High-Frequency Shoot Regeneration From Flower Bud Derived Callus of Gymnostachyum Febrifugum Benth., an Endemic Medicinal Plant to the Western Ghats

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Abstract

Gymnostachyum febrifugum Benth. is a small, scapigerous, rare and endemic medicinal herb indigenous to India belonging to the family Acanthaceae. This study reports an efficient protocol for high-frequency flower bud derived callus induction and shoot organogenesis in *G. febrifugum*. Flower buds at 7d before anthesis (dBA) were excised from the inflorescence and cultured on MS medium supplemented with various concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D; 0.5-2.0 mg/l) for callus induction. The optimum callus induction (78%) was obtained on MS medium supplemented with 1.5 mg/l 2, 4-D. The calli when subcultured on MS medium supplemented with different concentrations of thidiazuron (TDZ; 0.5-2.5 mg/l) or 6-benzylaminopurine BAP (0.5-2.5 mg/l) alone or in combination with 1-naphthaleneacetic acid (NAA; 0.2-0.7 mg/l) induced shoots. The highest frequency (94%) and number of shoots (44.6 shoots/unit callus) were obtained on MS medium supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA. The optimum rooting frequency (95%) and number of roots (10.2) were observed on ½ MS medium supplemented with 3.0 mg/l indole-3- butyric acid (IBA). The rooted plantlets were acclimatized and transferred to soil with 94% success.

Introduction

Gymnostachyum febrifugum Benth. is a rare endemic plant belonging to the family Acanthaceae (Thomas et al. 1996). This is a pretty, stemless, tiny and scapigerous herb with large long-petiolated ovate leaves and woody rootstock (Fig. 1; Gamble 1924). This plant is indigenous to India and is having a very beautiful attractive flower, which makes it a potential wild ornamental plant (Thomas et al. 1996). Inflorescence in *G. febrifugum* is a spike with limited flowered cymes and the flower is white tinged with purple colour and the lower lip appear yellow. The root of this plant is used as febrifuge and against blisters and sores on the tongue (Pattanayak 2019).

According to Mascarenhas (2010) the threat status of this plant is near threatened. Hence, there is an urgent need for the conservation of this plant. Conventional propagation of this plant is through seeds and our study revealed that the seed formation in fruits is comparatively low probably due to self-incompatibility. Additionally, low viability and formation of a high percentage of sterile seeds are the difficulties associated with its propagation. Further, vegetative propagation is not very promising due to the lack of erect stem in *G. febrifugum*. In this background, micropropagation is considered as a viable option to rapidly propagate this plant (Ramírez-Mosqueda and Iglesias-Andreu 2016).

Since this plant is devoid of an erect stem, the collection of explants like leaf and subterranean parts often cause high rate of contamination. The flower bud is considered as an ideal explant for micropropagation of this plant since it can reduce contamination rate substantially and also avoid destroying the mother plant by collecting other explants.

Although plant tissue culture related instability in regenerating plants is prevalent in several systems, somaclonal variation is considered as an additional tool for the improvement of agricultural as well as

horticultural crops along with well-established plant breeding methods (Arun et al. 2003; Santha and Mehta 2001; Anil et al. 2018). The cause of such variations is basically due to various reasons such as genetic, phenotypic and epigenetic alterations observed in plants growing in cultures (Karp 1995). Such variations may increase if the pathway is through callus organogenesis. Other factors which determine the variations include the medium and maintenance time in culture. The somaclonal variants usually appear due to the structural and numerical changes in chromosomes (Bairu et al. 2011; Delgado-Paredes et al. 2017).

