In-vitro regeneration and molecular characterization of critically endangered *Crinum brachynema* for conservation and secondary metabolite production

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2024

DECLARATION

I hereby declare that the thesis entitled, "*In-vitro* regeneration and molecular characterization of critically endangered *Crinum brachynema* for conservation and secondary metabolite production" submitted for my Ph.D. Biotechnology, Degree to Department of Biotechnology, Lovely Professional University. Completely original work and all ideas and references have been duly acknowledged. The research work has not been formed as the basis for the award for any other degree or fellowship previously.

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CERTIFICATE

This is to certify that the thesis entitled "***In-vitro* regeneration and molecular characterization of critically endangered *Crinum brachynema* for conservation and secondary metabolite production**", which id being submitted by **Mrs. Harmeet Kaur** for the award of the degree of Doctor of Philosophy in Biotechnology from the Faculty of Biotechnology, Lovely Professional University, Punjab, India is entirely based on the work carried out by her under my supervision and guidance. The work reported, embodies the original work of the candidate and has not been submitted to any other university or institution for the award of any degree or diploma, according to the best of my knowledge.

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Harmeet Kaur

Abstract

Commercialization of medicinal plants as recognized by pharmaceutical, nutraceutical, and cosmeceutical productions which is growing in popularity and interest around the world. Plants of the Amaryllidaceae family are well recognized for traditional medicine and have been used to cure a variety of ailments and they are recognized for its structurally varied compounds, which have a diverse variety of medicinal actions. *Crinum* is a well-known bulbous geophyte with ornamental and therapeutic benefits. 15 *Crinum* species are represented by India, each of the species contains various bioactive compounds having biological activities. Some medicinal properties of Indian *Crinum* species still require pharmaceutically or clinical investigation. Furthermore, a deeper study using various biological techniques is required to make sure for their long-term utilization and protection. More research is needed to increase our present understanding of the bioactive components, toxicity, clinical value, and conservation status. The current study summarizes research on *in-vitro* regeneration, somatic embryogenesis, enhancement, and extraction of a targeted compound from Indian *Crinum* species. Potential antioxidant activity, information gaps, and future views are also considered.

Crinum brachynema (Amaryllidaceae) is a critically endangered bulbous plant of the Northern-Western Ghats of India. This species contains an important compound called galanthamine (GAL), an anti-Alzheimer drug. This investigation aimed to establish a protocol for the regeneration of *C. brachynema* using twin scales as initial explants. Thus, cultures were established on MS medium with various hormones (BA, NAA in combination with BA and meta-*Topolin*) under the controlled conditions. Cultures were kept in a culture room with maintained temp. at 25 ± 2ºC, under a 16/8h (light/dark) and photo-period was maintained by cool white LED tubes at PPFD (photosynthetic photon flux density) of 40 µmol $m^{-2}s^{-1}$. The establishment of shoots from the explants was achieved. Media containing (2.0 mg/L) BA with (0.5 mg/L) NAA initiates the highest number of shoots. In general, *mT* proved superior as cultures produced a maximum number of regenerants compared to the control. The highest no. of shoots (11.10) with high shoot length (9.33 cm) and fresh shoot biomass was produced with 5.0 µM *m*T. The rooting frequency of *C. brachynema* shoots ranged from 65-100% over all the concentrations tested, and the highest no. of roots (13.47) with highest length of root (9.37 cm) was observed on (1.0 mg L-1) IBA. Rooted plants with bulblets were transferred to the greenhouse where they were phenotypically similar to the mother plants. Antioxidant activity was tested with ORAC (oxygen radical scavenging

capacity model). Cultures on *mT* exhibited a significant increase in antioxidant capacity compared to control. These findings highlight the beneficial effect and validate the rising importance of mT for *In-vitro* regeneration studies. This study will serve as a potential protocol to conserve and restore the medicinally important *C. brachynema*, while the regenerated *C. brachynema* may be subsequently manipulated to further increase the accumulation of antioxidant potential.

Medicinal plants play a significant role in enhancing human health and serve as a vital source for discovering new pharmacological possibilities. Plant-derived biomolecules represent the characterization of drug leads and have historically demonstrated their significance as a source of medicines. Naturally, the bioavailability of Amaryllidaceae alkaloids including GAL is low. As the demand for bioactive compounds is increasing in pharmaceutical industries *in-vitro* cultures is the alternative supply to their production. Thus, different biotechnological tools can optimize the *In-vitro* GAL biosynthesis for treating AD, such as manipulating PGRs, light-period, hormones (elicitors), and bio-reactors methods, besides being an environmentally sustainable approach, which protects the natural bio-diversity in circular bio-economy surroundings. In the present study, we highlight the GAL bio-synthesis by a plant *In-vitro* system including its mode of action. This report should also provide a starting point in the scaling-up of the biotechnological production of this valuable alkaloid.
Crinum brachynema, (Amaryllidaceae) is a bulbous plant that is endemic to India. Generating a productive plant re-generation protocol is urgently needed for conservation and large-scale propagation. In this study, regeneration was done through somatic embryogenesis from callus produced, media containing different conc. of Picloram and Thidiazuron (TDZ) and another combination of (2,4-D) with (BA) and 2,4-D alone. Advanced phases (cotyledonary, globular, and torpedo) of callus development were obtained on MS medium, had picloram (2.0mg/L) and TDZ (0.5mg/L), after 8-weeks higher frequency of somatic embryos was obtained. The highest number of somatic embryos (50.33±1.52) was obtained after eight weeks in the medium supplemented picloram (2.0 mg L−1) in combination with TDZ (0.5 mg L−1). MS medium with reduced concentration of salts in combination with GA3 (1.0 mg L−1) was used for somatic embryo germination. The maximum embryo germination frequency (82.22) was recorded on half-strength MS medium fortifed with 1 mg L−1 GA3. Scanning electron microscopic examinations showed the developmental stages of an embryogenic cluster. The genetic homogeneity of *in-vivo* and *in-vitro* plants was compared by using SCOT, RAPD, and ISSR marker studies. The amplification bands produced by

SCoT, ISSR, and RAPD applied were monomorphic across all the regenerated plants. This confirmed their genetic homogeneity compared to the *in-vivo* plant and demonstrated the dependability of our somatic embryogenesis method for *C. brachynema.* A rapid method for phytochemical analysis by LC-ESI/MS was utilized and standardized for investigating galanthamine as well as lycorine from methanolic extracts of *in-vitro*-raised plants. The protocol developed should be helpful in the reintroduction, restoration, and ex-situ conservation of *C. brachynema* in the native surroundings.

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Abbreviations

2,4-D 2,4-Dichlorophenoxyacetic acid

Ads Adenine sulphate

ANOVA Analysis of variance

BA 6-benzyladenine

CTAB Cetyl Trimethylammonium

DMRT Duncan's Multiple Range Test

DNA Deoxyribose Nucleic acid

dNTPs deoxynucleotide triphosphate

ESI-MS Electrospray Ionisation-Mass Spectrometry

GA_3 Gibberellic acid

GAL Galanthamine

HCL Hydrochloric Acid

HgCl2 Mercuric Chloride

HPLC High Performance Liquid Chromatography

HPTLC High Pressure Thin Layer Chromatography

IAA Indole-3- acetic acid

IBA Indole 3-butyric acid

ISSR Inter Simple Sequence Repeats

IUCN International union for conservation and nature

JA Jasmonic Acid

Kn Kinetin

LED light-emitting diode

MJ Methyl Jasmonate

MS Murashige and Skoog medium

NAA Naphthalene acetic acid

PCR Polymerase Chain Reaction

PGRs Plant growth regulators

PPFD Photosynthetic photon flux density

RAPD Random Amplification of Polymorphic DNA

SA Salicylic Acid

SCoT Start codon targeted polymorphism

SE Somatic embryogenesis

SEM Scanning Electron Microscope

TAE tris-acetate EDTA

TDZ Thidiazuron

UPLC Ultra Performance Liquid Chromatography

PPFD Photosynthetic photon flux density

AChE Acetylcholinesterase

LC-MS Liquid chromatography- mass spectrometry

***m*T** *meta*-Topolin

ORAC Oxygen radical absorbance capacity

Chapter 1

1. Introduction

Plants are one of nature's most significant suppliers of medicines. Medicinal plants have been employed for their therapeutic benefits since the dawn of human civilization. For many years, nature has been a source of medical plants, and today we have several medicines which have been extracted from herbal plants. Currently, most modern diseases are treated by conventional herbal plants which shows that traditional medicines have a significant effect in the public health sector. India offers a variety of traditional plant treatment methods, such as Siddha, Ayurveda, and Unani, that primarily use medications based on plants and have been practiced for centuries. The herbal traditional treatment systems comprise the history of indigenous herbal practices for the contribution towards the well-being of the majority of Indians. The Ayurveda structure in India has defined a significant number of medicines based on plant products, and determining their morphological and pharmacological characteristics may offer a better knowledge of their bioactive compounds. People rely on plants for their survival as they are essential for both shelter and supply of nutrients (carbohydrates, minerals, vitamins, and proteins). Plants have long been thought to be the source of essential and necessary primary metabolites [1]. Plants often synthesize an abundance of additional organic chemicals to build robust connections with the biotic environment and to activate defensive mechanisms, in addition to their core metabolites. These molecules are called secondary metabolites. [1-3]. Secondary metabolites are classified into six groups: phenolics (simple phenolics, flavonoids, coumarins, xanthones, chromone, tannins, lignans, stilbene), alkaloids (purines, quinolines, pyridines, indoles), saponins (, triterpenoids saponins, steroidal saponins), terpenes (farnesol, menthol, pinene), carbohydrates (mucilage) and lipids (waxes and essential oils) [4-6]. All these constituents are produced in specific plant tissue and their manufacturing relies on plant development stages.

It's widely accepted that items derived from plants have been used by humans in the form of herbal medicines for a long time and these compounds with distinct pharmacological benefits. Several illnesses have been successfully treated by using medicinal plants in both traditional and modern medicines [1,7]. People across the world are interested in herbal treatments. [8]. The rising demand reflects a growing concern about the safety and efficacy of herbal medications. Also, traditional methods of genetic enhancement and *in-vitro* propagation of plant species are time-consuming and challenging, However, plant-tissue cultures can be employed as another viable option for rapid growth of plant species. [9,1].

This method of generating and isolating secondary metabolites is far more efficient as compared to extraction from wild populations [10-14] which has all shown that cultures of plant cells and organs yield plant-specific physiologically active chemicals. When compared to plants cultivated in the wild, *In-vitro* methods are said to have elevated metabolic frequency owing to their higher rate of proliferation as well as shorter biosynthetic period [15].

1.1 Medicinal importance of *Crinum* species

The Amaryllidaceae family has an illustrious past in traditional medicine [16-19]. For centuries, people have relied on *Crinums* for various health benefits [20-22], including as a tonic, laxative, antipyretic, malaria preventative, tumor fighter, anti-aging agent, lymphocytic suppressant, and asthma treatment [23,24]. Furthermore, they are also used to treat headaches, backaches, swelling, haemorrhoids, wounds, and rheumatism [16,25,26]. Amaryllidaceae is a popular monocotyledonous family of herbaceous perennial plants [16,27], which can be from tropical to warm-temperate regions [28,29]. Thirteen of these have been reported from India, with five being endemic [30,31]. *Crinum L.* (Amaryllidaceae) was first recognized with four distinct species: *C. asiaticum L., C. africanum L., C. latifolium L., and C. americanum L.)* [32]. Currently, the genus *Crinum* contains 112 species that are found all over the world, including Asia, Africa, America, and Australia [33]. There are 15 species in India (*C. asiaticum, C. amoenum Roxb. ex Ker Gawl, C. brachynema Herb. C. humile Herb., C. lorifolium Roxb., C. latifolium, C. malabaricum). C. reddyi M. Patel &H. Patel, C. solapurense, Herb, C. stenophyllum, C. pusillum Herb, C. stracheyi Baker, C. viviparum R. Ansari &V.J. Nair, C. wattii Baker and C. woodrowii Baker)* [31,32,34]. Because of the bioactive alkaloids, *Crinum* species are well-recognized for their pharmacological actions such as antimicrobial, antimalarial, antitumor, antioxidant, antiviral, antiaging, anti-inflammatory, antidiabetic, and anti-Alzheimer [22,35-38]. Amazingly, due to their similar alkaloid constituents, a few *Crinum* species are used for the same medicinal purposes in different countries. Concerning the utilization of *Crinum* species in conventional medicine, India ranks second only after South Africa [20,39]. As a rubefacient, roast bulbs are used in the treatment of rheumatism. It is also used to treat haemorrhoids and suppuration [40]. *C. pratense Herb. (= C. lorifolium)* is used in Indian medicine to treat intestinal diseases such as dysentery and diarrhoea [41]. Its bulb extracts are effective in treating chest ailments as a bitter tonic [23]. *C.defixum Ker Gawl*., the bulb has been used to cure inflammatory conditions [42]. *C. latifolium* leaves are also used in

traditional systems such as Ayurveda to treat allergic disorders, piles, fever, earache, and skin diseases. In addition, fresh leaf juice is served to treat joint pain and vomiting. *C. viviparum* bulb paste is used to treat boils, skin allergies, and Herpes [16,43]. Tribal communities in Madhya Pradesh such as Bheel, Kol, Auranb, and Sahariya use *C. viviparum* bulb paste mixed with turmeric powder to treat bruises and swelling [44]. The bulb of *C. woodrowii* is also used to treat biliousness, vomiting, urinary discharge, bronchitis, night blindness, and gonorrhea [45,46,47]. According to several researchers, the presence of pharmacologically active alkaloids in various Indian *Crinum* species is expected to rise the interest of the pharmaceutical industry, which has an increasing need to discover more active constituents. *C. brachynema* Herb. is an indigenous and extremely rare bulbous plant found only in Maharashtra state in Western India [48], and it is known locally as "Karnaphul," and it is a potential supplier of GAL [33,49]. As same as other *Crinum* species, it is medicinal and ornamentally important (showy fragrant flowers) and it is often used commercially in the scents and medicinal industries. Because of the presence of isoquinoline alkaloid, GAL, this species has therapeutic and pharmacological activity [22,26,49,50]. GAL is a reversible persistent alkaloid derived primarily from the Amaryllidaceae species. It is an FDA-approved drug and is regarded as a potential first-line to treat moderate Alzheimer's disease [50-52]. It also acts as an acetylcholinesterase (AChE) inhibitor, resulting in positive allosteric modification of acetylcholine receptors [52-55]. This species population is dwindling in the wild due to excessive reaping of bulbs from their native regions for sale in local markets (for ornamental as well as medicinal activities), frequent forest fires, urban development, and the exhaustion of areas due to landslips.

According to the IUCN Red List [33], this plant is in the "most critical danger of extinction." Furthermore, due to its rareness, limited distribution, and human-caused habitat destruction, it is likely to become extinct. As a result of its recent status and overexploitation, researchers have an opportunity to develop novel approaches for *In-vitro* multiplication and preservation processes. *In situ* conservation is typically used to protect and manage the natural populations of threatened plants. Because of its occurrence in touristy areas of Maharashtra's Western Ghats, India, *in situ* conservation of *C. brachynema* is now a major concern, and *ex-situ* methods such as cryogenic preservation, germplasm conservation, and *In-vitro* approaches are critical. In this respect, *In-vitro* culture can ensure the long-term development of plant conservation applications while also providing numerous benefits such as mass propagation, disease-free plant production, and germplasm storage in a short period [27,52,56]. As a result,

we set out to develop an *In-vitro* regeneration protocol to alleviate the strain on the natural habitat. There has been not a single report on the conservation approach and *In-vitro* regeneration of *C. brachynema* to date. According to several researchers, the presence of pharmacologically active alkaloids in various Indian *Crinum* species is expected to increase the interest of pharmaceutical industries, which are under pressure to identify more active compounds.

1.2 Secondary metabolites from *Crinum* species

Secondary metabolites play an important role as they are used as food and medicine. The quality and safety assurance of herbal crops and drugs become a critical point in both underdeveloped and advanced nations. Medicinal herbs have been used for thousands of years to preserve, flavour, cure, and prevent illnesses. Secondary metabolites are formed by secondary metabolism and used in contexts, including the cure of infectious diseases.

These pharmaceutically significant bioactive compounds are extracted and isolated from medicinal plants used in drug synthesis. Plants provide the ingredients for blood thinners, antibiotics, laxatives, and anti-malarial drugs. Terpenoids and flavonoids, for example, are recognized for their biological actions as anti-viral, anti-cancer, anti-bacterial, anti-inflammatory, and antimalarial properties, whereas alkaloids are mostly known for their anesthetic properties. Phenolic bioactive compounds play a beneficial role in the neutralization of free radicals. Because of these properties, bioactive compounds are regarded as rich sources of antioxidants and have been crucial in the discovery and development of advanced therapies for a wide range of ailments, including cancer, tumor, arthritis, and hepatic disease. These phytochemicals are also used in cosmetics, fragrances, and flavouring agents and many of them are already commercially available in medicines and food supplements. Thus, in the modern era, bioactive compounds have emerged as an alternative system for resolving health issues all over the world. The quantitative and qualitative study of bioactive metabolites is primarily determined by the appropriate extraction technique [57,58]. If there are fewer bioactive chemicals in plant materials, it is difficult to extract, purify, and characterize them for use in drug development. Because the crude extract of plants contains a mixture of compounds (alkaloids, terpenoids, saponins, and so on), for optimal results in extracting, isolating, and purifying a desired bioactive chemical, one must choose an optimal extraction method and analytical approach. The facts show that advances in modern spectrometric and chromatographic techniques make secondary metabolite investigation

easier than ever, but dependent on a variety of factors such as input factors, nature of explants, and extraction techniques [59]. Solvent, time, temperature, plant matrix, and pressure are the most common parameters influencing the extraction process [60]. Several techniques exist for the extraction of bioactive substances. Aside from traditional extraction methods such as percolation, maceration, heat reflux, soxhlet, and infusion, non-conventional techniques have been developed in recent years. Unconventional procedures are considered more environment-friendly due to the use of fewer organic and synthetic solvents, shorter processing times, higher yield, and higher extract quality. Separation of phytochemicals from plant extracts remains a bigger challenge in the recognition and characterization of bioactive compounds because extracts of plants contain a wide number of phytochemicals. For the identification and determination of these phytochemicals, various techniques such as HPLC, HPTLC, TLC, UPLC, UFLC, GC-MS, LC-MS, and others have been used.

1.3 Biotechnological approaches for the conservation of *Crinum* species

Because medicinal plants are an important component of biodiversity, their consumption and conservation methods require strategic oversight to ensure long-term viability. Thus, organized efforts should be directed not only toward the conservation of plant populations but also toward raising awareness of the viability of using these plant species in drugs. It has been proposed that a combination of conventional techniques and biotechnology is important to meet future public diet and healthcare demands.

Biotechnological techniques not only enable rapid cloning and genotype conservation, but also genetic modification, gene expression, and gene regulation for the efficient production of profitable natural constituents in large quantities or with improved properties [61]. The arena of biotechnology has become a center of attraction for the preservation and viable source of herbal plants due to the numerous benefits of biotechnology in various sectors i.e. food, forestry, agriculture, pharmaceutical, etc. Several *ex-situ* and *in-situ* methods, including *in-vitro* techniques, plant, herbal gardens, seed, gene banks, and gene sanctuaries, have been proposed for the preservation of critically threatened cultivars for the long-term supply of ayurvedic plants or their raw materials [62].

The most hopeful biotechnological method for the preservation and sustainable cultivation of commercially significant and threatened medicinal plants is *In-vitro* culture, also known as plant tissue culture. It has become extremely important in the mass propagation of ornamental, horticultural, medicinal, and disease-free plant species, cryopreservation, plant

improvement, plant breeding, secondary metabolite production, and genetic transformation. Furthermore, *In-vitro* culture is now a well-developed method for cultivating and studying the biological activities of quarantined plant cells, organs, tissues, cell organelles, and protoplasts under carefully controlled chemical and physical conditions.

1.3.1 *In-vitro* regeneration and conservation of species

Because almost all applications of plant biotechnology require effective cultures for plant cells, organs, and tissues, micropropagation is the foundation for all biotechnological research. This method has several advantages over traditional vegetative propagation procedures such as air-layering, cutting, grafting, seeding, and the accelerated proliferation of a wide range of virus-free crops in limited duration with high homogeneity [63]. The success rate of micropropagation is determined by a variety of factors, including culture medium, medium composition, culture environment, plant growth hormones, and genotype. Rapid *in-vitro* clonal production methods for any plant might have a major economic effect if they can be developed. Some callus cultures may result in the development of commercially relevant improved varieties by creating clones with inheritable traits that vary from mother plants due to the chance of somaclonal inconsistency [64]. From a conservation standpoint, *in-vitro* regenerants should have the fewest somatic variations possible through the micropropagation process by reducing the number of subculturing and shoot tip or axillary bud cultures. Somaclonal variations in callus formations or cell suspension cultures are common, and they reduce the commercial value of *in-vitro* regenerants [65]. The presence of somaclonal variations during *in-vitro* propagation, secondary metabolite production, and genetically engineered plants can cause significant commercial concerns [66]. As a result, to detect soma-clonal inconsistency in tissue culture, need to search the genetic composition, and consistency of *in-vitro* plants. These procedures are used in a variety of methods to measure possible variations at various levels [67,68]. Chromosome counting and Flow cytometry are widely used to assess variations in chromosome number and ploidy, and various Molecular markers that rely on DNA sequence variation including AFLP, RAPD, ISSR, RFLP, SCoT, and microsatellite markers are utilized. By assisting in the perennial cultivation of plenty of identical plants, the production of non-pathogenic plants, and a significant increase in multiplying rates, *In-vitro* micropropagation has thus become a beneficial initiative over traditional agricultural proliferation practices [69]. At the moment, an enormous number of micropropagation protocols have been established for pharmaceutical plants [70,71].

1.3.2 Plant culture of cells for secondary metabolite production

In-vitro cultures are continuous sources of medicinal compounds since they are not affected by changes in climate. Current research is focused on the protocol of cell-suspension cultures and the extraction of compounds. Plant cell culture is required for secondary metabolites for a variety of reasons, including high market value, seasonal changes, and legal difficulties with utilization (exploitation of species). In light of these considerations, *In-vitro* cultures offer several benefits: independence over seasonal changes, simple downstream processing, alteration of biosynthetic pathways to enhance the production of metabolites, shorter production cycles, and control over genetic engineering. This plant cell culture approach allows researchers to explore the biosynthesis route of metabolites and learn about their molecular control. The use of different strategies to boost secondary metabolite synthesis has accelerated in recent years.

1.3.3 *In-vitro* production of secondary metabolites

In-vitro culture under aseptic conditions is a plan to form natural goods derivatives from plants. To generate plant tissue that may produce bioactive substances, micropropagation of plant organs, roots, or calluses is a viable option. The cultivation of plant cells has emerged as a reliable strategy for the generation of several valuable natural bioactive chemicals [72]. Extraction of bioactive constituents *In-vitro* techniques is dependent on a variety of factors, the most important of which are nutrients for the development of plants. The optimal concentration of nutrients is critical for explant production and bioactive compound accumulation. However, their production can be increased by making changes to the culture conditions, such as elicitors and precursors concentration, or by changing light and temperature conditions. Elicitors, as plant defense compounds, stimulate the production of bioactive compounds. Plant cell wall components (pectin and cellulose), microbial constituents (chitin and glucan), and plant-resistant signaling compounds are examples of elicitors that can boost bioactive compound production (methyl jasmonate, salicylic acid and jasmonic acid).

Another strategy is to include precursors that are intermediates in the desired metabolite's metabolic pathway. Precursors supplemented in culture media can improve the production of the desired compound [73] and have been successfully used in the production of flavonoids, phenolics, and triterpenoids [74]. Before selecting the appropriate precursor, it is necessary to examine the target compound's entire biosynthetic pathway. Other biotechnology ways

induce the production of economically significant bioactive constituents include culturing of hairy roots, bioreactor systems, endophytes, and immobilization techniques.

Many Indian *Crinum* species still require the established *in-vitro* and their conservation may be adversely affected leading to unsustainable harvesting of natural populations by local communities. *C. brachynema* is an endemic species that is critically endangered and is only found in the Satara district of Maharashtra. Similarly, the newly discovered *C. malabaricum* is only found in the Kerala district of Kasaragod [34]. As a result, these Indian *Crinum* species require immediate conservation efforts to ensure their survival in their natural habitats.

1.4 Aims and objectives of the study

Crinum brachynema is a severely endangered high-value therapeutic plant with enormous economic significance. However, much research, such as phytochemical screening for elite varieties, and plant and bioactive compound production using biotechnological techniques, was still lacking. Keeping its economic significance and the potential of well-known biotechnological techniques in mind, the current investigations were launched to develop alternatives for micropropagation, clonally identical *In-vitro* regenerants, sustainable production of components by elicitation and generation of molecular and chromatographic fingerprinting methods.

Objectives of the proposed work

1. Development of regeneration protocol for *Crinum brachynema* through *In-vitro* techniques.

2. Somatic embryogenesis of *Crinum brachynema*.

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

4. Assessment of genetic fidelity in regenerated *Crinum brachynema*.

5. Extraction and analysis of secondary metabolites.

Chapter 2

Literature review

2.1 Origin of Indian *Crinum* species

Crinum is widely distributed in Asia, America, Africa, and Australia [30,75], and 15 *Crinum* species in India such as *C. latifolium, C. malabaricum, C. brachynema, C. asiaticum, C. lorifolium Roxb., and C. amoenum Roxb., C. stracheyi Baker, C. reddyi, C. solapurense Herb., C. pusillum Herb., C. woodrowii, C. watti, C. viviparum, C. stenophyllum, and C. humile*. [31,34,35]. Two endemic species of *Crinum* can only be found near Mahabaleshwar in the Satara region of northern-western ghats India: *C. brachynema* and *C. woodrowii* (Table 2.3) [30]. It is divided into three subfamilies: Amaryllidoideae, Agapanthoideae, and Allioideae [16]. The Amaryllidaceae family contains approximately 1200 species and 80 genera that are distributed to tropical and subtropical regions, including warm temperate zones [22,76,77]. *Crinum latifolium* is the largest Amaryllidaceae genera over 112 species found worldwide, 13 of these have been reported from India and five are endemic (Table 2.1; Fig. 2.1) [30,31]. *Crinum* species have been shown various biological activities mainly as anti-Alzheimer because of the presence of bioactive alkaloids [16,22,30,31]. Indian *Crinum* species are a primary source of Galanthamine (GAL) which is known for the treatment of headaches, rheumatism, and hemorrhoids [16,21,52]. *Crinum brachynema* Herbert belongs to the family Amaryllidaceae used in folk medicines [16,52]. *Crinum brachynema* is distributed to Maharashtra regions of the Indian Northern-Western Ghats. The western ghats region is a very high rainfall area due to its proximity to the Indian Ocean and the Arabic Sea. Vegetation of the western ghat region is rich and luxuriant, especially on the slopes. The population of many valuable species of India is declining at an alarming rate due to several manmade reasons and due to environmental changes. The rare, vulnerable, endangered, critically endangered, and extinct plants are a major concern. In 1842 *Crinum brachynema* was described as a new species by William Herbert as it was first imported by Messrs Loddiges of Hackney to the UK from India. He imported the bulb of *C.brachynema* which was then declared a new species (Table 2.2). It was published for the first time in Edward's Bot.Reg.28(Misc.):36(1842) [32,78]. *C. brachynema* is commonly known as "Karnaphul" like other *Crinum* species it is a medicinal and ornamental plant and it can be utilized as economically in the pharmaceutical industries and perfume manufacturing sectors. [22,26,49].

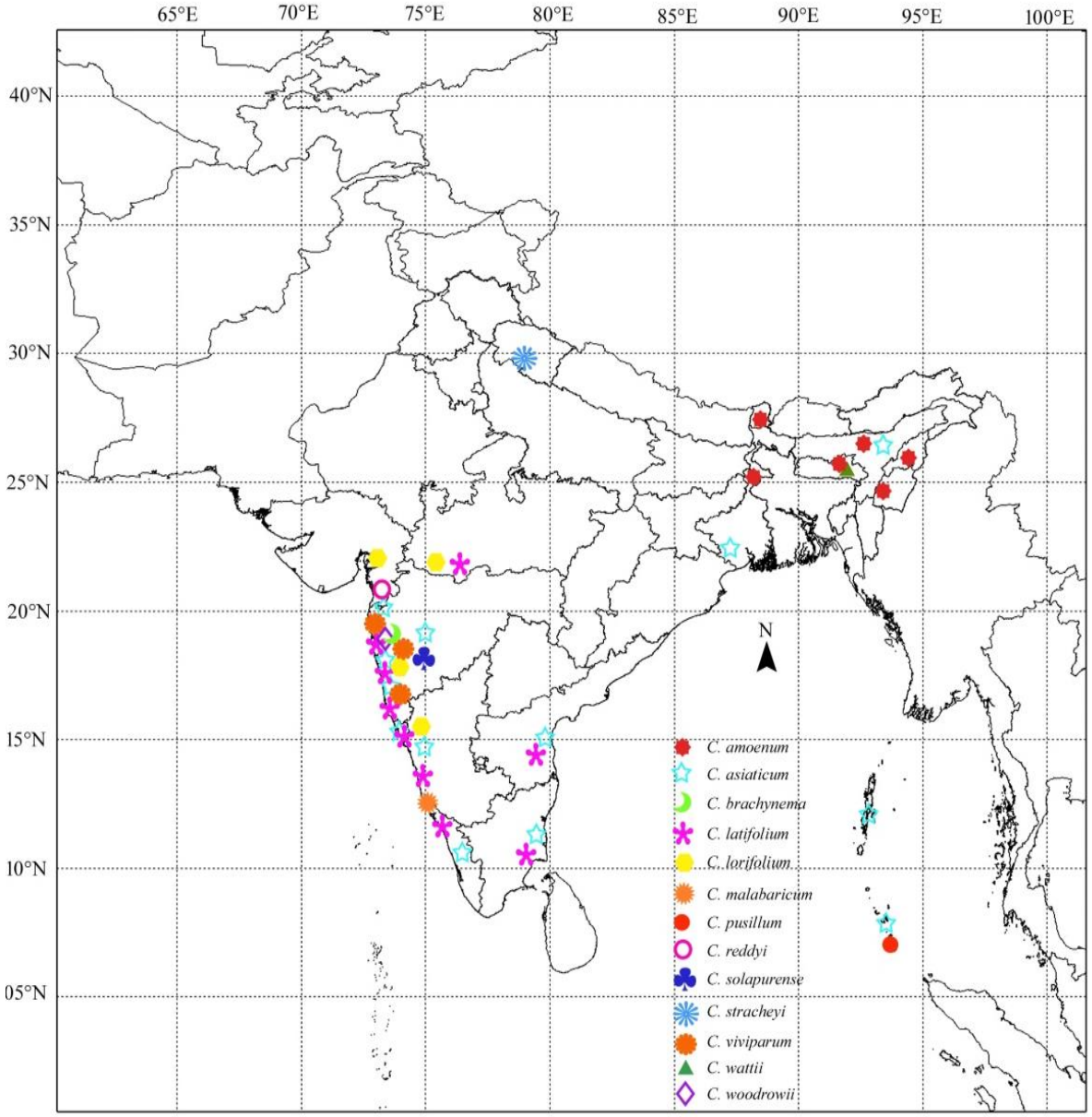


Figure 2.1 Distribution of Indian *Crinum* species

Table 2.1 Geographical distribution of Indian *Crinum* species

S.No	Species	Geographical distribution	Co- ordinates	References
1.	*C. brachynema*	Satara district, Mahabaleshwar, Maharashtra	17°56'.270''N,73°41'.488''E	[48,35]
2.	*C. malabaricum*	Periya Village, Kasaragod District, Kerala	12°24.526'N,75°06.571'E	[34]
3.	*C. woodrowii*	Katraj Ghat, District Pune, Maharashtra	18°27'15''N,73°51'45'E	[45,46,47]
4.	*C. latifolium*	Brajendranagar, Udaipur, Tripura	23°34'13.87''N,91°33'56.12''E	[79]
		Vandre, District Pune	18°31'019''N ,73°26'664''	[49]
5.	*C. asiaticum*	Throughout India	-	[21,35,80]
6.	*C. eleonorae*	Mahabaleshwar, Maharashtra	17°550.270''N,73°400.488''E	[30]
7.	*C. reddyi*	Ghuntvel, Tehsil Songadh, District Tapi, Gujarat	21°01'09.61''N,73°34'10.96''E	[81]
8.	*C. viviparum*	Ratnagiri, Maharashtra	16°52.793''N,073°19.593''E	[22,82]
		On the way to Castle rock, belgaum	15°26'314''N, 74°29'234''E	[49]
		Belgaum, District Belgaum	15°40'392''N,74°07'021''	[49]

9.	*C.solapurense*	Machnur, District Solapur, Maharashtra	17°33’58.8’’N,75°33’36.3’’E	[22,83]
10.	*C. pratese Herb.*	Borbet, District Kolhapur	16°31’070’’N, 73°53’586’’E	[49]
		Barki, District Kolhapur	16°44’673’’N, 73°50’824’’E	
11.	*C. humile*	Hyderabad, India	17.3850, 78.4867	[84]
12.	*C. pusillum*	NICOBAR ISLAND, INDIA	-	[85]
13.	*C. Stracheyi*	Kumaon, Uttrakhand, India	1676.40335313 altitude	[84]
14.	*C. amoenum*	Peninsular India, Central India, Arunachal Pradesh, Assam, Meghalaya	-	[86]
15.	*C. wattii*	Assam, Meghalaya, and Thailand	-	[87]

2.2 Morphology and medicinal uses of *Crinum brachynema*

Crinum is a genus comprised of evergreen herbaceous plants with funnel-shaped flowers, long-necked bulbs, and broad and narrow strap-shaped leaves. They are located in different regions across the world as distributed over Asia, Polynesia, Tropical America, Tropical Africa, South Africa, and Australia [16]. *C. brachynema* is restricted to Maharashtra, the Western region of India, and is usually located on hill slopes, evergreen forests, and lateritic plateaus. It is a bulbous plant of 30-60 cm high and 5-8 cm bulb, the flower is stout, 30-60 cm

long. There are 5-20 individual flowers that resemble an umbel. Six pure white lobes are cuspidate, oblong, and oblanceolate. The spathe is awl-shaped and 3-5cm long. Plants form funnel-shaped petals and sepals that are 3-5cm long. Sometimes the lobes are longer than the stamen. The *C.brachynema* species are easily differentiated by short stamens and style [35, 48]. Flowering occurs before fruiting that begins from May – June. Fruits are globular in shape (Table 2.3). Punekar et al., [89] studied these little-known taxa for their palynological, taxonomic, and distribution points of view. As per their report *C. brachynema* and *C. woodrowii* were restricted to the Mahabaleshwar Western ghats of India. (17° 56.270" N,73° 41.488" E). Pollen grains of both plant species were studied for their comparison.

