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Volume 5

BIOTECHNOLOGY & GENETIC ENGINEERING REVIEWS

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Potato Protoplasts and Tissue Culture in Crop Improvement

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Introduction

Potato (*Solanum tuberosum* L.) is one of the most important and widely grown food crops in the world. The cultivated forms originate from a narrow genetic base but 160 wild species are recognized and the global gene pool is relatively untapped (Hawkes, 1978). Commercial cultivars are tetraploid ($2n = 4x = 48$) and extremely heterozygous, with simplex inheritance (Aaaa) for many characters (Howard, 1978). Some do not flower easily, or have reduced fertility, and others are pollen sterile, thereby limiting the number of desired crosses that can be made (Howard, 1970). Cytological studies have helped towards understanding the relationships of different *Solanum* species, but the chromosomes are small and difficult to distinguish. Such knowledge as the chromosomal localization of important genes, which has aided cereal breeding, is therefore largely not available for potato.

Most new potato varieties are made by crossing together parents with useful characters followed by vegetative propagation of the F_1 plants to form clones. These clones and their tuber progenies are then screened in gradually increasing plots over several years for favourable combinations of agronomic traits. Effort has also been focusing on the use of true seed for breeding programmes.

Over the last 15 years a new approach to potato breeding has been emerging. At the outset, this approach should be viewed as a complement to conventional breeding practices, and not as a replacement. There have been technological advances in the developing fields of plant cell and molecular biology. Tissue culture systems provide the experimental system to which

Abbreviations: AEC, 5-(2-aminoethyl)cysteine; BAP, Benzylaminopurine; DDMH, doubled double monohaploid; DMH, double monohaploid; DNA, deoxyribonucleic acid; 5MT, 5-methyltryptophan; NAA, naphthalene acetic acid; PEG, polyethylene glycol; PLRV, potato leaf roll virus; PVY, potato virus Y.

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techniques of genetic manipulation can be applied. Potato is one of the more amenable crops for tissue culture. Whole plants can be regenerated from a range of cultured tissues (roots, leaves, tubers, stems) and from single cells. This technology offers new potential for potato breeding in a number of ways: some tissue culture techniques can be incorporated into breeding programmes to improve efficiency; new breeding strategies can be designed using tissue culture in conjunction with conventional breeding methods; new sources of variability are available, and a new type of genetics can be applied by coupling the regeneration systems with the advancing technology of genetic engineering.

In this review we briefly describe the tissue culture techniques that can be applied to potato, and the way in which some can be utilized in breeding programmes. We then examine some of the consequences of the culture techniques and the new source of variation that has unexpectedly arisen in certain tissue culture systems. We then review the progress that has been made in the developing areas aimed at more direct manipulation of the genome. Our emphasis is placed not so much on the techniques themselves as towards an assessment of their potential for crop improvement.

Tissue culture responses and their application

Potato is amenable to a number of tissue culture techniques, ranging from *in vitro* propagation via shoot cultures to regeneration of whole plants from protoplasts. In general terms, these all involve the growth of plants, cells, tissues and organs in sterile conditions, supported by an appropriate culture medium. Media normally contain a mixture of major and minor salts, vitamins, sugar (as a carbon source) and plant growth regulators. The most widely used formulations are based on that of Murashige and Skoog (1962), which is available commercially.

VIRUS ELIMINATION BY MERISTEM-TIP CULTURE

Potato is susceptible to many viral pathogens, some of which may be present without obvious symptoms, although causing gradual decrease in vigour and yield. It is therefore very important, in this vegetatively propagated crop, to be able to eliminate viruses and obtain disease-free plants.

Eradication of viruses can be achieved by culturing excised meristematic buds under appropriate conditions, a method that has been successfully applied in many countries. The techniques are described by Mellor and Stace-Smith (1977) who list 136 virus-free potato cultivars produced by meristem-tip culture. The general procedure involves pretreatment of potato shoots by growth at raised temperatures (32–37°C), surface sterilization and excision of apical and axillary meristems (length 0·3–0·7 mm) and transfer of the excised meristem to a filter paper bridge in a tube containing liquid culture medium. After culture at 20–25°C, shoots emerge and can be rooted and potted out. The treated plants are then tested for the presence of viruses.

