IN VITRO PROPAGATION AND MOLECULAR CHARACTERIZATION OF CRITICALLY ENDANGERED *Crinum malabaricum* FOR CONSERVATION AND SECONDARY METABOLITE PRODUCTION

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

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By

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Declaration

I, hereby declared that the presented work in the thesis entitled "***In vitro* propagation and molecular characterization of critically endangered *Crinum malabaricum* (Amaryllidaceae) for conservation and secondary metabolite production"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Vijay Kumar, working as Associate Professor, in the Department of Biotechnology, School of Biosciences and Bioengineering of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigators. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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Certificate

This is to certify that the work reported in the Ph. D. thesis entitled "***In vitro* propagation and molecular characterization of critically endangered *Crinum malabaricum* (Amaryllidaceae) for conservation and secondary metabolite production**" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Biotechnology, School of Biosciences and Bioengineering, is a research work carried out by Ms. Swati Chahal (11916700), is a bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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अज्ञान तिमिरान्धस्य ज्ञानाञ्जन शलाकया ।

चक्षुरुन्मीलितं येन तस्मै श्री गुरवे नमः ॥

(A Guru can save us from the pangs of ignorance by applying the us the balm of knowledge of the Supreme, I offer my salutations to such a Guru)

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Abbreviations

Abbreviations	Descriptions
2, 4- D	2, 4-Dichlorophenoxyacetic Acid
ANOVA	Analysis of Variance
BA	6-benzyl adenine
BAP	6-Benzylaminopurine
CM	*Crinum malabaricum*
CTAB	Cetyl Trimethylammonium Bromide
CYP96T1	cytochrome 450monooxygenase 96T1
GAL	Galanthamine
GA_3	Gibberellic Acid
GC-MS	Gas Chromatography- Mass Spectrometry
$HgCl_2$	Mercuric Chloride
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
IAA	Indole-3-Acetic acid
IBA	Indole-3-Butyric acid
IR	Infrared Spectroscopy
ISSR	Inter Simple Sequence Repeats
IUCN	International Union for Conservation of Nature
JA	Jasmonic Acid
KN	Kinetin

LC-MS	Liquid Chromatography- Mass Spectrometry
LY	Lycrorine
MeJa	Methyl Jasmonate
MS	Murshige & Skoog
MAE	Microwave Assisted Extraction
NaOH	Sodium Hydroxide
NAA	α-Naphthalene Acetic Acid
NBS	norbelladine synthase
NMR	Nuclear Magnetic Resonance
N4OMT	norbelladine-4'-O- methyltransferase
NR	noroxomaritidine reductase
PAL	Phenylalanine
PGR	Plant Growth Regulators
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
RFLP	Random Fragment Length Polymorphism
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SA	Salicylic Acid
SCoT	Start Codon Targeted Polymorphism
SPE	Solid Phase Extraction
TDZ	Thiadizuron

TLC	Thin Layer Chromatography
TYDC	Tyrosine Decarboxylase
UAE	Ultrasonic-Assisted Extraction
UHPLC	Ultra-High performance Liquid Chromatography

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Abstract

The Amaryllidaceae family has a long history in the traditional medicinal system and is used to treat diverse diseases. It is well known for its structurally diverse alkaloids which exhibit a wide range of pharmacological activities. The genus *Crinum* accommodates important bulbous geophytes and is famous for the ornamental and therapeutic properties of its species. In India, this genus is represented by 13 different species which contain several alkaloids with biological properties. However, some of the medicinal uses of Indian Crinum species still require pharmacological analysis. Further research is essential to improve our current knowledge about the bioactive components, toxicity, clinical relevance and their conservation status.

Medicinal plants are important for improving human health and represent an essential pool for the identification of novel pharmacological leads. Plant-derived biomolecules for a long time have proven their potential as probable contributors for the identification and characterization of novel drug leads. Many different alkaloids possessing a broad range of pharmacological activities have been isolated from plants belonging to the Amaryllidaceae family. One such compound is galanthamine. Galanthamine (GAL) is produced by the Amaryllidaceae family members and doctors use it to treat patients suffering from Alzheimer's among other conditions pertaining to dementia. Naturally, the bioavailability of Amaryllidaceae alkaloids including GAL is low. Recently there has been a significant increase in the demand for GAL by the pharmaceutical industries, but the inadequate availability of natural resources has proven to be a big hindrance leading to the chemical synthesis of this compound by the pharma industries which leads to the occurrence of various side effects like vomiting, headache, and fever in the patients. Herbal drugs are widely known for their negative side effects therefore, the implementation of biotechnological techniques could serve as a better approach for the broad-spectrum production of medicines from plants. Besides being an environmentally sustainable approach, this type of method also protects the native biodiversity in a circular bioeconomy context. Thus, different biotechnological tools can be implemented to optimize the *in vitro* GAL biosynthesis for treating AD.

Crinum malabaricum, Lekhak & S.R. Yadav (Amaryllidaceae) is a critically endangered bulbous plant endemic to India. Establishing an efficient plant regeneration protocol is necessary for its conservation and large-scale propagation. *Crinum malabaricum* is also a promising natural source of bioactive compounds including galanthamine (GAL), an anti-Alzheimer drug. *In vitro* regeneration in the Amaryllidaceae is often challenging. The purpose

of this study was to determine how *meta*-Topolin (*m*T) affected *C. malabaricum*'s *in vitro* regeneration. For six weeks, shoot explants were grown on plant tissue culture medium that had been supplemented with 0.5, 2.5, 5.0, 7.5, and 10.0 µM *m*T, whereby 7.5 µM *m*T resulted in the maximum multiplication of adventitious shoots, much higher than the control. These findings highlight the beneficial effect and validate the rising importance of *m*T for in vitro regeneration studies. This study will serve as a potential protocol to conserve and restore the medicinally important *C. malabaricum*.

Somatic embryos developed from callus grown on MS media supplemented with varied concentrations of 2,4-D alone and in conjunction with N6-benzyl-adenine (BA) were also used to accomplish regeneration. Different combinations of picloram and TDZ were employed on culture media for the advanced stages of embryo development (globular, torpedo and cotyledonary) and maturation. A high rate of somatic embryos (55±0.89) was obtained after eight weeks. SEM examinations showed the occurrence of cell clusters (embryogenic) which converted to somatic embryos. Well-developed cotyledon embryos were successfully germinated on PGRs free full and half strength MS medium, however, 94.03 % of somatic embryos germinated on MS medium of half-strength fortified using 1.0 mg L^{-1} GA_3. The true-to-type genetic conformity of regenerated plants was examined by SCoT, ISSR and RAPD primers based molecular analysis. This confirmed their genetic homogeneity compared to the mother plant and demonstrated the reliability of our somatic embryogenesis system for *C. malabaricum*. A rapid method for phytochemical analysis based on LC-ESI/MS exhibited better separation and analysis of galanthamine and lycorine from methanolic extracts of *in vitro* raised plants derived from somatic embryogenesis. The biochemical accumulation of eleven different phenolic acids was quantified by UHPLC-MS/MS analysis, and it appeared that *m*T-treated cultures exhibited the highest concentration of phenolic acids. In particular, increased concentrations of gallic acid, syringic acid, protocatechuic acid, vanillic acid, salicylic acid and p-hydroxybenzoic acid were detected compared to the control. *m*T (2.5 and 5.0 µM) produced the maximum amount of chlorogenic acid, ferulic acid, p-coumaric acid and sinapic acid. However, an increased content of caffeic acid was produced on PGR-free medium. The protocol developed should be helpful in reintroduction, restoration and *ex situ* conservation of this valuable plant in the natural condition as well as in the pharmaceutical sectors.

Chapter 1 | Introduction

Plants have always been the primary source of therapeutic agents since time immemorial. Existing texts dating back to several hundreds and thousands of years back suggests that the information has been preserved about the therapeutic effects of plants in conjunction with many therapies by Egyptians, Chinese, Romans, and Greeks. However, the Indian traditional medicinal system has records dating back to 5000 years and 10000 years if we consider "Siddha" system of medicine which is based on "*Saiva*" philosophy [1]. The best known examples of available records throughout the world are the "*Ebers Papyrus*" (1500 BC) from Egypt; the clay tablets of the Mesopotamia (1700 BC); *Erh-ya* (300 B.C), *Svu-ching* (1000 B.C) and *Ben-tsao* (1250 A.D) from China; "*Charka Samhita*" (100-800 B.C), "*Sushruta Samhita*" (800-700 B.C), "*Rigveda*" (1400-1800 B.C), and "*Atharva-Veda*" (4500-2500 B.C) from India. The works of Roman and Greek philosophers like Hippocrates (460-370 B.C.), Theophrastus (370-287 B.B.), Aristotle (384-322 B.C.), and Dioscorides (50-100 A.D) are highly recognized for their collection, storage and usage of traditional medicine and still being used today in many forms for various disease treatments. Additionally, the Ayurveda which has been an axiom of Vedic knowledge dated back to 1000 BC has been in practice for understanding the curative properties of the plants and is a pharmacopoeia of "*Charka Samhita*", "*Sushruta Samhita*" and "*Ashtanga Hridaya Samhita*" [2]. In 2022, a Host Country Agreement has been signed by the WHO and Ministry of Ayush, India to establish the world's first ever Global Centre for Traditional Medicine (GCTM) at Jamnagar, Gujarat [3]. Jamnagar will serve as the hub for devising and integrating new policies and standards on traditional medicine and in engaging to benefit all regions of the world, which paints quite a picture of the future with India playing a leading role in setting the trends for ethnomedicine. India and China are the leading Asian countries of the world when it comes to medicinal plant research and the collaborative research between India and first world countries involving the production of new plant derived medicines with scientific testing and efficacy is increasing [4]. This leads to the topic of preservation, conservation, and domestication of not only the natural habitats harbouring species contributing to advanced curative properties but the species themselves. A bibliometric survey of Scopus database done by Manzano et al. in 2020 of more than 100,000 publications indicated that the trend of comprehensive research has shifted more towards the search for new pharmaceutical or active compounds rather than the conservation or domestication of plant species. According to WHO about 40% of pharmaceutical products present in the market are derived from natural sources which emphasizes the importance of biodiversity conservation and sustainability.

1.1. Medicinal importance of *Crinum* species

Members of Amaryllidaceae J.St.-Hil. family are well known for their pharmacologically active alkaloids also known as Amaryllidaceae alkaloids. These members possess distinctive medicinal properties akin to anti-tumor, antioxidant, antimicrobial, anti-diabetic, anti-malarial, immuno-stimulating because of the presence of alkaloids like Crinamine, galanthamine, norgalanthamin, crisabetaine, gallic acid, catechin, hemanthamine, belladine, cholorrogenic acid, coumaric acid and many more [5]. The genus *Crinum* is prominent for their curative properties like tonics laxatives, anti-pyretics, anti-aging etc. The bioformulations concocted from boiled or powdered roots, leaf juice and roasted bulbs of crinum are used for the treatment of numerous disorders like haemorrhoids, rheumatism, herpes, swelling, earaches, ipecac, and night blindness [6].

The genus *Crinum* in India (Fig. 1) comprises of 15 species each possess unique medicinal properties with the addition of some newly discovered and lesser-known species like *C. malabricum* Lekhak & S.R. Yadav; *C. solapurense* S.P. Gaikwad, K.U. Gurad & R.D. Gore; and *C. brachynema* Herb. *Crinum malabaricum* is one of the largest fresh water, wet-land dependent plant species which is endemic to south – western India, particularly the western ghats. The name *Crinum malabaricum* is given because of its presence along the Malabar region of Kerela, India. Natively called 'Kanthanga', *C. malabaricum* is one of the rare aquatic species of *Crinums* available throughout the world. Being a member of the Amaryllidaceae family *CM* possesses medicinal properties of extreme importance. Available reports suggest the presence of alkaloids like Galanthamine ($C_{17}H_{21}NO_3$) and lycorine ($C_{16}H_{17}NO_4$) (Fig. 4.) in the different parts of this plant suggesting its importance as an important medicinal commodity. Residential development and human interference have significantly impacted the habitat of this species.

Due to their ability to be used to isolate secondary plant metabolites and use them in active pharmaceutical formulations, medicinal plants have attracted a lot of interest from pharmacologists. This interest is a result of the knowledge that these plants contain a wide range of secondary metabolites, which have the potential to be used as components for pharmaceutical products having pharmacological action. The increased consumption and harvesting of wild herbal medicines combined with habitat destruction by urbanization resulted in a decline in the natural populations of most medicinal plants over time [7,8]. This report aims to present the importance as well as morphological, developmental, and molecular studies

conducted for the conservation and secondary metabolite enhancement of critically endangered [9] *Crinum malabaricum* since its discovery in 2012 by Lekhak & Yadav [10] along with potential strategies to preserve this species.

1.2. Distribution and medicinal uses of *Crinum* species

The genus *Crinum* is widely distributed in Australia, America, Africa, and Asia [11,12]. In India, it is widely distributed throughout the country; however, few species are confined to the same places. Two endemic *Crinum* species, *C. brachynema* and *C. woodrowii* are widely distributed in the Northern-Western Ghats, Mahabaleshwar, Satara district, India [12]. A newly discovered *C. malabaricum* is restricted to 0.5 km^2 area in Periya village, Kasarsgod district, Kerela [10,13]. The rest of the *Crinum* species are widely distributed throughout the country. Table 1 and Figure 2 shows the distribution of different *Crinum* species throughout India.

For a large section of humanity throughout antiquity, plants have long been a main source of traditional medical medicines. At this time, they continue to be priceless resources for the synthesis and production of pharmaceutical medications. For centuries, the different plant parts (leaves, stem, root, flower, and bulb) have been used for the bioformulations or concoctions to treat various ailments. The Amaryllidaceae family has a long and rich history in traditional medicinal practices [14,15,16,17,18,19]. The use of the members of the genus *Crinum* as therapeutic plants dates back a long way [20,21,22,23,24,25,26]. Traditionally, *Crinums* are used as tonics, laxatives, antipyretics, anti-malarial, anti-tumor, anti-aging, anti-lymphocytic and anti-asthamatics [27,21,28]. In addition, they also used in treatment of several conditions such as headache, backache, swelling, haemorrhoids, wounds, and rheumatism [21,23,6]. Interestingly, a few *Crinum* species are used for the same medicinal purposes in different countries due to their alkaloid constituents, which in some instances are found to be common in a variety of species.

After South Africa, India comes second as far as the usage of *Crinum* species in traditional medicine is concerned (Table 1). In the case of *C. asiaticum*, both bulbs and leaves are used in the traditional medicine [20,24]. Roasted bulbs are used as a rubefacient for the treatment of rheumatism. In addition, it is also used for haemorrhoids and suppuration [14]. In the Indian medicine system, *C. pratense* Herb. (*C. lorifolium*) is used for the treatment of intestinal diseases such as dysentery and diarrhoea [22]. Its bulb extracts also used as a bitter tonic in popular medicine to treat chest ailments [27,22]. Traditionally, the bulbs of *C. defixum* Ker Gawl. and the juice from the leaves of *C. latifolium* has been used for treating several inflammatory conditions allergic disorders, piles, fever, earache, and skin diseases. In addition,

the fresh juice of leaves also served to cure joint pain and vomiting [29,30]. The paste of *C. viviparum* bulb is used to treat boils, skin allergies and for the treatment of Herpes [31]. In Madhya Pradesh tribal communities like Bheel, Kol, Auranb, Sahariya use the bulb paste of *C. viviparum* with turmeric powder in order to treat bruises and swelling [33]. The bulb of *C. woodrowii* is also used for the treatment of biliousness, vomiting, urinary discharge, bronchitis, night blindness and gonorrhoea [34,35,36]. As postulated by several researchers, the presence of pharmacologically active alkaloids in different Indian *Crinum* species is expected to stimulate more interest from the pharmaceutical industries where there is ever increasing need to discover more active compounds to meet consumer demands.

1.3. Pharmacological studies

As there is sufficient information available on the ethno-medicinal uses of several Indian *Crinum* species in traditional medicine, they have also been investigated for various pharmacological activities (Table 2). Use of methanolic and aqueous solvents and their preparation by several researchers from the leaves and bulbs extracts for different biological assays like the antioxidant activity, acetylcholinesterase inhibition activity has been described in the Table 2. All of these were *in vitro*-based assays, suggesting the need for *in vivo*-based assays to verify and complement the current evidence. The medicinal properties of the *Crinum* species are mainly due to the presence of pharmacologically active alkaloids [37]. Biologically active alkaloids such as lycorine (LY) presents anti-tumor, cytotoxicity, and plant growth-inhibitor activities, whereas GAL presents anticholinesterase properties used for the treatment of AD [38,39,40.41,42]. In addition, GAL has proven to be a long-lasting reversible, selective, and competitive acetylcholinesterase inhibitor. Researchers have also demonstrated that the antibacterial activity of *C. asiaticum* is mainly because of the presence of ample amount of alkaloids present in its bulbs [43,44,26].

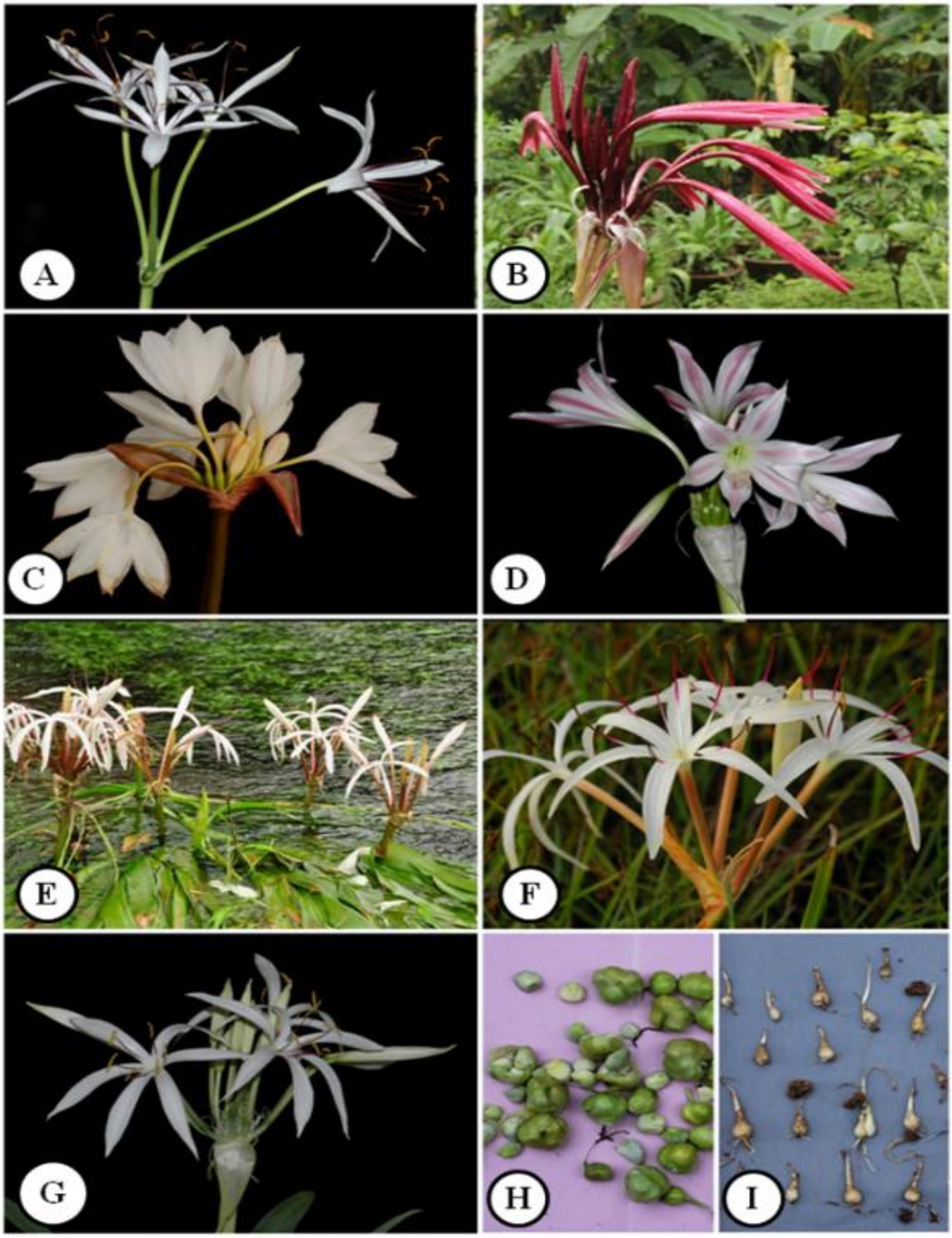


Fig 1. Indian *Crinum* species: (A) *C. amoenum*, (B) *C. asiaticum*, (C) *C. brachynema*, (D) *C. latifolium*, (E) *C. malabaricum*, (F) *C. viviparum*, (G) *C. woodrowii*, (H) *C. brachynema* seeds, (I) *C. malabaricum* bulb.

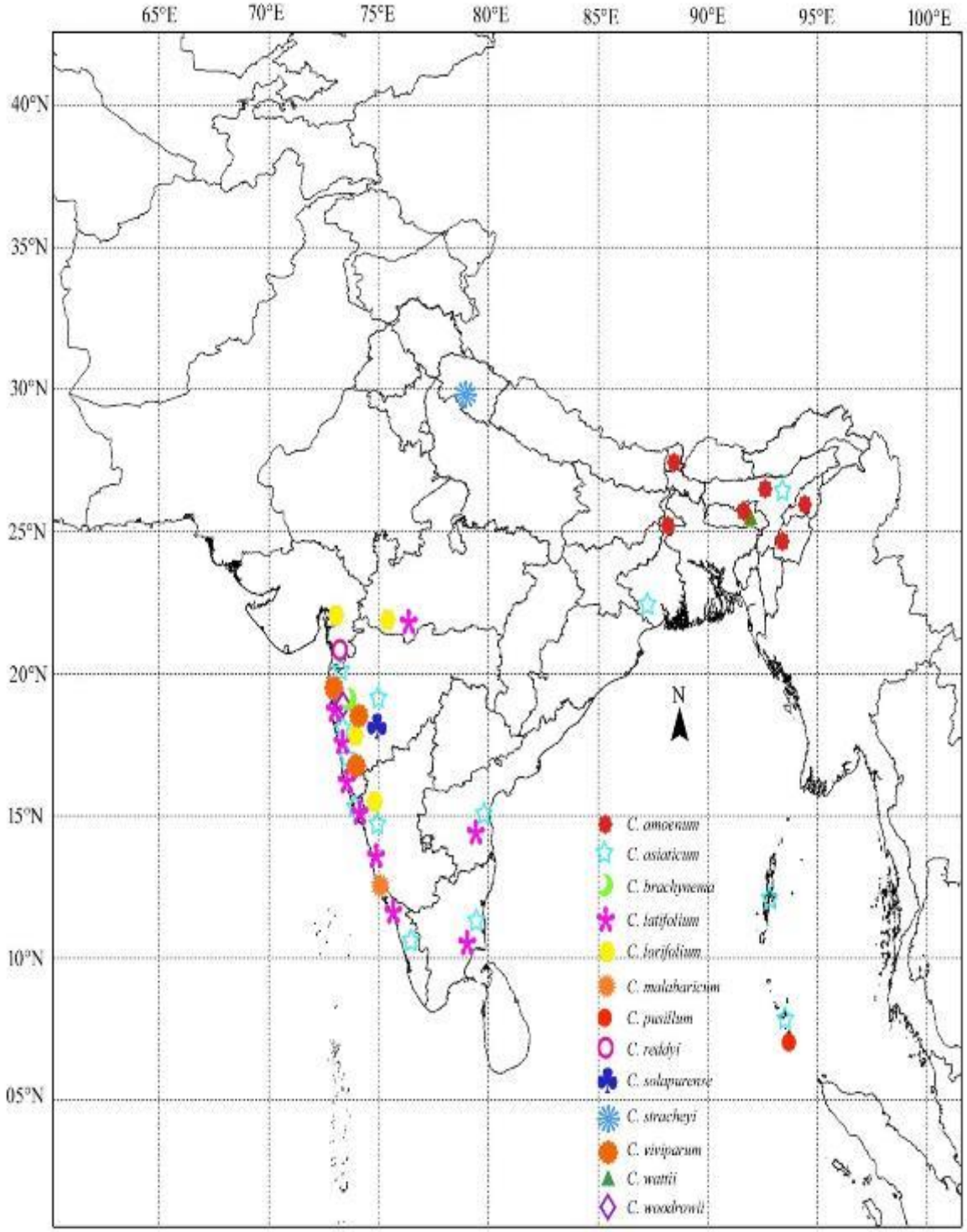


Fig 2. Geographical distribution of Indian *Crinum* species.

Table 1. Geographical distribution and ethnomedicinal uses of Indian *Crinum* species.

Plant Species	**Geographical Distribution**	**Co-ordinates**	**IUCN Status**	**Plant Part**	**Ethno-medicinal Use**	**Reference**
C. asiaticum	Throughout India		Not Listed	Bulbs; Seeds; Leaves	Serve as a drug and effective for headache, laxative, purgative; diuretic, skin infections and cough	[25,46,47]
C. brachynema	Satara district, Mahabaleshwar, Maharashtra	17°56'. 270" N, 73°41'. 488" E	Critically Endangered			[46,48]
C. defixum	Around Riverbanks and Swampy areas of Deccan and Bengal		Not Listed	Bulb; Leaves	Used in the treatment of burns, carbuncles, whitlow. The extracts also used to treat the pimples, paronychia, leprosy, diarrhoea, carbuncles, sweeling of dody, fever and leucorrhea	[29]
C. eleonorae	Mahabaleshwar, Maharashtra	17°55'. 270" N, 73°40'. 488" E	Endemic and Rare	Bulb	-	[12]

C. latifolium	Brajendranagar, Udaipur, Tripura	23°34′13.87″N; 91°33′56.12″E	Not Listed	Leaves	For treating allergies, piles, fever, earache, and skin diseases. In addition, fresh juice of leaves also served to cure joint pain and vomiting.	[30]
C. malabaricum	Periya Village, Kasaragod District, Kerala	12°24.526' N, 75°06.571' E	Critically Endangered	-	-	[10]
C. lorifolium (= *C. pratense*)	Barki Plateau, Kolhapur, Maharashtra; Karnataka and Madhya Pradesh	16°44.673″ N, 073°50.824″E	Least Concern	Bulbs	Bulb extracts used as a bitter tonic in popular medicine to treat chest ailments	[22,26,27]
C. reddyii	Ghuntvel, Songadh Tehsil, Tapi District, Gujarat	21°01′09.61″N, 73°34′10.96″E	Not Listed	-	-	[50]
C. solapurense	Machnur, Solapur district, Maharashtra	17°33'58.8" N 75°33'36.3" E	Endemic	-	-	[26,51]

C. viviparum	Ratnagiri, Maharashtra	16°52.793″ N, 073°19.593 ″E	Least Concern	Bulb; Root	The paste of bulb is used to treat boils and Skin allergy; Treatment of Herpes	[26,31,32]
C. woodrowii	Katraj Ghat, Pune District, Maharashtra	18°27'15"N , 73°51'45"E	Critically Endangered	Bulb	Biliousness, Vomiting, Urinary Discharge, Bronchitis, Night Blindness, Gonorrhoea	[34,35,36]

Previous research has shown that chloroform leaf extracts of *C. asiaticum* possess antinociceptive properties owing to the presence of bioactive compounds [52]. *C. asiaticum* leaves (ethanolic extract) also exhibited anti-inflamatory and analgesic effects in Swiss albino mice [53]. A study by [54] demonstrated that *C. asiaticum* leaf extracts also displayed anti-hepatotoxic effects in alloxan induced diabetic rats. In addition, it also exhibited significant anti-microbial potential against *Escherichia coli, Bacillus subtilis, Psuedomonas aeruginosa,* and *Staphylococcus aureus* [55].

Recently, Ghane et al. (2018) [26] investigated GAL and LY alkaloids from the leaves of different Indian *Crinum* species using RP-HPLC system. In addition, they also investigated several biological properties including anti-diabetic, acetylcholinesterase inhibitory and antioxidant potential from different Indian *Crinum* species. In their study, among all the species tested, *C. asiaticum* leaf extracts showed the strongest antioxidant (IC_{50} =111.54±2.03 mg TE g^{-1}) activities which indicates that for GAL and LY, *C. solapurense* and *C. latifolium* are the best sources [26]. DPPH activities of chloroform, ethyl acetate and methanol bulb extracts of *C. asiaticum* have also been found [56,57]. Similarly, remarkable DPPH activities were found for *C. asiaticum* ethanolic and aqueous leaf extract respectively (IC_{50} = 71.4 µg ml^{-1} and 196.9 µg ml^{-1} respectively) [58,59]. Considerable ORAC activity (IC_{50} =1610±150µmol TE/g) has been noted from the aqueous leaf extract of *C. latifolium* [60]. Recently, [26] reported remarkable ABTS scavenging activity (IC_{50} =39.65±0.91 mg TE g^{-1}) for the leaf extract of *C. latifolium*. The effect of different solvent extracts towards ABTS and DPPH scavenging radicals show the compounds present in the leaves can interact with free radicals via electron

donor or hydrogen atoms [61]. However, extensive pharmacological research is needed on all the Indian *Crinum* species for a better understanding of their chemical components and their possible applications in the pharmaceutical and/or nutraceutical industries.

1.4. Amaryllidaceae alkaloids

In a biosynthetic pathway the precursor formation for the alkaloid biosynthesis involves the condensation of phenylalanine and tyrosine derivatives. In the Amaryllidaceae alkaloids, biosynthesis is divided into five steps: (1) the shikimate pathway (2) the phenylpropanoid pathway (3) the core pathway (4) the intermediate pathway and (5) the late pathway [62]. A series of biochemical reactions such as bond formations, addition and modifications of functional group, oxide bridge formation and phenol-phenol coupling to produce a diverse range of alkaloids takes place in a biosynthetic pathway. Though the chemical signatures of Amaryllidaceae alkaloids have a wide range, they share a common biosynthetic intermediate (norbelladine), which gives the diverse skeleton of Amaryllidaceae alkaloids (Fig. 3) [62,63,64, 65]. The enzyme responsible for 3.4-dihydroxybenzaldehyde (3.4-DHBA) and tyramine biosynthesis is located in the clusters of phenylalanine ammonia lyase (PAL) and tyrosine decarboxylase respectively. Condensation of 3,4-DHBA and tyramine leads to the formation of norbelladine, which is catalyzed by the combination of norbelladine synthase (NBS) and noroxomaritidine reductase (NR) enzymes (Fig. 3) [66,67]. Norbelladine acts as a precursor to form cherylline-type Amaryllidaceae alkaloids, by undergoing a series of biochemical reactions such as dehydration, hydroxylation, methylation, tautomerization and cyclization. On the other hand, norbelladine generation is catalyzed by the norbelladine 4'-*O*-methyltransferase (N4OM) enzyme to form 4'-*O*-methylnorbelladine [68]. The cyclization of 4'-*O*-methylnorbelladine by three alternate ways (Para-ortho, Ortho-para and Para-para) of phenol-phenol coupling, yields crucial intermediate skeletons of Amaryllidaceae alkaloids (Fig. 3). These three crucial separations at 4'-*O*-methylnorbelladine are the para-ortho phenol coupling, which produce galanthamine-type alkaloids, the ortho-para phenol coupling leads to lycorine and homolycorine-types of alkaloids, whereas the para-para phenol coupling elaborates the crinine, narciclasine and montanine types of alkaloids (Fig. 3). All three phenol coupling reactions require cytochrome P450 enzymes for catalyzation [63,64,69]. By using comparative transcriptomics analysis of *Narcissus* sp. *aff. pseudonarcissus*, *Galanthus elwesii* and *Galanthus sp*., a candidate cytochrome P450 sequence was identified. Through heterologous expression, purification and *in vitro* characterization, cytochrome 450 monooxygenase 96T1 (CYP96T1) formed the products (10bS,4aR)-noroxomaritidine and (10bR,4aS)-

noroxomaritidine from 4'-*O*-methylnorbelladine, supporting its association as a para-para phenol coupling cytochrome P450 [70]. Following the separation of these three phenols coupling scaffolds, the para-ortho (nornarwedine), ortho-para (noroxolpluviine) and para-para (noroxomaritidine) are further modified to produce diverse range of end-products (**Fig. 3**).