Table 2.2 Classification of *Crinum brachynema*

Family	Amaryllidaceae (Amaryllis family)
Species	*Crinum brachynema* Herb.
Genus	*Crinum* Family – Amaryllidaceae
Order	Asparagales
Class	Monocotyledonae
Phylum	Anthophyta
Kingdom	Viridiplantae

Table 2.3 Comparative studies on Morphological description of *C. brachynema* and *C. woodrowii.*

Comparison		***Crinum brachynema***	***Crinum woodrowii***	**References**
Morphological description	Plant height	30-60cm high		[46,88]
	Bulb	5-8cm across	8.6-16.2 cm	

	Leaves	Developing after flower, Linear-oblong, entire margin, moderately firm, obtuse apex. Leaves are bright to dark green.	Contemporary with flowers, 18-17 cm, leaves are flat and bright green	
	Spathe valves	3-5cm long, lanceolate	8.7-10 x 2.7-3.9cm, deltoid and obtuse	
	Scapes	30-60cm long,sub-terete and stout.	53.5 -82.5 x 1-3 cm, purple in middle, green at base & apex	
	Lobes	5cm x 2cm, Lanceolate to oblong, cuspidate, obtuse	1.2 -1.5cm long	
	Style	Shorter than stamen	Terete, overtop the stamens	
	Flowers	5-20 umbel	10-20 umbel	
	Fruit	Sub- globose	3-7cm across, Irregular in shape	
Geographical distribution		Mahabaleshwar, Satara district, Maharashtra state, India.	Mahabaleshwar, Satara district, Maharashtra state, India	[46]

		At an elevation of ca.1275m on a lateritic plateau at the semi-evergreen forest and the hill slopes. (17°56.270" N,73°41.488) And Dharmapur forest, Gujarat, India, at an elevation of 700m (20°36'N,73°20E)	At an elevation of ca.1275m on hill slopes and a valley of semi – evergreen forest. (17°56.270" N,73°41.488" E) Bhimashankar wild life Sanctuary, district Pune, Maharashtra, India	
Flowering period		May and June	May and June	[88]
Fruiting period		June onwards	July onwards	[88]
Palynology	Pollen grains	Mono-aperturate pollen grain, ovoid	Mono- aperturate pollen grains, depressed globose	[88]
	Size	50 x 55 um	58.82 x 64.70 um	
	Exine	Bulbous excrescences, obtuse at apex, the base of echinate ca. 1.25 um across	Heteromorphic with echinulate excrescences, the base of echinate ca. 1.25 um across	
	Inter excrescence	Micro verrucae and almost areolate	Micro verrucae distant	

Threats		Commercially used for pharmaceutical purposes, Aesthetic and Medicinal purposes, use of flowers for cosmetic purposes and consumption by local habitants for health care, and commercially used for ornamental purposes.	Bulbs are used in cosmetic industries and for pharmaceutical purposes, Medicinal uses and Aesthetic purposes, Folk care medicine, selling for the treatment of respiratory diseases, and Consumption of raw buds for physical strength.	[46,88,]
IUCN status		Critically endangered	Critically endangered	[46,88]

2.3 Pharmacological importance of *Crinum* species

Crinum species have traditionally been used as anti-malarial, tonics, antipyretics, anti-aging, anti-tumor, laxatives, and anti-asthmatic agents [16,20,21,22, 23,24,25,39,89]. Furthermore, they are used to treat conditions including headaches, backache, swelling, hemorrhoids, wounds, and rheumatism [25,26,89]. Due to their similar alkaloid constituents, a few *Crinum* species are used for the same medicinal purposes in different countries. Indian *Crinum* species are well known for their elegant and beautiful flowers (for example, *C. asiaticum* and *C. latifolium*) which are generally grown with white and beautiful flowers. Because of the presence of pharmacologically active alkaloids, Indian *Crinum* species also have diverse clinical properties such as antimalarial, antimicrobial, anti-diabetic, antiviral, anti-inflammatory, anti-tumor, and immunostimulating effects [16,22,26,52,90,91]. In terms of

the use of *Crinum* species in traditional medicine, India ranks second and South Africa is at second rank. Both the bulbs and leaves of *C. asiaticum* are used in traditional medicine [16,20]. Roasted bulbs are used to treat rheumatism as a rubefacient, and also to treat hemorrhoids (Watt and Breyer-Brandwijk, 1962). *C. defixum* Ker Gawl. used to cure inflammatory conditions [42]. *C. pretense* Herb is used in Indian medicine to treat intestinal diseases such as diarrhea and dysentery [41] and its bulb extract is effective to cure chest pain [41,23]. Leaves of *C. latifolium* are used in traditional systems such as Ayurveda to treat allergies, fever, piles, skin diseases, and earaches. And the fresh juice of leaves is used to treat joint pain and vomiting [79]. The paste of *C. viviparum* bulb is used to treat skin allergies, herpes, and burns [16,43,83]. In Madhya Pradesh, tribal communities use the *C. viviparum* bulb as a paste mixed with turmeric powder to treat swelling and bruises [44]. The bulbs of *C. woodrowii* are used to treat urinary discharge, bronchitis, biliousness, vomiting, night blindness, and gonorrhea (Table 2.4) [45,46,4]. According to several researchers, the presence of pharmacologically active alkaloids in various Indian *Crinum* species is expected to gain the interest of pharmaceutical industries, which are under pressure to identify more bio active constituents to fulfill the consumer's demands.

Table 2.4. Ethno-medicinal properties of Indian *Crinum* species

Species	**IUCN Status**	**Ethnomedical uses**	**Reference**
C. brachynema	Critically endangered	-	[48,32]
C. malabaricum	Critically endangered	-	[34]
C. woodrowii	Critically endangered	To treat iliousness, vomiting, bronchitis, urinary discharge, Gonorrhoea, and night blindness.	[45,46,47]
C. latifolium	Not listed	Treatment of allergic disorders, fever, earache, piles, and skin diseases. Also to cure vomiting and joint pain.	[79]
C. asiaticum	Not listed	Effective for headache, laxative, purgative, diuretic,	[21,32,80]

		skin infection, and cough	
C. eleonorae	Endemic and rare	-	[34]
C . reddyi	Not listed	-	[31]
C. viviparum	Least concern	To treat skin allergy ad boils, also for Herpes.	[22,43,82]
C.solapurense	Endemic	-	[22,83]
C. pretense Herb.	Least Concern (LC)	Intestinal disease (Diarrhea and dysentery)	[20]
C. defixum	-	To treat painful infection of fingers	[20]

2.4 Biotechnological approaches for *In-vitro* cultivation

Plants are propagated through seed or vegetative plant parts. Plant sexual propagation uses seeds, whereas vegetative propagation uses vegetative plant parts. Plants multiply like their parents in both modes of propagation. These two methods are used in traditional plant propagation. However, these techniques are fraught with difficulties due to the slow rate of vegetative propagation and the short viability of fleshy seeds [20]. Because trade is the primary source of income especially for the poverty-ridden people, the enforcement of laws has not stopped indiscriminate plant harvesting. Attempts to overcome this obstacle using conventional methods of development have so far failed. The procedure also requires little in the way of resources or time, and it yields good results. Afolayan & Adebola [92] note that *In-vitro* plant culture may be utilized to augment natural plant resources for wild populations and to create a market for herbal medications.

2.4.1 Source of explants

Tissue culture techniques may grow an entire plant or several plant components from a single explant. Larger-sized explants may be better suited to culture, whereas smaller explants are more challenging to culture in media as they need more nutrients [93]. Tissue response can be affected by the explant's genotype, age, source and size, and physiological stage. The leaf is the most widely utilized explant, although almost any other plant element (nodes, internodes, seeds, root, stem, or leaf) may be employed [94]. Despite the difficulties of sterilizing, twin scales explants are mostly utilized for propagating most Amaryllidaceous species [20]. In one

study [95], *C. moorei* was successfully micro-propagated from early floral shoots and twin-scale explants, leading to the synthesis of secondary metabolites. Cultivating shoot explants on a modified MS medium result in the best adventitious shoot formation in *C. malabaricum* (Table 2.5) [27]. It has been observed that flower buds and bulb scales may be used to induce callus in *C. asiaticum*, and these explants are a great source for *In-vitro* research [96].

2.4.2 Culture condition

Tissues from plants need to be cultivated on the right medium with the right pH, humidity, temperature, and light levels [97] before they can be used for further multiplication or propagation. Temperature, have a significant impact on *Crinum spp*. in-vitro culture techniques. In *C. moorei*, and *C. macowanii*, temperatures as low as 15 ℃ were found to inhibit bulblet formation, but temperatures as high as 25 ℃-30 ℃ were optimized for bulb propagation [95,98]. The optimal temperature for culture chambers was determined to be 25 ℃ with a 16-hour photoperiod, 40-50 mol m 2s-1 light intensity, and an 8-hour dark period. In *C. macowanii* cultivation, Slabbert et al. [99] reported an SPFD (spectral photon flux density) of 12.5 mol m 2s1. Few studies indicated that a pH of 6.0 [100] and 70-80% relative humidity were optimal [102,103].

2.4.3 Surface sterilization

Before *In-vitro* inoculation, surface sterilization is a crucial step in plant tissue growth. Many different techniques of sterilization were used by Ulrich et al. [103] but the most successful were: the bulblets were submerged in warm water for 1 hour, cut into tiny pieces, and then dipped in sodium hypochlorite solution and tween 20 for one hour. Then the excised sample was washed with distilled water in LAF and the tri scale was sterilized with NaOCL 0.263%, with Tween 20 i.e 0.1% and cultures were incubated at appropriate conditions. To identify and isolate endophytic bacteria from *C. macowanii*, the bulblets were surface sterilized with Tween 80 and agitated for about 10 minutes by Rebotiloe et al. [104]. The bulbs were disinfected using alternative chemical treatments by Priyadarshini et al. (2020c). After soaking the bulbs in 0.1% HgCl2 for 60 minutes, they were first soaked in tap water for 1 minute, then in 70% ethanol (v/v) for 1 minute, then in sterile distilled water for 5 minutes. At last, the bulblets were soaked in sterile water for 3 intervals to ensure their sanitary condition. Ulrich [105] looked examined several techniques of sterilization and found that soaking the bulblets in warm water for 60 minutes before slicing was the most effective. After shaking for an hour in a flask containing 2mg/L surfactant with bleach, the chips were

rinsed and dried. After the sterilization, the explants were prepared as triscales. After incubation in a culture medium, they were rinsed once with sterile water, and incubated in bleach with 2 mg/L surfactant solution for five minutes.

2.4.4 Basal medium and supplements

Carbon, minerals, vitamins, plant growth regulators, various organic supplements, and agar; should all be present in media for growth of compounds from the plants [106]. Several different media are utilized in plant tissue culture [107,108,109,110]. Out of these mentioned mediums, MS media is used world-wide and was modified to 12 MS media (1/2 strength of MS media as needed in experimental studies). For *Crinum* spp., MS media with full strength is considered to be most appropriate [95,101,102,111]. Nevertheless, several supplements were often added to improvise the experiment. As a carbon source, such supplements may contain any organic substance, PGRs, carbohydrate, or any amino acid as a nitrogen source. Varied quantities of nitrogen, carbon, and PGRs were reported in the *in-vitro* cultivation of various *Crinum* species. Ranging levels of sucrose have been reported in various studies [95,111]. Inositol at 200 mg/L was used by Lien and Thrash [112] to induce callus in C. latifolium under different PGRs [98,103,111,113]. Nitrogen supplements, such as mT (*meta-topolin*) at the varying level for the production of shootlets and production of bulblets and L-arginine, adenine sulfate at 25 mg/L for induction of roots in *C. malabaricum* [111], are added to the medium to promote plant growth.

Several other supplements have been reviewed to improvise the medium for the production of plant organs in *Crinium* spp. such as activated charcoal, phytagel casein, citric acid, ascorbic acid, sodium alginate, and calcium chloride [95,111,112].

2.4.5 Callus induction

Callus formation in *C. americanum* occurred at cut made on explants revealing interior structures, whereas the callus didn't form from the other parts. Induction of callus began after 10 days of inoculation within 30 days of incubation, and good results were recorded on with 10 mg/L of 6-benzyl amino purine and 2.5-mg/L of 2,4-D. The callus cells were dense, opaque, and extended [114]. In the same species, potential callus induction has been observed in MS media with 2,4-D (2.26M) [115]. Bulbs, seeds, and roots of *C. americanum* were used as explants in an experiment, inoculated on ranging concentration of growth regulators [113]. In matured *C. lattifolium*, leaf and stem were utilized to induce callus by inoculating on media containing various conc. of cytokinin and auxin. The successful combinations were

casein-kinetin-inositol (400 mg/L, 1 mg/L, and 200 mg/L). Lien and Thanh, [112] successfully induced callus on MSQ2 media containing TSC1 medium having 0.2 mg/L NAA, 1 mg/L BAP, and 6 mg/L kinetin. In the case of *C. asiaticum*, calli were induced from scales of bulb and buds of the flower, with 2,4-D. A high volume of 2,4-D was effective in inducing callus from bulb scales and a lower concentration was better in the case of flower buds. Additionally, bulb scales produced soft, yellowish, and red pigmented callus whereas flower buds induced pale yellow and slow growing callus with no pigmentation [116].

2.4.6 Shoot organogenesis

BAP at 2.0 mg/L with MS-media to stimulate the meristematic area found between the scales of bulbs (Fig. 2.5). The concentration of 2.0 mg/L (BAP) was standardized for expansion of shoot among the various plant growth regulator concentrations tested. The sub-culture of these shootlets on mediums with the same concentration hastened the rate of shoot multiplication [101]. A study surveyed on *C. malabaricum* yielded the highest yield of 5.6 shoots from somatic embryos and 12.8 shoots per synthetic seed on MS-media having 2.0mg/L BAP, 0.5mg/L indole acetic acid, and additives [111]. The highest number of adventitious shoots were obtained from shoot explants grown on MS media containing several conc. of 0.5 M, 2.5 M, 5.0 M, 7.5 M, and 10.0 M *meta*-topolin [27]. A shoot regeneration study was conducted on *C. moorei* by using a modified MS-media. Shoots sprouted near the twin scales of the basal plate. On MS medium, sucrose (4g/L) resulted in the greatest shoot regeneration under dark conditions. External hormones in high concentrations caused abnormal organogenesis, so they were not required. Adding 10 mg/L BA resulted in the development of multiple shoots [95]. In a 4 3 factorial experiment, a combination of BA and NAA were used ranging from 0mM, 4.4mM, 8.9mM, and 22.2mM BA with 0, 2.7, and 5.3 (mM) NAA in a CRD (completely randomized design). Shoot numbers differed significantly between treatments. The highest frequency of shootlets was observed in BA (22.2 mM) with the combination of NAA [103].

2.4.7 Root organogenesis

Organogenesis of Root is a successful technique for producing newly regenerated roots from *In-vitro* plants in a nutrient-rich media, from which a whole plant can be propagated. Therefore, it can be used to mass-produce any plant. Root induction in tissue culture is dependent on the right phytohormones in the right amounts and concentrations, age, viability,

and source of the explants [117]. To date, various studies on successful root induction on several *Crinum* spp. have been published.

Earlier studies demonstrated that bulblets of *C. malabaricum* raised in the MS media augmented by phytagel (2g/L), sucrose (30g/L), IAA (0.5mg/L), BAP (2mg/L), and multiple nutrients like adenine sulfate (25mg/L), ascorbic acid (50mg/L), Arginine, and citric acid effectively produced better rooting frequency recorded after 8-weeks [111]. In a study on this species, Priyadharshini et al. [103] discovered that 1 mg/L IBA in ½-strength M.S.-media-induced roots outperformed the bulbs. In combination with IBA M.S. media derived better rooting frequency in *C. asiaticum* [118]. Plantlet formation studies on *C. macowanii* (Bak.) revealed that *in-vitro* propagated shootlets from explants showed better rooting frequency in M.S. media with sucrose (0.17M) [99].

Table 2.5. *In-vitro* studies on Indian *Crinum* species

Species	**Invitro studies**	**Explants**	**Major observation**	**Optimum concentration**	**References**
C. americanum		Bulbs	Callus induction	MS media +2.26 µM 2,4-D	[124]
C. moorei		Twin scales	Multiple shoot formation	MS media +10mg/L BA+ 4.0mg/L sucrose	[95]
C. americanum		Young leaves	Callus formation	MS media +2,4-D 2.5mg/L +BAP 10 mg/L	[114]
C. latifolium	Somatic embryogenesis	Leaf, stem	Callus formation	MS media +casein 400mg/L+1mg/L Kinetin +200mg/L inositol	[112]
C. asiaticum		Bulb scale	Root formation	Ms media +0.5mg/L IBA	[118]
C. malabaricu	*In-vitro* regeneration	Bulbs	Highest shoot frequency	MS media +(2.0mg/L)	[111]

m				BAP + (0.5 mg/L) IAA	
C. macowanii	Plant regeneration	Floral stem	Root formation	-	[99]
C. americanum	Somatic embryogenesis	Bulbs, roots, and seeds	Callus induction	MSmedia (BAP, 2,4-D)	[113]
C. Ellen Bosanquet		Bulbs and tri scales	Shoot proliferation	MS media +22.2Mm BA	[103]
C. asiaticum	*In-vitro* propagation	Bulb-scale	Reliable shoot proliferation protocol	4.0mg/L NAA +8.0mg/L BA	[125]
C. asiaticum	Somatic embryogenesis	Bulb-scale and flower bud	Somatic embryogenesis	2.68µM NAA +4.44 µM BA	[96]
C.asiaticum	Somatic embryogenesis	Bulb-scale	Somatic embryogenesis	2.68µM NAA +4.44 µM BA	[126]
C. malabaricum	In- vitro regeneration	Bulb-scale	Shoot regeneration	2.0mg/L BA	[102,103,111]
C.asiaticum	Somatic embryogenesis	Bulb-scale	Callus induction and somatic embryogenesis	2.68µM NAA +4.44 µM BA	[116]

2.4.8 *In-vitro* bulblets formation

Secondary bulbs, also known as bulblets, grow in the axils of leaflets or at the base of a flower or inflorescence and may produce new plants once they are separated. Lily plants benefit more from bulblet creation *In-vitro* than shoot formation since the bulblets are more manageable and plantable due to their compact size and strength. Bulblet production *In-vitro*

requires plant growth regulators (gibberellins, jasmonate, and abscisic acid), as well as sucrose (carbohydrates), and regulated conditions of culture including temperature and light intensities [119].

Ascoughet al. (2008) found that different temperatures affected *In-vitro* bulblet development, with 15 °C being detrimental to *C. moorei* and *C. macowanii* while 30 °C proved to block bulblet induction in *C. moorei* but to be suitable for *C. macowanii*. Most bulblets were formed by *C. malabaricum* twin scales within 8 weeks in a media containing 2.0mg/L of meta-topolin and 2.0mg/L of BAP, according to recent research by [121]. The maximum no. of bulblets developed from explants cultured for 3 months in MS medium with IBA (1.0mg/L) [101].

C. moorei explants in MS medium produced the most bulblets [95]. After maintaining twin scales explants under dark conditions, basal MS-media BA and charcoal generated bulblets (Table 2.6) [122].

In media with BAP and charcoal (5g/L), twin-scale *C. variable* explants produced big, numerous bulblets from regenerated shoots. (Table 2.6) [123]. In modified MS media containing 0.1 and 0.1 mg/L NAA, kinetin, and 1.25 mg/L ancymidol, *C. macowanii* produced bigger bulblets from flowering-size bulbs. In 12 months, a single bulb generated 700-1000 bulblets [98].

Table -2.6 *In-vitro* bulb generation of Indian Crinium spp.

Plants	**Media**	**Plant part**	**Results**	**References**
C. moorei	MS media +2mg/L BA+ 5mg/L AC	Twin scales	Bulbets induction	[122]
C. malabaricum	MS media +1mg/L IBA	Twin scales	Maximum bulblets formation	[101]
C. macowanii	-	Twin scales	Promote bulblets formation	[120]
C. moorei	MS media +5mg/L AC +6% sucrose	Twin scales	Promote a maximum number of	[95]

			bulblets	
C. Ellen Bosanquet	MS media +35.5 µM BA	Shoot and tri scales	Better bulbets induction	[103]
C. macowanii	MS media + NAA + KN +ancymidol	Bulbs	Large bulbet formation	[98]
C. malabaricum	MS media (meta-topolin and BAP)	Twin scales	The high frequency of bulblets formation	[127]
C. Variable	MS media +BA + 5mg/L AC	Twin scales	Better size bulblets	[20]

2.4.9 Somatic embryogenesis

Somatic embryogenesis was the most commonly studied experiment for plant regeneration. It is a potential technique for the production of soma-clones. This method can enhance and conserve higher plant genetics. Plant regeneration experiments focused on somatic embryogenesis and root or shoot organogenesis. This is the efficient way to produce somatic embryos and, then the entire plantlet. More than that, the method can be used for genetic improvement and conservation of germplasm. Additionally, various studies including ploidy evaluation of somatic cells, secondary metabolite formation and hybridization, and many others have been completed successfully [128]. Tri-scale explants from *C. malabaricum* produced 98.4% embryogenesis with 2,4-D, citric acid, L-arginine, adenine sulfate, and ascorbic acid (50 mg/L), produce synthetic seed with media containing 2% sodium alginate and 100 mM calcium chloride. They were also inoculated on IBA, which explants produce somatic embryos at the highest of 63.7% [111]. In addition to BAP, 2,4-dichlorophenoxyacetic acid was required for the production of somatic embryos from the callus of flower buds in *C. asiaticum* [126]. In *C. moorei*, twin scales showed better somatic embryogenesis with medium supplemented with BAP in combination with 2,4-D [95].

2.4.10 Hardening of plants

Many micro-propagated plants do not survive when moved from *in- vivo* environments in the field or greenhouses. Micro-propagated plants are delicate and may be damaged by changes

in humidity, light, and air quality. Therefore, most plants need acclimatization to live and quickly grow *In-vitro* [50]. Acclimatization is the process through which an organism adjusts to its new surroundings. The micropropagation environment and field conditions are vastly different. Plantlets under laboratory conditions were given varying environment conditions that caused little stress while additionally offering optimal growth conditions. That's why acclimatization is such a crucial part of plant tissue culture [129]. In various experiments, various hardening and acclimatization techniques were used. Manokari et al. [130] used microscopy to study the *In-vitro-to-in vivo* microstructural adaptation of *C. malabaricum* plantlets. Priyadharshini et al. [101] acclimatized plantlets under conditions of a greenhouse, and as the plants grew, the cups that held them were punctured to help with gas circulation and help the plant adapt to the outside surroundings. A month after, transferred to polybags for 4-weeks, then to pots including soilrite®, garden soil, and cocopeat in a 1:1:1 (w/w) ratio and watered on a daily-basis for a month for the hardening of plants. Saker et al. [125] studied *Hippaestrum vittatium*, *Crinum asiaticum*, *Hemerocallis aurantiaca L* and *Polianthus tuberosa*. The rooted plantlets were moved to pots and sealed in plastic bags for 14-days in a controlled growth condition at 25°C with 8 and 16 hours of dark and light. At the hardening stage, the rate of survival was around 95%, the surviving ones were transferred to greenhouse conditions. In an experiment, Ulrich et al. [103] noted bulblets were moved to a glasshouse and grown under intermittent mist for a month before being moved to a sheltered region for two weeks before exposing it to higher light conditions. Temperatures in the greenhouse ranged from 23 to 32°C, with relative humidity ranging from 65% to 98%. The fertilizer included soluble salt, phosphorus, and potassium, and the plant survival rate was reported at 100% after being watered and fertilized once a week.

2.5 Phytochemistry

Medicinal plants are primarily used as herbal medicine and play an important role in ethnomedicine, health care, and the production of appropriate drugs. Because of this increasing demand for various medicinal species can across national and international borders. The Amaryllidaceae family has a rich history and is composed of various alkaloids with medical properties benefiting humans worldwide [131-139]. All of these were *In-vitro* assays, indicating the need for *in vivo* assays to verify and supplement the existing evidence. *Crinum* species have been used for medicinal purposes, owing to their pharmacologically active alkaloids [50,52].

Because Plants belonging to the genus *Crinum* offer a broad variety of pharmacological effects because of the existence of several bio-active chemicals. Amaryllidaceae alkaloids are isoquinoline-type alkaloids with distinct structural properties. Tazettine, lycorine, galanthamine, crinine, arciclasine, hemanthamine, homolycorine, norbelladine, and montanine are only some of the alkaloids that may be broken down into these nine categories (Fig. 2.2) [22]. Because this species contains all of these phytochemicals, it is often considered a genuine example of the Amaryllidaceae family. Aside from these compounds, this plant contains a variety of alkaloids that do not belong to the family, including augustamine, sceletium, clivimine, phenanthridine, ismine, and –carboline (Table 2.7) [140]. The bulbs of *Crinum* spp. have high concentrations of crinine and lycorine, while the roots contain high concentrations of phenolic substances such as gallic acid and coumaric acid [35].

The bulbs of *C. ornatum* and *C. bambusetum* contain higher concentrations of chemical components (saponins and flavonoids) [50,52], crinamine, crinafolidine, crinamidine, and crinafoline have been identified in *Crinium latifolium* leaves. Additionally, several saponins, phytosterols, lipids, proteins, amino acids, and other compounds have been discovered (Table 2.7) [141]. Galanthamine, a tertiary isoquinoline alkaloid, is commonly used to treat Alzheimer's dementia [49]. It is found predominantly in *Crinium malabaricum* and is first found in bulbs of *Crinium malabaricum* before being concentrated in *C. viviparum* [142,143]. Cripowelline A and B, two galanthamine-type insecticidal alkaloids bound to methylated glucose, were isolated from a *C. powellii x hybrid*. Many species of *Crinum* contain the alkaloid lycorine-1-O-D-glucoside in their roots [90]. Terpenoids and sterols found in C. asiaticum var. japonicum bulbs include cyclolaudenol, etc In addition to alkaloids, *Crinum* spp. contains resins and polysaccharides. Carbohydrates found in C. annabile include pectins (8.7%), hemicelluloses, and water-soluble saccharides. Tram, 2002. Tyramine, apocynin, and acetophenone derivatives including 3-hydroxy-4-methoxyacetophenone were found during phytochemical screening of *C. macowanii* plant components [144].

Biologically active alkaloids such as Lycorine (LY) have anti-tumor, cytotoxicity, and plant growth inhibitory properties [50,145,146] whereas Galanthamine (GAL) has anticholinesterase properties that are used to treat Alzheimer’s disease [22,50,53]. Few reports have observed wide-benefits of GAL for Alzheimer's patients, and a large clinical trial has also reported positive results [49,50,147,148,149,150,151]. Rahman and colleagues [151] demonstrated that the high content of alkaloids in the bulb of *C. asiaticum* contributes

to antibacterial activity. Previous research has shown that *C. asiaticum* chloroform extracts have antinociceptive properties due to bioactive compounds [151]. Indradevi et al., [152] discovered that *C. asiaticum* leaf ethanolic extracts had anti-hepatotoxic effects in alloxan-induced diabetic rats. Furthermore, it demonstrated significant antimicrobial activity against various microbes [153]. Ghane et al., [22] used RP-HPLC (Reverse Phase-High Performace Liquid Chromatography) to extract GAL and LY alkaloids from the leaves of various Indian *Crinum* species (Table 2.9). They also looked into the biological properties of different Indian *Crinum* species such as antioxidant potential, anti-diabetic, and acetylcholinesterase inhibitory. Among all the species tested, *C.asiaticum* leaf extracts demonstrated the highest anti-oxidant activity and they also discovered *C. latifolium* and *C. solapurense* were the most abundant sources of GAL and LY, respectively [22].

The pharmaceutical industries are also concerned about the safety of plant-derived drugs. Plants are medically beneficial but still need ongoing toxicity and safety evaluations remain critical from a scientific point of view. Several studies have found that medicinal plants can be mutagenic and cytotoxic over time [50,52]. However, some studies include the potential cytotoxic risk associated with the long-term use of medicinal plants [128]. To distinguish between mutagenic/ toxic effects and pharmacological efficacies, a toxicity test must be performed while screening medicinal plants

As a result, evidence of the toxic/mutagenic effects of crude plant extracts and their chemical constituents is growing [154]. Even though Indian *Crinum* species have significant medicinal value, there is a significant gap in scientific knowledge about their toxicity and safety assessment. According to previous research, the Indian *Crinum* species are highly toxic and should be [25,155,156,157]. Methanol bulb extracts of *C. asiaticum* had an LC50 value of 94.06 mg/ ml1 in the Brine-Shrimp lethality test when compared to ascorbic acid (standard antioxidant). Furthermore, the methanolic leaf extracts of *C. latifolium* demonstrated mild cytotoxicity (LC50 value 15.652 mg ml1) [156]. However, unlike *C. latifolium*, the extract has no toxicity in cancer cells (lymphoma EL4-luc2) [158, 159]. However, the toxicity and mutagenic effects of various Indian *Crinum* species are unidentified. Furthermore, more effort is needed to test the toxicity and mutagenicity of Indian *Crinum* species. Furthermore, mechanism-based clinical tests are still required for the confirmation of the widespread use of Indian *Crinum*s in traditional medical systems. Although medicinal plant species have several advantages, toxicity, and safety must be better understood to distinguish between toxic/mutagenic effects and pharmaceutical relevance [27,160].

Table.2.7 Phytochemical compounds from *Crinum* species and biological activities

Plant species	Plant part	Alkaloids	Technique	Reference
Crinum latifolium	Leaves, stems, Bulbs	Galanthamine, Lycorine, Belladine, Latisoline, Cherylline, Latifine, Latisodine, Hippadine, Hippeastrine, Oxoassoanine	Anticancer, Antibacterial, Anti-inflammatory	[22,161,162]
Crinum brachynema	Bulbs, Leaves	Galic Acid, Salicylic Acid, Coumaric Acid Catechin, Galanthamine	Neuroprotective, Hepatoprotective, and Anti-cancer	[22]
Crinum asiaticum	Bulbs, Fleshy leaves, Fruits	Lycoriside, Palmilycorine, Criasbetaine, Ungeremine, Crinamine, Hamayne, Norgalanthamine, Platorinine, Hippadine, Kalbretorine, Lycoriside, Criasbetaine	Anti-tumor, Analgesic, Anti-oxidant, Anti-microbial, Anti-inflammatory	[90,151,152,163]
Crinum woodrowii	Tuber, Bulbs	Kaempferol, Catechin, Coumaric acid, Galanthamine,	Antioxidant	[22]

		Chlorogenic acid		
Crinum viviparum	Bulbs	Salicylic acid, Catechin, Chlorogenic acid, Coumaric acid, galanthamine	Antioxidant	[22,49]
Crinum pratense	Leaves	Lycorine, Ismine, Salicylic acid, Anhydrolycorine, Pratorinine, Catechin, Coumaric acid, Hippadine, Gallic acid	Anticancer, Antibacterial	[22,131]
Crinum zeylanicum	Bulbs	Galanthidine, Amarylline, Narcissine, Bellamarine	Anti-malarial	[131]
Crinum defixum	Leaves, bulbs, corms	Galanthine, Haemanthamine, Hippestrine, Homolycorine, Crinine, Caranine, Galanthamine	Anti-inflammatory, Anti-microbial, Anti-oxidant, and Analgesic,	[42,44]

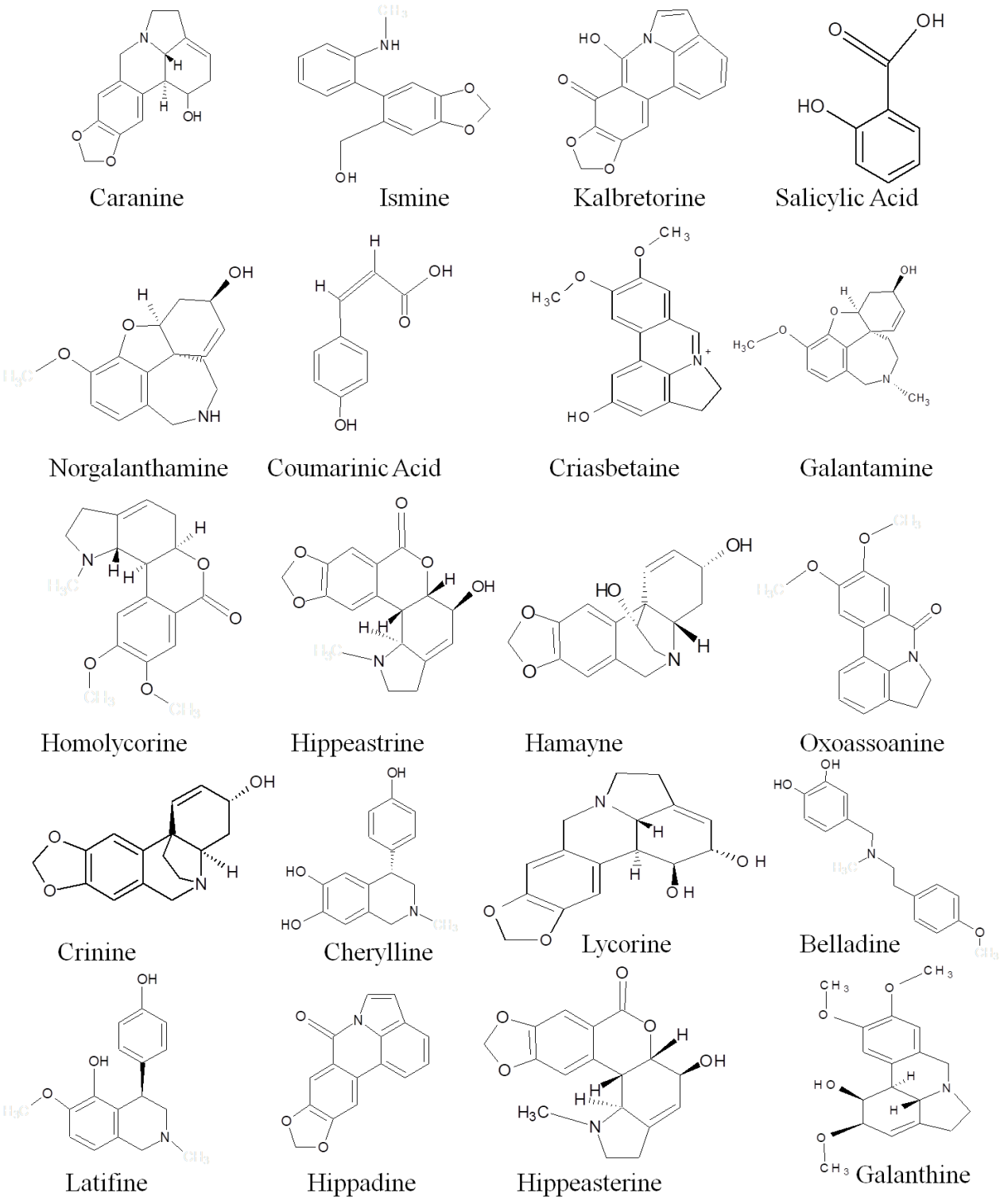


Figure 2.2 Chemical structures of important alkaloids of Indian *Crinum* species

2.6 Secondary metabolite production from *Crinum* species

Plants have significance to enhance human health and for discovering new pharmacological leads. Plant-derived biomolecules have long been recognised as a valuable source of therapeutic drugs, with significant potential for identifying and characterising new drug leads. Many different alkaloids with different medicinal properties have been isolated from Amaryllidaceae plants. Many studies have been directed toward medicinal plants due to the need for environmentally safe compounds as therapeutic agents against drug-resistant microorganisms [50,164,165,166]. One in every three people develops dementia, according to the World Alzheimer Report [167] and more than 10 million new cases are reported each year (Alzheimer Disease International 2020). Over 50 million people worldwide are estimated to have dementia in 2020, with this figure projected to increase to 152 million by 2050 [168,169]. Alzheimer's disease (AD) is characterised by mental disorientation, impairment of memory, and challenges in performing everyday activities [168,169]. Muscle function, object identification, competence to execute, ability to understand and express language, difficulty walking, and decision-making are all evidence of cognitive loss [169,170]. The main pathophysiological features are mutations in the Presenilin 1 and Presenilin 2 precursor genes, amyloid-protein (A) and tau protein accumulation, and the development of neurofibrillary tangles (Fig. 2.3) [169,170,171,172,173].

The pharmacological effects of Amaryllidaceae alkaloids have been thoroughly described in the scientific literature. These effects include antibacterial activity [50], cytotoxic activity, and antimalarial activity [50,128]. Because of their pharmacologically active principles, *Crinum*s have piqued the interest of phytochemists over the last decade. According to Refaat et al. [140], about 120 of the roughly 180 alkaloids that were extracted and identified from *Crinum*s are of the crinine and lycorine kinds. This is an important fact to keep in mind. *Crinum*s have been the subject of extensive chemical, cytological, and pharmacological research because of their high concentration of bioactive phytoconstituents.

