



## Research Article

# Optimization of NAA and BAP Concentrations for Callus Induction Response in *Manihot esculenta* Crantz

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### ABSTRACT

Efficient *in vitro* regeneration systems are essential for large-scale clonal propagation and biotechnology improvement of cassava (*Manihot esculenta* Crantz). Auxin-cytokinin is a critical determinant of callus formation and morphogenic competence; however, responses to concentration dependence remains genotype-specific and require empirical optimization. The present study investigated the effects of different concentrations of 6-benzylaminopurine (BAP: 0.5 - 3.0 mg L<sup>-1</sup>) combined with and  $\alpha$ -naphthaleneacetic acid (NAA: 1.0 – 4.0 mg L<sup>-1</sup>) on callus induction and growth responses in nodal explants cultured on Murashige and Skoog medium. Explants were arranged in a randomized design with three biological replicates per treatment. After seven weeks of culture, callus induction frequency, fresh and dry biomass, and cellular proliferation indices were assessed. Morphometric parameters including fresh weight, dry weight, callus length, width, cell proliferation, and biomass loss were assessed. Moderate hormone concentrations (0.5–1.0 mg L<sup>-1</sup> BAP combined with 1.0-2.0 mg L<sup>-1</sup> NAA) significantly enhanced callus induction and biomass accumulation compared with higher concentrations ( $p < 0.05$ ). Supra-optimal hormone levels resulted in reduced proliferation, compact callus formation, and lower cellular density, indicating possible hormonal imbalance and growth suppression. The findings demonstrate that precise auxin-cytokinin equilibrium is essential for maximizing callogenic response in cassava nodal tissues. The protocol provides a reproducible framework for improving cassava micropropagation and downstream regeneration systems.

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### INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a member of the Euphorbiaceae, is a major staple crop in tropical and subtropical regions, contributing significantly to food security and industrial starch production (Feyisa 2021; Yelli et al. 2023). Despite its economic importance, cassava

propagation through stem cuttings is limited by low multiplication rates and vulnerability to systemic diseases (Marius et al. 2018). *In vitro* regeneration systems, particularly callus induction, provide an effective alternative for rapid multiplication and genetic improvement. Callus represents a relatively undifferentiated

mass of plant tissue composed predominantly of parenchymatous cells. In plant tissue culture systems, callus can serve as a versatile experimental model to investigate fundamental aspects of plant biology, including pathology, cytology, biochemistry, anatomy, physiology, morphology, and genetics. Beyond its importance in basic research, callus cultures also provide a useful platform for addressing applied research challenges in plant biotechnology. These include studies related to organogenesis, micropropagation, and developmental processes associated with the regeneration of horticultural and agronomic crops (Caponetti, 2000). Callus tissues are additionally employed in investigations involving protoplast isolation, cellular selection, cell-type differentiation, and the production of valuable secondary metabolites (Kondamudi et al., 2009).

In cassava (*Manihot esculenta*), several studies have reported successful callus induction from various explant sources, demonstrating the feasibility of in vitro culture techniques for improving regeneration and propagation systems (Ogburia, 2002; Fletcher et al., 2011). The induction of callus followed by plant regeneration constitutes one of the most important tools in modern plant biotechnology, as it exploits the inherent totipotency of plant cells to regenerate whole plants from differentiated tissues (Bhojwani and Dantu, 2003).

Plant tissue culture systems are also fundamental to the development of transgenic plants, where efficient regeneration protocols are essential components of most transformation strategies (Stewart and Cardoza, 2008). Previous investigations on adventitious shoot formation from callus cultures within the family *Euphorbiaceae* have further demonstrated the potential of callus-mediated regeneration systems for crop improvement and biotechnological applications (Ahn et al., 2007; Kumari et al., 2008; Li et al., 2012).

The ratio and concentration of auxin and cytokinin are decisive factors influencing morphogenic pathways in cassava tissue culture (Demeke et al. 2014; Sukmadjaja and Widhiastuti 2011). Although several hormone combinations have been reported, genotype-

specific and concentration-dependent variations necessitate further optimization.

The objective of this study was to determine the optimal concentration of BAP and NAA for callus induction response in nodal explants of *M. esculenta* under controlled in vitro conditions.

## **MATERIALS AND METHODS**

### **Plant material and explant preparation**

Healthy and disease-free stem cuttings of cassava (*Manihot esculenta* Crantz) were obtained from field-grown plants and established under greenhouse conditions prior to explant collection. Actively growing nodal segments (approximately 1–1.5 cm in length) were excised and used as explants for in vitro culture. Explants were initially rinsed under running tap water for 10 min to remove surface debris. Surface sterilization was performed in a laminar airflow cabinet by immersion in 70% ethanol for 30 s. The explants were subsequently rinsed three to four times with sterile distilled water to remove residues.

