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Establishment of an Efficient *In vitro* Regeneration and Micro-propagation Systems of *Cassava* (*Manihot esculenta* Crantz)

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Key words: *Cassava,* Indirect regeneration, Nodal segments, Acclimatization

Abstract

An *in vitro* micropropagation protocol for cassava *Manihot esculenta* was developed through callus culture. The present investigation the highest callus induction was achieved on MS medium containing 8.0 mg/l 2,4-D. Inter node and leaf derived calli were transferred to MS medium supplemented with BAP and NAA for shoot regeneration. In this case 10.0 mg/l BAP alone produced highest number of shoots (4.8 ± 0.3) per explant having average length of 1.72 ± 0.2 cm. The combination of 10.0 mg/l BAP and 0.5 mg/l NAA further enhanced shoot proliferation, producing an average of 5.6 ± 0.6 shoots per explant with a mean shoot length of 3.6 ± 0.2 cm with a regeneration rate of 87%. Moderate BAP concentrations (4.0-10.0 mg/l) were optimal for indirect shoot regeneration, whereas NAA or Kn alone exhibited limited effects. Root induction was most effective on half-strength MS medium supplemented with 2.0 mg/l IBA, which resulted in 100% rooting, with the highest mean root number of 4.8 and root length of 2.8 cm. The regenerated plantlets were successfully acclimatized in pots containing a 1 : 1 mixture of cocopeat and autoclaved soil, exhibiting high survival rates under shade house conditions.

Introduction

Cassava (*Manihot esculenta* Crantz), a perennial woody shrub of the family Euphorbiaceae, is one of the important staple food crops in the tropics. Globally, cassava production reached 330 million tonnes in 2022 (FAO 2023). Native to South America, cassava is now extensively cultivated across tropical and sub-tropical regions of Africa, Asia, and Latin America (Santana et al. 2009). It is the third largest source of calories in the tropics after rice and maize, feeding nearly 600 million people worldwide (Cock 1982). Cassava's drought tolerance properties and its ability to remain unharvested in the soil for up to three years make it an important "food security reserve" crop, providing resilience during famine conditions (Ihemere et al. 2006). Despite its significance,

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cassava production faces major challenges, including low multiplication rates, vulnerability to pests and diseases, limited shelf life of stem cuttings, and restricted availability of improved cultivars (Escobar et al. 2006). Conventional vegetative propagation by stem cuttings is slow, with a multiplication ratio of approximately 1 : 10. This limitation hampers rapid dissemination of improved varieties. In this context, *in vitro* tissue culture offers a reliable strategy for large-scale propagation and year-round supply of planting materials (Shiji et al. 2014). Beyond its role as a staple food, cassava provides nutritional, medicinal, and industrial value. Cassava leaves contain proteins, vitamins, and bioactive compounds with reported antioxidant, anti-inflammatory, and analgesic properties (Boukhers et al. 2022). They are traditionally used to treat ailments such as fever, diarrhea, rheumatism, and loss of appetite (Miladiyah 2011), and have also been linked to improved lactation (Abidin 2020). Additionally, cassava starch is gluten-free, making it valuable for individuals with gluten intolerance and for industrial food applications. It has also antioxidant activity like Mulbery (Rahman and Islam 2021).

Plant tissue culture serves as a powerful approach for cassava improvement, facilitating rapid clonal multiplication, preservation of germplasm, and elimination of diseases (George et al. 2008). It also provides a foundation for somaclonal variation, genetic transformation, and metabolic engineering (Paul et al. 2013, Miri et al. 2016, Islam et al. 2017). Factors such as explant type, physiological state, medium composition, and plant growth regulator balance critically influence regeneration outcomes (Rahman and Islam 2021 and 2024). While auxin-cytokinin interactions have been shown to play key roles in cassava morphogenesis, reproducibility across genotypes remains challenging (Seran 2013). Callus culture, in particular, is a valuable system for studying morphogenesis and achieving indirect organogenesis and somatic embryogenesis in cassava (Sultana et al. 2009, Ali et al. 2016). Successful induction of friable callus and subsequent regeneration of shoots are essential steps in developing efficient propagation protocols and in facilitating transformation studies. The present study was therefore designed to establish an efficient *in vitro* regeneration system for cassava through optimization of plant growth regulator combinations for callus induction, axillary shoot proliferation, indirect regeneration, and rooting to establish a complete plantlet.

