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Isolation and fusion of protoplasts in *Vanilla* species

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Vanilla, an important flavouring material and spice, is the fermented and cured fruit of the orchid, *Vanilla planifolia* Andrews (*syn. V. fragrans* Salisb.). Origin of much of the planting material of vanilla from limited clonal source, coupled with the threat of destruction of its natural habitats, leads to a narrow genetic base being the major bottleneck in crop improvement programmes. Isolation and fusion of protoplasts was attempted to produce hybrids with desirable traits. Protoplasts were successfully isolated from *V. planifolia* and *V. andamanica* when incubated in an enzyme solution containing macerozyme R-10 (0.5%) and cellulase Onozuka R-10 (2%) for 8 h at 30°C in dark, with good yield and viability. PEG-mediated protoplast fusion between *V. andamanica* and *V. planifolia* was successful and the fusion product (heterokaryon) could easily be identified because the protoplasts of the two species differed in size and arrangement of chlorophyll. This can be helpful in gene transfer for helpful traits, especially the natural seed set and disease tolerance observed in *V. andamanica*.

Keywords: Isolation, protoplast fusion, *Vanilla andamanica*, *V. planifolia*.

VANILLA, an important and popular flavouring material and spice, is the fermented and cured fruit of the orchid,

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Vanilla planifolia Andrews (*syn. V. fragrans* Salisb.). Vanilla has been used as a flavouring agent since the 14th century by the Aztecs and is the second most expensive spice traded in the world market. The worldwide annual consumption was 1900 t in 1995, with 1400 t imported to USA alone. At present, Indonesia is the largest producer of vanilla[1], producing about 2102 mt from about 9689 ha.

Vanilla planifolia is little different from the wild progenitors. This can be attributed to limited breeding and recent domestication. Much of the planting material of vanilla originated from limited clonal source and hence practically no variability is available for crop improvement. This leads to monoculture, making vanilla susceptible to diseases and pests. Broadening the genetic base of vanilla by introduction from its native zone or from areas of diversity is difficult due to severe restrictions imposed by the host countries for germplasm exchange. This leaves behind no option but to look for other sources of variability. Vanilla is suspected to be highly heterozygous because of its cross-pollinated nature and this was exploited to harvest the variations segregating among its seedling progenies[2].

V. planifolia cultivation is severely hampered by the incidence of various diseases. The most common diseases are of fungal origin, viz. foot rot and wilting caused by *Phytophthora meadii*, *Fusarium oxysporum*, *Calospora vanillae*, *Sclerotium* rot[3], leaf rot, blights and brown spots or anthracnose by *Colletotrichum gloeosporioides*. Further, in its natural habitat in Central America, *V. planifolia* is pollinated by *Melipona* bees and tiny humming birds and in the absence of natural pollinators, fruit set is not observed until hand-pollinated. Hence, production of interspecific somatic hybrids between susceptible and resistant parents and species with natural fruit set could provide variants that could be used in crop improvement, especially to develop high quality, self-pollinating, disease-resistant genotypes.

Since protoplasts provide an ideal system for gene transfer because of the freely accessible plasma membrane that guarantees DNA to reach and enter every protoplast in a given population, an attempt was made to isolate protoplasts in two *Vanilla* species, namely *V. planifolia* (the cultivated vanilla) and *V. andamanica*, which gave indications of natural seed set[4]. Some of the desirable traits like self-pollination, higher fruit set and disease resistance have been reported in its near relatives[5]. The efficiency of DNA uptake can be further promoted using polyethylene glycol (PEG)[6]. Hence development of protoclonal hybrids was standardized for possible exploitation to broaden the available genetic base.

In the present study, mesophyll tissue of *in vitro*-derived leaves of *V. planifolia* and *V. andamanica* was used as source tissue. Young leaves were cut into small pieces to ensure proper enzymatic digestion, as it is difficult to peel-off the epidermis.

One-step method of enzyme digestion was used to release the protoplasts, i.e. the tissue was digested with a mixture of macerozyme and cellulase. The various enzymes tried were macerozyme R-10, hemicellulase, and Onozuka cellulase R-10 at different concentrations (Table 1). The enzyme solution was prepared in Cell Protoplast Washing (CPW) medium (Table 2).

During protoplast isolation osmotic strength of cytoplasm and the isolation medium needs to be balanced, to prevent plamolysis or bursting of the protoplasts. Osmotic pressure of the protoplast medium is generally manipulated by addition of sugars or sugar alcohols and in the present trial, mannitol was used at different concentrations (7, 8, 9 and 10%).

