Chapter

Plant Regeneration from Cassava Protoplasts

Feng Wen, Hai-Tian Fu, Yan-Chun Luo and Jian-Qi Huang

Abstract

Cassava is an important crop for food, feed, and industrial raw materials. Given that traditional conventional breeding is restricted by various factors, biotechnology breeding has become an important breeding method. Tissue culture regeneration is the basis of biotechnology breeding. This chapter reviews the establishment and development of cassava tissue culture and regeneration systems and the technical processes of tissue culture and regeneration starting from the induction of explants of tissue-cultured cassava plantlets to embryogenic calli, isolation to protoplasts, culture to embryogenic calli followed by differentiation into embryos, and then sprouting, stemming, and rooting into complete plants. This chapter focuses on the technical processes from protoplast to complete plant and summarizes the important influencing factors of protoplast regeneration, which is the key and difficult point in the entire regeneration process of cassava protoplasts. This chapter aims to provide technical guidance for cassava protoplast regeneration, offer useful inspiration and reference for cassava tissue culture, and lay a foundation for the genetic improvement of cassava.

Keywords: cassava, biotechnology, tissue culture, friable embryogenic callus, protoplast, somatic embryogenesis

1. Introduction

Cassava (*Manihot esculenta* Crantz), a perennial shrub of the Euphorbiaceae family, is widely cultivated in tropical and subtropical regions. It is a root crop that is a staple food for approximately 800 million people worldwide [1, 2]. It is also an important raw material for the production of starch, processed food, and biofuels [3, 4]. Its tubers, tender branches, and leaves are commonly used as animal feed [5]. Cassava also has some advantages, such as tolerance to adverse environmental conditions, adaptation to poor soils, flexible harvest times, and the capability for growth under marginal conditions [6].

Viral diseases, insect pests, toxic cyanogenic glucosides, postharvest physiological deterioration, and low root protein content roots are problems in cassava cultivation [7, 8]. Traditional conventional cassava breeding is restricted by several problems, such as high genotype heterozygosity, long life cycle, low natural fertility, poor seed set, and low seed germination rates [9–11]. Biotechnology breeding is a supplement to traditional breeding methods. With the development of molecular breeding and

genetic engineering, biotechnology breeding requires an effective regeneration system [12, 13].

This chapter provides a review of the establishment and development of cassava tissue culture and regeneration systems and the tissue culture and regeneration technology of cassava starting from the induction of the explants of tissue-cultured cassava plantlets into embryogenic calli and then into protoplasts, followed by culturing into complete plants in the cyclic process of plant regeneration from cassava protoplasts. It also provides information on experiences and skills in protoplast regeneration to lay a foundation for the genetic improvement of cassava.

2. Establishment and development of cassava tissue culture and regeneration systems

Cassava tissue culture and regeneration technologies have been continuously developed since Kartha et al. cultured the apical meristem of cassava and obtained complete plants of five varieties for the first time in 1974 [14].

2.1 Organogenesis

Cassava axillary buds were cultured on medium with a high concentration of 6-benzylaminopurine (6-BA, 10 mg L^{-1}) to form a round compact bulb-like structure and then transferred onto medium with a low concentration of 6-BA (1 mg L^{-1}) for multiple shoot production; this approach was an efficient mass propagation system for cassava [15, 16].

On the basis of the establishment of the plant regeneration pathway of cassava somatic embryogenesis, cotyledons formed from primary embryos, secondary embryos, and circulating embryos could regenerate plants through organogenesis in medium containing 1.0 mg L^{-1} 6-BA and 0.5 mg L^{-1} IBA [17]. The primary embryo has low ability for cotyledon organogenesis, whereas the circulating embryo has the highest ability for cotyledon organogenesis [18]. Cassava explants for organogenesis could be derived from the axillary buds, stem tips, young leaf lobes, and cotyledons of primary, secondary, and circulating embryos.

2.2 Somatic embryogenesis

Somatic embryogenesis has been widely developed since Stamp and Hemhaw first reported in 1982 that embryoids could be successfully induced from the cotyledons and hypocotyls of cassava zygotic embryos [19]. The four-step method of the somatic embryogenesis and plant regeneration of cassava has been established as follows: (1) induction of somatic embryos on medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and other auxins; (2) maturation or germination on medium containing a low concentration of 6-BA; (3) growth on medium containing a high concentration of 6-BA and development into stems; and (4) rooting in low naphthaleneacetic acid (NAA) concentration or hormone-free medium [20–22]. In steps 1 and 2 of circulation, secondary and cyclic somatic embryos could be generated, forming a cyclic somatic embryogenesis system, and cyclic embryo explants could be induced into embryos more significantly [20, 22].

Since then, most studies on somatic embryogenesis performed optimization in accordance with different factors, such as genotype, explant type, and hormone type.

Explants have also been developed from the hypocotyls and cotyledons of the initial zygotic embryo and the apical buds, young leaf lobes, axillary buds, flower tissues, and cotyledons of primary, secondary, and cyclic somatic embryos. The development of somatic embryogenesis and plant regeneration laid a foundation for the induction of friable embryogenic callus (FEC) [23] and genetic transformation in cassava [24].