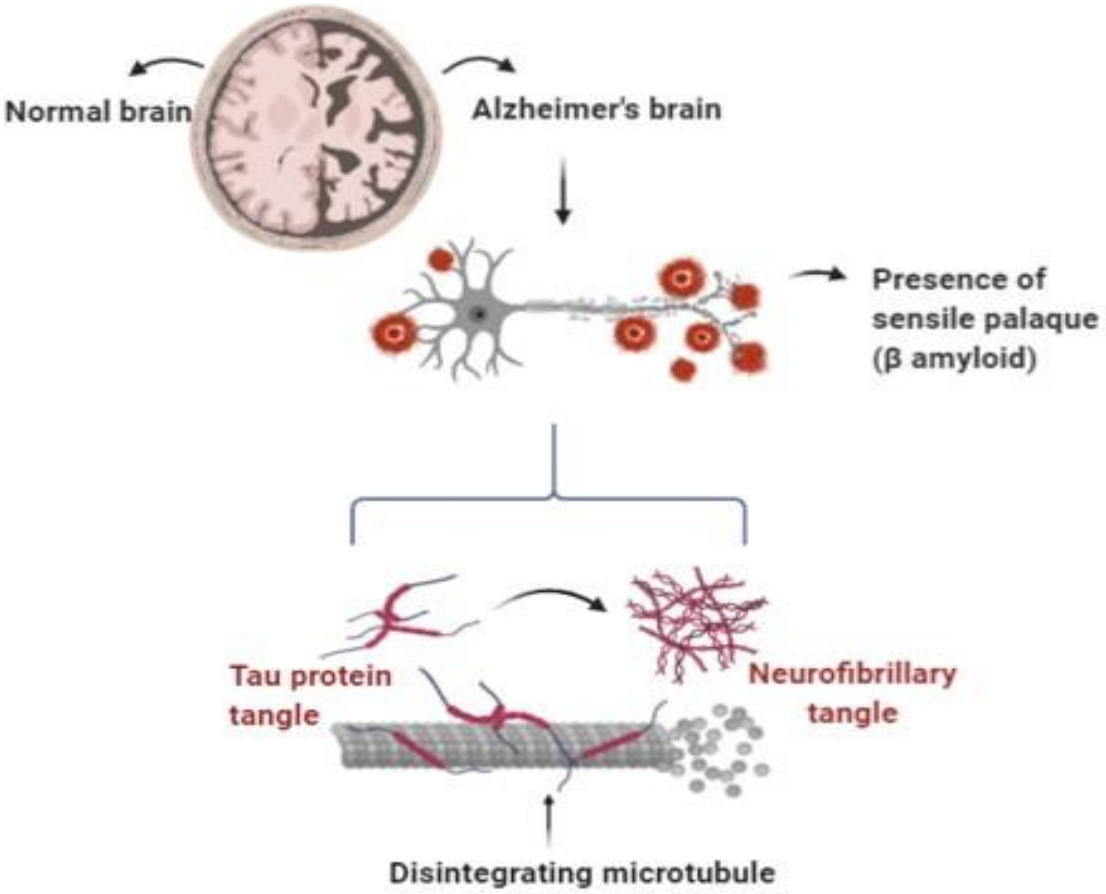


Fig. 2.3 Effect of Alzheimer's disease on the human brain

2.6.1 Galanthamine Production

GAL is an alkaloid that acts as an acetylcholinesterase (AChE) inhibitor as well as a modulator of nicotinic acetylcholine receptors (nAChRs [169,174,175]. It improves learning deficits by increasing nicotinic receptor concentration and inhibiting AChE due to its high selectivity and dual mechanism of action [176,177]. Interestingly, the GAL biological profile also demonstrates intraocular pressure reduction, respiration causation, analgesia, and weak antimalarial activity [17, 174]. GAL was first obtained from the Caucasus region's Galanthus woronowii [178]. GAL-based medicine is used in Bulgaria. Paskov,[179, 180]. Thus, plants continue to be the primary source of natural therapeutic drugs; however, screening and biosynthesis of GAL in various plant species face several challenges, as current methods for GAL isolation and purification are unsustainable and inefficient in terms of the environment. Genetic engineering could be a tool for increasing GAL levels, but it would be limited because it requires manipulating relatively few genes in the same transformation event and at

the same time in plants, and the GAL metabolic pathway hasn’t been completely described. Furthermore, chemical synthesis of GAL is expensive hence, new strategies are needed to induce the synthesis of GAL in-vitro to scale up the generation and production of GAL to cure a range of diseases.

This review summarises recent basic and applied research findings that would allow the GAL biosynthesis optimization techniques *in-vitro*, to achieve potentially efficient and sustainable scaled GAL production for the treatment of Alzheimer's disease.

2.6.2 *In-vitro* production of galanthamine

Galanthamine is an iso-quinoline alkaloid, found in *Crinum* spp. produced in higher amounts under a stress stimuli [181]. SMs help plants adapt to new or harsh environments and provide valuable pharmaceuticals. As a result, biotechnology allows *in-vitro* techniques for the generation of nutraceuticals, pharmaceuticals, and several other important components [182]. Chromatographic separation has revealed the highest content of Galanthamine of about 0.3080.004% in *C. malabaricum* bulbs [49]. In *C. asiatticum*, the highest galanthamine was found in xanthphyllum [35]. Other sources have also been discovered for the production of galanthamine and gal-derivatives from leaf cultures of *C. powellii* and *C. asiaticum*; and from bulb cultures of *C. moorei*, *etc. [128]*. Galanthamine was directly extracted through a proliferation of shootlets and bulbs from triple-scales on MSmedia enriched with IBA (for bulblet formation) and BAP (for shooting) of *C. malabaricum* [111].

In the same species, galanthamine was isolated from fresh shoots that were produced using shoot explants that had been cultivated on MS medium with 7.5 M meta-topolin [27] (Table 2.8). According to Fennell and colleagues' research from 2003, the synthesis of *Crinum* alkaloids may be boosted in charcoal-enriched MS medium, but it can decrease when BA-enriched media is used.

2.6.3 Biotechnological Production of GAL

Identification and extraction of natural compounds from their natural sources is expensive, time-consuming, and yields only small amounts, limiting future studies. The biological potential of Amaryllidaceae alkaloids has been recognized in recent decades with the commercialization of GAL, which has selective inhibitory activity as an Alzheimer's drug. GAL is well known for its pharmaceutical activities, including AChE properties [146]. GAL's commercialization as a potent drug resulted in a surge in demand from pharmaceutical

companies. To meet its demand, natural resource production has become a major challenge [139]. Long-term programs of genetic improvement and domestication of wild species, on the other hand, do not appear to be viable options. GAL levels in plants vary depending on cell or tissue type, developmental stage, and plant species [183].

As a result, the exploitation of native and wild plants is not recommended for long-term GAL production. Plant tissue culture is a non-pathway elucidation-based alternative production strategy. Against this backdrop, several approaches have been investigated, including *In-vitro* cell differentiation competence [184,185,191] medium components [174,186], light and dark conditions (photoperiod) [187,188] elicitation strategy [189,190,191]; manipulation of PGRs [184,190,191] and different bioreactor systems (Fig 2.4; Table 2.8) [192,193], significantly influenced GAL production [194,195]. Plant secondary metabolite production in large bioreactors should ensure well-defined and repetitive bioprocess parameters, paving the way for process validation and product registration.

The use of whole and photosynthetically active plants in temporary immersion bioreactor systems is another biotechnological alternative under development, this time for the productive scaling of secondary metabolites. The integrated management of these strategies during *In-vitro* cultures was shown to be successful in phenylpropanoid biosynthesis in temporary immersion bioreactors [196,197] and is now regarded as a methodological foundation for optimizing GAL production. In light of the study of the profile of phenylpropanoids and galanthamine metabolites in *Lycoris* species [198,199], we can speculate on the role of other elicitor molecules, such as ABA and H_2O_2, in increasing galanthamine yields in controlled systems such as temporary immersion bioreactors (TIBs). The use of this integrated strategy resulted in comparable phenylpropanoid yields for native maqui (*Aristotelia chilensis*) plants *In-vitro* and ex vitro [200]. On the other hand, biotic elicitation has the potential to be integrated into bioreactor systems. Inductors such as bacterial, fungal, and algal elicitors, as well as other polysaccharides derived from them, have been reported [201].

2.6.4 Relationship between GAL biosynthesis and cell differentiation competence *In-vitro*

Cell differentiation encompasses the physiological and structural development of cells to acquire particular functions within a plant [169,202]. The manipulation of several factors including nutritional elements and physical aspects of laboratory studies affects the secondary

metabolism of plant cells, tissues, and organs [2,169,203,204]. Many studies are underway globally on the isolation, identification, purification, and characterization of plant-derived biomolecules of pharmaceutical interest aiming at improving their yield. As evoked, some important plant-derived compounds are found in specific plant tissues in low amounts; therefore, it is necessary to develop biotechnology-based solutions. The same applies if the chemical synthesis approach is not possible or if the compounds of interest are produced by plant species which are difficult to grow/cultivate.

For example, secondary metabolite accumulation in callus culture is not as efficient as in bulblets and shoot cultures [191,192, 205-207,]. In *N. psuedonarcissus* it was 0.10-0.13% [187,210] and *in N. confusus* about 2.5 % DW [187,211]. Sellés and co-workers [206,208] reported that somatic embryos or regenerated sprout clusters could accumulate higher yields of GAL. A similar relationship between cell differentiation and GAL concentration has been observed by Laurain-Mattar et al. [209] in *L. aestivum*.

Crinum species are a rich source of several alkaloids with therapeutic action, including GAL (Table 2.7[16,20,26]. Recently discovered *Crinum malabaricum* Lekhak & S.R. Yadav (CR: Critically endangered), an aquatic medicinal plant species, is an additional promising source of GAL production. Efficient *In-vitro* regeneration technology has been established for this species [101,111]. In addition, inter-sample sequence repeats (ISSR) and start codon targeted (SCoT) molecular markers were used to evaluate the genetic stability of the regenerated *C. malabaricum* [101].

Several reports have shown that bulblet is a most promising tissue source for GAL production [184,187,191,207,213]. Despite the pharmaceutical interest in the Amaryllidaceae alkaloids including GAL, the corresponding biosynthetic pathway is not fully elucidated [146]. An overview of GAL production from different plant species is presented in Table 2.8.

2.6.5 The effect of medium composition on GAL biosynthesis *In-vitro*

In-vitro, biosynthesis of plant-specialized metabolites can be improved through various points such as media composition, PGRs, elicitors, sucrose concentration, photoperiod, and supplementation of precursors (Fig 2.4) [169,188,191,214,215,216,217]. Aiming to obtain high productivity and biosynthesis of secondary metabolites, a suitable nutrient medium selection and optimization of various parameters are crucial factors [218]. Mostly, the composition of culture media is optimized concerning the C/N ratio and/or other key components [219] and has been extensively addressed by multifactorial analysis to maximize

yields of the plant biomass and secondary metabolites [174,218]. The primary carbon source used *In-vitro* for the biosynthesis of GAL is sucrose [208,218]. The influence of different sucrose (30, 60, 90, 120, 150, and 180 g/L) concentrations on the GAL and biomass accumulation has been investigated in *N. confusus*, and the positive result was obtained with 180 g/L sucrose [208]. In addition, sucrose concentration also affected the photosynthetic ability and morphology of cultures, which were dark green and had 5-6 cm long shoots on media with less than 90 g/L sucrose, whereas those grown in higher sucrose concentration were pale green with a tendency to vitrification probably because sucrose at such high concentrations acted as a stressor changing the osmotic potential of the medium [208].

Georgiev et al. [218] investigated the combined effect of independent factors in the media on *In-vitro* GAL production by *L. aestivum* shoot cultures and found that an 8.6/1 ammonium/nitrate ratio produced the maximal level of GAL, whereas 6.5/1 ratio is the standard [218].On the other hand, EI Tachy et al. [220] assessed different sucrose concentrations (30, 60, 90, and 120 g/L)) in *L. aestivum* shoot cultures and reported that the highest quantity of Galanthamine (0.032-0.045% DW) was obtained with 30-60 g/L sucrose. Similarly, Schumann et al. [192] found that a 21/1 C/N ratio in the medium was optimal for GAL biosynthesis.

2.6.6 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 [221]. These elicitors could be abiotic or biotic and may include microbial cell wall extracts (chitosan, yeast extract), signaling molecules (salicylic acid, jasmonic acid, methyl jasmonate), heavy metals, inorganic salts, and UV radiation as a physical agent [221,222]. This multitasking capacity of elicitors is unique and multidimensional, hence there is increasing interest in their use as therapeutic utilization [193,223].

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 [224]. The addition of methyl jasmonate (25µM) initiates a 2-fold increase in GAL production. In addition, MeJA also stimulated GAL-accompanying alkaloids such as haemanthamine, homolycorine, N-formyl-norgalanthamine, and pretazetine [224]. However, other elicitors used in this study did not have any significant effect on GAL synthesis [225]. In line with results obtained by Colque et al. [224] the highest GAL level in *L. aestivum* shoot

cultures was found on a medium supplemented with methyl jasmonate. Ivanov et al. [189] report the jasmonic acid and methyl jasmonate on GAL biosynthesis by *L. aestivum* shoot cultures, cultivated in submerged conditions. Jasmonic acid elicitation fostered the production of the highest amount of GAL (226.9 µg/flask [189]. Different elicitors (salicylic acid: 5, 50 and 100µM, methyl jasmonate: 5, 50 and 100µM, 2-chloroethyl phosphonic acid (ethephon): 10 and 100µM, affecting GAL production, have been investigated by Ptak [193] Interestingly, in a more recent article, Ptak et al. [226] observed more accumulation of GAL that was obtained with 5 µM melatonin as an elicitor, while 10 µM melatonin increased the *L. aestivum* biomass and enhance the GAL content which is higher than that in the control. Recently, Ferdausi and co-workers [186] used an *In-vitro* elicitation approach and obtained an increase in GAL (44.41 µg/g) production in *Narcissus pseudonarcissus* callus culture cultured on media with methyl jasmonate. In addition, a 3-fold higher production of GAL (23.29 µg/g FW) with chitosan. In this context, using elicitors with *In-vitro* systems 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 confirm that elicitation could be an effective way to scale up formulation of natural biomolecules 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 for basic and applied knowledge to clarify the possible interrelationships with the signaling mechanism of phenylpropanoids, thus opening a way to implement a reproducible and cost-effective technology.

Table 2.8 Shows the effect of different plant growth regulators, elicitors, and different bioreactor systems in Galanthamine biosynthesis.

Plant Species	**Explant type**	**Bioreactor System**	**Optimum concentrations**	**Elicitors Used**	**Weight of Inoculum (g/L)**	**Maximum Yield**	**Reference**
Leucojum aestivum L. (Gravety	Callus	RITA®	25µM picloram+ 0.5µM BA	MeJa, SA, Ethephon (releases Ethylene)	-	0.8mg/g	[193]

Giant)							
L. aestivum L.	Shoot	Bubble column bioreactor	1.15mg/L NAA+ 2mg/L BAP	-	60g	6.0mg/L	[227]
L. aestivum L.	Shoot	Bubble column bioreactor	1.15mg/L NAA+ 2mg/L BAP	-	20.8g/L	1.7mg/L	[205]
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	[189]
L. aestivum L.	Shoot	Liquid-shake medium	1.15mg/L NAA+2.0mg/L BAP	-	17.8g/L	2.5mg/L	[207]
L. aestivum L.	Somatic embryos	RITA®	TDZ (varying conc.)	-	1g	0.025% D.W.	[190]
L. aestivum L.	Shoot	RITA®	1mg/L KN+ 0.03mg/L NAA	-	10g	19.416 mg	[225]
Narcissus confusus	Shoot-clumps	Liquid-shake medium	3mg/L BA	MeJa, SA, Arachidonic acid, Chitosan	100mg	3.4mg/g	[224]
N. confusus	Bulbs	Liquid-shake medium	BA and 2,4-D	-	4.25±1.3g	0.16mg	[208]
	Seeds			-	0.87±0.4g	0.182mg	
Pancratium	Shoot	Liquid-shake	1.15mg/L NAA+2.0m	-	200-300mg	3.3 mg	[228]

maritimum L.		medium	g/L BAP				

2.6.7 Light Conditions on GAL Biosynthesis

Light effects the development and initiation of cultures and the accumulation of natural compounds in *in-vitro* regenerated plants [214,215,217,221]. It regulates primary and secondary metabolites and affects the differentiated cells by altering the metabolism from heterotrophic to mixotrophic in both conventional *In-vitro* cultures and bioreactor systems [174,130]. Mainly, the accumulation and biosynthesis of bioactive compounds are triggered through photoreceptors that upregulate the signaling pathway leading to a modification in gene expression, activated by photons [217,231]. Light also stimulates the biosynthesis of alkaloid precursors by inducing the shikimate pathway enzymes, in particular GAL [232].

Pavlov et al. [207] studied the galanthamine biosynthesis of *In-vitro L. aestivum* cultures grown under dark and light conditions. The highest GAL accumulation of (15. and 17.8 g/L) was achieved within 35 days of culture under dark and light, respectively. However, the shoot growth rate was unaffected by illumination [207]. Berkov et al. [187] also found that *In-vitro L. aestivum* shoot clumps grown under illumination accumulated twice more GAL, but shoot clumps accumulated 5-times less GAL compared to the intact plants. Recently, Khonakdari and co-workers [188] reported that *Narcissus tazzeta* L. regenerated bulblets produced 40- 20 µg/g DW GAL when exposed to 16 / 8 h photoperiod and 24hrs dark respectively.

Therefore, light improve *In-vitro* photosynthesis and have a potential role in the accumulation of metabolic profiling in tissue-cultured plants. Therefore, a detailed study on photoperiod and photosynthetic photon flux (PPF) intensity is necessary to establish photo mixotrophic plant cultures producing GAL.

2.6.8 Manipulation of PGRs on GAL biosynthesis

In- vitro optimization of GAL biosynthesis and production is significantly affected by various plant growth regulators (PGRs) because of their particular link with cellular differentiation (Fig. 2.5). Role of different hormones in Galanthamine production was examined by several researchers [184,190,207]. In *L. aestivum* shoot cultures, the GAL production depends on light and liquid medium (Table 2.8) [207], as also shown by Berkov and colleagues [187].

However, the addition of silver nitrate and potassium permanganate significantly inhibited the GAL content by 0.27% - 0.8%. In another study, Yildirim et al. [234] found a higher accumulation of GAL of *L. aestivum* [235].

In *L. aestivum* shoot culture, *m*T, BAP, TDZ, Kn, and Zeatin for *In-vitro* GAL production in a temporary immersion system (RITA™) has been studied [190]. The same group also found that *In-vitro* GAL biosynthesis was significantly decreased by ethylene [233,236], although the precursor of ethylene increased GAL accumulation six-fold (2% DW) in cultures [233].

In a study with *N. pseudo narcissus* cultures, Ferdausi et al. [186] found that the maximum level of GAL (10-215 µg/g FW) was obtained in shoots and bulblets cultures. These results are in line with data obtained by Codina [212], where the highest GAL (1345 µg/g) in *N. confusus* cultures.

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

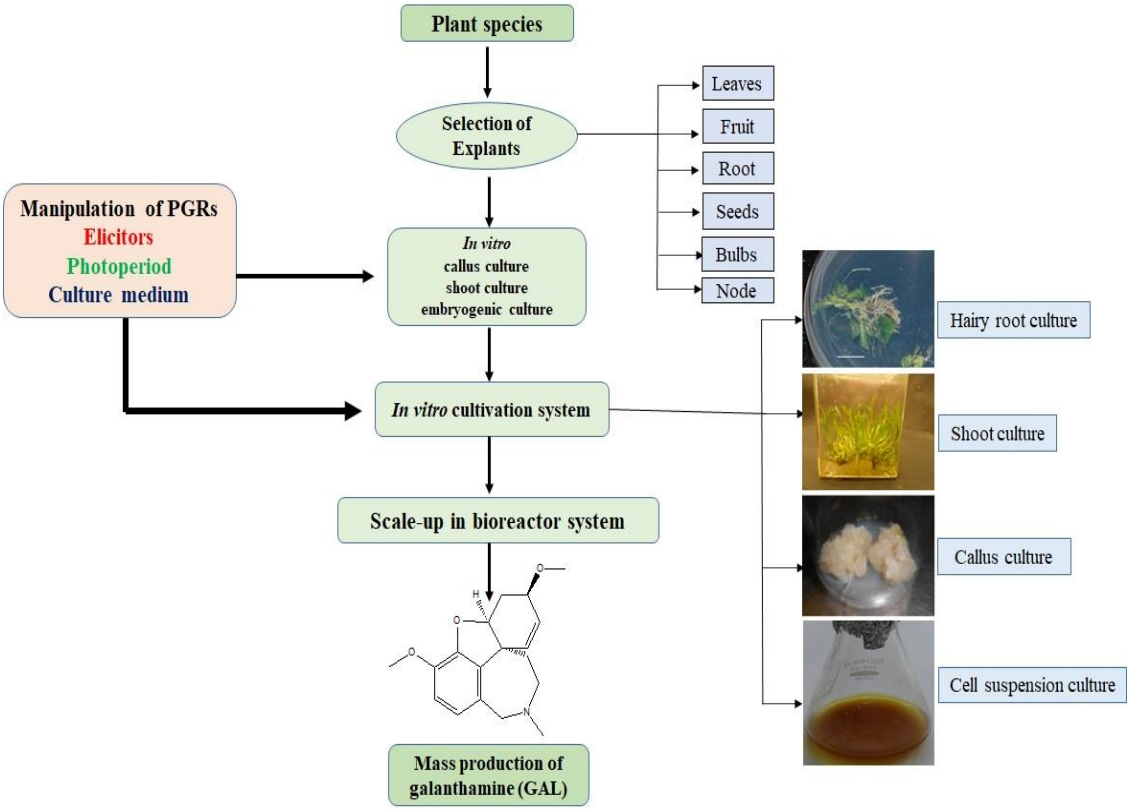


Fig. 2.4. Schematic representation for mass galanthamine production.

2.6.9 Bioreactor systems for GAL biosynthesis

Recently, plant biotechnology has increased interest plant tissue culture field and several studies were documented the scaled-up production of promising secondary metabolites [237,238,239,240]. Bioreactor-based production and scale-up have been used for bioactive compound accumulation and are the final step for the biosynthesis of plant-specialized metabolites. However, several technological and biological barriers such as cell size, cell heterogeneity, genetic stability, cell aggregation, foaming, and shear stress sensitivity are often challenging [241,242,243]. *In-vitro,* plant cell cultures require a special design of bioreactors for proper gas-exchange and nutrient transfer [196,197,243,244,245]. Notwithstanding the ever-increasing demand for producing pharmacologically active compounds including GAL, the main reason for the industrial potential of *In-vitro* technology is their cultivation on a large scale. In addition, inoculation with large workloads in the bioreactor systems is still a major concern [174]. However, temporary immersion bioreactor systems have been efficiently implemented to produce phenylpropanoid metabolites using SETIS® commercial and homemade models, reaching volumes of up to 5L capacity [196,197,200,244]. Concerning the scale-up of *In-vitro* GAL production, new biotechnology methods require for compound accumulation and to identify the most promising bioreactor system. The cultivation temperature and immersion frequency affect GAL biomass accumulation in *L. aestivum* [189]. The maximal GAL (265 µg/RITA) yield was achieved after 35 days of cultivation under 15 min flooding, 8 h of resting period, and at 26°C. These results indicate that both immersion frequency and temperature significantly influenced GAL production. Schumann et al. [192] examined the *In-vitro* GAL production of *L. aestivum* in a bubble bioreactor, air-lift column reactor, and shaking and non-shaking batch culture. The bioreactor size, culture medium, and inoculum size were optimized for GAL production. The maximum yield (19.416 mg GAL) was obtained in a temporary immersion bioreactor (Table 2.8) [192]. An advanced modified bioreactor investigation has logically led to the production of valuable alkaloids by Georgiev et al. [205]. The highest content of GAL (1.7 mg/L) and biomass (20.8 g/L DW) was obtained in a modified glass-column bioreactor when *L. aestivum* shoots were cultivated at 22°C with an incoming airflow of 18 (L/h) [192]. Despite considerable efforts undertaken to stimulate GAL production in bioreactors to date by optimizing several parameters, existing yields are still far from being industrially feasible. In future research, it is essential to breed high productive and genetically improved *L. aestivum* cell lines that can ensure a large-scale *In-vitro* technology for a high yield of GAL production. In addition, elucidation of biosynthetic pathway reactions will be obtained by

using sequencing methods correlated with bioinformatics studies and system biology analysis. This will not only help in the mass production of GAL but also support the identification and biosynthesis of other novel alkaloid derivatives.

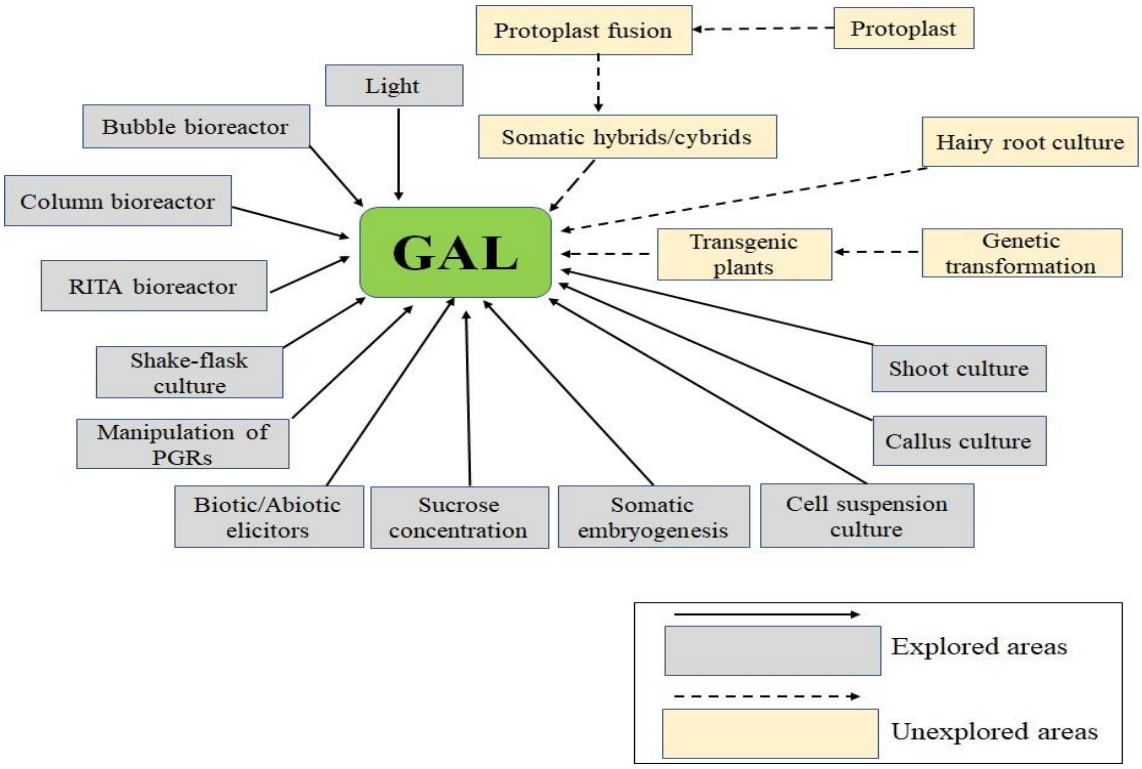


Fig 2.5 Explored and unexplored strategies for galanthamine production

2.7 Genetic diversity assessment

Genetic diversity is defined as the amount of genetic dissimilarity among individuals in a population or species, the genotype that produces new genotypes, and adaptability to changing ecological environments [50,52]. The molecular, physiological, morphological, biochemical, and cytogenetic traits all demonstrate this changeability between genotypes [128]. As a result, broad germplasm characterization includes morphological depiction as well as the determination of biochemical and molecular markers for genetic estimation. Over the previous two decades, molecular markers have created a new generation of markers, changing biological sciences. Molecular markers are used to determine genetic diversity, create genetic linkage maps, analyze genetic connections, and identify particular genes [247,248]. Because of molecular markers, assessing plant genetic diversity has become more reliable and simpler. Molecular marker techniques have the advantage of being highly polymorphic for each locus and not being affected by environmental influences, unlike

morphological and biochemical marker approaches. Molecular markers are costly, yet they reliably separate highly similar genotypes. As versatile tools, DNA-based molecular markers find their place in many fields such as genetic engineering, physiology, taxonomy, embryology, and so on [249,250].

The clonal fidelity studies help in the assessment and prediction of the genetic trueness between the mother plants and regenerated clones [251,252]. SCoT markers are an advanced type of more reliable and reproducible molecular markers [253] that can anneal specifically to the target region of DNA with ATG sequence which is considered the starting codon region of the translation process [254,255,256]. ISSR and RAPD are also considered advanced marker types [257,258,259,260,261]. Moreover, it is advantageous to DNA-based technique to confirm genetic fidelity of *In-vitro*-raised plantlets [259,262].

2.8 Analytical techniques for quantification and extraction

The determination of secondary metabolites is the essential way in medicinal plants, and the two main steps in sample preparation are extraction and quantification.

The extraction of bioactive compounds is the primary stage for separation, purification, isolation, and recovery. The plant material can be extracted in a variety of ways. In any extraction method, the extraction parameters (particle size, time, solvent composition, solid: solvent ratio, pH, temperature, and extraction solvent) are the primary parameters that define method efficacy and final product properties. Extraction solvents, sample pre-treatments, and extraction methods were a few significant steps during the extraction procedures, and they are important in their application and activity.

Table. 2.9 Extraction technique for secondary metabolites from *Crinum* species

Species	Explants	Technique	Compounds	Reference
C. latifolium	Bulbs, Leaves, Flower, Stems	RP-HPLC	Galanthamine, Lycorine, Belladine, Latisoline, Cherylline, Latifine, Latisodine, Hippadine,	[22,161,162]

			Hippeastrine, Oxoassoanine	
C. asiaticum	Fruits, leaves, Bulbs	IR, NMR	Lycoriside, Palmilycorine, Criasbetaine, Ungeremine, Crinamine, Hamayne, Norgalanthamine, Platorinine, Hippadine, Kalbretorine, Lycoriside, Criasbetaine	[90,246]
C. brachynema	Bulbs, leaves	RP-HPLC	Galic Acid, Salicylic Acid, Coumaric Acid Catechin, Galanthamine	[22]
C. asiaticum	Bulbs, leaves, fruits	NMR, HPLC, TLC		[151,152,163]
C. woodrowii	Bulbs, Tuber	HPTLC, Silica gel chromatography	Kaempferol, Catechin, Coumaric acid, Galanthamine, Chlorogenic acid	[22]
C. pratense	Leaves	RP-HPLC, NMR	Lycorine, Ismine, Salicylic acid, Anhydrolycorine, Pratorinine, Catechin, Coumaric acid, Hippadine, Gallic	[22,131]

			acid	
C. viviparum	Bulbs	RP-HPLC	Salicylic acid, Catechin, Chlorogenic acid, Coumaric acid, galanthamine	[22,24]
C. defixum	Corm, bulbs, leaves,	HPLC	Galanthine, Haemanthamine, Hippestrine, Homolycorine, Crinine, Caranine, Galanthamine	[42,44]
c. zeylanicum	Bulbs	-	Galanthidine, Amarylline, Narcissine, Bellamarine	[131]

Chapter 3

Material and methods

3.1 Collection of *Crinum brachynema* Development of regeneration protocol for *Crinum brachynema* through *In-vitro* techniques

3.1.1 Chemicals

MS Media with Sucrose and vitamins were from Hi-media®, agar powder, mercury chloride, Tween 20, Bavistin and, mT *(meta*-topolin), BA (6-benzyl adenine), IAA (Indole-3-acetic acid), (1-naphthaleneacetic acid) NAA, IBA (Indole-3-butyric acid). All chemicals used were of analytical grade.

3.1.2 Plant source, growth conditions and initiation.

Field-grown healthy plant material for this study was collected from its original and only location in the Mahabaleshwar region, Satara district, Maharashtra, India (17°56.270' N,73°41.488'E) (Fig 3.1). The collected plant material was maintained at the medicinal plant garden of the institute with standard practices. The maintained plants were regularly monitored and kept weed-free.

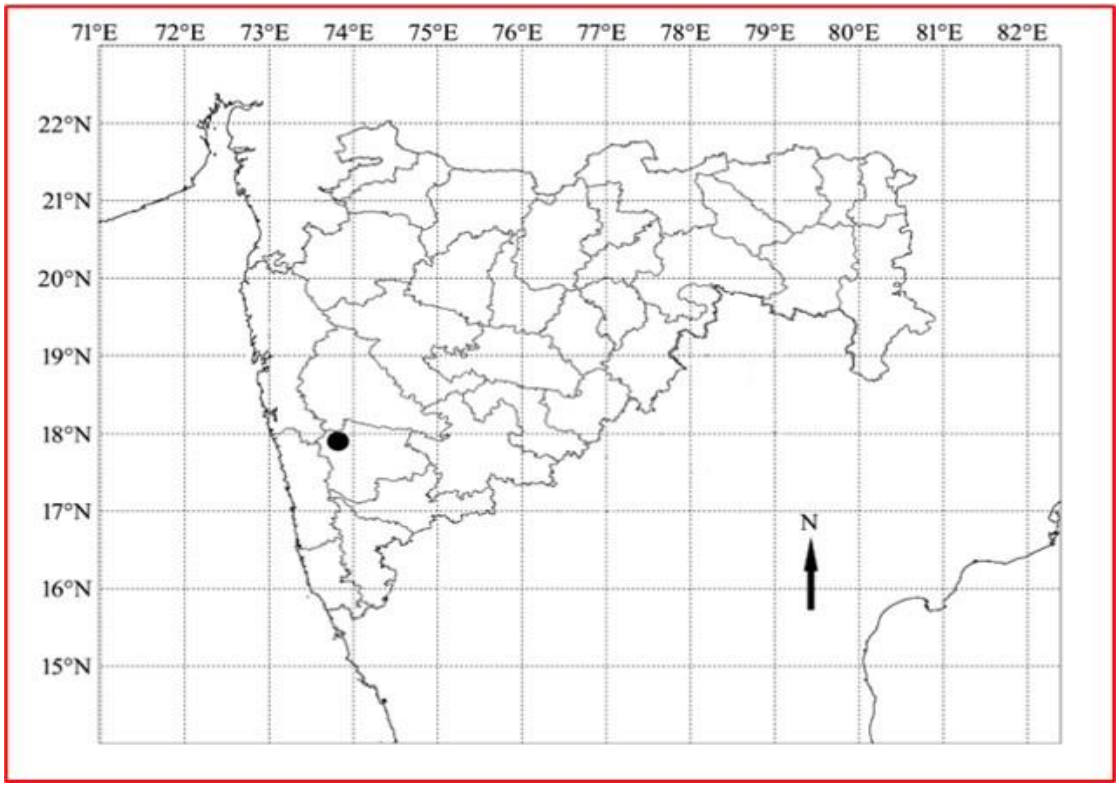


Fig 3.1. Collection site of *Crinum brachynema*.