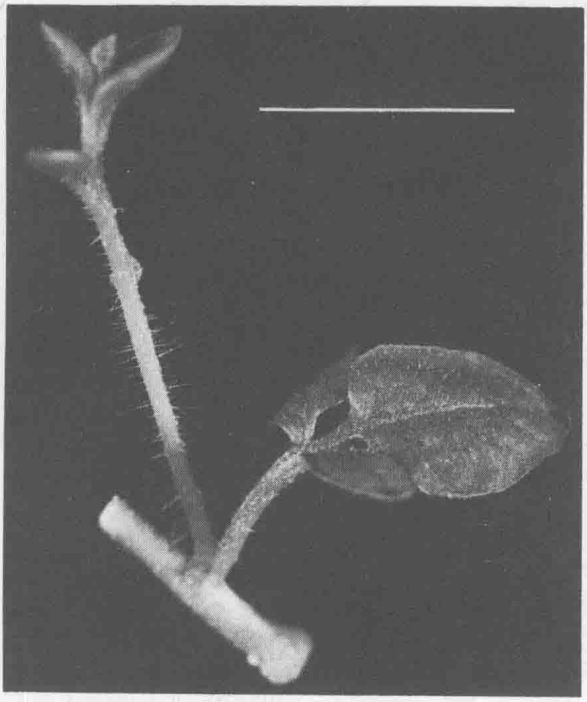

Figure 1. A shoot culture: development of a shoot from an axillary bud on a cut stem (Scale bar = 1 cm).

Critical factors are the size of the meristem and heat treatment. The addition of antimetabolites may also assist virus eradication.

MICROPROPAGATION AND GERMPLASM STORAGE

Axillary buds on excised stem segments in culture will grow out to form shoots (*Figure 1*). Shoot cultures of potato obtained in this way can be maintained indefinitely by repeated subculturing. They are a valuable source material for tissue culture and a means of keeping genetic stocks. They also provide the basis for rapid multiplication of potato stocks by micropropagation.

Following surface sterilization, stem segments are cultured on standard agar media, in the absence of growth regulators, or with low levels of cytokinin (Hussey and Stacey, 1981). Shoots which develop can, in turn, be cut into nodal segments to repeat the process. A multiplication rate of about × 10 per month occurs under continuous light (6000–8000 lux) at 25°C and, by 18 weeks, over 500 plants can be obtained from sprouts from one medium-sized tuber (Hussey and Stacey, 1981). Cultured shoots rapidly develop roots and can be transferred to soil after washing off the agar. Alternatively, stem segments can be cultured, rooted in liquid medium and transferred directly to soil, a method which is more convenient but which results in lower final multiplication rates (Hussey and Stacey, 1981).

After 3–4 months in culture, mini-tubers of 3–6 mm diameter may develop at the nodes. Production of one per node can be obtained effectively by culturing stems in short days on medium containing high benzylaminopurine (BAP) and sucrose levels (2·0 mg/l BAP, 6% sucrose). Mini-tubers exhibit dormancy and may be used for storage and transport of germplasm (Hussey and Stacey, 1984).

Micropropagation is a useful means of multiplying virus-free potato stocks and new or imported cultivars, for which few tubers may be initially available, under conditions where reinfection with virus or infection with fungus-borne diseases does not occur. It is used commercially, for example by Nickersons (Scotland) as a means of multiplying first-year stock for seed production. The propagation of virus-tested stem cuttings by conventional methods produces 800–900 plants from a single clone in 3 years (Hussey and Stacey, 1981). In contrast, by using *in vitro* micropropagation many thousands of plants can be produced from one clone in a single year, although at least one generation in the field is required before use.