The use of plant tissue culture techniques has been considerably increased over the past few decades for the mass propagation of rare, endangered, endemic and threatened plants (for review see Deepa and Thomas 2020). Micropropagation technique can give rise to large number of plants in a very limited period irrespective of season and climate (Liao et al. 2011). This technique is more effective in producing mass scale elite, healthy, disease-free plants as compared to that of conventional propagation protocols (Vieitez et al. 2007; Corredoira et al., 2017). Further, this technique can support the improvement of agriculture, horticulture and forestry plants. Various approaches like nodal segment culture, adventitious shoot formation, direct and indirect organogenesis and somatic embryogenesis are frequently used for the multiplication of various plant species (Demétrio et al. 2021; Kannan et al. 2021; Saavedra et al. 2021; Patel et al. 2021). To our knowledge, there is no report on in vitro propagation of *G. febrifugum*. Therefore, in this study, we report an efficient high frequency shoot induction protocol from flower bud derived callus.

Materials And Methods

Plant material and surface sterilization

G. febrifugum flowers from July to November. The plant bears beautiful flowers during this period. The flowers are arranged in a cymose inflorescence. Flower buds were collected from such inflorescences. For our study, we have selected flower buds at three different stages of development i.e. 4, 7, 10d before anthesis (dBA). The flower buds were excised from the inflorescence of field-grown plants and brought to the lab in the morning. The flower buds were excised and collected. Each flower bud has a small stalk at the cut end. The flower buds were washed thoroughly in sterilized distilled water for 5 min and then immersed in sterilized distilled water containing 1% Savlon solution for 8 min. followed by two rinses in sterilized distilled water. Finally, the explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 7 min. The explants were then washed three times with sterilized distilled water. The buds were then kept in sterilized glass Petri plates lined with blotting paper to remove traces of water.

Media and culture initiation

MS medium was invariably used throughout the experiment and is supplemented with various plant growth regulators. The pH of the medium was adjusted to 5.8 by using 0.1 N NaOH or 0.1 N HCl before gelling with 8.0 g/l bacteriological agar (Himedia, India). For callus induction from flower buds, different

concentrations of 2, 4-D (0.5 to 2.0 mg/l) was employed. The flower buds were horizontally placed in Petri plates and the basal stock portion of the flower buds was slightly pushed in the medium at the time of inoculation. The calli induced from explants were subcultured on MS medium supplemented with 0.5 mg/l 2, 4-D for multiplication in every 4 weeks.

For shoot regeneration from callus

The callus was subcultured on MS medium augmented with various concentrations of TDZ (0.5–2.5 mg/l) or BAP (0.5–2.5 mg/l) alone and in combination with NAA (0.2–0.7 mg/l) for shoot induction. For shoot induction, 1.0 g callus was inoculated in each container. The per cent response, number of shoots and mean shoot length were measured 45d after culture.

Culture conditions and Statistical analysis

The cultures were incubated at 22 ± 2°C, in the culture room with 16-h light conditions and 40–50 µmol m^2/s photosynthetic photon flux (PPF) provided by white, cool fluorescent lamps. For each experiment, at least 14 replicates were taken and each experiment was repeated three times. Statistical analysis was performed by using analysis of variance (ANOVA) through SPSS software. Data were analysed for the significance of differences of means among treatments using Duncan's multiple range test (DMRT) at $P \leq 0.05$.

Results And Discussion

Callus induction from flower buds

Our primary aim was to standardize the suitable stage of the flower bud and 2, 4-D concentration for optimum callus induction. The flower buds 4, 7 and 10 dBA were excised from the inflorescence in the morning hours and brought to the lab for inoculation (Fig. 2A-C). The flower buds were then subjected to four different callus induction treatments, which include MS medium supplemented with 0.5, 1.0, 1.5 and 2.0 mg/l 2, 4-D (Table 1). In almost all concentrations of 2, 4-D tested, calli induced with similar textures whereas on basal medium (control) there was no callus induction. Callus initiation started 2 weeks after culture (Fig. 2D). Initially, the calli appeared as small granules on the surface of the explant and later it slowly spread to the entire surface and after 6 weeks the calli completely covered the explants in responding cultures (Fig. 2E). Due to the presence of anthocyanin pigments in the explant, the calli showed pink spots occasionally (Fig. 2F, G).

