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Original Article

Callus mediated shoot organogenesis and regeneration of cytologically stable plants of *Ledebouria revoluta*: An ethnomedicinal plant with promising antimicrobial potency

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ABSTRACT

Ledebouria revoluta are important ethnomedicinal plant found in India and South Africa. Micropropagation via indirect shoot organogenesis had been established from three types of explant (i.e. scale leaf, leaf lamina and root) of *L. revoluta*. Scale leaf was found superior as compared to leaf lamina and root explant with respect to their organogenic callus induction potentiality. Murashige and Skoog (1962) [MS] media supplemented with 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.75 mg L^{-1} β-naphthoxyacetic acid were best effective for inducing organogenic callus. Maximum 17.0 ± 0.52 bulblets were induced from about 500 mg of callus within 42–46 days sub-culturing on a medium containing 0.75 mg L^{-1} kinetin. The bulblets were matured (86.7% success) after one month culture on the same medium composition. The best result of *in vitro* root induction with 100% response and 8.4 ± 0.31 roots per bulb was achieved after 18 days of implantation on MS medium containing 2.0 mg L^{-1} indole-3-butyric acid. Plantlets were acclimatized with a 96.0% survival rate. Chromosomal studies revealed cytological stability of callus cells and all regenerants containing 2n = 30 chromosomes, same as parental plants. Antimicrobial activity of *L. revoluta* was tested against two Gram-positive bacteria, three Gram-negative bacteria and two fungi. The methanol and ethanol extract proved more effective against bacteria, whereas acetone and chloroform extract shows potential anti-fungal activities. Present protocol can be applied reliably to produce uniform planting materials in large scale. In addition, this efficient indirect regeneration pathway via callus culture opens a way for improvement through genetic transformation.

1. Introduction

Ledebouria revoluta (L.f.) Jessop [Syn: *Scilla indica* (Wight) Baker] are bulbaceous species of the family Asparagaceae [1]. This plant is commonly known as 'jangali pyaaj' (wild onion) or 'Indian squill' and used as a substitute for onion by tribal peoples of Tamil Nadu, India [2]. Fresh squill yields several cardiac glycosides–Scillarin-A, Scillarin-B, 3-Benzyl-4-chromanones and traditionally used in ethno-medicinal system of India and South Africa, mainly for their cardioprotective potentialities [3–5]. Scaly bulbs of this species have antioxidant and antimicrobial properties and medicinally used as anthelmintic, cardiac stimulant, digestive, expectorant, diuretic [6–9]. It is also used in asthma, cough and bronchitis, paralytic attacks, ailments of the heart, calculous affections, rheumatism and skin diseases [5]. Traditionally used medicinal plants have recently attracted the attention of the pharmacist which involved the isolation of secondary metabolites produced by plants and their use as active principles in pharmaceutical preparations [7]. Due to the massive utilization in pharmaceutical industries, the demand for quality planting material of *L. revoluta* is increasing day-to-day, which necessitate the development of rapid and efficient *in vitro* propagation protocols for elite genotypes. Micropropagation methods are now widely accepted applied implementation of modern plant biotechnology, which being used for the clonal propagation of many medicinal plants in large scale. Manipulation of the composition and ratio of plant growth regulators (PGRs) is

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 6-(γ,γ-dimethylally lamino) purine; BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, kinetin; MS, Murashige and Skoog (1962); NAA, α-naphthaleneacetic acid; NOA, β-naphthoxyacetic acid; PGRs, plant growth regulators; TDZ, thidiazuron.

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often the primary experimental approach used for optimization of *in vitro* micropropagation methods [8,9]. Changes in chromosome number and structure are the most frequently observed chromosomal abnormalities in callus cells and the plants regenerated from them [10–13]. Therefore, the cytological fidelity of micropropagated plants needs to be checked before using this protocol at the commercial level [14]. Almost all of the previously published micropropagation methods of *L. revoluta* are at least 15–30 years old, and unfortunately the numerical value of the propagation rate is not provided in these reports [15–17]. Only one recent study has been published by us in 2016 [3], were somatic embryogenesis process was described thoroughly. However, plant can micropropagate through two different morphogenic pathways –somatic embryogenesis and shoot organogenesis, and both pathways have their own advantages and limitations. Therefore, propagation of *L. revoluta* through shoot organogenesis have certain importance and was not studied till date. The present study was thus aimed at the following: (1) develop an improved propagation system through indirect shoot organogenesis via callus formation. (2) Cytological assessment of regenerated plants as compared to parental plant. (3) Investigation of the antimicrobial activity of scaly bulb of *L. revoluta* against Gram-positive, Gram-negative bacteria and fungus.

2. Material and methods

2.1. Plant material and surface disinfection

The parental plants of *Ledebouria revoluta* (L.f.) Jessop were maintained inside the shade-net house (maintain relative humidity of 60–65% by misting system and avoid direct sunlight) of our experimental garden. Healthy young root, white bulb scale and green leaf lamina had used as initial explants. The explants were treated with 2.5% (w/v) fungicide (Bavistin®) for 15 min followed by 2% liquid detergent (Tween-20 solution) for 2 min and then surface disinfected with freshly prepared 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) for 10 min followed by washing three times with sterile distilled water to remove traces of $HgCl_2$.