2.3 Friable embryogenic callus (FEC) induction

FEC is an important research material in genetic and cell engineering. It has been developed for plants, and zygotic embryos are usually the preferred explant materials for FEC induction [25]. The zygotic embryos of cassava have extremely heterogeneous and unclear genotypes and are therefore unsuitable as explants.

Taylor et al. described the initiation of FEC for the first time. They utilized young leaves to induce embryogenic calli [23]. After embryogenic calli were generated, high-quality embryonic tissues were continuously subcultured on Gresshoff and Doy (GD) medium containing picloram to produce a small cell cluster that was composed of dozens of cells with diameters of 1 mm; these tissues were considered as fragile embryogenic calli from which highly totipotent embryogenic suspension cultures were established [23].

Since then, many reports on FEC induction have been published [26–29]. However, considering that cassava is a gene-dependent crop, FEC cannot be induced successfully for every variety [10, 30].

Successful FEC induction has laid a foundation for genetic transformation [28, 31, 32], CRISPR/Cas9 genome editing technology [33, 34], protoplast culture and regeneration [35, 36], and somatic hybridization [37].

2.4 Protoplast culture and regeneration

2.4.1 Culture and regeneration of mesophyll protoplasts

Protoplasts were separated from the leaves of tissue-cultured seedlings and cultured in a double-layered solid and liquid medium inserted with short glass rods evenly and vertically. No glass rod was inserted in the control culture, and the remaining cultures were all the same. The protoplasts divided continuously to form visible calli only in the medium inserted with glass rods [38].

After a long time, plant regeneration from the leaf mesophyll-derived protoplasts of cassava was reported in 2022 [39]. Prior to this report, only one successful report of plant regeneration from protoplasts isolated from cassava leaves had been published [40], and other scholars could not repeat this process [37, 38].

2.4.2 Culture and regeneration of protoplasts from embryos and embryogenic calli

The protoplasts separated from secondary embryos were tested in more than 50 media to form visible calli, a small number of which formed adventitious roots but never formed adventitious buds or embryos [41].

At present, most methods for protoplast regeneration involve inducing FEC and establishing an embryogenic suspension culture for the separation of protoplasts. In the 1990s, a breakthrough was made in the research on cassava protoplast regeneration. On the basis of establishing FEC induction technology and its suspension culture system, research on the isolation, purification, and culture of cassava protoplasts

from FEC was carried out, and plants were regenerated. However, regeneration efficiency was low mainly due to the low efficiency of protoplast-derived calli for differentiating into somatic embryos and the low germination efficiency of mature embryos, which is the bottleneck in cassava protoplast regeneration [35].

Subsequently, cassava protoplast regeneration was not reported for a long time. In 2012, Wen et al. improved the yield and activity of isolated protoplasts on the basis of their predecessors. Callus-derived protoplasts were first subjected to suspension culture in suspension culture medium (SH) liquid medium, and then cultured in somatic embryo emerging medium (MSN) solid medium [36]. The bottleneck mentioned by Sofiari et al. [35] was broken, and the regeneration efficiency of protoplasts was greatly improved. On the basis of the established protoplast regeneration technology system, tetraploid cassava plants were regenerated *via* protoplast electrofusion [37].

3. Plant regeneration from cassava protoplasts

In this section, cassava tissue culture and regeneration technology is mainly reviewed starting with the induction of explants of tissue-cultured cassava plantlets into embryogenic callus; followed by the isolation of protoplasts; the culture and differentiation into embryos of embryogenic calli; and sprouting, stemming, and rooting into a complete plant. This section focuses on the technical process from the isolation of protoplasts to the generation of a complete plant (**Figure 1**).