The leaves were incubated in CPW medium with mannitol for pre-plasmolysis. One gram each of mechanically macerated leaves from *in vitro*-cultured plants was immersed in 10 ml each of the isolation medium and incubated in dark up to 16 h. Changes occurring during incubation were observed at hourly intervals.

A combination of filtration, centrifugation, washing and floatation centrifugation was used to purify the protoplasts. After digestion, the enzyme solution containing protoplasts was filtered through a stainless steel mesh (60 mesh size, Sigma) to remove larger particles of undigested tissues and cell clumps.

A sample was observed under inverted microscope to confirm enzymatic digestion and release of protoplasts. The filtrate was distributed into sterilized, screw-capped centrifuge tubes and centrifuged in Beckman tabletop centrifuge for 10 min at 700 rpm. The protoplasts form as pellet at the bottom of the tube. The supernatant enzyme

Table 1. Composition of enzyme solutions (ES) for isolation of protoplasts in vanilla*

Code no.	Mannitol (%)	Macerozyme R-10 (%)	Hemicellulase (%)	Onozuka cellulase R-10 (%)
ES-1	10	0.5	–	1.0
ES-2	9	0.5	–	1.0
ES-3	8	0.5	–	1.0
ES-4	7	0.5	–	1.0
ES-5	6	0.5	–	1.0
ES-6	5	0.5	–	1.0
ES-7	10	0.5	0.5	2.0
ES-8	9	0.5	0.5	2.0
ES-9	8	0.5	0.5	2.0
ES-10	7	0.5	0.5	2.0
ES-11	6	0.5	0.5	2.0
ES-12	5	0.5	0.5	2.0
ES-13	10	1.0	–	3.0
ES-14	9	1.0	–	3.0
ES-15	8	1.0	–	3.0
ES-16	7	1.0	–	3.0
ES-17	6	1.0	–	3.0
ES-18	5	1.0	–	3.0

*Enzyme solutions were prepared in CPW medium.

Table 2. Composition of media used for protoplast isolation and culture in vanilla

Components	CPW medium (mg per l)	Floating media (mg per l)	Protoplast culture media (mg per l)	
			I	II
NH_4NO_3	–	–	1650	1650
KNO_3	101.0	101.0	1900	1900
$CaCl_2 \cdot 2H_2O$	1480.0	1480.0	440	440
$MgSO_4 \cdot 7H_2O$	246.0	246.0	370	370
KH_2PO_4	27.2	27.2	170	170
KI	0.16	0.16	0.83	0.83
H_3BO_3	–	–	6.2	6.2
$MnSO_4 \cdot 4H_2O$	–	–	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	–	–	8.7	8.7
$Na_2MoO_4 \cdot 2H_2O$	–	–	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	–	–	0.025	0.025
$FeSO_4 \cdot 7H_2O$	–	–	27.8	27.8
$Na_2EDTA \cdot 2H_2O$	–	–	37.3	37.3
Myo-inositol	–	–	100.0	100.0
Nicotinic acid	–	–	0.5	0.5
Thiamine HCl	–	–	0.5	0.5
Pyridoxine HCl	–	–	0.5	0.5
Glycine	–	–	2.0	2.0
Sucrose*	–	21%	2%	3%
Mannitol*	7–10%	–	7%	4%
Gibberellic acid	–	–	0.5	0.5
BA	–	–	0.5	1.0
NAA	–	–	0.5	1.0
2,4-D	–	–	0.5	–

*Were added in g l^{-1}.

Table 3. Composition of PEG solution* for protoplast fusion

Constituents	Molar concentration (mM)	g l^{-1}
NaCl	140	8.18
KCl	5	0.37
Na_2HPO_4	0.75	0.11
Glucose	5	0.90
$CaCl_2 \cdot 2H_2O$	125	18.40
PEG (MW 4000)		400.00

*pH was adjusted to 5.8.

solution was removed using a Pasteur pipette without disturbing the pellet. The pellet was suspended in CPW medium (Table 2).

Centrifugation and re-suspension in fresh medium was repeated three times so as to wash the protoplasts and remove traces of enzyme solution. After washing, the pellet of protoplasts was resuspended in 1 ml of the CPW medium and layered on top of 9 ml of floatation medium (Table 2), and centrifuged at 700 rpm for 10 min. Live protoplasts form a band at the interphase, which was collected with a Pasteur pipette and transferred to culture medium. Concentration of the protoplasts was estimated using a haemocytometer.

