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Potato *in Vitro* Culture Techniques and Biotechnology

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ABSTRACT

This review was prepared as a survey of key articles presenting development, achievements and interconnection of various lines of potato biotechnology research united through the common use of *in vitro* culture techniques. Starting with the early research on the induction and differentiation of callus tissues, review sequentially and chronologically presents the advance of various *in vitro* culture techniques and their practical applications in clonal propagation, germplasm storage, production of healthy virus-free plants and breeding. Main topics (sections) presented include: establishment and maintenance of callus, root and shoot cultures; variability in callus tissue and protoplast cultures; shoot differentiation and regeneration from various explant types and cultures, somatic embryogenesis and androgenesis; shoot and meristem tip cultures and their numerous applications; regulation of i*n vitro* tuberization, microtubers dormancy and sprouting and systems for production of micro and minitubers. Finally, review outlines the gradual technique merging and shifting of research interest from cellular to molecular biology. This approach enables us to comprehend developmental processes in cells and tissues not only through the interaction of plant growth regulators and environmental factors but also through sequential activation and expression of regulatory genes.

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Received: 11 June, 2008. Accepted: 17 September, 2008.

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INTRODUCTION

Potato is one of the most important crops in the world and therefore the subject of constant interest and numerous studies including those classified as plant biotechnology. In the rapidly developing field of plant biotechnology, the use of plant *in vitro* culture techniques is the common, integration feature. *In vitro* culture techniques enable us to excise from plants and under controlled, axenic laboratory conditions culture their cells, tissues, organs or even the whole plants. These techniques are also a starting point for many practical applications that we will briefly present and discuss.

The first report on the establishment of potato *in vitro* cultures by Stewart and Caplin (1951) was made now more than half a century ago. Since then, hundreds of research articles have been published and some of them can not be easily obtained today. For those who need to enter and join research on potato biotechnology reviews are by far the best choices for the first contact. Written in a digested mode they offer quick access to articles and data of interest. Some very good reviews covering various topics of potato biotechnology already exist but as the years pass by, they need to be updated and extended.

In this review, we will present a short overview covering all basic *in vitro* culture techniques and their applications in potato biotechnology. Apart from raw data, we hope to present a generalised outcome of certain research lines, establish their mutual interactions and point out some problems that remain to be solved in future research.

Basic research and practical applications

The early establishment of practical applications was highly characteristic for the development of potato biotechnology. Actually, this whole research field was from the beginning constantly driven by commercial interests and needs. Production of virus-free plants by meristem-tip culture was the first practical application of potato biotechnology. Starting in the 1950's it is still used even today, however in combination with other virus-eradicating techniques like thermotherapy and chemotherapy.

Prospects for the wide-scale use of potato biotechnology for practical purposes in the next step required establishment of (national, regional) potato centres and small commercial laboratories. Their main goal was production of elite, healthy, virus-free plants and germplasm storage. Mass nursery production of potato by micropropagation was possible but not so prominent as in other horticultural species. We must remember that the main propagule types in potato are tubers (seed potato) and not the rooted plantlets or seed as in other crops. Thus in potato, production of microtubers and later minitubers will be the main driving force for research. It will provide diverse techniques for mass production of small tubers including continuous and temporary immersion systems, hydroponics, aeroponics and others.

In vitro culture techniques will help maintain long-term potato germplasm collections and exchange of material intended for breeding. Useful techniques for this purpose are various types of sustained or slow growth propagation and cryopreservation.

Apart from the production of healthy, elite virus-eradicated plants and mass clonal propagation, biotechnology will offer solutions for improved potato breeding. Our commercial cultivars are highly heterozygous tetraploids imposing difficulties for classic breeding programs. Research on plant protoplasts coupled with protoplast fusion and creation of somatic hybrids will enable the creation of intra- and interspecific hybrids that could not be produced otherwise. Androgenesis is another technique interesting for breeding purposes since it offers production of haploid and homozygous plants directly form microspores of excised anthers.

Somaclonal variation comprising epigenetic changes imposed by the use of *in vitro* culture techniques was at first considered beneficial since it offered fast achievement of variability useful for breeding (Larkin and Scowcroft 1981). Unfortunately, later studies will show it to be highly detrimental due to the high incidence of aberrations triggered by the use of callus tissues. Thus, the promising research on plant protoplasts, although the object of intensive studies, failed to deliver the expected results. Practical application was achieved by another technique – *Agrobacterium*-mediated transformation which enabled direct transfer and stable incorporation of single genes of interest into the genome of recipient cultivars. Basic protocols, goals and prospects on this technique will be presented in a separate chapter of this volume.

Although much time has passed, there is still a very strong interest for the practical use even for the oldest *in vitro* culture techniques. All new cultivars and interesting genotypes need to be established as virus-free plants. For some of them well elaborated techniques for rapid clonal propagation are required. Maintenance of collections and germplasm storage is a permanent task of specialised institutions. Somatic hybridization can still solve breeding problems shifting traits between various *Solanum* species for breeding purposes. This are all situations in which *in vitro* culture techniques are invaluable. New studies often offer small improvements, which need to be recorded and evaluated. Finally, there is always the possibility of a major research breakthrough which may establish new, previously unforeseen (unexpected) applications for plant *in vitro* culture techniques.

Literature sources, general papers and reviews

Most of the data presented in this review comes from research articles published in leading journals specialised for basic or applied plant research. We also used a number of general papers, reviews and other useful articles that we highly recommend. Most useful among them were the early reviews presented by Miller and Lipschutz (1984) and Bajaj and Sopory (1986) covering the general *in vitro* culture research in potato from its beginning up to the mid 1980s. Both reviews offer a complete picture of the research on meristem tip culture, shoot cultures, germplasm storage, cold storage and cryopreservation; callus induction and regeneration, protoplast production, regeneration and fusion and evaluation of somaclonal variation. There are useful re-

views by Mellor and Stace-Smith (1977) and Styer and Chin (1983) presenting data on techniques for virus eradication in potato. Zuba and Binding (1989) presented a review containing data required for the understanding of research on potato protoplasts and their somaclonal variation. Problems related to somaclonal variation in potato have been presented by Pijnacker and Sree Ramulu (1990) and Jacobsen (1987).

Many publications in the period 1990-1995 were at the time unavailable to us for non-scientific reasons imposed by the political situation in the country. The later appearance of KOBSON (acronym standing for Serbian consortium of coordinated library acquisition) operated by the National library, solved most of our problems with missing literature.

Potato was used as one of the standard objects in extensive research on environmental factors and their impact on plant *in vitro* cultures done by T. Kozai and co-workers at the Faculty of Horticulture, Chiba University, Japan. Among numerous papers, we recommend reviews on autotrophic micropropagation – one of their key contributions (Kozai 1991).

Many articles can be recommended for the understanding of complex interactions involved in the regulation of tuberization: Ewing (1987), Vreugdenhil and Struik (1989), Vreugdenhil *et al.* (1998), Jackson (1999). Basic concepts presented here have been further investigated and then extended to the molecular level (Fernie and Willmitzer 2001; Kolomiets *et al*. 2001; Hannapel *et al*. 2004; Vreugdenhil 2004; Hannapel 2007).

Coleman *et al.* (2001) described the use of microtubers as a research tool while Donnelly *et al.* (2003) presented production and performance of microtubers. Seabrook (2005) presented a very useful review compiling all studies describing various effects of light on growth and development of potato cultures.

Last year a highly inspirational book dedicated to potato biology and biotechnology appeared. Edited by Vreugdenhil *et al*. (2007) it gives a complete insight of the potato as a crop and research object. Finally, the whole 2008 has been declared as the International Year of the Potato and we may therefore expect other useful scientific publications honouring this jubilee.

CALLUS TISSUE AND SUSPENSION CULTURES

In the multicellular body of higher plants consisting of several distinct types of tissues and organs, the ability to divide is highly restricted and reserved only for meristems and meristematic tissues. Such tight control over cell division enables plants to keep their body shape, structure and proportion, which are all required for proper functioning. In emergencies, nearly all tissue types can return to the meristematic state, and divide forming replacement tissues needed to repair wounds, broken and damaged shoots and roots. This fast dividing replacement tissue called callus can be induced in various *in vitro*-cultured explants by external addition of plant growth regulators (PGRs). In liquid media, friable callus quickly dissolves forming a mixture of individual cells and small cell aggregates called cell suspension.

Callus induction in potato was first reported by Stewart and Caplin (1951) and then confirmed by Chapman (1955) and Lingappa (1957). The number of studies dealing with callus induction, growth and differentiation increased slowly during the next two decades culminating in a short period lasting from 1977 till 1978. Some of the studies that we should mention include Bajaj and Dionne (1967), Okazawa *et al.* (1967), Anstis and Northcote (1973), Skirvin *et al.* (1975), Lam (1975) and Roest and Bokellman (1976). Attempts to develop practical applications for callus and suspension cultures were not very successful and research in this lane gradually declined during the 1980s. As we already mentioned the troublesome feature of potato callus was its genomic instability resulting in regeneration of aberrant shoots/plants rendering this whole technique unsuitable for clonal propagation and breeding purposes.

However, data collected from studies with callus and suspension cultures was a helpful starting point for other studies like those aimed at production of potato protoplasts or *Agrobacterium*-mediated transformation. The attention was turned towards the shoot regeneration from protoplast-derived callus (p-callus) and to the improvement of procedures enabling direct regeneration of shoots from various potato explants.

The first studies were done using explants excised from potato tubers. Leaves and other explant types would be investigated later. Tubers are predominantly built from parenchymatic cells with storage as the main function. Axillary buds ("eyes") are located at the tuber surface; therefore, samples of inner tissue contain no meristematic centers that could develop as false regenerants.

Early studies were done before cytokinins, gibberellic acids (GAs) and other PGRs were available. Apart from White's 1943 medium, there was not much choice for mineral media composition. MS (Murashige and Skoog 1962) medium appeared later and still a number of years will have passed before it became adopted as the best, wide-purpose medium in potato biotechnology. Early studies, although technically inferior, with inadequate experimental design, sample replication and lack of reliable statistics, provided invaluable data required for the understanding of basic requirements of potato *in vitro* cultures.

Early studies showed that potato tuber explants produce callus easily when cultured on media supplemented with auxins, 5-7 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) or 10-12 mg/l NAA (naphthylene acetic acid). Okazawa *et al.* (1967) investigated the effect of IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), NAA, 2,4-D and kinetin (KN) over a wide range of concentrations. Callus formation started after some 7 to 10 days requiring only the presence of auxins. KN however supported later callus growth. Roots were regenerated at lower auxin concentrations. KN induced shoot regeneration which was not observed in any of the early studies.

Bajaj and Dionne (1967) investigated growth of callus and suspension cultures using MS medium medium in combination with 0.2 mg/l KN and 2,4-D and 1000 mg/l yeast extract.

Anstis and Northcote (1973) performed extensive research on callus induction and growth of cv. 'King Edward's'. Using a 4 × 5 matrix comprising 4 KN and 5 auxin concentrations they investigated the effects of IAA, NAA, 2,4-D and KN. They also investigated the effect of medium pH, light colour and irradiance, temperature, agar concentration, sucrose concentration and replacement of sucrose with one of 8 different carbohydrate sources, cytokinin content of callus, antibiotics and finally production of suspension cultures. Optimal fresh callus weight increase was obtained on medium with 2.0 mg/l 2,4-D and 0.02 mg/l KN. Optimal concentrations and conditions were established for all investigated factors. Shoot regeneration was not observed in any of the numerous investigated treatments. Callus induction and maintenance were also studied by Dunwell and Sunderland (1973) and Wang and Huang (1975).

In a short period lasting from 1977-1978 there was a sharp increase in the number of studies dedicated to callus tissues (Bragdo-Aas 1977; Lam 1977a, 1977b; Shepard and Totten 1977; Wang 1977; Binding *et al.* 1978; Grout and Henshaw 1978; Melchers *et al*. 1978). Research covered various topics ranging from shoot regeneration up to protoplast culture. Some ten years later (1988-1989) there would be another similar surge in the number of published papers. This time their aim was to establish simple, feasible protocols for genotype independent *Agrobacterium*-mediated transformation of potato.

Callus and suspension cell cultures are still used and studied. The culture conditions and PGR balance of media did not change much. Studies are mostly aimed at shoot regeneration, somatic embryogenesis and problems related to the genetic stability (variability) of callus tissues.

Direct shoot regeneration

Shoot regeneration and multiplication was a long lasting problem of potato tissue culture. Shoots were produced sporadically but a simple and consistent regeneration procedure was missing. Apparently, potato callus can not be manipulated to regenerate shoots as easily as in other plant species.

If not from callus, shoot regeneration was attempted directly from tuber and other explant types. Okazawa *et al.* (1967) was the first to report that shoots could regenerate from large tuber explants. Direct shoot regeneration was of special interest since it was believed that avoiding the callus stage may prevent the formation of aberrant shoots. Direct shoot regeneration was later considered as the best approach for the production of transgenic plants.

Lam (1975) reported shoot formation in tuber disc explants cultured in the presence of 0.4 mg/l BA (6-benzyl adenine). Shoots appeared to develop from embryo-like bodies. In following studies shoot regeneration was achieved through the simultaneous use of several PGRs in the medium (Lam 1975; Skrivin *et al.* 1975).

Roest and Bokelmann (1976) reported shoot regeneration in rachis explants of a highly responsive potato genotype. They showed that GA_3 (gibberellic acid) is the factor favouring direct shoot regeneration.

In a study dedicated primarily to protoplast culture, Shepard and Totten (1977) showed that tuber explants of 'Russet Burbank' directly regenerated shoots when placed on protoplast medium C for 3-4 weeks with a transfer to medium D. Composition of media C and D is presented in the section on protoplast culture.

Jarret *et al.* (1980a, 1980b) studied the production of adventitious shoots on tuber explants of cv. 'Superior'. MS medium with 0.03 mg/l NAA, 0.3 mg/l GA_3 and 3.0 mg/l BA was recommended for shoot regeneration. The use of multiple cytokinins as in previous studies was not required, and the critical factor which supported both regeneration and subsequent shoot development was GA_3. The auxin/ cytokinin balance was required for the determination of regeneration as postulated by Skoog and Miller (1957).

Mix and Sixin (1983) reported the formation of callus from which embryos and shoots regenerated on tuber disc explants of three potato cultivars on a complex MS medium containing 1.0 mg/l IAA, 0.8 mg/l KN, 0.2 mg/l NAA, 0.5 mg/l BA, 0.4 mg/l ZR, 0.4 mg/l GA_3 and 50 ml liquid coconut endosperm.

Kikuta and Okazawa (1984) investigated direct regeneration from tuber explants of 'Irish Cobbler'. Shoot buds were regenerated on modified White's (1934) medium with 0.25 M mannitol and 0.5 mg/l zeatin and 0.1 mg/l IAA at 20°C and relatively high irradiance. Roots were regenerated on modified White's medium with 29 mM sucrose, 1.0 mg/l KN and 0.3 mg/l IAA at 30°C in darkness.

Sherman and Bevan (1988) reported direct shoot regeneration from tuber explants using 3C5ZR medium containing IAA conjugate and zeatin riboside (ZR). Direct shoot regeneration avoids the callus stage and occurrence of somaclonal variation. Medium was used for potato transformation.

Esna-Ashari and Villiers (1998) reported regeneration of shoots from tuber discs of cv. 'Désirée' in a single step on medium supplemented with 1.0 mg/l BA.

Callus production and shoot regeneration from leaf explants

The morphogenetic capacity of potato leaves was not much investigated until research on potato protoplasts became more popular. Even then, leaves were used only for the first, protoplast production stage. Among the rare reports on the production of callus from leaf explants is that by Gavinlertvatana and Li (1980).

During the 1980s, a number of protocols for *Agrobacterium*-mediated transformation would appear using leaves as initial explants. Since the production of transgenic shoots is the main outcome of such studies, there was an increased interest for the morphogenetic capacity of leaves as explants. Idea for the use of leaves as the explant source in plant transformation studies was postulated by the Monsanto research team (Horsch *et al.* 1985).

Fig. 1 This fast growing callus on leaf explants of cv. 'Dragačevka' was induced in two steps. Explants were first cultured on medium with 5.0 mg/l 2,4-D and after 4 weeks transferred to medium with 2.0 mg/l BA and 5.0 mg/l GA_3. Callus remained undifferentiated, failing to regenerate shoots at this high cytokinin concentration.

Fig. 2 Regeneration of shoots on leaf explants of cv. 'Dragačevka', (same as in Fig. 1), in a single step regeneration procedure on medium supplemented with 0.2 mg/l NAA, 2.0 mg/l BA and 10.0 mg/l GA_3. Shoots regenerated directly from explants without an intervening callus stage.

Webb *et al.* (1983) studied PGR interactions in the regeneration of shoots from potato leaf discs. The basic assumption was that shoot regeneration was a two-stage process at each stage requiring different PGRs. The two stage propagation procedure was superior to the control, single-stage system. The first stage required NAA and BA while the presence of GA_3 was beneficial. In the second stage, auxins were clearly inhibitory for shoot regeneration but the presence of GA_3 was obligatory. The timing of GA_3 application was not critical for shoot regeneration (**Figs. 1, 2**).

Wheeler *et al.* (1985) investigated direct shoot formation from leaf, rachis, shoot and tuber explants of fourteen potato cultivars. Experiments compared PGR combinations used in protocols developed by Shepard and Totten (1977), Jarett *et al.* (1980a, 1980b) and Webb *et al.* (1983). All cultivars regenerated shoots. Only two cultivars failed to regenerate shoots from leaf explants. Leaf explants were superior to tuber explants not only in shoot proliferation but also in a less pronounced production of aneuploid plants. Aberrant plants included only aneuploidy. Only one plant with a

high chromosome number (endoreplication) was detected.

Yaddav and Sticklen (1995) accurately pinpointed the cytokinin and GA_3 requirements for direct shoot regeneration of cv. 'Bintje' leaf explants. Using a two-stage shoot regeneration approach recommended by Webb *et al*. (1983), they found that the most responsive treatment was 6 days on medium with 0.5 mg/l zeatin or 0.8 mg/l ZR followed by a 6 day treatment on medium with 0.8 mg/l ZR + 2.0 mg/l GA_3.

Park *et al*. (1995) regenerated shoots from callus developing from edges of leaf explants of four North Dakota potato genotypes. Optimal shoot regeneration occurred in explants cultured on MS medium with 3.5 mg/l IAA and 3.0-4.0 mg/l ZR. Darkness favoured shoot regeneration, while callus developing in the dark was etiolated, i.e. cream-white in colour. There were apparent differences between genotypes in the time of shoot appearance and dark requirement.

Hansen *et al*. (1999) investigated proliferation and shoot regeneration of leaf explants in cvs. 'Posmo', 'Folva' and 'Oleva' encountering marked genotype effects. A wide range of PGR combinations and concentrations were investigated same as the initial position of explant on the leaf from which it was excised. Following protocol optimization and improved explant sampling shoot regeneration in 'Posmo' reached 97% while in 'Folva' it was only 32.1%. Cultivar 'Oleva' did not regenerate shoots even with the use of optimized protocols.