### **Culture medium and growth regulator treatments**

The basal culture medium consisted of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g L<sup>-1</sup> sucrose, 100 mg L<sup>-1</sup> myo-inositol and solidified with 7 g L<sup>-1</sup> agar. The pH of the medium was adjusted to 5.7 ± 0.1 prior to autoclaving at 121 °C and 15 psi for 15 min. Medium was supplemented with different concentrations of the 6-benzylaminopurine (BAP) and the auxin  $\alpha$ -naphthaleneacetic acid (NAA). The treatments included combinations of BAP (0.5–3.0 mg L<sup>-1</sup>) and NAA (1.0–4.0 mg L<sup>-1</sup>), while hormone-free MS medium which served as the control. The experiment was arranged in a completely randomized design (CRD) consisting of five treatments (including the control). Each treatment contained three replicates, with ten explants per replicate, resulting to the total of 30 explants per treatment. Randomization procedure was done to ensure that each explant had an equal chance of receiving any treatment. All explants were cultured under identical environmental and experimental conditions to maintain homogeneity. Cultures were maintained for seven weeks, after which quantitative and morphological assessments of callus development were conducted. Callus formation

was recorded weekly, and callus induction frequency was calculated at the end of the culture period using the following equation:

$$\text{Callus induction (\%)} = \frac{\text{(Number of explants producing callus)}}{\text{(Total number of explants cultured)}} \times 100$$

Callus morphology, including texture (friable or compact) and coloration, was also visually assessed

#### Culture conditions

The cultures were transferred into the sterile growth room and maintained at  $25 \pm 2^{\circ}\text{C}$  with a 16-hour photoperiod at a light intensity of 2500-3000 lux from cool white florescent 60-watt bulbs throughout the duration of the experiment.

#### Biomass and morphometric analysis

Thereafter, calli were carefully excised from the explants and weighed to determine the fresh biomass using Mettler weighing balance. For dry weight determination, calli were dried in a hot-air oven at  $60^{\circ}\text{C}$  for 48 h until constant weight was achieved.

Callus dimensions were measured using a digital Vernier caliper. Measurements included:

Fresh weight (mg), Dry weight (mg), Callus length (cm), Callus width (cm), Number of cells Absolute and percentage biomass loss.

Cellular proliferation assessment: Cellular proliferation within the callus tissue was estimated through microscopic cell counting. Small calli samples were macerated in sterile distilled water, and cell suspensions were observed under a compound microscope. Cell counts were obtained from randomly selected microscopic fields and averaged to estimate relative cellular density.

#### Statistical analysis

All quantitative data were expressed as mean  $\pm$  standard deviation (SD).

The results were analyzed using ANOVA tests with Turkey's multiple comparison tests and expressed as mean  $\pm$  standard error at  $p < 0.05$  level of significance.

#### RESULTS

The response of cassava nodal explants cultured on MS media supplemented with growth regulators NAA and BAP at different concentrations over a period of seven weeks resulted in the callus formation as seen in figure 8. Callus initiation was observed within 2–3 weeks of culture in all media supplemented with plant growth regulators, whereas the hormone-free control exhibited delayed and reduced callogenic response. The morphological changes observed during the seven weeks were recorded. Parameters taken include Fresh weight, Dry weight, Number of cells, Length of calli, and Width of calli.

Morphological results showed that treatment groups T1 and T2 cultured on MS medium supplemented with (0.5 mg/L BAP + 1.0 mg/L NAA and 1.0 mg/L BAP + 2.0 mg/L NAA) respectively showed the highest callus induction percentage. Treatments containing higher hormone concentrations (T3 and T4) produced significantly lower induction frequencies ( $P < 0.05$ ) (Fig. 5). Morphologically, calli produced under hormone free medium (control group) and moderate hormone concentrations and were friable and cream-colored, indicating active cellular proliferation, whereas calli from higher hormone treatments were compact and less proliferative.

