BIOTECHNOLOGY - ITS ROLE IN CONSERVATION OF GENETIC RESOURCES OF SPICES

K Nirmal Babu, S P Geetha, C Manjula, A Sajina, D. Minoo, K Samsudeen, P N Ravindran and K V Peter

Abstract Plant Genetic Resources are non-renewable and are most essential of the world's natural resources. Secure genetic stocks are fundamental requirements for plant breeding. The advances in biotechnology, brought about by combination of *in vitro* technology, genetics and molecular biology are changing the way that will look at gene pools and their utilisation for crop improvement. Most of the world's germplasm is currently maintained in field gene banks. *In vitro* genebank is a novel system of conservation of genetic resources especially those which are propagated vegetatively and/or have recalcitrant seeds. *In vitro* base gene bank where cultures are maintained cryopreserved it., stored in liquid nitrogen at or near a temperature of - 196^{0}C.

Progress of work in conserving spices germplasm in *in vitro* repositories at IISR is discussed. Protocols for successful conservation of black pepper, ginger, turmeric, cardamom, vanilla, seed and herbal spices etc. were standardised at IIRS for short and medium term conservation. Work is in progress for isolation of meristem and standardisation of cryopreservation protocols. At present the *in vitro* gene bank at IISR has about 300 accessions of various spices. All the accessions of spices germplasm currently available at IISR will be conserved in the *in vitro* gene bank.

I. INTRODUCTION

Secure genetic stocks are fundamental requirement for plant breeding. Any resources of value clearly needs to be protected and plant genetic resources are no exception. The awareness of the value of genetic resources has been sharpened in recent years by threats to biodiversity in an often hostile world. This realisation has come at the same time as the realisation that the new biotechnologies can greatly increase our ability to use genetic resources. The advances in biotechnology, that brought about by a combination of *in vitro* technology, genetics and molecular biology, are changing the way that we look at genepools and at the potential for crop improvement. The most dramatic benefit may well be reaped in the crops that are sterile and/or are traditionally propagated vegetatively. Genetic engineering can bring about

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improvement in their hitherto virtually inaccessible genomes and the vegetative habit, amplified by *in vitro* cloning, stabilises the improved genotypes (Withers 1991). Plant genetic resources are non-renewable and are among the most essential of the world's natural resources. It is essential that these be well conserved, be it at species, genepool or ecosystem level for the use of present and future generations (Ramanatha Rao and Riley 1994). Much of the world's germplasm is currently maintained as breeders' collections in plantations, orchards or even in so called evolution gardens. IPGRI (IBPGR) calls such collections 'field gene banks'. These field gene banks can conserve only the species which are suitable to the given agro-climatic zone. Hence any strategy for collection and conservation of samples of crops, more so in the case of vegetatively propagated plants or those that produce recalcitrant seeds which cannot be stored in seed gene bank at low temperatures, had to include a consideration of *in vitro* storage techniques. *In vitro* storage of such material should relate to cycling of material through multiplication, distribution of germplasm and also its characterisation and evaluation. Hence the development of the full potential of *in vitro* culture storage and associated biochemical techniques would revolutionise the handling of germplasm of crop species which are otherwise difficult to conserve (Withers and Williams 1986).

2. GENETIC RESOURCES OF SPICES - NEED FOR *IN VITRO* CONSERVATION

Indian Institute of Spices Research (IISR) is the national gene bank of spices and has the mandate to collect, evaluate, catalogue and conserve all genetic resources of spices in the country. It also acts as a nodal agency for germplasm exchange. At present the Institute maintains a very large collection of spices germplasm (Table 1).