Table 1

Effect of age of flower buds (4, 7 and 10-d before anthesis) and various concentrations of 2, 4-D on callus induction from flower bud explants of *G. febrifugum* after 6 weeks of culture. Minimum 24 explants were cultured in each treatment and all the experiments were repeated 3 times.

2, 4-D (mg/l)	4-d-BA		7-d-BA		10-d-BA	
	Explants with calli[a] (%)	Size of calli	Explants with calli (%)[a]	Size of calli	Explants with calli (%)[a]	Size of calli
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	13 ± 1.3b	*	27 ± 2.3c	*	-	-
1.0	28 ± 2.9a	*	62 ± 2.1b	***	10 ± 1.2b	*
1.5	31 ± 2.3a	**	78 ± 1.9a	***	23 ± 1.7a	**
2.0	20 ± 2.8ab	*	56 ± 2.8b	**	27 ± 1.9a	*
Callus size: * - small, ** - medium, *** - large						
[a] Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test						

Table 1 depicts callus induction using flower buds at 4, 7 and 10 dBA on MS medium supplemented with 0.5, 1.0, 1.5 and 2.0 mg/l 2, 4-D 45d after culture. There was no callus induction on MS basal medium. The per cent explants with calli and callus size were influenced by the age of the flower bud as well as concentration of 2, 4-D. Flower buds collected 4 dBA showed small to medium size calli in 13 to 31% explants on various concentrations of 2, 4-D (Table 1). When flower buds collected 10 dBA were cultured on 0.5 mg/l 2, 4-D, there was no callus initiation. However, on other 2, 4-D concentrations it exhibited small to medium size calli in 10 to 27% explants. The flower buds collected 7 dBA when cultured on 1.5 mg/l 2, 4-D exhibited the optimum response of 78% explants with large size calli (Table 1). This calli also had the highest biomass i.e. 2.6 ± 1.2 g fresh weight per callus mass. On this medium, the earliest callus induction was noticed (7-9d) and flower buds from 4 dBA and 10 dBA took longer to induce callus (12-14d). All the calli induced were of friable texture. Since the highest callus induction frequency and callus size was observed on 1.5 mg/l 2, 4-D in flower buds collected 7 dBA, all further callus induction experiments were done with this explant and media combination. The age of the flower bud is an important factor, which influences the callus induction and subsequent shoot organogenesis in some systems. In *Gerbera jamesonii* 7, 8, 9, 10 and 11-d-old flower buds were cultured to induce callus. In all four varieties, 7-9-d-old flower buds showed the best response in terms of both callus induction as well as shoot organogenesis (Akter et al. 2012). The auxin 2, 4- D is routinely used for callus induction from various explants in different systems (Rathore et al. 2011; Abraham and Thomas 2015; Bala et al. 2015; Patricia 2021). According to Zheng and Konzak (1999) 2, 4-D induced highest callus induction could be achieved not only by the use of proper concentration but also its duration of presence in the medium.

The calli during its initiation stage remained predominantly pinkish in colour whereas later as the calli grow further, it turned in to light yellow and green (Fig. 2D-G). However, pinkish spots were regularly noticed on all calli during the entire period of culture. This pinkish colour is due to the exudation of the flavonoid compound anthocyanins present in the explant. The presence of anthocyanins was reported in various parts of some plants especially in flowers, fruits and vegetables (Mihai et al. 2010). The induced calli were subcultured on MS medium supplemented with 0.5 mg/l 2, 4-D for multiplication after 45d.

