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Research Article

CALLUS INDUCTION AND PLANT REGENERATION IN THREE ECONOMICALLY IMPORTANT AROMATIC GRASSES: *CYMBOPOGON FLEXUOSUS*, *C. CITRATUS*, AND *VETIVERIA ZIZANIOIDES*

***Manju M. George**
Department of Botany, Union Christian College, UCC Post, Aluva,
Ernakulam District, Kerala 683102, India
**Author for Correspondence: manju@uccollege.edu.in*

ABSTRACT

Background: Aromatic grasses of the genera *Cymbopogon* and *Vetiveria* are economically valuable for their essential oils used in perfumery, pharmaceutical, and cosmetic industries. However, poor seed viability, limited genetic variability through vegetative propagation, and susceptibility to environmental stresses necessitate biotechnological interventions for crop improvement and mass propagation.

Methods: This study investigated callus induction and plant regeneration protocols for three aromatic grass species: *Cymbopogon flexuosus*, *C. citratus*, and *Vetiveria zizanioides*. Various explants, including mesocotyl segments from 4-5 day-old seedlings and immature inflorescences, were cultured on Murashige and Skoog (MS) medium supplemented with different combinations of growth regulators (2,4-dichlorophenoxyacetic acid, naphthalene acetic acid, kinetin, and benzyl adenine) at concentrations ranging from 0.1 to 4.0 mg/L. Additives, including polyvinylpyrrolidone (PVP) and casein hydrolysate (CH) were evaluated for callus maintenance, and riboflavin was tested for rooting enhancement.

Results: Mesocotyl explants of *C. flexuosus* and *V. zizanioides* produced morphogenic callus on MS medium supplemented with 1.0 mg/L 2,4-D and 0.1 mg/L kinetin, and 1.0 mg/L each of 2,4-D and kinetin, respectively. Immature inflorescences of *C. citratus* responded optimally to MS medium containing 1.0 mg/L each of 2,4-D and benzyladenine. Shoot regeneration was achieved on MS medium with benzyl adenine (1.0-2.0 mg/L) for *C. flexuosus* and *V. zizanioides*, yielding up to 34.17 ± 0.78 and 29.75 ± 0.69 shoots per explant, respectively. *C. citratus* required kinetin (0.5-1.0 mg/L) for shoot induction. Addition of 100 ppm each of PVP and CH maintained morphogenic potential through 7-8 subcultures. Rooting was achieved on half-strength MS medium or MS medium supplemented with low concentrations of auxins (0.1-0.5 mg/L NAA or IBA). Riboflavin (10 mg/L) suppressed callus formation at shoot bases in *V. zizanioides*, promoting direct rooting. Acclimatization success rate was 40-50%.

Conclusion: This study established efficient, reproducible protocols for callus induction and plant regeneration in three economically important aromatic grasses using readily available seedling and inflorescence explants. These protocols provide a foundation for germplasm conservation, mass propagation, and future genetic improvement programs to enhance essential oil yield and quality.

***Keywords**: Aromatic grasses, callus culture, Cymbopogon citratus, Cymbopogon flexuosus, essential oils, plant regeneration, tissue culture, Vetiveria zizanioides*

INTRODUCTION

Medicinal and aromatic plants constitute a vital component of global agriculture and industry, providing essential oils, pharmaceuticals, and natural products with diverse applications. Among these, aromatic grasses of the genera *Cymbopogon* and *Vetiveria* (family Poaceae) hold significant economic importance due to their high-value essential oils, which are used extensively in perfumery, cosmetics, pharmaceuticals, and the food industry (Boukhatem et al., 2014). Essential oils are complex mixtures of terpene derivatives, hydrocarbons, and straight-chain compounds that serve as antiseptics, stimulants, insecticides, insect repellents, and flavoring agents (Bassolé et al., 2011; Shah et al., 2011). The global essential oil market represents approximately 17% of the world's flavor and fragrance industry, with annual consumption estimated at 40,000 to 60,000 tonnes.