GAL derived from para-ortho synthesis pathway by phenol oxidative coupling enzyme [71]. The GAL biosynthesis entry point starts with the conversion of L-phenylalanine including the enzyme PAL to protocatechuic aldehyde and L-tyrosine, including TYDC to tyramine (Fig. 4) [65,72]. The junctions of the 3.4-DHBA and tyramine, converted into norbelladine. However, no enzymes have been reported until now in relation to downstream of nornarwedine. GAL biosynthesis also involves the phenol coupling of 4'-*O*-methylnorbelladine, which converts into N-demethylnarwedine and then produces norgalanthamine after stereospecific reduction. Further modifications of norgalanthamine lead to several GAL-type alkaloids. A proposed pathway of GAL biosynthesis is presented in Figure 4.

1.5. Biotechnological approaches for conservation of *Crinum* species in India.

In present times a major global challenge is devising new strategies and their implementation for the conservation of medicinal plant species [73,74,75]. Conservation practices involves the careful planning for the preservation of the natural resources as well as environment without over-exploitation and destroyinh the natural habitat [76,77]. Globally, several plant species with significant medicinal value (for example, *C. malabaricum* and *C. asiaticum*) are threatened, critically endangered or dwindling in the wild due to increased demand, urbanization, and other anthropogenic activities [10,76,78]. This ever-increasing demand has led to overexploitation and indiscriminate harvesting of the valuable plant species within all ecosystems [77,79].

The bulbous geophytes plants have always been important to human beings, and will continue to be so [80,81]. The knowledge of the medicinal potential of Indian *Crinum*s has led to a spike in their demand by the researchers. Consequently, overharvesting and urbanization by the local communities may lead to a decline in the natural populations. The number of wild Indian *Crinums* has declined significantly due to destructive and illegal harvesting of the bulbs. *C. malabaricum* is an endemic species which is listed as critically threatened and restricted to Kasaragod district, Kerala only [10]. Therefore, these Indian *Crinum* species need immediate measures to protect their continued existence.

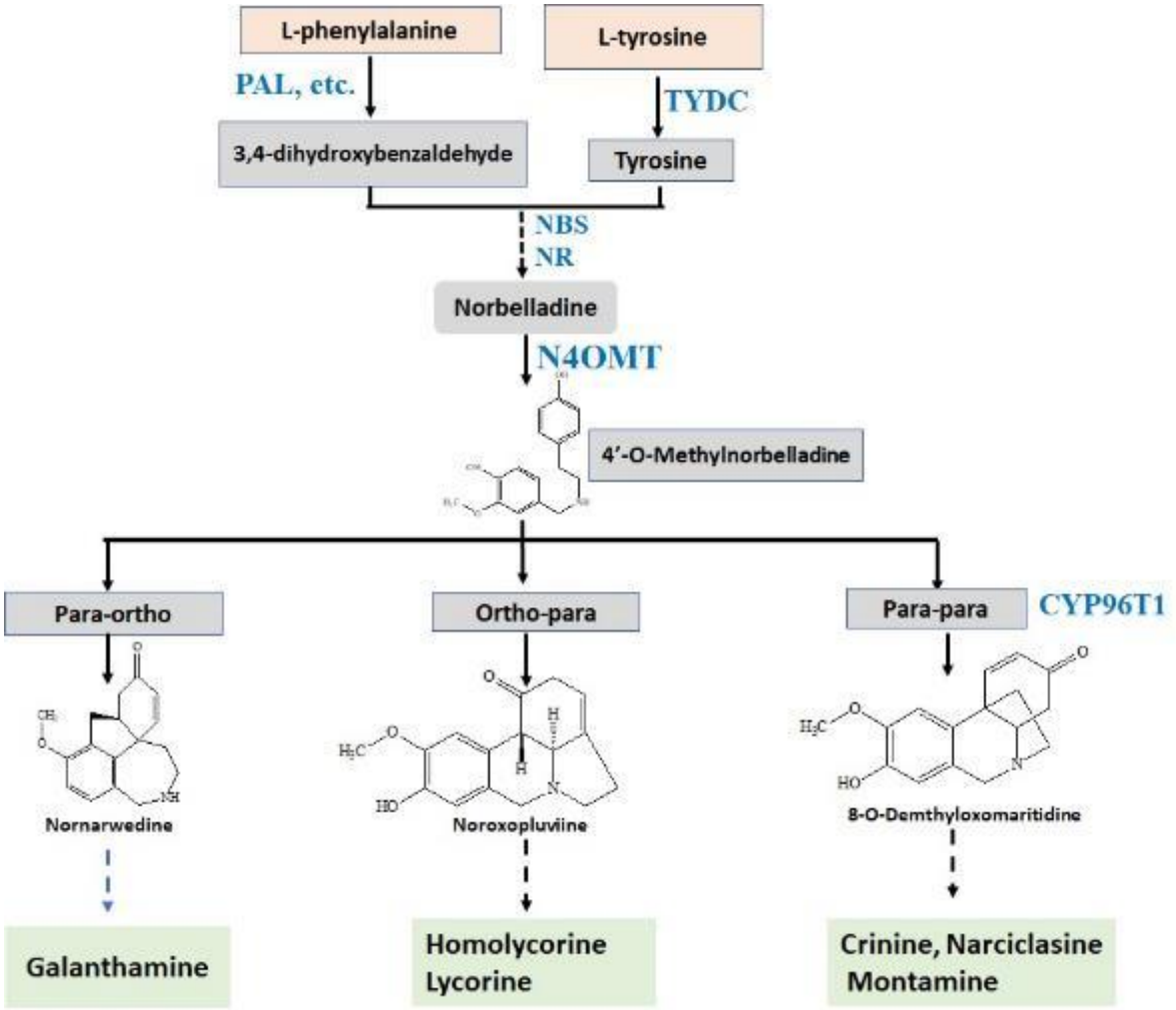


Fig 3. Major types of Amaryllidaceae alkaloid biosynthetic pathway. All enzymes are labelled in bold blue color. A solid arrow represents one enzymatic reaction, whereas dashed arrow represents multiple enzymatic reactions.

In the past few decades, the scenario of conservation of wild genetic resources has been completely transformed by the significant advancements and development of a myriad of novel techniques in the area of plant biotechnology and the tissue culture techniques in particular [82,83,84,85]. Direct and indirect micropropagation techniques like *in vitro* regeneration via somatic embryogenesis, bioactive compounds synthesis *in vitro*, cryopreservation has proven to be a great model in the preservation of some of the species from going extinct. Genetic transformational studies can also be used to re-introduce rare and endangered species back into the nature and to decrease the pressure on natural habitats. However, as depicted in the Table No.3 very few studies have been known to document the feasibility of *in vitro* regeneration of Indian *Crinum* species and their implementation in acclimatization of these species. Review

literature indicates that only a handful of protocols have been reported on *C. asiaticum* using bulb scale and flower bud explants. Some researchers suggests that true-to-type clones can also be produced via somatic embryogenesis approache. For instance, 4.2±0.82 somatic embryos were developed using bulb-scale and flower bud explants when propagated on the culture medium fortified by BA and NAA [86,87,88]. In contrast to the use of bulb-scale explants in aforesaid reports, *in vitro* propagation was also recommended by [89]. Based on this finding, up to nine shoots per explant were recorded from the developed protocol with 95% survival rate. Recently, an efficient in vitro regeneration system with maximum number of adventitious shoots (53) was recorded on culture medium fortified with 1.0 mgL^{-1} BA. Interestingly, a total of 83% of the produced adventitious shoots survived under field conditions [90].

Development and establishment of different *in vitro* studies without compromising biological potency is still a need for many Indian *Crinum* species. Therefore, techniques like micropropagation and development of protocols for somatic embryogenesis while manipulating PGRs could be established as alternate conservation strategies rather than depending on conventional methods for the conservation of Indian *Crinum* species.

1.6 Aims and Objectives of the study

- Collection of plant material and development of regeneraion protocol for *Crinum malabaricum* through *in vitro* techniques.
- Somatic embryogenesis of *Crinum malabaricum*.
- Maintenance of cell suspension and study the effects of elicitors on secondary metabolite production.
- Assessment of genetic fidelity in regenerated *Crinum malabaricum* and extraction and analysis of secondary metabolite

Table 2. Biological activities of Indian *Crinum* species.

Plant Species	Explant	Technique	Alkaloids	Activity	Reference
C. asiaticum	Bulbs, Leaves, Fruits	NMR, HPLC, TLC	Crinamine, Norgalanthamin, Hamayne, Hippadine, Platorinine, Crinasiadine, Lycobetaine, Kalbretorine, Criasbetaine	Analgesic, Anti-inflammatory, Anti-tumor, Antioxidant, Antimicrobial	[53,54,91]
C. asiaticum	Fruits, Fleshy leaves, Bulbs	IR, NMR	Palmilycorine, Lycoriside	Anti-tumor	[92,93]
C. asiaticum	Fruits, Fleshy leaves, Bulbs	IR, NMR	Ungeremine, Criasbetaine	Anti-tumor	[94]
C. brachynema	Bulbs, Leaves	RP-HPLC	Gallic Acid, Catechin, Salicylic Acid, Coumaric Acid, Galanthamine	Anticancer, Hepatoprotective and Neuroprotective	[26]
C. defixum	Corm, Bulbs, Leaves Elaiyaraja	HPLC	Homolycorine, Galanthine, Haemanthamine, Hippestrine, Caranine, Crinine, Galanthamine, 5α-hydroxyhomolycorine	Anti-inflammatory, Analgesic, Antioxidant, Antimicrobial	[29,33,95]

C. latifolium	Bulbs, Leaves, Flower Stems	RP-HPLC	Belladine, Latisodine, Latisoline, Hippeastrine, Cherylline, Latifine, Galanthamine, Lycorine, Hippadine, Oxoassoanine	Anti-inflammatory, Antibacterial, Anticancer	[26,96,97]
C. malabaricum	Bulbs	RP-HPLC	Lycorine, Gallic Acid, Catechin, Salicylic Acid, Coumaric Acid, Galanthamine	Acetylcholine esterase Inhibitor, Hepatoprotecti ve	[26]
C. pratense	Leaves	RP-HPLC, ^{13}C-NMR, ^{1}H-NMR	Ismine, Lycorine, Catechin, Salicylic Acid, Coumaric Acid, Hippadine, Pratorinine, Anhydrolycorine, Gallic Acid	Antibacterial, Anticancer	[26,98]
C. viviparum	Bulbs	RP-HPLC	Catechin, Salicylic Acid, Coumaric Acid, Chlorogeenic Acid, Galanthamine	Antioxidant	[26,99]
C. woodrowii	Bulbs, Tuber	HPTLC, Silica Gel Chromatogr aphy	Chlorogenic Acid, Kaempferol, Quercetin, Isoquercetin, Hesperidin	Antioxidant	[26]
C. zeylanicum	Bulbs		Narcissine, Galanthidine, Amarylline, Bellamarine	Anti-malarial	[98]

Chapter 2

REVIEW LITERATURE

2.1. Introduction

Plant cell, tissue, and organ bio-factories provides a significant alternative to the ever-increasing requirement for important plant-derived bioactive compounds. However, during *in vitro* maintenance of plant cultures there are few factors that inhibit the total yield, including loss of production. At present, there are no automated systems that are in commercial use for the large-scale production of plants through somatic embryos. Plant tissue culture offers potential alternate techniques of serving a large population as well as a means of increasing secondary metabolite synthesis. One of the most significant advantages of somatic embryogenesis is that it is an efficient way to preserve the biodiversity of plants from limited plant material. Amaryllidaceae J. St.-Hil. is a family of bulbous, perennial, monocots which is found in both tropical and sub-tropical areas and warm temperate regions of the African, American, South Asian and Australian continents, consisting of approximately 75 genera and 1600 species [6,100]. It is divided into three subfamilies Agapanthoideae, Amaryllidoideae and Allioideae [101] and is well known for its impressive alkaloid content and significant biological properties [18,19,98,102,103,104]. Some of the major alkaloids found in this family are- Galanthamine, Lycorine, Crinine, Homolycorine, Tazettine, Montanine, Pancrastatin, Narciclasine, Carboline, Isoquinoline, Lactone [18,24]. These alkaloids are found to exhibit various biological activities including both pharmacological and microbiological, some of them includes- anti-inflammatory, cytotoxic, anti-viral, anti-bacterial and anti-malarial, and plant growth inhibition in some cases [26].

The genus *Crinum* L. belongs to the subfamily Amaryllidoideae of Amaryllidaceae family. It is one of the largest pantropical genera of this family consisting of about 130 species, 2 sub species and 4 varieties of both terrestrial and aquatic, monocotyledonous, bulbous herbs. The genus is well known for its ornamental and medicinal properties such as immune-stimulating, antifungal, antiviral, analgesic, antitumor etc. all around the world [6,24,105]. Out of all the species of *Crinum* 14 are found in India collection and some are considered to be extinct [10,46]. Members of Amaryllidaceae family has been used for the treatment of various ailments like dysentery and ipecac, skin diseases, burns, carbuncles, rheumatism, leprosy, fever and leucorrhoea, fistula, bronchitis, night blindness, gonorrhoea, tumercle, diarrhoea, as well as for the treatment of Herpes, they are also used as laxatives and diuretics [6,25,26,27.29,30,46,47]. Also known as “Malabar River Lily” *Crinum malabaricum* Lekhak & Yadav is an aquatic medicinal plant which originated in the Western Ghats of India [10]. The area of distribution is limited to about 0.5 Km^2 and only 1000 plants in total are available. It is found in a seasonal

stream and is found to have cartilaginous, flat, strap-shaped leaves which lies horizontally to the length of the plant, the scape arises from beneath the water, and this floating umbel bear a resemblance to the water lily. Reproduction in plants is by vegetative process where small bulbs are born from the mother bulb and form visible cluster. The seeds are dispersed in the water due to the flow of the stream. Since the seed dispersal occurs in a stream the multiplication of the population and seed germination rate is questionable and needs to be evaluated.

It has been reported to possess compounds of medicinal value, some of which exhibits anticancer and anti-Alzheimer properties [6,26,58]. Amaryllidaceae alkaloids are specific and have a broad range of biological activities. Among all other alkaloids, galanthamine (GAL) have potent importance in medical system and has proven to be a selective, long-lasting and effective inhibitor of the acetylcholinesterase [44]. Besides, it is also used for the development of modern drug and doctors use it in the treatment of patients suffering from the Alzheimer's disease [43].

Since, CM grows across a running seasonal stream, fully immersed in the watercourse bed, conservation of this plant species via *in situ* mode is quite concerning. Hence, the conservation and sustainable development of *C. malabaricum* and its secondary metabolites can only be assured by *ex situ* methods. *In vitro* propagation methods have proved to be effective in overcoming problems like, the dwindling of the population and have provided just solutions for the conservation and rapid multiplication of the plant species. But very few studies have been done on Indian *Crinum* species when it comes to the *in vitro* propagation [13,90,106,107]. Therefore, more research is needed when it comes to the *in vitro* regeneration protocols of Indian *Crinum* species.

The *in vitro* micropropagation techniques can be helpful in overcoming the unsure and extended growth period of bulbous plants and can also provide possible solutions for the rapid multiplication of the endangered species [108]. In some Amaryllidaceae members like *Narcissus asturiensis*, *Hyacinthus orientalis*, *Tigridia pavonia*, *Muscari muscarimi* and *Cyranthus mackenii* propagation of bulb scales (twin and tri) have been used [90].

C. malabaricum is locally known as "Kanthanga" and is a critically endangered Indian aquatic *Crinum*. *Crinum* species exhibit diverse medicinal properties such as antioxidant, antimicrobial, antimalarial, antiviral, anti-inflammatory, anti-tumour, anti-diabetic, and immune stimulating. The above-mentioned medicinal properties are due to the presence of pharmacologically active alkaloids [6,26,58]. Alkaloids possessed by the members of the

amaryllidaceae family have diverse biological activities [44]. Besides, it is also for the development of modern drug and used by the doctors widely to treat the patients bearing Alzheimer's disease [43]. Few studies have shown global benefits of GAL for Alzheimer's patients and an extensive clinical trial has been reported with positive findings [99,109]. Natural medicinal plant resources are the primary source to exhibit various biological activities. Galanthamine is isolated from different plant species and in different continent such as *Lycoris radiate* in China, *Narcissus* and *Leucojum aestivum* in Europe, *Ungernia victoris* in Uzbekistan and Tadzhikistan [110,111]. Wild plants remain as untapped sources for identification, isolation, and characterization of GAL. In vitro multiplication of GAL producing plants has attracted the interest of many researchers for the sustainable synthesis of this very valuable molecule because to the rising demand and the limited supply of plant material [110].

Few reports have been found for GAL production through shake flask from *in vitro Leucojum aestivum* cultures [112,113,114]. However, RITA® bioreactor has been found to the enhance the *in vitro* cell biomass production as well as secondary metabolites. For GAL production this system has been found to be optimum for scale up of the compound via *in vitro L. aestivum* shoot cultures [115].

According to literature very few *Crinum* species have been investigated for GAL isolation and characterization [6,99]. However, there is no report available about bioreactor based *in vitro* cultivation and GAL production in *C. malabaricum* species. This plant is now classified as a critically endangered plant on the IUCN Red List as a result of over-harvesting and habitat loss caused by humans [10]. Due to farming and the tree lining the stream were being cut down, pushing this plant to the verge of extinction. Due to over-exploitation, an UN-led conservation network (Botanical Garden Conservation International) has requested Malabar Botanical Garden Institute of Plant Science (MBGIPS) to take necessary steps for the germplasm conservation of *C. malabaricum.* In order to preserve CM conventional methods of germplasm conservation are insufficient. As a result, the use of plant biotechnological techniques like micropropagation and somatic embryogenesis is now required for the preservation of the plant's genetic material, its sustainable use, and the enhancement of its most valuable galanthamine component in aquatic medicinal plants like *C. malabaricum.* In *in vitro* raised culture somaclonal variation can have a major issue in determining the quality of individual clones. Several factors such as long-term culture maintenance in *in vitro* conditions, and exposure to high concentrations of PGRs (plant growth regulators) could be potential inducers for somaclonal variation. A range of molecular markers such as RAPD, AFLP, ISSR, SSR and microsatellite have been generally used for the molecular stability of the *in vitro* derived

plantlets. Initiating a trend away from common DNA markers, a novel molecular marker called Start Codon Targeted Polymorphism (SCoT) has been reported which targets the short ATG start codon present in the plant genome [116]. It has several advantages over several random DNA markers, as it is reproducible, more stable and can be used to assess genetic diversity, DNA fingerprinting, population studies and genetic mapping in different plants. SCoT markers are directly involved in gene function and can be used in polymorphism and genotyping [117,118,119]. These markers have been successfully utilized for fingerprinting and diversity analysis in number of plant species [120,121]. Only preliminary studies have been conducted on CM and therefore very limited literature is available on it. No extensive work regarding micropropagation and somatic embryogenesis studies has been done till date. Lekhak and Yadav (2012) [10] discovered this new species in 2012 and reported a review of *C. malabaricum*. Ghane *et al*. (2018) and Chahal *et al.* 2021 [26] have reported the quantification and metabolic profiling estimation of lycorine and galanthamine CM. There is no report available on *in vitro studies* and isolation and characterization of galanthamine from *in vitro* culture of *C. malabaricum*.

2.2. Classification of *Crinum malabaricum*

Kingdom	Plantae
Phylum	Tracheophyta
Class	Liliopsida
Order	Asparagales
Family	Amaryllidaceae
Sub-family	Amaryllidoideae
Genus	Crinum

2.3. Distribution & Ecology of *Crinum malabaricum*

The first ever report of *Crinum malabaricum* appeared in 2012 and was reported to be endemic to Periya village in the Kasaragod District, Kerala, covering an area of about $0.5km^{-2}$ with a population estimate of 1000 individuals [10]. Also known as 'Malabar River Lily' *C. malabaricum* grows beneath dense canopies with indirect sunlight in a seasonal fresh water,

fairly fast-flowing running stream with a gravely bed and laterite soil at an elevation of 81 m above sea level (Fig. 4). The name *malabaricum* has been given because of its presence along the Malabar coastal region of Kerala. The soil analysis report of the laterite soil from the stream of Periya village reveals that the laterite soil is rich in Sulphur and Iron and deficient in Manganese, Potassium, Magnesium and Phosphorus (Table 8). The reproduction is mainly by vegetative means, as the capsules progressively increases in size they fall into the stream where the continuously flowing water helps in the gradual breakdown of capsule wall following dehiscence and seed dispersal (Fig. 5).

Fig 4. *Crinum malabaricum* in the fresh water stream of Periya. A-B) *Crinum malabaricum* in the Periya stream; C) Freshly harvested bulb of *C. malabaricum*

2.4. Pharmacology and Phytochemistry

Amaryllidaceae family plants contain diverse spectrum of compounds (Amaryllidaceae alkaloids) that have long been employed in the traditional folk medicine by Indigenous people because of their therapeutic properties [122]. Several Amaryllidaceae alkaloids have been isolated, identified, and investigated for their phytochemical properties over the years. Upon isolation the compounds were categorized into different structural types; galanthindole, haemanthamine, galanthamine, homolycorine and lycorine (Fig. 7). Galanthamine and

tazattine were incorporated into the structures related to the pallidiflorine type structures [123]. Galanthamine is well known over the counter drug used in the treatment of Alzheimer's patient for enhancing neurotransmission of the cholinergic neurons, which produce acetylcholine, a neurotransmitter, responsible for memory formation.

First characterized by "Alois Alzheimer" in 1906, Alzheimer's disease has a multifactorial etiology but histopathologically it is characterized by the presence of intracellular neurofibrillary tau tangles (NFTs) and amyloid plaques which results in the synaptic loss and neuronal atrophy throughout the hippocampus and cerebral cortex [124]. The degree of degeneration of cholinergic neurons is directly proportional to the severity of dementia. The exact mechanism of action for GAL is still unknown but research shows that GAL demonstrates agonistic properties and acts as allosteric potentiator of acetylcholine receptors like α4β2 and presynaptic α-7 nAChR (Nicotinic acetylcholine receptor). These receptors are responsible for the release of neurotransmitters like GABA (γ-aminobutyric acid), Norepinephrine, Ach (acetylcholine), Dopamine, glutamate, serotonin etc from the synaptic cleft by competitive selective and reversible inhibition which thereby blocks the hydrolytic degradation of acetylcholine [125,126].

Existing reports indicates that galanthamine is present in both roots (20.85 µg/g) and bulbs (0.308 %) it was not detected in leaves, whereas lycorine is present in both roots (2.28 mg/g) and leaves (1300.6 µg/g), studies related to the presence of lycorine in CM bulbs are extremely limited [26,99,127]. Very few reports of pharmacological studies conducted on the extracts of *C. malabaricum* are present till now [26,99,127]. Aqueous and methanolic extracts were prepared for biological assays like phytochemical, antioxidant, acetylcholinesterase inhibition Ghane *et al*. 2018 reported that TAC was highest in the aqueous extracts of *C. malabaricum* (5.41 mgg^{-1}) out of all the analysed crinum species (Table 3).

CM also showed considerable antioxidant activity, which indicates the presence of compounds like phenolic acids, tanins, flavonoids, quinones, coumarins, lignans and alkaloids. These bioactive compounds can protect the living organisms from diabetes, hypertension, ischaemia-reperfusion injury, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, systemic inflammatory response syndrome, atherosclerosis, Parkinson's, Alzheimer's, and cancer [128]. The brain has a high metabolic rate, and ageing reduces its ability to scavenge free radicals as well as the activity of glutathione peroxidase and catalases, limiting antioxidant availability and making the brain vulnerable to free radical damage [129].

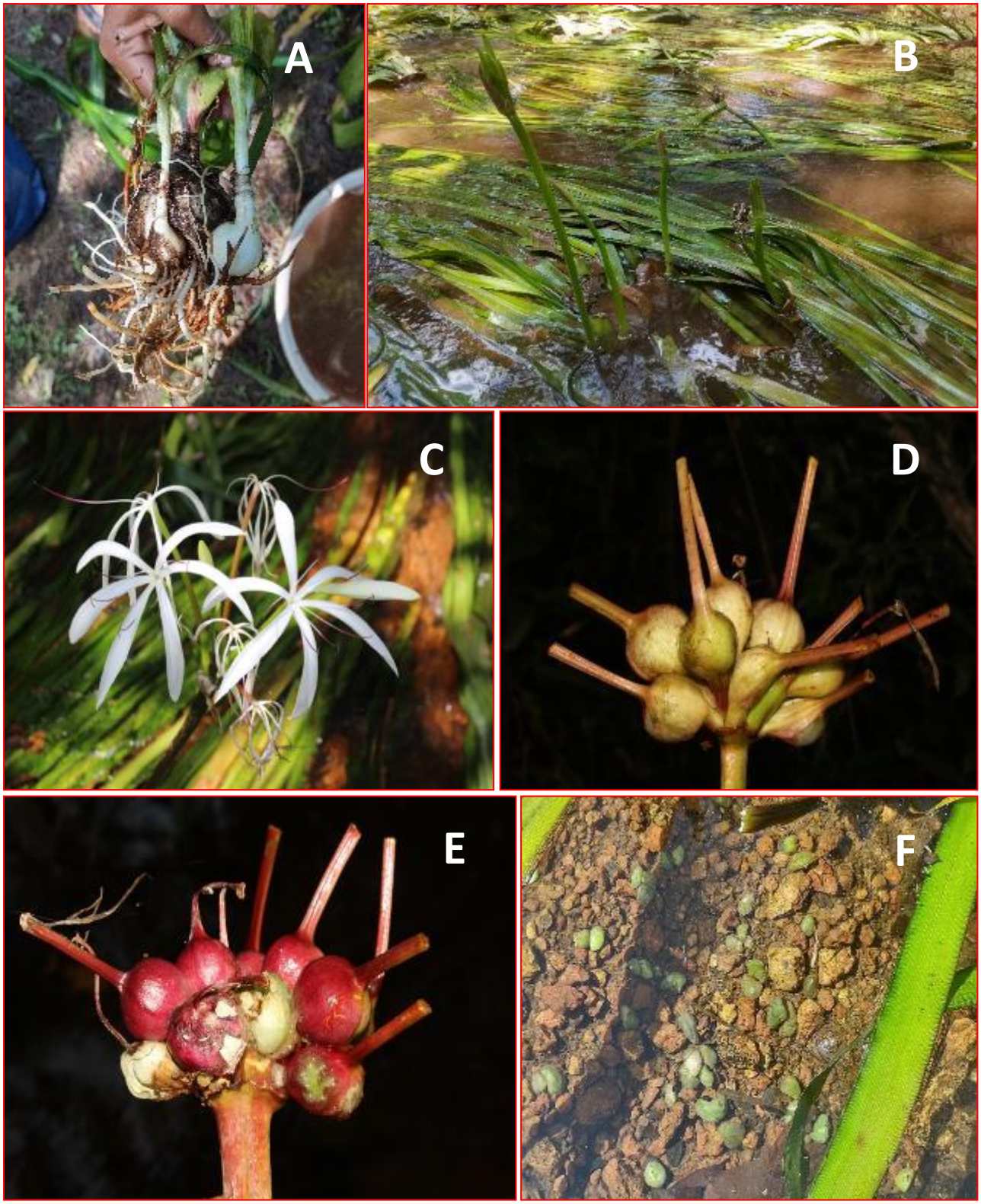


Fig 5. *Crinum malabaricum*: (A) Bulbs, (B) Scapes with umbels emerging from the underground bulbs, (C) Umbels open up to form Inflorescence of *Crinum malabariucm*, (D) Fertilized inflorescence turned into capsules, (E) Mature capsules and (F)seed dispersal.

Table 3: Phenolic acids found in *Crinum malabaricum.*

Plant part	Type of extract	Compound present	Concenteration (DW)	Analysis	Reference
In vitro Whole plant	MeOH	GA	0.199 µg/g	UHPLC-MS/MS	[106]
		p-Hydroxybenzoic acid	0.656 µg/g		
		PA	39.988 µg/g		
		SA	0.891 µg/g		
		Syringic acid	0.169 µg/g		
		VA	12.138 µg/g		
		CA	1.226 µg/g		
		Chlorogenic acid	510.995 µg/g		
		p-Coumaric acid	0.451 µg/g		
		FA	0.9 µg/g		
		Sinapic acid	0.102 µg/g		
Roots	MeOH	TPC	7.349 mg/g	Folin–Ciocalteu Method	[5]
		TFC	0.780 mg/g	Aluminium Chloride colorimetric method	
		TTC	0.558 mg/g	Vanillin−HCl method	
		TAC	1.674 mg/g	UV-Vis spectrophotometer	
		LY	2.28 mg/g	RP-HPLC	
		GAL	20.85 µg/g		
		LY + GAL	2.311 mg/g		

	Water	TPC	6.428 mg/g	Folin–Ciocalteu Method	
		TFC	0.977 mg/g	aluminium chloride colorimetric method	
		TTC	2.547 mg/g	Vanillin−HCl method	
		TAC	1.278 mg/g	UV-vis spectrophotometer	
Leaves	MeOH	TPC	60.84 mg/g	Folin–Ciocalteu Method	[26]
		TFC	105.36 mg/g	aluminium chloride colorimetric method	
		TTC	146.31 mg/g	Vanillin−HCl method	
		TAC	8.84 mg/g	UV-vis spectrophotometer	
	Water	TPS	56.09 mg/g	Folin–Ciocalteu Method	
		TFC	48.63 mg/g	aluminium chloride colorimetric method	
		TTC	115.24 mg/g	Vanillin−HCl method	
		TAC	5.41 mg/g	UV-vis spectrophotometer	
	MeOH	GAL	ND	RP-HPLC	
		LY	1300.6 µg/g		
		GA	289.3 µg/g		
		HBA	ND		
		VA	ND		

		CLA	ND		
		CAT	9066.6 µg/g		
		SA	3528.2 µg/g		
		COA	114.4 µg/g		
Bulbs	MeOH	GAL	0.308 %	HPLC	[99]

Exploring the pharmaceutical and nutraceutical properties of this aquatic species can open multiple doors for its conservation and shine light on the fundamental clues like (1) stress response and adaptation to harsh environmental conditions and (2) isolation of antioxidants with a functional flexibility for the treatment of heterogenous anthropological morbid afflictions [130].

2.5. Need for conservation!

Over the past few years, there has been a rapid decline in the number of individuals of *C. malabaricum* due to unsustainable collection, urban development, and over-harvesting of plants for bulbs, whereby the natural population of this species is dwindling, and it faces the danger of extinction. Therefore, IUCN has declared *C. malabaricum* as "critically endangered" and now is a part of the red list [131]. New constraints have emerged threatening to endanger the existence of *C. malabaricum.* One is in the form of a caterpillar, identified as the Indian Lily-moth (*Polytela gloriosae*) (Fig. 6). The larvae feed on the capsules of *Crinum malabaricum* and if occur in large numbers can completely wipe out the population even before the stream dries out. It was also observed that the stream banks were being used for washing clothes by the natives and construction has been going on around the streams in the form of building houses and concrete walls, which has significantly narrowed the stream. These factors indicate that the existence of this Indian aquatic species is still very much threatened and can become extinct if not properly dealt with. Moreover, the alkaloid content of this species also makes it medicinally and economically important as *C. malabaricum* has been found to exhibit elevated conc. of GAL which could turn it into a valuable pharmaceutical commodity. The propagation of this species is fairly low in nature, most of its leaves, bulbs and fruits are destroyed by moth caterpillars [106,132,133], and hence conventional propagation may not fulfil the demand for valuable alkaloid production. Hence, it is essential to develop rapid

propagation and conservation systems, as tools to save and expand the populations of *C. malabaricum*.