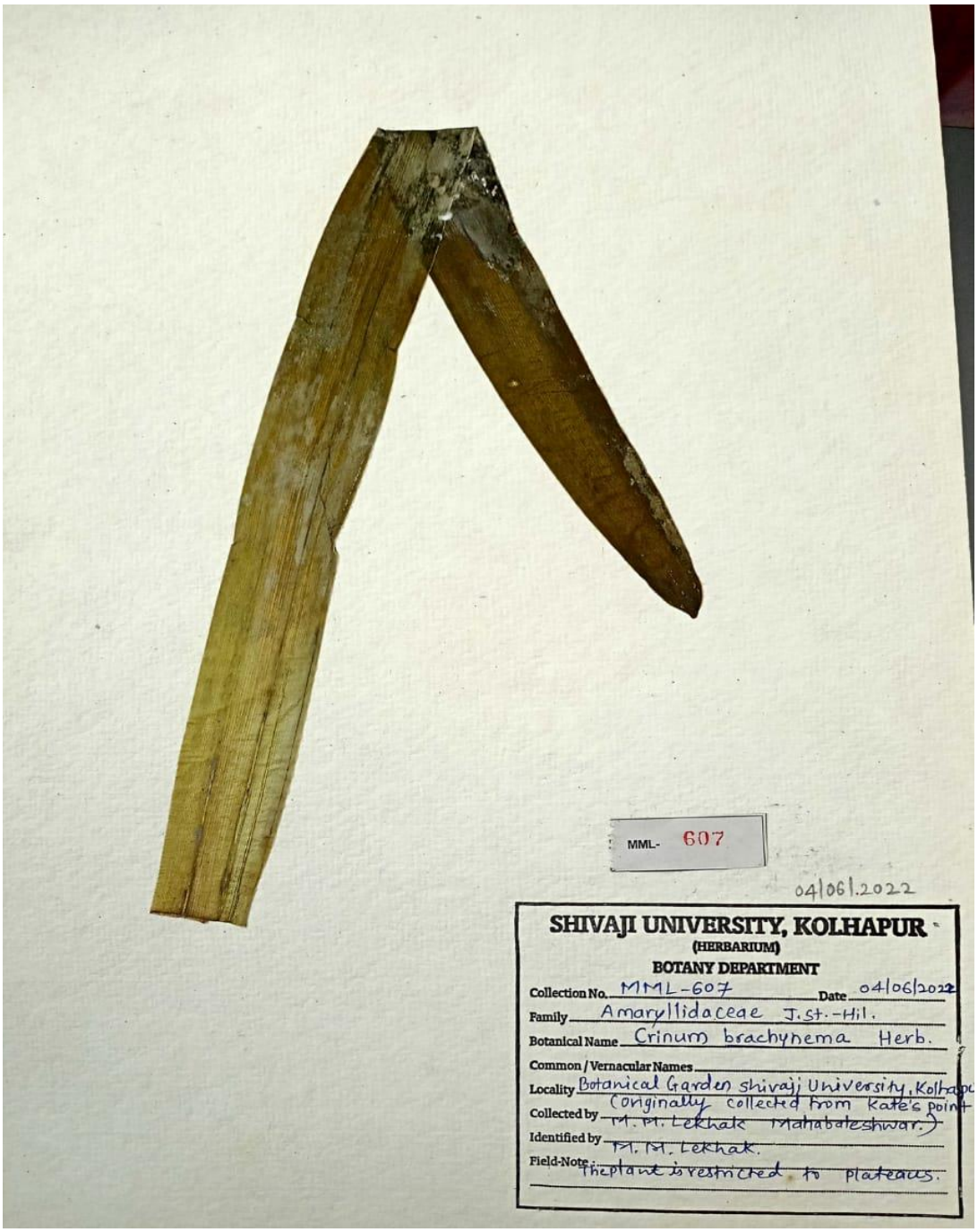


Fig 3.2 Herbarium of *Crinium brachynema* (Department of Botany) from Shivaji University, Kolhapur

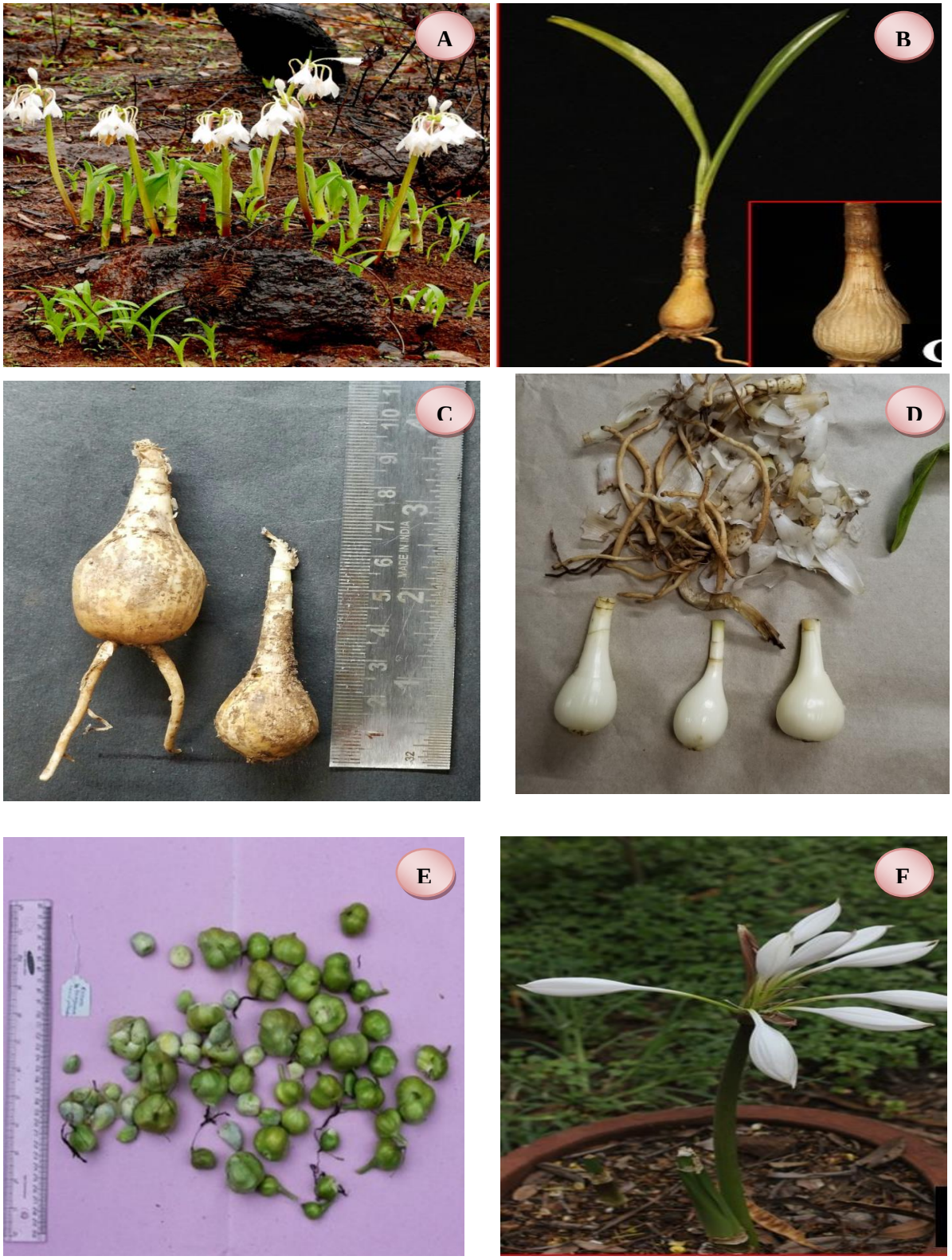


Fig 3.3. A. Habitat of *C. brachynema* B. Young bulbs C. C. Bulbs of different size. D. Bulb explants for Tissue culture. E. Fruits. F. brachynema plant maintained at the medicinal plant garden

3.1.2.1 Source of explants

Tissue culture requires an explant to grow a whole *in-vitro* plant. In this case, the source, age, physiological stage, and genotype of the explants can affect the development of tissue [263], as larger ones are easier to innoculate and need more nutrients in the medium [93]. Leaf explants are the most frequent, however, nodes, internodes, seeds, roots, shoot, and leaf may be employed [94]. Despite sterility issues, most Amaryllidaceous species are propagated via twin scales [123]. To conduct this study, bulbs of *C. brachynema* explants that were employed for regeneration and multiplication are shown in Fig 3.2; 3.3. The Mahabaleshwar area in the Satara district in the state of Maharashtra, India, was the source of the bulbs.

3.1.2.2 Conditions of Culture

Aseptic conditions should be maintained like humidity, pH, and temperature, and an aseptic medium should be prepared to culture the tissues or cells of plants [97]. Temperature affects *Crinum spp*. as in *C. moorei and C. macowanii*, bulblet production was inhibited at 15 °C, whereas bulb propagation was optimized at 25–30 °C [98,95].

The most favourable conditions in the culture chamber or vessel were determined to be 16-hour light with a 25°C, 40-50 µmol m^{-2} s^{-1} light intensity, and an 8 h dark period. Most research indicates that autoclaving conditions should be maintained at 121 °C for roughly 15 minutes, with a relative humidity of 50-60% [121,111]. Priyadarshini et al. [101,102] and others found that 70-80% relative humidity and a pH of 6.0 were optimal.

3.1.2.3 Media Sterilisation and Preparation

MS media [107] was utilized in this investigation, with the addition of $CaCl_2$ and premeasured packets of vitamins (Hi-Media) (Table 3.1 and 3.2). With constant stirring, we were able to dissolve MS medium and 30 grams of sucrose in one litre of double-distilled water. 5.8pH with 1N (Sodium hydroxide) or (Hydrochloric acid) and the necessary quantity of heat-stable growth hormones were added to the MS medium. Agar 0.8% (8 g) was dissolved in media by heating. The medium was then transferred to conical flasks, petri dishes, and test tubes that had been autoclaved at 121°C and 15 psi for 15 minutes.

Table 3.1 Composition of different reagents in MS media

Compounds	**Composition**
Macro-Salts (gm/L)	
Magnesium sulphate	7.4
Ammonium nitrate	33
Potassium dihydrogen phosphate	3.4
Potassium nitrate	38
Micro-Salts (mg/L)	
Cobalt chloride	25
Sodium molbdate	250
Boric acid	6300
Zinc sulphate	8600
Copper sulphate	25
Manganese sulphate	**22.3 g/L**
Calcium chloride	**88 g/L**
Potassium Iodide	**830 mg/L**
Myoinositol	**100 g/L**
Glycine	**2000 mg/L**
Vitamins	
Nicotinic Acid	500 mg/L
Pyridoxine HCl	500 mg/L
Thymine HCl	100 mg/L
Chelated EDTA	
Disodium salt of EDTA	6.550 g/L
Ferrous Sulphate	5.570 g/L

Table:3.2 Composition of MS medium

Compounds	Concentration
Calcium chloride	5ml/L
Fe EDTA salt	5ml/L
Glycine	1ml/L

Macro-salts	50ml/L
Manganese sulphate	1ml/L
Micro-salts	1ml/L
Myoinositol	1ml/L
Phytoagar	7g/L
Potassium iodide	1ml/L
Sucrose	30g/L
Vitamin	1ml/L

3.1.2.4 Stock 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 3.3 shows the solubility of these PGRs.

Plant hormone	**Formula**	**Molar mass (g/mol)**	**Solubility**
BA	$C_{12}H_{11}N_5$	225.2492	1N NaOH/EtOH
Meta-topolin	$C_{12}H_{11}N_5O$	241.25	1N NaOH/EtOH
NAA	$C_{12}H_{10}O_2$	186.2066	1N NaOH/EtOH
IAA	$C_{10}H_9NO_2$	175.184	1N NaOH/EtOH
IBA	$C_{12}H_{13}NO_2$	203.24	1N NaOH/EtOH
2,4-D	$C_8H_6O_3Cl$	211.04	1N NaOH
Picloram	$C_6H_3CL_3N_2O_2$	241.46	1N NaOH/EtOH
TDZ	$C_9H_8N_4OS$	220.25	1N NaOH/EtOH

Table 3.3 Plant growth regulators and their solubility

3.1.2.5 Preparation of aseptic condition in Laminar Air Flow (LAF)

LAF was thoroughly sterilized before inoculation by wiping with 70% ethanol, followed by a 15-minute UV treatment. A mask was used to cover the mouth and nose, and the hands were sterilized with 70% ethanol. All instruments, including forceps, scalpels, scissors, and surgical blades, were dipped in 70% ethanol and flame sterilized. Inoculation should be done near the flame, and a cotton plug or cap of culture test tubes/conical flasks should be kept in

hand during inoculation. After inoculation, the flasks or test tubes were recapped and wrapped in parafilm before being incubated in the culture room.

3.1.2.6 Sterilization of explants

For this experiment, the plant leaves were harvested, the outer dried scales and root were removed, and the bulbs (5-7 cm) were initially washed with tap water. The bulbs were sterilized with Bavistin 5% (w/v), (40-45 min) along with Tween 20 with a (few drops) (Himedia, India) for 30 minutes, and then surface disinfected with 70% ethanol (60 seconds) (Table 3.4). The washed explants were shifted to a laminar hood to continue the surface sterilization process, in which various sterilizing agents with different time intervals were used to standardize the sterilization protocol, as detailed below.

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 %	20	5 gm in 100 ml
			30	
			45	
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 HgCl2 dissolved in 1000 ml of D/w.
			4 m	
			5 m	

Table 3.4 Sterilising agents with different time intervals were used to standardize the sterilization protocol

After that, explants were cleaned with distilled water and immersed in mercuric chloride ($HgCL_2$) 0.1% (w/v) for 6-7 minutes, and washed with autoclaved water. The decontaminated

bulbs were cut and divided into the initial explants comprising twin scales (2.0 cm) (Fig 3.3). Such explants were then inoculated into MS media having myoinositol, agar, sucrose, and varying conc. of BA (0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg L-1) alone and with NAA (0.5 mg/L). Media without any hormone was planted as control, pH of all media was set to 5.8 before autoclaving at 15 psi pressure at 121 ºC for 20 minutes. Cultures were kept in a culture room with maintained temp. at 25 ± 2ºC, under a 16/8h (light/dark) and photo-period was maintained by cool white LED tubes at PPFD (photosynthetic photon flux density) of 40 µmol $m^{-2}s^{-1}$. The numbers and length of shoots obtained were recorded, with experiments replicated three times.

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 a hot-air oven.

3.1.3 *meta*-Topolin-mediated *In-vitro* shoot proliferation

C. brachynema shootlets induced were subcultured on media having varying conc. of *meta*-Topolin (*m*T) i.e. 0.5, 2.5, 5.0, 7.5, and 10.0 µM, and kept under the same growth conditions and pH range as used for shoot proliferation above. The shoot length and mean number of shoots after six weeks were recorded.

3.1.4 Callus induction

Extracted from *in- vitro* regenerated leaf explant, tuber explants cultured on media fortified with Picloram (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L) and (0.5 mg/L) TDZ and (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10mg/L) 2,4-D individually and with combination of (1.0 mg/L) BA. Both PGRs significantly influenced morphogenic responses such as callus culture percentage, callus nature, and callus amount. Increased concentrations of both PGRs resulted in a decrease in culture response and callus amount. Picloram and TDZ increased the amount of friable callus. The 2.0mg/L Picloram and 0.5mg/L TDZ were considered as optimum concentrations for friable callus formation while increasing the concentration of growth regulators lower culture response was observed.

3.1.5 Rooting and hardening

Well-established shoots of *C. brachynema* were transferred to concentrations of IAA or IBA (0.5, 1.0, 2.0, 3.0 mg/L). The response of roots was measured after eight weeks of culture. Well-developed complete plantlets obtained after eight weeks were recovered and cleaned. The plants were transferred in paper cups containing soilrite® and covered with a poly bag to provide humidity. After a few weeks, plants were placed to the greenhouse.

3.1.6 Oxygen radical scavenging capacity (ORAC) analysis

The oxygen radical scavenging capacity assay of *C. brachynema* was performed as detailed by Kumar et al. [165]. In triplicates, 100 µL of fluorescein reaction mixtures (500 mM) and *C. brachynema* extracts (25 µL) were added into 96 well microplates and pre-incubated at 37ºC. Thereafter, 2,2-azobis (2-methylpropionamidine) dihydrochloride (AAPH) (25 µL) was added and samples were mixed properly and shaken in a FluoroskanTM microplate fluorometer for 5 seconds. The fluorescence was read for 90 min (every 3 min). Finally, the antioxidant activity was calculated as per net area under the curve and was expressed in Trolox (3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid) equivalents (µmol g-1 TE).

3.1.7 Statistical analysis

In these experiments, Statistical analysis was done with ANOVA through the software SPSS. Mean values were calculated with DMRT (Duncan's multiple range test) at the probability level of 5% ($P \leq 0.05$).

3.2 Somatic embryogenesis of *Crinum brachynema*

3.2.1 Chemicals

MS Media with Sucrose and vitamins were from Hi-media®, agar powder, Bavistin and agar powder, Picloram, TDZ, 2,4-dichlorphenoxyacetic acid, 6-benzyladenine (BA), glutaraldehyde, gibberellic acid. All chemicals used were of analytical grade.

3.2.2 Plant Material and Methods

To obtain bulb explants, plant leaves were removed, and the root and outer scale were harvested. The bulbs (5-7 cm) were rinsed thoroughly and sterilized by using our recently published protocol [52]. The disinfected bulb explants were cut longitudinally to give twin

scales with basal plate (2.0 cm) under aseptic conditions. Sterilized twin-scale explants were placed in culture flasks containing MS media with the plant growth regulators (PGR) 2,4-D, BA, picloram, and TDZ, added individually or in a mixture under the sterile conditions of laminar airflow. The inoculated culture tubes were maintained and incubated at 26±2ºC in a culture room with a 16/8 h photoperiod from 40W fluorescent tubes (Philips, India) emitting a photon flux intensity of 50 µmol/m^2/s^1, with a relative humidity of 60% retained by using a humidifier. The medium without any PGR served as a control. The pH was adjusted to 5.8 and all cultures were kept under the same conditions as above. The percentage of embryogenic calli was calculated as the total number of embryogenic explants/total number of explants used x 100. Friable callus was transferred to media containing TDZ and Picloram. The mean number of somatic embryos was recorded after 8 weeks of culture.

3.2.3 Scanning Electron Microscopy (SEM)

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 a critical point dryer. After being coated with gold (to make the samples conductive) in an ion coater (D II-29030SCTR) the specimens were examined and images were captured under an SEM (FESEM, JSM-7610F, Oxford Instruments X-Max N) operated at 15-25 kv.

3.2.4 Somatic embryo germination

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

3.3 Cell suspension and study elicitors study on secondary metabolite production.

3.3.1 Material

MS Media with Sucrose and vitamins were from Hi-media®, agar powder, growth regulators as BA, 2,4-D, Picloram, TDZ, Elicitors as Jasmonic acid, methyl Jasmonate, and Salicylic acid were purchased from Sigma-Aldrich, USA. MS media with vitamins, glycine, and myoinositol were obtained from Hi-Media®.

3.3.2 Methods

3.3.2.1 Sterilization and preparation of media

For this experiment, Murashige and Skoog [107] (MS) supplemented with $CaCl_2$ and vitamins available in readymade packets (Hi-Media) were used. Heat-stable growth hormones were added in the desired amounts to the MS media, with 5.8 pH. The media was then heated to dissolve agar 0.8% (8g). The media was then poured into sterilized conical flasks, petri plates, and test tubes before being autoclaved for 15 minutes at 121 °C and 15 psi.

3.3.2.2 Elicitor Stock Preparation

Elicitors such as Jasmonic acid (JA), Salicylic acid (SA), and Methyl Jasmonate (MeJA) were used. For the initiation of suspension, JA, SA, and MeJA stocks were prepared and added to the MS media. Because all PGRs are insoluble in water, they were dissolved in a relative solvent before being mixed with double D/w to make the required volume (Table 3.5; Fig 3.5). The stock solution was prepared for the elicitors.

1. Jasmonic acid – Elicitors JA was produced by mixing 250ul of JA in a mixture of ethanol and water with a volume-to-volume ratio of 12:13 [264]. And 0.22 micrometer membrane filter was used to disinfect the elicitor solution. Different concentrations of jasmonic acid were prepared (0µm, 10µm, 25µm, 50µm, and 100µm) and added to autoclaved media.
2. Methyl-Jasmonate– Stock solution of elicitor was made by dissolving 250ul of methyl jasmonate in ethanol with a ratio of water (12:13 v/v) [264]. A membrane filter with a pore size of 0.22 micrometers was used to sterilize the elicitor solution. Different concentrations of jasmonic acid were prepared (0µm, 10µm, 25µm, 50µm, and 100µm) 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 (0 M, 0.5M, 1 M, and 1.5M) and added to the culture media.

   Samples were collected for HPLC analysis on the 0^{th},4^{th},8^{th},$12^{th,}$ and 16^{th} days. Along with-it media without any elicitor was served as control.

Elicitors	Formula	Molar mass (g/mol)	Solubility
Jasmonic acid	C12H18O3	210.27	EtOH/Water
Methyl jasmonate	C13H20O3	224.3	EtOH/Water
Salicylic acid	C7H6O3	138.121	EtOH/Water

Table 3.5 Different elicitors and their molar mass along with their solubility

3.3.2.3 Optimization of elicitors and elicitation

Elicitation from plant cell cultures is an important biotechnological method to increase secondary metabolites. Elicitors are chemicals that are required for the initiation of elicitation. Elicitors are substances from many sources, abiotic or biotic factors that promote the reaction, leading to the buildup of secondary metabolites. Biotic elicitors obtained from a plant itself or pathogens are more typically utilized as they are inexpensive [265]. Their makeup can be defined or complicated. Physical variables or chemical molecules, abiotic elicitors (salicylic acid, methyl jasmonate, lighting, pH, and sucrose) are more efficient. The optimization of elicitor specificity is a vital stage in the effectiveness of this approach. Because an elicitor can stimulate different cell cultures in different ways, choosing an elicitor that can elicit a desired response in the plant would be heavily reliant on the availability of such a chemical entity as well as the transduction pathways that each elicitor activates [266]. The improved activity of secondary metabolites by elicitors is a beneficial technology that may be scaled up to a commercial level. Secondary metabolite elicitors include salicylic acid, methyl jasmonate, jasmonic acid, and yeast extract.

3.3.2.4 Optimization of callus induction

Extracted 1 cm long surface sterile leaf explants were cultured in MS medium supplemented with different levels and combinations of PGRs for callus induction and establishment. The medium was then supplemented with (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10mg/l) 2,4-Dichlorophenoxyacetic acid (2,4-D) alone and in combination with (1.0 mg/l) 6-Benzyladenine (BA). All the cultures have been incubated at 22°C. After 4 weeks, the callus induction frequency and the rate of callus multiplication were reported. Subcultures were carried out every 10 days.

3.3.3 Suspension culture establishment

In the callus induction experiment, Media suspended with Picloram (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L) and TDZ (0.5mg/L) produced a high percentage of friable callus in the callus

induction experiment (Fig. 3.4). Friable callus (callus that easily falls apart) obtained from a medium containing 2mg/L picloram and 0.5mg/L TDZ was used to establish cell suspension as it was considered as best concentration for formation of friable callus. In a 250ml size conical flask, 1.0g friable callus was transferred to liquid media, and kept on orbital shaker at 110rpm for 16 hours and incubated at 28 °C. And the media was replaced with fresh on the 7th day.

3.3.4 Cell suspension culture

For the initiation of suspension, 150-250ml conical flasks were autoclaved for 50-60ml liquid media. 5-10 pieces of pre-established callus tissue (approx. wt 1gm) were added to the conical flask containing liquid media with PGRs (Picloram and TDZ) (Fig. 3.4). After flaming the neck, the conical flask was closed was covered with a cotton plug. And the flasks were placed in a rotary shaker moving at 100-150 rpm (revolution per min.) (Fig. 3.5). After 7 days, the callus from each flask was sieved through autoclaved sieve. And the 1ml filtrate containing free cells and cell aggregates was collected to sterilize the Eppendorf. Allow the filtrate to settle for 10-15 minutes and centrifuge the filtrate at 1000 rpm and the supernatant was stored for further analysis. And the remaining sieved solution of suspension was re-suspended in fresh liquid media supplemented with different concentrations of elicitors. On the shaker were set flasks containing the suspension to promote the growth of free cells as well as cell aggregates. During the subsequent subculture, move the cell suspension from each flask into a new flask that has 50 ml of freshly prepared elicitor medium.

3.3.5 Elicitation with Jasmonic acid and Methyl jasmonate

The batch of friable callus was cultured in media supplemented with PGRs. From the cell suspension culture media, the sieved suspension was transferred to a fresh batch of media of elicitors (JA, MeJA, and SA) (Sigma-Aldrich). Cultures were maintained under the same conditions. Suspension cells treated with elicitors (JA and MeJA) were harvested at different day intervals (0^{th}, 4^{th}, 8^{th}, $12^{th,}$ and 16^{th} days) after elicitation. In addition to that suspension without any growth regulator and elicitors served as control.

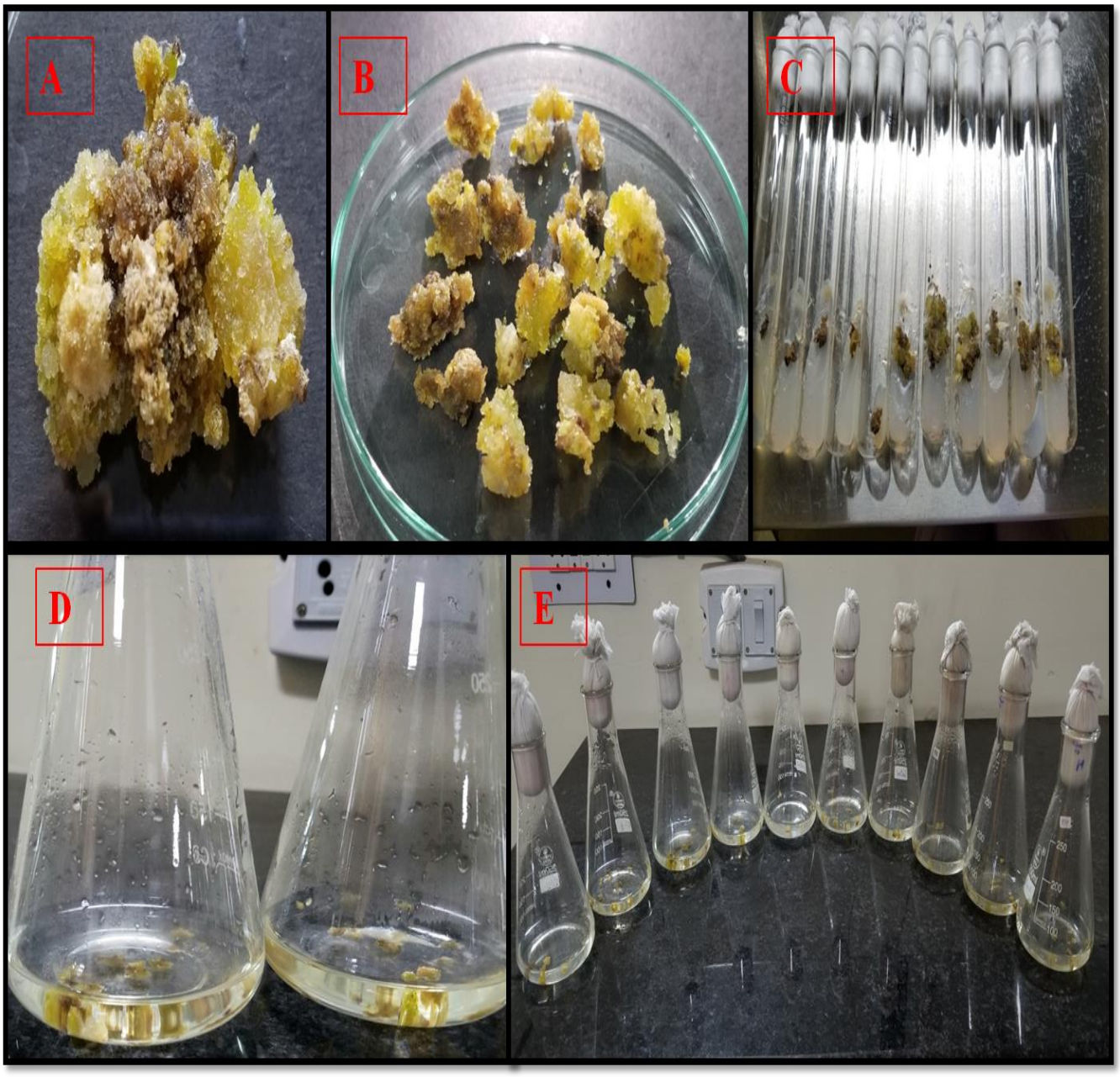


Fig 3.4 A.B. Friable callus C. Subculture of callus on different concentrations of 2,4-D and BA D. Establishment of cell suspension E. Callus suspended in liquid media

Fig. 3.5 A. Cell suspension after 7 days B. Sieving clump of callus from liquid media C. Seived liquid media D. Elicitor preparation E. Different concentrations of elicitors were added to suspension E. Cultures placed in rotator shaker.

3.3.6 Quantification Analysis

3.3.6.1 Biomass quantification

Biomass was quantified by weighing the fresh weight of the callus of both control and treated cultures after harvesting. The harvested sample was further used for analysis purposes.

3.3.6.2 Cell suspension extraction and chemical analysis

Friable callus was ground into fine powder using liquid nitrogen and suspension cells were sieved using autoclaved sieved. Powder of friable callus was extracted with 5ml of methanol for 45 min in a sonication water bath (UAE) at a temp. of 80°C with a frequency of 37 kHz. And the suspension cells were centrifuged for 5 mins at 400 rpm and the supernatant was collected and diluted with methanol. HPLC (High-Pressure Liquid Chromatography) platform was used to analyze the sample extracts. The sample was filtered via a 0.22-micron filter after being filtered, and samples were injected into an HPLC system. The retention periods of the chemical peaks in the samples were compared to the retention times of the standards.

3.4 Assessment of genetic fidelity in regenerated *Crinum brachynema*.

3.4.1 Material

Extraction buffer (2% N-cetyl-N, N, N-trimethylammonium bromide (CTAB; w/v), 0.2 % β-mercapto methanol (v/v), 100mM Tris- HCL with pH 8.0, EDTA 20mM ethylene diamine tetra acetic acid, 1.4mM NaCl), liquid nitrogen, chloroform and Isoamyl alcohol, Tris EDTA, TAE agarose gel, primers of SCoT, ISSR, RAPD marker, PCR reaction mixture.

3.4.2 Clonal Fidelity Analysis

Genomic DNA was extracted from 4-5 cm pieces of fresh leaves grown *In-vitro*. As a control *in vivo*-grown mother plants or bulbs were chosen to compare the fidelity of the plantlets raised. The genomic DNA was isolated from 4-5 cm pieces of fresh leaves grown *in-vitro*. As a control *in vivo*-grown mother plants or bulbs were chosen to compare the fidelity of the plantlets raised. A high-quality DNA was obtained using the Doyle and Doyle protocol [274]. Briefly, 1g of leaf tissue was grinded with liquid nitrogen and poured into falcon tubes with 10ml of extraction buffer (2% N-cetyl-N, N, N-trimethylammonium bromide (CTAB; w/v), 0.2 % β-mercapto methanol (v/v), 100mM Tris- HCL with pH 8.0, EDTA 20mM ethylene diamine tetra acetic acid, 1.4mM NaCl). Tubes were vortexed and kept at 65°C (1 hour)

during the lysis process. After the lysis step, the solution was centrifuged at 10,000 rpm at 22°C for 15 min in a combination of chloroform and Isoamyl alcohol (1:1, v/v). When RNase was added, the tubes were kept in a 37°C incubator for an hour. This RNase-treated sample was then mixed with the equal (1:1) mixture of isoamyl alcohol and chloroform.

After centrifuging the mixture (10,000 rpm; 15 minutes; 22°C), chilled isopropanol was used to precipitate the DNA. After centrifugation at 8000 rpm (10 min) and pellet was washed with 75% ethanol. Further, it was air-dried and resuspended in sterile water or Tris EDTA. The quality and concentrations of isolated DNA were checked on 1.0 % TAE agarose gel.

3.4.3 Polymerase Chain Reaction

Ten primers of SCoT and ISSR and eight primers of the RAPD marker were used for 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 mixture contains 10X buffer, 10mM dNTPs (Table 3.6), Taq DNA- polymerase, 2µl of SCoT, ISSR and RAPD primers. The amplification reaction was carried out using a cycling program consisting at 94ºC for initial denaturation for 3 min followed by 32 cycles each at 94ºC for the 30second, extension at 72ºC for 2 min, and final extension at 72 ºC for 10 min. During the PCR reaction, all conditions were kept as same without annealing temp., which was 50-58ºC (depending on the melting temp. of the primer) (Table 3.7). All the PCR products were mixed with gel loading dye and a total volume of 8µl of the mixture was loaded on the agarose gel, and a 100bp or 50 bp ladder (Thermo Fisher Scientific) was loaded to compare the size of the amplicons. A 2.5% 1X TAE Agarose gel was used to analyze the PCR products.

Table 3.6 The PCR reaction mix was preparation

Water	13.3µl
10X buffer	2 µl
10mM dNTPs (Takara, Japan),	0.4 µl
Taq DNA polymerase	0.3 µl
SCoT, ISSR, and RAPD primers (Operon Technologies, USA).	2 µl
DNA Template	2 µl

Total volume	20 µl

Table 3.7 The amplification reaction was carried out using a cycling program.

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

3.5 Extraction of secondary metabolites

3.5.1 Material

MS media with vitamins, glycine, and myoinositol were obtained from Hi-Media®, Methanol, Lycorine, and Galanthamine, Both the standards (lycorine and galanthamine) were procured from Sigma-Aldrich®. All reagents were of analytical grade.

3.5.2 Methodology

3.5.2.1 Liquid chromatography.

Liquid chromatography (LC) is an extremely important separation technology in the chemical, pharmaceutical, and biotechnological industries. A mixture of chemicals gets injected into a porous substance column and liquid is pushed through it. Variations in component migration rates across the column induced by a varying partition of the component substance between the stationary and mobile phases are utilized to separate the mixture's components. Based on the partition behaviour, distinct compounds elute at different times from the column outlet. MS Tswett, a Russian botanist, created the procedure in 1903 [275,276].

3.5.2.2 Ultra performance liquid chromatography-tandem mass spectrometry

A UV spectrum rarely enables a certain identification, even when the HPLC procedure and the PDA detector have sensitivity. It is a technique that considers the analyte's retention characteristics in both the sample and standard. The vast majority of the time, total certainty in identification is not achievable. The preferred method for confirming with absolute certainty the existence of an identified substance in an analyte is mass analysis.

The separation efficiency generally rises sharply as the column's particle size decreases. To execute separations using columns filled with tiny particles (1.7m), UPLC completely utilizes this chromatographic idea, which leads to faster processing and improved sensitivity [277]. UPLC is crucial to liquid chromatography because the effectiveness and speed of analysis have become increasingly crucial in many applications, particularly in quality control, and medicinal products.

By converting them into charged ions, MS is a technique that precisely calculates the molecular weight as well as atoms. It is perhaps the most adaptable and comprehensive analytical strategy available to Pharmacists and Biochemists right now. Since ions may be more easily controlled and identified experimentally than neutral species, ions are used in mass spectrometry experiments. Three fundamental mechanisms are involved in MS analysis.

1. First stage is ionization, which converts analyte atoms or molecules into a gas phase. In this phase, a proton and electron must be eliminated or added. The molecule may break into separate parts as a result of the additional energy given during an ionization event.
2. Depending on their m/z (mass-to-charge) ratios, the molecular ions, and their charged particles are separated and subjected to mass analysis.
3. Finally, a mass spectrum is calculated, amplified, and displayed for the ion current produced by these mass-separated ions.

When compared to other regularly used methods, with less sample preparation and analysis time, LC/MS/MS enables the isolation of molecules from mixtures easily. Electrospray ionization is a soft ionization technique, and ESI-MS/MS is an effective method for identifying a variety of plant components. [278,279].

3.5.2.3 LC-ESI-MS/MS analysis

Somatic embryo-derived plants were lyophilized and freeze-dried. The samples were pulverized to make a fine powder (0.1 g) and were homogenized for 30 min with methanol in

a sonication bath at 25ºC. Then the extracts were centrifuged at 20,000 rpm and the supernatants were stored for further analysis (Fig. 3.6).

An Agilent 1260 HPLC system with an autosampler, a pump, and a column heater was used for the analysis. The mobile phases comprise acetonitrile and formic acid. The flow rate was maintained at 0.5 min^{-1} and a column (RP-18e; 50mm x 3.0 mm) temperature was set at 30ºC. Mass spectrometry adjusted with electrospray ionization (ESI) was operated in the positive and negative ionization modes with a scan range of 100-600 (m/z). Ionization parameters were maintained as an ion source temperature of 300ºC, a capillary voltage of 3.5kV, nitrogen (collision gas), and nebulizer pressure at 50 psi. The lycorine and galanthamine peaks of samples were recorded corresponding to their standard.

