

Dendrobium protoplast co-culture promotes phytochemical assemblage in vitro

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Abstract The present study is intended to analyze the occurrence of potent, low produce, naturally occurring stilbenes in protoplasts of wild species and hybrids of *Dendrobium*. The wild species selected for the study was *Dendrobium ovatum*, endemic to Western Ghats of India. Protoplasts were isolated from leaves and tepal tissues of all the species and were cultured purely to generate homofusants and cross-cultured to raise heterofusants. Phytochemical composition of protoplast culture with atypical and pure microcolonies was performed using mass spectrometry. Enzyme cocktail of 4% pectinase together with 2% cellulase displayed the highest competence for protoplast isolations. Maximum protoplast density of $30.11 \times 10^4/\text{ml}$ was obtained from *D. ovatum* leaves in 2 h. Subcellular features such as the presence of partially formed cell wall, the position of the nucleus, chloroplast density, colony existence, and integrity of the plasma membrane were analyzed. Among the pure and cross-cultured

protoplasts, the number of heterofusants and homofusants formed were enumerated. The spectral feature extraction of the mass spectrometry indicated the presence of five phenolic marker compounds, viz., tristin, confusarin, gigantol, moscatilin, and resveratrol, some of them in pure and others in assorted protoplast cultures raised from *Dendrobium* leaves and tepals. The study demonstrated that protoplast fusion technique enabled phytochemical assemblage in vitro as stilbenes tend to get restricted either in a tissue or species specific manner. This is the first report showing the presence of resveratrol, moscatilin, tristin, gigantol, and confusarin in wild and hybrid species from cultured *Dendrobium* protoplasts in vitro.

Keywords *Dendrobium* · FACS · Fusants · Mass spectrometry · Protoplast · Stilbene

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Abbreviations

6-BAP 6-Benzylaminopurine
FACS Fluorescence-activated cell sorter
G Centrifugation speed in gravitational force
Rpm Revolutions per minute

Introduction

Dendrobium species of Orchidaceae such as *Dendrobium moscatum* (Majumdar and Sen 1987), *Dendrobium loddigesii* (Chen et al. 1994), *Dendrobium amoenum* (Majumdar et al. 1999), *Dendrobium nobile* (Miyazawa et al. 1999), and *Dendrobium densiflorum* (Fan et al. 2001) are reported to comprise potent phenolic compounds. The hybrids of *Dendrobium* are of high demand in the cut flower industry. Some hybrids and a few wild species have been extensively

screened for natural products only in Chinese traditional medicine (Committee of Chinese Pharmacopoeia 2005). Bibenzyl compounds such as moscatilin and other stilbenes have been sporadically reported to be present in few *Dendrobium* species. Moscatilin found in the fractionated extract of *Dendrobium loddigesii*, functions as an arachidonic acid inhibitor which revoked collagen-induced platelet aggregation, indicative of its anticoagulant function (Chen et al. 1994; Miyazawa et al. 1999; Song et al. 2012). Moscatilin, is also found to induce a time-dependent arrest of the cell cycle at G2-M (Gap 2- Mitotic) phase, with an increase of cells in sub-G1 (Gap 1) stage in HCT-116 (human colorectal cancer) cell line demonstrating its anti-cancer efficacy (Chen et al. 2008). However, there are no reports which analyzed the abundance of this compound or its stilbene partners in *Dendrobiums* endemic to Western Ghats of India, which is one of the 34 biodiversity hotspots of the world. Albeit recombinant DNA technology has revolutionized the impact on the biopharmaceutical industry; heterologous expression of plant genes in microorganisms has modeled imperfection, due to nonexistence of PTMs (post-translational modifications). Yeast, the GRAS (genetically regarded as safe) organism has an advantage for being a eukaryotic expression system with its ability to assemble DNA fragments in genomes by homologous recombination enduring the insertion of DNA sequences at specific locations. However, yeast system requires carefully controlled fermentation settings considering the shear sensitivity of the cells. Post-translational glycosylation pattern especially in yeast and fungal cells may also be inadequate or often different from plant systems. The challenges include choosing the precise transformation systems, adaptation of codon usage, gene silencing, design of transgenes with appropriate expression, tissue specificity, proper developmental regulation, subcellular localization of products which ensure correct folding and stability of the protein (Leinard et al. 2007). Therefore, it is always recommended to develop a whole new plant-based expression system, particularly protoplasts to improve yield and phyto-pharmaceutical assembly, which can be accomplished via cross-culturing procedures (Belarmino et al. 1996; Jarl et al. 1999). Protoplast cross-culturing studies are very limited in plants (Melchers et al. 1992). Cross-culturing of protoplasts has not been done in orchids till date. There are very few reports on the isolation of protoplasts from the leaves and tepals of *Dendrobiums* (Kunasakdakul and Smitamana 2003; Khentry et al. 2006). Moreover, there has been no such cross-culturing method reported for wild endemic orchids of Western Ghats, for phytochemical production, which makes this study relevant. Different plant organs such as root, seeds, leaves, and other plant subcellular compartments (endoplasmic reticulum, chloroplasts, vacuoles, and oil body) have been used to express different therapeutic proteins (Hellwig et al. 2004). For instance, in plants with large foliar volume such as

tobacco, alfalfa, and legume plants, expression studies are performed in leaves (Masumura et al. 2006). Protein accumulation in potato is achieved in the tubers, whereas in corn, safflower, soybean, wheat, or rice, it is achieved in seeds (Shirono et al. 2006). The most attractive feature of protoplast system is that it can be isolated from any tissue of a desired organ where phytochemicals preferentially accumulate. In the present study, we have developed an efficient procedure for isolation and maintenance of protoplasts from *Dendrobium* species which included two hybrids, viz., *Sonia* pink and *Sonia* white and wild *Dendrobium ovatum*. Protoplasts were co-cultured from hybrids and wild species of *Dendrobium* to raise atypical fusants. Phytochemical compositions of atypical and pure fusants were performed using electron spray ionization-quadrupole-time of flight-mass spectrometry (ESI-Q-TOF-MS) to detect the presence of moscatilin and other stilbene partners. In this study, we report that atypical microcolonies facilitated accumulation and assembly of very significant stilbene marker compounds in vitro.

