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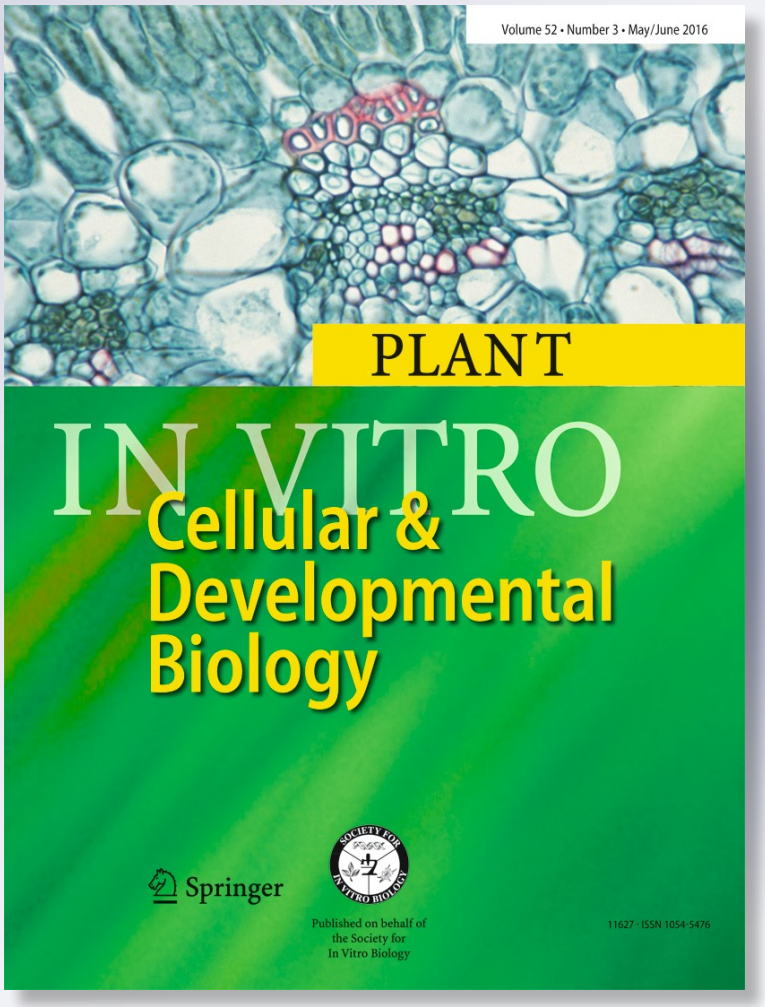
In Vitro Cellular & Developmental Biology - Plant

ISSN 1054-5476
Volume 52
Number 3

In Vitro Cell.Dev.Biol.-Plant (2016)
52:213-225
DOI 10.1007/s11627-016-9753-x



Springer

In Vitro Cell.Dev.Biol.—Plant (2016) 52:213–225
DOI 10.1007/s11627-016-9753-x

INVITED REVIEW

In vitro technology at the US Potato Genebank

J. B. Bamberg[1] · M. W. Martin[1] · J. Abad[2] · M. M. Jenderek[3] · J. Tanner[3] · D. J. Donnelly[4] · A M. K. Nassar[5] · R. E. Veilleux[6] · R. G. Novy[7]

Received: 28 September 2015 / Accepted: 22 February 2016 / Published online: 8 March 2016 / Editor: David Duncan

Abstract The US Potato Genebank in Sturgeon Bay, Wisconsin, is the national germplasm collection for the world's most important vegetable crop. It contains about 6,000 accessions of 100 species of tuber-bearing relatives of *Solanum tuberosum*. The potato of commerce is a clonal crop susceptible to many systemic pathogens, so the genebank routinely uses *in vitro* clonal maintenance and distribution for named cultivars. *In vitro* management is also the tool of choice for various breeding and genetic stocks of interest to breeders and researchers. Long-term backup cryopreservation of clones is done at the base collection in Fort Collins, Colorado. *In vitro* techniques also play an important role in virus elimination from clones. Recently, *in vitro* propagation has expanded to the temporary safekeeping of meristem propagules in antimicrobial medium during plant collecting expeditions in the southwest USA. The genebank's mission includes promoting technology that supports expanded use of the germplasm, in particular, finding ways to overcome interspecific hybridization barriers. Thus, *in vitro* techniques such as pollen viability testing, ploidy manipulation, protoplast fusion, and embryo rescue have contributed technology for major advances in interspecific hybridization, utilization of noncommercial species, and introgression of genes from wild near-relatives. Finally, advancing *in vitro* technology holds promise as a tool for mass bioassay and selection of seeds, pollen, or somaclones for useful traits.

Keywords Potato · *Solanum* · Genebank · Germplasm collection · *In vitro* conservation

✉ J. B. Bamberg
John.Bamberg@ars.usda.gov

[1] US Potato Genebank, 4312 Hwy 42, Sturgeon Bay, WI 54235, USA

[2] USDA-APHIS-PPQ-FO-Plant Germplasm Quarantine Program (PGQP), Beltsville, MD 20705, USA

[3] National Center for Genetic Resources Preservation, USDA-ARS, 1111 Mason Street, Fort Collins, CO 80521, USA

[4] Plant Science Department, McGill University, 21,111 Lakeshore Rd., Ste Anne de Bellevue, QC H9X 3V9, Canada

[5] Plant Protection Department, Faculty of Agriculture, Damanhour University, Damanhour, Albeheira 22516, Egypt

[6] Department of Horticulture, Virginia Tech, Blacksburg, VA 24061, USA

[7] Small Grains and Potato Germplasm Research Unit, USDA-ARS, Aberdeen, ID 83210, USA

Introduction

The US Potato Genebank (USPG), located in Sturgeon Bay, Wisconsin, is part of the National Plant Germplasm System (NPGS) administered by United States Department of Agriculture (USDA), Agricultural Research Service (ARS). The genebank program has close administrative and research ties with the University of Wisconsin Department of Horticulture and the USDA/ARS Vegetable Crops Research Unit in Madison, Wisconsin.

