eTechnical Bulletin No. 1
(Ref.: No.F.PME/3-3/2021 dated 4.7.2021)

Plant Tissue Culture and Somatic Hybridization in Potato

Authors

Jagesh Kumar Tiwari

Tanuja Buckseth

Dalamu

SK Luthra

Rajesh Kumar Singh

Vinay Bhardwaj

Vanishree G

Hemant B. Kardile

Vinod Kumar

Manoj Kumar

ICAR - CENTRAL POTATO RESEARCH INSTITUTE, SHIMLA (HP)

[I]. PLANT TISSUE CULTURE

1. Introduction

Potato (*Solanum tuberosum* L.), a member of the family *Solanaceae,* is one of the best examples of crop plants to which biotechnology has been most extensively applied in all aspects of genetic improvement, germplasm handling and high-tech seed production. Meristem culture was the first biotechnological approach successfully applied to obtain virus-free potato clones. During the last few decades, problem-driven use of *in vitro* technology in potato had been instrumental in addressing many inherent problems associated with this vegetatively propagated, heterozygous and tetraploid crop. The propagation method and genetic nature of this crop imposes several limitations on seed multiplication, conservation of genetic resources and genetic improvement. Micropropagation of disease-free potato clones combined with conventional multiplication methods has become an integral part of seed production in many countries including India. Several *in vitro* methods including the cryo-conservations have been applied in India for conservation of valuable potato genetic resources which is the largest in Asia. The developments in the fields of cellular selections, somaclonal variations, somatic hybridization and genetic transformation have not only improved present-day potato, but also generated novel genetic variability for selection of future potato cultivars. Recent advances in molecular breeding *vis-a-vis* marker/genomics-assisted selection have opened new avenues for the breeders to tap desired genetic variability more efficiently and to exploit it across trans-specific or generic barriers. In India, most of the technologies like tissue culture (micropropagation, meristem-tip culture, and long-term conservation), genetic engineering and molecular breeding are routinely being used for research and development in potato at Indian Council of Agricultural Research (ICAR) - Central Potato Research Institute (CPRI), Shimla, Himachal Pradesh. Efficient regeneration protocol has been standardized for genetic transformation work and application of recently developed genome editing technique in potato. Molecular markers are being utilized for characterization of potato genotypes, marker-assisted selection and QTL mapping. Gene cloning and designing of gene constructs for transformation work is also a priority area of the institute. Application of cellular biology techniques like protoplast fusion and regeneration of interspecific somatic hybrids is one of the key research area to utilize non-crossable wild species. Besides, immunological and molecular diagnosis of potato viruses is a major mandate of the institute. A brief account of the research work carried out along with achievements of ICAR-CPRI in the area of potato biotechnology is discussed here.

2. Micropropagation

Potato is a vegetatively propagated crop which is a good host for a large number of viruses besides other pathogens. Infection of planting material by pathogens mainly potato

viruses cause severe reduction in yield. Therefore, disease-free/healthy in vitro multiplied plantlets are used in germplasm conservation and conventional seed production. ICAR-CPRI, Shimla is designated as the National Active Germplasm Repository for conservation of potato germplasm in India. Presently more than 4500 accessions of cultivated and wild potato species, obtained from 30 countries, are conserved at the institute. Today nearly 2700 Tuberosum accessions of potato germplasm are maintained through tissue culture (in vitro) at the institute. Micropropagation allows large-scale multiplication of virus-free potato microplants. Nodal segments of virus-free potato microplants are cultured on semisolid or liquid medium under aseptic conditions for obtaining new microplants. Murashige and Skoog's (MS) medium supplemented with 2.0 mg/L D-calcium pantothenate, 0.1 mg/L GA3, 0.01 mg/L NAA and 30 g/L sucrose is best suited for propagation of potato microplants. Cultures are usually incubated under a 16-h photoperiod (50-60 μ mol $m^{-2}s^{-1}$ light intensity) at 24 ^{0}C. Usually, two-three nodal cuttings (1.0-1.5 cm) are inoculated per culture tube (25 x 150 mm), and the tubes are closed with cotton plugs. Within 3 weeks the axillary/apical buds of these cuttings grow into full plants. These plants can be further sub-cultured on fresh medium. At an interval of every 25 days of subculturing, theoretically 3^{15} (14.3 million) microplants can be obtained from a single virus-free microplant in a year.

Virus-free micro plants can be used for direct transplanting after hardening, in the fields or nursery beds for production of normal tubers or minitubers, respectively. Alternatively these plants can also be used for the production of microtubers in the laboratory. Microtubers are miniature tubers produced under tuber inducing conditions in vitro. These small dormant tubers are particularly convenient for handling, storage and distribution. Many protocols have been developed for induction of microtubers in vitro. Most of the published work on potato microtuberization is focused on the use of cytokinins, especially N6-benzyladenine (BA). Other substances like abscisic acid, chlorocholine chloride (CCC), NAA, triazoles, coumarine, acetic acid and jasmonic acid have also been used for induction of microtubers in potato. MS basal nutrient mixtures are universally used for potato microtuberization. Sucrose is the most effective carbon source, and an increase in its concentration to 8% induces early tuberization, whereas concentrations above 8% are inhibitory. Temperature and photoperiod are two important physical factors that affect potato microtuber induction in vitro. The optimum temperature for in vitro tuberization is 20 0C with a constant temperature being more effective than alternating day-night temperatures. Temperatures below 12^{0}C and above 28^{0}C have been found to be inhibitory to potato microtuber production. In general, optimum microtuberization occurs under continuous darkness during cytokinin-induced tuberization, but a longer photoperiod with higher light intensity is required when cytokinin is not used.

At ICAR-CPRI, Shimla microtubers are also induced in MS medium supplemented with 10 mg/L BA plus 80 g/L sucrose, and the cultures are incubated under complete darkness at 20 0C. Microtubers begin to develop epigeally 1-2 weeks after incubation depending on the genotype, and are harvested after 60-75 days of incubation. In general, 15-20 microtubers with an average weight of about 100-150 mg can be obtained from each flask/magenta box. Before

harvesting, the magenta boxes are shifted under diffused or artificial light at 20-30 0C for 10-15 days for greening the microtubers. Thereafter, green microtubers are treated with 0.2% Bavistin, dried at 20 0C, packed in perforated polythene bags, and stored under dark at 5-6 0C till dormancy release. These microtubers are planted on nursery beds under aphid-proof net houses (@ 50 microtubers/m^2) in seed producing areas of the Indian plains. The microtuber crop is allowed to mature in the nursery beds to produce minitubers. The virus-free minitubers thus produced constitute the pre-basic seed material for production of breeders' seed after 3-4 field multiplications.

In countries, where disease-free seed potatoes cannot be produced for want of vector-free production areas, micropropagation can play an important role to maintain and multiply commercial cutivars under disease-free conditions. In such countries, both microplants and microtubers can be used effectively for producing initial pre-basic (healthy) material in the form of minitubers. The ease with which microtubers are produced and handled also makes them more suitable for long distance transportation and use in potato seed production schemes. Minituber production is affected by genotype and crop husbandry including soil type, type of net house and planting density.

3. Meristem culture

Supply of healthy planting material is the backbone of seed potato industry. The only option to avoid losses caused by viruses is the selection of virus-free mother plants employing meristem tip culture followed by reliable and sensitive virus diagnosis techniques besides breeding virus resistant varieties. Potato is infected by over 30 viruses, which causes severe yield reduction. Potato viruses are systemic pathogens, and therefore, perpetuate through seed tubers. Since, there is no commercially available treatment to protect virus-infected plants, hence virus-free plant is necessary to ensure quality planting material by regenerating *in vitro* plantlets through meristem culture. The term 'meristem culture' denotes in vitro culture of meristematic dome of actively dividing cells located at the extreme growing tip of the shoot, along with a portion of the subjacent tissue containing one or two leaf primordia. This piece of tissue is about 0.1-0.3 mm in size. In the absence of chemical control of viral diseases, meristem culture is the only available method to eliminate viruses from systemically infected potato cultivars. This technique is based on the fact that in rapidly growing meristematic tips viruses are either absent or their concentration is very low. Despite the phenomenal success of meristem culture in elimination of plant viruses, it remains still unclear as to why the apical/axillary meristems contain a little or no virus? There are several hypotheses. Some of these are given below:

- Virus particles spread through vascular system but the vascular system is not developed in meristematic region.
- Chromosome replication during mitosis and high auxin content in the meristem may inhibit virus multiplication through interference with viral nucleic acid metabolism.

- Existence of virus-inactivating systems with greater activity in the apical region than elsewhere.

