Review Article

# Biotechnological Strategies for Boosting Sapogenin Yield in Safed Musli

**Pallavi B. More, Ashwinikumar B. Kshirsagar*, Rupali R. Taur**
Department of Plant Biotechnology, Institute of Biosciences and Technology, MGM University, Chh. Sambhajinagar, 431003; Maharashtra, India.
***Corresponding author's E-mail:** aashwinn9@gmail.com

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**ABSTRACT**

*Chlorophytum borivilianum* (Safed Musli), a valuable medicinal herb, is renowned for its rich content of bioactive compounds, particularly steroidal saponins, which have notable therapeutic properties. This study explores advanced techniques for optimizing the production of these compounds through *In-vitro* methods and elicitation strategies. *In-vitro* cultures were established using MS medium supplemented with growth regulators, achieving significant shoot and root induction. The production of key secondary metabolites, including stigmasterol and hecogenin, was enhanced in these cultures, with roots producing up to 46.4 mg/g DCW of stigmasterol and 685 mg/g DCW of hecogenin. Furthermore, elicitation techniques using chemical, biological, and physical agents were employed to stimulate secondary metabolite production, with notable increases in saponin yield and quality. Advanced extraction methods, particularly microwave-assisted solvent extraction (MASE), were optimized for saponin isolation, offering a rapid, high-yield approach suitable for industrial application. Additionally, the use of bioreactor cultures and hairy root systems provided a scalable and controlled environment for large-scale production of sapogenins. These methods not only enhance the yield of bioactive compounds but also ensure consistency and scalability, making them viable for commercial exploitation. This research underscores the potential of biotechnological approaches in maximizing the therapeutic and commercial value of *Chlorophytum borivilianum*, providing a foundation for future applications in phytopharmaceuticals.

**Keywords:** *Chlorophytum borivilianum*, Saponins, Elicitation, *In-vitro* culture, Bioreactor.

## INTRODUCTION

### *Taxonomy, Phylogeny, Diversity and Distribution*

The genus *Chlorophytum*, previously classified under the family *Liliaceae*, has recently been placed in the family *Anthericaceae* of the series *Coronariae* within the class *Monocotyledons* [1]. The name *Chlorophytum* is derived from the Greek words *chloros* meaning "green" and *phyton* meaning "plant" [2]. Hooker and Jackson [3], in their work Index *Kewensis*, have listed over 300 species within the genus *Chlorophytum*, suggesting that its probable center of origin and diversification is in tropical and subtropical Africa, where 85% of the species are found[4]. However, Poulsen (personal communication) noted that the listing of *Chlorophytum species* is still incomplete, estimating that about 125-150 species might exist. Most species of the genus are tuberous geophytes, well-adapted to light exposure and seasonal variations in precipitation, typically found in habitats such as savanna grasslands or open woodlands [4]. *Chlorophytum* species are predominantly distributed in tropical regions worldwide, with some species being cultivated for their attractive flowers [5]. In India, approximately 16 species of *Chlorophytum* have been recorded. Recently, a new species named *C. kolhapurense* was discovered in the Kolhapur region of Maharashtra [6]. Several *Chlorophytum* species, including *C. arundinaceum Baker* [7,8], *C. attenuatum Baker* [8], *C. borivilianum* [9,10], *C. brevescapum Dalaz* [11], *C. laxum R.Br.*[12], and *C. tuberosum Baker* [8], are collectively known by the trade name "Safed musli." There are several vernacular synonyms for Safed musli in India-Sanskrit: *Shweta musli,* Tamil-*Taniravi thang,* Hindi (U.P.)-*Khiruva,* Marathi/Hindi- *Safed musli,* Gujarati- *Dholi musli,* Telugu- *Tella nela tadi gaddalu,* Malayalam-*Shedheveli.* Among the species used as *Safed musli*, *C. arundinaceum* is particularly noted for its tuber quality in Ayurvedic texts[6]. However, *C. borivilianum* has recently gained more attention and is regarded as the main Safed musli crop by several researchers [5,13,14].

### *Discovery and Endemism*

*Chlorophytum borivilianum* was first discovered by Santapau and Fernandes [9,10] on June 14th, 1954, in the plains and lower slopes of the Krishnagiri National Park, Salsette Island, Borivali, Mumbai[15]. Following its initial discovery, the species has been reported from various localities across India, including the Dang forest in Gujarat, Aravali hills in Rajasthan, and various locations in Maharashtra such as Akola, Amaravati, Mumbai, Kolhapur, Pune, and Raigad [9,16]. It is considered endemic to India and has been categorized as a vulnerable species due to its decreasing population from overexploitation [17,18]. *C. borivilianum* is endemic to India and is listed as 'vulnerable' on the IUCN Red List [18].

### *Botanical Description and Morphology*

*C. borivilianum* is a small perennial herb with a full crown of radical leaves that emerge with the advent of summer rains. The roots are fleshy and fascicled, originating directly from the stem disc. The tubers, 5-20 in number, are 10-25 cm long and 1-2 cm in diameter, cylindrical, straw-colored on the outside, and white on the inside after peeling. There are 6 to 13 leaves, each 13 to 23 cm long and 1.75 cm wide, spirally imbricate, slightly narrowed at the base, sessile,

linear or ovate with an acute apex. The leaves spread horizontally with rough, wavy margins and parallel venation on the lower surface. The plant produces a solitary, unbranched scape, 15-30 cm long, bearing flowers on the upper three-quarters of its length. Small, white, bracteate, pedicellate, and usually arranged in alternate clusters of three. The floral bracts are linear, papery, purplish, and 1.0-1.5 cm long. The pedicel is whitish, jointed, and 6-10 mm long. Six tepals are arranged in two whorls of three, linear, membranous, acute, 3-5 nerved with imbricate aestivation. Six stamens in two whorls, opposite to tepals, united to the perianth, dithecus filaments glabrous, anthers yellow and linear, dehiscing by longitudinal slits. The style is slightly longer than the stamens, swollen at the apex. The ovary is green, globose, sessile, three-lobed with obtuse angles, and has axile placentation. The fruit is a loculicidal capsule, green to yellow, triquetrous to 3-sulcate, nearly equal in length and width, containing 14-16 black, endospermic, angular seeds. Floral Formula-⊕, ♂, ♀, P (3+3), A (3+3) [4], this detailed botanical description provides insight into the unique characteristics and reproductive structures of *Chlorophytum borivilianum*, highlighting its significance, therapeutic potential and the need for conservation efforts.

***Morphological Variability and Reproductive Biology***

Studies conducted under the all India coordinated project on medicinal plants at Indore on plant collections in various districts of Madhya Pradesh, Rajasthan, and Gujarat have revealed significant variability among different characteristics of *Chlorophytum borivilianum* [19, 20, 21 & 5]. This includes traits such as fleshy root yield per plant, dry root yield per plant, thickness of fleshy roots, leaf length and width, and the number of fleshy roots per plant. At the National Research Centre for Medicinal and Aromatic Plants (NRCMAP), considerable morphological variability was observed in *Chlorophytum borivilianum* collections. Two distinct plant types were identified- Prostrate/Spreading Type- In this type, the leaves emerge from the stem disc and immediately spread out. Erect Type- Newly produced leaves grow upwards for a short distance before spreading out. Variability was also noted in leaf length, leaf breadth, and maturity period. There were significant differences in root shape, size, and color. A plant with variegated leaves was identified, and it was observed that roots with blunt tips had better post-harvest keeping quality compared to those with tapering tips[22]. The NRCMAP initiated a program in 1999 for the collection, conservation, and cataloging of *C. borivilianum* biodiversity under the World Bank-funded National Agricultural Technology Project (NATP) [13]. This initiative underscores the potential for selecting promising strains of *C. borivilianum*.

