https://doi.org/ 10.33472/AFJBS.6.10.2024.3526-3539

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Effect of Jasmonic Acid on Somatic Embryogenesis in *Caralluma fimbriata*

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Article History

Volume 6,Issue 10, 2024

Received:20 Apr 2024

Accepted : 10 May 2024

doi: 10.33472/AFJBS.6.10.2024. 3526-3539

Abstract

Caralluma fimbriata is a succulent plant highly esteemed in traditional medicine for its medicinal properties. Plant hormones, also known as phytohormones, are chemical compounds that regulate several aspects of plant growth and development. The current study demonstrates the establishment of callus culture and investigate the effect of JA (jasmonic acid) on callus in development of somatic embryos and indirect organogenesis from the internodal explants of *C. fimbriata* through micropropagation techniques. MS medium (full-strength) was supplied with different concentrations of auxins (2,4-D, IAA, IBA, NAA) and the optimal callus growth was observed in MS medium + 2,4-D (2.0 mg/l) + NAA (1.0 mg/l) with 100% of callus growth response. JA influenced in producing somatic embryos from the callus at almost all concentrations. Highest shoot induction response (80%) was observed on MS medium+ 0.5 mg/l BAP with highest mean frequency of 9.53 ± 1.20^{a}, compared to Kn. Rooting was successfully accomplished on MS medium (half-strength) with supplementation of MS salts + 0.1 mg/l NAA. The survival rate of acclimatized plants was recorded at 80%.

Keywords: *Caralluma fimbriata*; jasmonic acid; Somatic embryos; Auxin; Cytokinin

Abbreviations

2,4-D: 2,4-Dichlorophenoxy acetic acid;
BAP: Benzyl amino purine;
CFE: *Caralluma fimbriata* extract;
IAA: Indole acetic acid;
IBA: Indole butyric acid;
JA: Jasmonic acid;
Kn: Kinetin;
MS: Murashige & Skoog;
NAA: Naphthalene acetic acid

Introduction

Caralluma fimbriata belongs to the family Apocynaceae that is native to India and other Asian countries which has been used for centuries in traditional medicine. People have long employed the *C. fimbriata* for its capacity to control hunger and aid in weight reduction. It has become more well-known recently as a dietary supplement for controlling weight with commercial brand names such as SLIMALUMA®.

It is believed to have a variety of therapeutic properties meaning that it may have a range of effects on the body and has been the subject of several scientific studies. In the recent research findings on *Caralluma fimbriata* extract (CFE) in preventing damage to the heart tissues induced by high-fat diet in comparison with metformin treated rats showed successful in conforming that CFE may be utilised as adjuvant treatment to treat or prevent heart damage brought on by a high-fat diet (Gujjala et al., 2022).

One study published in the journal Scientific Reports, 2021 to investigate the impact on body composition and hunger satisfactory indicators in obese people by *Caralluma fimbriata* extract (CFE), which is a randomised, double-blind, placebo-controlled found that supplementation with *C. fimbriata* extract resulted in significant reductions in waist circumference and appetite in overweight individuals (Rao et al., 2021). Other studies have examined the potential mechanism of *Caralluma fimbriata* in reducing stress and anxiety in adults by changing the cortisol levels significantly (Kell and Katsikitis, 2019).

Earlier studies have reported that, *Caralluma fimbriata* may have antioxidant (Vajha and Chillara, 2014; Gujjala et al., 2016), anti-inflammatory, antihyperglycemic activity (Ashwini and Anitha, 2017), hypolipidemic activity, renoprotective effect (Gujjala et al., 2016) anti-carcinogenic potential (Suhasini et al., 2019), nociceptive action, nootropic action and antimicrobial properties. These phytopharmacological activities, of *Caralluma fimbriata* are mainly due to the presence of certain active compounds. These compounds may include pregnane glycosides, flavonoids, saponins, and other phytochemicals that are found in the plant (Periyasamy, 2011; Vitalone et al., 2017). *Caralluma fimbriata* is safe to eat and has been endorsed by experts due to the lack of any negative side effects as it was approved by Therapeutic Good Administration, Australia (Saboo, 2011).

