High frequency plant regeneration of *Chrysopogon zizanioides* via organogenesis and somatic embryogenesis-an underutilized pharmaceutically valuable biofuel plant

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Research Article

Keywords: Vetiver, Callus, Node, Organogenesis, Somatic embryo

Posted Date: November 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-934878/v1

Abstract

Vetiver (*Chrysopogon zizanioides*) is an essential oil-producing plant that has tremendous application in cosmetics, perfumery, and herbal medicine. Natural sterility and indiscriminate harvests lead to the risk of extinction of plant species in natural habitats. Therefore, a protocol for regeneration systems via organogenesis and somatic embryogenesis using node, leaf, and root explants has been standardized. The highest shoot regeneration frequency (72.2%) through organogenesis was attained from node explants on MS (Murashige & Skoog) medium comprising 2.0 mg L^{-1} BAP ("6-benzylaminopurine"). Concurrently, leaf explants cultivated on MS medium augmented by 1.0 mg L^{-1}, 2, 4-D ("2, 4-dichlorophenoxyacetic acid") formed the optimal frequency (75.35%) of white friable compact (WFC) callus. However, the root explant was less responsive for WFC callus induction. Organogenic WFC callus cultivated on MS medium fortified by kinetin (1.0 mg L^{-1}) as well as BAP (1.0 mg L^{-1}) revealed the highest shoot regeneration efficiency (75.49%) with 48 shoots per callus. Adventitious shoots obtained from node and WFC callus of both leaf and root explants cultivated on MS medium increased by NAA (2.0 mg L^{-1} showed the optimal rooting of 76.97%. Concomitantly, an elevated frequency of somatic embryogenesis (52.50%) was recorded from leaf explants on MS medium using BAP (0.5 mg L^{-1}) & 2, 4-D (1.0 mg L^{-1}). Leaf explants were superior to node and root explants for somatic embryo initiation. The cotyledonary embryos were efficiently germinated into complete plantlets on a hormone-free MS medium. The plantlets gathered from organogenesis & somatic embryo genesis was effectively acclimatized into phenomenally similar plants. This technique may be applicable for wide-range propagation, genetic engineering, and the formation of bioactive compounds.

Introduction

Vetiver (*Vetiveria zizanioides)* is synonymously called as (*Chrysopogon zizanioides*) comes to the Poaceae family and is broadly cultured in tropical as well as sub-tropical areas for the production of essential oil. In spite of its economic importance, it has certain environmental significance such as soil moisture conservation, reduction in soil erosion, and deduction of heavy metals (Sudhishri et al. 2008; Minh and Khoa 2009; Leauagvutiviroj et al. 2010). Naturally, sterile plant and has no rhizome or stolons as seed material for cultivation, but conventionally propagated through root segments or slips.

The secondary metabolites present in vetiver root extract have been extensively used as a major ingredient in perfumery and cosmetic industries (Bhuiyan et al. 2008; Bhushan et al. 2013). Further, a variety of potential bioactivities have been notified previously from its extract including antimicrobial, herbicidal, and pesticide properties (Mao 2004; Koul 2008; Adam 2008; Sangeetha and Stella 2012). The pharmacological properties present in extract possess curative properties such as anti-inflammatory, antiseptic, sedative, vulnery, cicatrisant, aphrodisiac, digestive, haematinic, carminative, stomachic, antiasthmatic, antispasmodic, antihelmetic, antigout, and diuretic (Bhushan et al. 2013). Further, the viscous oil extorted from the roots of the vetiver has authenticated the presence of aroma and several potential therapeutic properties (Massardo and Pripdeevech et al. 2006; Rotkittikhum et al. 2010). The phytochemical components obtained from oil extracts include, khositone, vetivone, ß-Humulene, vetiverol, khusimol, vetivene, terpenes, benzoic acid, khusimone, Tripene-4-ol, ß-Humulene, epizizianal, vetivenylvetivenate, isokhusimol, ß- vetivone, vetivazulene (Pareek and Kumar 2011). Furthermore, the massive biomass derived from the entire plant could be an amenable and alternate resource for biofuel manufacture (Sun et al. 2014). Although *C. zizanioides* has a sufficient quantity of varied secondary

metabolites which could be utilized a novel phytomedicine, its population in natural habitat has become indiscriminately depleting due to pollen sterility, uncontrolled grazing, reaping by therapeutic professionals for making of folk medicine, deforestation, urbanization, and also due to unfavorable environmental factors.

Conventionally, vetiver has been propagated through root cuttings which have certain constraints such as inherent low viability, unsynchronized growth form, and also sluggish growth rate. These factors will impede the export of vetiver biomass across several nations. Thus, the actual requirement of biomass for oil extraction and biofuel production could not cope up with mounting commercial demands.

Henceforth, it is essential to propagate and conserve endangered or extinct germplasm by adopting the latest biotechnological technique *i.e.*, regeneration via plant tissue culture (Deo et al. 2010; Anis and Ahmad 2016). Environmentally independent and year-round generation of genetically similar and valuable populations through micropropagation technique is highly indispensable to meet out the ever-increasing demands on commercial and industrial-scale (Hussain et al. 2018).

Despite the fact that there are a few explants with its pre-existing meristem of vetiver has been used to accomplish micropropagationstudies, such as shoot segment (Code et al. 2012), adventitious bud and axillary bud (Zhenrang et al. 2006; Kanokporn and Chonnikarn 2016), and inflorescence (Sangduen and Prasertsongskun 2009) and reported with low regeneration frequencies. However, callogenesis and somatic embryogenesis have not been reported in this species. Herein, we developed efficient reliable and reproducible regeneration protocols through organogenesis and somatic embryogenesis from node, root, and leaf sheath explants of *C. zizanioides*.

Materials And Methods

Plant Materials

The propagating material clumps of *C. zizanioides* have been brought from a village, Nochikkadu, Cuddalore District, Tamil Nadu. The clumps were grown in the potting blend (compost, soil, and sand) in the proportion of 1:1:1 and maintained under shade in the pot culture yard of “Department of Genetics and Plant Breeding, Annamalai University, Annamalai Nagar”, India. Explants were collected from *ex-vitro* grown plants for optimization of regeneration methodology in *C. zizanioides.*

Explants preparation

Node, leaf sheath, and root explants were gathered from 30-day old plants and twenty minutes of washing is done with tap water and disinfected using 1% bavistin with continuous agitation. The materials are again rinsed with flowing tap water to eliminate residual bavistin. Again, explants were dipped in 0.1 percent “mercuric chloride” ($HgCl_2$) for 2 to 3 minutes, followed by 4 to 5 times cleaning with sterile filtered water to eliminate the traces of sterilizing agents. Explants such as nodal segments (5mm), leaf (5mm), and root segment (5mm) were cut away from the source plants with the support of sterilized sharp scalpels & forceps. Auxiliary buds and leaves and were cut off from the nodes and internodes by using a sharp scalpel blade. Identically, the cut may be given at the proximal end of the leaf sheath of explants.