The germplasm collection of various spices are at present conserved in field gene banks. Most of the spices are plagued by destructive diseases such as foot rot of black pepper caused by *Phytophthora capsici*, 'Katte' and 'Azhukal' diseases of cardamom caused by virus and *Phytophthora* species respectively, rhizome rot of ginger caused by *Pseudomonas* and *Pythium* species, the rhizome rot of turmeric caused by *Pythium graminicolum* etc. All the cultivars and their close relatives are highly susceptible to these diseases and thereby there is always a possibility of loss of purity of germplasm due to planting and harvesting year after year in the same field due to mixing up of left over rhizome pieces of the previous crop. The practical alternative to these problems is to conserve them using *in vitro* techniques. Majority of the spice crops are vegetatively propagated and hence *in vitro* conservation and cryopreservation of the apical meristems can be an ideal method, especially because in these crops seeds could not be used for conservation as they are heterozygous and recalcitrant. Due to the rampant soil born diseases, viral diseases and other crop specific problems, such a mode of conservation will be safer and also may be cost effective in a long run.

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3. GERMPLASM CONSERVATION APPROACHES IN *IN VITRO* GENE BANKS

There are two stages in *in vitro* gene banks: one, the *in vitro* active gene bank where cultures are maintained in growing state usually with growth slowed down by limiting physical, environmental or culture medium factors and two; *in vitro* base gene bank where cultures are cryopreserved i.e., stores in liquid nitrogen at or near a temperature of - 196^0 C (Withers 1980; 1985; B; 1987; 1989 and 1991).

In vitro conservation by slow growth method is reported earlier in various crops like coffee (Kartha et al 1981; Bertrand et al, 1992), cassava (Roca et al 1984a;b;), banana (Banerjee and De Langhe 1985; Banerjee 1989), colocasia (Staritsky et al 1985), potato (Espinoza et al 1986) etc. In spices, the possibility of *in vitro* conservation was reported in cardamom (Nirmal Babu et al 1994 a), ginger, turmeric, black pepper and its related species (Balachandran et al 1990; Dekkers et al 1991; Geetha et al 1995). This paper reviews the over all progress made in the *in vitro* conservation of spices germplasm at IISR.

3.1. Micropropagation

Plant regeneration and successful cloning of genetically stable plantlets in tissue cultures is an important pre-requisite in any *in vitro* conservation effort. Micropropagation protocols for stable cloning of elite genotypes of spice crops viz., black pepper and its related species, cardamom, ginger, turmeric and related genera, large cardamom, vanilla, cinnamon, camphor, cassia, seed and herbal spices like lavender, celery, thyme, mint, anise, savory, spearmint oregano etc. were standardised at IISR (Nirmal Babu et al 1992 a,b; 1993; 1994b; Rema et al 1995). The other reports on micropropagation of important spice crops are by Hosoki and Sagawa 1977; Broome and Zimmerman 1978; Nadgauda et al 1978, 1983; Du Manior 1985; Philip and Nainar 1986; Rech and Peres 1986; Repcakova et al 1986; Rai and Jagadishchandra 1987; Bhagyalakshmi and Singh 1988; Philip 1989; Balachandran et al 1990; Mathew and Hariharan 1990; Choi and Kim 1991; Dekkers et al 1991; Song et al 1991; Nazeem et al 1992; Philip et al 1992 etc. These techniques formed the base for establishing tissue cultures of various spice crops and conserving them in the laboratory. The basal medium used are MS (Murashige and Skoog 1962) for crops like cardamom, ginger, turmeric, kasturi turmeric, mango ginger, large cardamom, kaempferias, *vanilla* spp and herbal spices and WPM - woody plant medium (Mc Cown and Amos 1979) for black pepper and its related species, cinnamon, camphor and cassia. Simultaneously these tissue cultured plants are being evaluated for their morphological and genetic characterisation to estimate their genetic stability in culture (Lukose et al 1993).

Though micropropagation protocols were standardised using growth regulators, all the *in vitro* storage experiments were carried out using growth regulator free media to reduce the rate of multiplication which in turn will reduce the extent of variation.