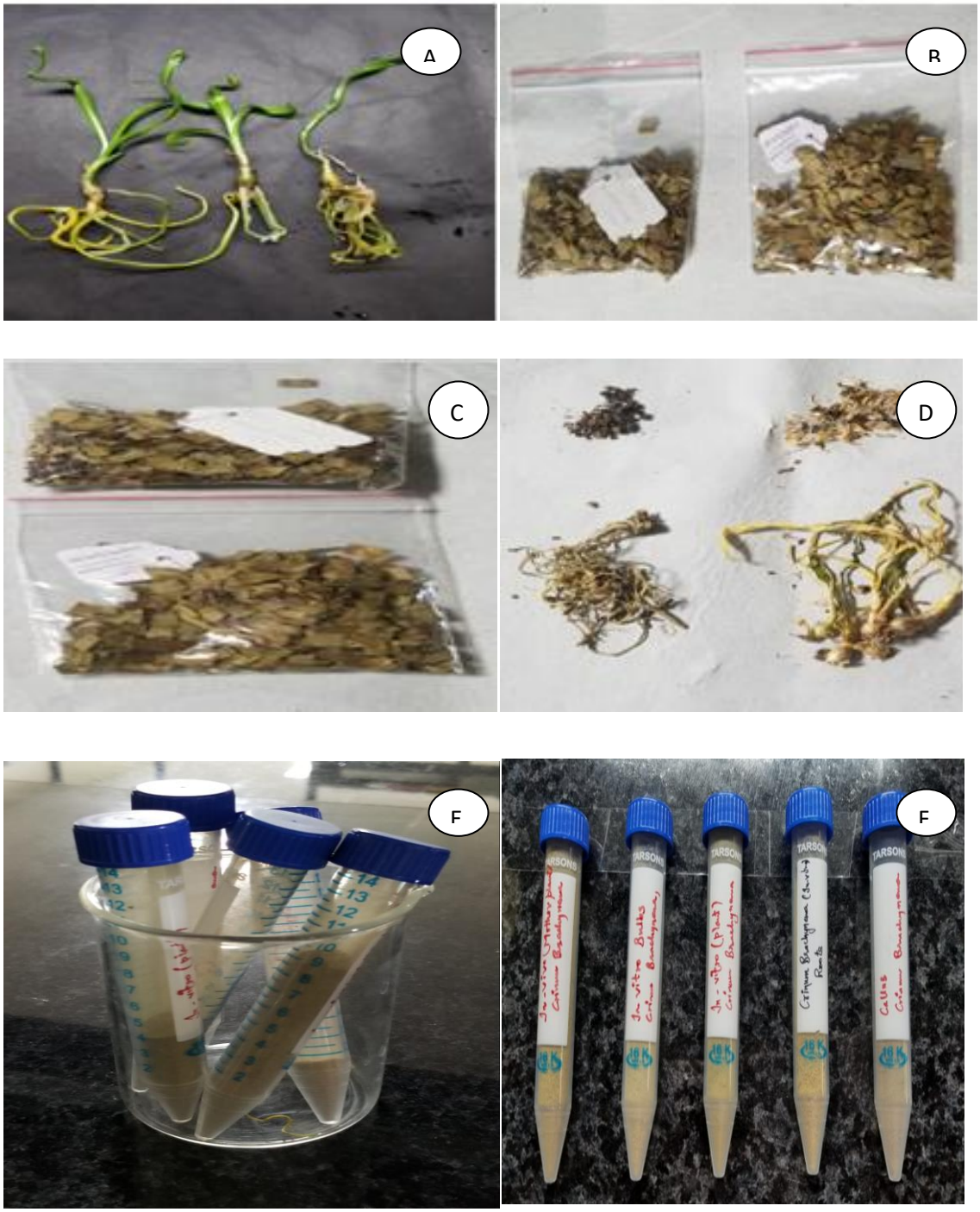


Fig. 3.6 A. *In –vitro* regenerated *Crinum* brachynema B.C *In –vivo* plant sample D. Dried *In- vitro* samples E. *In-vivo* dried samples F. *In- vitro* dried samples

Chapter 4

Result and discussion

4.1 Development of regeneration protocol for *Crinum brachynema* through *In-vitro* techniques.

In-vitro, regeneration of Amaryllidaceae species is time-consuming and often challenging. In the present study, a highly reproducible *In-vitro* regeneration system for *C. brachynema* was developed. The establishment of cultures was difficult but the used sterilization method comprising 70% ethanol, and 5% Bavistin with a few drops of Tween 20 and 0.1% $HgCl_2$ showed promising disinfection results. The bulbs of *C. brachynema* with inner juvenile scales comprising a piece of the basal plate were used to make twin-scale explants (Fig 4.1). The usage of twin-scale explants for regeneration was studied in several species such as *Narcissus asturiensis* (Jord.) Pugsley [114] and *Muscari muscarimi* (L.) Medik. [280], *Cyrtanthus mackenii* Hook. f. [281] *Narcissus tazetta* L. [188] and, more recently, in *Crinum malabaricum* Lekhak & S.R. Yadav [27]. In this study, it was noticed that twin-scale explants must be connected to the basal plate and the meristematic cells of the basal plate triggered the organogenesis (Fig 4.1). *In-vitro,* organogenesis through scale plants containing basal plates has been observed in several Amaryllidaceae species [101,123,213].

Shoots were regenerated on agar-gelled MS medium containing both BA alone or in combination with NAA (Table 4.1). Media containing (2.0 mg/L) BA plus (0.5 mg/L) NAA initiates the highest number of shoots (Fig 4.2 I). BA alone reduced the number of regenerated shoots and no growth was observed in control. Several studies have revealed a slightly higher concentration of BA with a lesser concentration of NAA to be optimum for regeneration in many species such as *Tectona grandis* L.f. [282], *Gloriosa superba* L. [283], *Rosa centifolia* L. [284] and *Croomia japonica* Miq. [285]. For example, the optimum shoot regeneration was observed with the supplementation of 5 mg/L BA + 0.1 mg/L NAA from twin-scale cultures of *Rhodophiala bifida* (Herb.) Traub [146].

Table 4.2 represents the role of *m*T (0.5 – 10 µM) on *In-vitro* shoot proliferation after six weeks of culture. The highest no. of shoots (11.10) with high shoot length (9.33 cm) and fresh shoot biomass (6.43gm) was produced with 5.0 µM *m*T (Fig.4.3 I), a concentration above which *m*T significantly reduced the shoot proliferation in *C. brachynema*. All the concentrations increased the shoot regeneration rate when compared to the BA and control. These results are in agreement with those of Amoo et al. [286], where 5.0 µM *m*T was most effective in shoot proliferation in *Aloe arborescens* Mill., whereas 7.5µM was best in *Corylus colurna* L. [52]. Recently, *m*T exhibited significant shoot multiplication in many plant

species such as *Scaevola taccada* (Gaertn.) Roxb. [287], *C. malabaricum* [27] and *Dioscorea pentaphylla* L. [29]. The significant effect of *m*T was also observed in *Aloe* spp. for shoot induction [270], *Pelargonium* cultivars [288], *Huernia hystrix* (Hook.f.) N.E.Br.[289], *Salvia sclarea* L. [290], *Allamanda cathartica* L. [291], *Santalum album* L. [121] and *Oxystelma esculentum* (L.f.) Sm. [292]. The positive impact of *m*T on shoot induction and proliferation has been recognized in a range of Amaryllidaceae including *Cyrtanthus* species, *Scadoxus puniceus* (L.) Friis & Nordal [173] and *C. malabaricum* [27,29]. Conversely, unfavourable responses in shoot proliferation were also detected in some species, like *Vaccinium corymbosum* L. [293], and *Sorbus torminalis* (L.) Crantz [294], *Citrus* hybrid [295]. Similar to this, the effect of mT in the Cyranthus species was less favourable than that of the other PGRs. In *Scadoxus puniceus*, mT performed less well than BA in terms of regeneration and bulblet production [173], while in a few experiments, other traditional cytokinins performed better than *mT* in terms of shoot proliferation [294].

In-vitro raised healthy shoots were transferred to IAA and IBA for rooting (Table 4.3; Fig 4.4). The rooting frequency of *C. brachynema* shoots ranged from 65-100% over all the concentrations tested, and the highest no. of roots (13.47) with highest length of root (9.37 cm) was observed on (1.0 mg L-1) IBA. IAA was less effective in root initiation than IBA, and in the PGRs-free control medium, no rooting was observed. These findings are consistent with previous reports on the positive effect of IBA on root induction in many plant species [261, 296-299]. In our recent report with *C. malabaricum*, IBA was also shown as the best auxin for the stimulation of root induction [27]. High auxin concentrations, however, resulted in thick, brittle roots and significantly inhibited rhizogenesis in *C. brachynema* shoots. Priyadharshini et al. [300], who utilized a high concentration of auxins, obtained the same results in *C. malabaricum*.

Amazingly, *in-vitro*, bulblets were produced after cultures were incubated over an extended period (eight weeks) with various auxin doses (Fig. 4.4, I; Table 4.3). When compared to IAA in *C. brachynema*, IBA was more effective at inducing bulblets (9.6) (Table 4.3). With IBA, similar outcomes in *Cyrtanthus mackenii* have been noted [281] and *C. malabaricum* [300]. Interestingly, although in Amaryllidaceae species *In-vitro,* bulblet induction is influenced by physiochemical parameters and PGRs [188, 301], it has also been reported on PGRs-free medium in *Amaryllis belladonna* L. [302], *Crinum macowanii* Baker [98] and *Pancratium maritium* L. [303], where it can be assumed that plantlets contained a high level of endogenous growth regulators for bulblet induction.

The ultimate success in *ex-vitro* acclimatization of regenerated plantlets within the least period is a crucial stage for the conservation of endangered species [304]. Well-developed rooted bulblets were gently removed from the culture medium and agar was carefully washed from plantlets. *In-vitro*, rooted plantlets were transferred to paper cups containing soilrite® and covered with polybags to provide humidity. After a few weeks, plants were maintained in the greenhouse (Fig.4.6 D, E), where 80% of the acclimatized plants survived. Importantly, no morphological abnormalities among *in-vitro* and *in-vivo* plants were observed.

In the present study, Callus regeneration was induced by 2,4-D (1.0-10mg/L) alone or in combination with BA (1.0mg/L). Afterwards, combination of picloram (1.0-5.0mg/L) and TDZ (0.5mg/L) were for maturation of somatic embroys.

4.1.1 ORAC activity of regenerated *C. brachynema*

The investigation of ORAC activity for *C. brachynema* was evaluated to determine the biological efficiency of the regenerated plants. All applied *m*T treatments had a substantial effect on the antioxidant capacity of the regenerated *C. brachynema*, which showed an improved ORAC activity relative to the control (223.55 µmol TE/g DW). The highest ORAC activity (494 µmol TE/g DW) was obtained in *m*T (7.5 µM) treated plants (Fig 4.7). The antioxidant capacity of medicinal plants is dependant on their type and concentration of phenolic acids [165, 305]. Several reports have demonstrated the influence of *m*T on the anti-oxidant acivity of *In-vitro* regenerated medicinal plants [160, 306] as also observed in the current investigation.

Table 4.1. *In-vitro* shoot proliferation of *C. brachynema* after six weeks of culture.

BA (mg/L)	NAA (mg/L)	The mean number of shoots	Shoot length (cm)
0.5	0.0	1.80 ± 0.50^{d}	3.5 ± 0.22^{d}
1.0	0.0	2.60 ± 0.47^{c}	4.1 ± 0.15^{c}
2.0	0.0	3.10 ± 0.41^{b}	5.8 ± 0.25^{b}
3.0	0.0	3.60 ± 0.75^{b}	4.5 ± 0.17^{c}
4.0	0.0	2.20 ± 0.24^{cd}	3.7 ± 0.10^{d}
5.0	0.0	1.00 ± 0.45^{e}	2.4 ± 0.36^{e}
0.5	0.5	2.7 ± 0.22^{c}	4.0 ± 0.25^{c}
1.0	0.5	3.4 ± 0.17^{b}	5.9 ± 0.11^{b}
2.0	0.5	6.0 ± 0.18^{a}	7.5 ± 0.15^{a}
3.0	0.5	2.0 ± 0.45^{cd}	4.0 ± 0.22^{c}
4.0	0.5	2.0 ± 0.45^{cd}	3.4 ± 0.19^{d}
5.0	0.5	1.9 ± 0.22^{d}	2.3 ± 0.13^{e}
PGR-free (Control)	0.00	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}

Table 4.2. *Meta*-topolin (*m*T)-mediated *In-vitro* shoot proliferation of *C. brachynema* after six weeks of culture

*m*T (µM)	The mean number of shoots	Shoot length (cm)	Shoots fresh weight (gm)
0.5	6.80 ± 1.15^{c}	8.60 ± 2.88^{b}	3.18 ± 3.76^{c}
2.5	6.40 ± 2.64^{c}	8.16 ± 1.99^{b}	3.00 ± 2.97^{c}
5.0	11.10 ± 2.64^{a}	9.33 ± 2.64^{a}	6.43 ± 2.03^{a}
7.5	8.60 ± 2.75^{b}	5.15 ± 3.33^{c}	4.36 ± 2.28^{b}
10	4.20 ± 1.84^{d}	5.0 ± 2.84^{c}	2.17 ± 1.23^{d}
PGR-free (Control)	1.00 ± 2.86^{e}	3.80 ± 1.79^{d}	0.63 ± 1.36^{e}

Table 4.3. Effect of different concentrations of auxins (IAA and IBA) on *In-vitro* rooting and bulblet formation in *C. brachynema* after eight weeks of culture.

IAA (mg/L)	IBA (mg/L)	Rooting frequency (%)	The mean number of roots	Root length (cm)	Number of bulblets
0.5	-	65 ± 0.72^{f}	5.80 ± 0.44^{f}	2.71 ± 0.33^{e}	1.0 ± 0.44^{f}
1.0	-	78 ± 0.49^{d}	7.10 ± 0.45^{d}	4.69 ± 0.40^{d}	2.5 ± 0.22^{d}
2.0	-	89 ± 0.78^{c}	8.40 ± 0.60^{c}	5.10 ± 0.60^{c}	4.0 ± 0.26^{c}
3.0	-	61 ± 0.71^{g}	3.80 ± 0.20^{g}	2.33 ± 0.80^{b}	2.0 ± 0.11^{d}
	0.5	94 ± 0.27^{b}	10.67 ± 0.24^{b}	6.69 ± 0.31^{c}	7.0 ± 0.49^{b}
	1.0	100 ± 0.00^{a}	13.47 ± 0.29^{a}	9.37 ± 0.78^{a}	9.6 ± 0.24^{a}
	2.0	100 ± 0.00^{a}	13.33 ± 0.19^{a}	9.87 ± 0.43^{a}	9.0 ± 0.19^{a}
	3.0	69 ± 0.44^{e}	6.87 ± 0.79^{e}	4.70 ± 0.22^{d}	1.8 ± 0.30^{e}
Control	0.0	50 ± 0.71^{h}	2.80 ± 0.20^{h}	1.71 ± 0.33^{f}	0.0 ± 0.00

Fig. 4.1 Explant preparation and inoculation on MS media A. Fresh bulbs B. Bulbs after sterilization C. Explant preparation as twin scales D. Twin scales were inoculated in MS media with BA. E.F. newly regenerated shoots from twin scales.

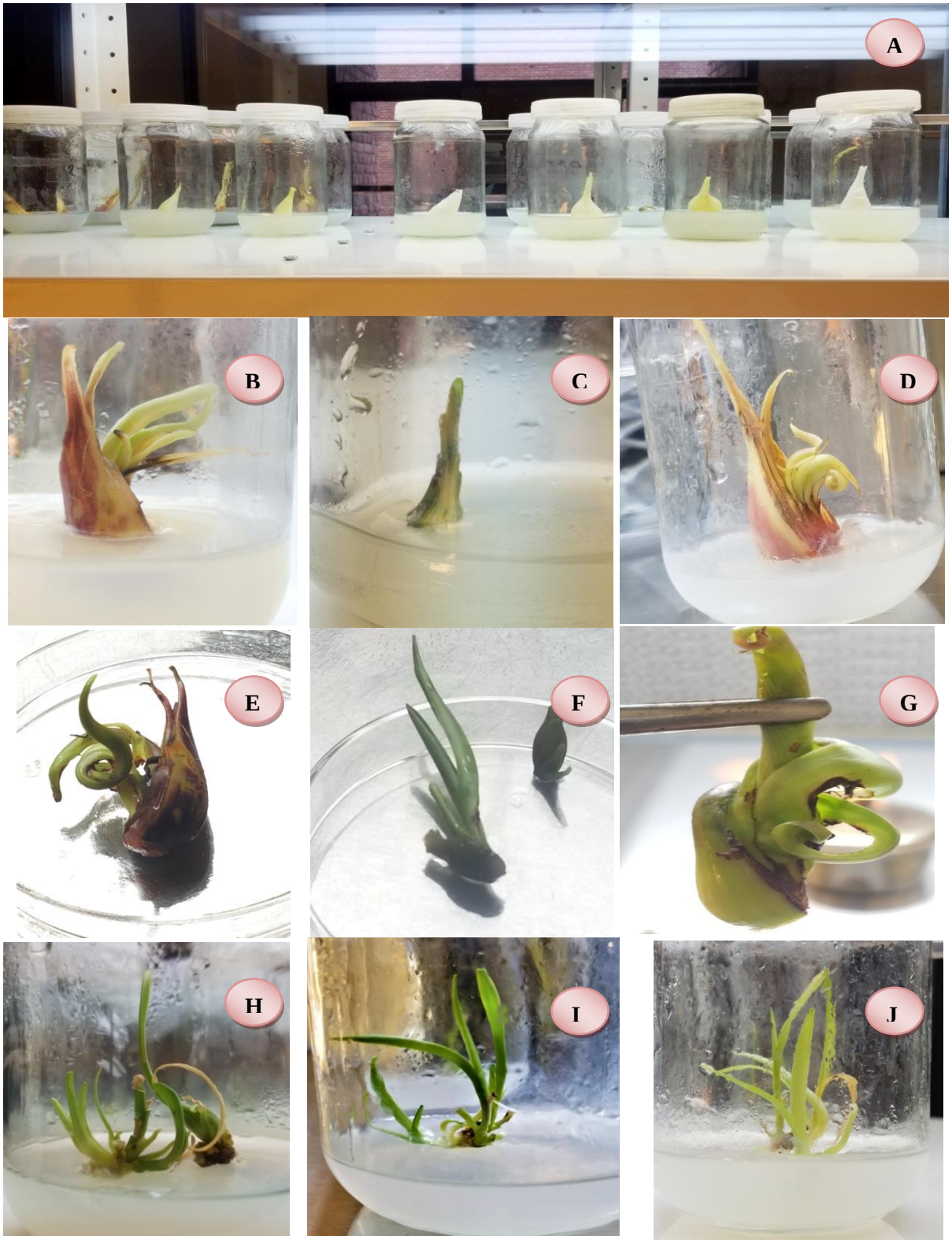


Fig. 4.2 *In-vitro* shoot regeneration with BA and NAA. A. Cultures maintained with different concentrations of BA B.C.D newly regenerated shoots were maintained on media with BA in combination with NA. (1mg/L, 2mg/L, 3mg/L BA +0.5 mg/L NAA) E.F.G Shoot proliferation after 3 weeks H. I. J *In-vitro* shoot proliferation with (1mg/L, 2mg/L, 3mg/L BA +0.5 mg/L NAA) after eight weeks.

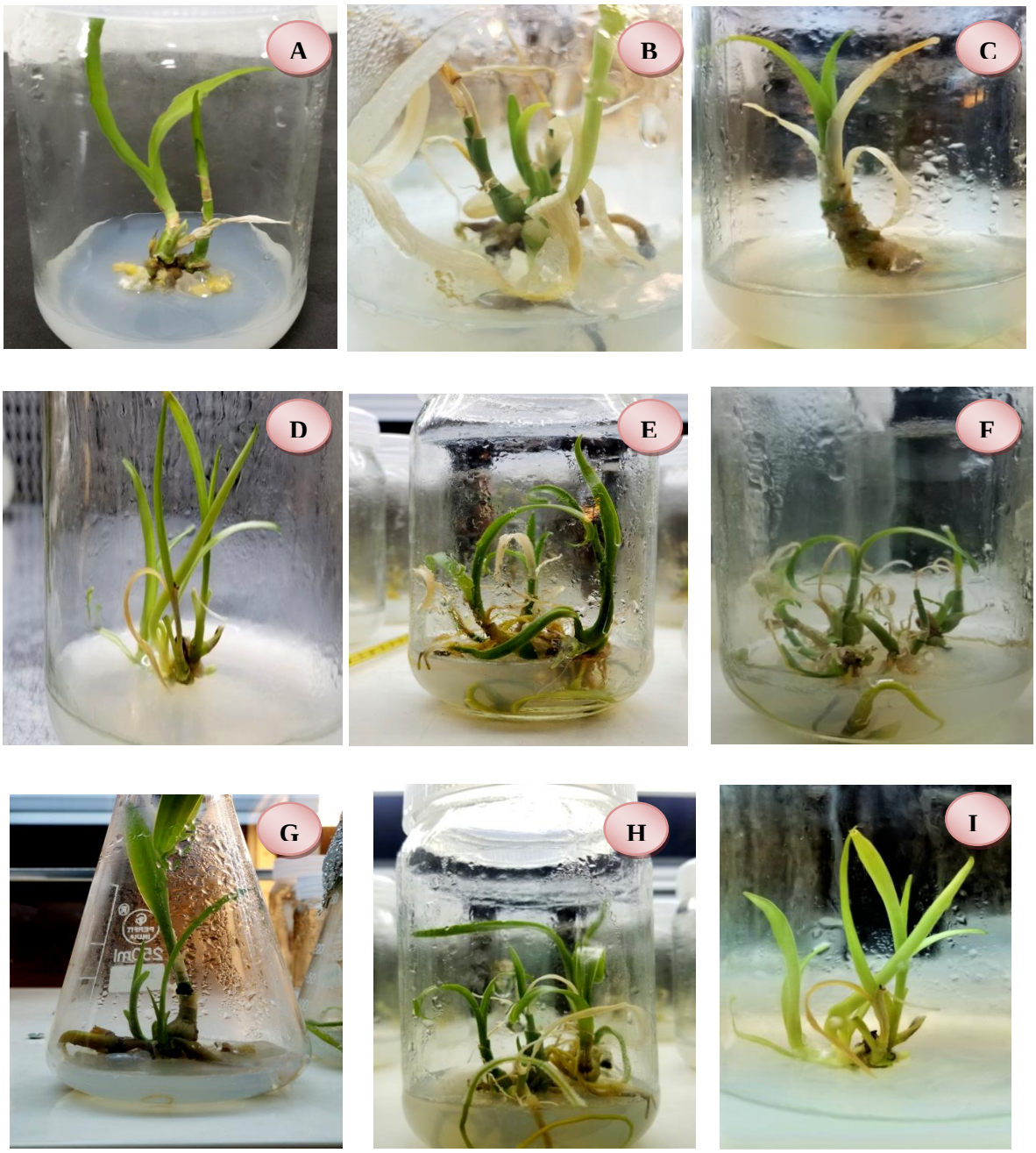


Fig.4.3 *In-vitro* shoot proliferation with *meta-topolin*. A. B. C *In-vitro* plants were maintained on MS media with different concentrations of *meta- topolin*. D.E.F Multiple shoot proliferation 3 weeks with (2.5, 5.0, 7.5 µM) concentrations G.I Shoot multiplication after eight weeks H. Multiple shoot proliferation with 5.0 µM *meta-topolin*

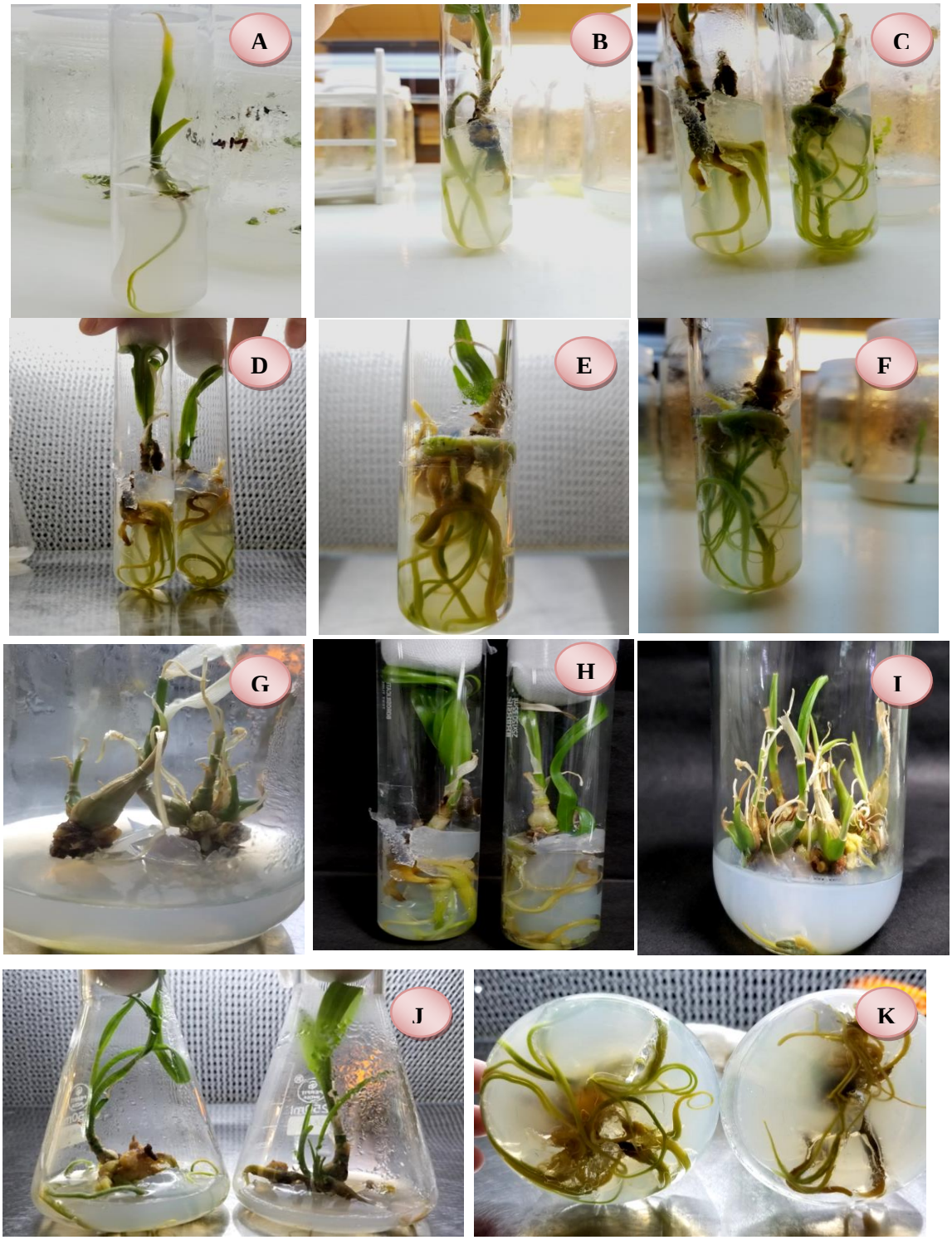


Fig. 4.4 *In-vitro* rooting and bulblet formation A. Root initiation B. Root multiplication C. Root multiplication with 1mg/L IBA and 2mg/L IBA D. Rooting frequency with 1mg/L IBA and 2mg/L IBA E. Img/L IBA E. *In-vitro* Rooting with 2 mg/L IBA F. Invitro rooting with 2mg/L IBA G.H *In-vitro* bulblets formation initiation. I. *In-vitro* proliferated bulbs J.K *In-vitro* matured plant with shoots, bulbs, and roots

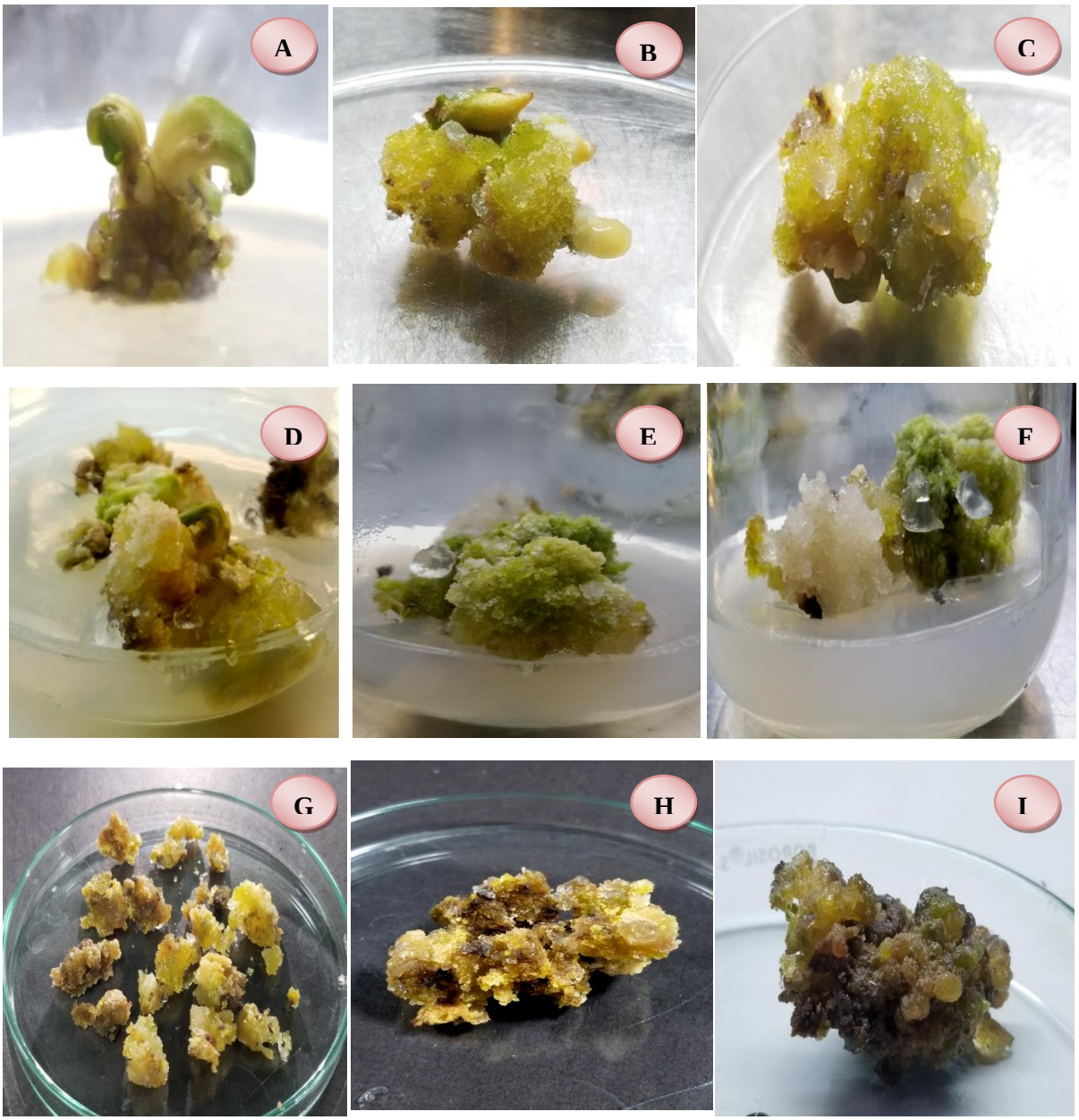


Fig 4.5 *In- vitro* Callus initiation and induction A. Callus initiation B. callus growth after 2 weeks C. Callus growth after 4 weeks. D.E.F Callus formation with 2,4-D in combination with BA. G. Friable culture. H. I. Callus for SEM analysis.

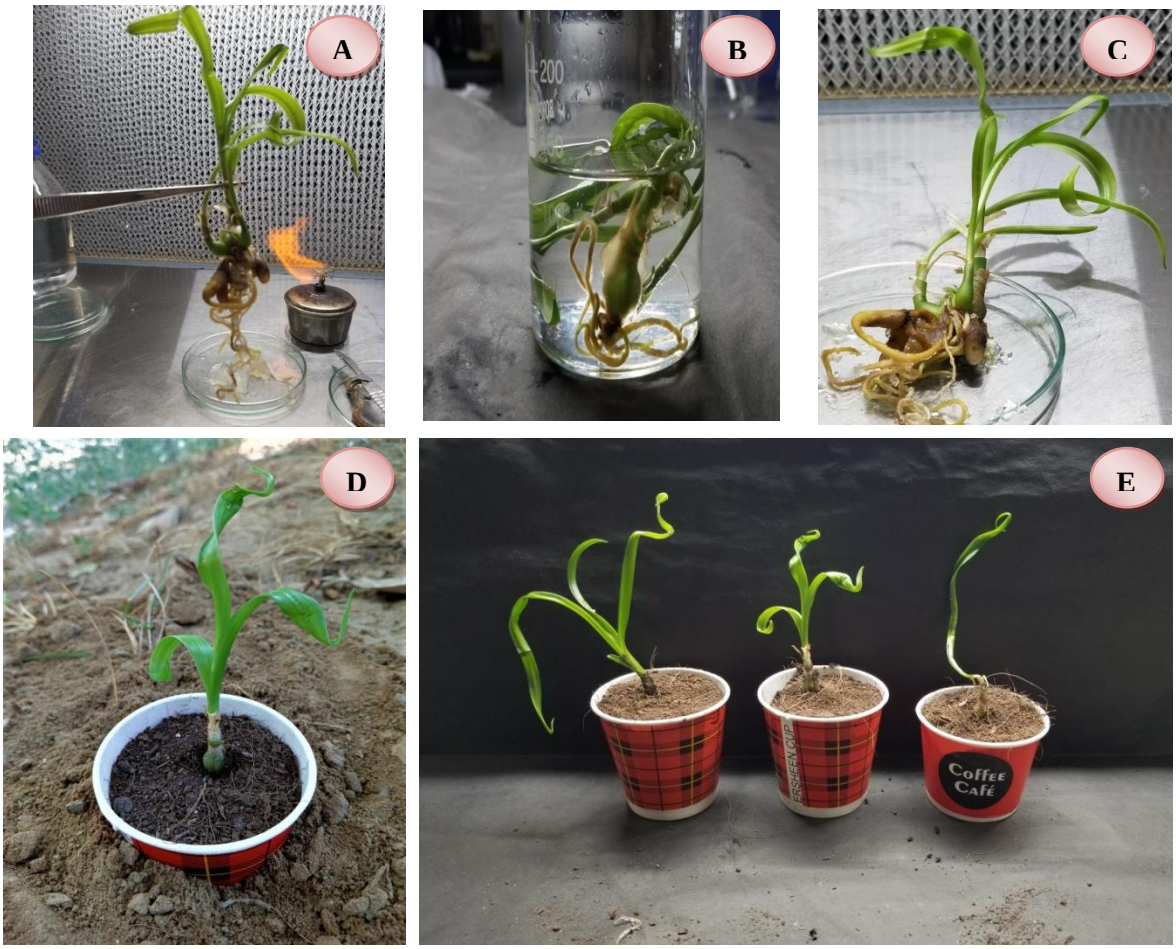


Fig. 4.6 A. *In-vitro Crinum* brachynema isolated from culture bottle. B. Cleaning to remove excess media C. Cleaned plant for harvesting D. E. Hardening of plant

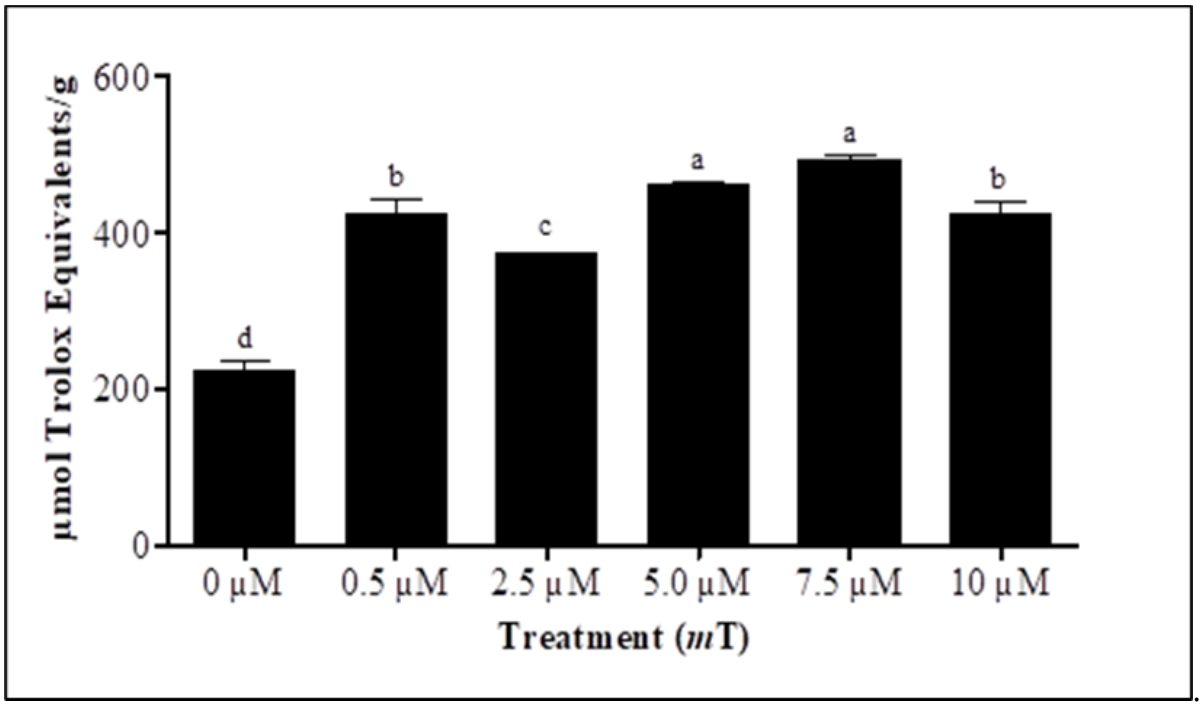


Fig 4.7. The oxygen radical scavenging activity of *C. brachynema*. Effect of different concentrations of mT on antioxidant capacity of micro propagated *C. brachynema*. In each graph, different letters direct significance differences between treatments as per DMRT ((P ≤0.05). Values represent mean ± SE (standard error)

4.2 Somatic embryogenesis of *Crinum brachynema*.

Somatic embryogenesis was the most commonly studied experiment for plant regeneration. It is a potential technique for the production of soma clones. This method may also be used to enhance plant genetics and preserve plant germplasm. Root and shoot organogenesis, along with somatic embryogenesis, was the most researched in the lab for regenerating plants. This is the efficient way to produce somatic embryos and, then the entire plantlet. More than that, the method can be used for genetic improvement and conservation of germplasm. Additionally, various studies including ploidy evaluation of somatic cells, secondary metabolite formation and hybridization, and many others have been completed successfully [128]. Globally, plant tissue culture techniques are efficiently used to conserve rare, endangered, and threatened species. Somatic embryogenesis (SE) offers several advantages to speed up the propagation rate with genetically uniform phenotypes [165,307]. Production of *in-vitro* plantlets via somatic embryogenesis in a short period is beneficial for rapid propagation and secondary metabolite production [308-311]. Thus, we designed a practical strategy for the generation of true-to-type plantlets employing somatic embryogenesis to satisfy the growing demand for this important medicinal species.