Yee *et al*. (2001) investigated shoot regeneration in leaf petioles developing a new explant source intended for *Agrobacterium*-mediated transformation. High shoot regeneration in all 7 investigated commercial cultivars was obtained on MS medium supplemented with 3.0 mg/l BA, 2.0 or 0.5 mg/l IAA and 1.0 mg/l GA_3. Calli from which shoots regenerated formed ~2 weeks after the initiation of cultures. Addition of 6.8 mg/l $AgNO_3$ in the form of silver thiosulphate was detrimental while the addition of 0.66 mg/l thidiazuron did not induce changes in shoot regeneration.

Cell suspension cultures

Suspension cultures can be easily obtained if friable potato callus is cultured on liquid medium supplemented with 2,4-D. The presence of cytokinins at very low concentration can be beneficial. Preparation of potato suspension cultures was described by Bajaj and Dionne (1967), Anstis and Northcote (1973) and Lam (1977b).

Cell suspension cultures were used successfully as a starting material for the establishment of protoplast cultures (Opatrny *et al*. 1980). It should also be noted that Vargas *et al*. (2005) used cell suspension cultures as starting material for production of somatic embryos.

Variability in callus cultures

The wide occurrence of somaclonal variation in potato callus tissue resulting in changes of chromosome number leading to aneuploidy and polyploidy was a well known fact in early 1980s. Somaclonal variation was a problem hindering all practical applications based on the use of callus tissue. As a consequence, serious research studies were soon launched aimed to discover factors and mechanisms responsible for the genomic instability of *in vitro* cultures.

Similar studies on somaclonal variation in callus tissue regenerated from protoplast cultures are presented separately in the next section dedicated to protoplast culture.

Sree Ramulu *et al*. (1985) studied variation in nuclear DNA content and chromosome numbers in callus and cell suspension cultures of mono-, di- and tetraploid potato. There was a high degree of instability during *in vitro* culture growth. Both polyploidy and aneuploidy were observed to occur in initial cell divisions leading to the proliferation of callus. Meristematic tissues were more stable that other tissue types. In leaf explants of monohaploid plants stained with Giemsa staining technique, the increase in the number of mitoses was observed on the third day (Pijnacker *et al*. 1986). Polyploidization occurred mostly through endoreduplication (Pijnacker *et al*. 1989). Analyzing the chromosome numbers in leaf explants of mono-, di- and tetraploid plants during callus induction Pijnacker and Ferwerda (1990) identified sucrose as the factor responsible for endoreduplication. Sucrose was most effective at 3% and in monohaploids. In di- and tetrahaploid plants sucrose-induced endoreduplication was less pronounced. These findings indicate that sucrose can identify and trigger cells of certain ploidy levels to enter an endoreduplication cycle instead of performing normal mitosis.

Rietveld *et al*. (1991) investigated variability in 1600 plants produced from adventitious meristems induced and regenerated from tuber disc explants of cv. 'Superior' using procedures elaborated by Lam (1977a) and Jarret *et al*. (1980a, 1980b, 1982). Direct regeneration of shoots from tuber disc explants resulted in variability but the frequency of aberrant plants was low. Somaclones with desirable alterations in yield, tuber number and shape, and plant vigour were observed, selected and studied in two or more consecutive tuber generations. This study actually indicates that adventitious shoot regeneration in the absence of callus induction produces stable regenerants which can be used in practical applications.

Sebastiani *et al*. (1994) investigated the potential use of somaclonal variation for production of plants resistant to *Verticillium dahlie*. Among 325 regenerated plants one was selected as being insensitive to fungal filtrate. The authors believed that the genetic variation induced in tissue culture could be used for selection of *Verticillium*-resistant potato plants.

Thieme and Griess (2005) studied 13,000 somaclones produced from *in vitro* cultures of 17 potato cultivars. Callus from leaf and stem explants was cultured on MS medium with 0.2 mg/l NAA, 2 mg/l zeatin and 5 mg/l GA_3. After 14 days primary callus was transferred to shoot induction media of Webb *et al*. (1983) and Wheeler *et al*. (1985). The conclusion of this study was that somaclonal variation can be used to modify one or a few characters in a leading cultivar preserving other important traits. Since somaclonal variation enables the production of aberrants with better performance for some desired traits, it should be exploited in potato breeding as an auxiliary method for some cultivars and specific breeding targets.

ROOT CULTURES

Culture of excised roots is one of the oldest *in vitro* culture techniques, which was gradually abandoned in the 1960s. Most of these studies were performed in monocotyledonous plants (cereals) with only few dicotyledonous species as models, which surprisingly did not include potato. Excised root cultures of potato were studied by Chapman (1956) and Bajaj and Dionne (1968).

Chapman (1956) tried to obtain potato root cultures which could not be established by the techniques of White (1934). With the addition of water extract from potato tubers, excised root tips could be maintained on agar solidified medium up to a year. The addition of auxin or KN could not improve the growth of root cultures. The stimulatory effect of the tuber extract on root growth could not be replaced with water extract from vegetative organs leaves, and shoots.

Bajaj and Dionne (1968) improved the growth of excised potato roots by adding 0.05 mg/l 2,4-D and 500 mg/l CH (casein hydrolysate) to 10% water extract of potato tubers.

PROTOPLAST CULTURE

Protoplasts are plant cells from which cell wall has been removed usually by enzymatic digestion or some mechanical action. It is a practical application for callus and suspension culture techniques. Protoplasts can also be isolated from non-axenic tissues, usually leafs. Viable protoplasts placed

in nutrient medium recover the missing cell wall. Some of them continue cell divisions creating protoplast callus (p-callus, Sheppard and Totten 1977) from which even whole plants can be regenerated (Zuba and Binding (1989). The temporary (transient) absence of cell wall enables protoplast to fuse and create hybrid cells. This process called somatic hybridization (protoplast fusion) may be used to create inter- and intraspecies hybrids, important for potato breeding. The transient absence of cell wall enables the conduction of various experimental manipulations including transfer of foreign DNA. Protoplast culture was the long-awaited technique intended to solve accumulated problems of potato breeding. Great efforts and many studies were dedicated to its development. Unfortunately, problems with increased somaclonal variation indicated a need for a technique with a better regeneration system (Stiekema *et al*. 1988).

Nutrient media used for protoplast cultivation are complex, containing more compounds than media intended for callus and suspension cultures. Protoplasts are usually isolated from leaf tissues with mesophyll as the target cell layer. In potato, apart from leaf tissue, protoplasts can be efficiently isolated from tubers and shoots. The use of explants from *in vitro*-cultured plants accelerates and simplifies the isolation procedure cancelling the need for the initial aseptic isolation of material. Cell walls can be easily removed by enzymatic digestion according to standardized procedures which we will not present here. Enzymatic digestion of the cell wall is not considered a critical step in potato protoplast preparation.

Protoplast culture technique is still interesting for its practical applications and we will take the liberty of presenting it with more details than other *in vitro* culture techniques.

Upadhya (1975) produced the first viable potato protoplasts working with cv. Sieglind. Protoplasts regenerated a cell wall, produced callus tissue and regenerated roots but not shoots. Butenko *et al*. (1977) also reported the production of potato protoplast cultures from leaf mesophyll.

Shepard and Totten (1977) made an important breakthrough by establishing a complete protocol enabling protoplast isolation, callus formation and shoot regeneration using cv. Russet Burbank as a model system. In the following years, Shepard somewhat improved the original protocol. Shepard's studies will markedly affect and influence the whole field of potato biotechnology. Shepard and Totten (1977) also presented a simple procedure for direct shoot regeneration from tuber explants.

Two points were obvious from the beginning, first the ability of protoplast to develop into p-callus and then to regenerate shoots were both highly genotype-specific. Thus, for the most important commercial cultivars protocols will have to be optimized. Second, it was also obvious that protocols for protoplast establishment generate unwanted variability resulting in regeneration of aberrant plants at frequencies which were not acceptable. Thus in some early studies (Thomas 1981) only 4% of protoplast-derived plants of cv. 'Maris Bard' had the normal, tetraploid number of chromosomes (Fish and Karp 1986). Utilisation of such technique for breeding purpose was therefore doubtful.

Only a tiny fraction of protoplasts usually survive to produce viable callus. According to Waara *et al*. (1991) only 0.01-0.2% of protoplasts succeed in producing callus. The next step, shoot regeneration, is highly genotype dependant. On any medium formulation, some cultivars will regenerate shoots while others will not. For example, medium recommended by Foulger and Jones (1986) for 'Désirée' and 'King Edward', was not suitable for 'Maris Bard'. In addition, Radke and Grun (1986), working with nine commercial cultivars using Shepard's (1980) protocol, obtained shoot regeneration only in two cultivars, 'Russet Burbank' and 'Lemhi'. Seven other cultivars were non-responsive and failed to regenerate shoots.

The procedure elaborated by Shepard and Totten (1977) for 'Russet Burbank' is a rather complex one. There are 5 different media to which cell suspension, protoplasts or their derivatives are transferred in a relatively short succession. These media have been designated by letters **A-D**. Leaves from shoot cultures are precultured on medium **A** for a day and exposed to enzymatic digestion for 4 h on a shaker with vacuum infiltration. Thoroughly washed and cleaned from debris they are transferred to medium **B** and maintained there at 24°C at 500 lux. Cell divisions start after 4-6 days proliferating into p-callus. Fragments reaching 2 mm in length are transferred to medium **C** two more weeks at the same temperature but at 5.000 lux. Shoot proliferation was triggered by transfer of p-callus to medium **D**. Callus with regenerated shoots was transferred to final medium **E**, from which rooted plantlets were collected and planted into vermiculite.

In later studies, the protocol was modified (Shepard 1980, 1982). Medium **A** was to be renamed **CL**-medium and medium **B**, **R**-medium. In addition, 0.2 mg/l abscisic acid (ABA) was first added to medium **D** and then removed in a later revision. The PGR balance of media **CL** and **R** is 1.0 NAA + 0.4 mg/l BA. In medium **C**, the NAA concentration decreases to 0.1 mg/l. The shoot induction, medium **D**, contains 0.1 mg/l IAA + 0.5 mg/l zeatin; finally medium **E** contains 0.2 mg/l GA_3. Gun and Shepard (1981) used this protocol to obtain more than 10,000 viable calli from 7 potato cultivars. The protocol was in general successful producing p-callus in 17 cultivars among which 10 regenerated shoots. A field test of regenerated plants unfortunately showed very high variations of morphological and agronomic traits. However, some 'Russet Burbank' regenerants surpassed the mother clone in agronomical traits including improved yield, photoperiod response, growth habit, maturity date and tuber morphology (Shepard *et al*. 1980). Some plants regenerated from mesophyll protoplasts of cv. 'Russet Burbank' showed increased and stable resistance towards *Alternaria solanii* (Matern *et al*. 1978).

Haberlach *et al*. (1985) investigated the use of Shepard and Totten's (1977) protocol to obtain protoplast cultures of 36 genotypes belonging to five different *Solanum* species. With minor modifications, this protocol enabled production of p-callus in 32 clones among which 22 produced shoots.

Binding *et al*. (1978) developed a less complex protocol based on Kao and Michayluk (1975) mineral formulation. Optimal PGR combination for callus production was BA 0.56 mg/l or zeatin 0.54 mg/l and 1.1 mg/l 2,4-D. For shoot regeneration, the optimal combination was 3.22 mg/l KN + 0.87 mg/l IAA on MS medium or 0.56 mg/l BA + NAA 0.93 mg/l (BA could be replaced with 5% coconut endosperm or 4.38 mg/l zeatin). Shoots were produced 3 months after protoplast isolation. Chromosomes were counted and although some tetraploid shoots were observed, the authors claimed karyotype stability.

Opatrny *et al*. (1980) described the use of cell suspension cultures for the production of protoplast cultures. They investigated various factors affecting the initial stages of protoplast induction including the age of cell cultures, precultivation, enzymatic mixture composition and incubation duration. Authors also studied the regeneration of cell walls and the effect of PGRs on cell divisions finding that 1.0-2.0 mg/l NAA and 2,4-D was the best combination for prolonged maintenance of cell divisions.

Thomas (1981) obtained protoplasts and regenerated shoots of cv. 'Maris Bard' using a simple protocol. Media of original mineral composition supplemented with 1.0–5.0 mg/l 2,4-D and 0.5 mg/l zeatin supported the development of p-callus. Shoots were regenerated only on medium with 0.5 mg/l zeatin. There was much variation among regenerated plants. From 45 clones established from regenerated plants, 20 were aberrant for growth rate and/or chlorophyll content.

Bokelmann and Roest (1983) developed a protocol for protoplast culture of cv. 'Bintje'. They combined media used by Binding *et al*. (1978) and Kao and Michayluk (1975). Medium for protoplast culture contained 0.2 mg/l 2, 4-D + 1.0 mg/l NAA + 0.5 mg/l zeatin. Highest shoot regeneration was on medium containing 0.01 mg/l NAA + 1.0

mg/l zeatin + 0.1 mg/l GA_3. There was much phenotypic variation among the several hundred regenerated plants grown to maturity.

In 1983, the high incidence of somaclonal variation among plants regenerated from potato protoplast became a widely accepted fact (Bright *et al*. 1983). Therefore, most of the further studies were done with the aim of explaining the appearance of this phenomenon. There were also attempts to avoid or to decrease the variation in protoplast-regenerated plants but without much success.

Haberlach *et al*. (1985) produced protoplast cultures of 36 different potato genotypes using the methods of Shepard and Totten (1977) and Shepard *et al*. (1980). They recommended the use of new culture vessel types that occupy less space and longer (overnight) enzyme digestion. Small modifications included an increase in zeatin concentration in the shoot differentiation medium and the omittance of ABA, which had no beneficial effect at the optimal zeatin concentration. Viable protoplast and macroscopic callus were obtained in all but four genotypes.

Foulger and Jones (1986) improved the protoplast production and regeneration protocol for cv. 'Désirée' increasing the $CaCl_2$ concentration in the medium from 0.3 to 6 .0 mM. With the improved protocol, 72% of 'King Edward' and 76% of 'Désirée' p-calli regenerated at least one shoot. Shoot regeneration of 'Maris Piper' at 12% was much lower indicating strong genotype effects. It is interesting to note that in the same year Sha *et al*. (1985) reported that an increase in $CaCl_2$ concentrations above the one in the MS medium formulation can decrease the appearance of apical necrosis in shoot cultures of 'Russet Burbank', 'Sperio' and 'Norland'.

Dai *et al*. (1987) reported production of potato protoplasts from hypocotyl and cotyledon explants of several potato breeding lines and wild species. The procedure was equally efficient as those using leaf explants.

Perl *et al.* 1988 used 2.0-8.0 mg/l silver thiosulfate to suppress ethylene production during the production of potato protoplasts cultures and PEG treatments.

Jones *et al*. (1989) isolated protoplasts from storage parenchyma of tubers. Cells full of amyloplasts started to divide only after they metabolised starch, which took 7 days. The procedure was completed for 6 cultivars and regenerated shoots were raised into plants. Most regenerants were octaploid- or octaploid-based aneuploids. Media for callus production and shoot regeneration were after Foulger and Jones (1986).

Lillo (1989) investigated the effect of various media components and environmental factors on shoot formation from p-callus of several potato genotypes. Shoot induction was stimulated by low sucrose concentration (3-15 mM) in moderated light irradiance and on media containing two times or more NO_3^- to NH_4^+ ions. Many compounds, including glutamine, proline, putrescine, spermidine, spermine, and adenine sulphate at 0.5-2.0 mg/l had no stimulatory effect on shoot induction.

Waara *et al*. (1991), working with dihaploid clones, improved the yield of viable p-calli by replacing KCl and NH_4NO_3 with 5 mM glutamine ammonium nitrate with glutamine and adding more dilution steps in the Carlsberg *et al*. (1987) protocol, which they employed. Most of the regenerated plantlets became tetraploid through chromosome doubling.

Variation in protoplast regenerants

Analysis of plants regenerated from protoplasts of 'Russet Burbank' showed a marked variation among the regenerants both in agronomical and morphological traits (Secor and Shepard 1981). Variation was soon confirmed in other potato cultivars regenerated from protoplasts including 'Désirée' (van Harten *et al*. 1981), 'Maris Bard' (Thomas *et al*. 1982), and 'Bintje' (Sree Ramulu and Roest 1983).

Analysing the variability in populations of plants regenerated from 'Maris Bard' protoplasts, Thomas *et al*. (1982) concluded that this variability arose during callus growth and that it was not present in the explant at the beginning of the propagation procedure, which was one of the possible explanations for the observed variation. Karp *et al*. (1982) analysed plants regenerated from protoplast of 'Maris Bard' and 'Fortyfold' and detected extensive variations in chromosome numbers. They observed that even shoots arising from the same callus fragment often had different chromosome numbers. Aberrant shoots apparently were subject to chromosome doubling leading to polyploidy and to chromosome loss leading to aneuploidy or to both processes. Creissen and Karp (1985) performed a karyological analysis of 200 plantlets regenerated from shoot-culture derived protoplasts of cv. 'Majestic' using the protocol of Shepard (1980) Regeneration frequency increased up to 71%. Observed morphological variations could be partly related to chromosome numbers. On average 57% of regenerants had the normal, euploid 2n = 4x = 48 number of chromosomes. In the remaining group of aneuploids only few plants with high (endopolyploid) chromosome number were detected. Thus, the use of shoot cultures as the source of explants for protoplast production seems to be an improvement in relation to other explant types. Authors also observed structural chromosome changes, which had no obvious phenotypic consequences. A check of morphological and agronomic characteristics affected by somaclonal variation (e.g., leaf size and shape, structure and chlorophyll content; Sree Ramulu and Roest 1983) enabled early identification of plants falling in the high chromosome class and their elimination prior to field trials.

Sree Ramulu and Roest (1983) observed significant phenotypic variation in plants regenerated from protoplasts of cv. 'Bintje' using the Bokelmann and Roest (1983) protocol. Regenerants were mostly aneuploid and mixoploid indicating that variation, at least partly, appeared in the culture stage. Aberrations included drastic changes in leaf size, shape, structure and chlorophyll content. There was a correlation between morphological aberrations and ploidy levels since 82% of plants with morphological aberrations had altered ploidy levels. Media based on ZR enabled higher shoot regeneration but increased also the number of aberrant plants. Sree Ramulu *et al*. (1984) showed that morphological variants are transmitted to the tuber progeny of aberrant plants. Some of the normal and variant protoclones produced new phenotypes or segregated into parental and new phenotypes after vegetative propagation. In most cases, ploidy levels were unchanged. Changes arising after tuber propagation were attributed to somatic segregation of chimeras resulting from gene mutations or chromosome structural rearrangements in parts of regenerated plants. Sree Ramulu *et al*. (1989) performed cytological and DNA cytophotometric analysis of protoplast derived regenerants from clones of various ploidy levels. The occurrence of instability of the nuclear process was observed in early stages of protoplast dedifferentiation and callus growth. In the interphase (G2), chromosomes undergo an extra DNA replication (S phase) resulting in formation of diplochromosomes (4 chromatid chromosomes) which have a doubled DNA content. Owing to a loose centromere connection, chromosomes separate forming polyploid cells.