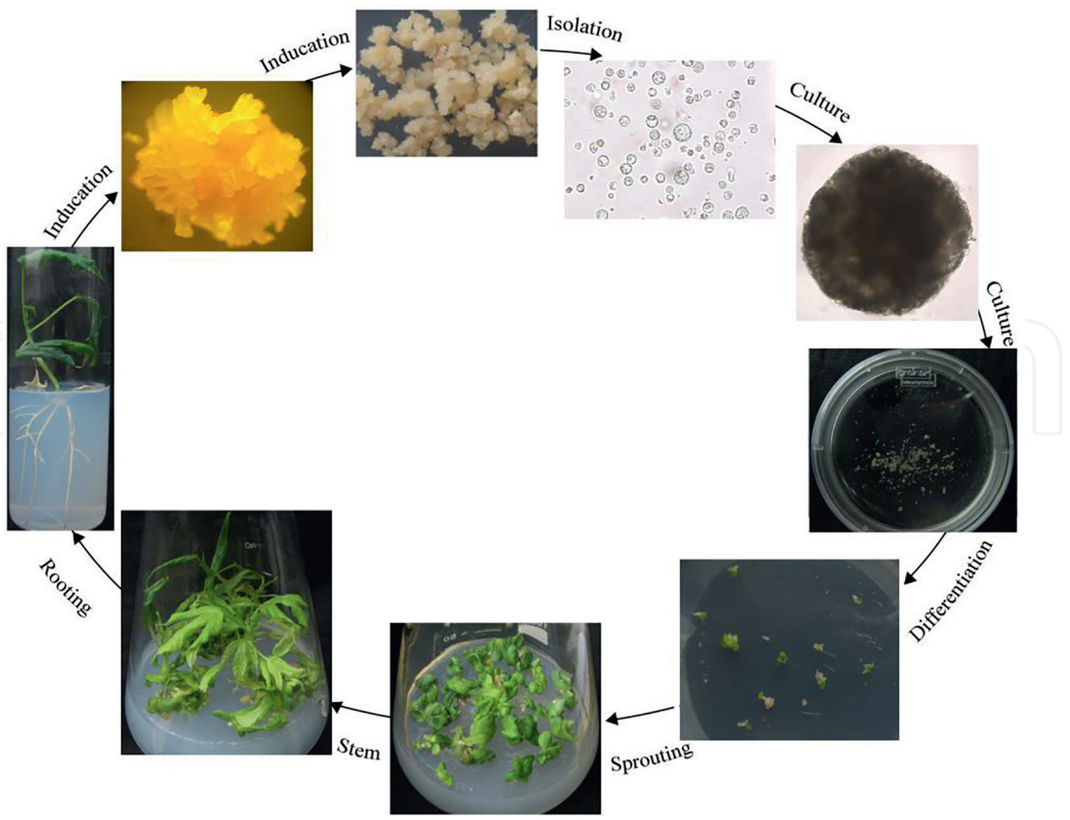

Figure 1.
Cyclic process of plant regeneration from cassava protoplasts.

3.1 Explants

Young leaf lobes of tissue-cultured cassava seedlings with an area of approximately 1 cm^2 and approximately 1 cm of stem cuttings with buds of tissue-cultured seedlings were used as explants.

3.2 Embryogenic callus induction

Embryonic calli were induced from young leaf lobe explants of tissue-cultured plantlets on somatic embryo induction medium (CIM) containing 12 mg L^{-1} picloram and were produced after 2–3 weeks. Embryogenic calli were picked out with tweezers and cultured continuously on CIM for 6–8 weeks for propagation. The medium was refreshed every 2 weeks. All cultures were kept in a growth chamber in the dark at 25°C. If the explants were stem cuttings with buds, they were cultured on axillary bud enlargement medium (CAM) for 2–4 days for bud enlargement before being cultured on CIM. The later cultures were the same as those used to culture young leaf lobe explants.

3.3 FEC induction

Embryogenic calli were cultured on GD for 2–4 weeks. The fine particles generated on the surfaces of embryogenic calli were separated and then propagated continuously on GD. FEC formed after 2–4 weeks of continuous circulation culture on GD. The medium was refreshed every 2 weeks. All cultures were kept in a growth chamber in the dark at 25°C.

3.4 Suspension culture

Cell suspension cultures were initiated by transferring approximately 1 g of FEC into a 100-mL flask with 30 mL of SH. The flask was agitated on a rotary shaker at 110–130 r min^{-1}. The liquid medium was refreshed every 2–3 days. All cultures were kept in a growth chamber at 25°C with a 12 h photoperiod and irradiance of 45 μmol^{-2} s^{-1}. Protoplast isolation was performed through 5 days of suspension culture in SH.

3.5 Protoplast isolation and purification

3.5.1 Enzymolysis

Large particles were removed from 5-day-old cell suspension cultures in SH with tweezers, and the liquid medium was aspirated out with a straw. Approximately 1 g of tissue was placed in a Petri dish (9 cm diameter) with 12 mL of cell digestion solution. The cell digestion solution contained a mixture of enzymes (10 g L^{-1} cellulase R-10, 400 mg L^{-1} macerozyme R-10, and 100 mg L^{-1} pectolyase from Yakult, Japan) and 1 mg L^{-1} NAA, 1 mg L^{-1} 2,4-D, 740 mg L^{-1} KNO_3, 368 mg L^{-1} $CaCl_2$, 34 mg L^{-1} KH_2PO_4, 492 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 19.2 mg L^{-1} Na-EDTA, 14 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 91 g L^{-1} D-mannitol, and 0.5 g L^{-1} MES. The suspension tissues were incubated in the enzyme solution for 18 h on a shaker at 40 r min^{-1} and 25°C in the dark.

3.5.2 Purification

Protoplasts were purified through the gradient centrifugation method. The digested tissues were filtered through a 45 μm stainless steel mesh to remove undigested cell clumps and debris. The filtrate was transferred into 10-mL centrifuge tubes and centrifuged for 6 min at 960 r min^{-1}. The supernatant was removed with a Pasteur pipet. The pellets were gently resuspended in 1.0–1.5 mL of 13% mannitol solution containing CPW nutrients (27.2 mg L^{-1} KH_2PO_4, 250 mg L^{-1} $MgSO_4$, 100 mg L^{-1} KNO_3, 150 mg L^{-1} $CaCl_2$, 0.2 mg L^{-1} KI, 0.003 mg L^{-1} $CuSO_4$). Then, the protoplast-containing 13% mannitol solution was slowly pipetted onto the top of 3–4 mL of 26% sucrose solution containing CPW nutrients while avoiding mixing and centrifuged for 6 min at 960 r min^{-1}. A band of viable protoplasts formed at the interface between the two layers. The protoplasts were carefully removed from the interface with a Pasteur pipet and resuspended in protoplast culture medium (TM2G). The protoplasts in TM2G were centrifuged for 6 min at 960 r min^{-1}. The supernatant was removed with a Pasteur pipet, and the protoplasts were resuspended in TM2G with 0.36 mol L^{-1} glucose at a density of 5×10^5 protoplasts mL^{-1}.

