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# Callus and cell suspension culture of *Viola odorata* as in vitro production platforms of known and novel cyclotides

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**Abstract** Cyclotides are unique plant cyclic-peptides that can serve as agrochemicals, pharmaceutical scaffolds for drug delivery, and therapeutic agents. Currently, cyclotides are obtained only via direct extraction from limited plants. Hence, they serve as valuable candidates for synthesis via plant cell bioprocesses. In this study, callus lines (47 in total) were successfully induced from the leaf and petiole explants of the Indian medicinal plant, *V. odorata*, on a solidified woody plant medium (WPM) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (4.5 mg/l). Two fast growing callus lines, VOP-4 and VOL-44, were selected for the development of cell suspension cultures having a doubling time of 8 and 6 days, respectively. Further, known (15) and novel (9) cyclotides were identified for the first time in the callus and cell suspension cultures of *V. odorata*, using liquid chromatography and Fourier transform mass spectrometry. The cyclotides were identified based on their monoisotopic mass (2.5–4 kDa), hydrophobic nature, disulfide bonds, circular structure and amino acid sequence. Some of the cyclotides identified in the study (vodo I96, vodo I97, vodo I98) were exclusively produced in callus/cell suspension cultures and not in the parent plant. The study revealed that besides germplasm conservation, plant cell bioprocessing of *V. odorata* could be a potential alternative for in vitro production of known and novel cyclotides.

## Introduction

Protein or peptide therapeutics has gained much attention as compared to small-molecule counterparts owing to its several advantages like, access to a much wider range of protein targets (Kintzing et al. 2016). To date, nearly 100 proteins with several therapeutic applications have entered worldwide pharmaceutical market (Uhlig et al. 2014). Currently peptide therapeutics is in use for the treatment of several human diseases including anemia, diabetes, gaucher, hepatitis, cancer etc. However, the manufacturing cost of protein drugs is very high ($140 billion in 2013) exceeding GDP of >70% of countries around the globe (Walsh 2014). Moreover, traditional production processes using microbial based systems have limitations and safety concerns such as product contamination by endotoxins and mycotoxins. On the other hand, production of biopharmaceutical proteins in plant cell cultures provide a safe and cost-effective solution (Huang and McDonald 2012). Plant cell culture is well-known for production of many commercial secondary metabolites like paclitaxel, shikonin, scopolamine and digoxin (Wilson and Roberts 2012). Recently, the scope of plant cell technology has extended toward the commercial production of protein therapeutics. Increase in the number of pharma-companies working in this direction unquestionably suggests the commercial viability of plant

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cell bioprocesses for the production of pharmaceutically important proteins.

Many peptides having high efficacy and pharmacokinetic parameters degrade before performing the desired function and hence lose their market potential. Stability of any protein therapeutic is an important feature for commercialization. Cyclotides are plant cyclic proteins that exhibit exceptional thermal, chemical and enzymatic stability owing to its unique cyclic cystine knot (CCK) motif, a knotted arrangement of three disulfide bond and a circular backbone (Craik and Conibear 2011). Remarkable stability of cyclotides makes them a potential candidate in diverse applications but not limited as agrochemicals, pharmaceutical scaffolds for drug delivery, and therapeutic agents (Daly et al. 2009).

The distribution of cyclotides is limited to few plants from family of Violaceae, Rubiaceae, Fabaceae and Cucurbitaceae (Craik and Conibear 2011). Violaceace represents the largest suite of cyclotides discovered so far. The first member of Violaceace for the discovery of cyclotides was *Viola odorata* (Craik et al. 1999). However, differential expression of cyclotides in different parts of the plant from different geographical locations has been reported (Trabi et al. 2004; Nguyen et al. 2011). Also, seasonal fluctuation is yet another factor associated with limitations of plant based extraction of cyclotides (Trabi et al. 2004). In India, *V. odorata* commonly known as "Banafsha" is a medicinal plant which has several ethanobotanical uses and the plant is reported to have phytochemicals like alkaloids, saponins and flavonoids (Singh 1965; Lim 2014). Owing to its extensive wild crafting, the plant has become endangered in some regions (Davazdahemami 2010; Malik et al. 2011). A major limitation in the cultivation of *V. odorata* is seed dormancy due to which the only feasible mode of propagation is through root cuttings.

This study therefore aims to establish callus and cell suspension culture of *V. odorata* as an alternative for production of cyclotides. Establishment of in vitro cultures of this commercially exploited medicinal plant will also facilitate its germplasm conservation. It is for the first time that known and novel cyclotides have been identified and reported in callus and cell suspension culture of *V. odorata*.

## Materials and methods

### Collection of *V. odorata* plant

*Viola odorata* Linn. plants were collected from the Survey of Medicinal Plants and Collection Unit, Emerald, Nilgiris district, Tamilnadu (India) and transplanted in the horticulture unit at Indian Institute of Technology Madras, Chennai, Tamilnadu, India. The plants were authenticated by

Dr. Suresh Baburaj, Survey Officer, Central Council for Research in Homoeopathy (CCRH), New Delhi, India.

### Establishment of in vitro cultures *of V. odorata*

#### Induction of callus cultures

*Viola odorata* explants (leaf and petiole) were washed in running tap water to remove dust and then surface sterilized with 70% (v/v) ethanol for 30 s followed by treatment with 0.2% (v/v) mercuric chloride for 6 min. The surface sterilized petiole and leaf explants (1 cm long) were placed on different media [Full and half strength MS medium (Murashige and Skoog 1962), Woody Plant Medium (WPM) (Lloyd and McCown 1980) and B5 medium (Gamborg et al. 1968)] supplemented with various phytohormones either alone or in combination [6-benzylaminopurine (BAP), 2,4-dichloro phenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA)] as mentioned in Table 1, along with 3% (w/v) sucrose and 0.4% (w/v) CleriGar (HiMedia, Mumbai, India), at an initial pH 5.7. Explants on different medium were incubated at 23 °C with 70% relative humidity (RH) in dark for callus induction.

The frequency of callus induction was calculated based on 120 days of incubation period using the following equation:

$$\text{Callus induction frequency} = \frac{\text{Number of explants producing callus}}{\text{Total number of explants}} \times 100$$

Data obtained from all treatments were presented as the mean of three independent replications. Statistically significant differences were determined by analysis of variance (ANOVA) and the Duncan multiple range test (DMRT) at $p < 0.05$ level of significance using IBM SPSS statistics ver. 24.