Culture media and conditions

MS basal medium (Skoog and Murashige 1962) was added with different doses of PGRs (“Plant growth regulators”) including kinetin, NAA:“α-naphthaleneacetic acid”, TDZ:“Thidiazuron”, 2, 4-D and BAP. The carbon source incorporated in the medium is sucrose (30 g L^{-1}) and phytagel was employed in all media as a gelling agent at a 4.0 g L^{-1} rate. These thermostable PGRs were applied before autoclaving of media. The pH of the medium was varied to 5.8 with 1 N HCl or1 N NaOH. The autoclaving of the culture media was done in an autoclave using steam sterilization on a 1.5 kg/cm^2 pressure and 121degrees Celsius for fifteen minutes. Twenty milliliters of medium were poured into sterile culture tubes (25 x 150 mm) and sealed with cotton plugs. Cool white fluorescent tubes were used in the chamber of plant growth to maintain a photoperiod of 16/8 hour with “light intensity”of 50 µmol $m^{-2}s^{-1}$ (“Panasonic, Japan”). The PGR, as well as chemicals, were imported from Hi-media, and glasswares were brought from “Borosil”, India.

Adventitious shoot culture

Nodal portions were inoculated in MS media enriched with many levels of kinetin (0.5, 1.0, 2.0 & 4.0 mg L^{-1}), or BAP (0.5, 1.0, 2.0 and 2.5 mg L^{-1}) or TDZ (0.1, 0.5, 1.0 & 2.0 mg L^{-1}) to achieve direct adventitious initiation. The control treatment was maintained by using MS medium without PGRs. Subculturing was performed with the same medium composition for every fortnight and kept until 8 weeks. Percentage of shoot organogenesis from explants and an average number of shoots/explants along with mean shoot duration were noted after six weeks of cultivation. On average, 20 explants were subjected to individual therapy and the tests were followed three times. The regeneration response was estimated with the help of the following formula. Regeneration response (%) = (explants showing shoot regeneration number ÷ explants cultured total number) × 100.

Shoot regeneration and callus induction

Root and leaf segments of suitable size were incubated in MS medium with the several intensities of 2, 4-D (0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0 & 4.0 mg L^{-1}) for initiation of callus. These cultures were incubated in dark at 28 ± 1ºC for 6 weeks and subcultured at 2 weeks intervals. Callus initiation Frequency of individual treatment was noted after 8 weeks of culture. The callus developmental nature was categorized as WFC (“white friable compact”), WNF (“white non-friable”), and no callus initiation (N). Twenty explants were subjected to individual replication, three replications per experiment, and treatment was performed three times. Organogenic calli resulting from the above trials were subjected to media with various intensities of NAA from 0.5 to 1.0 mg L^{-1} blended with numerous intensities of BAP (0.5, 1.0, 2.0, 3.0, 5.0 & 6.0 mg L^{-1}) respectively for initiation of adventitious shoots. Subculturing of these cultures was performed once in 2 weeks and continued for 6 weeks with the same medium concentration for adventitious shoot initiation. The fraction response of shoot organogenesis was observed from callus and a mean “number of shoots/callus pieces” after six weeks. The plantlet's nature was categorized as qualitatively +: “abnormal stunted shoots”, ++: “normal leaves shoots”, -: no shoots.

Hardening and Rooting

Transferring of shoots developed from “the nodes, internode explants, and shoots obtained from leaf sheath and root-derived calli to rooting media consisting of either hormone-free half-strength MS or half-strength MS + NAA (0.5 mg L^{-1}) or full-strength MS basal medium or half MS + NAA (1.0 mg L^{-1}) or full MS + NAA (2.0 mg L^{-1}) or full MS + NAA (2.5 mg L^{-1}) for 2-4 weeks. After root initiation from this culture, subculturing of inoculants into

the fresh medium after 2 weeks was done and kept until four weeks. After four weeks fully developed rooted shoots have been uprooted from the media and cleaned thoroughly thrice with" sterile water to eliminate the adhered material and transferred to plastic pots with the potting mixture for hardening. Then the plastic containers were coated with plastic bags and holes were created for gaseous exchange. These potted plants were kept for 2 weeks in the chamber of plant growth and then shifted on to the greenhouse condition. The polyethylene covers were gradually detached from well-established plantlets. The plant survival ratio in the greenhouse and at the field was assessed. After four weeks, the proportion of rooting response, the mean number of roots/shoots and root duration have been noted for each treatment. For each replication, ten adventitious shoots were utilized with 3 replications per experiment, and treatment was performed three times.

Plant regeneration and somatic embryogenesis

For somatic embryogenesis nodal, leaf, as well as root parts were surface cultivated on MS medium extended with BAP (0.5 & 1.0 mg L^{-1}) along with numerous intensities of 2, 4-D (0.5, 1.0, 3.0 & 5.0 mg L^{-1}) respectively. The lower cut end of the leaf sheath explants was placed in a resting position and embedded in the culture medium. Subculturing of inoculants was done once in two weeks and kept until 4 to 6 weeks at 23 ± 2ºC, RH of 60-65 % and "light intensity" of 30 µmol m^{-2} s^{-1} within cool white pipes of the fluorescent with a photoperiod of 16/8 hours in a growing chamber (Panasonic, Japan). The rate of explants developing "somatic embryos" was noted after four to 6 weeks and an average number of somatic embryos/explants was found after six weeks of inoculation. The different phases of somatic embryos such as cotyledonary, torpedo, heart, and globular, derived from MS medium consisting of 2, 4-D (1.0 mg L^{-1}) as well as BAP of (0.5 mg L^{-1}) were subjected to either MS + NAA of (0.5, 1.0 2.0 & 2.5 mg L^{-1}) or half or full strength for the transformation of "somatic embryos" into entire plantlets. Subculturing of cultures was done at the interval of two weeks and kept up to six weeks. The regeneration frequencies of somatic embryos were noted after four to six weeks. Twenty explants were used with three replications per experiment and treatment was replicated three times. The fully developed plantlets with a well-organized rooting system were detached from the culture tube, and cleaned with sterile water to detach adhering agar, and were moved to sterilized potting blend and maintained in a greenhouse for acclimatization. The plantlet's survival ability was examined in both greenhouse and in field conditions.