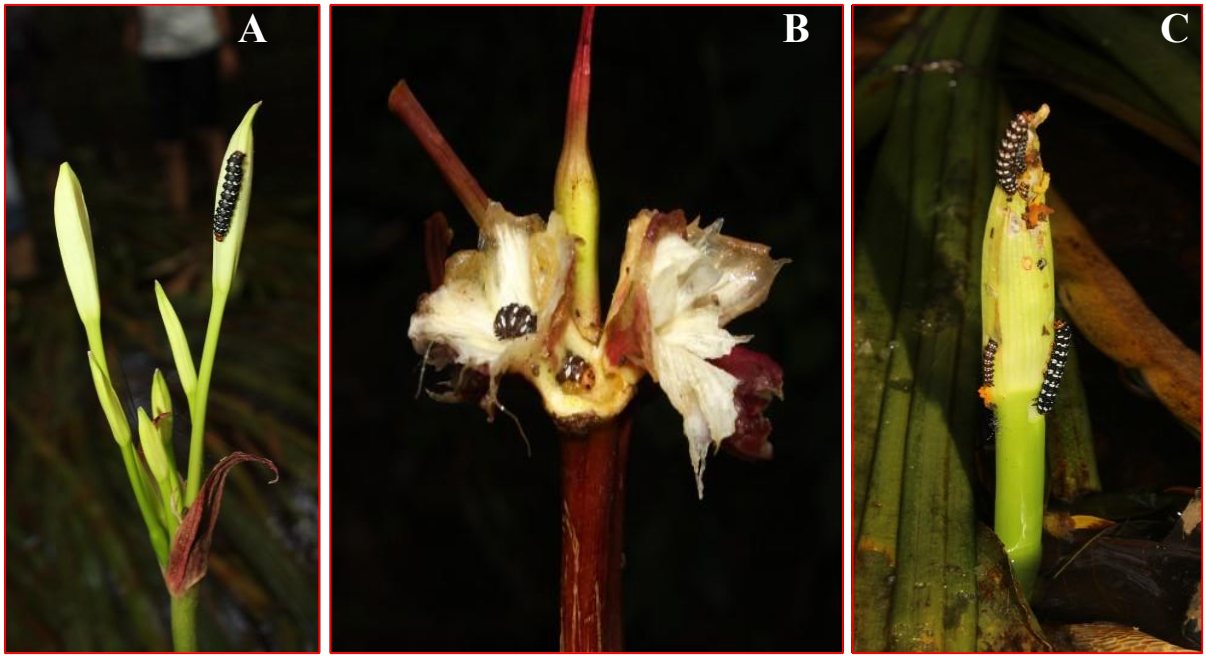


Fig 6. Indian Lily-moth (*Polytela gloriosae*) feeding on: (A)Flower pods, (B) Capsules and (C) Umbels of *Crinum malabaricum*.

2.6. Conservation strategies for *Crinum malabaricum*

For germplasm conservation *in vitro* strategies possesses certain advantages over traditional methods in the sole principle that it requires very little space in growth chambers for maintaining thousands of specimen cultures and the apparent lack of pest attacks and disease occurrence as compared to the vegetatively cultivated crops. Moreover, during the exchange of germplasm, in vitro storage reduces the need for arduous and drawn-out quarantine procedures [134]. Effective *in vitro* regeneration protocols through direct shoot induction have been successfully developed using twin scales and tri-scales from the bulbs of CM on a culture fortified with full-strength MS media and PGRs like BA, NAA, and *m*T [90,106]. *In vitro* raised` plants were sub-cultured on both half-strength and full-strength culture medium augmented with IBA for root induction. After acclimatization 83% plants survived in the natural stream [90].

In case of *ex vitro* conservation, although the reports of acclimatization of *in vitro* regenerated plants are present, reports related to the prolonged survival of acclimatized plants and long-

term effects of temperature, pH, and nutrient change from MS media to soil adaptation are still not available. However, a recent report by Mani et al. 2022 [135], presented the results of micro-morphoanatomical transitions that occurred during the in vitro cultures of CM. The study reported the evaluations done using a microscop to observe the developmental stages of both the *in vitro* and *in vivo* raised plants and described various structural anomalies in the form of underdeveloped wax deposition on the leaf cuticles, underdeveloped stomata in the leaves and reduced intercellular spaces and lumen in the parenchyma of the roots as well as some irregularities in the cell walls of *in vitro* propagated leaves. These plantlets show significant changes in their tissue systems when acclimatized *ex vitro* and were found to withstand increased light and temperature under in vivo conditions. Where reports regarding propagation through direct regeneration are available, in-depth research is required for indirect regeneration methods [133].

During *in vitro* regeneration, plant cells and tissues undergo several genetic alterations including chromosome rearrangement and/or doubling (polyploidy/aneuploidy), DNA methylation, morpho-physiological changes, generally termed as "somaclonal variation" [136,137,138]. Several factors such as source of explant, exogenous plant growth regulators (PGRs) and various intrinsic factors contribute differentially to somaclonal variation [119,139]. Several molecular markers including AFLP [140], RAPD [141,142], ISSR [143], SCoT [139,144] and DAMD [145,146] have been used extensively to evaluate the clonal variability of *in vitro* plant species. Genetically stable plants are important to protect the homogeneity of endangered species and are fundamental for the conservation and secondary metabolite production of a species. Molecular markers like SCoT and ISSR uses the same primer both as forward and reverse primers, which makes it really cost-effective, reproducible, simple, and quick and they can also be used as potential tools for screening somaclonal variations (Rohela et al. 2020). ATG start codon (coding region) is present in the primer sequence of SCoT marker and therefore can also be used to detect polymorphism. ISSR markers on the other hand are based on non-coding regions of the DNA sequences and can be used to analyse similarities and stability of the plants propagated in the controlled environment [116,147].

Another effective method for germplasm conservation is the development of somatic embryos from which proliferation of any species on a large scale can be achieved in very less time. In 2020, the development of somatic embryos was reported by Priyadarshini and colleagues with an induction rate 98.4%. MS media augmented with 8.0 mgL^{-1} 2,4 D (2,4-dichlorophenoxyacetic acid) gave the best results after four weeks of assessment whereas

induction rate in the nutrient medium supplemented with 6.0 mgL^{-1} IBA was 63.7% after six weeks. The nutrient media supplemented with 2,4-D in combination with BAP and IBA resulted in the low induction rate of somatic embryos, which was 40.7% and 34% respectively. This indicates that high concentrations of auxin give better results than lower concentrations. The somatic embryos formed in nutrient medium containing 2,4 D were initially white which later developed chlorophyll and turned green during the late globular stage and later formed heart shaped and torpedo stages, in contrast SEs developed on MS media supplemented with IBA were mostly white and globular in shape and chlorophyll development took prolonged incubation.

Most aquatic plant species are cultivated as ornamental and are either submerged or emerged. Moreover, the information regarding the micropropagation of aquatic plants in vitro is limited and the reports are far less when it comes to the molecular and biochemical studies. Propagation by seed takes a lot of time and are not constructive methods therefore, synthetic seed technology is helpful in joining the advantages of in vitro propagation method in producing high efficiency and pest safe plants [148]. Synthetic seeds or artificial seeds can be produced from the somatic embryos through alginate encapsulation. The goal of synseed technology is to mimic the natural seed germination process but with the advantage of producing viable, disease free, insect infestation free and true to type vegetative seeds which sometimes is not possible with natural seed germination. This technology is useful in the germplasm preservation of elite species that are endangered or rare species and commercially important [149].

Priyadarshini et al. 2020 in their research demonstrated the production of synthetic seeds by 2.0% sodium alginate with 100mM calcium chloride where the SEs were later on preserved in hydrogel beads at -4℃ for a year. It is noteworthy to optimize the ratio of sodium alginate and calcium chloride for the encapsulating matrix as it was observed that lower concentration ratio produces spongy seeds which is not ideal for cold storage and higher ratios will harden the matrix which will delay the germination rate. Encapsulating agents are essentially hydrogels with a high-water retention capacity that are made up of hydrophilic polymers formed by crosslinking polymer chains. These hydrogels act as endosperm, supplying nutrition while enclosing and protecting the explants. Because of its non-toxicity, low spin ability, and moderate viscosity, sodium alginate is the commonly used encapsulating agent. One drawback is that it produces sticky coatings and allows for quick dehydration [150]. A report by Qahtan

et al. from 2019 describes the different types of compounds that can be used as encapsulating agents for hydrogel preparation methods as well as their advantages and disadvantages.

Another method for long term storage and preservation of viable, genetically stable, and disease-free germplasm is cryopreservation, where explants like somatic embryos, shoot tips, seeds, dormant buds etc. are stored in liquid nitrogen at a temperature of -196℃ or liquid nitrogen vapour at -165 to -190℃, hypothetically for unlimited period of time [151]. In order to achieve successful cryopreservation several precautions must be taken into account where the water content of the plant during immersion into liquid nitrogen must be low enough to prevent intracellular ice crystallisation of the cytosol and high enough to enable regrowth of the explant following post-thawing process [152]. Therefore, different types of cryoprotectants like glycerol, DMSO, ethylene glycol, sucrose, sorbitol, PEG, and amino acids like proline are used for vitrification [151].

2.7. Mode of action of GAL

AD is the most common type of dementia and the leading dysfunction in aging adults [153,154], which is due to the presence of abnormal accumulated proteins in the patient's brain. In AD, amyloid beta (Aβ) peptides accumulate into amyloid plaques. [155]. GAL has an exceptional dual mode of action, combining the reversible, AChE inhibition and the modulation of nicotinic acetylcholine receptor (nAChRs) [156,157,158]. It is the only drug of plant origin exhibiting both of these modes of action that is actively commercialized for the treatment of mild to moderate AD, and GAL efficacy has been proved in several clinical trials [109]. In addition, it is also capable of rescuing changes in mitochondrial membrane potential and morphology induced by Aβ25/35 to prevent mitochondrial dysfunction and endoplasmic reticulum stress [159,160]. By inhibiting AChE activity and preventing mitochondrial dysfunction, GAL reduces oxidative damage to the cells being neuroprotective [161]. During Alzheimer, reduced cholinergic neurons are interrelated with cognitive deficits such as learning and memory loss [162]. Most importantly, GAL has inhibitory effects on AChE in the hippocampal regions and frontal cortex of the brain, which are the most affected areas in Alzheimer's patients [163]. GAL reversibly and competitively binds to the active site on AChE and significantly inhibits the breakdown of Ach [156,164], whereby the availability of ACh in the cholinergic neurons is increased [163,165].

In addition to inhibiting AChE, GAL also allosterically modulates nAChR [156]. GAL binds to nAChR on cholinergic neurons at another (allosteric) binding site, which is different to the one used by ACh [156,166]. When ACh and GAL bind simultaneously to their specific binding

site, nAChR becomes more sensitive to ACh [167,168] Activation of nAChR may increase the release of other neurotransmitters, indicating that nAChR is involved in behavioral and psychological aspects of AD [162,169,170,171]. Consequently, modulation of nAChR may provide essential clinical advantages in AD, by potentiating nicotinic acid neurotransmission [171].

2.8. Biotechnological production of GAL

The identification, extraction, and characterization of plant secondary metabolites from plants is costly, and hectic and subsequently takes far too much time to process the samples and the quantity generated is also very low, which hampers the likelihood of the research. Recent developments in plant biotechnology have transformed the accumulation and production scenario of plant secondary metabolites. In the past few decades, the biological potential of Amaryllidaceae alkaloids has been recognized through the commercialization of GAL, due to its selective inhibitory activity as well as an Alzheimer's drug. Thus, the alkaloid content was characterized in extracts from an array of wild species of the Argentinian Andean region. Results showed that all the tested species were harbouring GAL and exhibited a strong acetylcholinesterase inhibitory activity with IC_{50} levels ranging between 1.2 - 2 μg/mL [172]. GAL is well known for its pharmaceutical activities including AChE properties used for AD [39,42,173]. With the recognition of GAL as an efficient and commercially viable drug, there has been an increase in the demand for its larger production by the pharmaceutical industries. To fulfill its demand, production from natural source became a major challenge [174]. On the other hand, embarking on long-term programs of genetic improvement and domestication of wild species does not appear as a sustainable option. The amount of GAL in plants vary with, type of cell or tissue, developmental stage, and plant species (Table 5) [175]. Therefore, for a sustainable production of GAL, exploitation of growing native and wild plants should not be recommended.

In conclusion, there is still scope for conducting precise and thorough metabolomic and transcriptomic studies for the biosynthetic networks of GAL, which can be achieved by targeting and reconstructing the biosynthetic pathway in microorganisms with the target of making alternative technology to produce GAL [176,177,178,179,180].

Against this background, several biotechnological approaches such as *in vitro* cell differentiation competence (Fig. 8) [180,182,189], medium components [184,185], light and dark conditions (photoperiod) [110,186], elicitation strategy [115,185,187], manipulation of PGRs [181,183,188] and different bioreactor systems [187,189], significantly influenced GAL

production (Table 6). The integrated management of these strategies during *in vitro* cultures was shown to be successful in phenylpropanoid biosynthesis in temporary immersion bioreactors [190,191], being considered a methodological basis for the optimization of GAL production. In perspective and considering the study of the profile of phenylpropanoids and galanthamine metabolites in *Lycoris* species [192,193] we can hypothesize about the role of other elicitor molecules, e.g., ABA and H_2O_2, in the increase of galanthamine yields in controlled systems such as temporary immersion bioreactors (TIBs). The use of this integrated strategy has provided comparable yields of phenylpropanoids between *in vitro* and *ex vitro* plants for native maqui (*Aristotelia chilensis*) plants [194].

Table 4: *In vitro* studies on *Crinum malabaricum.*

Plant part used	**Explant type**	**Explant size**	**PGRs used**	**Optimum concentrations**	**Observation**	**Reference**
Bulbs	Twin scales	2 cm	MS + mT	7.5 µM	Shoot proliferation was inferior to BA	[106]
	In vitro shoots	-	MS + IAA	1.0 mg L^{-1}	Root synthesis and length was inferior to IBA	
			MS + IBA		Longest roots (8.67 cm per explant)	
Bulbs	Twin scales	1 cm	MS + mT	2.0 mg L^{-1}	Direct bulblet formation	[133]
			MS + BA		Direct bulblet formation	
Bulbs	Twin scales	1 cm	MS + Kinetin	2.0 mg L^{-1}	Shoot initiation	[90]
			MS + BA		Shoot initiation	

	In vitro shoots	-	½ MS + IBA+ BAP	1.0 mg L^{-1}	Bulblet formation	
			MS + IAA		promotes root formation	
			MS + BAP + NAA	0.1 mg L^{-1}	NAA promotes root formation	
			MS + IAA	1 mg L^{-1}	-	
			½ MS + IBA	1 mg L^{-1}	prolonged incubation results in bulblet formation	
			MS + NAA	1.5 mg L^{-1}	-	
	Tri scales	1.0 x 1.5 cm	2,4-D	8 mg L^{-1}	Development of SEs of various stages,	[13]
			IBA	6 mg L^{-1}	SEs were mostly white and globular	
			BA + 2,4-D	6 mg L^{-1} + 8 mg L^{-1}	Number of SEs were significantly less	
			BA + IBA	6 mg L^{-1} + 6 mg L^{-1}	SEs were significantly less in number	

Table 5. Galanthamine biosynthesis from different plant species.

Plant Species	**Explant type**	**Test System**	**Extraction and Quantification**	**Elucidation Technique**	**Concentration per D.W.**	**Reference**
Clivia miniata	Roots	*In vivo*	-	HPLC	0.0284 mg/g	[195]
	Leaves				0.1489 mg/g	
Galanthus elwesii	Shoot	*In vitro*	HPLC	GC-MS	0.1 %	[181]
Galanthus fosteri Baker	Bulbs	*In vivo*	GC-MS	-	0.33-0.94 %	[196]
Galanthus hortensis	Flowers	*In vivo*	-	HPLC	0.0126 mg/g	[195]
Galanthus nivalis	Bulbs	*In vivo*	-	HPLC	0.0124 mg/g	[195]
	Leaves				0.0010 mg/g	
	Stems				0.0140 mg/g	
	Roots				0.0178 mg/g	
	Flowers				0.0011 mg/g	
Galanthus woronowii L.	Bulbs	*In vivo*	Ultrasound-assisted extraction	HPLC	0.470% in 30-50 mL/g (solvent/material)	[197]
Leucojum aestivum	Bulbs	*In vivo*	LC-MS	-	0.03%	[198]
L. aestivum	Shoot	*In vitro*	HPLC	GC-MS	0.075 %	[181]
L. aestivum	Shoot	*In vitro*	HPLC	-	19.416 mg	[189]

L. aestivum	Bulb	*In vivo*	TLC and Flash-column Chromatography	1D- and 2D-NMR, IR and Mass Spectrometry	~9mg/6g	[199]
L. aestivum	Young fruits	*In vitro*	HPLC	GC-MS	100-200 µg/g	[113]
L. aestivum	Leaves	*In vitro*	RP-HPLC	LC-MS-MS	6.79 x 10-3 %	[112]
L. aestivum L. (Gravety Giant)	Callus	*In vitro*	LC-MS	HPLC-MS	0.8mg/g	[187]
L. aestivum L.	Shoot	*In vitro*	HPLC	GC-MS	2.5mg/L	[113]
L. aestivum L.	Bulbs	*In vivo*	Maceration	HPLC	0.0196 mg/mL	[195]
			Ultrasonic bath		0.0273 mg/mL	
			Both maceration and ultrasonic bath		0.0949 mg/mL	
L. aestivum L.	Bulbs	*In vivo*	-	GC-MS	28-2104µg/g	[200]
	Shoot-clumps	*In vitro*			454µg/g	
L. aestivum L.	Bulbs	*In vivo*	TLC	GC-MS	0.003-0.08%	[201]
L. aestivum L.	Bulbs		-	HPLC	0.0949 mg/g	[195]
	Leaves				0.6024 mg/g	
	Roots				0.0262 mg/g	

L. aestivum L.	Bulbs	*In vivo*	TLC-HPLC	-	0.13-0.14%	[202]
Leucojum vernum	Bulbs	*In vivo*	-	HPLC	0.0047 mg/g	[195]
	Leaves				1.6611 mg/g	
	Roots				2.3524 mg/g	
Narcissus confusus	Bulb derived shoot	*In vitro*	HPLC	-	0.16 MG	[203]
	Seed derived shoot				0.182 MG	
Narcissus pseudonarcissus	Shoot	*In vitro*	HPLC	GC-MS	0.02 %	[181]
N. pseudonarcissus	Callus	*In vitro*	GC-MS	NMR	13-50 µg/g	[183]
Narcissus papyraceus	Bulbs	*In vivo*	SPE	GC-MS and LC-MS	8 µg/g	[204]
	Bulbs	*In vitro*			6 µg/g	
Narcissus tazetta	Bulbs	*In vitro*	HPLC-UV-MS	-	20-40 µg/g	[186]
N. tazetta	Bulbs	*In vitro*	SPE	GC-MS	15 µg/g	[182]
	Roots				5.2 µg/g	
	Calli				1.9 µg/g	
N. tazetta	Bulbs	*In vitro*	SPE	GC-MS and LC-MS	82 µg/g	[204]
	Calli				58.5 µg/g	
Zephyranthes rosea	Bulbs	*In vivo*	-	HPLC	0.8384 mg/g	[195]

Table 6. Showing effect of different plant growth regulators, elicitors and different bioreactor system on Galanthamine biosynthesis.

Plant Species	**Explant type**	**Bioreactor System**	**Optimum concentrations**	**Elicitors Used**	**Weight of Inoculum (g/L)**	**Maximum Yield**	**Reference**
Leucojum aestivum L. (Gravety Giant)	Callus	RITA®	25µM picloram+ 0.5µM BA	MeJa, SA, Ethephon (releases Ethylene)	-	0.8mg/g	[187]
L. aestivum L.	Shoot	Bubble column bioreactor	1.15mg/L NAA+ 2mg/L BAP	-	60g	6.0mg/L	[205]
L. aestivum L.	Shoot	Bubble column bioreactor	1.15mg/L NAA+ 2mg/L BAP	-	20.8g/L	1.7mg/L	[206]
L. aestivum L.	Shoot	Liquid-shake medium	1.15mg/L NAA+2.0mg/L BAP	JA, MeJa	0.6-0.9g/flask	226.9µg/flask	[115]
L. aestivum L.	Shoot	Liquid-shake medium	1.15mg/L NAA+2.0mg/L BAP	-	17.8g/L	2.5mg/L	[113]
L. aestivum L.	Somatic embryos	RITA®	TDZ (varying conc.)	-	1g	0.025% D.W.	[207]
L. aestivum L.	Shoot	RITA®	1mg/L KN+ 0.03mg/L NAA	-	10g	19.416 mg	[189]
Narcissus confusus	Shoot-clumps	Liquid-shake medium	3mg/L BA	MeJa, SA, Arachidonic acid, Chitosan	100mg	3.4mg/g	[208]
N. confusus	Bulbs		1mg/L 2,4-D+ 5mg/L BA	-	4.25±1.3g	0.16mg	[203]

	Seeds	Liquid-shake medium	1mg/L 2,4-D+0.5 mg/L BA	-	0.87±0.4g	0.182mg	
Pancratium maritimum L.	Shoot	Liquid-shake medium	1.15mg/L NAA+2.0mg/L BAP	-	200-300mg	3.3 mg	[209]

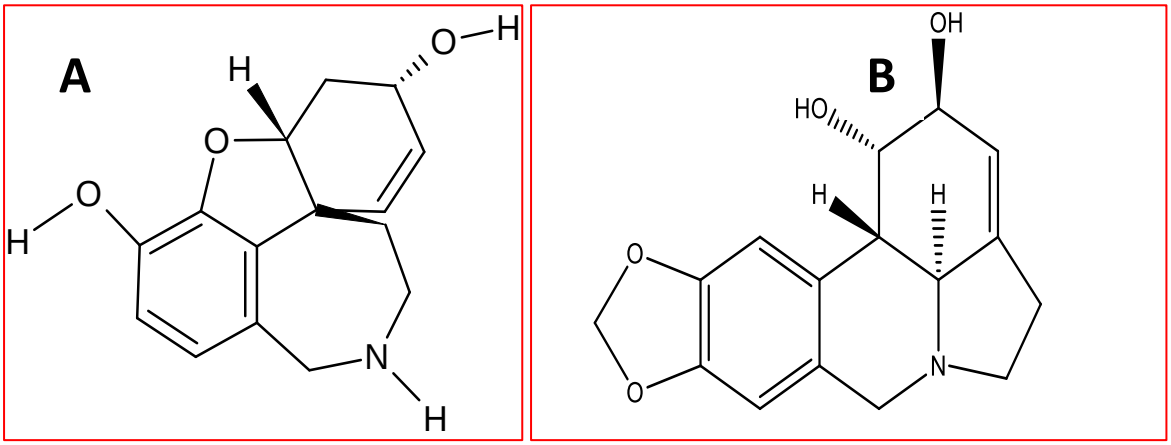


Fig 7. Chemical Structure: (A) Galanthamine; (B) Lycorine.

Table 7. *In vitro* studies on Indian *Crinum* species.

Plant species	***In vitro* studies**	**Explant type**	**Optimum concentrations**	**Major observation**	**Genetic fidelity Test (Molecular markers used)**	**References**
C. asiaticum	Somatic embryogenesis	Bulb-scale, flower bud	2.68 µM NAA + 4.44 µM BA	Somatic embryogenesis	-	[86]
C. asiaticum	*In vitro* propagation	Bulb-scale	4.0 mg/l NAA + 8.0mg/l BA	Reliable protocol shoot proliferation	-	[210]
C. asiaticum	Somatic embryogenesis	Bulb-scale	2.68 µM NAA + 4.44 µM BA	Somatic embryogenesis	-	[87]
C. asiaticum	Somatic embryogenesis	Bulb-scale	2.68 µM NAA + 4.44 µM BA	Callus induction and Somatic embryogenesis	-	[88]
C. malabaricum	*In vitro* regeneration	Bulb-scale	2.0 mg/l BA	Direct regeneration of shoots	**ISSR** Total band scored:35 Monomorphic bands:35 Polymorphic bands:00 **SCoT**	[90]

					Total band scored: 36 Monomorphic bands:36 Polymorphic bands:00	
C. malabaricum	*In vitro* regeneration	Bulb-scale	7.5 µm mT	Direct regeneration of shoots	-	[106]

Table 8: Soil analysis report of the Laterite soil of Kerala.

Lab No.	**Particular of Elements**	**pH**	**EC mm hos/cm**	**Organic Carbon (%)**	**P (ppm)**	**S (ppm)**	**K (ppm)**	**Ca (ppm)**	**Mg (ppm)**	**Fe (ppm)**	**Cu (ppm)**	**Mn (ppm)**	**Zn (ppm)**	**Mo (ppm)**	**B (ppm)**
	Concentration of Required Elements	**6.5-8.7**	**<0.80**	**0.40-0.75**	**5.0-9.0**	**10.00**	**55-135**	**300.00**	**120.00**	**4.50**	**0.20**	**3.50**	**0.60**	**0.15**	**0.50**
Sample No.	**Location**		**Quantity of Elements in Analysed Soil**												
3031	Kerala	7.60	0.18	0.90	7.52	50.26	39.32	204.90	22.96	60.00	2.70	2.54	0.88	0.11	0.53

2.9. Effect of plant growth regulators (PGRs) on GAL biosynthesis

Optimization of GAL biosynthesis, *in vitro* and production is significantly affected by various plant growth regulators (PGRs). The effects of quite a few auxins (2,4-D, NAA, and picloram) and cytokinins (BA, Kinetin, and Zeatin) has been investigated by several researchers in *L. aestivum* regarding *in vitro* GAL synthesis (Table 6 and Table 7) [113,181, 207]. In *L. aestivum* shoot cultures, it was observed that the accumulation of GAL depends on light and on the type of culture medium used for the propagation [113], as also shown by Berkov et al. (2009) [110]. The influence of picloram and BA on the GAL accumulation in *L. aestivum* somatic embryos was comprehensively evaluated by Ptak and colleagues in 2010 [211]. The maximum conc. hike which was about 2% was achieved in the experiments with given treatments of 0.5 µM BA, 25 µM picloram and 1-aminocyclopropane carboxylic acid (ACC). However, it was found that the GAL production was significantly inhibited (0.27% and 0.8% DW respectively) by addition of silver nitrate and potassium permanganate. In another study, it was found a higher accumulation of GAL in *in vitro* bulbs, followed by leaves and roots of *L. aestivum*. In the bulbs, 10 mg/L BA and 10 mg/L of putrescine were responsible for the maximum content of GAL which was found to be 0.290 % [212]. In *Narcissus tazetta* when the callus culture media was enhanced PGRs like with 2,4-D alone and combined with BA, researchers found that the total alkaloid content increased and reached a maximum of 2.070 µg/g DW for GAL with 0.1 µM conc. of 2,4-D and 1.0 µM conc. of BA [213].

In *L. aestivum* shoot culture, the effect of various cytokinins (*m*T, BA, TDZ, Kn and Zeatin) for *in vitro* GAL production in a temporary immersion system (RITA™) has been studied and it was observed that the highest GAL content (0.05%) was produced with TDZ [207]. The same group also found that *in vitro* GAL biosynthesis was significantly decreased by ethylene [198,211], although 1-aminocyclopropane-1-carboxylic acid increased GAL accumulation six-fold (2% DW) in cultures [211].

In a study with *N. pseudonarcissus* cultures, [185] found that the maximum level of GAL (10-215 µg/g FW) was obtained in shoots and bulblets inoculated into the cultured in medium enhanced with 4 mg/L NAA, while a lower level of GAL (1.0-7.0 µg/g FW) was observed in callus cultured with 20 mg/L NAA, and no GAL production occurred with a lower NAA concentration. These results are in line with data obtained by [214], where the highest conc. of GAL with a quantity of 1345 µg/g FW was found in *N. confusus* cultures inoculated onto the medium with 3 mg/L BA, but a small amount was found in absence of BA.

These studies indicate that PGRs significantly affect metabolic profiling in different cultures derived from the same species. PGRs, alone or combined, have a potential effect in the

metabolic profiling of *in vitro* raised plantlets. As changes in PGRs modify the concentration of bioactive compounds, a thorough study on their various types and concentrations for individual species is necessary to understand which of them will be most promising to synthesize the desired drug.

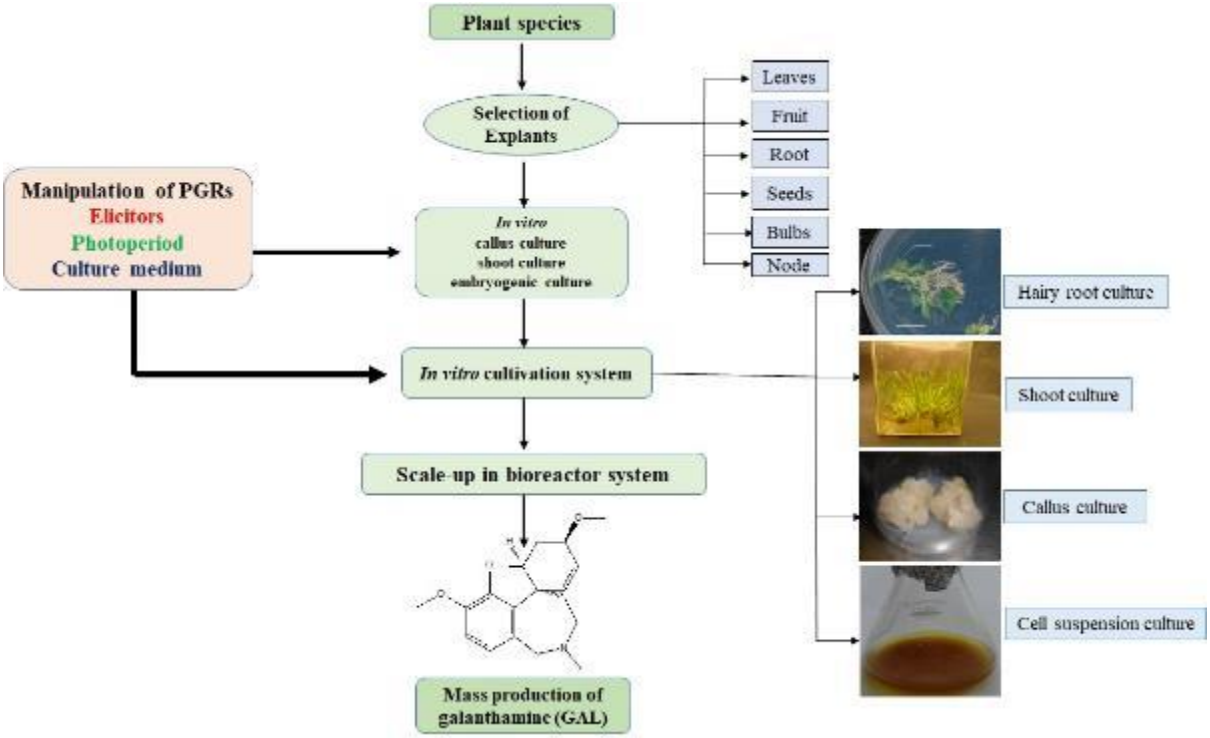


Fig 8. Schematic representation for mass galanthamine production.

2.10. Elicitation as an effective strategy to influence GAL biosynthesis

Plant-specialized metabolites are a unique source of pharmaceuticals produced by plant cells, tissues, and organ cultures as a defensive strategy against various pathogens, and the accumulation of these metabolites is often associated with several elicitors [215]. Elicitors are chemicals or biofactors that can trigger the expression of transcription factors, key genes and regulate a large number of biochemical reactions as well as physiological and morphological responses [215,216]. These elicitors could be abiotic or biotic and may include microbial cell wall extracts (chitosan, yeast extract), signalling molecules (salicylic acid, jasmonic acid, methyl jasmonate), heavy metals, inorganic salts, and UV radiation as a physical agent [215,217]. This multitasking capacity of elicitors is unique and multidimensional, hence there is increasing interest on their use for the development of therapeutic secondary metabolites [187,218].