Materials and Methods

Healthy explants of cassava were collected from the research field of the Institute of Biological Sciences, University of Rajshahi. Explants were washed under running tap water with Tween-20 (2-5 drops) for 10 min, followed by surface sterilization with 70% ethanol for 1.0 min, 7% sodium hypochlorite (NaOCl) for 10 min, and 0.1% mercuric chloride ($HgCl_2$) for 5.0 min. Sterilized explants were rinsed three times with sterile distilled water and trimmed into segments of approximately 1 × 1 cm.

For callus induction, explants were cultured on MS medium supplemented with 2,4-D (2.0-10.0 mg/l) and IAA (1.0-2.5 mg/l). The medium was further supplemented with 30 g/l sucrose and solidified with 7.0 g/l agar, adjusted to pH 5.6-5.8 before autoclaving

at 121°C (15 psi) for 20 min. Cultures were incubated at 25 ± 2°C under a 16 hrs photoperiod with cool white fluorescent light.

For shoot regeneration, calli were transferred to MS medium containing different concentrations of BAP (2.0, 4.0, 6.0, 8.0 and 10.0 mg/l), NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg/l) or Kn (0.75, 1.0, 1.25, 1.5 and 1.75 mg/l). Regenerated shoots were further sub-cultured onto MS media with BAP or NAA or Kn in different concentration range for evaluation of shoot multiplication and elongation. Rooting experiments were performed on half-strength MS medium supplemented with different concentrations of IAA (0.5-2.0) mg/l), IBA (0.5-2.0) mg/l), or NAA (0.5-2.0) mg/l). Rooting percentage, root number per explant and average root length were recorded.

The experiments were arranged in a completely randomized design (CRD). Each treatment consisted of 10 culture vessels, and all experiments were repeated three times. Data were analyzed using analysis of variance (ANOVA) in Microsoft Excel and treatment means were compared using the least significant difference (LSD) test at the 5% significance level ($p < 0.05$). Values are presented as mean ± standard error (SE).

Results and Discussion

After four weeks of culture initiation, cassava explants produced callus on MS medium supplemented with different concentrations of 2,4-D and IAA. The highest callus induction was observed on MS medium containing 8.0 mg/l 2,4-D (Table 1). Callus production in cassava has been widely reported using different auxins and cytokinins, in which 2,4-D and BAP identified as the most effective regulators (Sultana et al. 2009, Hossain et al. 2010, Ibrahim et al. 2015, Ali et al. 2016). In agreement with these studies, the present work confirmed the superior role of 2,4-D, at varying concentrations, and IAA for callus induction in cassava. Sultana et al. (2009) similarly reported that 2,4-D was more efficient in inducing friable callus compared to other auxins. Conversely, Hossain et al. (2010) and Ibrahim et al. (2015) demonstrated enhanced callus formation when BAP was combined with either IAA or 2,4-D. The present findings suggest that while multiple auxins can promote callogenesis, 2,4-D alone remains the most consistent and effective regulator for callus induction in cassava.

Callus weight is widely used as a quantitative parameter in plant tissue culture, including cassava, to evaluate the effectiveness of plant growth regulators (PGRs) for further regeneration and sometimes to compare the responsiveness of varieties. It reflects the growth rate and biomass accumulation, indicates better cell proliferation and more suitable hormonal balance. Callus growth (fresh weight) was measured to compare 2,4-D concentrations and determine the best conditions for friable embryogenic callus (Sofiari et al. 1997).