Cymbopogon citratus (DC.) Stapf, commonly known as West Indian lemongrass or citron grass, and *C. flexuosus* (Nees) Wats., commonly known as South Indian lemongrass or East Indian lemongrass, are perennial aromatic grasses cultivated primarily for their essential-oil-rich foliage (Negrelle & Gomes, 2007). Upon hydrodistillation, the leaves yield essential oils containing 75-80% citral as the major constituent, along with geraniol, which are widely used in medicinal, cosmetic, and pharmaceutical industries (Boukhatem et al., 2014). Citral serves as a precursor for the synthesis of vitamins A and E, ionones, methyl ionones, and synthetic violet compounds, in addition to its applications in perfumery and flavor industries (Nayak et al., 1996). The essential oil exhibits significant antibacterial activity (Bassolé et al., 2011), mosquito repellent properties (Shah et al., 2011), antifungal activity against dermatophytes (Boukhatem et al., 2014), and is increasingly used in aromatherapy.

Vetiveria zizanioides (L.) Nash, commonly known as vetiver or khus, is a perennial grass indigenous to India and cultivated mainly in tropical and subtropical regions for the essential oil produced in its roots. The oil, composed predominantly of sesquiterpenes, is highly valued in perfume, cosmetic, and pharmaceutical industries as a fixative to stabilize more volatile compounds (Belhassen et al., 2015; Champagnat et al., 2006). The fragrant roots are also used for making mats, baskets, fans, and ornaments, while the living grass serves as an effective soil binder for erosion control. Medicinally, vetiver oil is used as a diaphoretic and has been traditionally employed as a preservative against cholera (Kim et al., 2005).

Despite their economic importance, aromatic grasses face several cultivation challenges. Continued vegetative propagation and long gestation periods before yield have led to declining production (Nayak et al., 1996). Poor seed setting and low seed viability in many species necessitate cultivation through vegetative slips, which preclude genetic variability acquired during sexual reproduction (Mucciarelli et al., 1993). Consequently, the genetic pools of these plants become shallow, rendering them susceptible to diseases, pests, and environmental stresses, ultimately leading to yield decline and non-competitive pricing in international markets (Sreenath et al., 1994). Conventional plant breeding programs are time-consuming and labor-intensive, making plant improvement through tissue culture and molecular biology an attractive alternative approach (Nayak et al., 1996).

Plant regeneration from totipotent cultured cells is a prerequisite for genetic manipulation through somatic hybridization or genetic engineering (Leupin et al., 2000). In members of Poaceae, somatic embryogenesis and organogenesis are the most common methods of plant regeneration (Dey et al., 2010). Young seedlings, particularly immature organs such as embryos and mesocotyls, which contain undifferentiated cells, have been identified as optimal explant sources for inducing regenerable callus cultures in cereals and grasses (Nayak et al., 1996; Quiala et al., 2016). Immature inflorescences have also been recognized as important sources of totipotent cultures in many cereals, millets, and grasses (Sreenath et al., 1994).

The present investigation aimed to develop efficient protocols for callus induction and plant regeneration in three economically important aromatic grasses: *C. flexuosus*, *C. citratus*, and *V. zizanioides*. Specific objectives included: (1) identifying optimal explant types and culture conditions for callus induction, (2) determining appropriate growth regulator combinations for maintaining morphogenic potential during subculture, (3) establishing efficient shoot regeneration protocols, and (4) developing rooting and acclimatization procedures for complete plantlet recovery. These protocols are essential for establishing mass propagation systems, germplasm conservation, and future genetic improvement programs aimed at enhancing essential oil yield and quality in these valuable aromatic grasses.

MATERIALS AND METHODS

2.1 Plant Material and Explant Preparation

Vegetative slips of *Cymbopogon flexuosus*, *C. citratus*, and *Vetiveria zizanioides* were obtained from the Sardar Patel University Botanical Garden and maintained under field conditions. Seeds were collected during October-November and stored under ambient conditions until use. Various explant types were evaluated, including young leaves, immature inflorescences, basal stem portions from mature plants, and mesocotyl segments from germinating seedlings.

For mesocotyl explant preparation, seeds were soaked in distilled water for 24 hours, and viable seeds were selected based on their ability to sediment. Selected seeds were transferred to sterile Petri dishes lined with water-soaked Whatman filter paper and germinated under aseptic conditions. Seedlings of 4-5 days old were used as explant sources. Immature inflorescences were collected from field-grown plants at the appropriate developmental stage. Young leaves and basal stem portions were excised from mature plants.

All explants were surface-sterilized using 0.1% mercuric chloride ($HgCl_2$) solution. Mesocotyl segments from 4-5 day-old seedlings were treated for 1 minute, while immature inflorescences, leaves, and basal stem portions were treated for 3 minutes. Following sterilization, explants were thoroughly washed 4-5 times with sterile distilled water to remove residual sterilant.