In addition to the observed variations, some abnormalities in fasciculated roots of Safed musli were reported these abnormalities included [5]-

- Fusion of a pair of fleshy roots along their entire length.
- Fusion of one pair of fleshy roots in the upper portion up to half of their length.
- Fusion of two fleshy roots from the top part up to two-thirds of their length, forming a knot that then bifurcated into two parts.

Two small roots joined together up to their ends with one root extending further [23]. The root morphology of ten *Chlorophytum* species including C*. borivilianum, C. bharuchae, C. orchidastrum, C. arundinaceum, C. glaucum, C. attenuatum, C. glaucoides, C. breviscapum, C. laxum, and C. tuberosum.* They developed a key based on root morphology for these species [24]. The morphological variability in *Chlorophytum borivilianum* indicates potential for strain selection and conservation. Unique flowering patterns; inflorescences emerged within 4-6 days after rain, with plants from fleshy root bunches producing more flowers due to greater food reserves [5]. Inflorescence peduncles originate from the leaf whorl center. Safed musli is an insect-pollinated crop [25]. Various aspects of its reproductive biology have been studied by several researchers [5, 26 & 27]. Anthesis is not synchronous within the inflorescence; flowers opening on the same day in an inflorescence are arranged in different directions away from one another. Within a flower, the stigma is positioned away from the anthers, which favors cross-pollination. Anthesis occurs at around 4.00 a.m., followed by the longitudinal splitting of the anther lobes.

Pollen dispersal is facilitated by insects, particularly *Apis mellifera Linn.* and *Apis cerana indica Fabr* [27]**.** The maximum activity of pollinating agents is reported between 8.00 and 9.00 a.m. Pollen viability is 87.5% at 7.00 a.m. on the day of anthesis, gradually reducing to zero by 5.00 p.m. Pollen stored at approximately 6 ± 1°C maintains viability (83%) up to 30 hours after anthesis. Stigma receptivity is at its peak at 9.00 a.m. and decreases gradually to zero by 3.00 p.m. Although *C. borivilianum* is cross-pollinated in nature, artificial self-pollination is also effective, resulting in a 60% seed set. In this respect, *C. borivilianum* differs from African species of *Chlorophytum*, which are self-compatible with high auto deposition efficiency [2]. Each capsule contains 14-16 seeds [5]. Seed collection is challenging in this crop since all capsules do not mature simultaneously, and capsule dehiscence occurs as soon as they turn brown.

***Anatomical and Histochemical Characteristics***

Anatomical studies of different *Chlorophytum* species concerning tuber and leaf structures have been performed[28], but *C. borivilianum* was not included in these studies. Anatomical descriptions of *C. borivilianum* tubers have been obtained from a website [29]. A transverse section of the tuber reveals a circular outline with a yellowish, uniseriate epidermis of thick-walled, siliceous cells. Beneath this, a large cortex features rectangular outer cells and rounded, parenchymatous inner cells. The single-

layered endodermis is followed by a uniseriate pericycle. The vascular tissue includes exarch xylem with jointed vessels and abundant xylery fibers forming an irregular ring. Phloem and parenchyma are situated between xylery tissue arches, with a central, densely packed polygonal pith region. Made comparative histochemical studies of *Cynotis tuberosa* Roxb., *Chlorophytum laxum* Br., and *Chlorophytum borivilianum* [29]. They mentioned that the root tubers of Safed musli are very rich in proteins, raphides, and sphaeraphides. Among different species of *Chlorophytum*—namely, *C. attenuatum*, *C. borivilianum*, *C. capense*, *C. laxum*, *C. glaucum*, *C. orchidastrum*, *C. tuberosum*, *C. comosum* var. C-41, var. C-42 (variegatum), var. C-43 (pictratum), and var. C-47 (vittatum)—the maximum stomatal index (46.5) was observed in *C. borivilianum* leaves [30]. This species also exhibited the smallest stomatal size with respect to length and breadth.

### *Chromosomal Variability and Ploidy*

There are two basic chromosome numbers in the genus *Chlorophytum:* x=7 and x=8 [5, 31,32]. *C. borivilianum* is a diploid species with a chromosome number of 2n=2x=16 and a basic number of x=8 [32]. Notably, polyploidy has been reported within *C. borivilianum* populations, likely due to the predominance of vegetative propagation [34]. Precociously germinated somatic embryos in 6-8 and18-24 months old cultures of *C. borivilianum* exhibited a wide range of ploidy levels, from 3x-3 to 4x+4 and 5x+1 to 7x+2 [33]. Consequently, these somatic embryos were deemed abnormal and failed to develop into complete plantlets. Further observations, chromosomal variation was minimal (3x-3 to 3x+3) in regenerants from 1-4month-old cultures and increased (5x-1 to 7x) in regenerants from cultures older than 6 months [33]. The chromosome number in *C. borivilianum* is 2n=4x=28, indicating tetraploidy with a basic number of x=7 [35]. However, an octaploid number of 2n=8x=56 was also observed in some somatic cells. Confirmed the chromosome number of 2n=4x=28 in *C. borivilianum*[36]. They utilized fluorescence in situ hybridization (*FISH*) to analyze the physical localization and measurement of rDNA sites using two rRNA multigene families homologous to 45S and 5S rDNA. Results showed five pairs of 45S rDNA sites in *C. borivilianum* and three pairs in *C. comosum*, suggesting multi-genomic origins and discrete variations in rDNA loci. A karyomorphological study on seven accessions of *C. borivilianum* collected from Rajasthan and Madhya Pradesh [37]. They found that all accessions were tetraploid with 2n=4x=28 chromosomes, resolved into seven groups with seven homologous chromosomes each. Most accessions had a high proportion of submetacentric chromosomes, with metacentric, telocentric, and subtelocentrics being relatively rare. Nucleolar chromosomes were identified in five of the seven accessions (*PBL-1, PBL-2, PBL-4, PBL-5*, and *PBL-7*). According to, several selections of *C. borivilianum* are noted for their yield and saponin content, including varieties *RC-2, RC-16, RC-36, RC-20, RC-23, RC-37*, and *CT-1* [38]. These varieties are collected and maintained at the Rajasthan Agriculture University, Udaipur. Additionally, *MDB-13* and *MDB-14* are other varieties of Safed musli that are recognized for their high yield and resistance to insects and diseases.

### *Geographical Distribution of Chlorophytum Species in India*

*C. acaule* is found in Peninsular India. *C. arundinaceum* grows in northern and eastern peninsular India. *C. attenuatum* is located in the Western Ghats, extending southwards to Coimbatore and West peninsula. *C. bharuchae* is also native to Peninsular India. *C. borivilianum* is rare in moist places along plains and lower hill slopes in Maharashtra, including Dangs forest (Gujarat), Aravali hills, Mount Abu, Mahi (Rajasthan), and Bastar forests (M.P). *C. breviscapum*, *C. glaucum*, and *C. glaucoides* are distributed in Peninsular India. *C. heynei* is found in Tamil Nadu, *C. khasianum* in the central to eastern Himalaya, *C. laxum* in Peninsular India, *C. malbaricum* in Peninsular India and Karnataka, and *C. nepalense* in the subtropical Himalaya. C. nimmonii is found in Peninsular India, while *C. tuberosum* is distributed throughout India. *C. kolhapurense* is rare and sparsely distributed in the dry hilly tracts of Kolhapur district in Maharashtra and Belgum, Dharwar district of Karnataka[7].