Materials and methods

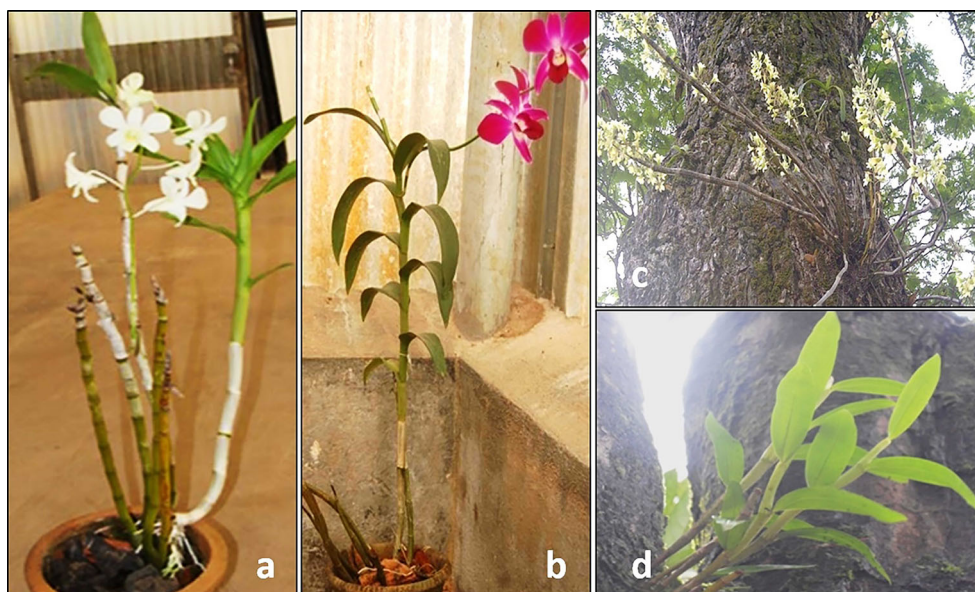
Explant source and explant preparation

Tender leaves and tepals from bloomed flowers of *Dendrobium cv. Sonia* (white and pink) and *D. ovatum* were chosen for protoplast isolations. The hybrids of *Dendrobium* were purchased from a nursery at Hebri, Karnataka, India (Fig. 1). *D. ovatum* species were handpicked from host trees such as *Pithecellobium dulce*, *Hopea parviflora*, and *Mangifera indica* from the buffer zones of Kudremukh Wild Life Sanctuary of Western Ghats, Karnataka, India. All explant sources were preconditioned for about 3 months in the greenhouse of School of Life Sciences, Manipal University, Manipal, India. Explants were surface sterilized with 1% Tween 20 for 15 min. Further procedures were carried out in laminar air flow cabinet, where the explants were given three washes with sterile distilled water and 5 min of 0.1% mercuric chloride treatment. Prior to protoplast isolation step, the explants were rinsed thrice, with sterile distilled water.

Protoplast isolation

Fresh tissue weighing 1 g was placed in a Petri plate with 8 ml of pre-plasmolysis solution for 15 min. The pre-plasmolysis solution included 0.3 M mannitol, 10 mM CaCl₂.2H₂O (calcium chloride dihydrate), and 10 mM MES [2-(*N*-morpholino) ethane sulfonic acid], with a pH 5.8. After incubation for 15 min in pre-plasmolysis solution, the tissues were finely chopped. The pre-plasmolysis solution was then replaced with 5 ml of freshly prepared enzyme solution. The

Fig. 1 *Dendrobium* plants used as source for protoplast isolations; white hybrid (a) and pink hybrid (b), *Dendrobium ovatum* at reproductive phase (c) and vegetative phase (d) of growth as seen in the wild



enzyme solution comprised of 0.5, 1, 2, 3, 4, and 6%, each of cellulase and pectinase. Individual concentrations were prepared in pre-plasmolysis solution and filter sterilized using 0.22 μm Millipore filter. The severed tissues were incubated up to 6 h in enzyme solution at 25 ± 2 °C. Subsequently, the tissues were kept in the rotospin (30 rpm) for digestion in the dark. The protoplast released from each Petri plate was segregated from the undigested tissue by filtering the solution through a nylon sieve of pore size 120 μm .

Protoplast purification

The filtrate was subjected to centrifugation at 750 rpm for 10 min, allowed to settle for 5 min and the supernatant was discarded. The pellet containing protoplasts was layered on to different gradient solutions. They are (a) Sucrose/Mannitol [0.3 M:0.3 M] (v/v) solution (b) 30% Percoll gradient in pre-plasmolysis solution. The solution was centrifuged at 500 rpm for 10 min. Protoplasts that were found at the interphase position of the osmoticum were collected. They were then subsequently washed twice by centrifugation with washing solution (pre-plasmolysis solution) at 750 rpm for 5 min. The purified protoplasts were suspended in 1 ml of osmoticum.

Protoplast count and viability assessments

After purification, the protoplast count and their viability percentage were estimated using hemocytometer and flow cytometer. In hemocytometer, the Evan's blue dye was used to check the viability percentage. Protoplast imaging was performed using a Motic BA400 binocular microscope. For fluorescence imaging, Olympus BX 51 fluorescence microscope with Luca-S camera equipped with Andor iQ software

system was used. Apart from Evan's blue, 0.05% fluorescein diacetate (FDA), a working concentration of 50 $\mu\text{g/ml}$ of 4',6-diamidino-2-phenylindole (DAPI), Hoechst 33342, thiazole orange (TO), and propidium iodide (PI) were also used independently to check the viability of the protoplasts. Protoplast suspension (20 μl) was mixed with an equal amount of dye, incubated for 1 h, washed, and was observed under the microscope. Different dyes were separately used to re-assess the accuracy of the protoplast yield and viability. Dual dye viability assessment was performed on a Becton Dickinson FACS Calibur machine. Phosphate-buffered saline (PBS) was added to the final pellet and was vortexed to attain a single cell suspension. To this, TO and PI were added and incubated at 4 °C in the dark for 30 min prior analysis. For each sample, 10,000 protoplasts were analyzed at a high flow rate to avoid protoplast sedimentation. Data acquisition was performed at 100–200 events/s.

Protoplast culture

Inoculum density of 1×10^4 protoplasts/ml were cultured in modified MS medium (Murshige and Skoog 1962) using two methods: (1) liquid culture and (2) agarose bead culture. Modified MS medium was glycine deprived; the macro salt $(\text{NH}_4)(\text{NO}_3)$ [ammonium nitrate] was replaced with $(\text{NH}_4)_2\text{SO}_4$ [ammonium sulfate], with half the concentration of macro and micronutrients (Shetty et al. 2015). Supplementary ingredients of 20% sucrose, 5% mannitol, 0.25% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.1% MES were used to maintain protoplast stability. 6-BAP (0.1 mg/l) was the plant growth regulator used for liquid culture. The pH of the medium was adjusted to 5.8. The protoplasts were cultured in a T25 flask under dark conditions at 25 ± 2 °C. For agarose bead culture, 20 μl of protoplast suspension was gently mixed with 100 μl

of the medium (mentioned above) containing 0.8% agarose. The mixture was pipetted into a T25 flask and allowed to gel, after which 4 ml of liquid medium was added for facilitating suspension of the beads. The final density of the embedded protoplasts was 1×10^4 /ml. The fresh medium was overlaid in the flask once in 10 days.