Protoplast fusion – somatic hybridization

Naked protoplasts with their cell walls removed can sometimes fuse creating a hybrid cell, the heterokaryon. Since this hybrid is created by somatic cells, the procedure is named somatic hybridization. Protoplast fusion is a technique of great practical use for potato breeding. It enables us to overcome breeding barriers and to establish hybrids, which could not be obtained by conventional breeding techniques.

Two main treatments used for protoplast fusion in potato are PEG (polyethylene glycol) medium supplementation and electroporation with strong electric pulses (Fish *et al*. 1988a). A difficult task immediately after fusion is identifi-

cation of hybrid cells, which can be performed in many different ways, using marker genes, fluorescent labelling and others.

The presence of two different complements of cell constituents in the same space can seldom function without problems. Heterokaryons try to solve this problem through elimination (sorting) of surplus chromosomes and organelles. Since there is no apparent priority rule as to which or what is eliminated first, heterokaryons will produce populations in which no two regenerants will be alike. Truly, the variation in somatic hybrids was higher than in protoplast regenerated plants (Fish *et al*. 1988b). Complex interactions of cell constituents and their elimination have been well presented in a review by Orczyk *et al*. (2003).

Binding *et al*. (1982) created somatic hybrids of *Solanum tuberosum* and atrazine resistant biotype of *Solanum nigrum* using 45% PEG. Protoplasts were cultured in accordance with previously published protocols (Binding and Nehls 1977; Binding *et al*. 1978). Some 2,705 clones regenerating shoots were screened. Most regenerants resembled *S. nigrum* and none of the plants were pure *S. tuberosum* type. A number of clones were investigated: 10 were found to be atrazine-resistant. Some plants were mosaics containing resistant and non-resistant parts.

Austin *et al*. (1985) reported the first intraspecific fusion of two *Solanum tuberosum* diploids. Tetraploid fusion progeny of *Tuberosum* × *Phureja* crosses, confirmed by isozyme analysis, had increased vigour. Results indicated that somatic fusion might be useful for transferring traits within the group.

Debnath and Wenzel (1987) regenerated tetraploid plants after polyethylene-glycol-induced protoplast fusion of dihaploids. Protoplast isolation and culture was done after Binding *et al*. (1978) and fusion in 40% PEG.

Puite *et al*. (1986) fused protoplasts of diploid *S. tuberosum* and *S. phureja* plants by electrofusion. Protoplasts were obtained by the Bokelmann and Roest (1983) protocol. A total of 840 heterokaryons selected on the basis of fluorescence were isolated to produce calli. Ultimately, 13 out of 41 calli regenerated shoots. From 7 calli, 18 plantlets were grown to maturity and investigated in detail. The eight regenerants appeared to be hybrids. Most of them were hexa- and octaploids and there were no tetraploid plants. Although hybrid calli showed increased growth vigour, many plants were short.

Rasmussen and Rasmussen (1995) used RAPD markers and isozymes for analysis of somatic hybrids produced by electrofusion of mesophyll protoplasts of male sterile dihaploid potato genotypes. Heterokaryon selection was based on hybrid vigour. Hybrid regeneration from callus was 6% and 45% of putative hybrids were confirmed as true. There was a strong correlation between the number of chromosomes and the number of chloroplasts in a cell. Scoring chloroplasts in guard cells enabled fast estimation of the plant's ploidy.

SHOOT AND MERISTEM TIP CULTURES

Shoot culture is the basic technique of potato biotechnology. It enables fast and efficient establishment of *in vitro* cultures using shoot tips as starting material. Once established, axenic shoots can provide explants required for other techniques including cell, tissue and organ cultures, protoplast culture, somatic embryogenesis and *Agrobacterium*-mediated transformation.

The main application shoot cultures have is in clonal propagation. They can be used for production of plants (micropropagation), tubers (microtubers), or both. Genotype storage (gene banks) is another important application achieved by sustained (minimal, slow) growth of shoot cultures. In cryopreservation, growth is completely arrested at the temperature of liquid nitrogen. Meristem tip culture is an important component in virus eradication used either alone or in combination with thermotherapy.

After the initial success in the establishment of *in vitro* cultures and meristem tip cultures (1951 and 1954, respectively), it took nearly three decades to obtain a complete picture of culture requirements and growth conditions for potato shoot cultures. Tempo was lost mostly in futile attempts to develop a propagation scheme based on cytokinin/auxin balance as in other dicotyledonous species. Potato has a very strong apical dominance (Kumar and Wareing 1972), which inhibits the growth of axillary buds (Levy *et al.* 1993). Multiplication *via* cytokinin-stimulated proliferation of axillary buds is possible but with prolonged subculture duration (Goodwin *et al*. 1980), and changes in shoot morphology. For fast shoot multiplication, a much better approach is to cut shoots into single node explants and culture them individually. Single node explants liberated from apical dominance develop at the highest possible rate.

Apart from strong apical dominance, potato has other unique features that need to be briefly explained in order to understand the life cycle and morpho-physiological adaptation of this unique species.

Life cycle and shoot polymorphism

Cultivars of potato (*S. tuberosum*) which we consume produce tubers, dual purpose organs functioning in storage and vegetative propagation (Viola *et al*. 2007). The tuber-bearing potato has an intricate life cycle (**Fig. 3**) in which the leafy plant present throughout the vegetative season alternates with dormant tubers throughout the resting period lasting from 1-15 months (Wiltshire and Cobb 1996). Early development steps from seed to seedlings and juvenile plant formation are missing. The final fruit and seed setting stage may be present but with no actual importance since tuber is the only valid propagule type. Most of the cultivars are highly heterozygous tetraploids and propagation by seed is out of the question.

The unfavourable period for growth lasting throughout winter plants spend time in storage in the form of tubers (Suttle 2004), which are modified shoots of axillary origin (Ewing 1987). In favourable conditions, new plants start to develop from axillary buds present in form of "eyes" on the tuber surface. Eyes develop into leafy sprouts (which may initially be etiolated) forming mature photosynthetically active plants. Some of the axillary buds on lower portions of potato stem develop as stolons, dia- (plagio) geotropic shoots that form underground a new generation of tubers. It is important to note that all transitions between stages in the potato life cycle are done only by axillary buds. The four characteristic stages in tuber formation are a) induction, b)

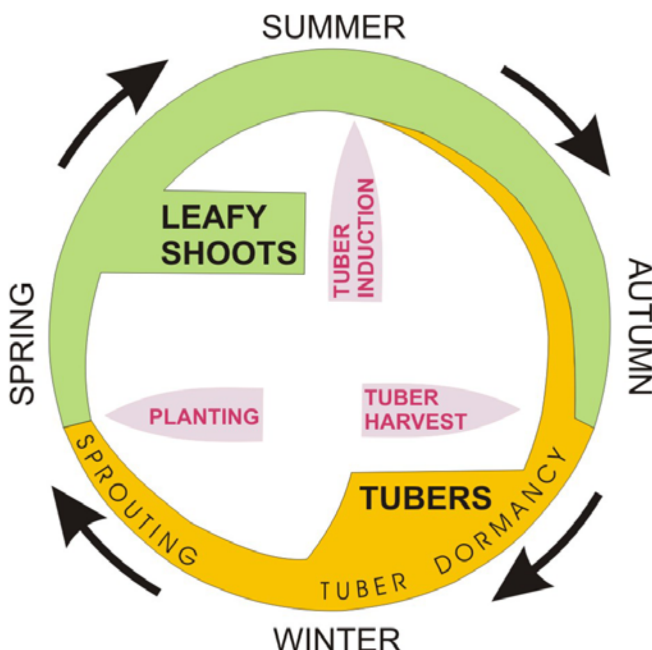

Fig. 3 Life cycle of a commercial, tuber-bearing potato cultivar.

storage, c) dormancy and d) sprouting (Hajirezai *et al.* 2003).

Another peculiar feature of potato is the existence of shoot polymorphism. There are three or maybe even four different shoot types in potato. First, there is the well known, leafy shoot, which develops, in full daylight, specially designed and adapted to perform photosynthesis. In the absence of light, the green leafy shoot changes into the white coloured, etiolated shoot of darkness with leaves reduced to scales. These etiolated shoots are heterotrophic; they often arise as white sprouts on improperly stored tubers in our households. Their main function is rapid elongation enabling them to get into light conditions as quickly as possible (Mohr and Schopfer 1995). In conditions of full daylight, etiolated shoots get rapidly transformed into the green, leafy, photosynthetically active shoots. Etiolated shoots should not be mixed with stolons, the third shoot type in potato. Stolons are diageotropic shoots of axillary origin with reduced leaf growth and their main function is to support formation of tubers, tuberization (Vreugdenhil and Struik 1989).

Etiolated shoots have a high negative gravitropic response in contrast to stolons, which under typical conditions are dia- (plagiogeotropic). However, problem is that under conditions of *in vitro* culture, some axillary buds develop into intermediate shoot types, difficult to distinguish and classify (Hussey and Stacey 1981). It should be noted that in some of the early studies, etiolated shoots were erroneously classified as stolons (Palmer and Smith 1969).

The stolon or etiolated shoot dilemma is solved by the level of available carbohydrates (starch). Thus, shoots emerging from starch-filled tubers as etiolated sprouts have a high negative gravitropic response and they can not be turned easily into stolons. In other shoots cultured *in vitro* in conditions of inadequate sucrose nutrition (low sucrose + low irradiance), shoots are prone to get transformed into stolons (Hussey and Stacey 1981). Kumar and Wareing (1972) pointed at a simple and obvious physiological difference between etiolated shoots and stolons of field grown plants. In light etiolated shoots will turn into green leafy shoots while stolons will not. GAs are also involved in the determination of the shoot type formation *in vitro*. They will stimulate stoloniferous shoot growth even in conditions of high sucrose nutrition (Vreugdenhil *et al.* 1998).

Finally, tubers can also be considered as a separate (fourth) shoot type (Ewing 1987) since they differ from stolons by the plane of cell divisions and other features. Tuberization occurs when a signal triggers a change in the plane of cell division and stops further stolon elongation. In tubers, most cells divide in a plane transverse in relation to the main axis of the stolon elongation. It is assumed that stolons always precede formation of tubers even in the case of sessile tubers in which there is no visible stolon formation and the axillary bud gets directly transformed into a tuber.

Fig. 4 Potato (cv. 'Désirée') shoot cultures can be cultured on a simple PGR-free MS medium supplemented with 2-3% sucrose. Medium supports shoot elongation and rooting but not axillary bud proliferation. To obtain multiplication, the shoot is cut (segmented) into single node cuttings, each containing an axillary bud. After transfer to fresh medium, the axillary buds of these explants rapidly develop into rooted plantlets consisting of several internodes. This process of axillary bud excision followed by rapid shoot elongation and rooting can be repeated indefinitely. It is the basic (but not the only) technique for shoot culture multiplication. Rooted plantlet originating from a single node explant in a Ø 18 mm test-tube after 4 weeks.

A question frequently placed in connection to the potato shoot polymorphism is the PGR balance required to maintain coherent potato body architecture. This question is subject to extensive research and data analysis. However, one thing is already clear, the green, leafy potato shoot is the main (first, starting, default) shoot type. With such a position, it has a unique privilege needing no external PGRs for its growth. Therefore, the leafy potato shoot cultures can complete the whole propagation protocol including the rooting stage on a simple, hormone-free medium (Hussey and Stacey 1981) (**Fig. 4**).

Propagation protocols

The establishment of proper protocols for the production and maintenance of potato shoot cultures was burdened by strong apical dominance along the shoot and by the poor response of excised explants to exogenously applied cytokinins. Goodwin *et al.* (1980a) showed that it can take many weeks before exogenous cytokinins can inflict a shoot proliferation response in subcultured shoots. Thus, although the division of shoots into single node cuttings was known and employed, multiple shoot production by activation of axillary buds was the favoured propagation approach in the early studies of Westcott *et al.* (1977) and Roca *et al.* (1978, 1979).

It is interesting to note that meristem tip culture, conceived as a practical application preceded the establishment of shoot culture as the basic technique for nearly three decades. Data collected by the early use of meristem tip culture of potato was useful for other plants species.

Westcott *et al.* (1977) investigated shoot cultures as an approach to potato germplasm storage. Shoot regeneration from callus cultures has been already demonstrated (Lam *et al.* 1975; Skirvin *et al.* 1975; Wang and Huang 1975; Roest and Bokelman 1976) but it was also evident that callus cultures were not suitable for germplasm storage due to the problems with their genetic stability (D'Amato 1975). Westcott *et al.* (1977) used meristem tips as initial culture explants. Meristem domes with 2-4 leaf primordia were cultured using filter bridges on liquid MS medium with 0.1 mg/l NAA or GA_3, or both. On the PGR-free medium none of the explants from the 14 different genotypes could be established *in vitro*. Culture success was improved when NAA concentration was increased to 0.5 mg/l and GA_3 to 0.2 mg/l. Cultures developed multiple shoots in which several shoots were joined at the common callus base. Multiple shoots were recommended for multiplication. Growth of multiple shoot cultures was tested with KN, BA and 2iP, all applied in the 0.1–10.0 mg/l concentration range. Cultures grew best on medium with 3.0 mg/l BA. Single node culture technique was suitable only for *S. tuberosum* clones and for other species authors recommended the initial use of multiple shoot explant cultures providing up to 1: 50 propagation rate. However, for prolonged germplasm storage they suggested single node explants and medium supplemented with 0.1 mg/l GA_3.

Roca *et al.* (1978) presented a procedure for rapid potato propagation. Single node explants were cultured on agar-solidified or liquid MS medium with 2% sucrose and 2.0 mg/l Ca-panthothenate. On BA-supplemented media they obtained two multi-meristem types of culture, "organogenic callus" and "rosette cultures". Both of these culture types contained some callus. The highest shoot production by multi meristem cultures was obtained on shaker-agitated liquid MS medium containing 0.5 mg/l BA, 0.4 mg/l GA_3 and 0.01 mg/l NAA. Subculturing was done at 2–3 week intervals. Single node explants were used in the final propagation stage on MS medium with 0.2 mg/l GA_3. Explants developed into complete plants, which were moved and further grown in a greenhouse. Electrophoretic separation of total proteins and esterases showed no differences between propagated and control plants. Roca *et al.* (1979) described how single node explants derived from multi-meristem cultures packed in polystyrene containers could be successfully

Fig. 5 Potato (cv. 'Désirée') shoot cultures can be maintained in various types of culture vessels depending on the purpose of the venture (research study or production). For small-scale studies we prefer Erlenmeyer flasks and cotton-wool plugs. Conical, slanted walls enable good culture illumination and cotton-wool plugs support good aeration critical for proper plant development. Flask in the figure set with five single node explants after 4 weeks.

used for long distance shipment.

Goodwin *et al*. (1980a) elaborated a procedure for shoot multiplication using tuber sprouts and agar-solidified or liquid MS medium with GA_3 and KN. Proliferation was best using a two-stage system with agitated liquid medium in the first stage followed by stationary liquid medium in the second stage. Up to 25-fold shoot multiplication could be obtained in 8 weeks. Highest shoot multiplication was on medium with 5.0 mg/l KN and 0.01 mg/l GA_3. At low light intensities leaves decreased in size, shoots became thin often producing a subapical hook. Rooting was achieved in a separate step (Goodwin *et al*. 1980b) in stationary liquid medium with 0.1 mg/l IAA or by direct *ex vitro* planting in potting mixture.

Data presented in the studies of Westcott *et al*. (1977), Roca *et al*. (1978, 1979) and Goodwin *et al*. (1980ab) attracted much attention and they can be considered as initial studies in potato shoot culture techniques. Most of the questions initiated by these studies will be answered by the work of Hussey and Stacey (1981).

Hussey and Stacey (1981) clearly demonstrated that PGRs are not required for successful propagation of potato by shoot cultures. Starting with apical or axillary buds, the whole micropropagation scheme based on the use of single node explants can be performed on a simple MS-based PGR-free medium. Shoots cut into separate nodes, each including 4 mm of stem, subcultured onto fresh medium produced within 4 weeks a new 30-50 mm–high upright positioned shoot with 6 to 12 nodes depending on the light and temperature regime. This procedure of serial shoot production by single node cuttings (segmentation) could be continued indefinitely (**Figs. 4, 5**).

The use of PGR-free medium was not new; it was frequently used in meristem tip culture studies to produce single rooted plants since the use of PGRs known at that time (auxins and early cytokinins) could be deleterious resulting in callus proliferation.

Working with PGR-free medium Hussey and Stacey (1981) investigated the effect of day length (8, 16 and 24 h of light) and temperature (15, 20 and 25°C) on the production of new nodes and stem thickness. Increased day length and temperature favoured the production of new nodes. The day length also stimulated stem thickness but the results on temperature increase although inconsistent for some cultivars still indicated 20°C as the optimal. They also pointed that a thicker and shorter stem was more desirable, facilitating the subculturing procedure.

Another important observation made in this study was that conditions combining low temperatures and short day lengths favour the formation of intermediate shoot types resembling stolons. Intermediate shoots grow at a 30–40° angle in relation to the vertically positioned main stem axis, forming reduced, scale-like leaves. Some of the intermediate shoots produced also subapical hooks characteristic for stolons. Intermediate shoot formation was favoured in conditions of reduced aeration imposed by the tightening of metal screw caps of the vessels used for culturing (Richardson's jars). The effect was attributed to ethylene build-up

Fig. 6 Rooting is concomitant with shoot elongation. Some 4-6 weeks after the subculture plantlets (cv. 'Dragačevka') have a well developed root system. Branching by activation of axillary buds also starts, but at higher internodes.

since it could be mimicked by placing a 2 ml of 10^{-4} M 2-chloroethylphosphonic acid in small vials inserted in the culture vessels. The effect was cancelled by including a vial with 2 ml of mercuric perchlorate solution – known to absorb ethylene.

No separate rooting stage was required (**Fig. 6**). However, since it was troublesome to remove agar from plantlets prior to planting, rooting was performed in Petri dishes with stationary, PGR-free liquid medium.

The propagation method presented by Hussey and Stacey (1981) is highly recommended for fast and continuous *in vitro* propagation of potato ensuring high genetic stability offered by the use of axillary buds as explants. Later investigations will further confirm that in the case of potato incorporation of cytokinins into the propagation medium offers no advantage if we have shoot propagation in mind (Vinterhalter *et al*. 1997).

If we would like to evaluate the propagation efficiency of serial shoot segmentation methods than we should calculate and compare the final shoot length and the number of new nodes produced during the subculture. Fresh and dry weight of shoots and other parameters are of far less importance in this propagation method.

