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Scale-up production of *Vigna caracalla* meristematic nodules in a pneumatically agitated, air-lift like bottle bioreactor system

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Abstract

In the present study, the large-scale production of meristematic nodules of *Vigna caracalla* L. Verdc. was achieved in a pneumatically agitated bottle bioreactor system. This species is valued both as an ornamental climbing plant, owing to its distinctive coiled flowers and strong fragrance, and as a potential model for biotechnological applications, thereby justifying efforts to develop efficient propagation systems. Leaf and stem explants from 14-day-old seedlings were cultured on Murashige and Skoog (MS) medium with different plant growth regulators (PGRs). 14-day-old leaf-derived calli were transferred to 250 mL Erlenmeyer flasks with 50 mL of diverse liquid media to optimize nodule production. According to the results for fresh weight (FW), specific growth rate (SGR), doubling time (DT), and fresh growth index (FGI), the SMN-3 (SMN: secondary meristematic nodule) medium [½ MSB5 (MS salts+B5 vitamins) +4.0 mg/L 6-benzylaminopurine (BAP)+100 mg/L ascorbic acid (AA)+100 mg/L glutamine+15 mg/L sucrose] provided the best response. Finally, large-scale production of meristematic nodules was optimized in a 2 L air-lift-like bottle bioreactor. 9.6 g/L inoculum in 1.6 L SMN-3 maximized FW, SGR, FGI, and biomass growth. These findings highlight a significant advancement in meristematic nodule production in *Vigna caracalla*, showcasing the potential of a cost-effective, air-lift bioreactor system for large-scale applications.

Introduction

The Fabaceae (*Leguminosae*) family is the third most extensive group of flowering plants worldwide, comprising approximately 19,400 varieties in roughly 730 classes (Ahmad et al., 2016). It is the second most important family after *Poaceae* (*Gramineae)* in terms of economic importance (Michaels, 2016). The Fabaceae family, encompassing a wide diversity of species, holds considerable importance across disciplines (Ahmad et al., 2016). Its members include legumes and oilseeds vital for human nutrition (Bagci et al., 2004), as well as species cultivated as ornamentals, forages, and green manures (Ahmad et al., 2016). They are further valued in phytoremediation (Hall et al., 2011), various industries such as timber and dye (Bagci et al., 2004), and medicine due to their phytochemical diversity (Sebastian et al., 2020). *Vigna caracalla* L. Verdc. (Fabaceae) is a tuber-like, coiling, perennial plant native to South and Central America. It has the most complex flowers among Papilionoideae (Fabaceae) species, with asymmetrical flowers that have a notably attractive fragrance. For this reason, this species is propagated as an ornamental plant and may also be used for forage (Etcheverry et al., 2008). It also has many potential features, such as unexplored secondary metabolite profiles, possible roles in phytoremediation, and applicability in *in vitro* culture systems, that have not yet

been systematically investigated. The synonym for *V. caracalla* L. Verdc. is *Cochliasanthus caracalla* (L.). *V. caracalla* is typicallypropagated via seeds and cuttings. It possesses the most advanced floral structure among asymmetrical Papilionoideae, and this highly asymmetrical floral feature complicates its pollination mechanism. Although it is self-compatible, it relies on pollinators for seed production, which can result in low seed formation (Etcheverry and Vogel 2018).

Plant tissue culture techniques have a wide range of applications, including large-scale production for agriculture and horticulture, regeneration of genetically modified plants with desired features, conservation of threatened plant species, secondary metabolite production, obtaining disease-free plants, and improving plant nutritional content, quality, yield, and stress resistance (Negi et al., 2024).

Nodule cultures arise through a transitional morphogenetic pathway that bridges organogenesis and somatic embryogenesis, with nodules typically exhibiting a strong potential for regenerating plants or organs via organogenesis (McCown et al., 1988; de Souza et al. 2017). Meristematic nodules, defined as self-sustained, round, tightly packed groups of cells that exhibit cell/tissue differentiation, have a considerable capacity for plant regeneration through organogenesis. The term "meristemoid" was first introduced by Bunning in 1952, and its use was later extended by Torrey (McCown et al., 1988). Meristemoids formed in woody perennials are also often referred to as nodules (Trigiano and Gray 2016). Cultures predominantly consisting of nodules are typically called nodule cultures (McCown et al., 1988). Nodules have the capacity to proliferate into large numbers of small, condensed other nodules, and plant tissue can be preserved in the nodule stage for extended periods. This process can be optimally synchronized in liquid cultures by sorting and categorizing the nodules based on size. These characteristics are crucial for automated micropropagation, making nodule culture a promising option for large-scale production (McCown et al., 1988; Kongbangkerd et al., 2005). In vitro regeneration from nodular/meristematic nodular structures has been developed for various herbaceous and woody plant species, such as *Cichorium intybus* L. (Piéron et al., 1993), *Lilium × formolongi* hort. (Godo et al., 1998), *Humulus lupulus* L. var. Eroica (Batista et al., 2000), *Eucalyptus globulus* (Trindade and Pais 2003), *Charybdis numidica* (Kongbangkerd et al., 2005), *Linum usitatissimum* (Salaj et al., 2005), *Sclerocarya birrea* subsp. *caffra* (Moyo et al., 2009), *Ananas comosus* var. *comosus* (Scherer et al., 2013), *Liquidambar orientalis* Miller (Bayraktar et al., 2015), *Billbergia alfonsi-joannis* Reitz and *Billbergia zebrina* (Herbert) Lindley (de Souza et al. 2017), and *Paeonia ostii* 'Feng Dan' (Xu et al., 2022). While these techniques are extensively documented in various species, several features of the evolution of the morphology and histology of nodules still require further clarification (de Souza et al. 2016).

Numerous reports have documented plant regeneration through organogenesis from different types of explants in leguminous species. However, many of these reports highlight the recalcitrant nature of leguminous species, especially those in the *Vigna* genus, such as V. *mungo* (L.) Hepper (Saini et al., 2002; Sen et al., 2002), *V. radiata* (L.) Wilczek (Sivakumar et al., 2010; Vats et al., 2014), and *V. unguiculata* (L.) Walp (Anand et al., 2000; Ramakrishnan et al., 2005; Bakshi and Sahoo 2013; Markin et al., 2023). Under *in vitro* conditions, this recalcitrance restricts their regenerative potential. To date, there have been few attempts to understand the in vitro regeneration capacity of *V. caracalla*. In our earlier study (Gungor et al., 2020), we reported, for the first time, an *in vitro* micropropagation protocol utilizing shoot tip and node explants derived from seedlings of *Vigna caracalla* L. Verdc. germinated under in vitro conditions. In the present study, we conducted the first attempt at scaling up *V. caracalla* meristematic nodule cultures in an air-lift-like bottle bioreactor.

Materials and Methods

Seed sterilization and *in vitro* germination

The seeds of *Vigna caracalla* (obtained from a commercial company in Turkey) were surface sterilized by rinsing with 70% (v/v) ethanol for 1 min, and 0.1% (w/v) mercury (II) chloride ($HgCl_2$) solution for 4 min, respectively. Finally, they were washed three times with sterile distilled water and subsequently kept in sterile distilled water for 7 h, following the sterilization procedure described by Gungor et al. (2020). The seeds were placed in 250 mL Erlenmeyer flask containing Murashige and Skoog (MS; Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose and solidified with 0.3% (w/v) Gelrite. 100 seeds per replicate were used for *in vitro* germination.