The induced callus lines were separated from the explants after 120 days of growth for subsequent subculture and maintenance on the same medium (favorable for callus induction) at 23 °C with 70% RH and 16/8 h light/dark (L/D) photoperiod.

#### Proliferation, screening and maintenance of callus cultures

The effect of 2,4-D on callus proliferation was calculated based on its growth index. About 1 g (FW) of callus was cultured in WPM supplemented with various concentrations of 2,4-D (1–6 mg/l), 3% (w/v) sucrose and 0.4% (w/v) CleriGar (HiMedia, Mumbai, India), at an initial pH 5.7. The callus was incubated in a plant growth chamber

**Table 1** Effect of medium and phytohormones on induction of *V. odorata* callus from petiole and leaf explants

| Exp. no | Medium | Phytohormone (mg/l) | | | Petiole explants | | | Leaf explants | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | NAA | 2,4-D | BAP | Mean frequency (%) | Induction Time (days) | Morphogenetic response | Mean frequency (%) | Induction time (days) | Morphogenetic response |
| 1 | Half MS | 1 | – | 3 | 06.57$^j$ | 100 | Dark green compact callus | 08.72$^{gh}$ | 120 | Dark green compact callus |
| 2 | Half MS | 2 | – | 3 | 60.05$^{ef}$ | 95 | Dark green compact callus | 52.66$^c$ | 120 | Dark green compact callus |
| 3 | Half MS | 3 | – | 3 | 75.44$^{bc}$ | 95 | Dark green compact callus | 54.99$^c$ | 120 | Dark green compact callus |
| 4 | Half MS | – | 1.5 | – | 77.70$^{bc}$ | 100 | Beige friable callus | 09.38$^{gh}$ | 95 | Beige friable callus |
| 5 | Half MS | – | 3 | – | 73.60$^{bcd}$ | 85 | Beige friable callus | 66.43$^b$ | 95 | Beige friable callus |
| 6 | Half MS | – | 4.5 | – | 06.30$^j$ | 95 | Beige friable callus | 21.42$^{ef}$ | 95 | Beige friable callus |
| 7 | Full MS | 1 | – | 3 | 08.99$^j$ | 100 | Dark green compact callus | 07.64$^{gh}$ | 120 | Dark green compact callus |
| 8 | Full MS | 2 | – | 3 | 48.39$^g$ | 100 | Dark green compact callus | 35.88$^d$ | 120 | Dark green compact callus |
| 9 | Full MS | 3 | – | 3 | 62.60$^{ef}$ | 100 | Dark green compact callus | 50.39$^c$ | 120 | Dark green compact callus |
| 10 | Full MS | – | 1.5 | – | 19.56$^i$ | 100 | Beige compact callus | 09.24$^{gh}$ | 120 | Beige friable callus |
| 11 | Full MS | – | 3 | – | 57.80$^f$ | 100 | Beige compact callus | 54.40$^c$ | 100 | Beige friable callus |
| 12 | Full MS | – | 4.5 | – | 64.64$^{def}$ | 95 | Beige friable callus | 58.25$^{bc}$ | 100 | Beige friable callus |
| 13 | B5 | 1 | – | 3 | 02.27$^j$ | 90 | Dark green compact callus | 00.00$^h$ | 90 | No callus formation |
| 14 | B5 | 2 | – | 3 | 27.55$^{hi}$ | 90 | Dark green compact callus | 09.33$^{gh}$ | 90 | Dark green compact callus |
| 15 | B5 | 3 | – | 3 | 23.20$^{hi}$ | 90 | Dark green compact callus | 30.96$^{de}$ | 90 | Dark green compact callus |
| 16 | B5 | – | 1.5 | – | 25.35$^{hi}$ | 85 | Beige compact callus | 03.22$^{gh}$ | 90 | Beige compact callus |
| 17 | B5 | – | 3 | – | 30.99$^h$ | 85 | Beige compact callus | 14.23$^{fg}$ | 90 | Beige compact callus |
| 18 | B5 | – | 4.5 | – | 30.74$^h$ | 85 | Beige friable callus | 29.43$^{de}$ | 90 | Beige friable callus |
| 19 | WPM | 1 | – | 3 | 06.88$^j$ | 80 | Dark green hard callus | 03.15$^{gh}$ | 90 | Dark green compact callus |
| 20 | WPM | 2 | – | 3 | 27.35$^{hi}$ | 80 | Dark green compact callus | 22.57$^{ef}$ | 75 | Dark green compact callus |
| 21 | WPM | 3 | – | 3 | 68.74$^{cde}$ | 75 | Dark green compact callus | 48.04$^c$ | 75 | Dark green compact callus |
| 22 | WPM | – | 1.5 | – | 77.44$^{bc}$ | 65 | Beige friable callus | 12.21$^{fg}$ | 70 | Beige friable callus |
| 23 | WPM | – | 3 | – | 79.04$^{ab}$ | 60 | Beige friable callus | 79.86$^a$ | 60 | Beige friable callus |
| 24 | WPM | – | 4.5 | – | 87.26$^a$ | 50 | Beige friable callus | 82.30$^a$ | 60 | Beige friable callus |
| 25 | WPM | – | 6 | – | 55.45$^{fg}$ | 65 | Beige friable callus | 57.21$^{bc}$ | 70 | Beige friable callus |

Mean frequency (%) in a column with *same letters* (a–j) demonstrate no significant statistical variation (p < 0.05 level)

[a–j]Obtained from comparison by Duncan's multiple range test

maintained at 23 °C with 70% RH and 16/8 h L/D photo-period. Growth index of various cell lines was determined after 30 days of growth using the following equation,

$$Growth\ index = \frac{Final\ fresh\ weight - Initial\ fresh\ weight}{Initial\ fresh\ weight}$$

The induced callus lines were maintained on the favorable medium giving highest growth index value, hereafter referred as callus maintenance medium. Among the 17 callus lines under maintenance, two fast growing callus lines were further selected (based on growth index on callus maintenance medium) for the development of cell suspension culture.