Statistical analysis

The CRD ("Completely randomized design") was employed as an experimental design for all experiments conducted in this research. The data collected from these experiments were analyzed through ANOVA ("analysis of variance") with the aid of SPSS Version 10 (SPSS, Chicago, IL) software. The significance differences within the treatment means were compared with the aid of DMRT ("Duncan's Multiple Range test") at a 5 percent probability level. The resulted data were represented as mean ± SE ("Standard Error").

Results

Induction of multiple shoots

Nodal segments from one-month-old seedlings (Fig. 1a) were subjected to MS medium augmented with numerous kinetin, TDZ, and BAP levels for proliferation and induction of adventitious shoots. Shoots appeared from explants after two weeks of culture (Fig. 1b-f). However, completely emerged shoots and shoot elongation

was found after four weeks of culture. Here the direct organogenesis pathway from the nodal segments without production of callus stage and visible shoot buds was identified within 4 weeks. The nodal explants cultivated on MS media augmented using BAP (1.0 mg L^{-1}) recorded high frequency (72.20 %) of shoot initiation with the maximum shoots number (10.04) and with the greater shoot duration of 8.1 cm (Table 1; Fig. 1h).

Table 1. Effect of different concentrations of BAP, TDZ and kinetin on regeneration of adventitious shoots from nodal segments of *C. zizanioides*

Phytohormones (mg/L)			Regeneration response (%)	Mean no. of shoots/ explants	Mean shoot length (cm)	Nature of plantlets
BAP	TDZ	Kinetin				
0.0	0.0	0.0	0.0 ± 0.0i	0.0 ± 0.0f	0.0 ± 0.0g	–
0.5	-	-	31.45 ± 1.3d	1.0 ± 0.5e	2.5 ± 0.1d	+
1.0	-	-	72.20 ± 3.1a	10 ± 0.4a	8.1 ± 0.4a	++
2.0	-	-	53.00 ± 1.6b	6.0 ± 0.3b	2.0 ± 0.1d	++
2.5	-	-	25.43 ± 1.0e	1.0 ± 0.1e	2.3 ± 0.1d	+
-	0.1		21.89 ± 1.0f	1.0 ±0.2e	1.8 ± 0.1e	+
-	0.5		43.63 ± 2.0c	3.0 ± 0.2c	4.0 ± 0.1b	++
-	1.0		19.87 ± 0.9f	0.5 ± 0.2e	0.8 ± 0.0f	+
-	2.0		06.89 ± 0.3h	1.2 ± 0.4e	1.2 ± 0.1e	+
-	-	0.5	11.45 ± 0.5g	1.0 ± 0.1e	0.9 ± 0.0f	+
-	-	1.0	04.66 ± 0.2h	2.2 ± 0.1d	2.9 ± 0.1d	+
-	-	2.0	35.28 ± 1.5d	3.0 ± 0.2c	3.4 ± 0.2c	++
-	-	2.5	07.88 ± 0.3h	1.0 ± 0.5e	1.7 ± 0.1e	+

Means having the same letter in columns are not significantly different by Duncan's multiple range test ($P < 0.05$).

Each data represent mean ± SE of three independent experiments

TDZ and Kinetin indicated less response than BAP for shoot induction as well as proliferation. No shoot induction without cytokinin (control) was found on MS medium. The shoot induction frequency was compared with other PGRs as well. Since the findings, it was found that nodal segments exhibited 43.63% of regeneration efficiency TDZ (0.5mg L^{-1}) and 35.28 % with TDZ at 2.0 mg L^{-1}.

Callus Induction And Shoot Organogenesis

Leaf and root explants were nurtured onto MS medium amended with several levels of 2, 4-D (0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0 mg L^{-1}) for callus induction. It was detected after 2 weeks (Fig. 1i) and a well-developed callus was noted after 4 weeks (Fig. 1j). Callus induction frequency of both explants was raised with the rise in levels of 2, 4-D from 0.2 to 0.5 mg L^{-1} for root and from (0.5 to 1.0 mg L^{-1}) for leaf explants. Further rise in concentration decreased the potential of callus formation (Table 2). After eight weeks of cultivation, the highest rate of callus formation (75.35%) was identified to be 2, 4-D (1.0 mg L^{-1}) in (Fig. 1k) from leaf explants on MS and 64.28% from root explants cultivated on MS augmented by 2, 4-D (0.5 mg L^{-1}) in (Fig. 1O). Thus, leaf explant shows a better callus induction response than that root explants. There was no callusing response in case of nodal explants in the similar media containing 2, 4-D.

Table 2
Effect of 2, 4-D concentrations on callus induction from leaf and root explants of *C. zizanioides*

2,4-D (mg/L)	% of leaf forming callus	Nature of callus	% of root forming callus	Nature of callus
0.0	0.0 ± 0.0h	N	0.0 ± 0.0h	N
0.1	18.96 ± 0.9g	WNF	45.36 ± 2.1c	WFF
0.2	33.29 ± 1.4e	WNF	50.70 ± 2.4b	WFC
0.5	36.73 ± 1.8d	WNF	64.28 ± 3.4a	WFC
1.0	75.35 ± 3.7a	WFC	42.60 ± 2.1c	WNF
1.5	50.98 ± 1.5b	WFC	32.00 ± 1.6d	WNF
2.0	46.23 ± 2.2c	WNF	26.20 ± 1.3e	WNF
3.0	29.60 ± 1.3f	WNF	18.90 ± 0.8f	WNF
4.0	20.02 ± 1.0g	WNF	11.45 ± 0.5g	WNF
Means having the same letter in columns are not significantly different by Duncan's multiple range test ($P < 0.05$).				
N-no response, (N), WFC- white friable compact, WNF-white non friable				
Each data represent mean ± SE of three independent experiments				

Calli obtained from root explant at 2, 4-D from 0.2 mg L^{-1} to 0.5 mg L^{-1} along with leaf explant at 2, 4-D from 1.0 mg L^{-1} to 1.5 mg L^{-1} were"observed to be compact, friable as well as white in nature, whereas the rest were noted to be non-friable, white and spongy in nature (Table 2). These embryogenic WFC callus cultures procured from root explants and leaf explants were moved onto MS medium comprising numerous levels and blends of NAA along with BAP (Table 3). Both leaf sheath and root-derived calli showed the highest shoot regeneration rate on MS medium augmented using NAA of (1.0 mg L^{-1}) and BAP of (1.0 mg L^{-1}). Callus obtained from leaf demonstrated an optimum shoot regeneration frequency (75.49%) with 40 shoots per callus after six weeks of culture (Fig. 1l-n). Similarly, root-derived calli also recorded the maximum frequency of shoot generation (60.20%) with 25 shoots per callus (Fig. 1p - r).This observation indicated that indirect organogenesis pathway is achievable from leaf and root explants of *C. zizanioides*

Table 3
Effect of different concentrations of BAP, TDZ and kinetin on regeneration of adventitious shoots from callus cultures of *C. zizanioides*

