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Plant regeneration from embryogenic callus-derived from immature leaves of Momordica charantia L

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ABSTRACT

Bitter melon (*Momordica charantia* L.), a widely cultivated food and medicinal plant native to the world's subtropics and tropics, is a *Cucurbitaceae* rich in carotenoids. However, the low seed germination frequency and progeny variability associated with the production of this plant have a substantial impact on its growth and yield. These constraints affect the availability and exploitation of this crop, especially the fruits, which are rich in secondary metabolites such as β-carotene and α-carotene. *In vitro* regeneration would help overcome the obstacle linked to the germination of this plant and increase its yield and utilization. A reproducible *in vitro* organogenesis protocol was established using bitter melon embryogenic callus derived from immature leaf explants of *in vivo* grown seedlings and *in vitro* plantlets. Regeneration via callus was conducted on MSB5 media augmented with different plant growth regulator concentrations. The maximum frequency of callus formation (95.09 %) was produced in MSB5 media incorporated with 1.2 mg L^{-1} NAA augmented with 0.5 mg L^{-1} TDZ. MSB5 medium with no growth regulators was observed to be the most suitable for the shoot and root formation from the callus, producing a significantly high shoot percentage of 90.91 % and 21.53 shoots per explants, and the highest rooting frequency and root number of 88.92 % and 6.23 roots per explant, respectively, from leaf-derived callus of *in vitro* plantlets. The elongated plantlets had grown to a significantly higher average height of 12.20 cm on media added with 0.75 mg L^{-1} GA_3. This reproducible method for regenerating bitter melon plantlets could facilitate mass multiplication, conservation, and commercial field production.

1. Introduction

Bitter melon is grown in tropics and subtropics such as Africa, Asia, and South America, where this species is taken as food and utilized medicinally [1,2]. Bitter melon fruit and leaf are used as food worldwide, and the entire plant is commonly used as a resource for pharmaceuticals and constitutes a tropical vegetable market [3]. Vegetables and medicinal species are of enormous interest to

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biotechnology researchers, as the majority of farmers and pharmaceutical firms rely on them. The use of herbal plants as medicine cum vegetables in health care management programs has been recognized internationally. Bitter melon is an important plant mainly valued for its medicinal properties [4]. This herbaceous is considered to be an essential alternative source of β-carotene [5]. β-carotene is a vitamin A precursor [6], critical for human body growth and development, as its deficiency is a severe problem that leads to malnutrition, particularly in developing countries.

However, local bitter melon species are near extinction in Africa because they are being replaced by commercially cultivated plants. They are, however, frequently gathered from the environment as a vegetable or one of the essential indigenous medicinal herbs for both ritual and traditional usage [7,8]. Moreover, when it comes to mass multiplication, thick seed coats and a high seed breakdown rate lead to low field germination [9]. It was observed that most bitter melon seeds exhibited 100 % dormancy, and none of the seeds imbibed water even after 30 days of plating using the between-paper method [10]. Physical dormancy is caused by the seed coat's inability to absorb water [11]. Consequently, the inherent productivity of bitter melon secondary metabolites is affected [9], as well as the availability of bitter melon fruits and leaves to cover the increased demand of the population, even though they are already overexploited [12,13].

One requirement to be sorted out for this genus is the germination issues resulting from the hard seed coat to establish a quick and effective mechanism for breeding and targeted introduction of novel genes into the plant. Breeding conventionally is time-consuming and ineffective for some species, like bitter melon. Alternative plant propagation techniques, such as plant tissue culture, are frequently utilized for the commercial multiplication of numerous medicinal species [14]. Consequently, the recommended strategy for large multiplication is plant regeneration through micropropagation, usually applied to produce thousands of clones from a single plant part for commercial purposes. Indirect regeneration via callus frequently results in somaclonal variation, which is essential for genetic manipulation [15]. As a tool of biotechnology, indirect regeneration is essential for breeding and the recovery of genetic resources and has become a priority and a novel breeding approach with the potential to reintroduce traits that have been lost or have never been present in the cultivated species via transformation tools [16].

To our knowledge, no research has compared the regeneration of bitter melon via organogenesis from immature leaves collected from direct organogenesis and *in vivo*-produced seedlings. Therefore, this investigation aimed to set up an efficient protocol for regeneration through embryogenic callus from immature bitter melon leaves derived from either *in vitro* plantlets or *in vivo* grown greenhouse seedlings.

2. Methods

2.1. Plant material

Two sources of explants were considered: immature leaves of *in vitro* plantlets of bitter melon (*Momordica charantia*) and immature leaves from *in vivo*-grown greenhouse seedlings, according to Naitchede et al. [17]. Bitter melon seeds (accession GBK027049) were collected from the Genetic Resources Research Institute (GeRRI) of Kenya. The *in vivo* plantlets were raised into seedlings from seeds sown in plastic pots containing a mix of 3/4 sand +1/4 manure in the greenhouse of the Kenyatta University plant transformation laboratory, while *in vitro* plantlets were previously produced from direct organogenesis using nodal explants [18].

2.2. Explant preparation

The immature leaves from direct organogenesis were harvested from 14-day-old *in vitro* plantlets, as well as the immature leaves from *in vivo* grown seedlings were harvested from proliferating plants growing in the greenhouse (21-day-old after germination). The first two leaves were cut off from the top of each plant, whether it was an *in vitro* or *in vivo* plantlet. Leaf explants from the greenhouse were rinsed for 30 min with tap water and cleaned for 10 min with a commercial disinfectant soap (Savlon, South African) containing pine (Bina+), followed by six rinses with tap water. Then, explants were disinfected for 2 and 3 min in 70 % ethanol (C_2H_2OH) and 1 % sodium hypochlorite (NaClO), including Tween-20, according to Ref. [18]. They were then cleaned six times with sterile deionized water prior to being put into the culture medium. Explants from *in vitro* cultured plantlets were not subjected to sterilization protocol because they were already clean.

Based on prior bitter melon studies [19,20], potential optimal concentrations were chosen for callus induction and maturation, shoot, and root formation.