### Development of cell suspension cultures

Fresh callus mass (3 g/l DW) was suspended in 20 ml of WPM in a 100 ml flask containing 2,4-D (3 mg/l) and sucrose [3% (w/v) (HiMedia, Mumbai, India)], at an initial pH 5.7. The cultures were incubated at 85 rpm and 23 °C in a gyratory shaking incubator with a photoperiod of 16/8 h L/D conditions. After 10 days of growth (cultivation), the cells were passed through a buchner funnel and were transferred to a fresh medium (20 ml) and incubated at same culture conditions for 15 days to obtain a synchronous culture. Further, the cells were filtered through Whatman no. 1 filter paper under vacuum conditions and the fresh weight was measured after transfer to a pre-weighed Petri-dish. The inoculum thus obtained was suspended in 50 ml of fresh medium in 250 ml flasks for development of fine suspension culture. Cells (3 g/l DW) in suspension were sub-cultured on a fresh liquid medium periodically (every 8 days of cultivation) for maintenance.

### Confirmation of cyclotides using liquid chromatography–Fourier transform mass spectrometry (LC-FTMS)

Callus and cell suspension cultures were harvested and freeze-dried. The biomass (0.5 g) was then homogenized and macerated in 20 ml of 60% (v/v) aqueous ethanol for 6 h. The extract was centrifuged at 9056g for 10 min and the supernatant was then lyophilized to dryness for further analysis. All samples (each comprising of two biological replicates) were analyzed by LC-FTMS in triplicates on an Orbitrap Elite Mass Spectrometer (Thermo, Bremen, Germany) coupled to EASY-nLC™ liquid chromatography (Thermo Fisher Scientific, Odense, Denmark) via a Nano Electrospray ion source equipped with a stainless steel nano-emitter. Reversed phase liquid chromatography was performed using a Thermo EASY-nLC™ with a binary buffer system consisting of water/acetonitrile (95:5 v/v)

with 0.1% (v/v) formic acid (Solvent A) and water/acetonitrile (5:95 v/v) with 0.1% (v/v) formic acid (Solvent B) over a 100 min gradient at a flow rate of 300 nl/min. Samples were automatically loaded from a 96-well microplate auto sampler by using an EASY-nLC system at 3 µl/min. The peptides were concentrated on a trapping column (Easy-column™, L 2 cm, ID 100 µm, 120 Å, C18-A1; Thermo Scientific, SanJose, CA, USA) and were eluted and directed onto a reverse phase column (Easy-column™, L 10 cm, ID 75 µm, 120 Å, C18-A2; Thermo Scientific, SanJose, CA, USA). The Orbitrap Elite instrument was operated in data-dependent mode, automatically switching between MS and MS/MS.

### Intact mass analysis and indication for hydrophobic nature of cyclotides

The supernatant collected after ethanolic extraction of the biomass was diluted with 20% (v/v) acetonitrile for LC-FTMS analysis. The mass scan range used for this analysis was m/z 500–2000. Monoisotopic mass of cyclotides were determined using Protein Deconvolution Software (version 1.0; Thermo Fisher Scientific, SanJose, CA, USA) and were also manually verified by direct calculation from the peak positions and charge states using Xcalibur Qual Browser (version 2.2 Thermo Fisher Scientific, San Jose, CA, USA). Also, retention time on LC was used as a preliminary indicator for ascertaining hydrophobic nature of putative cyclotides.

### Presence of disulfide bonds

The number of disulfide bonds present in the peptides were identified by reducing and alkylating the disulfide bonds and verifying the expected mass gain as per the protocol adapted from literature (Koehbach et al. 2013). Briefly, the lyophilized extract was suspended in 5 ml of 100 mM ammonium bicarbonate buffer (pH: 8). Further, to an aliquot (500 µl) of this (extract solubilized in the buffer), 50 µl of 100 mM Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri, USA) was added. This mixture was incubated at 60 °C for 1 h. The reaction mix was brought to room temperature, 50 µl of 200 mM iodoacetamide (IAA) (Sigma-Aldrich, St. Louis, Missouri, USA) was added, and the mixture was further incubated under darkness for 45 min. The solution was then de-salted using C18 Zip-Tips (Millipore, Carrigtwohill, Ireland) and subjected to further analysis using LC-FTMS. The mass scan range used for this analysis was m/z 500–2000. Monoisotopic mass of chemically modified cyclotides were determined using Protein Deconvolution Software and were also manually verified as

described in "Intact mass analysis and indication for hydrophobic nature of cyclotides" section.

### Assessment of circular structure

The circular nature of the peptides was detected by observing mass gain acquired after linearizing the putative cyclotides by hydrolyzing the peptide bond as per the protocol adapted from literature (Craik et al. 1999). Briefly, 100 µl of reduced and alkylated cyclotides (obtained from "Presence of disulfide bonds" section prior to the desalting step) were treated with 10 µl of 50 µg/ml endo-Glu-C (Promega, Milan, Italy) for 2 h at 37 °C. The solution was then de-salted using C18 Zip-Tips (Millipore, Carrigtwohill, Ireland) and subjected to further analysis using LC-FTMS. The mass scan range used for this analysis was m/z 500–2000. Monoisotopic mass of chemically linearized cyclotide(s) was determined using Protein Deconvolution Software and were also manually verified as described in "Intact mass analysis and indication for hydrophobic nature of cyclotides" section.

### Amino acid sequence of cyclotides

To determine the sequence of cyclotides using LC-FTMS/MS analysis, 100 µl of the reduced and alkylated cyclotide samples (obtained from "Presence of disulfide bonds" section prior to the desalting step) were treated separately with either of the following enzymes, 10 µl of 50 µg/ml endo-Glu-C for 2 h at 37 °C or 10 µl of 50 µg/ml TPCK-treated trypsin (St. Louis, Missouri, USA) for 3 h at 37 °C or 10 µl of 50 µg/ml chymotrypsin (Promega, Milan, Italy) for 3 h at 25 °C. The solution was then de-salted using C18 Zip-Tips (Millipore, Carrigtwohill, Ireland) and subjected to further analysis using LC-FTMS. The mass scan range used for this analysis was m/z 100–2000. The six most intense peaks were subject to collision-induced dissociation (CID). Normalized collision energy was set to 35%. The MS/MS spectra were manually examined and sequenced based on the presence of both b and y series of ions (N- and C-terminal fragments) using de novo sequencing approach.

