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Conservation of *Vanilla* species, *in vitro*

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Abstract

Vanilla (*Vanilla planifolia*) is a crop of great commercial importance as the source of natural vanillin, a major component of flavor industry. The primary gene pool of *V. planifolia* is narrow and is evidently threatened due to destruction of its natural habitats making the secondary gene pool important as a source of desirable traits especially for resistance to diseases. Many species of vanilla are considered rare and endangered hence an urgent need to conserve them, arises. Effective procedures for micropropagation and *in vitro* conservation by slow growth in selected species of vanilla, are described. Synthetic seed technology was standardized by encapsulating 3–5 mm *in vitro* regenerated shoot buds and protocorms in 4% sodium alginate, which could be stored up to 10 months with 80% germination in sterile water at 22 ± 2 °C. *In vitro* conservation technology of *Vanilla* was standardized and shoot cultures could be maintained for more than 1 year without subculture, on slow growth medium, i.e. Murashige and Skoog medium supplemented with 15 g l^{-1} each of sucrose and mannitol in sealed culture vessels at 22 ± 2 °C. These cultures were maintained *in vitro* for more than 7 years with yearly subculture. The conserved material could be retrieved and multiplied normally in MS medium with 1.0 mg l^{-1} BA and 0.5 mgl $^{-1}$ IBA. The *in vitro* conserved plants showed good growth and developed into normal plants. This synseed and *in vitro* conservation system can be utilized for conservation and exchange of vanilla genetic resources.
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Keywords: *In vitro* conservation; Micropropagation; Slow growth storage; Synseeds; *Vanilla planifolia*; *V. aphylla*; *V. pilifera*; *V. andamanica*; *V. wightiana*

1. Introduction

The genus *Vanilla* comprises of about 110 species, distributed in tropical parts of the world (Purseglove et al., 1981) and commercial vanilla, an important and popular flavouring material and spice, is obtained from the fully grown and processed fruit of tropical climbing orchid, *Vanilla planifolia* Andrews, (syn. *V. fragrans* Salisb.). Areas of megadiversity are on the verge of threatening processes leading to plant endangerment and extinction, hence conservation procedures need to be implemented to establish long-term *ex situ* collections containing the maximum genetic diversity of the species.

Vanilla is one of the most economically important spice across the globe with over 37,525 ha cultivation and a production of 4403 tonnes. In the international market, world demand for vanilla was around 32,000 tonnes and the demand for natural vanillin is increasing at 7–10 per annum as the world is shifting towards herbal products (Anil Kumar, 2004). Introduced into India in the 18th century, in recent years, vanilla has gained importance with doubled cultivation in the last 2 years, from 1600 ha in 2001–2002 to 3427 ha in 2003–2004, and the production from 60 to 131 tonnes during this period (http://www.foodindianews.com). Intense work of selection, breeding and conservation of genetic resources, are required to overcome the narrow genetic base of this vegetatively propagated crop. The species diversity in the country is represented by five species *viz.*, *V. aphylla*, *V. walkeriae*, *V. wightiana*, *V. pilifera* and *V. andamanica* and most of them are considered endangered. Although vanilla is cultivated throughout the tropics, its natural populations in Southern Mexico – the most critical sources of novel genetic diversity – are on the verge of disappearing due to deforestation and over collection (Lubinsky, 2003). Since the narrow primary gene pool is evidently threatened, the secondary gene pool comprising of the close relatives of *V. planifolia*, which is also equally threatened, becomes important as a source of desirable traits – especially for self-pollination, higher fruit set and disease resistance (Rao et al., 2000; Minoo, 2002). Many

Abbreviations: BA, benzyl aminopurine; IBA, indole-3-butyric acid; MS, Murashige and Skoog's medium

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species of vanilla are considered endangered and there is urgent need to conserve them. The recent International Congress on vanilla emphasized the need to conserve these species before they go extinct (International Congress on Vanilla 2003). Thus a major challenge is to conserve the vanilla gene pool from the onslaught of habitat destruction, over collection, climate changes and destructive diseases in monocultures (Gopinath, 1994; Suseela and Thomas, 2000; Thomas and Suseela, 2000). Recent advances in conservation have paved the way to safeguard plant biodiversity with a biotechnological approach, which can be regarded as complementary to the traditional clonal orchards and seed banks. Traditionally, vanilla germplasm is conserved in clonal repositories belonging to botanical gardens and in scientific institutions. However, the high costs of this traditional conservation system limit the number of accessions that can be preserved.