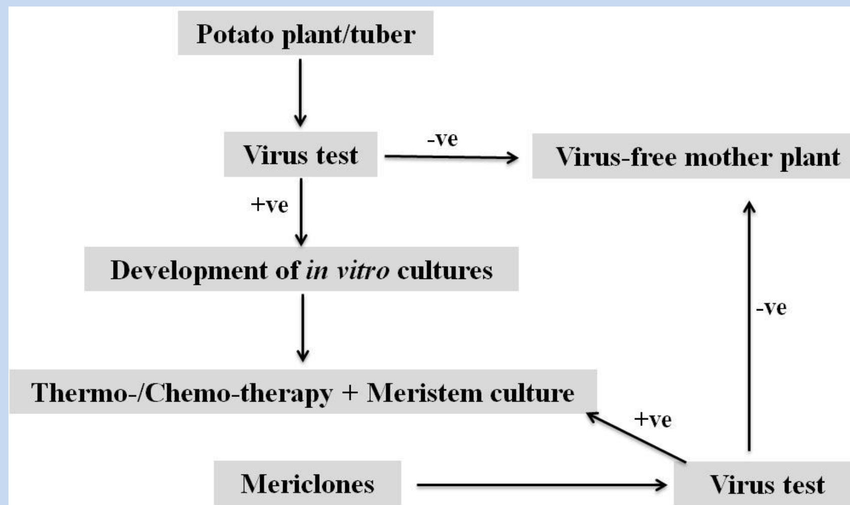

Fig. 1 An outline of major steps involved in potato meristem culture

Fig. 2. Figure showing meristem culture and conservation in vitro plantlets of potato

Various factors, like size of the explant, meristem location and cultural factors largely affect success of virus elimination by meristem culture. 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. As the distribution of a virus within a plant is uneven, especially towards the shoots tips, meristem of varying sizes are used to regenerate virus-free plants depending on the genotype and virus strains under consideration. It is difficult to excise apical meristems from terminal buds, because they have more rudimentary leaves and leaf primordia than the axillary buds. There is, however, no difference between the apical (terminal) and axillary meristems in terms of survival or freedom from virus infection. Therefore, axillary meristems are preferred to apical meristems in many laboratories for virus elimination. Although it is possible to eliminate viruses from potato plants following meristem culture alone, plant regeneration from meristems takes four to eight months, and sometimes depending on the nature of the virus, the percentage of virus-free plants obtained from regenerated meristems is low. As a result, meristem culture procedure is often combined with thermotherapy and/or chemotherapy to increase the likelihood of obtaining virus-free plants.

3.1 Thermotherapy

Meristem culture combined with thermotherapy is widely used for virus elimination in potato. The source plants infected with viruses are incubated in a growth chamber under light intensity of 30-50 μ mol $m^{-2}s^{-1}$ at 35-37 ^{0}C for 2-6 weeks. After respective periods of thermotherapy, the meristems are excised and cultured on nutrient medium for regeneration. Cold therapy followed by apical meristem culture has also been shown to successfully eliminate several viruses from infected plants. Viroids, some of which are quite resistant to elevated temperatures, have been effectively eliminated by cold therapy. Low temperature therapy (4-7 ^{0}C) followed by meristem excision and regeneration has been used to eliminate potato spindle tuber viroid (PSTVd) from infected potato plants.

3.2 Chemotherapy

Chemotherapy involves the use of chemicals like antiboitics, plant growth regulators, amino acids, purine and pyrimidine analogues to inactivate viruses or inhibit replication/movement of viruses in tissues. These chemicals can either be sprayed on growing plants prior to excision of meristems or incorporated into tissue culture media. As early as in 1954, eradication of PVX from potato tissue cultures by malachite green and thiouracil treatments was reported. Of all the chemicals tested for plant virus elimination, synthetic nucleotide analogues like ribavirin (Virazole: 1-D-ribofuranosyl-1, 2, 4- triazole-3-3carboxamide) and DHT (5-dihydroazauracil) have been particularly effective in inhibiting different plant viruses.

3.3 Electrotherapy

Electrotherapy of explants of infected potato plants has recently been reported to be an effective means for virus elimination. Potato stems infected with PVX were exposed to 5, 10 or 15 mA

for 5-10 minutes followed immediately by culturing of the shoot tips in vitro. The highest efficiency was obtained at 15 mA for 5 min, and about 60-100 % of the regenerated plantlets tested negative against PVX.

4. Virus detection

Even after taking all precautions to excise small meristem tips and subjecting them to various treatments favouring virus elimination, ultimately very few virus-free mericlones are obtained. Therefore, meristem-derived plants must be tested for virus freedom before using them as mother plants in micropropagation. Accurate, sensitive and rapid detection of potato viruses is critical for identifying virus-free mother plants and their integration into seed production programme. Several serological and nucleic acid-based assays are available for accurate detection and diagnosis of potato viruses at the institute.

In India, over 30 viruses are reported, of which PVX, PVS, PVY, PVA, PVM, PLRV, TolCNDV (PALCV) and GBNV are more important in potato. ICAR-CPRI is the first institute, among plant science research institutes of India, to introduce ELISA in 1984 and ISEM in 1987 for plant virus diagnosis. The virus diagnosis laboratory is now equipped with automated ELISA system, PCR and NASH facilities. Initially, immunodiagnostic protocols were standardized for detection of important viruses like PVY, PLRV and PVX that contributed largely in production of disease free seed stock. ELISA is still being used as the primary protocol for potato virus detection in seed production. Nucleic acid spot hybridization (NASH) protocol has been applied for potato spindle tuber viroid (PSTVd) that does not have a coat protein. The institute is also developing molecular diagnosis protocols for PVX, PVS, PVM and PVA. RT-PCR based detection of the above viruses has been achieved and validated for routine use in post-entry quarantine, screening of mericlones and *in vitro* microplants for virus freedom. Very recently, multiplex detection methods for multiple virus detections have been standardized using both PCR and RT-PCR techniques and are being used routinely for the detection of potato viruses. Lateral flow techniques (dip stick test) have been developed for the on field detection of multiple viruses and are made available to the end users at very reasonable rates at the institute.

[II]. Tissue Culture Protocols

1. Regeneration of *in vitro* plants from explants/TPS

Explants / true potato seeds (TPS) are the basic materials for regeneration of *in vitro* plants of potato genotypes. Explants/TPS are regenerated under aseptic conditions in laminar air flow to avoid contaminations in tissue culture of plants. These in vitro plantlets are used for conservation or mass multiplication through tissue culture methods.

Protocol

- All the materials to be used for in vitro regeneration should be sterilized in autoclave except explants/TPS.
- Prepare the explants from field/glass-house grown plants and cut into small pieces. If TPS, take it instead of cuttings.
- Wash the cuttings under the tap water for 20-30 min.
- Transfer the explants in a sterile beaker and rinse with sterile Milli-Q water.
- Sterilize the cuttings with 5% sodium hypochlorite for 20-30 min.
- Rinse the cuttings with sterile Milli-Q water (3-4 times).
- Again sterilize the explants with 0.1-1.0% mercuric chloride ($HgCl_2$) (0.1 g/100 ml water) for 5-10 min.
- Again rinse the cuttings with sterile Milli-Q water (3-4 times) to clean the mercuric chloride.
- Dry the cuttings and regenerate *in vitro* onto MS medium (pH 5.8) under aseptic condition in laminar air flow.

2. Murashige and Skoog (MS) Medium

Murashige and Skoog (MS) medium is the basic medium for tissue culture of potato. We can prepare MS medium as summarised below or can use ready-to-use MS medium from any firm.

Stock solution

MS Stock- 1

Chemical	**Strength (× 50)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
NH_4NO_3 1650 mg/L	20 ml MS Stock 1 for 1L medium	8.250 g	20.625 g	41.250 g	82.500 g
KNO_3 1900 mg/L		9.500 g	23.750 g	47.500 g	95.0

MS Stock- 2

Chemical	**Strength (× 100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
$MgSO_4.7H_2O$ 370 mg/L	10 ml MS Stock 2 for 1L medium	3.700 g	9.250 g	18.500 g	37.000 g
$MnSO_4.H_2O$ 16.9 mg/L		169 mg	423 mg	845 mg	1690 mg
$ZnSO_4.7H_2O$ 8.6 mg/L		86 mg	215 mg	430 mg	860 mg
$CuSO_4.5H_2O$ 0.025 mg/L		0.25 mg (1.0 ml)	0.625 mg (2.5 ml)	1.25 mg (5.0 ml)	2.5 mg (10.0 ml)
Dissolve 25 mg $CuSO_4.5H_2O$ in 100 ml dH_2O and then add required volume (within parenthesis) to MS 2 Stock					

MS Stock- 3

Chemical	**Strength (× 100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
$CaCl_2.2H_2O$ 440 mg/l	10 ml MS Stock 3 for 1L medium	4.400 g	11.000 g	22.000 g	44.000 g
KI 0.83 mg/L		8.3 mg	21.0 mg	41.5 mg	83.0 mg
$CoCl_2.6H_2O$ 0.025 mg/L		0.25 mg (1.0 ml)	0.625 mg (2.5 ml)	1.25 mg (5.0 ml)	2.5 mg (10.0 ml)
Dissolve 25mg $CoCl_2.6H_2O$ in 100 ml dH_2O and then add required volume (within parenthesis)					

to MS 3 Stock

MS Stock- 4

Chemical	**Strength (× 100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
KH_2PO_4 170 mg/L	10 ml MS Stock 4 for 1L medium	1.700 g	4.250 g	8.500 g	17.000 g
H_3BO_3 6.2 mg/L		62.0 mg	155 mg	310 mg	620 mg
$NaMoO_4.2H_2O$ 0.25 mg/L		2.5 mg (1.0 ml)	6.25 mg (2.5 ml)	12.5 mg (5.0 ml)	25.0 mg (10.0 ml)