3.2. Short and Medium term Conservation by Slow growth

3.2.a Principal Factors : The principle of slow growth storage is that the safety of *in vitro* cultures could be utilised without disadvantage of frequent subculturing, thus reducing cost of labour and consumables. The risk of contamination in each transfer interval is also reduced. The subculture intervals in some cases can be extended without significant modifications of the culture procedures. Another important factor is that these cultures should readily be restored to normal culture conditions for growth, multiplication and distribution. The stress imposed on cultures to reduce its growth may affect its vitality and selective loss of viability may lead to genetic drift. Some of the notable successes in routine maintenance of germplasm by slow growth are in yam, cassava (Espinoza et al 1984; Ng and Hahn 1985; Roca et al 1984a;b; Withers and Williams 1986) and banana (Banerjee and De Lange 1985; Banerjee 1989).

Various factors like temperature, culture medium use of osmoticum, physiological state of the explant, culture vessel, minimisation of evaporation loss encapsulation, dessication etc. are shown to influence slow growth (Withers 1980; Kartha et al 1981; Staritsky et al 1985; Espinoza et al 1986; Engelman 1991 and Agrawal et al 1992).

3.2.b. Effect of different factors : Three temperature regimes (5,10 and 22$\pm$°C), four types of culture vessel closures to minimise evaporation loss (cotton plug, screw cap, polypropylene cap and aluminium foil), use of full strength and half strength basal medium, use of osmoticum (Mannitol) by partial replacement of sucrose at three different levels ($5gl^{-1}$, $10gl^{-1}$ and $15gl^{-1}$) and reduction in sucrose ($10gl^{-1}$, $25gl^{-1}$, $20gl^{-1}$, $15gl^{-1}$, $10gl^{-1}$ and $5gl^{-1}$) were tested for their efficiency in inducing minimal growth and increasing subculture intervals of various spices. Only plantlets/shoot tips/meristems are used as explants as these tissues were reported to be most stable in in vitro cultures (Withers 1980; Hu and Wang 1983).

Growth reduction can generally be achieved by lowering the culture temperature, but the scope for temperature reduction depends upon the species to be conserved. Tissue cultures of cardamom could not be stored under low temperatures of 5°C and 10°C (Nirmal Babu et al 1994a). Deleterious effects of low temperatures were reported in *Rauvolfia serpentina* (Sharma and Chandel 1992) *Punica granatum, morus nigra* and *Actinidia chinensis* (Wilkins et al 1988). Normal culture room temperature of 22 $\pm$ 2°C is suitable for the storage of spices germplasm (Nirmal Babu et al 1994a; Geetha et al 1995). Storage of multiple shoot cultures of ginger at ambient temperatures of 24-29° C was reported by Dekkers et al (1991).

Though cotton plugs, used to cover the mouth of culture vessel, allowed comparatively better gaseous exchange, there is a faster rate of loss of moisture, depletion of culture media and drying up of cultures between 120 and 180 days depending upon the species. However use of screw caps, polypropylene caps or aluminium foil as vessel closures minimised the moisture loss, helped in retention of the medium and thereby resulted in increased longevity of cultures and the subculture

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period could be prolonged upto 360 days (Nirmal Babu et al 1994 a; Geetha et al 1995). Sealing the culture tube with parafilm helped in reducing the chance of contamination and moisture loss. The use of polypropylene caps as vessel closures to minimise evaporation and to enhance the longevity of culture was reported earlier in ginger and turmeric (Balachandra et al 1990) and in *Rauvolfia* (Sharma and Chandel 1992).

In the present study, reducing the basal medium concentration to half and partial replacement of sucrose by the addition of mannitol helped in inducing slow growth in spices like black pepper, cardamom, ginger, turmeric and some of their related species. In some others full strength with sucrose alone helped in inducing slow growth.

All the cultures were stored under 12h photoperiod of 2500 lux. The culture conditions and media for each species are given in Table 1.