In order to stem the flow of loss of biodiversity, an attempt to conserve *Vanilla* species, *in vitro*, has been made in this study. Shoot explants of different species were introduced *in vitro*, induced to multiply, and the micropropagated shoots were used as source of plants for *in vitro* conservation experiments. The development of conservation protocols in the present study focused on shoot apices of vanilla and was extended to conserve its endangered species. Shoot cultures required a regular 3-week subculturing at 22 °C and 12 h photoperiod, hence the possibility of achieving long-term storage is crucial. A significant extension of the interval between subcultures was successful. Thus, a protocol for the medium-term (by slow growth storage, SGS) conservation of selected species was standardized and about 300 lines could be conserved in a 5 m × 2 m area.

2. Materials and methods

2.1. Establishment of in vitro cultures

Shoot tips and nodal segments from field grown plants of different species of *Vanilla* viz., *V. planifolia*, *V. andamanica*, *V. aphylla*, *V. pilifera* and *V. wightiana* at Indian Institute of Spices Research, were established *in vitro*. Murashige and Skoog's (MS) medium (1962) fortified with 2% sucrose and supplemented with auxins and cytokinins and gelled with 0.7% agar was used as basal medium.

Cultures were initiated in MS medium supplemented with low cytokinin concentration, i.e., BA (0.5 mg l^{-1}), for induction of bud break. These explants were transferred to MS medium supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} IBA standardized earlier (IISR, 1993; Minoo et al., 1997) for multiplication. Each multiplication treatment consisted of 20 tubes with 1 shoot per tube. The experiment was repeated twice. All the cultures were maintained at 22 ± 2 °C, under 12 h photoperiod. The organogenic efficiency and number of shoots induced per explant was recorded 60 days after culture initiation.

The study was in progress with a total of five species. After introduction *in vitro*, the species were kept in standard proliferative conditions (Minoo, 2002), i.e., in MS medium, containing 1.0 mg l^{-1} BA and 0.5 mg l^{-1} IBA, at 22 ± 2 °C with 12 h photoperiod, and subcultured every 3 weeks or at the first signs of culture decline.

2.2. Preparation of synthetic seeds

In vitro regenerated shoot buds and protocorms were used as propagules and 4% sodium alginate was used for encapsulation. The propagules along with sodium alginate matrix were dropped into a solution of calcium chloride ($CaCl_2 \cdot 2H_2O$) so as to form round beads. The solution (1.036 g/150 ml) so as to form round beads. The propagules were allowed to remain in the solution for 30–40 min with gentle shaking, for proper bead formation. The beads were recovered by decanting the $CaCl_2$ solution and later washed in one or two changes of sterile water. The synthetic seeds or 'synseeds' thus produced were stored in 50 ml flasks in sterile water at 5, 15 and 22 °C. The synthetic seeds were transferred to culture medium and growth of propagules was used as an index of their viability after storage.

2.3. In vitro conservation by slow growth

To standardize suitable conditions for inducing minimal growth in vanilla cultures and to increase subculture intervals substantially, the basal media was used in full and half strength, with carbon source, sucrose, at different concentrations (30, 20, 15, 10, 0 g l^{-1}), with gradual reduction of sucrose and substitution with mannitol (10, 15 g l^{-1}). The cultures were maintained at 22 ± 2 °C. Shoots (2 cm) were isolated from the proliferating cultures and transferred to sterile glass tubes containing 10 ml of medium (one shoot per tube), closed with aluminum foils to minimize evaporation rate.