Dissolve 250mg $NaMoO_4.2H_2O$ in 100 ml dH_2O and then add required volume (within parenthesis) to MS 4 Stock

MS Stock- 5

Chemical	**Strength (× 100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
$FeSO_4.7H_2O$ 27.8 mg/L	10 ml MS Stock 5 for 1L medium	278 mg	695 mg	1390 mg	2780 mg
$Na_2EDTA.2H_2O$ 37.3 mg/L		373 mg	933 mg	1865 mg	3730 mg

Store in amber colour bottle

MS Stock- 6 (Vitamins)

Chemical	**Strength** (× **1000)**	**100 ml**
Thiamine-HCl (0.1 mg/L)	1 ml MS Stock 6 for 1L medium	10.0 mg
Pyridoxine-HCl (0.5 mg/L)		50.0 mg
Nicotinic acid (0.5 mg/L)		50.0 mg
Glycine (2.0 mg/L)		200.0 mg

Store at 0°C

Weigh and add directly

- Myo-Inositol: 100 mg/L
- Sucrose: 20 g/L
- pH: 5.8
- Gelrite: 2 g/L
- Autoclave-sterilize: 121 °C for 20 min

[III]. SOMATIC HYBRIDIZATION

1. Introduction

A huge genetic diversity is available in *Solanum* species for various desirable traits. Wild *Solanum* species have been used in potato breeding but they represent only a small fraction to the total *Solanum* diversity. Huge efforts are involved in successful utilization of wild species to widen the narrow genetic base of the cultivated potato. Many useful genes of wild sources cannot be transferred to common potato through conventional breeding because of sexual barriers caused by the differences in ploidy level and endosperm balance number (EBN). However, it is now possible to overcome the sexual barriers using methods such as manipulation of ploidy and EBN, bridge crosses, mentor pollination, embryo rescue, hormone treatment, reciprocal crosses, and somatic hybridization. Somatic hybridization aims to strengthen the potato gene pool by introducing genes from wild species. This technique allows several advantages over conventional breeding and transgenic methods, such as: *i*) produces fertile somatic hybrids with target traits of wild *Solanum* species, *ii*) provides access to basic pre-breeding material for effective utilization in breeding, *iii*) enables easy transfer of monogenic and polygenic traits in one step, *iv*) results recombination of nuclear and cytoplasmic genomes, and *v*) avoids biosafety regulatory issues associated with transgenics. During the past 40 years, hundreds of somatic hybrids have been produced in plants including potato.

2. Protocols

Somatic hybridization is a method of protoplast fusion and regeneration of post-fusion products i.e. putative somatic hybrids between non-crossable wild species and cultivated potatoes. At ICAR-CPRI, following brief protocols are used for somatic hybridization.

- *In vitro*-grown microplants of 3 weeks old are used to isolate mesophyll protoplasts under sterile condition.
- *In vitro* plants are grown at 20°C for 48 h under a 16-h photoperiod in the dark prior to protoplast isolation.
- Cut young leaf tissues in a Petri dish containing Protoplast Digestion Solution (PDS) (10.0 ml digestion solution for 1.0 g leaf tissue).
- Incubate the chopped leaves under 16 h/dark/25℃ (on gyratory shaking at 40-50 rpm).
- Next day: Add 0.3 M KCl to the digestion medium in a 1:1 ratio.
- Filter the suspension through a 40-µm nylon mesh, and collect in 10 ml centrifuge tubes.
- Centrifuge the filtrate at 50 ×g (60 RCF) for 5 min, and then resuspend the pallet in 10 ml of 0.6 M sucrose (filter sterilized).
- Layer 1.0 ml of 0.3 M KCl onto this protoplast suspension, and centrifuge at 50 g for 5 min.

- Recover the live protoplasts (green) from the sucrose: KCl interface, and dilute with 10.0 ml of 0.3 M KCl.
- Centrifuge at 50 ×g for 5 min to pellet the protoplast. Resuspend the pellet in 0.5 M mannitol (sterile) to a final density of 1×10^6 protoplasts ml^{-1} for electrofusion.
- Electrofusion medium: 0.5 M mannitol (FS)/ pH 7.0-7.3/adjust pH with sterile 0.1 N NaOH.
- Symmetric fusion: 1:1 of each species under laminar work station.
- Dispense 50 µl Na-alginate 2.8%, prepared in 0.5 M mannitol in each box of 'castor rack' and mix well the 50 µl post-fusion products with sodium alginate.
- Grow post-fusion products in 5 ml VKMG (VKM Glucose) liquid medium and tightly wrapped the 'castor rack' with parafilm.
- Incubate the castor racks (containing post-fusion products mixed with sodium alginate into VKMG under dark at 25 °C for regeneration of microcalli for 4-6-12 months.
- Before transfer the microcalli onto solid medium, dissolve the sodium alginate film surrounding post-fusion products/microcalli in Dissolving Solution.
- After dissolving sodium alginate, wash microcalli in Washing Solution.
- Dissolving solution→ MS_{13K} Medium (Without hormones + Coconut water + Gelrite) + 20 mM or 50mM Na-Citrate dehydrate (pH=7.0)
- Washing solution→ MS_{13K} Medium (Without hormones + Coconut water + Gelrite) (pH=5.8)
- Finally transfer microcalli onto MS_{13K} Medium (solid) in a Petri dish for development of micro-shoots/plants.
- Transfer of microshoots/plants onto the MS Medium for *in vitro* regeneration and multiplication of putative somatic hybrids' plantlets.

A. Protoplast Digestion Solution (PDS) Preparation

SN	Chemicals	Strength	Medium volume (500 ml)
1.	MS Macronutrients		
	KNO_3	0.95 g/L	475.0 mg
	KH_2PO_4	0.085 g/L	42.5=43.0 mg
	$MgSO_4.7H_2O$	0.185 g/L	92.5=93.0 mg
	$CaCl_2.2H_2O$	0.660 g/L	330.0 mg
2.	MS Micronutrients (To be used as half strength)	× 1000	250 µl
3.	MS Fe-EDTA(MS stock 5) (To be used as full strength)	× 100	2.5 ml
4.	MS Vitamins (MS stock 6) (To be used as half strength)	× 1000	0.5 ml
5.	Myo-Inositol	100 mg/L	50.0 mg
6.	Polyvinylpyrrolidone (PVP) [AVM 10,000]	5.0 g/L	2.5 g
7.	MES (FW 195.24 for MES hydrate)	5.0 mM	488.0 mg
8.	D-Glucose (FW 180.16)	0.1 M	9.0 g
9.	D –Mannitol (FW-182.17)	0.4 M	36.434 g
10.	Cellulase 'ONOZUKA' RS or Cellulase ONOZUKA' R-10	1.0 %	Not to be added here
11.	Macerozyme R-10	0.5 %	Not to be added here

Notes:

- pH 5.7 to be adjusted using 0.1/1.0 N NaOH.
- Prepare the above digestion medium without digesting enzymes, adjust the pH and filter-sterilize.
- Store the medium in aliquots (20-25 ml) at -20°C.
- Before using, thaw the medium, add enzymes at required amount to medium aliquots, centrifuge to dissolve and check/adjust the pH (5.7), if required.
- Filter-sterilized (0.2 µm filter) and use.

B. Other Solutions

SN	Chemicals	Strength	Medium volume (500 ml)
1.	KCl (FW 74.55)	0.3 M	11.183 g
2.	Sucrose (FW 342.30)	0.6 M	102.69 g
3.	D-Mannitol (FW 182.17) (pH 7.0)	0.5 M	45.543 g
4.	Solution 2 (pH 7)		
	$CaCl_2.2H_2O$ (MW 147.02 g)	10 mM	220.53 mg
	D-Mannitol (FW 182.17 g) (pH 7)	0.5 M	150 ml

5.	Solution 3 (pH 7)		
	$CaCl_2.2H_2O$ (MW 147.02 g)	50 mM	1.1026 g
	D-Mannitol (FW 182.17 g) (pH 7)	0.5 M	150 ml
6.	Sodium Alginate solution		
	Na-Alginate (alginic acid sodium salt)	2.8%	2.8 g
	D-Mannitol (FW 182.17 g) (pH 7)	0.5 M	100 ml
7.	NaOH (FW 40)	1N	4.0 g/100 ml

Notes:

- Autoclave all solution at 121°C for required duration depending on the volume, and then filter-sterilize using a 0.2 µm filter.
- Use 1N NAOH to prepare 0.1N NAOH and filter-sterilize the solution in small aliquots.
- pH adjustment of Mannitol (pH 7) may take longer time, adjust with 0.1N NaOH.
- Na-Alginate solution may take longer time while dissolving solution.