The basic aspects of the genebank and potato germplasm summarized below are presented in detail with primary references in Bamberg and del Rio (2005), with updates at the genebank website (http://www.ars-grin.gov/nr6) and in the Potato Crop Germplasm Committee 2014 "Vulnerability Statement" (http://www.ars-grin.gov/npgs/cgclist.html#Potato).

USPG is the site responsible for the US collection of wild and cultivated *Solanum* species. These species have their

natural origin in the western mountainous highlands of the Americas stretching from southern Chile to southern Utah and Colorado in the USA. Current taxonomy recognizes about 100 species. One major taxon (*Solanum tuberosum*) is cultivated, but most of the remaining wild species are also relatively amenable to incorporation into the breeding pool. The genebank clonally maintains *in vitro* about 1,000 named commercial cultivars and breeding stocks of interest from around the world, as well as some cultivated landraces (Fig. 1*a*). In contrast, about 5,000 of the USPG holdings are in the form of botanical seeds. When the number of seeds of these accessions gets low or their germination is reduced, they are sown in the greenhouse as parents (Fig. 1*b*) of a new seedlot, which will be stored in sealed packets at −20°C for up to 50 yr.

Potato is subject to numerous systemic pathogens, so international quarantine restrictions are stringent. In the USA, potato is categorized as "prohibited", which means that no germplasm can be imported without a USDA-APHIS official permit and a period of testing (and possibly disease eradication) at the federal quarantine facility in Beltsville, Maryland, described in detail below. Imported germplasm may also be subject to ownership rights of the country of natural origin, restricting collection and exchange. These factors make technology that ensures the efficient safekeeping and deployment of the germplasm already at USPG even more important.

Potato is the most important non-cereal food crop in the USA and worldwide. In recent years, it has expanded greatly into new growing regions and gained cultural acceptance, particularly in Asia. Its capacity to produce many more food calories per unit of land than other major crops (Ensminger and Ensminger 1993), combined with outstanding palatability, makes it one of the best available options for feeding an increasingly hungry world. Yet, potato has numerous producer-oriented challenges such as susceptibility to diseases, pests, and abiotic stress. There is also great potential for improving consumer-oriented traits such as nutritional value, appearance, taste, and processing quality. The readily available genetic tools found in exotic germplasm in the USPG are extremely valuable for improving the potato crop. Many of the traits to be improved are known, but the genebank must also be able to provide the resources to overcome unknown challenges of the future. When a mechanic is facing an unknown problem, the best strategy is to have a very diverse set of tools. For the genebank, the parallel is in acquiring and organizing the maximum diversity of genetic tools, keeping them sharp, and doing the characterization, research, and development needed to use them efficiently.

Thus, the basic USPG mission matches the 5-fold goals of most genebanks to acquire, classify, preserve, evaluate (characterize), and distribute germplasm. “Characterize” often represents one of the many possible forms of evaluation research that may be done in-house or by external germplasm cooperators who contribute expertise in their particular disciplines.

Figure 1. (*a*) Tuber diversity requires clonal preservation. (*b*) Flowering for botanical seed reproduction.

In vitro technology can be used to facilitate each of these genebank goals.

This summary aims to highlight some examples of how genebank staff and cooperators have used *in vitro* techniques

to more effectively provide genebank services. These include tissue culture methods for collecting propagules from the wild, disease-free preservation of clonal stocks, virus elimination, pollen viability assays, and cryopreservation. Finally, no treatment of the role of *in vitro* technology in potato germplasm would be complete without mentioning some key examples of how *in vitro* methods such as selective bioassays, somaclonal variant selection, protoplast fusion, ploidy manipulations, and embryo rescue have facilitated germplasm introduction, enhancement, deployment, and breeding.

Acquisition and Preservation Methods for Genebank Service

Disease-free preservation and distribution of clonal stocks Potato cultivars are highly heterozygous clones and are maintained vegetatively, so when a genotype needs to be preserved, *in vitro* methods are most practical. At the USPG, cultivars are maintained in 20 × 150-mm glass culture tubes containing 10 mL of Murashige and Skoog (1962) BC Potato Medium (PhytoTechnology Laboratories®, Shawnee Mission, KS) supplemented with 30 g L^{-1} D-sorbitol, covered with disposable caps (Kim-Kap®, Kimble-Chase, Rockwood, TN) and closed with sealing film (Paraflim M®, Curwood-Bemis, Oshkosh, WI). A total of 624 clones (three copies per clone) fit in a 0.65-m^3 growth chamber (Power Scientific Inc., Pipersville, PA, DS33SD). Plants are multiplied by axillary bud cuttings placed into the above-described culture tubes and allowed to establish for 2 wk in a growth room with 16-h photoperiod at 20–22°C. After the plants are established, they are moved to a long-term growth chamber set at 16-h photoperiod (~25 μmol m^{-2} s^{-1} light intensity with Philips F17T8/T841 17-W fluorescent tubes) at 8–10°C (Fig. 2). Under these conditions, plantlets will remain viable for 1–1.5 yr. The collection is tested for viruses (PVA, PLRV, PVM, PVS, PVX, PVY), viroid (PSTVd), and bacteria (BRR) every 5 yr. For security, copies of each clone are stored at more than one location at the USPG, and most also exist in other US locations and abroad.

Virus elimination and secure germplasm importation through quarantine The most reliable method for obtaining virus-free stocks from clonally propagative infected plants is viral eradication by using *in vitro* tissue culture techniques, including thermo- and/or chemotherapies. Tissue culture plantlets are subcultured after arrival at the Plant Germplasm Quarantine Program (PGQP) by excising the growing tips and placing them into new tissue culture tubes with fresh *Solanum* maintenance medium (SMM), which is MS medium supplemented with thiamine (1 mg L^{-1}), sucrose (30 g L^{-1}), NaH_2PO_4 •H_2O (1 mg L^{-1}), and Gelrite™ (1.5 g L^{-1}), adjusted to pH 6.0 with KOH or HCl. Prior to therapy, each variety is multiplied to six clones and grown on SMM at 23°C with a 16-h photoperiod

Figure 2. Long-term preservation of clones *in vitro* at the genebank.