2.2. Medium preparation and culture condition

Surface disinfected root explant was cut into 1 cm long pieces from the tip portion, whereas bulb-scale and leaf explants were cut to about 8 mm × 10 mm pieces and implanted on callus induction medium. All the experiments had carried out on Murashige and Skoog (MS) basal medium [18] containing 3.0% (w/v) sucrose as a carbon source and various concentrations and combinations of cytokinins [6-benzylaminopurine (BAP), kinetin (KIN), 6-(γ,γ-dime thylallylamino) purine (2iP), thidiazuron (TDZ)] and auxins [2,4-dichlorophenoxyacetic acid (2,4-D), β-naphthoxyacetic acid (NOA), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA)] as experimental requirements. The pH of the medium was adjusted to 5.7 before the addition of 0.8% (w/v) agar powder for solidification. The medium without any PGR was used as a control. The medium was sterilized by autoclave at 1.04 kg cm^{-2} pressure and 121 °C for 18 min. All cultures were incubated at 24 ± 1 °C in the dark for callus induction and rest of the experiments under a 16 h photoperiod with a photosynthetic photon flux density of approximately 50 μmol m^{-2} s^{-1} emitted from cool fluorescent tubes (Philips India Ltd.). All cultures were subcultured to their respective fresh medium at 3-week intervals.

2.3. Organogenic callus induction

The explants were implanted on slant MS medium supplemented with different concentrations (1.0, 2.0, 3.0 and 4.0 mg L^{-1}) of 2,4-D alone or in combination with NOA or NAA (0.25, 0.5, 0.75 and 1.0 mg L^{-1}) and incubated in complete dark condition at 24 ± 1 °C for 3 weeks and subcultured in the same medium for every 3 week intervals. The percentage of explant responding and the degree of induced callus per explant were recorded after 8 weeks of culture

2.4. Induction of shoots (bulblets) and their maturation

Organogenic calli (about 500 mg) was cultured on MS medium supplemented with different concentration of four altered cytokinins (KIN, BAP, 2iP and TDZ at 0.1, 0.25, 0.5, 0.75, 1.0 mg L^{-1}) alone. The medium without any PGR was used as a control. At 3 week intervals, the organogenic calli were subcultured in their respective fresh medium for induction and further development of bulblets. The percentage of calli responds and the number of bulblets per 500 mg of calli was recorded after 45 days of culture.

2.5. In vitro root induction

Regenerated bulblets (2.0–2.5 cm long) were separated from clumps into single ones and were cultured on MS medium supplemented with auxins (IAA or IBA) at four different concentrations (1.0, 2.0, 3.0, and 4.0 mg L^{-1}). Generally agar are used as solidifying agent for routine experiments, but in special case Phytagel™ (SIGMA®) are used for better photography of *in vitro* rooting.

2.6. Acclimatization and field evaluation of in vitro derived plants

A total of 75 *in vitro* derived plantlets were acclimatized for 30 days by transferring to small earthen pots containing 'Soilrite' (chemically inert, sterile, horticultural grade perlite), and covered with clear polybags to maintain 90 to 99% relative humidity. Ultimately the acclimatized plants were transferred to earthen tubs containing mixture of soil and vermin compost (3:1 ratio) and kept inside the shade-net house.

2.7. Cytological studies of regenerants and parental plants

For the somatic chromosome study, young and healthy root tips (0.5 cm long) were pre-treated with a saturated solution of paradichlorobenzene (*p*DB) at 17 ± 1 °C for 4.5 h. For callus cytology, highly deviding cells from the peripheral regions of the callus mass were pre-treated with a saturated solution of *p*DB at 17 ± 1 °C for 4.0 h. Then pre-treated root tips and callus cells were fixed in an ethanol/acetic acid solution (3:1; v/v) for 24 h at 4 °C. Following overnight fixation, the root-tips and callus cells were stained with 2.0% aceto-orcein and squashed following the methods described by Haque and Ghosh [19]. All the mitotic plates were observed in Leica DM750 microscope and photographed with Leica DFC295 camera.

2.8. Antimicrobial activity

2.8.1. Plant extract preparation for antimicrobial test

Scaly bulb of tissue culture derived one year old *ex vitro* plants and naturally propagated *in vivo* parental plants are air dried separately and made fine powder. Then plant extracts are prepared from 5.0 g of dry powder in four different organic solvent extracts (ethanol, methanol, acetone, chloroform) using a Soxhlet apparatus for 8 h. The extracts were completely dried and were finally dissolved in dimethyl sulfoxide (DMSO) to prepare dilution for further study.