Medium and culture conditions

The pH of all culture media was calibrated to 5.8 with 1 N HCl or 1 N NaOH before adding the gelling agent [0.3% (w/v) Gelrite] and autoclaving at 121 °C for 15 min for semi-solid cultures. For liquid cultures, the step of adding the gelling agent was omitted. All the cultures were incubated at 24 ± 2 °C under a 16-h photoperiod (3500 lux) provided by white light emitting diodes (LED).

Callus formation

Leaf and stem explants, approximately 1 cm in length, were excised from 14-day-old *in vitro* seedlings of *V. caracalla* and used for callus induction (Fig. 1a). Both types of explants were cultured in glass jars (210 mL) each containing 20 mL of MS medium supplemented with various concentrations (1 and 2 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with Indoleacetic acid (IAA) (0.2 mg/L) or Kinetin (Kin) (0.2 mg/L), 3% (w/v) sucrose and 0.3% (w/v) Gelrite (Table 1). Ten explants were used in each replication in the experiments, which were conducted in

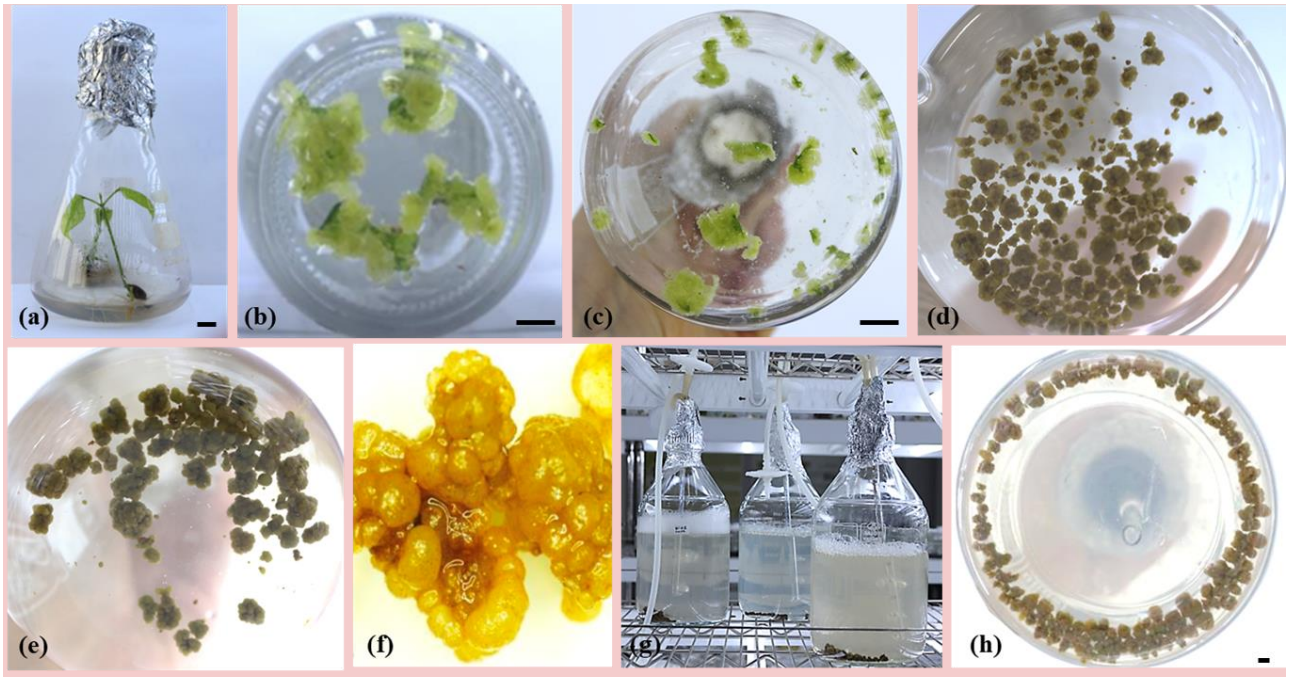

Figure 1. Scale-up production process of meristematic nodules of *Vigna caracalla* L. Verdc. in a bottle bioreactor system. **(a)** 14-day-old *in vitro* seedlings, **(b)** Callus formation on leaf explants after 14 days of culture on CI-6 medium (MS + 2.0 mg/L 2,4-D + 0.2 mg/L IAA), **(c)** 14-day-old leaf-originated calli at the beginning of liquid culture, **(d)** Meristematic nodules after 14 days of liquid culture in MN-6 medium (½ MSB5 + 4.0 mg/L BAP + 100 mg/L AA), **(e)** Secondary meristematic nodules after 14 days of liquid culture in SMN-3 medium (½ MSB5 + 4.0 mg/L BAP + 100 mg/L AA + 100 mg/L Glu), **(f)** Secondary meristematic nodules observed under a stereomicroscope, **(g)** Bottle bioreactor system used for scale-up production of meristematic nodules, **(h)** Meristematic nodules obtained from the bottle bioreactor. Scale bar = 1 cm

three replications. Data were collected 14 days after culture initiation.

Callus formation rate (CFR) was calculated as follows:

$$CFR(\%) = \frac{Number\ of\ explants\ showed\ callus\ formation}{Total\ number\ of\ flasks\ cultured} \times 100$$

Adaptation of callus tissues to the liquid medium environment

In a preliminary experiment, callus tissues derived from leaf and stem explants were evaluated for their adaptability to liquid suspension cultures. The results indicated that leaf-derived callus tissues exhibited greater viability and a more homogeneous distribution. Consequently, leaf-derived callus tissues were selected for subsequent adaptation experiments in liquid suspension cultures. (Fig. 1b). Fourteen-day-old leaf-originated calli (1.5 g) were transferred to a 250 mL Erlenmeyer flask containing 50 mL liquid medium to adapt the callus tissues to the liquid medium environment. The experiment was conducted in two sequential applications based on the outcomes of the previous application. Each experiment was conducted with three replications.

Experiment 1: The first experiment evaluated the effects of various concentrations of different plant growth regulators (PGRs) and sucrose on maintaining the viability of the culture and increasing biomass accumulation. The calli were cultured in liquid MS supplemented with various concentrations of PGRs (0.25 mg/L 2,4-D or 0.2 and 0.5 mg/L 6-Benzylaminopurine: BAP) and sucrose (30 and 60 g/L) (Table 1).

Experiment 2: After determining an efficient PGR and its concentration and sucrose concentration, a second experiment was conducted to find an appropriate basal medium for biomass accumulation. For this purpose, the calli were cultured in different liquid basal media (MS, ½ MS, MSB5, and ½ MSB5) supplemented with 0.5 mg/L BAP. MSB5 contains full-strength MS macro- and micro-salts and B5 vitamins (Table 1).

Meristematic nodule formation in the liquid suspension culture

The nodules were weighed for their fresh weight prior to being transferred to the Erlenmeyer flask. The leaf-originated calli (1.5 g) were placed into a 250 mL Erlenmeyer flask containing 50 mL liquid medium to promote the development of meristematic nodules. The experiment was designed in the form of two consecutive experiments based on the result of the previous experiment.

Experiment 1: The objective of the first experiment was to induce meristematic nodule formation. In this experiment, the calli were cultured in liquid ½MSB5 supplemented with various concentrations of BAP (1.0, 2.0, and 4.0 mg/L) and 15 mg/L sucrose (Table 1).

Experiment 2: After determining an efficient BAP concentration for meristematic nodule formation, a second experiment was conducted to prevent browning in the meristematic nodules. For this purpose, the calli were cultured in liquid ½MSB5 containing 4.0 mg/L BAP, 15 mg/L sucrose, and various concentrations of different

Table 1. Composition of culture media used for callus induction (CI), callus adaptation to liquid medium (CA), meristematic nodule induction (MN), secondary meristematic nodule induction (SMN), and regeneration (R) of *Vigna caracalla* L. Verdc.