### *Economic and Medicinal Significance*

The fleshy roots (tubers) of *Chlorophytum borivilianum* are used as a raw drug, while its leaves serve as a leafy green vegetable in Gujarat, Madhya Pradesh, and Chhattisgarh [38, 14]. Local traditional healers use fresh and dried leaves for treating various conditions, including enhancing resistance against sex-related diseases and delaying menopause in Bastar [38]. Ancient Indian texts like the Sarngadhar Samhita and Bhavaprakasha highlight the tuber's medicinal value as an aphrodisiac, adaptogen, and health restorative [28]. Safed musli is documented for its uses in treating sexual dysfunction, diabetes, arthritis, physical weakness, and improving brain development and immunity. It is included in Ayurvedic, Unani, and allopathic products such as Paurush and Chavanprash [38]. The Gujarat State Forest Development Corporation markets a potency drug, 'Nai Chetna,' containing Safed musli (The Indian Express, 1st Dec 1999). The annual foreign demand for the tubers ranges from 300 to 700 tonnes, with a market price of 0.8 to 1.2 million Indian rupees (US $0.02-0.03 million) per ton[5, 39].

### *Pharmacological Studies*

Aqueous extracts from *Asparagus racemosus*, *Chlorophytum borivilianum*, *Curculigo orchioides*, *Dactylorhiza hatagirea*, and *Orchis latifolia* (200 mg/kg) significantly enhanced pendiculatory activity and *In-vitro* sperm count in male rats after 14 days [40]. Comparatively, extracts of *Asparagus racemosus*, *Chlorophytum borivilianum*, and *Curculigo orchioides* showed pronounced anabolic effects, increasing body and reproductive organ weight and improving sexual behavior parameters like reduced latency and enhanced mating frequency [40]. *Chlorophytum borivilianum* also

demonstrated protective effects against testicular damage induced by high temperatures, improving histo-architecture, spermatogenesis, and sexual behavior parameters [41]. Extracts at 125 mg/kg and 250 mg/kg improved sexual vigor, arousal, and sperm count, with the higher dose showing a saturation effect[42]. The root powder of *Chlorophytum borivilianum* (0.75 and 1.5 g/rat/day) improved HDL-cholesterol levels and antioxidant enzyme activity in hyperlipidemic rats, though it had no effect on normocholesterolemic animals[43]. Antioxidant activity of ethanolic extracts was significant, supporting the plant's therapeutic use[44]. Additionally, extracts exhibited antidiabetic, immunomodulatory, and spermatogenic effects, with improvements in sexual behavior and reproductive organ weight at higher doses [45, 46].

***Phytochemical Studies***

The phytochemical analysis of *Chlorophytum borivilianum* tubers has been carried out by various organizations involved in Safed musli cultivation, with relevant data available online. According to Malbar Herbs and Musli Grower's Society (MHMGs), the physicochemical properties of Safed musli tubers are as follows- Total ash: 3.02%,Acid-insoluble ash: 0.25%,Water-soluble ash: 50.70% of total ash, Hot extraction: 20.48%,Cold maceration: 0.35%,Water and volatile matter: 7.60%,Foaming index: 166.67%,Swelling index: 4.60ml, Mass fraction of tannins: 1.20% .The following organic and inorganic constituents in Safed musli tubers [5]- Organic constituents (%)- Carbohydrates: 42.0,Proteins: 8-9.0,Fibers: 3-4.0, Saponins: 2-17.0. Inorganic constituents (mg/g dry weight)- Sodium: 0.040, Potassium: 0.800, Calcium: 6.600, Magnesium: 1.900, Phosphorus: 3.200, Zinc: 0.002, Copper: 0.148, The tubers also contain 0.63% polyphenols and a very low amount of ascorbic acid (0.12%) on a fresh weight basis [47]. Highlighted that among all *Chlorophytum* species in India, *C. borivilianum* yields the highest root and saponin content[5]. They observed that the saponin content is influenced by genotype and environmental conditions, with genotype *RC-14* producing up to 9.3% saponin, whereas genotype *RC-28* yielded only 1.8% at the same site. The effect of soil fertility on *C. borivilianum* tubers and found the highest saponin (6.93%). and protein (9.28%) percentages with the highest fertility level of NPK at 60:80:8086. Applying vermicompost (5 t/ha) also increased saponin (6.27%) and protein (9.17%) contents compared to other organic manuring treatments. Wider plant spacing (30 × 20 cm) resulted in significantly higher saponin (6.17%) and protein (9.21%) contents compared to closer spacings (30 × 10 cm and 30 × 15 cm) (86). Successfully isolated and standardized sapogenins from *C. borivilianum* using HPTLC [44]. Isolated fructo-oligosaccharides from the tubers and identified them as O-β-D-fructofuranosyl-(2→1)-(β-D-fructofuranosyl)n-(2→1)-α-D-glucopyranoside (n = 5-30) using high-pressure anion exchange chromatography, MALDI-MS, NMR, GC, HPTLC, and chemical analysis. While the chemical composition of *C. borivilianum* tubers has been extensively studied, the chemical composition of its leaves has not been as thoroughly investigated [45]. The phytochemical analysis of *Chlorophytum borivilianum* leaves shows the following results: Organic Constituents- Reducing sugar is 308.0 mg/100 g dry weight, soluble sugar is 459.0 mg/100 g dry weight, starch is 1316.0 mg/100 g dry weight, and total nitrogen is 1910.0 mg/100 g dry weight. Inorganic Constituents-Potassium is 1988.0 mg/100 g dry weight, calcium is 1236.0 mg/100 g dry weight, magnesium is 278.0 mg/100 g dry weight, phosphorus is 215.45 mg/100 g dry weight, sodium is 94.0 mg/100 g dry weight, sulphur is 36.4 mg/100 g dry weight, iron is 13.56 mg/100 g dry weight, manganese is 1.61 mg/100 g dry weight, copper is 0.56 mg/100 g dry weight, and zinc is 2.16 mg/100 g dry weight [47].

***Eco physiological and Biochemical Studies***

The seeds of *Chlorophytum borivilianum* exhibit a dormancy period of approximately 9-10 months [13]. Similar dormancy duration of about 10 months [48], approximately 13% germination in seeds that were one year old [25], while [13] reported a seed germination rate of about 28-62% after 37 days of sowing in Petri dishes at Indore. Further germination trials of seeds of *C. borivilianum* [48].

***Traditional and Enhanced Propagation Methods***

For large-scale cultivation of Safed musli ***(****Chlorophytum borivilianum****),*** vegetative propagation is currently the most practical method. A dormancy period of approximately 7-8 months in the fleshy roots [48]. Investigated the impact of phytohormone treatments on sprouting percentage and seedling vigor [49]. They treated 400 small unsprouted roots (weighing 0.3-0.5 g) by soaking them for 24 hours in solutions of 100 ppm $GA_3$ + 50 ppm Kinetin, and 50 ppm Kinetin. The roots were then placed in petri dishes with filter papers moistened with the respective solutions. The control group was soaked in distilled water. The phytohormone treatments significantly enhanced sprouting percentage, emergence index, speed of germination, mean germination time, and coefficient of velocity, with 50 ppm Kinetin proving to be the most effective [49]. Evaluated the sprouting ability of fleshy roots with crowns in raised beds under various treatments, including 'Gomutra' (cow urine), aqueous leaf leachate of *Calotropis gigantea****,*** and 0.2% Bavistin (Carbendazim) [50]. The roots treated with *C. gigantea* leaf leachate exhibited the highest sprouting rate (87%), surpassing other treatments [50]. Growth features and mineral nutrition studies, a continuous increase in biomass up to 120 days, with biomass partitioning towards the tuber observed from 45 days onwards. Leaf area increased up to 75 days before declining due to leaf senescence. Leaf nitrogen followed a similar trend [47]. Maximum phosphorus content was recorded in young plant leaves at 15 days after planting (DAP), with a subsequent decline. Calcium and magnesium levels in leaf and tuber tissues increased with plant age, while nitrogen, phosphorus, and potassium accumulated in tuber tissues during the later stages of plant growth [47]. Traditional methods involve using seeds for propagation, but the seeds of Safed Musli are very

small and have low viability. Seeds exhibit low germination rates due to dormancy or poor seed viability [52]. Seedlings develop slowly, and establishment from seeds can be inconsistent [53]. This is the most common method where tubers are divided into smaller pieces, each with one or more buds, and then planted. Tubers can have a dormancy period of 7-8 months, complicating planting schedules [52]. Tubers may carry diseases or pests, leading to the spread of these issues to new plants [50]. Manual tuber division and planting require significant labor and are inefficient for large-scale cultivation [54]. Small segments of roots or offsets are used for propagation by planting directly in the soil. Root segments may not always produce uniform growth or yields [55]. This method offers limited control over the quality and uniformity of the plants [56].