Phytohormones (mg/L)		Leaf			Root		
BAP	NAA	Shoot regeneration (%)	Mean no. of shoots/callus	Nature of plantlets	Shoot regeneration (%)	Mean no. of shoots/callus	Nature of plantlets
0.0	0.0	0.0 ± 0.0i	0.0 ± 0.0g	–	0.0 ± 0.0k	0.0 ± 0.0j	–
0.5	0.5	33.80 ± 1.0f	4.0 ± 0.1d	+	33.53 ± 1.5f	3.1 ± 0.2f	+
0.5	1.0	36.00 ± 2.2e	2.0 ± 0.8e	+	38.70 ± 1.9d	5.1 ± 0.2d	+
1.0	0.5	52.23 ± 2.3b	20.5 ± 1.5b	++	45.65 ± 2.2b	12.6 ± 0.9b	++
1.0	1.0	75.49 ± 4.0a	48.0 ± 0.4a	++	60.20 ± 2.5a	25.6 ± 1.2a	++
2.0	0.5	48.30 ± 0.5c	15.3 ± 1.5c	+	42.63 ± 2.0c	9.5 ± 0.6c	+
2.0	1.0	42.03 ± 2.4d	4.0 ± 0.5d	+	38.31 ± 1.8d	4.1 ± 0.2e	+
3.0	0.5	37.57 ± 1.8e	2.7 ± 0.5e	+	35.78 ± 1.6e	3.1 ± 0.2g	+
3.0	1.0	34.82 ± 1.6f	2.2 ± 0.4e	+	29.23 ± 1.3g	2.5 ± 0.1h	+
5.0	0.5	25.78 ± 1.2g	2.0 ± 0.8e	+	25.56 ± 1.2h	2.0 ± 0.1h	+
5.0	1.0	22.54 ± 1.1g	2.0 ± 0.5e	+	20.44 ± 1.0i	1.0 ± 0.0i	+
6.0	0.5	22.34 ± 1.4g	1.1 ± 0.6f	+	19.28 ± 0.8i	1.0 ± 0.0i	+
6.0	1.0	15.65 ± 1.0h	1.5 ± 0.5f	+	11.21 ± 0.4j	1.0 ± 0.0i	+

Rooting Of Adventitious Shoots

Shoots obtained from indirect as well as direct organogenesis were removed from cultivation and subjected to the rooting medium comprising of full-strength MS using NAA (2.0, 2.5 mg L^{-1}),half and full MS medium with NAA (0.5 & 1.0 mg L^{-1}), hormone-free half-strength, for effective root regeneration (Table 4). Root initiation was detected at the base of *in vitro* shoots after 2 weeks of inoculation (Fig. 1s) and a well-developed root system was visible after 4 weeks (Fig. 1t and u). Results indicated that maximum response of root initiation (76.97%) was found with full-strength MS amended by NAA (2.0 mg L^{-1}) with an average of 16 roots per plant with the root duration of 4.01 ± 0.2 cm. However, there is less response of root initiation in the half-strength hormone-free medium.

Table 4
Effect of different levels of NAA and MS medium strengths on rooting of adventitious shoots regenerated through direct and indirect organogenesis of *C. zizanioides*

Culture medium	Rooting (%)	Mean no. of roots /shoot	Average root length (cm)
Half MS	12.25 ± 0.6f	2.00 ± 0.1f	0.98 ± 0.0d
Full MS	26.62 ± 1.3e	3.00 ± 0.2e	1.20 ± 0.0d
Half MS + NAA (0.5 mg/L)	35.34 ± 1.7d	5.50 ± 0.3d	1.56 ± 0.1d
Half MS + NAA (1.0 mg/L)	40.01 ± 2.0c	8.76 ± 0.4c	2.08 ± 0.1d
Full MS + NAA (2.0 mg/L)	76.97 ± 3.8a	16.00 ± 0.8a	4.01 ± 0.2a
Full MS + NAA (2.5 mg/L)	54.21 ± 2.6b	10.50 ± 0.5b	3.14 ± 0.2b
Values followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range tests.			
Each data represent mean ± SE of three independent experiments			

Regeneration Via Somatic Embryogenesis

Node, leaf, and root explants were surface sterilized and inoculated in the MS medium augmented with many mixtures and levels of 2, 4-D as well as BAP for initiation of somatic embryos (Table 5). The proximal part of the nodal segments was placed vertically in the culture tube, whereas leaf and root surface were allowed to firmly contact in the medium.

Table 5
Somatic embryogenesis from node, leaf, and root explants of *C. zizanioides*

PGRs (mg/L)		Node		Leaf		Root	
2, 4-D	BAP	R (%)	MNS	R (%)	MNS	R (%)	MNS
0.0	0.0	00.00 ± 0.0i	0.0 ± 0.0	00.00 ± 0.0h	0.0 ± 0.0g	00.00 ± 0.0i	0.0 ± 0.0i
0.5	0.5	4.23 ± 0.6gh	5.26 ± 0.3i	13.26 ± 1.5e	4.1 ± 0.2g	5.64 ± 0.6h	6.1 ± 0.4h
0.5	1.0	5.36 ± 0.5f	8.03 ± 0.2gh	15.34 ± 0.3d	10.1 ± 0.4e	7.61 ± 0.8g	12.1 ± 0.5ef
1.0	0.5	32.88 ± 1.9a	23.6 ± 0.5a	52.50 ± 2.5a	46.2 ± 1.5a	40.80 ± 2.5a	38.2 ± 1.7a
1.0	1.0	17.55 ± 1.5b	18.1 ± 0.4b	27.44 ± 1.8b	28.5 ± 0.6b	33.50 ± 1.2b	25.5 ± 2.0b
3.0	0.5	14.0 ± 0.8c	16.6 ± 0.6c	18.25 ± 1.2c	15.1 ± 1.8c	25.13 ± 1.4c	22.1 ± 1.5bc
3.0	1.0	13.6 ± 0.8cd	14.4 ± 0.2d	15.61 ± 0.9d	13.1 ± 1.2d	20.22 ± 2.1d	15.1 ± 1.4d
5.0	0.5	12.26 ± 0.6de	13.5 ± 0.5de	11.45 ± 0.6ef	10.6 ± 1.4e	17.50 ± 1.2e	13.6 ± 1.2e
5.0	1.0	9.43 ± 0.4f	9.52 ± 0.6f	10.62 ± 0.5g	9.1 ± 0.8ef	15.60 ± 1.2ef	10.1 ± 0.6g

Means having the same letter in columns are not significantly different by Duncan's multiple range test ($P < 0.05$).