Somatic embryogenesis is a promising tool 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 development of an efficient and long-term conservation strategy needs serious consideration. In this study, an efficient plant regeneration system through somatic embryogenesis was established (Fig 4.7). Different concentrations and combinations of 2,4-D (1.0-10 mg L^{-1}) alone and in a mixture with BA (1.0 mg L^{-1}) were used for somatic embryo induction from *C. brachynema* bulbs. Further, combinations of Picloram (1.0-5.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}) were examined to achieve the maturation of somatic embryos. The initiation and production of somatic embryos varied with the PGRs used (Tables 4.4 and 4.5). Thus, 2,4-D alone initiated embryogenic callus and had a positive influence on the somatic embryo development, in agreement with similar findings in somatic embryogenesis of other Amaryllidaceae species [101, 190, 312, 313]. However, a conc. combination of 8.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA had a significant impact on embryogenic calli from *C. brachynema* (78.9 %) and was coupled with a significant effect on the total number of somatic embryos (35.44) produced. This finding is similar to reports on the embryogenic potential of various plant

species including *Chrysanthemum* sp. [314, 315], *Prosopis laevigata* [316], *Vitis vinifera* [317], Hypoxis *hemerocallidea* [307], and *C. brachynema* [101].

Interestingly, the mean number of somatic embryos was increased (50.55) with picloram and TDZ (2.0mg/L + 0.5 mg L^{-1}) (Fig 4.8). Although 2,4-D is widely used as the most important growth regulator in somatic embryogenesis [307, 318, 319], the use of picloram for induction and proliferation of somatic embryogenesis has become commonplace in several plant species [320-322] and has also been widely used in species such as *Lilium longiflorum* [323], *Lachenalia virdiflora* [324,325-327]. This report revealed that picloram and TDZ are necessary for somatic embryo production in *C. brachynema*. A similar result was observed for somatic embryogenesis of *Merwilla plumbea* [326], *Drimia robusta* [327], *Lachenalia virdiflora* [324], *Cyrtanthus mackenii* [328], and *Stewartia sp*. [329].

SEM analysis of *C. brachynema* embryogenic calli indicated the development stages on the surface and revealed the presence of many tightly packed globular embryo clumps, as well as cotyledonary-shaped embryos characterized by well-developed cotyledons (Fig 4.9 D-F).

4.2.1 Embryo germination and plantlet development

Successful somatic embryo germination and its conversion into a complete plant are crucial for the regeneration of plants. Somatic embryo germination was 40% on full-strength media and 51.11% on half-strength MS medium, while as many as 82.22 % of somatic embryos germinated when half-strength MS media was fortified with (1 mg L^{-1}) GA_3 (Fig 4.12). Root growth and expansion of leaves were effective in half-strength when compared to full-strength media. The same utilization has been observed in several other plant species [307, 322, 330-333]. Changing the medium strength (half and full) can influence the osmotic stress [307, 334]. Similarly, the supplementation of GA_3 for somatic embryo germination has been well-documented for several plant species [165, 335].

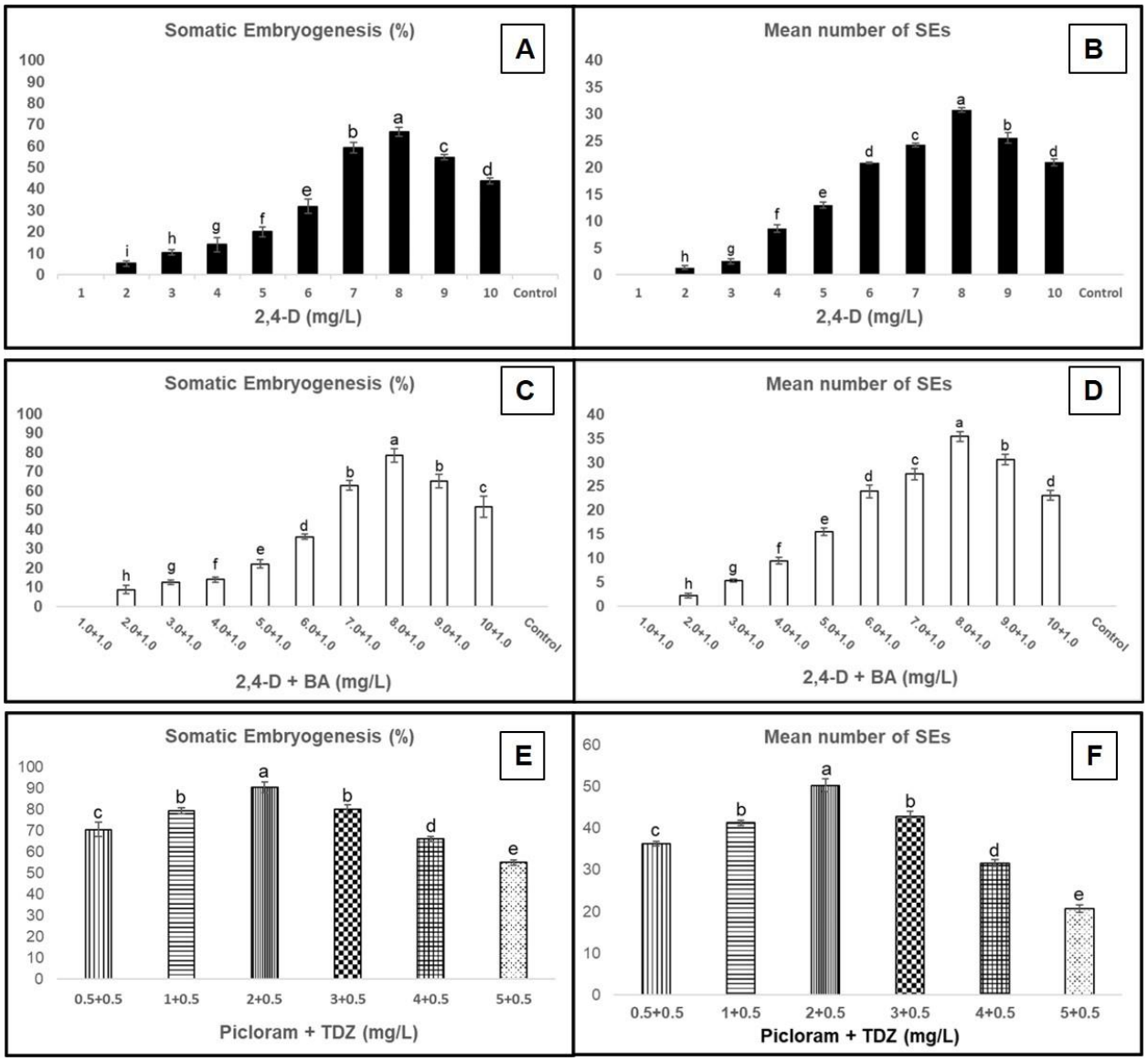


Figure 4.8. A and **B:** Effect of 2.4-D alone on somatic embryogenesis induction in *Crinum brachynema.* **C** and **D**: Showing the effect of 2,4-D in combination with BA on somatic embryogenesis induction in *Crinum brachynema*. **D** and **E**: The effect of picloram and thidiazuron on somatic embryo development in *Crinum brachynema*. Mean ± S.D followed by different letters indicate significant differences analysed by Duncan's multiple range test at a 95 % confidence level (P≤0.05).

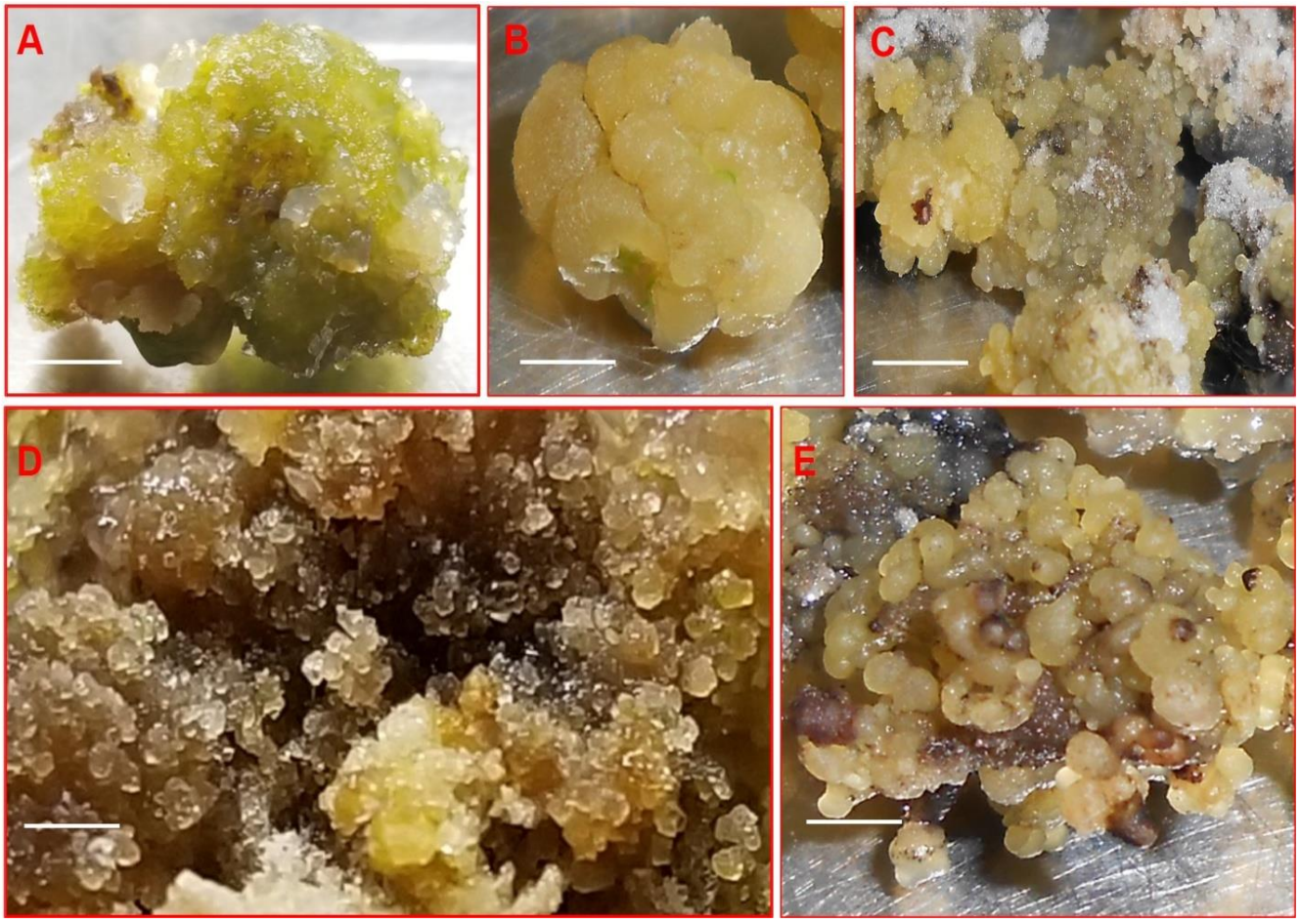


Figure 4.9. Somatic embryogenesis showing embryogenic callus induction and formation of somatic embryos in *C. brachynema*. **A-B.** Induction and proliferation of embryogenic callus. **C-D.** Embryogenic callus with cluster of emerging globular somatic embryos. **E.** Development and maturation of various somatic embryos *Scale Bar: A -E = 5mm*

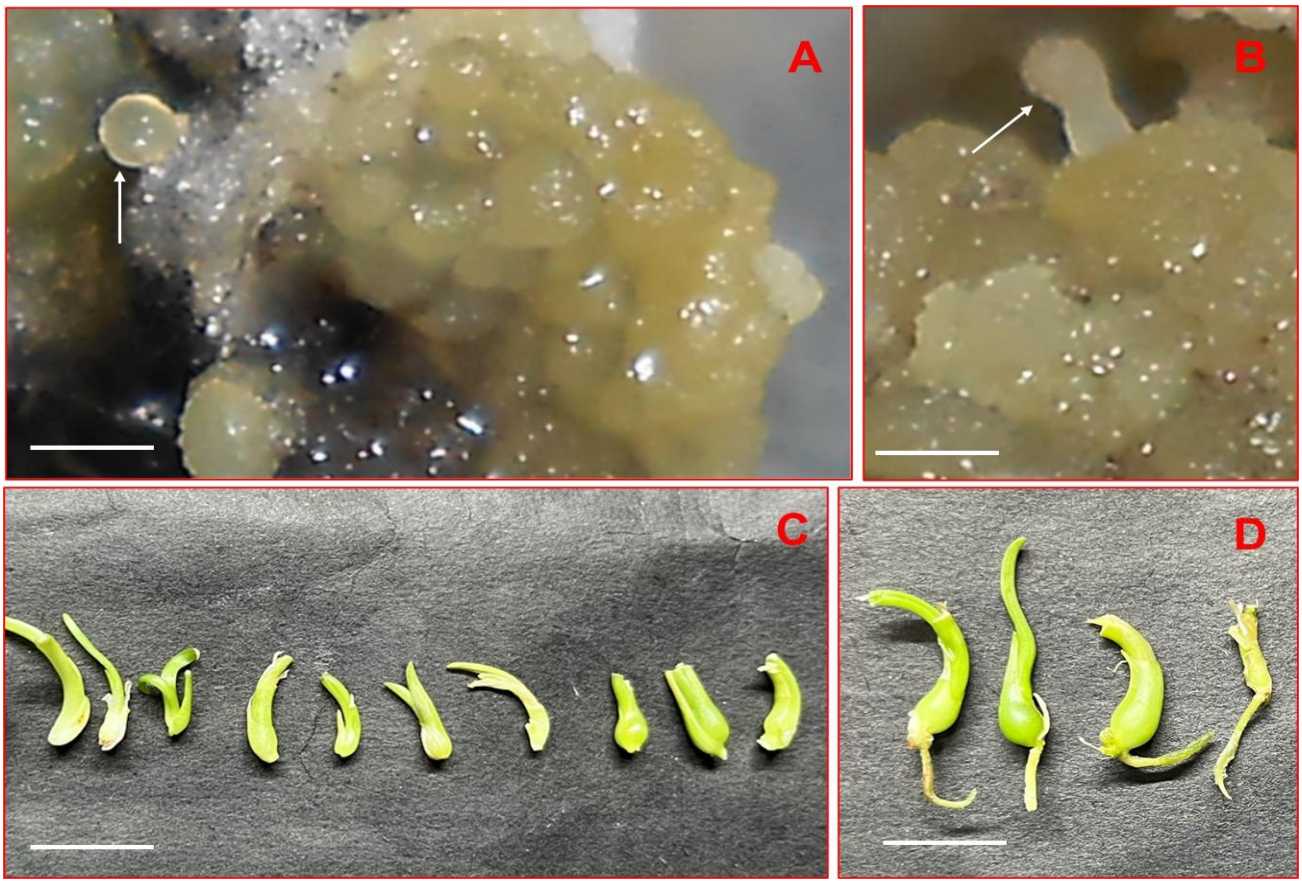


Fig. 4.10 A. Translucent globular-shaped embryo (arrow indicating the globular embryo). **B.** Initiation of cotyledonary-shaped somatic embryo globular-shaped embryo (arrow indicating the appearance of cotyledonary embryo). **C.** Cotyledonary-shaped somatic embryos **D** Cotyledonary embryos with radical

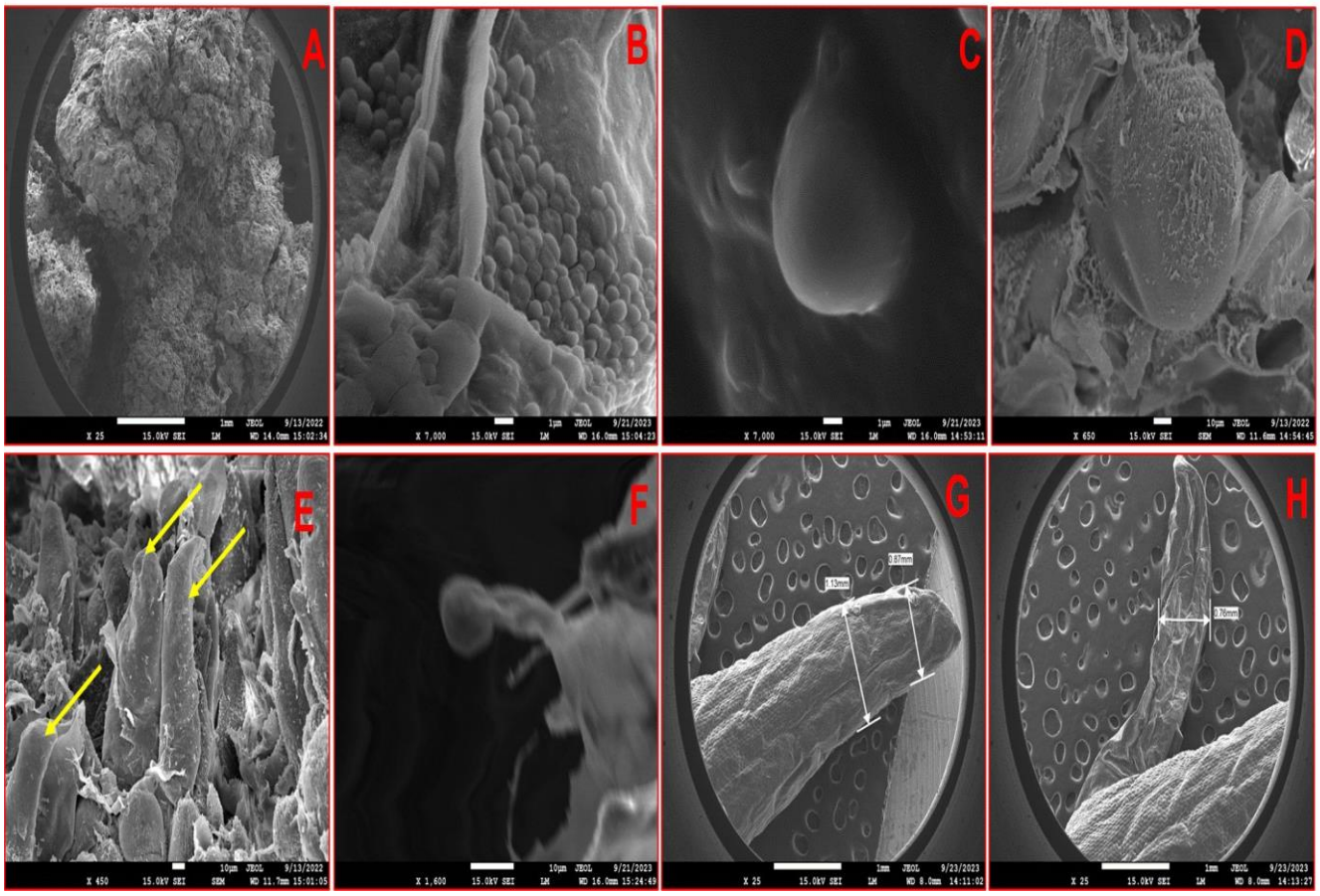


Figure 4.11 Different stages of *C. brachynema* somatic embryos viewed under scanning electron microscope**. A.** SEM micrograph showing the appearance of embryogenic calli. **B.** Globular-shaped somatic embryos emerging from the surface of embryogenic calli. **C-D.** Single globular-shaped somatic embryo. **E.** Cluster of globular-shaped somatic embryos **E-F.** SEM micrograph of developing cotyledonary-shaped somatic embryos. **G-H.** Well-developed cotyledonary-shaped embryo with single shoot *Scale Bar: A = 1mm; B-D= 1 µm; E-F = 10 µm; G-H = 1 mm.*

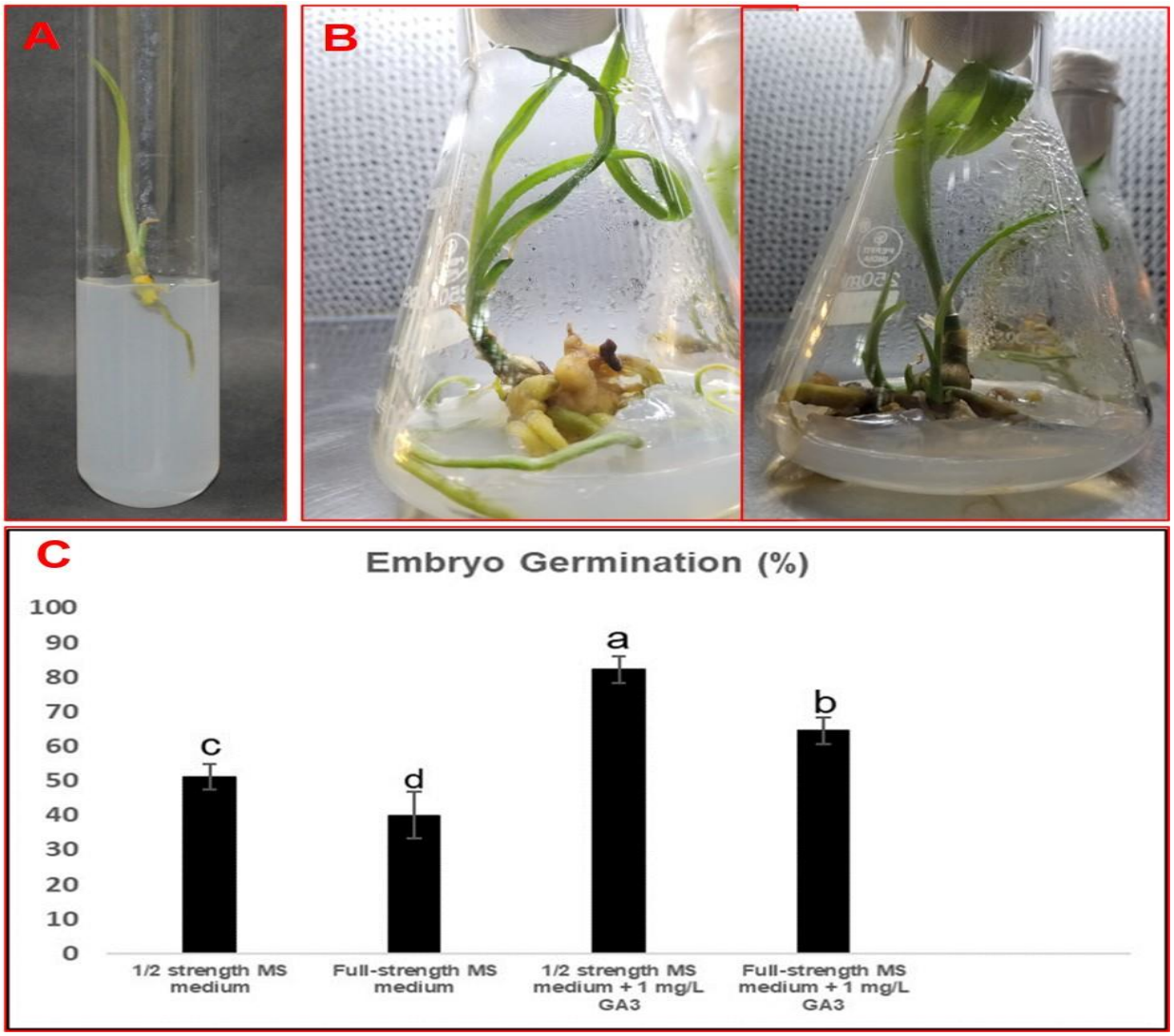


Figure 4.12 *C. brachynema* somatic embryos germination and plantlet conversion**. A.** Germinated cotyledonary-shaped embryos in germination medium**. B.** Well-developed rooted plantlets of *C. brachynema*. **C.** The effect of MS medium (full strength and half strength + 1.0 mg L^{-1} GA_3) on the germination of somatic embryos of *C. brachynema*. All the experiments were repeated thrice with at least three replicates. The significant difference between mean values was obtained via Duncan's multiple range test at a 95 % confidence level ($P \leq 0.05$).

4.3 Maintenance of cell suspension and study effects of elicitors on secondary metabolite production

Plant cell or organ cultures, as a technology for secondary metabolite production, offer advantages over bioactive constituents extracted from plants or chemical synthesis [73]. However, commercial application of plant cell cultures has met with limited success, owing to low product yield, instability of biosynthesis, and scaling-up challenges [182]. The universal phenomenon among them is low bioactive compound productivity. Many strategies, such as elicitation, precursor feeding, medium optimization, and so on, have been developed in recent years to address this issue [336]. Elicitation is the most effective strategy for stimulating the production of bioactive compounds [336-339]. Elicitors, which can be either biotic or abiotic, are external stimuli molecules capable of stimulating stress response. Exogenous elicitors are useful *In-vitro* cultures for improving the biosynthesis of desired secondary metabolites and studying plant responses to pathogen attacks. According to Largia et al. [337], low elicitor concentrations in the medium increase the number of bioactive compounds. Cultures elicited with chemical substances are more consistent in producing high levels of bioactive substances [340]. SA and MeJA derived from these chemical elicitors are well known for increasing bioactive compound production [336, 337]. In response to abiotic and biotic stressors, plants release MeJa, a volatile methyl ester of jasmonic acid [341]. Yu et al. [342] report that MeJa interacts with the signal transduction pathway to stimulate the production of certain enzymes that create defensive chemicals. SA, another stress signalling molecule, is widely used in plants to induce resistance against pathogens [343]. To meet the demand for galanthamine and lycorine, elicitors must be used. As a result, in this study, elicitation feeding was attempted on various parameters such as exposure time, elicitor type, and concentration. Currently, we are specifically aiming to develop an effective elicitation approach for increased biomass production and improved compound accumulation using two different elicitors, Jasmonic acid, MeJa, and SA individually. This is the first research that we are aware of that investigates the effects of elicitor on the generation of galanthamine and the development of biomass in *C. brachynema* cultures grown *In-vitro*.

4.3.1 Establishment of suspension culture and elicitation study

In this experiment, the elicitation process was accomplished in liquid media. The application of elicitors has shown a significant effect on biomass accumulation. The batch of friable callus was cultured in MS liquid media supplemented with 2,4-D (8.0mg/L) in combination with BA (0.5mg/L) as the best concentration for callus induction. From the cell suspension culture media, the sieved suspension was transferred to a fresh batch of conical flasks containing 50 ml fresh medium supplemented with different concentration elicitors (JA, MeJA, and SA) (Sigma-Aldrich). Cultures were maintained under the same conditions. Suspension cells treated with elicitors (JA and MeJA) were harvested at different day intervals (0^{th}, 4^{th}, 8^{th}, $12^{th,}$ and 16^{th} days) after elicitation. In addition to that suspension without any growth regulator and elicitors served as control.

This work has been accompanied by the objective of enhancing the production of secondary metabolites *In-vitro* cultures of *C. brachynema* by the method of elicitation. The plant cultures of *C. brachynema* were elicited with JA, MeJA, and SA as elicitors. The outcome of the present study revealed that friable calli clumps which were grown on 8.0 mg/L 2,4-D + 1.0mg/L BA were used for initiation of cell suspension and cell growth with 2,4-D and BA was recorded as 0^{th} day. The cell suspension culture was subcultured with fresh media with different concentrations of elicitors. According to Stafford and Warren [344], it is preferable to subculture of cells required after linear growth, the medium was depleted and the cells started to create hazardous chemicals [345]. Because the cultures tend to aggregate into small cell clusters, subcultures are required to maintain the fine suspension culture [346]. Two different cell types, both spherical and elongated, made up the suspension. The number of giant round-shaped cells in the culture was typically increased during periods of time greater than 7 days (exponential phase). These findings demonstrate that throughout cultivation, the cells' elongated form altered to a round one.

The combination of Picloram (2.0mg/l) and TDZ (0.5 mg/l), out of the total 5 combinations and 10 combination sof 2.4-D individually and in combination with BA tested for *C. brachynema* callus culture, produced the most callus in a short length of time (28 days). Even softer callus was produced by subculturing after a 7-day break. The other plant growth regulator combinations took longer to proliferate and produced less callus overall.

4.3.2 Growth monitoring of suspension cultures

The PCV (%) is displayed in graphs that were plotted to track the growth of *C. brachynema* cell lines in the suspension medium. For the *C. brachynema* cultures, day 8 saw the greatest cell development. According to one study, light irradiation had little to no impact on cell proliferation in strawberry cell suspension cultures [354] but had a negative impact on *Vitis vinifera* cultures. Depending on the plant cell lines, the difference can exist.

4.3.3 Elicitors effect on Suspension culture

The addition of jasmonic acid during elicitation had the greatest effect on cell proliferation. Initially, elicitor concentration had no effect on cell development, but as time passed, cell biomass decreased. On the eighth day, growth was boosted by up to 6% when compared to the 0th and 4th-day cultures (Fig. 4.11). Jasmonic acid inhibited cell development in Mentha x piperita cell suspension cultures grown under light [355].

Methyl jasmonate had a lower effect on cell proliferation than jasmonic acid. Cell growth was comparable to the control until the fourth day when it grew by 3.5% and reached a maximum of 5.5% until the eighth day. (Fig. 4.12). Methyl jasmonate had no effect on cell growth in *Vaccinium pahalae* (ohelo) and sweet potato cell suspension cultures [356,357], but marginally decreased cell growth in strawberry suspension cultures [358]. A recent transcriptomics study that used elicitation and fast-growing Arabidopsis cultures discovered that jasmonate treatments inhibited cell cycle suppression in the M phase. [360], which has been connected to jasmonates' potential to block cell cycle progression [359]. Cell proliferation that was unaffected during elicitation was unaffected by the rising salicylic acid concentration (Fig. 4.13). A minor increase in cell growth was seen in *Daucus carota* when salicylic acid was added to the culture medium, but lower biomass was seen when MeJA and SA was combined [361].

The suspension culture inoculated with different elicitors indicated the growth rate and cell volume were initially slow till day 4^{th} with jasmonic acid, methyl jasmonate, and salicylic acid. However, the culture proceeded it show cell volume increased rapidly after the 4^{th} to 8^{th}-day interval with all elicitors. The maximum increase was recorded on the 8^{th} day with all elicitors and due to depletion of nutrients it comes to a stationary phase till the 12^{th}-day interval and gets declined after 16^{th}-day intervals.

4.3.4 Identification of secondary metabolites of suspension culture

In the experiment, Secondary metabolites that were targeted to release an enhancement by elicitation in the cell suspension culture were quantified using HPLC. Galanthamine and Lycorine were found in the culture filtrate.

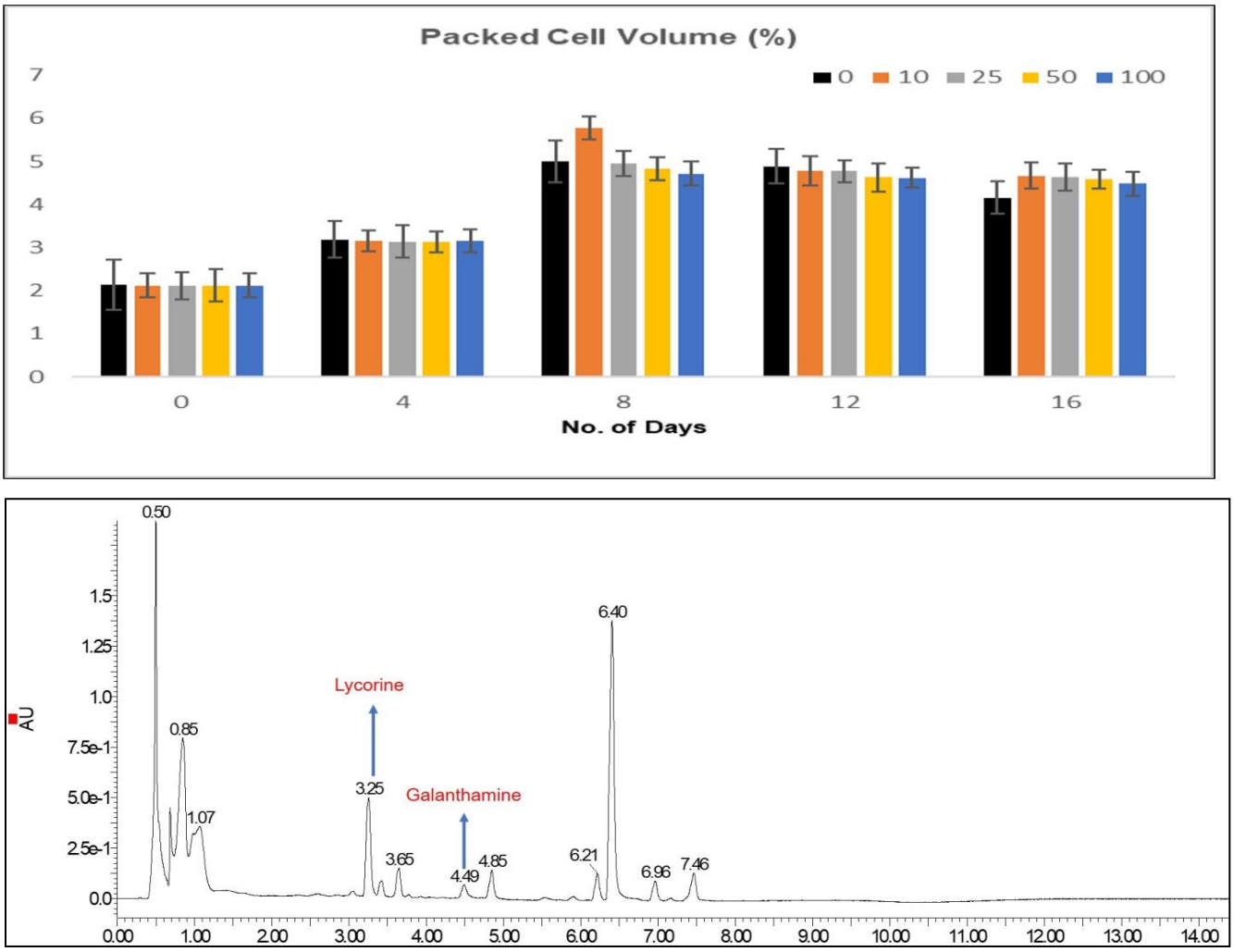
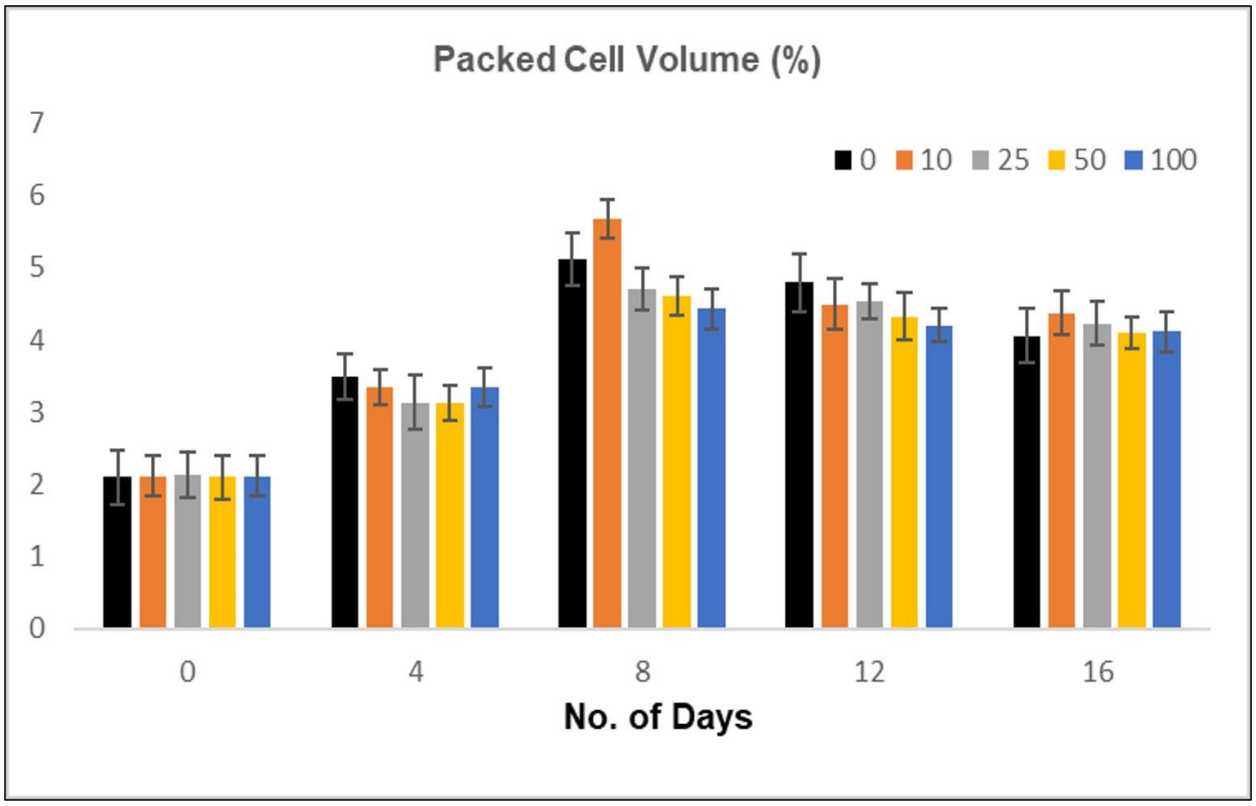


Figure.4.13 The bar graph depicts elicitation enhancement with jasmonic acid as the elicitor and chromatogram of jasmonic acid.