3.2c Black Pepper and Related Species : Black pepper (*Piper nigrum* L.) shoot tip cultures could be stored up to 360 days without subculture in half strength WPM with 15 gl each of sucrose and mannitol in screw capped culture tubes with 85% survival (Fig. 1 C). Half strength of the basal medium supplemented with mannitol was insufficient for the survival of the related species like *P. barberi*, *P. colubrinum* and *P. longum*. *P. barberi* cultures could be stored up to 360 days in full strength WPM with $25 gl^{-1}$ sucrose and 5gl mannitol with 80% survival. The survival rate after 360 days found reduced to 70% when half strength of the basal medium with 30 gl sucrose was used (Table - 1). In higher concentration of mannitol, the growth rate was highly reduced with poor rooting and the cultures showed yellowing, shoot tip necrosis, decaying etc. and the cultures could not be maintained more than 180 days. Culture abnormalities in higher concentration of mannitol was reported earlier in cassava (Roca et al, 1982) and in colocasia (Bessemінder et al 1993). Shoot tip cultures of *P. Colubrinum* (Fig. 1 D) and *P. longum* could be stored up to 360 days in full strength WPM with 20 gl sucrose and $10 gl^{-1}$ mannitol with 70% and 75% survival respectively (Table 1). The technique standardised is presently used to conserve other *Piper* species also.

3.2d Cardamom: Tissue cultures of cardamom could be stored up to 360 days with 85% survival (Fig 1 A) and up to a maximum period of 420 days with 70% survival in half strength MS medium with 10gl each of sucrose and mannitol in screw capped culture tubes (Table 2). Under these conditions the cultures attained miniature but healthy appearance (Nirmal Babu et al 1994a; Geetha et al 1995). Similar growth patterns were noticed during second and third years of storage.

3.2e Ginger, turmeric and other related genera of family Zingiberacae: Slow growth method for short term conservation of ginger (Fig. 1 B), turmeric (Fig. 1 C) Kasturi turmeric, mango ginger, *Kaempferia* spp. (*K. galanga* and *k. rotunda*) were also standardised. All these taxa could be stored up to 1 year without subculture in half strength MS medium with 10gl each of sucrose and mannitol in sealed culture tubes.

(Geetha et al 1995). *Alpinia purpurata* and *Amomum subulatum* cultures could be stored up 8 and 6 months respectively in the same medium (Table 1). Refining the technique is in progress to increase the subculture interval for over 360 days.

3.2f Vanilla species : Shoot tip cultures of *V. planifolia* (Fig. 1A) and *V. aphylla* (Fig 1B) could be stores in full strength MS medium with $10gl^{-1}$ each of sucrose and mannitol, without subculture easily up to 1 1/2 years (Table 2). In *V. aphylla* growth is much more reduced as it is slow in growth under normal conditions also. In vitro storage of another species of *Vanilla (V. walkeriae)* was reported by Agrawal et al (1992). Both these species *(V. aphylla and V. walkeriae)* are considered endangered.

3.2g Seed and Herbal Spices : Cultures of seed and herbal spices like anise oregano (Fig 1 E) lavender, mint, spearmint, thyme (Fig 1 F), savory, chives, Ocimum (Fig 1 G) etc could be established in minimal media (i.e., half strength MS medium with $20gl^{-1}$ sucrose and full strength MS medium with $15gl^{-1}$ each of sucrose and mannitol). Based on the preliminary observations these cultures can be maintained only for around 90 days after which transfer to fresh medium is required. Studies on increasing the subculture interval by further alterations in culture conditions are in progress.

4. GENETIC STABILITY OF THE CONSERVED GERMPLASM

The principle of germplasm conservation is that the material retrieved should represent the material conserved (Withers and Williams 1986). Storage of organised structures like shoot cultures under medium term conditions appears to be safer, but due to prolonged storage in more or less detrimental conditions may lead to changes in the genetic constitution, thus leading to the loss of a part of the germplasm stored (Engelman 1991).