Initiation of somatic embryos from explants was observed from all the treatments of 2, 4-D & BAP blends, indicating the optimization of PGRs for somatic embryogenesis. However, the greatest rate of somatic embryogenesis was notified from leaf (52.50%), followed by root (40.80%) and node segments (32.88%) on MS medium expanded by 2, 4-D (1.0 mg L^{-1}) as well as BAP (0.5 mg L^{-1}) (Table 5). Somatic embryos of all stages were noted after 6 weeks of incubation. Concomitantly, leaf explant formed a substantially highest number of somatic embryos (46.2), followed by root (38.2) and node explants (23.6). Heart shape and torpedo embryos from node (Fig. 2a), cotyledonary stages from leaf (Fig. 2b), and roots (Fig. 2c) were observed during the 4th week of the culture period. Entire somatic embryos of all phases were acquired and transported to a hormone-free MS medium for germination into complete plantlets. Germination and proliferation of cotyledonary stage embryos (Fig, 2 d and e) into complete plants with an efficient rooting system were observed after 6 weeks (Fig. 2f). Thereafter, *in vitro* established somatic plantlets were moved to sterile pot blend & adjusted in the greenhouse (Fig. 2h).

Hardening Of In Vitro Raised Plantlets

The better survivability of regenerated plantlets was achieved through hardening. In this study, the plantlets regenerated via organogenesis as well as somatic embryogenesis was moved to plastic containers covered with sand, sterilized soil along with FYM mix in a 1:1:1 ratio for acclimatization. These hardened plantlets were coated with a plastic sheet and drenched with a half-strength MS medium twice a week. Acclimatized plants

were cultured in the chamber of a plant growth for 15 days and subsequently transferred to a greenhouse and then on the field condition. The survival rates of regenerated plants were evaluated in the greenhouse as well as in field conditions. Findings indicated that organogenic plantlets survived 70 percent in the greenhouse and 60% at field conditions, whereas, somatic embryogenesis derived plantlets survived 78% at greenhouse conditions and 65% at field conditions (Table 6). The complete protocol involving regeneration by organogenesis as well as somatic embryogenesis of *C. zizanioides* was presented in Fig. 3.

Table 6
Acclimatization and survival rate capability of *in vitro* regenerated plantlets of *C. zizanioides*

Mode of regeneration	Survival rate (%)	
	Greenhouse	Field condition
Organogenesis	70	60
Somatic embryogenesis	78	65
CD @ 5%(0.05)	2.49	4.20
*Mean of three replications		

Discussion

About four million people in rural regions have entirely depended upon medicinal plants as a primary source of health care (Petrovska 2012). Plant tissue culture can be an alternate methodology for germplasm conservation and mass production of quality bio-active compounds (Ahmed et al. 2019; Grigoriadou et al. 2019). Hence, the current study was set to regulate reliable regeneration protocols via organogenesis & “somatic embryogenesis” in *C. zizanioides*.

Regeneration through direct organogenesis

Some of the reports on in vitro regeneration of vetiver using explants like leaf crown (Leupin et al. 2000), leaves (Mucciarelli et al. 1993), young inflorescence (Sangduen et al. 2009), meristem tip culture (Chitra et al. 2014) and mesocotyl culture (George et al. 1999). In this direct shoot organogenesis involving nodal segments has been successfully established from *in vivo* grown seedlings of *C. zizanioides*. Several reports have authenticated the superior role of nodal explant over the other types to accomplish micropropagation in medicinal plants such as *Mentha piperita* (Sujana 2011), *Withania somnifera* (Fatima 2012)*, Agastache foeniculum* (Moharami 2014), *Teucrium scorodonia* (Makowczyńska 2016), and *Spermacoce hispida* (Deepak et al. 2019). Earlier literature has proved the existence of positive correlations among explant types, the shoot proliferation rate, and influences of cytokinin (Sajid et al. 2011; Dantu and Bhojwani 2013; Lebedev et al. 2018).

Here, cytokinins such as kinetin, TDZ, and BAP were used to assess the shoot induction efficacy from nodal explants of *C. zizanioides*. Among the three cytokinins tested, BAP exhibited maximum performance for adventitious shoot regeneration than TDZ and kinetin.

Yang et al. (2013) also stated about the effect of BAP in MS medium on direct regeneration in vetiver. The effect of BAP in shoot organogenesis was previously noted in several medical plants like *Canscora*

decussate (Loganathan 2016), *Scutellaria alpine* (Grzegorczyk 2016), *Dracocephalum forrestii* (Weremczuk 2018), and *Gymnema sylvestre* (Isahet al. 2019). Interestingly, we noticed maximum shoot proliferation (72.2%) from node explant cultured onto MS fortified with BAP @ 1 mg L^{-1}. Similar trends of findings were also observed in *Securidaca longipedunculata* (Lijalem et al., 2020) and *Trichosanthes dioica* (Tiwari et al. 2010). The nodal segment is considered to be the best explant type when compared to other types for initiation of direct shoot regeneration and proliferation and also requires only a lesser concentration of cytokinin, according to the Algabri et al. survey (2019).

Callus induction

Callus culture offers a wide variety of applications in pharmacological research, rather than direct organogenesis (Efferth 2018). The efficacy of callus initiation is reliant on the medium composition and types of the explants (Romano and Gonçalves 2013; Nordine et al. 2014). Leaf and root parts from *in vitro* seedlings of *C. zizanioides* were used as explants for callogenesis. In this study, even inflorescence of vetiver also used for callus development (Somporn 2003)using synthetic 2, 4-D, and auxin. 2, 4-D performs a significant part in plant tissue culture methods for callogenesis, which has been already stated in *V. zizanioides* (Mucciarelli et al. 1993; yang et al. 2008) proliferation as well as somatic embryogenesis (Gao et al. 2011). Further, it has been said that 2, 4-D has a noteworthy effect on the biochemical and molecular processes of callus with stimulating certain gene expression, controlling the metabolism of endogenous IAA as well as regulating DNA methylation (Pan 2010). The maximum frequency of friable compact callus was found in MS medium fortified using 2, 4-D at 0.5 mg L^{-1}& 1.0 mg L^{-1} from root and leaf explants, respectively. Similar studies on callogenesis using leaf and root explants were reported in several medicinally important herbs such as *Trachyspermum ammi* (Fazeli-nasab 2018), *Vitex negundo* (Chowdhury 2011), *Piper betel* (Junairiah 2019), *Ledebouria revoluta* (Haque 2018), *Celastrus paniculatus* (Moola et al. 2020) and *Rhinacanthus nasutus* (Reshi et al. 2018), and *Rosa hybrida* (Liu et al. 2018). Further, callus is the most amenable and optimal tissue for the formation of novel secondary metabolites (Benjamin et al. 2019). It has been noticed that the leaf explants showed a better callusing response than root explants.