3. Results and discussion

3.1. Establishment of in vitro cultures and micropropagation of Vanilla species

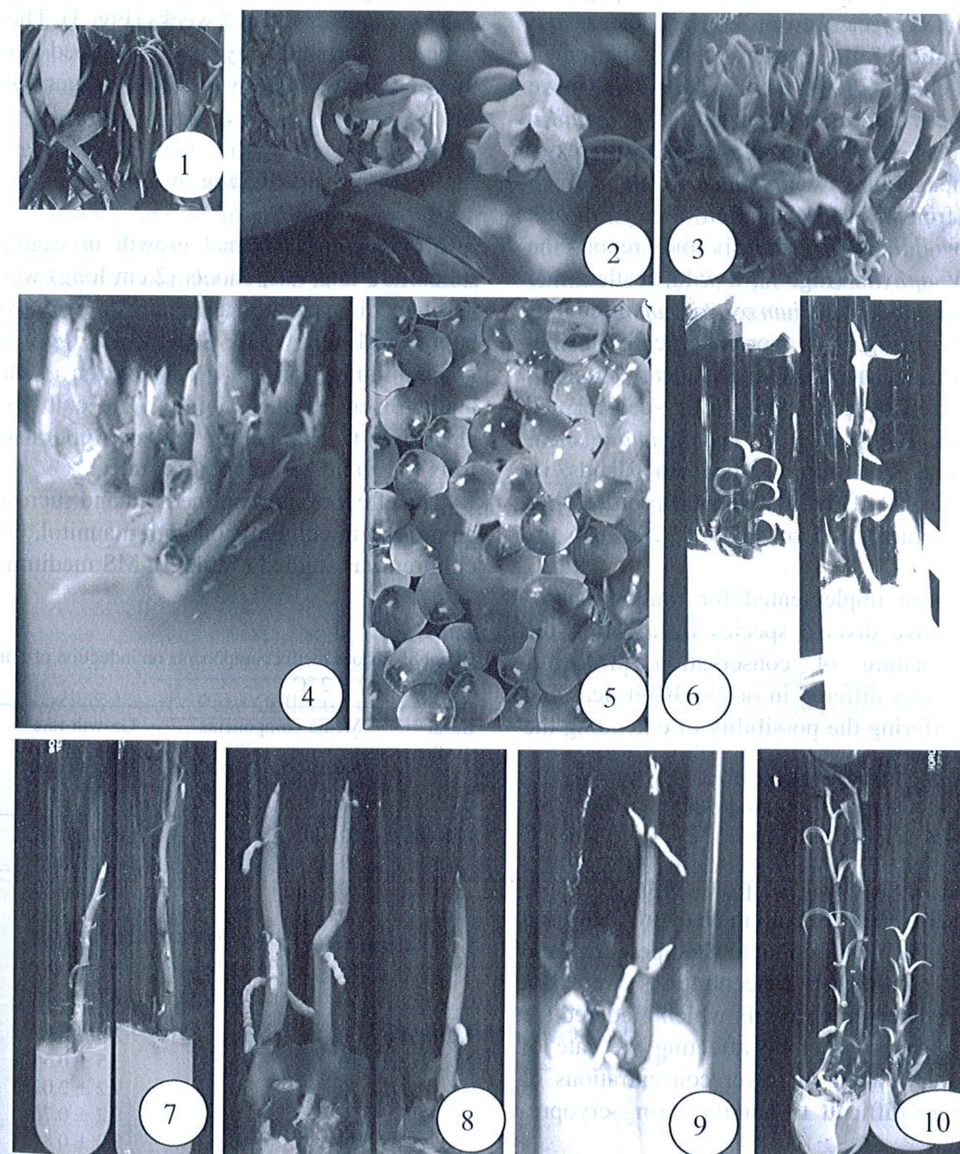
Micropropagation of vanilla was essential to generate uniform, disease free plantlets for conserving the genetic resources, *in vitro*. The subculture of the explants onto the proliferation medium, i.e., MS medium supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} IBA standardized earlier (IISR, 1993; Minoo et al., 1997) for multiplication, promoted the organogenic response and the highest shoot number was obtained in explants induced in the presence of BA combined with IBA. Initiation of preexisting buds to grow *in vitro*, could be induced in MS medium with low cytokinin however, a combination of cytokinins and auxin promoted multiple shoot formation. The number of shoots induced on the nodal segments varied with the species studied (Table 1).

