Original Research Article

Development of an efficient *in vitro* regeneration system in Cassava [*Manihot esculenta* C.] through axillary bud's explants for cryopreservation

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 the present study, two cassava genotypes namely YTP-1 and Sree Athulya were evaluated for their *in vitro* regeneration response through optimization of various factors influencing organogenesis. The results indicated that, 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 (CMSI 2) for YTP 1 and Sree Athulya respectively. Overall, 70-80% of explants produced 3 to 5 multiple shoots from a single shoot apex 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. Thus, a simple and genotype independent *in vitro* regeneration system was developed which could be well adopted for conservation of cassava germplasm in the cryo gene bank.

Key words: Axillary bud, Cassava, Cryopreservation, *In vitro* regeneration, Shoot apices

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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 (Immanuel et al., 2024). Worldwide, 800 million people depend on cassava as their primary staple food (McCallum et

al., 2017). Cassava was originated in Latin America and the crop was said to be found by the Native Indians around 4000 years ago (Akinpelu et al., 2011). 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 (FAOSTAT, 2020). 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 TamilNadu ranks first in term of production 2603.2m.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 (Rajasekharan and RamanathaRoa, 2019). 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 (Nair et al., 2017). 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 (Aribi, 2024).

As such conditions, *in vitro* culture techniques provide some important tools for improved conservation and management of vegetatively propagated crop species (Sharma et al., 2022). The advantages of in *vitro* conservation are maintenance of material in pathogen-free condition that facilitates safer distribution, conservation of vegetatively propagated plants (Agrawal et al., 2024). Further, the cultures are not subjected to environmental disturbances (Engelmann, 2011). *In vitro* options provide short to medium (slow growth) and long-term (cryopreservation) conservation of genetic resources (Kaviani, 2011).For cryo-preservation, an *in vitro* regeneration system for the target species should be available for long-term conservation of vegetatively propagated crop species (Shankar et al., 2018). As

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cassava is a very important commercial crop, breeding research demand continuous supply of cassava germplasm for long period of time (Amelework and Bairu, 2022). Therefore, the present investigation has been undertaken with a view to establish an efficeint*in vitro* plant regeneration protocol in cassavausing axillary buds as explants that could support the establishment of a functional cryogene bank facility for cassava germplasm on a large scale.

MATERIALS AND METHODS

The *in vitro* experiments were carried out in the Tissue culture unit of Department of Plant Genetic Resources, Tamil Nadu Agricultural University, Coimbatore. Stem cuttings of two cassava genotypes YTP-1 and Sree Athulya collected from the Tapioca and Castor Research station, TNAU, Yethapurand planted in 'F' block of new area at Department of Forage crops, TNAU, Coimbatore. Cassava stem cuttings were planted in six rows by adopting the spacing of 90 cm between rows. The recommended agronomic practices and need based plant protection measures were adopted as per the crop production manual.

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

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supplemented with different concentrations of growth hormones (**Fig. 2**). Details of different media compositions tested for multiple shoot induction and proliferation is given in **Table 1**. 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. The shoot induction percentage was calculated by using the formula given below.

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

After two months of shoot elongation, the individual shoots were removed and transferred to rooting medium supplemented with different combination of growth hormones. The media compositions used for successful rooting of shoots are given in the **Table 2**. The regeneration frequency was calculated by using the formula given below.

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

All the cultures were maintained at a temperature of 25±2˚C with continuous illumination supported by Light Emitting Diodes (LED) lights providing a light intensity of 2500 lux with a photoperiod cycle of 16 h light and 8 h dark.The experiments were performed following Completely Randomized Design (CRD) and the data were subjected to statistical analysis as per the method suggested by Panse and Sukhatme (1978). Each treatment was replicated thrice. An analysis of variance (ANOVA) was computed using statistical software AGRES.

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 (Babu et al., 2012). For any

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vegetatively propagated crops to be made amenable for cryo-conservation, the first and foremost requirement is that the *in vitro* regeneration system should be sufficiently operational for the target species (Engelmann and Dussert, 2012). Upon culture of appropriate explants *in vitro*, the tissue culture protocol should facilitate the induction of multiple shoots and subsequent plantlet production. 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.

Various factors such as genotype, explants and nutrient composition of the culture medium and culture conditions greatly influence the regeneration of plantlets during *in vitro* culture (Bidabadi and Jain, 2020). As a first step, studies on standardization of all these parameters were undertaken to develop a comprehensive protocol to achieve successful *in vitro* regeneration in cassava germplasm.Among these different plant tissues, the shoot apices excised from the growing shoots are widely used for cryo-conservation of vegetatively propagated crops (Gowthami et al., 2024). Shoot apices are preferred as they contain pre-formed meristematic regions providing genetic stability potential and also possess ability to recover a plant (Srivastava et al., 2013). Furthermore, since regenerants produced through organogenesis should be identical to the mother plant, a small piece of material extracted from the mother plant can be considered as germplasm and can be used as a storage material (Osena, 2017). Therefore, shoot apices were selected as explants in the present study. Previously various authors have reported *in vitro* regeneration through shoot apices in cassava (Kartha et al., 1974; Konan et al., 1994; Rodrigues et al., 2010; Mapayi et al., 2013; Cacai et al., 2023). Nevertheless most of these protocols could not be adopted to wide array

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of genotypes as they are genotype and cultivar dependent. Therefore, the present study focused on the development of genotype independent *in vitro* regeneration protocol for cassava using axillary buds as explants though manipulation of nutrient composition of the culture medium.