Micropropagation is a method of plant propagation that involves growing plant tissue in a sterile laboratory environment, using techniques such as tissue culture and genetic engineering. This approach may rapidly generate several genetically identical plants in vast number for a variety of purposes, including conservation, research, and commercial production. There have

been several studies that have explored the use of micropropagation techniques for the production of *Caralluma* species.

The research findings published by Rajaram et al. (2012), has demonstrated that callus was obtained from the internodal segments of *C. fimbriata* on the MS media supplemented with 0.5 mg/L 2, 4-D +1.0 mg/L NAA and highest shoot number (6.2 shoots) have been regenerated from the green organogenic calli by transferring onto the media with 2, 4-D (0.5 mg/l) +(BAP 4.0 mg/l). Another study reported indirect organogenesis through somatic embryoids in the presence of NAA developed from the callus generated on MS medium fortified with 2 mg/L 2iP + 0.5 mg/L Zeatin in *Caralluma pauciflora* (Kiranmai et al., 2015).

In 2017 an investigation on micropropagation studies on *C. fimbriata* resulted in highest shoot proliferation (91%) from the explants regenerated from MS media provided with BA (2.0 mg/L) + Kn (0.5 mg/L) + IBA (0.2 mg/L) and callus induction was reported in combination with NAA (1.5 to 2.0 mg/L) + 2, 4-D (2.0 mg/L) (Ramachandra Naik et al., 2017) . Present study focusses on to investigate the effect of various cytokinins and auxins along with elicitor, jasmonic acid in micropropagation of *Caralluma fimbriata* on solid MS medium.

Materials and Methods

C. fimbriata plants were collected from the wild near Udayagiri hills, Nellore and transplanted into pots maintained in the shade net house, Vikrama Simhapuri University, SPSR Nellore (Andhra Pradesh) India. To get rid of dust, the collected newly formed juvenile shoots of *C. fimbriata* were thoroughly washed under running water from the faucet. After gently stirring in Tween-20 (2% v/v) for 10 minutes to clean the explant's surfaces, they were rinsed with distilled water. Following their transfer to a laminar airflow (LAF) area, the explants were transferred to conical containers with a capacity of 1/2 litre for further surface sterilisation. After 60 seconds in 70% ethanol, the items were repeatedly washed (for 3 to 4 rinses) in sterile water. These were subsequently washed with disinfected water (3–4 rinses) after being treated with 0.1% mercuric chloride ($HgCl_2$) for 5 minutes to remove any remaining mercuric chloride from the material. The disinfectants like H_2O_2 and NaOCl resulted in contamination. Throughout sterilisation, the flask containing the explants was vigorously shaken. Then they were set on medium for the callus induction after the cut edges were trimmed horizontally.

MS nutrient media is taken as the culture media for this experiment. The nutrient medium is comprised of macronutrients and micronutrients supplemented with 3% sucrose, various concentrations of auxins (2,4-D, IAA, IBA, NAA), cytokinins (BAP, Kn), and JA in addition to other combinations. All of the plant growth regulators were incorporated into the medium prior to autoclaving. Before subjecting the medium to autoclaving for 15 minutes at 108 kPa and 121^{o}C, its pH was adjusted to 5.7. Equal amounts of media (10ml) were loaded into culture tubes that were covered in aluminium foil. Every culture was kept alive in a growth chamber. Parallel fluorescent tubes that were put above the cultures provided artificial lighting. 2000 lux of light was present, and the surrounding temperature ranged from 25^{o}C ± 2. At a 4-week interval, subculture was periodically performed. At intervals of 4 weeks, the type of response and its percentage were also noted. The induced callus was transferred to MS media fortified with cytokinins (BA and Kn) for further response. On MS medium (half-strength) treated with different auxin concentrations (IAA, IBA and NAA), the regenerated shoots were rooted.

Statistical Analysis

Each experiment had fifteen replicates, and each experiment was performed three times. At a significance level of 0.05 percent, the means were analysed via the Tukey test. In order to statistically assess the data, one-way analysis of variance was implemented. The Pearson correlation analysis was performed using Python programming language, employing Pandas library for data frame administration (Virtanen, 2023) between different jasmonic acid concentrations and weights of callus. This included computing a correlation matrix to measure the extent of correlation between the two variables. The correlation coefficient, a statistical metric, was used to analyse the magnitude and orientation of this association.