In cassava, somatic embryogenesis (SE) can be initiated not only from zygotic embryos but also from vegetative explants. Leaves, petioles, roots, nodes and stems have

Table 1. Effects of 2,4-D and IAA on callus induction after 28 days of culture initiation.

PGRs	Treatment	PGRs concentration (mg/l)	Types of explants	Callus formation frequency (Mean± SE)	Callus weight (mg/explant) (Mean ± SE)	Quality of calli
2,4-D	T_1	4	LD	71.3 ± 1.3	220 ± 20	Moderate callusing
			NS	61.1 ± 1.2	120 ± 15	
	T_2	6	LD	75.1 ± 1.2	350 ± 25	Friable
			NS	62.4 ± 1.1	250± 25	
	T_3	8	LD	92.5 ± 1.3	480 ± 30	Friable
			NS	87.7 ± 0.9	380 ± 30	
	T_4	10	LD	78.3 ± 1.6	410 ± 35	Slight decline, watery
			NS	65.5 ± 1.3	130 ± 20	
IAA	T_5	1.0	LD	62.3 ± 2.5	110 ± 12	Little callusing
			NS	60.5 ± 2.1	95 ± 10	
	T_6	1.5	LD	68.7 ± 1.9	150 ± 18	Little callusing
			NS	64.4 ± 1.7	125 ± 15	
	T_7	2.0	LD	70.8 ± 1.6	200 ± 20	Compact
			NS	65.2 ± 1.8	165 ± 18	
	T_8	2.5	LD	69.2 ± 2.0	160 ± 15	Loosely compact
			NS	67.8 ± 2.3	130 ± 14	

LD = Leaf disc, NS = Nodal segment, SE = Standard Error.

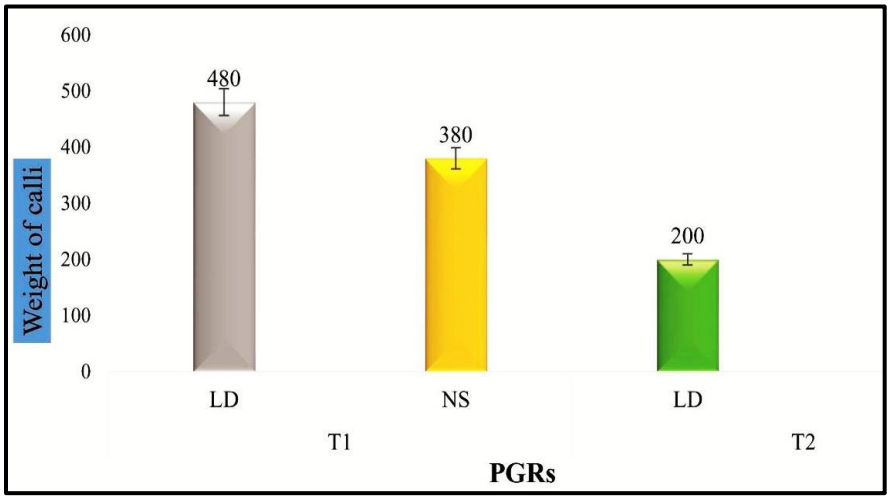

Fig. 1. Effects of different concentrations of 2,4-D and IAA on callus weight. Here, T_1 = 2,4-D (8.0 mg/l), T_2 = IAA (2.0 mg/l), LD = Leaf disc, and NS = Nodal segment.

to support SE when cultured on auxin-supplemented media (Raemakers et al. 1999). In this study, leaves and nodal segments were used. But vigorous calli derived from leaf explants (Fig. 1). Both greenhouse and *in vitro* grown leaves are suitable for SE induction

(Sofiari et al. 1997). Although cytokinin combinations are often reported to improve shoot proliferation in other species (Baskaran and Jayabalan 2008, Islam and Bhattacharjee 2015), in cassava BAP + NAA did not significantly outperform BAP alone. This contrasts with Ayenew et al. (2012), who achieved maximum proliferation at lower cytokinin concentrations.