Research dedicated to clonal propagation of potato by shoot cultures continued slowly for some time with small modifications and improvements. Soon it will be challenged by other approaches and partly replaced with the idea to use microtubers as propagules for clonal propagation.

Shoot cultures - continued research

Potato shoot cultures on PGR-free medium have been used as a starting point in many later studies in which the effect of various culture conditions and medium supplements were investigated and evaluated. We will present here briefly some of the studies presenting data which improved our knowledge on culture conditions and requirement of potato shoot cultures.

Evaluation

Amirouche *et al*. (1985) compared single node explant micropropagation using MS and modified Knop's (1865) medium formulations with or without vitamins. MS was far superior to modified Knop's in supporting the growth of

potato cultures. Interestingly, there was no significant overall effect of the presence/absence of vitamins in the medium. Miller *et al*. (1985) continued this line of research comparing the growth of 20 potato cultivars on MS PGR-free medium. A great variation in shoot length and production of new nodes among different cultivars was observed. In some cultivars, shoots were too short for subculturing and this problem was corrected by the addition of PGRs. A medium with 1.0 mg/l GA_3 and 0.1 mg/l NAA was suitable since it increased shoot length but only in cultivars developing short shoots under *in vitro* culture conditions like 'Record' and 'Foxton'. Authors also showed that CCC (chlorcholine chloride, chlormequat choline) at 500 mg/l could reduce shoot elongation. CCC in combination with GA_3 + NAA reduced shoot length to the size roughly found in the controls.

Estrada *et al*. (1986) suggested media with 0.25 mg/l GA_3 for propagation of single node cuttings on agar solidified media and a liquid medium with 0.4 mg/l GA_3 + 0.5 mg/l BA + 0.1 mg/l NAA for propagation of shoots layered on liquid tuberization medium. Layering enables explants to increase their contact with nutrient medium, often increasing the growth rate of explants.

Liquid, agar- and Gelrite-solidified media

Potato shoot cultures grow quite well both on liquid and agar solidified medium (Goodwin *et al*. 1908). Several studies, however, further compared growth characteristics of these systems.

de Ávila *et al*. (1996) compared the growth of shoot cultures in liquid and agar-solidified media. Liquid media was far superior increasing the shoot length, number of nodes, shoot dry weight and root dry weight in cvs. 'Spunta', 'Kennebec' and 'Huinkul'. The effect of liquid medium was attributed to increased sucrose and nitrogen nutrition in liquid medium resulting from an improved incorporation rate. Authors also considered a possibility that the presence of agar gel matrix physically reduced the diffusion of nutrients through the medium.

Veramendi *et al*. (1997) showed that Gelrite is an advantageous alternative to agar for micropropagation and microtuberization. Gelrite was found to be superior or equal to agar in all growth aspects of shoot cultures. It was superior in shoot elongation, rooting and microtuber production.

Gopal *et al*. (2002) investigated prolonged culture of potato at normal temperatures on media supplemented with mannitol and sorbitol in addition to sucrose as osmotica. Among many different combinations the best results were obtained with 2% sucrose + 4% sorbitol. In a detailed discussion, covering various treatments used to slow down *in vitro* growth of potato cultures authors were against the use of low temperatures for a number of reasons. Apart from energy cost, especially in subtropical regions they point at instances when low temperatures triggered genotype instability as showed by Lizarraga *et al*. (1989), Roca *et al*. (1989), Dodds *et al.* (1991), Harding (1994) and Lopez-Delgado *et al* (1998).

Effect of co-factors

Co-factors are substances usually not recognized as PGRs which can still under certain circumstances affect the growth of *in vitro* plant cultures.

Feray *et al*. (1993) investigated the effects of 0.5 mM polyamines (putrescine, spermidine, spermine) on the growth of potato shoot cultures. Polyamines stimulated growth of aerial parts expressed as an increase in the fresh weight (FW). Hourmant *et al*. (1998) investigated the presence of catecholic compounds in potato in *in vitro* plants. HPLC analysis confirmed the presence of DOPA, dopamine and caffeic acid. The addition of exogenous dopamine caused a large increase in the FW of leaves and stems. Addition of MGBG (inhibitor of spermidine synthesis) decreased dopamine content of aerial parts and increased presence of caffeic acid stimulated tuberization.

Fluridone (FLD, norflurazon), an inhibitor of ABA biosynthesis strongly stimulates rooting of potato single node explants in darkness (Harvey *et al*. 1994). In the light, FLD is toxic since it inhibits carotenoid biosynthesis inducing photobleaching. The addition of ABA in the presence of FLD prevented rooting. A stimulative effect of FLD was most apparent at high sucrose concentrations. Replacement of sucrose with mannitol resulted in decreased rooting.

Mineral nutrition

Zarrabeitia *et al*. (1997) investigated the effect of ammonium nitrate nutrition on the growth and microtuberization of potato shoot cultures. Media which they investigated contained 8 times more or 10 and 100 times less NH_4NO_3 than the full strength MS. A decrease in ammonium nitrate strongly promoted the production of new nodes, leaves and increased internode length and chlorophyll content in all four investigated cultivars. Therefore, MS medium in its standard formulation contains supraoptimal concentration of ammonium nitrate for growth of potato shoot cultures.

Abdulnour *et al*. (2000) investigated the effect of boron on Ca^{2+} uptake showing that increased boron (B) in the medium can results in signs of Ca^{2+} deficiency. Keeping B at a quarter of the concentration present in the MS medium and using B-free Gelrite instead of agar can improve micropropagation of potato preventing Ca^{2+} deficiency symptoms.

Habib *et al*. (2004) investigated the effect of calcium availability on potato micropropagation and microtuberization. They found that when the Ca^{2+} levels in the MS medium were increased from 3 to 5 or 15 mM then all aspects of the growth of shoot cultures were improved. A similar increase of Ca^{2+} concentration helped some cultivars to overcome problems with apical necrosis Sha *et al*. (1985).

Aeration of cultures and photosynthetic ability

Genoud-Gourichon *et al*. (1993) observed that hermetic sealing of culture vessels impaired the development of potato shoot cultures, similarly to the findings previously reported by Hussey and Stacey (1981). Plantlets cultivated in non-sealed vessels were more developed and their photosynthetic potential was 4.7 times higher. Ethylene released by potato cultures can be efficiently removed by the addition of silver thiosulftahe (Perl *et al*. 1988).

Zobayed *et al*. (2001) studied the levels of ethylene, carbon dioxide and oxygen in culture vessels as affected by various treatments including the addition of silver nitrate, ACC (1-aminocyclopropane-1 carboxylic acid) and different closure types and ventilation systems. Good (forced) ventilation substantially improved the growth of cultures eliminating hyperhydricity and its related effects. Ethylene removal was the key factor leading to the improved growth of cultures. Chanemougasoundharam *et al*. (2004) studied the growth and morphology of potato shoot cultures in relation to the type of closures used for stopping test tubes. Polypropylene caps and aluminium foil increased the FW and shoot length but lowered the chlorophyll content, induced senescence and triggered the appearance of abnormalities. Cotton wool plugs and Steristoppers® were the best closure types supporting good growth without morphological abnormalities like small folded leaves, hooked stolon type stems and callus at the shoot base.

Inoculation density

Sarkar *et al*. (1994) reported that the density (number) of explants placed in a 25 × 100 test tube closed with polypropylene closures significantly affected the early culture development measured after 2 weeks. Shoot length, number of new internodes and number of roots per plants were highest in test tubes with 5 explants and linearly decreases with the number of explants. There is no reasonable explanation for this phenomenon but its impact on the efficiency of potato micropropagation should be taken into consideration.

Synseeds – artificial seeds

Sarkar and Naik (1998) investigated a system enabling the use of potato single node cuttings encapsulated in alginate matrix as a new propagulum type concurrent to microplants and microtubers. This novel approach under favourable conditions enabled a 57% survival rate. The use of single node cutting synseeds offers advantages avoiding complex propagation methods used for other propagule types. However, this method needs more testing and some improvements for mass application.

Effect of light

Light has two major functions in plants. First, it is a free and unlimited source of energy efficiently harnessed by photosynthesis. Second, it provides information enabling plants to become oriented in space and time.

Talking about the importance of light for potato *in vitro* cultures we should first completely forget the whole segment dedicated to photosynthesis. Plant *in vitro* cultures in general are heterotrophic, avoiding photosynthesis as long as there is a suitable carbohydrate source (usually sucrose) available in the medium. In the case of shoot cultures the situation is a bit different since these are the structures functionally equipped and ready to perform photosynthesis at short notice. Cultures of this type are called mixotrophic denoting their ability to perform both heterotrophic and autotrophic metabolism. Two requirements for shoot cultures to perform photosynthesis are sufficiently high irradiance and a constant supply of CO_2. Irradiance required for photosynthesis to occur is estimated at 150–375 $\mu mol^{-2}\ s^{-1}$ (Seabrook 2005). This is much more then some 33–80 $\mu mol^{-2}\ s^{-1}$ provided by a couple of fluorescent lamps in growth rooms of most *in vitro* culture facilities. The penalty for the use of high irradiance is also the high cooling cost. However, the true limiting factor for *in vitro* photosynthesis is the low CO_2 concentration in culture vessels. Fujiwara *et al.* (1987) showed in a number of species (not including potato) that in shoot cultures photosynthesis starts at the beginning of the day (lights go on). Cultures quickly spend most of the available CO_2 and photosynthesis stops after the CO_2 concentration falls under the CO_2 compensation point. Thus, to maintain autotrophic micropropagation, shoot cultures need to be cultured on a carbohydrate-free medium, at high irradiance and with a constant supply of CO_2. After the addition of 2–3% sucrose to the nutrient medium, the whole concept of autotrophic micropropagation is compromised.

For potato *in vitro* cultures light is much more important as a source of information then as a photosynthesis driving force. For this purpose plants are equipped with additional pigment systems absorbing the red/far red and blue light. The three pigments (groups) probing the light conditions of the environment are phytochrome, cryptochrome, phototropin and a yet unidentified pigment absorbing in the UV range (Nagy *et al.* 2001). Phytochromes are very well known for their ability to measure time laps – duration of day and night. Phytochromes are therefore involved in the timing of flowering and in potato additionally in the timing of tuberization. Cryptochromes and phototropine are both pigments absorbing blue light. The main function of cryptochrome is to regulate the shoot length in different light conditions. In plants growing in light cyptochrome reduces shoot elongation. In dark conditions, phytochrome is inactive and plants elongate becoming etiolated. Phototropine enables plants to detect the spatial location of the light source illuminating them. Photototropic reaction is performed by shoot bending (curving) probably as a consequence of auxin redistribution within the shoot (Cholodny-Went theory). It should be noted that there is constant interaction (cross-talk) between the various pigment systems.

Fluorescent lamps are still the most commonly used light source for plant *in vitro* cultures. Their spectral emission is wide, usually mimicking the daylight conditions most pleasant for the human eye. Spectral composition of fluorescent lamps can be adjusted to provide more blue or red expressed as the lamp temperature in °K. There are also types specially designed for plant growth having in mind mostly the needs of photosynthesis. Seabrook (1987) demonstrated that the size and shape of potato shoot cultures is significantly affected by the type and combination of fluorescent and incandescent lamps used in these studies. The most apparent differences were observed in shoot length, mean number of leaves and leaf area, while the differences in the dry weight were less pronounced.

The effect high-pressure sodium (HPS) and metal halide lamps (MHL) on the growth of *in vitro* propagated potato plantlets using hydroponics with nutrient film technique (Wheeler *et al.* 1990) has been investigated by Yorio *et al.* (1995). Research showed that a new HPS type, HPS SON-Agro although designed to provide more light in the blue region of the spectrum, failed to decrease the shoot length of potato plants in comparison to standard HPS lamp types.

Seabrook and Douglass (1998) used yellow filters to subtract the blue light from the spectre provided to the plants. As expected, in the absence of blue light shoot cultures increased their length. Increased length is desirable for cultivars with short shoots. Absence of blue light helped also to suppress oedemata formation characteristic for some potato genotypes like 'Kennebeck' and 'Russet Burbank' (Pettite and Ormrod 1986).

The formative effect of blue light was also demonstrated by Aksenova *et al.* (1994). Here the blue light applied in long day conditions prevented shoot elongation as compared to red light both in short and long days and blue light in short day conditions.

Thus in potato shoot cultures light of common fluorescent lamps rich in blue prevents the excess shoot elongation, correlating and synchronizing the growth of all meristematic centres and zones within the shoot. This effect should not be interpreted as growth inhibition. Conversely, increased elongation of potato shoots in conditions of low irradiance and in some zones of monochromatic lights should not be interpreted as stimulation.

A problem encountered sometimes in studies with light is that its effects are rarely independent and unequivocal. Reactions to light are often expressed only as tendencies, indicating strong interaction of light with other regulatory factors, mostly PGRs. For instance, Hussey and Stacey (1981) could not clearly separate the effects imposed by photoperiod and temperature. Photoperiod also affects the KN-induced tuberization (Pelacho and Mingo Castel 1991b). The joint effect of light and PGRs in the regulation of development of stolons and tubers has been well documented Dragićević *et al.* (2008).

We must keep in mind that one of the basic light effects expressed as "no light" or absence of light induces gross morpho-physiological changes in shoot cultures leading to the formation of etiolated cultures. Whole metabolic pathways will be temporarily shut off and the histological structure of shoots will be changed to adjust to new needs (Pristley and Ewing 1923; Venning 1954) At certain low irradiance levels shoots will manifest poor growth just from the fact that they can not decide which of the two light-dependent pathways to follow. For plants this is the famous Shakespearian dilemma "To be… or not to be". However, once it starts, etiolation will strongly stimulate both shoot elongation and production of new nodes.

There seem to be no recent studies dedicated to etiolated shoot cultures of potato. In many other plant species the use of etiolated shoot cultures has been strongly recommended to increase shoot multiplication (Vinterhalter and Vinterhalter 2002). Etiolated potato shoot cultures also have a high shoot multiplication rate combined with very fast shoot elongation. Unfortunately, this combination of fast growth responses is not suitable for mass propagation since the slender etiolated potato shoots are far too fragile and damage-prone during aseptic operations.

Etiolated shoot cultures are a good model system to de-

Fig. 7 Potato (cv. 'Désirée') shoots are highly phototrophic bending to a transversely positioned light source. Etiolated shoot bends towards a blue LED diode (470 nm) but not towards the red LED diode (660 nm) of the same irradiance. Shoot tip bending required continuous illumination. It starts after 13-15 h reaching a 90° angle 23-24 h after the beginning of illumination at room temperature.

monstrate high phototropic feature of potato. When single node explants are placed in darkness on PGR-free medium with 2% sucrose axillary buds develop as etiolated shoots and not as stolons. After some 4-6 days in darkness, the well elongated shoots manifest a strong phototrophic reaction enabling them to bend and grow towards a laterally positioned light source (**Fig. 7**). Using blue LED diodes (NSPB, Nichia, Japan) as the only transversely positioned light source we observed a complete 90° bending within 24 h of continuous illumination. Bending was obtained with blue (~470 nm) but not with red (~660 nm) LEDs (Vinterhalter and Vinterhalter 2002; **Fig. 7**). Bending started after 16 h at a point lying 7 mm from the shoot tip. Light-grown potato shoot cultures manifest also a high phototrophic response in growth rooms with classic fluorescent lights. They often detect subtle differences and choose to grow towards the light source providing the highest irradiance.

Studies investigating the effects of irradiance, photoperiod and spectral composition of light in potato *in vitro* cultures have been reviewed by Seabrook (2005).

Storage

Shoot cultures were the first successful biotechnological solution for clonal propagation and maintenance of valuable potato clones (Westcott *et al*. 1977). However, subculturing performed at monthly intervals can be time- and labour-consuming if large collections are to be maintained. To solve this problem it was necessary to prolong the subculture duration to avoid deleterious effects on the survival rate and genetic stability of propagated clones. The problem of slowing down the shoot culture growth rate can be tackled by many different strategies or approaches. It should be noted that most of the procedures developed and recommended for shoot cultures can be also be used for prolonged storage of microtubers since these are left in flasks in which they were produced to fulfil their dormancy requirements.

Shoot culture as a technique enables indefinite maintenance of potato shoots in a condition of active growth. A critical step in the maintenance of shoot cultures is subculturing, which requires direct human attention and manipulation, increasing the overall maintenance expenses and creating manipulation risks (mistakes) which can jeopardize the whole process. The two most common types of mistakes that occur during subculturing are labelling mistakes leading to the mix-up of cultures in the collection, and contaminations, which are more or less fatal for the cultures. There are also subtle seasonal and other changes in the quality of cultures, which may lead to long-term (gradual) culture deterioration requiring highly experienced operators to counteract.

Standard subculture duration of shoot cultures lasting 4–6 weeks can be somewhat prolonged using larger culture vessels with more medium and less explants. Sarkar *et al*. (1994) found that the initial growth rate of shoot cultures decreases with the number of explants in the culture vessel. In emergencies, cultures can be briefly stored in household refrigerators with low light irradiance set at 4°C (Pruski *et al*. 2000). The ultimate goal for long-term storage is to develop protocols which require subculturing once a year providing also high survival frequency of explants.

There are several basic approaches which used alone or combination with other treatments can significantly prolong storage duration of shoot cultures.

Use of low temperatures – cold storage

The growth rate of shoot cultures dramatically decreases with ambient temperature. Thus in refrigerated growth rooms (with sufficient irradiance and a light regime) the average subculturing time can be prolonged at least 3 to 4 times. Westcott (1981a) showed that a reduction of temperature from 22 to 6–12°C extended the subculture duration from 4 weeks up to 12 months. Increased media volume and an increase in sucrose concentration from 3 to 8% additionally increased survival efficiency of cultures. Sealing culture vessels with closures can prevent fast media evaporation. In some ornamental species, it can be a helpful treatment but in potato, attempts to reduce aeration are detrimental (Hussey and Stacey 1981).

Cryopreservation

Cryopreservation is a technique for storing material at the temperatures of liquid nitrogen, –196°C. Potato is not a frost-hardy species and it suffers irreplaceable damage at temperatures lower than –3°C. But when immersed in solutions with cryoprotectants it can withstand freezing and spend an extended period of time conserved at very low temperatures. First reports of successful cryopreservation in potato were made by Bajaj (1977), Grout and Henshaw (1978), Bajaj (1981) and Towill (1981). Cryopreservation will be presented separately in another chapter in this publication.

Use of plant growth retardants

The growth of shoot cultures can be decreased by addition of plant growth retardants. Westcot (1981b) showed that the addition of ABA at 5.0 mg/l enables subculture duration to be extended to 12 months providing 96% efficient survival of plants cultured for 6 months and 66% survival of plants cultured for 12 months. Plants treated with ABA were stunted with reduced internodes. Some were also chlorotic and with retarded root development in comparison to the control. Upon transfer to ABA-free media, cultures resumed their normal growth. Miller *et al*. (1985) showed that CCC at 500 mg/l can be used to reduce the growth rate of potato shoots cultures without any apparent deleterious effects. Acetylsalicylic acid (ASA) at 18 mg/l can also be used to reduce the growth of potato shoot cultures (Lopez-Delgado *et al*. 1998). It is slightly less efficient than 4% mannitol but it induces no aberrations characteristic for the use of mannitol. Supplementing media with low ASA concentrations may be considered as a mild growth retardation treatment.

