Tissue Cultural Innovations for Production of Quality Potato Seed in Asia-pacific Region

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MICROPROPAGATION FOR PRODUCTION OF QUALITY POTATO SEED IN ASIA-PACIFIC

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Cover page: From top clockwise: Inoculation of potato explants during sub-culturing, growth of explants in culture room, *in-vitro* plants ready for planting in greenhouse, potatoes raised from *in vitro* plantlets crop in greenhouse, harvested minitubers from greenhouse crop, and healthy seed crop raised from minitubers (Courtesy: M/S KF Bioplants Pvt. Ltd., Pune, India)

Center: Tubers of early maturing Indian potato variety Kufri Jawahar

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FOREWORD

The potato (*Solanum tuberosum* L.) is a major world food crop, next in production only to maize, rice and wheat. Short duration and wide flexibility in planting and harvesting time are potato's other valuable traits that help adjusting this crop in various intensive-cropping systems without putting much pressure on scarce land and water resources. In the Asia-Pacific region, potato is grown on about 7.3 million hectares, producing about 121.7 million tonnes of potatoes with an average productivity of 16.49 t/ha. The contribution of the Asia-Pacific region to the world area and production of potato is 39.3% and 37.7%, respectively. However, potato cultivation in the region is unevenly distributed with China and India alone accounting for about 79% area as well as production.

Shortage of good quality seed has been recognized as the single most important factor limiting potato production in the developing countries. Fortunately, potato has been an early beneficiary of advances in conventional and modern biotechnologies resulting in their use for solving practical problems relating to potato cultivation and improvement. Meristem culture was possibly the first biotechnological approach used to eliminate viruses from systemically infected potato clones. Over the years, this technique has been successfully combined with micropropagation to produce disease-free potato seed.

Production of quality planting material is essential not only for improving domestic potato productivity but also to ensure minimum commercial quality as required under international agreements. According to the International Plant Protection Convention under the World Trade Organization's Sanitary and Phytosanitary Agreement, presence of pathogens in seed potatoes is a major quality concern. Many countries in the region are, therefore, developing/modifying potato certification standards to harmonize them with international seed standards. Such initiatives are likely to facilitate fair international trade by avoiding technical barriers, and encourage production of high quality seed to ensure farmers' profitability.

The Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB) was established in 2003 under the umbrella of Asia-Pacific Association of Agricultural Research Institutions (APAARI) that has been promoting appropriate use of emerging agri-technologies and tools in the region. One of the activities of APCoAB is to bring out status reports of biotechnological applications that have proved useful to the farmers and other stakeholders in the region. The present publication, "Micropropagation for Production of Quality Potato Seed in Asia-Pacific", is one such report in this series. It provides detailed information on various micropropagation techniques and status of their application for large-scale production of disease free planting material. Selected success stories involving application of these techniques in the Asia-Pacific

region have also been narrated. I am sure that this publication will be of great help to the scientists, research managers, policy planners and the seed industry in the region in evolving suitable potato seed production systems that help in improving productivity as well as sustainability of this important crop.

(Raj Paroda)
Executive Secretary,
APAARI

ACRONYMS AND ABBREVIATIONS

APAARI	Asia-Pacific Association of Agricultural Research Institutions
APCoAB	Asia-Pacific Consortium on Agricultural Biotechnology
APSA	Asia Pacific Seed Association
BA	benzyladenine
CCC	chlorocholine chloride
CIAT	Centro Internacional de Agricultura Tropical, Colombia
CIP	Centro Internacional de la Papa, Peru
CPRI	Central Potato Research Institute
CS	certified seed
CSO	Civil Society Organization
DA-DHT	diacetyl-5-dihydroazauracil
DHT	5-dihydroazauracil
DNA	deoxyribonucleic acid
ELISA	enzyme linked immuno sorbet assay
FAO	Food and Agriculture Organization
FFS	farmer field school
FS-I, FS-II	foundation seed-I, foundation seed-II
FYM	farm yard manure
G-1, G-2, G-3	generation-1, generation-2, generation-3
GA_3	gibberellic acid
ha	hectare
HEPA	high efficiency particulate air
ICAR	Indian Council of Agricultural Research
ICARDA	International Center for Agricultural Research in the Dry Areas.
IFPRI	International Food Policy Research Institute
ISF	International Seed Federation
IITA	International Institute of Tropical Agriculture
IPGRI	International Plant Genetic Resources Institute
IPPC	International Plant Protection Convention
ISPM	International Standards for Phytosanitary Measures
ISTA	International Seed Testing Association
LB medium	liquid broth medium
mha	million hectares
MS	Murashige and Skoog
mt	million tonnes
NAA	naphthalene acetic acid

NSC	National Seeds Corporation
OECD	Organization for Economic Cooperation and Development
PAMV	*Potato aucuba mosaic virus*
PLRV	*Potato leaf roll virus*
PMTV	*Potato mop top virus*
PRA	pest risk analysis
PSTV	*Potato spindle tuber viroid*
PVA	*Potato virus A*
PVM	*Potato virus M*
PVS	*Potato virus S*
PVX	*Potato virus X*
PVY	*Potato virus Y*
RH	relative humidity
rpm	revolutions per minute
RNA	ribonucleic acid
SAU	State Agriculture University
SFCI	State Farms Corporation of India
SNCs	single node cuttings
SPS	Sanitary and Phytosanitary Measures
t	tonnes
TBT	Technical Barriers to Trade
TNV	*Tobacco necrosis virus*
TPS	true potato seed
TRV	*Tobacco rattle virus*
UPOV	The International Union for Protection of New Varieties of Plants
UPWARD	User's Perspective with Agricultural Research and Development
VBSE	Village Based Seed Enterprise
WTO	World Trade Organization

1. INTRODUCTION

The potato (*Solanum tuberosum* L.) is a major world food crop. In world food production, potato (322 mt) is exceeded only by maize (637 mt), rice (585 mt) and wheat (549 mt). Potatoes are consumed by over one billion people world over, half of whom live in the developing countries. Potato gives an exceptionally high yield and also produces more edible energy and protein per unit area and time than many other crops. While the developed countries make the most diversified use of potatoes as food, feed and raw material for processed products, starch and alcohol; the developing countries are increasingly adopting potato cultivation primarily as a food crop. The share of developing countries in world potato area rose from 15.1% in 1961 to 51.0% in 2005. In 1961, potatoes produced in the developing countries accounted for 10.5% of the global output. Today, they produce about 47.2% of potatoes in the world.

Averaged over the three-year period of 2003-2005, potato was grown on 7.3 mha in 28 countries of the Asia-Pacific region, the latter producing about 121.7 mt with an average productivity of 16.49 t/ha (Table 1). The share of the Asia-Pacific region in the world area and production of potato was 39.27% and 37.71%, respectively. Although potato productivity in the region varied widely from 2.50 t/ha in Timor-Leste to 44.25 t/ha in New Zealand, the average potato productivity (16.49 t/ha) was just a little lesser than world average (17.18 t/ha). China and India alone accounted for about 79% area and production of potato in the region.

Potato is a semi-perishable crop susceptible to many diseases and insect pests. Shortage of good quality seed has been recognized as the single most important factor limiting potato productivity in the developing countries. The availability of tissue culture technology for rapid multiplication of disease-free planting material has facilitated potato seed production to a great extent (Dodds 1988). Meristem culture is being successfully employed to obtain virus-free potato clones (Mori *et al.* 1969). Rapid multiplication of these disease-free clones using micropropagation coupled with conventional multiplication methods has now become an integral part of seed production in many countries (Donnelly *et al.* 2003).

This publication analyses the current status and future prospects of potato micropropagation for producing quality planting material in the Asia-Pacific region. It provides detailed information on protocols for production of virus-free plants, their rapid multiplication and, microtuber and minituber production. Integration of micropropagation with conventional seed production, seed certification and quality standards for tissue culturally grown potato seed are also discussed. Success stories of quality potato seed production in some Asia-Pacific countries using micropropagation have been detailed.

Table 1. Potato production in the Asia-Pacific region (Triennial averages for 2003-2005).

Country	Area ('000 ha)	Yield (t/ha)	Production (mt)
Australia	36.01	35.71	1.29
Bangladesh	262.23	14.23	3.73
Bhutan	4.57	8.80	0.04
China	4408.93	16.04	70.65
DPR Korea	188.72	10.85	2.05
Fiji Islands	0.01	8.00	0.0001
India	1390.00	17.99	25.00
Indonesia	64.74	15.47	1.00
Iran (Islamic Rep. of)	186.67	21.66	4.04
Japan	87.13	33.38	2.91
Kazakhstan	166.40	13.76	2.29
Kyrgyzstan	85.80	15.81	1.36
Lao PDR	5.50	6.55	0.04
Mongolia	9.17	8.89	0.08
Myanmar	32.23	12.44	0.40
Nepal	143.33	11.42	1.64
New Zealand	11.30	44.25	0.50
Pakistan	112.51	17.52	1.97
Papua New Guinea	0.19	4.46	0.0008
Philippines	5.45	12.70	0.07
Rep. of Korea	23.74	25.34	0.60
Sri Lanka	5.72	13.47	0.08
Tajikistan	27.76	18.06	0.50
Thailand	6.89	14.11	0.10
Timor-Leste	0.40	2.50	0.0010
Turkmenistan	29.00	5.31	0.15
Uzbekistan	50.45	17.05	0.86
Viet Nam	34.29	10.67	0.37
Asia-Pacific Total/Average	7379.14	16.49	121.72
All World	**18792.68**	**17.18**	**322.75**

(Source: http://faostat.fao.org/, accessed February 2006)

2. POTATO SEED PRODUCTION

The conventional planting material for potato (potato seed) is the vegetative tuber. Potato tuber is a modified stem adapted for food storage and reproduction. Rudiments of the leaves (eyebrows), axillary buds (eyes) and lenticels can be seen on the skin of potato tuber (Figure 1a). Each eye has more than one bud. At harvest, potato tubers are dormant and do not show any bud growth. Depending upon the variety, the dormancy period may vary from 6 to 10 weeks, which can be prolonged to several months at low temperatures of cold stores (4 °C). At the end of the dormant period tubers begin to sprout. The number of sprouts growing on a tuber is determined by the size of the tuber, temperature and duration of storage. Generally, more sprouts develop on large tubers stored for longer periods. Well-sprouted whole tubers with 2-3 cm long sprouts (Figure 1b) are usually used for planting @3-4 t/h. Sometimes the sprouted tuber is cut into two or more pieces before planting, each piece having at least one eye. There is, however, a risk of spreading diseases from one tuber to another during the cutting process.

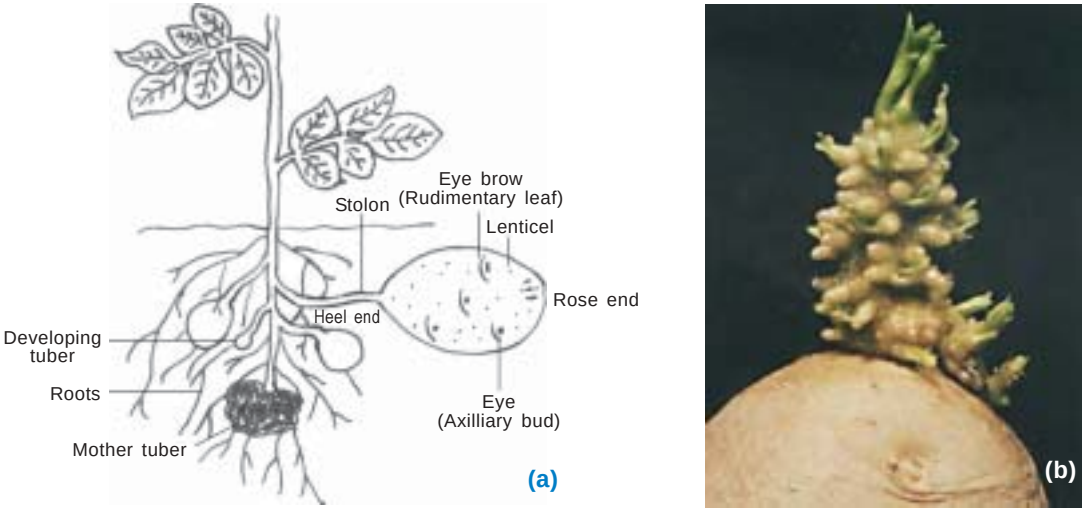

Figure 1. Potato plant with tubers (a) and sprout growing on a potato tuber (b).

Conventional potato seed production involves production of basic seed (also called breeders' seed) on special seed farms, which is further multiplied by seed agencies and registered seed growers to produce certified seed. Most seed production programs operate a "flush through" system starting each year with fresh true to type and healthy tubers which have been indexed for freedom from viruses. These tubers are further multiplied 4-6 times to produce basic seed under strict management practices. Production of certified seed from the basic seed requires inspection by certification agencies to ensure the required quality of the seed being distributed for commercial cultivation. A typical example of conventional seed production system in practice in India is given in Annexure-I.