Protoplast fusion and imaging

A 6 ml of protoplast suspension containing density (6×10^4 protoplasts/ml) from every species was overlaid on a Petri dish and was allowed to sediment. The fusion was facilitated by adding 6 ml of 40% polyethylene glycol (PEG) to protoplast suspension. The treated protoplasts were incubated for 3 h at room temperature. For observing the fusion, the treated protoplasts (20 μ l) was overlaid on a glass slide covered with a coverslip and the washing solution was perfused across the slides. The slides were viewed in Motic BA400 binocular microscope to visualize the fusion products (fusants).

Data collection and statistical analysis

Protoplast yield was calculated using both hemocytometer and flow cytometer. Every treatment was carried out in three replicates. In FACS, each sample was analyzed four times and the most repeated value was tabulated.

Extraction and screening of stilbenes in pure and hybrid protoplasts

Protoplast solutions were frozen in liquid nitrogen with five volumes of 95% methanol and were then homogenized. After homogenization, the samples were pulse sonicated with 95% methanol for 10 min in ice. It was further centrifuged for $15,000 \times g$ for 10 min. The supernatant was collected, mixed and were filter sterilized using a Millipore filter of 0.2- μ m pore size. The samples were subsequently subjected to lyophilization. After the solvent has been evaporated, the powder was reconstituted with 10 μ l of methanol and was subjected to metabolite profiling using mass spectrometry. The sample extracts were analyzed using Agilent 1200 LC-MS (liquid chromatography coupled with mass spectrometry) system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 6520 electrospray ionization (ESI) interface with quadrupole-time of flight (Q-TOF). Small molecules from the protoplast cultures were resolved using HPLC column C18 (pore size, 1.7 μ m; length, 4.6×250 mm; Waters analytical column) and was gradient eluted with a flow rate of 500 μ l/min using water with 1% formic acid/acetonitrile, 95:5 v/v for 1 min, 45:35 v/v for next 5 min, 47:75 v/v for 5 min, 52:75 v/v for 5 min, 54:5 v/v for 5 min, 5:0 v/v for last 2 min. The mass analyzer was scanned over a range of 50–1700 m/z (mass by charge ratio). The electron spray ionization was used in a

positive mode with following parameters-temperature 25 $^{\circ}$ C, MS detection: capillary voltage (V Cap) +3 eV, source temperature (gas temperature) 350 $^{\circ}$ C, desolvation temperature 300 $^{\circ}$ C, cone gas flow 3.0 l/min, desolvation gas flow (drying gas) 8 l/min. The data was analyzed using Agilent Mass Hunter Workstation Data Acquisition software B.02.01 (Agilent Technologies, Santa Clara, CA, USA) and spectra were aligned and processed using Agilent Mass Hunter Qualitative analysis software (Version B.04.01). Molecular feature extractor was used to find features in the chromatogram.

Results

Protoplast yield and viability

Dendrobium hybrid leaves were initially used as test samples to standardize the individual effects of pectinase and cellulase. Enzyme efficiency was calculated based on its ability to retrieve the highest protoplast density with a viability percentage of 75 and above. We encountered heterogeneity in protoplast size and shape, because of the presence of partially digested cell wall. Combination of 4% pectinase and 2% cellulase had the highest efficiency in protoplast isolation (Fig. 2), both in terms of density and viability. Consistent protoplast density was recorded from 30% Percoll gradient when compared to sucrose-mannitol gradients. The isolation studies indicated that the density of protoplast retrieval was more from the leaves when compared to tepals (Table 1), where the time of incubation ranged between 3 and 4 h. Maximum protoplast density of 30.11×10^4 /ml was obtained from *D. ovatum* leaves with a viability percentage of 76.97. *Dendrobium* hybrids both the pink and white varieties provided similar protoplast density of 13.7×10^4 protoplasts/ml and 13.45×10^4 protoplasts/ml in 3 and 4 h of enzyme cocktail incubations, respectively. However, the viability percentages were higher in protoplasts derived from pink *Sonia* hybrid (85.65) than the white hybrid (77.36) (Table 1).

Fusion products and their density

The protoplasts isolated from tepals were relatively colorless, when compared to protoplasts obtained from the leaves (Fig. 3). This color difference among the protoplasts was exploited to visualize the fusants in light microscopy without subjecting them to staining procedures. Furthermore, to have a consistent protoplast density count and viability percentage, the hemocytometer-based protoplast yield was re-analyzed with different fluorescent dyes. The protoplast density was then post-validated with flow cytometer for an absolute density count and viability percentage (Table 1). The use of variant fluorescent dyes for protoplast visualization and imaging gave us the percentage of live protoplasts. Fluorescent