2.8.2. Anti-bacterial and anti-fungal test

The antibacterial activity was screened by agar-well diffusion method against microbial type culture collection (MTCC) strain of two Gram-positive bacteria [*Staphylococcus aureus* (MTCC-3160), *Bacillus subtilis* (MTCC-441)], three Gram-negative bacteria [*Pseudomonas aeruginosa* (MTCC-3542), *Klebsiella pneumoniae* (MTCC-109), *Escherichia coli* (MTCC-443)] and two fungus (*Fusarium verticillioides* (MTCC-10726), *Aspergillus flavus* (MTCC-277)]. All bacterial strain were tested in Muller Hinton agar medium and fungi were in Saborouds dextrose agar medium. The experiment was carried out following the methods previously described by Haque et al. [20]. The dried plant extracts were dissolved in DMSO for dilution and DMSO alone used as negative control. Cultures were incubated for 24 h at 37 °C and 72 h at 26 °C for bacteria and fungi respectively. After incubation, the zones of inhibition (ZI) were measured with HiAntibiotic ZoneScale™ (HIMEDIA®).

2.9. Statistical data analyses

Each treatment contained three replicates with 10 explants per replicate. All data were subjected to one-way analysis of variance (ANOVA) using SPSS software for Windows (IBM® SPSS, version 19.0, Chicago, IL). After conducting an ANOVA, the means were further separated using Tukey's test at $P \leq 0.05$.

3. Results

3.1. Effect of PGRs and explant type on callus induction

All the three types of explant showed response to callus induction in *L. revoluta*, but in different manners e.g., bulb scale showed maximum response followed by leaf lamina and roots (Table 1; Fig. 1A–C). When cultured on PGR-free controlled medium, the de-differentiation process failed to occur in all three types of explant and finally the culture was rotted after 3–4 weeks. Usually callus induction initiated after 10–12 days of implantation. A white, friable, nonorganogenic callus was induced in the medium containing 2,4-D alone. Fascinatingly, if NAA or NOA was supplemented together with 2,4-D, the explants responded remarkably by inducing a whitish yellow organogenic calli within 60 days of culture, which further proliferate to a huge mass of callus within next 45 days following two subcultur on the same medium. In combination with 2,4-D, the NOA gave better results than NAA with a high degree of callus induction. Callus induction frequencies ranged from 25.6 ± 1.1 to 94.5 ± 2.2% in different concentrations of PGRs (Table 1). Optimum response (94.5 ± 2.2) for the induction of organogenic calli was recorded in 3.0 mg L^{-1} of 2,4-D and 0.75 mg L^{-1} of NOA.

3.2. Regulatory effect of PGRs on shoot induction and their maturation

Low concentrations of cytokinin stimulate organogenesis process to re-differentiate a unipolar shoot bulb from the callus (Fig. 1D). Among all four cytokinins, KIN and 2iP both individually at low concentration have the ability to induced shoot bulbs (Table 2). Whereas, TDZ and BAP proved unproductive for shoot organogenesis; even at low concentrations ($\geq$0.5 mg L^{-1} TDZ or $\geq$0.75 mg L^{-1} BAP), only somatic embryos were induced instead of shoot bulbs (Table 2). Though the aim of our present study is to standardized a regeneration protocol via shoot organogenesis, therefor the somatic embryogenesis path was not addressed here. Only 60.0 ± 0.54% of calli responded with 14.4 ± 0.45 bulblets per 500 mg of calli in presence of 0.75 mg L^{-1} 2iP. Whereas highest 73.3 ± 0.85% response with maximum 17.0 ± 0.52 bulblets per 500 mg of callus was noted in presence of 0.75 mg L^{-1} KIN (Table 2). Interestingly, if auxins (NAA or NOA even in low concentration, i.e. only 0.2 mg L^{-1}) are added along with the low concentration of KIN and 2iP, the developmental path always shifted to embryonic route, so the total withdrawal of auxin is must needed criteria for organogenesis in *L. revoluta*. Again, the developmental path also shifted from organogenic to embryonic in presence of higher concentration (1.0 mg L^{-1}) of KIN. However, the bulblets induced in 0.75 mg L^{-1} KIN were successfully matured (86.7% success) after one month culture on the same medium composition (Fig. 1E).

3.3. In vitro root induction

Auxins play an important role on *in vitro* root induction of the mature bulblets. Both IAA and IBA exhibits root inductive ability when supplemented with MS medium. On the basis of root inducing ability, IBA proves better than IAA. Minimum 93.3% to maximum 100% response was noted in all four concentration of IBA; however, the best result with 100% responses and 8.4 ± 0.31 roots per bulb was achieved after 18 days of implantation on MS medium containing 2.0 mg L^{-1} IBA (Fig. 1F). Whereas, merely 80.0 ± 0.86% responses with only 5.7 ± 0.18 roots per bulblet was achieved in presence of 3.0 mg L^{-1} IAA after same duration (Table 3).

3.4. Acclimatization and field evaluation of in vitro derived plants

From a total of 75 *in vitro* derived plantlets were hardened for 30 days with 96.0% (72 out of 75 plants) survival rate. Ultimately the acclimatized plants were established inside the shade-net house with cent percent (72 out of 72 plants) survival rate. After

Table 1
Effect of explant types and different auxins on callus induction of *Ledebouria revoluta*. [Data recorded after 8 weeks of culture.]