2.3. Callus induction

Callus induction media contained Murashige and Skoog (MS) salts [21] plus B5 vitamins [22], 30g sucrose, and solidified with 4.4g gelrite (for 1L solution) and augmented with different plant growth regulators (PGRs). Experiments were conducted in a Completely Randomized Design (CRD) with three replicates in MSB5 media augmented with thidiazuron (TDZ) in combination with naphthaleneacetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D). One experiment contained three levels of each PGR as follows: NAA (1.4 mg L^{-1}; 1.2 mg L^{-1}; 0.8 mg L^{-1}); TDZ (0; 0.5 mg L^{-1}; 0.8 mg L^{-1}), and 2,4-D (1.0 mg L^{-1}; 1.5 mg L^{-1}; 2.0 mg L^{-1}). Each callus formation medium was distributed into three culture vessels (50 ml) with twelve explants. Explants cut into 1-cm^2 sections, including the midrib portions, were spread down flat into direct contact with the media. All cultures have been maintained in darkness for eight (8) weeks with in-between fortnight subculturing. After eight weeks, the percentage of leaf explants that induced callus and the number of days to calli induction were recorded. Callus induction rates were calculated by the number of leaves that formed calli as the

proportion of the total number of leaves inoculated in the media expressed as a percentage (%).

2.4. Embryo maturation

After eight weeks of callus induction and maintenance, all formed calli were subcultured into embryo maturation media consisting of MSB5 with 30g.L^{-1} sucrose, 4.4g.L^{-1} gelrite, and augmented with varying amounts of TDZ (0.8 mg L^{-1}; 1.2 mg L^{-1}) combined with kinetin (KIN) or 6-benzylaminopurine (BAP) at different doses (0; 0.5 mg L^{-1}; 0.8 mg L^{-1}) and left in the dark for two (2) weeks and subcultured again after two weeks. Data were collected on the percentage, colour, and friability of mature embryogenic calli after a period of four weeks. The embryogenic callus formation rates were calculated by expressing the number of calli that formed embryogenic calli as a proportion of the total number of calli transferred to embryo maturation media expressed as a percentage (%).

2.5. Adventitious shoot and root formation

The embryogenic calli obtained on the previous embryo maturation media were transferred to MS media containing vitamins (nicotinic acid, pyridoxine HCl, glycine, myo-inositol), 30 g L^{-1} sucrose, 4.4 g L^{-1} gelrite, various concentrations of BAP (0; 0.5 mg L^{-1}; 0.75 mg L^{-1}; 1.0 mg L^{-1}; 1.25 mg L^{-1}) either singly or together with NAA (0.25 mg L^{-1}; 0.5 mg L^{-1}; 0.75 mg L^{-1}), 2,4-D (0.25 mg L^{-1}; 0.5 mg L^{-1}; 0.75 mg L^{-1}), and TDZ (0.25 mg L^{-1}; 0.5 mg L^{-1}; 0.75 mg L^{-1}). The same media were used to subculture calli with regenerated adventitious buds twice at intervals of 15 days to avoid a lack of nutrients impacts on the plantlets. After six weeks, the number of callus-forming shoots was calculated by expressing the number of calli that formed shoots as a proportion of the total number of calli transferred to shoot formation media expressed as a percentage (%), the shoots number per explant, the length of the shoots, the frequency of rooting, the mean root number, and the average root length/shoot were counted.

2.6. Shoot elongation

Regenerated shoots (2.0 cm and above) were collected and transferred to MSB5 containing 15g.L^{-1} sucrose, 4.4g.L^{-1} gelrite, without PGRs or supplemented with gibberellic acid (GA_3) at various doses (0.25; 0.50; 0.75; 1.0; 1.25 mg L^{-1}) for plantlet elongation. All cultures were kept at 25±2 °C with 16 h of light and 8 h of dark. Philips fluorescent tubes (36W) produced the light (45 μmol $m^{-2}s^{-1}$). Data were recorded on the mean shoot length after 3 weeks of culture.

2.7. Plantlet acclimatization

Healthy elongated plantlets were removed from the culture bottles and washed to remove any agar residue. They were put in peat-moss-filled plastic pots (Kekkilia, Finland). Clear plastic bags of 8 cm were used to cover the pots, and the plantlets were watered every five days to maintain 85 % humidity. Before being placed into the greenhouse, *in vitro* plantlets were kept for three weeks with a 16-h

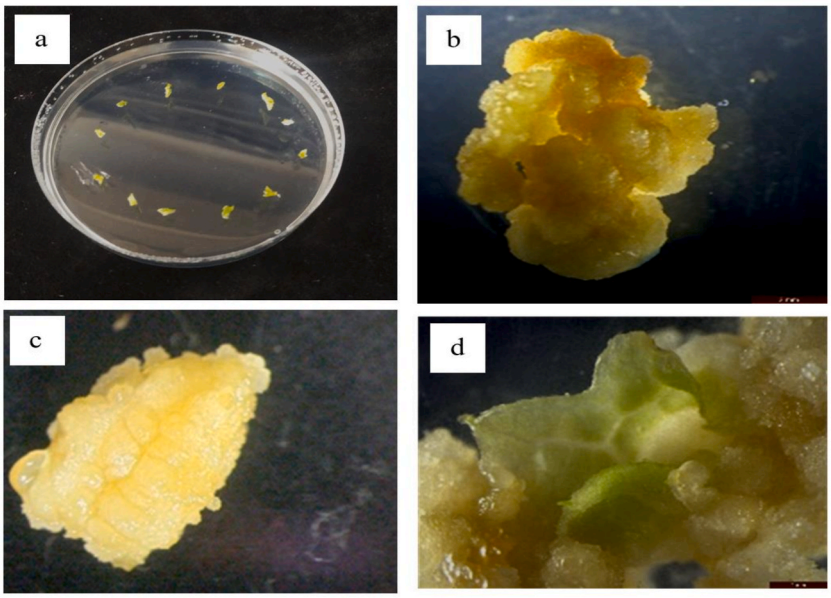

Fig. 1. Callus induced and matured from leaf of *Momordica charantia* on MS media added with various doses of hormones. (a) Immature leaf lobes used as explants. (b) Four weeks old callus induced at 0.5 mg L^{-1} TDZ and 1.2 mg L^{-1} NAA. (c) Four weeks old callus induced at 1.5 mg L^{-1} 2,4-D with 0.8 mg L^{-1} TDZ. (d) Compact embryogenic callus obtained at 1.2 mg L^{-1} TDZ + 0.8 mg L^{-1} BAP.

photoperiod. After two weeks, the plastic bags were gradually removed to allow the plantlets to acclimate to the field environment. After removing the covers, the hardened plants were placed in plastic pots with a mixture of soil, vermiculite, and sand (1:1:2, v/v/v) following the previous study of Naitchede et al. [18]. Plant survival was examined after three weeks in the greenhouse.