In the present study all the plant materials conserved were brought back to normal conditions after 1,2 or 3 year cycles. They were multiplied normally (Fig. 1 D) and transferred to soil with 80-90% survival (Table 2) They developed into normal plantlets without any deformities. Preliminary morphological observations suggest that they resemble the parent plant at the nursery stage. These plants are being field evaluated for estimating their genetic stability. Molecular and biochemical markers like isozyme and RAPD profiles are being worked out to detect minor changes in the genotypes which cannot be observed in whole plant.

5. LONG-TERM STORAGE BY CRYOPRESERVATION

Cryopreservation is a very different phenomenon from slow growth with its own advantages, disadvantages and challenges. Since all metabolism is suspended at the temperature of liquid nitrogen (-196°C) or liquid nitrogen vapour (-150°C), time is not a limiting factor in the design and application of cryopreservation regimes (Withers 1991). Under storage conditions the only known cause of damage is during free radical

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A cryopreservation process comprises a series of steps like choice of material, pretreatments for cryoprotection, freezing, storage, thawing, recovery and re-growth (Withers 1980, 1991, Engelman 1991).

As a general rule, material will be chosen as young and as meristematic as possible. Since meristem culture techniques have been widely used for the clonal propagation and production of genotypically identical progenies in a pathogen free state, plant meristems offer an excellent system for long term preservation of germplasm (Kartha 1981).

5.1. Meristem/embryo isolation and culture

As a preliminary step to initiate cryopreservation of spices, meristem isolation in cardamom (Fig. 2 F) ginger and turmeric and embryo isolation from black pepper seeds (Fig. 2 E) were done. The meristems were cultured on MS medium containing 0.5 mgl Kinetin and the embryos were cultured on WPM growth regulator free medium and they were developed into whole plants.

5.2 Encapsulation of somatic embryos/shoot tips

Encapsulation is a technique used in the production of 'synthetic seeds', by coating somatic embryos/shoot tips in alginate beads. Encapsulation of somatic embryos/shoot tips were successfully achieved at IISR, in ginger, cardamom, vanilla, camphor and cinnamon. The possibility of short term storage of encapsulated buds and somatic embryos were demonstrated in different crops like mulberry, sandalwood and *Podophythim* (Bapat et al 1987; Bapat and Rao 1988; Arumugham and Bhojwani 1990). Cryopreservation of encapsulated meristems and somatic embryos were reported in carrot (Dureuddre et al 1991) and Potato (Fabre and Dureiddre 1990) respectively. Encapsulation of embryos or meristems protect the material embedded and make it resistant to treatments during cryopreservation which otherwise would be deleterious.

Suitable techniques for cryopreservation of shoot tips, meristems, somatic embryos, zygotic embryos, synthetic seeds etc. are being standardised.

7. CONCLUSION

In vitro conservation is a package of techniques connecting with collection and storage of disease free germplasm and for their distribution and utilisation. *In vitro* collection helps in overcoming problems relating to availability, condition and portability of germplasm to be collected from the field. Exchange of germplasm through *in vitro* means is one of the safest ways for the movement of germplasm without the fear of introducing new pests and pathogens. *In vitro* conservation is highly valuable for crops like spices which are propagated vegetatively and in which conventional conservation in field gene bank is hazardous. At present a total number of 295 accessions

of various spices including three endangered species (*P. barberi, P. hapnium* and *V. aphylla*) are maintained in the *in vitro* repository (Fig. 1 H) under slow growth (Table 3). However in vitro conservation may never replace conventional technologies entirely but will complement them within a strategy that balances traditional approaches with the best of the modern technology. Foundation for a comprehensive *in vitro* conservation strategy for spices have already been laid. IISR proposed to establish the world repository of spices germplasm, for posterity.