The use of retardants in storage may have some disadvantages but it represents a simple, efficient and relatively cheap way for controlling culture growth, which is as routine employed in several institutions that keep *in vitro* germplasm collections (Dodds *et al*. 1991).

Reduced nutrition and manipulation of osmotic stress

Slow growth of potato shoot cultures can be induced by reduced carbohydrate and mineral nutrition. Sucrose can be partly replaced with sugar alcohols (sorbitol, mannitol) which are not metabolised but increase the osmotic value of the medium. Cultures also manifest decreased growth in conditions of low, dim irradiance.

The addition of 4–6% mannitol is very efficient since it induces water stress decreasing the growth rate of cultures. Westcott *et al*. (1977) showed that the addition of 1–6% mannitol enables 100% survival of single node cultures if

storage did not exceed 6 months. Mannitol can unfortunately induce appearance of phenotypic abnormalities (Lopez-Delgado *et al*. 1998) probably connected with the increased tissue hypermethylation (Harding 1994).

An interesting new approach in storage of potato shoot tips is their encapsulation in calcium alginate hollow beads. This technique enabled Nyende *et al*. (2003) to store this "synthetic seeds" with 100% recovery for 180 days at 10°C or 270 days at 4°C.

Meristem tip culture – virus eradication

Meristem tip culture is a technique in which small, 0.3–1.0 mm long apical meristem tips with 2 to 4 leaf primordia are excised and cultured *in vitro.* This is an applied technique used for eradication of harmful viruses. The technique is based on the observation of a low number of virus particles in meristem cells. A drawback of this technique is the need for a compromise between the explant size and the efficiency of virus eradication. Only small explants have a chance to be really virus-free but they have a low survival rate.

Meristem tips were traditionally cultured in liquid medium with filter paper bridge supports. Tips can actually float on top of liquid medium but they can be cultured almost equally well on agar-solidified medium. Meristem tips can be cultured on PGR-free medium but they benefit from the addition of some PGRs at very low concentrations. Novak *et al*. (1980) reported the beneficial effect of 0.17 mg/l IAA and 0.2 mg/l KN, while GA_3 was beneficial at 1.73–3.46 mg/l. Higher cytokinin concentrations prevented plant development while higher auxin concentrations induced callusing.

Among early studies dedicated to meristem tip culture, we would like to mention the following reports and studies: Norris (1954), Morel and Martin (1955), Kassanis (1957), Morel and Muller (1964), Goodwin (1966), Mellor and Stace-Smith (1966, 1969), Morel *et al*. (1968), Pennazio and Redolfi (1973), Gregorini and Lorenzi (1974), Penazzio and Vecchiati (1976), Novak *et al*. (1980).

The efficiency of virus eradication by meristem tip culture was variable depending on virus type, potato genotype and procedure details. Thermotherapy, another technique for virus eradication was also used. In comparison to meristem tip culture, thermotherapy is a much simpler procedure requiring only plant material to be continuously maintained at an increased temperature (32–36°C). The efficiency of virus eradication could be improved by combining thermotherapy as the first and meristem tip culture as the second eradication step (Smith and Mellor 1968; Šip 1972). This combination is still widely used. Ravnikar and Gogala (1989) reported that meristem tip culture alone could not eradicate PVM from Slovenian cv. 'Vesna'. Eradication was obtained after 4-8 weeks of thermotherapy at 36°C.

Another interesting approach to virus eradication was supplementation of media with viricidal substances. This technique is known as chemotherapy. Positive results were reported by the use of malachite green (Norris 1954), virazol (Klein and Livingstone 1982) and jasmonic acid (JA) Ravnikar and Gogala (1989). Recently Faccioli and Colalongo (2002) showed that chemotherapy alone can be used for eradication of PVY and PLRV (potato leaf-role virus) in a number of potato cultivars. Viricidal compounds added to MS medium include 100 mg/l DHT (2,4-dioxo-hexahydro-1,3,5 triazine and 50 mg/l ribavirin. Presence of viruses is tested by ELISA after 4 weeks with recultivation of infected explants for another 4 week long viracidal treatment. The percentage of virus-free plants after 8 week treatments ranged from 13.8% for PVY in 'Liseta' to 50% for PVY in 'Monnalisa'. Same authors previously demonstrated the use of chemotherapy in eradication of PVX and PVS (Faccioli and Colalongo 1998).

Of interest is also the approach reported by Wang and Huang (1975). They produced callus tissue from shoot tips and then regenerated shoots and whole plants from callus finding that 17 out of 37 regenerants were free from PVX, which infected the mother plant.

Mass production of virus-free plants

Production of virus-free potato plants was a tremendous achievement of plant biotechnology. It opened yet another great possibility – mass propagation of virus-free plants, which could be used as seed for the routine establishment of a potato crop in the field. However, there is no guarantee that plants in the field would stay virus-free for any length of time. On the contrary, plants usually get infected at soon as they are planted into the soil and the production yield decreases through the season. Having in mind the high cost of the whole virus eradication + mass propagation scheme there was a question over the feasibility of this scheme. Both methods used in this production line are sound and the problem lies not in the methods employed but in the local presence of leaf aphids, the main virus-spreading vectors.

In the fight against potato viral diseases a much better approach would be to introduce resistance directly into the leading commercial cultivars. This can be done successfully using techniques of genetic engineering which are presented in a separate chapter in this publication.

Acclimatization and weaning

Individual shoots with well developed root system can be transferred from culture vessels and planted *ex vitro* in soil or in some adequate planting substrate. A well drained substrate like a 1:1:1 mixture of peat, soil and sand is recommended (Faccioli 2001). Agar is gently washed of (if possible) to prevent points of infection by bacteria and fungi. However, the most important condition required by small plantlets is high humidity (90-100%) provided by protective plastic sheet wrapping or glass plates covering containers or beds with plantlets. High air humidity is maintained during the first 10-15 days and it is gradually decreased in the following 2-4 weeks. Leaves formed on shoot cultures are actually adapted to high air humidity characteristic for *in vitro* culture conditions. Their cuticle is thin and stomata are closed and dysfunctional. Plants can be considered as adapted when they start to develop new leaves.

Autotrophic micropropagation

Basic concepts for autothrophic micropropagation in potato presented by Kozai *et al*. (1988) were extended and elaborated in later studies and reviews (Kozai 1991a, 1991b). In this *in vitro* culture system media contains no sucrose or related carbohydrates. Plants are expected to assimilate CO_2 and perform photosynthesis *in vitro* in order to acquire energy and carbon atoms for their growth. Thus facilities for autotrophic micropropagation require increased irradiance and CO_2 supplementation.

Kozai *et al*. (1988) studied establishment of photoautotrophy in shoot cultures of cv. 'Benimaru'. Plantlets were maintained by subculturing 25 mm long shoot tips with several leaves (internodes) cultured on MS medium with 0.4% agar, 3.0% sucrose and 250 µmol $m^{-2}s^{-1}$. Plantlets were cultured at three irradiance levels 65, 210 and 400 µmol $m^{-2}s^{-1}$, and three sucrose concentrations 0, 1.5 and 3% with or without CO_2 enrichment. At lower irradiance (65 µmol $m^{-2}s^{-1}$) and in the absence of CO_2 enrichment, sucrose strongly promoted increase of shoot culture FW. At both higher irradiances with CO_2 enrichment, 210 and 400 µmol $m^{-2}s^{-1}$, the effect of sucrose on FW weigh was minimal indicating successful establishment of photoautotrophy. In CO_2 non-enriched treatments with 65 µmol $m^{-2}s^{-1}$ irradiance internal CO_2 levels through the experiment were less than 100 ppm and it prevented (inhibited) photosynthesis. Actually, sucrose was the factor contributing to the increase of shoot dry weight in these conditions. Authors also noted that plants at high irradiances appeared short (dwarfed) and hardy. This is a well known, direct and basic consequence of high irradiance in plants, intended to prevent excessive

shoot elongation.

In nearly air-tight culture vessels with chlorophyllous plantlets (shoot cultures), CO_2 concentration often drops to less than 100 ppm, much less than the normal atmospheric value at 350 ppm (Kozai 1991a). During the night, CO_2 values increase to up to 3.000–9.000 ppm but then sharply decrease to 100-200 ppm some 2 h after the beginning of the day. This means that through most of the daytime cultures are unable to perform photosynthesis on basis of low available CO_2.

Among the numerous advantages listed for the use of autotrophic micropropagation (Kozai 1991b) the most prominent are: strong promotion of growth and development of shoot cultures under autotrophic conditions, decreased contamination risks in absence of sucrose, increased control over environmental factors, reduced mineral salts and PGR content of media, possible use of automated subculturing equipment and increased survival rate in the acclimatization stage.

Other studies performed within the project of autotrophic micropropagation include: study of forced ventilation (Kubota and Kozai 1992), study on the relative humidity within the culture vessel (Kozai *et al*. 1993), study on the effect of medium volume per explant and mineral salt content of the medium (Kozai *et al*. 1995), study on light intensity and illumination direction (Kitaya *et al*. 1995), study on photoperiod and light intensity (Niu *et al*. 1996), effect of red light and LEDs as light sources (Miyashita *et al*. 1997) and others. These latter studies did not make such a high impact on the scientific community as the initial appearance of the autotrophic micropropagation concept.

Autrophic micropropagation did not prove to be superior than the routine *in vitro* culture techniques. Techniques are considered as improvements if they present technical or some other form of simplification and reduction in operation costs. With increased costs of facility equipment installation, operation and energy consumption, gains offered by this concept are not justified in potato micropropagation. However, autotrophic micropropagation applied in the final stage can improve acclimatization of micropropagated plants.

Potato shoot cultures tolerate high levels of CO_2 supplementation. Mackowiak and Wheeler (1996) showed that in *ex vitro* plantlets of cvs. 'Denali' and 'Norland' grown hydroponically in environmental growth chambers, shoot biomass increased linearly as the CO_2 partial pressure increased from 50 to 1000 Pa.

Automated micropropagation

Another interesting approach at the end of the 1980s and early 1990s was automatization of the micropropagation process Aitken-Christie *et al*. (1995). Manual labour accounted for 60–75% of the overall cost of micropropagation. Automation if proved acceptable could significantly decrease these expenses. Technology had already mastered serial production manufacturing based on the use of robots. The process was supposed to be monitored by cameras and computer programmes which made manipulation decisions by video imaging input data. Problems encountered in this approach were mostly handling of delicate plant material and the need for accurate and justified decision-making concerning manipulation of plant material. An interesting contribution to this approach in potato biotechnology was a study by Alchantis *et al*. (1994). Potato shoot cultures were treated with 0.25-0.5 mg/l ancymidol in order to increase the visual colour contrast of cultures, to regulate shoot size and to induce tuberization. Automated micropropagation is possible, but its feasibility is a completely different question. However, production of potato microtubers in large bioreactors can be done with a great reduction in manual labour approaching therefore a semi-automatic operational mode.

SOMATIC EMBRYOGENESIS

Potato as a species has been earlier considered recalcitrant not only to shoot regeneration but also to somatic embryogenesis (SE). Both techniques were significantly improved by the use of GA_3 and modern cytokinin types. It will be shown that GA_3 in general supports the development of shoots after cytokinins trigger cell divisions leading to activation of existing meristems or induce the new ones. For SE, proper timing of stages in combination with certain PGR balances will be key factors for its success.

SE is considered as an applied technique providing means for mass clonal propagation of some species same as micropropagation. In potato this will not be the case, arising from single cells in callus-like tissues which are burdened by somaclonal variation and the creation of aberrant plants.

Embryogenic-like structures were first reported by Lam (1975) developing on tuber explants cultivated on media supplemented with a number of different PGRs. Formation of bipolar embryoid-like bodies were also reported by Bragdø-Aas (1977) in callus from tuber explants of 2 out of 23 potato clones cultured on a complex medium containing 0.4-1.2 mg/l BA, 0-0.8 mg/l KN, 0.4 mg/l IAA and 1000 mg/l CH.

In the next 15 years there was no success with SE in potato. In the meantime another type of embryogenesis was extensively studied in isolated anthers and microspores. Apparently, the findings obtained with androgenic embryogenesis did not function in SE that was considered recalcitrant until the 1990's.

The first study in which SE was obtained on purpose and not as a side effect was the one by Pretova and Dedicova (1992). They reported early stages of SE in surface layers of cotyledons and hypocotyls of zygotic embryos. The initial culture medium containing 5% sucrose, Gamborg B5 mineral salts, 0.05 mg/l BA, 1000 mg/l yeast extract and 0.4 mg/l glutamine produced several embryos after 20 days. On MS medium with 2 mg/l 2,4-D or 2.0 mg/l dicamba explants produced friable yellow callus within 10–14 days. After 3–4 subcultures on medium with 2.0 mg/l 2,4-D performed at two-week intervals granular embryogenic tissue developed. Full embryos developed 14–18 days after transfer to PGR-free MS medium with 5% sucrose. SE appeared in roughly 50% explants producing some 300 somatic embryos from a single zygotic embryo.

De García and Martínez (1995) obtained all stages of SE in single node shoot explants. They were initially cultured on medium with 0.5–4.0 mg/l 2,4-D. After 3 subcultures on medium with 2.0 mg/l 2,4-D, each lasting for a month, compact callus was obtained. After a further 2 months, embryos developed in media with 0.1 mg/l GA_3 or 1.0 mg/l BA. Embryos were formed some 6 months after the culture initiation and it took them 45 more days to develop into plantlets.

JayaSree *et al.* (2001) investigated the production of SEs in leaf explants of cv. 'Jyothi'. For SE explants were cultured two weeks on medium with 0.2 mg/l 2,4-D and 2.25 mg/l BA medium on which nodular callus developed. On medium with 2.25 mg/l BA and 5.0 mg/l zeatin nodular callus developed somatic embryos. On media containing GA_3 nodulary callus regenerated not embryos but shoots. Also the replacement of 2,4-D with NAA in the initial callus induction step resulted in the production of compact callus, which, unlike nodulary callus, could not produce shoots and embryos.

Seabrook and Douglass (2001) obtained somatic embryos (SEs) from various explants of 18 potato cultivars. Explants included stem internodes, leaves, microtubers and roots. A two-step protocol produced SEs within 14–28 days. The first (step) medium contained 3.32 mg/l IAA and 0.033 mg/l TDZ or 0.033 mg/l BA. The second (step) medium contained 0.0875 mg/l IAA, 2.63 mg/l zeatin and 0.19 mg/l. GA_3. On the first medium a pale yellow nodular callus developed on cut surfaces of stem internodes after 1–2 weeks in culture and a week later for other explant types. After 7–

14 days on the first medium stem internode explants were transferred to the second medium on which SEs developed from explants within 2–3 weeks. Thus, SEs could be produced in 5 weeks, which was much faster than the 6 months in the previously published protocol of de García and Martínez (1995). An interesting observation was that SEs reaching 0.5 cm or more in length exhibited strong apical dominance inhibiting the growth of other nearby positioned somatic embryos present on the explants. Development of SEs from early torpedo stage to plantlets took 2–4 weeks. After transfer and acclimation in a greenhouse minitubers could be harvested in 3 months. Auxin in the second medium was not necessary for SE production of most cultivars. SE productivity was strongly influenced by the genotype. It affected SEs production time, number of responding explants and the number of embryos per explant. Clonal differences were also observed in the appearance of aberrant plants.

Sharma and Millam (2004) performed a detailed histological examination of somatic embryogenesis in potato. Their main effort was to establish connections between the histological structure and key developmental stages of SEs.

Vargas *et al*. (2005) produced SEs from suspension cultures established from nodal section explants. This is a complex 5-step protocol in which callus initially induced with 4.0 mg/l 2,4-D is processed into cell suspension cultured on medium with 0.5 mg/l 2,4-D and 0.5 mg/l KN and transferred to media with 1.0 and later 0.5 mg/l zeatin. SEs were analysed histologically and were shown to be of unicellular origin. This protocol is very productive in comparison to similar protocols elaborated for other species. Frequent reculturing (within 15 days) helped to maintain the SE capacity of cells. Their protocol shows that GA_3 is not required in any of the propagation steps. Stages in SE development were characterized by proteins released into the medium.

Sharma *et al*. (2007) improved SE applying short (1 h) pulse 2,4-D treatments to internode explants. A single pulse with 4.42 mg/l 2,4-D could replace a two-week long induction phase of SE, accelerating and improving the practicability of potato SE procedures.

HAPLOID CULTURE

Androgenesis and gynogenesis are techniques in which gametophytes are *in vitro* cultured with the aim to obtain haploid cells/plants. Explants in androgenesis are young anthers or pollen excised from flower buds. In gynogenesis explants are ovules. Cells, tissues, embryos or whole plants obtained from andro- and gynogenesis are expected to be haploid – which is not always the case. In species like potato with prominent breeding barriers, andro- and gynogenesis are highly valuable techniques.

Androgenesis

In potato, androgenesis usually results from microspores entering the embryogenesis pathway. Androgenic embryos pass through characteristic stages of embryogenesis ending with the production of plantlets. Microspores may also produce organogenic callus. Ploidy levels of these plantlets are not reduced in comparison to somatic tissues due to endoreduplication or failure in meiotic divisions. Most of the plantlets obtained by androgenesis are diploid with a small percentage of monoploids and tetraplods. Since the plantlets develop from tissue which is initially haploid, their final ploidy level is best described as dihaploid.

Early studies started with the reports by Dunwell and Sunderland (1973), Sunderland and Dunwell (1973), Irikura (1975), Sopory and Rogan (1976), Foroughi-Wehr *et al.* (1977), Jacobsen and Sopory (1978) followed by many studies of Sopory and Wenzel at the Max-Plank Institute in Germany in the late 1970s. Bajaj and Sopory (1986) reviewed the research in this early period. The major problem initially encountered was that only few out of many investigated genotypes responded positively to androgenesis. This was the highest genomic effect encountered in potato biotechnology. The term androgenic competence indicates the possible existence of heritable androgenic traits (Wenzel and Uhrig 1981; Singsit and Veilleux 1989; Sonnino *et al.* 1989). The second major problem of androgenesis was extremely high variability among regenerated plantlets. Other minor problems related to the efficiency and yields were mostly solved by later protocol improvements.

Sopory *et al*. (1978) as routine for androgenesis recommended the following medium: MS with 0.8% agar, 6% sucrose, 0.5% activated charcoal (AC) and 1.0 mg/l BA.