C. VKM Medium Preparation

1. Macroelements

Chemical	Medium volume: 1 lit
KNO_3	1480 mg/L
KH_2PO_4 (FW 136.09)	68 mg/L
$MgSO_4.7H_2O$ (FW 246.48)	984 mg/L
$CaCl_2.2H_2O$ (FW 147.02)	735 mg/L

No need to prepare stock solution, add directly during medium preparation

2. VKM Stocks

Chemical	Medium volume: 1 lit
VKM stock-I	1 ml/L
MS stock-5	10.0 ml/L
VKM stock II	10.0 ml/L
VKM stock III	10.0 ml/L
VKM stock IV	10.0 ml/L
VKM stock-V	10.0 ml/L
VKM stock-VI	400 µl/L

3. Complex additives

Chemical	Medium volume: 1 lit
Casein Hydrolysate	250 mg/L
Coconut water (store at -20°C)	20 ml/L

Add directly during medium preparation. Coconut water to be added inside laminar

4. Growth hormone

Chemical	Medium volume: 1 lit
2,4-D (FW 221) (0.2 mg/L) (store at 0℃)*	200 µl/L
α-NAA (1.0 mg/L) (store at 0℃)*	1000 µl/L = 1 ml/L
Zeatin (trans) (FW 219.2) (0.5 mg/L) (store at -20℃)**	500 µl/L

*Separate stock of 1 mg/ml (dissolved in NaOH and water)
**Dissolved Zeatin-vial, supplied by manufacture, in 100 µl KOH and make up water volume to prepare stock of 1 mg/ml. (e.g. 10 mg/10 ml)

5. Sugar: Glucose (0.5M) - 90.1g for 1 litre medium
Note:

- Adjust medium pH: 5.6-5.7 with 0.1 M KOH.
- VKM medium is used for the division and growth of protoplast.
- Please note, that-VKM medium must be filter-sterilized using 0.2µ membrane filter (cellulose acetate/cellulose nitrate + 0.4µ pre filter (glass fiber).
- NO AUTOCLAVING

D. VKMG (VKM Glucose) medium

- VKM medium must be filter-sterilized using 0.2 µ membrane filter (cellulose acetate) + 0.4 µ prefilter (glass fibre)
- Weigh 9.01 g of 0.5 M Glucose in 100 ml VKM and adjust pH 5.6-5.7 with 0.1 M KOH

E. VKM STOCKS

Composition of VKM Stock for preparation of VKM Medium

a) VKM Stock-I

MICRO ELEMENTS	mg/L	Stock (×1000)
H_3BO_3 (FW 61.83)	3.0	300 mg/100 ml
$MnSO_4.H_2O$ (FW 169.02), OR $MnSO_4.4H_2O$, OR $MnSO_4$ (anhyd)	8.0 10.0 7.0	800 mg/100 ml 1000 mg/100 ml 700 mg/100 ml
$ZnSO_4.7H_2O$ (FW 287.54)	2.0	200 mg/100 ml
KI (FW 166.01) (Store in amber colour bottle)	0.75	75 mg/100 ml
$Na_2MoO_4.2H_2O$ (FW 241.95)	0.25	25 mg/100 ml
$CuSO_4.5H_2O$ (FW 249.68)	0.025	2.5 mg/100 ml*
$CoCl_2.6H_2O$ (FW 237.93) (store in amber colour bottle)	0.025	2.5 mg/100 ml*

1.0 ml VKM Stock-I for 1.0 L medium, Store at 2-8ºC

* Prepare 1 mg/ml separate stock of each and add 2.5 ml to prepare VKM Stock-I

b) MS Stock-5

Chemical	mg/L	Stock (×100)
$FeSO_4.7H_2O$ (FW 278.01)	27.8	278 mg/100 ml
$Na_2EDTA.2H_2O$ (FW 372.24)	37.2	373 mg/100 ml
10.0 ml MS Stock-5 for 1.0L medium		

c) VKM Stock-II

Sugar & Sugar alcohol	mg/L	Stock (×100)
d-Mannitol (FW 182.17)	250.0	2.5g/100 ml
d-Sorbitol (FW 182.17)	250.0	2.5g/100 ml
Sucrose (FW 342.3)	250.0	2.5g/100 ml
Fructose (FW 180.2)	250.0	2.5g/100 ml
Ribose (FW 150.1)	250.0	2.5g/100 ml
Xylose (FW 150.1)	250.0	2.5g/100 ml
Mannose (FW 180.2)	250.0	2.5g/100 ml
Rhamnose (FW 182.2)	250.0	2.5g/100 ml
Cellobiose (FW 342.3)	250.0	2.5g/100 ml
m-Inositol (180.16)	100.0	1.0g/100 ml
Glucose*	90,000 (90 g)	9.0 g/100 ml
10 ml VKM Stock-II for 1.0 L medium, Store at 2-8℃		

*Glucose not to be added in the stock; to be added while preparing the medium at the end.

- Filter-Sterilize (FS) this stock and to be added inside the laminar work station (while preparing the medium)

d) VKM Stock-III

Organic acid	mg/L	Stock (×100)
Sodium-pyruvate (FW 110.04) (Pyruvic acid-sodium salt)	20.0	200 mg/100ml
Citric acid (FW 192.12)	40.0	400 mg/100ml
Fumaric acid (FW 116.07)	40.0	400 mg/100ml
Malic acid (FW 134.09)	40.0	400 mg/100ml
10ml VKM Stock-III for 1.0 lit medium, Store at 0ºC		

Filter-Sterilize this stock and to be added inside the LFCA Work station (while preparing the medium

e) VKM Stock-IV

Vitamin	mg/L	Stock (×100)
Calcium-D-pontothenic acid (FW 283.3)	1.0	10 mg/100ml
Choline chloride (FW 139.63)	1.0	10 mg/100ml
Ascorbic acid (FW 176.12)	2.0	20 mg/100ml
p-Aminobenzoic acid (FW 137.14)	0.02	0.2 mg*
Nicotinamide	1.0	10 mg/100ml
Pyridoxine-HCl (FW 205.6)	1.0	10 mg/100ml
Thiamine-HCl (FW 337.27)	10.0	100 mg/100ml
Biotin (FW 244.3) (Vit H)	0.01	0.10 mg**
10 ml VKM Stock-IV for 1.0 lit medium, Store at 0ºC		

- Filter-Sterilize and to be added inside the LFCA Work sterilize (while preparing the medium)

* 200 µl from 1mg/ml separate stock of p-Aminobenzoic acid.

** 100 µl from 1mg/ml separate stock of biotin in KOH/NaOH.

f) VKM Stock-V

Vitamin	mg/L	Stock (×100)
Vitamin A	0.01	0.1 mg
10 µl from stock of 10 mg/ ml OR 100 µl from stock of 1 mg/ml for 100 ml VKM Stock-V. Dissolve in 100% ethanol only		
Vitamin B_{12}	0.02	0.2 mg
200.0 µl from 1mg/ml for 100 ml VKM Stock-V		
Vitamin D_3	0.01	0.1 mg
10 µl from stock of 10 mg/ ml OR 100 µl from stock of 1 mg/ml for 100 ml VKM Stock-V. Dissolve in 100% ethanol only		
10ml VKM Stock-V for 1.0 lit medium, Store at -20ºC		

g) VKM Stock-VI

Vitamin	mg/L	Stock
Folic acid (FW 441.4)	0.4 mg	1 mg/ml
400 µl VKM Stock-VI for 1.0 lit medium, Store at 0°C in amber colour bottle		

- This stock must be kept in an amber colour vial/or wrap Al-foil.

F. MS_{13K} Medium Preparation

1. Macroelements

Chemical	mg/L	Medium Volume (1000 ml)
NH_4NO_3	1650	1650 mg/1000 ml
KNO_3	1900	1900 mg/1000 ml

$CaCl_2.2H_2o$	440	440 mg/1000 ml
$MgSO_4.7H_2O$	370	370 mg/1000 ml
KH_2PO_4	170	170 mg/1000 ml

Add directly during medium preparation

2. Microelements

Chemical	mg/L	Stock (×1000)
H_3BO_3	6.2	620 mg/100 ml
$MnSO_4.H_2O$	16.9	1690 mg/100 ml
$ZnSO_4.7H2O$	10.59	1059 mg/100 ml
KI	0.83	83 mg/100 ml
$Na_2MoO_4.2H_2O$	0.25	25 mg/100 ml
$CuSO_4.5H_2O$	0.025	2.5 mg/100 ml*
$CoCl_2.6H_2O$	0.025	2.5 mg/100 ml*
1 ml for 1.0 lit medium		

* Prepare 1 mg/ml stock and add 2.5 ml to prepare Microelements stock

3. Iron –EDTA (or MS stock-V)

Chemical	mg/L	Stock (×100)
$Na_2EDTA.2H_2O$	37.2	373 mg/100 ml
$FeSO_4.7H_2O$	27.8	278 mg/100 ml
10.0 ml for 1 lit medium		