In order to maintain genetic resources in potato, there is a need to store primitive potato cultivars and related wild species which cannot be stored as seed. In conventional propagation there is always a risk of loss (Westcott, Henshaw and Roca, 1977). Clones may be stored by the techniques outlined above, but using additional modifications to slow the growth in culture and to lessen the need for subculturing. This can be usefully achieved by growth at low temperature (e.g. 6–8°C) and low light intensity, which requires only annual subculturing. In future the need to subculture may be eliminated completely by cryopreservation in liquid nitrogen (Withers, 1983).

ORGAN AND EXPLANT CULTURE

Whole potato plants can be regenerated from cultured organs, such as anthers and ovaries, and from cultured explants, such as pieces of leaf, stem, rachis and tuber. Regeneration procedures generally involve the production of adventitious shoots from disorganized cell growth, or callus, unlike meristem-tip culture and micropropagation, in which callus formation is avoided.

Regeneration from cultured explants has been achieved by use of a single medium for both callus initiation and shoot formation (Roest and Bokelmann, 1976; Jarret, Hasegawa and Erickson, 1980). A simple two-stage procedure has been applied to a broad range of cultivars (Webb, Osifo and Henshaw, 1983; Wheeler *et al.*, 1985) for monohaploid and dihaploid lines (Karp *et al.*, 1984). The cell proliferation phase, of about 2 weeks' duration, requires the presence of auxin and cytokinin in the medium. In a second morphogenetic phase, adventitious shoots appear on a medium containing cytokinin and gibberellic acid (Webb, Osifo and Henshaw, 1983; Wheeler *et al.*, 1985). Callus formation occurs at the cut surfaces of explants by the 10–14th day. The callus becomes nodular with the emergence of shoots after 24 days and large numbers of shoots (more than 50) may form on 1 cm leaf discs or rachis pieces (*Figure 2*). The shoots can be rooted by transfer to medium containing 0·06 mg/l naphthalene acetic acid (NAA) and then transplanted into soil to produce full-size potato plants.

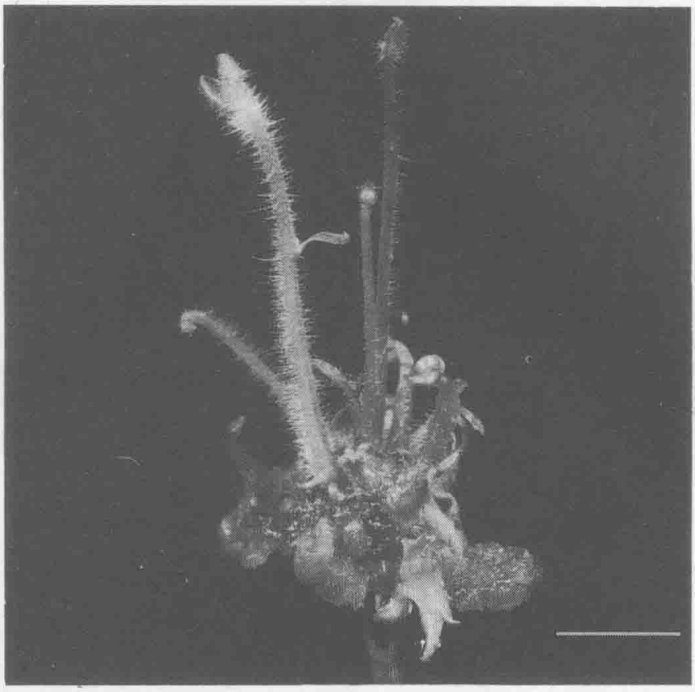

Figure 2. Shoots regenerated from callus formed on cultured leaves of a monohaploid potato (Scale bar = 1 cm).

Anther and ovary culture for the production of haploids

Anther and ovary culture can be used as a means of producing potato plants with a gametic chromosome constitution (or haploid potatoes). As potato is a tetraploid, two successive levels of haploidy are possible: the first, or dihaploid (2n = 2x = 24) is obtained after reduction from the tetraploid; the second, or monohaploid (2n = x = 12) after reduction from the dihaploid. Both levels are useful in potato breeding (Hermsen and Ramanna, 1981).