Table 2. Effects of different concentrations and combinations of BAP, NAA and Kn on shoot proliferation and elongation after 28 days of culture initiation.

PGRs (Concentration)			No. of shoots per explant	Length of shoots
BA	NAA	Kn	(Mean ± SE)	(Mean ± SE)
2.0	-	-	2.4 ± 0.3	2.0 ± 0.2
4.0	-	-	3.1 ± 0.4	2.4 ± 0.2
6.0	-	-	3.8 ± 0.5	2.2 ± 0.2
8.0	-	-	3.5 ± 0.4	1.9 ± 0.2
10.0	-	-	4.8 ± 0.3	1.7 ± 0.2
-	0.1	-	0.8 ± 0.2	1.5 ± 0.1
-	0.2	-	0.9 ± 0.2	1.4 ± 0.1
-	0.3	-	1.0 ± 0.3	1.3 ± 0.1
-	0.4	-	1.7 ± 0.2	1.2 ± 0.1
-	0.5	-	0.5 ± 0.2	1.1 ± 0.1
2.0	0.1	-	3.0 ± 0.4	2.3 ± 0.2
4.0	0.2	-	3.9 ± 0.4	2.6 ± 0.2
6.0	0.3	-	5.1 ± 0.5	2.8 ± 0.2
8.0	0.4	-	4.2 ± 0.5	2.5 ± 0.2
10.0	0.5	-	5.6 ± 0.6	3.6 ± 0.2
-	-	0.75	1.6 ± 0.2	1.8 ± 0.3
-	-	1.00	2.2 ± 0.3	2.1 ± 0.2
-	-	1.25	3.8 ± 0.5	2.4 ± 0.2
-	-	1.50	2.5 ± 0.3	2.5 ± 0.3
-	-	1.75	2.7 ± 0.4	2.4 ± 0.2
2.0	-	0.75	2.3 ± 0.4	1.6 ± 0.3
4.0	-	1.00	2.1 ± 0.5	2.0 ± 0.2
6.0	-	1.25	2.2 ± 0.6	2.1 ± 0.2
8.0	-	1.50	1.9 ± 0.6	1.8. ± 0.2
10.0	-	1.75	2.8 ± 0.5	2.3 ± 0.2

Calli derived from leaf explants were transferred to MS medium supplemented with varying concentrations of BAP (2.0, 4.0, 6.0, 8.0, 10.0 mg/l), NAA (0.1-0.5 mg/l), or Kn (0.75-1.5 mg/l) for adventitious shoot regeneration. The morphogenic response of plants *in vitro* is highly dependent on the balance between auxin and cytokinin in the medium (Schaller et al. 2015). After four weeks, the highest regeneration response was obtained with 10.0 mg/l BAP, where 87% of calli produced plantlets (Table 2). BAP consistently outperformed NAA, confirming its superiority as a cytokinin for cassava morphogenesis.

These results align with earlier reports that highlighted the positive role of BAP, alone or in combination with NAA, in enhancing cassava shoot regeneration and elongation (Sultana et al. 2009, Islam and Bhattacharjee 2015).

A combination of BAP and NAA has been reported to promote multiple inductions in *Prunella vulgaris* explants (Rasool et al. 2009) and also in *Morus alba* (Rahman and Islam 2024). In the present study, 10.0 mg/l BAP combined with 0.5 mg/l NAA promoted greater elongation, producing an average of 5.6 ± 0.6 shoots per explant with an average length of 3.6 ± 0.2 cm and a regeneration rate of 87% (Figs 2-3).