Type of culture	Code of medium		Media composition												
			Basal medium composition			Plant growth regulators						Antioxidants		Amino acids	
			Macro- and micro-salts	Vitamins	Sucrose g/L)	2,4-D mg/L	Kin (mg/L)	IAA (mg/L)	BAP (mg/L)	(GA_3) (mg/L)	NAA (mg/L)	CA (mg/L)	AA (mg/L)	Pro (mg/L)	Glu (mg/L)
Callus induction (CI)	CI-1		MS	MS	30	1.0	-	-							
	CI-2		MS	MS	30	2.0	-	-							
	CI-3		MS	MS	30	1.0	0.2	-							
	CI-4		MS	MS	30	1.0	-	0.2							
	CI-5		MS	MS	30	2.0	0.2	-							
	CI-6		MS	MS	30	2.0	-	0.2							
Callus adaptation to liquid medium (CA)	CA-1	Exp. 1	MS	MS	30										
	CA-2		MS	MS	30				0.2						
	CA-3		MS	MS	30				0.5						
	CA-4		MS	MS	30	0.25									
	CA-5		MS	MS	60										
	CA-6		MS	MS	60				0.2						
	CA-7		MS	MS	60				0.5						
	CA-8		MS	MS	60	0.25									
	CA-9	Exp. 2	½ MS	½ MS	15				0.5						
	CA-10		MS	B5	30				0.5						
	CA-11		½ MS	½ B5	15				0.5						
Meristematic nodule induction in liquid medium (MN)	MN-1	Exp.1	½ MS	½ B5	15				1.0						
	MN-2		½ MS	½ B5	15				2.0						
	MN-3		½ MS	½ B5	15				4.0						
	MN-4	Exp. 2	½ MS	½ B5	15				4.0			50			
	MN-5		½ MS	½ B5	15				4.0			100			
	MN-6		½ MS	½ B5	15				4.0				100		
Secondary meristematic nodule induction in liquid medium (SMN)	SMN-1		½ MS	½ B5	15				4.0				100	50	
	SMN-2		½ MS	½ B5	15				4.0				100		20
	SMN-3		½ MS	½ B5	15				4.0				100		100
Regeneration in semi-solid culture (R)	R1	Exp. 1	½ MS	½ B5	15				0.1	1.0			100		
	R2		½ MS	½ B5	15				0.1	2.0			100		
	R3		½ MS	½ B5	15				0.1	3.0			100		
	R4		½ MS	½ B5	15				0.2	1.0			100		
	R5		½ MS	½ B5	15				0.2	2.0			100		
	R6		½ MS	½ B5	15				0.2	3.0			100		
	R7		½ MS	½ B5	15				0.3	1.0			100		
	R8		½ MS	½ B5	15				0.3	2.0			100		
	R9		½ MS	½ B5	15				0.3	3.0			100		
	R10		½ MS	½ B5	15				1.0	2.0			100		
	R11		½ MS	½ B5	15				2.0	2.0			100		
	R12		½ MS	½ B5	15				4.0	2.0			100		
	R13	Exp. 2	½ MS	½ B5	15				0.2		0.5		100		
	R14		½ MS	½ B5	15				1.0		0.5		100		
	R15		½ MS	½ B5	15				2.0		0.5		100		
	R16		½ MS	½ B5	15				4.0		0.5		100		
	R17		½ MS	½ B5	15				0.2		1.0		100		
	R18		½ MS	½ B5	15				1.0		1.0		100		
	R19		½ MS	½ B5	15				2.0		1.0		100		
	R20		½ MS	½ B5	15				4.0		1.0		100		

MS: Murashige and Skoog medium (Murashige and Skoog 1962), **MSB5**: MS and B5 media combination (full-strength MS macro- and micro-salts and B5 vitamins) (Murashige and Skoog 1962; Gamborg et al. 1968), **2,4-D**: 2,4-dichlorophenoxyacetic acid, **Kin**: Kinetin, **IAA**: Indoleacetic acid, **BAP**: 6-Benzylaminopurine, **GA3**: Gibberellic acid, **NAA**: Naphthaleneacetic acid, **CA**: Citric Acid, **AA**: Ascorbic Acid, **Pro**: Proline, **Glu**: Glutamine

antioxidants (50 and 100 mg/L citric acid: CA; or 100 mg/L ascorbic acid: AA) (Table 1).

Same-age green calluses were used for each experiment (Fig. 1c). The flasks were maintained on a shaker at a speed of 100 rpm. All experiments were performed using three independent flasks, each representing a biological replicate. Each replication consisted of one 250 ml Erlenmeyer flask. Data were recorded 14 days after culture initiation.

Meristematic nodule formation rate (MNFR) and browning rate (MNBR) were calculated as follows:

$$MNFR = \frac{Number\ of\ flasks\ showed\ callus\ formation}{Total\ number\ of\ flasks\ cultured} \times 100$$

$$MNBR = \frac{Number\ of\ flasks\ showed\ browning}{Total\ number\ of\ flasks\ cultured} \times 100$$

In this context, each flask serves as a replicate to assess the darkening rate per explant. Therefore, it is defined as the total mass of all calluses introduced into each flask, while the extent of darkening may either be influenced by or remain independent of the darkening factor.

Meristematic nodule multiplication through secondary meristematic nodule formation

To enhance the multiplication of meristematic nodules, secondary nodule formation was induced in the medium (MN-6) which previously showed the most effective nodule development. Meristematic nodule pieces (1.0 g) were transferred to a 250 mL Erlenmeyer flask containing 50 mL of ½ MSB5 liquid medium supplemented with 4 mg/L BAP, 100 mg/L AA, 15 g/L sucrose and amino acid (proline or glutamine) (Table 1). The cultures were incubated on a shaker at a speed of 100 rpm. The experiments were carried out with three replications. Each replication consisted of one 250 mL Erlenmeyer flask. Fresh weight (g) (FW), Specific growth rate (week^{-1}) (SGR), Doubling time (week) (DT) and Fresh growth index (FGI) were identified as growth parameters for all three media.

After determining the best medium for secondary meristematic nodule formation, another culture was established with SMN-3 to determine the growth kinetics of meristematic nodule culture. Meristematic nodules (1 g) were transferred to 50 mL of liquid SMN-3 medium, and FW, SGR, DT, and FGI were measured weekly for up to 4 weeks. According to the results obtained from the growth parameters, meristematic nodules were subcultured at 2-week intervals by transferring 1 g of fresh weight of meristematic nodules to 50 mL of liquid SMN-3 medium for multiplication.