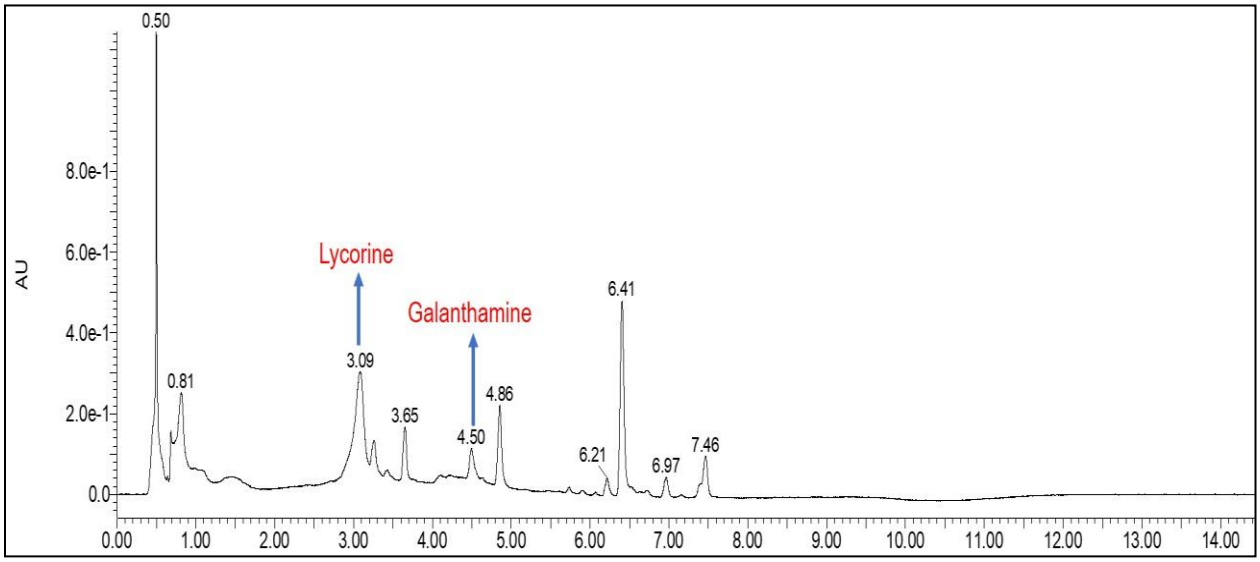
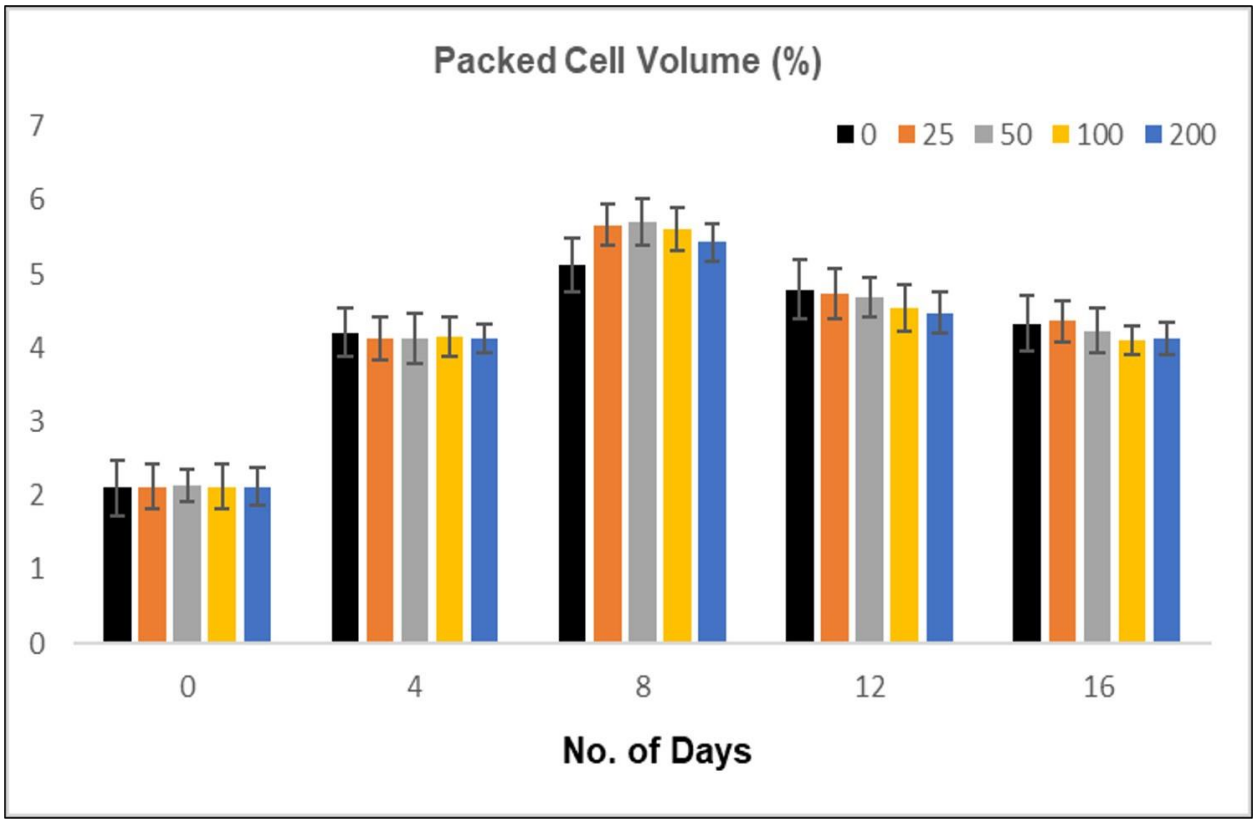


Figure 4.14 The bar diagram depicts the growth pattern of cells during elicitation with methyl jasmonate as the elicitor and a Chratogram of methyl jasmonate. Values represent mean ± SD



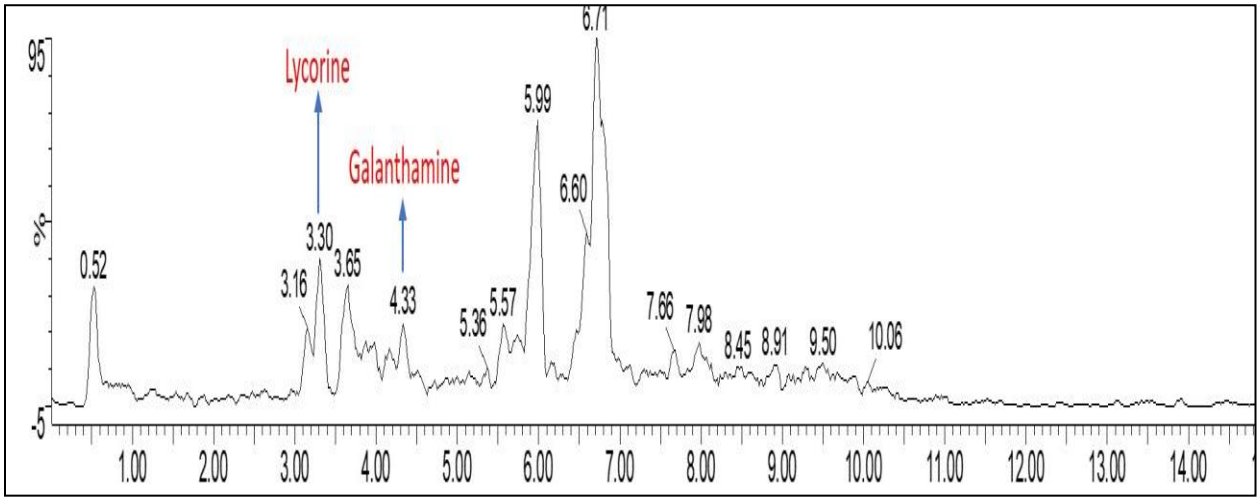


Figure 4.15 The bar graph depicts the enhancement with Salicylic acid as the elicitor and chromatogram of Salicylic acid.

Sr No.	Sample Name	LY (mg/gm DW)	GAL (mg/gm DW)
1	Control	5.09±0.60	1.78±0.53
2	Suspension culture + JA acid	5.22±0.62	1.62±0.58
3	Suspension culture + MJ	4.12±0.47	0.72±0.49
4	Suspension culture +SA acid	2.10±1.19	0.54±0.90

Table4.4- HPLC analysis of lycorine (LY) and galanthamine (GAL) from the elicitated sample collected on 8th day of *Crinum brachynema*

4.4 Assessment of genetic fidelity in regenerated *Crinum brachynema.*

4.4.1 Clonal fidelity assessment

The clonal fidelity studies help in the assessment and prediction of the genetic trueness between the mother plants and regenerated clones [251, 252]. SCoT markers are an advanced type of more reliable and reproducible molecular markers [347] that can anneal specifically to the target region of DNA with ATG sequence which is considered the starting codon region of the translation process [254-256]. In regenerated plants, it is crucial to evaluate the genetic stability, especially when plants are produced through somatic embryogenesis with an intermediate callus phase. Moreover, it is advantageous to use different DNA-based technique for genetic fidelity of *In-vitro*-raised plantlets [259, 262]. The current report validates the genetic study assessment of plant compared with the mother plant using different markers (SCoT, ISSR, and RAPD) (Fig 4.14). A total of ten SCoT and ISSR primers and eight RAPD primers-based fingerprinting were performed by randomly selecting seven regenerants derived from somatic embryos *In-vitro* compared with the mother plant (Table 4.6-4.9). While verifying the genetic stability, SCoT amplified primers had shown good amplification with the generation of a total of 18 scorable DNA bands with amplified band sizes of 220 bp to 2000 bp (Table 4.7). The ISSR primers scored 22 bands with sizes of 250 bp to 950 bp (Table 4.8), while the RAPD primers produced 10 bands in a range of 400bp to1300 bp (Table 4.9). The generated bands produced with SCoT, ISSR, and RAPD were monomorphic and since true-to-type plantlets were obtained via somatic embryogenesis no polymorphism was observed (Fig 4.14). These markers analyses were also

utilized in other plant species such as *Eclipta alba* [271], *Helicteres isora* [348], *C. malabaricum* [101] *Cicer arietinum* L. [258], *Lippia javanica* [261], *Iris x hollandica* [256], Congenesis-Robusta (CxR) cultivar [349]. The present strategy describes the efficient utilization of more than one marker which is also validated by the results of Bhattacharyya et al. [350], Sadhu et al. [258], and Mood et al. [261], which indicates that a single marker is not much effective to screen genetic variations of *In-vitro* regenerated plantlets.

Table 4. 5 Genetic homogeneity analysis of *C. brachynema* using SCoT markers

S. No.	Primer code	Primer Sequence (5'-3')	Number of Scorable bands	Band length of Amplicons
1	SCoT-1	CAACAATGGCTACCACCA	1	500
2	SCoT-2	CAACAATGGCTACCACCC	2	700, 300
3	SCoT-3	CAACAATGGCTACCACCG	2	600, 220
4	SCoT-4	CAACAATGGCTACCACCT	1	510
5	SCoT-5	CAACAATGGCTACCACGA	2	700,400
6	SCoT-6	CAACAATGGCTACCACGC	4	2000,1100,700,400
7	SCoT-7	CAACAATGGCTACCACGG	2	1100, 250
8	SCoT-8	ACGACATGGCGACCAACG	1	250
9	SCoT-9	ACCATGGCTACCACCGAC	1	2000
10	SCoT-10	CCATGGCTACCACCGCAG	2	1000, 800

Table 4.6 Genetic homogeneity analysis of *C. brachynema* using ISSR markers

S. No.	Primer code	Primer Sequence (5'-3')	Number of Scorable bands	Band length of Amplicons
1	ISSR-1	AGAGAGAGAGAGAGAGC	3	400,300,250
2	ISSR-2	AGAGAGAGAGAGAGAGG	1	450
3	ISSR-3	GAGAGAGAGAGAGAGAC	2	580,420
4	ISSR-4	TCTCTCTCTCTCTCTCC	4	700,600,500,450
5	ISSR-5	AGAGAGAGAGAGAGAGYT	2	700,480
6	ISSR-6	AGAGAGAGAGAGAGAGTC	3	950,900,600
7	ISSR-7	CACACACACACACACARC	-	-
8	ISSR-8	TGTGTGTGTGTGTGTGRA	1	500
9	ISSR-9	ACACACACACACACACT	4	800,600,430,300
10	ISSR-10	CTCCTCCTCCTCCTCCTC	3	900,650,550

Table 4.7 Genetic homogeneity analysis of *C. brachynema* using RAPD marker

S. No.	Primer code	Primer Sequence (5'-3')	Number of Scorable bands	Band length of Amplicons
1	OPAA-01	AGACGGCTCC	2	700,450
2	OPAA-02	GAGACCAGAC	2	750, 600
3	OPAB-01	CCGTCGGTAG	3	1300, 900, 400
4	OPAB-02	GGAAACCCCT	3	900, 700, 400
5	OPAD-01	CAAAGGGCGG	-	-
6	OPAD-02	CTGAACCGCT	-	-
7	OPAH-01	TCCGCAACCA	-	-
8	OPAH-02	CACTTCCGCT	-	-

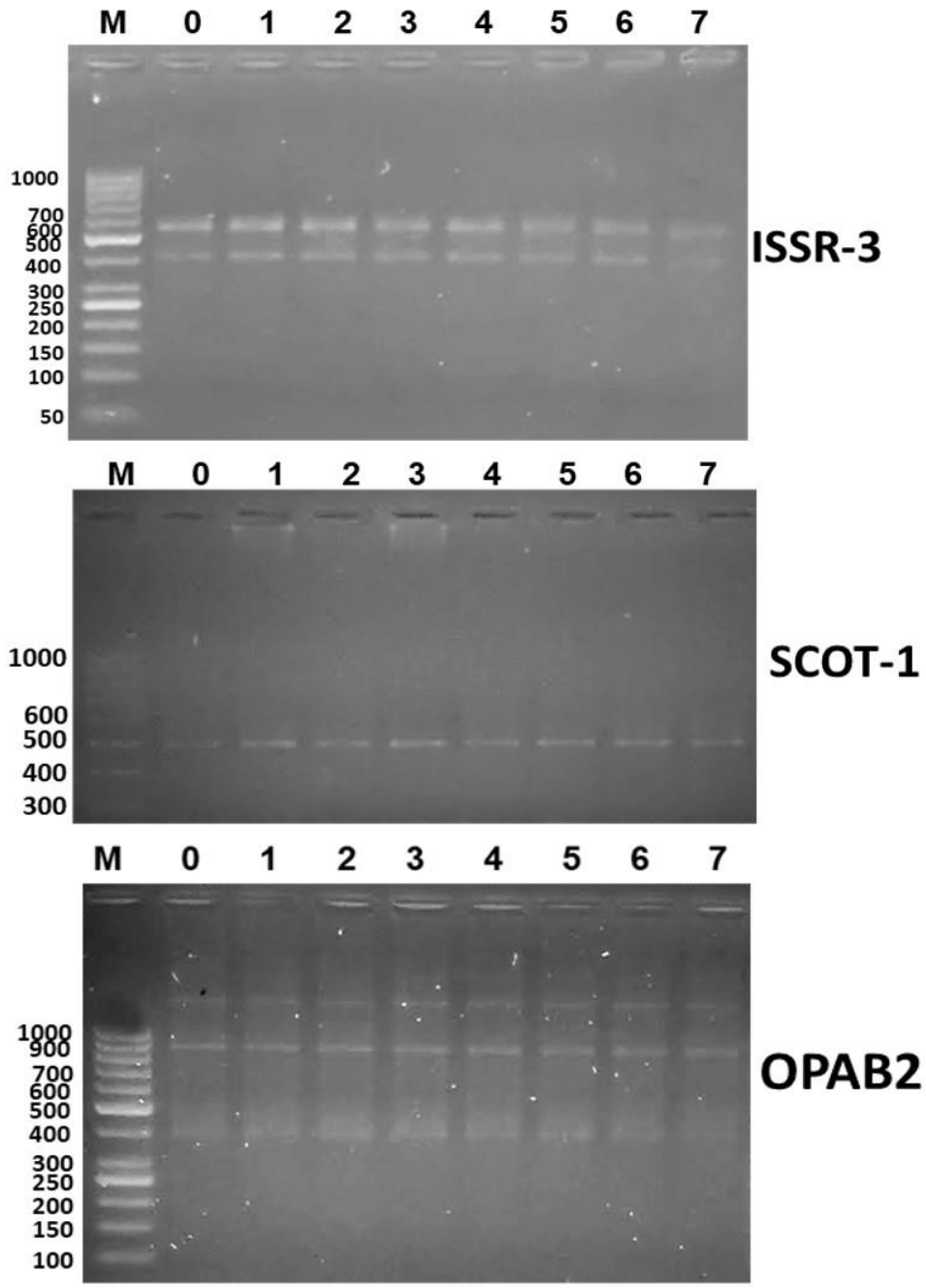


Figure 4.16 Genetic stability assessment in somatic embryogenesis derived plantlets and mother plant of *C. brachynema*. **A.** ISSR amplification profile with primer ISSR-3. **B.** SCoT amplification profile with primer SCoT-1. **C.** RAPD amplification profile with primers OPAB-2. Lane 1 represents the 100bp DNA ladder (M) followed by the mother plant (0) in lane 2. Lanes 3-9 labeled 1-7 are the *In-vitro* raised plant lines via somatic embryogenesis.

4.5 Extraction and analysis of secondary metabolites.

It is expected that advancement in the field of biotechnology, especially in the area of plant tissue cultivation, will open up more opportunities for the commercialization of rare plantlets and bioactive compounds. Climatic or soil conditions are one of the primary advantages of micropropagation for their ability to enhance bioactive compounds in a controlled microenvironment. Biotechnological methods may be used to solve the long growing period of plants in their native environment, and also play an important step in the conservation and quick multiplication of rare, endangered, and threatened plants [267,268,269] The genetic fidelity of *Crinum* sp. was also evaluated for commercial micropropagation. The benefits of *In-vitro* growth, include the ability to cultivate all year round due to the lack of a seasonal variation and resistance to stresses. This strategy may have some advantages such as availability in all seasons, improvised processing, and separation ways, and enhanced secondary metabolite production [273].

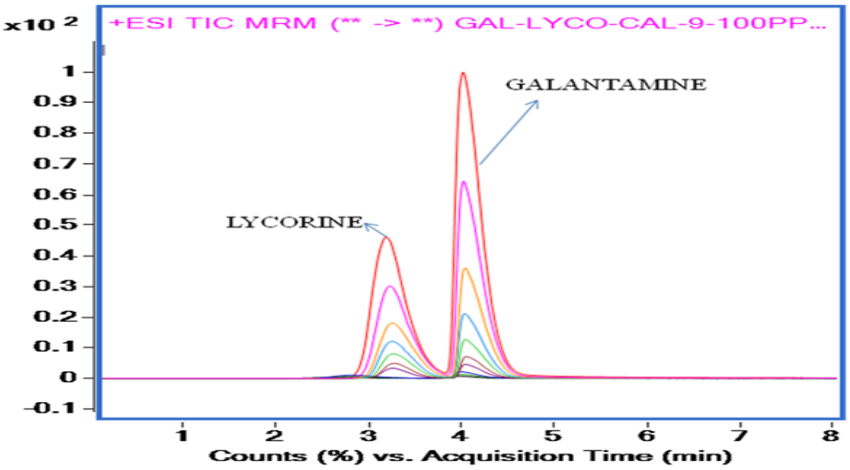
This study provides a current overview of *in-vitro* micropropagation, organogenesis, and hardening, in *Crinum* spp., also focusing on its main phytoconstituent galanthamine.

Chromatographic separation has revealed the highest content of Galanthamine of about 0.3080.004% in *C. malabaricum* bulbs [49]. In *C. asiatticum*, the highest galanthamine was found in xanthphyllum [35]. Other sources have also been discovered for the production of galanthamine and gal-derivatives from leaf cultures of *C. powellii* and *C. asiaticum*; and from the bulb cultures of *C. moorei*, *C. Amabile, and C. defixum* [128]. Galanthamine was directly extracted through the proliferation of shootlets and bulbs from triple-scales on MS media enriched with 1.0 mg/L IBA (for bulblet formation) and 2.0 mg/L BA (for shoot formation) of *C. malabaricum* [101].

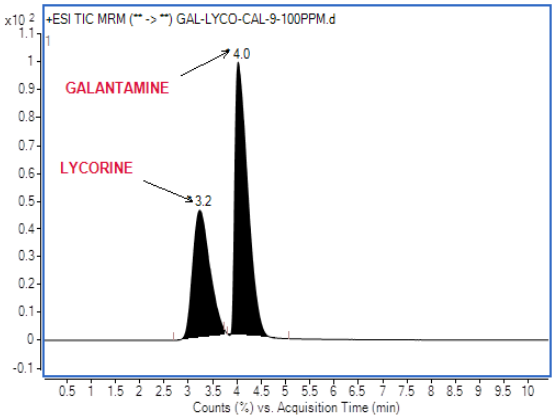
To date very limited studies, including our recent report [27] have been reported on conservation and *In-vitro* regeneration of *Crinum species* [101]. Production of *In-vitro* plantlets via somatic embryogenesis in a short period is beneficial for rapid propagation and secondary metabolite production [307-311, 351]. We have also developed, for the first time, a rapid method for phytochemical analysis by (LC-ESI/MS) of galanthamine and lycorine from methanolic extracts of *In-vitro* raised plants derived from somatic embryogenesis.

4.5.1. LC-ESI-MS/MS analysis

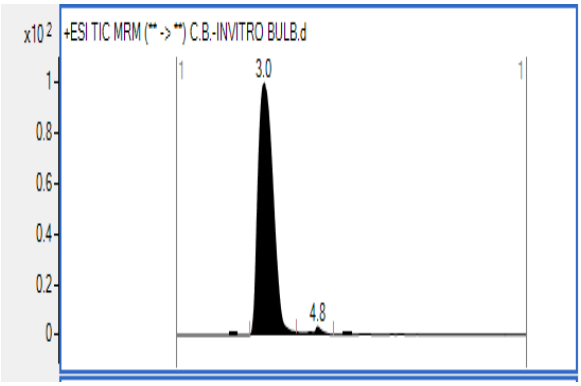
The efficiency of in-vitro plants to produce specialized molecules is an important and great application of tissue culture. There are well-documented reports demonstrating that *In-vitro* cultured plants are important for the formation and production of secondary metabolites [165, 188, 352, 353]. Lycorine and galanthamine were identified and compared with peaks of MRM and retention time (Fig 4.15). According to the data, the methanolic extracts of *In-vitro* raised plantlets confirmed the presence of galanthamine ($C_{17}H_{21}NO_3$) and lycorine ($C_{16}H_{17}NO_4$). The exact mass for galanthamine and its retention time are 287.15 and 4.0 min, whereas for lycorine, the exact mass and retention time are 287.12 and 3.2 min respectively (Fig 4.15,4.16). The MRM peak of galanthamine and lycorine was used for futher quantification analysis. The concentration of galanthamine and lycorine in the *In-vitro*-raised plants were 69 µg/DW and 1600 µg/DW, respectively. Considerable differences were obtained in the content of galanthamine and lycorine. Our results are in line with [22] who showed a high content of lycorine in naturally grown *C. brachynema* whereas, galanthamine was low. Production of galanthamine and lycorine through *In-vitro* culture of other Amaryllideace species such as *Leucojum aestivum* [192], *Pancratium maritimum* L., *Narcissus pseudonarcissus* cv. Carlton [191], and *Narcissus tazzeta* L. [188] have been also reported. However, there is no report available on galanthamine and lycorine production from somatic embryogenesis-derived plantlets of *Crinum brachynema*.



Calibration curve for LYCORINE and GALANTHAMINE



Standard chromatogram of Lycorine and Galanthamine



Chromatogram of *In-vitro* Bulb extracts of *C. brachynema*

Fig. 4.17 Standard chromatogram of Galanthamine and Lycorine, and chromatogram of *In-vitro* bulbs

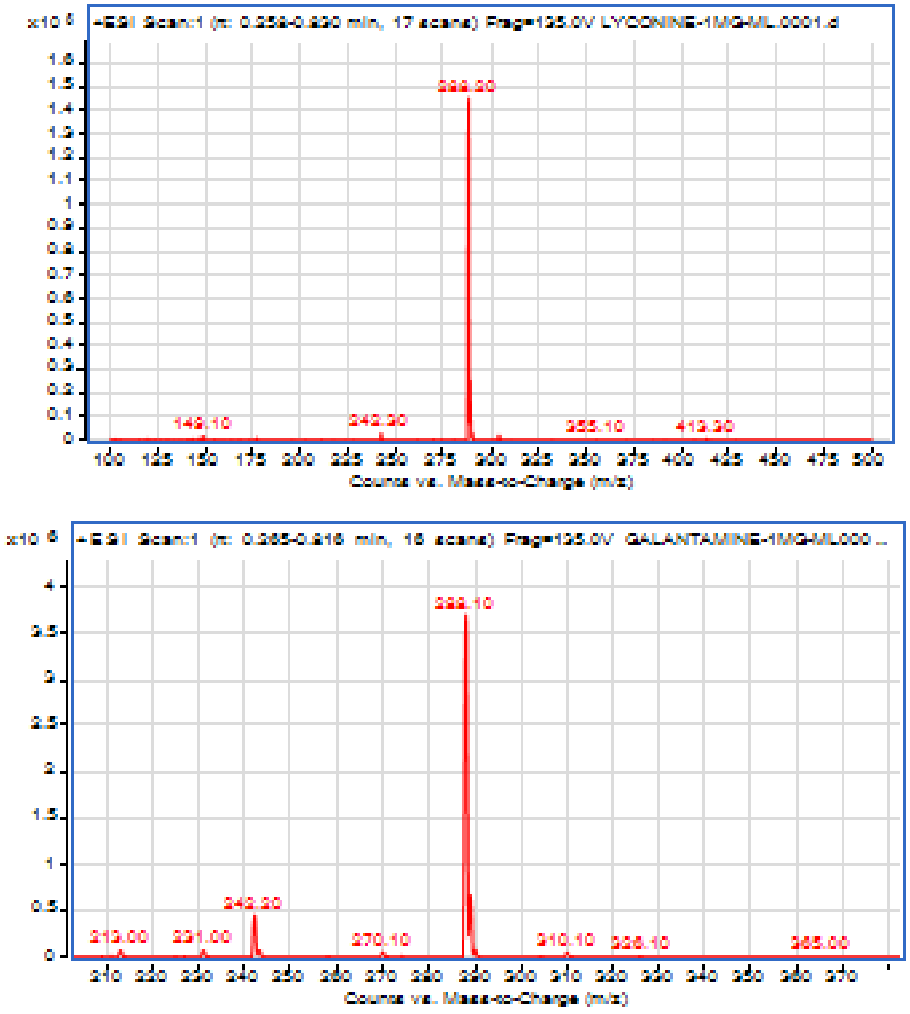


Fig. 4.18 Mass spectra of compound Galanthamine and lycorine

Chapter 5

Conclusion

Humans have traditionally relied on plants to remedy a broad variety of issues, leading in many cases to an unanticipated decline in natural population owing to over-harvesting and environmental deterioration. New possibilities for the commercialization of even uncommon plants and their components are anticipated with advancement in the biotechnological process. The decorative and medicinal plant *Crinum* spp. is discussed here because it contains various significant compounds with therapeutic potential, and this report addresses their relevance. Overharvesting has put this plant at risk of extinction in its native range. In light of the importance of *Crinum* sp. for therapeutic purposes, this study analyses the present status of *In-vitro* biotechnology strategies for its conservation and extensive proliferation for the commercial production of galanthamine. Artificial seeds, *ex-situ* plantings, and roots, on the other hand, have received less attention in the scientific community. However, more research is needed to address several concerns with micropropagation methods and give answers to the obstacles connected with *In-vitro* optimization. In conclusion, the *Crinum* genus (Amaryllidaceae) has a long history of therapeutic usage. Surprisingly, several species have been tried for a single illness with varying degrees of success. This work exemplifies the Indian *Crinum*'s tremendous therapeutic significance, demonstrating its amazing potential on a worldwide scale.

- Development of regeneration protocol for *Crinum brachynema* through *In-vitro* techniques.

The findings in Chapter 3 explain how a highly efficient and improved plant regeneration protocol for *Cinum brachynema* was developed. In this study, bulb explants were inoculated into an MS medium supplemented with various PGRs, which resulted *In-vitro* regenerated plantlets, callus initiation, and direct shoot and root induction. As an economically significant but endangered plant, the current protocol for *Crinum brachynema,* micropropagation may be useful for the mass production of clonal plants for *ex-situ* germplasm conservation, re-establishment in wild habitats, and commercial use in pharmaceutical industries. In this study regeneration of microshoots via *bulb* explants in *C. brachynema* was established using different concentrations of BA and NAA. *mT*-mediated regeneration significantly influenced the regeneration efficiency. The protocol could be useful for mass propagation and genetic transformational stuides.

Only a few Indian *Crinum* species have been subjected to rigorous scientific evaluation, including *In-vitro* conservation, toxicity, and safety testing. According to the evidence, several Indian *Crinum* species are at risk of over-exploitation, and many have become endangered as a result of a lack of knowledge in both conservation and traditional usage. As a result, the development of various biotechnological approaches ensures that these species are available when needed. Aside from traditional techniques, the use of *in-vitro* studies model systems will be beneficial for the conservation and improvement of mass production of these species, which often grow slowly. Finally, new research may improve understanding of the therapeutic and safety factors in the use of Indian *Crinum species*, while various biotechnological tools are required for their long-term use and conservation.

- Somatic embryogenesis of Crinum brachynema.

This is the first report for the development of somatic embryogenesis system of *C. brachynema* offers an opportunity to ensure germplasm conservation, to control destruction threats, and it also delivers a potential system for the analysis of secondary metabolites. The induction and production of somatic embryos were significantly influenced by the combination of picloram (2.0 mg/l) and TDZ (0.5 mg/L). The SEM analysis confirms the development differentiation of somatic embryos. The banding pattern from SCoT, ISSR and RAPD markers analysis proved that the in-vitro clones highly resembled to their mother plant which ruled out any somaclonal variation induced during somatic embryogenesis. The established model system enables the mass multiplication of genetically stable *C. brachynema* plants and could be employed as a potential source of active biomolecules in several ailments. In addition to the considerable work carried out, in-depth studies including genetic and metabolic engineering should also be performed in future to maximize the productivity of valuable alkaloids present in this species

The pharmaceutical, nutraceutical, and cosmetics sectors are only a few examples of the expanding market for commercialized medicinal plants. The Amaryllidaceae plant family has been used to cure a wide range of illnesses for centuries in traditional medicine. It is well-known for the wide variety of pharmacological effects produced by its structurally distinct alkaloids. The *Crinum* bulbous geophyte is well appreciated for its aesthetic and medicinal value. There are

15 species of *Crinum* in India, and each one of them includes several alkaloids with useful biological effects. However, further pharmacological research is needed into the therapeutic potential of several Indian *Crinum* species. To ensure its continued use and protection, additional in-depth study is needed, ideally using some different biotechnological techniques. The developed somatic embryogenesis protocol may also facilitate mass propagation of high quality true-to-type *C. brachynema* plants for conservation and can be utilized as a potent source of valuable alkaloids to treat several diseases. Our existing knowledge of the bioactive components, toxicity, clinical significance, and conservation status needs to be strengthened by more studies.

- Maintenance of cell suspension and study effects of elicitors on secondary metabolite production.

There has been significant interest in the phytochemical and pharmacological properties of natural products for quite some time. One of the most common places to find them is in plants since they offer a rich, untapped supply of bioactive compounds. Medicinal plants have the potential to magically give us the solution to our greatest problem: illness. The pharmaceutical, cosmetic, and culinary sectors also made extensive use of them. Despite breakthroughs in synthetic organic chemistry, natural plant products are gaining attention for their potential to combine ancient wisdom with modern scientific understanding to address a wide range of healthcare issues. The negative connotation attached to synthetic pharmaceuticals is largely attributable to the public's awareness of the harm they cause. Therefore, it is crucial to produce pharmaceuticals that are both naturally derived and highly effective, safe, and affordable, and medicinal plants seem to provide these desirable advantages.

Crinum is presented as an inexhaustible supply of bioactive components, as shown by the aforementioned research. This assertion is supported by four pieces of evidence. To begin, just roughly 35 out of 130 species have been researched phytochemically, while the great majority are still unknown. Second, it is believed that hybridization between *Crinum* species is rather prevalent. New hybrids and previously unknown species may pave the path for the discovery of previously unknown chemicals. Additionally, chemotaxonomic examination of these unstudied species will be of considerable help in clarifying the complicated taxonomic situation that currently exists within the genus. The third piece of evidence is the underexplored potential of non-alkaloidal and polar components in chemistry and biology. Fourth, further pharmacological

research is to confirm the effectiveness of various *Crinum* extracts and purified components from a biological perspective. Positive and original actions are eagerly awaited.

GAL is a specialized alkaloid uniquely present in Amaryllidaceae plant species. Due to its high medicinal importance, studies on the 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 scaling process to achieve the crucial step from the laboratory to the market.