The yield of obtained protoplasts (cells g^{-1}) was calculated by using the following formula: $N \times 5 \times 10^4 \times V/m$; where N = number of protoplasts counted in a hemocytometer chamber; V = volume of diluted protoplasts; and m = fresh weight of plant material for protoplast isolation.

3.5.3 Viability test

The viability of the obtained protoplasts was checked with fluorescein diacetate (FDA). A total of 12 μL of 5 mg mL^{-1} FDA solution was added to 0.5 mL of the protoplast suspension. After 5 min, the protoplasts were examined with an Olympus IX71 inverted fluorescence microscope (green fluorescence, Olympus, Japan). The viability of obtained protoplasts (%) was calculated as follows: number of protoplasts with green fluorescence/Total protoplasts in the field × 100.

3.6 Protoplast culture

Initially, protoplasts were cultured through the thin liquid layer culture method in 1.5 mL of TM2G with 0.30–0.36 mol L^{-1} glucose in a 6-cm plastic Petri dish in the dark at 28°C. The medium was refreshed every 10 days: twice with TM2G with 0.30–0.33 mol L^{-1} glucose, then twice with a medium with reduced levels of glucose (0.27–0.30 mol L^{-1}). It was refreshed again two times with reduced glucose levels (0.25 mol L^{-1} glucose). The osmotic pressure of the culture was reduced by gradually reducing the glucose concentration of the TM2G medium to promote cell division.

3.7 Suspension culture

Protoplasts were cultured in TM2G with gradual dilution for approximately 6–10 weeks. Then, protoplast-derived compact calli were transferred into SH for suspension culture, and the other calli were cultured further. The liquid medium was refreshed every 7–15 days, and the calli were propagated in SH for 2–3 weeks.

3.8 Somatic embryogenesis

For embryo differentiation, the calli propagated in SH were transferred to MSN under light. The differentiated embryos were cultured on embryo maturation medium (CMM) for 1–3 weeks to develop large green cotyledon embryos. Then, the mature large green cotyledon embryos were transferred to shoot elongation medium (CEM). Shoot elongation began after 4–8 weeks. When the length of the elongated shoot reached 2–3 cm, the shoot was cut off for rooting on Murashige and Skoog (MS) medium. Rooting could occur in 7 days, and the protoplasts usually took 5–7 months to develop into complete plants.

3.9 Influencing factors of plant regeneration from cassava protoplast

3.9.1 State of FEC

The isolation of high-quality protoplasts is a prerequisite for protoplast culture, and the state of FEC directly affects the quality of isolated protoplasts, including yield and activity. FEC is characterized by a loose structure, the presence of spherical particles on its surface, and a milky white or yellow color. It can be used to establish suspension systems. When suspended in SH, numerous fine particles are dispersed.

In general, subculturing FEC on GD for 15–20 days results in FEC in the best state, i.e., friable and loose, and increases the yield to the maximum. After suspension culture, protoplasts are separated from FEC. This approach is conducive to plant regeneration. The protoplasts isolated from the embryogenic callus suspension of cassava subcultured for 5–15 days have high activity and few impurities.

3.9.2 Protoplast extraction and purification

The extraction and purification of cassava protoplasts separated in cell digestion solution are a key step. The cell digestion solution may not flow automatically when it is filtered through a stainless steel screen due to the effect of its surface tension, and filtration generally takes a long time. The longer the protoplasts stay in the enzyme solution, the lower their activity and the higher their impurity content. Therefore, the enzyme solution should be filtered through a stainless steel screen immediately. An external force can be exerted on the stainless steel screen to enable the enzyme solution to flow down and filter quickly. Purification through gradient centrifugation provides protoplasts with high yield and activity.

3.9.3 Protoplast culture

Cassava protoplasts were cultured in TM2G at a density of 5×10^5 pieces mL^{-1}. The initial concentration of glucose in TM2G can be within the range of 0.30–0.36 mol L^{-1}. Subsequently, the medium must be constantly refreshed and its glucose concentration must be reduced gradually to promote cell cluster division and growth. After protoplasts were cultured in TM2G for 6–8 weeks, 1–2 mm compact calli visible to the naked eye were selected, and other calli were used for further culture.

3.9.4 Embryo differentiation and germination

Sofiari et al. [35] believed that the differentiation of cassava protoplast-derived calli into embryos and the germination of embryos constitute the bottleneck of plant

regeneration from protoplasts. Therefore, somatic embryogenesis is a key step in plant regeneration from cassava protoplast. The medium is an important factor in this process.