Shoot regeneration from callus

Well-developed friable yellowish-white calli with pink spots were subcultured on MS medium supplemented with TDZ (0.5 to 2.5 mg/l) or BA (0.5 to 2.5 mg/l) alone or in combination with NAA (0.2–0.7 mg/l) for shoot induction (Table 2). The calli were inoculated (2.0 gm fresh weight per bottle) in culture bottles containing 150 ml medium. The data for per cent cultures responding, number of shoots and mean shoot length were recorded 45d after culture. In this study, two cytokinins were used for shoot organogenesis from callus i. e. BAP and TDZ. Comparatively, TDZ showed better response than BAP. BAP at 1.5 mg/l produced an optimum 56% response with 15.4 shoots per unit calli. The calli showed maximum percentage response (68) and number of shoots (22.5) on MS medium supplemented with 2.0 mg/l TDZ when used individually. However, the addition of an auxin NAA significantly improved the percentage response and number of shoots. BAP at 1.5 mg/l in combination with 0.2 mg/l NAA produced optimum 67% response. However, the shoot number was optimum (28.4) on 0.5 mg/l NAA in combination with 1.0 mg/l BAP. The highest per cent shoot induction and number of shoots was observed on MS medium supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA. On this medium 94% cultures produced shoots from callus with a mean number of 44.6 after 45d of culture (Table 2). Although the per cent response and number of shoots varied depending on the type of plant growth regulators and concentrations, there was not much difference in mean shoot length. Some regions of the calli turned green after 2 weeks of culture and the shoot initiation started from such regions (Fig. 2F, G). Later the shoots were formed all over the surface of the calli (Fig. 3A).

Table 2
Effect of various concentrations of TDZ or BAP alone or in combination with NAA on shoot organogenesis from callus.

PGRs			% response*	No of shoots*	Mean shoot length*
TDZ	BAP	NAA			
0.0	0.0	0.0	0.0	0.0	0.0
0.5			45 ± 6.3de	10.4 ± 1.9f	1.2 ± 0.08a
1.0			58 ± 7.8c	14.2 ± 2.3e	1.4 ± 0.09a
1.5			60 ± 4.2c	19.2 ± 2.2d	1.2 ± 0.06a
2.0			68 ± 5.1c	22.5 ± 2.5cd	1.0 ± 0.08a
2.5			55 ± 6.6d	14.7 ± 2.1e	1.2 ± 0.05a
	0.5		42 ± 7.9e	9.6 ± 1.9f	0.7 ± 0.05a
	1.0		49 ± 6.2de	12.4 ± 1.6ef	0.9 ± 0.08a
	1.5		56 ± 6.6d	15.4 ± 1.8d	1.2 ± 0.09a
	2.0		28 ± 4.2f	12.8 ± 1.4ef	1.3 ± 0.07a
	2.5		16 ± 2.8g	12.5 ± 2.1ef	1.0 ± 0.05a
2.0		0.2	79 ± 9.7b	31.5 ± 2.2b	1.2 ± 0.09a
2.0		0.5	94 ± 8.4a	44.6 ± 2.8a	1.0 ± 0.06a
2.0		0.7	76 ± 9.8b	31.3 ± 2.9b	1.2 ± 0.07a
	1.5	0.2	67 ± 8.5c	22.9 ± 2.6cd	1.0 ± 0.05a
	1.5	0.5	55 ± 9.1d	28.4 ± 2.8bc	1.1 ± 0.04a
	1.5	0.7	51 ± 8.7d	25.3 ± 2.5c	1.2 ± 0.08a

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Table 2
Efficiency of rooting in *G. febrifugum* on ½ MS medium supplemented with various concentrations of IBA after 45d of culture.