2.2 Culture Medium and Growth Regulators

Murashige and Skoog (1962) basal medium supplemented with 3% (w/v) sucrose (Hi Media, India) was used for all experiments. The medium pH was adjusted to 5.8 before adding 0.8% (w/v) agar (Hi Media, India). The medium was autoclaved at 15 psi (121°C) for 15-20 minutes. Growth regulators, including 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), kinetin (KN), and benzyl adenine (BA) were filter-sterilized and added to autoclaved medium at concentrations ranging from 0.1 to 4.0 mg/L, either alone or in various combinations.

Additives, including polyvinylpyrrolidone (PVP) and casein hydrolysate (CH), were evaluated for callus maintenance at 100 ppm each. Riboflavin was tested in rooting media to suppress callus formation at shoot bases. For rooting experiments, half-strength MS medium and full-strength MS medium supplemented with various auxin concentrations were evaluated.

2.3 Callus Induction and Maintenance

Two mesocotyl explants were used per culture vessel. For immature inflorescences, young leaves, and basal stem portions, individual explants were cultured in separate vessels. Cultures were maintained under a 14-hour photoperiod provided by cool white fluorescent lamps (3000 lux) at 25 ± 2°C. Callus induction was monitored over 4-6 weeks, and the frequency, color, texture, and morphogenic potential of induced calli were recorded.

For callus maintenance, morphogenic calli were subcultured every 35-40 days onto fresh medium. The effect of additives (PVP and CH) on maintaining morphogenic potential during prolonged culture was evaluated. Callus growth, texture, color changes, and regeneration capacity were monitored over successive subcultures (up to 8).

2.4 Shoot Regeneration and Rooting

Morphogenic calli (40-50 days old) were transferred to shoot induction media consisting of MS basal medium supplemented with various concentrations and combinations of cytokinins (BA, KN) and auxins (NAA, IBA). Cultures were maintained under the same light and temperature conditions as callus induction. Shoot formation was monitored weekly, and the number of shoots per explant was recorded after 4-6 weeks of culture.

For rooting experiments, healthy shoots (2-3 cm in length) were excised and transferred to rooting media. Various treatments were evaluated, including half-strength MS basal medium, full-strength MS medium, and MS medium supplemented with different concentrations of NAA or IBA (0.05-0.5 mg/L). The effect of riboflavin (10 mg/L) on basal callus suppression and direct rooting promotion in *V. zizanioides was evaluated*. Rooting frequency and root quality were assessed after 3-4 weeks

2.5 Acclimatization

Well-rooted plantlets were carefully removed from culture vessels, and residual agar was gently washed from roots using tap water. Plantlets were transferred to small pots containing a sterile mixture of soil, sand, and vermiculite (1:1:1 v/v/v). Potted plantlets were maintained in a small polyhouse with high relative humidity (80-90%) for 12-15 days to facilitate gradual acclimatization. Humidity was gradually reduced over the following 2-3 weeks by increasing ventilation. Survival rate was recorded after 4-6 weeks, and successfully acclimatized plants were transferred to larger pots or field conditions.

2.6 Statistical Analysis

All experiments were conducted with at least 12 replicates per treatment. Data on shoot number per explant were expressed as mean ± standard error (SE). Statistical analysis was performed to determine significant differences among treatments where applicable.

RESULTS

3.1 Explant Selection and Callus Induction

Among the various explants evaluated for callus induction in the three aromatic grass species, mesocotyl segments from 4-5 day-old seedlings and immature inflorescences proved most responsive. In contrast, basal stem portions, young leaves from mature plants, and culms showed poor response to all media combinations tested. These mature tissue explants released a dark leachate, presumably phenolic compounds, into the culture medium, which inhibited callus growth and eventually led to explant necrosis. In *V. zizanioides*, basal stem portions induced minimal callus initially, but growth was subsequently retarded due to the accumulation of leachate in the medium.

For *C. flexuosus* and *V. zizanioides*, mesocotyl segments from 3-4 day-old seedlings were identified as the most suitable explants for callus induction. In *C. flexuosus*, MS medium supplemented with 1.0 mg/L 2,4-D and 0.1 mg/L kinetin induced pale yellow, nodular, morphogenic callus within 2-3 weeks of culture initiation. The callus exhibited compact texture and showed high regeneration potential. In *V. zizanioides*, MS medium containing 1.0 mg/L each of 2,4-D and kinetin produced morphogenic callus with similar characteristics. Higher concentrations of 2,4-D (>2.0 mg/L) resulted in friable, non-morphogenic callus with reduced regeneration capacity.