Given that the compact callus of protoplast origin is in the same state as the FEC used for cassava genetic transformation, compact callus of protoplast origin and FEC are considered as cell clusters, and the MSN used as the medium for genetic transformation is also used as the medium for embryo differentiation.

Before the differentiation of embryos on MSN, compact calli are first suspended in SH for 2–4 weeks. The compact calli become loose after being cultured in SH. This effect is advantageous for further somatic embryogenesis or proliferation on MSN or GD.

3.10 Composition and function of cassava culture medium

Nine kinds of cassava media are discussed in this chapter. **Table 1** shows the functions of nine kinds of media, and **Tables 1–3** present the composition of the nine kinds of media.

Cassava culture media containing MS, CAM, CIM, MSN, CMM, and CEM have basically the same compositions and differ only by hormone type or dosage. They are all composed of MS salt and vitamins, plus 20 g L^{-1} sucrose, 8 g L^{-1} agar, and 2 μM $CuSO_4$ (or not). They play different roles in the tissue culture and regeneration of cassava due to the different kinds or dosage of hormones that they contain (**Table 1**).

Although CIM and MSN media could be used to induce cassava embryos, they induce different explants. The explants often induced on CIM can be young leaf lobes, apical buds, and axillary buds used for the induction of primary, secondary, and circulating somatic embryos, which are in the beginning stages of somatic

Medium	Culture stage/function	Components
CAM	Axillary bud enlargement	MS salts and vitamins (**Table 2**), 2 μM $CuSO_4$, 10 mg L^{-1} 6-BA, 20 g L^{-1} sucrose, 8 g L^{-1} agar
CIM	Embryo induction	MS salts and vitamins (**Table 2**), 2 μM $CuSO_4$, 12 mg L^{-1} picloram, 20 g L^{-1} sucrose, 8 g L^{-1} agar
MSN	Embryo induction	MS salts and vitamins (**Table 2**), 2 μM $CuSO_4$, 1 mgL^{-1} NAA, 20 g L^{-1} sucrose, 8 g L^{-1} agar
CMM	Embryo maturation	MS salts and vitamins (**Table 2**), 2 μM $CuSO_4$, 0.1 mg L^{-1} 6-BA, 20 g L^{-1} sucrose, 8 g L^{-1} agar
CEM	Shoot elongation	MS salts and vitamins(**Table 2**), 2 μM $CuSO_4$, 20 g L^{-1} sucrose, 8 g L^{-1} agar, with 1.0 mg L^{-1} 6-BA
MS	Rooting and subculture of tissue culture seedlings	MS salts and vitamins (**Table 2**), 0.02 mg L^{-1} NAA, 20 g L^{-1} sucrose, 8 g L^{-1} agar
GD	FEC induction, maintenance, and proliferation	GD salts and vitamins, 12 mg L^{-1} picloram, 20 g L^{-1} sucrose, 8 g L^{-1} agar (**Table 3**)
SH	FEC suspension culture, maintenance, and proliferation	SH salts and vitamins, 12 mg L^{-1} picloram, 60 g L^{-1} sucrose (**Table 3**)
TM2G	Protoplast culture	TM-2 salts and vitamins, 1 mg L^{-1} NAA, 0.5 mg L^{-1} ZT, 54–64.8 g L^{-1} glucose (**Table 3**)

Table 1.
Medium components and function.

Macro-elements (g L^{-1})		**Micro-elements (mg L^{-1})**		**Iron salts (mg L^{-1})**		**Vitamins (mg L^{-1})**	
KNO_3	**19**	**KI**	**0.83**	**$FeSO_4{\cdot}7H_2O$**	**27.8**	**Glycine**	**2**
NH_4NO_3	16.5	H_3BO_3	6.2	Na_2·EDTA	37.3	Myo-inositol	100
KH_2PO_4	1.7	$ZnSO_4{\cdot}7H_2O$	8.6	/	/	Nicotinic acid	0.5
$MgSO_4{\cdot}7H_2O$	13.7	$Na_2MoO_4{\cdot}2H_2O$	0.25	/	/	Pyridoxine HCl	0.5
$CaCl_2{\cdot}2H_2O$	4.4	$CuSO_4{\cdot}5H_2O$	0.025	/	/	Thiamine HCl	0.1
/	/	$CoCl_2{\cdot}6H_2O$	0.025	/	/	/	/
/	/	$MnSO_4{\cdot}H_2O$	16.9	/	/	/	/

Table 2.
MS salts and vitamins.

embryogenesis and can grow into different types of embryoids, such as globular, torpedo, and cotyledon embryos. The explants often induced on MSN are FEC or calli derived from protoplast division. When cultured on MSN under light, they can grow into different types of embryoids, such as globular, torpedo, and green cotyledon embryos. CIM is used as a somatic embryo induction medium under dark conditions, whereas MSN is used as a somatic embryo induction medium under light conditions.

The regeneration processes of globular, torpedo, and cotyledon embryos induced on CIM and MSN are the same. They all undergo and complete maturation, bud elongation, and rooting on CMM, CEM, and MS, respectively. These processes should be conducted under light conditions.