Sequences of known cyclotides were retrieved in ERA format from CyBase database (Wang et al. 2008) (http://www.cybase.org.au) and the manually deciphered sequences of novel cyclotides were included. The raw data obtained from the experiments performed as mentioned above was subjected to two different search engines namely, "PEAKS DB" in Peaks (version 7; Bioinformatics Solutions Inc., Waterloo, Canada) and "SEQUEST" in Proteome Discoverer software (version 1.4.0.228; Thermo Fisher Scientific, SanJose, CA, USA) for the validation of cyclotides. The following parameters were used

for searching on databases: 15 ppm precursor tolerance; 0.5 Da fragment tolerance; static modification: cysteine carbamidomethylation; dynamic modifications: methionine oxidation.

A standalone application of CyPred (http://biomine.cs.vcu.edu/servers/CyPred/) was used for sequence based prediction of the cyclic proteins (Kedarisetti et al. 2014).

## Results and discussion

### Establishment of in vitro cultures of *V. odorata*

### Induction of callus cultures

Different standard growth media were evaluated containing different phytohormones for the induction of *V. odorata* callus as mentioned in Table 1. It was observed that the explants, leaf and petiole, cultured on different growth medium (Half MS, Full MS, B5 and WPM) without any phytohormone could not result in callus induction. However, a varied callus induction frequency from leaf and petiole explants was observed in media supplemented with phytohormone combinations as reported in Table 1.

In this study, favorable role of 2,4-D in the medium for callus induction was evident irrespective of the medium used. However, as shown in Table 1, the concentration of 2,4-D required for successful callus induction varied with the basal medium tested and the type of the explants used. Notably, 2,4-D mostly induced beige colored
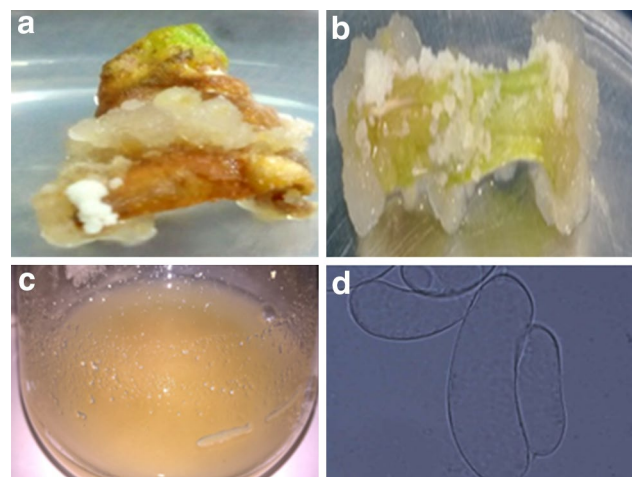


**Fig. 1** Induction of *V. odorata* calli in woody plant medium with 4.5 mg/l, 2,4-D from **a** leaf and **b** petiole explants. **c** Cell suspension culture of *V. odorata* and, **d** micro images of cells in suspension culture

friable callus irrespective of the medium used (Fig. 1a, b). Among the various medium and phytohormone combination tested, highest frequency and early callus induction (in 50 days) was obtained when WPM was supplemented with 2,4-D (4.5 mg/l). The highest frequency obtained was 87.26% from the petiole explants and 82.30% from the leaf explants. The significance of the nature of the explant in callus induction was evident from the fact that induction frequency varied with the type of explant used. In most of the favorable medium compositions tested, the petiole explants resulted in better callus induction frequency in comparison to that from the leaf explants (Table 1). Muhammad et al. (2013) have achieved a callus induction efficiency of 85% (after 40 days of incubation) in *V. odorata* on Half MS medium supplemented with BAP (2.5 mg/l) and 2,4-D (0.15 mg/l). Variation in callus induction frequency with different type of explants has also been previously reported in other plant species including *Eucalyptus camaldulensis* and *Allium chinense* (Prakash and Gurumurthi 2009; Yan et al. 2009). Overall, 47 callus lines of *V. odorata* were established in this study.

### Proliferation, screening and maintenance of callus cultures

The concentration of phytohormone in the growth medium significantly impacts the growth of the callus and its indefinite proliferation. Hence, the effect of 2,4-D concentration (1–6 mg/l) on the growth of 17 relatively fast growing callus lines was determined based on their growth index. As shown in Table S1, in the concentration range tested, highest growth index could be obtained at 2,4-D concentration of 3 mg/l (6.24 for a leaf derived callus, VOL-44 and 5.45 for a petiole derived callus line, VOP-4). Hence, WPM containing 3 mg/l of 2,4-D was subsequently used as callus maintenance medium and the two callus lines VOL-44 and VOP-4 were selected for further studies on cyclotide analysis.

### Development of cell suspension cultures

Callus lines, VOL-44 and VOP-4, were used for initiation of cell suspension cultures. The cells in suspension (Fig. 1c) exhibited elongated morphology (Fig. 1d). The cells in suspension were found to be uniformly spread giving rise to a homogeneous cell suspension, allowing rapid and uniform access to nutrition, growth hormones and signal compounds to all the cells thereby facilitating mass cultivation in bioreactors. As expected, the culture doubling time in the liquid medium (6 days) was found to be ~4-fold higher than that on the solid medium (25 days).

### Identification and characterization of cyclotides in the extracts

Cyclotides present in the callus and cell suspension cultures of *V. odorata* were analyzed using a high resolution Fourier transform mass spectrometry (FTMS) coupled to a liquid chromatography (LC) and confirmed based on five different approaches (Koehbach et al. 2013; Burman et al. 2014), (a) the late eluting property of cyclotides on a reverse phase chromatography, (b) their molecular mass ranging from 2500–4000 Da (c) presence of three disulfide bonds, (d) presence of cyclic structure and (e) sequence of cyclotides with defined number of amino acids in each loop. Each of these approaches is discussed in detail in the following sections.

### Intact mass analysis and indication for hydrophobic nature of cyclotides

Cyclotides have 28–37 amino acids and thus correspond to a molecular mass in the range of 2500–4000 Da. Moreover, unlike other proteins, cyclotides have their hydrophobic residues exposed to the exterior as the core is occupied by CCK knot. Due to this unusual characteristic, cyclotides have relatively late elution time on a reverse phase liquid chromatography. Hence, to confirm the presence of cyclotides in the ethanolic extracts of callus and cell suspension cultures, the extracts were subjected to LC-FTMS as per the protocol adapted from literature (Poth et al. 2011). The sample analysis revealed the presence of 98 peptides with cyclotide-like masses that had late elution time on a reverse phase (gradient) LC [eluting between 30–50% (v/v) of ACN]. The intact masses thus obtained were then compared with CyBase database (Wang et al. 2008) to match the identity of known cyclotides. In addition to the known cyclotides, the samples also comprised several other peptides with the above mentioned characteristics but their masses did not match with the existing literature. Hence, based on these preliminary indications such peptides were presumed to be novel cyclotides and subsequent confirmation was done based on the investigations as described below.