Visualization

For an intuitive understanding of the correlation results, we employed a heatmap visualization. The heatmap was generated using Seaborn, a Python-based data visualization library, which provided a color-coded representation of the correlation matrix (Rogel-Salazar, 2023). The colour intensity in the heatmap corresponded to the strength of the correlation, with a scale ranging from -1 (perfect negative correlation) to +1 (perfect positive correlation), and zero indicating no correlation. This visualization facilitated an easy interpretation of the correlation between jasmonic acid concentration and callus weight.

Results

Callus Studies

Different concentrations of 2,4-D, IAA, IBA and NAA (0.5, 1.0, 2.0, and 4.0 mg/l) were supplied to the MS medium to induce callus formation from *C. fimbriata* internodal explants. After three weeks of culturing, callus reaction was found in the majority of concentrations. However, various amounts induce callus formation to varying degrees. The highest degree of callus formation was found in MS medium supplemented with 2,4-D (80 %) at a concentration of 2.0 mg/l compared with other auxins. No callus formation was seen with 2,4-D, IAA, IBA and NAA (0.5 mg/l), the lowest concentration tested. 20% to 40% of callus initiation, was noted with 2,4-D, IAA, IBA and NAA at 1.0 mg/l. With concentration of 4.0 mg/l for 2,4-D, IAA, IBA and NAA 20% to 60% of callus formation was observed (Table 1).

Table 1: Effect of different concentrations of 2, 4- D, IAA, IBA and NAA in callus induction.

MS + Auxin	Concentration (mg/l)	% of response	Degree of callusing
2,4-D	0.5	10	-
	1.0	30	+
	2.0	80	+++
	4.0	60	++
	0.5	-	-

IAA	1.0	20	+
	2.0	40	++
	4.0	30	+
IBA	0.5	10	-
	1.0	20	+
	2.0	30	+
	4.0	30	+
NAA	0.5	10	-
	1.0	30	+
	2.0	30	+
	4.0	20	+

Note: The experiment was repeated thrice with (n=15) replicates.

Among all other concentrations, 2,4-D (2.0 mg/l) is considered in producing maximum degree of callusing. Hence, other auxins like NAA, IAA and IBA with different concentrations of 0.5, 1.0 and 2.0 mg/l were added to test further callus induction. After a week from the day of subculturing the callus growth was observed. 100 % callusing was observed in MS media fortified with 2,4-D (2.0 mg/l) + NAA (1.0 mg/l), which is maximum among all other various concentrations of auxins. 80% and 70% of callusing was observed from 0.5 and 2.0 mg/l concentrations of NAA respectively (Table 2).

Among different concentrations of IAA 60%, 40% and 20% and with IBA 70%, 40% and 30% in degree of callusing was observed in 0.5, 1.0 and 2.0 mg/l concentrations respectively. Maximum degree of callusing was resulted from lowest concentrations of auxins supplemented. As the concentration of auxins increased the callus growth gradually decreased, except with NAA (Table 2).

Table 2: Effect of various auxins at different concentrations on callus induction from internodal explants of *C. fimbriata* on MS medium.

MS + Auxins				**% of response**	**Degree of callusing**
2, 4- D (mg/l)	**NAA (mg/l)**	**IAA (mg/l)**	**IBA (mg/l)**		
2.0	0.5	-	-	80	++

	1.0	-	-	100	+++
	2.0	-	-	70	++
	-	0.5	-	60	++
	-	1.0	-	40	+
	-	2.0	-	20	+
	-	-	0.5	70	++
	-	-	1.0	40	+
	-	-	2.0	30	+

Note: The experiment was repeated thrice with (n=15) replicates.