2.8. Experimental setup and statistical analysis

A completely Randomized Design (CRD) was used to design experiments [23]. PGRs-free MS media was utilized as a control. Twelve explants were considered for each treatment, which was repeated three times. Statistical analysis software R studio and SAS (ver. 9.2, SAS institute, Cary, NC) were used to run the data analysis. Analysis of variance (ANOVA) was achieved to determine the significance of variation in callus induction, shooting, rooting, and plantlet elongation. Tukey multiple comparison tests (TMCT) were used for the separation of means by Least significant difference (LSD) at $P \leq 0.05$. P-value is the probability of getting a result that is either the same or more extreme than the actual observations.

3. Results

3.1. Callus induction and embryo maturation

Calli was formed from leaf segments on Murashige and Skoog (MS) media, including TDZ coupled with either 2,4-D or NAA (Fig. 1a). The maximum callus formation frequency (95.09 %) was found in media incorporated with 1.2 mg L^{-1} NAA together with 0.5 mg L^{-1} TDZ (Fig. 1b, Table 1) in *in vitro*-plantlet explants and calli were induced within a short amount of time of 11.5 days (Fig. 2). Among the numerous doses of 2,4-D employed either alone or with TDZ, the co-incorporation of 1.5 mg L^{-1} 2,4-D and 0.8 mg L^{-1} TDZ produced a significantly higher frequency (75.49 %) of callus formation (Fig. 1c) from explants of *in vitro* plantlets. Most combinations exhibited a decrease in callus frequency above 0.5 mg L^{-1} TDZ. However, MS containing 0.8 and 0.5 mg L^{-1} TDZ associated with 2.0 mg L^{-1} 2,4-D or 1.4 mg L^{-1} NAA induced no calli (Table 1). The shortest time (8.5 days) to callus induction was noted on MSB5 media supplemented with the couple (0.8 mg L^{-1} TDZ; 2.0 mg L^{-1} 1,5-D) when the largest time (14 days) was noted on seven different media such as 0.8 mg L^{-1} NAA; 1.2 mg L^{-1} NAA; 1.4 mg L^{-1} NAA; 0.8 mg L^{-1} NAA + 0.8 mg L^{-1} TDZ; 1.0 mg L^{-1} 2,4-D; 1.5 mg L^{-1} 2,4-D; 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} TDZ (Fig. 2).

The most effective treatment for callus maturation was noted under TDZ and BAP (Fig. 1d). Explant from *in vitro* plantlets cultured in MSB5 media augmented with 1.2 mg L^{-1} TDZ coupled with 0.5 mg L^{-1} BAP resulted in 100 % embryogenic calli. TDZ and KIN were also tested together for callus maturation producing the maximum response of 80.25 % embryogenic calli from leaf explant of *in vitro* plantlets recorded on MSB5 media augmented with 0.5 mg L^{-1} KIN associated with 0.8 mg L^{-1} TDZ. The lowest formation frequency (66.19 %) of embryogenic calli formation was noted in explants of *in vivo* plants on the media containing 0.8 mg L^{-1} KIN associated with 0.8 mg L^{-1} TDZ (Table 2). Of the two growth regulators tested in combination with TDZ, BAP produced brilliant yellow-green

Table 1
Effect of various concentrations of TDZ combined with NAA and 2,4-D on callus formation from leaves of *Momordica charantia* L.

Sr. No	Growth regulators	Concentration (mg.L^{-1})	Percentage of callus induction ± S.E	
			Immature leaf from *in vitro* plantlets	Immature leaf from *in vivo* plants
1	NAA + TDZ	0.8 + 0.0	62.74 ± 1.96de	45.09 ± 1.96 fg
2		0.8 + 0.5	83.33 ± 0.98 ab	65.20 ± 0.49b
3		0.8 + 0.8	68.63 ± 1.96dc	51.96 ± 2.59de
4		1.2 + 0.0	65.69 ± 0.98cde	47.55 ± 0.47ef
5		1.2 + 0.5	95.09 ± 0.97a	77.45 ± 0.98a
6		1.2 + 0.8	82.84 ± 1.14 ab	65.19 ± 1.14b
7		1.4 + 0.0	41.67 ± 2.15 fg	42.15 ± 0.75g
8		1.4 + 0.5	00.00 ± 00.00 h	00.00 ± 00.00j
9		1.4 + 0.8	00.00 ± 00.00 h	00.00 ± 00.00j
10	2,4-D + TDZ	1.0 + 0.0	29.41 ± 0.19g	12.26 ± 0.94i
11		1.0 + 0.5	53.43 ± 0.49ef	36.28 ± 0.57 h
12		1.0 + 0.8	00.00 ± 00.00 h	00.00 ± 00.00j
13		1.5 + 0.0	59.80 ± 0.97de	42.16 ± 0.98g
14		1.5 + 0.5	66.67 ± 0.82cd	48.04 ± 0.89ef
15		1.5 + 0.8	75.49 ± 2.51BCE	57.35 ± 2.24c
16		2.0 + 0.0	71.57 ± 0.77bcd	53.92 ± 0.93cd
17		2.0 + 0.5	00.00 ± 00.00 h	00.00 ± 00.00j
18		2.0 + 0.8	00.00 ± 00.00 h	00.00 ± 00.00j
Mean		–	47.57 ± 7.95	44.39 ± 4.04
F Value		–	58.17	211.21
P-value		–	<0.0001	<0.0001
LSD (0.05)		–	12.69	5.22
CV,%		–	16.11	8.81
R^2		–	0.002	0.007

Values are the mean ± Standard deviation (S.E) of 12 replications per treatment, and all experiments were conducted thrice. No difference between means with the same letter when compared with Tukey multiple comparison method.

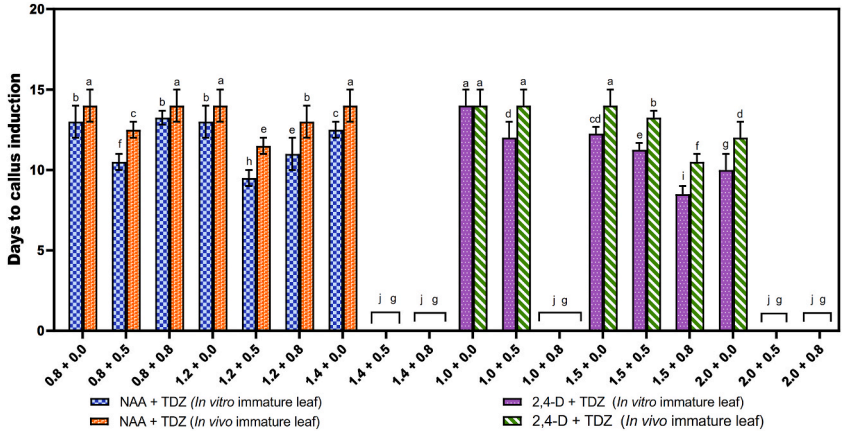

Fig. 2. Effect of varying doses of NAA with TDZ and 2,4-D on days to callus formation from immature leaves of *Momordica charantia* L. No difference between values with the same letters when evaluated using Tukey's method for multiple comparisons ($P \leq 0.0001$; *In vitro* immature leaf LSD (0.05) = 0.48; *In vivo* immature leaf LSD (0.05) = 0.37).

compact callus, greyish-yellow-green friable, and brilliant yellow-green friable (Table 2).