### Presence of disulfide bonds

Cyclotides have six conserved cysteine residues that form three disulfide bonds (Craik et al. 1999). The presence of disulfide bonds in the cyclotide-like peptides were investigated based on mass shift observed before and after chemical modifications (reduction and alkylation) during the LC-FTMS analysis. The ethanolic extracts of callus and cell suspension cultures were reduced using DTT and alkylated with IAA to prevent oxidation of free thiol

groups. As a result of alkylation, cysteine groups present in the peptides become carbamidomethylated, and thus each cystine converted cysteine group gains a mass of 58.03 Da. The masses of peptides before and after said chemical modifications were compared. A mass shift of 348.18 Da was observed in the chemically modified peptide extracts, which confirmed the presence of six cysteine residues and three disulfide bonds. Additionally, in comparison to the native cyclotides, the retention time of the chemically modified cyclotides was observed to be much ahead in LC, confirming the presumable breakdown of disulfide bonds in the knotted topology resulting in the exposure of hydrophilic residues of cyclotides to the exterior (Fig. 2). Unlike other conventional proteins, these chemically modified cyclotide-like peptides also displayed poor fragmentation patterns on MS/MS indicating the presence of head-to tail cyclic backbone (Koehbach et al. 2013). These observations provided supportive evidence that the cyclotide-like masses were most likely cyclotides.

*Assessment of circular structure*

The cyclic structure of the cyclotide-like peptides were assessed by selectively cleaving the chemically modified peptides (as described earlier in "Assessment of circular structure" section) at a single amino acid using endoproteinase to linearize them. Subsequently, using LC-FTMS, the mass shift (by 18 Da) was verified, in the putative cyclotides, due to the hydrolysis of the peptide bond by endoproteinase. Literature suggests that most of the cyclotides contain only one glutamic acid residue except few like circulin D (Gustafson et al. 2000), circulin E (Gustafson et al. 2000), cter K (Poth et al. 2011) and cter L (Poth

et al. 2011). Hence, endoproteinase Glu-C was used to specifically cleave only at carboxyl terminal of glutamic acid (Glu) residue. Interestingly, most of the cyclotide-like peptides identified earlier displayed a mass shift of 18 Da after treatment with Glu C which further confirmed the identity of known and novel cyclotides (Table S2) in the extract samples. It is noteworthy that some of these novel cyclotides identified in the in vitro cultures were also identified earlier in the natural plant (unpublished data). However, cyclotides vodo I96, vodo I97, vodo I98 were found to be exclusively present in callus and cell suspension cultures of *V. odorata*.

*De novo sequencing of cyclotides*

Conventional methods of protein sequencing include Edman degradation and N-terminal residue identification which requires pure protein samples. On the other hand, mass spectrometry based protein sequencing strategies such as de novo sequencing using tandem mass spectrometry offers advantage of sequencing peptides even from crude samples. In this study, Orbitrap elite mass spectrometer was used that has unsurpassed resolution (Resolving power >240,000 FWHM) with extreme mass accuracy (<3 ppm RMS) which is especially useful when dealing with complex and low abundance samples. However, intact cyclotides are poorly fragmented and hence they were reduced and alkylated and further digested using endoproteinase such as Glu-C, trypsin and chymotrypsin. The digested samples were subjected to tandem mass spectrometry and sequence of some novel cyclotides (vodo I66 and vodo I98) identified in this study were deciphered using de novo sequencing approach described below as examples.



**Fig. 2** LC chromatograms and mass spectra showing shift in the retention time and mass of **a, b** native cyclotides and **c, d** chemically modified cyclotides from *V. odorata* cell cultures
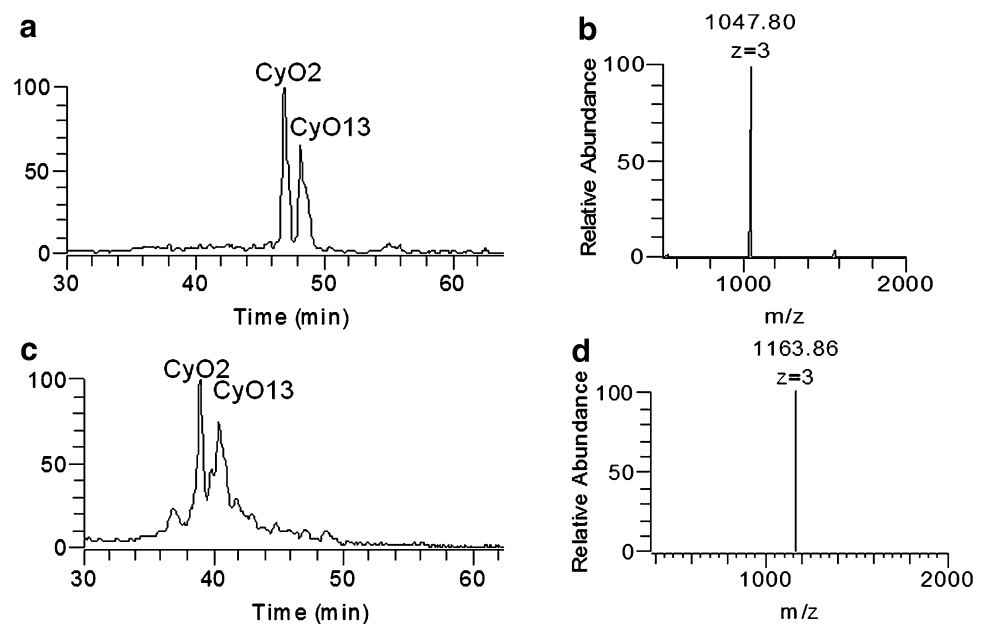
Sequences of known cyclotides were also identified and compared (with CyBase database and literature) which further confirmed the robustness of the analysis.