All conventional potato seed production systems are characterized by low multiplication rate and progressive accumulation of degenerative viral diseases during clonal propagations. About 30 viruses and virus like agents infect potato. These being systemic pathogens, are perpetuated through seed tubers and pose a major threat to potato seed production. Potato viruses X, S, Y and PLRV are ubiquitous in the Asia-Pacific region. Other viruses reported from major potato growing countries of the region are PVA, PVM, PMTV, TRV, PAMV, TNV and PSTV from China and PVA, PVM, Geminivirus and Tospovirus from India. Details of some important potato viruses, their mode of transmission, symptoms and potential yield losses are given in Table 2. Further, in contrast to seed propagated crops, the multiplication rate in potato is low varying from 1:4 to 1:15 (one tuber yields 4 to 15 tubers) depending upon variety, agro-climatic conditions and crop management practices. Therefore, to build up sizeable seed stocks, the initial disease-free tuber material needs to be field multiplied for a number of years. With each such multiplication cycle, viral diseases accumulate progressively causing degeneration or "running out" of seed stocks. Consequently, non-availability of quality planting material in adequate quantities and at affordable prices is the major bottleneck in potato cultivation in many countries. The problem is further aggravated by high seed rate (3 to 4 t/ha) due to which the cost of seed potatoes alone accounts for about 40% to 60% of the total production costs in many parts of the world (Sawyer 1979).

Well-developed potato seed production programs and seed certification standards are in place in only a few countries of the Asia-Pacific region, viz. Australia, India, Japan and New Zealand. In several other countries there is limited quality control on production and distribution of potato seed. In these countries, potato seed is either imported or multiplied locally and supplied through informal distribution systems. However, in view of the recent global developments including those initiated through WTO-SPS-TBT to protect human, plant and animal life from the risks arising from pests or disease causing organisms, major potato producing countries in the region are making serious efforts to develop and implement quality standards for potato seed production. These efforts need to be supported by integration of modern disease elimination and micropropagation techniques with the conventional seed production systems.

Table 2. Some important potato viruses, their mode of transmission and symptoms.

Potato virus	Mode of transmission	Symptoms
X (PVX)	Mainly mechanical through contact between the plants and due to the passage of machinery.	Generally symptomless. Chlorosis, mosaics and decreased leaf size. PVX can interact with PVY and PVA to cause severe mosaic symptoms and higher yield losses. **Potential yield losses:** 10-20%.
Y (PVY)	By aphids (mainly *Myzus persicae*). It is a non-persistent virus (vectors remain viruliferous only for a short period).	PVY^{0} is common strain that causes mosaic symptoms; PVY^{C} causes stipple streak; and PVYN causes necrosis in leaves. Mixed infections of common and necrotic strains produce hybrid strains (i.e. $PVY^{N:0}$ and PVY^{NTN}). PVY^{NTN} strain causes severe symptoms including tuber necrosis. The secondary infections are more severe than primary infections. PVY strains can interact with PVX and PVA to result in heavier losses. **Potential yield losses:** 10-80%.
A (PVA)	By aphids (mainly *Myzus persicae*). It is a non-persistent virus.	Slight and transient mosaics that are particularly visible in cloudy weather. These mosaics appear as a discoloration of leaf portion not containing veins. In secondary infection, the symptoms are more pronounced, resulting in waffling/ embossing of the leaves combined with a glassy appearance. In case of mixed infection with PVY or/and PVX the symptoms are severe. **Potential yield losses:** 15%.
S (PVS)	Contact as well as non-persistent trans-mission by aphids.	Lightening of foliage; deepening of veins on the upper side of the leaves; reduction in leaf size; and bronzing and necrotic spots on leaves. **Potential yield losses:** 10-40%.
Potato Leaf Roll Virus (PLRV)	By aphids in a persistent manner (vectors remain viruliferous for a long time).	**Primary infection (current year):** The leaves at the top of the plant are slightly curled and show yellowing. Purple pigmentation can sometimes be seen on the edge. **Secondary infection (previous year):** The leaves at the base are tightly curled and hardened; the plant growth habit is straighter and the internodes are shorter. The plant is yellowed and sometimes dwarfed. In certain varieties internal necrosis can appear as network on tubers.**Potential yield losses:** 50-90%.
Potato Spindle Tuber Viroid (PSTV)	Mechanically through contact. PSTV is also transmitted through pollens and true potato seed.	Smaller leaves that curl downwards giving a stiff and upright growth habit. Plants can be stunted with more branches. There is a reduction in tuber size combined with tuber deformation giving rise to spindle-shaped tubers with prominent eyebrows. **Potential yield losses:** 10-40%.

TRUE POTATO SEED

True potato seed (TPS) produced through sexual mode is used as an alternative planting material in some parts of Bangladesh, China, India, Republic of Korea, Nepal, the Philippines, Vietnam and some countries of Central Asia. Since TPS is produced through fertilization, it is also called botanical seed and resembles seeds of other solanaceous plants like tomato, brinjal and chilly. A single fruit or berry contains on an average 150-200 seeds. TPS has several advantages; it can be produced easily, is not bulky and thus easy to store and transport, only about 50-125 g TPS is sufficient to plant one hectare area and most of the viruses are eliminated during the process of sexual seed formation.

TPS is used to grow commercial potato crop by two methods; (i) transplanting of seedlings and (ii) use of seedling tubers. In the former method, potato seedlings are raised in nursery beds and transplanted in the field at 4-5 leaf stage. Potato crop raised from seedlings is harvested at maturity. This method requires about 125 g TPS and 75 m^2 nursery bed area for growing seedlings sufficient for transplanting in one-hectare area. In the second method, small size tubers are produced by allowing the seedlings to mature in nursery beds. Seedlings are dehaulmed (cutting of plants at ground level) at maturity and harvested in another 10-15 days when the tuber skin becomes firm and well developed. These seedling tubers are stored and used as planting material for growing the commercial crop in the following crop season. About 50-60 g TPS and 250-300 m^2 nursery bed area are required for producing seedling tubers sufficient for planting one hectare. Various stages of TPS production and utilization are shown in Figure 2.

The disadvantages with TPS technology are that the seeds are not genetically pure and exhibit high heterogeneity; the crop is late in maturity as compared to the crop grown from seed tubers; and the technology is labour intensive.

Figure 2. Stages of TPS production and utilization. (a) Pollination. (b) Berries. (c) Extracted TPS. (d) Seedling transplanting in field. (e) Crop grown from seedling tubers. (f) Harvested potato crop grown from TPS.

3. MICROPROPAGATION FOR PRODUCING QUALITY POTATO SEED

Micropropagation is a tissue culture (*in vitro*) method used for rapid and true to type multiplication of plants on artificial nutrient media under controlled environment. The controlled and aseptic environment of the tissue culture laboratory (Annexure-II) provides optimum conditions for multiplication of plant cultures. Further, the culture medium, the light and the temperature can be adjusted to meet specific requirements for growth and development of specific plants and plant parts. Micropropagation is the most commercially exploited area of plant tissue culture, having been widely used for production of quality planting material in vegetatively propagated species. The most significant advantages offered by micropropagation are: (i) large numbers of disease-free propagules can be obtained from a single plant in a short period, (ii) propagation can be carried out throughout the year, and (iii) the propagating material can be accommodated in a small space. Micropropagation has been successfully used in almost all potato seed producing countries to speed up initial stages of seed production. The process typically consists of: (i) production of virus-free potato plants using meristem culture, (ii) micropropagation of virus-free plants, (iii) production of micro-and/or mini-tubers from micropropagated plants, and (iv) growing healthy seed crop using minitubers as a planting material.

Availability of pathogen free starting material is a pre-requisite for any micropropagation program. Produced using meristem culture, the pathogen free plants can be maintained indefinitely in tissue culture and a constant flow of disease-free plants for micropropagation becomes available from this stock. However, for confirmation and maintenance of clonal identity, such *in vitro* stock needs to be subjected to grow-out tests regularly, at least once in a year.

3.1 Production of Virus-free Potato Plants Using Meristem Culture

Meristem culture is a procedure in which apical or axillary growing tips (0.1-0.3 mm) are dissected and allowed to grow into plantlets on artificial nutrient media under controlled conditions. Details of the protocol for potato meristem culture are given in Annexure III. The meristem culture for virus elimination is essentially based on the principle that many viruses are unable to infect the apical/axillary meristem of a growing plant and that a virus-free plant can be produced if a small (0.1-0.3 mm) piece of meristematic tissue is propagated (Morel and Martin 1952). However, despite the phenomenal success of meristem culture in elimination of plant viruses, it remains still unclear as to why the apical/axillary meristems contain little or no virus? Some of the explanations are: (i) virus particles spread through vascular system which is not developed in the meristematic region; (ii) chromosome replication during mitosis and high auxin content in the meristem may inhibit virus multiplication through interference with viral nucleic acid metabolism; and (iii) existence of a virus-inactivating system with greater activity in the apical region than elsewhere.

In general, larger the size of the meristem, better the chances of its survival *in vitro*, whereas, smaller the size of the meristem, better the chances of its being virus-free (Wang and Hu 1980). The presence of leaf primordia also appears to determine the ability of a meristem to develop into a plantlet. Hence, it has been suggested that the excised meristem should include meristematic dome plus one or two leaf primordia. Both terminal and axillary buds are suitable for excision of meristems since there is no difference in their survival or freedom from viruses. However, it is difficult to excise apical meristems from terminal buds, because they have more rudimentary leaves and leaf primordia than the axillary buds. Therefore, axillary meristems are often preferred over apical meristems for virus elimination purposes.

Excision of very small meristems requires a high degree of expertise and the development of plants from these small meristems (mericlones) takes as long as four to eight months. Moreover, the percentage of virus freedom in regenerated mericlones is very low. As a result, meristem culture is mostly combined with thermotherapy (high temperature treatment) or chemotheraphy (treatment with antiviral compounds) to increase the likelihood of obtaining virus-free plants (Figure 3). Replication of many plant viruses is significantly reduced when the host plant is grown at elevated temperatures (Spiegel *et al*. 1993). It has also been suggested that disruption in the production or activity of virus-encoded movement proteins which are involved in cell-to-cell movement of viruses through plasmodesmata may also play a role in the effectiveness of heat therapy for virus elimination (Martin and Postman 1999). Therefore, heat therapy of infected plants followed by meristem culture improves virus freedom (Table 3).

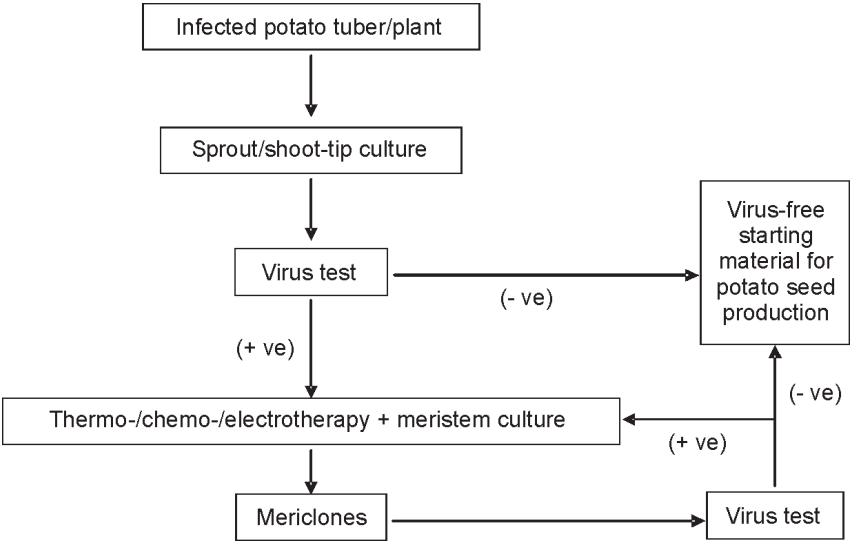

Figure 3. Schematic presentation of virus elimination in potato.

Production of virus-free plants from infected stock is also improved by applying chemicals that inhibit or interfere with virus replication or movement. These antiviral chemicals are generally incorporated into meristem culture media. A variety of natural or synthetic compounds have been tested for their potential to eliminate both DNA and RNA plant

Table 3. Effectiveness of thermotherapy for virus elimination in potato.

Genotype	Virus	% virus freedom	
		Meristem culture alone	Meristem culture + Thermotherapy (34 ^{0}C, 60 days)
Cardinal	PVX	4	35
	PVS	0	8
	PVY	2	46
Desiree	PVX	1	48
	PVS	0	9

(Source: Sajid *et al.* 1986)

viruses. The guanosine analogue of ribavirin (Virazole, 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), the uracil analogue of DHT (5-dihydroazauracil) and DHT derivative, diacetyl-5-dihydroazauracil (DA-DHT) are the three substances that have proved to be particularly effective in inhibiting different plant viruses (Hansen 1988). Cassells (1987) observed that *in vitro* chemotherapy of meristematic explants with antiviral chemical ribavirin was most promising for virus elimination in potato (Table 4).

Table 4. Efficacy of Virazole for virus elimination in potato.

Cultivar	Infected by	Virazole (µM)	% Virus-free mericlones
Mary Queen	PVX, PVY & PVM	0	3
		205	87
King Edward	PVY, PVS & PVM	0	0
		205	71
Kerr's Pink	PVY, PVS & PVM	0	21
		205	91
Golden Wonder	PVM	0	59
		205	100

(Source: Cassells 1987)

As stated earlier, the major constraints of meristem culture are difficulty in dissecting small meristems, lower survival and long time taken by them to develop into mericlones. To circumvent these problems, thermotherapy of mother plants followed by culturing of nodal cuttings (0.1 to 0.5 cm) on nutrient medium containing ribavirin have been used to obtain virus-free plants. Sanchez *et al*. (1991) evaluated nodal cuttings and meristems after combining heat treatment with ribavirin therapy in 34 potato genotypes infected with PVS, PVX, PVY and PLRV. They observed that the combined therapy generated twice as many virus-free plants as thermotherapy alone (Table 5).