4. Vitamins (or MS stock-VI)

Chemical	mg/L	Stock (×1000)
Nicotinamide	0.5	50 mg/100 ml
Pyridoxine-HCl	0.5	50 mg/100 ml
Thiamine-HCl	0.1	10 mg/100 ml
Glycine	2.0	200 mg/100 ml
1.0 ml for 1 lit medium		

5. Choline chloride

Chemical	mg/L	Stock
Choline chloride	8.0	1 mg/ml

- 8 ml from stock of 1 mg/ml for 1 lit medium

6. Amino acids

Chemical	mg/L	Stock (×100)
Arginine-HCl	6.24	62.4 mg/100ml

Asparaginic acid*	4.8	4.8 mg/100ml
Cystein**	1.2	12.0 mg/100ml
Glutamic acid	11.2	112.0 mg/100ml
Histidine	2.08	20.8 mg/100ml
Isoleucin	8.2	82 .0mg/100ml
Leucin	12.48	124.8 mg/100ml
Lysine	12.48	124.8 mg/100ml
Methionine	10.4	104 .0mg/100ml
Phenylalanine	4.0	40 .0mg/100ml
Proline	4.0	40.0 mg/100ml
Threonine	10.4	104.0 mg/100ml
Tryptophan	3.2	32.0 mg/100ml
Valine	10.4	104.0 mg/100ml
10.0 ml for 1 lit medium		

- Asparagine, isoleucine, tryptophan and valine take time to dissolve so use warm water

7. Myo-Inositol: 100 mg for 1 lit medium
8. Sucrose: 30.0 g for 1 lit medium
9. Coconut milk/Water: 50 ml for 1 lit medium

10. Growth Hormones (to be added by sterile - filtration)

Chemical	mg/L	Stock (1mg/ml stock)
IAA	0.1 mg/l	100 µl/ 1 lit
Zeatin-riboside	2.0 mg/l	2 ml/ 1 lit

11. Gelrite: 2.5 g for 1 lit medium

- Prepare MS13K medium by mixing amount/volume of SN 1 to 11
- Adjust pH: 5.8; Autoclave: 121℃ for 20 min
- After autoclave, add the required amount of growth hormones by sterile filtration
- This medium is used for Callus regeneration and growth of post-fusion products.

G. Solubility Parameters of Growth Hormones

SN	Hormones	Solubility	Stock solution	Storage
1.	2,4-D (2,4 dichlorophenoxy acetic acid)	At 60ºC in water bath (ddH_2O)/ 100% EtOH/ 1N KOH or NaOH	20 mg/20 ml	0ºC
2.	α-NAA (Naphthalene acetic acid)	At 60ºC in water bath (ddH_2O)/ 1N KOH or	20 mg/20 ml	0ºC

		NaOH		
3.	Zeatin mix isomer*	1N KOH or NaOH	10 mg/10 ml	-20ºC
4.	Zeatin riboside (ZR)*	1N KOH or NaOH	10 mg/10 ml	-20ºC
5.	BA (N^6Benzyl adenine)	1N KOH or NaOH	20 mg/10 ml	0ºC
6.	GA_3	100% EtOH/1N KOH or NaOH	20 mg/10 ml	0ºC
	*Directly dissolve in the vial supplied by manufactures			

H. Solubility Parameters of Vitamins

SN	Vitamins	Solubility	Stock solution	Storage
1.	4 (para) amino benzoic acid (PABA) (Vit Bx/Vit H1)	At 60ºC in water bath (ddH_2O)	20 mg/20 ml	2-8 ºC (0ºC)
2.	Foliac acid (Vit M)	1N KOH	20 mg/20 ml	2-8 ºC (0ºC)
3.	Biotin (Vit H/VitB_7)	At 60ºC in water bath (ddH_2O)/ 1N KOH	20 mg/20 ml	2-8 ºC (0ºC)
4.	Vit A (Retinol acetate)*	100% EtOH (@ 25 mg/ml of EtOH)	20 mg/2 ml	-20ºC
5.	Vit B_{12} (Cyanocobalamine)	At 60ºC in water bath (ddH_2O)/ 1N KOH	20 mg/20 ml	2-8 ºC (0ºC)
6.	VitD_3 (Cholcalciferol)*	100% EtOH (@ 10 mg/2ml of 100% EtOH)	20 mg/2 ml	2-8 ºC (0ºC)
7.	Pyrodoxin-HCl (Vit B_6)**	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
8.	Thiamine-HCl (Vit B_1)**	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
9.	Nicotinamide (Vit B_3)**	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
10.	Nicotinic acid (Niacin)** (Vit B_3)	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
	*May encounter difficulties in dissolving			
	** Weigh directly while preparing the vitamins stock			

3. Application of somatic hybridization at ICAR-CPRI, Shimla

3.1 Somatic hybrid (*Solanum tuberosum* 'C-13' + *Solanum cardiophyllum*) for late blight resistance

At ICAR-CPRI, Shimla we have produced potato somatic hybrids of three wild species. Interspecific potato somatic hybrids (4 clones) have been produced via protoplast fusion between potato dihaploid *Solanum tuberosum* dihaploid 'C-13' ($2n=2x=24$) and wild *Solanum cardiophyllum* (PI 341233) for late blight resistance with wider genetic base to develop new varieties. The interspecific potato somatic hybrids are tertraploid, male fertile, resistant to late blight introgressed from wild *Solanum cardiophyllum* and have wider genetic base possessing W/α, W/γ and T/β types diverse cytoplasm types. Somatic hybrids (Crd 6, Crd 10 and Crd 16) were identified based on the field performance. They possess high tuber dry matter content (≥ 24%), excellent keeping quality and high late blight resistance along with adaptability under sub-tropical plain condition.

Fig. 1. Steps involved in protoplast isolation, regeneration and development of somatic hybrids in potato

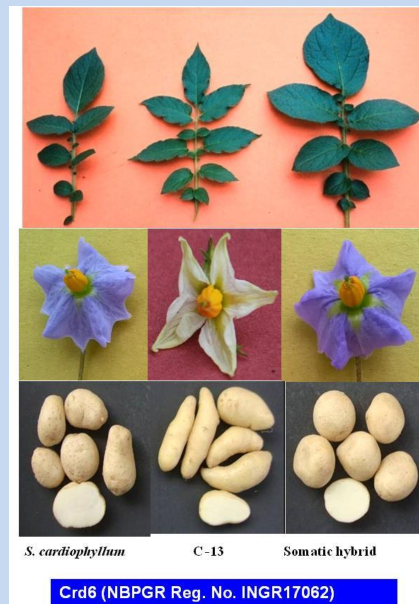

Fig. 2. Somatic hybrid (‘C-13’ + *Solanum cardiophyllum*) for late blight resistance

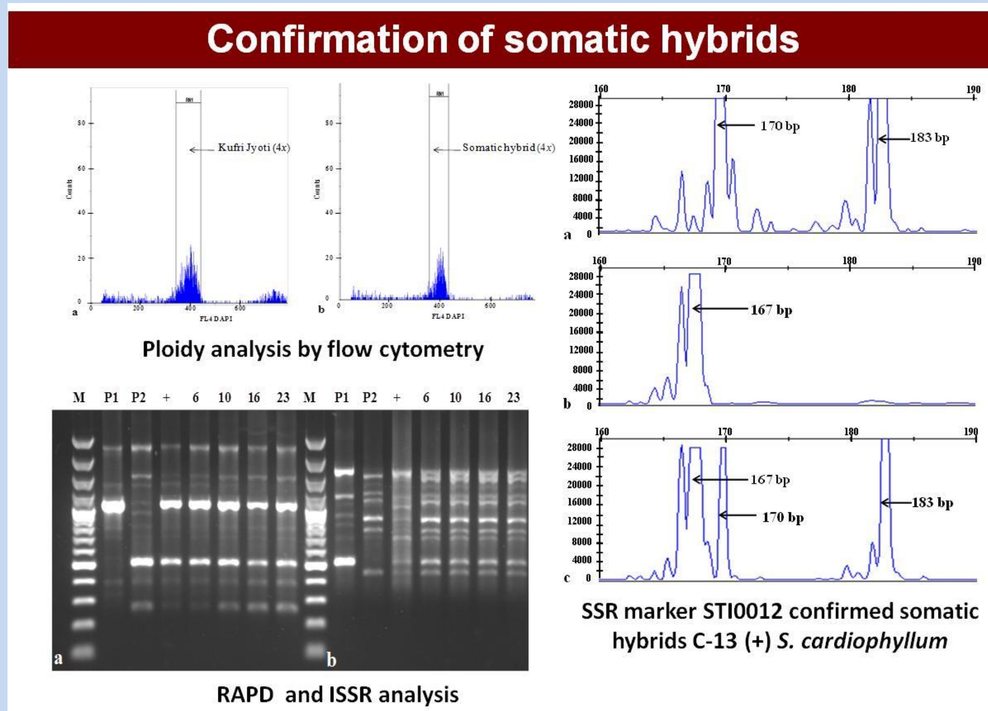

Fig. 3. Confirmation of somatic hybrids (‘C-13’ + *Solanum cardiophyllum*) by ploidy analysis, RAPD, ISSR and SSR markers.