Chemically modified vodo I66 (a novel cyclotide identified in the study) upon treatment with endo-Glu-C resulted in one fragment with mass 3643.61 Da indicating the presence of only one Glu. This precursor was further fragmented (MS/MS) to assign the b and y ions to decipher the sequence as 'SCVFXPCXTGXFGPCACKSKV-CYYNSXPCGE', where "X" indicates the presence of either isoleucine (Ile) or leucine (Leu) (Fig. S1a). Upon digestion with trypsin, one detectable fragment with mass 3428.48 Da was obtained which was further subjected to MS/MS and the fragmentation pattern was used for de novo sequencing (Fig. S1b). Concordant sequence results were obtained using trypsin digest. Since, Ile and Leu are isobaric, they cannot be distinguished in a mass spectrometer and hence their assignment was done based on the cleavage by chymotrypsin. Chymotrypsin cleaves a peptide at Leu, Phe, Tyr and Trp but not at Ile. As deciphered, vodo I66 has a sequence comprising four Ile/Leu. It should be noted that two among the four Ile/Leu in the sequence is followed by a proline (Pro) residue and hence no cleavage can occur even if it is Leu (as no cleavage occurs if a Pro residue is on the carboxyl side). Based on the sequence similarity with the previously identified cyclotides, the amino acid at both the positions is assigned to be Ile. Fragments obtained from chymotrypsin digestion, 919.47 and 1258.51 Da, indicated the presence of two Ile residues. Further, MS/MS of 919.47 Da (Fig. S1c) and 1258.51 Da (Fig. S1d) confirmed the presence of two Ile residues. Thus, the sequence of vodo I66 was deciphered as 'cyclo-CGESCVFIPCITGIFGPCACKSKVCYYNSIP'.

Another novel cyclotide, vodo I98, when treated with endo-Glu-C after reduction and alkylation resulted in one fragment with mass 3522.54 Da. De novo sequencing was done on the MS/MS fragmentation pattern of this precursor and the sequence was deciphered as 'SCVWX-PCFSAAXGCSCKSKVCYRNGXPCGE' (Fig. S2a). Tryptic digests of vodo I98 resulted in one detectable fragment with mass 2729.15 Da. De novo sequencing of the MS/MS fragmentation pattern (Fig. S2b) resulted in the sequence 'NGIPCGESCVWXPCFSAAXGCSCK' and the amino acids sequence matched that of the Glu-C digests. Among the three Ile/Leu residues found in the sequence, two of the Ile/Leu residues have a Pro following them in the sequence and hence no chymotrypsin cleavage can occur. Based on the sequence homology with the sequences available till date, X in 'VWXP' was assigned as Ile. However, X in 'YRNGXP' remained ambiguous as both Ile/Leu are reported in the literature. From the chymotrypsin digests, a fragment of 1589.70 Da was obtained indicating 'X' in 'SAAXGCSCKSKVCY' as

'I'. De novo sequencing of MS/MS fragmentation pattern of precursors 1433.62 and 1589.70 Da resulted in consistent sequence 'RNGXPCGESCVW' (Fig. S2c) and 'SAAIGCSCKSKVCY' (Fig. S2d), respectively. Thus, the sequence of vodo I98 is deciphered as 'cyclo-CGESCVWIPCFSAAIGCSCKSKVCYRNGXP'.

The framework of cyclotides is composed of six cysteine residues that form three disulfide bonds. The disulfide bond connectivity was given as Cys$^{I}$–Cys$^{IV}$, Cys$^{II}$–Cys$^{V}$, Cys$^{III}$–Cys$^{VI}$ based on the disulfide bond connectivity reported earlier in cyclotides (Saether et al. 1995). The segments between successive cysteine residues termed as 'loops' have unique number of amino acids. For example, only three amino acids are reported in loop-1 and the number of amino acids present in the sequences deciphered in this study is in agreement with the literature. In accordance with the literature, loop-1 contained one conserved Glu residue occupying the second position. Glu residue is known to form hydrogen bonds with loop-3 amino acids and restrict flexibility of the protein structure (Craik and Conibear 2011). Loop-4 is known to have only one amino acid. Vodo I66 contains an alanine residue while vodo I98 contains a serine residue in loop-4. An asparagine or aspartic residues in loop-6 are presumed to be required for the cyclization of cyclotides via an asparaginyl endopeptidase and is found in all cyclotides (Craik and Conibear 2011). In accordance with this statement, an asparagine is found along with other residues in loop-6 of the novel cyclotides. The novel cyclotides, vodo I66 and vodo I98, were classified within the bracelet subfamily on the basis of the absence of a *cis*-Pro peptide bond in loop-5 in the circular peptide backbone. The bracelet sub-family is known to be more prevalent than the möbius subfamily representing a large suite of cyclotides.

*Validation of cyclotides using search engines*

In the recent years, many database search engines have been developed to analyze large volumes of proteomics data in less time and high accuracy. In this study, well-known search engines such as SEQUEST and PEAKS DB have been used. These search algorithms compare the experimentally obtained MS/MS spectra to an in silico digested cyclotide from a sequence database to acquire and confirm exact protein match, if any. Table S3 lists known and novel cyclotides identified in the study, which were validated using one or both of these search engines. It was anticipated to obtain matches for all the cyclotides identified using the above-mentioned approaches, however, SEQUEST and PEAKS DB failed to identify cter G and kalata B17, respectively.

The cyclic nature of the cyclotides identified earlier was also predicted using CyPred tool (Kedarisetti et al. 2014). A positive score of 1.73 and 0.99 was obtained for the novel cyclotides, vodo I66 and vodo I98, respectively, indicating their cyclic nature.

### *Viola odorata* cell culture as an alternative strategy for production of cyclotides

It was observed in the study that the callus and the cell suspension culture generated from the corresponding callus line demonstrated similar cyclotide profile. However, the cyclotide profile varied among the two callus lines, VOP-4 and VOL-44. For example, cyclotides such as vodo I44, vodo I62, vodo I96 were detected in VOL-44 but not in VOP-4. Further, as shown in Table 2, callus and cell suspension cultures could not only express cyclotides specific to the parent explant (leaf and petiole) but also those specific to the other parts of the plant (flower, runner and root) (unpublished data). Thus, runner specific cyclotides like, cycloviolacin O22, vodo I42, vodo I66

can be easily produced in vitro which will be rather difficult to do in nature as the plant is routinely propagated via runners in the field. The study demonstrates that the developed cell suspension culture can serve as a sustainable in vitro source of cyclotides present in *V. odorata*.