**Table 1:** Effect of BAP and NAA on callus induction and Biomass

Treatment mg/L	Fresh weight (mg)	Dry weight (mg)	Length (cm)	Width (cm)	No of cells	Weight Loss (g)
Control	210.38 $\pm$ 50.68 <sup>ab</sup>	27.00 $\pm$ 5.87 <sup>b</sup>	1.80 $\pm$ 0.33 <sup>a</sup>	0.70 $\pm$ 0.13 <sup>b</sup>	648.75 $\pm$ 200.04 <sup>a</sup>	183.38 $\pm$ 53.81
T1-0.5BAP+1NAA	385.42 $\pm$ 123.38 <sup>a</sup>	46.50 $\pm$ 14.66 <sup>a</sup>	1.48 $\pm$ 0.21 <sup>a</sup>	1.01 $\pm$ 0.23 <sup>a</sup>	371.42 $\pm$ 62.75 <sup>ab</sup>	338.92 $\pm$ 109.85
T2-1BAP+2NAA	550.25 $\pm$ 216.65 <sup>a</sup>	43.83 $\pm$ 15.68 <sup>a</sup>	1.88 $\pm$ 0.16 <sup>a</sup>	1.04 $\pm$ 0.22 <sup>a</sup>	264.17 $\pm$ 23.80 <sup>ab</sup>	506.42 $\pm$ 207.32
T3-2BAP+3NAA	124.00 $\pm$ 15.50 <sup>b</sup>	16.17 $\pm$ 4.68 <sup>b</sup>	1.68 $\pm$ 0.26 <sup>a</sup>	0.43 $\pm$ 0.02 <sup>b</sup>	199.33 $\pm$ 20.09 <sup>b</sup>	107.83 $\pm$ 19.49
T4-3BAP+4NAA	126.40 $\pm$ 16.73 <sup>b</sup>	9.27 $\pm$ 2.41 <sup>b</sup>	1.90 $\pm$ 0.20 <sup>a</sup>	0.60 $\pm$ 0.05 <sup>b</sup>	232.00 $\pm$ 34.20 <sup>b</sup>	117.13 $\pm$ 18.81
Mean	327.58	33.01	1.73	0.83	350.11	294.56
LSD ( $p \leq 0.05$ )	5.63	1.32	0.64	1.54	5.50	1.33

Values represent mean  $\pm$  SD ( $n = 30$ ). Means followed by different letters within columns differ significantly at  $p < 0.05$  (Tukey HSD).

Table 1 shows the fresh biomass accumulation varied significantly among treated groups ( $F=3.39$ ,  $p=0.03$ ). The highest fresh weight was recorded in treatment T2, followed by T1, while treatments with higher concentrations of BAP and NAA exhibited reduced biomass accumulation. The control treatment produced significantly lower biomass compared with hormone-supplemented media, confirming the stimulatory effect of auxin–cytokinin combinations on callus proliferation.

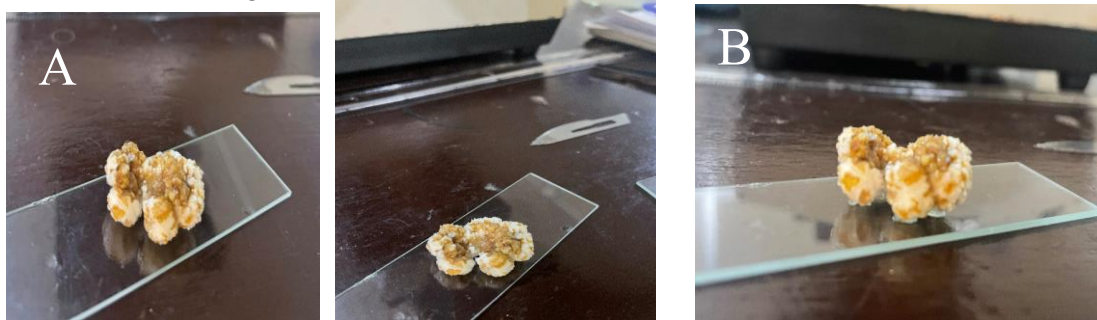
The dry weight followed a similar trend to the fresh biomass (Table 1). Statistical analysis indicated significant differences among treatments ( $P < 0.05$ ). Explants cultured under moderate hormone concentrations (T1 and T2) produced the highest dry matter accumulation, indicating enhanced cellular proliferation and tissue development. In contrast, higher regulator concentrations resulted in significantly lower dry weights.