3.2 Somatic hybrid (‘C-13’ + *Solanum pinnatisectum*) for late blight resistance

Interspecific potato somatic hybrids (11 clones) were produced via protoplast fusion between ‘C-13’ and wild *Solanum pinnatisectum* (CGN No.: 17745) for very high resistance to late blight disease. These somatic hybrids have immense potential in the potato breeding to transfer durable resistance to late blight by breeding methods and to widen the gene pool of the cultivated potato. Somatic hybrids were analyzed for cytoplasm types using organelles (chloroplast and mitochondria) genome-specific markers. Study showed that above somatic hybrids possess W/α, W/γ and T/β types diverse cytoplasm types. Besides, protocols have been standardized for an efficient cell system in potato for somatic cell genetic manipulations from stoloniferous shoot protoplast. For symmetric somatic hybridization (electrofusion) between ‘C-13’ and diploid wild species *S. pinnatisectum*, protoplasts isolated from 0.1M sucrose-induced stoloniferous shoots were also found to be most responsive. Somatic hybridization has unique potential to widen the cytoplasm types of the cultivated gene pools from wild species through introgression by breeding methods.

Further, genetic improvement of these somatic hybrids (C-13 + *S. pinnatisectum*) has been done through breeding. The promising somatic hybrids namely P-4, P-8 and P-10 were identified and can be utilized in breeding to transfer important characters such as high tuber dry matter, resistance to late blight and excellent keeping quality into the cultivated potato. Three promising advance stage hybrids namely MSH/14-7, MSH/14-129 and MSH/14-131 were developed using somatic hybrids as a parent. Five promising hybrids namely MSH/14-112, -113, -115, -122 and -123 (P8 × Kufri Jyoti) were selected for very high resistance to late blight and high dry matter content (20.75-22.10%). These advance stage hybrids have been developed till F_1C_6 stage and MSH/14-7 has been introduced into AICRP trial. SSR alleles- linked to late blight resistant somatic hybrid (C-13 + *S. pinnatisectum*) parent P8 (103 and 144 bp) and Kufri Jyoti (132 and 144 bp) their progenies (103 and 144) were identified. Progenies were generated by cross of P8 × Kufri Jyoti. SSR allele 103 bp of P8 was transmitted into the progenies and was identified for genetic fidelity testing and breeding application.

Fig. 4. Somatic hybrid ('C-13' + *Solanum pinnatisectum*) for late blight resistance

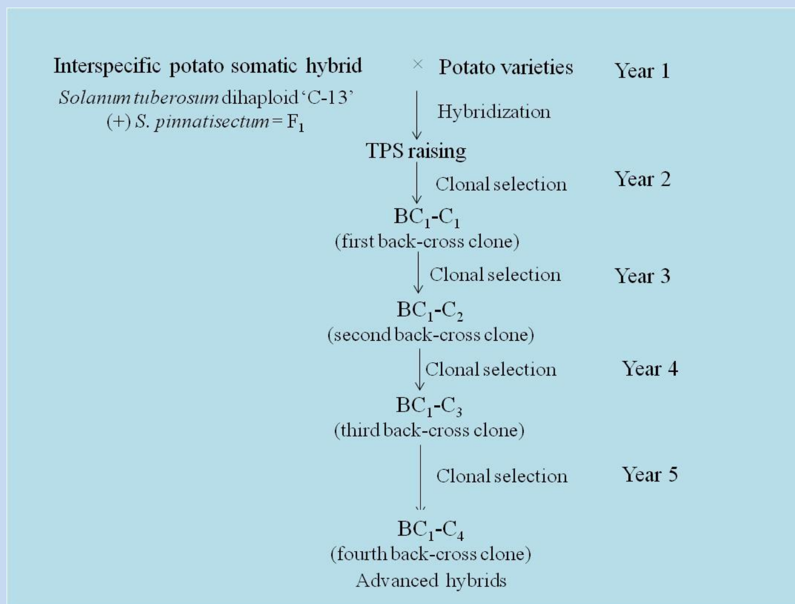

Fig. 5. Development of advance breeding lines of somatic hybrids by breeding methods.

Fig. 6. Development of advance stage potato hybrids (F_1C_6) by crossing somatic hybrids ('C-13' + *Solanum pinnatisectum*) with indigenous potato varieties

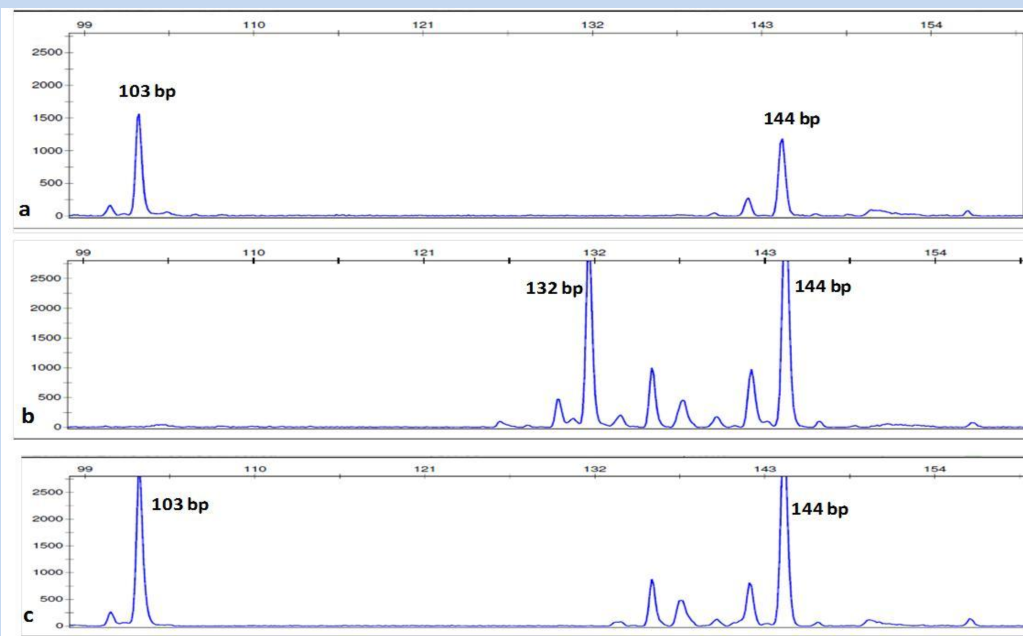

Fig. 7. Development of SSR alleles in somatic hybrid progenies

3.3 Somatic hybrid (‘C-13’ + *Solanum etuberosum*) for Potato Virus Y resistance

We developed interspecific potato somatic hybrids (21 clones) via protoplast fusion between dihaploid *Solanum tuberosum* ‘C-13’ and wild *Solanum etuberosum* (CGN No.: 23066) for Potato Virus Y resistance. The wild species *Solanum etuberosum* is no-tuberous species, whereas somatic hybrids form tubers but not like tetraploid potato. Somatic hybrids were analyzed for cytoplasm types using organelles (chloroplast and mitochondria) genome-specific markers and showed diverse cytoplasm types (W/α, W/γ and T/β).

Fig. 8. Somatic hybrid (‘C-13’ + *Solanum etuberosum*) for Potato Virus Y resistance

4. Status of the development of somatic hybrids in the world

We have summarised the potato somatic hybrids developed world wide using wild species in Table 1.

Table 1. Summary of potato somatic hybrids developed worldwide using *Solanum* species

SN	Somatic hybrid/fusion parents		Target trait
1.	*S. acaule* (+) *S. tuberosum*		
	S. acaule dihaploid (2*x*)	*S. tuberosum* (4*x*) dihaploid (2*x*)	Potato virus X (PVX) resistance
	S. acaule (4*x*) and dihaploid (2*x*)	*S. tuberosum* (4*x*) and dihaploid (2*x*)	Resistance to bacterial ring rot (*Clavibacter* spp.) and potato leaf roll virus (PLRV)
	S. acaule dihaploid (2*x*)	*S. tuberosum* (4*x*)	Glycolkaloids
2.	*S. berthaultii* (+) *S. tuberosum*		
	S. berthaultii (2*x*)	*S. tuberosum* dihaploid (2*x*)	Resistance to insects, PVY, soil-borne pathogens (*Fusarium*, *Pythium* and *Rhizoctonia* spp.) and salinity tolerance
3.	*S. brevidens* (+) *S. tuberosum*		