Wenzel *et al*. (1979) compared techniques of microspore and protoplast cultures. Over 2000 *S. phureja* plants were produced by androgenesis using the protocol after Sopory *et al*. (1978). The vast majority of plants produced by androgenesis were diploid although at the beginning of the procedure they were monoploid. Diploid state appeared through the *in vitro* culture. Only 8 plants raised in the greenhouse were true monoploids. Very high variation was a major characteristic of plants obtained by androgenesis. On the other hand, among plants obtained from protoplast culture variation was almost absent. This finding is not in accordance with later findings of somaclonal variation in protoplast derived plants.

Uhrig (1985) significantly improved androgenesis protocols introducing agitated liquid medium. For the initial anther culture liquid Linsmaier and Skoog (1965) medium at ½ concentration, supplemented with 6% sucrose, 0.05% AC, 0.1 mg/l IAA and 2.5 mg/l BA on a rotary shaker is recommended. After 2 to 3 weeks, free embryoids collected at the bottom of the flask were harvested and replanted on agar solidified ½ LS medium with 2% sucrose and 1.0 mg/l GA_3. Plantlets were transferred to PGR-free medium and rooting was performed *ex-vitro* in a greenhouse. Linsmaier and Skoog (1965) medium differs from MS medium only in thiamine-HCl content which in LS is increased to 0.4 mg/l.

Johansson (1986) studied protocol improvements using AC in a double phase medium and pre-treatment of donor plants with Alar 85 and cold treatment of buds (several days at 7°C). There was a positive response with these improvements in a number of potato cultivars, including ‘Bintje’.

Singsit and Veilleux (1989) studied the inheritance of androgenic competence analysing hybrids between clones competent and incompetent for androgenesis. The authors confirmed the existence of androgenic competence and showed that it can be transmitted by sexual hybridization between diploid potato species. The authors discuss the possibility that a simple inherited dominant gene can be responsible for both the callus regeneration capacity (Simon and Peloquin 1977) and androgenic competence (Uhrig and Salamini 1987).

Tianinen (1992) investigated some common culture conditions and pre-treatments and their effect on anther culture production in commercial potato varieties. Comparison of high (30°C) and low (6°C) temperature pre-treatment showed that the latter had a beneficial effect in some cultivars while the former had no positive effect at all. Tianinen (1992) showed also that sucrose is superior in supporting androgenic embryogenesis in comparison to maltose, melbiose and mannitol. In addition, the efficiency of androgenic embryo production in cv. ‘Pito’ showed seasonal variation being highest during September and October.

Tianinen (1993) investigated the effect of various PGRs on the anther culture response of tetraploid potatoes. In comparison to studies performed previously by other authors, productivity was high, having a positive response in 10.9% of treated anthers with 4.3% of anthers producing plantlets on MS medium with 2.27 mg/l BA.

The problem encountered in this this study was not the productivity but the insufficient specificity of PGRs in the regulation of this process. A positive androgenic response was obtained here both with cytokinins and auxins applied either alone or in combination. Such a lack of specificity towards PGRs is unusual, indicating perhaps the existence of other regulatory mechanisms. Converging signal pathways in the initiation of androgenesis has been proposed by

Maraschin *et al*. (2005) with the aim to explain why so many different stress factors can trigger androgenesis in plants. Maraschin *et al*. (2005) further pointed at insufficient PGR specificity in the regulation of potato SE affected by auxins, cytokinins and ABA. The situation is actually analogous to meristem tip culture where plantlet production benefits by addition of various PGRs at low concentration. Thus, at 0.1 μM potato meristem tip development was promoted either with KN or GA_3 (Novak *et al.* 1980). It possible that androgenic embryogenesis actually does not require any PGRs (Chani *et al*. 2000), as for SE in many plant species (Krikorian 1982) or shoot cultures of potato.

Říhova and Tupý (1996) studied the combined effect of 2.0 mg/l 2,4-D and 6% lactose on the production of embryoids. On callogenic medium supplemented with 2,4-D embryo production was low but it increased if sucrose was replaced with lactose.

Říhova and Tupý (1999) investigated the symmetry in divisions of potato microspores as affected by media constituents. Symmetric division required for microspores to enter somatic embryogenesis pathway were supported on media supplemented with 0.5 mM cytidine, 1.0 mM uridine, 3.0 mM glutamine and 1% lactalbumin hydrolysate present in M1 medium (Tupy *et al.* 1991). The principal factor responsible for symmetric cell division was the presence of glutamine and lactalbumin hydrolysate.

Valkonen *et al*. (1999) used anther culture to obtain dwarf dihaploid mutants of cv. 'Pito'. Dwarf plants were genetic mutants containing a recessive dwarf gene *pito*.

Aziz *et al.* (1999) screened androgenesis of 23 diploid potato clones on four different media formulations. Anther derived plants/tissues were obtained only in 7 clones using agitated liquid media. Thus, the genotype related control was the most important factor in the anther culture response.

Asakavičiūtė *et al*. (2007) investigated the androgenic response in a number of Lithuanian potato (*S. tuberosum* L.) cultivars. Using media elaborated previously for barley (Jacquard *et al*. 2006) supplemented with 2.0 mg/l NAA and 1.0 mg/l NAA they obtained regenerants from three out of five investigated cultivars with 17.4% as the highest anther response in cv. 'Nida'.

According to Chani *et al*. (2000) the androgenenic response in potato is limited mostly by the genotype while the techniques can be considered as well elaborated. The end of the 1990's brings a strategic change in research with the research interest shifted from *S. tuberosum* to related potato species *S. phureya*, *S chackoensis*, *S. acaule* and their hybrids (Teparkum and Veilleux 1998; Rokka *et al.* 1998; Naseer Aziz *et al*. 1999; Chani *et al*. 2000; Boularte-Medina and Veilleux 2002). There is much less research dedicated to technique improvement. Instead, androgenic response of different potato interspecies hybrids is investigated with the aim of making preparations for specific DNA introgression.

Recent findings and research prospects in potato androgenesis have been briefly reviewed by Pret'ová *et al*. (2006).

Gynogenesis

In potato, gynogenesis can be obtained by conventional cross-pollination using *S. phureya* as a pollen donor. Seed forming on mother plants is supposed to arise by parthenogenesis – enabling the whole process to be classified as gynogenesis. In such a process, there is only a limited need to use *in vitro* culture techniques for storage and maintenance of interesting genotypes.

Uijtewaal *et al*. (1987) described production of monohaploids, plants with 2n = x = 12 n by pollination with *S. phureya*. Pollination is done with special clones homozygous for an easily observable morphological trait (colour spots at leaf base) enabling early selection of plants resulting from parthenogenesis (no visible markers). Working with 2 million seeds, they recovered 33,000 "spotless" seeds ending with 500 monohaploid plants. All monohaploid plants were established and stored *in vitro* as shoot cultures. Thus, *in vitro* culture techniques were not needed for the initial production of monohaploids but they were useful for mass propagation and storage.

Lough *et al*. (2001) compared the characteristics of monoploid plants produced by androgenesis and gynogenesis. Androgenic monoploids were superior in most agronomic traits including leaf size (13–18%) and tuber yield (26–27%).

Androgenesis, somatic embryogenesis and somatic hybridization are all biotechnologies highly promising for use in potato breeding. Serious problems encountered through research did not discourage investigators – on the contrary, research continued only with small changes in methods, strategies and goals, considering also positive results obtained with other plant species. Attention at the moment is shifted from commercial potato cultivars to related potato species. Identification of gene loci supporting various types of embryogenesis could perhaps be used for introgression of these traits into *S.tuberosum*, which would then greatly facilitate and boost potato breeding.

TUBERIZATION

Potato tubers are underground organs with storage and vegetative propagation as their main functions (Rodríguez-Falcón *et al*. 2006) Tubers are modified shoots closely associated with stolons from which they develop (**Fig. 8**). Tubers and stolons differ by planes of cell divisions which in stolons promote elongation while in tubers increase their thickness. Signal that the plant is competent to produce tubers generated in leaves is transmitted to other plant parts by the phloem system. Target cells are located in the subapical zone of stolons. Signal induces a change in the plane of cell elongation and division, Cell division plates become parallel to the elongation axis of stolons promoting radial growth. At the subcellular level, transition in the plane of cell divisions is connected with the arrangement of microtubules (MT) (Struik *et al*. 1999).

Under *in vitro* culture conditions, the change in the MT arrangement in the subapical zone of stolon outgrowth can be observed on the fifth day on the tuberization medium (Sanz *et al*. 1996). Change in the MT orientation is followed by a change in the plane of cell elongation and formation of cell division plates.

A tuber is a typical sink organ (tissue) in which surplus photosynthetic assimilates are stored (Fernie and Willmitzer 2001). The main storage components are starch (Visser *et al*. 1994; Farre *et al*. 2000) and proteins (Hednriks *et al*. 1991; Shewry 2003) as the main storage components. Assimilate in the form of sucrose reaches tubers by phloem stream

Fig. 8 Tubers of cv. 'Désirée'. Tubers usually form on the tip of stolons, tuber bearing shoots (**A**). In sessile tubers, stolon is not visible, since the axillary bud directly develops into a tuber. In some cases apical bud on well developed tubers can resume growth forming a leafy shoot. In this photograph a sessile tuber (**B**) resumes growth.

where it gets processed into starch (Fernie *et al.* 2002a). Up to 70% of sucrose reaching tubers gets processed into starch (ap Rees and Morrell 1990). Potato has a complex and versatile carbohydrate metabolism extensively investigated by genetic engineering techniques enabling accurate gene silencing or overexpression (Fernie *et al.* 2002b; Geigenberger *et al.* 2004).

Tuber storage and photosynthesis in leaves are directly linked and functionally co-regulated by phloem transport (Fernie *et al.* 2002). Assimilates reach tubers in the form of sucrose where, for storage, it needs to be converted into starch. Sucrose to starch conversion in potato is one of the major research foci of plant metabolism (Fernie and Willmitzer 2001). Tubers should also be considered as specialized propagule since they enable efficient vegetative propagation.

Many different factors are involved in the establishment of tuberization competence. In the field grown plants main tuberization-inducing factors are photoperiod, temperature, nitrogenous nutrition and most likely changes in endogenous PGRs (Cutter 1978; Ewing 1987; Hannapel 1991; Hendriks *et al.* 1991; Sanz *et al.* 1996; Ulloa *et al.* 1997; Fernie and Willmitzer 2001). Tuberization is also genotype dependant. Some potato species do not produce tubers at all. Among them is *Solanum brevidens*, important for breeding as a species carrying resistance to virus strains (PLRV, PVX, PVY) and fungal diseases (Liu *et al.* 2001). *S. brevidens* and *S. tuberosum* are sexually incompatible but some of their hybrids obtained by somatic hybridization produce rhizome-like tubers in the field (Fish *et al.* 1988b). However, tuber production and quality is the main goal in the selection of current commercial cultivars to which out attention in this review is turned.

In field-grown plants tuberization occurs when plants reach certain size or degree of physiological competence, enabling them to store surplus assimilates (Ewing and Struik 1992). The process is known as tuber induction and plants competent to produce tubers are tuber-induced. Information (signal) that a plant has become competent for tuber production is generated in leaves from which it gets transmitted to stolons as target organs where it initiates tuber development (Jackson and Willmitzer 1994). The tuber induction signal strongly resembles the flower induction signal. It is also a chemical signal, transmittable by grafting.

After a well organized and long search, the tuberization signal was finally identified as tuberonic acid, a substance similar to JA (Koda and Okazawa 1988; Koda *et al.* 1988). This substance is active *in vitro* inducing tuberization when supplemented at 0.01 mg/l (Yoshihara *et al.* 1989). JA was not the ultimate cause of tuberization, rather only one of the many compounds which affect tuberization. Studies on the interaction of PGRs and environmental factors in tuberization continued but studies in the mid 1990's were rapidly refocused on the molecular level with the aim to identify genes active in tuberization. Some of them, like *StBEL5*, active in leaves of short day induced plants, produce mRNA that gets translocated by phloem from leaves down to axillary buds and stolon tips (Banerjee *et al.* 2006).

Factors affecting *in vitro* tuberization

Those expecting that plants in highly favourable *in vitro* culture conditions will quickly attain tuberization competence are bound to be disappointed. Tuberization *in vitro* is greatly delayed in comparison to tuberization of field-grown plants. On standard (maintenance), PGR-free MS medium with 2-3% sucrose, shoot cultures start to develop tubers only after some 4-5 months in culture (Hussey and Stacey 1984) (**Fig. 9**). They even speculated that tuberization in shoot cultures is perhaps connected with senescence. However, tuberization in shoot cultures can be significantly accelerated and improved by adding PGRs or by altering culture conditions.

The most efficient factors for the induction of in *vitro* tuberization are increased sucrose concentration of the medium and the addition of cytokinins. Highly potent tuberization inducing factors are inhibitors of gibberellin biosynthesis and in general all compounds that inhibit or retard shoot elongation.

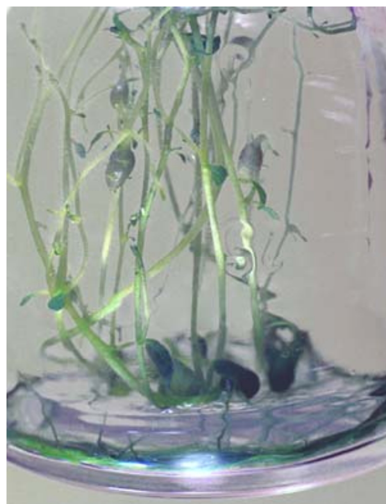

Fig. 9 On PGR-free medium with 2-3% sucrose, tuber (cv. 'Désirée') development is greatly delayed, occurring after 3-4 months. These tubers are usually aerial, positioned high above the medium. *In vitro* tuberization can be significantly accelerated by the addition sucrose, cytokinins, inhibitors of GA synthesis, various growth-retarding substances and others.

In vitro tuberization is usually obtained by the addition of 2-10 mg/l cytokinins and/or an increase in sucrose concentration to 5-8% (Mes and Menge 1954; Palmer and Smith 1969; Mingo-Castel *et al.* 1976; Wang and Hu 1982; Abbott and Belcher 1986; Estrada *et al.* 1986; Gopal *et al.* 2004). For large-scale tuber production in fermentors, Akita and Takayama (1994) used PGR-free liquid MS medium containing only 9% sucrose.

An extensive early study of tuberization in single node cuttings was performed by Hussey and Stacey (1984) using cvs. 'Ulster Sceptre' and 'Red Craigs Royal'. They showed that on medium containing 2.0 mg/l BA and 6% sucrose, tuberization was accelerated, occurring in some 6-8 weeks. BA strongly stimulated tuberization, which was highest at 2.0 mg/l. With an increase in BA concentration (2.0 mg/l) the upright leafy shoot was transformed into a horizontally growing stolon. In long days + BA, stolons entered medium forming tubers in the medium. In short days + BA, most of the tubers formed above the solidified agar medium. The effect of photoperiod on tuberization was inconclusive although short-day treatments had a weak promotive effect on tuberization. Photoperiod strongly affected shoot morphology with strong, leafy shoots in long days and thin, upright, branched, stoloniferous shoots with small leaves and terminal hook in short days. At 0.1-1.0 mg/l, GA_3 completely inhibited tuberization maintaining this inhibition for months. It also converted BA induced horizontal stolons into upright growing leafy shoots. CCC at 500-1000 mg/l promoted tuberization manifesting a strong synergistic effect when applied together with BA. When applied alone ABA stimulated tuberization at 0.015 and 0.06 mg/l but at higher concentrations tuberization was reduced. ABA at 0-1.0 mg/l reduced tuber number when applied together with 2.0 mg/l BA. However, at 32.0 mg/l ABA promoted tuberization in 'Ulster Sceptre' the same as CCC.

The superiority of sucrose over glucose, fructose and maltose in tuberization can be best observed from the studies of Khuri and Moorby (1995). Previously Lo *et al.* (1972) compared tuberization in media supplemented with sucrose and the sugar alcohol mannitol. They showed that the stimulative effect of sucrose on tuber formation is nutritional and not the result of increased osmotic concentration since mannitol at concentrations equimolar to 2, 9 and 12% failed to induce tubers.

Most of the findings made by Hussey and Stacey (1984) were confirmed in later studies. Thus GAs are considered as PGRs that strongly inhibit tuberization (Vreugdenhil *et al.* 1994; Dragićević *et al.* 2008) and stimulate stolon elongation (Kumar and Wareing 1972; Xu *et al.* 1998). Thus it seems that GAs have an unequivocal role in the determination of

stolon/tuber development pattern (Fernie and Willmitzer 2001).

A large group of GA antagonists and PGRs classified as growth retardants strongly stimulate tuberization under conditions of *in vitro* culture.

Paclobutrazol (PBZ, (2RS, 3RS)-1-(4-chlorphenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol)) significantly promoted tuberization both in light and darkness (Šimko 1993; Pelacho *et al.* 1994; Šimko 1994). The effect was visible at a wide range of concentrations, from barely observable stimulation at 0.001 mg/l with long stolons and tubers at the surface of the medium up to strong inhibition by 1000 mg/l, which induced development of sessile tubers instead of apical shoot buds (Šimko 1994). Ancymidol at 5 mg/l in combination with 8% sucrose and 2.5 mg/l 2iP (2 isopentenyl adenine) was used in tuberization medium by Levy *et al.* (1993) and Seabrook *et al.* (1993). Tetcyclacis (TET) also strongly promotes tuberization in potato. Vreugdenhil *et al.* (1994) used 1.35 mg/l TET to efficiently induce tuberization in stolons developing from axillary shoots pre-cultured on medium with 1.12 mg/l BA. TET induced tuberization even in "droopy" (ABA-deficient) potato mutant recalcitrant for *in vitro* tuberization. Dragićević *et al.* (2008) showed that the strong stimulative effect of 0-2.7 mg/l TET on *in vitro* tuberization could be cancelled by the presence of 0.35 mg/l GA_3. Results obtained with ABA were inconsistent. In some reports ABA was found to stimulate tuberization while in others it was inhibitory (Xu *et al.* 1998; Gopal *et al.* 2004). In general, ABA is considered as a tuberization promotor (Chandra *et al.* 1988). Destefano-Beltrán *et al.* (2006) showed that ABA plays a critical role in the establishment and control of dormancy in tubers which are sites of active ABA biosynthesis.

CCC, a well known plant growth inhibitor, is a potent tuber-inducing agent at 500 mg/l (Hussey and Stacey 1984; Estrada *et al.* 1986; Lentini and Earle 1991). In combination with 5.0 mg/l BA, it manifests a strong synergistic effect in tuber induction but only in darkness (Yamamoto and Nakata 1997).

The effect of auxins on *in vitro* tuberization has been less investigated (Ewing 1987). Dragićević *et al.* (2008) showed that 0-1.75 mg/l IAA could increase tuberization up to 80% but only in darkness. This dark-stimulated IAA tuberization was completely cancelled in the presence of 0.35 mg/l GA_3 but promoted in the presence of 0.8 mg/l TET.

Various aromatic compounds are also known to stimulate tuberization (Chandra *et al.* 1988). Stallknecht (1972) showed that coumarin at 25-50 mg/l induces tuber formation *in vitro*.