Two droplets of protoplast suspension from *V. planifolia* and *V. andamanica* were added to another droplet of PEG solution (Table 3), and allowed to mix at room temperature for 30 min. They were maintained as droplet cultures on glass slides and were periodically observed for fusion of protoplasts, cell-wall regeneration and cell division.

Protoplasts were cultured initially in liquid medium in petri dishes. Five drops of culture medium containing protoplasts were placed in the bottom half of the petri dish, sealed with parafilm and incubated in dark at 25°C. Fresh culture medium with low osmoticum was added after every seven days to replenish the nutrients. The samples were periodically observed for cell-wall regeneration and cell division. After 40–60 days of culture, they were plated on 0.25% agarose medium and monitored for microcallus formation and further development.

Since it was difficult to peal-off lower epidermis in vanilla, the plasmolysed leaf tissue was mechanically macerated by scraping the lower surface of the leaf with a sharp blade and incubating in different concentrations and combinations of enzyme solutions (Table 4). Periodical microscopic observations showed the liberation of cell clusters and individual cells after 2 h of incubation in enzyme solution (Figure 1 *a* and *b*). In vanilla, macerozyme at 0.5% was sufficient to digest the middle lamella and separate the cells. Among the enzyme solutions tried, only one containing macerozyme R-10 (0.5%) and cellulase Onozuka R-10 (2%) was found to digest the cell wall and liberate protoplasts after incubation for 8 h.

Table 4. Effect of enzyme concentration and incubation conditions on yield of protoplasts

Species	Enzyme solution	Incubation conditions	Protoplast yield	Viability (%)
Vanilla planifolia	0.5% Macerozyme R-10 + 2% Onozuka cellulase R-10	8 h at 30°C in dark	2.5×10^5/g of leaf	72
V. andamanica	1% Macerozyme R-10 + 3% hemicellulase + 6% Onozuka cellulase R-10	8 h at 30°C in dark	1×10^5/g of leaf	55

The tissues were incubated initially for 8 h at 30°C in dark (Table 4). Among the cellulases tested, only cellulase Onozuka R-10 was found to be effective in releasing the protoplasts. The isolation solution containing 9% mannitol was found necessary for releasing and maintaining viable protoplasts. The isolated protoplasts were round and filled with chloroplasts (Figure 1 *c* and *d*). The protoplast yield was 2.5×10^5 per g of leaf tissue. The protoplast viability as assessed by FDA staining was 72% in *V. planifolia* and 55% in *V. andamanica*.

The successful isolation of protoplasts, their culture and subsequent regeneration into complete plants depend on a number of factors, the important ones being genotype, tissue from which protoplasts are isolated, physiological conditions under which the plant cultures have been raised, purity of the enzymes and the osmoticum, period of incubation, culture media and growth regulators, milieu of protoplasts/plating density, method of culture (liquid/solid) and incubation conditions.

The enzymatic (cellulase) isolation of protoplasts was first reported in tomato from root tips by Cocking[7]. The easy availability of commercial, purified enzymes such as cellulase, cellulysin, pectinase, macerozyme, driselase, rhozyme and hemicellulase has now resulted in increase in the yield and viability of protoplasts and their subsequent response in the culture medium. The period of treatment is reduced and thus deleterious effects on the plasma membrane are avoided. Usually a combination of pectinase and cellulase is used to macerate cells and also liberate protoplasts in a single cell[8]. The concentration and combination of enzymes required depend upon a number of factors such as age, genotype and stage of differentiation of the tissue from which the protoplasts were to be isolated. Though protoplasts can be isolated from a variety of tissues, young *in vitro*-grown plants[9], tissues and explants such as root tips[10], hypocotyl, cotyledons[11] and shoots[12] require low concentration of enzymes and relatively short period of treatment compared to leaves from old or mature plants.

Protoplasts were isolated from *V. planifolia* and *V. andamanica* (Figure 1 *a* and *b*) and were visibly different (Figure 1 *c* and *d*). *V. planifolia* protoplasts were bigger in size (0.031 mm) than *V. andamanica* (0.022 mm). In the former, chloroplasts were arranged around the periphery, whereas in the latter they remained scattered within. When subjected to PEG-mediated fusion, protoplasts of the two species came nearer and formed a passage gradually leading to the transfer of the cell contents of one species into another (Figure 1 *f–h*). The fusion product or heterokaryon has chloroplasts both at the periphery and in the middle, making its identification and selection easier (Figure 1 *i*). This can be helpful in gene transfer of useful traits, especially the natural seed set and disease tolerance observed in *V. andamanica* to *V. planifolia*. The fused protoplasts were cultured as droplet cultures in MS[13] liquid medium with 0.5 mg l^{-1} BA, 0.5 mg l^{-1} IBA supplemented with 3% sucrose and 7% mannitol for 20 days. The cell-wall development around the fusion product was observed after 36 h. The contents became dense by one week and fresh medium needs to be added at 7-day interval. The possibility of protoplast systems in spice crops such as cardamom, ginger and vanilla has been studied earlier[14,15].