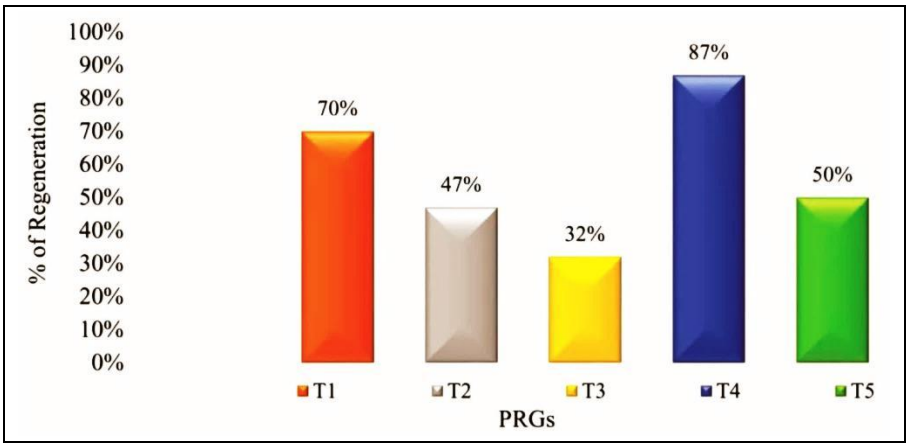

Fig. 2. Regeneration of cassava from callus on MS medium with different concentrations of BAP and Kn (mg/l) T_1 = BAP (10.0), T_2 = NAA (0.4), T_3 = Kn (1.25), T_4 = BAP (10.0) + NAA (0.50), T_5 = BAP (10.0) + Kn (1.75).

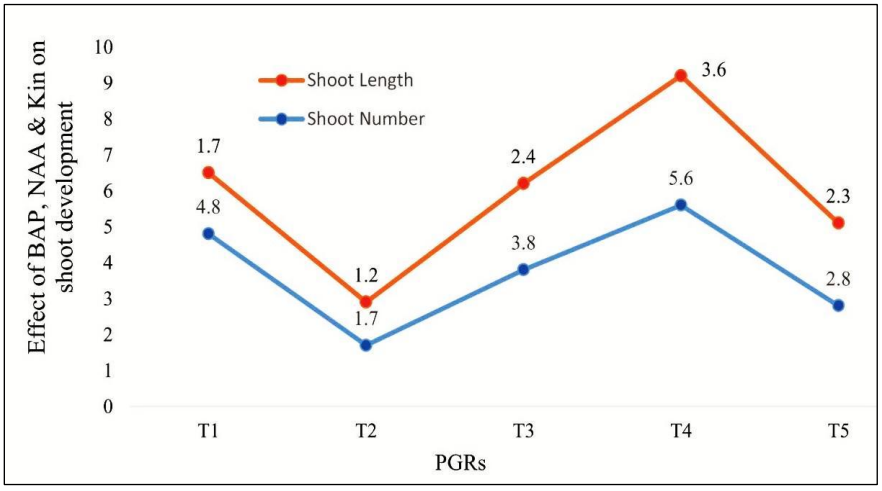

Fig. 3. Effects of BAP and Kn on shoot number and shoot length of cassava cultured on MS medium supplemented with different concentrations of plant growth regulators (PGRs) either singly or in combination (mg/l). Treatments: T_1 = BAP (10.0), T_2 = NAA (0.4), T_3 = Kn (1.5), T_4 = BAP (10.0) + NAA (0.5), T_5 = BAP (10.0) + Kn (1.75), T_6 = BAP (2.0) + Kn (1.0), T_7 = BAP (4.0) + Kn (1.75).

Overall, moderate BAP concentrations (2.0-10.0 mg/l) proved optimal for indirect shoot regeneration, whereas NAA or kinetin alone showed weaker effects. Regenerated plantlets were subsequently sub-cultured onto MS medium containing different concentrations and combinations of BAP, NAA, and Kn to evaluate shoot multiplication and elongation (Table 2). Initial high proliferation was obtained with 10.0 mg/l BAP, yielding 4.8 ± 0.3 shoots per explant with an average length of 1.72 ± 0.2 cm. However, the best proliferation was achieved with the combination of 10.0 mg/l BAP and 0.5 mg/l NAA, producing 5.6 ± 0.6 shoots per explant with an average length of 3.6 ± 0.2 cm. After 4 weeks of transferring calli to regeneration medium it shows becoming greenish and develop healthy plantlets and finally, healthy plants in the tub (Fig. 4A-H). Interestingly, while higher BAP + NAA concentrations enhanced shoot elongation, they did not significantly increase shoot number, reflecting the commonly observed trade-off between proliferation and elongation under elevated cytokinin levels (Erfani et al. 2017, Sessou et al. 2020).