Medium components		**GD**	**SH**	**TM2G**
Macro-elements (mg L^{-1})	$Ca(NO_3)_2{\cdot}2H_2O$	208.81	/	/
	KCl	65.00	/	/
	KH_2PO4	300.00	/	170
	KNO_3	1000.00	2500	1500
	$MgSO_4{\cdot}7H_2O$	35.00	400	370
	NH_4NO_3	1000.00	/	/
	$CaCl_2{\cdot}2H_2O$	/	200	440
	$NH_4H_2PO_4$	/	300	/
Micro-elements (mg L^{-1})	$CoCl_2{\cdot}6H_2O$	0.025	0.1	0.025
	$CuSO_4{\cdot}5H_2O$	0.025	0.2	0.025
	H_3BO_3	0.30	5.0	6.20
	KI	0.80	1.0	0.38
	$MnSO_4{\cdot}H_2O$	1.00	10	16.9
	$Na_2MoO_4{\cdot}2H_2O$	0.025	0.1	0.25
	$ZnSO_4{\cdot}7H_2O$	0.30	1.0	8.60
	$FeSO_4{\cdot}7H_2O$	0.278	15	13.9
	Na_2·EDTA	0.336	20	18.5

Medium components		GD	SH	TM2G
Vitamins (mg L^{-1})	Glycine	4.00	/	0.50
	Myo-inositol	100.00	0.5	4600
	Nicotinic acid	1.00	0.5	2.5
	Pyridoxine HCl	1.00	0.1	1
	Thiamine HCl	10.00	/	10
	Folic acid	/	/	0.5
	Biotin	/	/	0.05
	D-Ca-pantothenate	/	/	0.50
	Choline chloride	/	/	0.10
	Casein hydrolysate	/	/	150
	L-cysteine	/	/	1
	Malic acid	/	/	10
	Ascorbic acid	/	/	0.50
	Adenine sulfate	/	/	40
	L-glutamine	/	/	100
	Riboflavin	/	/	0.25
Others (g L^{-1})	Sucrose	20	60	/
	Agar	8	/	/
	Glucose	/	/	54–64.8
	Mannitol	/	/	4.56
	Xylitol	/	/	3.80
	Sorbitol	/	/	4.56
	MES	/	/	0.098
Hormone (mg L^{-1})	Picloram	12	12	/
	NAA	/	/	1
	Zeatin	/	/	0.5

Table 3.
GD, SH, TM2G components.

GD can be used for the induction, maintenance, and proliferation of cassava FEC. SH can also participate in the maintenance of the embryogenic proliferation of FEC. GD is a solid medium, whereas SH is a liquid medium. FEC can be converted between GD and SH cultures, and its properties do not change. FEC cultured on SH has better cell consistency and faster proliferation than that cultured on GD. TM2G is used as a medium for culturing cassava callus protoplasts. Its osmotic pressure is reduced during culture by decreasing its glucose concentration gradually to promote cell division.

4. Conclusion

Cassava is a food crop, and the biotechnology research on cassava lags behind that on major food crops, such as rice and wheat. The establishment and development

of cassava tissue culture and regeneration technology have promoted the application of biotechnology techniques, such as genetic transformation, genome editing, and somatic hybridization, to cassava. However, deficiencies remain. Cassava tissue culture and regeneration technology still need development and optimization to establish an efficient regeneration system without genotype dependence.

In cassava, protoplast regeneration technology could be applied to somatic hybridization and protoplast transformation. Somatic hybridization technology could break through the barriers of sexual hybridization and represents a direction for cassava breeding with protoplast regeneration technology as the basis. The disadvantages of cassava protoplast regeneration technology are high technical requirements and time consumption. We hope the chapter will be beneficial for the genetic improvement of cassava.

Acknowledgements

This work was supported by the Guangxi Scientific and Technological Development Subject, China (AB21196071), and the Basic Scientific Research Fund of Guangxi Academy of Agricultural Sciences, China (2021YT149).

Abbreviations

FEC	friable embryogenic callus
CAM	axillary bud enlargement medium
CIM	somatic embryo induction medium
MSN	somatic embryo emerging medium
GD	Gresshoff and Doy (1974) medium
SH	suspension culture medium
TM2G	protoplast culture medium
CMM	embryo maturation medium
CEM	shoot elongation medium
MS	Murashige and Skoog (1962) medium
2,4-D	2,4-dichlorophenoxyacetic acid
6-BA	6-benzylaminopurine
NAA	naphthaleneacetic acid

Author details

Feng Wen*, Hai-Tian Fu, Yan-Chun Luo and Jian-Qi Huang
Guangxi Subtropical Crops Research Institute, Nanning, P.R. China

*Address all correspondence to: wenfengw83@163.com

References

[1] Howeler R, Lutaladio N, Thomas G. Save and Grow: Cassava, a Guide to Sustainable Production Intensification. Rome: Food and Agriculture Organization of the United Nations; 2013