For *C. citratus*, immature inflorescences were found to be the most suitable explants for callus induction and subsequent regeneration. MS medium augmented with 1.0 mg/L each of 2,4-D and benzyl adenine induced morphogenic callus from both floret primordia and spikelets within 3-4 weeks. The callus was initially pale yellow and gradually became nodular over time in culture. Mature inflorescences and other explant types from *C. citratus* showed poor response or failed to produce morphogenic callus

3.2 Callus Maintenance

During prolonged culture, the morphogenic potential of calli from all three species declined progressively, with regeneration capacity often lost within 3-4 subcultures. After this period, calli either failed to grow or reverted to root-forming cultures. To address this limitation, various additives were incorporated into maintenance media.

The addition of 100 ppm each of polyvinylpyrrolidone (PVP) and casein hydrolysate (CH) to MS medium containing 1.0 mg/L 2,4-D and 1.0 mg/L kinetin significantly extended the morphogenic potential of *V. zizanioides* callus. With these additives, morphogenic capacity was maintained through 7-8 subcultures (one subculture every 35-40 days), compared to only 3-4 subcultures without additives. PVP appeared to adsorb phenolic compounds leached into the medium, while CH provided amino acids and potentially unknown growth-promoting factors. For *C. flexuosus* and *C. citratus*, the additives were less critical, and morphogenic potential could be maintained for 5-6 subcultures without supplementation, though PVP addition reduced browning in *C. flexuosus* cultures.

3.3 Shoot Regeneration

Shoot regeneration patterns differed among the three species, reflecting genotype-specific responses to growth regulators. In *V. zizanioides*, microtillering (multiple shoot formation) was observed when 40-50-day-old calli derived from mesocotyl explants were transferred to MS basal medium devoid of growth hormones or to MS medium supplemented with 0.5-2.0 mg/L benzyl adenine. Maximum shoot formation

(29.75 ± 0.69 shoots per explant) was achieved on MS medium containing 1.0 mg/L BA (Table 1). Higher BA concentrations (2.0 mg/L) resulted in fewer shoots (22.67 ± 0.93), while lower concentrations (0.5 mg/L) produced 13.2 ± 1.03 shoots per explant. Addition of low concentrations of NAA (0.2 mg/L) to BA-containing medium suppressed shoot number, with MS + 1.0 mg/L BA + 0.2 mg/L NAA yielding only 19.67 ± 0.55 shoots per explant.

Table 1. Effect of growth hormones on shoot formation in *Vetiveria zizanioides* and *Cymbopogon flexuosus* from mesocotyl-derived callus

Medium composition	Average number of shoots (Mean ± SE)*	
	V. zizanioides	*C. flexuosus*
MS only	12.75 ± 0.82	7.50 ± 0.77
MS + 0.5 mg/L BA	13.2 ± 1.03	12.10 ± 0.69
MS + 1.0 mg/L BA	29.75 ± 0.69	23.40 ± 1.75
MS + 2.0 mg/L BA	22.67 ± 0.93	31.10 ± 0.95
MS + 1.0 mg/L BA + 0.2 mg/L NAA	19.67 ± 0.55	-
MS + 2.0 mg/L BA + 0.2 mg/L NAA	14.75 ± 0.64	-
MS + 1.0 mg/L BA + 0.2 mg/L IBA	-	34.17 ± 0.78
MS + 2.0 mg/L BA + 0.5 mg/L IBA	-	20.67 ± 1.35

Mean ± Standard error (SE) calculated from 12 replicates. BA = benzyl adenine; NAA = naphthalene acetic acid; IBA = indole-3-butyric acid.