PGRs (mg L^{-1})			Bulb scale explant		Leaf explant		Root explant	
2,4-D	NOA	NAA	Explant response (%)	Callus growth*	Explant response (%)	Callus growth*	Explant response (%)	Callus growth*
–	–	–	0^{a}	–	0^{a}	–	0^{a}	–
1.0	–	–	25.6 ± 1.1^{bc}	++	23.3 ± 0.9^{b}	+	0^{a}	–
2.0	–	–	54.4 ± 1.1^{cd}	+++	50.0 ± 1.1^{d}	++	25.6 ± 1.5^{bc}	+
3.0	–	–	62.2 ± 2.9^{c}	+++	54.4 ± 1.7^{d}	++	30.0 ± 1.8^{c}	++
4.0	–	–	55.3 ± 1.0^{cd}	+++	43.3 ± 1.2^{c}	++	23.3 ± 2.0^{b}	+
3.0	0.25	–	80.0 ± 1.9^{f}	++++	75.6 ± 1.7^{g}	+++	50.0 ± 1.6^{gh}	++
3.0	0.50	–	90.0 ± 1.9^{fg}	++++++	83.3 ± 2.1^{h}	+++++	54.4 ± 1.8^{hi}	+++
3.0	0.75	–	94.5 ± 2.2^{g}	++++++	85.6 ± 2.0^{h}	+++++	56.6 ± 2.3^{i}	+++
3.0	1.0	–	83.3 ± 1.9^{efg}	+++++	74.4 ± 2.4^{g}	+++	46.6 ± 2.0^{fg}	+++
3.0	–	0.25	75.6 ± 2.9^{ef}	++++	65.6 ± 1.9^{ef}	+++	36.6 ± 1.8^{d}	++
3.0	–	0.5	84.4 ± 1.1^{efg}	+++++	70.0 ± 1.2^{fg}	+++	40.0 ± 2.2^{de}	++
3.0	–	0.75	80.0 ± 1.9^{f}	+++++	75.3 ± 1.6^{g}	++++	45.5 ± 1.8^{efg}	+++
3.0	–	1.0	72.2 ± 1.1^{ef}	++++	63.3 ± 1.3^{e}	+++	42.2 ± 1.6^{def}	++

* The increase in '+' signs indicates a progressive induction and growth of the callus. Each value represents the mean ± standard error, n = 10 × 3 (3 sets, 10 samples in each set). Mean followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to Tukey's multiple range tests.

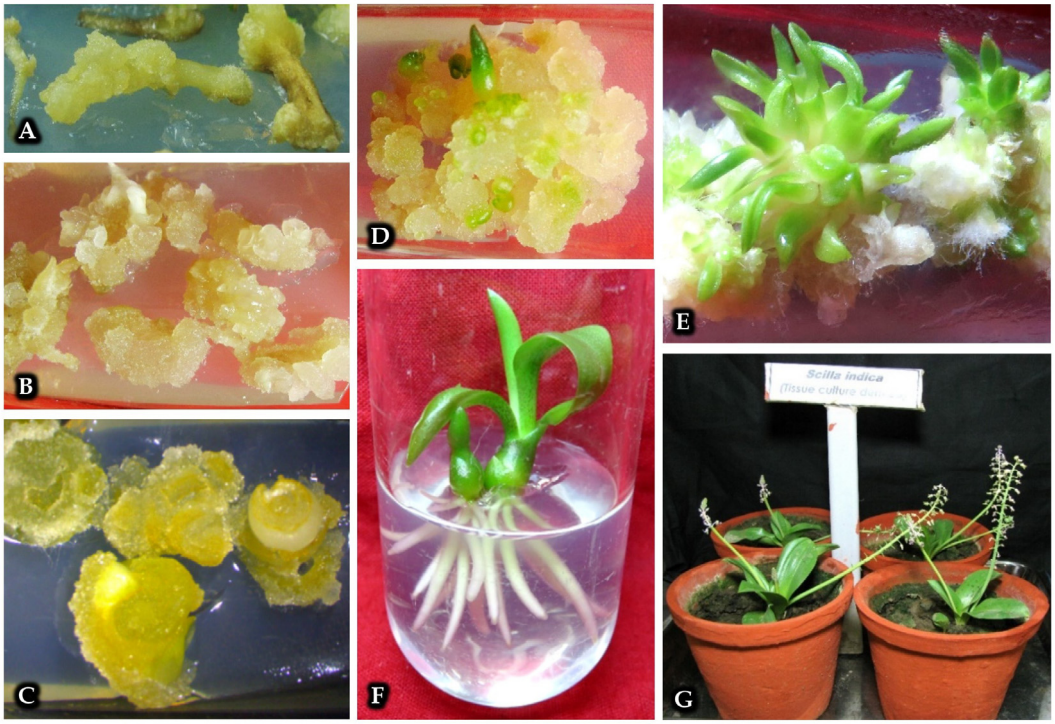

Fig. 1. *In vitro* regeneration of *Ledebouria revoluta* through callus mediated indirect shoot-organogenic pathway. (A) Root explants derived callus grown in 3.0 mg L^{-1} 2,4-D plus 0.75 mg L^{-1} NOA (after 45 days of implantation). (B) Leaf derived callus grown in same medium composition (after 60 days of implantation). (C) Bulb scale derived callus in same medium composition (after 30 days of implantation). (D) Shoot induced from the surface of the callus cultured on MS medium containing 0.75 mg L^{-1} KIN alone after 42 days of implantation. (E) Maturation of unipolar shoots (or bulblets) on same medium after 70 days of inoculation. (F) Root induction of *in vitro* derived shoots after 18 days of implantation on MS medium containing 2.0 mg L^{-1} IBA (medium solidified with Phytagel®). (G) Tissue culture derived *ex vitro* plants in flowering stage after 14 months of field transfer.