Effect of jasmonic acid on callus and somatic embryogenesis

The callus developed from MS + 2,4-D (2.0 mg/l) + NAA (1.0 mg/l) was tested with same media composition along with different concentrations of jasmonic acid which is a potential chemical elicitor. Callus was transformed in the presence of various concentrations of JA and sub-cultured twice, among them nodular callus was observed after 6th week at 0.25 mg/l (Figure 1A), embryogenic callus was observed in all remaining concentrations from 0.5 mg/l to 2.0mg/l (Figure 1B-D), whereas the concentration of JA increased the proliferation of embryogenic callus was also increased from 0.5 mg/l to 2.0mg/l (Table 3) after 8th week.

Table 3: Effect of jasmonic acid along with 2, 4- D and NAA on callus regeneration of *C. fimbriata* on MS medium.

MS + Auxins	**Chemical elicitor**		**Weight of the callus (gms)**
2, 4- D (2 mg/l) + NAA (1 mg/l)	jasmonic acid (mg/l)	0.25	1.74 ± 0.15
		0.5	2.21 ± 0.16
		1.0	2.74 ± 0.11
		1.5	2.96 ± 0.09
		2.0	3.14 ± 0.14

Note: The experiment is repeated thrice with (n=15) replicates.

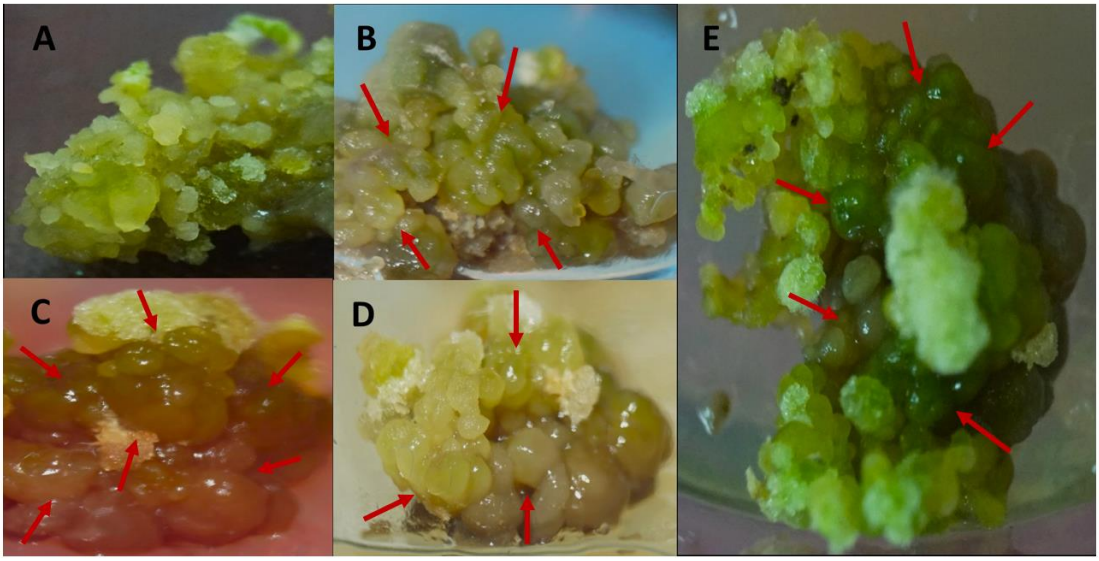

Figure 1: Effect of jasmonic acid at different concentrations on the callus of *C. fimbriata*. A- Nodular callus (JA, 0.25 mg/l), B, C, D and E- Embryogenic callus (JA, 0.5 to 2.0mg/l).

Correlation analysis

The correlation matrix between different concentrations of jasmonic acid and weight of the callus obtained was depicted using heatmap for visualization (Figure 2). The value of correlation coefficient is obtained as '0.95'. Which suggests a very strong positive correlation as the concentration of jasmonic acid increased (from 0.25 mg/l to 2.0 mg/l) the weight of the callus gradually increased.