(53.55 μmol m^{-2} s^{-1} light intensity, Philips F17T8/TL841, 17-W fluorescent tubes). The tissue culture clones remain on SMM until the shoot length is approximately 5 cm and roots are formed. Two clones of each variety remain on SMM under normal growth conditions to serve as backup clones in case the others do not survive disinfection. The remaining four clones are individually identified and exposed to thermotherapy for 4 wk using four cycles daily of 35°C with light and 31°C without light. After thermotherapy, a small apical cutting (0.25–0.50″) from each clone is transferred to a chemotherapy medium (SMMR) containing the antiviral ribavirin (PhytoTechnology Laboratories®) (Klein and Livingston 1982). These clones are grown under normal conditions (23°C with a 16-h photoperiod) for 8 wk; clones are subcultured onto fresh SMMR every 2–3 wk. Varieties may react differently to the chemotherapy medium, so if the chemotherapy medium is lethal to a particular variety, the ribavirin concentration or the duration on SMMR is reduced. After 8 wk of chemotherapy, the clones are transferred to SMM. When the clones have generated sufficient shoots and roots for testing, they are tested for pathogens one more time.

After testing is complete, plants found to be free of the original pathogens are released from the program. Subclone identification within each variety is important because the therapy is not always successful. One subclone may test negative for the pathogen, while other subclones test positive. Normally more than 60% success is obtained by thermo- and/or chemotherapy.

The importation of tuber-bearing *Solanum*, either as true seed or plant parts for propagation, is prohibited from all countries with the exception of some regions of Canada, the tenth (X) region of Chile (*i.e.*, between lat 39°S and 44°S), and New Zealand under the USDA-APHIS Code of Federal Regulations (C.F.R. §319.37-2, 2015). However, small quantities of tubers and true seed may be introduced into the USA through the PGQP. In order to import this material, a requestor (US citizen or permanent resident) should contact the PGQP. The potato accession may come to the PGQP as tubers or most commonly as *in vitro* plantlets. Eventually all accessions are propagated from tissue culture prior to their establishment in pots. PGQP will test all accessions for the presence of pathogens, particularly viruses, viroids, and phytoplasmas, using state-of-the-art technologies. Serology by ELISA and ImmunoStrips, conventional PCR and RT-PCR, quantitative PCR, sequence analyses, electron microscopy, biological indexing, and most recently, next-generation sequencing are among the technologies applied. After testing, all the requested accessions that have been found negative are released to the requestor. If an accession is found to be infected, it goes immediately to therapy. When distribution of the introduced accession is not restricted, it is shared with the USPG. Otherwise it will go only to the requestor.

Tissue culture methods for collecting propagules from wild plants The quality of germplasm samples from wild plants is often unpredictable. The local environment at the exact time the collector visits the natural population may preclude the easy collection of viable true seeds, shoots, or tubers. Thus, temporary storage of axillary meristems in antimicrobial medium (Fig. 3) can allow survival of samples for several days until they can be established at the genebank in sterile culture or as potted plants in the greenhouse. For species native to the southwest USA such as *Solanum jamesii*, a treatment that prevents contamination for at least 10 d is the agitation of the collected sample for 10 min in 5% (*v*/*v*) aqueous solution of household bleach (5% hypochlorite) followed by culture on medium with 3.0 g L^{-1} Plant Preservative Mixture (PPM) biocide (www.plantcelltechnology.com).

Pollen viability assays A major service function of USPG is the multiplication of botanical seed of the 5,000 accessions maintained in this form. Species vary greatly in how readily they sexually reproduce in the artificial greenhouse environment. In the wild, they may not be subject to natural selection for seed reproduction, but may typically survive from season to season as

Figure 3. A. del Rio (USPG) collecting meristems in the wild in Arizona, USA.

tuberlings (Bamberg *et al.* 2009). When seeds do not readily form in response to pollination, but pollen morphology and shed appear to be normal, pollen viability can be tested *in vitro*. Bamberg and Hanneman (1991a) established that a simple aerated 20% (*w*/*v*) lactose/water solution with 50 mg L^{-1} boron promotes pollen tube growth of viable pollen up to the length of a typical style (~1 cm). When this is applied to pollen in small (*e.g.*, 3 × 5 cm) resealable plastic pouches, the progress and ultimate success of germination may be repeatedly observed under a microscope through the pouch without disturbing the culture or sampling it onto a microscope slide (Fig. 4). Řihová *et al.* (1996) have suggested using cultures with PEG 1000 as the osmoticum, but USPG staff have not found the addition of PEG to give superior results (unpublished). Pollen culture has intriguing possibilities beyond simple viability testing. For example, it might be used to systematically test treatments such as greenhouse chemicals for toxic effects, or to test hormones and related compounds to improve weak pollen growth and overcome stylar barriers.

Cryopreservation Cryopreservation (storage at −196°C) is one of the safest methods of preserving plant genetic resources. It effectively safeguards germplasm from biotic and abiotic stress, making the germplasm available for future use by breeders and researchers. Once successfully cryopreserved, the plant material may be stored for a very long time without recurrent transfers or replanting. While the initial cost of introducing a totipotent tissue into liquid nitrogen (LN) storage is high, the cost of maintaining the material in LN is much lower than in field planting or as tissue culture. This method of plant preservation requires very little storage space. Together, these attributes of low maintenance

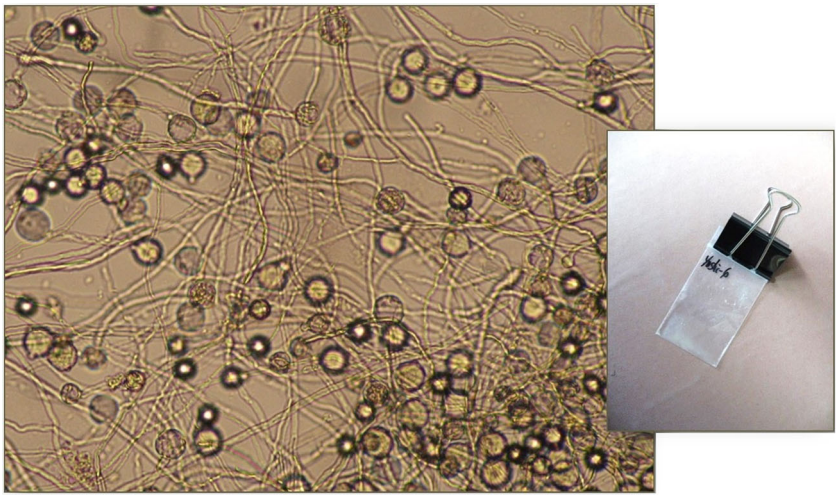


Figure 4. Pollen germination assay.

cost and long term storage make cryopreservation a very useful tool for genebank preservation.