IBA (mg/l)	% response*	Mean number of roots*	Mean root length*
0.0	0.0	0.0	0.0
1.0	77 ± 8.6c	4.6 ± 1.6c	1.8 ± 0.6b
2.0	84 ± 7.9b	6.8 ± 1.4bc	2.1 ± 0.9a
3.0	95 ± 9.6a	10.2 ± 1.9a	2.6 ± 0.8a
4.0	81 ± 8.4bc	8.4 ± 1.8b	1.4 ± 0.7b

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

In the present study, a combination of TDZ and NAA produced the highest shoot induction from callus. The synergistic action of TDZ and auxin-induced shoot induction has been reported in several systems including *Solanum khasianum* (Chirumamilla 2021), *Ficus carica* (Abdolinejad 2020) and *Dieffenbachia* (Shen 2008). TDZ is an active cytokinin extensively used for shoot induction from various explants in different systems (Deepa et al. 2018). TDZ was found more efficient than other cytokinins in some systems (Zhang et al. 2011; (Bhattacharyya et al. 2016). TDZ stimulated shoot organogenesis from various systems including medicinal plants, trees and endangered plants were reported by many workers (for review see Deepa et al. 2018).

In the present study, we used flower bud as explant. There are various reports in which young tissues like flower buds and inflorescence segments were used as explants for shoot induction. In *Arnebia hispidissima* of the various explants like leaf, immature inflorescence and mature and immature seeds, employed for callus induction and shoot regeneration, only immature inflorescence responded (Phulwaria and Shekhawat 2013). Flower buds were used for micropropagation in some systems (Akter et al. 2012; Liao et al. 2011; Yang et al. 2011). Young tissues like immature inflorescence, flower buds etc have high meristematic activity and form excellent material for micropropagation studies (Kackar and Shekhawat 1991; Yadav et al. 2009; Głowacka et al. 2010). There are some reports, which suggest that floral structures have more ability to reproduce vegetatively than other plant parts (Gingas 1991; Lopez-Baez et al. 1993; Merkle et al. 1997; Steinmacher et al. 2007).

Rooting of shoots

For root induction, micropropagated shoots measuring a size of about 1.0 cm or above were used. The shoots were cultured on ½ MS medium supplemented with various concentrations of IBA (1.0–4.0 mg/l). On MS basal medium (control) there was no root induction. Half strength MS medium supplemented with 3.0 mg/l IBA possessed the highest frequency of root induction (95%) and number of roots per explant (10.2) as well as mean root length (2.6 cm). IBA induced root induction has been reported in other

systems like *Plumbago zeylanica* (Sharma and Agarwal 2018), *Pterocarpus marsupium* (Ahmad et al. 2021) and *Swertia minor* (Kshirsagar et al. 2021)

In conclusion, a robust shoot regeneration system through flower bud derived callus organogenesis has been standardized for *G. febrifugum.* This protocol could be utilized for the mass multiplication of this plant for commercial purpose.

Declarations

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Author contributions

SP performed tissue culture experiments, analyzed data, wrote the manuscript. TDT designed the experiment, contributed to writing and corrected manuscript.

Compliance with ethical standards

Ethics approval

This research paper does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figures

Figure 1

Habit of G. febrifugum showing the plant and inflorescence.