Table 2. Various medium combinations evaluated for shoot regeneration in *Cymbopogon citratus*

Medium composition	Response/Observations
MS	Shoots and roots
MS + 0.5 mg/L BA	Greening of calli, root formation
MS + 1.0 mg/L BA	Greening of calli, root formation
MS + 1.0 mg/L BA + 0.5 mg/L NAA	Greening of calli, root formation
MS + 0.5 mg/L BA + 1.0 mg/L NAA	Greening of calli, root formation
MS + 1.0 mg/L BA + 0.5 mg/L IBA	Greening of calli, root formation
MS + 0.5 mg/L BA + 1.0 mg/L NAA	Greening of calli and profuse rooting
MS + 0.5 mg/L KN	Shoot formation
MS + 1.0 mg/L KN	Shoot formation
MS + 1.0 mg/L KN + 0.5 mg/L NAA	Shoots and roots
MS + 0.5 mg/L KN + 1.0 mg/L NAA	Shoots and roots
MS + 1.0 mg/L KN + 0.5 mg/L IBA	Shoots and roots
MS + 0.5 mg/L KN + 0.5 mg/L IBA	Shoots and roots
MS + 1.0 mg/L 2-iP	Nil

BA = benzyl adenine; KN = kinetin; NAA = naphthalene acetic acid; IBA = indole-3-butyric acid; 2-iP = 2-isopentenyladenine.

In *C. flexuosus*, shoot regeneration was also achieved with benzyl adenine, but the optimal response differed from that in *V. zizanioides*. MS medium supplemented with 1.0 mg/L BA and 0.2 mg/L IBA produced the highest number of shoots (34.17 ± 0.78 shoots per explant) (Table 1). Adding low concentrations of IBA increased shoot number, whereas higher concentrations (>0.5 mg/L) suppressed shoot formation. MS medium with 2.0 mg/L BA alone yielded 31.10 ± 0.95 shoots per explant, while 1.0 mg/L BA alone produced 23.40 ± 1.75 shoots per explant. Even MS basal medium without growth regulators supported some shoot formation (7.50 ± 0.77 shoots per explant), indicating the presence of endogenous growth regulators in the callus tissue.

C. citratus exhibited a distinctly different regeneration pattern compared to the other two species. Shoot formation was achieved using kinetin rather than benzyl adenine. MS medium supplemented with 0.5-1.0 mg/L kinetin induced shoot formation from callus derived from immature inflorescences (Table 2). With benzyl adenine, calli greened but failed to regenerate shoots, instead forming roots profusely. The transformation of floral primordia into vegetative shoots, a phenomenon known as vivipary, was observed in *C. citratus* cultures. This suggests that phytohormones in the culture medium triggered the conversion of reproductive structures into vegetative shoots.

3.4 Rooting and Acclimatization

Rooting requirements varied among the three species and depended on the composition of the culture medium. In *C. flexuosus*, half-strength MS basal medium without growth regulators was sufficient for rooting of healthy shoots. In *C. citratus*, supplementation with 0.2-0.5 mg/L NAA or IBA was essential for efficient rooting. Interestingly, somaclonal variants of *C. citratus* (observed occasionally in regenerated plants) rooted readily on MS basal medium without auxin supplementation, suggesting altered endogenous hormone levels.

In *V. zizanioides*, rooting was achieved on MS medium supplemented with 0.05-0.5 mg/L NAA. However, higher auxin concentrations (>0.5 mg/L) induced callus formation at the base of shoots, with roots sometimes emerging from the callus rather than directly from shoot bases. To suppress this undesirable callus formation, riboflavin (vitamin B_2) was added to the rooting medium at 10 mg/L. The addition of riboflavin to MS medium containing 0.1-0.5 mg/L NAA effectively prevented basal callus formation while promoting direct root emergence from shoot bases. This resulted in healthier root systems with better morphology and improved subsequent acclimatization success.

White's rooting medium was also evaluated for all three species, but performed inferiorly to MS-based media. Higher auxin concentrations (>0.5 mg/L) consistently produced callus at shoot bases across all species, and in some cases, roots emerged from the callus without connection to the shoots, rendering such plantlets unsuitable for acclimatization.

Well-rooted plantlets were transferred to a sterile soil mixture and maintained in a polyhouse with high humidity (80-90%) for 12-15 days. During this period, plantlets adapted to ex vitro conditions, developing thicker cuticles and functional stomata. Humidity was gradually reduced over the following 2-3 weeks. Approximately 40-50% of plantlets survived the acclimatization process and were successfully established in pots or field conditions. Plantlets with well-developed root systems and multiple shoots showed higher survival rates compared to those with fewer roots or single shoots.

DISCUSSION

4.1 Explant Selection and Callus Induction

The selection of appropriate explants is critical for successful callus induction and plant regeneration in tissue culture systems. In the present study, mesocotyl segments from young seedlings and immature inflorescences proved to be the most responsive explants for the three aromatic grass species investigated. This finding is consistent with numerous reports in cereals and grasses, which emphasize the importance of using explants from immature organs containing undifferentiated cells (Nayak et al., 1996; Quiala et al., 2016).

