Journal of Experimental Agriculture International

Volume 46, Issue 9, Page 404-411, 2024; Article no.JEAI.122830
ISSN: 2457-0591
(Past name: American Journal of Experimental Agriculture, Past ISSN: 2231-0606)

Development of a Robust *In vitro* Regeneration Protocol in Cassava (*Manihot esculenta* C.) Using Axillary Bud Explants for Cryopreservation

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/jeai/2024/v46i92836

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/122830

Original Research Article

Received: 01/07/2024
Accepted: 02/09/2024
Published: 02/09/2024

ABSTRACT

Cassava is an important crop as it is the source of energy in the diet for the most of the tropical countries. Field gene bank is the only viable option for the maintenance of cassava germplasm. Cryopreservation offers the long-term conservation of vegetatively propagates crops without losing its viability through an effective *in vitro* regeneration system. *In vitro* regeneration response in YTP-1 and Sree Athulya genotypes of cassava was standardized through optimization of various factors influencing organogenesis. Among the different media tested, highest shoot induction frequencies of 81.4% and 77.8% were recorded in medium supplemented with 0.5 mg/l BAP + 0.2 mg/l NAA

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Cite as: *Shankar, M., V. Thiruvengadam, and PL Viswanathan. 2024. "Development of a Robust In Vitro Regeneration Protocol in Cassava (Manihot Esculenta C.) Using Axillary Bud Explants for Cryopreservation". Journal of Experimental Agriculture International 46 (9):404-11. https://doi.org/10.9734/jeai/2024/v46i92836.*

(CMSI 2) for YTP 1 and Sree Athulya respectively in the present study. 3 to 5 multiple shoots from a single shoot apex obtained from 70-80% of explants within 50-60 days. Highest regeneration frequencies of 56.6%and 46.6% were recorded in medium supplemented with 1 mg/l IAA (CRM 2) for YTP1 and Sree Athulya. As a result, an easy *in vitro* regeneration method that is genotype independent was created and may be effectively used to preserve cassava germplasm in the cryogen bank.

Keywords: Axillary bud; cassava; cryopreservation; In vitro regeneration; shoot apices.

1. INTRODUCTION

Cassava (*Manihot esculenta* C.) is an important commercial crop grown in tropical region. It is an important source of energy and food security in the developing countries of the globe to meet out the demand of increasing population [1]. Worldwide, 800 million people depend on cassava as their primary staple food [2]. Cassava was originated in Latin America and the crop was said to be found by the Native Indians around 4000 years ago [3]. Cassava is an important dietary supplement to the many under developed countries like Brail, Congo, Indonesia. Nigeria produces around 60 Mt of cassava every year consider as the largest cassava producer in the world [4]. International Centre for Tropical Agriculture (CIAT) conserves over 43,000 of diverse set of germplasms collected around the world (CIAT, 2023). Cassava is majorly cultivated in India at an area of 1962.0 m. ha with production of 4095.6 m.t. and productivity of 20.9 Mt/ha. Among the states Tamil Nadu ranks first in term of production 2603.2 m.t. followed by Kerala and Andhra Pradesh (Horticultural statistics, 2017). Conservation of vegetatively propagated crops such as potato, cassava, yams, sweet potato, sugarcane, coffee, rubber are usually carried out in field gene banks [5]. The advantages of field gene banks are that the material is easily available for utilization, and that evaluation can be undertaken while the material is being conserved [6]. However, maintenance of clonal gene banks is quite expensive due to increasing cost on land and labour and also presents risks of loss of germplasm through natural calamities and pest and diseases [7].

As such conditions, *in vitro* culture techniques provide some important tools for improved conservation and management of vegetatively propagated crop species [8]. The advantages of in *vitro* conservation are maintenance of material in pathogen-free condition that facilitates safer distribution, conservation of vegetatively propagated plants [9]. Further, the cultures are not subjected to environmental disturbances [10]. Genetic resources can be preserved *in vitro* in both the short- to medium-term (slow growth) and long-term (cryopreservation) durations [11]. For the long-term preservation of vegetatively propagated crop species, an *in vitro* regeneration system for the target species should be accessible for cryopreservation [12]. Given the importance of cassava as a commercial crop, breeding research requires a steady supply of cassava germplasm over an extended period of time [13]. In order to facilitate the creation of a viable cryo gene bank facility for cassava germplasm on a broad scale, the current study aims to design an effective *In vitro* plant regeneration methodology in cassava employing axillary buds as explants.