The influence of different concentrations of four elicitors (arachidonic acid, chitosan, methyl jasmonate and salicylic acid) on *in vitro* accumulation of GAL in *N. confusus* cultures has been investigated [208]. The addition of methyl jasmonate (25µM) promoted 2-fold increase of GAL production. In addition, methyl jasmonate also stimulated GAL accompanying alkaloids such as haemanthamine, homolycorine, N-formyl-norgalanthamine and pretazetine [208]. However, other elicitors used in this study did not have any significant effect on biosynthesis of GAL and other alkaloids. The effect on the GAL biosynthesis as observed in previous studies has been reenacted by the use of elicitors like methyl jasmonate, silver nitrate, copper sulphate and salicylic acid in *L. aestivum* shoot cultures [219]. The results obtained by Colque and colleagues (2004) [208], indicates that the highest GAL level in *L. aestivum* shoot cultures was found on medium supplemented with methyl jasmonate. Another study conducted by Ivanov and colleagues back in 2013 [115] exposed the effects of commonly used elicitor compounds like methyl jamonate and jasmonic acid on the GAL biosynthesis of *L. aestivum* during the shoot cultures, which were cultivated in submerged conditions. Jasmonic acid elicitation fostered production of the highest amount of GAL (226.9 µg/flask), which was found to be 1.36-fold higher than the control, and the reason behind such increase in the conc. was because of the expression of gene responsible for tyrosine decarboxylation, which enhanced the precursor (tyramine) of Amaryllidaceae alkaloids [115]. Different elicitors (salicylic acid: 5, 50 and 100µM, methyl jasmonate: 5, 50 and 100µM, 2-chloroethylphosphonicacid (ethephon): 10 and 100µM, and 1-aminocyclopropane-1-carboxylic acid: 10 and 100µM) affecting GAL biosynthesis in *L. aestivum* shoot culture have been investigated by [187]. They observed the highest yield of GAL which is 0.8 mg/g of DW when plants were treated with 5.0 µM of SA for 10h, and also an increased amount of 0.10 mg/g per DW in the presence of 10 µM of ACC while ethephon was not found effective to induce GAL production. Interestingly, in a more recent article, [220] it was observed that there was 58.6 times greater accumulation of GAL with 5 µM melatonin as an elicitor which is 46.88 µg/L,higher than that of the control, while 10 µM melatonin increased the *L. aestivum* biomass and increased the conc. of the GAL content by 15.7 times higher as compared to the control. Recently, Ferdausi and co-workers (2021) [185] used an *in vitro* elicitation approach which resulted in the increase of GAL concentration by 5.6-fold and was estimated to be 44.41 µg/g of FW, in *Narcissus pseudonarcissus* callus culture inoculated onto the tissue culture media enhanced with methyl jasmonate as an elicitor. In addition, chitosan supplemented media resulted in a 3-fold higher production of GAL which was assessed to be 23.29 µg/g of FW. In this context, using elicitors with *in vitro* plant cultures instead of callus cultures in controlled systems would improve the photosynthetic activity *in*

vitro, and could therefore pave the way for a subsequent scaling process for the efficient production of GAL in such automated TIBs. Hence, these findings confirms that elicitation could be an effective approach to scale up the synthesis of natural secondary metabolites from *in vitro* cultures. They also indicate that, although the mechanism of activation of the production of alkaloids is not yet clear, there is the potential of basic and applied knowledge to clarify the possible interrelationships with the signalling mechanism of phenylpropanoids, thus opening a way to implement a reproducible and low cost technologies for the synthesis of GAL in *in vitro* production/culture systems.

Chapter 3

RESEARCH METHODOLOGY

1: Collection and development of regeneration protocol for *Crinum malabaricum* through *in vitro* technique

3.1.1. Chemicals

Bavistin, Agar powder, BA (6-benzyladenine), IBA (indole-3-butyric acid), IAA (indole-3-acetic acid), *meta*-Topolin (*m*T), vitamins, sucrose and myoinositol were obtained from Hi-media, India. All reagents used were of analytical grade.

3.1.2. Plant Collection and Authentication

Plant material for the present study was collected from its place of origin, which is a seasonal freshwater stream in the village of Periya (12°24.5260N, 75°06.5710E, 102 m asl), and comes under the authority of Kasaragod district, Kerala, India. The species was identified by taxonomist Dr. Manoj Lekhak and a voucher specimen was submitted in the herbarium of Shivaji University, Kolhapur, Maharashtra, India.

3.1.3. Sterilization of explants

The collected plant material was maintained in the greenhouse in plastic pots. For this experiment, the plant bulbs of CM were harvested, the outer dried scales, dried leaves, and roots were removed (Fig. 15). The bulbs (5-7 cm) were thoroughly washed with running tap water several times to remove any trace of soil or dirt. The bulbs were sterilized with Bavistin (fungicide) 5% (w/v) (for 45 min) along with 0.1% (v/v) Tween 20 (detergent) (Himedia, India) for 20 minutes, and then surface disinfected with 70% ethanol (60 s) (Table 10.) and rinsed with double D/W. The rest of the sterilization process was carried out in the laminar air flow bench where the treated bulbs were sterilized with 0.1% mercuric chloride for 5 minutes and later on rinsed with autoclaved double D/W 3-4 times. The sterilization protocol was standardized by treating the CM bulbs with different sterilizing agents at different time intervals and the treatments with least contamination was chosen as standard protocol as given in the table 10.

3.1.4. Culture Media Preparation

The MS medium was prepared by adding macronutrients, micronutrients and vitamins and inositol (Table 9). Using IN NaOH and 1N HCl the pH of the medium was adjusted to 5.8 and the medium was solidified with 0.8% agar microwaved to mix the agar powder. Thereafter, 30

ml of preheated culture medium was poured into the tissue culture vessels and sterilized by autoclaving at a temperature of 121 °C and 103 kPa pressure for 20 min. After a thorough wash with autoclaved water, the sterilized bulbs were cut and split into twin scales (2.0 cm) with basal plates and inoculated onto the culture medium [221] with 3% (w/v) sucrose and 4.44 µM initial conc. of BA. The cultures were then kept in the growth chamber where the temperature was maintained at 25±2 °C, with a 16 hrs light and 8 hrs dark photoperiod with a light intensity (PPFD) of 50 µmol m^{-2} s^{-1} from cool white, fluorescent lamps. *In vitro* grown cultures were used in further experiments.

3.1.5. Glassware sterilization

Before use, all glassware, including flasks, petri plates, test tubes, beakers, culture bottles, forceps, and scalpels, were cleaned. They were first sprayed with liquid detergent to remove any dirt particles and washed with tap water. They were dipped in chromic acid and washed with double distilled water a few hours later. Finally, they were sterilized by drying them at 60-80°C in hot-air oven for further usage.

Table 9. MS media composition.

Stock	Salt Component	Mass/500 ml Stock (g)	Volume stock (ml/L) final medium
1	NH_3NO_3	82.5	5
2	KNO_3	47.5	10
3	$CaCl_2.2H_2O$	22	5
4	$MgSO_4.7H_2O$	18.5	5
5	NaFe EDTA	2	5
6	KH_2PO_4	8.5	5
7 (a)	H_3BO_4	0.31	5
	$ZNSO_4.7H2O$	0.430	5
	KI	0.0415	5
(b)	$MnSO4.4H_2O$	1.115	5
8	$NaMoO_4.2H_2O$	0.0125	5
	$CuSO_4.5H_2O$	0.00125	5
	$CoCl_2.6H_2O$	0.00125	5
9	Thiamine HCl	0.005	5
	Nicotinic Acid	0.025	5
	Pyridoxine HCl	0.025	5
	Glycine	0.1	5
10	Inositol	0.05	-
11	Sucrose	15	-

Table 10. Sterilising agents with different time intervals were used to standardize the sterilization protocol

S.No.	Sterilization agents	Concentration (%)	Treatment duration (min/sec)	Preparation
1	Tween 20	0.1 %	15 m	2 drops in 400 ml distilled water
			20 m	
			30 m	
2	Bavistin	5 %	25m	5 gm in 100 ml
			35 m	
			45 m	
3	Ethanol	70 %	40 s	70 ml ethanol in 30 ml distilled water
			50 s	
			60 s	
4	Mercuric chloride ($HgCl_2$)	0.1 %	3 m	1 g $HgCl_2$ dissolved in 1000 ml of distilled water
			4 m	
			5 m	

3.1.6. Stock solution preparation

Auxin/cytokinins are the primary hormones used in this study. For the induction of callus, shoot, and root, varying conc. of BA, IBA, IAA, NAA, 2, 4-D, Picloram, and TDZ were prepared. Because all PGRs are insoluble in water, they were dissolved in a relative solvent before being mixed with D/W to make the required volume. Table 11 shows the solubility of these PGRs.

Table 11. Plant growth regulators used for micropropagation and their solubility.

Plant hormone	**Formula**	**Molar mass (g/mol)**	**Solubility**
BA	$C_{12}H_{11}N_5$	225.25	1N NaOH/EtOH
Meta-topolin	$C_{12}H_{11}N_5O$	241.25	1N NaOH/EtOH
NAA	$C_{12}H_{10}O_2$	186.21	1N NaOH/EtOH
IAA	$C_{10}H_9NO_2$	175.18	1N NaOH/EtOH
IBA	$C_{12}H_{13}NO_2$	203.24	1N NaOH/EtOH
2,4-D	$C_8H_6ClO_3$	221.03	1N NaOH
Picloram	$C_6H_3Cl_3N_2O_2$	241.5	1N NaOH/EtOH
TDZ	$C_9H_8N_4OS$	220.25	1N NaOH/EtOH

3.1.7. Culture Initiation

After sterilization, the bulbs were cut and divided into twin scales (2.0 cm) (Fig 11) with basal plate intact and not damaged. Twin scales were then inoculated into the MS media comprising of macro-nutrients, micro-nutrients, vitamins, myoinositol, sucrose (3%), and 4.44 µM. Media without any hormone served as control and the pH of the culture medium was set to 5.8 with the help of 1N NaOH and 1N HCl and was solidified using agar with a conc. of 0.8%. Then 30 ml of culture medium was distributed in tissue culture vessels and autoclaved at 15 psi pressure and 121 ºC for 20 minutes. Cultures were maintained in the culture room with a sustained temp. at 25 ± 2 ºC, under a 16/ 8h (light/dark) photoperiod was maintained by cool white LED tubes at PPFD (photosynthetic photon flux density) of 50 mmol $m^{-2}s^{-1}$.

3.1.8. *meta*-Topolin mediated *in vitro* shoot proliferation

The *C. malabaricum* shootlets induced previously were used as explants and the meristematic regions of the bulbs with intact basal plate were transferred to the semi-solid nutrient medium having varying concentrations of *meta*-Topolin (*m*T) (0.5, 2.5, 5.0, 7.5 and 10 µM). The growth conditions and pH range for the cultures as detailed above were used during shoot proliferation.

Subculturing was done using the previously described concentrations of *m*T at a interval of six-weeks. The average of number of shoots, length of shoots and fresh weight of shoots calculated and recorded, while each experiment was replicated three times. The culture medium without any hormones served as control.

3.1.9. Rooting and acclimatization of CM *in vitro*

The *C. malabaricum* shoots micropropagated *in vitro* were excised and transferred to the fresh medium supplied with varying concentrations of both IAA (0.5, 1.0, 2.0, and 3.0 mg L^{-1}) and IBA (0.5, 1.0, 2.0, and 3.0 mg L^{-1}) alone and also in combination with each other. Cultural growth conditions were maintained as stated above. After eight weeks the mean number of roots per shoot and the rooting frequency were assessed.

After eight weeks, *in vitro* regenerated plants, with well-developed roots were removed from culture jars and thoroughly washed for the removal of any residue of the culture medium, and transferred to paper cups containing sand, soil, and vermiculite mixture 1:1:1 (v/v/v) and covered with plastic bags to maintain humidity (Fig. 20). The plantlets were then transferred in a mist house under natural photoperiod conditions with 90-100% RH (relative humidity). The healthy plants were later on transferred to the greenhouse.

3.1.7 Statistical analysis

In these experiments, Statistical analysis was done with ANOVA through software SPSS. The DMRT (Duncan's multiple range test) was used to calculate the means at a probability level of 5% ($P \leq 0.05$).

2: Somatic embryogenesis of *Crinum malabaricum*

3.2.1. Chemicals

Bavistin and agar powder, 2,4-D, BA, Picloram, TDZ, GA_3, macro-nutrients, micro-nutrients, vitamins, and myo-inositol were obtained from Hi-Media®, India. All reagents were of analytical grade.

3.2.2. Development of somatic embryos

To obtain the bulb explant, plant leaves were removed, and the root and outer scale were harvested. The bulbs (5-8 cm) were rinsed thoroughly under running tap water and sterilized by using our previously published protocol [106]. The sterilized bulbs were cut longitudinally and twin-scale plants (2 cm) with basal plates were made under aseptic conditions. Sterilized twin-scale explants were inoculated into standard MS medium [221] supplemented with $8gL^{-1}$ agar and multiple concentrations, either alone or in combination with different PGRs (2,4-D, BA, picloram and TDZ) for SE induction. Vitamins, macro-, and microelements for medium preparation were introduced in the form of previously prepared aqueous solutions stored at 4 ^{o}C. The culture media and the PGRs were prepared in accordance with our recently published protocol [107] and the media without the presence of PGR served as a control. All the cultures were maintained in the growth chamber with the optimum conditions as stated above. The percentage of embryogenic calli was calculated as the total number of embryogenic explants/total number of explants used x 100. Selective friable embryogenic calli were added to a culture medium that contained TDZ and picloram in various quantities and 8 weeks later, the average number of somatic embryos was recorded.

3.2.3. Somatic embryo germination

To study the regeneration of complete plantlets, cotyledonary-shaped embryos were transferred to a media (Full strength and half strength) supplemented with gibberellic acid (1.0 mg/L). The inoculated cultures were maintained and incubated under the same conditions as above. The embryo germination rate was determined after 8 weeks.

3.2.4. Scanning Electron microscopy

For SEM analysis, embryogenic calli with developing embryos were selected and fixed in glutaraldehyde (2.5 %) followed by dehydration with ethanol series (10-100%) and dehydrated samples were dried by critical point dryer. After being coated with gold (to make the samples conductive) in sputter ion coater (D II-29030SCTR) the specimens were examined and images

were captured under a SEM (FESEM, JSM-7610F, Oxford Instruments X-Max N) operated at 15-25 kv.

3: Maintenance of cell suspension and study the effects of elicitors on secondary metabolite production.

3.3.1 Chemicals

MS media, Growth regulators, Jasmonic acid, methyl Jasmonate, and Salicylic acid were purchased from Sigma- Aldrich, USA. Both the standards (lycorine and galanthamine) were procured from Sigma-Aldrich®, India. All reagents were of analytical grade.

3.3.2. Preparation of stock solutions for elicitors.

The elicitors utilized in the present study included salicylic acid, methyl jasmonate, and jasmonic acid. The cell suspension cultures were initiated on different concentrations of JA, MeJA, and SA were prepared and added to the MS media. Because most PGRs are insoluble in water, they were dissolved in a relative solvent before being mixed with double D/W to makeup the required volume (Table. 12). The stock solution was prepared for the elicitors.

1. Jasmonic acid – Elicitors JA was produced by mixing 250 milligrams of JA in a mixture of ethanol and water with a volume-to-volume ratio of 12:13 [222,223]. This produced a stock solution with a volume of 100 ml. This produced a stock solution with a concentration of 100 millimeters and 0.22 micrometer membrane filter was used to disinfect the elicitor solution. Different concentrations of jasmonic acid were prepared (15µm, 25µm, 50µm) (Table. 12) and added to autoclaved media.
2. Methyl Jasmonate – A 100M stock solution of elicitor methyl jasmonate was made by dissolving 250ml of methyl jasmonate in ethanol: water (12:13 v/v) [222,223]. A 0.22 micrometer membrane filter was used to disinfect the elicitor solution. Different concentrations of jasmonic acid were prepared (15µm, 25µm, 50µm) and added to autoclaved media.
3. Salicylic acid – Elicitor salicylic acid solution was prepared in water to prepare a 5M stock solution. The elicitor solution was autoclaved. Different concentrations of Salicylic acid were prepared (15µm, 25µm, 50µm) and added to autoclaved culture media.

   Samples for HPLC were collected on the 7^{th}, 14^{th}, and $21^{st,}$ days. MS media without any elicitor served as control for all the samples.

Table 12. Elicitors used for cell suspension and their solubility.

Elicitors	Formula	Molar mass (g/mol)	Solubility
Jasmonic acid	$C_{12}H_{18}O_3$	210.27	EtOH/Water
Methyl jasmonate	$C_{13}H_{20}O_3$	224.3	EtOH/Water
Salicylic acid	$C_7H_6O_3$	138.121	EtOH/Water

3.3.3 Callus induction and establishment

Explants from plants that had been *in vitro* propagated were employed in this experiment. Different concentrations and combinations of PGRs were added to MS medium to cultivate the explants in order to induce and establish callus growth. MS medium with 2,4-D (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10mg l^{-1}) alone and in combination with BA (2.5 mg l^{-1}) as well as Picloram (0.5,1.0,2.0,3.0,4.0, 5.0 mgL^{-1}) and TDZ (0.5 mgL^{-1}) was used as culture medium. All the cultures were maintained at a temperature of 22°C and white light intensity of 50 mmol $m^{-2}s^{-1}$. Subcultures were carried out every 10 days and after 4 weeks the multiplication rate and the frequency (%) of callus formation from the explants was recorded.

3.3.4 Elicitation and establishment of suspension culture

To establish cell suspension culture friable callus was used. In a 250 ml conical flask pour 50 ml of MS medium, enriched with different concentration elicitors (Table 13) (JA, MeJA, and SA) and add approximately 1.0g of pre-established callus of CM into it. The suspension cultures were maintained in an incubator shaker at 120rpm at 28°C. Samples of suspension cells treated with elicitors were harvested at different day intervals (7^{th}, 14^{th}, and 21^{st} days) after elicitation and filtered using 0.22 micron nylon membrane filter (Fig. 9). The callus was sieved and further used for the analysis. The MS media without any PGR or elicitors served as control.

Table 13. Different Elicitors and their concentrations used for elicitation in cell suspension.

Elicitors	Stock Concentration Used (µM)	Total volume used
Jasmonic Acid	15, 25, 50	165 µl, 220 µl, 550 µl
Methyl Jasmonate	150, 250, 500	1.65 ml, 2.75 ml, 5.5 ml
Salicylic Acid	15, 25, 50	165 µl, 220 µl, 550 µl

3.3.5. Sample preparation for HPLC analysis

Callus harvested from the cell suspension experiment was dried for 16 hrs in an oven at 50℃ and using a pestle and mortar were ground into fine powder. The powdered sample was dissolved in 95% ethanol and kept in an incubator shaker for 12 hrs, 120 rpm at 28℃. At room temperature, the solvent was evaporated, and the dry residue was dissolved in 20 ml of 2% H_2SO_4 followed by a defatification procedure through petroleum ether. An aqueous layer will form which was later basified using liquid ammonia (23%). The pH of the solution was maintained between 9.5-10 and the alkaloid fraction thus obtained was separated with HPLC grade chloroform. Methanol was used to dissolve the chloroform layer and then filtered through 0.22 µm micron membrane syringe filter and used for HPLC analysis.

3.3.6. Quantification of LY and GAL

Identification and quantification of GAL and LY from the *in vitro C. malabaricum* plant extracts was accomplished using HPLC analysis, by following the protocol established by researchers like Ghane and colleagues in 2018, and Lekhak and colleagues in 2021 [26,127]. Jasco chromatographic system (Model No. LC-2000 plus) equipped with an UV detector (UV 2070), autosampler, column guard, and binary pump was used to perform the HPLC analysis. C18 Hiber column (5µm, 250-4.6 mm) was used for separating the compounds from the sample. Acetonitrile (CAN) (10%), Acetate buffer (pH – 3.6) diethanolamine (DEA) (0.025M) comprised the mobile phase for the column. The total run time for the sample was 20 minutes with a flow rate of 1ml/minute and the loading volume of the plant extract for the injection was 20ml. The peaks were observed at a wavelength of 240 nm. The retention time was compared for both the samples and the standards to identify the alkaloids GAL and LY. The calibration

curve was plotted with alkaloid concentration expressed in terms of milligrams per gram of dry weight (mg/g DW).

Fig 9. Cell suspension culture of Callus of *C. malabaricum*. A) Callus used for the initiation of cell suspesnsion; B) Cell suspension culture on Day 7; C) Cell suspesnsion culture on Day

14; D) Cell suspension culture on Day 21; E) Callus used for the initiation of cell suspension; F) Preparation of elicitors using 0.22-micron membrane filter.

4: Assessment of genetic fidelity in regenerated Crinum malabaricum and extraction and analysis of secondary metabolites

3.4.1. Plant Sample

A 4-5 cm length of fresh leaves grown in the *in vitro* conditions were used as explants to isolate genomic DNA. *In vitro* grown plants of *C. malabaricum* and mother plant grown in *in vivo* conditions were chosen to compare for the genetic fidelity analysis.

3.4.2. DNA isolation from *C. malabaricum*

A high-quality DNA was isolated from the 4-5 cm *in vitro* leaves of *C. malabaricum* using CTAB method following the Doyle and Doyle, 1987 protocol [224]. CTAB buffer was prepared freshly with a composition of 1.0 M Tris-Cl and pH was adjusted to 8.0. To this buffer 5.0 M NaCl, 0.5 M sodium EDTA, 2% CTAB and 0.2% β-mercaptoethanol were added. Approximately 50–200 mg of leaf tissue was grounded to fine powder by freezing in liquid nitrogen and poured into the 1.5 ml centrifuge tubes along with 750 µl of CTAB pre-warmed at 65 ◦C and 0.2% v/v β-mercaptoethanol (2 ml). The grounded tissue samples were mixed by using a vortex mixer and incubated in a water bath at 65 ◦C for 1 hr to facilitate the lysis procedure. After lysis the samples were cooled down and centrifuged for 10 min at 13,000 rpm.

Treatment with 5 µl of RNase A (10mg/ml) was used to remove the RNA from genomic DNA which was then incubated at 37 ◦C for 1 h and later on centrifuged for 10 min at 13,000 rpm. The upper aqueous layer was separated and transferred into a new centrifuge tube. to this separated aqueous phase an equal volume of Phenol: Chloroform: Isoamyl alcohol (P: C: I 25:24:1) was added. This was mixed properly by inverting the tube 15–20 times. This mixed suspension was then again centrifuged at 13, 000 rpm for 10 min. From the separated phases the upper layer was again separated and the RNase-treated samples were mixed with 750 µl of Chloroform : Isoamyl alcohol (C : I; 24 : 1) in a new tube and again centrifuged for 10 min at 13,000 rpm room temperature in a tabletop microcentrifuge. After centrifugation chilled 0.6 vol of Isopropanol (6000 µl) was added to the aqueous phase and mixed properly by gently inverting the tubes to facilitate the precipitation of DNA. After centrifugation at 12,000 rpm (20 min), the supernatant was decanted from the centrifuge tube and the DNA pellet was washed with 5 ml of 70% Ethanol. After washing the DNA pellet was resuspended in 100 –

200 µl of sterile DW. By running the DNA across a 1.0% TAE agarose gel in a gel electrophoresis system allowed researchers to evaluate and assess the purity and concentrations of the isolated DNA.

3.4.3. Polymerase Chain Reaction

Eight RAPD marker primers, ten SCoT and ten ISSR primers were used in the PCR analysis. All the reactions were set in a 96 well plate in a Gradient Thermal Cycler (iGene Labserve or G-storm). The PCR reaction mix was prepared by the addition of 10X buffer, 10 mM dNTPs (Takara, Japan), (3.0 u/µl) Taq DNA polymerase, 2.0 µl of primers (SCoT, ISSR and RAPD) (Operon Technologies, USA) (Table 14). The following conditions were met during the cycle program-based PCR amplification reaction: Denaturation was first carried out at 94 ºC for 3 min, followed by 32 cycles of annealing followed by extension at 72 ºC for 2 min, and finally denaturation at 94 ºC for 10 min (Table 15). During the PCR reaction, annealing varied between 50 and 58 ºC based on the melting temperature of the primer. All the PCR products were mixed with gel loading dye and a total volume of 8 µl of the reaction mixture and a 100 bp or 50 bp ladder (Thermo Fisher Scientific, USA) was loaded onto the agarose gel to compare the size of the amplicons. A 2.5% 1X TAE Agarose gel was used to analyse the PCR products.

Table 14. Composition of PCR reaction mix.

Components	**Quantity (µl)**
Water	13.3 µl
10X Buffer	2 µl
10mM dNTPs (Takara, Japan)	0.4 µl
ISSR, SCOT, and RAPD primers (10µM) (Barcode Biosciences)	2 µl
Taq DNA polymerase (3U/µl) (Merck)	0.3 µl
DNA Template (~25ng)	2 µl
Total volume	**20 µl**

Table 15. The amplification reaction carried out using PCR.

Temperature	Time	Activity	
94°C	3 min	Initial denaturation	
94°C	20 sec	Denaturation	32 cycles
37°C (RAPD) **52-58°C (ISSR/SCOT)**	30 sec	Annealing	
72°C	2 min	Extension	
72°C	10 min	Final extension	
4 °C	∞	Storage	

3.4.4. Agarose gel electrophoresis

All the PCR products were mixed with gel loading dye and a total of 8µl volume of the mixture was loaded on the agarose gel. 100bp or 50 bp ladder was loaded to compare the size of the amplicons. A 2.5% 1X TAE Agarose gel was used to analyze the PCR products.

3.4.5. Quantification of phenolic acid profiling using UHPLC-MS/MS and LC-ESI-MS/MS analysis

3.4.5.1. Sample Preparation

In vitro regenerated plantlets were freeze-dried and extracted with methanol (80%; 100 mg/mL) and deuterium-labelled internal standards. In an Eppendorf tube (2 mL), the extraction was done using an oscillation ball mill for 3 minutes at a frequency of 27 Hz. Following centrifugation at 20,000 rpm for 10 min. after centrifugation a 0.45 µM nylon membrane microfilter (Whatman^{TM} UNIFLO^{TM}, GE Healthcare, Buckinghamshire, UK) was used to filter the supernatants and used for the analysis.

3.4.5.2. UHPLC-MS/MS Analysis

C. malabaricum phenolic acid profiles including different hydroxybenzoic and hydroxycinnamic derivatives were quantified as reported [225,226]. Analysis was carried out using an ACQUITY UHPLC (Waters, USA) system attached with micro mass Quattro micro^{TM} API triple quadrupole mass spectrometer.

Based on MS/MS transitions of target compounds with standards and the comparison of RT identification was done. Each treatment included three replicates.

3.4.5.3 LC-ESI-MS/MS analysis

a) Preparation of stock solution of standard compound

Lycorine and Galanthamine standards were purchased from Sigma. Stock solution of Lycorine and Galanthamine (1 mg ml^{-1}) was prepared in methanol. The concentration utilized for the preparation of ten point calibration curve for LYCORINE and GALANTHAMINE ranged between 5 and 50 µg ml^{-1} (1/2 dilution pattern), respectively. Standard solutions were filtered through a 0.2 µm membrane filter (Millipore). The stock and working solution were stored at 4 °C.

b) Sample preparation

Somatic embryo-derived plants were lyophilized and freeze-dried. The samples were pulverized to make a fine powder (0.1 g). 100 mg of the powdered sample each of leaves, bulbs and roots was weighed and dissolved in 10 ml 60% methanol (HPLC grade) (Fig. 10) and further used for extraction techniques like microwave-assisted, ultrasonication and maceration. After extraction the sample solution was concentrated using rotary evaporator (Buchi, Switzerland) and the residue was dissolved in 1ml methanol. After that, the produced sample was centrifuged for 10 minutes at 10,000 rpm. Using a 0.22 M nylon membrane filter, the supernatant was purified and kept at 4 °C. The experiment was carried out thrice.

Microwave assisted Techniques- 100 mg of the powdered material each of leaves, bulbs and roots was dissolved in the 60% 10 ml of HPLC grade methanol and microwaved for 60 sec. The rest of the extraction was carried out as stated above.

Maceration- For maceration, 100 mg of the dried and powdered sample was dissolved in 10 ml of 60% methanol for 24 hrs with intermittent shaking in between. The rest of the extraction was carried out as stated above.

Ultrasonication- Again the 100 mg of powdered samples were dissolved in 10 ml of 60% methanol and sonicated in an ultrasonic bath at 40KHz for 10 mins at room temperature. The rest of the extraction was carried out as stated above.

c) Compound Extraction

An Agilent 1260 HPLC autosampler system fitted with a column heater and a quaternary pump was used for the LC-ESI-MS/MS analysis. Formic acid (0.1%) and acetonitrile comprised the mobile phase. A column (RP-18e; 50mm x 3.0 mm) temperature was set at 30 ºC with 0.5 min^{-1} of flow rate. The scan range of a hybrid quadrupole mass spectrometry equipped with electrospray ionization (ESI) was 100–600 (m/z), and it was operated in both positive and negative ionization modes. Ionization parameters were maintained as ion source temperature 300 ºC, 4000 V charging, capillary voltage 3.5 kv, nitrogen (collision gas) and nebulizer pressure at 50 psi. The lycorine and galanthamine were acknowledged by the retention time in correspondence to the retention time of the standards.