Young seedlings are particularly advantageous as explant sources because they can be grown in vitro, providing a frequent and reliable supply of uniform explants (Dey et al., 2010). The mesocotyl region, located between the seed and the first leaf, contains meristematic cells with high regenerative potential. In the present study, mesocotyl explants from 3-4 day-old seedlings of *C. flexuosus* and *V. zizanioides* produced morphogenic callus efficiently, consistent with previous reports on related species. Nayak et al. (1996) successfully used mesocotyl explants for somatic embryogenesis in *C. flexuosus*, while Mucciarelli et al. (1993) reported callus induction from various explants, including nodes in *V. zizanioides*.

The failure of mature tissue explants (basal stem portions, mature leaves, and culms) to produce viable callus cultures was attributed to the release of phenolic compounds into the medium. Phenolic oxidation and subsequent browning are common problems in tissue culture of woody and herbaceous plants, particularly those with high phenolic content (Mucciarelli et al., 1993). These compounds can be toxic to cultured tissues and inhibit growth. Similar observations have been reported in *Cymbopogon winterianus*, where mature tissue explants released leachates that inhibited callus growth (Sreenath et al., 1994).

Immature inflorescences proved to be excellent explants for *C. citratus*, consistent with their recognized importance as sources of totipotent cultures in cereals, millets, and grasses (Sreenath et al., 1994). The floret primordia and spikelets in immature inflorescences contain actively dividing cells with high morphogenic potential. Similar success with inflorescence explants has been reported in various grass species, including *Oryza sativa*, *Triticum aestivum*, *Cenchrus* spp., *Eleusine coracana*, *Sorghum* spp., and *Poa pratensis* (Sreenath et al., 1994). In *Cymbopogon winterianus*, Sreenath et al. (1994) achieved plant regeneration from immature inflorescences cultured on MS medium containing 1.0 mg/L each of 2,4-D, BA, or kinetin, similar to the optimal combination identified for *C. citratus* in the present study.

The requirement for 2,4-D in callus-induction media is well established in grass tissue culture systems (Dey et al., 2010). This synthetic auxin promotes cell division and dedifferentiation, leading to callus formation. The optimal concentration of 1.0 mg/L 2,4-D identified in this study is consistent with reports in related species. However, the cytokinin requirement varied among species: *C. flexuosus* required only 0.1 mg/L kinetin, while *V. zizanioides* required 1.0 mg/L kinetin, and *C. citratus* responded best to 1.0 mg/L benzyl adenine. This variation reflects genotype-specific differences in endogenous hormone levels and sensitivity to exogenous growth regulators (Leupin et al., 2000)

4.2 Role of Growth Regulators in Regeneration

The regeneration patterns observed in this study highlight the critical role of the balance of growth regulators in determining morphogenic responses. In *V. zizanioides* and *C. flexuosus*, benzyl adenine was effective for shoot induction, whereas *C. citratus* required kinetin. This differential response to cytokinins reflects species-specific variations in hormone perception and signal transduction pathways (Quiala et al., 2016).

The optimal BA concentration for shoot regeneration in *V. zizanioides* (1.0 mg/L) is consistent with findings by Sompornpailin et al. (2016), who reported synergistic effects of BA and kinetin on vetiver regeneration. However, Mucciarelli et al. (1993) reported regeneration on MS medium with 2.0 mg/L kinetin and 0.2 mg/L 2,4-D from nodal explants, indicating that different explant types may have different optimal requirements. The present study's use of mesocotyl explants may explain the preference for BA over kinetin in *V. zizanioides*.

In *C. flexuosus*, the addition of low concentrations of IBA (0.2 mg/L) to BA-containing medium enhanced shoot number, achieving up to 34.17 ± 0.78 shoots per explant. This synergistic effect of cytokinin and low auxin concentrations has been reported in other grass species. Nayak et al. (1996) reported plant regeneration from nodal cultures of *C. flexuosus* using MS medium with 5.0 mg/L 2,4-D, 0.5 mg/L kinetin, and 0.1 mg/L NAA, though the present study achieved efficient regeneration with BA alone or BA with low IBA, suggesting that mesocotyl-derived callus may have different hormonal requirements than nodal explants.