The techniques of protoplast isolation and fusion are important in studies of plant improvement by cell modification and somatic hybridization. Since the first report on the regeneration of complete plants from isolated protoplasts[16], tremendous progress has been made and a number of crops of commercial importance such as potato, tomato, tobacco, rice, linseed, alfalfa, cucumber, eggplant, lettuce, *Brassica* species, etc., have been routinely regenerated; this has paved the way for their genetic manipulation. The refinement in the technology has also enabled the successful culture of somatic hybrids/cybrids at interspecific and intergeneric levels with far-reaching implications in crop improvement programmes, for widening the pool of genetic resources[17].

The present study indicated the requirement of low concentrations of macerozyme R-10 and Onozuka cellulase R-10 and shorter incubation period for release of protoplasts. The visibly distinguishable nature of protoplasts can be exploited for genetic transformation and incorporation of important desirable traits by cell modification and somatic hybridization, which could have far-reaching implications in improvement programmes of this commercially important spice. Micropropagation and *in vitro* conservation techniques for different species of *Vanilla*[18] and interspecific hybridization as a tool for gene flow of desirable characters from wild species into cultivated species, through pollen, have been reported[19]. This study reports the isolation of viable protoplasts in *Vanilla* species, i.e. in *V. andamanica* and that of PEG-mediated protoplast fusion between *V. planifolia* and *V. andamanica*. The practical implications of scaling up the technology for gene transfer of useful traits, especially natural seed