Elongated shoots from proliferation medium were rooted on MS growth regulator free medium containing 30 g l^{-1} sucrose and *in vitro* plantlets with well-developed roots were acclimated with a survival percentage of over 70%. Root initiation on microcuttings started between 4 and 6 days after culture, reaching 100% of the cultures after 2 weeks in all the species studied except *V. pilifera* (Fig. 9), where 80% of the

Table 1
Organogenic responses of diverse *Vanilla* species

Species	Origin	Habit	*In vitro* responses			
			Multiple Shoots[a]		Root growth[a]	
			% response	No. of shoots	% response	No. of roots
V. planifolia	South Eastern Mexico, Guatemala	Cultivated, fleshy subsessile leaf, pale yellowish green flowers	100	13.5 ± 2.0	100	2.46 ± 0.71
V. andamanica	Middle, South and Little Andamans	Wild/cultivated with glossy green leaves, white, fragrant, pink lipped flowers	100	6.0 ± 1.5	100	2.27 ± 0.31
V. pilifera	Malaya, North Eastern India	Wild, leafless near base; leaves only near top, white ovary, flowers greenish white with purple hairs at base of lip	80	3.0 ± 1.7	80	1.00 ± 0.0
V. aphylla	Thailand, North Eastern India	Wild, scale leaves, greenish yellow flowers with reddish-violet tuft of hairs at the base of lip	100	9.0 ± 2.6	100	1.00 ± 0.0
V. wightiana	South India	Wild, leafless, flowers white, lip 3 lobed	80	5.2 ± 2.5	100	1.66 ± 0.47

[a] Mean of 20 replicates.



Figs. 1–10. 1. *Vanilla planifolia* vine; 2. *V. pilifera* (a) flower in comparison with *V. aphylla* (b) flower; 3. *In vitro* multiplication of *V. planifolia*; 4. Multiple shoots in *V. aphylla*; 5. Synseeds of Vanilla, 6. Slow growth in *V. planifolia* cultures; 7. Slow growth in *V. aphylla* cultures; 8. Normal shoots of *V. wightiana* in comparison with slow growing shoot; 9. Slow growth in *V. pilifera*; 10. Slow growing culture in comparison with normal shoots in *V. andamanica*.

cultures responded. The spontaneous rooting observed in the explants cultured on media without any growth regulators is an indication that optimal endogenous levels of plant growth regulators required for rooting are already present in the original tissue/explants. Uniform sized plantlets could be harvested and used for *in vitro* conservation experiments. All species could be induced to produce multiple shoots and develop roots in the same media combination and culture conditions, except for differences in the number of multiple shoots (Table 1).

Micropropagation of *V. planifolia* was reported earlier by various workers (Cervera and Madrigal, 1981; Kononowicz and Janick, 1984; Philip and Nainar, 1986; Giridhar and Ravishankar, 2004). However, the protocol developed in the present study is efficient in the production of more multiple shoots and extendable to different species, hence can be used for large-scale multiplication of disease free plants in all these species. A commercially viable protocol for mass propagation of *V. tahitensis*, a cultivated species of vanilla, was standardized with a multiplication ratio of 1:4.7 over a culture period of 60–70 days (Mathew et al., 2000). Rao et al. (2002) have reported the occurrence and micropropagation of *Vanilla wightiana* Lindl., an endangered species. Earlier reports are not available for micropropagation of *V. aphylla*, *V. andamanica* and *V. pilifera*. Apart from micropropagation of *V. planifolia* (Figs. 1 and 3) and *V. wightiana* (Fig. 8), this study reports the micropropagation of *V. aphylla* (Fig. 7), a wild, leafless and endangered species, tolerant to *Fusarium oxysporum* infections (Minoo, 2002), *V. pilifera* (Fig. 9), a morphologically distinct species with juvenile leafless shoots and mature leafy branches with flowers characterized by pure white ovary-pedicel and violet hairs on the base of lip (Fig. 2) and *V. andamanica* (Fig. 10), a leafy species, endemic to Andaman islands, its collections forming a distinct clade in molecular profiles, thus indicative of possible natural seed set (Minoo, 2002) in the species.

This protocol has been implemented for routine micropropagation of all the five diverse species overcoming the species/clonal-specific nature of conservation protocols, though the different species differed in origin, habit, leaf and floral characters, thus offering the possibility of extending the protocol to the genus *Vanilla*.

3.2. Synthetic seeds

In vitro regenerated shoot buds and protocorms (3–5 mm size) were encapsulated in 4% sodium alginate to produce good quality rigid beads ideal for withstanding low temperatures and cryopreservation (Fig. 5). Higher concentrations were not suitable as they produced very hard matrix which hindered the emergence of shoot buds and thereby affecting the rate of germination and recovery, while at lower concentrations of alginate, the beads were difficult to handle during cryopreservation and retrieval.