Settled cell volume (SCV)

Settled cell volume (SCV) was determined on the basis of the data obtained from the cultures established in liquid SMN-3 medium (Table 1). The cultures were incubated on a shaker at a speed of 100 rpm. The experiment was carried out with three replications. Each replication consisted of three 250 mL Erlenmeyer flasks and each flask was observed separately. The meristematic nodule suspension culture was poured into a sterile Falcon tube and left for 1 hour until all meristematic nodules had settled. The height of the meristematic nodule mass along the Falcon tube wall represented the volume of the culture. This measurement was repeated at the 1st, 2nd, 3rd and 4th weeks of culture. The SCV was calculated as follows:

$$SCV\ (\%) = \frac{Settled\ cell\ volume\ (mL)}{Total\ volume\ (meristematic\ nodule + medium)(mL)} \times 100$$

Fresh growth index (FGI)

Fresh weight of biomass was recorded at the end of the culture period. FGI was calculated according to Hayta et al. (2011) as follows:

$$FGI = \frac{Final\ fresh\ weight\ of\ biomass\ (g) - Initial\ fresh\ weight\ of\ inoculum\ (g)}{Initial\ fresh\ weight\ of\ inoculum\ (g)}$$

Specific growth rate (SGR) and doubling time (DT)

Specific growth rate (μ) and doubling time (dt) were calculated according to Farjaminezhad et al. (2013) as follows:

$$\mu = \frac{ln\ Xt - lnX0}{t}$$

$$dt = \frac{ln2}{\mu}$$

where μ is specific growth rate, X0 is the initial fresh weight (g) of the inoculum, Xt is the final fresh weight (g) of the inoculum at time t, t is culture period (week) and dt is the doubling time.

Screening of meristematic nodule clusters

The meristematic nodules were captured using a digital camera (Olympus E-330, Olympus Corp. Japan) connected to an Olympus compact stereo microscope (Olympus SZX7 with SZX-LGB DF PLAPO observation tube, Olympus Corp. Japan).

Large scale production of meristematic nodules in bottle bioreactor systems

For large-scale production, a 2-L borosilicate glass vessel, suitable for autoclave sterilization, was utilized. In our previous experiments, the continuous agitation provided by an orbital shaker (100 rpm) facilitated the diffusion of air into the nutrient medium, which allowed the proliferation of meristematic nodules. When scaling up, the culture also needed homogeneous mixing and ventilation equally effectively. Consequently, the system was closed with a screw cap having two ports for air inlet and outlet. A cylindrical glass pipe with a diameter of 1 cm was attached to one of the ports to carry the air to the bottom of the bottle. Two 0.22-μm hydrophobic (polytetrafluoroethylene) membrane filters were attached to the inlet and outlet ports to allow sterile air. The aeration rate was provided by an aquarium pump with an air capacity of 4 L/min during cultivation. The air flow entered through sterile filters from the top of the bioreactor, passed through a pipe, and reached the bottom of the bottle (Fig. 1g). Scale up was performed in 2 L bottle bioreactors containing 1.6 L

of ½ MSB5 liquid medium supplement with 4 mg/L BAP, 100 mg/L AA, 15 g/L sucrose and 100 mg/L L-glutamine (SMN-3) (Table 1). The bioreactor was inoculated with 2.4, 4.8 and 9.6 g/L of green meristematic nodules. Meristematic nodule biomass was submerged in liquid medium throughout the culture period. Continuous immersion culture system was operated with batch mode. The large-scale studies carried out with three replications, a total of 3 pneumatically agitated bottle bioreactor systems were used, with 1 pneumatically agitated bottle reactor in each replication. Data were recorded 14 days after culture initiation.

Investigation of regeneration potential of meristematic nodules

Meristematic nodules (0.5 g) harvested from bioreactors were transferred to glass jars (210 mL) each containing 25 mL semi-solid medium to understand the regeneration potential of meristematic nodules. The experiment was conducted in the form of two consecutive experiments based on the result of the previous experiment.

Experiment 1: The objective of the first experiment was to induce shoot regeneration. In this experiment, meristematic nodules were cultured in ½ MSB5 medium supplemented with 100 mg/L AA, various concentrations of BAP + GA3 combination, 3% (w/v) sucrose and 0.3% (w/v) Gelrite (Table 1).

Experiment 2: In the second experiment, the calli were cultured in ½ MSB5 medium supplemented with 100 mg/L AA, various concentrations of BAP + Gibberellic acid (GA3) combination or BAP + naphthaleneacetic acid (NAA) combination, 3% (w/v) sucrose and 0.3% (w/v) Gelrite to induce shoot regeneration (Table 1). Ten explants were used in each replication in the experiments, which were conducted in three replications. Data were recorded 14 days after culture initiation.

Statistical analysis

The experiments were conducted using a completely randomized design, with all treatments performed in triplicate. Data were statistically analyzed using standard ANOVA procedures. Significant differences among the experiment means were evaluated by Tukey's test, at P = 0.05 using MINITAB 17.0 Statistical Software (Minitab Inc., State College, PA).

Results and Discussion

Callus formation

All seeds cultured in MS medium germinated, and 14-day-old *in vitro* seedlings were used as sources of stem and leaf explants for callus formation (Fig. 1a).

Callus induction was observed in both types of explants within 5 days of culture, independent of the applied doses of plant growth regulators. The mean callus formation rates for both explants cultured in MS medium containing different concentrations of 2,4-D, either alone or combined with IAA or Kin, ranged between 90% and 100% with no substantial differences among the treatments (Fig. 2).

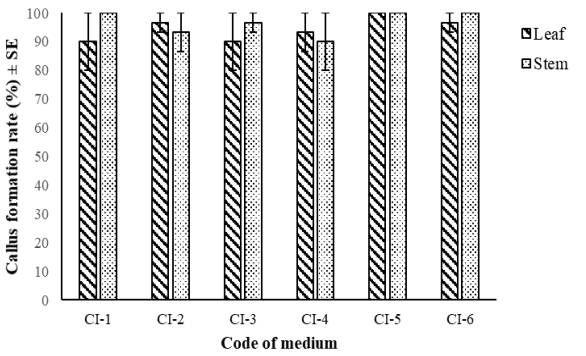

Figure 2. Effects of different explant types and media supplemented with various concentrations of PGRs (2,4-D, Kin, IAA) on callus induction in *Vigna caracalla* L. Verdc. Each value represents the mean ± SE of three replicates.

Both explant types produced green calli in all treatments. The green callus rate reached 100% in the CI-1 medium (MS + 1.0 mg/L 2,4-D) and the CI-6 medium (MS + 2.0 mg/L 2,4-D + 0.2 mg/L IAA) for stem explants, and in the CI-5 medium (MS + 2.0 mg/L 2,4-D + 0.2 mg/L Kin) for both explant types (Fig. 1b) (Table 1). Callus formation in leaf explants from young seedlings cultured in medium containing 2,4-D has also been observed in different Vigna species, such as *Vigna radiata* (L.) Wilczek (Sivakumar et al., 2010), *Vigna unguiculata* (L.) Walp (Anand et al., 2000; Ramakrishnan et al., 2005), *Vigna aconitifolia* (Jacq.) Marechal (Choudhary et al., 2009). In our study, leaf-originated green calli were more friable than stem-originated green calli. Consequently, the leaf-originated callus tissues were more homogeneously distributed and remained more viable (data not shown), and they were used in the establishment of liquid cultures.

Auxins and cytokinins are plant hormones that influence growth and development. Auxins, both natural (such as indolebutyric acid and indole-3-acetic acid) and synthetic (such as 1- naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid), promote expansion, root induction, and bud genesis. Cytokinins affect shoot formation and cell division. These hormones often work together, and their ratio is crucial for plant growth and development. The induction of callus formation can be regulated by a combination of these hormones (Ren et al., 2020). In this study, the combination of 2,4-D (auxin) and Kin (cytokinin) produced the best response in terms of callus formation for both explant types.

Adaptation of callus tissues to the liquid medium environment

The aim of the suspension culture was first to adapt callus tissues to liquid suspension culture to maintain tissue viability and increase biomass accumulation. For this purpose, two experiments were conducted.

Table 2. Experiment 1: Effects of liquid media supplemented with various concentrations of PGRs (2,4-D, BAP) and sucrose on growth in *Vigna caracalla* L. Verdc.