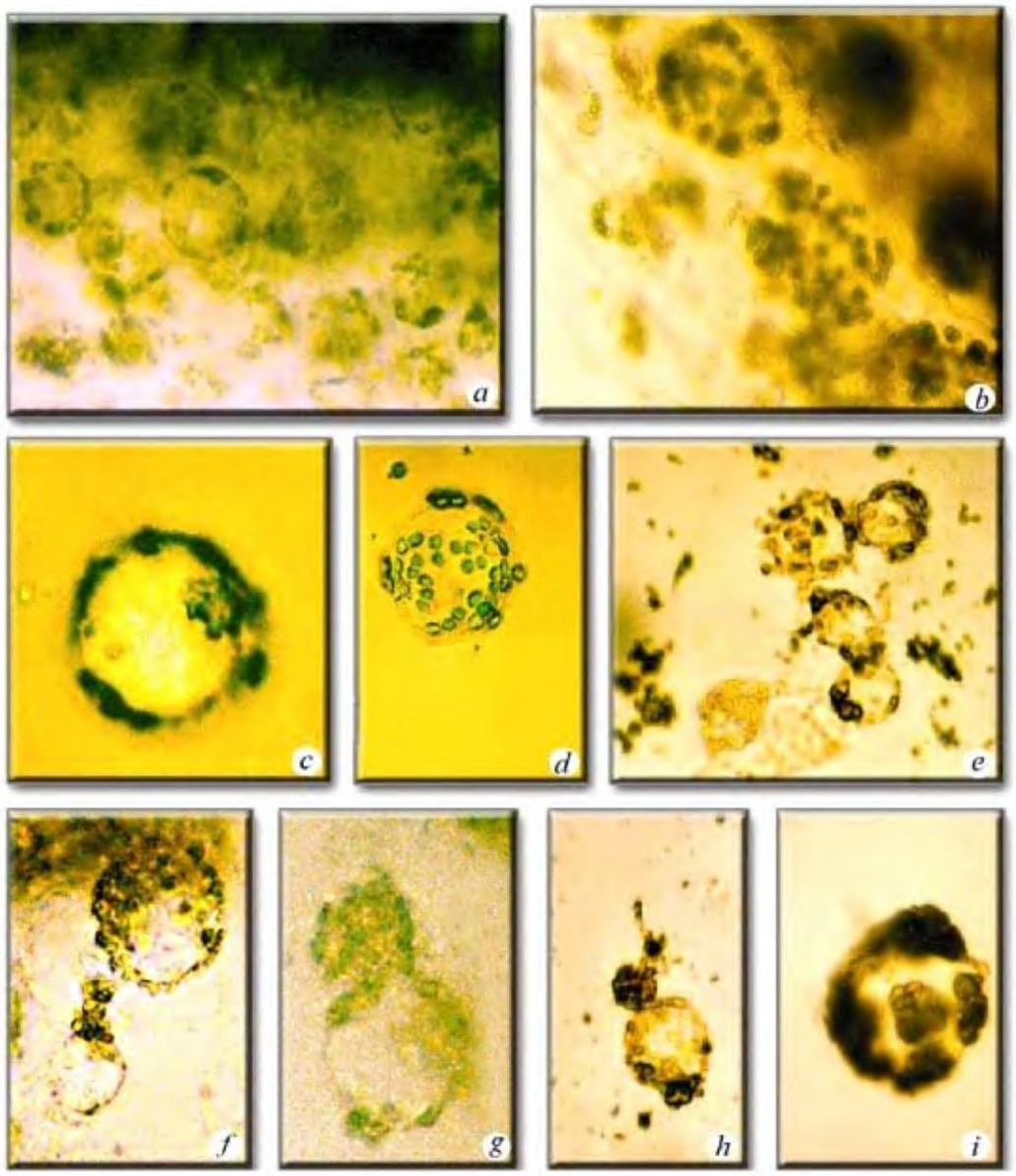

Figure 1. Isolation and fusion of protoplasts in *Vanilla* species. ***a***, ***b***, Release of protoplast from *V. planifolia* leaf tissue (***a***) and *V. andamanica* leaf (***b***). ***c***, Isolated protoplasts from *V. planifolia* (note concentration of chloroplasts around the periphery). ***d***, Isolated protoplasts from *V. andamanica* (note concentration of chloroplasts in the centre). ***e***, Protoplasts coming closer in PEG-incorporated medium. ***f***, Formation of a passage by dissolving of the two plasma membranes, for transfer of protoplasmic contents from one to other. ***g***, ***h***, Fusion of protoplasts of the two species. ***i***, Heterokaryon of *V. planifolia* and *V. andamanica* (noted by the presence of chloroplasts both at the centre as well as the periphery).

set and disease tolerance are tremendous, more so since technology for further *in vitro* multiplication of different species as well as hybrids is already available.

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Computer simulation studies on Delhi Iron Pillar: Estimation of weight

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A computational approach for estimation of weight of the world-famous Delhi Iron Pillar has been presented. The CATIA V5R16 software was used to prepare component drawings based on dimensions from the literature. The weight of the decorative bell capital was estimated as 646 kg and the main body as 5865 kg, thereby giving the entire weight of the pillar as 6511 kg. The methodology utilized in this communication can be employed to determine precise weights of significant historical objects.

Keywords: Computer modelling, Delhi Iron Pillar, simulation studies, weight estimation.

THE Delhi Iron Pillar located in the courtyard of the Quwwat-ul-Islam mosque near Qutub Minar, New Delhi stands testimony to the high level of skill achieved by ancient Indians in the metallurgy of iron and steel[1,2]. A recent photograph of the Delhi Iron Pillar and its computer-simulated model are shown in Figure 1.

The pillar was originally erected around AD 402, in front of a Vishnu temple complex to serve as a 'Standard of Vishnu' at Udayagiri (situated close to modern-day Sanchi near Bhopal, Central India) by Chandragupta II Vikramaditya (AD 375–413). Udayagiri was known as Vishnupadagiri during the Gupta period[2]. The pillar, in its original location, also served an interesting astronomical function, namely the early morning shadow from the pillar fell in the direction of the foot of Vishnu's image in one of the important temples at Udayagiri in the time period around summer solstice[3].

The pillar was shifted by Iltumish (AD 1210–1236) from Udayagiri to its present location in the Qutub Complex[3,4], sometime around AD 1233.

The Delhi Iron Pillar has attracted the attention of archeologists, corrosion scientists and engineers because it has withstood atmospheric corrosion for more than 1600 years. The earliest scientific studies on the pillar were undertaken by Robert Hadfield in 1912. In the late 1950s and early 1960s, the buried underground region of the pillar was excavated and examined by a team of archaeologists and scientists[5]. New insights on historical, scientific and technological aspects of the pillar are available in Balasubramaniam[2].

It is important to understand the physical nature of engineering materials and in this regard, the use of computer

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