Additionally, differentiating aspects of the potential production of GAL using *In-vitro* cultures in TIBs 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 CO_2; (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, and thus generate active plant molecules to be purified more easily. At the same time, research on the genetic and molecular mechanisms of different alkaloids should be continued as a crucial point to optimize GAL production. This approach will help in overcoming challenges associated with lower yields. The pharmacogenomics research initiatives to understand GAL biosynthesis using genetic and biochemical approaches are crucial.

In the future, knowledge about specific Amaryllidaceae species selection will lead to the optimization of industrial GAL production. Furthermore, the identification of other functional enzymes for GAL biosynthesis will surely lead to more strategies for the effective mass production of GAL. Finally, new, and in-depth research may allow a better understanding of different factors in GAL biosynthesis, while different biotechnological approaches are also required for its high accumulation and sustainable production.

This work has been accompanied by the goal of increasing galanthamine production *In-vitro* cultures of *Crinum brachynema* using the elicitation method. Plant cultures of *Crinum brachynema* were elicited using different concentrations of MeJa, JA, and SA as elicitors. The current study's findings revealed that elicitation was a more significant method for increasing galanthamine and lycorine production. The study also found that galanthamine and lycorine

content in *C. brachynema* cultures varied significantly depending on the type and concentration of elicitor used, as well as exposure time. Furthermore, elicitor treatment can be used to increase the production of bioactive compounds and the mass propagation of medicinal plants. Furthermore, more research is needed to develop metabolic engineering methodologies that would improve the production of valuable secondary metabolites in *C. brachynema in-vitro* cultures. In the present study elicitation experiments were done which provided the preliminary idea regarding the elicitor concentration and its optimization. These studies lead towards a successful elicitation experiment on pilot scale.

Crinum brachynema is a rich source of bioactive compounds, but it has been designated as critically endangered. To meet the high industrial demand for this species, conservation strategies for *Crinum* brachynema or the identification of an elite source/good substitute are required. Because *Crinum brachynema* is an unexplored species, screening major bio-active compounds in this plant could lead to the discovery of additional important sources in the future. The use of molecular and phytochemical tools like those used in our study would be beneficial in identifying useful genotypes for herbal drug formation. Optimized concentrations of various elicitors effectively increase the significant bioactive compounds found in *Crinum brachynema*. These findings will be useful in the future for large-scale production of galanthamine, lycorine, and various alkaloids from different *In-vitro* cultures of *Crinum brachynema* or related genera.

- Assessment of genetic fidelity in regenerated *Crinum brachynema*

The selection and screening of top genotypes, which would further enrich the gene pool of this species, is significantly aided by the evaluation of genetic diversity with the association of molecular and phytochemical markers. ISSR, RAPD, and SCOT primers were used to conduct this study to evaluate the genetic diversity of *Crinum* species that were gathered in the area of the Indian Western Ghats. This is the first study of its kind. The banding pattern from SCoT, ISSR, and RAPD markers analyses of *In-vitro* clones highly resembled their mother plant which ruled out any somaclonal variation induced during somatic embryogenesis. The genetic true-to-typeness of regenerated plants was confirmed by ISSR, SCoT and RAPD primers based molecular analyses. This confirmed their genetic homogeneity of in vitro and in vivo plant. The developed protocol may also be effective for *C. brachynema* 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, in-depth studies including genetic and metabolic engineering should also be performed in the future to maximize the productivity of valuable alkaloids present in this species. The results of this research showed that there is a significant connection between the genetic makeup, chemical profiles, and geographic locations of plant accessions. In addition, the results of this research will be helpful in the formulation of urgent conservation plans and breeding programs for the *Crinum brachynema* species.

- Extraction and analysis of secondary metabolites.

The investigations of the Indian *Crinum*'s phytochemistry, pharmacology, and *In-vitro* efficacy are summarised in the present report. Prospects, potential toxicity, and gaps in knowledge are also explored. People have turned to the ancient remedy *Crinum* for help with their health problems for a long ago. The analgesic, anticholinergic, anticancer, and antiviral effects of the most well-known Amaryllidaceae alkaloids have only lately been discovered by phytochemical studies. Micropropagation methods are being studied by scientists as a means to enhance phytochemical production in a laboratory setting for these reasons.

The efficiency of *in-vitro* plants to produce specialized molecules is an important and great application of tissue culture. There are well-documented reports demonstrating that *in vitro* cultured plants are important for the formation and production of secondary metabolites. We developed an efficient protocol of plant regeneration through direct and indirect organogenesis via *in vivo* bulb explant in *C. brachynema* . Two major secondary metabolites (Lycorine and Galanthamine) were analyzed and quantified using high performance liquid chromatography. Amount of secondary metabolites was found significantly higher, in *in vitro* plantlets compared to *in vivo* plantlets. Effect of three elicitors: Jasmonic acid, Methyl jasmonate and Salicylic acid on cell growth as well as secondary metabolite accumulation in cell suspension cultures of *C. brachynema* was investigated. The galanthamine and lycorine were detected. A rapid and convenient LC/ESI-MS technique has been investigated for the extraction and identification of alkaloids (galanthamine and lycorine) from *in vitro* extract of *C. brachynema*.

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ORIGINAL ARTICLE

Meta-topolin induced in vitro regeneration in *Crinum brachynema* (Amaryllidaceae): a critically endangered and endemic medicinal plant of India

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Abstract
Crinum brachynema (Amaryllidaceae) is an endemic and critically endangered bulbous medicinal plant of the Northern Western Ghats of India. This species contains an important and promising alkaloid called galanthamine (GAL), an anti-Alzheimer drug. This investigation aimed to establish a reproducible protocol for in vitro regeneration of *C. brachynema* using twin scales as initial explants for the first time. Thus, cultures were established on Murashige and Skoog (MS) medium with cytokinins (6-benzyladenine (BA), and *meta*-Topolin (*m*T). The establishment and growth of shoots from the explants was achieved on MS medium supplemented with BA alone and in combination with 1-naphthaleneacetic acid (NAA). In vitro shoots were cultured on MS medium containing 0.5–10 µM of *m*T, of which 5.0 µM *m*T gave the highest shoot regeneration frequency (11.10). In general, *m*T proved superior as cultures produced a maximum number of regenerants compared to the control. Rooted plants with bulblets were successfully acclimatized in the greenhouse where they were phenotypically similar to the mother plants. Antioxidant activity was estimated using oxygen radical scavenging capacity (ORAC) model. Cultures on *m*T exhibited a significant increase in antioxidant capacity compared to 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. brachynema*, while the regenerated *C. brachynema* may be subsequently manipulated to further increase the accumulation of antioxidant potential.

Key Message
An efficient regeneration strategy was developed for conservation of *Crinum brachynema*, a critically endangered medicinal plant, using twin-scale explants; meta-Topolin enhanced in vitro shoot proliferation and improved regenerants antioxidant activity.

Keywords *Crinum brachynema* · Critically endangered · Conservation · *meta*-topolin · Plant growth regulators · Regeneration

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REVIEW

Harnessing plant biotechnology-based strategies for in vitro galanthamine (GAL) biosynthesis: a potent drug against Alzheimer's disease

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Abstract
Medicinal plants are important for improving human health and represent an essential pool for the identification of novel pharmacological leads. Plant-derived biomolecules have historically proven their value as a source of therapeutic drugs and hold an important potential 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. Galanthamine (GAL) is a selective, reversible and an Amaryllidaceae-derived acetylcholinesterase inhibitor used for the treatment of Alzheimer's disease (AD) and other neurological diseases. Naturally, the bioavailability of Amaryllidaceae alkaloids including GAL is low. Due to the significantly increased demand of GAL by the pharmaceutical industries and the inadequate availability of natural resources, in vitro culture offers an alternative approach for its sustainable production. Thus, different biotechnological tools can optimize the in vitro GAL biosynthesis for treating AD, such as manipulation of plant growth regulators, photoperiod, elicitors, and bioreactors systems, besides being an environmentally sustainable approach, which protects the native biodiversity in a circular bioeconomy context. In the present review, we highlight the biosynthesis of GAL by plant in vitro systems including its mode of action. This article should also provide a starting point in the scaling-up of the biotechnological production of this valuable alkaloid.

Key message
Galanthamine biosynthesis by plant in vitro systems and its mode of action are reviewed to provide a starting point for scaling-up of the biotechnological production of this valuable medicinal alkaloid.

Keywords Alzheimer disease · Bioreactors · Biosynthesis · Elicitors · Galanthamine · In vitro system

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ORIGINAL ARTICLE

Somatic embryogenesis and genetic homogeneity assessment of regenerated plants of *Crinum brachynema* (Amaryllidaceae): an endemic critically endangered medicinal plant

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Abstract

Crinum brachynema is a bulbous plant belonging to the family Amaryllidaceae which is restricted to Western India. Due to its high rarity and low distribution range, it has been classified as "critically endangered". Establishing an efficient and unprecedented somatic embryogenesis protocol is necessary for its conservation and large-scale propagation. In this study, regeneration was achieved through somatic embryogenesis using bulb explants on MS media supplemented with various concentrations of 2,4-D alone and in combination with N^6-benzyl-adenine. Different advanced phases with maturation of somatic embryo were obtained on MS medium with different ratios of picloram and thidiazuron (TDZ). The highest number of somatic embryos (50.33 ± 1.52) was obtained after eight weeks in the medium supplemented picloram (2.0 mg L^{-1}) in combination with TDZ (0.5 mg L^{-1}). MS medium with reduced concentration of salts in combination with GA_3 (1.0 mg L^{-1}) was used for somatic embryo germination. The maximum embryo germination frequency (82.22) was recorded on half-strength MS medium fortified with 1 mg L^{-1} GA_3. The genetic true-to-typeness of regenerated plants was confirmed by ISSR, SCoT and RAPD primers based molecular analyses. This confirmed their genetic homogeneity compared to the mother plant and it also demonstrated the reliability of our somatic embryogenesis system for *C. brachynema*. The protocol developed may facilitate efforts in reintroduction, restoration, and ex situ conservation of *C. brachynema* in its natural habitat and its potential commercial utilization.

Key message

We provided the first report on somatic embryogenesis system in *C. brachynema*. SEM indicated the morphogenesis and several molecular markers revealed genetic homogeneity of the regenerated plants.

Keywords Conservation · *Crinum Brachynema* · Critically endangered · Genetic fidelity · Molecular markers · Somatic embryo

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Abbreviations

ANOVA	Analysis of variance
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
CTAB	Cetyl trimethyl ammonium bromide
DMRT	Duncan's multiple range test
dNTP	Deoxynucleotide triphosphate
GAL	Galanthamine
GA_3	Gibberellic acid
IUCN	International union for conservation and nature
ISSR	Inter simple sequence repeat
LED	Light-emitting diode
MS	Murashige and Skoog
PCR	Polymerase chain reaction

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Meta-topolin-mediated regeneration and accumulation of phenolic acids in the critically endangered medicinal plant *Crinum malabaricum* (Amaryllidaceae): A potent source of galanthamine

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ABSTRACT

Crinum malabaricum (Family: Amaryllidaceae) is a critically endangered aquatic medicinal plant endemic to India. This species is a promising natural source of bioactive compounds including galanthamine (GAL), an anti-Alzheimer drug. *In vitro* regeneration in the Amaryllidaceae is often challenging. This study assessed the use of meta-Topolin (mT) on *in vitro* regeneration of *C. malabaricum*. Shoot explants were cultured on Murashige and Skoog (MS) medium supplemented with 0.5, 2.5, 5.0, 7.5 and 10.0 μM mT for six weeks, whereby 7.5 μM mT resulted in the maximum multiplication of adventitious shoots, much higher than the control. The biochemical accumulation of eleven different phenolic acids was quantified by UHPLC-MS/MS analysis, and it appeared that mT-treated cultures exhibited the highest concentration of phenolic acids. In particular, increased concentrations of gallic acid, protocatechuic acid, syringic acid, p-hydroxybenzoic acid, salicylic acid and vanillic acid were detected compared to the control. mT (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. These findings highlight the beneficial effect and validate the rising importance of mT for *in vitro* regeneration studies. This study will serve as a potential protocol to conserve and restore the medically important *C. malabaricum*.

1. Introduction

The Amaryllidaceae J.St.-Hil. is a monocotyledonous family comprising herbaceous and perennial flowering plants (Christenhusz and Byng, 2016; Chahal et al., 2021). These bulbous geophytes typically occupy tropical to warm-temperate locations of the globe (Christenhusz and Byng, 2016). With over 1600 species in around 100 genera, it is one of the more prominent members in the order Asparagales (Christenhusz and Byng, 2016), where Crinum with more than 100 species globally to its name, is one of the largest genera of Amaryllidaceae (Meerow and Snijman, 2001; Govaerts et al., 2012). About 13 *Crinum* species are known to inhabit the Indian region, including 5 endemic species (Lekhak and Yadav, 2011).

The 'Malabar River Lily' *Crinum malabaricum* Lekhak & S.R. Yadav, a critically endangered bulbous species is a promising source of galanthamine (GAL) (Lekhak and Yadav, 2012; Lansdown, 2016). *C. malabaricum*, locally known as "Kanthanga", is restricted to a natural habitat in Periya village, Kasaragod district of Kerala, India and only around 1000 plants are available in a 0.5 km^2 area (Lekhak and Yadav, 2012; Chahal et al., 2021; Priyadharshini et al., 2021) (Fig 1A). It is well known for its medicinal properties and therapeutic action, including anticancer and anti-Alzheimer, which are due to the occurrence of various important alkaloids (Refaat et al., 2013; Uddin et al., 2012; Ghane et al., 2018; Jin and Yao, 2019). In this regard, GAL (Fig 1G) is recognized as one of the most successful drugs of choice for mild to moderate Alzheimer's disease (Heinrich and Teoh, 2004; Heinrich, 2010). The effect of GAL is played out via its potent inhibitory action on acetylcholinesterase (AChE), thereby increasing the availability of acetylcholine for synaptic transmission (Heinrich, 2010). A few studies have shown that GAL has been used as a

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Unraveling the medicinal potential and conservation of Indian *Crinum* (Amaryllidaceae) species

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ABSTRACT

The commercialization of medicinal plants as recognized in their uses by nutraceutical, cosmeceutical and pharmaceutical industries is gaining more popularity and interest around the globe. The plant family Amaryllidaceae has a long history in the traditional medicinal system and used to treat diverse diseases. It is well known for its structurally-diverse alkaloids which exhibit a wide range of pharmacological activities. *Crinum*, an important bulbous geophyte is well known for its ornamental and therapeutic properties. In India, *Crinum* is represented by 15 different species which contain several alkaloids with biological properties. However, some of the medicinal uses of Indian *Crinum* species still require pharmacological analysis. In addition, more extensive research including different biotechnological approaches is needed in order to guarantee its sustainable utilization and long-term conservation. Further research is essential to improve our current knowledge about the bioactive components, toxicity, clinical relevance as well as its conservation status. The present review summarizes the research that has been done on the phytochemistry, pharmacology and in vitro studies of the Indian *Crinum*. Potential toxicity aspects as well as knowledge gaps and future perspectives are also discussed.

1. Introduction

Medicinal plants are mainly used as herbal medicine and play an essential role in primary health care, ethno-medicine system and production of appropriate drugs. Due to this multi-usage, trade network demand for the different medicinal species can extend to national as well as international boundaries. The Amaryllidaceae J. St.-Hil is a monocotyledonous plant family, widely distributed around the globe and well known for its exceptional alkaloid principles and unique structural features, which have a diverse range of biological properties (Dahlgren et al., 1985; Meerow and Snijman, 2006; Habartová et al., 2016; Nair et al., 2017; Nair and Van Staden, 2018, 2019). It comprises three different subfamilies including Agapanthoideae, Amaryllidoideae and Allioideae (APG III, 2009). The Amaryllidaceae includes about ± 80 genera and ± 1200 species distributed throughout the tropical and subtropical regions including warm temperate zones (Bastida et al., 2011; Rønsted et al., 2012; Jin and Xu, 2013; Christenhusz et al., 2017; Ghane et al., 2018). Amaryllidaceae alkaloids are specific types of isoquinoline alkaloids and have a broad range of biological activities(Nair et al., 2017; Habartová et al., 2016; Nair and Van Staden, 2018, 2019). Among the Amaryllidaceae alkaloids, galanthamine (GAL) is well known for its pharmacological effects, having importance in medical systems where it is a proven selective, long-lasting and competitive inhibitor of the acetylcholinesterase enzyme (Maelicke et al. 2001; Habartová et al., 2016). Besides, it is also utilized for the development of modern drugs and in the treatment of Alzheimer's disease (AD) (Scott and Goa, 2000; Heinrich and Lee, 2004; Marco and do Carmo Carreiras, 2006). Some studies have shown the global benefits of GAL for Alzheimer's patients and an extensive clinical trial has reported positive findings (Olin and Schneider, 2002; Jagtap et al., 2014).

Abbreviations: AD, Alzheimer's disease; ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); BA, N^6-benzyladenine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing anticoxidant power; GAL, galanthamine; HPLC, high performance liquid chromatography; IR, infrared spectroscopy; ISSR, inter simple sequence repeats; IUCN, international union for conservation of nature; MS, Murashige and Skoog medium; NAA, α-naphthalene acetic acid; NMR, nuclear magnetic resonance; ORAC, oxygen radical antioxidant capacity; PTC, plant tissue culture; SCoT, start codon targeted polymorphism; TLC, thin layer chromatography

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MINI-REVIEW

Biotechnological interventions of in vitro propagation and production of valuable secondary metabolites in *Stevia rebaudiana*

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Abstract

Plant cell and tissue culture makes provision of a sustainable and nature-friendly strategy for the production of secondary metabolites, and modern progress in gene editing and genome engineering provides novel possibilities to improve both the qualitative and quantitative aspects of such phytochemicals. The ever-expanding quest for plant-based medicine to treat diabetes facilitates large-scale cultivation of *Stevia rebaudiana* to enhance the yield of its much-coveted low-calorie sweetener glycosides. The potential to process stevia as a "natural" product should enhance the acceptance of steviosides as a natural calorie-free sweetener especially suitable for use in diabetic and weight control drinks and foods. Besides sweetener agents, *S. rebaudiana* is a potent source of many antioxidant compounds and is used to cure immunodeficiencies, neurologic disorders, inflammation, diabetes mellitus, Parkinson's disease, and Alzheimer's disease. This comprehensive review presents the research outcomes of the many biotechnological interventions implicated to upscale the yield of steviol glycosides and its derivatives in in vitro cell, callus, tissue, and organ cultures with notes on the use of bioreactor and genetic engineering in relation to the production of these valuable compounds in *S. rebaudiana*.

Key points

- *Critical and updated assessment on sustainable production of steviol glycosides from Stevia rebaudiana.*
- *In vitro propagation of S. rebaudiana and elicitation of steviol glycosides production.*
- *Genetic fidelity and diversity assessment of S. rebaudiana using molecular markers.*

Keywords Steviol glycosides · Phytochemistry · Micropropagation · Elicitation · Biotechnology

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Introduction

With the advancing world and modernization, we have been introduced to new eating habits, foods that are heaven in taste but are the reason behind the diseases that are fatal in nature. Out of these diseases, diabetes is one of the most commonly occurring diseases around the globe (Genco et al. 2020). Obesity brings elevated risk of type 2 diabetes in comparison to the healthy individuals (Ortega et al. 2020). India, presently with 67 million confirmed diabetic cases and 30 million prediabetic patients, is facing diabetes almost as an epidemic. Diabetes mellitus (type 2 diabetes) presents an array of metabolic disorders. Diabetes II represents a number of metabolic disorders in which affected people exhibit high level of blood glucose or blood sugar, either due to insufficient insulin production or due to the unresponsiveness of the body cells to insulin, or both. In case of diabetes I though, the body does not produce insulin (Eizirik et al. 2020). Approximately

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Review

CRISPR/Cas9-mediated genome editing is revolutionizing the improvement of horticultural crops: Recent advances and future prospects

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ABSTRACT

In modern agriculture, horticultural crops are an integral component and play a vital role in sustaining human life. Although conventional approaches have contributed to the enhanced agricultural production, innovative breeding technology is required to further increase horticultural and nutritious crop worldwide. Genome editing has revolutionized the plant world by precisely edit the targeted modification of plant genome, including model plants, agricultural and horticultural crops. Recent advances in Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR/associated protein 9 (Cas9) genome editing tool have emerged as an efficient targeted modification in most fruit, vegetable and ornamental plants thus promising to accelerate crop improvement due to its high accuracy and efficiency. Several reports of the development of improved fruit, vegetable and ornamental plants using CRISPR/Cas9 tool has been documented. CRISPR/Cas9 genome editing efficiency depends on several factors such as promoter sequence, RNA polymerase specificity, Cas9 expression, types of vectors, guide RNA expression etc. In the present review, we summarize the applications of CRISPR/Cas9 genome editing system in fruit, vegetable and ornamental crops. This article also provides its origin, mechanism, an existing pitfall and future possibilities for bright future in development of horticultural crops.

Introduction

In the next few decades, the total population is expected to reach around 9.8 billion and global demand for food will be increased by almost 110% than those of 2005 (Tilman et al., 2011; Mishra et al., 2018; Chennakesavulu et al., 2021). In the present scenario, the most critical challenging problem confronting is to ensure food security to the rising population in a sustainable way under escalating climate change (Schaart et al., 2016; Biswas et al., 2021a). There is an urgent need to enhance global food production, however, agriculture is facing multiple constraints such as potential environmental hazards, emergence of pathogens and pests, universal climate change and rapid population growth (Mishra et al., 2018). Conventional breeding techniques based on random selection and crossing, lack of gene knowledge is a very time consuming and laborious method as well as drawbacks with genetic outputs (Schaart et al., 2016). Therefore, an advanced and improved technique may allow us to design and develop strategies to increase sustainable agriculture and accelerate global food production.

The existence of genome sequences for many crops, as well as recent advancements in plant genome editing techniques, have made it possible to breed any desirable trait. In the last decades, genome editing with site-specific nuclease allows accurate and effective reverse genetics, genetic engineering and the targeted transgene integration experiments. It involves using engineered nuclease to introduce targeted DNA Double Strand Breaks (DSB) which stimulate cellular DNA repair processes. Depending on the availability of the repair template and the

Abbreviations: ALC, Alcobaca; ALS, Acetolactate synthase; AP1, APETALA1; BSV, Banana streak virus; CCD, Carotenoid cleavage dioxygenase gene; CRISPR, Clustered regularly interspaced short palindromic repeats; crRNAs, CRISPR RNA; DML2, DNA demethylase 2; DNA, Deoxyribonucleic acid; DSB, Double-strand breaks; dsDNA, Double-stranded DNA; eIF4E, Eukaryotic translation initiation factor 4E; FSH, Flavanone 3-hydroxylase; FT, Flowering locus; GABA, γ- aminobutyric acid; GBSS, Granule-bound starch synthase; MLO, Mildew resistance locus; NHEJ, Non-homologous end joining; NPR3, Non- expressor of pathogenesis-related genes3; PAM, Protospacer adjacent motif; PDS, Phytoene desaturase; PL, Pectate lyase; RIN, Ripening inhibitor; RNA, Ribonucleic acid; SGAs, Steroidal glycoalkaloids; SP, Self Pruning; sgRNA, Single-guide RNA; TALENs, Transcription activator-like effector nucleases; TFL, Terminal flowering; tracrRNA, Transactivating CRISPR RNA; ZNFs, Zinc-finger nucleases.

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REVIEW ARTICLE

Single-cell transcriptomics is revolutionizing the improvement of plant biotechnology research: recent advances and future opportunities

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ABSTRACT

Single-cell approaches are a promising way to obtain high-resolution transcriptomics data and have the potential to revolutionize the study of plant growth and development. Recent years have seen the advent of unprecedented technological advances in the field of plant biology to study the transcriptional information of individual cells by single-cell RNA sequencing (scRNA-seq). This review focuses on the modern advancements of single-cell transcriptomics in plants over the past few years. In addition, it also offers a new insight of how these emerging methods will expedite advance research in plant biotechnology in the near future. Lastly, the various technological hurdles and inherent limitations of single-cell technology that need to be conquered to develop such outstanding possible knowledge gain is critically analyzed and discussed.

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Plants; plant transcriptomics; single-cell technology; scRNA-seq

Introduction

Over the past few decades, biologists have been attempting to classify and characterize cells within multicellular organisms. Plants, like all other multicellular organisms need to provide fine tunes in their complex transcriptional networks to deal with several environmental stressors and other daily challenges [1,2]. Plant tissues are made up of cells with specific spatial architecture and viewed as layers of information, which helps to translate genetic information. Within a tissue it is often critical to determine each cell function, and a comprehensive analysis of spatial gene expression is therefore necessary to elucidate the role of cell interaction and localization from development in regulatory networks. By understanding the: cell identity, cell-to-cell communication and gene expression by specific cells in different tissue locations, it is possible to unveil the complex spatial gene expression network. This information could be used to improve plant research including crop yield and quality, plant resistance to biotic factors and plant adaptation to climate change. In plants, the transcriptional regulation of gene expression is mostly obtained by mapping a large subset of the transcriptome using microarray [3] and bulk RNA-sequencing [4] approaches. However, these technologies limit our knowledge of molecular/regulatory mechanisms involved in complex transcriptional networks by providing bulk gene expression pattern information. It is well recognized that specific cells have distinct roles in plant growth and development and environmental adaptation [5–7]. Hence, there is a need to escalate novel emerging technologies to obtain more accurate transcriptome responses and programs of specific cell types.

Modern biology is going through a new era of plant science with advanced omics technologies. Recently, researchers have risen to several pioneering studies in the single-cell RNA sequencing (scRNA-seq) for mapping transcriptional regulation at single-cell resolution. scRNA-seq has emerged as a promising technique to describe with high and accurate trajectories of cell transcriptome dynamics enclosed in individual cells [8,9]. This technique has an immense potential in animal-based research and human life to resolve inter-cellular transcriptomics heterogeneity of cells at the individual level [10–15]. In 2017, under the Human Cell Atlas international collaborative project, this approach has led to the discovery of: novel human cell types, expression

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Nardostachys jatamansi (D.Don) DC.: An invaluable and constantly dwindling resource of the Himalayas

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ABSTRACT

The use of medicinal plant species for different therapeutic effects is well recognized around the globe. *Nardostachys jatamansi* (D.Don) DC. (Family: Caprifoliaceae Juss.), commonly known as Indian spikenard is a critically endangered medicinal plant which grows at high altitudes in the alpine and sub-alpine regions of the Himalayas. Its medicinal use is well-recognized in the Bhutanese, Chinese, Indian, Japanese, Nepalese and Tibetan medicine. Moreover, its medicinal properties are well established in traditional medicines including Ayurveda, Ben-Cao- Shi-Yi, Homer's Iliad, the Old Testament, as in conventional systems. The increasing national and international demand of *N. jatamansi*, mostly for the rhizomes (underground tissue), as well as illegal/unsustainable harvesting has brought this valuable species to the edge of extinction. Therefore, more research input including in vitro biotechnological approaches is required to ensure its sustainable utilization and long-term conservation. Further research is also needed to improve our current knowledge about its conservation status, clinical relevance, and bioactive components. This review comprehensively summarizes the currently available information on the ethnomedicinal uses, pharmacology, phytochemistry, trade value and potential role of modern plant biotechnology tools for the conservation of this high value plant.

1. Introduction

About 80% of the world population totally depends on traditional medicine systems for their primary health care needs (WHO, 2008; Moyo et al., 2015; Yemele et al., 2015; Chen et al., 2016; Kumar et al., 2016). Indeed, medicinal plant-based herbal medicines have always been important to treat different therapeutic diseases as traditional preparations, with an ever-increasing demand due to their safety and lack of side effects (Dhiman and Bhattacharyya, 2020). Market surveys predicted that by the end of 2023, herbal medicine will constitute a 111 billion-dollar industry globally (Anonymous, 1997; Dhiman and Bhattacharyya, 2020). Himalayan biodiversity hotspots have a high value of endemic floras with potential medicinal value, of which very few have been scientifically studied and used in the herbal medicine market. Several Himalayan species with rich potential have not been used commercially, mainly due to lack of knowledge and poor scientific research.

Nardostachys jatamansi (D.Don) DC. (Caprifoliaceae Juss.: Dipsacales Batsch) has a long history of use in ayurveda and traditional medicine system (Sharma et al., 2000; Chen and Mukherji, 2013). Phytochemical analysis have revealed the presence of essential oils (coumarins and sesquiterpenes) in both the roots and rhizomes of *N. jatamansi*, which are directly associated with different medicinal purposes (Mishra et al., 1995; Nautiyal, 2013; Singh et al., 2015; Rekha et al., 2013; Liu and Liu, 2014). It is also listed in the top 20 most traded plants in India and is largely exploited (Rai et al., 2000; Olsen, 2005; Chauhan et al., 2011; Dhiman and Bhattacharyya, 2020). Due to its

Abbreviations: ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); AE, acid, above sea level aqueous extract; BA, N6-benzyladenine; CR, critically endangered; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ECD, electronic circular dichroism; EtOH, ethanol; FRAP, Ferric Reducing Antioxidant Power; IEJ, intron splice junction; IUCN, international union for conservation of nature; Kn, kinetin; mT, meta-topolin; MeOH, methanol; MS, Murashige and Skoog medium (1962); MS, mass spectrometry; NAA, α-naphthalene acetic acid; NMR, nuclear magnetic resonance; PMo, phosphomolybdenum; PGRs, plant growth regulators; PTC, plant tissue culture; RAPD, random amplified polymorphic DNA; SCoT, start codon targeted polymorphism; UV, ultraviolet

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Review

Arundina graminifolia (D.Don) Hochr. (Orchidaceae): A review of its medicinal importance, phytochemistry and pharmacology activities

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ABSTRACT

Traditional medicines play an important role in protecting the health of all ethnic groups around the world, and their importance in the development of medical and health services cannot be overstated. The Orchidaceae family includes some of the most beautiful flowers in the world, each with its own unique shape, color, and form. Potted and cut flower orchids are big business because the bioactive components in them are great for your health. *Arundina graminifolia* (Orchidaceae) has been extensively used for wide range of ethnopharmacological activities, including rheumatism, food poisoning, snake bites, traumatic injuries, detoxification and heat clearance. It is also utilized to treat pneumonia, tuberculosis and bronchitis by the ethnic people. The recent two decades of fruitful achievements in its chemical constituent investigations have laid a foundation for the study of its therapeutically material basis and action mechanism, which will help clarify its broad-spectrum detoxification mechanism and develop into a modern pharmaceutical preparation. This suggests that the whole plant of *A. graminifolia* is more important than other orchid species for extracting bioactive constituents such as flavonoids, phenylpropanoid, phenanthrene, glycosides, bibenzyl and phenolic compounds for medicinal purposes. The bamboo orchid is a promising species for studying the efficacy to obtain pharmaceutical products due to its short life cycle and accumulation of bioactive ingredients in multiple plant parts. This review summarizes the current medicinal usage, phytochemistry and pharmacological activities of this high value species.

1. Introduction

Arundina is a terrestrial orchid genus with cattleya-like blossoms that is one of the largest old-world orchids. From India, Thailand, Nepal, Malaysia, Singapore, Indonesia, South China, and the Pacific islands this genus can be found. And genus has a lot of variation in its color which is thought to be caused by polyploidy (Thomas and Blakey, 1993). The Orchidaceae family comprises ornamental orchids which are the largest angiosperm group (Cai et al., 2015; Wong et al., 2017; Comber, 1990; Chen et al., 1999). More than 100,000 orchid species have been cultivated due to their horticultural value. Most of the commercial orchid varieties are Dendrobium, Cymbidium and Phalaenopsis grow at specific seasons at temperatures between 5 and 10°C (Yang et al., 2019). The Bamboo orchid *Arundina graminifolia* (D. Don.) Hochr. (syn. *Arundina bambusifolia* Lindl.) Unlike these orchids, bloom all year with the peak between September and January and are mainly found in Asia's tropical and sub-tropical regions (Hooker, 1890; Wu et al., 2009; Seidenfaden et al., 1992; Auberon et al., 2016). It was first documented in South and Central America in the 1960s (Wester, 1992; Oppenheimer and Bartlett, 2000; Oakeley, 2003; Wood et al., 1993).

A. graminifolia (bamboo orchid) is an evergreen terrestrial species of orchid and is known as the sole member and only recognized species in the genus *Arundina*. This plant is thought to have anti-arthritic, detoxification, and anti-irritant properties, and it is used as an

Abbreviations: BA, 6-benzyladenine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ROS, reactive oxygen species; GC-MS, gas chromatography-mass spectrometry; HIV, human immunodeficiency virus; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kn, Kinetin; TMV, tobacco mosaic virus; MMP9, metalloproteinase-9; AMACR, α-methylacyl-CoA-racemase

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REVIEW PAPER

The Beneficial Role of Silicon Alleviating Heavy Metal and Disease Resistance Stress in Crops

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Abstract
Silicon (Si), as a quasi-essential element, is the second richest element in the surface of earth crust and in the soil with a vital role in alleviating the damaging effects of several environmental cues on plants. Over the last few decades, heavy metal contamination in water and soil has increased due to rapid anthropogenic activities such as irrigation, mine exploration, metal processing, urbanization, low quality water and metalloids pollution. The maximum exposure to heavy metal stress significantly reduces the yield and growth of crop plants. In this regard, the use of Si offers a promising way in mitigating stresses imposed by heavy metal stress as well as biotic (insect, pest, and pathogenic diseases)/abiotic factors (drought, salt heat etc.) in Crop plants. Recent years have seen the advent of unprecedented technological advances in the field of plant biology to study the beneficial role of Si in plants by improving plant tolerance mechanism at various levels. This review focuses on the potential role and mechanism involved in the Si-mediated alleviation of heavy metal toxicity as well as modern advancements of Si in crop plants over the past few years. In addition, it also offers a new insight of how Si will expedite advance research in disease resistance stress.

Keywords Crop · Heavy metal · Silicon · Toxicity

Abbreviations

ABA	Abscisic acid
Al	Aluminum
APX	Ascorbate peroxidase
B	Boron
bASi	Biogenic Amorphous Silica
BOR2	Boron transporter 2
CAT	Catalase
Cd	Cadmium
Chl	Chlorophyll
Cl	Chlorine
Co	Cobalt
Cr	Chromium
Cu	Copper
ET	Ethylene
Fe	Iron
GA	Gibberellic acid
GR	Glutathione reductase
Hg	Mercury
JA	Jasmonic acid
LOX	Lipoxygenases
MDA	Malondialdehyde
Mn	Manganese
Mo	Molybdenum
N	Nickel
Na	Sodium
NIP	Nodulin-26 Proteins
NPR1	Nonexpressor of Pathogenesis-Related Proteins 1
PAL	Phenylalanine ammonia-lyase
Pb	Lead
PIP1	Plasma membrane intrinsic protein 1
PME	Pectin methylesterase
POD	Peroxidases
PPO	Polyphenol oxidases
SA	Salicylic acid
Se	Selenium
Si	Silicon
SOD	Superoxide dismutase
Zn	Zinc

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CHAPTER

Biostimulants, the cinderella for plant development

2

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Introduction

The need to grow plants with better yields and overall productivity has always had a challenging presence in modern day agriculture. In recent years, during the early days of the biotechnology era, a variety of biostimulants have come to the fore to aid agricultural systems in enhancing the overall productivity of various crops. The earliest definition of biostimulants was "materials, other than fertilizers, that promote plant growth when applied in small quantities" (Kauffman et al., 2007). The fact that they are required only in minute quantities distinguishes biostimulants from soil amendments and nutrients which are required in greater quantities to bring about significant differences in the plant (Du Jardin, 2015). Biostimulants are known to mitigate biotic and abiotic stresses to optimize plant growth conditions (Yakhin et al., 2017).

As a result of increasing demand for quality of crop and better yield, the search for sustainable and environmental friendly methods to create fertilization reagents of biological origin has already become a key objective in agriculture. Biostimulants are the products capable of acting on the enzymatic and metabolic processes of plants for improving plant quality and productivity. These products are helpful for plants to deal with stress conditions, especially during the early stages of plant growth and development. To influence growth of plant, improving their tolerance to abiotic stress and enhancing seed yield and quality, these compounds cause changes in structural and vital processes and reduce the use of fertilizers. Biostimulants are generally produced as intersection of synthetic and natural substances composed hormones or plant hormone precursors (Yakhin et al., 2017). It acts on physiological processes when correctly applied to crop by giving potential for development, growth, and response to stress conditions and toxic elements.

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