Table 2
Effect of different cytokinins on shoot (bulb) organogenesis from callus of *Ledebouria revoluta*. [Data recorded after 45 days of culture]

Concentration of PGRs (mg L^{-1})				Explant response (%)	Number of shoot bulbs/500 mg callus	Number of embryos/500 mg callus
KIN	BAP	2iP	TDZ			
–	–	–	–	0^{a}	–	–
0.10	–	–	–	0^{a}	–	–
0.25	–	–	–	33.3 ± 0.55^{c}	5.7 ± 0.43^{b}	0^{a}
0.50	–	–	–	56.7 ± 0.63^{f}	10.6 ± 0.60^{d}	0^{a}
0.75	–	–	–	73.3 ± 0.85^{g}	17.0 ± 0.52^{g}	0^{a}
1.00	–	–	–	70.0 ± 0.72^{g}	0^{a}	15.3 ± 0.75^{e}
–	0.10	–	–	0^{a}	–	–
–	0.25	–	–	0^{a}	–	–
–	0.50	–	–	0^{a}	–	–
–	0.75	–	–	30.0 ± 0.82^{bc}	0^{a}	6.6 ± 0.31^{c}
–	1.00	–	–	40.0 ± 0.95^{d}	0^{a}	10.8 ± 0.43^{d}
–	–	0.10	–	0^{a}	–	–
–	–	0.25	–	0^{a}	–	–
–	–	0.50	–	46.7 ± 0.65^{e}	7.6 ± 0.57^{c}	0^{a}
–	–	0.75	–	60.0 ± 0.54^{f}	14.4 ± 0.45^{e}	0^{a}
–	–	1.00	–	60.0 ± 0.73^{f}	12.8 ± 0.31^{f}	0^{a}
–	–	–	0.10	0^{a}	–	–
–	–	–	0.25	0^{a}	–	–
–	–	–	0.50	26.7 ± 0.75^{b}	0^{a}	4.5 ± 0.30^{b}
–	–	–	0.75	40.0 ± 0.88^{d}	0^{a}	8.2 ± 0.36^{c}
–	–	–	1.00	46.7 ± 1.04^{e}	0^{a}	13.4 ± 0.47^{e}

Each value represents the mean ± standard error, n = 10 × 3 (3 sets, 10 samples in each set). Mean followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to Tukey's multiple range tests.

12–14 months of field transfer, 91.6% (66 out of 72 plants) of the survived plants flowered normally (Fig. 1G).

3.5. Cytological status of regenerants and parental plants

Cytological analysis was done for the evaluation of the cytological fidelity among parental and *in vitro* derived plants. The diploid chromosome number of the parental plants of *L. revoluta* was found 2n = 30 chromosomes (Fig. 2A). Callus cytology revealed chromosomal stability of the callus cells with 2n = 30 chromosomes (Fig. 2B). Randomly selected *in vitro* plants and one year old field-grown *ex vitro* plants are also shown stable chromosome number 2n = 30, same as parental plants (Fig. 2C and D), which confirmed all the regenerants are diploid, there was no proof of variation in ploidy level.

Table 3
Effect of different auxins on root induction of *Ledebouria revoluta*. [Data recorded after 18 days of culture.]

Concentration of auxins (mg L^{-1})		Explant response (%)	Number of roots/bulblet
IAA	IBA		
–	–	63.3 ± 1.45^{d}	3.9 ± 0.32^{abc}
1.0	–	70.0 ± 0.93^{de}	4.4 ± 0.21^{abc}
2.0	–	76.7 ± 0.65^{ef}	4.9 ± 0.25^{bc}
3.0	–	80.0 ± 0.86^{f}	5.7 ± 0.18^{cd}
4.0	–	66.7 ± 1.05^{d}	4.0 ± 0.22^{abc}
–	1.0	100 ± 0.00^{g}	5.6 ± 0.20^{cd}
–	2.0	100 ± 0.00^{g}	8.4 ± 0.31^{e}
–	3.0	100 ± 0.00^{g}	7.5 ± 0.34^{e}
–	4.0	93.3 ± 1.33^{g}	4.8 ± 0.23^{bc}

Each value represents the mean ± standard error, n = 10 × 3 (3 sets, 10 samples in each set). Mean followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to Tukey's multiple range tests.