Table 5. Effectiveness of combined virus elimination therapies using meristems and nodal cuttings in 34 potato genotypes.

Infected by	Explants	% virus-free plantlets			
		MS-RT*	MS-HT	MSR-RT	MSR-HT
Single virus	Nodal cutting	5	14.5	19.6	22.3
	Meristem	7	29.6	42.3	65.0
Multiple viruses	Nodal cutting	2	6.8	11.1	15.1
	Meristem	13	23.3	26.9	48.0

*MS= Murashige and Skoog (1962) medium; RT= Temperature in tissue culture room (23 °C); HT= Thermotherapy at 35 °C for 30 days; MSR= MS medium + 20 mg/l ribavirin.
(Source: Sanchez *et al.* 1991)

3.2 Micropropagation

In vitro multiplication of the mericlones and use of *in vitro* plants thus obtained for microtuber and minituber production are the next steps in potato micropropagation. These steps are described below and their protocols are given in Annexure-III.

3.2.1 In vitro Multiplication of Mericlones

Nodal segment culture in which the axillary and apical buds grow into new plants is predominantly used for initial shoot multiplication of mericlones. The axillary/apical buds of these nodal segments grow into full plants within a period of about 3 weeks. These plants can be further sub-cultured on fresh medium. Assuming effective generation of minimum 3 nodal cuttings by an *in vitro* plant and sub-culturing interval of 25 days, theoretically 3^{15} (14.3 million) microplants can be obtained from a single virus-free microplant in a year.

Considerable research has been done on chemical and physical requirements of micropropagation and their effects on explant growth. MS medium is most widely used in potato micropropagation, and growth regulators are usually omitted from culture media. In general, semisolid medium (0.8% agar) is used for initial propagation. Agar is the most frequently used gelling agent for semisolid cultures; however, some laboratories use 0.24% Gelrite, a synthetic polysaccharide, as a substitute for agar. Once sufficient number of cultures on semisolid medium are developed, liquid MS medium without agar is used for further mass micropropagation. Temperatures in the range of 22-25 °C and relatively high light intensity (50-60 $\mu E/m^2/s$) at 16 h photoperiod are beneficial for axillary shoot development in potato (Wang and Hu 1982). Although polypropylene caps are widely used to close the culture tubes in many a laboratories, it has been observed that the use of these caps leads to the plants becoming highly branched, hairy and with aerial roots developing all over the stem, perhaps due to the accumulation of ethylene. Optimum growth of the plants is obtained when the culture tubes are closed with cotton plugs.

The above microplants are used for potato seed production by diverse means. The simplest procedure is to harden and transplant them under aphid proof net house on nursery beds for production of minitubers (Naik 2005). The *in vitro* plants can also be used for the production of microtubers in the laboratory (Wang and Hu 1982). To attain commercial scale

production, specialized minitubers like Technitubers® or Quantum Tubers™ are produced from micropropagated disease-free plants under special screen houses.

3.2.2 Production of Microtubers

Microtubers are miniature tubers developed *in vitro* under tuber inducing conditions. They are very small (average weight 100-150 mg) and convenient for handling, storage and long distance transportation. Protocol for production of microtubers is given in Annexure-III.

The general microtuber production procedure consists of mass multiplication of *in vitro* plants in liquid propagation medium in flasks/magenta boxes. Stem segments (each with 3-4 nodes) obtained from 5-6 *in vitro* plants are cut and inoculated in each container containing 20 ml liquid propagation medium and the containers are incubated stationary under normal potato propagation conditions (22-25 °C temperature and 50-60 $\mu E/m^2/s$ light intensity for 16 h). Under these culture conditions, a number of plantlets grow from axillary buds and fill the container within 15-20 days. For microtuber production, the liquid propagation medium from the container is decanted under laminar flow conditions and replaced by microtuber induction medium (Annexure-III). Microtubers begin to develop epigeally within 1-2 weeks depending on the genotype, and are harvested after 60-75 days of incubation. Microtubers being delicate and dormant need to be stored for 3-4 months at 5-6 °C before they are used for planting. Heavy losses may occur during storage due to shrinkage, rotting and excessive sprouting. Greening of the microtubers for 10-15 days under diffused or artificial light before harvesting is highly beneficial in avoiding these losses (Naik and Sarkar 1997). The skin of the microtubers hardens during greening and accumulation of glycoalkaloids on exposure to light provides protection against fungal and bacterial diseases. Harvested green microtubers are washed, treated with fungicide, dried under dark, packed in perforated polythene bags and stored at 5-6 °C till the release of dormancy. The process of microtuber production is depicted in Figure 4.

Microtuber production technology is evolving rapidly. Fementors (containers in which plant material is in contact with nutrient solution continuously or at intervals) and bioreactors (vessels containing plants on screens or porous substrate subjected to nutrient mist and aeration cycles of varying durations) have been described for commercial scale rapid and synchronous microtuberization. These include 8-10 l jar fermentor (Akita and Takayama

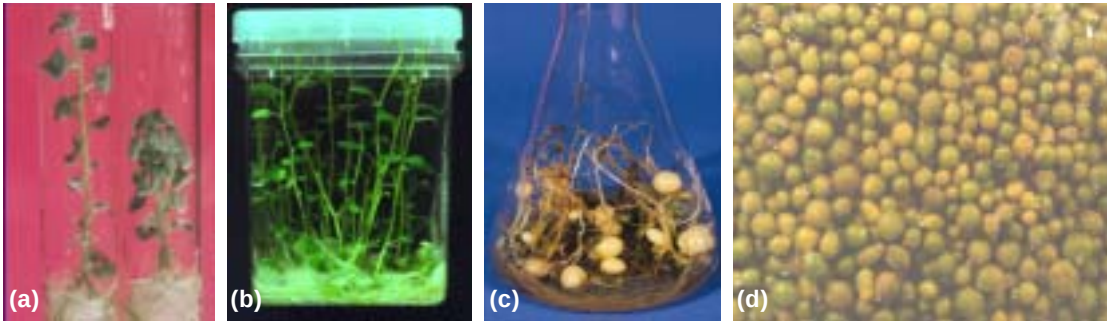

Figure 4. Microtuber production in potato. (a) Virus-free *in vitro* plants. (b) Mass micropropagation on liquid culture medium. (c) Production of microtubers in special medium under dark at 20 °C. (e) Harvested microtubers that have been greened before harvesting.

1994), nutrient mist bioreactor (Hao *et al.* 1998), temporary immersion container (Teisson and Alvard 1999) and continuous immersion system with or without net (Piao *et al.* 2003). Akita and Takayama (1994) reported yield of 500-960 microtubers of uniform weight from 100 nodal cuttings cultured in 10 l fermentor. Microtuber yields could be increased by extending microtuber growth period and refreshing the medium at intervals. With increased microtuberization time, 1,653 microtubers with total fresh weight of 1,420 g were produced. About 30% of these microtubers weighed more than 1 g. Teisson and Alvard (1999) could produce up to 3 microtubers per original node and 90 microtubers per vessel in 1 l RITA® system, 50% of which were above 0.5 g. Two types of low cost automated bioreactor systems, viz. continuous immersion (with or without net) and temporary immersion using ebb and flood (Figure 5) were developed by Piao *et al.* (2003) for production of potato microtubers in two steps. These were 10 l capacity bioreactors containing 1.5 l medium. In the first step, nodal cuttings were inoculated into the system for growth and multiplication of plantlets. After 4 weeks the propagation medium was replaced by microtuber induction medium. Inoculation density of 50 nodal cuttings/vessel, inclusion of 6-benzyladenine in microtuber induction medium and medium renewal during microtuber growth were observed to produce about 90 more than 1.1 g microtubers/vessel. Immersion type bioreactor with net was observed to be more valuable for large scale application.

A simple system for mass propagation and microtuber production was developed using a bioreactor without forced aeration (Akita and Ohta 1998). In this system, explants were cultured in 1 l bottles equipped with an air-permeable membrane on the cap and these bottles were slowly rotated on a bottle roller. Microtubers of potato were induced using a two-step culture method. In the first step, potato plantlets were grown from nodal cuttings under static

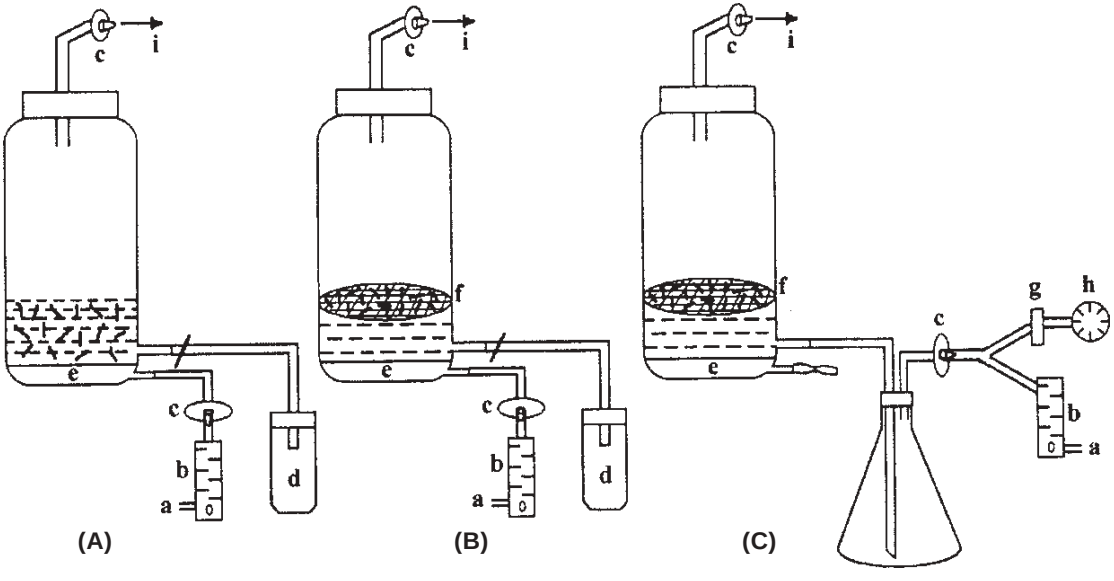

Figure 5. Schematic layout of continuous immersion bioreactor without net (A) or with net (B) and temporary immersion bioreactor using ebb and flood (C). [a, Air inlet; b, Airflow meter; c, Membrane filter; d, Sampling port; e, Sparger; f, Supporter (net); g, Solenoid valve; h, Timer; I, Air outlet]. (Figure reproduced from Piao *et al.* 2003, with permission)

conditions. After shoot proliferation, the culture medium was replaced with a microtuber induction medium and the bottles were rotated at 1 rpm. One hundred microtubers were produced per bottle having 200 ml medium, a higher number of microtubers than produced in static cultures without rotation. The slowly rotating containers appear to be simpler and less expensive than airlift or immersion type fermentors.

The increasing interest and rapid development in the field of commercial microtuber production has resulted in patenting of some of the microtuber production technologies (Donnelly *et al.* 2003). Aseptic production and possibility of reducing costs through automation are important factors that are likely to promote use of microtubers in potato seed production world over (Hoverkort and van der Zaag 1989). Further, any breakthrough technology that increases microtuber size will eliminate intermediary step of minituber production.

3.2.3 Production of Minitubers

3.2.3.1 Minituber production from *in vitro* plants

Minituber is an intermediate stage of potato seed production between laboratory micropropagation and field multiplication. The simplest method of producing minitubers from *in vitro* plants particularly suited to North Indian plains has been described by Naik (2005). In this method, 15-20 day old cultures are kept in glasshouse or polyhouse for 8-10 days for hardening without removing culture tube plugs or lids of magenta boxes. The hardened *in vitro* plants are removed from culture vessels with the help of forceps, washed to remove adhering medium and cut into two pieces after cutting out the root zone. The lower portion (about 0.5 cm) of the cuttings is dipped in rooting hormone powder (soft wood grade) and planted in pre-prepared nursery beds (soil: FYM: sand 1:1:1 v/v) at plant to plant and row to row distance of 10 cm in a vector-free net house (Figure 6a and b). It is beneficial to drench nursery beds with fungicide solution before planting. Three to four water sprays are given daily with a sprayer to keep the soil moist and maintain humidity for initial one week. If available, mist irrigation is also suitable. Once the plants establish and start growing (Figure 6c), normal irrigation with a watering can or any other means can be followed. With progressive growth of the plants, additional soil substrate is added on the nursery beds to bury lower leaves. This is important to optimize minituber production from buried axillary buds. The crop is allowed to mature on the nursery bed and minitubers are harvested (Figure 6d). The minitubers are cold stored and used as planting material in the next crop season. In general, 80-90% cuttings establish and produce about 8-12 minitubers per plant of average

Figure 6. Minituber production from *in vitro* plants under vector-free net house during October-January in North Indian plains. (a) Cuttings of *in vitro* plants and treatment with rooting hormone powder. (b) Planting of treated cuttings in nursery bed. (c) One month old crop from *in vitro* cuttings. (d) Harvested minitubers.

10-15 g depending upon variety. Bigger size of the minitubers as compared to microtubers facilitates robust post harvest handling and ease in field planting.