3.2. Effect of PGRs on adventitious shoot induction and rooting

In regeneration media, embryogenic calli gradually changed from light yellow to green (Fig. 3a and b), resulting in shoot bud formation. Shoot buds (Fig. 3b and d) of callus derived from *in vitro* explants and *in vivo* plantlets were induced on media augmented with varying amounts of BAP (0.00–1.25 mg L^{-1}) either alone or in combinations with 2.4-D, TDZ, and NAA (0.25 mg L^{-1} – 0.75 mg L^{-1}) and reached an average height of 7.13 cm within 42 days in growth regulator-free MS medium (Fig. 3d, e & f). The highest shoot formation frequency (90.91 %) and significantly the highest number of shoots (21.53 shoots/explants) occurred in leaf explants of *in vitro* plantlets on MS with no PGRs. Within the same medium, leaf explants from *in vivo* plants produced a low shoot frequency of 68.18 %, shoot length of 5.63 cm, and shoot number of 12.46 shoots per explant (Fig. 4 & Table 3). Among the different growth regulator combinations, the association of 0.25 mg L^{-1} TDZ together with 0.5 mg L^{-1} BAP was noted to be most efficient, producing a significantly high percentage of shoots (76.47 % from explant of *in vitro* plant and 61.76 % from explant of *in vivo* plant), shoot lengths of 5.36 cm from explant of *in vitro* plant and 3.83 cm from explant of *in vivo* plant, and shoot number (17.53 shoots/explants from explants of *in vitro* plantlets and 11.53 shoots/explants from explant of *in vivo* plant), followed by the couples 0.25 mg L^{-1} 2.4-D/0.5 mg L^{-1} BAP and 0.25 mg L^{-1} NAA/0.5 mg L^{-1} BAP. The shoot formation frequency, shoot number, and shoot length decreased with the doses of BAP (0–1.25 mg L^{-1}) alone or in conjunction with different hormones (NAA, 2,4-D, and TDZ). However, 1.25 mg L^{-1} BAP and 1.00 mg L^{-1} BAP, either alone or associated with 0.75 mg L^{-1} 2,4-D, 0.75 mg L^{-1} TDZ and 0.75 mg L^{-1} NAA, induced no shoots (Fig. 4 & Table 3).

Table 2
Effect of various concentrations of TDZ, kinetin, and BAP on embryo maturation of calli from immature leaves of *Momordica charantia* L.

Sr. No	Growth regulators	Concentration (mg.L^{-1})	Percentage of embryogenic calli ± S.E		Nature of embryogenic calli	
			Callus-derived from immature leaf of *in vitro* plantlets	Callus-derived from immature leaf of *in vivo* plants	Callus-derived from immature leaf of *in vitro* plantlets	Callus-derived from immature leaf of *in vivo* plant
1	TDZ + KIN	0.8 + 0.0	75.25 ± 0.25d	67.26 ± 0.20d	LYGOC	GYGC
2		0.8 + 0.5	80.25 ± 0.14c	72.17 ± 0.08c	GYGC	MYGC
3		0.8 + 0.8	66.19 ± 0.47^{e}	58.28 ± 0.15f	GYGC	GYGC
4	TDZ + BAP	1.2 + 0.0	75.30 ± 0.19d	66.27 ± 0.14e	BYGC	BYGC
5		1.2 + 0.5	100.00 ± 0.00a	90.19 ± 0.10a	GYGF	BYGF
6		1.2 + 0.8	91.19 ± 0.29b	85.43 ± 0.22b	BYGC	BYGC
Mean		–	81.36 ± 4.99	73.27 ± 5.72	–	–
F Value		–	2103.29	5868.16	–	–
P-value		–	<0.0001	<0.0001	–	–
LSD (0.05)		–	0.82	0.49	–	–
CV,%		–	0.57	0.38	–	–
R^2		–	0.02	0.01	–	–

Values are the mean ± Standard deviation (S.E) of 12 replications per treatment, and all experiments were conducted thrice. No difference between means with the same letter when compared with Tukey multiple comparison method. LYGOC light yellow-green olive compact, GYGC greyish yellow green compact, BYGC brilliant yellow-green compact, GYGF greyish yellow green friable, MYGC moderate yellow green compact, brilliant yellow-green friable (Royal Horticultural Society colour chart, London, UK, sixth edition, 2015).