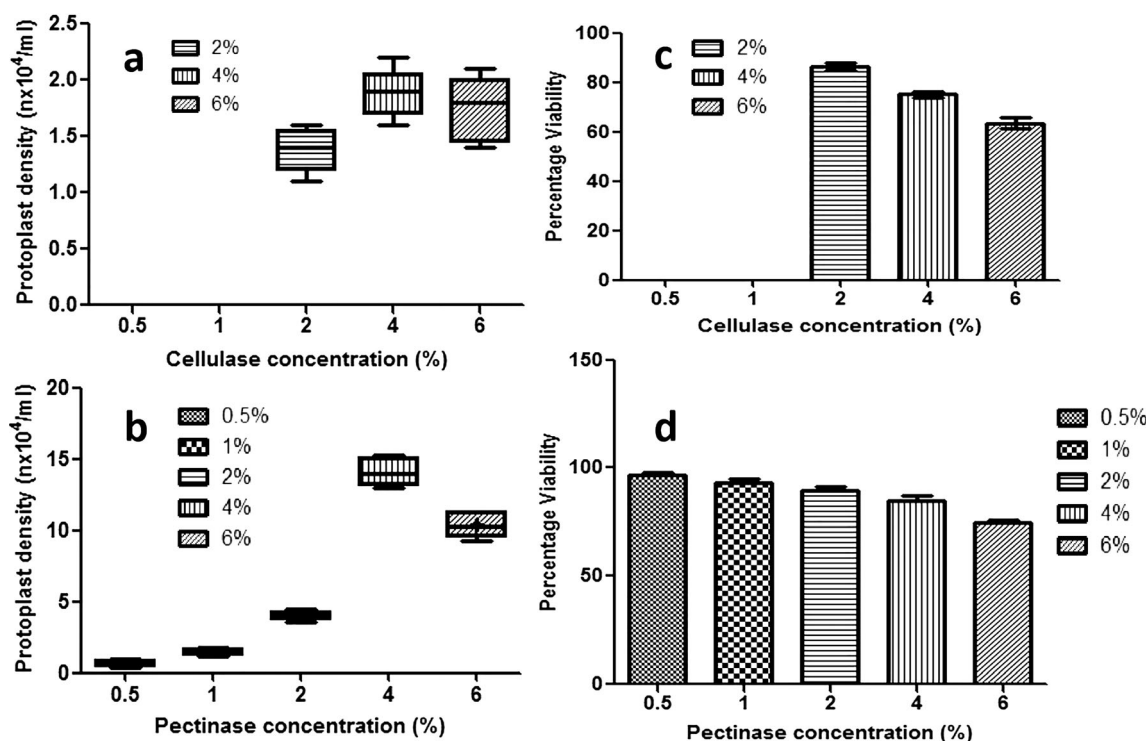


Fig. 2 Protoplasts density obtained from different concentrations with individual treatment of cellulase (a) and pectinase (b). Viability percentages obtained from individual treatments of various

concentrations of cellulase (c) and pectinase (d). Sample used were leaves of *Dendrobium* pink hybrid. Differences between the groups according to one-way ANOVA are indicated ($P < 0.0001$)

imaging showed the presence of partially formed cell wall, the position of the nucleus, chloroplast density, colony existence, and integrity of the plasma membrane (Fig. 4). The dual dye staining method with thiazole orange along with propidium iodide could thus validate the live and dead populations of protoplasts more precisely than their individual use. The events in the lower left quadrant of the dot plot (Fig. 5) denote the population of dead protoplasts specific for propidium iodide, and the lower right quadrant depicts thiazole orange specific live protoplasts. We fused green leaf protoplast of hybrids to non-green tepal-derived protoplasts of wild species and vice versa. This enabled easy identification of atypical fusants even in bright field microscopy. We witnessed occurrences of karyokinesis, homokaryons, heterokaryons, and budding of protoplasts (Fig. 6) in vitro. The number of fusants was estimated (Table 2) using hemocytometer. The fusion efficiency can further be increased by media optimizations and change of fusogens. Our experiments had both homokaryons and heterokaryons along with some unfused protoplasts. Both liquid and agarose bead method had exhibited similar results in protoplast fusion.

Identification of stilbenes in protoplast cultures

We performed metabolomic analysis using mass spectrometry to monitor the presence of phenolic marker reference compounds, viz., moscatilin, resveratrol, tristin, confusarin, and

gigantol in protoplasts of *Dendrobium* hybrids and wild species that were maintained in medium for 2 weeks. Moscatilin was below the limit of detection in protoplasts, isolated from leaves and tepals of *Dendrobium ovatum* (Fig. 7). It was detected only in leaves and tepals of *Dendrobium* hybrid (Table 2). Feature extractions of the mass spectra revealed the relative abundance of important reference phenols by percentage volume. In leaf-derived protoplasts of *Dendrobium* pink hybrid, the percentage volume of resveratrol and moscatilin was 0.28 and 0.26, respectively. Gigantol, confusarin, and tristin were below the limit of detection. However, the leaf-derived protoplasts of *D. ovatum* showed 1.59% of tristin (Fig. 7a). Protoplasts isolated from *Dendrobium* hybrid pink had 0.12% confusarin and 0.3% resveratrol. Tepal-derived protoplasts of *Dendrobium* white hybrid and *D. ovatum* showed 0.58% moscatilin and 0.26% resveratrol, respectively. When fusion products were analyzed, more compounds, viz., tristin, confusarin, gigantol, and resveratrol apart from moscatilin could be detected in the protoplast fusion (Fig. 7b, c). Co-cultured protoplasts of *Dendrobium* hybrid leaves together with *D. ovatum* tepals had yielded 0.9×10^1 /ml homokaryons and 0.6×10^1 /ml of heterokaryons (Table 2). The spectral feature extraction of this co-cultured protoplast combination generated 0.14% of tristin, 0.22% resveratrol, and 0.46% gigantol in vitro. Pure cultures of *D. ovatum* tepals earlier showed only 0.26% resveratrol, and there was a very little change in its production, when it

Table 1 Yield of protoplasts and their percentage of viability estimated from leaves and tepals of *Dendrobiums* (wild and hybrids) using 4% pectinase together with 2% cellulase

Plant source	Tissue type	Time of incubation (h)	Protoplast density $n \times 10^4/\text{ml}$		Viability (%)		
			By hemocytometer	By FACS			
<i>Sonia pink</i> (hybrid)	Leaf	0.5	4.45 ± 0.42	5.4	96.71 ± 1.57		
		1.0	6.33 ± 0.12	7.8	91.94 ± 2.39		
		2.0	<i>13.48 ± 0.60</i>	<i>13.7</i>	85.65 ± 2.35		
		3.0	10.72 ± 0.57	12.6	75.83 ± 2.18		
		4.0	10.34 ± 0.95	11.3	66.91 ± 3.01		
		6.0	8.29 ± 0.60	9.8	56.51 ± 2.99		
	Tepal	0.5	1.64 ± 1.38	2.5	91.01 ± 2.84		
		1.0	2.86 ± 0.60	4.6	87.11 ± 4.49		
		2.0	2.58 ± 1.12	3.8	78.02 ± 4.11		
		3.0	<i>5.05 ± 0.34</i>	<i>6.5</i>	66.74 ± 3.18		
		4.0	3.18 ± 0.36	5.4	55.10 ± 2.87		
		6.0	1.07 ± 0.28	3.26	47.62 ± 4.62		
		<i>Sonia white</i> (hybrid)	Leaf	0.5	4.04 ± 0.35	6.11	91.08 ± 2.56
				1.0	6.93 ± 0.22	8.51	89.88 ± 2.26
2.0	7.69 ± 0.54			6.24	83.97 ± 2.41		
3.0	<i>13.37 ± 0.44</i>			<i>13.45</i>	77.36 ± 3.12		
4.0	12.09 ± 0.41			11	67.96 ± 4.47		
6.0	11.01 ± 0.41			13	57.62 ± 3.38		
Tepal	0.5		1.02 ± 0.38	3.22	91.55 ± 2.16		
	1.0		2.75 ± 0.62	2.89	88.46 ± 2.25		
	2.0		2.16 ± 0.63	3.6	76.97 ± 2.63		
	3.0		4.27 ± 1.40	5.11	66.74 ± 3.18		
<i>Dendrobium ovatum</i> (wild)	Leaf	4.0	<i>4.50 ± 1.08</i>	5.7	56.12 ± 3.73		
		6.0	1.70 ± 0.82	3.24	48.02 ± 1.27		
		0.5	0.97 ± 0.23	1.1	91.55 ± 2.16		
		1.0	10.52 ± 1.34	12	88.46 ± 2.25		
		2.0	<i>30.25 ± 3.52</i>	<i>30.11</i>	76.97 ± 2.63		
		3.0	18.63 ± 2.14	19	66.94 ± 3.18		
	Tepal	4.0	19.82 ± 2.63	22	57.10 ± 3.97		
		6.0	13.17 ± 0.94	12	52.20 ± 3.90		
		0.5	1.09 ± 0.25	3.22	77.27 ± 2.67		
		1.0	<i>4.05 ± 0.28</i>	4.56	62.04 ± 2.53		
	Tepal	2.0	3.25 ± 0.35	5.99	56.12 ± 3.73		
		3.0	1.95 ± 0.43	3.34	48.02 ± 4.02		
		4.0	2.16 ± 0.51	4.66	42.00 ± 7.58		
		6.0	0.84 ± 0.22	1.5	40.60 ± 7.73		