Cryopreservation of potato is based on *in vitro* culture and on *in vitro* procedures. Reports on storing potato at ultra-low temperature go back almost 40 yr, the first being by Bajaj (1977), Grout and Henshaw (1978, 1980), and Towill (1981, 1983, 1984). The cryogenic techniques for potato have evolved from two-step cooling, through ultra-rapid cooling, vitrification, encapsulation–dehydration, and encapsulation-vitrification to droplet vitrification. The progression of potato cryopreservation research, development of applicable techniques, and level of success (measured by the percentage of regenerated plants after storage in LN) were reviewed and summarized by Kaczmarczyk *et al.* (2011).

In 2001, the USDA-ARS National Center for Genetic Resources Preservation (NCGRP), part of the NPGS, started cryopreservation of cultivated potato (*S. tuberosum* Group Tuberosum) as a routine backup of plant variety protection sample vouchers. Currently 280 accessions are stored in LN according to the program's minimum standard of ≥40% of post-cryopreservation viability and ≥60 viable shoots (estimated number) recovered from LN storage (referred to as "40/60"). In the first years of the program (2001–2003), the two-step-cooling and vitrification techniques were used. For several accessions, the post-cryopreservation viability was below the standard of 40% and for such accessions, the cryopreservation process was repeated using a different technique. Application of the droplet-vitrification technique (Kim *et al.* 2006) resulted in viability of 45–100%. In the droplet-vitrification technique, shoot meristems (0.6–1.3 mm in length including the apical dome and two-leaf primordia) are excised from 4- to 8-wk-old aseptic nodal segments, cultured in 0.3 M sucrose (16–24 h) and then in 0.7 M sucrose (7–8 h) at 25°C, and dehydrated in droplets of PVS2 (standard "plant vitrification solution 2" containing 30% glycerol, 15% ethylene glycol, and 15% dimethylsulfoxide [all *w*/*v*]) for 20 min at room temperature on an aluminum foil strip. The foil strip is put into a cryopreservation vial (Fig. 5*a*) containing LN and plunged into LN. Rewarming is done in 0.8 M sucrose (30 min). Shoots are plated on MS medium (M519, PhytoTechnology Laboratories®); plantlets develop in approximately 5–8 wk (Fig. 5*b*, *c*). All steps of the cryogenic procedures are carried out aseptically under a laminar flow hood. The procedural details are posted at http://www.ars.usda.gov/Services/docs.htm?docid=17024.

All 280 accessions are also maintained *in vitro* as minitubers (5°C, dark) and are rejuvenated approximately every 3 yr. The same droplet-vitrification technique was used to cryogenically back up 32 non-*tuberosum* species from the USPG with a 45–100% plant recovery rate. Future work of the NCGRP program will focus on cryopreservation of other non-*tuberosum* species and accessions managed by the USPG as *in vitro* cultures.

Methods for Germplasm R&D and Utilization

Much work has been done with genetic transformation of potato with non-potato DNA; this of course is usually facilitated by *in vitro* technology. The topics below, however, are restricted to *in vitro* methods that facilitate germplasm utilization without introduction of non-potato DNA. Even within that boundary, the treatment below is not intended to be exhaustive. Other techniques that may depend on aspects of *in vitro* technology, such as the very old applications of chemical and radiation-mediated mutagenesis, or quite new methods of intra-genetic modification and gene editing are also being used. Some *in vitro* genetic modification is a combination of natural and artificial manipulation, *e.g.*, when an agent such as a transposon induces spontaneous mutants only in culture (Duangpan *et al.* 2013).

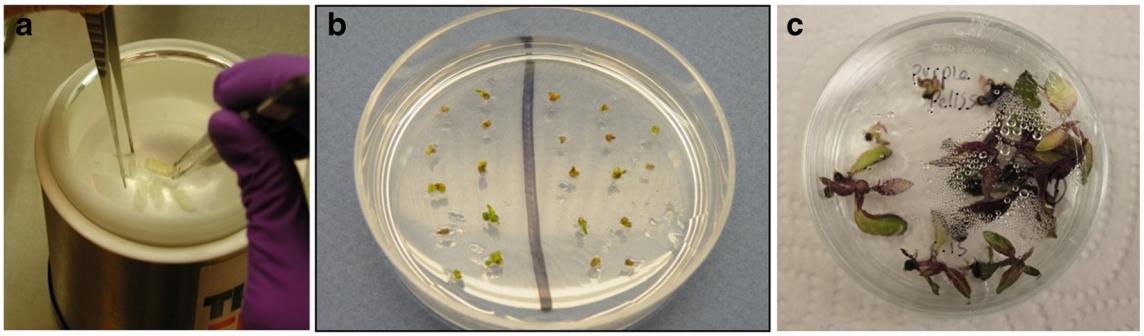


Figure 5. Cryopreservation of shoots. (*a*) Loading into cryopreservation vial. (*b*) Plating. (*c*) Plantlet grow-out of cryopreserved meristems.

Bioassays and selection The *in vitro* environment, although artificial, can be precisely formulated and controlled to differentiate the responses of genotypes to diseases and stress. *In vitro* culture has tremendous potential as a selection tool, considering that the most costly, imprecise, and time-consuming aspect of potato germplasm improvement and breeding is evaluating and selecting the one individual among hundreds of thousands of progeny that is outstanding enough to become a new cultivar—a process that typically takes 10–20 yrs.

The potential of the many and varied applications of *in vitro* selection for potato has long been recognized (Bolik *et al.* 1986), although there has been no recent review of the topic. The technique has been used to select for resistance to heat, drought, and salinity stress (Khrais *et al.* 1998; Albiski *et al.* 2012; Khan *et al.* 2015; Zaki *et al.* 2016), to fungi (Cristinzio and Testa 1999; Rodríguez *et al.* 2007), and to bacterial rots of the stem and tuber (Khu *et al.* 2007; Hudák *et al.* 2009; Habe and Ohbayashi 2014). Mutants such as the gibberellin-deficient stocks identified at the USPG can provide sensitive *in vitro* bioassays and thus serve as research tools (endogenous negative controls) for the study of the effects of exogenously applied compounds (Vega *et al.* 2006).