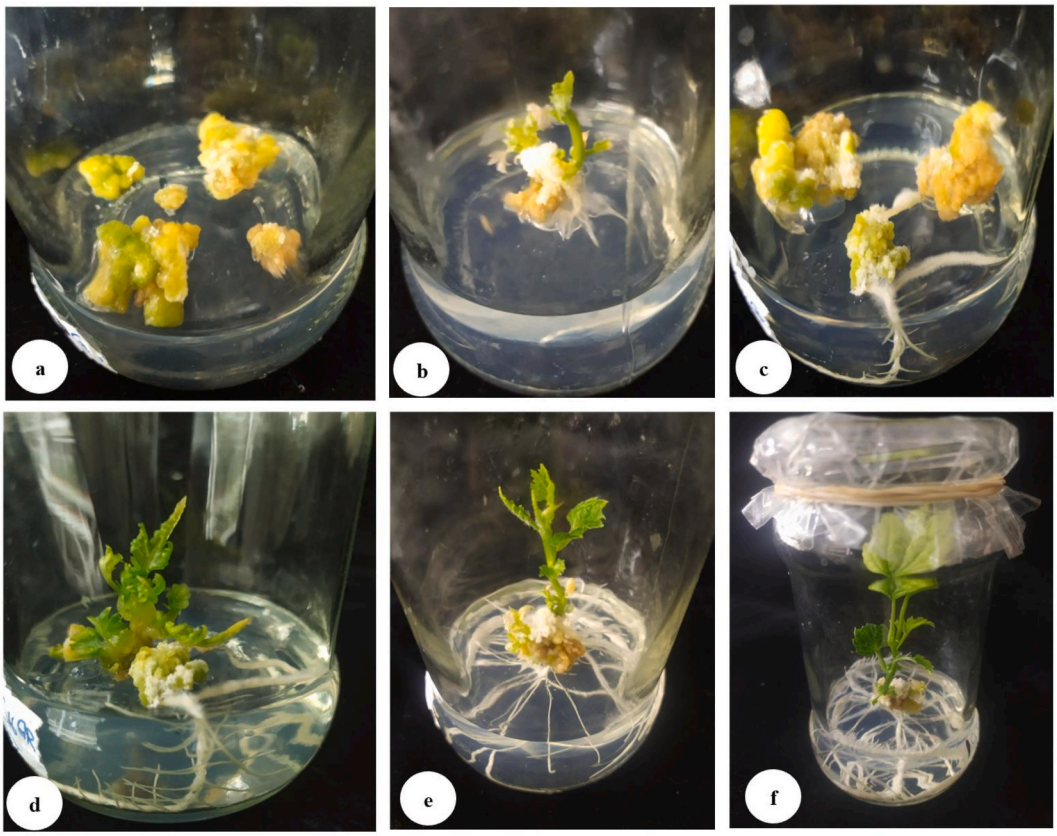

Fig. 3. Shoots formation from immature leaf-derived callus of *bitter melon.* (a) Embryogenic calli greening after transferring to light. (b) Shoot bud initiation and rooting MS medium with no PGRs (21-days-old culture). (c) Calli rooting without shoots (0.75 mg L^{-1} NAA + 1.00 mg L^{-1} BAP). (d) Proliferation of adventitious shoots and roots on PGR-free MS medium. (e, f) Shoots elongated on MS medium with no PGRs (28-days-old and 42-days-old cultures).

Root-forming calli were also counted from the same medium where shoots were induced (Fig. 3). In calli derived from explants of *in vitro* plants, the highest rooting frequency (88.92 %), length of root (4.13 cm), and number of roots (6.23 roots/explant) were recorded on media without plant growth regulators (Table 4 & Fig. 5). Among the three hormones combinations used, 0.50 mg L^{-1} BAP associated with 0.25 mg L^{-1} TDZ was significantly the most appropriate for rooting, with root formation frequency of 77.81 %, root number of 5.33 roots/explants, and a root length of 3.23 cm (Table 4 & Fig. 5). In calli derived from leaf explant of *in vivo* plant, MS without plant growth regulators was still noted to be the most efficient for root formation producing the maximum frequency of root (55.59 %), root length (2.78 cm), and root number (4.23 roots per explant). The rooting frequency, length, and number of roots decreased with the doses of BAP either alone or in association with NAA, 2,4-D, and TDZ. However, above 0.75 mg L^{-1} BAP, no roots formed (Table 4). It was also observed that 1.0 mg L^{-1} BAP combined with 0.75 mg L^{-1} TDZ or 0.75 mg L^{-1} NAA induced root without shoot formation (Fig. 3c, Table 4).

3.3. *Effect of GA_3 on plantlet elongation and plantlet acclimatization*

Within four weeks, shoots elongated on MS media either without growth regulators or augmented with GA_3 (Fig. 6a and b). The elongated shoots had grown to a significantly highest average height of 12.20 cm (explant from *in vitro* plantlets), and 8.70 cm (explant from *in vivo* plant) in MS media added with 0.75 mg L^{-1} GA_3 (Fig. 7). The length of elongated shoots rose with increasing GA_3 concentrations up to 0.75 mg L^{-1} but declined thereafter. The control (MS without PGRs) was found to be also efficient in producing shoot lengths of 11.53 cm (explants from *in vitro* plant) and 8.03 cm (explants from *in vivo* plant). No significant differences were observed between MS media with no hormones and MS augmented with 0.75 mg L^{-1} GA_3. The smallest elongated plantlet (2.73 cm) was recorded on media augmented with 1.25 mg L^{-1} GA_3 from explants of *in vivo* plants (Fig. 7).

The acclimatization protocol applied to elongated plantlets was successful, with a 90.13 % survival rate. Acclimatized plants revealed no morphological alterations and appeared normal (Fig. 6c, d, and e).

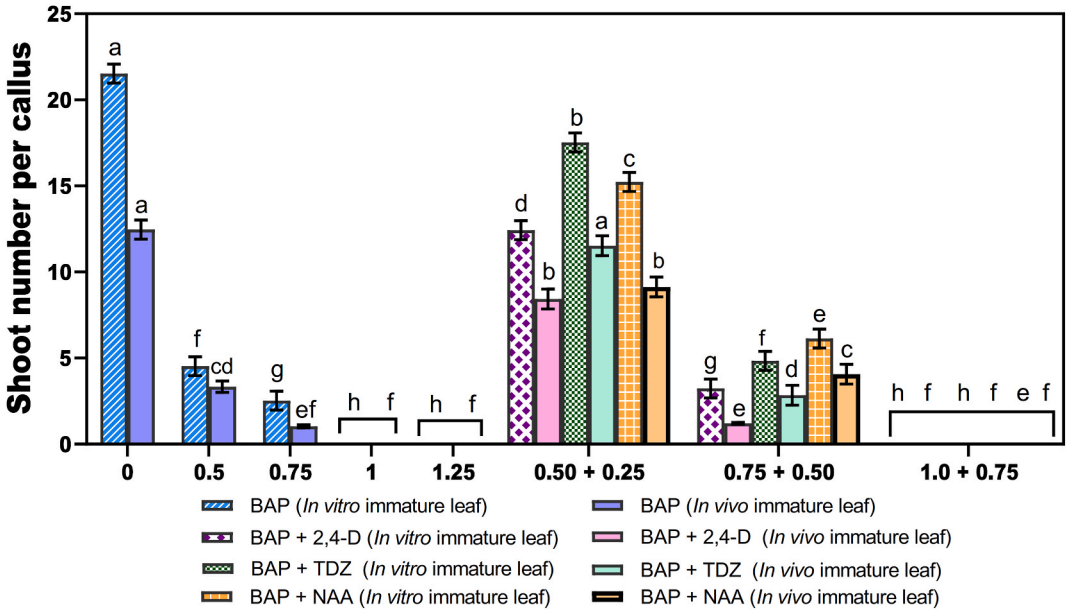

Fig. 4. Effect of various amounts of BAP with TDZ, 2,4-D, and NAA on adventitious shoot bud number on callus of immature leaves of bitter melon. No difference between values with the same letter when compared with Tukey multiple comparison method ($P < 0.0001$; *In vitro* immature leaf LSD (0.05) = 1.27; *In vivo* immature leaf LSD (0.05) = 1.12).