The synthetic seeds were stored at 5, 15 and 22 °C to study the effect of temperature on their storage and viability. Low temperatures (5 and 15 °C) were not suitable for synthetic seed storage as they lost their viability within 30 days. But at 22 ± 2 °C, synthetic seeds could be stored successfully upto 10 months in sterile water (Table 2). The beads germinated normally when transferred to MS medium supplemented with BAP (1 mg l^{-1}) and IBA (0.5 mg l^{-1}), maximum germination (80%) was observed in 2 weeks (Fig. 3). They multiplied in the same medium in 60 days and developed into normal plantlets. Synseed technology was ideal for germplasm conservation and exchange.

Table 2
Viable storage of *Vanilla* synseeds

Storage temperature (°C)	Storage time (days)	Germination (%)
5	30	21
15	30	22
22	30	88
	90	88
	120	87
	300	85
	330	45
	360	10

3.3. In vitro conservation by slow growth

For inducing minimal growth in vanilla and to increase subculture intervals, shoots (2 cm long) were cultured on MS basal medium in full and half strength, with carbon source being used singly (30, 20, 15, 10, 0 g l^{-1}) as well as with gradual reduction and substitution with an osmoticum, mannitol, at 10 and 15 g l^{-1}. The cultures were maintained at 22 ± 2 °C. The culture vessels were closed with aluminum foils to minimize evaporation rate.

The effect of nutrient medium and sucrose at various levels, alone, and in combination with mannitol, on growth of vanilla cultures was studied (Table 3). MS medium when used in half strength supported 'normal' growth and development of plantlets. The rate of growth was higher in full strength MS medium. In full strength MS medium with 30 g l^{-1} sucrose, the cultures grew faster and filled the culture vessel within 180 days and this overgrowth resulted in drying up of cultures and exhaustion of nutrients. In media containing full strength MS salts, the cultures could be stored only to a maximum of 200 days with 70% survival without the addition of mannitol.

Table 3
Effect of various media components on induction of minimal growth in vanilla cultures at 22 ± 2 °C

Basal medium	Media components: Sucrose (g l^{-1})	Media components: Mannitol (g l^{-1})	Growth rate[a]: Plant height (cm)	Survival %	Storage period (days)[a]
Full MS	0	0	2.4 ± 0.46	20	30
	10	0	4.7 ± 0.73	50	100
	20	0	9.3 ± 0.80	40	160
	30	0	11.3 ± 1.03	40	180
	20	10	9.0 ± 0.68	70	280
	15	15	8.2 ± 0.87	90	360
	10	10	7.7 ± 0.56	80	360
1/2 MS	0	0	2.5 ± 0.72	20	20
	10	0	3.5 ± 0.54	40	120
	20	0	9.2 ± 2.02	60	240
	30	0	9.2 ± 0.76	60	180
	20	10	8.2 ± 0.84	70	360
	15	15	7.8 ± 0.93	90	360
	10	10	6.7 ± 0.87	80	360

[a] Mean of 20 replicates, initial plant height = 2 cm.

Addition of mannitol (10–15 g l^{-1}) and reduction of sucrose to lower levels (15–10 g l^{-1}) induced slow growth and subsequently 80–90% of the cultures could be maintained for a period of 360 days, when the culture vessels were closed with aluminum foil. Supplementing mannitol and sucrose in equal proportions at 10 or 15 g l^{-1}, could maintain the cultures for 1 year (Table 3). Thus out of 14 different combinations tested only five, i.e. full or half strength MS medium supplemented with 10, 15 and 20 g l^{-1} each of sucrose and 10–15 g l^{-1} mannitol, allowed the cultures to be maintained for over 360 days. The plantlets maintained in this medium showed reduced growth rate and maximum survival (Fig. 4).

These cultures were maintained *in vitro* for more than 7 years with yearly subculture. The conserved material was transferred to MS medium fortified with 30 g l^{-1} sucrose and supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} IBA, for retrieval of normal shoots and their multiplication. Each accession is represented by twenty 18 mm × 25 mm test tubes, and the entire collection occupies a 5 m × 2 m area. Temperature and illumination have been set at 22 ± 2 °C and 2000 lx, respectively. Under these conditions, subculture interval ranges between 12 and 18 months. After more than 7 years of slow growth storage, involving over five subculture cycles, the genotypic stability of few species was assessed using molecular markers. No changes were observed in DNA fingerprinting vis-à-vis non-conserved controls in the authors' laboratory (unpublished). Though *in vitro* conservation cannot be viewed as a method to replace *in situ* conservation, the present study has demonstrated the advantages of *in vitro* conservation as a component which can be incorporated into an overall vanilla conservation strategy (Fig. 11), for safe and economical means for long-term storage of the germplasm.