***Advancements in In-vitro Propagation***

Attempts at *In-vitro* propagation of *C. borivilianum* have been made by several researchers. Variability in morphological and yield attributes among germplasm, with *CB/MS-6* producing the highest number of tubers [54]. Clonal multiplication of Safed musli using young shoot bases as explants on Murashige and Skoog's (MS) medium supplemented with 22.2 µM BA[55]. This method resulted in four-fold higher multiplication rates every 3 weeks. All shoots rooted when transferred to MS medium with ¾ strength inorganic and organic constituents, 9.8 µM IBA, and 67% of micro propagated plants were successfully established in pots, producing normal fasciculate storage roots [55]. Further studies focused on multiplication through somatic embryogenesis from young shoots on MS medium with 4.53 µM 2,4-D, and maintenance of somatic embryos on 1.13 µM 2,4-D [59]. About 20% of the embryos produced plantlets when transferred to a medium without growth regulators [59]. Further improved the protocol for *C. borivilianum* by focusing on somatic embryogenesis and plantlet regeneration [60]. They developed a method for inducing callus from immature zygotic embryos using MS medium with 1.0 mg $l^{-1}$ 2,4-D. Initially, the callus was slow-growing, soft, and slimy. However, after six weeks and subsequent subculturing on MS medium with 0.5 mg $l^{-1}$ 2,4-D, yellow compact, hard, nodular, shiny somatic embryos were observed. The embryogenic cultures were maintained by repeated subculturing every four weeks on a medium with 0.25 mg $l^{-1}$ 2,4-D and 100 mg $l^{-1}$ ascorbic acid. Ascorbic acid was found beneficial for healthy callus growth. Reducing 2,4-D concentration to 0.1 mg $l^{-1}$ proved optimal for embryo growth under dark conditions. Mature somatic embryos were transferred to a medium without growth regulators for germination and exposed to light. About 21% of the embryos produced shoots and roots, with some showing profuse rhizogenesis. The germinating embryos were separated and transferred to fresh media for further development. Although the plantlets exhibited profuse tillering, only a few reached maturities in soil and displayed poor growth and storage root development [60]. Developed an enhanced method for large-scale multiplication through shoot base and stem disc cultures[61]. They achieved *In-vitro* multiplication on MS medium supplemented with 2 mg/l BA. Up to 90% of plantlets were established in pots through a hardening treatment, which involved transferring plants to sterile sand and maintaining them in a mist chamber under high humidity. The effectiveness of various explants was evaluated, concluding that leaf bases were the best, followed by stem discs [62]. Regenerated *C. borivilianum* plants via organogenesis and embryogenesis, studying the influence of auxins and cytokinin's on these processes [63]. Somatic embryos using MS medium with 2.25 µM 2,4-D and 1.15 µM Kinetin [64]. The medium, supplemented with KNO3 and (NH4)2SO4, showed significant effects on somatic embryogenesis, with lower total nitrogen levels (250 or 500 mg N $l^{-1}$) being favorable. Among cytokinin's, 2-iP had the most stimulatory effect on somatic embryogenesis, with the order of effectiveness being 2-iP > TDZ > Kinetin > BAP. The optimal medium for regeneration and long-term maintenance included 2880 mg $l^{-1}$ KNO3, 471.4 mg $l^{-1}$ (NH4)2SO4, 0.18 mM adenine, and 22 µM 2-iP, producing 58 shoots per inoculum in the first passage. Rooting of isolated shoots was optimal in B5 liquid medium with 0.49 µM IBA, leading to de novo tuberous roots. These shoots showed 100% survival after transfer to soil and were comparable to in vivo cultivated plants [64]. Somatic embryogenesis from long-term calluses of seedling and leaf explants. They observed high levels of morphological and cytological variation among the regenerants, which increased with the age of cultures [65]. This included occasional variegated plants and variations in leaf size, stomatal number, epidermal cell size, and chromosomal number. They noted that instability and a decline in embryonic potential were major issues with seedling-derived callus for long-term maintenance. To address this, they recommended using callus from leaf explants, which supports a cyclic system of shoots → callus → embryogenesis → germinated embryo → leaf → callus [65]. Developed a highly reproducible, field-tested, and cost-effective micropropagation scheme for *Chlorophytum borivilianum* [56]. They achieved optimal shoot multiplication on MS medium with 22.2 µM BA and 3% sucrose, producing over 15,000 plantlets within 20 weeks. Plantlets hardened under agro-shadenet conditions during the monsoon months of high humidity exhibited better survival rates and growth compared to those hardened *In-vitro* and later acclimatized in a greenhouse. Survival rates were 87% under open field conditions and 90% under agro-shadenet conditions, with plantlets producing tuberous roots [56]. The *In-vitro* propagation of *C. borivilianum* using encapsulated shoot buds [66]. They found that 4 mm long shoot buds encapsulated in a 3% sodium alginate matrix, polymerized with 100 mM $CaCl_2.2H_2O$, produced the best results. Storage conditions, gel matrix media, and storage duration significantly influenced the *In-vitro* regrowth potential of shoot buds. Shoot buds stored in wet agar-gel under light conditions (45 µmol $m^{-2}$ $s^{-1}$ and 28 ± 2°C) sprouted more than 80% within 3 weeks. However, those stored in the dark (4°C) on agar-gelled wet medium exhibited over 90% sprouting after 7 days of storage. The regrowth potential declined to 60% after 30 days and

dropped below 20% after an additional 30 days of storage. Supplementing the alginate matrix with sucrose and MS salts yielded better results compared to MS salts alone or no supplements. All sprouted shoot buds, regardless of storage conditions, produced normal shoots on standard multiplication (SM) medium and multiplied at a rate of 2.5-fold per 21-day subculture. Rooted plantlets successfully hardened and developed normal tuberous roots under greenhouse conditions. This method facilitates off-season storage and easy transport of germplasm [66]. Developed a method for shoot regeneration from immature floral buds and inflorescence axes. They established axenic cultures with minimal contamination (10%) [67]. MS medium with 2 mg $l^{-1}$ kinetin and 0.1 mg $l^{-1}$ 2,4-D was optimal for multiple shoot induction, with MS medium containing 2 mg $l^{-1}$ BAP yielding the maximum number of shoots [10]. Shoots (86.7%) rooted with the highest number of fasciculated roots [5] on Knops medium with 2 mg $l^{-1}$ IBA and 0.1% activated charcoal. Approximately 80% plant survival was achieved 4 weeks after removal from *In-vitro* conditions, with 34 hardened plants generated per explant within 50 weeks. This protocol is beneficial for large-scale clonal multiplication and germplasm conservation of this rare medicinal herb without destroying the mother plant [67].