Mean ± SD values were calculated using Graph Pad Prism version 5.01, acquired from triplicate readings of three independent isolations. The most repeated value estimated by FACS is represented as the absolute protoplast density count. The highest protoplast density count obtained from each tissue type is italicized

was co-cultured with *Dendrobium* hybrid leaf-derived protoplasts (0.22%). Whereas *Dendrobium* hybrid white tepal protoplasts, when co-cultured with *Dendrobium ovatum* leaf protoplasts, accumulation of 0.14% tristin, 0.03% confusarin,

1.18% resveratrol, and 0.17% moscatilin was noticed. A productivity descent of tristin was observed in cultures in a co-cultured protoplast to 0.14%. Moscatilin, confusarin, and resveratrol were below the limit of detection in the pure

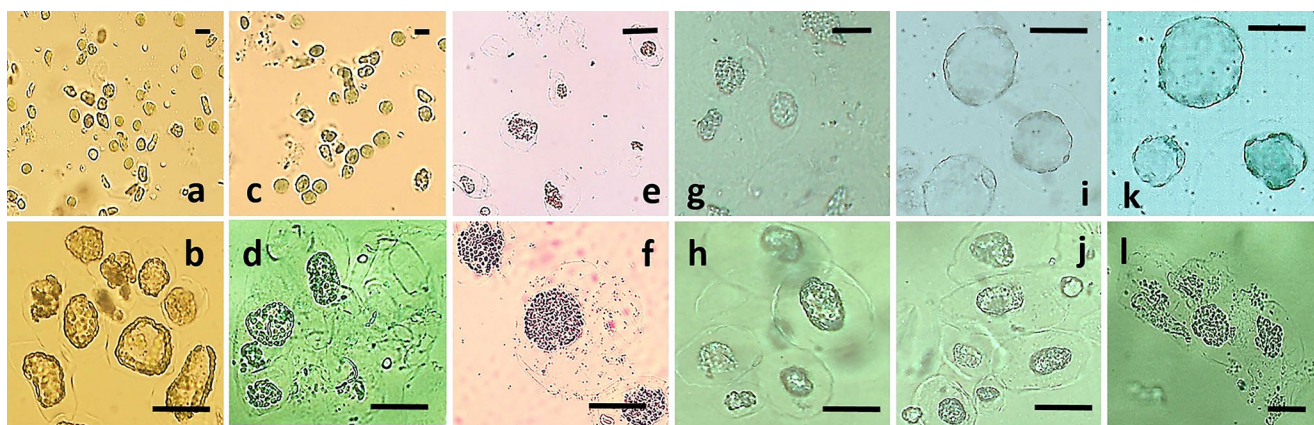


Fig. 3 Protoplasts isolated from *Dendrobium* leaves of pink hybrid (a, b), leaves of white hybrid (c, d), tepals of pink hybrid (e, f), tepals of white hybrid (g, h). Protoplasts isolated from *Dendrobium ovatum* tepals (i, j) and leaves (k, l). [Scale bar = 20 μm]

protoplast cultures derived from *D. ovatum* leaves. But these compounds were detected in a mixed population of protoplasts.

Discussion

To improve the efficiency of interspecific crosses and also to investigate their potency, we need a unique platform. It has been shown that somatic hybridization among incompatible ornamental species was resolved through protoplast fusion (Pati et al. 2008). Protoplast isolation and the fusion facilitated the production of many high yielding varieties in crops (Liu et al. 2007; Jiang et al. 2013). The basic idea of improved productivity via molecular pharming concepts includes cost reduction in developing a novel transgenic system with desirable characters. Few pertinent traits include the attainment of complete PTMs that could modulate (accentuate, diminish, or eliminate) the activity of desired enzymes. Protoplast derived from specific tissues of desired plants is a unique cell system in all aspects, including its biology, kinetics, physiology, and

performance, where bottlenecks connected with heterologous expression can be effortlessly resolved. To improve its productivity, we have to equip the system with stronger promoters and channelize the target protein expression in specific subcellular compartments for commercial scale-up (Hellwig et al. 2004; Moloney 2000).