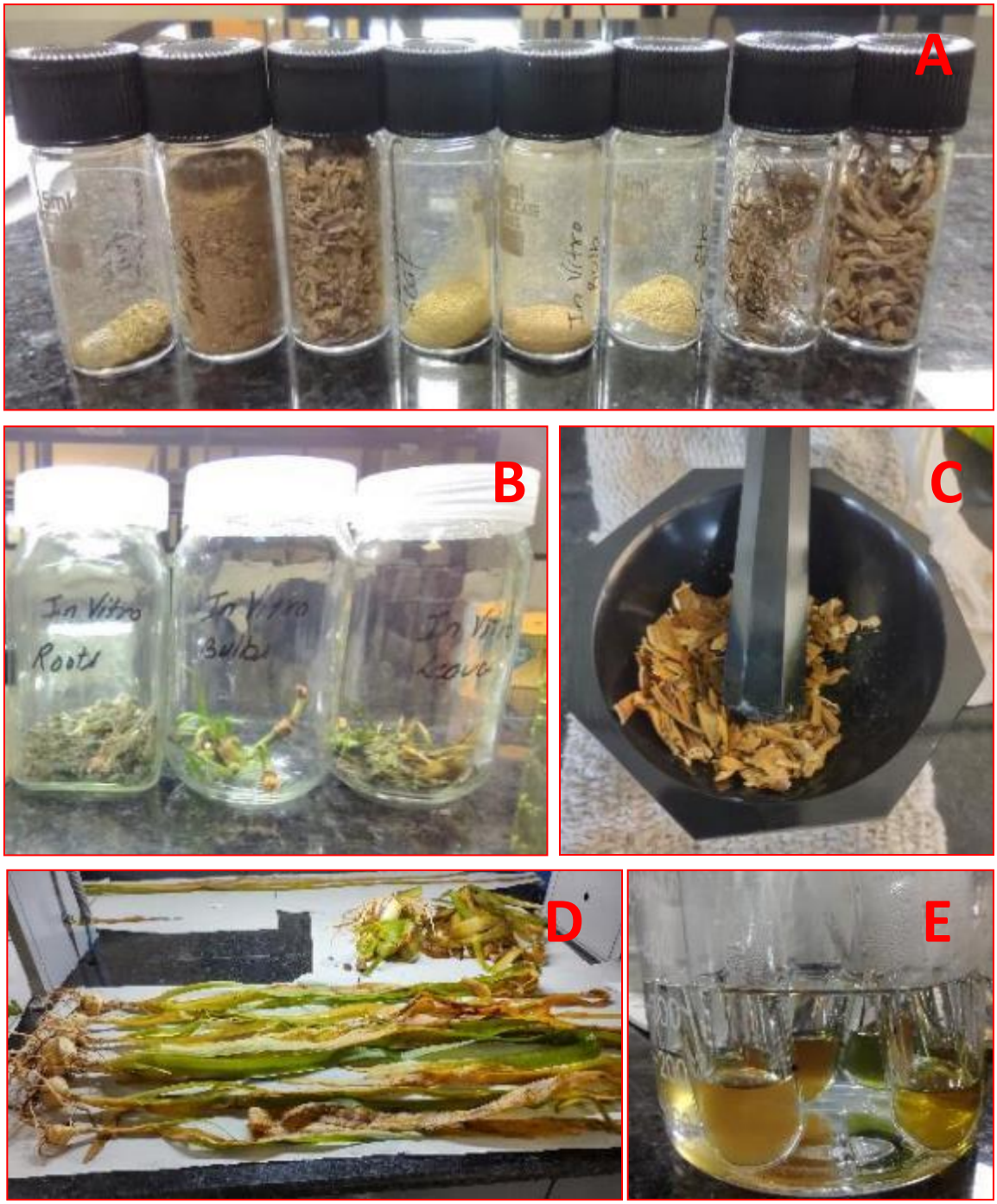


Fig 10. Samples Used for the LC/ESI-MS analysis: A) *In vitro* fresh smples; B) *In vivo* fresh samples; C) Crushing of dried *in vivo* leaves; D) Dried and powdered samples for extraction; E) Methanolic extract preparation for LC/ESI-MS analysis

Chapter 4 RESULTS AND DISCUSSION

Objective 1: Collection of plant material and development of regeneration protocol for *Crinum malabaricum* through *in vitro* technique

4.1.1. Collection of Plant Material

C. malabaricum plants were collected from the location described by Dr. Manoj Lekhak & Dr. S. R. Yadav in 2012 which is Periya Village, Kasaragod, Kerala, India (Fig. 11). A satellite view and GPS coordinates are given in Fig. 12 (12°24.545'N, 75°06.562'E). The species was identified by the botanist Dr. Manoj Lekhak who first discovered it in 2012 and a voucher specimen (voucher no. – SC-001) (Fig. 13.) was deposited at the Shivaji University herbarium in Kolhapur, Maharashtra, India. The harvested samples were than brought to the laboratory for further experimentation (Fig. 14).

Fig 11. Sample Collection. (A) Collection team consisting of Swati Chahal, Dr. Manoj Lekhak and Rupali Chougule; (B) Collection site (Periya Village, Kasaragod District, Kerala)

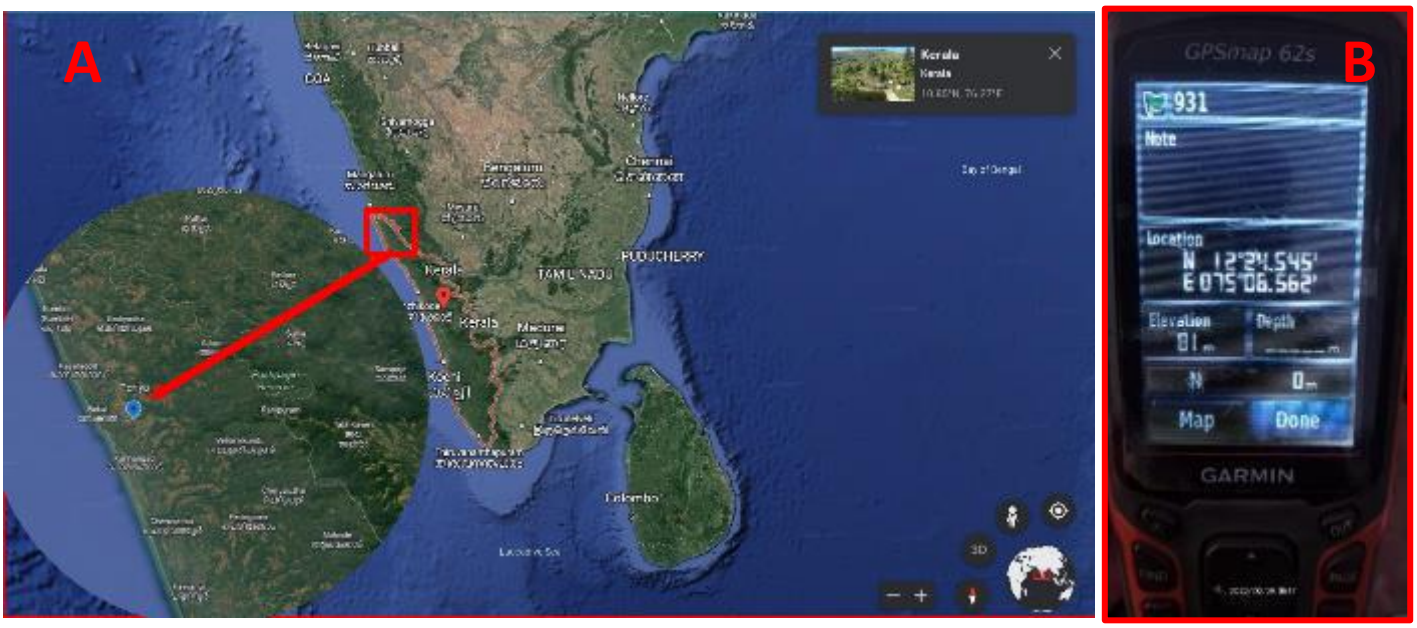


Fig 12. Collection site. (A) Satellite view of the collection site; (B) GPS coordinates of Periya village.

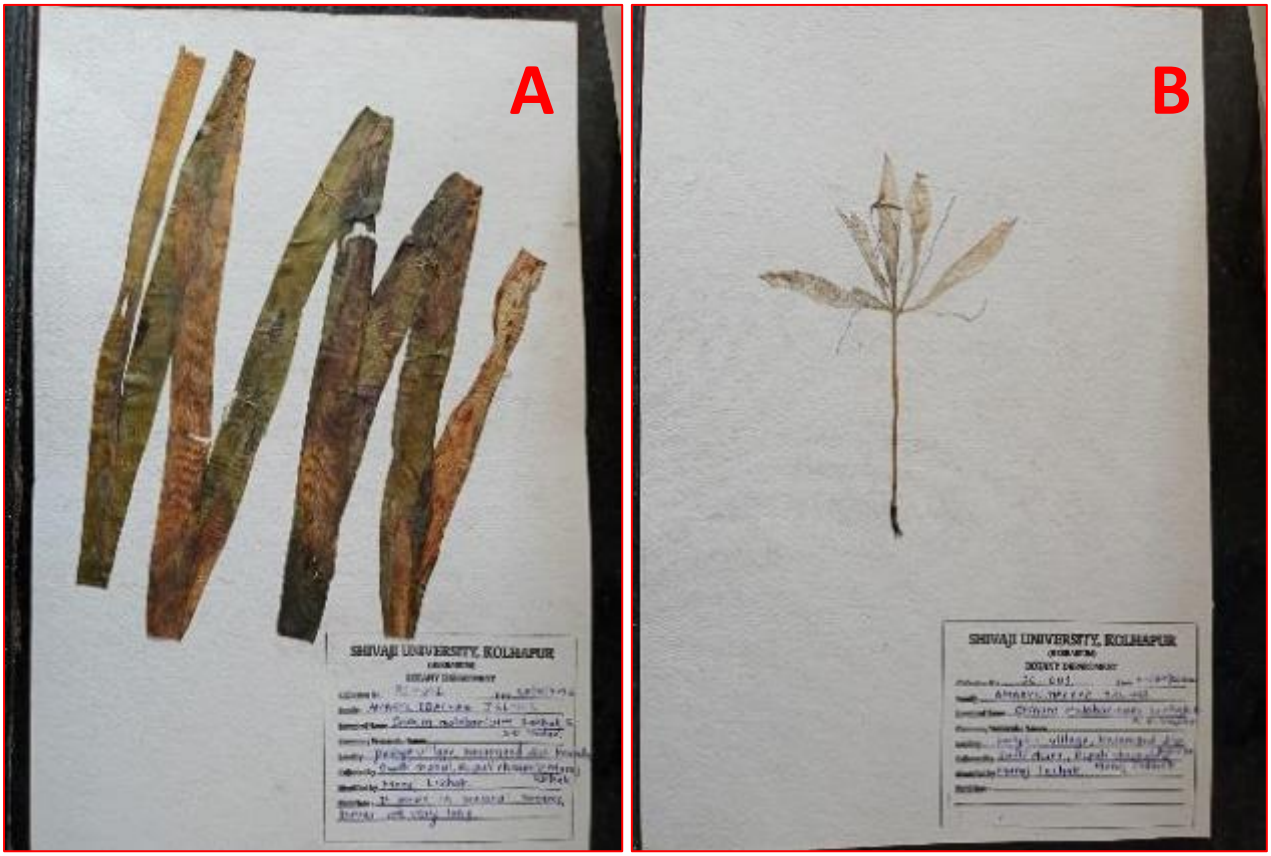


Fig 13. Herbarium submitted at the Shivaji University, Kolhapur, Maharashtra. A) Leaves; B) Flowers.

Fig 14. A) *Crinum malabaricum* in its natural habitat of Periya village, Kerala, India, during monsoon season; B) Freshly harvested bulbs of *C. malabaricum* from the collection site; C) Collected bulbs of *C. malabaricum* brought to the laboratory for further experimentation.

4.1.2. *meta*-Topolin-mediated *in vitro* shoot proliferation

A regeneration protocol for *C. malabaricum* was established from the MS medium fortified with macro-nutrients, micro-nutrients, vitamins and myo-inositol at a conc. of 0.1 g L^{-1}, 30 g L^{-1} sucrose and 4.44 µM BA which was used for the initiation of in vitro shoots used as explants (Fig. 15). The effect of *m*T on adventitious shoot regeneration after 6 weeks of culture is represented in Table 16. All concentrations (ranging from 0.5-10 µM) of mT generally increased shoot regeneration in *C. malabaricum* when compared to the control (Fig. 16). The

optimum shoots/explant (38.6) with a shoot length greater than 9.0 cm was produced at 7.5 µM *m*T. The highest fresh shoot biomass was obtained for *C. malabaricum* plantlets grown on medium supplemented with 7.5 µM *m*T. On the other hand, the highest concentration of *m*T significantly reduced the shoot proliferation rate, shoot length and fresh biomass. *Aloe polyphylla* and *Aloe arborescens* produced considerably more adventitious shoots when the amount of mT in the medium was increased. [227,228]. Recently, it was demonstrated that an increased *m*T concentration significantly enhanced regeneration frequency in *Scaevola taccada* [143]. The least amount of shoots per explant were produced by the explants cultured with mT 0.5 µM and shortest shoot length were produced by explants cultured with *m*T 10.0 µM, respectively. Overall, *m*T at 7.5 µM was found to be optimum for shoot proliferation. Similarly, [228] observed that *m*T (5 µM) was best for shoot proliferation in *A. arborescens*, whereas 7.5 µM favoured the highest shoot production in *Corylus colurna* [229]. The beneficial effect of *m*T in adventitious shoot production has been documented in several Amaryllidaceae such as *Cyrtanthus* species [230], *Allium schoenoprasum* [231], *Scadoxus puniceus* [232]. Despite studies emphasizing the superiority of *m*T to affect *in vitro* regeneration in several species [143,233,234], its ability to influence shoot proliferation in *C. malabaricum* was inferior compared to a previous report [236], where the highest number of shoots (53 shoots/explant) were produced by BA (2.0 mg/L) [235]. These reports are in agreement with those of [230], where *m*T was less effective in three Cyrtanthus species compared with the other PGRs tested, and in *Scadoxus puniceus*, where BA was also more effective for *in vitro* regeneration and bulblet induction [232]. Thus, in some cases, conventional cytokinins may produce better response than *m*T [236]. Recently, direct in vitro bulblets (26.0) formation was produced in *C. malabaricum* at 2.0 mg L^{-1} *m*T [235]. The number of scales per bulblet and diameter of bulblets was also increased by the variable concentrations of *m*T used in the study. In *C. moorei*, MS medium supplemented with BA and NAA stimulated *in vitro* bulblets from twin-scale explants [237].

Table 16. Effect of *m*T on the *in vitro* shoot proliferation of *C. malabaricum*.

mT (µM)	Mean shoots	Shoot length (cm)	Fresh weight of shoots (gm)
0.5	16.80 ± 2.15^{d}	8.00 ± 3.18^{b}	7.58 ± 3.36^{b}
2.5	26.60 ± 2.84^{c}	8.40 ± 2.99^{b}	6.48 ± 1.97^{c}
5.0	28.10 ± 2.64^{b}	7.80 ± 2.24^{c}	7.43 ± 2.03^{b}
7.5	**38.60 ± 3.75^{a}**	**9.95 ± 4.73^{a}**	**8.76 ± 2.28^{a}**
10	27.20 ± 1.84^{b}	7.20 ± 1.24^{c}	7.13 ± 1.23^{b}
PGR-free (Control)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

4.1.3 *In vitro* rooting and acclimatization

Table 17 illustrates the impact of varied concentrations of the root inducing hormones like IAA and IBA on the *in vitro* rooting of the *C. malabaricum* shoots propagated on the culture medium containing different concentrations of shoot-inducing aromatic cytokining *m*T. All treatments of IAA (0.5 - 3.0 mg L^{-1} and IBA (0.5 - 3.0 mg L^{-1}) favoured a rooting frequency ranging from 51%-100% (Table 17). Use of IBA as a root induction hormone at a conc. of 1.0 mg L^{-1} was found to induce the highest number of roots (13.07) per shoot, with an aggregate of longest roots (8.67 cm) in *C. malabaricum* (Fig. 19). Regenerated shoots i*n vitro* showed no rooting in the control experiments, devoid of PGRs. This is in line with previous reports where IBA induced root induction in different plant species [235,238,239,240,241,242]. Auxins at high concentrations reduced the length and number of roots. In addition, thick and brittle roots were observed with higher auxin concentrations (IAA and IBA) (Fig. 19). Priyadharshini et al. (2021) also reported a similar phenotype in roots with high auxin concentration. Interestingly, prolonged incubation of regenerated shoots with IAA and IBA produced *in vitro* bulblets (Fig 17). Such in vitro rooted bulblets of *C. malabaricum* were successfully transferred to the

greenhouse and acclimatized ex-vitro (Fig 20). The acclimatized plants exhibited 80% survival rate and there was no significant variation was observed when compared to the mother plant.

Table 17. Effect on *in vitro* rooting of *C. malabaricum* exhibited by IBA and IAA

IAA (mg/L)	IBA (mg/L)	Rooting frequency (%)	Mean number of roots	Root length (cm)
0.5	-	75	4.60 ± 0.22f	2.69 ± 0.71e
1.0	**-**	**87**	**6.44 ± 0.25d**	**4.10 ± 0.44c**
2.0	-	69	3.60 ± 0.20g	2.10 ± 0.40f
3.0	-	51	3.20 ± 0.24h	2.33 ± 0.40f
	0.5	91	11.47 ± 0.19b	4.00 ± 0.38c
	1.0	**100**	**13.07 ± 0.07a**	**8.67± 0.58a**
	2.0	83	9.67 ± 0.39c	6.67 ± 0.34b
	3.0	71	5.67 ± 0.39e	3.00 ± 0.25d
Control	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

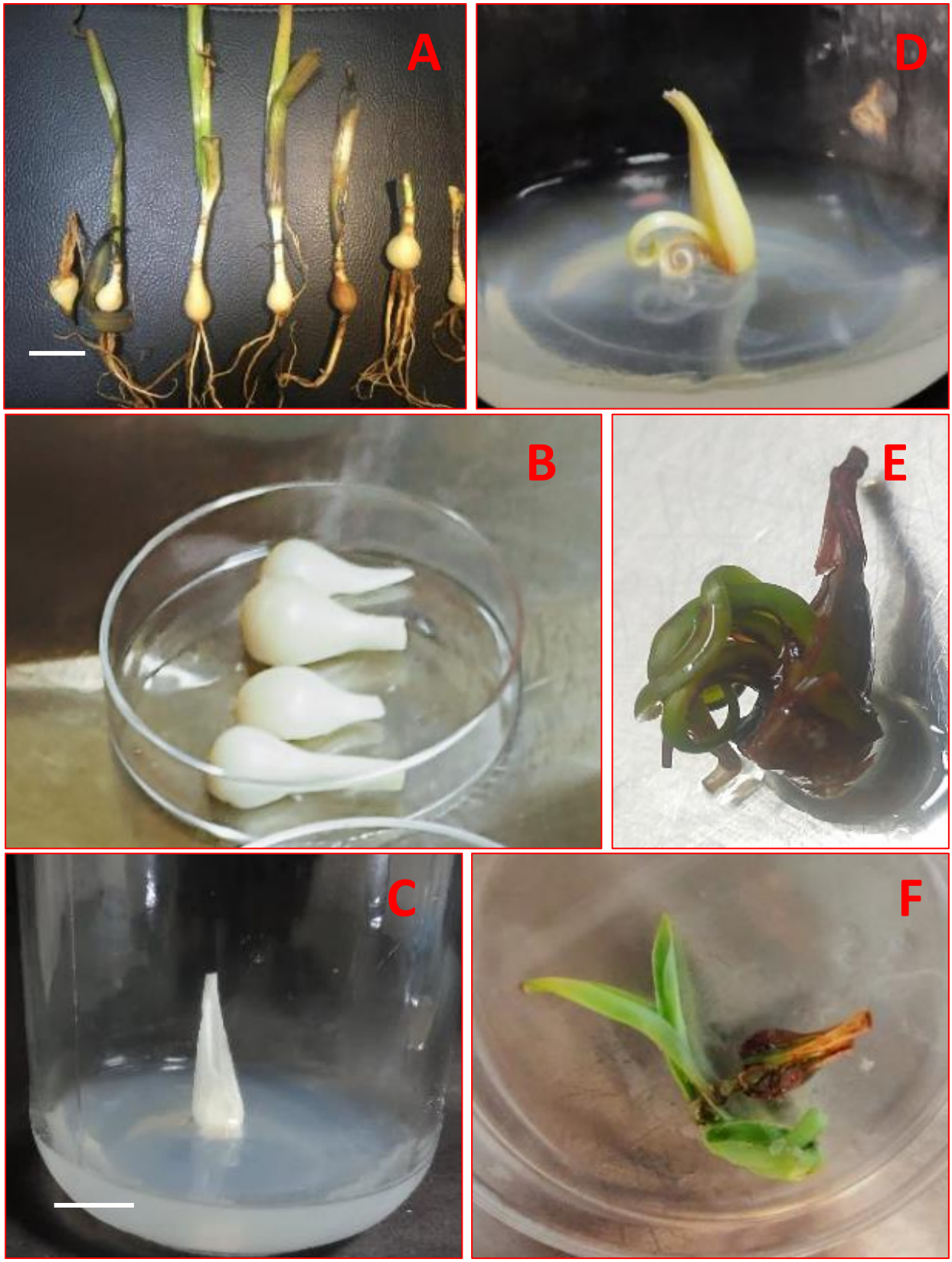


Fig 15. *In vitro* micropropagation (primary culture); A) Bulbs of different sizes collected from the Periya village, Kasaragod, Kerela (scale bar5 cm); B) Sterilized bulbs cut into half; C) Twin scales inoculated into the MS media + BA (scale bar 2 cm); D-F) Shoots regenerated from the twin scales of *C. malabaricu.*

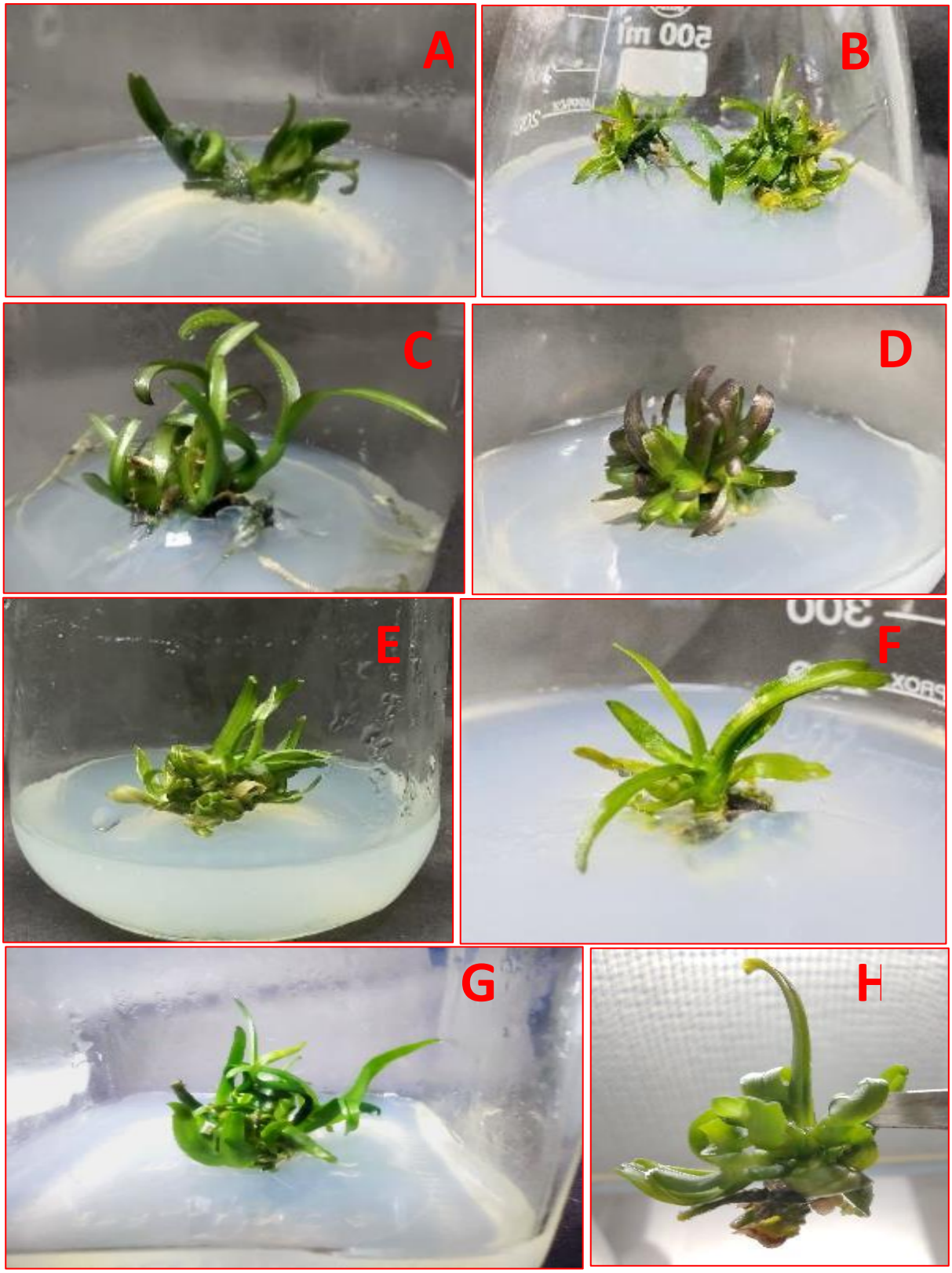


Fig 16. Micropropagation (secondary culture); A) *In vitro* plants were maintained on PGR free MS media; B-F) In *vitro* plants were maintained on culture media with Meta topolin (0.5 - 10 µM); C & E) Multiple shoot proliferation with 7.5 µM *meta*-topolin

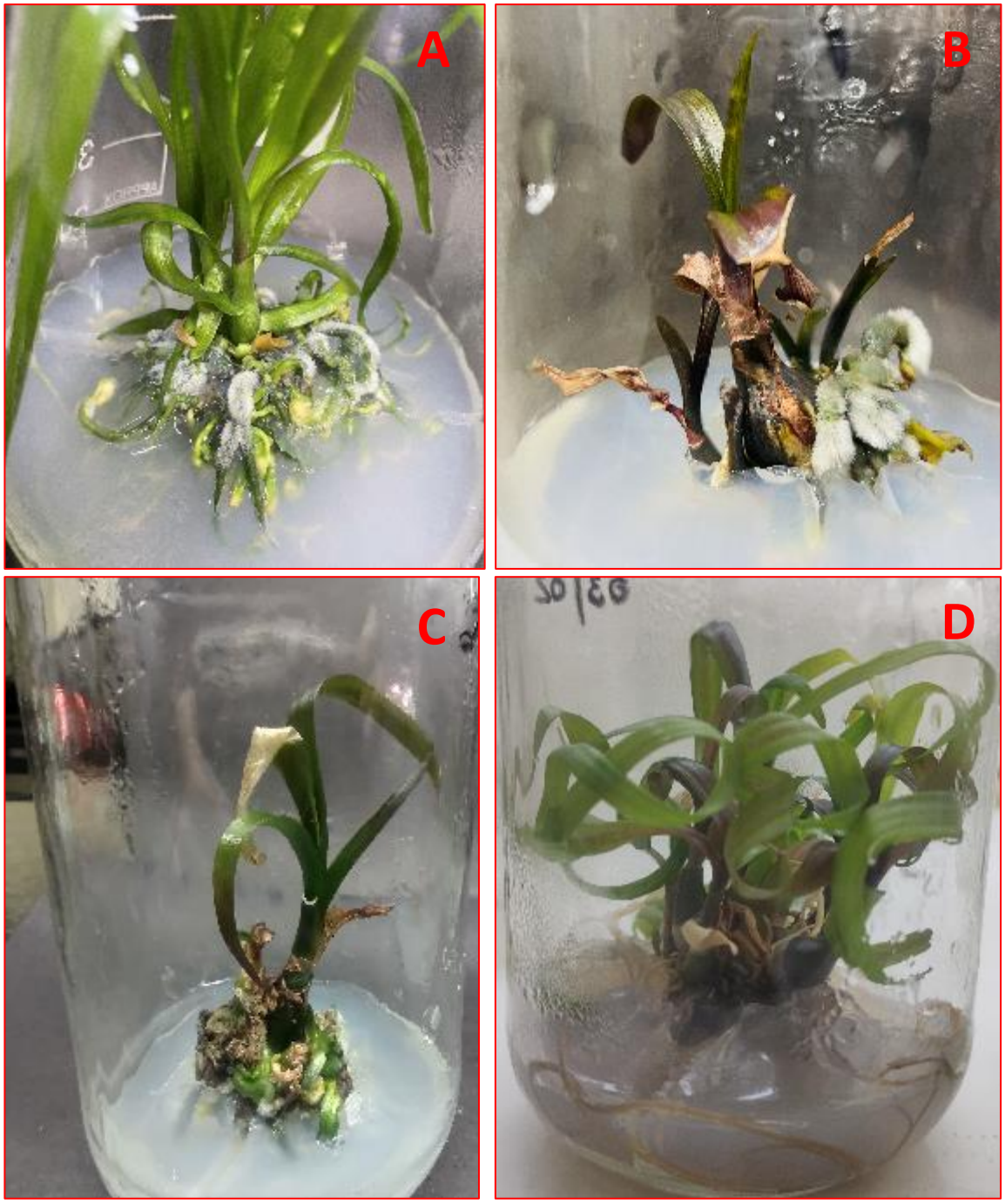


Fig 17. *In vitro* bulblet formation after eight weeks in MS media supplemented with *m*T.


A
B
C
D

Fig 18. A) Control; **B)** Shoot elongation and maturation of *in vitro* regenerated *Crinum malabaricum* on MS supplemented with *m*T + IBA after 8 weeks.

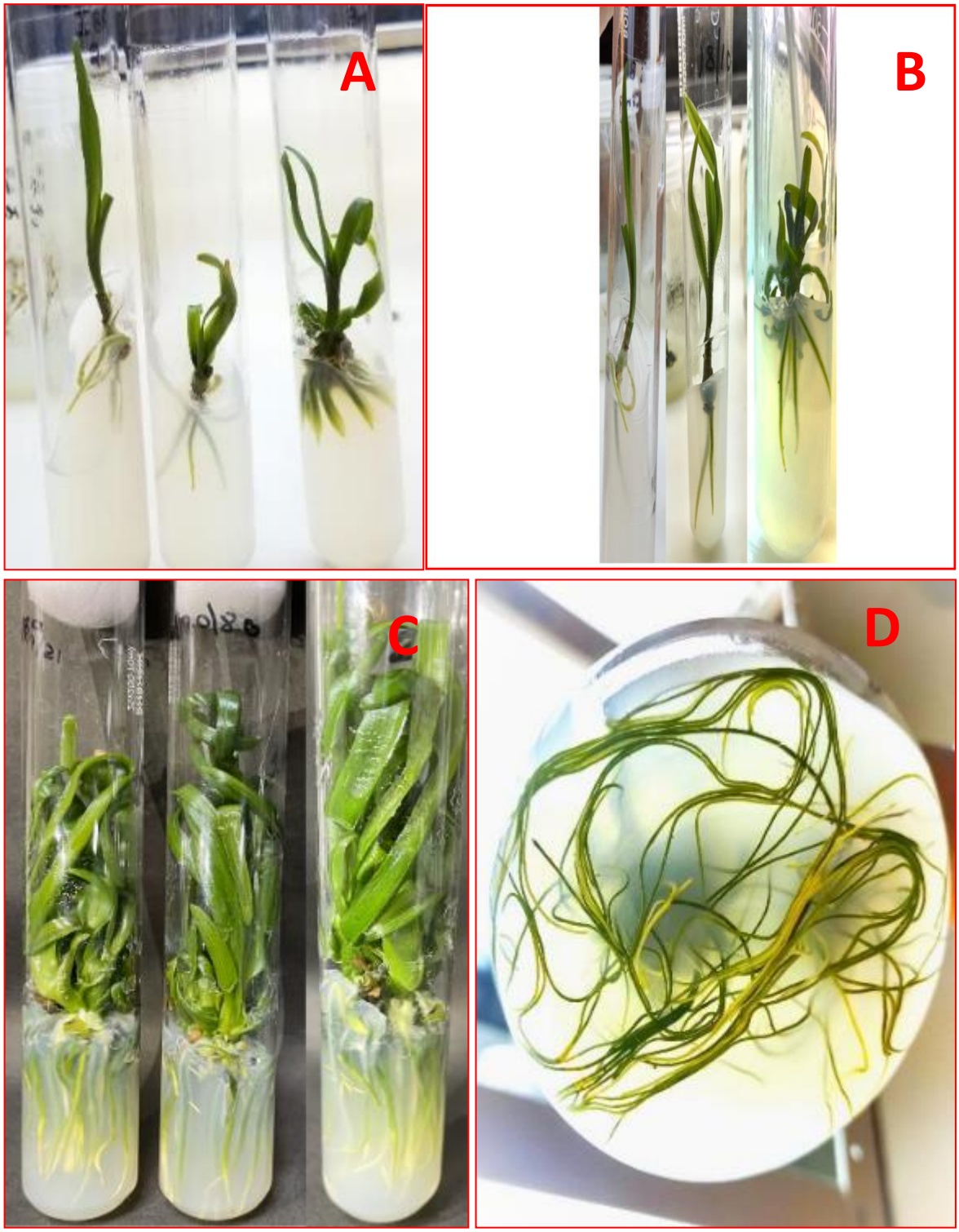


Fig 19. Root initiation in *Crinum malabaricum* on MS media supplemented with different concentrations of IBA: A) Root Initiation; B) Root elongation; C) Multiple root formation and shoot elongation on nutrient medium enriched with IBA (1 - 3 mg/L); D) Root multiplication.