Recently, hydroponic and aeroponic systems have been developed for production of minitubers from *in vitro* plants. In addition to reducing the cost of production, these systems enable round the year production and adoption of phytosanitary standards. Hydroponically developed minitubers, such as Technitubers® are produced under stringent sanitary conditions in high density plantings and harvested at intervals from plants growing in nutrient film (Gable *et al.* 1990). Technitubers® are miniature seed potatoes measuring 10-15 mm in diameter. These are ideal for storage, shipment and mechanized planting with the help of vacuum seeders. Agronomic packages have been developed and field trials conducted over years in several countries have demonstrated that a healthy and vigorous potato crop can be raised from Technitubers® (Gable *et al.* 1990). The technology is well suited for Asia and currently Technituber® units are operating in Australia, China and India.

Commendable efforts are being made to integrate *in vivo* and *in vitro* rapid multiplication techniques in potato seed production in China. Use of aeroponic technique for producing minitubers from *in vitro* plants is employed in some Chinese provinces (Sun and Yang 2004). The modified device consists of culture channel, pump, spraying tube, timer and nutrient solution tank. A tube with several nozzles passes through culture tunnel and sprays nutrient solution on root zone of plants. The culture channel has a removable top with holes for holding potato plants (Figure 7). *In vitro* plantlets are transplanted in these holes and fixed by sponge. The nutrient solution is sprayed for 30 seconds after every 3 minutes in initial growing stages. The spraying interval is prolonged up to once in 15 minutes with progressive growth of the plants. The system allows repeated harvesting of minitubers of desirable size. In this aeroponic system, it was possible to produce 2,094 minitubers/m^2 as compared to 771 minitubers/m^2 in nursery beds/substrate culture (Sun and Yang 2004).

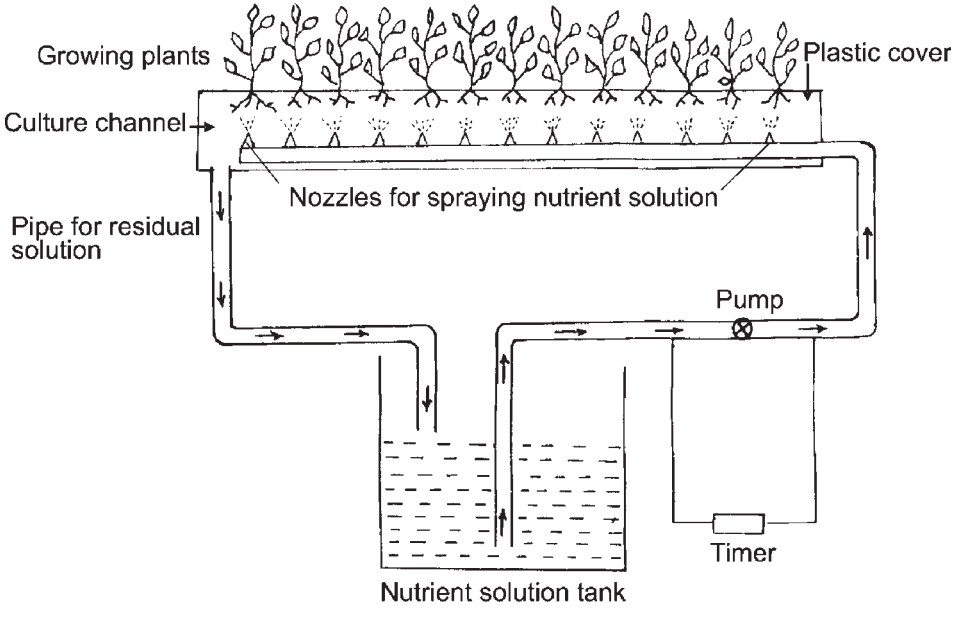

Figure 7. A simple aeroponic device used for minituber production in China.

3.2.3.2 Minituber production from microtubers

Minitubers can also be produced from microtubers under high density planting on nursery beds as in the case of *in vitro* plants (Naik 2005). The main advantages of microtubers over *in vitro* plants are that they are less delicate, easy to handle and transport, and require less care during planting and post planting operations (Hoque *et al.* 1996, Naik *et al.* 2000, Wang and Hu 1982). However, it takes 2-3 months longer in laboratory for production of microtubers which also need to be stored for dormancy breaking. Further, yields from microtubers on nursery beds are similar to those of *in vitro* plants though microtubers tend to produce larger sized minitubers (Ahloowalia 1994).

SYNTHETIC OR ARTIFICIAL POTATO SEED: A NEW POSSIBILITY

The use of somatic embryos (embryos developed from a single or group of somatic cells in tissue culture without fertilization) as artificial or synthetic seeds (encapsulated embryos which can be used for planting as seed) was long felt as a promising alternative to conventional potato seed (O'Hair *et al.* 1987). The main advantages of somatic embryos are that their production could be completely automated and large quantities of embryos could be produced in a closed system. However, somatic embryogenesis is not common in potato. Therefore, a possibility of encapsulating *in vitro* stem segments was explored at the Central Potato Research Institute, Shimla, India (Sarkar and Naik 1998). As high as 57% encapsulated segments survived in the soil in Shimla hills, when they were incubated under light for 3 days and treated with rooting hormone powder prior to planting. However, their performance under high temperatures of low lands was not encouraging. Nyende *et al.* (2003) produced hollow calcium alginate beads averaging 0.78 cm in diameter from pre-cultured 3-4 mm shoot tips (pre-culturing on solid MS medium for 2 days). The encapsulated shoot tips stored at 4 and 10 °C for up to 390 days gave 100% regeneration on MS solid medium. Germination of 93-100% was recorded for non-stored encapsulated shoot tips, directly transferred on soil in the greenhouse after a 2-week pre-culturing on MS solid medium with an added fungicide (carbendazim) in the encapsulating gel. The germinated shoot tips produced plants showing normal development.

The technique offers tremendous opportunities for production of large quantities of disease-free potato propagules from *in vitro* plantlets in protected condition within a limited time and space. The method is also highly cost-effective. However, additional research is required for field level application of this technology.

3.3 Field Performance of Mini- and Micro-tubers

Apical and axillary cuttings obtained from *in vitro* plants are sometimes used directly for raising commercial potato crop (Uyen and Zaag 1983). In some cases, microtubers are also used for direct field multiplication (Hoverkort and van der Zaag 1989). Direct field planting of these propagules is, however, not practicable at most places and hence are pre-planted in greenhouses or polythene bags and then transplanted in the field (Naik *et al.* 1998).

Ranalli *et al.* (1994) produced micro- and mini-tubers of cv. Monalisa and compared their yield potential with normal seed tubers in a field experiment. The tubers were planted at similar plant densities with row to row distances of 60 and 90 cm. Complete ground cover by foliage was observed in the crop developed from normal tubers and decreased with the reducing size of the mother tubers. Averaged over two spacings, normal seed, mini- and micro-tubers yielded 50.8 t/h, 31.7 t/h, and 17.0 t/h, respectively. At 60 and 90 cm spacings,

microtubers yielded 27.3 t/h and 16. 7 t/ha and minitubers yielded 38.9 t/h and 24.4 t/ha., respectively. Row spacing did not influence the yields from normal seed tubers. Total number of tubers per m^2 ranged from 107.8 with microtubers, 122.1 with minitubers, to 142.9 with normal tubers.

At Central Potato Research Institute, Shimla, India, a team led by one of the present authors (Naik) conducted field trials in North Indian plains with minitubers (av. weight 10-15 g) produced from *in vitro* plants and microtubers. For comparison, the minitubers were planted with normal seed tubers at recommended spacings of 60 cm (row to row) x 20 cm (plant to plant). Recorded yield performances indicate good possibility of producing normal seed crop from minitubers (Table 6).

Table 6. Yield performance of minitubers vs. normal seed tubers in north Indian plains.

Cultivar	Performance of minitubers produced from microtubers		Performance of minitubers produced from *in vitro* plants		Performance of normal seed tubers	
	Yield/ plant (g)	No. of tubers/ plant	Yield/ plant (g)	No. of tubers/ plant	Yield/ plant (g)	No. of tubers/ plant
Kufri Badshah	366	7.2	355	7.4	408	7.9
Kufri Bahar	363	8.5	337	7.2	439	7.3
Kufri Chipsona-1	-	-	319	9.6	386	9.2
Kufri Chipsona-2	303	9.8	315	9.4	402	10.4
Kufri Pukhraj	369	8.6	380	8.7	418	9.2
Kufri Sutlej	345	6.8	-	-	414	7.0

(Source: Naik *et al.* unpublished data)

3.4 Integration of Micropropagation in Seed Production System

The ease with which virus-free mericlones are produced and later multiplied through nodal cuttings, microtubers and minitubers make micropropagation a very viable option for initial stages of potato seed production, especially in countries where disease-free seed potatoes can not be produced for want of vector-free production areas. In most cases, integration of micropropagation in potato seed production scheme involves: (i) production of virus-free stocks using meristem culture, (ii) micropropagation and/or microtuber production in laboratory, (iii) production of minitubers or specialized minitubers (Technitubers®/Quantum Tubers™) in polyhouses or specialized structures, and (iv) field multiplications of minitubers or specialized minitubers. A typical integration module is depicted in Figure 8.

Elaborate procedures for integrating micropropagation in potato seed production have been described for China (Sun and Yang 2004) and India (Naik and Khurana 2003). These procedures involve production of disease-free plants using meristem culture, their rapid *in vitro* mulplication, production of micro-/mini-tubers and use of minitubers as planting material for further field multiplications. In China, two systems, one for summer crop region and

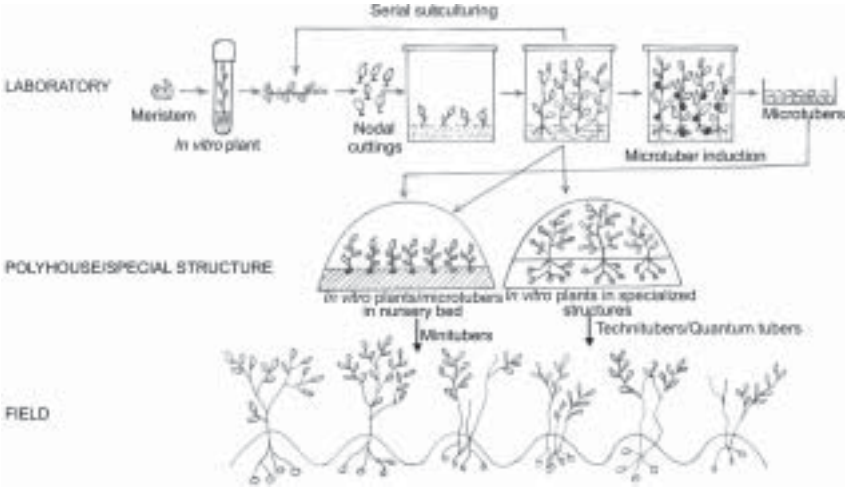

Figure 8. Typical integration of micropropagation in potato seed production.

another for double cropping region are operating. In both these systems, *in vitro* plants and microtubers are used to produce pre-elite minitubers. The latter are further multiplied under aphid-proof nethouses and the produce is field multiplied to generate certified seed (Figure 9). In India, the proposed potato seed production system consists of production of nucleus seed from *in vitro* plants and/or microtubers under vector-free nethouses followed by two field multiplications for production of basic seed. The basic seed is further multiplied in fields to obtain certified seed (Figure 10).

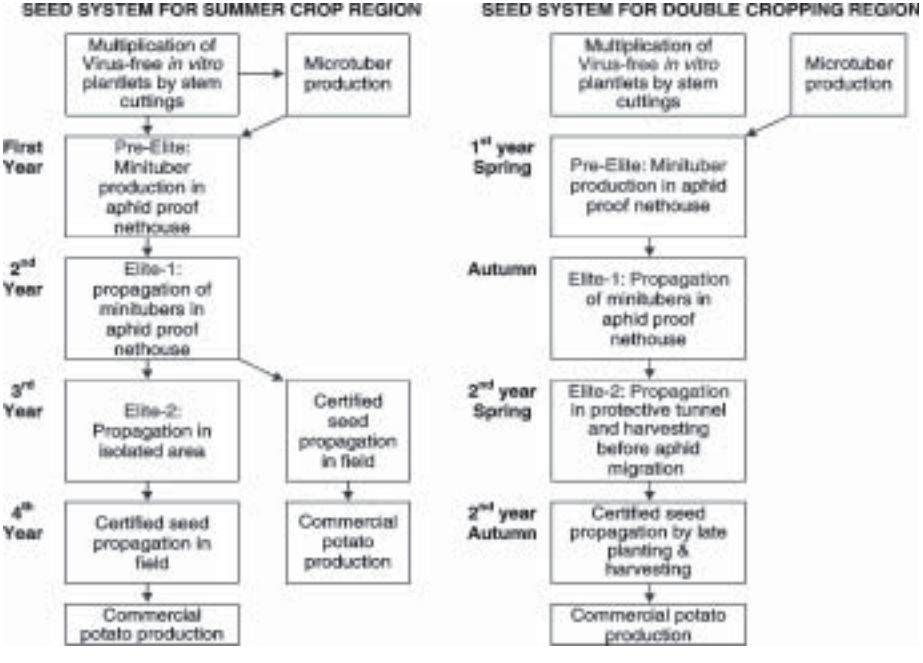

Figure 9. Potato seed production systems in China. (Source: Sun and Yang 2004)