[2] Ojola OP, Nyaboga EN, Njiru PN, Orinda G. Overexpression of rice thaumatin-like protein (*Ostlp*) gene in transgenic cassava results in enhanced tolerance to *Colletotrichum gloeosporioides* f. sp. *manihotis*. Journal of Genetic Engineering & Biotechnology. 2018;**18**:125-131. DOI: 10.1016/j.jgeb.2017.12.002

[3] Ceballos H, Kulakow P, Hershey C. Cassava breeding: Current status, bottlenecks and the potential of biotechnology tools. Tropical Plant Biology. 2012;**5**:73-87. DOI: 10.1007/s12042-012-9094-9

[4] Parmar A, Sturm B, Hensel O. Crops that feed the world: Production and improvement of cassava for food, feed, and industrial uses. Food Security. 2017;**9**:907-927

[5] Mongomake K, Doungous O, Khatabi B, Fondong VN. Somatic embryogenesis and plant regeneration of cassava (*Manihot esculenta* Crantz) landraces from Cameroon. Springerplus. 2015;**4**:477. DOI: 10.1186/s40064-015-1272-4

[6] Jaramillo AM, Sierra S, Chavarriaga Aguirre P, Castillo DK, et al. Characterization of cassava ORANGE proteins and their capability to increase provitamin A carotenoids accumulation. PLoS ONE. 2022;**17**(1):e0262412. DOI: 10.1371/journal.pone.0262412

[7] Chavarriaga-Aguirre P, Brand A, Medina A, Prías M, Escobar R, Mar-tinez J, et al. The potential of using biotechnology to improve cassava: A review. In Vitro Cellular & Developmental Biology-Plant. 2016;**52**:461-478. DOI: 10.1007/s11627-016-9776-3

[8] Beyene G, Chauhan RD, Gehan J, Siritunga D, Taylor N. Cassava shrunken-2 homolog *MeAPL3* determines storage root starch and dry matter content and modulates storage root postharvest physiological deterioration. Plant Molecular Biology. 2022;**109**:1-17

[9] Bredeson JV, Lyons JB, Prochnik SE, Wu GA, Ha CM, Edsinger-Gon-zales E, et al. Sequencing wild and cultivated cassava and related species reveals extensive interspecific hybridization and genetic diversity. Nature Biotechnology. 2016;**34**:562-570. DOI: 10.1038/nbt.3535

[10] Brand A, Quimbaya M, Tohme J, Chavarriaga-Aguirre P. Arabidopsis *LEC1* and *LEC2* orthologous genes are key regulators of somatic embryogenesis in cassava. Frontiers in Plant Science. 2019;**10**:673. DOI: 10.3389/fpls.2019.00673

[11] Souza LS, Alves AAC, de Oliveira EJ. Phenological diversity of flowering and fruiting in cassava germplasm. Scientia Horticulturae. 2020;**265**:109253

[12] Liu J, Zheng Q, Ma Q, Gadidasu KK, Zhang P. Cassava genetic transformation and its application in breeding. Journal of Integrative Plant Biology. 2011;**535**:52-69

[13] Ceballos H, Kawuki RS, Gracen VE, Yencho GC, Hershey CH. Conventional breeding, marker-assisted selection, genomic selection and inbreeding in clonally propagated crops: A case

study for cassava. Theoretical Applied Genetics. 2015;**128**:1647-1667

[14] Kartha KK. Regeneration of cassava plants from apical meristems. Plant Science Letters. 1974;**2**:107-113

[15] Konan NK, Sangwan RS, Sangwan-Norreel BS. Efficient in vitro shoot-regeneration systems in cassava (*Manihot esculenta* Crantz). Plant Breeding. 1994;**113**:227-236

[16] Konan NK, Schöpke C, Cárcamo R, Beachy RN, Fauquet C. An efficient mass propagation system for cassava (*Manihot esculenta* Crantz) based on nodal explants and axillary bud-derived meristems. Plant Cell Reports. 1997;**16**:444-449

[17] Li HQ, Sautter C, Potrykus I, Puonti-Kaerlas J. Genetic transformation of cassava (*Manihot esculenta* Crantz). Nature Biotechnology. 1996;**14**:736-740

[18] Li HQ, Guo JY, Huang YW, Liang CY, Liu HX, Potrykus I, et al. Regeneration of cassava plants via shoot organogenesis. Plant Cell Reports. 1998;**17**:410-414

[19] Stamp JA, Henshaw GG. Somatic embryogenesis in cassava. Zeitschrift für Pflanzenphysiologie. 1982;**105**:183-187

[20] Raemakers CJJM, Amati M, Staritsky G, Jacobsen E, Visser RGF. Cyclic somatic embryogenesis and plant regeneration in cassava. Annals of Botany. 1993;**71**:289-294

[21] Raemakers CJJM, Schavemaker CM, Jacobsen E, Visser RGF. Improvement of cyclic somatic embryogenesis of cassava(*Manihot esculenta* Crantz). Plant Cell Peports. 1993;**12**:226-229