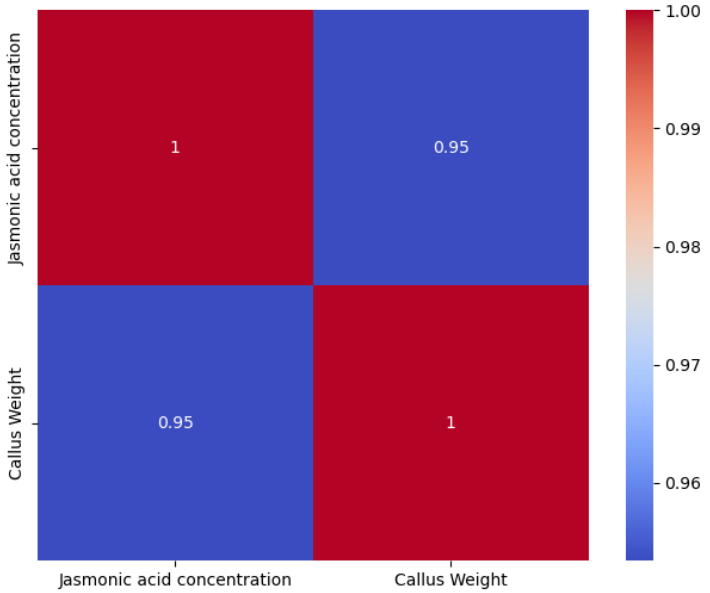

Figure 2: Heatmap for the correlation between jasmonic acid concentrations and weight of the callus.

Shoot Organogenesis

For organogenesis, the growth regulators that induce callus exert a significant influence. Callus produced on MS medium supplemented with 2,4-D (2.0 mg/l) + NAA (1.0 mg/l) + JA (1.5, 2.0

mg/l), was chosen for morphogenesis due to the highest degree of callusing being observed. The embryogenic callus induced was sub-cultured on to the MS media supplemented with different concentrations of cytokinins (BAP and Kinetin). After 5 weeks of sub-culturing, cells are differentiated into shoot buds from the surface of callus. Shoot bud proliferation from callus was seen mostly in the lowest concentrations of cytokinins (Kiranmai et al., 2015).

Among different concentrations of cytokinins, the highest response with 80% of shoot regeneration and maximum number of shoots with mean frequency 9.53 ± 1.20^{a} were recorded at MS medium fortified with 0.5 mg/l BAP (Figure 6 E,F & G) (Table 4), followed by 60% of shooting frequency with mean average of 6.93 ± 0.92^{b} from MS medium containing 1.0 mg/l BAP. Least shoot response of 30% with mean frequency 3.40 ± 0.89^{d} was observed in MS medium with 0.5 mg/l Kn.

Table 4: Effect of BAP and Kinetin in regeneration of shoots from the embryogenic callus induced from internodal explants of *C. fimbriata* after 5 weeks.

MS + Cytokinins		**% of shoot response**	**Morphogenic response (Shoot number) Mean ± S. D**
BAP (mg/l)	**Kn (mg/l)**		
0.5	-	80 %	9.53 ± 1.20^{a}
1.0	-	60 %	6.93 ± 0.92^{b}
-	0.5	30 %	3.40 ± 0.89^{d}
-	1.0	50 %	5.86 ± 0.80^{c}

Note: The experiment was repeated thrice with (n=15) replicates. The values followed by different alphabets are statistically significant ($P<0.05$) and those with same alphabet are not significant.

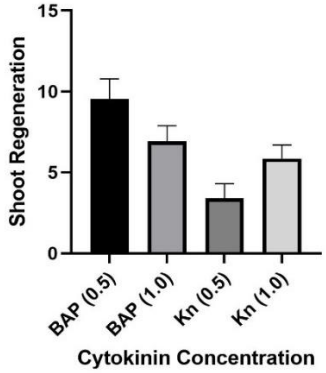

Figure 3: Effect of cytokinin concentration on shoot regeneration in *Caralluma fimbriata.*

Rooting of regenerated shoots

The callus-regenerated shoots were removed and transplanted to half-strength MS media containing auxin for rooting *in vitro*. The impact of auxin concentration on *in vitro* rooting was variable depending on the medium supplied with auxins (Table 5). The highest root per shoot response of 65% with maximum number of roots generated from single shoot with mean frequency of 6.33 ± 0.89^{a} and the maximum root length with mean frequency of 2.88 ± 0.20^{a} cm was observed on ½ MS medium supplemented with 0.1 mg/l NAA (Figure 6 H). Rooting frequency of 50% with mean frequency (5.13 ± 0.74^{a}) of roots per shoot and with root length with mean frequency (1.99 ± 0.14^{c} cm) was observed on medium containing 0.1 mg/l IAA. Lesser concentrations of IBA result in root development, whereas greater concentrations prevent rooting.