Somatic embryogenesis in potato Somatic embryos (SE) can be produced on almost any potato tissue in an indirect process that involves a thin layer of callus (reviewed by Nassar *et al.* 2015). Micropropagated plantlets or microtubers are more readily used as a source of explants than field-grown material, as surface disinfestation is not required. Variations in cultivar and source-tissue responses are well documented (Nassar *et al.* 2015).

In general, explants spend 2 wk on callus induction medium with 2, 19, and 0.15 mg L^{-1} of 2,4-D, indole acetic acid (IAA), and thidiazuron (TDZ), respectively, before transfer to SE-induction medium containing 0.05, 12, and 0.55 mg L^{-1} of IAA, zeatin, and gibberellin (GA_3), respectively (Nassar *et al.* 2015). Microtuber and field tuber explants showed a similar response for 'Russet Burbank'; a prolific "forest" of new shoots developed within 3–4 wk (Fig. 6). The plants regenerated come from a mixture of *de novo* adventitious bud formation or "calliclones" (since indirect shoot formation and callus are involved) and somatic embryogenesis or "somaclones" (rooted shoots with a developmental pathway similar to that of zygotic embryos). The latter developmental pathway is preferred and these plants are harvested since the expectation is that less variation and potential loss of commercial characteristics will occur if the callus phase is limited and the SE production interval short. In practice, as growth proceeds, it becomes difficult to distinguish adventitious shoots from rooted plantlets so harvest of SE-derived plantlets is done on a regular basis.

Somaclones are placed individually into micropropagation medium and maintained *in vitro* through single-node cuttings. This is a major limiting feature for somatic breeding, as hundreds of somatic lines, each represented by one or more test tubes, must be kept for months (or years, for advanced lines) during the field assessment phase. Once yield, processing, phytonutrient, or molecular selection is accomplished, only the best lines are retained. Ideally, the same somatic population could be retested in different geographic locations or for further characteristics. However, maintenance of these potentially valuable somatic populations for further exploitation is not a simple matter. In this respect, somatic breeders face problems similar to those of large germplasm repositories.

Somaclonal variation was first described by Larkin and Scowcroft (1981) and results from complex endogenous (genetic variation between cells present in the original explant) and/or exogenous (variation caused by wounding or culture) processes. Endogenous and exogenous variation could result from genetic and/or epigenetic processes occurring in meristematic tissues in culture.

Nassar *et al.* (2015) recommended incorporation of SE technology into potato breeding programs for improvement of advanced lines. Important additional roles for SE technology include molecular transformation activities, where plants are often regenerated through the SE route, and synseed (artificial seed) technology, which relies on SE but has been little studied in potato.

Protoplast fusion The fusion of potato protoplasts (Fig. 7) and the regeneration of somatic hybrids in the late 1970s (Melchers

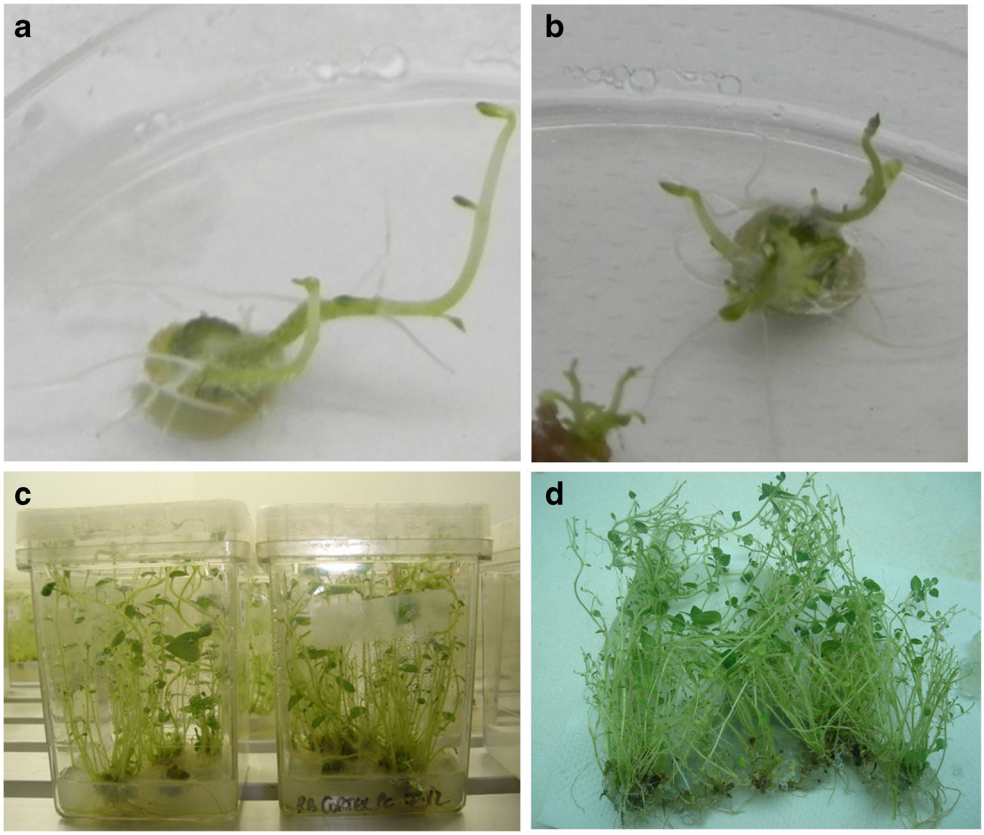


Figure 6. Somaclone production in Russet Burbank. (*a*, *b*) Early stage; (*c*, *d*) later stage.

et al. 1978; Butenko and Kuchko 1980) demonstrated the potential of such techniques in the genetic improvement of potato. Bypassing sexual reproduction allowed researchers the opportunity to generate intraspecific, interspecific, and even intergeneric somatic hybrids of potato. The generation of such hybrids was conducted to facilitate the genetic improvement of potato by (1) the incorporation of genetic resistances from other genera/species previously sexually isolated from potato, and (2) genetic improvement of potato at the dihaploid/diploid level with the utilization of cell fusion to combine complementary traits and maximize heterosis (Wenzel *et al.* 1979).