Critical steps in protoplast isolation

The most critical step in protoplast isolation is to optimize the enzyme concentration that would generate large biomass of healthy, viable protoplasts and also maintain them in an osmoticum-based growth medium for fusion experiments. Hemicellulose and pectin constitute a plant cell wall matrix in which cellulose fibers are embedded. The use of individual enzymes may result in partial dissolution of the cell wall resulting in low protoplast density. Therefore, combination of both cellulase and pectinase was found to be efficient in the present report. A study in winged bean and crown gall cell line of *Parthenocissus tricuspidata* reported that Percoll gradient gave satisfactory results in protoplast retrieval than sucrose-mannitol gradient (Fakhrai et al. 1988). Percoll

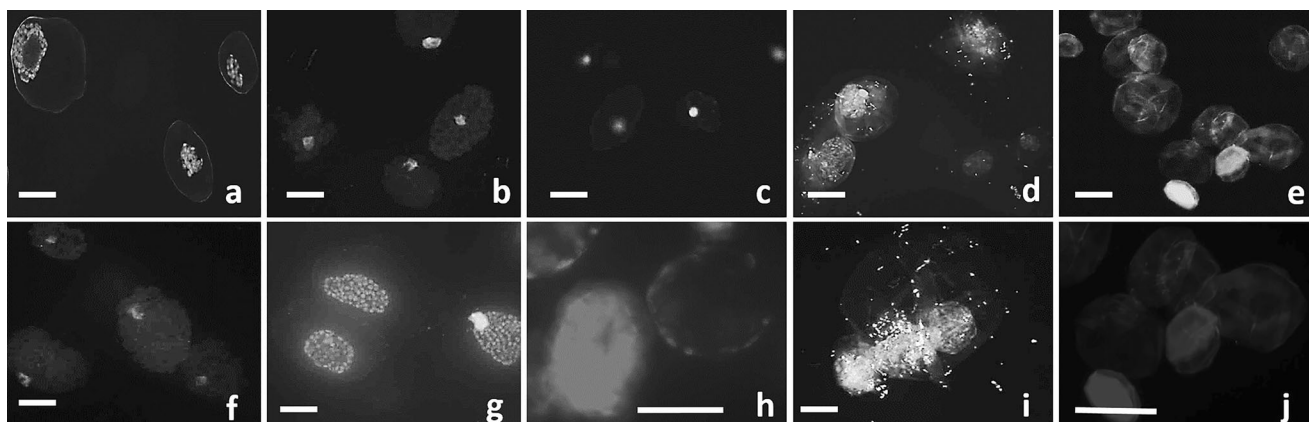


Fig. 4 *Dendrobium* protoplasts depicting homofusants, heterofusants and non-fusants stained with fluorescein diacetate (a, b, f, g), DAPI (c, h), thiozole orange (d, i), Hoechst 33342 (e, j). [Scale bar = 20 μm]

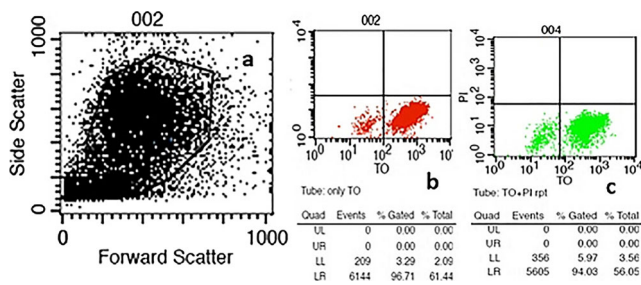


Fig. 5 Dot plot of gated live protoplast using Cell Quest Pro software in FACS (a), quadrant statistics representing live protoplasts in *lower right* (LR) quadrant stained with thiozole orange (TO) alone showing a gated percentage of 96.71 (b), quadrant statistics depicting protoplasts stained with both propidium iodide (PI) and thiozole orange (TO) with 5.97% of gated dead protoplasts in the *lower left* (LL) quadrant and thiazole orange (TO) stained 94.03% gated live protoplasts in the *lower right* (LR) quadrant (c)

gradients are osmotically inert (Pertoft 2000) and were shown to exhibit sharp rise in viscosity usually above 20% and therefore more preferred than sucrose or mannitol, which was in consonance with our study. High protoplast density was obtained from the leaves of *D. ovatum* owing to their slender texture, when compared to that of hybrids. Despite having slender texture, the tepals yielded low protoplast density, which is due to its low cell biomass relative to leaves. Viability of the protoplasts is dependent on factors such as

age of the protoplast tissue source, concentration of the enzyme mixture, incubation time in the enzyme cocktail, isolation medium, tonicity, and strength of the osmoticum. Critical factors that contribute to the protoplast fusion and division are pH, medium nutrient composition, plant growth regulator concentrations, and initial plating density of protoplasts. Genetic manipulation for obtaining somatic hybridization, cybridization, and direct gene transfer can all be exploited for plant improvement if a reliable and efficient plant regeneration system is developed.

The necessity for protoplast fusion and its growth

In the present study, the protoplasts were stained with a panel of fluorescent dyes which aided to reveal their vigor prior to fusion experiments. The use of different panels of dyes helped us to identify heterofusants and atypical microcolonies. There has been a pursuit to raise interspecific crosses in orchids, where only limited success was seen, due to sexual incompatibility which is witnessed both at the intervarietal and interspecies levels. Protoplast fusion has been continually used to improve genetic traits in plants (Melchers et al. 1992; Belarmino et al. 1996; Jarl et al. 1999), mushrooms (Sunagawa and Miura 1992; Zhao and Chang 1995, 1996), and many fungi (Kiyohara et al. 1990; Kirimura et al. 1997)