The requirement for kinetin rather than BA in *C. citratus* is noteworthy. When BA was used, calli greened but failed to regenerate shoots, instead forming roots profusely. This suggests that BA may promote root formation in this species, possibly by altering the auxin:cytokinin ratio in favor of rooting. Kinetin, in contrast, promoted shoot formation effectively. This differential response to cytokinin types has been reported in other plant systems and may reflect differences in cytokinin receptor specificity or downstream signaling pathways (Quiala et al., 2016).

The phenomenon of vivipary, observed in *C. citratus* cultures in which floral primordia transform into vegetative shoots, is well documented in grasses (Sreenath et al., 1994). This transformation is triggered by phytohormones in the culture medium and represents a natural mode of reproduction in some grass species. Multiple shoot production from cultured inflorescences has been suggested as an effective means of clonal propagation in grasses (Sreenath et al., 1994).

4.3 Callus Maintenance and Morphogenic Potential

The loss of morphogenic potential during prolonged culture is a common problem in grass tissue culture systems (Dey et al., 2010). In the present study, morphogenic capacity was typically lost within 3-4 subcultures in the absence of additives. This decline may result from genetic and epigenetic changes, accumulation of toxic metabolites, or depletion of essential nutrients (Leupin et al., 2000).

The addition of polyvinylpyrrolidone (PVP) and casein hydrolysate (CH) significantly extended the morphogenic potential of *V. zizanioides* callus to 7-8 subcultures. PVP is commonly added to tissue culture media to adsorb phenolic compounds released into the medium, which can inhibit growth (Mucciarelli et al., 1993). Phenolic oxidation products can be particularly problematic in aromatic grasses, which naturally contain high levels of phenolic compounds. By adsorbing these compounds, PVP maintains a more favorable culture environment.

Casein hydrolysate provides a complex mixture of amino acids and may contain unknown growth-promoting factors (Dey et al., 2010). Amino acids can serve as nitrogen sources, osmotic agents, and precursors for various metabolic pathways. The use of CH at 100-500 mg/L has been reported to enhance callus proliferation in various plant species, including *Nigella sativa* (Dey et al., 2010). In red fescue, the addition of CH along with 2,4-D helped develop high-density embryogenic aggregates within 2 weeks of culture (Dey et al., 2010).

The differential requirement for additives among the three species studied suggests variation in phenolic content and metabolic characteristics. *V. zizanioides*, which showed the greatest benefit from PVP and CH addition, may produce higher levels of phenolic compounds or have greater sensitivity to their accumulation. *C. flexuosus* and *C. citratus* maintained morphogenic potential for 5-6 subcultures without additives, though PVP addition reduced browning in *C. flexuosus* cultures.

4.4 *Rooting and Acclimatization*

Rooting is a critical step in micropropagation, and the requirements vary considerably among species and even among different regeneration pathways within a species (Quiala et al., 2016). In the present study, *C. flexuosus* shoots rooted readily on half-strength MS basal medium without exogenous auxin, suggesting that endogenous auxin levels were adequate. This is consistent with reports in *C. winterianus*, where shoots rooted on half-strength MS basal medium or White's medium supplemented with auxins (Sreenath et al., 1994).

C. citratus required supplementation with 0.2-0.5 mg/L NAA or IBA for efficient rooting, indicating lower endogenous auxin levels or reduced auxin sensitivity. The observation that somaclonal variants rooted on MS basal medium without auxin suggests that these variants may have altered hormone biosynthesis or sensitivity, a common consequence of somaclonal variation (Bhattacharya et al., 2008).

The use of riboflavin (vitamin B_2) to suppress callus formation at shoot bases in *V. zizanioides* is particularly noteworthy. Riboflavin has been reported to inhibit callus formation while promoting root formation in several plant species (Leupin et al., 2000). The mechanism may involve interference with auxin-induced callus formation or direct effects on root initiation pathways. In *Eucalyptus ficifolia*, riboflavin prevented rooting by stopping IBA-induced basal callus formation, which was essential for rooting in that species (Leupin et al., 2000). In contrast, riboflavin increased rooting frequency in *Carica papaya* by promoting root growth and decreasing callus formation (Leupin et al., 2000). The present results in *V. zizanioides* are consistent with this pattern, in which riboflavin promoted direct rooting while suppressing callus formation.