The use of protoplast fusion for the introgression of traits from reproductively isolated potato species into cultivated potato expanded rapidly over the next several decades. Ferreira and Zelcer (1989) presented a thorough review of somatic hybridization in potato from its inception until 1987, with more recent reviews by Pandey *et al.* (2010), Rokka (2015), and Cho and Park (2014). Eeckhaut *et al.* (2013) also presented a thorough review of progress in plant protoplast research and the current and future outlook for the use of somatic hybridization for crop improvement. These reviews and additional reports in the literature detail a diversity of *Solanum* species having been used in the generation of somatic hybrids with haploids/tetraploids of cultivated potato: *Solanum acaule*, *Solanum berthaultii*, *Solanum brevidens* (synonym of *Solanum palustre*), *Solanum bulbocastanum*, *Solanum chacoense*, *Solanum circaeifolium* (synonym of *Solanum stipuloideum*), *Solanum commersonii*, *Solanum etuberosum*, *Solanum melongena*, *Solanum nigrum*, *Solanum papita* (synonym of *Solanum stoloniferum*), *Solanum phureja* (synonym of *Solanum tuberosum* Andigenum Group), *Solanum pinnatisectum*, *Solanum sanctae-rosae* (synonym of *Solanum boliviense*), *Solanum stenotomum* (synonym of *Solanum tuberosum* Andigenum Group), *Solanum torvum*, and *Solanum verrucosum*.

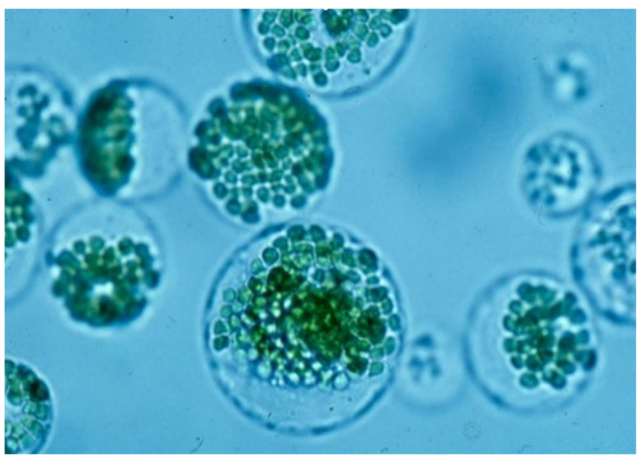


Figure 7. Protoplasts for fusion.

Somatic hybrids have not always had adequate fertility for continued use in potato breeding. However, fertility was documented in several somatic hybrids, which allowed for the successful introgression of wild species traits into cultivated potato. Some notable examples of success in the introgression of resistance genes into cultivated potato from sexually isolated wild potato species using protoplast fusion are detailed below.

Late blight and columbia root-knot nematode resistance derived from *S. bulbocastanum* Somatic hybrids between *S. bulbocastanum* resistant to Columbia root-knot nematode (CRKN: *Meloidogyne chitwoodi*) and tetraploid *S. tuberosum* were obtained (Austin *et al.* 1993) and shown to have nematode resistance (Mojtahedi *et al.* 1995). Brown *et al.* (1996) analyzed the second backcross (BC_2) progeny of the nematode-resistant somatic hybrid using RFLPs and localized CRKN resistance to chromosome 11. Resistance was monogenic, dominant, and mapped to a region not previously associated with resistance to CRKN, with the resistance gene subsequently given the designation $R_{Mc1(blb)}$. This gene conferred resistance to CRKN in the root system of breeding clones derived from the *S. bulbocastanum* somatic hybrid. Brown *et al.* (2009) subsequently also identified tuber resistance to CRKN derived from *S. bulbocastanum* that was loosely linked to $R_{Mc1(blb)}$ in coupling phase and was designated $R_{Mctuber(blb)}$. Analysis also indicated a close relationship between these resistance genes from *S. bulbocastanum* and CRKN resistance genes of *Solanum fendleri* and *Solanum hougasii*, suggesting a common ancestry among the three species (Brown *et al.* 2014).

S. bulbocastanum somatic hybrids were also found to have high levels of resistance to metalaxyl-resistant isolates of *Phytophthora infestans*, responsible for the resurgence of late blight in North America in the 1990s (Helgeson *et al.* 1998). Late blight resistance was heritable, with some sexual progeny of an *S. bulbocastanum* somatic hybrid displaying resistance comparable to that of the somatic hybrid (Dorrance *et al.* 2001). Naess *et al.* (2000, 2001) analyzed the BC_1 and BC_2 progenies from late blight-resistant *S. bulbocastanum* somatic hybrids with RFLP and RAPD markers. They found that the synteny of RFLP markers noted in tomato, potato, and *S. brevidens* was largely maintained in *S. bulbocastanum*, and were able to localize the late blight resistance in *S. bulbocastanum* to chromosome 8. Subsequent research resulted in the cloning of the late blight *R* gene, *RB* (also known as *Rpi-blb1*), which confers broad-spectrum resistance to multiple isolates of the pathogen (Song *et al.* 2003; van der Vossen *et al.* 2003). Breeding clones and transgenic plants carrying *RB* have shown high levels of resistance to late blight (Halterman *et al.* 2008; Meier *et al.* 2015).

Insect, virus, and fungal resistances derived from *Solanum etuberosum* and *S. berthaultii* Protoplast fusion was used in the generation of somatic hybrids between *S. etuberosum* and a Group Tuberosum dihaploid × *S. berthaultii* hybrid clone (Novy and Helgeson 1994a). The somatic hybrids and their progenies have been shown to have resistances to PVY (Novy and Helgeson 1994b; Novy *et al.* 2002), PLRV (Novy *et al.* 2002, 2007), pink rot and *Pythium* leak (Thompson *et al.* 2007), and defense against green peach aphid (Novy *et al.* 2002) and potato psyllid (Butler *et al.* 2011; Diaz-Montano *et al.* 2014). Unpublished data also suggests resistance to wireworm and Colorado potato beetle. A major gene for resistance to PLRV derived from *S. etuberosum*, Rlr_{etb}, has also been mapped (Gillen and Novy 2007; Kelley *et al.* 2009) with markers developed for use in marker-assisted selection for PLRV resistance.