2. MATERIALS AND METHODS

The tissue culture facility of the Department of Plant Genetic Resources at Tamil Nadu Agricultural University in Coimbatore was the site of the *in vitro* studies. The Tapioca and Castor Research Station, TNAU, Yethapur provided stem cuttings of two genotypes of cassava, YTP-1 and Sree Athulya, which were then planted in the 'F' block of the new area at the Department of Forage Crops, TNAU, Coimbatore. Six rows of cassava stem cuttings were planted, with a 90 cm gap between each row. In accordance with the crop production handbook, the suggested agronomic methods and need-based plant protection measures were implemented.

The healthy and actively growing axillary buds of 1.0 cm length with leaf sheaths were excised from two to three months old cassava field grown plants using a sterile blade. The collected buds were washed repeatedly with tap water containing two drops of tween 20. The cleaned buds were taken to the laminar air flow chamber in double distilled water and disinfected with 70% ethanol for 10 seconds followed by washing three times with sterile distilled water. The buds were then surface sterilized with 0.1 % $HgCl_2$ for six to seven minutes and rinsed four to five times with doubled distilled water to remove the traces

of $HgCl_2$ before excision of shoot apex for *in vitro* culture (Fig. 1).

Under laminar air flow cabinet, using the sterile forceps and scalpel, the outer leaf sheaths surrounding the axillary bud were removed first. Subsequently, the shoot apices explants of 2 mm in size were excised out and placed in the shoot induction medium supplemented with different concentrations of growth hormones (Fig. 2). Table 1 provides specifics of several media compositions investigated for multiple shoot induction and proliferation. The ability of the explants to establish and respond in the shoot induction medium was observed after a month and observations were recorded regularly with sub culturing at 15 days interval. Utilizing the method, the shoot induction % was determined by given formula below.

$$\text{shoot induction (\%)} = \frac{\text{No. of explants with shoots}}{\text{Total no. of explants cultured}} \times 100$$

The individual shoots were taken out and placed in rooting media treated with various combinations of growth hormones after the shoots had grown for two months. Table 2 lists the medium compositions needed for shoots to root successfully. The regeneration frequency was calculated by using the formula given below.

$$\text{Regeneration frequency (\%)} = \frac{\text{No. of explants with shoots \& roots}}{\text{Total no. of explants cultured}} \times 100$$

Light Emitting Diodes (LED) lights were used to provide continuous lighting and maintain a temperature of 25±2˚C. The lights produced a light intensity of 2500 lux, with a photoperiod cycle of 16 hours of light and 8 hours of darkness. Completely Randomized Design (CRD) was used in the studies, and Panse and Sukhatme [14] recommended technique of statistical analysis was applied to the data. Each treatment was replicated thrice. An analysis of variance (ANOVA) was computed using statistical software AGRES.

3. RESULTS AND DISCUSSION

The success of any cryo-conservation procedures for problem species is purely judged based on the recovery of plantlets after cryo-storage [15]. The primary prerequisite for any vegetatively grown crop to be suitable for cryopreservation is that the *in vitro* regeneration mechanism must function adequately for the intended species [16]. The tissue culture approach should make it easier to induce numerous shoots and produce plantlets after the proper explants have been cultured *In vitro*. This could be well achieved through direct organogenesis process which involves no callusing phase thus enabling the regeneration of true to type plants. As the maintenance of genetic integrity of the germplasm is very important during germplasm conservation and regeneration, direct organogenesis would be the method of choice for adoption in the cryo-conservation of vegetatively propagated crops.