Jarret and Fernandez (1992) have reported storage of *V. planifolia* shoot tips as tissue cultures for 10 months and Philip (1989) has discussed the possibility of use of root cultures for conservation of vanilla germplasm for assured genetic stability. *In vitro* conservation of *V. walkeriae* using slow growth method was reported by Agrawal et al. (1992) and effects of polyamines on *in vitro* conservation of *V. planifolia* has been studied by Thyagi et al. (2001). In the present study, the subculture intervals were substantially increased with simple addition of mannitol in the culture media and without any growth regulators which may affect genetic fidelity of conserved materials. The conventional and *in vitro* genebanks are complementary, as the active and base collections. All accessions can thus be represented at all times, in the *in vitro* slow growth collections. Protocols for conservation of vanilla has been developed for slow growth as well as cryopreservation as a means for maintaining genepools *viz.*, accessions as encapsulated shoot-tips and genepools as pollen and DNA (Minoo, 2002). Cryopreservation, once fully implemented will provide an expeditious and cheaper means to duplicate the base collection for safety reasons, as well as for the distribution of germplasm sets to other countries/continents. Vanilla being practically reproduced by vegetative means, the role of micropropagation is also key to the management of the

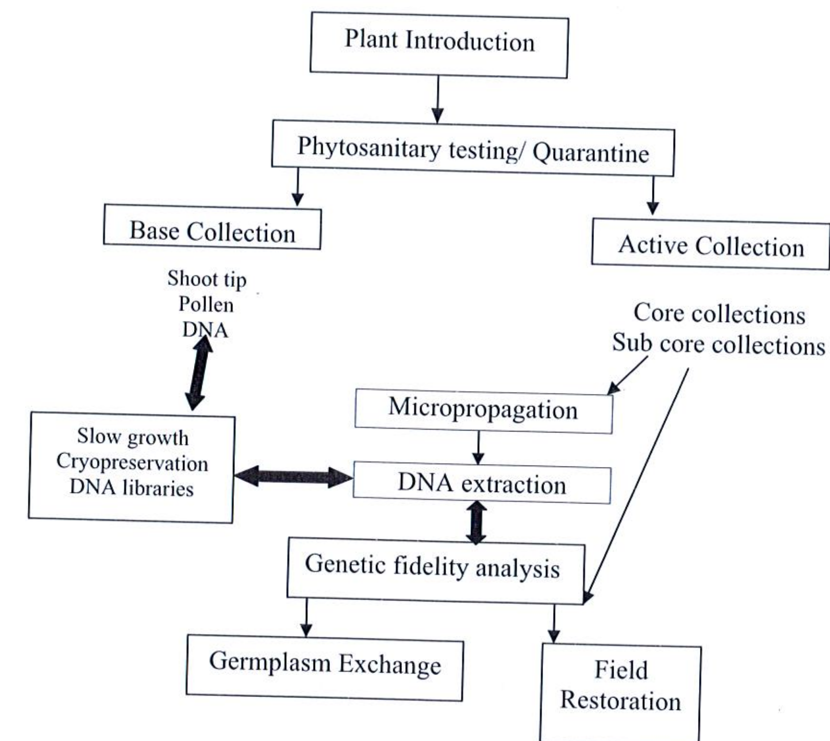


Fig. 11. Integrated *in vitro* conservation strategy for vanilla.

in vitro conservation strategy (Fig. 11). Despite the species-specific nature of conservation protocols, the procedure developed for *V. planifolia* has been effectively applied to four endangered and species with desirable traits, from different geographical zones. This provides an economical means for the long-term *ex situ* conservation of endangered species and possible effective species translocation as a later date. This protocol has now been implemented into the conservation programmes at the *In vitro* gene bank of Indian Institute of Spices Research with more than 300 genotypes, including *Vanilla* species (Figs. 7–10), exotic, indigenous collections, seedling progenies and somaclones.

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