Code of medium	Final fresh weight (g/50 mL) ± SE	Browning rate (%) ± SE
CA-1	1.50 ± 0.00 b	100.00 ± 0.00
CA-2	1.60 ± 0.06 b	100.00 ± 0.00
CA-3	1.80 ± 0.00 a	33.30 ± 16.70
CA-4	1.53 ± 0.03 b	100.00 ± 0.00
CA-5	1.50 ± 0.00 b	100.00 ± 0.00
CA-6	1.50 ± 0.00 b	100.00 ± 0.00
CA-7	1.53 ± 0.03 b	66.70 ± 33.30
CA-8	1.60 ± 0.06 b	100.00 ± 0.00

In the first experiment, the effects of different PGRs (2,4-D and BAP) and sucrose concentrations on biomass accumulation were tested (Table 1). Except for CA-3 (MS + 0.5 mg/L BAP + 30 g/L sucrose), the FW results of the remaining media were statistically the same. The highest FW (1.80 g) was observed on the CA-3 (Table 2). Due to the intense browning in the cultures, the browning rate was also considered as a parameter. All media gave statistically similar responses regarding the browning rate. Except for the CA-3 (MS + 0.5 mg/L BAP + 30 g/L sucrose) and the CA-7 (MS + 0.5 mg/L BAP + 60 g/L sucrose), the browning rate reached 100% in the remaining media. The lowest browning rate (33.30%) was observed on the CA-3 followed by the CA-7 (66.70%). No meristematic nodule formation was observed in the cultures. For the next experiment, the 0.5 mg/L BAP concentration that provided the highest biomass increase was selected and used. A key factor contributing to explant browning *in vitro* is the high salt content in culture media, particularly MS medium (Bayraktar et al., 2020). Although MS macro- and micro-nutrient composition is widely applied, its standard formulation can be excessive or even toxic to certain species (Han et al., 2010). Issues like shoot tip necrosis and hyperhydricity have been reported, necessitating reductions in MS salt strength for better growth responses (Arteta et al., 2022). Essential vitamins such as thiamine (B1), nicotinic acid (B3), pyridoxine (B6), and myo-inositol are also critical for plant development, acting as enzymatic cofactors and growth facilitators (Sudheer et al., 2022). Their addition to culture media has shown beneficial effects on callus formation and embryogenesis (Kintzios et al., 2001). Compared to MS, B5 vitamin formulation includes significantly higher concentrations of B1, B3, and B6.

We hypothesized that full-strength MS medium may negatively impact *V. caracalla* callus cultures by promoting browning, while elevated vitamin content could enhance biomass. MSB5 medium has been used for regeneration purposes in different *Vigna* species (Anand et al. 2000; Saini et al. 2002; Amutha et al. 2003; Mao et al. 2006; Tie et al. 2013; Markin et al. 2023). Therefore, MS, MSB5 (MS salts + B5 vitamins), and their half-strength versions (½ MS and ½ MSB5) were evaluated in liquid culture supplemented with 0.5 mg/L BAP. Fresh weight (FW) and browning rates were recorded (Table 1). Statistical analysis showed no significant differences in FW among treatments, with values ranging between 1.63 g (CA-9) and 1.83 g (CA-11). Browning was also not statistically different; however, CA-9 exhibited the highest browning rate (66.70%), while others showed 33.30%. No meristematic nodule formation was observed in any treatment. Although FW was not markedly improved by vitamin enrichment or salt dilution alone, the ½ MSB5 medium slightly increased biomass compared to MS. Based on these results, ½ MSB5 was selected as the basal medium for subsequent experiments due to its marginal advantage in supporting callus growth.

Table 3. Experiment 2: Effects of different liquid basal media supplemented with 0.5 mg/L BAP on growth in *Vigna caracalla* L. Verdc.

Code of medium	Final fresh weight (g/50 mL) ± SE	Browning rate (%) ± SE
CA-3	1.80 ± 0.00	33.30 ± 16.70
CA-9	1.63 ± 0.09	66.70 ± 33.30
CA-10	1.70 ± 0.10	33.30 ± 16.70
CA-11	1.83 ± 0.12	33.30 ± 16.70

Meristematic nodule formation in the liquid suspension culture

Since no differentiation was observed in the callus tissues in the suspension culture in our previously mentioned experiments, we investigated the effect of BAP concentration on the differentiation of callus tissues in the first experiment. The medium from which the highest callus FW was obtained (CA-11: ½ MSB5 + 0.5 mg/L BAP + 15 g/L sucrose) was used as a reference for subsequent experiments (Table 3). For this purpose, in the first experiment, the leaf-originated calli were cultured in liquid ½ MSB5 supplemented with various concentrations of BAP (1.0, 2.0, and 4.0 mg/L) and 15 mg/L sucrose (Table 1). Although the FW contents were slightly increased in MN-2 (½ MSB5 + 2.0 mg/L BAP + 15 g/L sucrose) and MN-3 (½ MSB5 + 4.0 mg/L BAP + 15 g/L sucrose), the differences among the treatments were not statistically significant. The mean FW results ranged between 1.73 (MN-1) and 2.06 g (MN-3) without significant differences among treatments. The browning rate also did not show significant differences among the BAP concentrations. However, the browning rate of the callus increased following an increase in the concentration of BAP in the medium (66.70%, 100.00% and 66.70% at the concentration of 1.0, 2.0 and 4.0 mg/L BAP, respectively). The rate of meristematic nodule formation exhibited significant variation across the different BAP concentrations. Calli cultured in medium containing 4.0 mg/L BAP showed the highest meristematic nodule formation rate (100.00%), indicating a positive response to high BAP concentration in terms of nodule formation (Table 4). The presence of cytokinins in the culture medium has been identified as a factor contributing to nodule formation and shoot regeneration (Ferreira et al., 2009). The promotive effect of BAP on meristematic nodule formation has also been noted in *Eucalyptus globulus* (Trindade and Pais 2003), *Charybdis numidica* (Kongbangkerd et al., 2005),

Azadirachta indica A. Juss. (Arora et al., 2009). Nodules are described as cell clusters that demonstrate a typical arrangement of internal cellular and tissue differentiation. They have been differentiated from callus or directly from explant in different woody and herbaceous plants (McCown et al. 1988; Teng 1997). We obtained meristematic nodules from callus tissues grown in liquid medium. Similar report has been reported for *Humulus lupulus* L. var. Eroica, in which the organogenic nodule formation was only found when calli were cultured in liquid medium (Batista et al., 2000). Explants may undergo the process of oxidative stress brought on by cellular harm or environmental fluctuations occuring during the transfer to culture media or other procedural manipulations during explant preparation (Permadi et al., 2024). Transfer of explants from semi-solid medium to liquid medium causes changes in the environmental conditions of the explants (Dong et al., 2015). In our study, despite an increase in biomass and the highest meristematic nodule formation rate (100.00%), intense darkening (66.70%) occurred in the liquid MN-3 medium (½ MSB5 + 4 mg/L BAP + 15 g/L sucrose) (Table 4). To address this issue, we aimed to improve the medium to prevent the browning in the meristematic nodules and established the second experiment. Antibrowning reagents like CA and AA were tested to suppress browning. For this purpose, the calli were cultured in liquid ½MSB5 fortified with 4.0 mg/L BAP and various concentrations of different antioxidants (CA or AA) (Table 1).

Table 4. Experiment 1: Effects of different BAP concentrations on growth and meristematic nodule formation in *Vigna caracalla* L. Verdc.