In addition to anther and ovary culture, haploids can be obtained by parthenogenesis or chromosome elimination and can also arise spontaneously. To be of value, tissue culture should therefore be a more efficient alternative. Parthenogenetic extraction of dihaploids by crossing tetraploid potatoes with *Solanum phureja* (Hougas and Peloquin, 1957) has been so improved by the use of 'superior pollinators' that production by this method is relatively routine and has superseded anther culture. Recent studies by Johansson (1986), however, have shown a much improved efficiency of dihaploid production in anther culture of several potato cultivars. Out of 20 tetraploid clones, 19 produced embryoids and more than 90% of the regenerated plants were dihaploid. These studies indicate that tissue culture may still make a contribution to dihaploid production.

Parthenogenetic extraction has been used to obtain monohaploids from dihaploids (Jacobsen, 1978), but efficiency is low and advances in the culture of dihaploid anthers have led this to be the favoured technique for certain

genotypes (Binding *et al.*, 1978; Jacobsen and Sopory, 1978; Sopory, Jacobsen and Wenzel, 1978; Wenzel *et al.*, 1979).

The factors that are important for anther culture include background genotype, plating at the correct developmental stage (uninucleate microspores, i.e. flower buds 4–6 mm in length), pretreatment (e.g. incubation of flower buds at 6°C for 48 h) and the media components (e.g. sucrose 6%, activated charcoal 0·5%, BAP, 1 mg/l (Sopory, Jacobsen and Wenzel, 1978; Wenzel and Uhrig, 1981). Donor plants for anther culture can be grafted on to tomato to prolong flowering (Wenzel and Uhrig, 1981).

Using such techniques Sopory, Jacobsen and Wenzel (1978) achieved microspore embryogenesis in 35% of cultured dihaploid anthers. Of 22 embryos examined cytologically, seven were found to be monohaploid, 14 had 24 chromosomes and one contained the full tetraploid complement. In a more exhaustive study, Wenzel and Uhrig (1981) produced 6000 clones from cultured dihaploid anthers: about 90% of these appeared to have doubled up spontaneously in culture to give fertile double monohaploid clones ($2n = 2x = 24$); the remainder were monohaploids. Hybrid donor clones carrying resistance genes to *Globodera rostochiensis* and potato virus Y (PVY) yielded homozygous resistant double monohaploid lines.

The main limitation of anther culture is that progress so far has been restricted to few genotypes, although attempts to breed in responsivity (i.e. higher recovery from anther culture) have met with some success (Uhrig, 1983).

PROTOPLASTS

Potato plants can be regenerated from wall-less cells (or protoplasts) (*Figure 3*). This is very important for direct genome manipulation by mutation, fusion or transformation, where the aim, to obtain plants in which all cells contain the altered genotype, can be achieved most satisfactorily through regeneration from single cells. These applications are discussed in some detail later.

In general terms, to obtain viable leaf protoplasts of potato it is necessary to control leaf growth conditions carefully. Plants must either be grown in controlled-environment cabinets with specific light, nutrient and humidity regimes, or else as shoot cultures. After plasmolysis the protoplasts are released by enzyme digestion of the cell wall (*Figure 3a*) and then protected from bursting by provision of a suitable osmotic environment. They are washed and cultured in an appropriate medium which allows the synthesis of new cell walls followed by cell division. Repeated divisions result in the formation of colonies (*Figure 3b,c*). When these are large enough to be visible to the naked eye they are transferred to a solid medium on which further growth occurs to form callus (*Figure 3d*). This is followed by transfer to media that induce differentiation and shoot emergence (*Figure 3e*) and then to a 'rooting' medium on which roots are established on the shoots (*Figure 3f*). The yield of regenerated plants may be 5% of the protoplasts originally cultured (Nelson, 1983). Plants can be maintained and multiplied