Regeneration from callus

The compact friable calli obtained from culturing of leaf and root explants were transferred onto shoot initiation medium augmented with varied levels and mixtures of BAP and NAA. It has been found that both the explants exhibited the highest frequency of shoot redevelopment on MS by NAA of 1.0 mg L^{-1} and BAP of 1.0 mg L^{-1} as stated in *V. zizanioides* (Sompornpailin et al. 2016; Sompornpailin 2009). Also the Zinc Oxide Nanoparticles showed some impact on calli derived regenerants in *V. zizanioides* (Khunchuay et al. 2017). Similar effects of auxin and cytokinin combinations for shoot initiation were well studied from several medicinal plant types like *Rauvolfia serpentine* (Singh et al. 2009), *Celosia argentea* (Bakar et al. 2014), *Lallemantia iberica* (Ozdemir et al. 2014), and *Indigo feraviscosa* (Rajabudeen 2016). Further, leaf-derived calli documented a better response that was produced from root explants on shoot regeneration over the calli.

Rooting of adventitious shoots

The rooting efficiency and survival rate of hardened plants at *in vivo* conditions are the factors for successful *in vitro* propagation (Reshi 2018). *In vitro* rooting hastened the adaptability of plants to *ex vitro* conditions (Krupa-

Malkiewicz and Mglosiek 2016). Regardless of medium, auxin enhances the root initiation to *in vitro* regenerated adventitious shoots (Martins et al. 2013; Shekhawat and Manokari 2018). In the current work, full-strength MS medium amended with 2.0 mg L^{-1} NAA illustrated a better-rooting response than other levels. Concomitantly, the results of Widoretno et al. (2017) also corroborated the present results that NAA @ 1.0 mg L^{-1} noted better-rooting efficiency on *C. zizanioides*. 154 Concomitantly, the results of Widoretno et al. (2017) also corroborated the present results that NAA @ 1.0 mg L− 1 noted better-rooting efficiency on *C. zizanioides*. The NAA efficiency in the roots of *in vitro* regenerated shoots was well recognized in *Rotula aquatica* (Chithra 2004), *Phyllanthus caroliniensis* (Catapan et al. 2012), *Andrographis paniculata* (Hossain et al. 2016), *Tinospora cordifolia* (Mridulaet al. 2017).

Regeneration via somatic embryogenesis

Somatic embryos are the unicellular origin and can be maintained as an embryogenic culture for a long period (Tomiczak 2019). Here the somatic cells are differentiated into the whole plant by the embryogenesis process. *In vitro* embryogenesis is the direct approach where the somatic embryos are directly developed from explant tissues (Mazri and Meziani 2015). Many diverse and complex factors affect somatic embryogenesis (Deo et al. 2010). The concentration, type, and combination of regulators for plant growth are the main variables that influenced somatic embryogenesis as well as plant regeneration (De Almeida et al. 2012).

Here, node leaf, and root segments as explants for somatic embryogenesis have been accomplished. High-rate somatic embryogenesis was detected at BAP (1.0 mg L^{-1}) and 2, 4-D (0.5 mg L^{-1}) combinations from all explants. The mixtures of auxins with cytokinins were accounted for to induce considerable somatic embryogenesis in *Fraxinus excelsior* (Capuana et al. 2007),*Eremochloa ophiuroides* (Liu et al. 2008), *Swertia chirayita* (Chandra and Kumar 2014), and *Lachenalia viridiflora* (Kumar et al. 2016). The current research demonstrates that MS medium augmented with 2, 4-D and BAP is essential for friable embryogenic callus development in *C. zizaniodes.* The results of this research correspond to previous studies on somatic embryogenesis of *Chrysanthemum indicum* (Datta and Mandal 2005), "*Brachiaria brizantha*" (Cabral et al. 2011), *Rosa hybrida* (Bao et al. 2012), "*Prosopis laevigata"* (González-Buendíaet al. 2012), *Vitis vinifera* (Dai et al. 2015) and *Curcuma amada* (Raju et al. 2015).

The transformation of somatic embryos into mature plantlets is crucial for a successful *in vitro* plant regeneration system. The torpedo and cotyledonary embryos were harvested from 2, 4-D, and BAP treatments and were subjected to germinations. Results showed significant conversion and germinations of somatic embryos were noticed on half-strength MS medium. Published literature has confirmed the effectiveness of half-strength MS for "somatic embryo germination" in other types as well (Paul 2011; Raju 2013; Kumar, 2015; Khan et al. 2015). The simultaneous transformation of the shoot and root axis from the matured somatic embryos into plantlets is crucial in the somatic embryogenesis mode of regeneration (Pandey et al. 2013).

Hardening of somatic plants

Success in micropropagation is based on the successful *ex vitro* establishment of a fully regenerated *in vitro* plant. Fully grown healthy plants from experimental tubes were carefully removed and transferred to the potting mixture for acclimatization. The plantlet's survival rate was judged in the greenhouse and subsequently in field conditions. Results revealed that the plantlets derived from somatic embryogenesis survived at higher rate when

compared to organogenic plantlets (Table. 6). Regardless of the acclimatizing environment, similar observations on survival rate and adaptability were recorded from organogenesis and somatic embryogenesis derived plants (Bhagya et al. 2013; Deepak et al. 2019).

Conclusion

The demonstrated protocol of plant regeneration via organogenesis and somatic embryogenesis using node, root, and leaf explants of *C. zizaniodes*. Callogenesis from leaf and roots explants may facilitate the extraction of novel bioactive compounds. The present protocol might open the ways to scale up research on genetic engineering to synthesize novel drugs, germplasm conservation, and micropropagation at industrial level.

Abbreviations

2, 4-D 2, 4-dichlorophenoxyacetic acid

BAP 6-benzylaminopurine

IBA Indole-3-butyric acid

IAA Indole-3-acetic acid

NAA α-Naphthaleneacetic acid

Declarations

Conflict of interest

The manuscript was approved by all authors after reading it and declared that there is no conflict of interest.