Investigated biochemical changes leading to shoot regeneration in *C. borivilianum* derived from bud pedicels on MS medium with 1.0 mg $l^{-1}$ BAP and 1.0 mg $l^{-1}$ NAA **[68]**. They observed that starch content and reducing sugars were high in the control callus and increased significantly in shoot-differentiating cultures. Total soluble sugars, free amino acids, total soluble proteins, and total phenols were lower in the control but increased in shoot-differentiating cultures. Activities of α-amylase, acid-protease, acid phosphatase, and peroxidase peaked on the 16th day, coinciding with shoot appearance, while acid invertase activity decreased until shoot emergence [68]. Achieved cost-effective micropropagation using a liquid MS medium [69]. They established *In-vitro* cultures from young shoot apices of field-grown tuberous roots. Liquid medium resulted in a 7.5-fold increase in shoot multiplication compared to 4.5-fold on solid medium and reduced single shoot production costs by 92.31%. Liquid culture medium proved more efficient for large-scale multiplication of *C. borivilianum* [69]. Investigated biological hardening and genetic fidelity of micro-cloned progeny of *C. borivilianum* [70]. They found that micro-cloned plantlets established more than 95% in soil treated with bio-inoculants like *Glomus aggregatum*, *Trichoderma harzianum*, and *Piriformospora indica*. Inoculation with *Azospirillum* sp. (CIM-azo) and *Actinomycetes* sp. (CIM-actin) showed up to 85% plantlet establishment. Un-rooted shoots treated with these bio-inoculants also exhibited in vivo rooting (50%) with *Glomus aggregatum* and *Trichoderma harzianum*. Genetic fidelity testing using RAPD analysis showed strong uniformity in relation to the parent genotype [70].

The production of plantlets via tissue culture has emerged as a crucial biotechnological approach for the improvement of Safed Musli (*Chlorophytum borivilianum*), offering a pathway to enhance propagation efficiency and obtain high-quality planting material. Tissue culture techniques enable the rapid multiplication of plantlets from a small number of initial explants under controlled conditions, facilitating the large-scale production of uniform and disease-free plantlets. This method is particularly advantageous for Safed Musli, which is known for its slow seed germination and limited natural propagation rates. Recent advancements in tissue culture have led to the development of optimized protocols for the successful micropropagation of Safed Musli. For instance, demonstrated an effective protocol for the *In-vitro* production of plantlets using shoot tip explants cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg $L^{-1}$ BAP (6-Benzylaminopurine) and 0.5 mg $L^{-1}$ IBA (Indole-3-butyric acid) **[71]**. This combination resulted in a high frequency of shoot proliferation and rooting, producing vigorous plantlets suitable for field transplantation. Additionally, plantlet production efficiency was enhanced by using an alternative rooting medium with 0.5 mg $L^{-1}$ IBA and activated charcoal, which improved root development and the overall quality of the plantlets **[72]**. This method also addressed issues related to root proliferation and acclimatization, leading to higher survival rates upon transplantation. Overall, tissue culture not only facilitates the mass production of genetically uniform plantlets but also serves as a valuable tool for preserving and enhancing the genetic traits of Safed Musli. By refining these protocols, researchers can ensure the continuous availability of high-quality plant material, thus supporting the sustainable cultivation and improvement of this important medicinal plant.

***Indirect Organogenesis for Safed Musli Improvement***

Indirect organogenesis represents a significant biotechnological approach for the improvement of Safed Musli (*Chlorophytum borivilianum*), focusing on the development of shoots and roots through callus formation from explants. This method involves the initiation of callus tissue from various explants, followed by the induction of organogenesis, leading to the formation of shoot and root structures. Indirect organogenesis is particularly advantageous for Safed Musli, as it allows for the generation of a large number of plantlets from a single explant and can facilitate the incorporation of desirable traits through genetic manipulation. Recent research has demonstrated effective protocols for indirect organogenesis in Safed Musli. For instance, the successful regeneration of shoots from callus cultures derived from leaf explants on MS medium supplemented with 1.0 mg $L^{-1}$ 2,4-D (2,4-Dichlorophenoxyacetic acid) and 0.5 mg $L^{-1}$ BAP (6-Benzylaminopurine) [73]. This protocol resulted in a high frequency of shoot formation and subsequent plantlet development, showcasing the potential for large-scale propagation and trait improvement. In addition, improved the efficiency of indirect organogenesis by using a two-step process, where initial callus induction was achieved on MS medium with high levels of 2,4-D, followed by transfer to a medium with lower 2,4-D and higher

concentrations of BAP to promote shoot differentiation [74]. This approach not only enhanced the quality of the regenerated plantlets but also increased the rate of successful acclimatization.

Overall, indirect organogenesis offers a valuable strategy for the mass propagation of Safed Musli, facilitating the development of high-quality plantlets with improved traits. By optimizing these protocols, researchers can advance the production of Safed Musli and enhance its genetic potential, supporting sustainable cultivation and conservation efforts.

**Table 1:** Summarizing *In-vitro* plant regeneration studies in *Chlorophytum borivilianum*

| Explant Used | Morphological Response | Media Composition |
|---|---|---|
| Leaf explants [73] | Shoot regeneration, high frequency of plantlets | MS medium with 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ BAP |
| Stem disc explants [75] | High shoot proliferation, improved organogenic potential | B5 medium with 0.18 mM adenine and 22 µM BAP, enhanced with (NH4)2SO4 and KNO3 |
| Immature floral buds[76] | High shoot induction, effective for large-scale multiplication | MS medium with 2 mg $L^{-1}$ kinetin and 0.1 mg $L^{-1}$ 2,4-D |
| Shoot apices [77] | Efficient shoot multiplication, improved growth rates | MS medium with 22.2 µM BA and 3% sucrose |
| Young shoot apices [78] | Increased shoot multiplication in liquid medium | MS basal liquid medium |
| Callus from seedling and leaf explants [79] | Somatic embryo formation, high morphological variability | MS-9 medium with 2.25 µM 2,4-D and 1.15 µM Kinetin |

***Direct Organogenesis of Safed Musli***

Direct organogenesis involves the development of shoots or roots directly from the explant tissue without an intervening callus phase. In Safed Musli (*Chlorophytum borivilianum*), several studies have successfully utilized this technique to produce plantlets, demonstrating its potential for large-scale propagation. Developed a protocol for somatic embryogenesis and plantlet regeneration using immature zygotic embryos as explants [80]. Callus development was induced on MS medium containing 1.0 mg/L 2,4-D. The initial callus was slow-growing, soft, watery, and slimy, but after six weeks and subsequent subculturing on MS medium with 0.5 mg/L 2,4-D, a yellow, compact, hard, nodular callus with somatic embryos formed. The inclusion of 100 mg/L ascorbic acid was found beneficial for the healthy growth of the callus. Reducing the 2, 4-D concentration to 0.1 mg/L promoted embryo growth under dark conditions, and 21% of the embryos produced shoots and roots when transferred to a regulator-free medium and exposed to light. Another study, enhanced the protocol using stem disc explants [81]. They treated the explants with fungicide and antibiotics to reduce contamination. The stem discs produced a large number of shoots (15 per explant) on $B_5$ medium supplemented with 0.18 mM adenine and 22 µM BAP. Subsequent passages increased shoot numbers significantly, reaching up to 58 shoots per inoculum in the third passage. The study found that increasing the concentrations of (NH4)2SO4 and KNO3 in the medium further enhanced shoot proliferation in long-term cultures. They observed the effectiveness of different cytokinin's in the order TDZ > BAP > Kinetin > 2-iP, with the optimal medium containing 2880 mg/L KNO3, 471.4 mg/L (NH4)2SO4, 0.18 mM adenine, and 22 µM 2-iP. This medium supported the regeneration and long-term maintenance of organogenetic potential. Rooting of isolated shoots was optimal in B5 liquid medium containing 0.49 µM IBA, producing de novo tuberous roots and achieving 100% survival upon soil transfer. Developed a method for large-scale multiplication using shoot base and stem disc cultures [82]. They achieved up to 90% plantlet establishment in pots through a hardening treatment that involved transferring plants to sterile sand in a mist chamber under high humidity. Compared the efficiency of various explants (seedling, root, stem disc, and leaf basal half) and found that the leaf base was the best explant for regeneration, followed by the stem disc [62]. Introduced a novel method using immature floral buds along with the inflorescence axis for shoot regeneration [80]. They achieved multiple shoot induction on MS medium containing 2 mg/L kinetin and 0.1 mg/L 2, 4-D, with a maximum of 35 shoots on MS medium with 2 mg/L BAP. Rooting (86.7%) with maximum fasciculated roots was observed on Knops medium containing MS medium's iron and vitamins, 2 mg/L IBA, and 0.1% activated charcoal. The protocol yielded 80% plant survival after 4 weeks of hardening, generating 34 hardened plants per explant within 50 weeks. Demonstrated an efficient and cost-effective micropropagation method using liquid MS basal medium [83]. They established *In-vitro* cultures from young shoot apices and found that liquid medium resulted in a better shoot growth and multiplication response than solid medium, with a 7.5-fold increase in shoot multiplication. The use of liquid medium reduced single shoot production costs by 92.31% compared to solid medium, making it advantageous for large-scale multiplication. These studies highlight the effectiveness of direct organogenesis for the propagation of Safed Musli, providing valuable protocols for the mass production of this medicinal species.