The acclimatization success rate of 40-50% achieved in this study is comparable to rates reported for other grass species (Quiala et al., 2016). Acclimatization is often challenging because in vitro-grown plantlets possess succulent stems and leaves, poorly developed cuticles, reduced palisade cell differentiation, large intercellular spaces, and defective stomata, leading to rapid water loss and desiccation when exposed to ambient conditions (Quiala et al., 2016). The gradual acclimatization protocol employed in this study, involving initial maintenance in high humidity followed by gradual reduction, allowed plantlets to develop functional cuticles and stomata before exposure to ambient conditions.

4.5 *Comparative Analysis with Related Species*

Comparison of the present results with previous reports on related aromatic grasses reveals both similarities and important differences. In *C. winterianus*, regeneration was obtained on MS medium containing 1.0 mg/L IAA and 0.5 mg/L kinetin, with auxin concentration higher than cytokinin (Sreenath et al., 1994). In contrast, the present study achieved efficient regeneration in *C. flexuosus* and *V. zizanioides* using BA alone or BA with low auxin concentrations, suggesting that mesocotyl-derived callus may have different hormonal requirements than callus from other explant types.

In *C. martinii*, Sreenath et al. (1994) reported plant regeneration using MS medium with 1.5 mg/L BA and 0.2 mg/L kinetin, requiring both cytokinins. In the present study, however, single cytokinins (either BA or kinetin) were sufficient for shoot induction in all three species, simplifying the regeneration protocol and reducing costs.

The high shoot multiplication rates achieved in this study (up to 34.17 shoots per explant in *C. flexuosus* and 29.75 shoots per explant in *V. zizanioides*) compare favorably with previous reports. Nayak et al. (1996) reported rapid propagation of *C. flexuosus* through somatic embryogenesis, while Monisha et al. (2021) achieved 75.49% shoot regeneration efficiency with 48 shoots per callus in *Chrysopogon zizanioides* (synonym of *Vetiveria zizanioides*) using organogenic callus. The present protocols thus represent efficient systems for mass propagation of these valuable aromatic grasses.

The establishment of these protocols provides a foundation for future biotechnological applications, including somatic hybridization, genetic transformation, and somaclonal variation studies aimed at improving essential oil yield and quality (Bhattacharya et al., 2008). The ability to maintain morphogenic potential through multiple subcultures is particularly important for genetic transformation experiments, which often require extended culture periods (Leupin et al., 2000).

CONCLUSION

This study established efficient, reproducible protocols for callus induction and plant regeneration in three economically important aromatic grasses: *Cymbopogon flexuosus*, *C. citratus*, and *Vetiveria zizanioides*. Key findings include:

Explant selection: Mesocotyl segments from 4-5 day-old seedlings were optimal for *C. flexuosus* and *V. zizanioides*, while immature inflorescences were most suitable for *C. citratus*. Mature tissue explants were unsuitable due to phenolic compound release.

Callus induction: MS medium supplemented with 1.0 mg/L 2,4-D combined with 0.1 mg/L kinetin (*C. flexuosus*), 1.0 mg/L kinetin (*V. zizanioides*), or 1.0 mg/L benzyl adenine (*C. citratus*) induced morphogenic callus efficiently.

Callus maintenance: Addition of 100 ppm each of PVP and CH extended morphogenic potential to 7-8 subcultures in *V. zizanioides*, addressing the common problem of declining regeneration capacity during prolonged culture.

Shoot regeneration: High shoot multiplication rates were achieved using benzyl adenine for *C. flexuosus* (up to 34.17 ± 0.78 shoots per explant) and *V. zizanioides* (up to 29.75 ± 0.69 shoots per explant), while *C. citratus* required kinetin for shoot induction.

Rooting and acclimatization: Species-specific rooting requirements were identified, with riboflavin effectively suppressing unwanted callus formation in *V. zizanioides*. An acclimatization success rate of 40-50% was achieved through gradual adaptation protocols.

These protocols provide practical tools for mass propagation, germplasm conservation, and genetic improvement programs in aromatic grasses. The high multiplication rates and ability to maintain morphogenic potential through multiple subcultures make these systems suitable for commercial micropropagation. Furthermore, these protocols establish a foundation for future biotechnological applications, including genetic transformation, somatic hybridization, and somaclonal variation studies aimed at developing improved varieties with enhanced essential oil yield, quality, and stress tolerance. Such improvements are essential for maintaining the competitiveness of these valuable crops in international markets and ensuring sustainable production of high-quality essential oils for the perfumery, pharmaceutical, and cosmetic industries.

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