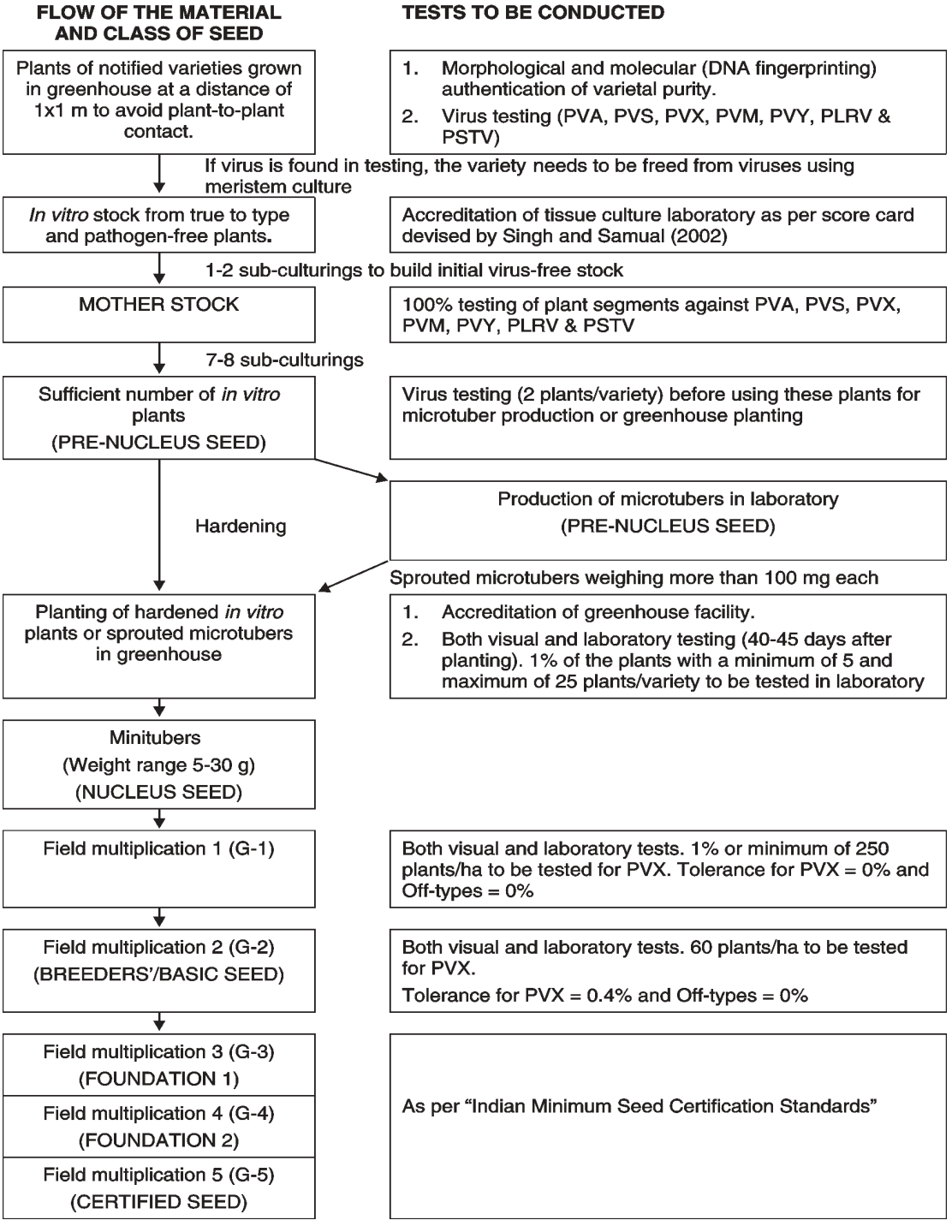

Figure 10. Micropropagation based system for potato seed production proposed for India. (Source: Naik and Khurana 2003)

3.5 Seed Certification and Quality Standards for Potato Seed Produced through Micropropagation

During potato production, the plant is constantly exposed to pathogens and the probability of a seed tuber becoming infected progressively increases every year. There is thus considerable risk of spreading diseases by transboundary movement of seed potatoes. However, micropropagated material can be rendered disease free and multiplied under protected conditions to produce tubers which can also be traded with minimum risk of spreading diseases.

Minimum commercial quality requirements for trade in potato seed fall under the WTO-SPS-TBT agreement. In line with the SPS requirements, several countries have recently revised their certification schemes for seed potatoes. These schemes focus on micropropagation as the recommended method for initial seed production (nuclear stock). Therefore, there is an urgent need to develop certification standards for various categories of seed potatoes in such a way that these are aligned as far as possible with standards in developed countries (Naik and Khurana 2003). Such seed standards should also aim at, (i) facilitating fair international trade by avoiding technical barriers, (ii) encouraging production of high quality seed to ensure farmer's profitability, (iii) protecting consumer's interests, and (iv) gaining confidence of importing countries that the imported seed lots have been monitored using internationally accepted protocols.

The ideal seed certification system, therefore, should have following salient features:

(a) Pest risk analysis should form the basis for potato seed certification, import and export. PRA should be carried out in accordance with ISPM.No.11 and ISPM No.21 to identify the pests of national and international importance.

(b) All seed potatoes must be limited to specified multiplications. The number of multiplications may vary from country to country depending upon agro-climatic and pest conditions. However, the first two multiplications must be confined to laboratory/greenhouse.

(c) The classes of the limited generation system should be; pre-nuclear, nuclear, generation 1, generation 2 (breeders'/basic seed), generation 3 and so on till certified seed; where pre-nuclear is essentially from the laboratory, nuclear seed is produced in greenhouse, while others are open field multiplications.

(d) Pre-nuclear seed stocks must originate from tissue cultured plantlets, minitubers or microtubers.

(e) Except for varietal mixtures, seed lots should be downgraded or advanced in generation if they do not meet the disease tolerances for that generation. Such seed lots, if meeting the standards of certified seed, should be sold as certified seed.

(f) The certified seed class should not be eligible for re-certification. However, if seed availability is low for a specific potato variety, seed lots with more field multiplications may be eligible for re-certification after prior approval of the Seed Certification Authority.

The minimum quality requirements at each stage of potato seed production using tissue culture are given in Annexure-IV.

4. PROSPECTS OF POTATO MICROPROPAGATION FOR QUALITY SEED PRODUCTION IN ASIA-PACIFIC

According to recent estimates, developing countries are likely to have higher growth rates in production and productivity of potatoes during 1993-2020 than the world average growth rate (Scott *et al.* 2000). According to these estimates, the developing countries are likely to produce 48% world potatoes by 2020 as against 10.5% in 1961 and 44% during the current years (Table 7). The increase will be more pronounced in India, Sub-Saharan Africa, China and other Asian countries. Thus, the Asia-Pacific region with two major potato producing countries, viz. China and India is likely to be a focus for potato production.

Table 7. Production and expected growth rates of potato during the period 1993-2020 in different parts of the world.

Country/Region	Potato production (mt)		Expected growth rate
	1993	2020	1993-2020
			(% per year)
China	42.5	87.8	2.72
Other East Asia	2.4	3.3	1.18
India	16.3	43.3	3.67
Other South Asia	3.5	7.7	2.98
Southeast Asia	1.3	2.3	2.08
Latin America	12.6	20.2	1.76
W. Asia/N. Africa (WANA)	13.0	23.4	2.21
Sub-Saharan Africa	2.6	6.0	3.06
Developing countries	94.3	194.0	2.71
Developed countries	191.0	209.5	0.34
World	285.3	403.5	1.29

(Source: Scott *et al.* 2000)

4.1 Some Success Stories

In Dalat, Lam Dong, Vietnam where the environment conditions for growing of *in vitro* raised potato plantlets are highly favorable (mean maximum and minimum temperatures 23 °C and 14 °C, respectively; seasonal range of 2 °C; and relative humidity above 70%), farmers have adopted innovative approaches to use *in vitro* plants for producing rooted single node cuttings as a planting material. These include tissue culture facilities developed from

domestically available articles like autoclaves made from a gas cylinders cut near the top, and plastic inoculation hoods fitted with UV light. Incubation of cultures is done in the corridors of homes under diffused sunlight. *In vitro* plants produced in these facilities are planted in the nursery beds. After establishment, these are cut into single node cuttings at 5-6 leaf stage. These cuttings are rooted in sand or any other suitable substrate and, after going through two rounds of cuttings, transplanted in fields to grow into a regular potato crop (Uyen and Zaag 1983). Using this innovative method, the production cost of one *in vitro* plantlet comes to just US $ 0.0017. The farmers can obtain 10,000 rooted cuttings from one *in vitro* plant in a period of 8 months by integrating micropropagation with *in vivo* single node cuttings as shown in Figure 11.

Figure 11. Innovative potato seed production technique used by Vietnamese farmers.

Researchers in Taiwan have reported production of 36,000 microtubers from 1,200 culture flasks in a period of 4 months (Wang and Hu 1982). After 3 field multiplications, these microtubers produced 1,800 t potato seed, which was enough for 2000 ha on a schedule of one-third rotation per year. In India, Naik (2005) reported the possibility of producing 264,500 basic seed tubers after one nursery bed and two field multiplications of microtubers produced from one *in vitro* plant.

In the Republic of Korea, use of virus-free planting material produced through tissue culture has increased the national potato yield from 11.9 t/ha in 1980 to 20.3 t/ha in 1986 (Chung 1989). Subsequently, an *in vitro* tuberization system was also established and became an integral component of the potato seed industry in the country (Choi *et al.* 1994).

With the technical support from the CIP, China established an industry scale microtuber production facility with a production capacity of 10 million microtubers per annum. To start with, this facility produced one million microtubers in 1988, which increased to 4 million in 1989 (Li *et al.* 1991). The production costs per microtuber were US$ 0.009 and US$ 0.008 in 1989 and 1990, respectively.

The ICAR initiated a self sustaining "Revolving Fund Scheme for Potato Breeders' Seed Production" with the objectives of integrating micropropagation and sensitive virus detection techniques in the initial stages of potato breedres' seed production. Virus-free stocks of Indian varieties were developed which led to 2-3 fold improvement in health standards of resulting breeders' seed. During the 1st decade of its operation, the scheme generated a revenue of US$ 4.06 million. The total expenditure, including that incurred on development of modern infrastructure and amounting to US$ 3.12 million was met from these receipts. During this period, potato breeders' seed production (27,904 t) and supply (20,801 t) increased by 37.5% and 34.5%, respectively. Physical and financial performance of the scheme is shown in Figure 12

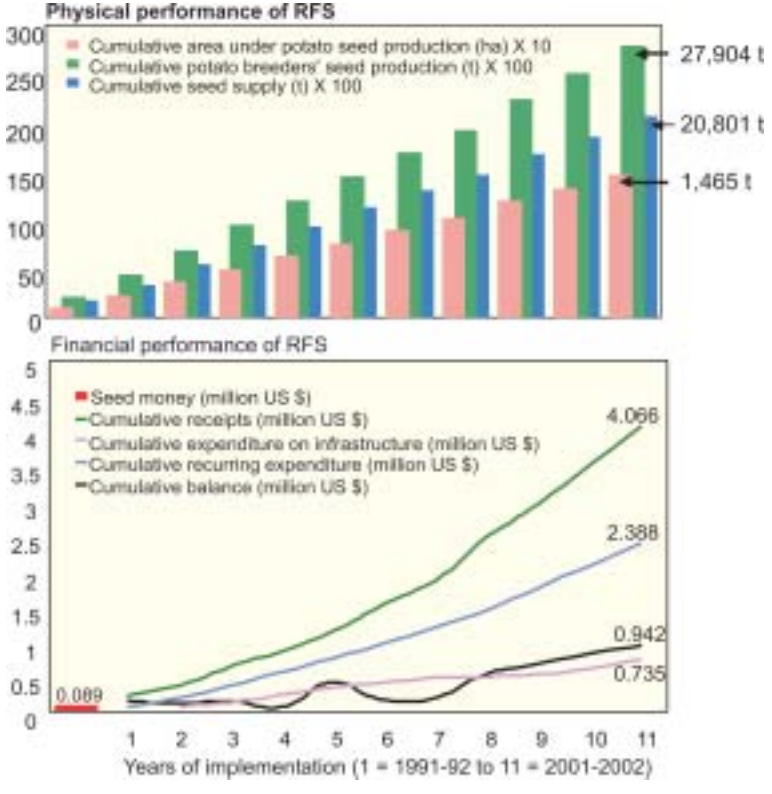

Figure 12. Success story of Revolving Fund Scheme for potato breeders' seed production in India.

4.2 The Way Ahead

Shortage of good quality seed is recognized as the single most important factor hindering potato production in Asia. Availability of quality planting material of improved potato varieties in adequate quantities, therefore, is the major issue that needs to be attended by respective national agricultural research systems in order to attain the projected production targets. Several FAO reports suggest that about 75% of the global seed requirement is met through informal seed system comprising exchange among neighbors, friends, relatives, tribes and villages, indicating its importance in world agriculture. Further, the formal seed system mainly relies on local extension services for diffusion of quality planting material. Such services, are either poor or non-existent in many countries of the Asia-Pacific region. The major task for improving potato productivity and production in this region, therefore, is to strengthen formal seed system, integrate it with socially efficient informal seed system and also to develop public-private partnership in quality seed production.

Strengthening of formal potato seed production system by integrating micropropagation is a sound technological option. Large-scale micropropagation and microtuber production, however, still continues to be time consuming and labour intensive. Automation of micropropagation is expected to reduce the production cost as well as increase the production per unit time and space. The success of such adoption, however, will rely heavily on simultaneous development of low cost pathogen diagnostic methods and development of seed certification standards. Many countries in the region do not have necessary infrastructure and capabilities for adopting these technological advancements. It is, therefore, necessary that national and international research institutes, and public and private sector seed industries join hands to develop time bound action plans to ensure seed security. The important components of seed security that need immediate attention are; (i) capacity and infrastructure building, (ii) developing national seed policies (encompassing variety development, evaluation and registration, plant variety protection legislation, seed certification standards, quarantine etc.) (iii) harmonizing regional seed policies (standards for seed purity and testing, plant protection and quarantine regulations, plant variety protection, uniform seed certification and marketing regulations) to facilitate exchange of seed. Organizations such as ISF, UPOV, ISTA, APSA and OECD can play a pivotal role to help countries in the Asia-Pacific region on these issues.