Table 3
Effect of various concentrations of BAP with TDZ, 2,4-D, and NAA on shoot bud formation from immature leaves of (*Momordica charantia*).

Sr. No	Growth regulators	Concentration (mg. L^{-1})	Percent of calli formed shoots ± S.E		Shoots length (cm) ± S.E	
			Immature leaf of *in vitro* plantlets	Immature leaf of *in vivo* plants	Immature leaf of *in vitro* plantlets	Immature leaf of *in vivo* plants
1	BAP	0.00	90.91 ± 2.62a	68.18 ± 1.95a	7.13 ± 0.54a	5.63 ± 0.54 a
2		0.50	46.97 ± 1.15^{e}	24.24 ± 1.51f	2.56 ± 0.65d	1.43 ± 0.68d
3		0.75	27.27 ± 2.54g	7.57 ± 1.51g	2.40 ± 0.45d	1.23 ± 0.25de
4		1.00	00.00 ± 00.00 h	00.00 ± 00.00 h	00.00 ± 00.00e	00.00 ± 00.00e
5		1.25	00.00 ± 00.00 h	00.00 ± 00.00 h	00.00 ± 00.00e	00.00 ± 00.00e
6	BAP + 2,4-D	0.50 + 0.25	70.59 ± 1.96c	55.88 ± 1.57c	3.61 ± 0.24cd	1.86 ± 0.97cd
7		0.75 + 0.50	35.29 ± 1.69f	20.58 ± 1.69f	2.85 ± 0.32d	1.60 ± 1.25d
8		1.00 + 0.75	00.00 ± 00.00 h	00.00 ± 00.00 h	00.00 ± 00.00e	00.00 ± 00.00e
9	BAP + TDZ	0.50 + 0.25	76.47 ± 1.58b	61.76 ± 1.49b	5.36 ± 0.58b	3.83 ± 0.38b
10		0.75 + 0.50	44.44 ± 1.60^{e}	30.55 ± 1.95e	3.40 ± 0.54cd	3.83 ± 0.62cd
11		1.00 + 0.75	00.00 ± 00.00 h	00.00 ± 00.00 h	00.00 ± 00.00e	00.00 ± 00.00e
12	BAP + NAA	0.50 + 0.25	73.68 ± 1.15BCE	60.52 ± 1.89b	4.52 ± 0.29BCE	3.03 ± 0.57BCE
13		0.75 + 0.50	57.90 ± 1.51d	44.73 ± 1.38d	3.34 ± 0.77cd	2.03 ± 0.43cd
14		1.00 + 0.75	00.00 ± 00.00 h	00.00 ± 00.00 h	00.00 ± 00.00e	00.00 ± 00.00e
Mean		–	37.39 ± 4.50	26.71 ± 6.65	2.51 ± 0.61	1.62 ± 0.33
F Value		–	488.35	365.04	26.42	139.43
P-value		–	<0.0001	<0.0001	<0.0001	<0.0001
LSD (0.05)		–	4.37	4.04	1.28	1.30
CV,%		–	6.99	9.05	30.61	17.40
R^2			0.59	0.48	0.61	0.60

Values are the mean ± Standard deviation (S.E) of 12 replications per treatment and all experiments conducted thrice. No difference between means with the same letter when compared with Tukey multiple comparison method.

4. Discussion

It has been reported that leaf explants induce more calli than nodal cuttings and can proceed via somatic embryogenesis or organogenesis on inductive media [24]. However, callus induction effectiveness depends on the plant growth regulators employed and the conditions of plant cultures. Among the eighteen treatments tested, 0.5 mg L^{-1} TDZ together with 1.2 mg L^{-1} NAA was recorded to be the most appropriate for callus induction. This investigation finding was similar to the report of Malik et al. [25] in *M. charantia* explants (stem, internodal, shoot-tip, nodal, cotyledon, and leaf) cultured on MS media supplemented with 2,4-D, NAA, or BAP; however, percentage of callogenic was low, and non-organogenic calluses were produced. The outcome showed that TDZ and NAA are

Table 4
Effect of different doses of growth regulators on various parameters of bitter melon roots.

Sr. No	Growth regulators	Concentration (mg. L^{-1})	Percent of calli formed roots ± S.E		Roots length (cm) ± S.E	
			Immature leaf of *in vitro* plant	Immature leaf from *in vivo* plant	Immature leaf from *in vitro* plant	Immature leaf from *in vivo* plant
1	BAP	0.00	88.92 ± 1.18a	55.59 ± 2.91a	4.13 ± 0.54a	2.78 ± 0.31 ab
2		0.50	33.36 ± 1.16g	22.25 ± 2.31d	1.63 ± 0.65c	1.53 ± 0.26cd
3		0.75	22.25 ± 1.20 h	11.14 ± 1.51e	1.53 ± 0.45c	1.23 ± 0.36d
4		1.00	00.00 ± 00.00i	00.00 ± 00.00f	00.00 ± 00.00d	00.00 ± 00.00e
5		1.25	00.00 ± 00.00i	00.00 ± 00.00f	00.00 ± 00.00d	00.00 ± 00.00e
6	BAP + 2,4-D	0.50 + 0.25	55.58 ± 1.24d	44.47 ± 3.86b	2.20 ± 0.24BCE	2.13 ± 0.37BCE
7		0.75 + 0.50	38.92 ± 1.12f	16.70 ± 1.96de	2.17 ± 0.32BCE	1.43 ± 0.25cd
8		1.00 + 0.75	00.00 ± 00.00i	00.00 ± 00.00f	00.00 ± 00.00d	00.00 ± 00.00e
9	BAP + TDZ	0.50 + 0.25	77.81 ± 1.10b	47.81 ± 1.98b	3.23 ± 0.58 ab	2.63 ± 0.39 ab
10		0.75 + 0.50	55.58 ± 1.12d	47.81 ± 3.30b	2.82 ± 0.54abc	1.53 ± 0.35cd
11		1.00 + 0.75	24.94 ± 0.35 h	21.99 ± 0.50d	1.93 ± 0.08BCE	1.20 ± 0.05d
12	BAP + NAA	0.50 + 0.25	66.70 ± 1.18c	42.25 ± 3.47BCE	2.64 ± 0.29BCE	2.93 ± 0.48a
13		0.75 + 0.50	47.81 ± 1.05^{e}	36.70 ± 2.36c	2.24 ± 0.77BCE	2.13 ± 0.15BCE
14		1.00 + 0.75	31.89 ± 0.71g	20.01 ± 0.96d	1.53 ± 0.13c	1.66 ± 0.08cd
Mean		–	38.84 ± 8.47	26.19 ± 5.21	1.86 ± 0.37	1.51 ± 0.26
F Value		–	854.65	68.57	6.29	14.88
P-value		–	<0.0001	<0.0001	<0.0001	<0.0001
LSD (0.05)		–	2.81	6.82	1.41	0.74
CV,%		–	4.33	15.57	25.55	29.31
R^2			0.35	0.26	0.28	0.28

Values are the mean ± Standard deviation (S.E) of 12 replications per treatment and all experiments conducted thrice. No difference between means with the same letter when compared with Tukey multiple comparison method.