Table 5: Effect of various auxins on rooting response from *in vitro* regenerated shoots of *C. fimbriata* cultured on ½ MS medium (after 4 weeks).

½ MS+ Auxins			% of root/shoot response	No. of roots/shoots Mean ± S. D	Length of roots (cm) Mean ± S. D	Degree of callusing
NAA (mg/l)	IAA (mg/l)	IBA (mg/l)				
0.1	-	-	65%	6.33 ± 0.89^{a}	2.88 ± 0.20^{a}	-
0.5	-	-	40%	3.93 ± 0.79^{ab}	2.46 ± 0.21^{b}	-
1.0	-	-	-	CP	-	++
-	0.1	-	50%	5.13 ± 0.74^{a}	1.99 ± 0.14^{c}	-
-	0.5	-	26%	2.66 ± 0.97^{b}	1.46 ± 0.81^{d}	-
-	1.0	-	-	NR	-	+
-	-	0.1	30%	2.93 ± 0.79^{ab}	0.39 ± 0.24^{e}	-
-	-	0.5	-	CP	-	+
-	-	1.0	-	NR	-	+

Note: The experiment was repeated thrice with (n=15) replicates. The values followed by different alphabets are statistically significant ($P<0.05$) and those with same alphabet are not significant.

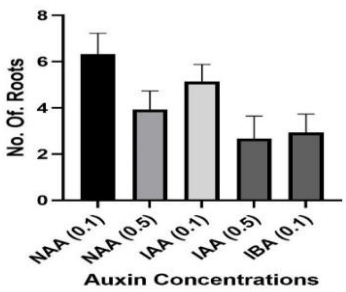

Figure 4: Effect of auxin concentrations on root regeneration in *Caralluma fimbriata.*

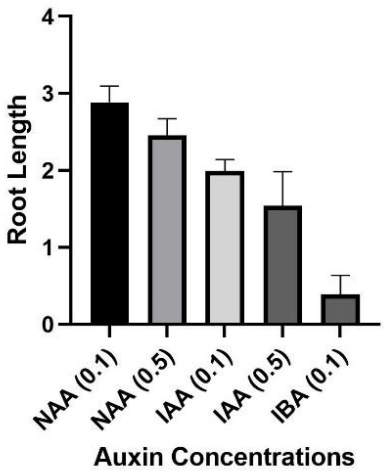

Figure 5: Effect of auxin concentrations on root length in *Caralluma fimbriata.*