***Somatic embryogenesis***

Somatic embryogenesis is crucial for the clonal propagation of Safed Musli (*Chlorophytum borivilianum*), enabling the production of uniform, disease-free plants essential for commercial cultivation and conservation. A protocol for somatic embryogenesis and plantlet regeneration was developed using immature zygotic embryos [86]. Callus induction on MS medium with 1.0 mg/L 2,4-D initially produced a slow-growing, soft callus, which later developed into yellow, compact somatic embryos after six weeks on a medium with 0.5 mg/L 2,4-D. Embryogenic cultures were maintained by subculturing on a medium with 0.25 mg/L 2,4-D and 100 mg/L ascorbic acid. Reducing 2,4-D to 0.1 mg/L promoted embryo growth under dark conditions, and germination was achieved on a medium without growth regulators, with 21% of embryos producing shoots and roots. Significant factors for embryogenesis included low nitrogen levels and the incorporation of amino acids like proline, leading to a maximum of 63 somatic embryos per inoculum [84]. Continued refinement of these protocols will enhance the efficiency of large-scale Safed Musli production.

***Rooting and Hardening of Tissue-Culture-Raised Plants***

Successful rooting and hardening are critical in the micropropagation of Safed Musli (*Chlorophytum borivilianum*), ensuring the acclimatization and establishment of plantlets in soil. Root induction typically involves transferring regenerated shoots to a rooting medium. Effective rooting has been observed on MS medium supplemented with 2.0 mg/L Indole-3-butyric acid (IBA), with roots forming within 2-3 weeks [85]. The addition of 0.1% activated charcoal further enhanced root quality and length. Rooting was also achieved on ¾ MS medium containing 2.0 mg/L IBA, with micro shoots rooting successfully within 2 weeks [86]. Knops medium supplemented with 2.0 mg/L IBA and 0.1% activated charcoal led to 86.7% rooting efficiency with maximum fasciculated roots [87]. Hardening, or acclimatization, gradually adapts tissue-cultured plantlets to external conditions. A protocol using plastic cups with a soil, sand, and vermicompost mixture under high humidity resulted in an 80% survival rate [86]. High humidity and shade during hardening further improved survival rates to 87% in open fields and 90% under agro-shadenet conditions [88]. Bio-hardened plantlets treated with bio-inoculants like *Glomus aggregatum* and *Trichoderma harzianum* exhibited over 95% establishment in soil [89].

***Varying Ploidy Level under In-vitro Conditions***

Manipulating ploidy levels in *Safed Musli* (*Chlorophytum borivilianum*) through *In-vitro* techniques can develop cultivars with increased biomass, enhanced secondary metabolite production, and improved stress resistance. Polyploidy is induced using chemicals like colchicine or oryzalin. Colchicine treatment involves immersing explants in solutions (0.05% to 0.5%) for 12 to 72 hours, followed by culturing on regeneration media. Tetraploidy has been achieved in *Safed Musli*, resulting in larger tubers and higher saponin content. Oryzalin, which is less toxic than colchicine, is used at concentrations of 0.001% to 0.01% [90]. Oryzalin-induced polyploid plants show better growth and disease resistance. Polyploid plants typically exhibit larger leaves, thicker stems, and increased biomass [91]. Enhanced secondary metabolite production and stress tolerance are also observed. Mutagenesis followed by *In-vitro* regeneration involves callus induction, shoot regeneration, root induction, and acclimatization. EMS has been used for mutations, enhancing saponin content and growth [92]. Gamma irradiation has improved disease resistance and tuber yield [93].

***Responses to Environmental Constraints***

*C. borivilianum* is sensitive to waterlogging, and its responses to environmental stresses are not extensively studied. Research on its response to sodium chloride salinity revealed a reduction in phosphorus content and enzyme activities in leaf and tuber tissues, except for an increase in leaf acid phosphatase activity under salt stress [95]. Under water stress, leaf nitrogen, phosphorus, and potassium levels declined, while calcium, magnesium, and sulfur levels increased. Waterlogging led to decreased phosphorus and potassium in leaves, but increased nitrogen, calcium, magnesium, and sulfur. Both stresses reduced potassium and sulfur in tubers, with water stress increasing phosphorus and magnesium content. Micronutrient levels were altered, with increases in copper, zinc, and manganese in leaves under both stresses, though copper decreased under water stress and increased under waterlogging. Tuber tissue showed increased iron and reduced sodium levels, with prolonged waterlogging significantly impacting nutrient levels [95].

***Biochemical and Molecular Analyses for Trait Identification***

Identifying genotypes with desirable traits is crucial for enhancing the efficiency and effectiveness of Safed Musli (*Chlorophytum borivilianum*) breeding programs. Key traits include high tuber yield, high saponin content, disease resistance, and stress tolerance. Field evaluations are essential for assessing these traits across diverse environmental conditions, providing insights into the performance and adaptability of different genotypes. Techniques such as biochemical assays and molecular markers play a significant role. For instance, one study explored the effects of environmental stress on the saponin content and growth of Safed Musli, highlighting the importance of stress tolerance [96]. Molecular characterization to identify genotypes with desirable traits, demonstrating the utility of molecular markers used in breeding programs [97]. Additionally, genotype-environment interactions, emphasizing the need for comprehensive assessments to select genotypes that perform well under various conditions [98]. Overall, combining field trials, biochemical and molecular analyses, and genetic assessments is vital for identifying and utilizing superior

genotypes, ultimately enhancing Safed Musli breeding and commercial production.

***Enhancing saponin content***

Increasing saponin content is vital due to its medicinal importance; selective breeding and metabolic engineering are effective strategies for achieving this [99]. Disease resistance is another key target, with a focus on developing varieties resistant to fungal and bacterial pathogens through marker-assisted selection and genetic transformation [100]. Enhancing stress tolerance to abiotic factors like drought and salinity improves adaptability, as shown by research on stress responses [101]. Yield enhancement involves selecting high-yielding genotypes and optimizing cultivation practices [102]. Improving nutrient and mineral content in tubers boosts quality and market value, guided by studies on nutrient composition [103]. Additionally, optimizing root and tuber characteristics, including size and storage capacity, is essential for yield and quality improvement [104]. Identifying superior genotypes with high saponin content and adaptability is crucial. Identified such genotypes through biochemical and field evaluations, improving breeding selection [105]. Singh et al. emphasized the use of molecular markers to select genotypes with disease resistance and stress tolerance, crucial for stable production [106]. Combining traditional breeding with modern biotechnological approaches will advance Safed Musli cultivation and utilization.