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Figures

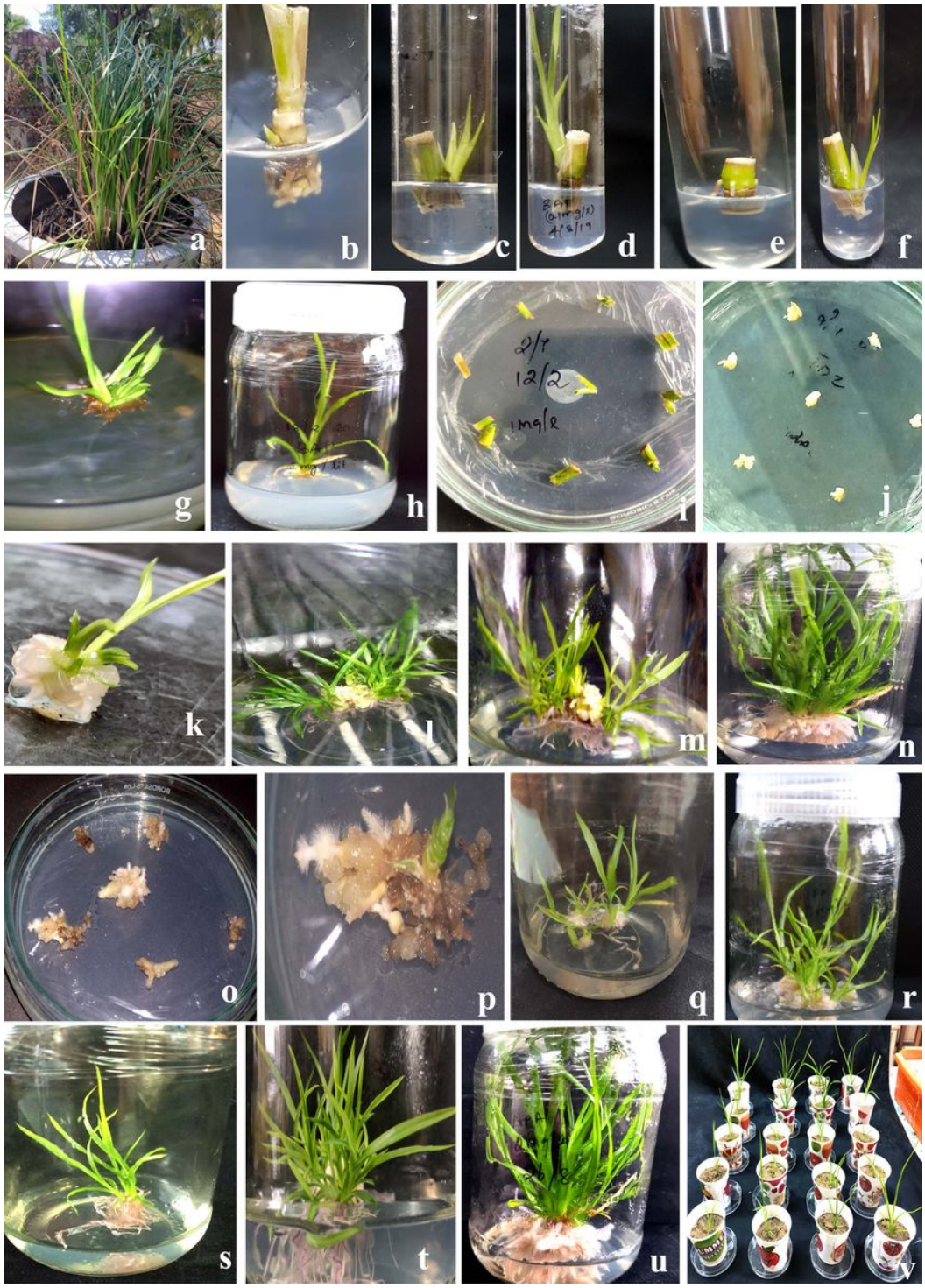

Figure 1

In vitro regeneration of C. zizanioides through direct (a-h) and indirect organogenesis (i-v). (a) Stock plants established in pot culture; (b & c) Shoot initiation from node on MS using 1.0 mg L− 1BAP after 1week (b) 2 weeks (c); (d, e & f) Response of nodal segments on different cytokinins i.e single shoot on MS with BAP (0.1 mg L− 1 (d)), only rooting at 2.0 mg L− 1TDZ (e) and stunted shoot on 4.0 mg L− 1kinetin; (g & h) Various shoots on MS using BAP(1.0 mg L− 1) after four weeks (g) and elongated shoots after six weeks (h); (i& j) Callus initiation from leaf segments on MS using 2, 4-D (1.0 mg L− 1) after 2 weeks (i) four weeks (j); (k-n) Shoot initiation from leaf derived callus on MS with NAA (1.0 mg L− 1) and BAP (1.0 mg L− 1) after four weeks

(k), Shoot elongation and proliferation after eight weeks (l - n); (o) Callus induction form root segments on MS using2, 4-D (0.5 mg L− 1) after four weeks; (p - r) Shoot redevelopment from root-originated embryogenic callus on MS with BAP of (1.0 mg L− 1) and NAA of (1.0 mg L− 1) after six weeks (p), Many shoots initiation and elongation after 8 weeks (q & r); (s - u) Rooting of shoots on MS using NAA(2.0 mg L− 1) after two weeks (s) & four weeks (t and u); (v) Somatic plants acclimatized in pot mixture. Scale bars (a = 1 cm, b -f = 1 mm, g - i = 0.5 cm, k - n = 1 cm, q & p= 1mm, q – u= 1 cm, v = 2 cm).