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Table 1. *In*

Species	Basa Medi
Elettaria cardamum	MS
	1/2
	1/2
	1/2
Zingiber officinale	1/2
Curcuma longa	1/2
C. aromatica	1/2
C. amada	1/2
Kaempferia galanga	1/2
K. rotunda	1/
Alpinia purpurata	1/
Amomum subulatum	1/
Piper nigrum	1/
	1
	1
Piper barberi	V
	1
Piper colubrium	V
Piper longum	V
Vanilla spp. V. planifolia V. aphylla	
Seed and herbal spices	

S : Sucrose
M : Manitol
MS : Murashi
WPM : Woody
* : In screw

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Table I. *In vitro* **Storage of Spices Germplasm - Effect of Media Constituents on Survival of Cultures***

Species	Basal Medium	Carbon Source (gm/1)	Growth	Survival period (days)	Survival (%)	Establishment in nursery
Elettaria cardamum	MS	30S	Fast	200	55	
	1/2 MS	20S	Normal	250	45	
	1/2 MS	10S + 10M	Slow	360	85	
				420	70	85
	1/2 MS	15S + 15M	Slow	360	90	
Zingiber officinale	1/2 MS	10S + 10M	Slow	360	85	85
Curcuma longa	1/2 MS	10S + 10M	Slow	360	90	90
C. aromatica	1/2 MS	10S + 10M	Slow	360	85	90
C. amada	1/2 MS	10S + 10M	Slow	360	80	85
				450	70	
Kaempferia galanga	1/2 MS	10S + 10M	Slow	360	90	100
				450	85	
K. rotunda	1/2 MS	10S + 10M	Slow	360	80	90
Alpinia purpurata	1/2 MS	10S + 10M	Slow	240	85	90
Amomum subulatum	1/2 MS	10S + 10M	Slow	180	90	90
Piper nigrum	1/2 WPM	30S	Fast	250	60	
	1/2 WPM	20S	Normal	360	70	
	1/2 WPM	15S + 15M	Slow	360	85	80
Piper barberi	WPM	25S + 5M	Slow	360	80	
	1/2 WPM	30S	Normal	360	70	85
Piper colubrium	WPM	20S + 10S	Slow	360	75	80
Piper longum	WPM	20S + 10M	Slow	360	70	80
Vanilla spp. V. planifolia V. aphylla	MS	10S + 10M	Slow	450	100	90
Seed and herbal spices	MS	20S 15S + 15M	Slow	90	95	

S : Sucrose
M : Manitol
MS : Murashige & Skoog Medium
WPM : Woody Plant Medium
* : In screw capped culture tubes