***Production of Uniform High-Quality Planting Material and Saponin Production***

Achieving uniform, high-quality planting material and optimizing saponin production are crucial in Safed Musli (*Chlorophytum borivilianum*) cultivation. Uniformity is ensured through tissue culture techniques like micropropagation. Developed a micropropagation protocol that enhances plantlet quality and uniformity using optimized growth regulators [107]. To maximize saponin content, which is vital for medicinal value, selective breeding and optimal cultivation practices are essential. Selecting high-saponin genotypes and optimizing agronomic practices significantly boost saponin production [108]. Integrating these methods ensures high-quality planting material and maximizes saponin yield, supporting both commercial viability and medicinal efficacy.

***Pest and disease Resistance in Safed Musli***

The productivity of Safed Musli (*Chlorophytum borivilianum*) is affected by pest and disease vulnerabilities. Developing pest-resistant genotypes is crucial, with genetic screening for traits like leaf toughness and biochemical defenses proving effective [109]. Combining these genotypes with integrated pest management (IPM) strategies reduces reliance on chemical pesticides and enhances yield and quality [110]. Disease resistance is also vital, achieved through traditional breeding and biotechnological methods. Research has shown that using molecular markers for disease resistance improves protection against pathogens like *Fusarium* and *Alternaria* [111]. *CRISPR/Cas9* technology further enhances disease resistance by introducing specific resistance genes into Safed Musli [112]. Integrating disease-resistant varieties with integrated disease management (IDM) practices, including crop rotation and disease-free planting material, supports sustainable farming and improves economic returns.

***Increasing Storage Life of Safed Musli***

Extending the storage life of Safed Musli (*Chlorophytum borivilianum*) is vital for maintaining tuber quality and viability. Controlled atmosphere storage effectively prolongs shelf life by regulating temperature, humidity, and oxygen levels, thus reducing physiological deterioration and preserving biochemical stability [113]. Coating tubers with natural preservatives like chitosan and essential oils provides a protective barrier against moisture loss and microbial growth, enhancing storage longevity [114]. Proper post-harvest handling, including thorough cleaning, drying, and storage in cool, dry conditions, further reduces spoilage and maintains tuber viability. Integrating these strategies ensures a steady supply of high-quality planting material and medicinal products.

***Advances and Challenges in Safed Musli Transformation***

*Agrobacterium*-mediated transformation has proven effective for enhancing Safed Musli (*Chlorophytum borivilianum*). Significant advancements include increasing saponin content by introducing a squalene synthase gene [115] and developing fungal-resistant plants through a chitinase gene [116]. These improvements demonstrate the potential of genetic transformation for boosting secondary metabolite production, disease resistance, and other beneficial traits. However, several limitations hinder progress in plant regeneration and transformation. Low regeneration efficiency from explants, variable responses across cultivars, and callus induction issues pose significant challenges [117 & 118]. Additionally, the understanding of optimal growth regulator concentrations and combinations is insufficient[120]. Transformation efficiency is often low due to poor infection rates and high bacterial contamination [115&116], while the lack of reliable selection markers restricts the development of stable transformants [78]. Transgenic plants also exhibit high phenotypic variability, affecting stability and performance [121&77]. Regulatory and ethical concerns further complicate the commercialization of genetically modified varieties [119]. Addressing these limitations through improved protocols, better transformation techniques, and robust selection markers is crucial for advancing Safed Musli's genetic improvement.

***Molecular Analysis Techniques***

Molecular approaches are crucial for analyzing genetic diversity and improving Safed Musli (*Chlorophytum borivilianum*). Techniques such as Restriction Fragment Length Polymorphisms (*RFLPs*), Random Amplified Polymorphic DNA (*RAPDs*), Amplified Fragment Length Polymorphisms (*AFLPs*), and Simple Sequence Repeats (*SSRs*) provide valuable insights into genetic variation. In Safed Musli research, AFLP methods have been employed

to detect adulterants in herbal preparations, ensuring product integrity and reliability [122]. Molecular analyses have also revealed new taxa within the *Chlorophytum* genus, based on nuclear and chloroplast DNA sequences [123]. *RAPD* markers have been used to profile genetic diversity among various *Chlorophytum* species and genotypes, helping to clarify genetic relationships and diversity [124,125,126]. *AFLP* analysis of *Chlorophytum borivilianum* accessions from central India highlighted genetic variations influenced by propagation methods and mutations [128]. These molecular techniques are essential for understanding genetic diversity, trait inheritance, and improving Safed Musli varieties, contributing to better breeding strategies and conservation efforts.

**Table 2:** Molecular markers used for Safed Musli (*Chlorophytum borivilianum*)

| Genotypes Accessions | Techniques | Primers/Microsatellite Markers Used | Purpose of Study |
|---|---|---|---|
| 20 | SSR, ISSR | 10 SSR markers, 12 ISSR primers | Genetic diversity studies [129] |
| 15 | SSR, ISSR | 8 SSR markers, 10 ISSR primers | Genetic characterization and fingerprinting [130] |
| 12 | SNP analysis | 50 SNP markers | Gene mapping and functional genomics [131] |
| 25 | Gene expression analysis | 15 microsatellite markers | Gene expression and functional genomics [132] |
| 30 | Whole genome sequencing | 200 SNP markers | Whole genome sequencing and SNP analysis [133] |

***Limitations of Molecular Techniques in Safed Musli Research***

While molecular techniques have advanced our understanding of genetic diversity in Safed Musli (*Chlorophytum borivilianum*), they have notable limitations. Markers like *RAPDs* and *AFLPs* can offer limited resolution in distinguishing closely related species or varieties, leading to inconsistent results due to their reproducibility issues [125, 127]. Additionally, methods such as *AFLPs* and *SSRs* require sophisticated equipment and expertise, which may not be accessible in all research settings, and their high costs can be prohibitive for extensive studies [128]. Some studies may not fully capture the species' genetic diversity due to the reliance on a limited number of markers or accessions, resulting in an incomplete understanding of genetic variability [126]. The complexity of molecular data analysis can lead to misinterpretation, especially when data do not align with phenotypic observations or lack comprehensive reference genomes [122]. Furthermore, ethical and legal concerns arise with molecular research, particularly regarding genetic resource ownership and data sharing, impacting biodiversity conservation and intellectual property rights [124]. These challenges indicate that while molecular techniques are valuable, they should be complemented with traditional methods and careful consideration of their limitations.

***Hairy Root culture***

The successful initiation of *In-vitro* culture in *Chlorophytum borivilianum* was achieved by inoculating surface-sterilized explants onto MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l NAA. After 20 days, multiple shoot buds developed from the explant base and elongated into shoots upon maturation [134]. For root induction, young shoot buds were transferred to MS media with various concentrations of IBA, NAA, and IAA, with optimal results observed on MS media supplemented with 3 mg/l IBA, inducing roots after two weeks. Growth kinetics in shake cultures revealed peak biomass of 12.1±2.18 mg/g DCW after 25 days. Stigmasterol production reached 46.4±0.47 mg/g DCW, and hecogenin reached 685.68±0.51 mg/g DCW after 25 days. This study is the first to report the production of stigmasterol and hecogenin from *In-vitro* root cultures of *C. borivilianum* [134]. Auxins, particularly IBA, play a crucial role in root induction, though effectiveness can vary; for instance, IBA was more effective than NAA and IAA here, but other studies found NAA more effective in some species [137,138]. Auxins have been noted for their role in root induction and elongation, with comparable results in other species, such as *Panax ginseng* [139], *chicory* [136], and *Andrographis paniculata* [140]. Additionally, saponin content in field-grown plants was significantly enhanced by mycorrhizal fungi [141].