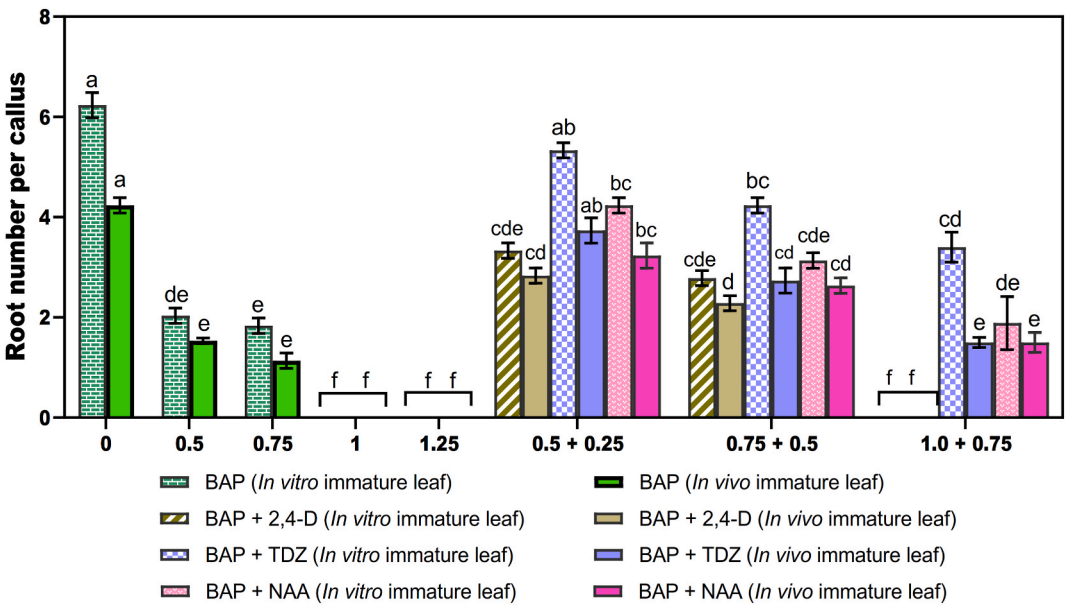

Fig. 5. Effect of various doses of BAP with TDZ, 2,4-D, and NAA on root number on callus of immature leaves of bitter melon. No difference between values with the same letter when compared with Tukey multiple comparison method ($P < 0.0001$; In vitro immature leaf LSD (0.05) = 1.56; In vivo immature leaf LSD (0.05) = 0.74).

the key hormones in callus induction from bitter melon in this study. These findings are similar to Devendra et al. [26] and Chung & Ouyang [27] studies, where they observed that MS medium supplemented with TDZ combined with auxin exhibited a high induction rate and produced organogenic callus in *M. dioica* and *Fragaria vesca* after 4 weeks of culturing. Moreover, above 0.5 mg L^{-1} TDZ, the frequency of calli decreased in most of the hormone combinations. These results are similar to the studies of Pai & Desai [28] on various crops, where they observed that TDZ was more successful at low levels. The findings of this study exhibited higher callus induction response from leaf explants of *in vitro* plantlets than explants of *in vivo* plants. This difference might be due to sterilization undergone by the explants of *in vivo* plants as well as the physiological differences between the two types of donor plants (culture and stress conditions).

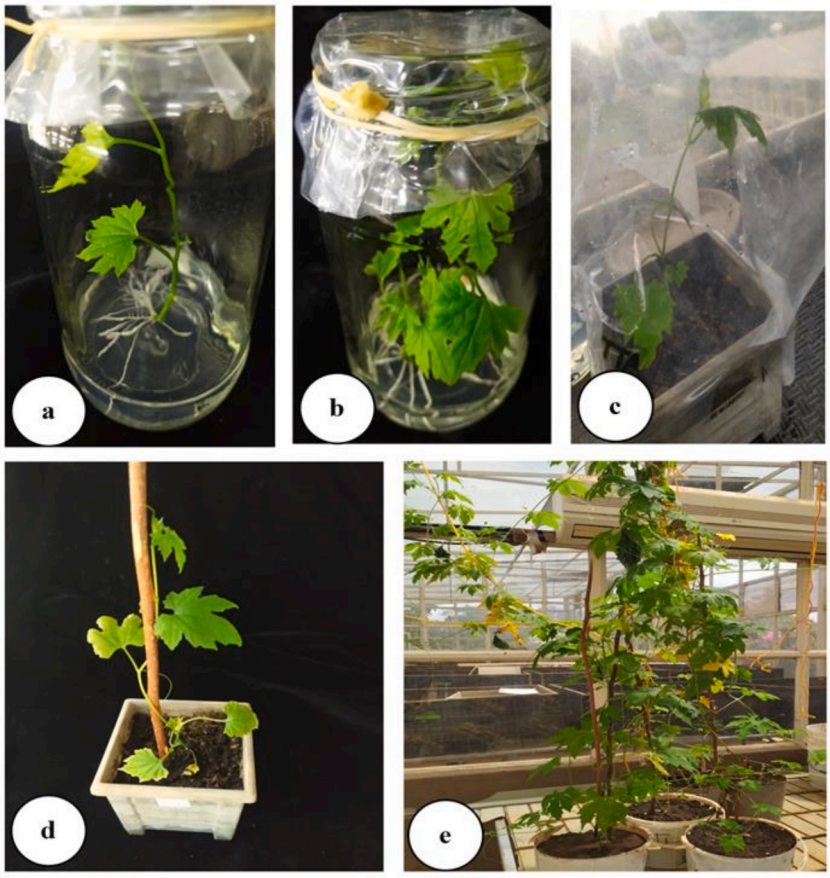

Fig. 6. Elongation of shoots. (a) Shoot elongated on hormone-free MS media. (b) Shoot elongated on MS with a supplement of 0.75 mg L^{-1} GA_3 at 21 days of culture. (c) Hardened plant. (d) Acclimatized plant. (e) Acclimatized plants showing normal fruits and flowers.