JA is a potent tuber inducer. Pelacho and Mingo-Castel (1991a) showed that JA was much more efficient than KN in tuber induction. Thus on medium with 10.5 mg/l JA cultures produced 2.4 times more tubers per treatment than on medium with 2.5 mg/l KN. According to Koda (1992), apart from potato, JA and related compounds can induce *in vitro* tuberization in other tuber-bearing plant species like Jerusalem artichoke (*Helianthus tuberosus*) and Chinese yam (*Dioscorea batatas*).

Ethylene and CO_2 may also affect *in vitro* tuberization. Garcia-Torres and Gomez Campo (1973) found increased tuberization with 50 mg/l ethrel, an ethylene-liberating compound. Palmer and Barker (1973) and Mingo-Castel *et al.* (1974) could not confirm this effect. Mingo-Castel *et al.* (1974, 1976) showed that CO_2 stimulates while ethylene inhibits tuberization and that therefore these two substances act as antagonists in tuberization.

In vitro tuberization is promoted by the presence of AC. Bizzari *et al.* (1995) reported that 0.2% AC in combination with 8% sucrose had a very strong stimulatory effect on *in vitro* tuber formation. Thus a medium supplemented with 8% sucrose and 0.2% AC supported much higher *in vitro* tuberization than media supplemented with cytokinins or other tuberization-promoting compounds.

If the tuberization signal is strong, axillary buds will directly develop into sessile tubers without the preceding stolon formation (Ewing and Wareing 1978). Strong tuberization signals can also shift the position of tuber appearance from lower to higher shoot internodes (Šimko 1994). Thus PBZ at very low concentrations (0–0.001 mg/l) promoted tuber formation on stolons developing from the lowest positioned shoot internodes at the level of medium. Increase of PBZ concentration to 0.01–10 mg/l caused stolon shortening and shifting tuber formation to higher positioned internodes. At highest PBZ concentrations, sessile tubers developed directly from the uppermost, apical shoot bud.

Tuberization markers

Stages in the *in vitro* tuber development resemble development of tubers on field grown plants with cessation of stolon elongation, swelling of the subapical stolon region and further increase in tuber diameter. Much effort was done in order to connect visible stages of tuberization with the events at molecular levels as in the study of Uloa *et al.* (1997). They connected changes in morphological appearance and histological structure with the activity of protein kinases. Other more or less reliable markers for the transition of stolons into tubers are changes in the levels of carbohydrates (Visser *et al.* 1994; Pevalek-Kozlina and Berljak 1997), patatin (Hendriks *et al.* 1991) and ascorbic acid (Viola *et al.* 1998).

With the advance of molecular methods, it became possible to identify and then monitor the appearance and changes in the activity of genes involved in tuberization. Genes involved in tuberization were identified through analysis of cDNA libraries established from tissues in early tuberization stages (Struik *et al.* 1999). In this way, a number of genes involved in the regulation of potato development have been identified. *POTM1* is a potato MADS box gene affecting apical dominance (Rosin *et al.* 2003a). Suppression lines with decreased *POTM1* mRNA had decreased apical dominance, compact growth habit, reduced leaf size and increased production of cytokinins. Tuber yields of suppression lines were reduced from 79% to 97%. *POTH1* is a class1 KNOX gene affecting plant development by the control of GA production (Rosin *et al.* 2003b). Plants with overexpressed POTH1 were dwarfs with malformed leaves. Their synthesis of bioactive GAs was decreased and consequently tuber production was increased. Another group of transcription factors in potato are the seven members of the BEL1 family identified through their interaction with POTH1 protein as bait (Chen *et al.* 2003). BEL (StBEL) and KNOX (POTH1) proteins perform a joint repression of *ga20oxidase*, binding to a specific promoter sequence (Chen *et al.* 2004). Their joint overexpression enhances the tuber-forming capacity in transgenic plants (Hannapel 2007). RNA of one of them, *StBEL5*, accumulates in leaves and stolons under inductive short day conditions. Banerjee *et al.* (2006) showed that *StBEL5* mRNA enters phloem vessels in potato leaves and then gets translocated down to the axillary buds and stolon tips. Therefore, apart from plant growth regulators, mRNA molecules may also function in long distance signal transduction.

Differences between *in vitro* and *ex vitro* tuberization

Ewing (1987) pointed that several different *in vitro* culture systems are currently in use for tuberization studies and that we must be cautious in extrapolating results of this research to the whole, intact plants. To avoid misunderstandings, in the present review the term *in vitro* tuberization is reserved only for tubers developing on shoot cultures, implying that at least one subculturing was performed.

In many studies classified as *in vitro* tuberization, *in vitro* techniques are used only to accelerate the response of mature explants. In studies of this type, field grown or greenhouse grown plants are submitted to tuber-inducing conditions *ex vitro*. Tuber-induced shoots are then sectioned

into single or multiple node explants, surface sterilized and cultured *in vitro*. The response of these explants can be scored after very short periods lasting for several days (Gregory 1956; Chapman 1958), 8-10 days (Hendricks *et al.* 1991; Vreugdenhil *et al.* 1998) or 3 weeks (Koda and Okazawa 1983).

The single-node "*in vitro*" system of Hendriks *et al.* (1991) became very popular in tuberization studies since it enables a very fast and highly synchronised response of explants. Plants of cv. 'Bintje' providing explants were induced for tuberization growing in a greenhouse at short day conditions. Medium contained 1/10 of MS nitrogenous salts, 8% sucrose and 1.12 mg/l BA. In darkness etiolated shoot/stolon explants produced visible tuber swelling after 5-6 days in comparison to explants from long-day plants swelling after 7 to 8 days.

The single-node tuberization system although not performed completely under *in vitro* culture conditions (explants were tuber induced on the mother plant *ex vitro*) provide a very good insight in stolon to tuber transition (Vreugdenhil *et al.* 1994, 1998).

Using the same single-node tuberization system Vreugdenhil *et al.* 1998 accurately showed that sucrose, and GAs are principal factors inducing tuberization under conditions *in vitro*. If the sucrose level in the medium was decreased from 8% to 1%, then there was no tuberization and axillary buds developed upward growing into etiolated shoots. Although 8% sucrose strongly promoted tuberization, addition of 0.17 mg/l $GA_{4/7}$ to this medium prevented tuberization, promoting formation of stolon-like shoots. Authors showed that axillary buds could be manipulated into three different structure types: tubers (high sucrose no GAs), shoots (low sucrose) and stolons (high sucrose + GAs). They also demonstrated that GA inhibits tuberization even when all other factors are inductive.

The single-node tuberization system of Hendriks *et al.* (1991) has a hidden disadvantage illustrating why this system can not be used as a representative for *in vitro* tuberization *sensu stricto*. According to Vreugdenhill *at al.* (1994) this system was not suitable for *in vitro* cultured material since the tuberization obtained in 'Bintje' *in vitro* explants was poor, requiring the addition of TET to the medium.

Tuberization of *in vitro* cultured material (shoot cultures) shows several important differences when compared to tuberization of field-grown, intact plants.

First, JA is a tuber-inducing agent *in vitro* (Yoshihara 1989; Pelacho and Mingo-Castel 1991a) but has little or no effect on *ex vitro* plants (Jackson and Willmitzer 1994; Jackson 1999). Also at 5 μM, JA applied together with 10 μM FLD synergistically reduced tuber dormancy *in vitro*, inducing precocious sprouting in 90% of tubers checked for dormancy after 6 weeks of development (Suttle and Hulstrand 1994).

Cytokinins are important tuberization factors *in vitro* (Palmer and Smith 1968; Forsline and Langille 1976; Koda and Okazawa 1983) but not in *ex vitro* plants, where this role belongs to the photoperiod (Pelacho *et al.* 1994, Rodriguez-Falcón *et al* 2006). While the photoperiod is important for *ex vitro* plants, *in vitro* plantlets are relatively insensitive and the effect of photoperiod is not clear-cut (Ewing 1987; Vecchio *et al.* 2000; Rodriguez-Falcón *et al* 2006).

In vitro tuberization can be regulated by the photoperiod only in the absence of sucrose and PGRs when it can be triggered by inductive short daylengths alone (Seabrook 2005). Various photoperiodic treatments can also affect the microtuber shape and morphology (Seabrook *et al.* 1993).

Sucrose is the most powerful tuber inducing substance of *in vitro* cultures (**Fig. 10**). It also supports and promotes the tuber-inducing effect of cytokinins and various inhibitors (Struik *et al.* 1999). Sucrose action can not be mimicked in field-grown plants same as the temporarily decreased nitrogen nutrition which strongly promotes tuberization in hydroponic systems (Krammer and Marschner 1982).

Sucrose may actually be the only compound necessary for induction of tuberization *in vitro* (Leclerc *et al.* 1995) and our studies in potato and other plants species indicate that sucrose *per se* may exert cytokinin-like effects (Vinterhalter *et al.* 1977; Vinterhalter and Vinterhalter 1999).

Fig. 10 In field-grown plants, lower internodes are "reserved" for tubers same as in these strong shoots of Serbian cv. 'Dragačevka' on medium with increased sucrose nutrition (5%). Sucrose stimulates tuberization and activation of axillary buds.

We must not forget that in plants sucrose is the mobile, transportable carbohydrate translocated as assimilate from sources to sinks. Source-to-sink transport to tubers of in *vitro*- and *ex vitro*-cultured plants apparently moves assimilate in opposite directions. In *ex vitro* plants the assimilate moves down, from the upper positioned functional leaves into the lower positioned tubers. In *in vitro*-cultured plants the main direction of assimilate flow is up, from the low positioned medium within the culture vessel to the up positioned aerial tubers (**Fig. 9**). Also in mixotrophic potato shoot cultures, photosynthesis is almost completely missing due to the lack of available CO_2 during the daytime (Kozai 1991b), and the sucrose present in the medium contributed to the dry matter build-up (Kozai *et al.* 1988; Cournac *et al.* 1991).

Care must be taken that the most frequently used single node shoot explants and cuttings are not the structural units of shoots and perhaps not a good representative of the plant as a whole. Such structural units are phytomers (Evans and Groover 1940; Khurana *et al.* 2004) and it takes a two-node shoot cutting to have a full, functional non-damaged phytomere explant. A single-node cutting consists of two phytomere units both damaged by excision. The upper unit contains axillary bud and part of the internode above the bud with the leaf missing. The lower unit contains only the leaf with the node and the rest (lower part) of the unit is missing. Such explants require some time to recover (growth delay) but the only possible growth located at the axillary buds covers the putative excision damage. Damage made to single node explants by excision can be best evaluated from the study of Leclerc *et al.* (1995) who demonstrated much higher tuberization in explants consisting of six nodes than in single-node explants. Differences in the tuberization response between single and two-node cuttings were described by Ewing and Wareing (1978). They claimed that in single–node cuttings good tuberization required maximum removal of the shoot stump below the internode (lower phytomere). Delayed tuberization induced by the excision damage of the upper phytomere of the single-node explant has been demonstrated by Papathanasiou *et al.* (1994). Delayed development of axillary buds and their derivatives was also observed after the removal of the leaf lamina from single-node cutting explants (Seabrook and Douglass 1994). The effect of leaf lamina of single-node explants on microtuber production was much less apparent affecting only decreased

FW and percentage of dry matter production (Seabrook *et al*. 2004). A somewhat different view was offered by Osaki *et al*. (2004). They showed that according to photoassimilate partitioning, shoots of a number of plant species, including potato, appear to be organised in nodes as structural and functional units, suggesting a modification for the phytomer concept.

Finally, a factor equally important in the regulation of tuberization in explants of *in vitro* shoot cultures and intact field-grown plants are GAs. In all cases presence of GAs inhibits tuberization promoting elongation of stolons (Smith and Rappaport 1969; Kumar and Wareing 1972; Vreugdenhil and Struik 1989; Xu *et al*. 1998). Extensive studies performed by Suttle (2004a, 2004b) showed that endogenous GAs in contrast to exogenous GAs are not intimately involved with tuber dormancy control, playing a critical role only in subsequent sprout elongation.

Microtubers, microplants and minitubers

Small tubers produced *in vitro* by shoot cultures are called *microtubers*. Their size is variable depending on propagation techniques. Their FW is usually 0.1-0.4 g and the diameter is less than 15 mm. Microtubers can be efficiently used as propagule showing some advantages in comparison to propagation by *in vitro* culture produced plantlets, *microplants*. Microtubers can be very easily stored, transported and then planted directly in the field without previous acclimation. Microtubers can be mass-propagated by various techniques based mostly on the use of liquid media in bioreactors. Following successful acclimation, microplants planted in the field or in a protected glasshouse develop their own system of stolons and tubers, which, by the end of the season, overpass in size *in vitro* produced microtubers. These small tubers produced by acclimated, *in vitro* propagated plantlets (microplantlets) are named *minitubers*. Minitubers are typically larger than microtubers but they are both smaller than seed tubers which typically weight about 50-70 g (Watad *et al*. 2001).

Micropropagated plants do not need to be removed from disposable containers in which they were cultured *in vitro*. It is sufficient to add soil in the containers and puncture container bottoms (Ahloowalia 1994). Micropropagated plantlets produce minitubers 5-25 mm in diameter within 70 to 90 days of growth in soil (**Fig. 11**).

Fig. 11 Minitubers produced by a plant (cv. 'Désirée') grown from a microtuber.

According to Ahloowalia (1994), minitubers have a number of advantages over microtubers. Thus, microtubers are too small, *in vitro* tuberization is too long, and their germination in soil is uneven. In contrast, the relatively larger minitubers allow better handling and resemble parental cultivars in tuber shape, skin colour and texture. Minitubers can be classed as Super Elite or Elite Seed and used for production of Certified Seed.

Microplants can be cultured in systems specially designed to increase the productiion of minitubers. We will later briefly present the main techniques for production of both micro- and minitubers and discuss their advantages and weak points.

Microtuber dormancy

Tubers induced on field-grown potato plants steadily grow till the end of the vegetative season increasing their size and weight by the input of surplus photoassimilate. All through this period tubers gradually enter dormancy a period of rest lasting through the cold winter period. Dormancy is initiated concurrently with tuber initiation (Destefano-Beltrán *et al*. 2006). At the time of harvest tubers are completely dormant meaning that they can not sprout even in placed in favourable conditions. Also at the time of harvest, tubers rapidly (in a matter of days) change their metabolic activity becoming source organs in contrast to their previous sink function (Viola *et al*. 2007).

Microtubers produced by potato shoot cultures and their explants are supposed to be initially dormant same as tubers of field grown plants and minitubers. Microtuber dormancy is a very useful trait if we intend to use them for germplasm storage (Mix-Wagner 1999). Nevertheless, if our intention is to plant microtubers in the filed or in a glasshouse, then we first must overcome their dormancy in order to obtain good and synchronized sprouting.

Microtuber dormancy is strongly genotype dependant (Leclerc 1995; Veccchio 2000; Pruski *et al*. 2003)

Microtuber dormancy decreases with the time spent in cold storage. Le (1999) reported over 50% sprouting after 3 months and 100% sprouting after 6 months in microtubers of 8 cultivars stored at 4°C.

Small microtubers with low FW (size) manifest a significantly higher dormancy then larger microtubers (Rannali *et al*. 1994; Leclerc *et al*. 1995). Thus tuber size plays a role equally important as the genotype in microtuber dormancy.

Physiological processes underlying dormancy in microtubers and field-grown plants appear to be similar (Leclerc *et al*. 1995) but similarity can not be extended over dormancy mechanisms (Coleman *et al*. 2001). Microtubers demonstrate a very wide range of dormancy responses including reduced dormancy and even complete absence of dormancy (Leclerc *et al*. 1995; Coleman *et al*. 2001).

Thus microtubers produced under light had a short dormancy and sprouted prematurely (Gopal *et al*. 1997). Similarly light applied even as short day treatments reduced the dormancy duration in comparison to microtubers produced in dark (Coleman and Coleman 2000). Increased sprouting was also demonstrated in microtubers produced from single node cuttings cultured in long day as compared by short day conditions (Vecchio *et al*. 2000). During microtuber storage the situation may be different. Thus in two out of three investigated cultivars microtuber dormancy was higher in light- (20 µM m^{-2} s^{-1}) than in dark-stored microtubers (Gopal *et al*. 2003).

To ensure early and synchronised sprouting tubers are submitted to dormancy-removal treatments prior to planting. Pruski *et al*. (2003) found that soaking microtubers in 100 ppm GA_3 was a better treatment, giving higher tuber yields than the Rindite treatment. Rindite and similar chemicals (ethylene chlorcohydrin, ethylene dichloride, carbon disul-

phide and carbon tetrachloride) used to break tuber dormancy are toxic and present a health risk. Better choice is to plan and keep microtubers in cold storage until they reach over 80% sprouting or to pre-culture them in a safe, protected and controllable place.

The effect of PGRs on the dormancy in potato micro- and minitubers has been thoroughly investigated by Suttle in a serial of studies performed on cv. 'Russet Burbank'. With information provided by other research groups this result presents the backbone of our current knowledge on hormonal regulation of potato tuber dormancy.

Suttle and Hulstrand (1994) investigated microtuber dormancy exposing them to exogenously supplied ABA and FLD recognised as an ABA inhibitor. Continuous exposure to 10 μM FLD resulted in microtubers devoid of endogenous ABA exhibiting precocious sprouting. Application of 50 μM exogenous ABA to FLD-treated microtubers increased endogenous ABA levels and decreased precocious tuber sprouting. Continuous ABA application restored endogenous ABA levels to control values abolishing precocious sprouting. Authors concluded that endogenous ABA is directly involved in the initiation and possibly maintenance of dormancy in potato microtubers.

Later studies will confirm that among all PGRs ABA plays a key role in the control of tuber dormancy (Destefano-Beltran *et al.* 2006). Authors identified and studied a number of ABA biosynthetic gene orthologs (*StZEP*, *StNCED1*, *StNCED2*, *StCYP707A1-A4*) all present and active in tuber tissues and meristems. Meristems were both target and principle site of ABA metabolism of potato tubers. Through the dormancy period ABA levels were modulated by the dynamic balance between biosynthesis and catabolic processes.

The involvement of ethylene in the regulation of microtuber dormancy was investigated by Suttle (1998a). Single-node explants used in this study generated detectable amounts of ethylene producing microtubers completely dormant for at least 12-15 weeks. Precocious sprouting could be obtained by continuous exposure of explants to either 25-50 μM $AgNO_3$ or 2.0 ml/l (gas phase) norbornandiene (NBD), both recognised as inhibitors of ethylene synthesis. Both compounds showed a marked dose response. Exogenous addition of ethylene inhibited precocious sprouting of NBD-treated microtubers. NBD was efficient only at the beginning of tuber formation i.e., within the first 7 days after the start of experiments. Suttle concluded that ethylene plays an essential role in the regulation of potato tuber dormancy similar to the one previously established for ABA (Suttle and Hulstrand 1994). However, endogenous ethylene is required only during the earliest stages of tuber dormancy. The nature and the extent of interaction between ABA and ethylene was not followed.