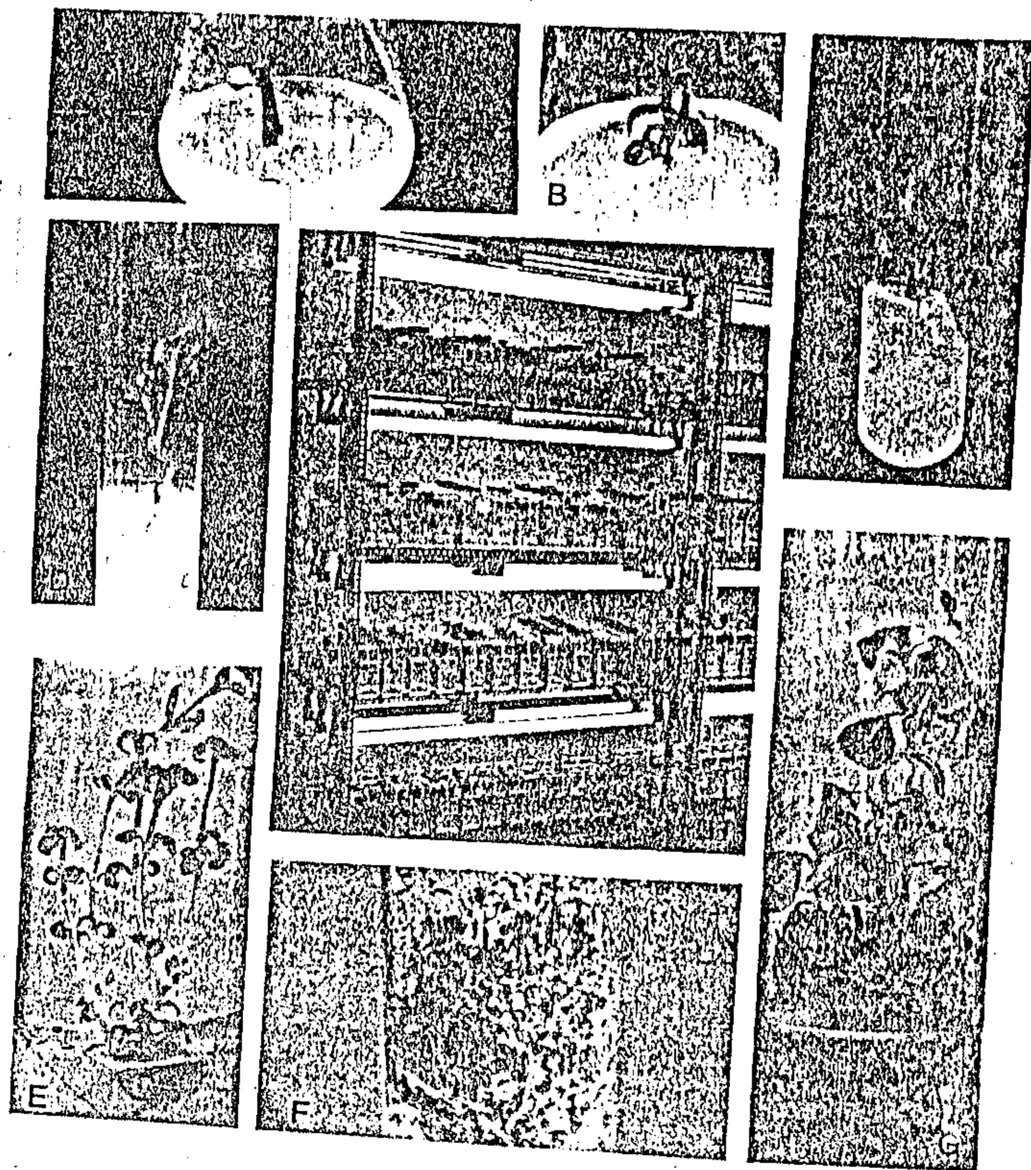
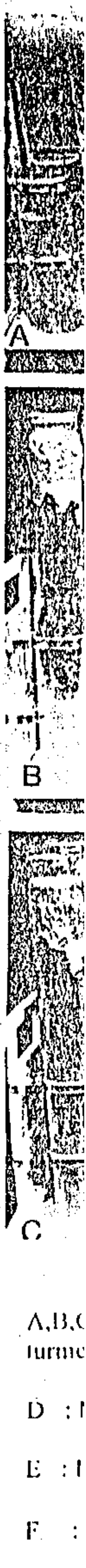


Fig. 1. *In vitro* conservation of spices germplasm - species maintained in the *in vitro* gene bank

A : *Vanilla aphylla*
B : *Vanilla fragrans* (Vanilla)
C : *Piper nigrum* (Black pepper)
D : *P. Colubrinum*
E : *Oreganum vulgare* (oregano)
F : *Thymus vulgaris* (Thyme)
G : *Ocimum sanctum* Sacred basil)
H : A view of the *in vitro* repository



A,B,C
turme

D : I

E : I

F :

nplasm - species
bank

Fig. 2 : *In vitro* conservation of spices germplasm

A,B,C : 2 year old cultures (with yearly subculture) of cardamom, ginger and turmeric respectively.

D : Multiplication and conservation of *Piper barberi* and endangered species

E : Isolation of Black pepper embryos for cryopreservation

F : Isolation of cardamom shoot meristem for cryopreservation