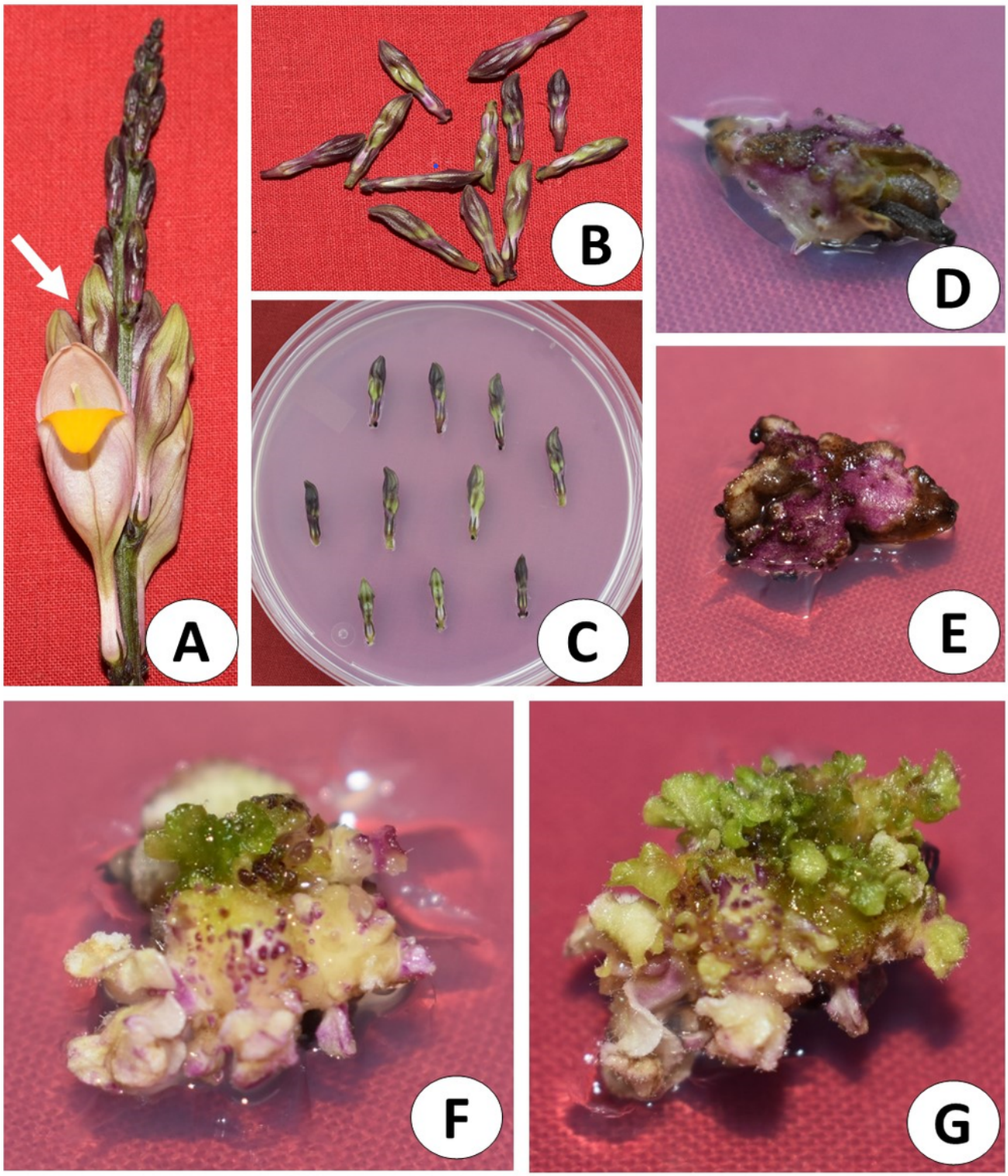

Figure 2

Callus induction and shoot organogenesis from flower bud derived callus in G. febrifugum. A. A close up view of the inflorescence with flower buds. B. Flower buds 7 dBA just before culture. C. Flower buds at the time of culture in Petri plates. D. Flower bud 2 weeks after culture. Calli started emerging from the surface of the explant. E. Same as in fig. 1D, 3 weeks after culture. Thick layer of calli developed all over the

surface of the explant. F. Shoot initiation from calli on MS medium supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA 1 week after culture. Tiny green coloured shoots were emerged from the calli. G. Same as in fig. 1F, 2 weeks after culture. More number of elongated shoots were developed from the calli.