Code of medium	Final fresh weight (g/50 mL) ± SE	Meristematic nodule formation rate (%) ± SE	Browning rate (%) ± SE
CA-11	1.83 ± 0.12	0.00 ± 0.00 b	33.30 ± 16.70
MN-1	1.73 ± 0.12	33.30 ± 16.70 b	66.70 ± 33.30
MN-2	1.85 ± 0.03	0.00 ± 0.00 b	100.00 ± 0.00
MN-3	2.06 ± 0.12	100.00 ± 0.00 a	66.70 ± 33.30

The mean FW ranged from 1.70 g (MN-5: ½ MSB5 + 4.0 mg/L BAP + 100 mg/L CA + 15 mg/L sucrose) to 2.09 g (MN-6: ½ MSB5 + 4.0 mg/L BAP + 100 mg/L AA + 15 mg/L sucrose) with no significant differences among treatments. CA slightly reduced biomass and meristematic nodule formation and had no positive effect on preventing browning. Unlike CA, AA completely prevented browning and no browning was observed in the meristematic nodules (Fig. 1d). The highest FW (2.09 g) and meristematic nodule formation (100.00%) were obtained from the MN-6 medium (Table 5). Ascorbic acid is an antioxidant extensively studied for its potential to inhibit the oxidation of phenolic substances that induce browning; it is added to the medium as a substance that reduces the pathway of browning. Although AA does not directly interact with the polyphenol oxidase (PPO) enzyme, it effectively inhibits enzymatic browning through the reduction of oxidized substrates (Permadi et al., 2024). In our study, tissue browning was also effectively suppressed by AA.

Table 5. Experiment 2: Effects of various concentrations of different antioxidants (50 and 100 mg/L CA or 100 mg/L AA) on growth and meristematic nodule formation in *Vigna caracalla* L. Verdc.

Code of medium	Initial fresh weight (g/50 mL)	Final fresh weight (g/50 mL) ± SE	Meristematic nodule formation rate (%) ± SE	Browning rate (%) ± SE
MN-3	1.5	2.06 ± 0.12	100.00 ± 0.00 a	66.70 ± 33.30
MN-4	1.5	1.73 ± 0.03	33.30 ± 16.70 b	66.70 ± 33.30
MN-5	1.5	1.70 ± 0.10	33.30 ± 16.70 b	66.70 ± 33.30
MN-6	1.5	2.09 ± 0.17	100.00 ± 0.00 a	0.00 ± 0.00

Meristematic nodule multiplication through secondary meristematic nodule formation

Meristematic nodule pieces (1.0 g) of *V. caracalla* that were harvested after two weeks of culture establishment, were transferred to 250 mL Erlenmeyer flasks each containing 50 mL of ½ MSB5 liquid medium supplemented with 4 mg/L BAP, 100 mg/L AA and different amino acids (proline or glutamine) to multiply meristematic nodules through secondary meristematic nodule formation (Fig. 1e,f and Table 1). According to ANOVA, significant differences were observed among treatments for the FW. SMN-3 (½ MSB5 + 4 mg/L BAP, 100 mg/L AA and 100 mg/L glutamine) showed the highest FW (2.13 g) after two weeks of culture. The minimum FW (1.16 g) was recorded for SMN-1 (½ MSB5 + 4 mg/L BAP, 100 mg/L AA and 50 mg/L Proline). Glutamine increased the FW more effectively than proline. Depending on the applied glutamine doses, the FW at 100 mg/L was higher than at 20 mg/L (Fig. 3). A comparable reaction was noted in the liquid shake culture of embryogenic calluses of *Vigna mungo* L. Hepper. L-Glutamine at 20 mg/L significantly stimulated the induction and development of all somatic embryo stages. However, proline in the medium diminished the degree of somatic embryo induction and maturation (Muruganantham et al., 2010). L-glutamine has been used as an organic supplement (amino acid) in the plant tissue culture media for many species to serve as an alternative organic nitrogen source that stimulates cellular metabolism, facilitates the synthesis of storage proteins, and supports differentiation and cellular proliferation. It is well known that L-glutamine plays an important role in the enhancement of cell proliferation in cell suspension cultures, somatic embryo induction, maturation and germination (de Mello et al., 2024; Singh et al., 2024). In addition to these effects, in the present study, L-glutamine supported the formation of secondary meristematic nodules in *V. caracalla*. Proliferation from meristematic nodules into new nodules (secondary meristematic nodules) has also been reported for *Ananas comosus* Merr. (Teng, 1997), *Humulus lupulus* L. (Batista et al., 2000), *Charybdis numidica* (Kongbangkerd et al., 2005), *Populus euphratica* Oliv. (Ferreira et al., 2009), *Sclerocarya birrea* subsp. *caffra* (Moyo et al., 2009). Our results in *V.*

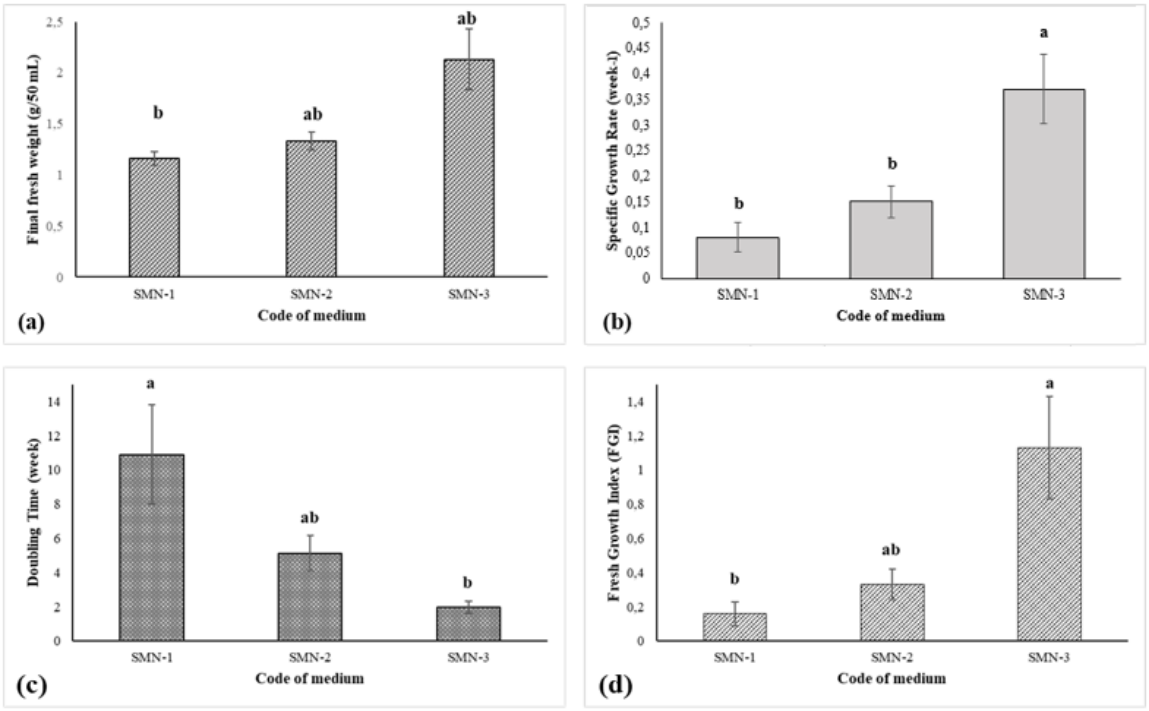

Figure 3. Effects of various concentrations of different amino acids (50 mg/L proline or 20 and 100 mg/L glutamine) on growth in *Vigna caracalla* L. Verdc. **(a)** Fresh weight (g), **(b)** Specific growth rate (week^{-1}), **(c)** Doubling time (week), **(d)** Fresh growth index. Each value represents the mean ± SE of three replicates. In columns, different letters denote significant differences ($P \leq 0.05$) between applications according to Tukey's test.

caracalla showed that the addition of 50 mg/L proline in the SMN medium did not enhance the secondary meristematic nodule formation and hence did not increase FW. Moreover, meristematic nodules lost their viability. In contrast, Girija et al. (2000) reported different stages of somatic embryos grown in liquid medium containing 50 mg/L proline in mung bean. The external application of proline, a stress-associated amino acid, enhanced somatic embryo maturation and shoot differentiation in *Vigna* spp. (Sen et al., 2002). Sivakumar et al. (2010) obtained embryonic callus, all stages of somatic embryos and mature cotyledonary-stage somatic embryos from the media containing 150 mg/L glutamine, 50 mg/L proline and 20 mg/L proline, respectively, in mung bean (*Vigna radiata* L.).