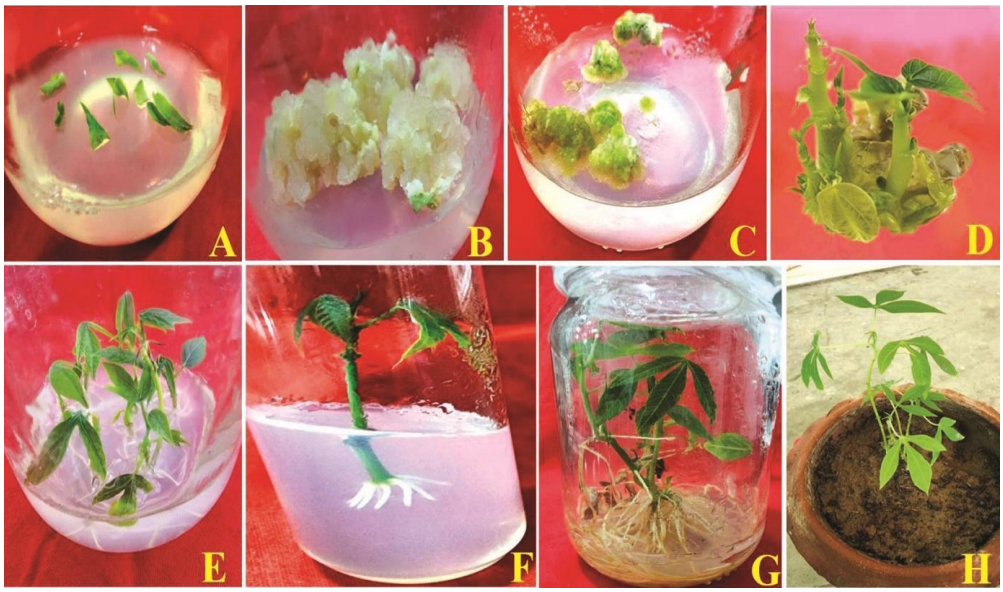

Fig. 4(A-H). *In vitro* regeneration of cassava: (A) callus initiation from leaf disc on MS + 8.0 mg/l 2,4-D after 7-10 days of culture, (B) callus proliferation after 2-4 weeks, (C) callus greening and maturation after 4-6 weeks, (D) shoot formation on MS + 10.0 mg/l BAP after3-5 weeks, (E) shoot multiplication after 4-10 weeks, (F) root induction on MS + 2.0 mg/l IBA after 2-4 weeks, (G) matured plantlets (10-15 cm) after 4-6 weeks, and (H) acclimatized plantlets transfer to tub containing soil in field after a 6-8 weeks.

Although cytokinin combinations are often reported to improve shoot proliferation in other species (Baskaran and Jayabalan 2008). BAP + NAA did not significantly outperform BAP alone in cassava. This observation contrasts with the findings of Ayenew et al. (2012), who reported maximum proliferation at lower cytokinin concentrations.

Rooting was assessed on half-strength MS medium supplemented with IAA, IBA, or NAA (0.5-2.0 mg/l). The highest rooting response (100%) occurred with 2.0 mg/l IBA, which also produced the greatest number of roots per explant 4.8 and the longest mean root length 2.8 cm (Fig. 5). A well-developed root system is critical for successful vegetative propagation and acclimatization of *in vitro* plantlets. Auxin supplementation is widely known to promote root initiation and elongation (Krupa-Małkiewicz and Mgłosiek 2016). In this study, root number and length were strongly influenced by auxin type and concentration. Spontaneous rooting was occasionally observed at lower BAP concentrations, likely due to endogenous IAA accumulation at the shoot base (Martin et al. 2012).