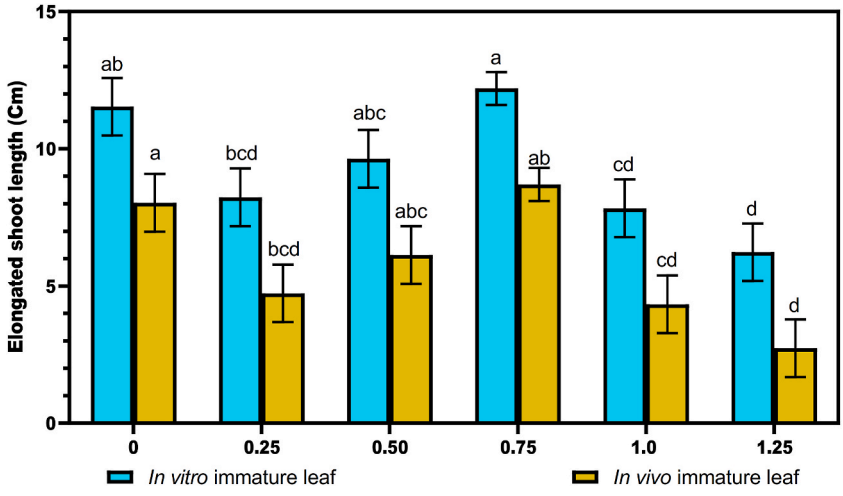

Fig. 7. Effect of GA_3 on shoot elongation from regenerated shoots. No difference between values with the same letter when compared with Tukey multiple comparison methods (P = 0.016).

The combination of BAP with TDZ was found to be more efficient in callus maturation than the combination of KIN and TDZ. It has been reported that no embryogenic callus is produced using auxin only, while various compositions of auxin combined with cytokinins (CKs), especially BAP, showed embryogenic responses [29]. The colour and the friability of the calli varied between the concentration

of growth regulators applied. The 1.2 mg L^{-1} TDZ combined with 0.5 mg L^{-1} BAP produced greyish yellow-green friable callus and brilliant yellow-green friable, similar to the findings of Malik et al. [25].

Shoot regeneration is a critical stage for plant improvement using tissue and cell culture techniques. Auxins are necessary for callus induction [30,31] but negatively affect plant regeneration. Therefore, auxin is lowered or removed from shoot regeneration media. In this study, BAP alone was less effective than when combined with other PGRs, similar to previous reports on *Asparagus racemosus* Willd where BAP induced few multiple shoots. However, the association of 0.5 mg L^{-1} BAP and 0.25 mg L^{-1} TDZ produced a better response for shoot organogenesis than other growth regulator combinations. This could be due to the interaction of growth regulators, resulting in improved cell growth and multiplication. Reports revealed that the combination of BAP and auxin or another CK increased shoot proliferation and multiplication [23,24]. For shoot induction in plant tissue culture, TDZ is also widely regarded as one of the most active CK [32]. Several studies report that an appropriate dose of TDZ stimulates shoot regeneration more effectively than any other CK (BAP, KIN) [25–27]. The results corroborate earlier studies in *Momordica charantia* and rice genotype SR4, in which many shoot buds were formed with the combination of TDZ/NAA and TDZ/BAP respectively [28,29]. However, MS media with no growth regulators showed the highest shoot induction rate, number, and length in this study. Plant growth regulators might negatively affect the induction of shoots in bitter melon, similar to the study on *Citrus sinensis,* in which growth regulator combinations showed relatively lower shoot induction compared to hormone-free MS medium [33]. The shoot induction frequency recorded in this investigation is higher than the one obtained by Naitchede et al. [18] from direct organogenesis via nodal explant in bitter melon. Plantlet regeneration via callus might be the most appropriate methodology for this important medicinal species.

The highest frequency, root number, and length of root formation on calli were observed on MS medium without hormones. These results corroborated the findings of Ghaderi et al. [34] in *Perovskia abrotanoides*. Verma et al. [35] reported that the hormone-free MS media showed good proliferation of rooting in *Oryza sativa* L. compared to MS supplemented with hormones. Among the various hormone combinations, the (0.25 mg L^{-1} TDZ; 0.50 mg L^{-1} BAP) produced the highest rooting frequency and root number. It has been reported that the combinations of BAP and auxin or other CK result in root formation from calluses [36,37]. The outcome of this investigation also showed root formation and the absence of shoot initiation on media, such as the combinations of (0.75 mg L^{-1} TDZ; 1.00 mg L^{-1} BAP) and (0.75 mg L^{-1} NAA; 1.00 mg L^{-1} BAP). Pawar et al. [38] recorded no shoot initiation from callus on media augmented with a high level of auxin even after 45 days and 60 days of culture.

The shoots elongated on MS media either without hormones or augmented with GA_3. The latter at 0.75 mg L^{-1} GA_3 elicited a higher average height; however, there is no significant difference compared to MS with no PGRs. The role of GA_3. in shoot elongation has been reported in several studies. Nagai et al. [39] found in the rice study that the internode elongation is stimulated in the presence of GA_3. In the same way, Thiruvengadam et al. [20] observed that within two weeks, the elongated shoots in *Momordica charantia* had grown to an average height of 9.2 cm when GA_3 was applied. However, the plantlet shoot length decreased with increasing GA_3 dose above 0.75 mg L^{-1}. Reports on *Asparagus officinalis* showed that GA_3 induced maximum shoot length at low concentrations [40].

5. Conclusions

The MS medium containing 0.5 mg L^{-1} TDZ combined with 1.2 mg L^{-1} NAA resulted in the highest callus formation frequency within the shortest time (11.5 days). The MS medium without hormones was noted to be the most appropriate for the shoot and root induction from calli, yielding the highest shoot percentage and root formation frequency. The MS medium supplemented with 0.75 mg. L^{-1} GA_3 has been recorded as the most convenient medium for shoot elongation. Leaf explants from *in vitro* plantlets produced significantly higher callus and shoot formation frequency than *in vivo* raised seedling explants.

Declarations

Ethical approval

None of the authors of this paper conducted any investigations using human or animal participants.

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Data availability statement

Data associated with our study has not been deposited into a publicly available repository. Data will be made available on request.

CRediT authorship contribution statement

Labodé Hospice Stevenson Naïtchédé: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aggrey Bernard Nyende:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Steven Runo:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Allen Johnny Borlay:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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