Once a strong formal seed system is in place, it needs to be integrated with informal seed system and private sector so that benefits of new varieties and technological advances percolate to the farming community. In this direction training of the extension officers and farmers through FFS in Nepal, Pakistan, Bangladesh, Sri Lanka, Bhutan and the Philippines by CIP in late nineties is a step forward. These trainings and FFS helped farmers to produce and maintain seed quality through better seed management (www.eseap.cipotato.org). In most of these countries trained farmers now buy small quantities of seed from formal seed system and multiply and distribute it under appropriate management practices.The sucess of these initiatives indicates that training of extension functionaries/farmers and injection of clean planting material of new improved varieties into informal seed system can circumvent seed shortage to a greater extent. UPWARD, a partnership program with CIP is also active in the Asia-Pacific region for developing regional capacity for participatory research and development concepts in root crop livelihood covering integrated crop management, genetic resources conservation, processing, marketing and consumption (www.cip-upward.org).

Private sector is emerging as a major participant in potato seed production in Australia, India, China and other countries of the region. This sector can contribute further by developing and delivering new technologies and creating marketing chains. The key motivation for seeking partnership with the private sector is to strengthen linkages between technology generation and its delivery to the end users. This sector can also sensitize policy makers for creating competitive, efficient and sustainable seed industry. ICARDA is working in collaboration with the private sector for the establishment and promotion of village-based seed enterprises (VBSE) in the Asia-Pacific region. These are farmers-led production entities that produce high quality planting material of improved varieties adapted to local conditions. Under VSEB, ICARDA organizes farmer groups; provides technical support for preparing business plans; sources high quality seeds of improved varieties; builds capacities of farmers and staff of participating organizations; and assists in procuring fertilizer, equipment and credit. In collaboration with CIP, Ministry of Agriculture and Animal Health, NGOs and farmers, ICARDA has developed 19 potato seed production groups in Afghanistan (www.icarda.org).

It is hoped that with these developments, many developing countries in the region will integrate micropropagation techniques in the formal seed system to meet the shortage of quality seed. Availability of quality planting material in adequate quantities is likely to improve potato productivity by about 25-30% in the region as demonstrated by a micropropagation project on production and distribution of virus-free sweet potatoes in China's Shandong Province that led to an increase in yields up to 30% valued at US$ 145 million annually (www.scidev.net). Such integration in the Asia-Pacific region needs to be watched with enthusiasm and optimism. APCoAB will continue to play its role of organizing deliberations and disseminating information on biotechnology policy and technical issues impacting agricultural development in the region. Networking of research programs in the field of agricultural biotechnology, human resource development and promotion of pubic-private partnership would be priority activities of the consortium.

Annexure-I

Conventional Potato Seed Production and Seed Certification Standards in India

In India, Central Potato Research Institute, Shimla produces entire potato breeders' seed (basic seed), which is distributed to various agencies for further multiplications as foundation-I, foundation-II and certified seed. The procedure for potato seed production (Singh 1993) and some of the seed certification standards (Tunwar and Singh 1988) are given in Tables 8 and 9.

Table 8. Conventional potato seed production procedure in India.

Stage		Procedure	Multiplication rate
Plant selection and tuber indexing	  	True to type, healthy, and high yielding plants are selected from growing potato crop in different stages of seed production. These plants are harvested individually and four large sized tubers from each plant (one clone) are selected for indexing. Single scooped eye from each tuber is grown in the glasshouse and the emerging plants are tested for presence of PVX, -S, -A, -Y, -M and PLRV by ELISA. Clones showing virus freedom in all the four scooped eyes are declared healthy and used for further multiplication.	0
Stage-I	 	Above four tubers of healthy indexed clones are field multiplied during low aphid periods (April-September in hills and October-January in plains) at spacing of 1m x 1m. In addition to two/three visual inspections for roguing, all Stage-I plants are tested by ELISA. Long duration of the crop in hills allows three visual inspections in field while it is done only twice in the plains. The disease-free clones are harvested and stored individually.	15

Stage		Procedure	Multiplication rate
Stage-II		The produce of Stage-I is planted clone-wise in separate rows at a wider inter-row spacing of 1 m-1.2 m. Three visual inspections and ELISA tests on 4-5 plants/row are performed for roguing. Healthy clones are harvested and the produce is bulk stored.	8
Stage-III		The bulk produce of Stage-II is multiplied in next season at normal spacing of 60 cm x 20 cm. Three visual inspections and ELISA test for PVS and PVX on 300 plants/ha are done for roguing. All precautions are taken to maintain a healthy crop through pesticide sprays.	6
Stage-IV		The produce of Stage-III is field multiplied at normal spacing as above. ELISA testing is done on 100 plants/ha. A team comprising scientists and representatives from national and state seed certification agencies inspects the crop. The produce of Stage-IV is designated as breeders' seed, which is distributed by the Ministry of Agriculture to state departments of agriculture and horticulture, and the National Seeds Corporation for its further multiplication as foundation-I, foundation-II and certified seed.	6
Foundation-I (FS-I)	 	Crop under these seed classes is grown at normal spacing during low aphid periods taking adequate precautions to protect it from biotic infections. These seed classes are subjected to 3 field inspections by seed certifying agencies during the crop season. In plains the crop is inspected at 30-35, 60-65 and 75-80 days while in the hills at 40-45, 75-80 and 90-95 days. These inspections are carried out as per Indian Minimum Seed Standards (Tunwar and Singh 1988). Some of these standards are given below. For assessing post harvest quality, the seed lots are inspected for physical abnormalities or defects, size of tubers, sprouting, occurrence of storage pests and diseases etc. before supply.	6
Foundation-II (FS-II)			6
Certified seed (CS)			6

Table 9. Some seed certification standards for potato in India.

(A) Permissible limits for purity and diseases in potato seed crop

Class of seed crop	Maximum permissible % of plants showing					
	Off type	Mild mosaic	Severe mosaic, leaf roll and yellows	Total viruses	Brown rot	Re-growth of plants after haulm cutting
FS-I	0.05	1.0	0.50	1.0	-	0.5
FS-II	0.05	2.0	0.75	2.0	-	0.5
CS	0.10	3.0	1.00	3.0	3 plants/ha	0.5

(B) Permissible limits of damages and diseases in potato seed tubers

Seed class	Maximum permissible % of tubers (by number) showing				
	Common scab*	Black Scurf	Late blight, dry rot, charcoal rot	Wet rot	Total disease
FS-I	3.0	5.0	1.0	0.0	5.0
FS-II	3.0	5.0	1.0	0.0	5.0
CS	5.0	5.0	1.0	0.0	5.0

(C) Grade/size standards for seed tubers

Seed source	Seed grade	Size in mm	Corresponding tuber weight (g)
Hill seed	Seed	30-60	25-150
	Large	> 60	> 150
Plains seed	Seed	30-55	25-125
	Large	> 55	> 125

Following this system of seed production, ≈ 930,000 tubers weighing ≈ 70 t certified seed (@ 75g/tuber) are produced from one indexed tuber after 7 field multiplications. In India, potato is grown in about 1.39 mha and in general farmers replace potato seed stocks once in every 5 years (in certain areas the replacement is after every 2-3 years). The certified seed production program meets about 46% of the country's seed requirement.

Annexure-II

Organization of Plant Tissue Culture Laboratory

A standard tissue culture laboratory is required to have four separate rooms, viz. washing room, media (preparation and storage) room, inoculation room and culture room. In addition to these basic facilities, separate rooms for general-purpose laboratory and electric installations are needed.

Washing room: The washing room should be in a corner of the laboratory, well isolated from inoculation and culture rooms. It should be provided with a workbench fitted with a large washing sink, running water and a water-heating unit. The room should also have plastic buckets, plastic tubs to wet the laboratory wares before washing, ovens to dry the washed laboratory wares and cupboards/cabinets to store them. Although overnight incubation of glasswares in chromic acid solution prior to washing in soap solution is a recommended practice in many tissue culture laboratories, it is advisable to exclude such type of acidic treatment. If not thoroughly washed, these substances detrimentally affect cell and tissue growth *in vitro* besides being hazardous to the persons involved in washing. Use of laboratory detergent followed by thorough washing in tap water with a final rinse in deionized water may be adequate to cleanse most of the glassware and plasticware. However, a modern laboratory can well simplify the setup as well as increase the efficiency by installing an automated washing, drying and disinfecting machine. In any case, it is strongly recommended that the contaminated laboratory wares must be autoclaved before washing, and appropriate safety measures are adopted for waste disposal.

Media room: Preparation of nutrient media and their sterilization is carried out in the media room. This room is required to be adjacent to the inoculation room for ease in transporting supplies. Besides, this room should be kept free of dust as far as possible. The facilities required for media preparation and storage include: (i) work-benches, (ii) refrigerators for storing thermo-sensitive chemicals and stock solutions, (iii) deep freezers (-20 °C) for storing chemicals and growth regulators requiring sub-zero storage, (iv) water distillation unit, (v) pH meter, (vi) weighing balances, (vii) vertical or horizontal autoclave for steam sterilization of culture/nutrient media (depending on the scale of micropropagation, it is better to have two autoclaves installed in a separate autoclave room, one big for regular use and another small for occasional use), (viii) magnetic stirrer-cum-hotplate for dissolving chemicals, (ix) filter sterilization units and a vacuum pump for sterilization of thermo-labile chemicals, (x) automatic media dispenser for repeated dispensing of measured volumes of media into culture vessels, and (xi) cabinets or cupboards for storing chemicals and prepared media.

Inoculation room: The inoculation room is the place where aseptic operations are carried out. An interior small room away from the flow of air currents and just adjacent to the culture room is an excellent location for aseptic procedures. This room is provided with laminar flow workstation, which provides a gentle flow of ultra filtered (through a pre-filter plus 0.2 μ

HEPA filter assembly) sterile air across the working area in the cabinet. The inoculation room is also provided with high temperature ovens for sterilization of glassware and other metal accessories required for aseptic operation. A better, although expensive, choice is to install programmable hot-air sterilizers for sterilization of glassware and metal accessories. In addition, this room needs to have cabinets for storing culture media. This room should always be kept clean, and special care should be taken to arrange regular waste disposal. The pre-filter assemblies of the laminar flow need thorough washing at least once a year.

Culture room: This room is a simulated environment facility where the tissue cultures are grown under regulated light and temperature regimes. There must be at least two independent culture rooms for potato micropropagation; one for *in vitro* propagation and another for microtuber production. Basically, this room is required to be provided with: (i) air-conditioners for the maintenance of room temperature around 20-24 °C as per specific requirements, (ii) temperature controllers for regulating the culture room temperature, (iii) sequential timers for alternating the operation of air-conditioners at a given interval, (iv) photoperiodic controllers for setting the photoperiod duration, and (v) tissue culture trolleys fitted with fluorescent lights. Cool white fluorescent lights are usually used for providing artificial light for plant tissue cultures. However, depending upon the requirements of cultures which can vary from as low as 20 $\mu E/m^2/s$ to as high as 200 $\mu E/m^2/s$. On an average one 40 W cool white fluorescent tube provides a radiation intensity of about 20 $\mu E/m^2/s$. In the absence of sophisticated tissue culture trolleys, ordinary metal shelves can well be adapted for culture-incubation purpose. For potato tissue culture, the recommended photoperiod regime is 16 h light and 8 h dark with radiation intensity varying from 20 $\mu E/m^2/s$ to 100 $\mu E/m^2/s$. Sophisticated laboratories can install ozone disinfecting units for the maintenance of clean environment inside the culture room. However, regular washing of culture room with appropriate disinfectants and strict regulation of visitors is necessary to maintain the culture room hygiene at the desired level. The incubation shelves can be periodically cleansed with 70 % alcohol. However, alcohol solution or other disinfectants should never be sprayed inside the culture room since these are toxic to the growing cultures.

Besides the basic infrastructure listed above, following equipment and other infrastructure are required in a tissue culture laboratory for diverse uses.