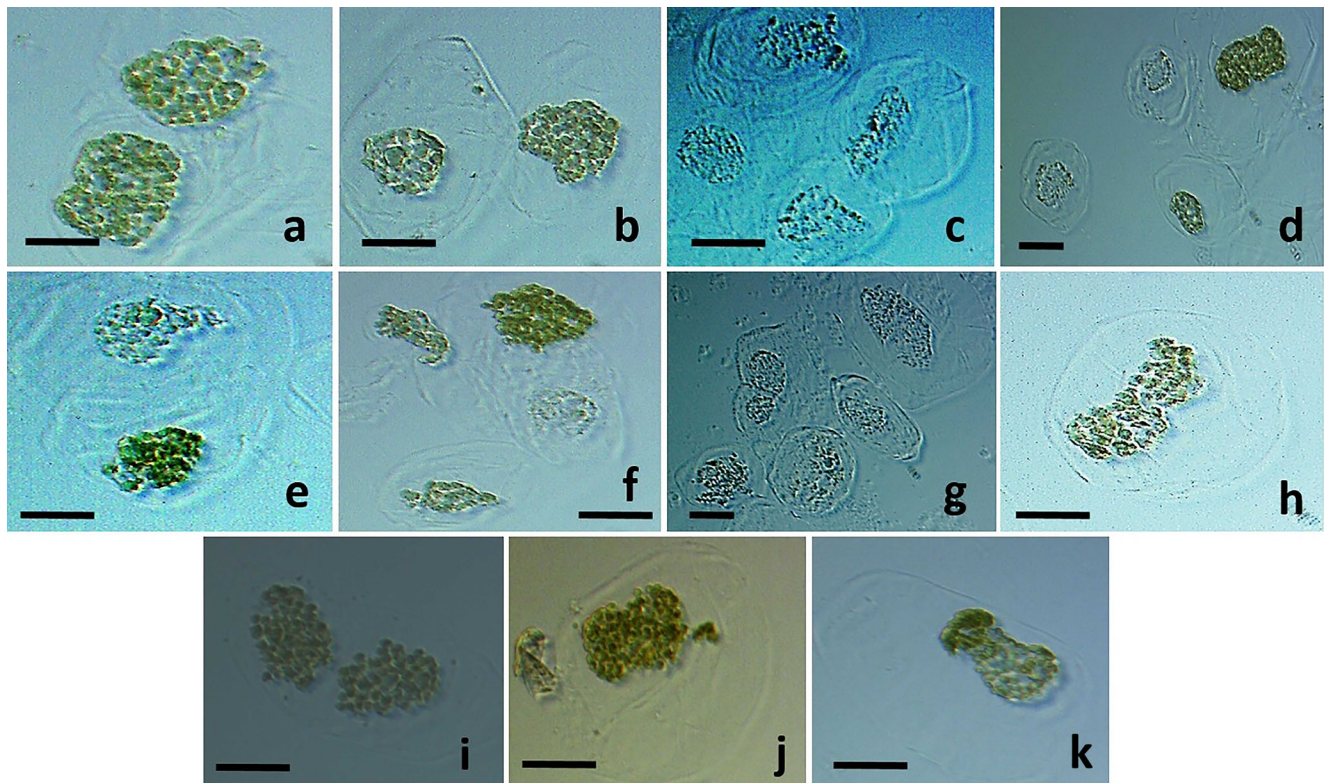


Fig. 6 Fusion products obtained in modified MS medium. Homotypic fusion of leaf-derived protoplasts from hybrid (a, b), protoplast budding in leaves of *Dendrobium ovatum* (c), heterotypic fusion of leaf and tepal protoplasts forming atypical microcolonies (d, e, f), budding of tepal

protoplasts (g), karyokinesis in leaf protoplast (h), post-karyokinesis event in leaf protoplast (i), onset of karyokinesis (j), heterokaryon (k). [Scale bar = 20 μ m]

Table 2 Number of fusants obtained from protoplasts after 3 h of incubation

Source	Homokaryons in regenerating medium	Heterokaryons witnessed	Stilbenes identified ^a
Leaf protoplasts of <i>Dendrobium Sonia</i> pink hybrid	0.4×10^2 /ml	ND	Resveratrol, moscatilin
Leaf protoplasts of <i>Dendrobium Sonia</i> white hybrid	3×10^2 /ml	ND	Moscatilin
Leaf protoplasts of <i>Dendrobium ovatum</i>	1×10^2 /ml	ND	Tristin
Tepal protoplasts of <i>Dendrobium Sonia</i> pink hybrid	0.3×10^1 /ml	ND	Confusarin, resveratrol
Tepal protoplasts of <i>Dendrobium Sonia</i> white hybrid	0.1×10^1 /ml	ND	Moscatilin
Tepal protoplasts of <i>Dendrobium ovatum</i>	0.24×10^1 /ml	ND	Resveratrol
Leaf protoplasts of <i>Dendrobium pink</i> hybrid \times Tepal protoplasts of <i>Dendrobium ovatum</i>	0.9×10^1 /ml	0.6×10^1 /ml	Tristin, resveratrol, gigantol
Tepal protoplasts of <i>Dendrobium white</i> hybrid \times Leaf protoplasts of <i>Dendrobium ovatum</i>	0.3×10^1 /ml	0.2×10^1 /ml	Tristin, confusarin, resveratrol, and moscatilin

Initial seeding density was 1×10^4 protoplasts/ml

ND not detected

^a Analyzed by mass spectrometry after 2 weeks of culture

for years. It is recommended to eliminate these homokaryons and unfused protoplasts and only permit heterokaryons to survive in culture medium. This could be done in two ways—(a) by inactivating the nuclear genome of one of the protoplast sets, by irradiating it and (b) by inactivating the cytoplasm of another, by treating it with a metabolic inhibitor such as iodoacetamide (Veilleux and Compton 1996). This method would eliminate unfused protoplasts and homokaryons as they will fail to regenerate in the culture medium. Since we were interested to analyze the metabolites, we retained all homofusants, heterofusants and non-fusants in the medium. Our previously published study indicated that replacement of ammonium nitrate with ammonium sulfate in MS medium was appropriate for symbiotic hypergeneration of *D. ovatum* microseeds (Shetty et al. 2015) in vitro. Hence, we attempted to use the same medium for protoplast fusion and maintenance as well. The protoplast displayed good division rate in modified MS medium. However, we could get very low heterofusants in

the cross-cultured samples. The low yield of fusants could be due to the low seeding density. The initial seeding density was deliberately kept low, as it reduced crowding of fusants which in turn assisted in immaculate imaging of fused protoplasts.

Stilbene undulation in cross-cultured protoplasts

Moscatilin, tristin, gigantol, and confusarin excluding resveratrol are regarded as reference phenols and were studied earlier in other *Dendrobium* species (Yang et al. 2004, 2006; Zhang et al. 2005). Our study is the first report showing the presence of resveratrol, moscatilin, tristin, gigantol, and confusarin in *Sonia* hybrids and wild species from cultured *Dendrobium* protoplasts in vitro. Possibilities of rise and fall of stilbenes in protoplast culture were compared with the general flavonoid pathway in plants, as the moscatilin biosynthetic pathway is not reported yet. L-Phenylalanine and L-tyrosine are precursors for a wide range of plant-derived natural products. The frequent first step in plants would be the elimination of ammonia from the side-chain to generate cinnamic acid, if the start compound is phenylalanine or else 4-coumaric acid, if it is tyrosine. Cinnamic acid notably their coenzyme A esters like 4-hydroxycinnamoyl-CoA may also function as initiation units for chain extension with malonyl-CoA, thereby linking shikimate and acetate pathways. Based on the nature of enzymes (stilbene synthase or chalcone synthase), the bond foldaway of their respective substrates is hypothesized to have resulted in aromatization forming either stilbenes or chalcones. Resveratrol and chalcone synthases apparently compete or utilize the same substrates, but their intermediates and products differ in the newly formed ring systems (Schröder and Schröder 1990). This is attributed to the nature of enzymes and their substrate availability, which might again vary both at intergeneric or interspecies levels. During this biosynthetic mechanism, many intermediates and products might have lost their hydroxyl groups or might have undergone changes like modifications in the pattern of aromatization, methylation, glycosylation, and dimethylallylation, thereby increasing the range of compounds. The structural comparison of compounds, viz., confusarin, tristin, gigantol, moscatilin, and resveratrol identified in the protoplast cultures further strengthens our hypothesis (Fig. 7). Substrate utilization and differential enzyme activity is hypothesized to play a vital role in the rise and drop of natural products in cultures. Committee of Chinese Pharmacopoeia 2005 has regarded *Dendrobium* species highly medicinal as it has antipyretic, ophthalmic drug, and possesses some immunomodulatory benefits. Considering its benefits, it has become a key ingredient of many pharmaco-nutraceutical formulations. More than 50 such phenolic marker compounds have been screened that were proven to display anti-tumorigenic (Lee et al. 1995), anti-inflammatory (Lin