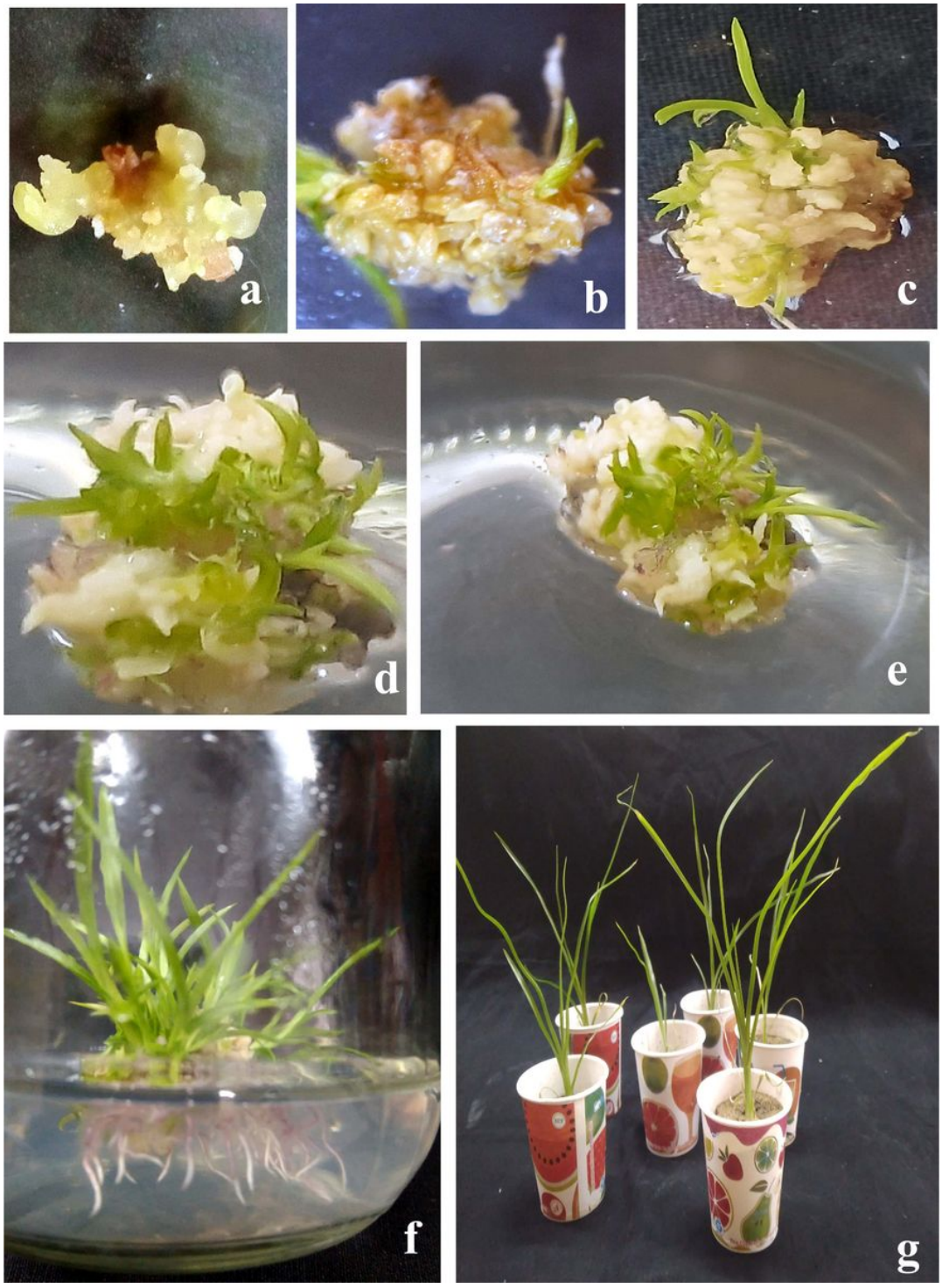

Figure 2

Somatic embryogenesis in C. zizanioides from explants cultivated on MS fortified using 2, 4-D (1.0 mg L− 1) as well as BAP (0.5 mg L− 1). (a-c) Clusters of somatic embryos from the node (a), asynchronous cotyledonary embryos from leaf (b) and root (c) after six weeks; (d - f) Germination of cotyledonary staged embryo on MS medium (d & e) after two weeks, complete plants with shoots and roots after four weeks (f); (g) Somatic plants acclimatized in pot mixture before transferring to the soil. Scale bars (a, b, c, d and e = 1 mm, f = 0.5 cm, g = 1 cm).

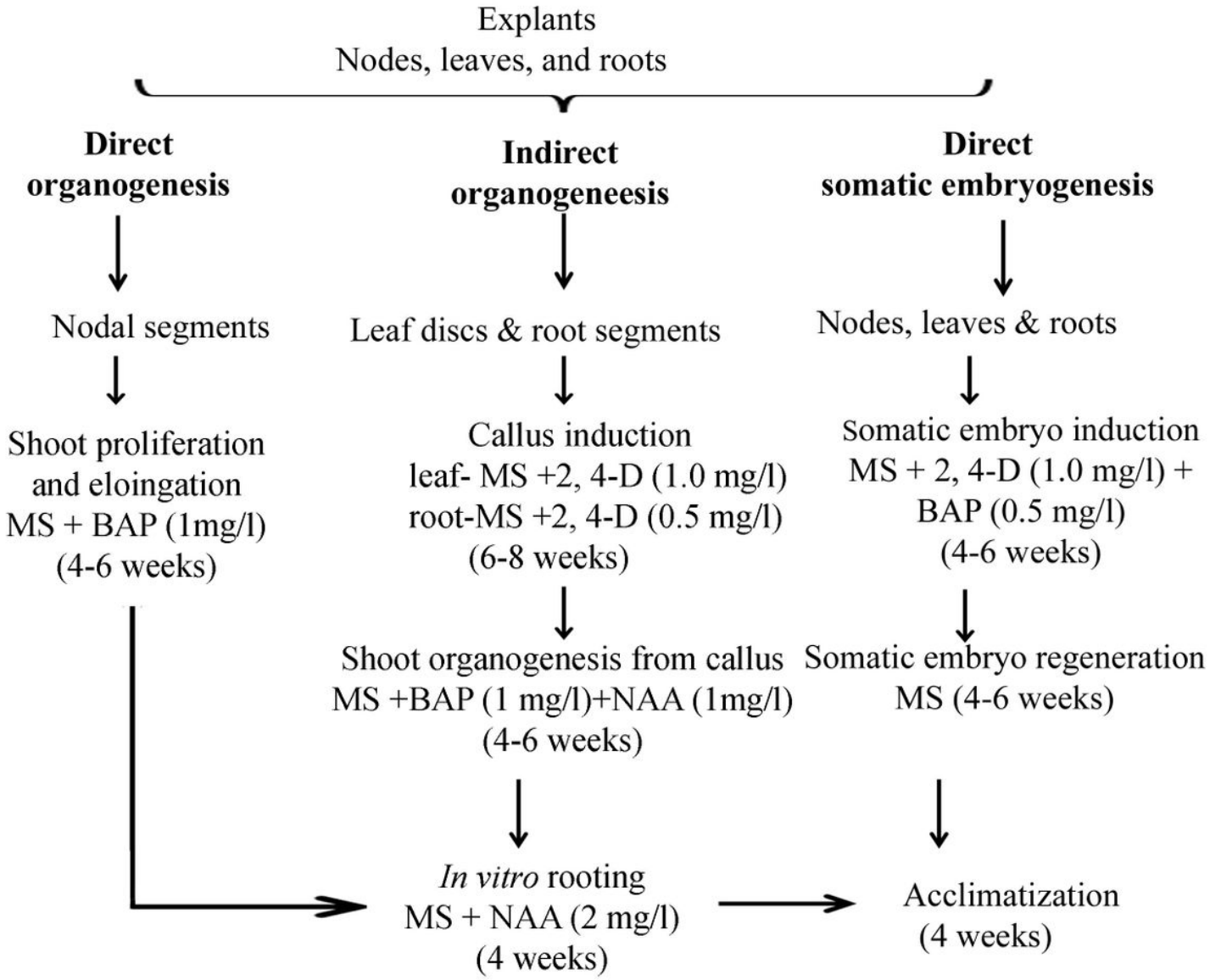

Figure 3

Outline of morphogenic pathways involved in micropropagation of C. zizanioides from different explants