The regeneration of plantlets during *In vitro* culture is significantly influenced by a number of parameters, including genotype, explants, the nutritional content of the culture medium, and culture conditions [17]. To successfully achieve *in vitro* regeneration in cassava germplasm, a thorough procedure was developed by first studying the standardization of all these factors. Among these different plant tissues, the shoot apices excised from the growing shoots are widely used for cryo-conservation of vegetatively propagated crops [18]. Shoot tips are the best since they have the capacity to restore a plant and pre-formed meristematic areas that may provide genetic stability [19]. Additionally, a little fragment of material taken from the mother plant can be regarded as germplasm and employed as a storage material since regenerants created during organogenesis should be similar to the mother plant [20]. Therefore, shoot apices were selected as explants in the present study. Previously various authors have reported *in vitro* regeneration through shoot apices in cassava [21,22,23,24,25]. However, because they depend on both genotype and cultivar, the majority of these techniques could not be applied to a broad range of genotypes. As a result, the current work concentrated on creating a genotype-independent *in vitro* regeneration strategy for cassava utilizing axillary buds as explants and adjusting the culture medium's nutritional content.

In the present study, cassava shoot apices of 2 mm in size were cultured on MS medium supplemented with different concentrations and combinations of BAP, NAA and kinetin for the shoot induction and proliferation. Best multiple shoot formation response was obtained on MS medium supplemented with 0.5 mg/l BAP + 0.2 mg/l NAA invariably in both YTP 1 and Sree Athulya genotypes. The shoot apices were sub

cultured once in 15 days and shoot induction was observed after 20 days of inoculation in the shoot induction medium. Raspor et al. [26] reported that a variety of phytohormone combinations frequently control how shoot organogenesis, or morphogenesis, proceeds. Shoot tips for multiple shoot regeneration were significantly impacted by the kinds and amounts of auxin and cytokine applied. The cytokinin BAP was more effective than kinetin and NAA. Gupta et al. [27] discussed in detail how various hormone combinations affect the growth and elongation of shoots.

The findings indicated that in YTP1, the shoot induction frequency varied from 15.7% to 81.4%, whereas in Sree Athulya, it ranged from 5.7% to 77.8%. Out of all the examined media, the ones supplemented with 0.5 mg/l BAP + 0.2 mg/l NAA (CMSI 2) for YTP 1 and Sree Athulya had the greatest shoot induction rates, at 81.4% and 77.8%, respectively (Table 3). Shoot elongation was observed after 30 days of inoculation on CMSI 2 medium. 3 to 5 multiple shoots from a single shoot apex obtained from 70-80% of explants within 50-60 days (Fig. 3). An average of 4 shoots was formed per explant. During all stages of shoot induction and subsequent multiple shoot development, callus formation was not noticed on this medium. Hemmati et al. [28] reported that NAA and BAP were important for micropropagation. The use of NAA and BAP growth regulators during shoot growth initiated from meristem culture of different cassava varieties were also reported by Cacai et al. [25].

The efficiency of rooting in the shooted plantlets was tested by transferring the individual shoots to four different culture media (Fig. 4). Rooting of the shoots may be affected by pH, auxin level and nutrient concentration of the rooting media [29]. In the present study, rooting was observed after 20 days shoot culture. Rooting regeneration was recorded higher when shoots are cultured in half strength MS medium showed poor performance as compared to cultures in full strength MS medium. According to the data, the range of regeneration frequency for genotype YTP1 was 3.3% to 56.6%, whereas for genotype Sree Athulya, it was 3.3% to 46.6%. Highest regeneration frequencies of 56.6%and 46.6% were recorded in medium supplemented with 1 mg/l IAA (CRM 2) for YTP1 and Sree Athulya. (Table 4). This may be attributed to the fact that auxins stimulate cell elongation and division and rhizogenesis is achieved by treatment with auxin [30]. Genotypes YTP1 and Sree Athulya showed lowest regeneration frequency of 3.3% when inoculated on Half MS medium (CRM 1). Fully established plantlets with typical branches and roots were created after 30 days of cultivation on rooting media, and they were then moved to screen house conditions for additional establishment.