The numerous disease and pest resistances identified in the somatic hybrids and sexual progenies of *S. etuberosum*, *S. berthaultii*, and *S. bulbocastanum* highlighted in this section provide examples of the usefulness of protoplast fusion in the genetic improvement of potato. Protoplast fusion, which depends on the use of *in vitro* technology, allows the use of wild species in breeding that, due to sexual incompatibilities, cannot be readily hybridized with cultivated potato. In conjunction with the introgression of desirable traits into the cultivated potato gene pool, protoplast fusion also has led to the discovery of new genes for resistance. Such resistance genes can prove useful in better understanding the mode of gene action, including host and pest/pathogen interactions, and can also be used in the developing field of intragenic improvement of potato.

Ploidy manipulation The natural ploidy variation for cultivated potatoes ranges from 2*x* to 5*x*, with most cultivars of worldwide economic significance being 4*x* ($x = 12$, the basic [monoploid] chromosome number of potato). However, the plethora of wild potato species ranges from 2*x* to 6*x*, with most being 2*x* (Hawkes 1990; Ortiz 2001; Machida-Hirano 2015). The common occurrence of male and female unreduced gametes has facilitated both naturally occurring and breeder-imposed sexual polyploidization to intercross potatoes that differ in ploidy (Mendiburu and Peloquin 1977; Johnston *et al.* 1980; Veilleux 1985). Mismatch of endosperm balance number (EBN) refers to a situation in which plants of the same ploidy that would otherwise hybridize fail to produce viable seed due to genetic incompatibility for endosperm development (Ortiz and Ehlenfeldt 1992). Ploidy reduction can be accomplished by the use of so-called prickle pollination using specific diploid pollinators that induce paternal chromosome elimination in a low frequency of zygotes, resulting in gynogenic haploid seedlings. In crosses with tetraploid female parents, the "triploid block" results in the production of mostly haploid seedlings from 4*x*–2*x* crosses with a prickle pollinator (Hougas *et al.* 1964; Marks 1966). In 2*x*–2*x* crosses, most of the seedlings will actually be diploid hybrids with the occasional maternal haploid. The prickle pollinator is homozygous for a gene contributing a selectable phenotype (embryo spot) visible through the seed coat. This spot is present only in hybrid seed; thus, haploids can be selected by the absence of

an embryo spot (Hermsen and Verdenius 1973; Uijtewaal *et al.* 1987; Liu and Douches 1993).

Anther and microspore culture offer an alternative method to develop androgenetic haploids by redirection of the immature pollen grain along an embryogenic route (androgenesis) (Snider and Veilleux 1994; Ferrie and Caswell 2011; Santra *et al.* 2012). The ability to respond to anther culture is genotype dependent and reasonably rare, with anthers of most potatoes demonstrating little or no response to this *in vitro* technique (Powell and Uhrig 1987; M'Ribu and Veilleux 1990; Boluarte-Medina and Veilleux 2002). Haploids have only been obtained from a few tetraploid cultivars using anther culture (Fig. 8) whereas haploids have been more easily obtained from a wider range of cultivars using prickle pollination (Batty and Dunwell 1987; Kotch and Peloquin 1987; Johansson 1988; Upadhya and Cabello 1997; Asakavičiūtė *et al.* 2007). Diploid *Solanum tuberosum* Group Phureja, *Solanum chacoense*, and a few other species have been used to develop monoploid potatoes with only 12 chromosomes (Cappadocia *et al.* 1984; Veilleux 1990; Aziz *et al.* 1999). The success of monoploid extraction depends both on the genetic predisposition of a sporophyte to anther culture and on the genetic load of the diploid progenitor (Hougas *et al.* 1964; Meyer *et al.* 1993). The more Group Tuberosum in the pedigree, the greater the genetic load and the poorer the quality of the monoploids. The primarily diploid status of members of Group Phureja tends to result in a reduced genetic load and may give rise to somewhat more vigorous monoploids according to expectation based on population genetics (Haldane 1930; Bever and Felber 1992). Occasionally, these monoploids may double spontaneously during routine *in vitro* subculture (Meyer *et al.* 1993).

Clones may be induced to double by a simple *in vitro* leaf disc regeneration technique without the use of a specific doubling agent (Karp *et al.* 1984; Rao and Suprasanna 1996). Such doubled monoploids may express somaclonal variation as a result of the *in vitro* passage, so it is generally recommended to regenerate several diploids from any given monoploid to be able to select the least variant types (Karp *et al.* 1989; Veilleux and Johnson 1998). Chromosome doubling can also be achieved by callus induction of *in vitro* internodes on autoclaved MS medium supplemented, after autoclaving, with filter-sterilized zeatin (1.75 mg L^{-1}) and IAA (0.5 mg L^{-1}). Internodes (~1 cm) are pushed onto the surface of the medium in Petri dishes, sealed with Parafilm®, and checked monthly for callus production. Callus is then broken into pea-size pieces and pressed into shoot induction medium consisting of MS medium supplemented with folic acid (0.25 mg L^{-1}), D-biotin (0.05 mg L^{-1}), and naphthalene acetic acid (NAA, 0.2 mg L^{-1}). Developing shoots are grown in pots and ploidy is determined by pollen size (Bamberg and Hanneman 1991b) or by counting chromosomes of microspore mother cells.

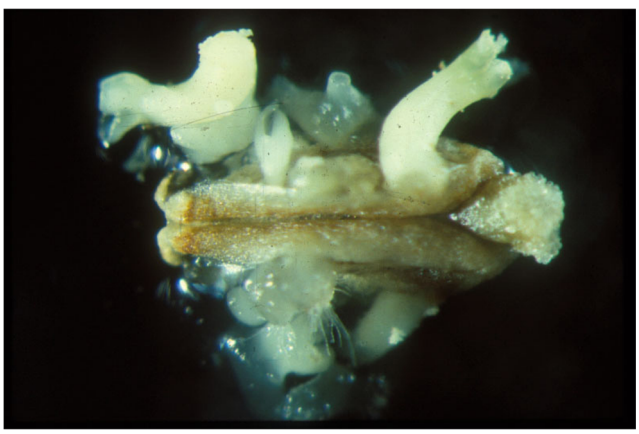


Figure 8. Embryos from anther culture.