Apart from FW, Specific growth rate (SGR), Doubling time (DT) and Fresh growth index (FGI) were also observed as growth parameters for all three media. According to results of these parameters, the highest SGR (0.37 week^{-1}), FGI (1.13) and the shortest DT (1.80 week) were observed in SMN-3 medium. Among the amino acids, 50 mg/L proline (SMN-1) showed a poor stimulatory effect on nodule multiplication with a lowest SGR (0.08 week^{-1}) and FGI (0.16) (Fig. 3). When the doubling time of the meristematic nodules were compared, the nodules grown in SMN-3 containing 100 mg/L glutamine were 5.50-fold faster than the nodules grown in SMN-1 containing 50 mg/L proline (Fig. 3).

After determining that SMN-3 was the best medium for meristematic nodule multiplication, another culture was conducted with SMN-3 to determine the growth kinetics of meristematic nodule culture. This culture was observed for 4 weeks and Settled cell volume (SCV), FW, SGR, DT and FGI parameters were measured every week. According to the results, the growth of meristematic nodules continued until the 2nd week and remained constant between 2-4 weeks (Fig. 4). The FW, SGR and FGI achieved their peak value at the end of the 2nd week and remained unchanged until the fourth week was over. FW, SGR and FGI of the second week increased 1.48-fold, 2.06-fold and 2.57-fold compared to the first week, respectively (Fig. 4a, 4c, 4e). The highest SCV (7.67%) was identified at the end of the second week (Fig. 4b). Based on DT results, meristematic nodules multiplied 1.9-fold faster in the 2nd week compared to the first week, and doubled more steadily in the 3rd week and 4th week after the 2nd week (Fig. 4d). According to these results, a two-week culture period is sufficient to maintain biomass viability and provide continuation of the log phase in large-scale biomass production experiments.

Large scale production of meristematic nodules in bottle bioreactor systems

Nodules typically possess a strong ability to regenerate plants or organs through organogenesis. This process is most effectively synchronized in liquid cultures through the classification and measurement of the nodules. These characteristics are crucial for automated micropropagation and make nodule culture a promising option for large-scale production (Teng, 1997). We optimized an effective large-scale production system for meristematic nodules of *V. caracalla* using a bottle bioreactor system.

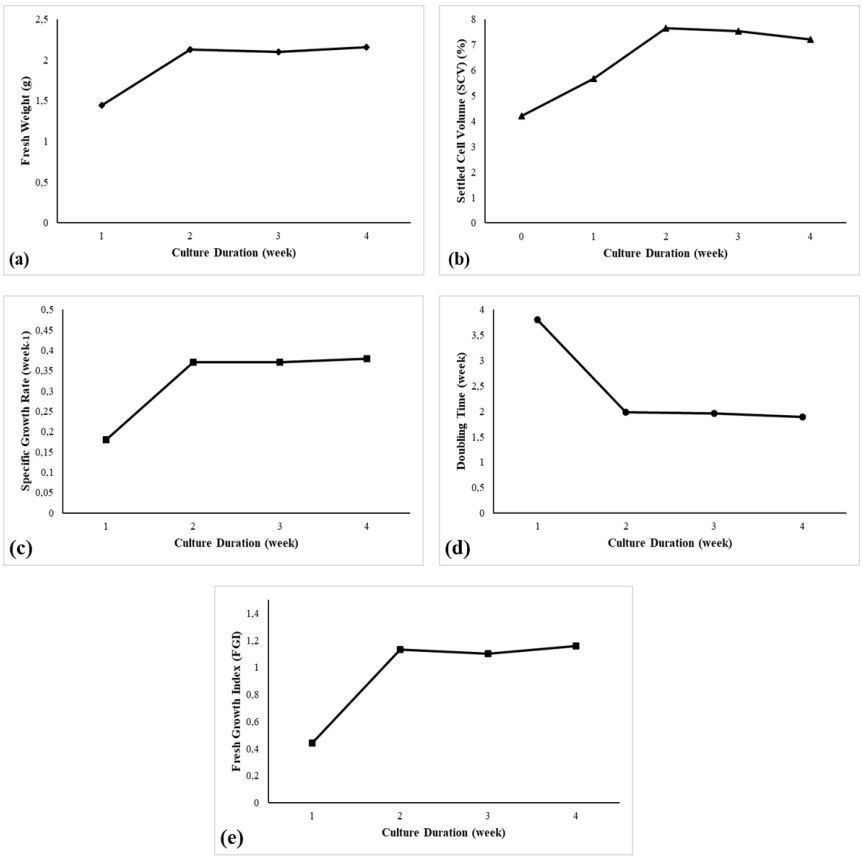

Figure 4. Growth characteristics of *Vigna caracalla* L. Verdc. meristematic nodules cultured in a 250 mL Erlenmeyer flask containing liquid SMN-3 medium (½ MSB5 + 4 mg/L BAP + 100 mg/L AA + 100 mg/L glutamine): **(a)** Fresh weight (g), **(b)** Settled cell volume, **(c)** Specific growth rate (week^{-1}), **(d)** Doubling time (week), **(e)** Fresh growth index.

Large scale production of meristematic nodules was carried out with 2 L bottle bioreactors. For this purpose, different inoculum densities (2.4, 4.8 and 9.6 g/L) of green meristematic nodules were transferred to bioreactors containing 1.6 L of SMN-3 (Table 1).

According to ANOVA, FW, SGR, DT, and FGI were significantly affected by different inoculum densities. The 9.6 g inoculum density resulted in the highest FW (27.19 g) after two weeks of culture. The minimum FW (4.35 g) was recorded for the 2.4 g inoculum density. The increases in FW were 1.81-, 1.97-, and 2.83-fold for the 2.4 g, 4.8 g, and 9.6 g inoculum densities, respectively (Fig. 5a). The SGRs of bioreactor cultures varied between 0.30 (2.4 g inoculum density) and 0.52 week^{-1} (9.6 g inoculum density) depending on the density of the meristematic nodules inoculated (Fig. 5b). The fastest biomass increase (1.33 week) was obtained with 9.6 g inoculum density followed by 4.8 g inoculum density (2.06 week). In the large-scale system established with 9.6 g/L inoculum density, the meristematic nodules doubled 1.8-fold faster than the culture established with 2.4 g inoculum density (Fig. 5c). In parallel with the highest FW, SGR, the highest FGI (1.83) was also observed with 9.6 g inoculum density followed by 4.8 inoculum (0.96) density (Fig. 5d).