A


B
500 ml


C


D


E

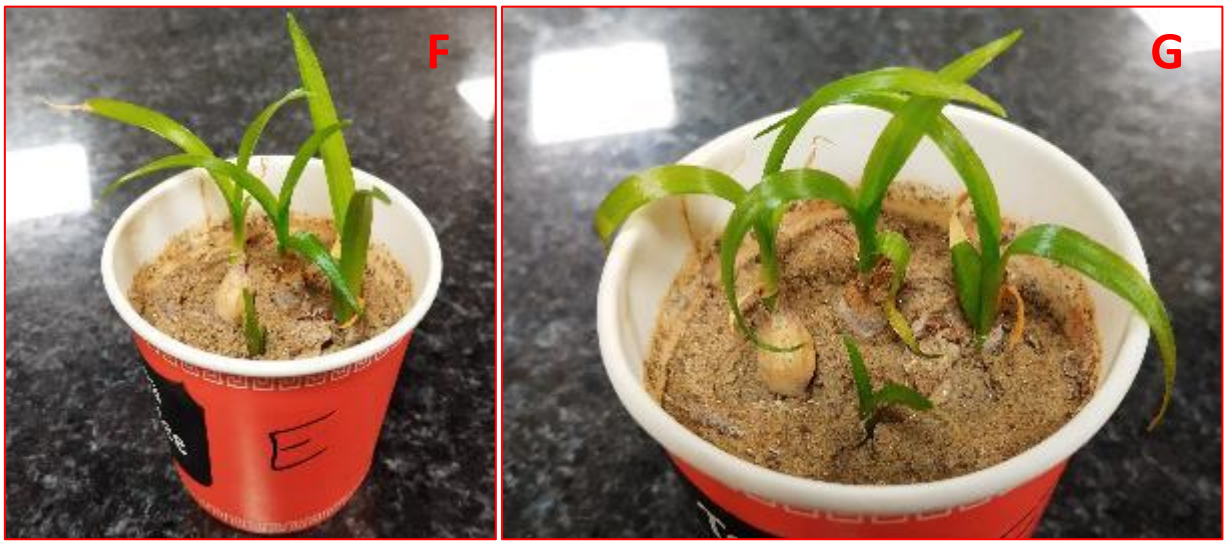


Fig 20. Establishment of rooting and acclimatization of i*n vitro* regenerated plants. A) *In vitro* bulblets of *Crinum malabaricum* used for acclimatization; B-C) Mature in vitro regenerated plants used for acclimatization; D-E) In vitro plants potted in soil mixture; F-G) Fully acclimatized *In vitro* bulblets.

Objective 2: Somatic embryogenesis of *Crinum malabaricum*.

4.2.1 Somatic embryo development

Somatic embryogenesis approach is a promising way to obtain *in vitro* regenerated plantlets and has the potential to revolutionize the conservation practices for rare, endangered and threatened species. As an endemic and critically endangered species, the establishment of an efficient embryogenesis system for *C. malabaricum* needs serious consideration. In this study, an efficient and highly reproducible somatic embryogenesis system was developed (Fig 21). Different concentrations and combinations of Picloram (1.0-5.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}); 2,4-D (1.0-10 mgL^{-1}) with BA (2.5 mg L^{-1}), were examined to induce somatic embryos from *C. malabaricum* bulb explants (Table. 18 and 20). In the initiation of embryogenic callus and formation of somatic embryos, a positive response was shown by the 2,4-D alone. This outcome is consistent with observations made in the somatic embryogenesis of other Amaryllidaceae species. [13,86,207,243]. The significance of 2,4-D is also well-documented for inducing embryogenesis in several other plant species [244,245,246]. However, the production of *C. malabaricum* embryogenic calli was significantly effected (87%) by the combination of 2,4-D and BA at concentrations of 8.0 mg L^{-1} and 2.5 mg L^{-1} respectively, and resulted in the somatic embryos being produced in large numbers which was calculated to be 45 (Table 18). This finding is similar to studies on somatic embryogenesis of various different plant species including *Chrysanthemum* sp. [247,248], *Prosopis laevigata* [244], *Vitis vinifera* [249]. *Hypoxis hemerocallidea* [85], and *C. malabaricum* [13].

Interestingly, the mean number of somatic embryos significantly increased (55.89) on plant tissue culture medium enriched with picloram at a conc. of 2.0 mgL^{-1} and TDZ at a concentration of 0.5 mg L^{-1} (Table. 20). Table 19 shows the effect of different concentrations of Picloram and TDZ on the morphology of the callus of *C. malabaricum*. Although the most widely used and an important growth regulator in somatic embryogenesis is 2,4-D [85,250,251,252], the use of picloram for induction and proliferation of somatic embryogenesis has become commonplace in several plant species [253,254,255,256] and has also been widely used other monocotyledonous and bulbous species like those of *Lilium longiflorum* [257], *Leucojum aestivum* [188,207], *Lachenalia virdiflora* [258]. Additionally, research on several other plant species have revealed that TDZ significantly affect the development of somatic embryos [84,259,260,261,262]. The current research revealed that MS medium fortified with picloram and TDZ is necessary for somatic embryo production in *C. malabaricum*. A similar result was observed for somatic embryogenesis of *Merwilla plumbea* [260], *Drimia robusta*

[261], *Lachenalia virdiflora* [258], *Cyrtanthus mackenii* [263], and *Stewartia* sp. [264]. In *M. plumbea*, [260] obtained maximum somatic embryos per settled volume in MS medium containing picloram (8.3 µM) and TDZ (2.3 µM). Intriguingly, it was discovered that the medium that included 2.5 µM picloram and 1.0 µM TDZ produced the most somatic embryos [258]. Our research also demonstrated that the development of somatic embryos might be affected by varying the quantities of exogenously administered auxins or by mixing both the auxin and cytokinin.

4.2.2 SEM Analysis

Scanning Electron Microscopy of the embryogenic callus derived from *C. malabaricum* explants indicated the presence of several somatic embryos on the surface of embryogenic calli and revealed the presence of many tightly packed globular embryo clumps, as well as cotyledonary-shaped embryos characterized by well-developed cotyledons (Fig 22).

4.2.3 Germination and plantlet development from somatic embryos

For the successful regeneration conversion of somatic embryos into complete plantlets is crucial. It was observed that, in *Crinum malabaricum* somatic embryos cultured on full-strength MS media had a germination rate of 52.76% and 64.22% on half-strength MS media, whereas the germination rate was observed to be 94.03% when the half-strength media was spiked with 1 mgL^{-1} GA_3 (Fig 23). Root formation and expansion of leaves was found to be more effective in half-strength as compared to the full-strength medium. It has been noted that a number of different plant species use half-strength MS media for somatic embryo germination. [85,265,266,267,268,269]. Changing the medium strength (half and full) can influence the osmotic stress [85,270]. Similarly, several studies reported the supplementation of GA_3 for somatic embryo in multiple plant species [84,271,272].

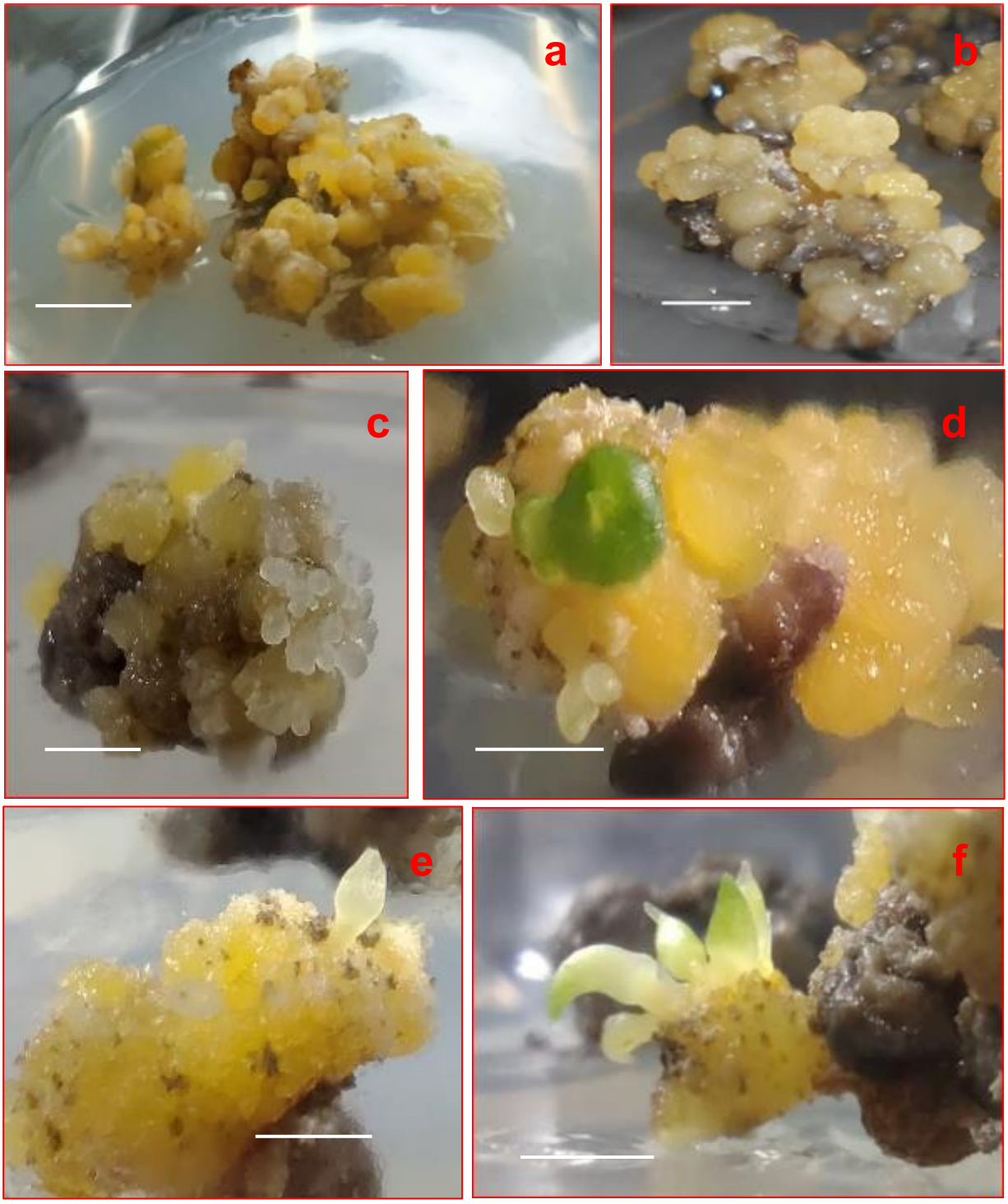


Fig 21. Somatic embryogenesis: Induction of embryogenic callus and different stages of SE development in *C. malabaricum*. a) Induction and multiplication of embryogenic callus. b) Cluster of globular-shaped SEs. c) Development of various globular and heart-shaped somatic embryos. d) Initiation of torpedo shaped-somatic embryo E. Juvenile-shaped somatic embryo F. Cotyledonary-shaped somatic embryos. *Scale bars: a-b = 10mm; c = 5 mm; d-f = 2 mm.*

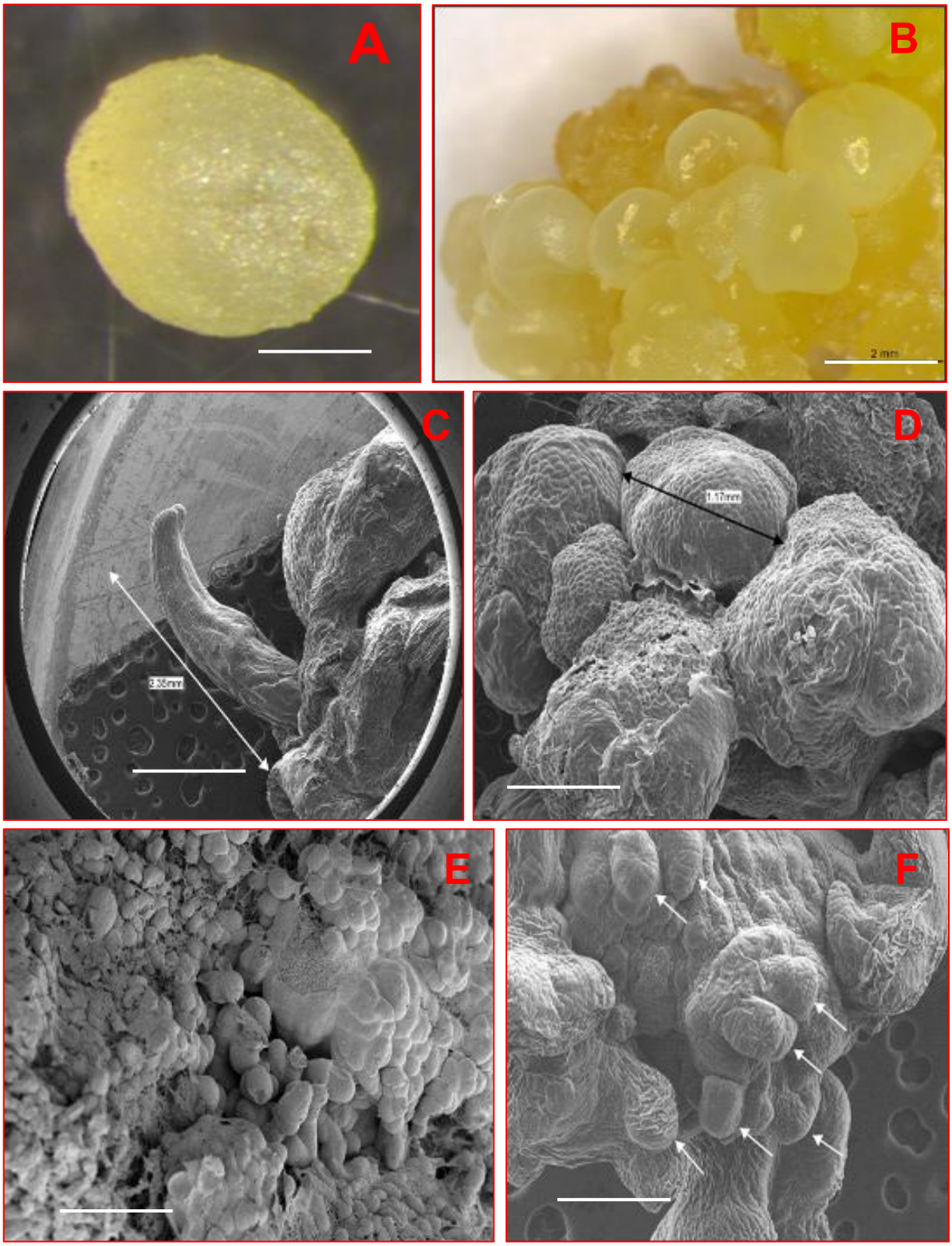


Fig 22. Scanning electron microscope view of *C. malabaricum* somatic embryos. A-B) Stereo micrograph of translucent globular embryo, C) Cotyledonary-shaped embryos, D-E) Cluster of the globular-shaped embryos, F) Appearance of embryogenic callus. *Scale Bar: A = 1.17mm; B = 2mm; C= 100 µm; D-E = 200 µm; F = 1mm.*

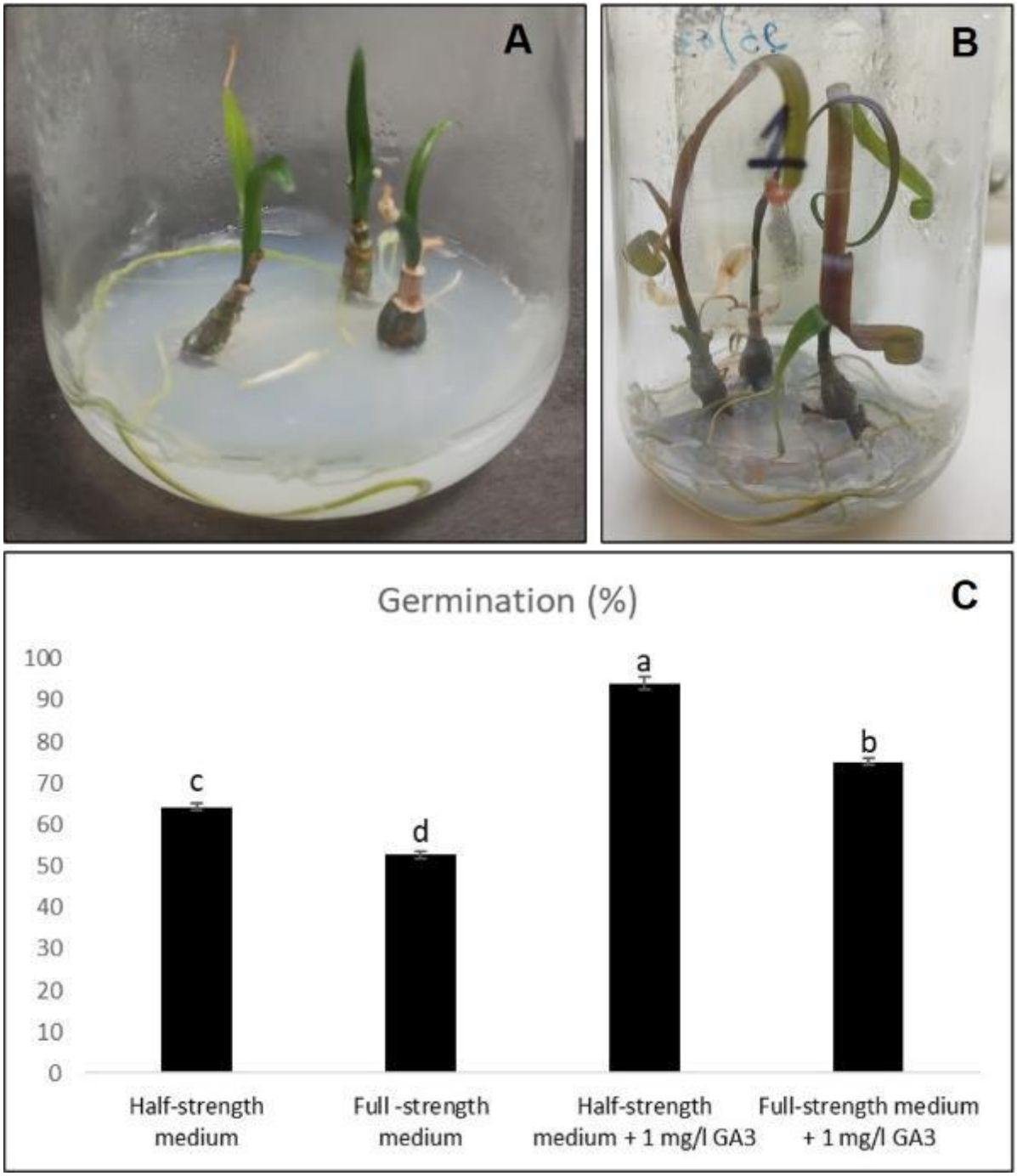


Fig 23. *C. malabaricum* somatic embryos germination and plantlet conversion. A) Germinated embryos in germination medium, B) Well-developed rooted plantlets, C) The effect of different types of culture medium (full strength and half strength + 1.0 mg L^{-1} gibberellic acid) on the germination of somatic embryos of *C. malabaricum*. All the experiments were repeated thrice with at least three replicates.

4.2.4. Development of Callus

Table 18. Effect of different concentrations of 2,4-D alone and in combination with BA on somatic embryo induction in *Crinum malabaricum.*

2,4-D (mg L^{-1})	**BA (mg L^{-1})**	**Somatic embryogenesis (%)**	**Mean number of somatic embryos**
1.0	0.0	0.00	0.00
2.0	0.0	14^{f}	5.60 ± 0.76^{g}
3.0	0.0	17^{e}	11.76 ± 0.88^{f}
4.0	0.0	47^{d}	15.79 ± 0.90^{e}
5.0	0.0	60^{c}	27.00 ± 0.80^{c}
6.0	0.0	61^{c}	28.20 ± 0.50^{c}
7.0	**0.0**	**77^{b}**	**29.77 ± 0.88^{b}**
8.0	0.0	64^{c}	25.80 ± 0.50^{d}
9.0	0.0	62^{c}	29.28 ± 1.00^{b}
10.0	0.0	61^{c}	27.80 ± 0.50^{c}
6.0	2.5	78^{b}	30.44 ± 0.22^{b}
7.0	2.5	86^{a}	44.13 ± 0.36^{a}
8.0	**2.5**	**87^{a}**	**45.00 ± 0.19^{a}**
9.0	2.5	77^{b}	31.00 ± 0.80^{b}
10.0	2.5	64^{c}	29.00 ± 0.60^{b}
Control	-	0.00	0.00

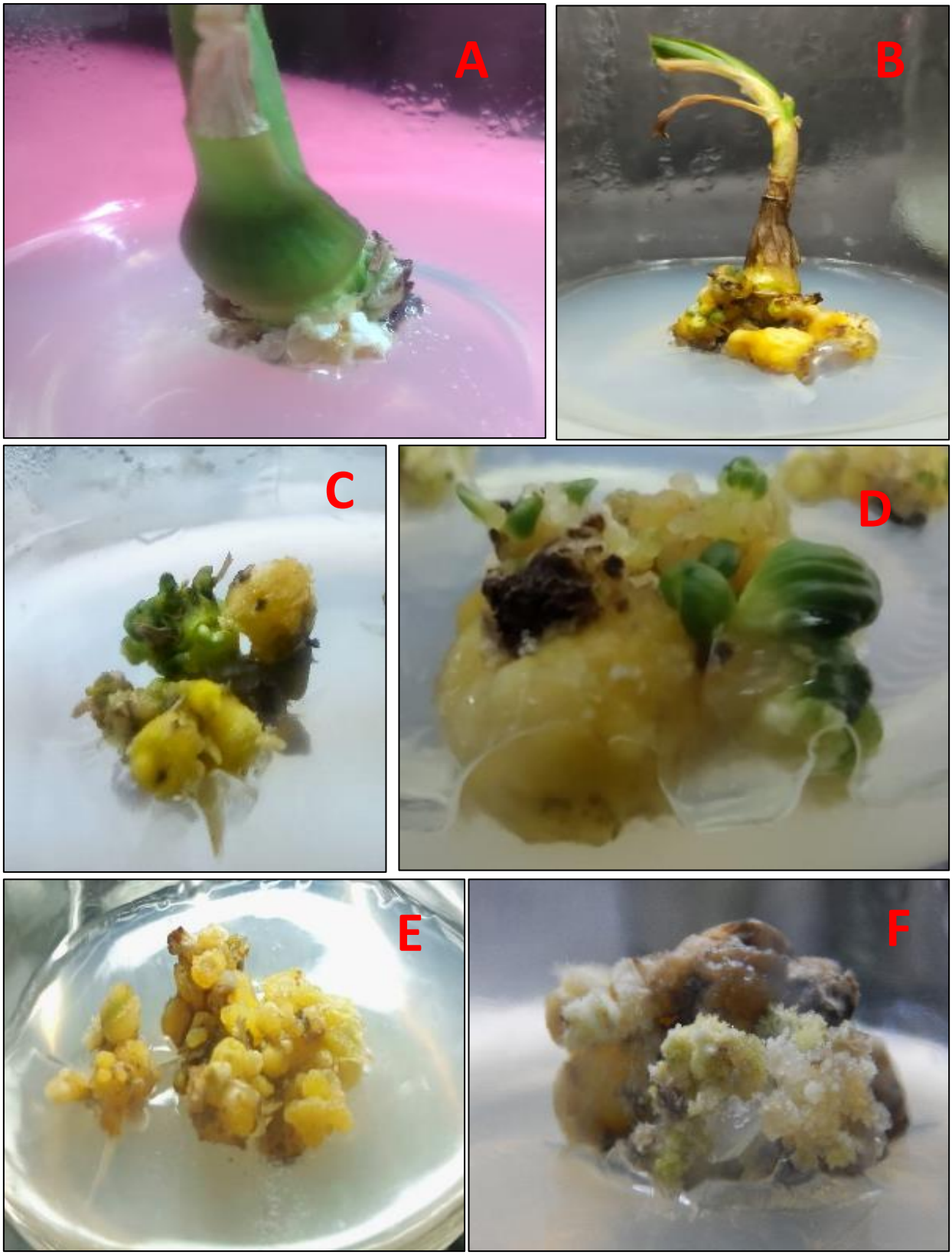


Fig 24. Development of callus in *C. malabaricum*. A-B) Initiation of callus after one week from the basal plate; C-D) Callus development after two weeks on nutrient media enriched with picloram (2.0 mg/L) + TDZ (0.5 mg/L); E-F) Friable callus formation after 4 weeks.

Table 19. Influence of Picloram and TDZ on the morphology of *C. malabaricum* callus.

MS + PGR (mg L^{-1})		Plant Part Used	Days Required for callus induction	Nature of callus after 2 weeks	Nature of callus after 4 weeks
Picloram	**TDZ**				
0	0	Basal Plate	7	No callus	No callus
0.5	0.5	Basal Plate	7	Explant deformation	Completely deformed explant
1	0.5	Basal Plate	7	Green, Yellow, compact	Green,White, Fragile
2	0.5	Basal Plate	7	Embrogenic, Yellow, white, Compact	Embryogenic white, fragile
3	0.5	Basal Plate	7	Green, white, compact	Embryogenic white, fragile
4	0.5	Basal Plate	7	Green, white, compact	Yellow, Compact
5	0.5	Basal Plate	7	Green, white, compact	Yellow, Compact

Table 20. The influence of picloram and thidiazuron on *Crinum malabaricum* somatic embryogenesis.

Picloram (mg L^{-1})	TDZ (mg L^{-1})	Somatic embryogenesis (%)	Mean number of somatic embryos
0.5	0.5	93^{c}	47.22 ± 0.52^{b}
1.0	0.5	96^{b}	49.33 ± 0.88^{b}
2.0	**0.5**	**100^{a}**	**55.89 ± 0.60^{a}**
3.0	0.5	90^{d}	42.44 ± 0.70^{c}
4.0	0.5	81^{e}	30.0 ± 0.22^{d}
5.0	0.5	77^{f}	25.69 ± 0.21^{e}

Objective 3: Maintenance of cell suspension and study the effects of elicitors on secondary metabolite production.

4.3.1. Initiation of cell suspension

In this experiment, the elicitation of callus was accomplished in liquid media enhanced with different concentrations of PGRs. The application of elicitors has shown a significant effect on biomass accumulation.

The friable callus was cultured in MS liquid media enhanced with Picloram (2.0 mgL^{-1}) + TDZ (0.5 mgL^{-1}) as it was optimized as the best concentration for somatic embryogenic formation. From the cell suspension culture media, the sieved suspension was transferred to a fresh batch of conical flasks having 50 ml fresh medium amended with different concentration elicitors (JA, MeJA, and SA) (Sigma-Aldrich, USA). Cultures were maintained under the same conditions. Suspension cells treated with elicitors (JA, SA and MeJA) were harvested at different day intervals (7^{th}, 14^{th}, and 21^{st} days) after elicitation. In addition to that suspension without any growth regulator and elicitors served as control.

