic virus (CaMV 35S) and taro bacilliform virus (TaBV-600) promoters has been examined in both bombarded leaves [45] and embryogenic suspension cultures of cocoyam. A comparison of promoters in stably transformed cocoyam is essential towards assessing their strength and tissue specificity. Priorities for cocoyam transformation should target resistance to TLB and CRRD, which are considered the most serious threats to global production. Approaches for resistance to microorganisms such as antimicrobial proteins [46] and anti-apoptotic genes [47] may be more effective. Identifying a suitable promoter driving these genes in cocoyam may be an issue requiring considerable experimentation on gene expression pattern and stability in transgenic cocoyam. Although a preliminary promoter study has been undertaken [24], a greater range of promoters in stably transformed plants needs to be assessed in the field. Extensive field trials of plants generated via somatic embryogenesis is required to ascertain the frequency of somaclonal variation. In addition, the vigor and feasibility of plants generated via somatic embryogenesis in terms of rate of growing, biotic and abiotic stress resistance and corm morphological and proximate quality needs to be ascertained [15]. Emerging genome engineering techniques like a RNA-guided endonuclease technology for sequence-specific gene expression known as clustered regularly interspaced short palindromic repeats (CRISPR) aids in selectively perturbing gene expression on a genome-wide scale [58] This approach has led to engineering genes that encode compatible organic osmolytes, plant growth regulators, antioxidants, heat shock proteins and transcription factors involved in gene expression in potato [59,60]. Only a few genes or their coding proteins that are related to yield, quality or disease resistances have been isolated and identified. There is need to saturate the currently existing genetic map. Future efforts should include a larger number of co-dominant and informative markers such as SSR or SNP markers. The current high-throughput sequencing techniques is quite promising for gene mapping. Optimization of QTL detection through the use of large populations of progenies as well as trial replications is important and critical when low heritability traits are considered.

The lifting of legislative barriers and establishment of biosafety laws in many developing countries will be quite helpful in bringing transgenic technology to the benefit of resource poor farmers while providing opportunities for extensive field testing in diverse environments. The limited success achieved with improving cocoyam can be linked to the weak institutional capacity of most NARs engaged in cocoyam crop improvement. This trend is not sustainable for the future of the crop and needs an urgent reappraisal. In optimizing methodologies, the consolidation and development of already existing regional and internationational network is important. The International Network for Edible Aroids (INEA) is presently leading this step but more efforts are needed within the cocoyam research community to translate these into practical field improvement that will lead to varietal release in participating countries. In addition the CGIAR Research Program on Roots, Tubers and Bananas (RTB) that has part mandate to develop the underutilized minor root and tuber crops like cocoyam should take a lead. The implementation of recommendations in the recent report by Onyeka could serve as a working document and should be given utmost priority.

Conclusion

One of the great challenges for sustainable cocoyam production is to mitigate any effect of biotic and abiotic stress while ensuring increased production levels with reduced cropping area. The future of cocoyam lies in adding value to its organoleptic characteristics and nutritional properties, widening of export markets, diversification of use and promotion of more intensive consumption in peoples diet. A combination of expertise including geneticists, breeders, pathologists, taxonomists, food scientists, chemists, ecologists and physiologists will be critical in sustaining cocoyam production. Natural variation in wild and cultivated cocoyam germplasm provides an excellent platform for the discovery of diagnostic markers for marker-assisted selection (MAS) and for cloning and insertion of economic resistance genes applicable to diverse agrarian zones. Efforts should be channelled towards exploiting advances in “omics” technologies for exciting innovations.

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