	S. brevidens (2*x*)	*S. tuberosum* (4*x*)/ dihaploid (2*x*)	PVY, PLRV, late blight, early blight, bacterial soft rot (*Erwini* spp.) and frost resistance
	S. brevidens (2*x*)	*S. tuberosum* (4*x*)	PLRV and cold-stress resistance
	S. brevidens (2*x*)	*S. tuberosum* (4*x*)	Bacterial soft rot (*Erwini* spp.) resistance, common scab (*Streptomyces* spp.) resistance
	S. brevidens (2*x*)	*S. tuberosum* (4*x*)	Glycoalkaloids
4.	*S. bulbocastanum* (+) *S. tuberosum*		
	S. bulbocastanum (2*x*)	*S. tuberosum* (4*x*)	Nematode (*Meloidogyne* spp.) resistance
	S. bulbocastanum (2*x*)	*S. tuberosum* haploids (2*x*)	Late blight resistance
	S. bulbocastanum (2*x*)	*S. tuberosum* (4*x*)	Late blight resistance
5.	*S. cardiophyllum* (+) *S. tuberosum*		
	S. cardiophyllum (2*x*)	*S. tuberosum* (4*x*)	Late blight resistance
	S. cardiophyllum (2*x*)	*S. tuberosum* (4*x*)	Late blight, PVY and Colorado potato beetle resistance
	S. cardiophyllum (2*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
6.	*S. chcoense* (+) *S. tuberosum*		
	S. chcoense (2*x*)	*S. tuberosum* dihaploid (2*x*)	Colorado potato beetle resistance
	S. chcoense (2*x*)	*S. tuberosum* (4*x*)	Bacterial wilt (*Ralstonia* spp.) resistance
7.	*S. circaefolium* (+) *S. tuberosum*		
	S. circaefolium (2*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
8.	*S. commersonii* (+) *S. tuberosum*		
	S. commersonii (2*x*)	*S. tuberosum* dihaploid (2*x*)	Verticillium wilt, tuber soft rot (*Erwinia* spp.), bacterial wilt resistance and cold-stress/freezing tolerance
9.	*S. etuberosum* (+) *S. tuberosum*		
	S. etuberosum (2*x*)	*S. tuberosum* dihaploid (2*x*)	PVY, PLRV and green peach aphid resistance
	S. etuberosum (2*x*)	*S. tuberosum* dihaploid (2*x*)	PVY resistance
10.	*S. melongena* (+) *S. tuberosum*		
	S. melongena (2x)	*S. tuberosum* dihaploid (2*x*)	Bacterial wilt resistance
11.	*S. × michoacanum (+) S. tuberosum*		
	S. × michoacanum (2*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
12.	*S. nigrum* (+) *S. tuberosum*		
	S. nigrum (6*x*) (non-tuberous)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
	S. nigrum (6*x*)	*S. tuberosum* (4*x*)	Atrazine resistance

13.	*S. phureja* (+) *S. tuberosum*		
	S. phureja (2*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance, bacterial wilt resistance
	S. phureja monoploids (1*x*)	*S. phureja* monoploids (1*x*)	Long photoperiods
14.	*S. pinnatisectum* (+) *S. tuberosum*		
	S. pinnatisectum (2*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
	S. pinnatisectum (2*x*)	*S. tuberosum* dihaploid (2*x*) and *S. phureja* (2*x*)	Late blight resistance
	S. pinnatisectum (2*x*) & Hybrid clone (*S. pinnatisectum* × *S. bulbocastanum*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
	S. pinnatisectum (2*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance
	S. pinnatisectum (2*x*)	*S. tuberosum* (4*x*)	Late blight resistance
15.	*S. tuberosum* (+) *S. tuberosum*		
	S. tuberosum dihaploid (2*x*)	*S. tuberosum* dihaploid (2*x*)	Resistance to late blight, PVX, PVY, potato cyst nematode, soft rot (*Erwiniai* spp.), and storage rot (*Pythium aphanidermatum*)
	S. tuberosum (4*x*)	*S. tuberosum* (4*x*)	Cytoplasmic male sterility
	S. tuberosum dihaploid (2*x*)	*S. tuberosum* dihaploid (2*x*)	Resistance to *Globodera* spp. and metribuzin herbicide
16.	*S. sanctae-rosae* (+) *S. tuberosum*		
	S. sanctae-rosae (2*x*)	*S. tuberosum* (4*x*)	Potato cyst nematode resistance
17.	*S. spegazzinii (+) S. tuberosum*		
	S. spegazzinii (2*x*) & Hybrid clone (2*x*) (*S. microdontum* x *S. vernei*)	*S. tuberosum* (4*x*) and dihaploid (2*x*)	-
18.	*S. stenotomum* (+) *S. tuberosum*		
	S. stenotomum (2*x*)	*S. tuberosum* dihaploid (2*x*)	Bacterial wilt resistance
19.	*S. tarnii* (+) *S. tuberosum*		
	S. tarnii (2*x*)	*S. tuberosum* (4*x*)	Late blight, Colorado potato beetle and PVY resistance
20.	*S. torvum* (+) *S. tuberosum*		
	S. torvum (2*x*)	*S. tuberosum* dihaploid (2*x*)	*Verticillium* wilt resistance
21.	*S. vernei* (+) *S. tuberosum*		
	S. vernei (2*x*)	*S. tuberosum* dihaploid (2*x*)	Salt tolerance
22.	*S. verrucosum* (+) *S. tuberosum*		
	S. verrucosum (2*x*)	*S. tuberosum* dihaploid (2*x*)	Resistance to PLRV

23.	23. *S. villosum* (+) *S. tuberosum*		
	S. villosum (4*x*)	*S. tuberosum* dihaploid (2*x*)	Late blight resistance

S. brevidens, *S. commersonii*, *S. etuberosum* and *S. villosum* are non-tuberous wild potato species. Cytoplasm types are described as: ALC1/ALC3: 381 bp (T/β), 622 bp (W/α and W/γ); *ii*) ALM1/ALM3: 1.2 kb (W/α and W/γ); *iii*) ALM4/ALM5: 1.6 kb (T/β), 2.4 kb (W/α); and *iv*) ALM6/ALM7: 2.4 kb (W/γ) (Lössl et al. 2000).

5. Ploidy analysis by Flow Cytometer

Flow cytometry is a technique for the measurement and counting of small particles in a fluid stream. A flow cytometer comprises three systems: fluidics, optics and electronics. In essence, every single particle is excited by a light source and is finally displayed on a graph. Common flow cytometers detect multiple parameters: forward scatter (FSC), sideward scatter (SSC) and a number of fluorescent wavelengths (FL1 & FL2), depending on the excitation source and the complexity of the instrument. FSC and SSC signals provide information about the size, shape and complexity of the cell. FSC is the narrow angle light scatter and is dependent on the size and refraction index of the cell. SSC is the right-angle light scatter and depends on the external granularity, internal complexity and shape of the cell. The sensitivity of each photo-multiplier tube (PMT) can be adjusted separately to suit the application. During the analysis, the instrument can be triggered on one of its parameters and particle positive for the triggering parameter will be displayed on the outputs.

5.1 Methodology for estimation of DNA ploidy of potato

Ploidy estimation is one of the most frequent applications of flow cytometry. The methodology consists of three major steps namely preparation of suspensions of intact nuclei, labeling with the fluorochrome and analysis of nuclear DNA content either with external or internal standards.

5.1.1 Ploidy analysis with the external standard method

External standardization involves the individual analysis of a plant with known ploidy (reference standard) followed by the analysis of an unknown specific plant with the same instrument settings. It results in a histogram of DNA content who's relative G1 peak position indicates the plant ploidy. It is suitable for large-scale screening. The test samples and reference standard (the reference standard for the ploidy determination must be from the same species, whose ploidy is already known) were analysed separately on the flow cytometer with same instrument gain settings. The diploid, *S. chacoense* is used as the reference standard for potato clones. The suspensions of intact nuclei were prepared from the young leaf using the modified HPI buffer. As, buffer itself contains the DNA fluorochrome, Propidium iodide (PI), the nuclei get stain during the isolation process itself. The reference samples were first analysed in the flow cytometer and positioned the G_1 peak on the absicca. Similarly, stained nuclear suspension from

the potato samples introduced in the flow cytometer and positioned the G_1 peak at the required position on the abscissa with same instrument gain settings. The ploidy was calculated as per the formula:

Sample ploidy (integer) = Reference ploidy × mean position of the G_1 sample peak\ Mean position of the G_1 reference peak

5.1.2 Ploidy analysis with the internal standard method

Internal Standardization involves simultaneous analysis of a reference and unknown plant of different ploidy results in a histogram having two G1 peaks. The ratio of the G1 peak positions reflects the ratio of ploidy levels. It is more precise since it eliminates most of the sources of variation. In this method, the test samples and reference standard (the reference standard for the ploidy determination must be from the same species, whose ploidy is already known) were analysed separately on the flow cytometer with same instrument gain settings. The rest of the methodology remains same as that of external method.