Callus width differed significantly among treatments ( $P < 0.05$ ). Explants cultured under T1 and T2 treatments produced broader callus structures, whereas higher hormone concentrations resulted in smaller and more compact tissues. However, callus length did not differ significantly among treatments ( $P > 0.05$ ), suggesting that radial expansion was more responsive to hormonal balance than longitudinal growth (Table 1). The tissues treated with 0.5 mg/L BAP + NAA and 1.0

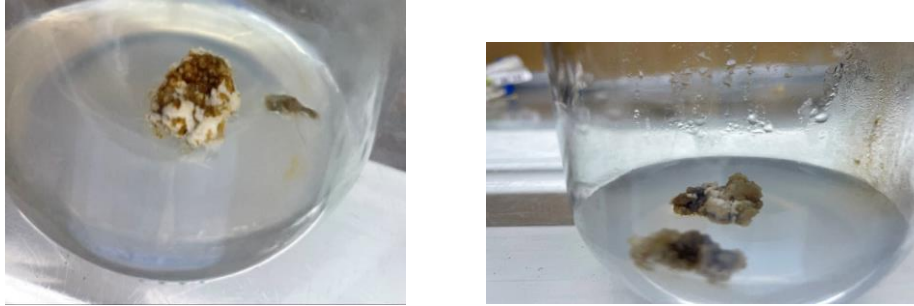
mg/L BAP + 2.0 mg/L NAA showed considerable higher width growth when compared with the control.

The loss in weight between the fresh weight and the dry weight were recorded following drying in the air oven. The weight loss was recorded by replicates to get the mean values for the weight loss. The results showed that 0.5 mg/L BAP + 1.0 mg/L NAA demonstrated considerable weight loss after drying while the higher concentrations showed lesser weight loss as observed in figure 5. Compared to the control, 2.0 mg/L BAP + 3.0 mg/L NAA and 3.0 mg/L BAP + 4.0 mg/L NAA showed less weight loss after drying ( $F=1.39$ ,  $p=0.02$ ). This supports the hypothesis that there was less tissue growth which will lead to less weight loss (Table 1).

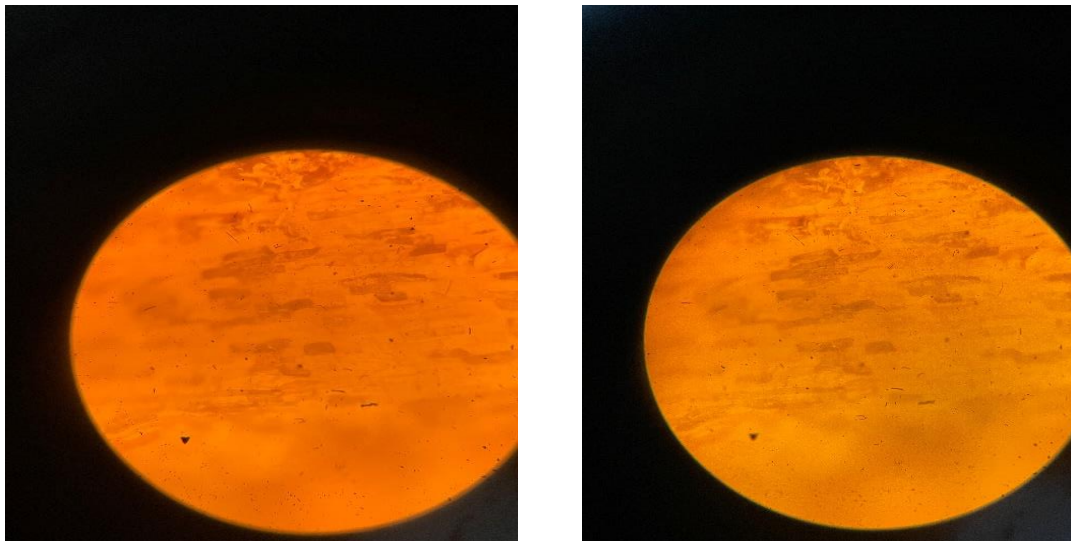
The number of cells that grew on the tissue was counted per replicates and the mean values were calculated. The control had the highest number of cells showing that it encouraged the highest number of cell growth but with lesser biomass accumulation. The results of the lower concentrations were statistically different from that of the higher concentrations based on the number of recorded cells ( $F=1.46$ ,  $p=0.03$ ). The number of cells observed were lower for the higher concentrations. This shows that the control and lower concentrations of the treatment induces higher number of new cell growth in the tissues. This result as shown in Table 1.



**Figure 1:** A, B, C: Callus formation from NAA and BAP treatment



**Figure 2:** A, B, Callus formed in 100ml growth bottles



**Plate 1:** A, B; Microscopic view of callus cells

## DISCUSSION

The present study demonstrates that callus induction and proliferation in cassava nodal explants are strongly influenced by the concentration and balance of auxin and cytokinin in the culture medium. Moderate concentrations of BAP combined with NAA significantly enhanced callus formation and biomass accumulation compared with higher hormone concentrations. The synergistic interaction between auxins and cytokinins is widely recognized as a critical factor controlling cell division, differentiation, and morphogenic responses in plant tissue culture.