3.6. Antimicrobial activity of regenerated plants

Different solvent extracts were dissolved in DMSO and being negative control the DMSO showed no ZI against any of the investigated bacteria or fungus. All four types of organic solvent extract show more or less antibacterial activities against both Gram-positive and Gram-negative bacteria (Table 4; Fig. 3A, B). However the overall result indicates an ethanolic and methanolic extract has better antibacterial activity than acetone or chloroform extract. Inversely in fungus, acetone or chloroform extract has great activity against *F. verticillioides* whereas the ethanolic and methanolic extract has no activity (Fig. 3C). Another fungus *A. flavus* is resistance against all four types of extract. The present study indicates that compounds responsible for anti-fungal activity are well extracted in chloroform and acetone solvent, but not present in the methanolic or ethanolic extracts (Table 4). All the tissue culture derived *ex vitro* field grown plants have little better antimicrobial activity as compared to naturally propagated *in vivo* parental plants (Fig. 4).

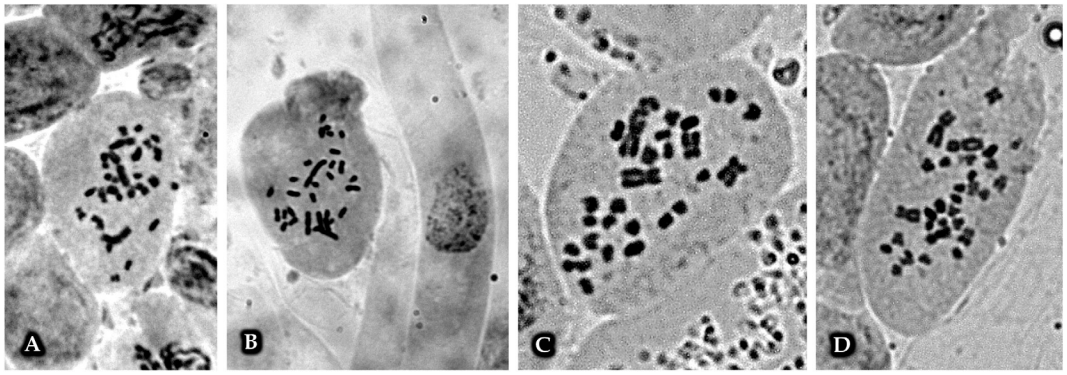

Fig. 2. Mitotic metaphase plate of *Ledebouria revoluta* showing 2n = 30 chromosomes. (A) parental plant, (B) callus cell, (C) *in vitro* plant, (D) tissue culture derived field-grown *ex vitro* plant.

Table 4
Antimicrobial activity of tissue culture derived *ex vitro* plants of *Ledebouria revoluta.*

Type and concentration of extract		Zone of Inhibition (mm)						
		Gram positive bacteria		Gram negative bacteria			Fungus	
Solvent type	mg/cup	MTCC 441	MTCC 3160	MTCC 109	MTCC 443	MTCC 3542	MTCC 277	MTCC 10,726
Ethanol	0.75	–	13 ± 0.88	15 ± 0.58	–	–	R	R
	1.5	9 ± 0.58	15 ± 0.58	17 ± 0.33	–	–	R	R
	3.0	10 ± 0.47	17 ± 0.47	17 ± 0.47	12 ± 0.58	–	R	R
	4.5	11 ± 0.33	19 ± 0.33	17 ± 0.33	13 ± 0.47	14 ± 0.58	R	R
	6.0	11 ± 0.33	19 ± 0.33	17 ± 0.33	13 ± 0.33	17 ± 0.33	R	R
Methanol	0.75	8 ± 0.88	11 ± 0.58	13 ± 0.88	11 ± 0.58	13 ± 0.58	R	R
	1.5	10 ± 0.58	13 ± 0.47	15 ± 0.53	12 ± 0.88	15 ± 0.88	R	R
	3.0	12 ± 0.47	15 ± 0.88	16 ± 0.33	14 ± 0.58	17 ± 0.47	R	R
	4.5	12 ± 0.33	16 ± 0.33	16 ± 0.33	14 ± 0.47	17 ± 0.33	R	R
	6.0	12 ± 0.33	16 ± 0.47	16 ± 0.47	14 ± 0.33	17 ± 0.33	R	R
Acetone	0.75	–	11 ± 0.47	–	–	–	R	17 ± 0.88
	1.5	–	13 ± 0.58	10 ± 0.88	10 ± 0.58	–	R	21 ± 0.47
	3.0	8 ± 0.58	12 ± 0.58	12 ± 0.47	12 ± 0.47	–	R	23 ± 0.33
	4.5	9 ± 0.88	16 ± 0.33	13 ± 0.33	13 ± 0.33	10 ± 0.58	R	23 ± 0.33
	6.0	10 ± 0.47	16 ± 0.33	13 ± 0.33	13 ± 0.33	13 ± 0.47	R	23 ± 0.33
Chloroform	0.75	–	10 ± 0.58	–	–	10 ± 0.58	R	13 ± 0.88
	1.5	–	12 ± 0.58	08 ± 0.47	–	12 ± 0.47	R	19 ± 0.58
	3.0	–	14 ± 0.88	11 ± 0.58	8 ± 0.58	14 ± 0.47	R	22 ± 0.47
	4.5	9 ± 0.58	15 ± 0.47	14 ± 0.88	10 ± 0.47	14 ± 0.33	R	22 ± 0.33
	6.0	11 ± 0.33	15 ± 0.33	16 ± 0.47	10 ± 0.33	14 ± 0.33	R	22 ± 0.33
DMSO	50 µl	0	0	0	0	0	0	0