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 SreeAthulya 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. (2021) reported that combination of phytohormones often determine the course of morphogenesis *i.e.,* shoot organogenesis. For multiple shoot regeneration, shoot tips were remarkably influenced by types and concentration of the auxin and cytokine used. The cytokinin BAP was more effective than kinetin and NAA. Gupta et al. (2020) elaborated on the effects of different hormone combinations in inducing shoot proliferation and elongation.

The results showed that the shoot induction frequency was ranged from 15.7% to 81.4% in YTP1 and 5.7% to 77.8% in Sree Athulya. 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 (CMSI 2) for YTP 1 and Sree Athulya, respectively (**Table. 3**). Shoot elongation was observed after 30 days of inoculation on CMSI 2 medium. Overall, 70-80% of explants produced 3 to 5 multiple shoots from a single shoot apex 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. (2020) reported that NAA and BAP were important for micropropagation. The use of NAA and BAP growth regulators during shoot

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growth initiated from meristem culture of different cassava varieties were also reported by Cacai et al. (2023).

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 (Khuna et al., 2023). 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. The results indicated that regeneration frequency was ranged from 3.3% to 56.6% in genotype YTP1 and 3.3% to 46.6% in genotype Sree Athulya. 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 (Cantabellaet al.,2022).Genotypes YTP1 and Sree Athulya showed lowest regeneration frequency of 3.3% when inoculated on Half MS medium (CRM 1). After 30 days of culture on rooting medium, fully developed plantlets with normal shoots and roots were formed and transferred to screen house condition for further establishment.

Conclusion

A simple and genotype independent *in vitro* regeneration system using shoot apex explants was developed in the present study. The regeneration frequencies achieved in the two genotypes of cassava are comparable with other studies. This could provide an impetus for successful deployment of shoot apices as an appropriate storage tissue under liquid nitrogen as efficient recovery could be accomplished. Furthermore, this genotype independent tissue culture protocol offers significant scope towards its implementation across many research

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station/laboratories dealing with micropropagation as well as cryo-conservation of cassava germplasm.

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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 (Murashige and Skoog, 1962)

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 $p > 0.01$

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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.

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

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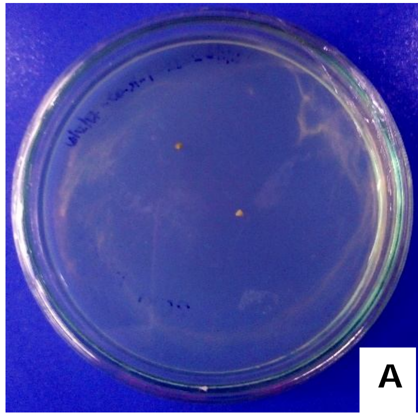

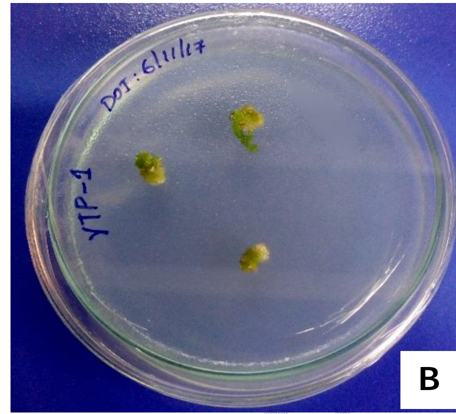

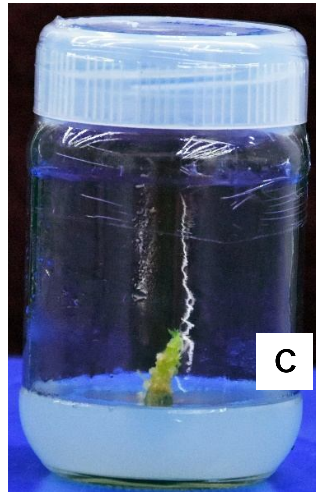

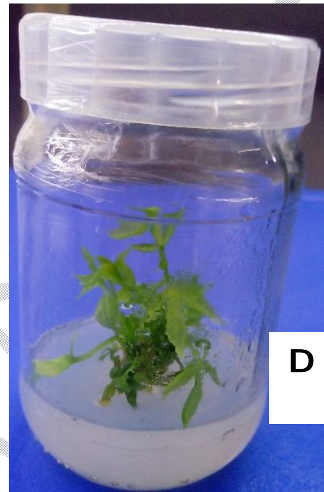

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Fig. 3. (A-E): *In vitro* regeneration of cassava genotypes using shoot apex explants**.** (A) Shoot apices inoculated on CMSI 2 medium.(B) Shoot initiation after 20 days of culture in the CMSI 2 medium**.** (C) Elongation of shoots after 30 days of 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.

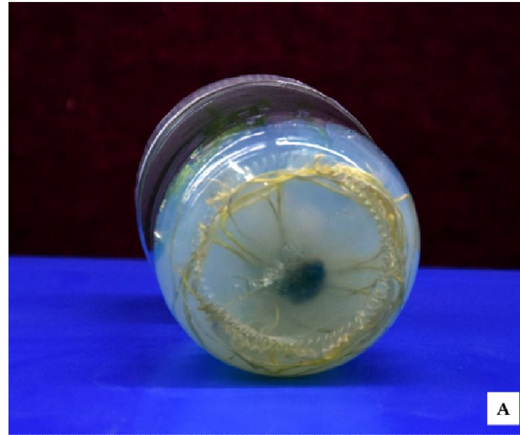

Fig. 4. (A-B): Rooting and regeneration of cassava (A). Root proliferation (B). Regenerated plant

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