Effect of cytokinins was studied in minitubers (Suttle 1998b; Suttle and Banowetz 2000). Contrary to ABA and ethylene, exogenous application of cytokinins resulted in termination of dormancy and sprouting (Suttle 1998b). Dormancy break was accompanied by increase of endogenous cytokinins. During the postharvest storage cytokinin levels increased more than 7-fold due mainly to the *de novo* synthesis. Endogenous cytokinin increase precedes the onset of sprouting. Eight different cytokinins of both zeatin and isopentenyl adenine type were detected in bud extracts.

Involvement of *cis*- and *trans*-Z and their ribosides in maintaining dormancy in minitubers was also studied (Suttle and Banowetz 2000). Minitubers 3-5 g stored at 3°C in darkness were totally dormant for the first 81 days. At 116 days sprouting was 43% reaching 100% after 165 days. Exogenous *cis*-Z and *cis*-ZR occurred in potato tuber extract. Endogenous *cis*-Z levels increased in tuber prior to the termination of dormancy while exogenous *cis*-Z promoted premature dormancy termination. Thus, dormancy could be terminated by injection of both *cis*-Z and *trans*-Z. Immediately after harvest tubers were insensitive to the addition of both zeatin isomers, *trans*-ZR and 2iP (Suttle 1998b, 2001). Cytokinin sensitivity of tubers gradually increased until the end of dormancy. Continued studies showed that no apparent changes in the cytokinin metabolism occurred as tuber spontaneously exited dormancy (Suttle 2001). Thus postharvest cytokinin levels seem to be regulated via changes in *de novo* synthesis and not through catabolism. Similar but inverse situation was previously demonstrated for ABA which decline by the approach of sprouting (Suttle 1995).

Suttle (2004) extended the studies on potato microtuber dormancy to GAs following endogenous GA levels through the storage and after addition of AMO16-18, ancymydol and TET recognised as GA inhibitors. Endogenous GA content is associated more with the sprout growth than with the dormancy termination. Results therefore do not support a role of endogenous GAs in the release from tuber dormancy, supporting the view that GAs are involved in the regulation of subsequent sprout growth. Continuous treatments with CCC, AMO, and ANC had no apparent effect on dormancy duration. Only TET at 30 μM increased microtuber sprouting to 50%.

Microtubers and storage

Dormant microtubers are excellent material for germplasm storage. Once they are formed, they can be either harvested or just left over in the same culture vessels after the leaves and stems dye off (Mix-Wagner 1999). Microtuber stored at 4°C sprout in the culture vessel after 12-15 months. Potato germplasm storage by microtubers has been also described by Rossel *et al.* (1987) and Thieme (1992).

Microtuber field performance

Yield comparisons between microtubers, *in vitro* propagated plantlets, minitubers and conventional seed tuber pieces in the field production do not always agree (Donnely *et al.* 2003). They are confounded by numerous variables involved in their production systems and perhaps even insufficient experience with this novel propagulum types. Microtubers can hardly be expected to reach the size required for food consumption and processing but they can be used for potato seed production.

Comparison studies performed by Struik and Lommen (1990), and Lommen and Struik (1994), on basis of their higher weight indicated minitubers as a material more suitable for direct field planting than microtubers. On the other side, predictions of reduced production costs accompanying improvements in the efficiency of microtuber production seem to favour potato propagation *via* microtubers (Donnelly *et al.* 2003). They also consider that improvements resulting in increased microtuber size could completely eliminate the use of minitubers. However, minituber production systems also rapidly evolve, as we will see in the next section. A different view was offered by Pruski *et al.* (2003). Comparing performance of *in vitro* plantlets and microtubers in field trials, they concluded that unlike *in vitro* plantlets, microtubers at present stage of knowledge could not be recommended for production of seed tubers in the field. The main problem encountered in microtubers in this study was release from dormancy

The most serious problem and disadvantage of microtubers as propagules is their fairly small size in comparison to minitubers and conventional seed potato tubers. Development of plants arising from microtubers in the field is delayed in comparison to microtubers and seed potato and as a consequence their growth and yield parameters are also lower (Ranalli 1994). However, with decreased row spacing enabling microtuber planting at higher densities the delayed early growth is compensated at least in late maturing cultivars.

One of the possible improvements in microtuber utilization is their preculture in glasshouses or in other protected locations prior to the final field planting (Haverkort *et al.* 1991; Rannali *et al.* 1994; Ranalli 1997). This approach provides more control over the early stage of sprout deve-

lopment in which future plantlets are most sensitive to environmental factors and conditions.

The second approach is microtuber recycling, as proposed by Khuri and Moorby (1996), based on the observation that small microtubers (5 mm in diameter or less) perish in field conditions. Here small microtubers and their nodal segments are used to produce a new generation of *in vitro* plantlets and for rapid production of new microtubers. Thus, instead of wasting nearly a 1/3 of an average microtuber production, by planting small sized tubers in the field, they are reused by subculturing to a medium with 4% sucrose where they produce a new generation of plantlets in two weeks. Explants from these plantlets can be used for further shoot multiplication or for production of new microtubers. Instead of usual 12-14 weeks at 8% sucrose, explants originating from microtubers require only 10 weeks to produce new, useful microtubers.

Microtuber dormancy is the second major problem responsible mostly for non-synchronized microtuber sprouting. Pruski *et al*. (2003) showed that in field trials 24 h treatment with GA_3 at 100 ppm was superior to Rindite.

In a field trial, Haverkort *et al*. (1991) compared crops raised from conventional seed tubers and microtubers of various sizes. The final concusion of this study was that the use of microtubers for direct planting is not a practical option. Direct field planting of small microtubers (0.2-0.4 g) was hazardous due to their vulnerability to night frosts. Also, crops grown from microtubers with an initial weight under 0.5 g had lower yields than crops grown from conventional seed tubers. Covering soil with plastic films increased the yields of larger microtubers and those pre-grown in a glasshouse. Plastic foil reduced the night frost damage but increased the risk of infections caused by *Rhizoctonia solani* and viruses.

Ranalli *et al*. (1994) performed field trials comparing the growth and production of crops established from microtubers, minitubers and normal tubers (seed tubers). Plants from microtubers emerged 8-9 days later than those from minitubers and 14-15 days later than those from seed tubers. Total yield after 110 days with 60 cm row spacing expressed in t/ha was 27.3, 38.9 and 47.5 for microtubers, minitubers and seed tubers, respectively. At 90 cm row spacing yield was 6.7, 24.4 and 54.0 t/ha, respectively. Therefore, microtubers should be planted only in narrow rows. According to this study, microtubers are an inferior propagule type in comparison to minitubers and seed tubers. The authors believed that microtuber yields could be improved by preplanting of minitubers in a greenhouse prior to their final planting in the field. Thus there is still space for the improvement of microtuber yields based on planting techniques.

Gopal *et al*. (1997) compared the performance of crops raised from microtubers produced in darkness and in light. A study of 22 characters performed on 18 different genotypes showed that light-grown microtubers are superior to dark-grown ones in most characters including tuber yield. Pruski *et al*. (2003) also reported that microtubers produced at short day (8 h photoperiod) performed better in field trials than microtubers produced in darkness. Microtubers produced in light were greenish and less juvenile then microtubers from dark treatments.

According to Rolot and Seutin (1999) the use of microtubers for direct field planting is currently in little use due to the irregular shoot emergence and lack of sturdiness required for growth in open-field conditions. The main disadvantage of microtubers is their high dormancy, which needs to be overcome before planting.

Kawakami *et al*. (2003) performed field trials with microtubers comparing their growth with conventional seed potato. The yield of microtuber-derived crops was high, only 18% lower than in plants from conventional tubers. Microtubers could be an alternative in situations when environmental conditions are not suitable for potato seed tuber production. High tuber yields obtained in this study are the consequence of trials established with much larger microtubers weighing 0.5-1.0 g or more. These results are a good indication of the high potential of microtuber-originating crops if the production technologies improve their size/weight.

The choice of microtubers, microplants or minitubers is a dilemma left to producers. They need to calculate and decide individually which one of these approaches suits them best.

Microtuber production systems

Microtubers can be produced on PGR-free medium supplemented with 8% sucrose but only after prolonged periods of time lasting 4-5 months (Wang and Hu 1982; Hussey and Stacey 1984; Garner and Blake 1989). For large-scale propagation, tuberization needs to be radically accelerated. Microtuberization can be both increased and accelerated using large (long) shoot explants consisting of six nodes layered in liquid medium (Leclerc *et al.* 1994). MS medium in the first stage was supplemented with 0.4 mg/l GA_3, 0.5 mg/l BA, 100 mg/l Ca-panthothenate and 2% sucrose. After 4 weeks medium was drained and replaced with MS based tuberization media containing 8% sucrose. Treatments were done with 50 ml liquid media in 400 ml plastic containers and 16 h light period at 22°C. The procedure enabled rapid and extensive tuberization with up to 2.0 g microtubers FW per starting shoots for all three investigated cvs., 'Kennebec', 'Russet Burbank' and 'Superior'. Microtuber weight from layered shoots was 3-5 times higher than from single-node explants. Nodal cuttings produced much more tubers, none of them exceeding 250 mg in weight. The authors point that the use of liquid medium, absence of growth regulators and lower labour input required to handle shoots could reduce the production cost of microtubers. Microtuberization system of Leclerc *et al*. (1994) is a simplified version of protocols developed by Estrada *et al*. (1986) and Meulemeans *et al*. (1986).

Production of microtubers in agitated liquid medium and airlift type bioreactors was investigated by Akita and Takayama (1988, 1994). According to Akita and Takayama (1994) propagation should be divided into two steps. In the first, an 8000 ml capacity jar filled with 2000 ml of PGR-free liquid medium with 3% sucrose is inoculated with single node cuttings and exposed to weak light (9.5 μmol $m^{-2}s^{-1}$) to stimulate the formation of shoots, which will bear tubers in the next stage. When shoots reach 15-20 mm in length, the whole medium of the first stage is replaced with 6000 ml of the second stage MS PGR-free medium with sucrose increased to 9%. The second stage was performed in darkness. Aeration was from the 200 ml min^{-1} in the first stage increased to 600 ml min^{-1} in the second stage. Productivity of the system was 223 ± 29 tubers and 1670 ± 216 axillary buds both calculated per the whole fermentor. Most of the tubers formed within the first two weeks of stage 2 and this number did not increase further. Tubers were preferentially formed on the shoots in surface layers of the medium. The absence of tubers deep in the medium clearly decreased the overall productivity of the system. Induction of tubers was clearly repressed under continuously submerged conditions (Akita and Takayama 1988). Factors responsible for the spatial distribution of tubers within the fermentor were not disclosed. Tuber fresh and dry weights increased steadily over time. The addition of BA at a low concentration increased tuber weight while GA_3 inhibited tuberization. Decrease of temperature from 25 to 17°C clearly decrease tuber number and size. Important observation was that tuber induction and their subsequent development are separate processes. Development (size) of tubers in the fermentor can therefore be separately regulated once the tubers were initiated.

Akita and Ohta (1998) presented a simplified procedure for microtuber propagation. A large 8 l bioreactor was replaced with 1000 ml plastic bottles (Ø90 × 209 mm), equipped with a stainless steel net on a polyurethane foam. Bottles with passive aeration, mounted on a bottle roller

were slowly rolled at 1 rpm. The procedure also uses separate shoot proliferation and microtuber induction stages as in Akita and Takayama (1994). Bottles supplemented with 200 ml media produced an average of 100 microtubers – 500 tubers per liter of medium. This procedure seems to be less complex and a more flexible production system than the air-lift type bioreactor (Akita and Takayama 1994).

Ziv and Sheemesh (1996) studied potato tuberization in airlift type bioreactors and double-phase agar solidified/liquid medium using ancymidol as the main tuberizing inducer. Potato tuberization in liquid medium was sensitive to aeration since callus developed around lenticels.

Jiménez *et al.* (1999) presented a microtuber propagation system in which cultures in their culture vessels/jars were briefly immersed in liquid medium. After the immersion, liquid media was withdrawn from jars and stored in large tanks until the next immersion cycle. Individual jars and tanks for liquid media storage were connected by plastic silicone tubing enabling media pumping too-and-fro. The best growth rate was obtained with a 5-min immersion repeated every 3 h (8 immersions per day). Tuberization was performed on a MS PGR-free medium with 8% sucrose. Production rate after 9 weeks in culture was 3.1 and 2.8 tubers per single node for cvs. 'Désireé' and 'Atlantic', respectively.

Teisson and Alvard (1999) also propagated potato microtubers by temporary immersion using a special type of culture vessels, RITA® and double RITA®, developed and used for somatic embryogenesis of banana, coffey and rubber tree (Teisson and Alvard 1995). Thirty single node explants of cvs. 'Désireé', 'Bintje' and 'Ostara' were placed in each container with 200 ml MS medium with 3% sucrose in the first shoot elongation stage, and 8% sucrose in the second, tuber inducing stage. First stage lasted two weeks and the second stage 8 more weeks with the last six weeks in darkness. Light was provided at 27 $\mu mol\ m^{-2}s^{-1}$, 12-h photoperiod. Immersion rates were 4 × 1 min per day during shoot multiplication and 4 × 60 min in the second, tuber-inducing stage. In average for cv. 'Bintje', Rita® vessels produced 1.7 microtubers per single node explant, with 50.8 microtubers weighing 20.6 g per vessel. Weight distribution was good with 31.2% of microtubers under 0.25 g and 60% of tubers in the 0.25-0.75 g range. Double RITA® vessels were more productive in total numbers but less productive if parameters were calculated per employed medium volume. A more general study on the use of temporary immersion systems in plant micropropagation has been presented by Etienne and Merthouly (2002).

Piao *et al.* (2002) compared the production of potato microtubers in several different types of bioreactors. A two-stage propagation system similar to that of Akita and Takayama (1994) was used. Tuber-inducing medium contained 8% sucrose with or without 1.0 mg/l BA. The second stage, conducted in darkness, produced microtubers harvested after 8 weeks. Very good results were obtained with a continuous immersion system in which a net kept explants in the surface area of the medium. The addition of BA increased the number of desirable tubers weighing 1.1 g or more.

Soilless production of minitubers

Minitubers in large quantities can be produced by acclimated, *in vitro* propagated plants cultured in well protected and controlled conditions. Instead, in the field, acclimated microplantlets can be cultured by one of several soilless production techniques. These techniques enable accurate adjustment of conditions to meet the physiological needs of microplantlets providing high sanitation levels. Soilless systems can be adjusted to accept plants developing from microtubers.

Hydroponics is an old, well known technique. The lower part of the plants with roots in a water-filled container is enclosed in a chamber in darkness while the shoot is free to develop in the light. Nutrients are added in a liquid solution directly to the root system. According to Muro *et al.* (1997) a hydroponic culture system is superior to the traditional minituber propagation system both in total production (yield) and number of produced tubers. Wan *et al.* (1994) reported that short periods of decreased solution pH can trigger synchronised tuberization in hydroponic systems.

The aeroponic system differs from hydroponics in the way in which water and nutrients are applied to the root system. In the dark chamber containing (enclosing) the lower part of the plant body with a root system, nutrients are applied in the form of a mist. Mist spray is produced by a special device.

In the Nutrient Film Technique (NFT) plants are in containers with clay granules or similar neutral support providing a kind of mechanical substrate for roots. Over the table with plant containers, a thin layer film on nutrient solution is enabled to flow. Plants in containers have a free and unlimited access to the nutrient solution by their root systems. The excess, overflowing nutrient solution is collected, sterilised and reused. Comparing the NFT with classic minituber production by microplants Rolot and Seutin (1999) showed NFT technique to be highly productive.

Ritter *et al.* (2001) compared minituber propagation by hydroponic and aeroponic production systems. Plants in the aeroponic system showed increased vegetative growth and delayed tuber formation extending the vegetative cycle. Thus, in the same season two hydroponic cycles could be performed compared to only one aeroponic-based cycle. Still, the tuber yield in the aeroponic system was nearly 70% higher and the tuber number was 2.5 times higher than in the hydroponic system. Only the average tuber weight was reduced by 33%. Apparently, in a direct comparison, aeroponic systems were more productive than hydroponic systems.

Farran and Mingo-Castel (2006) optimised the use of aeroponic minituber production systems for different plant densities and harvesting intervals. Plants also showed extended vegetative cycle duration. A comparison of crops produced from minitubers propagated by aeroponic and hydroponic system did not establish difference between these two propagation systems.

FUTURE PERSPECTIVES AND GOALS

Techniques of *in vitro* culture have been successfully used in potato biotechnology for nearly 60 years. Some of these techniques like culture of callus, root and shoots are considered mature, well elaborated, routine techniques in which no major improvements have been implemented for a number of years. However, some of their practical applications are still widely used as for instance clonal propagation, germplasm storage and virus eradication. Somatic embryogenesis and androgenesis on the other side did not reach their full potential and they can yet be improved. We still do not know the growth conditions and PGR balances that regulate these processes. The same can be said for protoplast culture and somatic hybridization, additionally hampered by chromosome instability and somaclonal variation. Strong genotype effects and variability in general still prevent the full-scale use of *in vitro* culture techniques for breeding purposes. We can only hope that in years to come a breakthrough may occur solving the instability problem and enabling breeding applications with no genotype restrictions.

For *in vitro* culture techniques it was always characteristic that their empirical approach and success in practical applications preceded theoretical explanations of processes underlying the regulation of plant growth and development. However, this situation is rapidly changing. Molecular methods enable us to accurately identify factors (genes) active in the regulation of certain developmental stages. Thus, we will soon be in position to know in advance the genome location and regulatory nature of key developmental points of our target cells. With this knowledge, we will be in a position to create specific manipulation strategies enabling us to control and change the developmental patterns within tissues according to our needs and interest.

In vitro culture techniques will remain basic, initial steps in most studies of molecular biology and genetics. They provide uniform explants produced under strictly defined and rigorously controlled experimental conditions. Thus, they will remain unsurpassed, routine experimental approach in years to come.

Techniques for *Agrobacterium*-mediated transformation of potato have been presented in a separate chapter of this volume. They are all based on the use of *in vitro* culture techniques independently of the vector system employed. They are also considered mainly as routine although any improvement in the transformation efficacy would be a welcome event.

In vitro culture techniques in future may be combined more with classic genetic and breeding techniques. It as a pity that haploid culture techniques are so strongly genotype restricted. We can only hope that the orchestrated use of various techniques may result in creation of diploid varieties of breeding interest, more amenable to *in vitro* culture techniques. Only then the full potential of androgenesis, somatic embryogenesis and other techniques may be expected.

ACKNOWLEDGEMENTS

Potato is one of the most important research objects in our main project No. 143026B, sponsored by the Serbian Ministry of Science. Help in collecting articles for this presentation from Borut Bohanec (Slovenia), Vladimir Orbović (USA) and Milica Ćalović (USA) was welcome and highly appreciated. **Figs. 1** and **2** were provided by Aleksandar Cingel (Serbia)

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