Experiment repeats 3 times and each value represents the mean ± standard error. [R – Resistant; MTCC 441 – *Bacillus subtilis;* MTCC 3160 – *Staphylococcus aureus;* MTCC 109 - *Klebsiella pneumoniae;* MTCC 443 - *Escherichia coli;* MTCC 3542 - *Pseudomonas aeruginosa;* MTCC 277 – *Aspergillus flavus;* MTCC 10,726 – *Fusarium verticillioides.*]

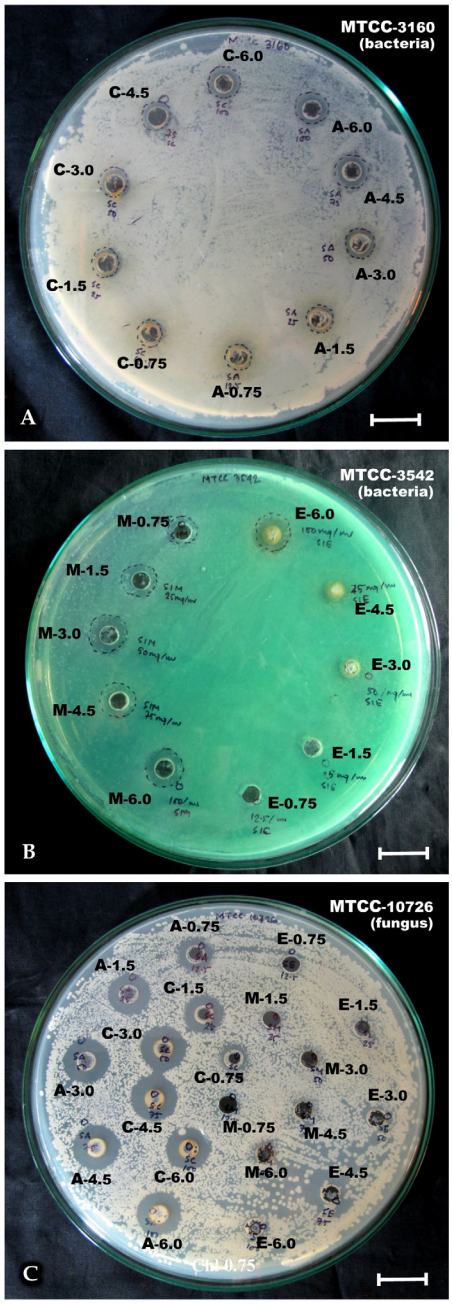

Fig. 3. Antimicrobial activities of *Ledebouria revoluta* (A, Acetone extract; C, Chloroform extract; M, Methanol extract; E, Ethanol extract). (A) *Staphylococcus aureus* [Gram positive bacteria], (B) *Pseudomonas aeruginosa* [Gram negative bacteria]. (C) *Fusarium verticillioides* [fungus]. *bar = 25 mm.

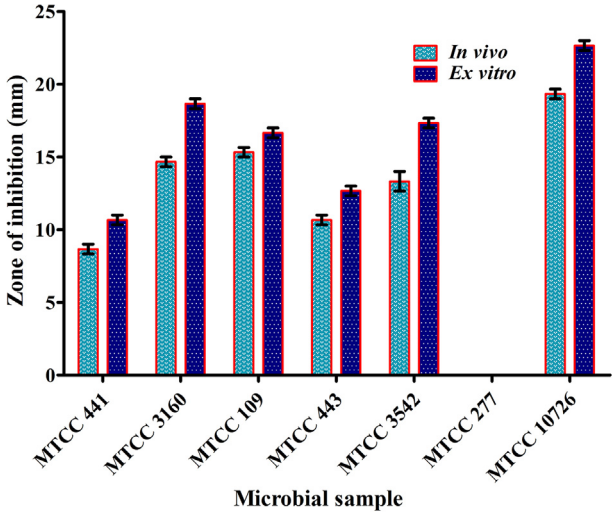

Fig. 4. Comparative antimicrobial activities of *in vivo* and ex vitro raised plants of Ledebouria revoluta against standard bacterial strains (MTCC 441, 3160, 109, 443, 3542) and standard fungal strains (MTCC 277 and 10726). *Results of ethanol extract against bacteria and acetone extract against fungus are presented here.