In many cases, the research and crossing goal can be facilitated by doubling of genetically heterogeneous sprouting seeds. Since colchicine and other doubling agents are often somewhat toxic and make the sprouting seeds vulnerable to rotting, sterile culture is helpful. Seeds are surface sterilized and placed on sterile filter paper in Petri plates. A 0.3% solution of sterile colchicine applied shortly before the radicle emerges is effective for chromosome doubling of most germplasm, but there is a range of sensitivity among different species and populations, so some customization of the protocol may be needed. A preliminary sprouting test to predict when the seeds begin to grow can be used to determine when to apply the colchicine. If the particular germplasm treated is sensitive to colchicine toxicity or is less vigorous when doubled, success will be enhanced by transferring seedlings to nutrient agar medium on which they can be nursed to a stage ready for transplanting to the greenhouse.

Homozygous lines are extremely rare in potato, and anther culture has provided the most reliable route to develop them. An anther-derived monoploid that was doubled by leaf disc regeneration was used to develop a draft genome for potato (Potato Genome Sequencing Consortium 2011). Anther-derived doubled monoploids have exhibited various levels of female fertility and have been used in half-diallel analyses (M'Ribu and Veilleux 1992; Paz and Veilleux 1999). However, their lack of male fertility has been an impediment to extensive breeding adaptation. An attempt to improve fertility by genetic complementation using somatic hybridization between genetically distinct monoploids resulted in male- and female-fertile tetraploid somatic hybrids (Lightbourn and Veilleux 2007) that were only marginally fertile upon chromosome reduction to the diploid level. Subsequent monoploid extraction and leaf disc regeneration did not result in doubled monoploids with any greater fertility than the original first-cycle doubled monoploids.

Another impediment to the utilization of diploid building blocks in hybrid breeding strategies is that most diploid potato lines are self-incompatible, with only a few sources of self-compatibility available in some poorly adapted clones (Carson

Fig. 9 Plantlet developed from an immature embryo nursed *in vitro*

and Howard 1942; Cipar *et al.* 1964, 1967; Cappadocia *et al.* 1986; Phumichai *et al.* 2005). It may require expansion of the germplasm pool to develop true-breeding, self-pollinating diploids that would facilitate hybrid breeding.

The potato genome Since its publication in 2011, the draft potato genome (generated from a homozygous doubled monoploid) has been extensively used in genomic approaches to potato improvement. The original genome assembly was improved by developing a dense genetic map from a backcross population [(DM × heterozygous diploid) × heterozygous diploid]; segregation of DM specific DaRT (diversity arrays technology) and microsatellite markers permitted the orientation and reassembly of scaffolds into 12 more complete linkage groups (Sharma *et al.* 2013). Alignment of sequence obtained from a heterozygous diploid clone RH89-039-16 (RH) and from potato cultivars to the improved draft genome has provided millions of single-nucleotide polymorphisms (SNPs) with highly confident positions on the physical map and variation in nearly every gene of interest. These tools have facilitated the discovery of resistance gene clusters (Jupe *et al.* 2012; Lozano *et al.* 2012), stress response genes (Muñiz García *et al.* 2012; Zhang *et al.* 2014; Kitazumi *et al.* 2015), distribution of microRNA genes (Zhang *et al.* 2013; Lakhotia *et al.* 2014), genes for photoperiod adaptation (Kloosterman *et al.* 2013), the distribution of loci associated with distorted segregation (Felcher *et al.* 2012; Hackett *et al.* 2013; Obidiegwu *et al.* 2014), and genes for resistance to the fungal cause of potato wart disease, to name just a few. The ability to identify genes in potato orthologous to any that have been described in model crops will pave the way for targeted breeding strategies.

True potato seed The possibility of propagating potato from true seed (TPS) in order to bypass the encumbrance of bulky, perishable sections of tubers (seed pieces) for planting has been envisioned for many years (Macaso-Khwaja and Peloquin 1983; Golmirzaie *et al.* 1994). The International Potato Center (CIP) released some open-pollinated TPS varieties that were too variable for wide adaptation (Almekinders *et al.* 2009). The use of diploid inbred lines either to produce diploid F_1 hybrids or to generate diploid hybrids that can be recombined again through bilateral sexual polyploidization to generate double-cross tetraploid hybrid varieties has been hampered by the paucity of available homozygous lines as building blocks (Lindhout *et al.* 2011). The advantage of the double-cross hybrid scheme is that it accommodates the perceived requirement for multiallelic heterozygosity thought to underlie vegetative vigor in potato. The simplicity of the single-cross hybrid scheme would need to overcome the bias that the tetraploid state is optimum for productivity in potato. Recent evidence of the potential of diploid hybrids is promising (Jansky *et al.* 2014).

Embryo rescue Many attempted matings of wild potato species with cultivated forms cause abortion of the seed at a very early stage. USPG staff have also noted (unpublished) that in other cases, seeds formed within the berries are plump with normal seed coats, but when dried, are flat with nearly zero germination. Microscopic examination shows that in such cases, the endosperm does not develop normally, although embryos may progress to a stage at which they can be rescued on artificial media. To do so, berries are surface sterilized and opened under a dissecting microscope in a sterile hood, and the embryos removed with insulin syringes or microsurgical needles. Embryos plated on MS medium in Petri plates typically develop root hairs and green expanding cotyledons within 1 wk (Fig. 9) and can then be transferred to individual culture tubes. Although time consuming, embryo rescue appears to be the only practical way to obtain certain hybrids.

Conclusions

In vitro technology has useful applications in nearly every germplasm service component of the USPG: acquisition, classification, preservation, evaluation (characterization), and distribution. It also has had very productive applications to germplasm use, allowing the synthesis of novel hybrids and variants for breeding and the development of bioassays and selective environments for basic research and germplasm improvement.

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