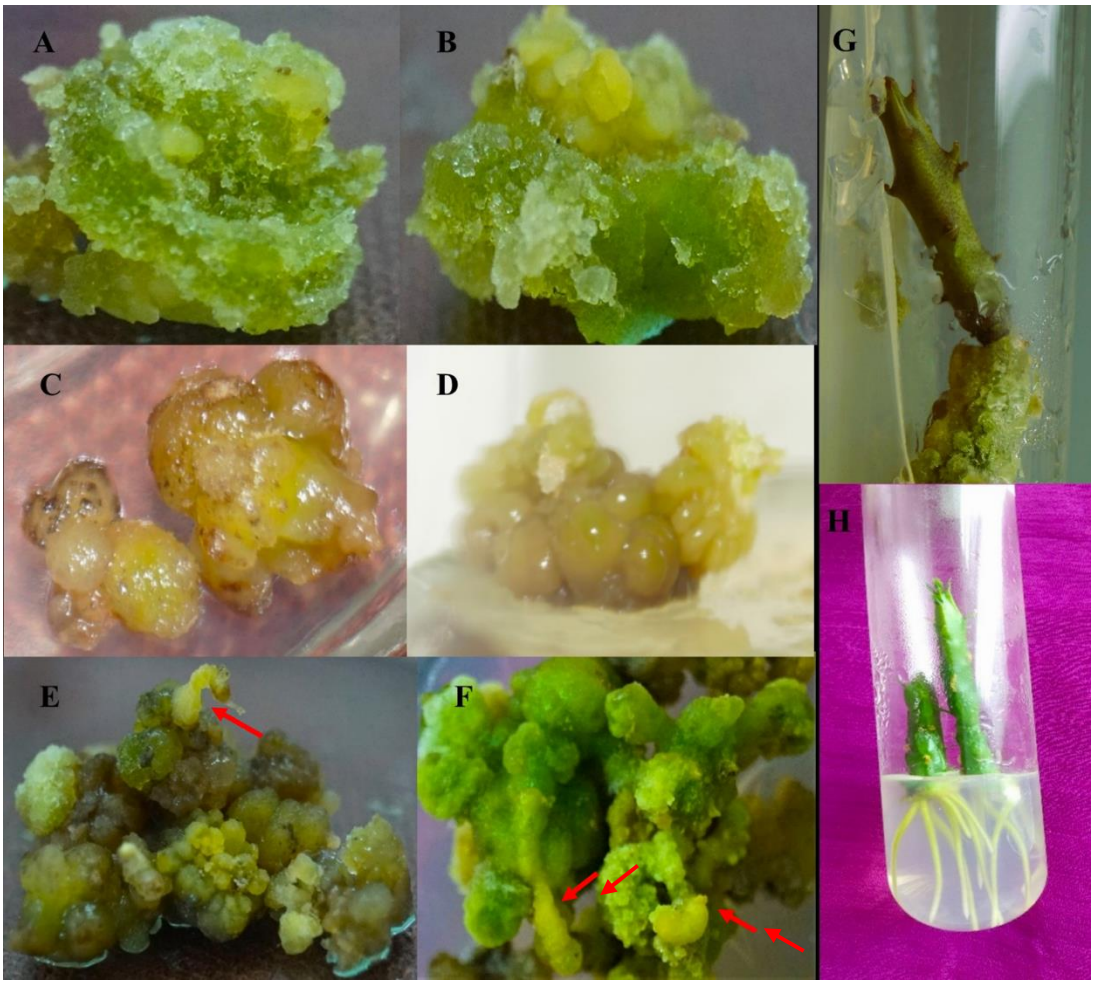

Figure 6: A, B: Fresh green callus developed on MS + 2, 4-D (2.0 mg/l) + NAA (1.0 mg/l); C: Somatic embryoids developed on MS medium + JA (1.0 mg/l) + 2, 4-D (2.0 mg/l) + NAA (1.0 mg/l); D: Somatic embryos developed on MS medium + JA (1.5, 2.0 mg/l) + 2, 4-D (2.0 mg/l) + NAA (1.0 mg/l); E, F: Shoot bud initiation and, G: Shoot organogenesis from the embryogenic callus on MS medium + BAP (0.5 mg/l); H: *In vitro* rooting on ½ MS medium+ NAA (0.1 mg/l).

Acclimatization

After removing rooted plantlets from the culture tube with care, they were thoroughly rinsed with sterile distilled water to eliminate any remaining agar. These plants were transplanted into plastic cups containing sterilised peat moss and sand in a ratio of 3:1 (Figure 7). Pots were first put in a culture chamber with a temperature of 25^{o}C, a photoperiod of 16 hours, and a relative humidity of 85 %. The potted plants were watered every five days for three weeks with a one-fourth-strength MS basal salts solution free of sucrose and myo-inositol. After 25 days, the hardened plants were moved to clay pots, maintained in the shade, and acclimatised. A total of 80 % of *C. fimbriata* plants were effectively field adapted.

Figure 7: Acclimatization of rooted *C. fimbriata* plants in cups containing coco peat and sand with 3:1 ratio.

Discussion

Jasmonic acid (JA) and its derivatives, referred to as jasmonates, which are essential for plant stress responses, somatic embryo development, and plant regeneration. These lipid-derived messenger molecules modulate multiple physiological processes in plants via diverse signalling networks, within and exogenously (Wang et al., 2020).