***Optimized Extraction of Saponins***

A rapid, high-yield extraction method for saponins from Safed Musli was developed and optimized using both conventional and modern microwave-assisted solvent extraction (MASE) techniques[142]. The roots were extracted through maceration, Soxhlet, sonication, and microwave methods. The extracts were fractionated to obtain total saponins, which were quantified using High-Performance Thin-Layer Chromatography (HPTLC). Optimization of MASE was performed using a Taguchi L9 orthogonal array design, focusing on factors such as temperature, irradiation time, power, and powder size. Under optimal conditions, MASE achieved a saponin yield of 5.11% with a notable reduction in extraction time to just 4 minutes, outperforming

conventional methods. The yield comparison ranked the methods as - MASE, Maceration, Soxhlet, and Sonication. The optimized MASE method demonstrated significant potential for industrial application, offering a more efficient and effective approach for saponin extraction from Safed Musli.

***Bioactive Compounds***

*Chlorophytum borivilianum*, commonly known as Safed Musli, is rich in bioactive compounds that contribute to its therapeutic properties, making it a valuable herb in both traditional and modern medicine. Steroidal saponins, particularly borivilianoside, are the most prominent, enhancing sexual health and vitality [122]. Alkaloids present in Safed Musli offer analgesic and anti-inflammatory benefits, aiding in pain management, especially in conditions like arthritis [143]. The herb's flavonoids act as potent antioxidants, providing anti-inflammatory and cardioprotective effects, and supporting overall cardiovascular health[144]. Phenolic compounds add to its antioxidant capacity, helping prevent diseases related to oxidative stress, such as cancer and neurodegenerative disorders [145]. Terpenoids, known for their antimicrobial and anticancer activities, further enhance Safed Musli's medicinal value by inhibiting pathogen and cancer cell growth [146]. Additionally, the roots contain significant carbohydrates and proteins, contributing to the herb's nutritional value [147].

Root cultures of *Chlorophytum borivilianum* were established using MS media supplemented with 3 mg/L IBA, achieving a twenty-four-fold increase in fresh biomass under shake culture conditions. These cultures produced significant amounts of stigmasterol (46.4 mg/g DCW) and hecogenin (685 mg/g DCW) [134]. Phytochemical screening and secondary metabolite profiling using ESI-MS and GC-MS revealed a high percentage of saponins, especially in *In-vitro* roots, with compounds such as β-Sitosterol and Taraxerone being identified [148].

Safed Musli's roots are traditionally used to treat physical weakness, diabetes, arthritis, and to enhance immunity, among other conditions [149]. The roots are rich in saponins, alkaloids, vitamins, proteins, and essential minerals, all contributing to its therapeutic effects. The commercial potential of C. borivilianum lies in its exploitation for secondary metabolites and germplasm conservation, with *In-vitro* techniques playing a key role in enhancing the production of these valuable compounds [152]. The impact of UV-B radiation on *Chlorophytum borivilianum* was studied, revealing that low UV-B doses enhanced saponin content and altered steroidal components, highlighting the role of UV-B in stimulating medicinal compounds [150]. The role of secondary metabolites in plants, which are crucial for industrial and pharmaceutical applications, was emphasized, with biotechnological methods like tissue culture and gene transfer being highlighted as ways to boost production [151]. This comprehensive overview underscores the therapeutic and nutritive value of Safed Musli, stressing the importance of *In-vitro* techniques for maximizing the plant's medicinal potential.

***Elicitation***

Elicitation is a technique used to stimulate the production of secondary metabolites in plants by applying specific chemical, biological, or physical agents known as elicitors. This method enhances the biosynthesis of valuable compounds in *Chlorophytum borivilianum* (Safed Musli), a medicinal plant known for its therapeutic saponins [153].

Chemical elicitors, such as jasmonic acid (JA), salicylic acid (SA), and methyl jasmonate (MJ), mimic stress conditions and activate the plant's defense mechanisms, leading to increased secondary metabolite production. These chemicals are typically added to the culture medium or sprayed onto plant tissues, with optimized concentrations and exposure times to achieve the best response without causing damage. Biological elicitors include microorganisms like fungi, bacteria, and yeast, which can stimulate the plant's defense response, enhancing secondary metabolite production. For instance, introducing fungal pathogens or beneficial microorganisms can trigger saponin production in Safed Musli. Physical elicitors, such as UV light, temperature fluctuations, and mechanical injury, induce secondary metabolite production as part of the plant's stress response. These factors are applied by adjusting environmental conditions or physically manipulating plant tissues, like exposing cultures to UV light or varying temperatures to boost metabolite production. Elicitation can significantly increase the production of secondary metabolites, including saponins, in Safed Musli, both *In-vitro* and in vivo. This process not only enhances the quantity of secondary metabolites but can also improve their quality and therapeutic efficacy. Elicitors activate the plant's defense mechanisms, potentially increasing its resilience and capacity to produce secondary metabolites in response to stress. Overall, elicitation is a valuable approach for enhancing secondary metabolite production in *Chlorophytum borivilianum*. By applying chemical, biological, or physical elicitors, it is possible to significantly increase the yield and quality of bioactive compounds such as saponins, offering a promising strategy for optimizing the production of valuable metabolites for medicinal and commercial applications.

***Bioreactor cultures***

Bioreactor cultures provide a controlled environment for the large-scale production of secondary metabolites, including sapogenins, offering enhanced control over environmental conditions, increased yield, and scalability [154]. Commonly used for cultivating plant cell suspensions or hairy roots, bioreactors ensure efficient aeration, mixing, and temperature control. Hairy root cultures offer a stable, high-yield method for producing secondary metabolites, while bioreactors provide a scalable, controlled environment, contributing significantly to the commercial production of valuable compounds in medicinal plants. Bioreactor cultures represent an advanced technique for

optimizing the production of secondary metabolites in *Chlorophytum borivilianum* (Safed Musli), utilizing controlled environments to maximize plant cell or tissue growth and metabolite production [155]. These specialized vessels offer precise control of environmental factors, improved aeration, and automated nutrient delivery. The benefits of bioreactor cultures in producing secondary metabolites in Safed Musli are substantial, making them essential for scaling up production [156]. Their ability to control environmental factors and increase output makes bioreactors vital for both research and commercial applications in producing bioactive compounds from Safed Musli.

## CONCLUSION AND FUTURE PROSPECTS

*Chlorophytum borivilianum* (Safed Musli) holds significant promise as a source of bioactive compounds, particularly steroidal saponins, which contribute to its wide range of therapeutic applications. Advances in in vitro culture techniques, including the establishment of callus and root cultures, have enabled the controlled production of these secondary metabolites. The use of elicitation methods, both chemical and biological, has further enhanced the yield and quality of these metabolites, demonstrating the plant's potential for large-scale production. Furthermore, bioreactor systems have emerged as a powerful tool for scaling up the production of valuable compounds such as stigmasterol and hecogenin, offering controlled environments that maximize metabolite output.

Future research should focus on optimizing these biotechnological approaches to enhance metabolite production even further. Exploring the synergistic effects of different elicitors, refining bioreactor conditions, and applying genetic engineering techniques to boost specific metabolite pathways are promising areas for continued investigation. Additionally, the development of rapid, high-yield extraction methods like microwave-assisted solvent extraction (MASE) offers great potential for industrial applications, making the large-scale production of Safed Musli's bioactive compounds more viable. Continued exploration of these techniques could lead to more efficient and sustainable production processes, positioning *Chlorophytum borivilianum* as a key resource in the pharmaceutical and nutraceutical industries.

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