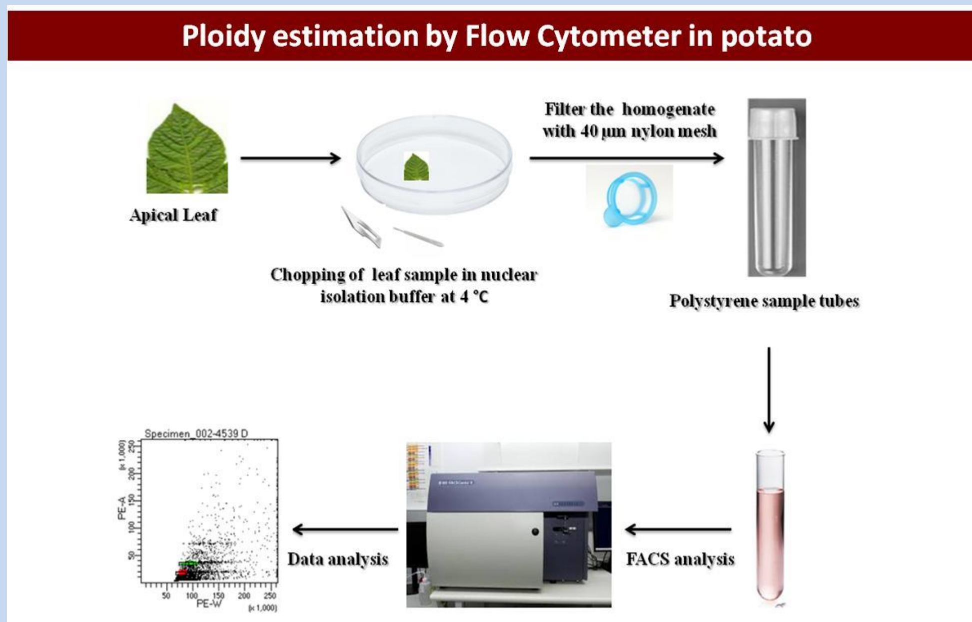

Fig. 9. Steps involved in ploidy analysis by flow cytometer in potato

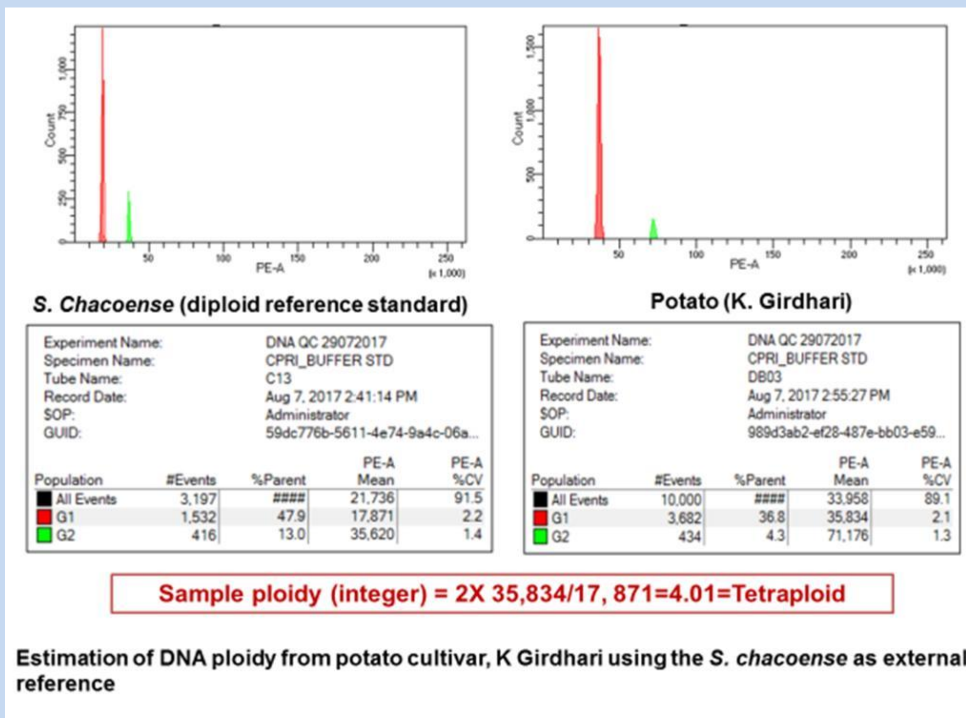

Fig. 10. Estimation of ploidy level in diploid and tetraploid potatoes by flow cytometer

5.2 Protocols

In potato, ploidy estimation of putative somatic hybrids or other genotypes are done by flow cytometry analysis following procedures described by Arumuganathan and Earle (1991).

Protocol #1:

1. Macerate fresh leaf samples (~100 mg) from 3- to 4-week-old *in vitro* plants 1 ml Nuclei Isolation Buffer (NIB) by fine-chopping on ice.
2. Filter the macerates through a 41-µm nylon sieve and collect in 1.5 ml Eppendorf tube followed by centrifugation at 5,000 rpm for 5 min.
3. Resuspend the white pellets (nuclei) in 200 µl $MgSO_4$ buffer followed by RNAse (2 µl) treatment at 37 ºC for 15 min.
4. Add 300 µl propidium iodide (PI) on ice under the dark. Add 2 µl CRBC (chicken red blood cell) in the same tube of each sample. Use CRBC (2C value of DNA = 2.33 pg) as an internal standard.
5. Measure the nuclear DNA content of PI-stained nuclei in a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, USA)
6. Analyze the samples by relative fluorescence for forward (FSC) versus side (SSC) scatter signals for at least 10,000 nuclei in each sample.
7. Adjust the peak corresponding to the CRBC nuclei to around channel 250 set on a linear scale of fluorescence intensity.

8. Estimate the nuclear DNA amount (2C value in pg) by direct comparison of the mean position of nuclear peak of somatic hybrids to that of CRBC. For each sample, measure at least three independent replicates.

$MgSO_4$ buffer (pH 8.0)

Reagents	Final Concentration	Amount for 100 ml stock
$MgSO_4.7H_2O$ (MW 246.47)	10 mM	246.47 mg
KCl (MW 74.55)	50 mM	372.75 mg
HEPES (MW 238.30)	5 mM	119.15 mg
Milli-Q water		Up to 100 ml

Filter through a 22-µm sieve, sterilize in autoclave and store at 4°C.

Nuclei Isolation Buffer (NIB)

Reagents	Volume (15 ml)
$MgSO_4$ buffer (pH 8.0)	14.325 ml
Dithiothretol (DTT)	15 mg
Triton X-100	375 µl
Propidium Iodide (PI)	300 µl

Protocol #2

Materials required:

- Plastic Petri dishes (5.5 cm diameter) kept at 4 °C before sample preparation.
- Razor blade kept at 4 °C before sample preparation and razor blade holder.
- Nylon filters/cell strainer of 40-micron mesh size.
- Polystyrene sample tubes suitable for the flow cytometer (e.g. BD Falcon).
- Sample tube holder.
- Ice container.
- Appropriate software (BD FACS Diva) for the evaluation of flow cytometric data
- FACS tubes and BD FACS Canto II^{TM} Flow cytometer.
- Nitrile or latex gloves, safety glasses

Composition of nuclear isolation buffer (Modified HPI buffer)

- 0.1 % trisodium citrate dehydride,
- 3 µl/ml Triton X-100,
- 1% PVP-40,
- Filter through a 0.22-mm filter.
- Add 1% of b-mercaptoethanol, 2mg/ml RNase A, 25 µg/ml of propidium iodide.
- Store at 4 ^{0}C in 10 ml aliquots.

A. ***Preparation of fluorochrome labeled intact nuclear suspension***

1. Take an approximately of 20 mg young leaf from the apical meristem of potato plants
2. Chop the leaf samples in 200 ul ice-cold modified HPI buffer with new razor blade and add another 600 ul modified HPI buffer to the chopped samples. Mix the homogenate by pipetting up and down for several times.
3. Filter the homogenate through a 40-mm cell strainer into a labeled sample tube
4. Incubate the sample tube on ice for 5-10 minutes, with occasional shaking in the dark.

B. Ploidy analysis with external standard

5. Run a sample prepared from a reference standard, *S. Chacoense* (diploid potato) by introducing the samples into the flow cytometer. Analyze the sample at low flow rate (the sample rate usually does not exceed 20–50 nuclei/s). The DNA peak(s) of G_1 and G_2 cells were positioned to the required position on the abscissa by adjusting the instrument gain settings. About 10000 populations were measured and the data were saved for the analysis.
6. Analyze unknown samples with same instrument gain settings by following just above step.
7. Calculate the DNA ploidy of the unknown sample as follows:

Sample ploidy (integer) = 2 × mean position of the G_1 sample peak\ Mean position of the G_1 reference peak

B. **Ploidy analysis with internal standard**

8. Prepare the sample by simultaneous chopping and staining tissues from a reference plant with known ploidy (chromosome number) and from the unknown plant sample as described earlier.
9. Perform sample analysis following Step 5
10. Determine the DNA ploidy of the unknown sample following Step 7

Estimation of ploidy level based on nuclear DNA contents in potato

Sample (ploidy)	nDNA content (pico gram)	
	1C value	2C value
CRBC (2X)	~1.25	~2.5
Human	~3.50	~7.0
S. tuberosum (2X)	~0.88-0.90	~1.7-1.80
S. tuberosum (4X)	~1.60-2.10	~3.20-4.20

- There are 12 haploid chromosomes (840 MB) in potato genome

- C value = amount of nuclear DNA content within haploid set of chromosome
- 1C value = constant value and symbolize DNA content of haploid genome
- 2C value = DNA of diploid somatic cell