Novel cyclotides, vodo I96, vodo I97 and vodo I98 were found to be present only in the in vitro culture extract and not in the whole plant extract which establishes plant cell/tissue cultures as a potential source of novel cyclotides. Moreover, cyclotides (viba 11, vigno 6, globa B, viba 5, cter G, kalata B17 and hyfl I) that have been reported to be present in other plant species (Wang et al. 2008) were also found to be present in the in vitro cultures of *V. odorata*. Similar observation has also been reported by Slazak et al. (2015), where cyclotides previously reported in other plant species (such as cycloviolacin O3, cycloviolacin O8 and cycloviolacin O13 and mram8) were detected in cell suspension cultures of *Viola uliginosa*. This differential expression of cyclotides in *V. odorata* plant cells cultivated under in vitro conditions was presumably due to the influence of controlled environmental parameters, medium

**Table 2** Comparison of cyclotides detected in cell culture of *V. odorata* with whole plants of *V. odorata*

| Cyclotide | Plant parts detected[a] | Cell line VOP-4 | Cell line VOL-44 |
|---|---|---|---|
| Kalata S | Leaves, petioles, flowers, runners and roots | Y | Y |
| Kalata B1 | Leaves, petioles, flowers, runners and roots | Y | Y |
| Cycloviolacin O22 | Runners and roots | Y | Y |
| Cycloviolacin O13 | Leaves, petioles, flowers, runners and roots | Y | Y |
| Cycloviolacin O6 | ND | Y | Y |
| Cycloviolacin O2 | Leaves, petioles, flowers, runners and roots | Y | Y |
| Cycloviolacin O17 | Leaves, petioles, flowers and runners | Y | Y |
| Cycloviolacin O4 | Petioles, flowers and runners | Y | Y |
| Cycloviolacin O8 | Leaves, petioles, flowers and roots | Y | Y |
| Viba 11 | Leaves, petioles, flowers, runners and roots | Y | Y |
| Vigno-6 | Flowers and roots | Y | Y |
| Globa B | Runners and roots | Y | Y |
| Hyfl I | Runners and roots | Y | Y |
| Viba 5 | Leaves and petioles | ND | Y |
| Cter G | Petioles and flowers | ND | Y |
| Kalata B17 | Petioles, flowers and roots | ND | Y |
| Vodo I77 | Petioles | Y | Y |
| Vodo I36 | Runners, leaves and flowers | ND | Y |
| Vodo I42 | Runners and roots | Y | Y |
| Vodo I44 | Leaves and roots | ND | Y |
| Vodo I62 | Flowers and leaves | ND | Y |
| Vodo I66 | Runners and roots | Y | Y |
| Vodo I96 | ND | Y | Y |
| Vodo I97 | ND | ND | Y |
| Vodo I98 | ND | Y | Y |

*ND* not detectable, *Y* presence

[a]Unpublished data

**Fig. 3** LC chromatograms showing relative abundance of cyclotides from *V. odorata* cell cultures **a** VOP-4, **b** VOL-44 and **c** whole plant. CyO2-cyclo-violacin O2, CyO13-cycloviol-acin O13, SM-small molecules. (Color figure online)
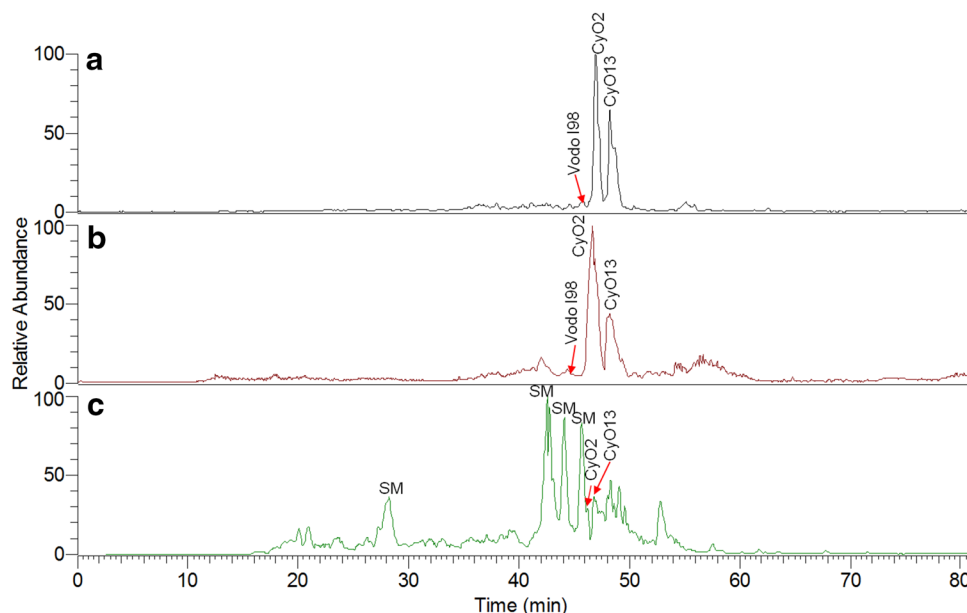


composition and aseptic culture conditions (Dörnenburg 2010; Slazak et al. 2015).

Figure 3 shows the LC chromatograms of callus lines (VOP-4 and VOL-44) and the whole plant extract. As can be observed, the callus culture extracts demonstrate relatively less number of small molecule impurities than the whole plant extract which can make downstream processing much easier and more economical for cyclotide extraction at large scale. Also, as measured by LC-FTMS (Fig. 3), the relative abundance of some cyclotides like cycloviolacin O2 was found to be higher in the cell lines in comparison to that in the whole plant extract. These preliminary observations indicate possible diversion of the metabolic flux in the in vitro cultures favorably toward cyclotides production than toward the competing pathways of other secondary metabolites (referred as small molecule impurities).