The current study illustrates the influence of jasmonic acid on both mass and morphogenic characteristics of callus in *C. fimbriata.* Rudus et al. (2009) documented the function of jamonic acid in addition with abscisic acid (ABA) internally on somatic embryogenesis in *Medicago sativa*. Mira et al. (2016) demonstrated the modulation of jasmonic acid along with nitric oxide (NO) in development of somatic embryos in *Arabidopsis.* The research describes the effectiveness of jasmonic acid in production of somatic embryos by down regulating MYC2 and enhancing the activity of JAZ1 (Jasmonate-Zim-Protein1) and PDF1 (Plant defensin 1) which is regulated by NO through activity of JA promoting its pathway related key enzyme coding genes (Allene oxide synthase and Lipoxygenase 2).

In *C. fimbriata* the significant correlation coefficient of 0.95 indicates that variations in the concentration of jasmonic acid may explain around 90.25% (since 0.95^2=0.9025) of the variability observed in callus weight. This percentage is substantial, indicating that the concentration of jasmonic acid is a significant factor in determining not only morphology but also the weight of callus. A strong correlation between the concentration of jasmonic acid and the weight of callus is indicated.

Our research follows the development of callus and somatic embryos from internodal explants of *C. fimbriata*, just as *Euphorbia hirta* did (Shekhawat et al., 2018). However, previous investigations on Euphorbiaceae members have also documented somatic embryo formation from other explants, including immature anthers in *Hevea brasiliensis* (Jayasree et al., 1999) and floral parts of *Manihot esculenta* (Woodward and Puonati-Kaelas, 2001). A study by

Sreelatha and Pullaiah (2015) reported initiation of somatic embryos (globular stage) in *Caralluma sarkariae* in the medium supplemented with 2.0 mg/l picloram and later observed different stages (torpedo and cotyledon) upon subculturing onto different composition of media. However, in the present study different stages of somatic embryos from the *C. fimbriata* calli are observed in the same medium after subculturing.

Recent studies reported somatic embryogenesis in plants *Buchanania lanzan* (Jha et al., 2022) and *Euphorbia hirta* (Shekhawat et al., 2018) in presence of both auxin and cytokinin combination along with the MS salts whereas somatic embryogenesis in *C. fimbriata* is seen in the presence of auxins (2,4-D & NAA) along with the JA. Several studies reported the formation of embryos in the presence of cytokinins along with auxins compared to auxins alone. Martin et al. (2005) reported somatic embryogenesis in *E. nivulia* using BAP and 2,4-D and Singh et al. (2015) found 2,4-D alone generated least number of somatic embryos in *Sapindus mukorossi.* Indeed, the addition of JA to auxins (2,4-D, NAA) in the induction of somatic embryos has been supported by many research findings. The maximum percentage of shoot regeneration from embryogenic callus was observed in BAP at lower concentrations than in kinetin. In *C. fimbriata,* BAP is more sensitive than Kn to shoot organogenesis from callus. NAA exhibits eventual root regeneration with greater maximal height and greater number at low concentrations than IAA and IBA. As the increasing auxin concentrations inhibited the development of root. Following this, the *in vitro*-regenerated plant undergoes three weeks of acclimatisation under controlled conditions prior to being hardened.

Conclusion

Studies of *in vitro* propagation demonstrate that *Caralluma fimbriata* may be effectively micropropagated, allowing numerous herbal manufacturers and companies to produce the plant via tissue culture and avoid their economic exploitation in the wild. The somatic embryogenesis induced by jasmonic acid in *C. fimbriata* provides a viable and effective method for achieving rapid multiplication while maintaining genetic stability and uniformity. Additionally, year-round production is facilitated through the elimination of pathogens and diseases, which enhances both yield and quality. This guarantees pharmaceutical industries a steady and sustainable supply of raw materials. By means of propagation and preservation, this strategy aids in the conservation of biodiversity. As *C. fimbriata* may have potential as a weight reduction aid, cultivating plants *in vitro* facilitates the further research required to fully comprehend its effects and establish the optimal dose and duration of usage for therapeutic purposes.

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