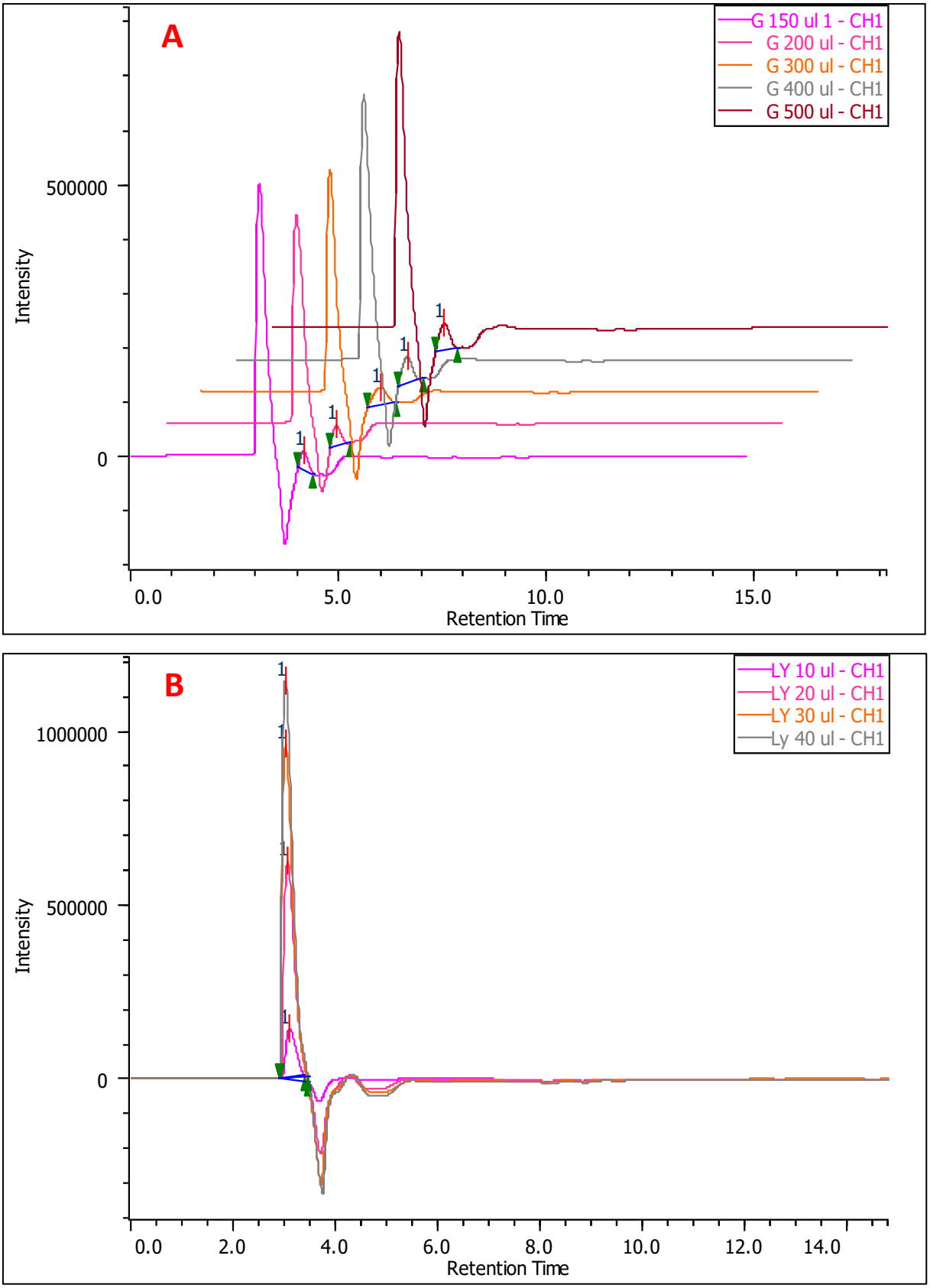


Fig 25. HPLC calibration curves of A) Galanthamine, B) Lycorine

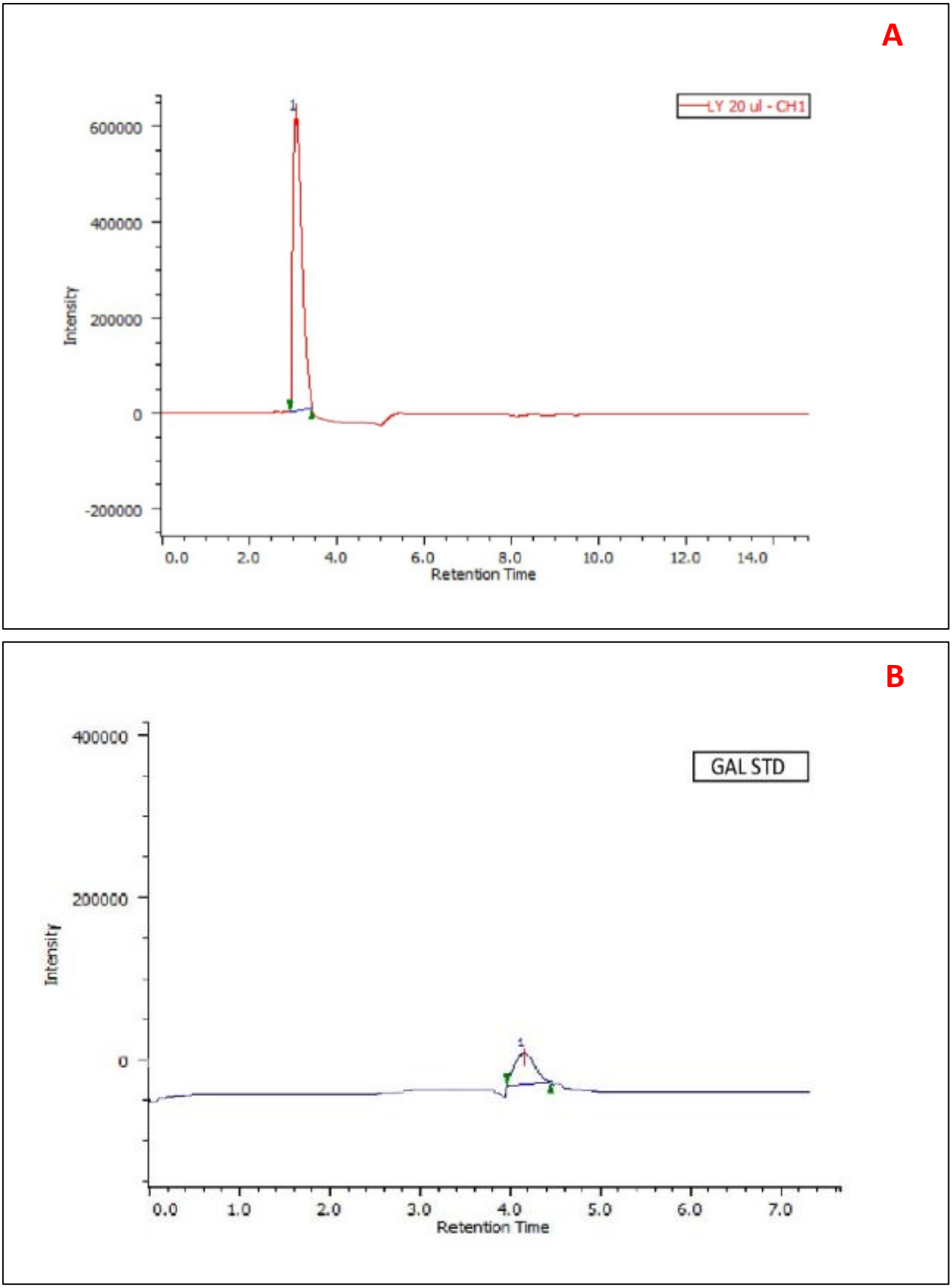
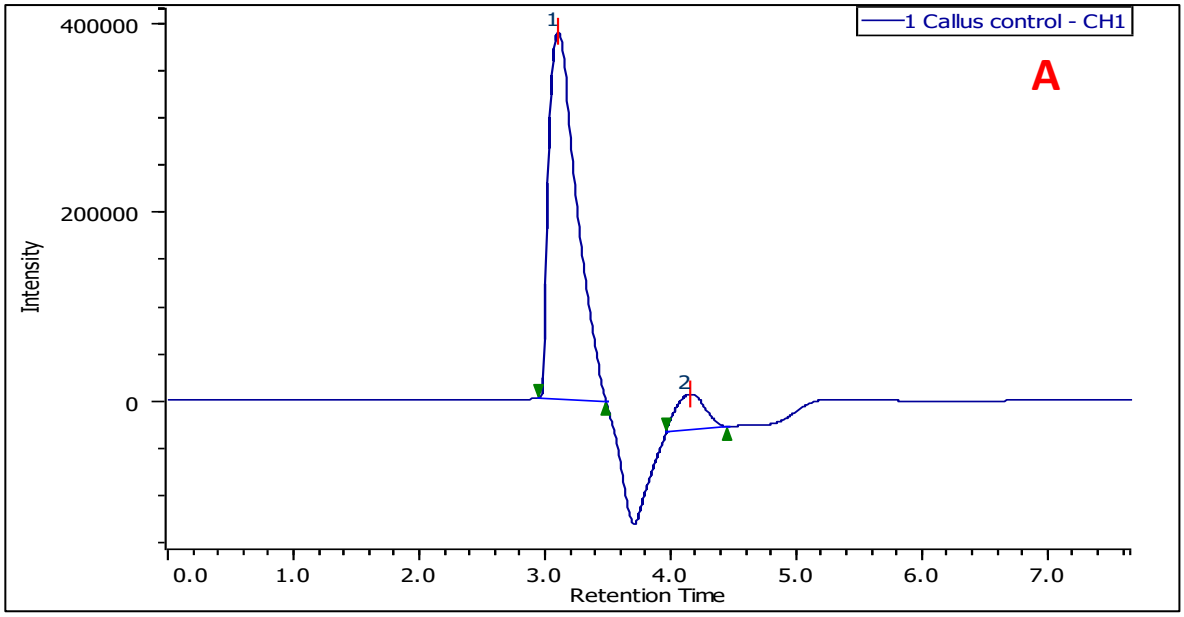
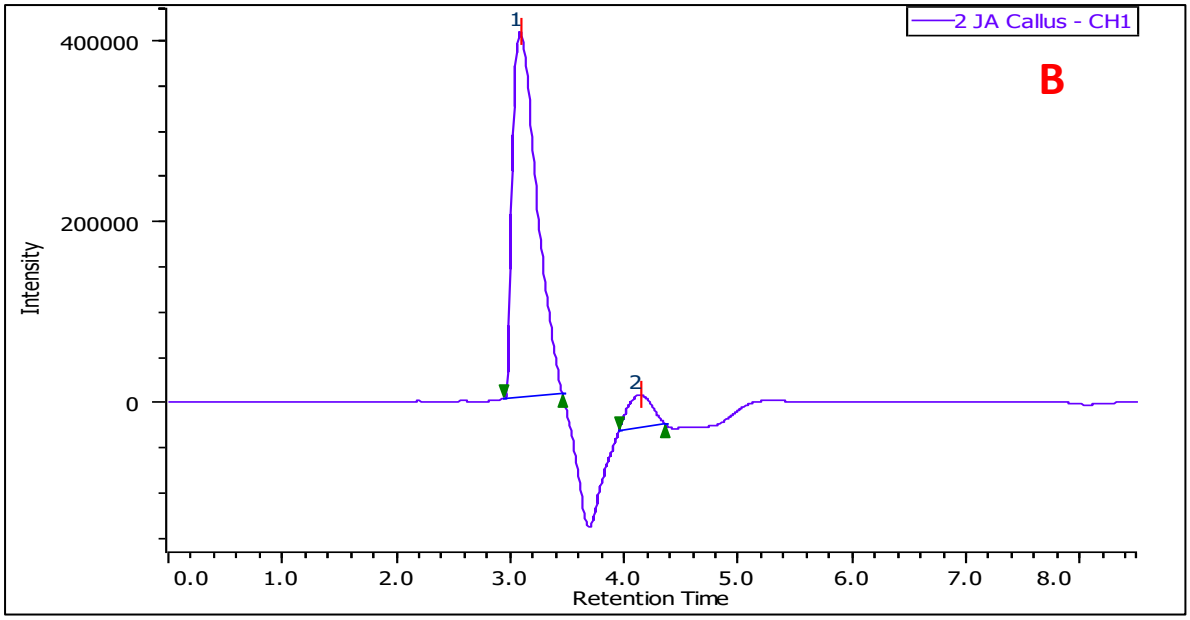


Fig 26. Standard HPLC chromatogram of A)Lycorine; B) Galanthamine


1 Callus control - CH1
A
400000
200000
0
Intensity
1
2
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Retention Time


2 JA Callus - CH1
B
400000
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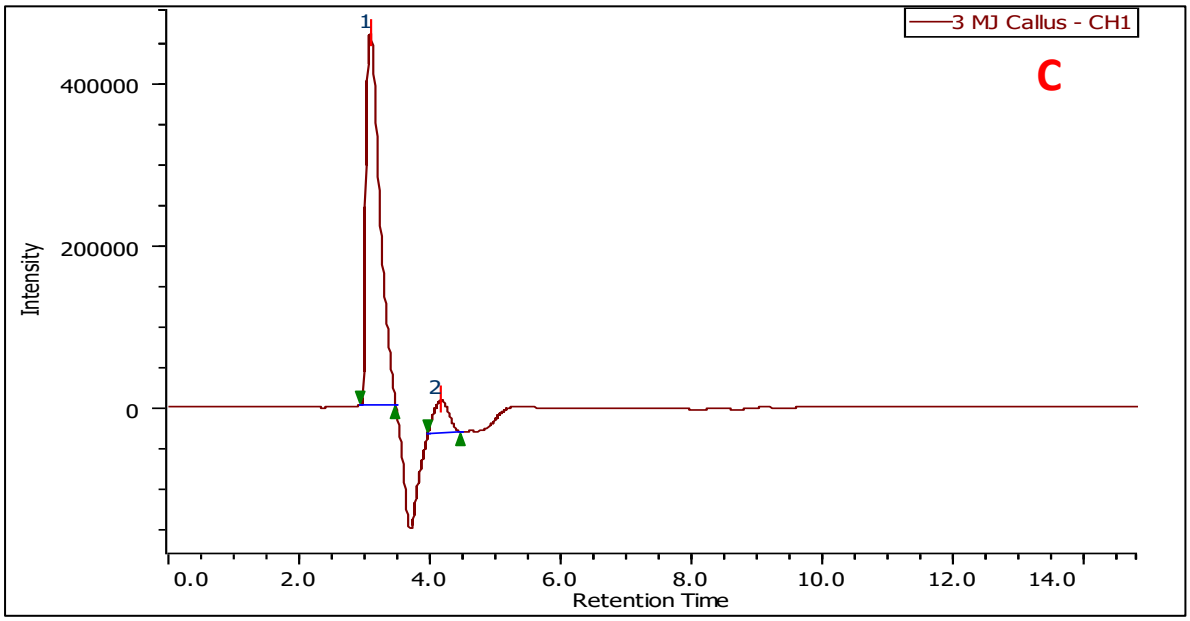
3 MJ Callus - CH1
C
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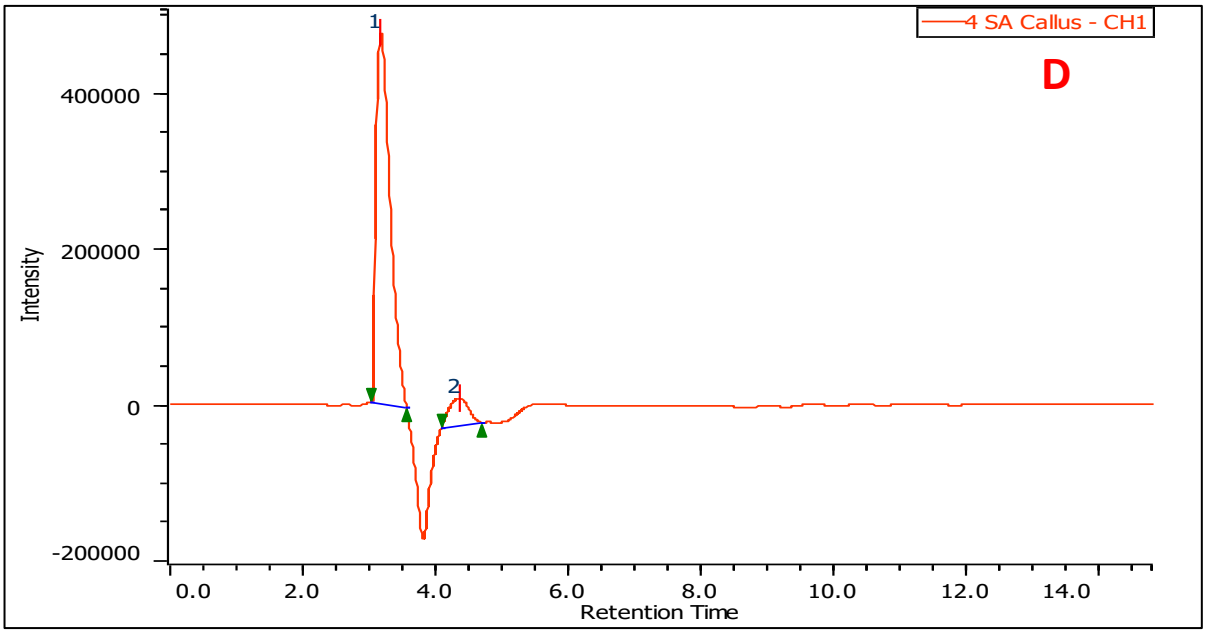

Fig 27. Standard chromatogram of suspension callus; A) Control callus, B) Jasmonic acid callus, C) Methyl Jasmonate callus, D) Salicylic Acid Callus

4.3.2. HPLC Analysis

The effect of methyl jasmonate, jasmonic acid and salicylic acid was observed on the galanthamine and lycorine concentrations on the *in vitro* callus of *Crinum malabaricum* was studied using suspension cultures system in a shake flask method. Galanthamine and Lycorine were quantified using HPLC analysis. The callus which was suspended in the control media was found to possess the highest conc. of galanthamine was found in which indicates that there is no significant effect of the elicitors on the enhancement of galanthamine. However, Lycorine was found in highest concentrations in the suspension media elicitated with Salicylic acid.

Table 21. HPLC analysis of lycorine (LY), galanthamine (GAL) from the elicitated callus of *Crinum malabaricum*.

Sr No.	Sample Name	LY (mg/gm DW)	GAL (mg/gm DW)
1	Callus Control	6.13±0.50	**2.85±0.35**
2	JA Callus	1.62±0.42	0.62±0.38
3	MJ Callus	3.92±0.49	1.72±0.43

4	SA Callus	**6.20±1.07**	2.74±0.90

Objective 4: Assessment of genetic fidelity in regenerated *Crinum malabaricum* and extraction and analysis of secondary metabolites.

4.4.1. Genetic Fidelity Assessment

The clonal fidelity studies help in the assessment and prediction of the genetic homogeneity between regenerated clones and the mother plants [120,147]. In this context, SCoT markers are simple, cost effective and designed to have a short ATG-start codon in plants for a better reproducibility of genetic polymorphism [116,273,274]. Similarly, the advanced marker types ISSR and RAPD are also based on the non-coding sections of the DNA. [119,275,276,277,278]. In regenerated plants, it is crucial to evaluate genetic stability especially when plants are produced through somatic embryogenesis with an intermediate callus phase. Moreover, it is advantageous to use more than one DNA-based technique to assess the genetic fidelity of in vitro raised plantlets [276,279]. The current report validates the genetic homogeneity assessment of mother plants in comparison with the regenerated plants using DNA markers like SCoT, ISSR and RAPD (Fig 5). For this study a total of 10 primers each of SCoT and ISSR and 8 RAPD primers-based fingerprinting was performed by randomly selecting seven somatic embryos derived in vitro regenerants and with the mother plant (Table 3-5). While verifying the genetic stability, a total of 21 reproducible bands with the amplified band size of 200 bp to 1600 bp were yielded by SCoT amplified primers (Table 3) and 24 bands with size of 200 bp to 1200 bp were yielded by the ISSR primers (Table 4), while 13 bands in a range of 100bp to1200 bp were yielded by the RAPD primers (Table 5). Monomorphic banding patterns were produced with SCoT, ISSR and RAPD markers and since true-to-type plantlets were obtained via somatic embryogenesis no polymorphism was observed (Fig 5). SCoT, ISSR and RAPD markers analysis were also utilized in other plant species such as *Eclipta alba* [280], *Helicteres isora* [281], *C. malabaricum* [13], *Cicer arietinum* L. [119] *Lippia javanica* [27], *Iris x hollandica* [274]. The present strategy describes efficiently utilization of more than one marker as also validated by the results of Bhattacharyya et al. (2018) [139], Sadhu et al. (2020) [119] and Mood et al. (2022) [278], which indicates that a single marker is not much effective to screen genetic variations of in vitro regenerated plantlets.

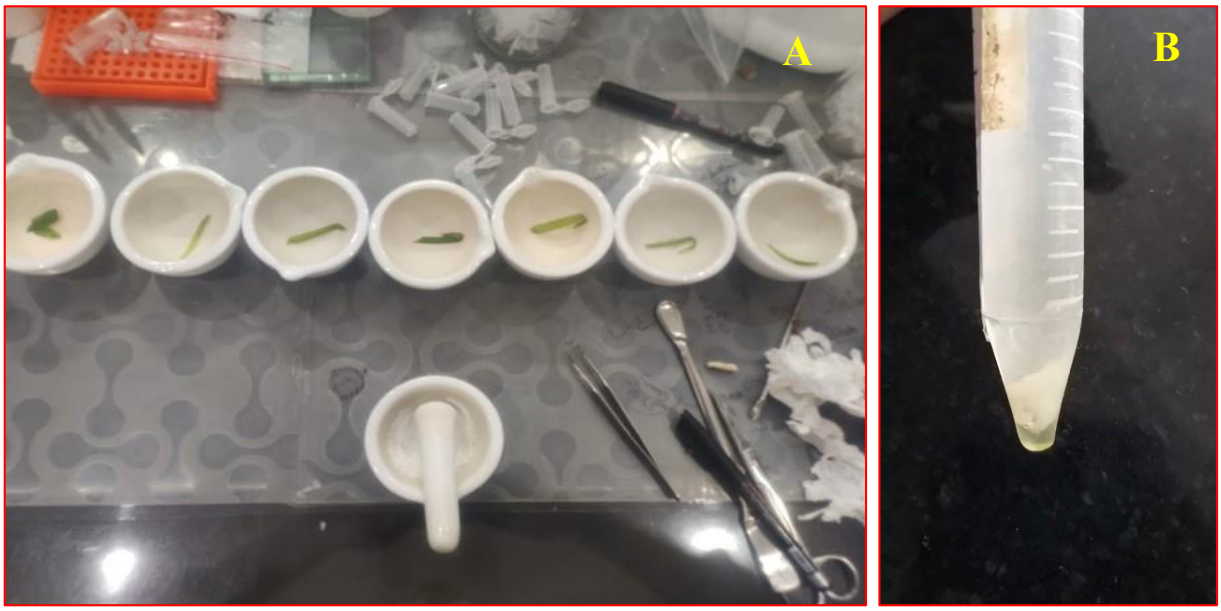

Fig 28. Isolation of DNA. A) DNA isolation setup; B) Isolated DNA pellet (seen at the bottom of falcon tube)

Table 22. Genetic fidelity assessment using RAPD markers on the *in vitro* regenerated *C. malabaricum*.

S. No.	Primer code	Primer Sequence (5'-3')	Number of Scorable bands	Band length of Amplicons
1	OPAA-01	AGACGGCTCC	1	460
2	OPAA-02	GAGACCAGAC	5	750, 700, 650, 600, 500
3	OPAB-01	CCGTCGGTAG	3	1200, 1000, 400
4	OPAB-02	GGAAACCCCT	4	900, 600, 150, 100
5	OPAD-01	CAAAGGGCGG	-	-
6	OPAD-02	CTGAACCGCT	-	-
7	OPAH-01	TCCGCAACCA	-	-
8	OPAH-02	CACTTCCGCT	-	-

Table 23. Genetic homogeneity assessment using SCoT markers on the *in vitro* regenerated *C. malabaricum*.

S. No.	Primer code	Primer Sequence (5'-3')	Number of Scorable bands	Band length of Amplicons
1	SCoT-1	CAACAATGGCTACCACCA	1	510
2	SCoT-2	CAACAATGGCTACCACCC	2	700, 300
3	SCoT-3	CAACAATGGCTACCACCG	2	350, 200
4	SCoT-4	CAACAATGGCTACCACCT	2	524, 430
5	SCoT-5	CAACAATGGCTACCACGA	3	1100, 850, 500
6	SCoT-6	CAACAATGGCTACCACGC	1	200
7	SCoT-7	CAACAATGGCTACCACGG	3	1100, 400, 250
8	SCoT-8	ACGACATGGCGACCAACG	2	490, 200
9	SCoT-9	ACCATGGCTACCACCGAC	1	350
10	SCoT-10	CCATGGCTACCACCGCAG	4	1600, 1300, 760, 550

Table 24. Genetic fidelity assessment using ISSR markers on the *in vitro* propagated *C. malabaricum*.

S. No.	Primer code	Primer Sequence (5'-3')	Number of Scorable bands	Band length of Amplicons
1	ISSR-1	AGAGAGAGAGAGAGAGC	4	650, 600, 500, 325
2	ISSR-2	AGAGAGAGAGAGAGAGG	2	500, 200
3	ISSR-3	GAGAGAGAGAGAGAGAC	1	600
4	ISSR-4	TCTCTCTCTCTCTCTCC	2	600, 400
5	ISSR-5	AGAGAGAGAGAGAGAGYT	1	490
6	ISSR-6	AGAGAGAGAGAGAGAGTC	3	1200, 800, 700
7	ISSR-7	CACACACACACACACARC	3	750, 520, 450
8	ISSR-8	TGTGTGTGTGTGTGTGRA	-	-
9	ISSR-9	ACACACACACACACACT	3	570, 550, 350
10	ISSR-10	CTCCTCCTCCTCCTCCTC	5	800, 600, 540, 500, 400

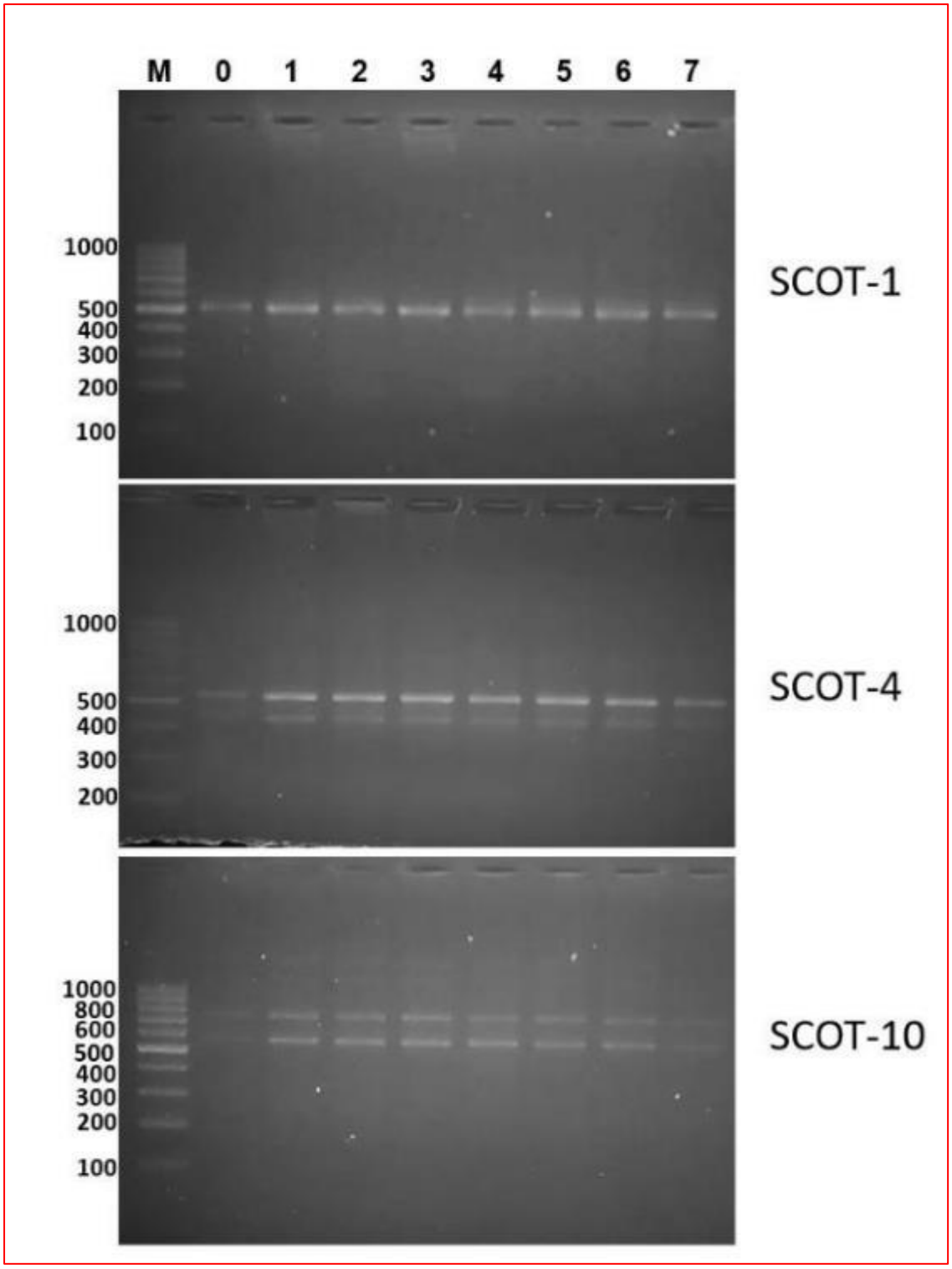

Fig 29. Genetic stability assessment in mother plants and somatic embryogenesis derived plantlets of *C. malabaricum*. using SCoT amplification profile with primers SCoT-1, SCoT-4 and SCoT-10. Lane 1 represent 100bp DNA ladder (M) followed by mother plant (0) in lane 2. Lane 3-9 labelled 1-7 are the *in vitro* raised plant lines via somatic embryogenesis.

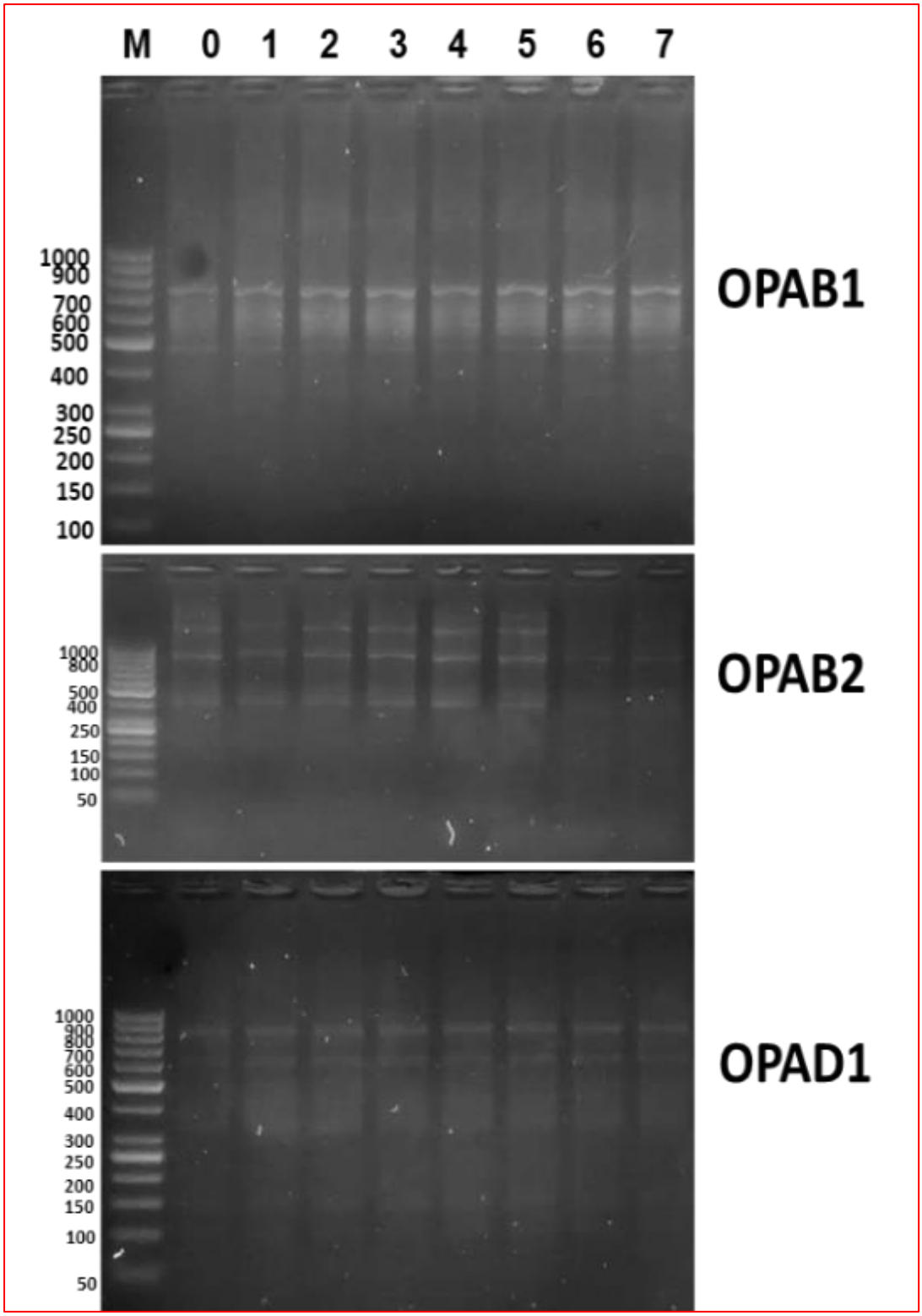

Fig 30. Genetic homogeneity analysis of mother plants and somatic embryogenesis derived plantletsof *C. malabaricum*. using RAPD amplification profile with primers OPAB-1, OPAB-2 and OPAD-1. Lane 1 represent 100bp DNA ladder (M) followed by mother plant (0) in lane 2. Lane 3-9 labelled 1-7 are the *in vitro* raised plant lines via somatic embryogenesis.

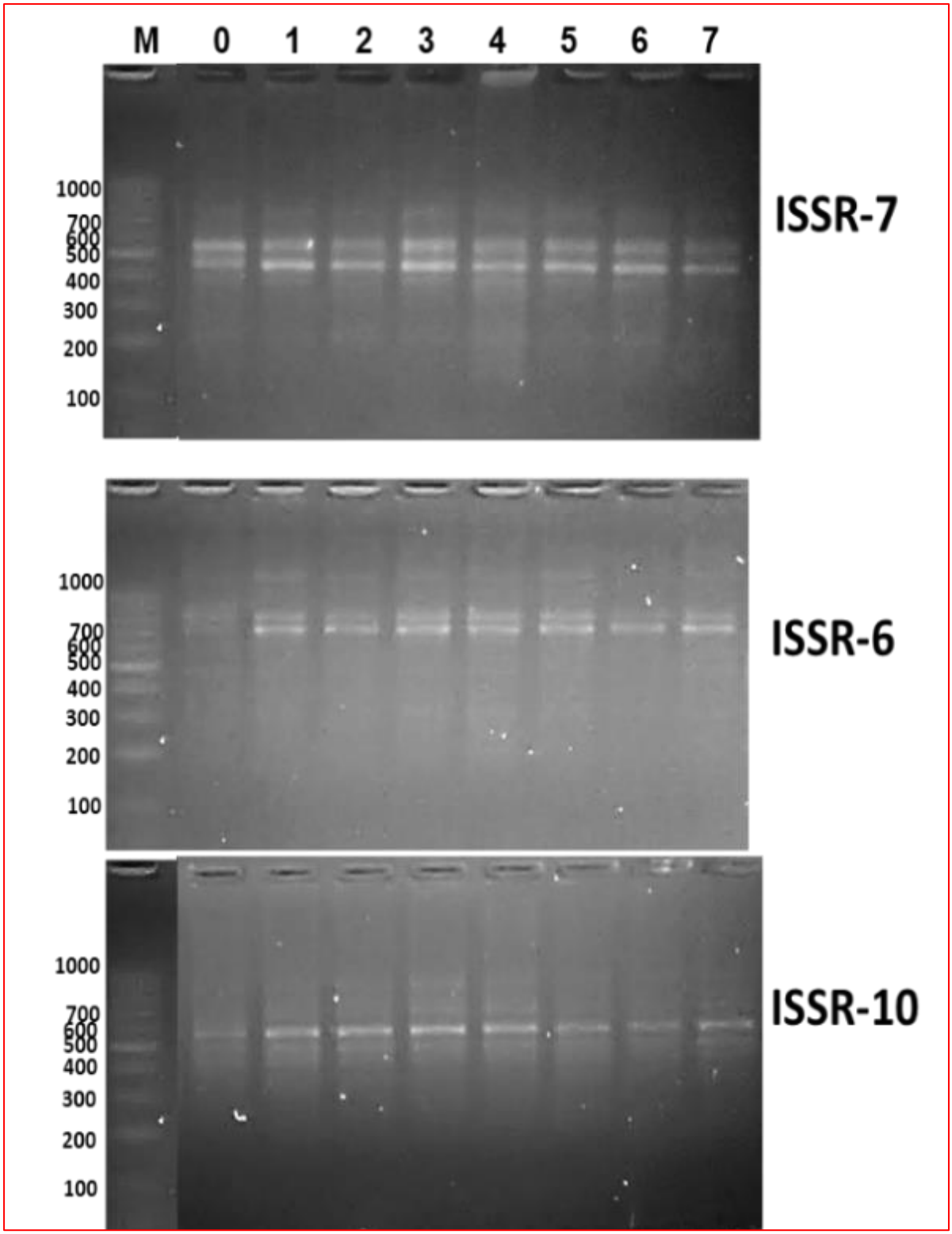

Fig 31. Genetic stability assessment of mother plants and somatic embryogenesis derived plantlets of *C. malabaricum*. using ISSR amplification profile with primers ISSR-7, ISSR-6 and ISSR-10. Lane 1 represent 100bp DNA ladder (M) followed by mother plant (0) in lane 2. Lane 3-9 labelled 1-7 are the *in vitro* raised plant lines via somatic embryogenesis.