Table 1. List of media compositions used for shoot induction and proliferation in cassava

S. No	Code	Media composition
1	CMSI 1	MS + 0.05 mg/l BAP + 0.01mg/l NAA+ 30g/l sucrose + 8 g/l agar
2	CMSI 2	MS + 0.5 mg/l BAP + 0.2 mg/l NAA + 30g/l sucrose + 8 g/l agar
3	CMSI 3	MS + 0.2mg/l kinetin + 30g/l sucrose + 8 g/l agar

**MS medium [31]*

Table 2. List of media composition used for rooting in cassava

S. No	Code	Media Composition
1	CRM 1	Half MS + 30g/l sucrose + 8 g/l agar
2	CRM 2	Full MS + 1 mg/l IAA + 30g/l sucrose + 8 g/l agar
3	CRM 3	Full MS + 0.2 mg/l IAA + 30g/l sucrose + 8 g/l agar
4	CRM 4	Full MS + 0.5 mg/l IAA + 30g/l sucrose + 8 g/l agar

Table 3. Effect of growth regulators on shoot induction and proliferation in the selected cassava genotypes

Media compositions	Shoot induction frequency (%) (mean of three replicates)	
	YTP 1	Sree Athulya
CMSI 1	15.7^{b}	22.8^{b}
CMSI 2	81.4^{a}	77.8^{a}
CMSI 3	32.8^{b}	5.7^{c}
SE (d)	7.03	6.29
Cd (0.01)	20.26	18.12

Values followed by the same letter are not significantly different at p > 0.01

Table 4. Effect of growth regulators on regeneration frequency in the selected cassava genotype

Media	Regeneration frequency (%) (mean of three replicates)	
	YTP 1	Sree Athulya
CRM 1	3.3[d]	3.3[c]
CRM 2	56.6[a]	46.6[a]
CRM 3	13.3[c]	13.3[b]
CRM 4	33.3[b]	23.3 [b]
SE (d)	5.08	5.12
Cd (0.01)	17.05	17.19

Values followed by the same letter are not significantly different at p > 0.01

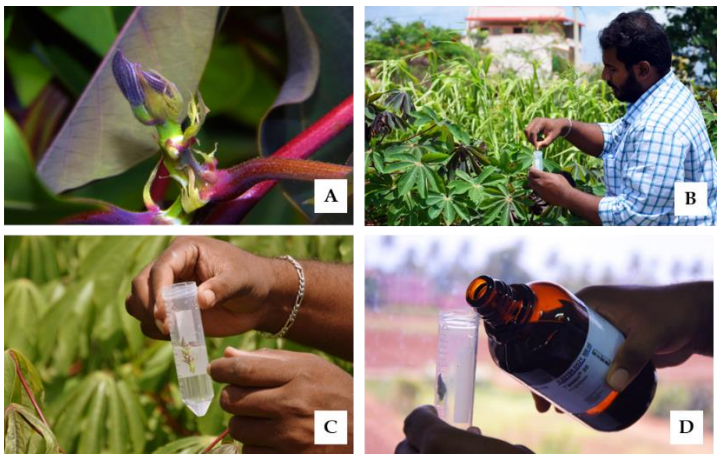

Fig. 1. (A-D): Collection and preparation of shoot apex explants from field grown cassava plants. (A) Selection of explant. (B) Explant isolation. (C) Collection of explants in falcon tube (Sterilized water). (D) Tween 20 wash