It was observed that optimum callus formation occurred in the growth bottles that were supplemented with 0.5 BAP+ 1 NAA and 1 NAA+2 BAP concentrations of growth

hormones. The results of this research were consistent with the study carried out by Sukmadjaja and Widhiastuti (2011) on the effects of plant growth regulators on shoot multiplication and root induction of Cassava varieties *in vitro* culture. The largest number of shoots was obtained from the explants when treated with BAP 1 mg/L + thidiazuron 0.1 mg/L. This is not in tandem with Feyisola *et al.*, (2017) whose study identified that nodal proliferation of the cassava plantlets is not dependent on hormone supplementation, however, hormones in various concentrations are required for early germination and elongation of cassava plantlet's height.

Callus formation occurred in all hormone-supplemented treatments within 2–3 weeks of culture. Treatments T1 and T2 produced friable,

cream-colored calli with greater expansion. Higher concentrations (T3 and T4) resulted in compact, less proliferative calli.

BAP and NAA concentrations showed negative correlations with cell number at supra-optimal levels. The results confirm that auxin–cytokinin balance critically influences morphogenic response in cassava. Moderate concentrations of BAP and NAA enhanced dedifferentiation and callus proliferation, consistent with previous findings in cassava tissue culture systems (Demeke et al. 2014; Sukmadjaja and Widhiastuti 2011). Another study by Isalar, (2026), confirmed that auxin treatments increased calli biomass accumulation, while cytokinin treatments restricts proliferation but optimized structural compactness and colour, indicative of partial differentiation.

High concentrations of growth regulators suppressed growth, possibly due to hormonal toxicity, metabolic imbalance, or ethylene accumulation, which can inhibit morphogenesis (Wójcik et al. 2020). Similar inhibitory effects of excessive BAP concentrations have been reported in cucurbits and woody species (Azizan, 2017). The findings demonstrate that a balanced concentration of 0.5–1.0 mg/L BAP with 1.0–2.0 mg/L NAA provides optimal conditions for callus proliferation in *M. esculenta*. Auxins such as NAA promote cellular dedifferentiation by activating transcriptional pathways associated with cell cycle re-entry and acquisition of totipotency. Cytokinins, particularly BAP, stimulate mitotic activity and enhance cell proliferation by regulating DNA synthesis and cytokinesis. The interaction between these two classes of plant growth regulators determines the morphogenic outcome of cultured plant tissues (Skoog and Miller, 1957; Ikeuchi et al., 2013).

The enhanced callogenic response observed at moderate hormone concentrations in this study suggests that an optimal auxin–cytokinin balance promotes cellular dedifferentiation and sustained mitotic activity. Similar hormone-dependent responses have been reported in cassava tissue culture systems, where balanced concentrations of auxins and cytokinins promote callus proliferation and improve regeneration potential (Taylor et al., 2012; Chauhan et al., 2015). Although the control treatment produced

a significantly higher number of cells, explants cultured in media supplemented with auxin–cytokinin combinations exhibited greater biomass accumulation. This suggests that hormonal balance influenced not only cell proliferation but also cell expansion and metabolite accumulation, leading to fewer but larger and denser cells. The observed variation between cell number and biomass among treatments may be attributed to the differential roles of auxin and cytokinin in regulating cell division and expansion. Previous studies have demonstrated that plant growth regulators significantly influence callus morphology and biomass accumulation, with certain hormone combinations promoting compact and heavier callus despite reduced proliferation (Isalar, 2026; Rahman et al., 2019). Furthermore, auxin–cytokinin interactions have been reported to significantly affect callus fresh weight, indicating that biomass accumulation is not solely dependent on cell number (Lee and Huang, 2013).

Conversely, the reduced biomass accumulation observed at higher hormone concentrations may reflect disruption of endogenous hormonal homeostasis. Excess cytokinin has been reported to interfere with auxin transport and induce ethylene accumulation, both of which can inhibit cell division and suppress callus growth (George et al., 2008; Fehér, 2019).

Morphometric analysis revealed that callus width varied significantly among treatments, whereas callus length exhibited comparatively limited variation. This observation suggests that radial cellular expansion may be more sensitive to hormonal balance than longitudinal growth under the tested conditions. Such anisotropic growth patterns have been observed in plant tissue cultures where localized hormone gradients regulate cellular expansion and proliferation (Ikeuchi et al., 2013).

Although this study focused on callus induction and biomass proliferation, the optimized hormonal regime identified here provides an important foundation for improving cassava micropropagation and regeneration systems. Future studies should evaluate the regenerative competence of the induced callus and explore their potential applications in genetic transformation and crop improvement programs.

**CONFLICTS OF INTEREST**

The authors declare that they have no conflict of interest.

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