Name of the instrument	**Purpose**
Automatic media dispenser	For dispensing measured volume of culture media into tissue culture tubes, vessels and containers
BOD incubator, oven, microwave	For general laboratory use
General purpose trolley	For transporting cultures
Growth chamber (with temperature and humidity controls)	For acclimatization of *in vitro* microplants before *ex vitro* transplanting, thermotherapy, etc.
Glass beads/ infrared sterilizer	For aseptic operation inside the laminar flow workstation
Micropipettes	For liquid handling and dispensing
Research microscope	For routine microscopic observation

Stereoscopic binocular zoom microscope	For dissecting meristems, shoot tips, etc. and for observing cultures
Tissue culture castor racks	For culture incubation
Voltage stabilizer	For use with all instruments sensitive to voltage fluctuation
Walk-in-Chamber	For storing cultures and microtubers at low temperature

A list of laboratory consumables other than chemicals required in a tissue culture laboratory is given below:

Name of the consumable	**Purpose**
Borosilicate culture tubes (25 × 150 mm)	For semisolid culturing of microplants in vitro
Erlenmeyer flasks (150 and 250 ml capacity)/Melli-Jar bottles	For liquid culturing of microplants and microtuber production in vitro
Glassware/ plastic ware like conical flasks, volumetric flasks, beakers, measuring cylinders, graduated pipettes, Pasteur pipettes, test tubes, Petri dishes, reagent bottles.	For media preparation and other laboratory activities
Parafilm.	For sealing the culture tube mouths and Petri dishes.
Stainless steel forceps, scissors, scalpel handle (No. 11), sterile surgical blades (No. 11), sterile syringes, needles.	For aseptic operations inside the laminar flow workstation, and meristem excision and culture.
Filter papers Watman (No. 1), net filters, autoclave indicator tapes, polypropylene closure caps, cotton plugs, aluminum foil, steristoppers, tissue papers, blotting papers.	For general purpose laboratory use in the tissue culture laboratory.
Plastic pots, earthen pots, polythene packets, autoclavable packets, aluminum tags, labels.	For transplantation and laboratory disposable purpose.
Lab coats, protective goggles, slippers, disposable latex gloves, protective hoods.	For general purpose laboratory uses.
Glass marker pens, laboratory notebooks, measuring ruler, data sheets.	For recording data, observations.
Fire extinguishers, first aid boxes.	For general laboratory safety.

Annexure-III

Protocols for Quality Potato Seed Production Through Micropropagation

Protocol 1: Potato meristem culture

The protocol for potato meristem culture consists of (i) selection and testing of apparently healthy plants from the field or harvested tubers, (ii) establishment of *in vitro* cultures, and (iii) virus elimination through meristem culture

Selection and testing of plants/tubers

- Select apparently healthy plants from the field or sample tubers.
- Test these plants/tubers for freedom from viruses using enzyme-linked immunosorbent assay (ELISA) or any other method.
- If no plant/tuber is found free from all viruses then one has to resort to meristem culture.
- Select a plant/tuber that is infected with minimum viruses for use in meristem culture.

Establishment of in vitro cultures

- *From infected plant:* Excise nodal stem segments from the third and fourth nodes from the stem apex. Each nodal cutting should be 1-2 cm long, and the leaves should be detached. Such single node cuttings (SNCs) are used to initiate *in vitro* cultures.
- *From infected tuber:* Treat the freshly harvested tubers with a fungicide for 15 min and dry them. If not required for immediate use, the tubers can be stored at 4 °C till dormancy breaking. For immediate use, give dormancy breaking treatment and allow the tubers to sprout in dark at 24 °C. Harvest sprouts measuring about 2-3 cm long.
- In the laminar flow clean air work station, surface sterilize the SNCs/sprouts for 8-10 min in 20% of commercial sodium hypochlorite solution (4% w/v available chlorine), rinse in sterile distilled water three times, trim both ends of the explants by a scalpel and place the explants inside culture tubes (25 x 150 mm) each containing 13 ml of semisolid propagation medium. The propagation medium is based on MS (Murashige and Skoog, 1962) basal nutrients supplemented with D-calcium pantothenate (2 mg/l), gibberellic acid (0.1 mg/l), a-naphthaleneacetic acid (0.01 mg/l) and 30 g/l sucrose. The medium is solidified with 7.0 g/l agar.
- Incubate the cultures under a 16 h photoperiod using cool white fluorescent lights (50-60 $\mu E/m^2/s$ light intensity) at 24 °C.

- Allow the explants to grow up to 6-8 nodes stage, and then subculture through SNCs on fresh medium under the cultural conditions described above. Shoot cultures can be maintained and multiplied *in vitro* by subculturing on fresh medium after every 3 weeks.

Virus elimination

Thermotherapy

Thermotherapy is given to *in vitro* cultures or tubers prior to meristem culture. This is done as under:

- Place 7-day-old cultures in a thermotherapy chamber or BOD incubator at 37 °C under a 16 h photoperiod at 20-30 $\mu E/m^2/s$ light intensity, and incubate for 3 weeks.
- Treat infected tubers with GA_3 (2mg/l) and allow them to sprout at 37 °C under dark till 2-3 cm long sprouts are formed.
- Dissect meristems from the *in vitro* plantlets/sprouts by the method described below.

Meristem excision and culture

- Excise meristems (terminal as well as axillary) from thermo-treated *in vitro* plantlets/ sprouts under laminar flow cabinet using a stereoscopic zoom microscope, scalpel and needle. Protective leaves on the buds should be removed carefully using a needle. Use a drop of sterile distilled water to avoid meristem desiccation during excision.
- Trim the meristematic dome plus one set of leaf primordia with a scalpel to 0.2-0.3 mm.
- In case of sprouts, surface sterilization of sprouts using 20% of commercial sodium hypochlorite solution is essential before dissecting meristems.
- Place the excised meristems on semi-solid meristem culture medium in a culture tube (1 meristem/culture tube), and incubate the cultures at 24 °C under a 16 h photoperiod with 50-60 $\mu E/m^2/s$ light intensity).
- Several meristem culture media have been reported in the literature. The meristem culture medium used at the Central Potato Research Institute, Shimla, India is based on MS basal nutrients supplemented with 2mg/l D-calcium pantothenate, 0.1 mg/l GA_3, 0.01mg/l NAA and 30 g/l sucrose, and solidified with 6.0 g/l agar. For chemotherapy, meristem culture medium is also supplemented with suitable concentration of anti-viral compounds.
- It takes about 5-6 months for meristems to grow into full plantlets (mericlones). At this stage sub-culture the plantlets individually for maintaining their clonal identity.
- Test meristem-derived plantlets for freedom from viruses by ELISA.
- Multiply and maintain virus-negative counterparts of meristem-derived clones by single node culture *in vitro* as described above.

Protocol 2: *In vitro* multiplication of mericlones

- Multiply disease-free stock plants obtained through meristem culture using nodal cuttings. Cuttings with 1-2 nodes each are cultured in tubes (25 x 150 mm) on semisolid propagation medium following the procedure described under Protocol 1. To economize on inputs, use of high quality commercial sugar in place of sucrose and 3 nodal cuttings in each tube are recommended.
- Incubate the cultures at 24 °C under a 16 h photoperiod using cool white fluorescent lights (50-60 $\mu E/m^2/s$ light intensity).
- Sub-culture plants after every 20-25 days depending on growth response.
- Large number of *in vitro* plants produced by this method are used for microtuber/ minituber production.

Protocol 3: Microtuber production

Microtuber production technology involves: (i) mass multiplication of *in vitro* plantlets in liquid medium, (ii) production of microtubers, and (iii) harvesting and storage.

Mass micropropagation in liquid medium

- Initiate liquid propagation cultures in 250 ml Erlenmeyer flasks or magenta boxes. In each flask or magenta box, pour 20 ml liquid propagation medium (composition same as propagation medium given in protocol 1, except no agar is used) and autoclave.
- Inoculate 10-12 stem segments (each having 3-4 nodes) obtained from 20-25-day-old plantlets in each flask/box.
- Incubate the liquid cultures stationary at 24 °C under a 16 h photoperiod with 50-60 $\mu E/m^2/s$ light intensity ensuring that the explants are not submerged in liquid medium.
- In about 3 weeks all the axillary buds grow into full plants and fill the container.

Microtuber production

- After 21 days of incubation, decant the liquid propagation medium from the Erlenmeyer flask or magenta box under aseptic conditions of a laminar flow workstation, and pour in 40 ml of microtuber induction medium. The microtuber induction medium is based on MS basal nutrients supplemented with 10 mg/l N6-benzyladenine (BA), 500 mg/l chlorocholine chloride (CCC) and 80 g/l sucrose.
- Incubate these induction cultures under complete darkness at 20 °C. Microtubers start developing epigeally at the terminal or axillary ends of the shoots within 8-10 days and they are ready for harvesting after 60-90 days depending upon genotype. In general, 15-20 microtubers with an average weight of 100-150 mg are produced in each flask or magenta box.

Harvesting and storage

- Before harvesting, green the microtubers by exposing the induction cultures (without removing plugs/lids) to white fluorescent lights or diffused sunlight for 10-15 days.
- Hand-harvest the green microtubers in plastic trays. Avoid damaging the microtubers during harvesting.
- Wash harvested microtubers under running water to remove adhering constituents of the medium. Treat harvested microtubers with fungicide (mancozeb 78% WP @ 0.2%) for 10 min. and allow them to dry in the dark at 20 °C for 2 days.
- Pack the dried microtubers in perforated polyethylene bags and store at 5 °C in a refrigerator for 4-5 months under dark for breaking the dormancy.
- After 3-4 months of storage, the microtubers are taken out for sprouting and field planting

Protocol 4: Minituber production

Healthy minitubers can be produced from *in vitro* plants as well as microtubers by growing on nursery beds under aphid proof net houses.

- Harden 15-20 day old *in vitro* plants by keeping culture vessels in greenhouse under normal sunlight for 8-10 days without removing culture tube plugs or lids of magenta boxes.
- Remove hardened plants with the help of forcep, cut and remove root zone, wash lower portion of plants to remove adhering medium, cut plants into two pieces from middle, dip 0.5-1 cm lower portions of the cuttings in commercially available rooting hormone powder and transplant on pre-prepared nursery beds at 10 x 10 cm spacing.
- Irrigate the transplants with cotton swabs pre-soaked in water or with sprayer for initial one week and thereafter irrigate judiciously with the help of sprinkler/watering can/mist/sprayer, taking care to avoid over-or under-irrigation.
- The ideal conditions for establishment are temperatures in the range of 20-25 °C and humidity above 75%. Shading of the plants for initial 4-5 days to protect them from direct sunlight is beneficial.
- Sprouted microtubers are hardy and can be directly planted on nursery beds at 10 x 10 cm spacing. Microtubers can be irrigated with the help of watering can/sprayer.
- With progressive growth of plants, add soil-FYM mixture in the rows to bury maximum number of nodes under the soil to get maximum tuber yield.
- Allow the crop to mature on nursery bed and at maturity harvest minitubers (8-12 per plant) having an average weight of about 10 g.
- The harvested minitubers are cold stored and used for growing seed crop in next season.

Annexure-IV

Proposed Standards for Tissue Culturally Grown Potato Seed

The following standards for tissue culturally grown potato seed as proposed by Naik and Khurana (2003) are under the consideration of Department of Biotechnology, Ministry of Science & Technology, Government of India.

Laboratory and Greenhouse Facility Requirements

- All micropropagation and greenhouse facilities must be approved by an appropriate certification authority. Score card for assessing tissue culture and greenhouse facilities developed in India is given below.
- Laboratory and greenhouse facilities used for production of plantlets/microtubers or minitubers should be maintained free from potato pests or vectors of potato pathogens.
- All potting or growth media should be sterile and free from pathogens, pests and volunteers. Water sources used in laboratory or greenhouse operations should also be free of potato pathogens and pests.
- Suitable precautions should be taken in micropropagation practices, potting, planting, irrigating, and other laboratory/greenhouse practices to guard against the spread of diseases or pests within the facilities.
- All details of the material propagated must be maintained for inspection by the certification authority. These may include inventory of all plant material in the laboratory, origin of initial material, individual records for each line showing step-by-step flow through various multiplication stages, number of plants/microtubers/minitubers on inventory, test reports etc.

Score card for assessing tissue culture and greenhouse facilities in India

A group constituted by the Department of Agriculture and Cooperation, Ministry of Agriculture, Government of India, has designed a score card for evaluating tissue culture and greenhouse facilities for distribution of micropropaged planting material. The parameters and scores for various components are given in Table 10. The laboratory facilities including hardening facilities carry maximum marks of 60 followed by quality control with 30 marks. These facilities are most crucial for production and supply of disease free planting material. Technical supervision and monitoring are also important which involve strict supervision of all the activities that are performed in the tissue culture unit (media room, inoculation room, growth room and hardening area). The technical competence of the supervisory staff will also have a bearing on the output.

The tissue culture units scoring an overall of less than 65 are not be considered eligible for distribution of micropropagated planting material till such time when the facilities are improved as per norms.

Table 10. Parameters developed in India for evaluating micropropagation facilities.