Bioreactors create optimal environments for combining cultivated tissues with a culture medium and assuring ideal environmental parameters such as aeration (carbon dioxide, oxygen, several vital gases), the outside temperature, pH level, and light periods. These conditions support the growth and development of cultured tissues for plant regeneration through organogenesis or embryogenesis. The benefits of using a liquid medium and bioreactor for plant tissue cultures are: i) they allow for the efficient production and scaling up of a large number of plantlets; ii) the process of handling cultures, encompassing inoculation and harvesting, is simplified, reducing labor and time; iii) constant exposure to the medium ensures easy nutrient absorption, thereby promoting faster growth; and iv) The introduction of forced air in bioreactor cultures stimulates the development and metabolic processes of the cultured cells and organs. Different bioreactors are used for different types of cultures (Murthy et al., 2023). One drawback of using a bioreactor system is the high cost of setup, as these systems are expensive and have only a few manufacturers (Abu Hassan et al., 2022). For the large-scale production of meristematic nodules of *V. caracalla*, we used an air-lift-like, low-cost bioreactor system designed with glass bottles, which is easy to set up, as detailed in the Materials and Methods section. Meristematic nodules could be produced on a massive scale using this bioreactor technology.

Regeneration potential of meristematic nodules

To understand the regeneration potential of meristematic nodules, meristematic nodules (0.5 g) harvested from bioreactors, were transferred to glass jars (210 mL) each containing 25 mL of ½ MSB5 medium

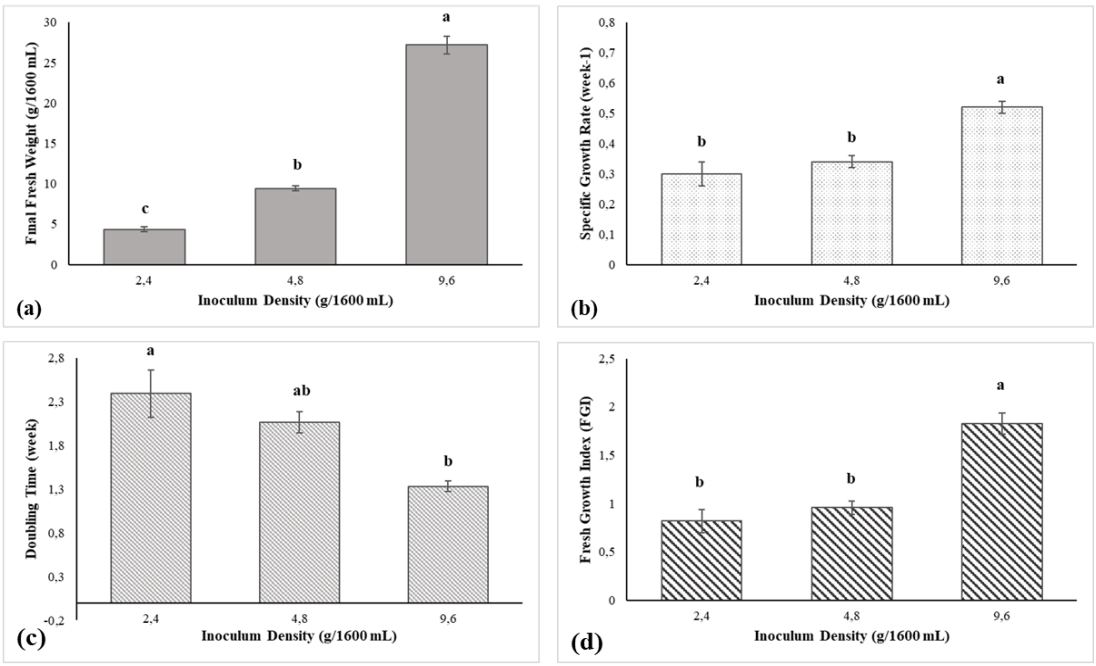

Figure 5. Growth characteristics of *Vigna caracalla* L. Verdc. meristematic nodules cultured in a 2 L bottle bioreactor containing liquid SMN-3 medium (½ MSB5 + 4 mg/L BAP, 100 mg/L AA and 100 mg/L glutamine) and inoculated with 2.4, 4.8 and 9.6 g/L of meristematic nodules: **(a)** Fresh weight (g), **(b)** Specific growth rate (week^{-1}), **(c)** Doubling time (week), **(d)** Fresh growth index. In columns, different letters denote significant differences (P ≤ 0.05) between applications according to Tukey's test.

supplemented with 100 mg/L AA, various concentrations of BAP + GA3 combination or BAP + NAA combination, 3% (w/v) sucrose and 0.3% (w/v) Gelrite (Table 1).

It has been documented that some legume crops, particularly *Cajanus cajan, Cicer arietinum, Pisum sativum,* other crucial *Vigna* species like *Vigna unguiculata*, *V. radiata*, *V. mungo*, and several forage legumes, exhibit recalcitrance to *in vitro* regeneration and display significant genotype dependency in transformation studies (Ramakrishnan et al., 2005; Sivakumar et al., 2010; Tie et al., 2013; Choudhury and Rajam 2021). To our knowledge, to date there has been a single report published regarding *in vitro* study on *V. caracalla*. Gungor et al., (2020) performed a micropropagation study by culturing shoot tip and node explants in different basal media with different levels of PGRs. They obtained the best response from shoot tip explants cultured in MS medium containing 1 mg/L IBA + 0.5 mg/L BAP. In the present study, we did not observe conversion from the meristematic nodules into *in vitro* shoots. Meristematic nodules of *V. caracalla* re-callused and then turned brown **(Data not shown)**. Recallusing in somatic embryos was reported in *V. unguiculata* L. (Kulothungan et al., 1995, Ramakrishnan et al., 2005), *V. radiata* L. (Sivakumar et al., 2010), *Cicer arietinum* L. (Dineshkumar et al., 1995). Moyo et al. (2009) reported that plant regeneration via nodular meristemoids offers a reliable organogenic pathway. However, the primary difficulty lies in regenerating these nodular meristemoids into plantlets. In our opinion, there may be recalcitrance to *in vitro* shoot regeneration in *V. caracalla*, which may complicate nodule-to-shoot regeneration.

Conclusion

In conclusion, this study achieved large-scale production of meristematic nodules of *Vigna caracalla* for the first time, using an air-lift-like, low-cost bioreactor system designed with glass bottles. Green callus regenerated 100% in the CI-1 medium (MS + 1.0 mg/L 2,4-D) and the CI-6 medium (MS + 2.0 mg/L 2,4-D + 0.2 mg/L IAA) in the stem explants, and the CI-5 medium (MS + 2.0 mg/L 2,4-D + 0.2 mg/L Kin) in both explant types. Due to its more friable texture, leaf-originated callus tissue was used to establish liquid cultures. Liquid SMN-3 (½ MSB5 + 4 mg/L BAP + 100 mg/L AA and 100 mg/L glutamine) was identified as the best medium for the induction and production of meristematic nodules in small-scale 250 mL Erlenmeyer flasks and large-scale 2 L bottle bioreactor cultures without browning problems after two weeks. The conversion of meristematic nodules into *in vitro* plantlets remained inefficient. Future studies may focus on inducing regeneration from meristematic nodules to plantlets. Moreover, the findings of this study can be used as a guide for future investigations in genetic engineering and the synthesis of secondary metabolites.

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Author Contributions

All authors participated in the conception and design of the study. SS: Data Curation, Formal Analysis,

Investigation, Methodology, Visualization and Writing. MB: Formal Analysis, Visualization and Writing -review and editing. AG: Supervision, Writing - review and editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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