Among the auxins used, IBA proved to be most effective which produced the highest rooting percentage and most vigorous roots. This aligns with reports by Abbas et al. (2011) and Kambaska and Santilata (2009), who highlighted the stimulatory effect of auxins on cassava root induction. While some studies identified NAA as effective, the present results demonstrate that IBA at 2.0 mg/l is the most efficient auxin for inducing both root initiation and elongation in cassava plantlets.

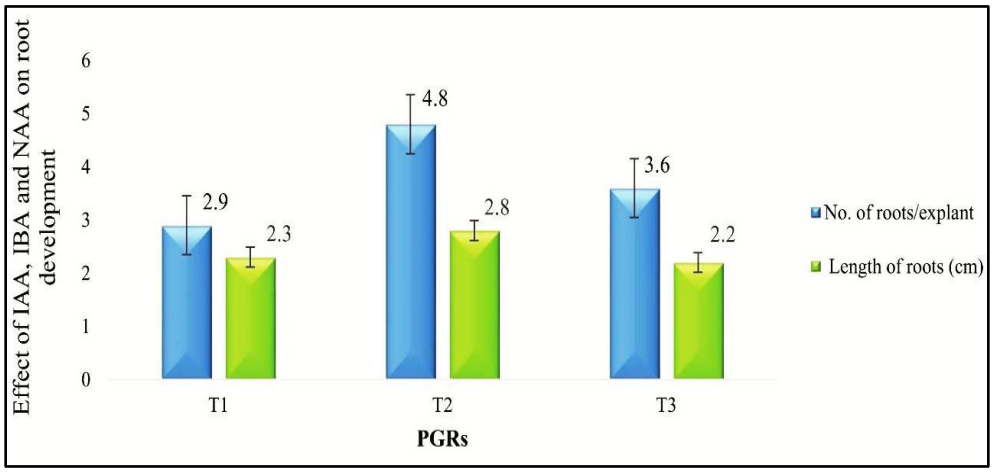

Fig. 5. Rooting on half MS medium with IAA, IBA and NAA showing root number and root length. Treatments (mg/l): T_1 = IAA (1.0), T_2 = IBA (2.0), T_3 = NAA (1.5).

After four weeks, the well-rooted plantlets were gently removed from the jars and rinsed thoroughly with running tap water (Fig. 4G-H). They were then placed in little pots with a 1 : 1 mixture of sterilized cocopeat and autoclaved soil. To maintain humidity, the pots were covered with polythene bags and sprayed with water every two days. They were placed in a greenhouse for six weeks to acclimate. The average daily greenhouse temperature and relative humidity were set at 20 ± 2°C and 75%, respectively. The polythene bags were gradually removed to acclimatize the plantlets to the natural environmental condition.

This study established a reproducible *in vitro* micropropagation protocol for cassava. Here, the plant growth regulators were optimized for callus induction, shoot regeneration, rooting, and acclimatization.

Among auxins, 8.0 mg/l 2,4-D was most effective for friable callus induction, while BAP outperformed NAA and Kn in shoot regeneration. A combination of 10.0 mg/l BAP with 0.5 mg/l NAA achieved 87% regeneration with enhanced shoot proliferation. For rooting, half-strength MS medium with 2.0 mg/l IBA yielded vigorous roots and 100% response. The regenerated plantlets were successfully acclimatized in a cocopeat-soil mixture, exhibiting high survival rates and demonstrating the practical effectiveness of the protocol. Overall, this optimized system provides a reliable platform for large-scale clonal propagation of cassava, supporting multiplication and year-round supply of disease-free planting material. It also offers a basis for advanced applications, including somatic embryogenesis, genetic transformation, and trait improvement, contributing to cassava breeding, conservation, and food security.

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