4.4.2. Profiling of hydroxybenzoic acid derivatives from *in vitro C. malabaricum*

The accumulation of phenolic acids is greatly influenced by the different types and multiple used concentrations of the PGRs in the regenerated plant organs. Recent studies have explored plant tissue/organ culture as potential tools for high contents of phytochemical accumulation and production that could be explored broadly for industrial applications [282,283,284,285,286]. In particular, the capacity of cytokinins to foster the accumulation of bioactive phytochemicals has been explored in other plant species [286,287,288,289]. In this study, different *m*T treatments significantly influenced the content of hydroxybenzoic acid derivatives in regenerated *C. malabaricum* (Table 25). Particularly, the maximum production of p-hydroxybenzoic acid and salicylic acid were observed in the plants subcultured on the MS medium which was enhanced using 0.5 mM *m*T. In addition, *m*T (2.5 µM) induced the highest production of gallic acid, protocatechuic acid, syringic acid and vanillic acid, compared to the control. Previously, [283] had demonstrated in *Hypoxis hemerocallidea* the influence of *m*T and confirmed the increased accumulation of different hydroxybenzoic acid derivatives *in vitro*, later extended to studies in other plant species [288,289]. Recently, it was also observed that *m*T also stimulated polyphenols, flavonoids, and other secondary metabolites in different plant species [290,291]. Hydroxybenzoic acid derivatives are associated with several health benefits, including antioxidant, anti-inflammatory, antibacterial, antimicrobial and cholinesterase inhibitors [226,292,293,294,295] and thus to increase their amount in plant cells is highly desired. The administration of *m*T in *C. malabaricum* was then investigated to see if it had a good impact on the generation of hydroxybenzoic acid derivatives.

Table 25. Influence of *m*T on the hydroxybenzoic acid derivatives in regenerated *C. malabaricum*.

Treatments	**Phenolic acid concentration (µg/g DW)**					
mT (µM)	**Gallic acid**	**p-Hydroxybenzoic acid**	**Protocatechuic acid**	**Salicylic acid**	**Syringic acid**	**Vanillic acid**
0.5	0.163±0.032^{bcd}	**0.656±0.075^{bc}**	33.313±1.806^{cd}	**0.891±0.044^{bc}**	0.15±0.018^{a}	8.067±0.125^{c}
2.5	**0.199±0.019^{b}**	0.653±0.047^{bc}	**39.988±4.812^{abc}**	0.869±0.048^{bc}	**0.169±0.027^{a}**	**12.138±0.561^{a}**
5.0	0.149±0.015^{cd}	0.489±0.007^{e}	27.788±2.488^{d}	0.845±0.015^{bcd}	0.136±0.013^{ab}	4.881±0.484^{e}
7.5	0.17±0.006^{bcd}	0.591±0.029^{cd}	38.505±2.275^{bc}	0.823±0.071^{bcd}	0.152±0.01^{a}	8.12±0.24^{c}
10	0.19±0.008^{bc}	0.587±0.014^{cd}	38.772±1.168^{bc}	0.888±0.022^{bc}	0.157±0.022^{a}	8.618±0.771^{c}
Control	0.138±0.014^{d}	0.542±0.026^{de}	33.981±2.549^{bcd}	0.847±0.021^{bcd}	0.076±0.008^{c}	2.358±0.054^{f}

4.4.3. Hydroxycinnamic acid derivatives accumulation in *C. malabaricum*

C. malabaricum extracts had five hydroxycinnamic acid derivates (caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid and sinapic acid) at varying concentrations (Table 26). With the exception of caffeic acid, all other hydroxycinnamic acid derivatives were significantly high in the MS medium supplemented with *m*T. For caffeic acid, the highest content (1.226 mg/g DW) was accumulated in the control. The maximum content of ferulic acid and sinapic acid were obtained with 2.5 mM *m*T. Furthermore, with *m*T (5.0 µM) the highest quantity of chlorogenic acid and *p*-coumaric acids were produced. Previous studies reported the significance of *m*T on *in vitro* production of hydroxycinnamic acid derivatives in different plant organs had been reported in *Hypoxis hemerocallidea* [283] and, more recently, in two *Brachystelma* species [286]. Interestingly, sinapic and ferulic acids have been reported to have a higher antioxidant activity than other hydroxybenzoic acid derivatives [296]. Ferulic acid also accounts for several biological properties, notably antiallergic, antiviral, hepatoprotective, vasodilatory actions and in modulation of enzyme activity [297]. Additionally, sinapic acid has been attributed several pharmacological properties including anticancer, anti-anxiety, antioxidant and antimicrobial activity 298]. Nonetheless, both hydroxycinnamic and hydroxybenzoic acid derivatives account for cardioprotective and anti-diabetic activity and have also been shown to be potent anti-cancer agents [299].

Table 26. Influence of mT on the hydroxycinnamic acid derivatives in regenerated *C. malabaricum.*

Treatments	**Phenolic acid concentration (µg/g DW)**				
mT (µM)	**Caffeic acid**	**Chlorogenic acid**	***P*-Coumaric acid**	**Ferulic acid**	**Sinapic acid**
0.5	1.015±0.174^{ab}	135.334±18.525^{e}	0.326±0.042^{b}	0.543±0.036^{bcd}	0.096±0.012^{ab}
2.5	0.849±0.042^{bc}	254.674±46.78^{cd}	0.296±0.061^{bc}	**0.9±0.112^{a}**	**0.102±0.01^{a}**
5.0	0.859±0.097^{bc}	**510.995±46.059^{a}**	**0.451±0.026^{a}**	0.503±0.017^{bcde}	0.095±0.018^{ab}
7.5	0.801±0.035^{bc}	331.914±37.435^{bc}	0.301±0.022^{bc}	0.471±0.01^{cde}	0.085±0.009^{ab}
10	0.754±0.085^{bc}	180.858±19.782^{de}	0.225±0.018^{c}	0.613±0.063^{b}	0.081±0.011^{abc}
Control	**1.226±0.208^{a}**	404.58±58.683^{b}	0.412±0.062^{a}	0.403±0.047^{e}	0.04±0.013^{d}

4.4.4. LC-ESI-MS/MS analysis

The capacity of regenerated plantlets to synthesize and produce specialized compounds is an important phenomenon in plant tissue culture. There are well-documented reports demonstrating that *in vitro* cultured plants are important for the formation and production of secondary metabolites [186,300,301,302]. Based on a comparison of the peak observed in the sample MRM and retention time, lycorine and galanthamine were found to be the two substances present in the tested samples (Table 27). The presence of galanthamine ($C_{17}H_{21}NO_3$) and lycorine ($C_{16}H_{17}NO_4$) had been verified by performing the LC/ESI-MS analysis of the methanolic extracts of in vitro raised plantlets. The exact mass for galanthamine and its retention time are 288.10 and 4.0 min respectively, whereas for lycorine, the exact mass and retention time are 288.20 and 3.3 min respectively (Fig 35). For the quantification analysis, a total peak area obtained using the MRM of galanthamine and lycorine was employed. The concentration of galanthamine and lycorine in the *in vitro* raised plants were 73 µg/DW and 1400 µg/DW, respectively. Considerable differences were obtained in the content of galanthamine and lycorine. Our results are in line with those of Ghane et al. (2018) which showed a high content of lycorine in naturally grown *C. malabaricum* whereas, galanthamine was not detected. Production of galanthamine and lycorine through in vitro culture of other Amaryllideace species such as *Leucojum aestivum* [189], *Pancratium Maritimum* L. [303], *Narcissus pseudonarcissus* cv. Carlton [183], and *Narcissus tazzeta* L. [186] has been also reported. However, there is no report available on galanthamine and lycorine production from somatic embryogenesis-derived plantlets.

Table 27. Quantification data of GAL and LY from LC-ESI/MS analysis.

Sample Code	**Wt. of samples (mg)**	**GAL (ppm)**	**LY (ppm)**
CM *In Vitro* Bulb	100.18	**0.314**	**73.205**
CM *In Vitro* Roots	100.18	0.009	1.845
CM *In Vitro* Leaves	100.10	0.025	41.254
CM Callus	100.56	0.003	0.179
L-UAE	-	**0.255**	23.816
B-UAE	-	0.081	**32.365**
R-UAE	-	0.047	6.573
L-MAE	-	0.007	1.253
B-MAE	-	**0.049**	**17.191**
R-MAE	-	0.002	2.346
L-CE	-	0.009	6.732
B-CE	-	**0.036**	**7.191**
R-CE	-	0.001	3.048

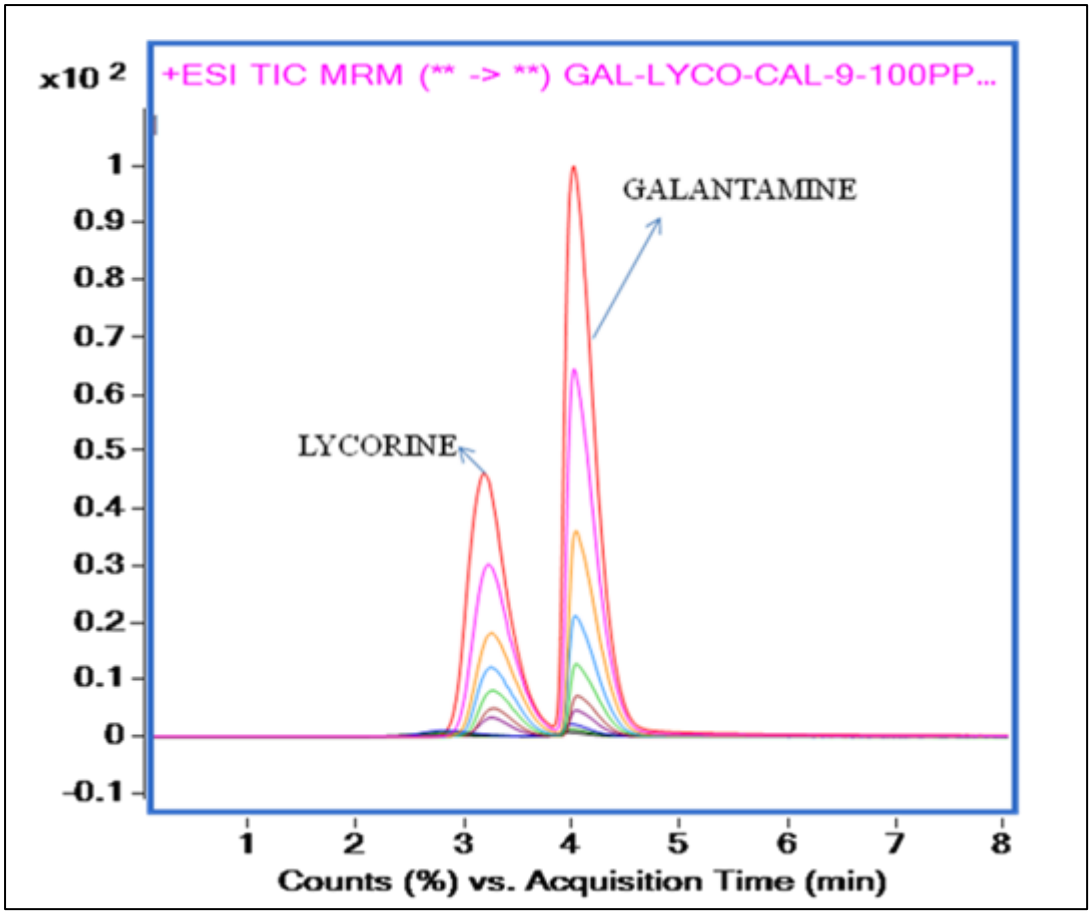

Fig 32. Calibration curve for Lycorine and Galanthamine.

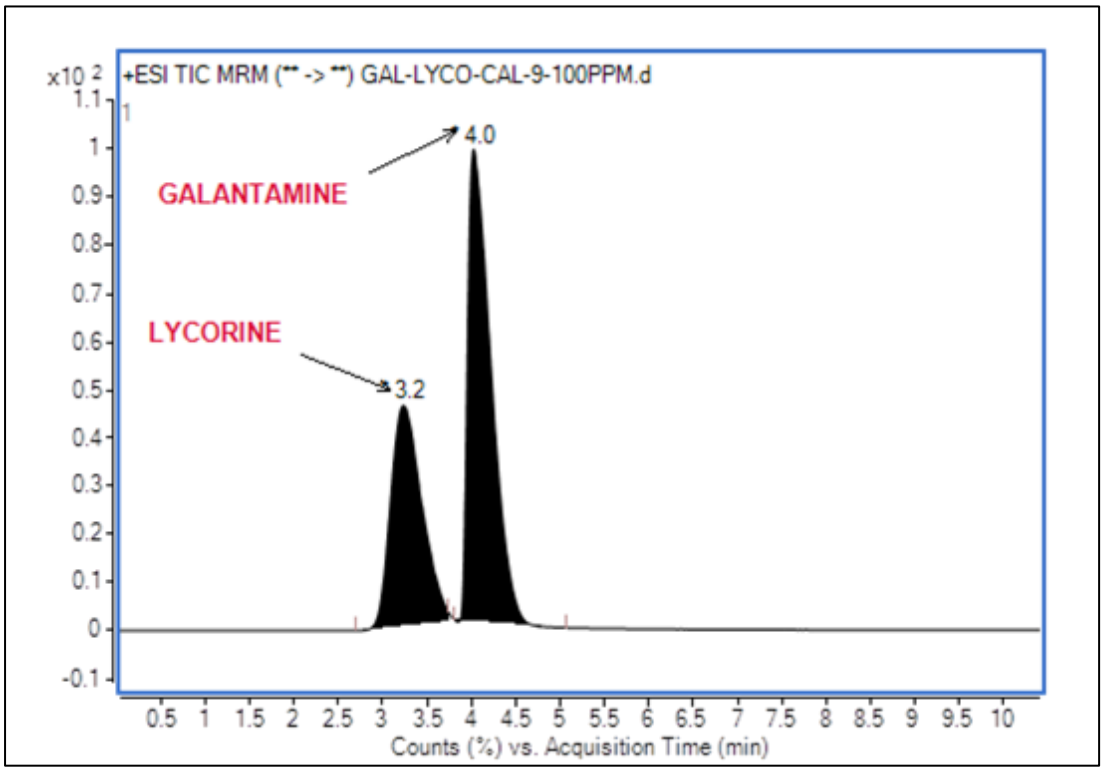

Fig 33. Standard Chromatogram of Lycorine and Galanthamine.

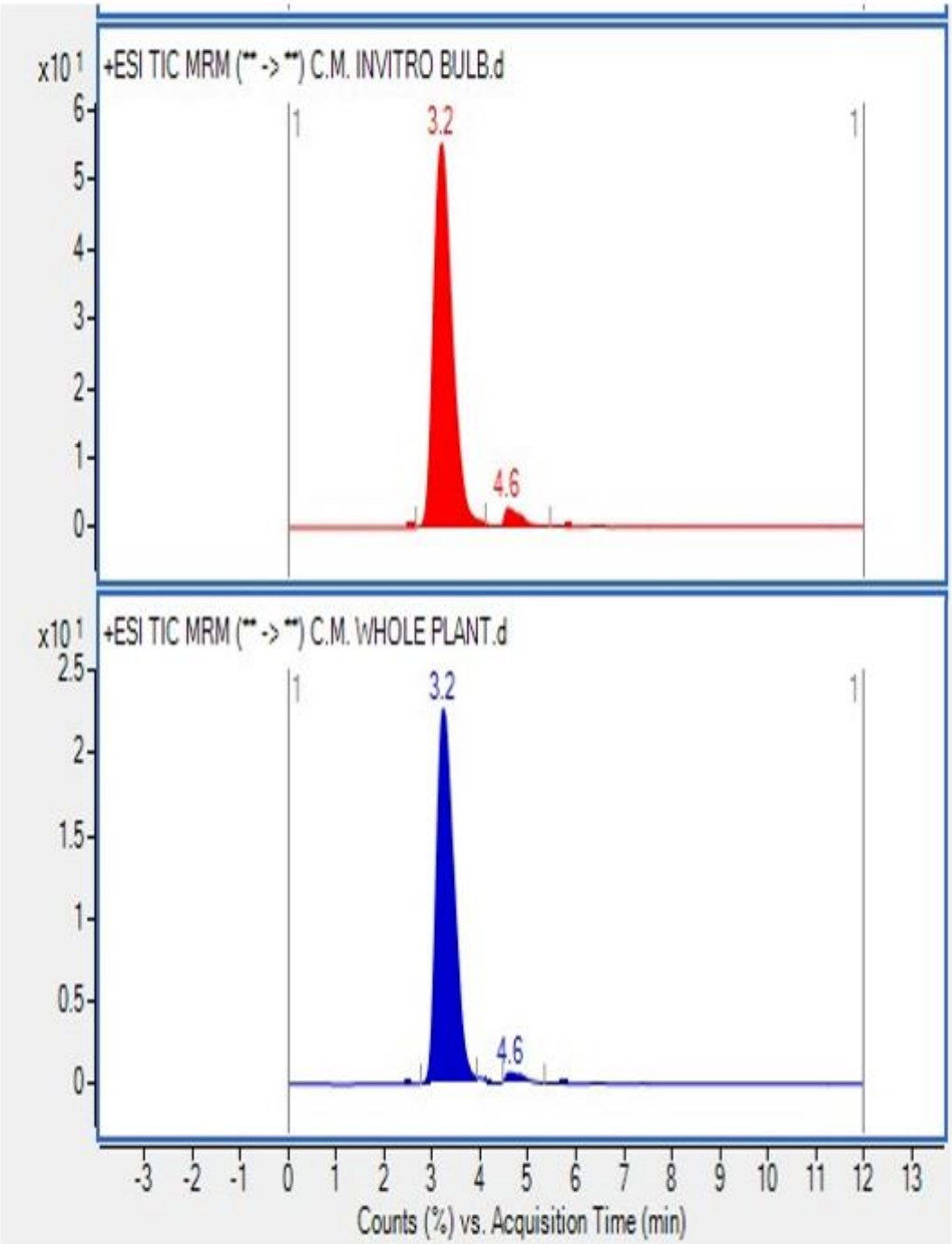

Fig 34. Standard Chromatogram of In vitro Bulbs and Whole plant Of *Crinum malabaricum.*

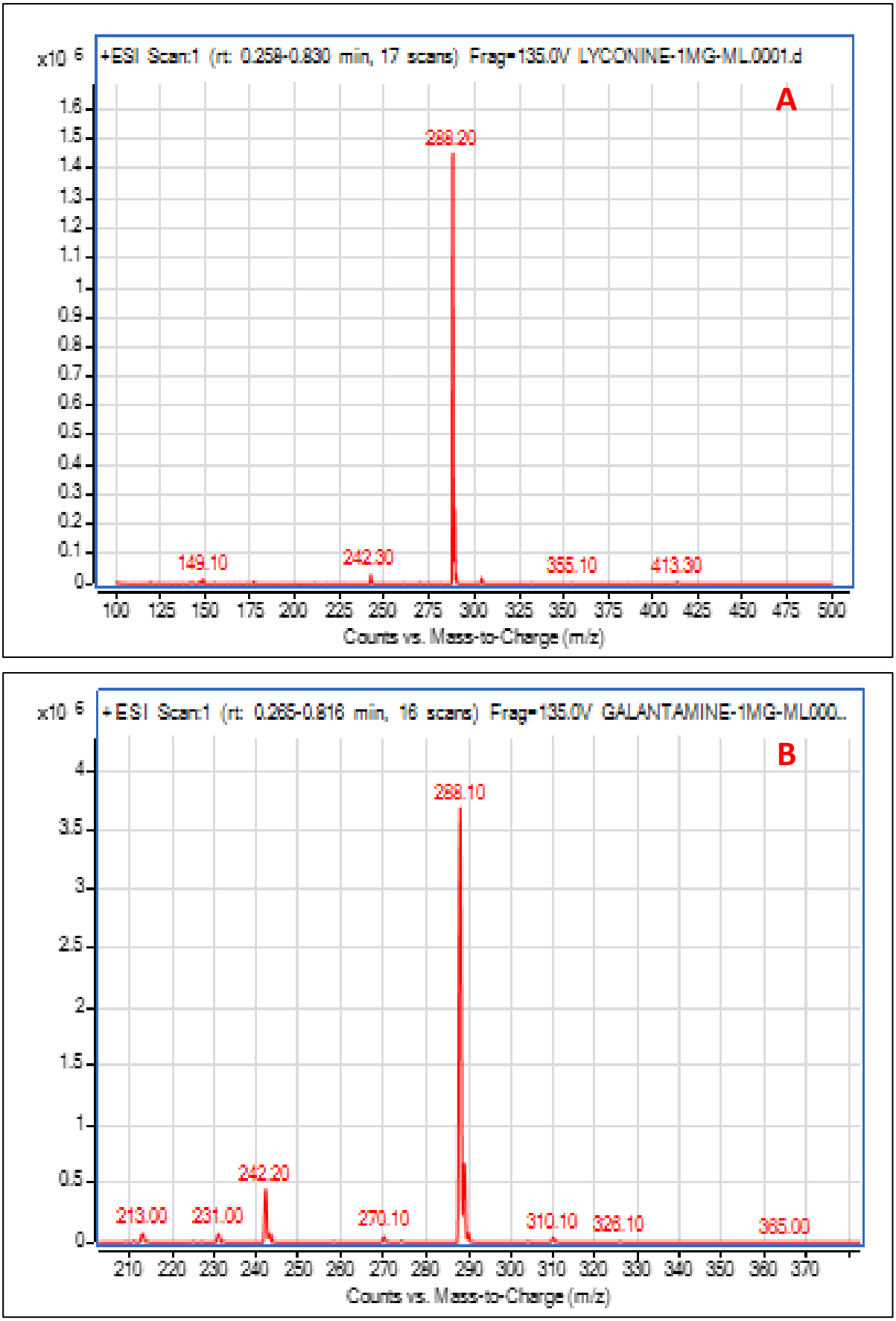

Fig 35. Mass Spectra. (A) Lycorine and (B) Galanthamine.

Chapter 5

CONCLUSION

The *Crinum* species (Amaryllidaceae) have long been utilized to treat several diseases around the globe. Interestingly, different species have been used in treating the same disease. As highlighted in the review above, the immense medicinal value of the Indian *Crinum* is a signal of their remarkable potential globally. Among the several Indian *Crinum* species, only few have been subjected to rigorous scientific evaluation including *in vitro* conservation, toxicity, and safety evaluation. Based on the evidence, several Indian *Crinum* species are at risk of over-exploitation, and many become endangered due to a lack of knowledge from both conservation and traditional usage perspective. Apart from the conventional techniques, the use of *in vitro* studies including micropropagation and somatic embryogenesis model system will be useful for the conservation and to improve the mass production of these species which often grow very slow.

The current findings highlighted for the first time the major role of *m*T on *in vitro* regeneration and phenolic acid profiles in regenerated *C. malabaricum*. *m*T influenced the growth and development of *C. malabaricum*, including higher shoot proliferation, shoot length and fresh weight. Our results also confirmed the biochemical potential of *in vitro* cultures of *C. malabaricum* to accumulate and synthesize free phenolic acids, with *m*T greatly influencing the accumulation of hydroxybenzoic and hydroxycinnamic acid derivatives during *in vitro* propagation in C. malabaricum. This investigation would serve for a better understanding of *in vitro* culture production for conservation of the Malabar River Lily genetic resources and coupled with an unrestricted and sustainable production of its secondary metabolites.

This research also confirms *in vitro* regeneration of critically endangered species *C. malabaricum* by utilizing somatic embryogenesis system. The induction and production of somatic embryos was significantly influenced by the combination of picloram and TDZ, while half-strength MS medium with 1 mg L^{-1} GA_3 significantly enhanced the conversion of somatic embryos into complete plantlets. The SEM analysis affirms the developmental differentiation of somatic embryos. The clonal fidelity of *in vitro* raised *C. malabaricum* plants was evaluated to rule out any somaclonal variation induced during somatic embryogenesis cycle. The banding pattern of *in vitro* clones highly resembled their mother plant which was evident from SCoT, ISSR and RAPD markers analysis. Galanthamine and lycorine were efficiently identified by using LC-ESI-MS/MS analysis. Therefore, isolation and production of valuable alkaloids (viz. galanthamine and lycorine) in *in vitro* cultures will lead to commercialization of somatic embryo-derived plantlets and that may be considered as a natural source of drug for several pharmacological applications. This developed protocol may facilitate mass propagation of high quality true-to-type *C. malabaricum* plants for conservation and can be utilized as a potent

source of valuable alkaloids to treat several diseases. In addition to the considerable work carried out, further, in-depth studies including genetic and metabolic engineering should be performed to maximize productivities of these valuable alkaloids.

GAL is a specialized alkaloid uniquely present in Amaryllidaceae plant species. Due to its high medicinal importance, studies on improvement of in vitro production have received much attention. There is a need to increase the yields and efficiency in the production of GAL to make them more attractive to the pharmaceutical industry; therefore, it is mandatory to apply an integrated biotech approach to the scale process to achieve the crucial step from the laboratory to the market. Plant cell tissue and organ culture is a promising approach to synthesize and scale-up of therapeutically valuable metabolites such as GAL. The use of various PGRs, elicitors and carbon sources are promising approaches for the optimization and biosynthesis of *in vitro* GAL production. *In vitro* accumulation of GAL mostly depends on different differentiation levels and type of culture medium among several other factors. Additionally, differentiating aspects of the potential production of GAL using *in vitro* cultures in TIBs (Temporary Immersion Bioreactor) are presented: (1)- it would be based on complete and photosynthetically active plants originated by improving both the intensity (and quality) of light, and the supplies of sterile air that can be enriched with CO2; (2)- the initial materials would be a reduced number of meristematic explants from selected donors, making possible the production of sterile plant biomass without the need for intensive exploitation from wild plantations, usually from native species. At the same time, research on the genetic and molecular mechanisms of different alkaloids should be continued to optimize GAL production in culture systems to develop metabolic engineering approaches.

. The pharmacogenomics research initiatives to understand GAL biosynthesis using genetic and biochemical approaches are crucial. In future, knowledge about specific Amaryllidaceae species selection will lead to optimization of industrial GAL production, thus providing this potent drug to individuals affected by AD without diminishing natural population of the species. Furthermore, identification of other functional enzymes for GAL biosynthesis will surely lead to more strategies for effective mass-production of GAL. Finally, new and in-depth research may allow a better understanding of different factors in GAL biosynthesis, therapeutic and safety factors in the use of Indian *Crinum* species, while different biotechnological tools are required for their sustainable use and long-term conservation.

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Chapter 6 | List of Publications

- **Swati Chahal**, Manoj M. Lekhak, Harmeet Kaur, Mahipal S. Shekhawat, Abhijit Dey, Priyanaka Jha, Devendra Kumar Pandey, Vijay Kumar. South African Journal of Botany, 2020. Unraveling the medicinal potential and conservation of Indian Crinum (Amaryllidaceae) species. South African Journal of Botany 136, 7-15. (Impact Factor 3.11)
- **Swati Chahal**, Manoj M. Lekhak, Harmeet Kaur, Mahipal S. Shekhawat, Umesh Goutam, Sachin Kumar Singh, Sergio J. Ochatt, Vijay Kumar, 2022. *Meta*-topolin-mediated regeneration and accumulation of phenolic acids in the critically endangered medicinal plant *Crinum malabaricum* (Amaryllidaceae): A potent source of galanthamine. South African Journal of Botany 149, 853-859. (Impact Factor 3.11)
- **Swati Chahal**, Manoj M. Lekhak, Ajai Prakash Gupta, Sergio J. Ochatt, Vijay Kumar, 2023. True-to-typeness and phytomedicinal potential in somatic embryo-derived plants of *Crinum malabaricum* (Amaryllidaceae): A medicinally important source of pharmaceutical biomolecules. Industrial Crops and Products 204, 117329. (Impact Factor 5.9)
- Harmeet Kaur, **Swati Chahal**, Priyanka Jha, Manoj M. Lekhak, Mahipal S. Shekhawat, Devashan Naidoo, Ariel Arencibia, Sergio J. Ochatt, Vijay Kumar, 2022 Harnessing plant biotechnology-based strategies for in vitro galanthamine (GAL) biosynthesis: a potent drug against Alzheimer's disease. Plant Cell Tissue & Organ Culture 149**,** 81–103 2022. (Impact Factor 3.76)
- Saikat Sena, Harmeet Kaur, **Swati Chahal**, Priyanka Jha, Umesh Goutam, Vijay Kumar, 2023. The Beneficial Role of Silicon Alleviating Heavy Metal and Disease Resistance Stress in Crops. Silicon 15, 2973-2988. (Impact Factor 3.4)
- Harmeet Kaur, Manoj M. Lekhak, **Swati Chahal**, Umesh Goutam, Priyanka Jha, Devashan Naidoo, Serhio J. Ochatt, Vijay Kumar, 2020. *Nardostachys jatamansi* (D. Don) Dc.: An invaluable and constantly dwindling resource of the Himalayas. South African Journal of Botany 135, 252-267. (Impact Factor 3.11)
- Priyanka Jha, **Swati Chahal**, Devendra Kumar Pandey, Joginder Singh, Ram Prasad, Vijay Kumar, 2020. Conservation strategy for African Medicinal Species: In Vitro Biotechnological Approach. Phyton International Journal of Experimental Botany 89, 779-794. (Impact Factor 1.7)

- Harmeet Kaur, **Swati Chahal**, Manoj M. Lekhak, Priyanka Jha, Sergio J. Ochatt, Vijay Kumar, 2022. Meta-topolin induced in vitro regeneration in *Crinum brachynema* (Amaryllidaceae): A critically endangered and endemic medicinal plant of India. Plant Cell, Tissue and Organ Culture 151, 663-672. (Impact Factor 3.76)
- Harmeet Kaur, **Swati Chahal**, Priyanka Jha, Devendra Kumar Pandey, Ariel D. Arencibia, Vijay Kumar, 2021. “Biostimulants, the Cinderella for plant development” book chapter in Biostimulants for Crops from Seed Germination to Plant Development: A practical approach, 61-72.