## Conclusion

Callus and cell suspension cultures of *V. odorata* were successfully established for germplasm conservation and in vitro production of cyclotides. Presence of 15 known and 9 unknown cyclotides was confirmed in callus and cell suspension cultures of *V. odorata*. It is noteworthy that cyclotide production from in vitro callus/cell suspension culture of *V. odorata* has been reported for the first time. Novel cyclotides, vodo I96, vodo I97 and vodo I98 were found to be present only in in vitro cultures and not in the whole plants. Sequence of two novel cyclotides, vodo I66 and vodo I98, has been reported using de novo sequencing

approach. Based on the results it can be hypothesized that culture conditions can play a major role in the expression of cyclotides and manipulating culture conditions can result in cyclotides with diverse sequence, which can be subsequently used for several applications. Furthermore, the study demonstrates that *V. odorata* cell culture system can be used as a model system to study biosynthesis of cyclotides in plants, and the factors that regulate them under controlled conditions. The results of the study suggest that cell culture of *V. odorata* can serve as an in vitro production platform of cyclotides (like cycloviolacin O2) which have several reported applications like cytotoxic, antimicrobial, antiviral, anti-biofouling and anthelmintic.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

# References

Burman R, Gunasekera S, Strömstedt AA, Göransson U (2014) Chemistry and biology of cyclotides: circular plant peptides outside the box. J Nat Prod 77:724–736. doi:10.1021/np401055j

Craik DJ, Conibear AC (2011) The chemistry of cyclotides. J Org Chem 76:4805–4817. doi:10.1021/cr60113a001

Craik DJ, Daly NL, Bond T, Waine C (1999) Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J Mol Biol 294:1327–1336. doi:10.1006/jmbi.1999.3383

Daly NL, Rosengren KJ, Craik DJ (2009) Discovery, structure and biological activities of cyclotides. Adv Drug Deliv Rev 61:918–930. doi:10.1016/j.addr.2009.05.003

Davazdahemami S (2010) Collection, cultivation and establishment rare and endangered medicinal plants of Iran in order to conservation and reclamation of them. http://agris.fao.org/agris-search/search.do?recordID=IR2012070062

Dörnenburg H (2010) Cyclotide synthesis and supply: from plant to bioprocess. Biopolymers 94:602–610. doi:10.1002/bip.21466

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158. doi:10.1016/0014-4827(68)90403-5

Gustafson KR, Walton LK, Sowder RC et al (2000) New circulin macrocyclic polypeptides from *Chassalia parvifolia*. J Nat Prod 63:176–178. doi:10.1021/np990432r

Huang T-K, McDonald KA (2012) Bioreactor systems for in vitro production of foreign proteins using plant cell cultures. Biotechnol Adv 30:398–409. doi:10.1016/j.biotechadv.2011.07.016

Kedarisetti P, Mizianty MJ, Kaas Q et al (2014) Prediction and characterization of cyclic proteins from sequences in three domains of life. Biochim Biophys Acta J 1844:181–190. doi:10.1016/j.bbapap.2013.05.002

Kintzing JR, Filsinger Interrante MV, Cochran JR (2016) Emerging strategies for developing next-generation protein therapeutics for cancer treatment. Trends Pharmacol Sci 37:993–1008. doi:10.1016/j.tips.2016.10.005

Koehbach J, Attah AF, Berger A et al (2013) Cyclotide discovery in Gentianales revisited: identification and characterization of cyclic cystine-knot peptides and their phylogenetic distribution in rubiaceae plants. Pept Sci 100:438–452. doi:10.1002/bip.22328

Lim T (2014) Edible medicinal and non-medicinal plants. Springer, New York

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot-tip culture Comb. Proc Int Plant Prop Soc 30:421–427

Malik AR, Siddique MAA, Sofi PA, Butola JS (2011) Ethnomedicinal practices and conservation status of medicinal plants of North Kashmir Himalayas. Res J Med Plants 5:515–530. doi:10.3923/rjmp.2011.515.530

Muhammad N, Naveed I, Saqlan Naqvi SM, Mahmood T (2013) Standardization of tissue culture conditions and estimation of free scavenging activity in *Viola odorata* L. Pak J Bot 45:197–202

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Nguyen GKT, Zhang S, Nguyen NTK et al (2011) Discovery and characterization of novel cyclotides originated from chimeric precursors consisting of albumin-1 chain a and cyclotide domains in the fabaceae family. J Biol Chem 286:24275–24287. doi:10.1074/jbc.M111.229922

Poth AG, Colgrave ML, Philip R et al (2011) Discovery of cyclotides in the Fabaceae plant family provides new insights into the cyclization, evolution, and distribution of circular proteins. ACS Chem Biol 6:345–355. doi:10.1021/cb100388j

Prakash MG, Gurumurthi K (2009) Effects of type of explant and age, plant growth regulators and medium strength on somatic embryogenesis and plant regeneration in *Eucalyptus camaldulensis*. Plant Cell Tissue Organ Cult 100:13–20. doi:10.1007/s11240-009-9611-1

Saether O, Craik DJ, Campbel ID et al (1995) Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. BioChemistry 34:4147–4158

Singh P (1965) Pharmacognosy of *Viola odorata* l. and its adulterants. Q J Crude Drug Res 2:712–719

Slazak B, Jacobsson E, Kuta E, Göransson U (2015) Exogenous plant hormones and cyclotide expression in *Viola uliginosa* (Violaceae). Phytochemistry 117:527–536. doi:10.1016/j.phytochem.2015.07.016

Trabi M, Svangård E, Herrmann A et al (2004) Variations in cyclotide expression in *Viola* species. J Nat Prod 67:806–810. doi:10.1021/np034068e

Uhlig T, Kyprianou T, Martinelli FG et al (2014) The emergence of peptides in the pharmaceutical business: from exploration to exploitation. EuPA open. Proteomics 4:58–69. doi:10.1016/j.euprot.2014.05.003

Walsh G (2014) Biopharmaceutical benchmarks 2014. Nat Biotechnol 32:992–1000. doi:10.1038/nbt0910-917

Wang CKL, Kaas Q, Chiche L, Craik DJ (2008) CyBase: a database of cyclic protein sequences and structures, with applications in protein discovery and engineering. Nucleic Acids Res 36:206–210. doi:10.1093/nar/gkm953

Wilson SA, Roberts SC (2012) Recent advances towards development and commercialization of plant cell culture processes for synthesis of biomolecules. Plant Biotechnol J 10:249–268. doi:10.1111/j.1467-7652.2011.00664.x.Recent

Yan MM, Xu C, Kim CH, et al (2009) Effects of explant type, culture media and growth regulators on callus induction and plant regeneration of Chinese jiaotou (*Allium chinense*). Sci Hortic (Amsterdam) 123:124–128. doi:10.1016/j.scienta.2009.07.021