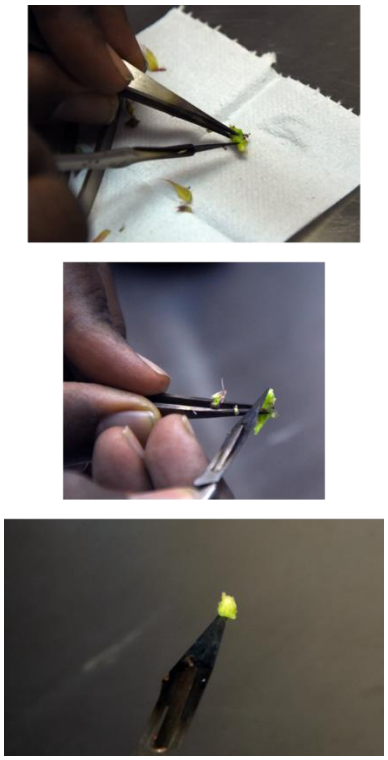

Fig. 2. Isolation of cassava shoot apex under laminar air flow cabinet

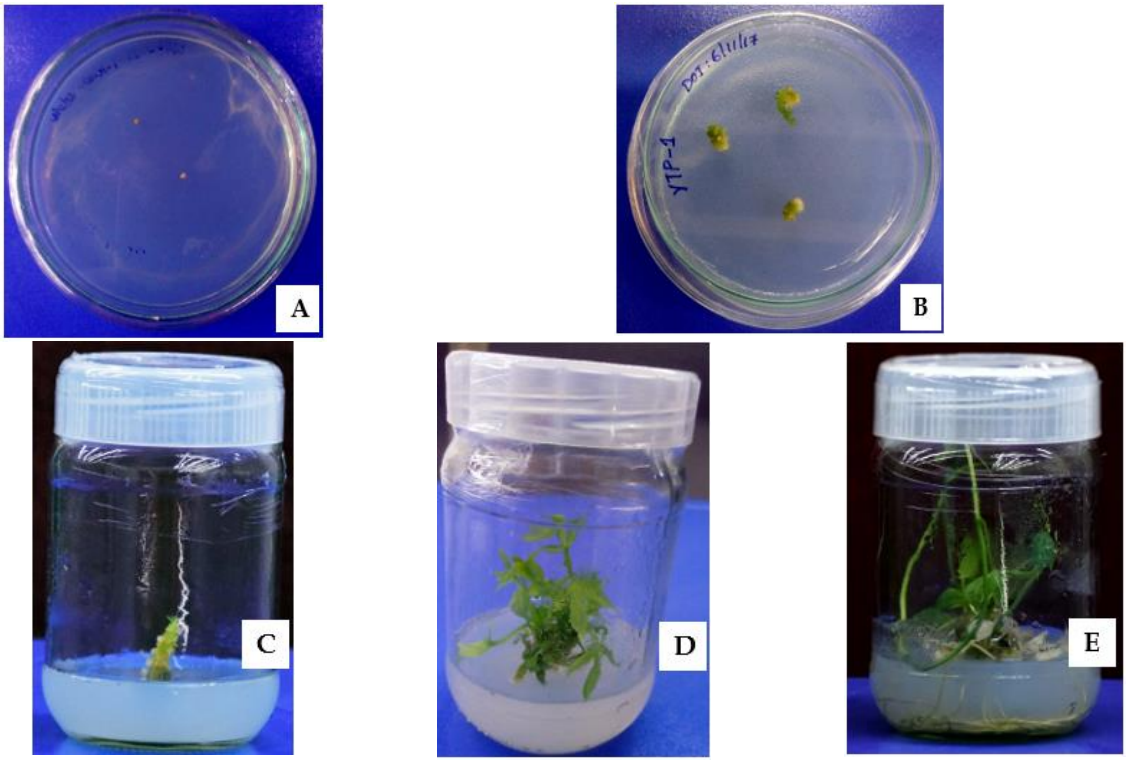

Fig. 3. (A-E): *In vitro* regeneration of cassava genotypes using shoot apex explants. (A) Shoot apices inoculated on CMSI 2 medium. (B) Shoot initiation after 20 days of culture in the CMSI 2 medium. (C) Elongation of shoots after 30 days of inoculation on CMSI 2 medium. (D) *In vitro* regenerated multiple shoots after 60 days of culture on CMSI 2 medium. (E) Plantlet establishment after 20 days of transfer to rooting medium CRM 2

Fig. 4. (A-B): Rooting and regeneration of cassava (A). Root proliferation (B). Regenerated plant

4. CONCLUSION

The current work devised a straightforward, genotype-independent in vitro regeneration method employing shoot apex explants. The two genotypes of cassava's regeneration frequencies are equivalent to those found in earlier investigations. As effective recovery could be achieved, this might serve as motivation for the successful deployment of shoot apices as a suitable storage tissue under liquid nitrogen. Moreover, a wide range of research stations and laboratories dealing with the micropropagation and cryopreservation of cassava germplasm might potentially adopt this genotype-independent tissue culture methodology.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/122830