1. INFRASTRUCTURE		
(A) Laboratory Facilities ***Washing room*** • Facilities for washing, drying and storing of glassware • Quality of washing • Overall cleanliness	(a) Depending on the volumes, washing may be done manually or through a machine but the quality of washing must be good. (b) Contaminated cultures should not be stored. They should be washed as soon as possible. (c) All the contaminated cultures must be autoclaved before washing with a detergent. If the contamination levels are very high then the glassware (infected cultures only) should be left overnight in chromic acid after autoclaving and washed with detergent the following day. (d) The glassware must be washed under running tap water to ensure that no traces of media or detergent are left behind (e) After washing with ordinary water, the culture vessels should be rinsed with deionized water before drying. (f) Drying may be done by leaving the jars in an inverted position overnight. Petriplates and other glassware may be dried in an oven. (g) There should be a proper mechanism for disposal of used agar. (h) Overall cleanliness must be maintained.	5 Marks
Media preparation room • Availability of equipment for media preparation and autoclaving • Quality of chemicals • Quality of culture vessels • Maintenance of records • Operational efficiency of media preparation (amount of media prepared everyday, proper labeling of media, etc.) • Cleanliness	(a) The media preparation room must have all the basic equipment such as weighing balance (electronic), pH meter, conductivity meter, microwave oven, deionizer/ distillation unit/RO water facility, autoclave, etc. (b) The chemicals should be of analytical grade from a reputed company. (c) The details of the media must be recorded and the trays/racks containing media should be properly labeled. (d) All the parameters pertaining to autoclaving such as the time when the autoclave was switched on, when the desired pressure was achieved, autoclaving time, etc. must be recorded. (e) As much as possible, high operational efficiency should be maintained to save on manpower. (f) After autoclaving, the medium should ideally be stored for 2-3 days so that if something has gone wrong with autoclaving, microbial contamination is detected before the medium is put to use, (g) The medium must be stored in clean area where very high level of sterility (at least Class 1000) is maintained.	10 Marks

Inoculation room • Equipment • Sterility levels • Technical competence of the operators • Operational efficiency (number of cultures handled by each operator, labeling of cultures, contamination losses, etc.)	(a) The inoculation room should have at least sterility level of Class 1000. (b) The room must be fumigated periodically with sterilant. (c) The airflow of the laminar airflow cabinet should be checked periodically. (d) Besides flaming, the tools (forceps, scalpels, etc.) should also be autoclaved periodically. (e) Instead of rectified spirit, use of glass bead sterilizers should be favoured as the former is a potential fire hazard. (f) Regular monitoring of air borne microbes in the laboratory is must. (g) Operators working in the laboratory must remove their foot wears outside the room and wear clean (preferably autoclaved) laboratory coats. (h) During sub-culturing, at a time only one clone/genotype should be handled to avoid any mixing. (i) Due emphasis should be given to the efficiency of the operators (the number of jars handled, multiplication rates, contamination losses, etc.) (j) Proper record of species, clone, passage number, media operator names, etc. should be maintained.	10 Marks
Growth room • Availability of equipment such as culture racks, shakers, etc. • Adequate facilities to maintain stringent conditions for temperature and RH. • Sterility levels.	(a) The growth room should be equipped with racks, AC, heat convector, temperature and humidity controller, photo period stimulator, shakers etc. (b) High sterility levels (Class 10000) should be maintained with periodic check on airborne contaminants. (c) The room must be fitted with UV lights. It should also be fumigated periodically especially during the rainy season to keep the contamination under control. (d) Restricted entry.	10 Marks
(B) Hardening Facilities ***Transfer area*** • *Ex vitro* management. • Selection of proper container and potting mix.	(a) Only one clone to be washed at a time (b) Hardening trays should be properly labeled (c) Selection of the hardening container and potting mix to be done as per the requirement of the species (d) Drying of plants should be avoided by transferring them to the mist room/greenhouse immediately after transfer to the potting mix. (e) Water used for irrigation must not be hard (rich in salts) (f) Excessive watering of plants to be avoided. (g) Due consideration should be given to the texture and pH of the soil used for hardening. (h) All records pertaining to number of plants transferred, date of transfer, etc. should be maintained for future reference.	10 Marks

Greenhouse/polyhouse/ shade area • Necessary facilities for proper hardening of plants through adequate control of temperature and RH	(a) Stringent control of temperature and RH (b) There should not be any leakage for the inside air to escape. (c) Facility for ventilation to control excess RH during rainy season (d) Excessive watering of plants to be avoided (e) It must be ensured that direct sunlight does not fall on the plants but at same time there should be sufficient natural light in the greenhouse. (f) Adequate provision for artificial light for species that are high light demander (g) Plants should be monitored regularly for their growth and presence of any disease or pest. (h) Dead plants should be removed immediately to avoid any possible attack of saprophytic fungi. (i) Fungal infection in greenhouse particularly during rainy season is very common. If present, the plants should be sprayed with suitable fungicides. (j) Wherever possible, use of compost at the greenhouse stage should be avoided because that may invite contamination. (k) Any kind of treatment given to the plant such as fertilizer, fungicides, pesticides, etc. must be recorded for reference just in case something goes wrong with the plants. (l) All mortalities taking place in the greenhouse/ polyhouse should be recorded to arrive at the transplantation losses.	10 Marks
Nursery • Adequate space and facilities for irrigation • Proper management	(a) Nursery should have some shade area where the plants could be kept till they are hardened enough to be kept under direct sunlight. (b) Only fully decomposed organic manure to be used. Partially decomposed manure will do more harm than any good to the plant. (c) There should be adequate facilities for irrigation. (d) Nursery beds should be properly leveled so as to avoid any water-logging (e) Regular weeding (f) Regular shifting of plants to prevent the roots from entering the ground.	5 Marks
2. QUALITY CONTROL		
Selection of clones and maintenance of germplasm • Selection of high yielding clones. • Maintaining the germplasm in proper disease-free conditions	(a) Following points must be recorded while selecting the mother plant: - Geographical location of the mother plant or the area where mother plant is growing - Micro-climatic conditions prevailing in that area - Various growth attributes of the mother plant (height, diameter of the stem, yield, etc.) - Origin of the mother plant (seedling raised or vegetatively raised)	

	(b) High yielding clones should only be used for micropropagation work (c) The mother plants should be maintained in disease-free environment so the chances of initiating aseptic cultures remain high.	5 Marks
Explant • Apical or axillary bud	(a) Choice of the explant is a critical factor in the success of the micropropagation protocol. Since axillary branching method is the most favoured method for *in vitro* clonal propagation, only apical or axillary bud should be used as the explant for micropropagation work. While excising the explant from the mother plant, following points must be properly recorded:- - Location of the explant on the mother plant. - Season (month) in which the explants have been derived - Any pre-treatment given to the mother plant before excising the explant.	5 Marks
Virus indexing • Testing the plants for known viruses and ensuring their elimination before micropropagation	(a) Before starting with the micropropagation work, the material should be tested for the presence of the known viruses. (b) If the presence of virus is established then these must be eliminated through meristem culture or a combination of techniques (c) Only virus-free tissue should be used for further micropropagation work.	5 Marks
Number of multiplication cycles and clonal uniformity • Number of multiplication cycles • Ensuring that multiplication is only through axillary shoots and not adventitious • Ensuring clonal uniformity of plants by molecular methods • Carrying out field trials and confirming the yield before undertaking mass distribution of TC plants.	(a) In general the multiplication cycles should not exceed 10 passages. However, this number is not fixed and would vary with the species under consideration. (b) Operators should be thoroughly trained so that they can draw a distinction between the adventitious and axillary shoots. Only axillary shoots should be used for micropropagation work. (c) The plant tissue should be tested for the presence of systemic bacterial contamination by culturing the tissue after every 3-4 passages on LB medium. (d) Clonal uniformity may be established morphologically through field trials. (f) Proper field data must be collected and appropriately analysed.	10 Marks

Overall quality of the plants	(a) At the time of dispatch it must be ensured that the plants are fully hardened and are of transplantable size. (b) A small handout giving all necessary information about after-care of the tissue cultured plant of that particular species should be provided to all growers for reference.	5 Marks
3. TECHNICAL SUPERVISION AND MONITORING		
Monitoring of the production process and the staff involved therein	Strict monitoring of the entire production process covering all the activities that are performed in media room, inoculation room, growth room and hardening area is a must.	10 Marks
Technical competence of the production supervisory staff	(a) The managers, scientists and the supervisory staff involved in production must have very sound technical knowledge of the subject so that they could deal with any eventuality that may arise during course of production. (b) There should be at least two supervisors (one in the clean area to monitor laboratory activities and one in the hardening area for after care and for monitoring field activities) in the production facility	
Operators	The operators may or may not have very sound scientific background but they must be thoroughly trained by the supervisors and the professional staff before they undertake any skilled job such as media preparation or inoculations.	

Note: Besides various parameters indicated above, the cost of plantlet production would also be very important.

FARM REQUIREMENTS

- Seed potatoes can not be grown in areas/fields having history of wart (*Synchytrium endobioticum*), bacterial wilt (*Ralstonia solanacearum*) and nematodes (*Globodera rostochiensis* and *G. pallida*)
- Boundaries of seed potato fields must be clearly defined.
- Adequate separation (isolation) from fields growing uncertified potato must be maintained.
- Seed potatoes should not be planted on the farm that was cropped with potatoes the previous year, unless the farm is fumigated.
- There must be clear demarcation between different varieties and classes of seed potatoes.
- All equipment and storage facilities in the potato operation must be used only for the field entered for certification. In case these have been used on other farms, they need to be properly disinfected before reuse in seed plot.
- Equipment and stores must be clean and disinfected periodically but at least once annually.

Minimum Quality Requirements

Pre-Nuclear (In vitro Production)

Any potato material can be used to initiate Pre-nuclear stock, provided the following requirements are met:

- The material being initiated must be of a known varietal/clonal identity and must be duly documented with respect to origin.
- All samples of a potato variety/clone being initiated must be tested by an accredited laboratory and found free from PVA, PVS, PVM, PVY, PVX, PLRV, PSTV, endophytic/epiphytic bacteria and fungi and also nematodes.
- The initiating facility uses recognized aseptic initiation and propagation procedures (i.e. follows procedures and uses equipment, which will maintain sterile conditions).
- The initiating facility must maintain information on variety/clone identification, date of initiation, origin and testing results from accredited laboratory for each variety/clone for review and audit by the Seed Certification Authority.
- Pre-nuclear stocks need to be developed every year from certified *in vitro* plants after subjecting them to "Grow Out" test in greenhouse and authentication of varietal purity and pathogen-freedom. Varietal purity needs to be established by an accredited organization using morphological characters and DNA fingerprinting.

Certification of Pre-Nuclear Stock

On application for inspection, the Pre-nuclear *in vitro* stocks as developed above are eligible for certification. The micropropagation facility to be inspected must have been approved by the Seed Certification Authority. Each variety/clone must have a valid disease-testing report at any time during multiplication process. Tests must be carried out on a minimum of two plantlets for each variety/clone by an accredited laboratory. Such tests will be valid for a period of one year. No plant should contain PVA, PVS, PVM, PVY, PVX, PLRV, PSTV, endophytic/epiphytic bacteria and fungi and nematodes.

Valid disease testing results are required prior to the initiation of micropropagation and microtuber production cycle.

Every container showing visual evidence of bacterial or fungal contamination must be removed from the micropropagation facility.

Nuclear (Greenhouse or Controlled Environment) Seed Production and its Certification

The following requirements must be met for the multiplication of Nuclear stock in a protected environment:

- The grower must notify the Seed Certification Authority of the production plans well in advance of the planting.
- The crop must be grown from certified Pre-nuclear *in vitro* plants or microtubers. Small minitubers of certified Nuclear stage produced previous year, which are too small for field

planting can also be used for planting. But these minitubers must be planted in a separate greenhouse.

- If pathogen testing is required, it must be carried out on well grown plants on a representative sample, which consists of 1% of the plants or tubers with a minimum of 5 and a maximum of 25 plants or tubers sampled for each variety/clone.
- The protected environment must be "vector proof" and be equipped with a double-door entrance, provision for footwear disinfecting prior to entering the protected environment and aphid proof ventilation screening on air inlet and outlet openings.
- Effective sanitation practices including insect and disease monitoring and prevention must be adhered to.
- Nuclear stock can be planted in commercially available medium, which has not been recycled. If nursery beds are used, a new or clean physical barrier from the growing medium must separate the underlying soil. If containers are used, they must be new or clean.
- The facility must be free from all potato and solanaceous plant debris before planting.
- No field-produced seed potatoes (including pathogen tested clonal selections), non-seed potatoes, nor any other solanaceous species of plants can be grown in the protected environment while used to produce Nuclear Stock.
- Varieties/clones must be separated by physical barriers, which will prevent varietal mixture.
- The crops and the facility must be got inspected by the Seed Certification Authority, at least once during the growing cycle. The inspection must take place on well grown plants. Depending on the condition of the crop, the inspector may take leaf samples for laboratory testing to determine if the crop is free of pathogens.
- If testing reveals the presence of PVA, PVS, PVM, PVY, PVX, PLRV or PSTV, the crop needs to be assigned next appropriate class but should not be certified as Nuclear stock.
- If testing performed by an accredited laboratory reveals the presence of banned virus(s), fungus or bacteria all the crop in the protected environment will be ineligible for certification.
- In the eventuality of detection of insect vectors by Seed Certification Authority, the grower must provide post harvest test results to this authority. A representative sample, representing each variety/clone grown in the protected environment must be post harvest tested and if the results are negative for PVS, PVM, PVA, PVY and PLRV, the crop should be assigned a Nuclear stock status. Any crop, testing positive for one of the above mentioned pathogens should be assigned appropriate seed class and allowed to be grown for the next generation by the owner only.

Transfer of Pre-Nuclear and Nuclear Stocks

An official Nuclear stock certificate issued by the certifying agency must accompany pre-nuclear or Nuclear stock being transferred to a new owner. The certificate may contain information on the date and number of certificate, name of the variety, type of propagules

transferred (i.e. microtubers, micro-cuttings, or minitubers), amount of propagules transferred, grower's name and address and consignee's name and address.

Nuclear stock once transferred to a new owner, should not be re-used to produce nuclear stock again.

Field Inspections and Certification of Field Generations

The nuclear seed is further field multiplied twice (G-1 and G-2 stages) to produce basic seed. In G-1 and G-2 stages, visual inspections will be carried out twice during the season for pathogen freedom. In G-1 stage a minimum of 250 leaves collected from separate plants or 1% of the hills will be tested for PVX by an accredited laboratory. Tolerance for PVX in this stage shall be 0%. In G-2, 60 plants/ha shall be tested for PVX in the laboratory and the permissible tolerance should be 0.4%, (the possible tolerance limits for G-1 and G-2 will vary from country to country but should be within acceptable international standards). Further field generations for production of certified seed should be subjected to existing seed certification standards of the country.

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