[22] Saelim L, Phansiri S, Netrphan S, Suksangpanomrung M, Narangajavana J. Optimization of in vitro cyclic somatic embryogenesis and regeneration of the Asian cultivars of cassava (*Manihot esculenta* Crantz) for genetic manipulation system. Global Journal of Biotechnology & Biochemistry. 2006;**1**(1):07-15

[23] Taylor NJ, Edwards M, Kiernan RC, Davey CDM, Blakesley D, Henshaw GG. Development of friable embryogenic callus and embryogenic suspension culture systems in cassava (*Manihot esculenta* Crantz). Biotechnology. 1996;**14**:26-730

[24] Syombua ED, Adero MO, Mbinda WM, Wanyonyi CN, Ngugi MP, Oduor RO. A system for rapid gene introgression into cassava immature leaves and subsequent recovery of transformed lines. Plant Biotechnology Reports. 2021;**15**:27-37. DOI: 10.1007/s11816-020-00654-1

[25] Lin HS, van der Toorn C, Raemakers KJJM, Visser RGF, De Jeu MJ, Jacobsen E. Development of a plant regeneration system based on friable embryogenic callus in the ornamental Alstroemeria. Plant Cell Reports. 2000;**19**(5):529-534. DOI: 10.1007/s002990050768

[26] Taylor NJ, Masona MV. Production of embryogenic tissues and regeneration of transgenic plants in cassava (*Manihot esculenta* Crantz). Euphytica. 2001;**120**:25-34

[27] Taylor N, Chavarriaga P, Raemakers K, Siritunga D, Zhang P. Development and application of transgenic technologies in cassava. Plant Molecular Biology. 2004;**56**:671-688

[28] Utsumi Y, Utsumi C, Tanaka M, Ha VT, Matsui A, Takahashi S, et al. Formation of friable embryogenic callus in cassava is enhanced under conditions of reduced nitrate, potassium and phosphate. PLoS One.

2017;**12**(8):e0180736. DOI: 10.1371/journal.pone.0180736

[29] Ibrahim AB, Heredia FF, Pinheiro CB, Aragao FJL, Campos FAP. Optimization of somatic embryogenesis and selection regimes for particle bombardment of friable embryogenic callus and somatic cotyledons of cassava(*Manihot esculenta* Crantz). International Journal of Histology and Cytology. 2020;7(16):2790-2797

[30] Elegba W, McCallum E, Gruissem W, Vanderschuren H. Efficient genetic transformation and regeneration of a farmer-preferred cassava cultivar from Ghana. Frontiers in Plant Science. 2021;**12**:668042. DOI: 10.3389/fpls.2021.668042

[31] Bull SE, Owiti JA, Niklaus M, BeechingJR,GruissemW,VanderschurenH. Agrobacterium-mediated transformation of friable embryogenic calli and regeneration of transgenic cassava. Nature Protocols. 2009;**4**:1845-1854. DOI: 10.1038/nprot.2009.208

[32] Wang YJ, Lu XH, Zhen XH, Yang H, Che YN, Hou JY, et al. A transformation and genome editing system for cassava cultivar SC8. Genes. 2022;**13**:1650. DOI: 10.3390/genes13091650

[33] Odipio J, Alicai T, Ingelbrecht I, Nusinow DA, Bart R, Taylor NJ. Efficient CRISPR/Cas9 genome editing of phytoene desaturase in cassava. Frontiers in Plant Science. 2017;**8**:1780

[34] Devang M, Alessandra S, Ravi BA, Shan-e-Ali ZS, Matthias H, Wilhelm G, et al. Linking CRISPR-Cas9 interference in cassava to the evolution of editing-resistant geminiviruses. Genome Biology. 2019;**20**:1-10

[35] Sofiari E, Raemakers CJJM, Bergervoet JEM, Jacobsen E, Visser RGF. Plant regeneration from protoplasts isolated from friable embroygenic callus of cassava. Plant Cell Reports. 1998;**18**:159-165

[36] Wen F, Xiao SX, Nie YM, Ma QX, Zhang P, Guo WW. Protoplasts culture isolated from friable embryogenic callus of cassava and plant regeneration. Scientia Agricultura Sinica. 2012;**45**:4050-4056. (In Chinese)

[37] Wen F, Su W, Zheng H, Yu B, Ma Z, Zhang P, et al. Plant regeneration via protoplast electrofusion in cassava. Journal of Integrative Agriculture. 2020;**19**(3):632-642

[38] Anthony P, Davey MR, Power JB, Lowe KC. An improved protocol for the culture of cassava leaf protoplasts. Plant Cell, Tissue and Organ Culture. 1995;**42**:229-302

[39] Mukami A, Juma BS, Mweu C, Ngugi M, Oduor R, Mbinda WM. Plant regeneration from leaf mesophyll derived protoplasts of cassava (*Manihot esculenta* Crantz). PLoS One. 2022;**17**(12):e0278717. DOI: 10.1371/journal. pone.0278717

[40] Shahin EA, Shepard JF. Cassava mesophyll protoplasts: Isolation, proliferation and shoot formation. Plant Science Letters. 1980;**17**:459-465

[41] Sofiari E. Regeneration and Transformation of Cassava. Netherlands: Wageningen Agricultural University of Wageningen; 1996