4. Discussion

An effective and suitable callus regeneration system had been established from the non-reproductive organ derived explant of *L. revoluta*. For indirect shoot organogenesis, the explant type is an important factor for callus initiation efficiency [21]. Out of three different types of explant, bulb scale of *L. revoluta* showed better callusing ability. A similar observation was noted in *Scilla indica* and *Lilium* where bulb-scale responded better than young leaf-tips or root explant [16,22]. In present study, the strong synthetic auxin 2,4-D plays an important role on de-differentiation of highly organized explant tissue to form an unorganized cell-mass or callus. This findings were supported by a lots of previous reports including several bulbaceous plants like *Scilla indica*, *Zephyranthes rosea*, *Lachenalia montana* [15,23,24]. Many of the previous studies suggested that application of 2,4-D along can induces non-morphogenic friable calli, whereas supplementation of other PGRs along with 2,4-D had improved the quality of morphogenic callus. Findings of the present studies revealed that along with optimum 2,4-D, the additions of low dose of NOA or NAA directly induces morphogenic callus instead of non-morphogenic fragile callus. Similar findings were reported in another monocot *Spathoglottis*, where synergistic effect of 2,4-D and NAA had improved the morphogenic callus induction from leaf, stem, and root explants [25]. The course of acquisition of competency, de-differentiation, and re-differentiation is initially regulated by PGRs, viz, auxins and cytokinins [26]. In our previously published study on somatic embryogenesis of *L. revoluta* [3], we found that high concentration of cytokinins along with low auxin was needed for somatic embryo induction. The modulation of concentration and combination of PGRs (mainly by auxins and cytokinins) regulate the morphogenic paths. Presently, interesting findings of regulating the influence of PGRs are observed during shifting of the morphogenic path from embryonic to organogenic. When the cytokinin concentration was reduced to very low and auxin was totally withdrawn from the medium, the callus were entered into organogenic path instead of the embryonic path. Complete withdrawal of auxin as well as lowering the concentration of cytokinin, both are equally important for indirect shoot organogenesis in *L. revoluta*. Out of four different cytokinin tested (KIN, 2iP, BAP, TDZ), only KIN and 2iP have the ability to induce bulblets from calli of *L. revoluta*, whereas BAP and TDZ even in low concentration always induce somatic embryo. Hence present investigation revealed that organogenic path was induced by comparatively weak cytokinins (both KIN and 2iP), whereas relatively strong cytokinins (both BAP and TDZ) are responsible for somatic embryogenesis.

The root development is a complex organogenic process controlled by various endogenous and environmental factors, among which one of the key regulatory factors is auxin [27,28]. The induction of *in vitro* rooting by exogenously supplied auxin, mainly IBA is well accepted in numerous plant species [29]. Between IBA and IAA, the best results of root induction in the *in vitro* regenerated bulblets were achieved in presence of IBA. Similar fruitful effect of IBA on *in vitro* root induction was reported in several bulbaceous monocots like *Allium hookeri*, *Leucocoryne* spp., *Zephyranthes*

grandiflora, Z. rosea, etc. [24,30–32]. The majority of the *in vitro* micropropagation protocols do not trials with the acclimatization procedure, but we studied it thoroughly up to the flowering of the regenerants. Similar approaches to study the acclimatization process and field evaluation up to the flowering of the tissue culture derived plants are also reported in *Aloe*, *Tylophora*, *Bacopa, Spathoglottis* [14,25,33–35].

All the regenerated and parental plants of our experiments contained 2n = 30 chromosomes, which are corroborated with the previous cytological report of *L. revoluta* [36]. Variations in chromosome number and structure are the commonly observed chromosomal abnormalities in callus cells and the plants regenerated from them [10,13]. There are spontaneous endoreduplications in the cells during the callus proliferation, particularly ageing of the culture is highly mutagenic and causes chromosomal rearrangements and somaclonal variation [11,12]. But our present study revealed all the regenerants are cytologically stable, there was no evidence of any ploidy change. This present findings are corroborated with some other studies where callus derived regenerants are found cytologically stable [37–40].

A group of secondary compounds was responsible for antimicrobial properties [41]. Bulb of *L. revoluta* contains many secondary metabolites with strong antimicrobial activity. However, our findings confirm all the tissue culture derived *ex vitro* plants have better antimicrobial activity than naturally propagated plants. These results are in agreed with reports in *Bacopa monnieri*, *Drimia robusta* and *Cotyledon orbiculata* where tissue-culture-derived *ex vitro* acclimatized plants showed maximum antimicrobial activity [20,42,43]. This preliminary findings ensure the bulb of *L. revoluta* have potential antimicrobial activity.

5. Conclusion

The scaly bulb of the regenerated plants of *L. revoluta* had potential antibacterial (against both Gram-positive and Gram-negative bacteria) and antifungal activity. Regeneration through callus mediated shoot organogenesis and cytological stability of the regenerants of this protocol make it well suited for propagation of *L. revoluta* and can be applied to produce bulk quantity of identical planting materials for pharmaceutical industries. Besides the mass propagation of elite plants, a highly efficient indirect regeneration pathway via callus culture opens a way for improvement of this ethnomedicinal plant through genetic transformation strategies.

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Author contributions

B.G. designed the experiments. S.M.H. performed all the tissue culture experimentations, cytological works and statistical analysis. A.C. and S.M.H. jointly performed the antimicrobial tests. The manuscript was initially written by S.M.H. and critically revised by B.G. and finally checked and approved by all three authors.

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