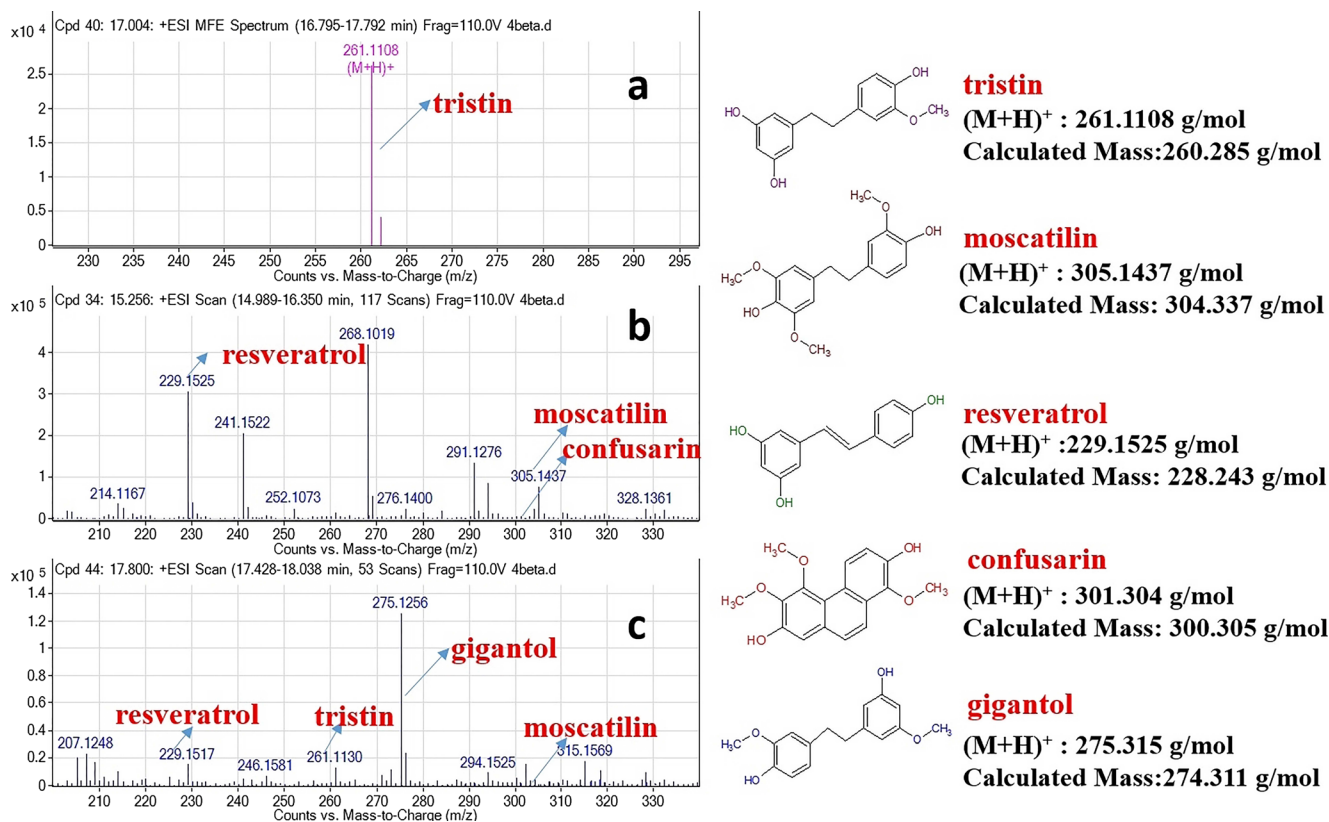


Fig. 7 Mass spectrometric profiles of leaf protoplasts derived from *Dendrobium ovatum* showing the presence of tristin (a), protoplasts from *Dendrobium Sonia* hybrid tepals \times *Dendrobium ovatum* leaves showing the presence of resveratrol, moscatilin, and confusarin (b), *Dendrobium Sonia* hybrid leaf \times *Dendrobium ovatum* tepals showing

the presence of tristin, resveratrol, gigantol, and moscatilin (c). Chemical structures drawn using ACD/ChemSketch are depicted with the protonated mass (M+H⁺) obtained when run in a positive mode by the spectral feature extraction and the calculated mass

et al. 2001; Yang et al. 2006), anti-mutagenic (Miyazawa et al. 1997, 1999), anti-platelet (Fan et al. 2001), and anti-angiogenic effects (Gong et al. 2004). It has been shown in studies that many species show chemical diversity, as they are picked from different ecological zones. It is difficult for one to estimate the right harvest time of such medicinally significant herb. The present study thus has demonstrated that protoplast fusion could bring about natural product assemblage, which otherwise may tend to get restricted either in a tissue or species specific manner. Chemical fingerprinting studies such as this is significant as they would aid in identifying key chemotaxonomic markers and biosynthetic pathway of stilbenes in genus *Dendrobium*.

Conclusions

Our study indicated that cross-culturing of protoplasts brings about rare metabolite combinations and phytochemical assemblage in vitro. To achieve consistent yield of rare and low produce natural products, there is a need to standardize the yield, viability, and growth maintenance medium for live

and dividing protoplasts. By providing selection medium, the division rate of atypical protoplast colonies from heterokaryons and their biomass growth can be assessed. We believe that this protoplast fusion technique would serve as an excellent platform to accumulate compounds, which tend to get constrained in a tissue/species specific manner. Since protoplasts are single plant cells in isolation, this platform opens up new avenues for studying biosynthesis of potent natural products. In the future, this study may also be adopted to generate novel hybrids in *Dendrobium* species.

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Compliance with ethical standards

Declaration All authors declare that the content of the manuscript is original and it has not been submitted elsewhere for publication. All authors agree on the content of the manuscript. Laws on nature protection were not violated during this study.

Conflicts of interest The authors declare that they have no conflict of interest.

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