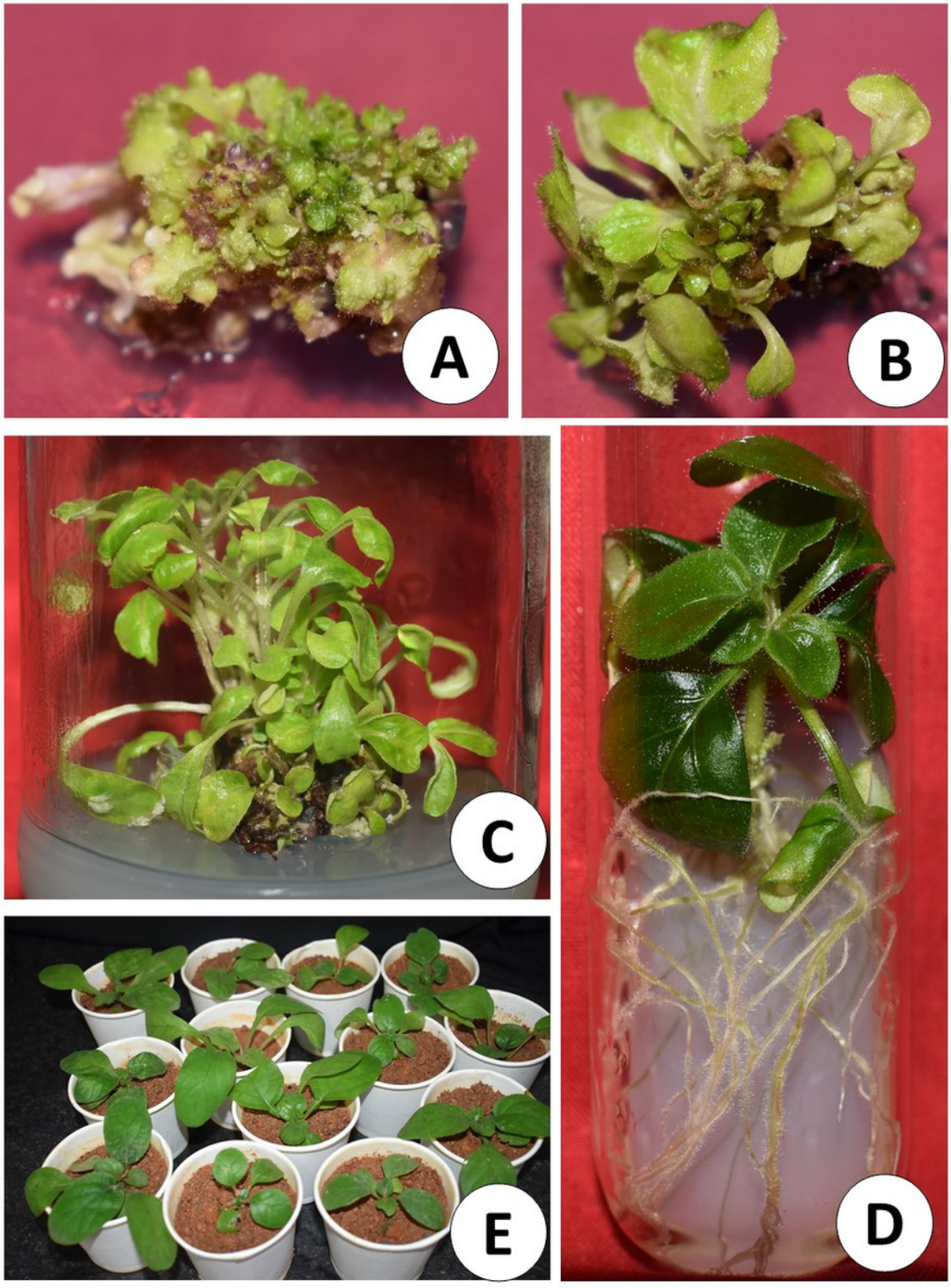

Figure 3

Shoot organogenesis from callus, rooting and transplantation of G. febrifugum. A. Shoots induced from callus on MS medium supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA 3 weeks after culture. More

shoots are visible. B. Same as in fig. 2A 4 weeks after culture. The shoots elongated further. C. Several elongated well developed shoots originated from callus 45d after culture on MS medium supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA. D. Rooting of shoots on ½ MS medium supplemented with 3.0 mg/l IBA 45d after culture. Several roots were emerged from the cut end of the shoot. E. Transplanted plantlets one month after transfer to soil.