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In vitro cultures for producing pathogen-free plants and selecting disease resistant genotypes

by

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Abstract

Plant tissue culture has a major impact in phytopathology for the production of pathogen-free plants. Various factors affect the elimination of viruses in explants, including cultural conditions and inhibitors of viral replication.

Tissue culture methods were also used in plant improvement for the production and selection of new genotypes resistant to diseases. In this specific field, the benefits to be expected from tissue culture depend on the availability of suitable screening methods : application of toxic compounds produced by the pathogen, co-culture of the pathogen with plant tissues, or field selection of regenerated plants.

1. Introduction

Plant tissue culture has a major impact in phytopathology for the production of pathogen-free plants. This technology was also used for the production and selection of plants resistant to diseases [TOMES, 1984]. Our purpose is to highlight the prospects and limitations of plant tissue culture in disease control strategies. We selected a few case studies to illustrate the possible experimental schemes to be used.

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2. Production of pathogen-free plants by tissue culture

The first regeneration of a virus-free (i.e. dahlia) plant from an infected donor was based on the assumption that the apical dome excised from an infected plant was free of virus [MOREL and MARTIN, 1952].

Recent evidence suggests that with some host-virus combinations, dome cells do contain virus particles, but nevertheless regenerate virus-free plants through meristem culture. Meristems were considered an ideal material for the production of virus-free material because of their inherent genetic and developmental stability. However, it is also possible to regenerate virus-free plants from callus cultures of several economic crops, including tobacco [CHANDRA and HILDEBRAND, 1967] and potato [WANG and HUANG, 1975].

The elimination of viruses from explants cultured *in vitro* depends on a combination of factors : heat-pretreatment of the donor plant, size of the explant, cultural conditions, and addition of inhibitory chemicals to the culture medium.

Although detailed studies are not available to elucidate the mechanisms involved, it has been observed that the presence of high concentrations of growth regulators in a culture medium may decrease the virus content of plant tissue. Nodes, excised from potato plantlets (cv. Roxane) infected with virus Y (PVY), were micropropagated by placing subcultures alternately in liquid MS medium [MURASHIGE and SKOOG, 1962] either hormone-free, or supplemented with benzyladenin (BAP), isopentonyladenin (2iP), kinetin (Kin), zeatin, adenin sulfate or gibberellic acid (GA_3). No effect on virus content was observed, except that GA_3 at 10 ppm reduced PVY concentration. After two cycles of culture with GA_3, potato explants appeared virus-free on the basis of the ELISA test (KUMMERT and VAN DEN HAUTE, unpublished results).

A number of chemicals, including purine and pyrimidine analogs, amino acids, and antibiotics, have been tested *in vitro* or *in planta* for potential use in virus eradication. Although some degree of inhibition was obtained, most compounds were either too phytotoxic, or showed insufficient activity [KARTHA, 1986].

Ribavirin (trade name Virazole®), a nucleoside analog, showed a broad spectrum of antiviral activity against animal or plant viruses. KUMMERT and TOUSSAINT [1984] tried to cure *Cymbidium* from Odontoglossum ringspot virus (ORSV), a virus which could not be eradicated by meristem tip culture, while thermotherapy was unavailable because of the heat sensitivity of *Cymbidium*. Incorporation of 25 ppm ribavirin in the culture medium progressively decreased the concentration of ORSV in infected protocorms. After the 4th subculture in a medium containing the inhibitor, some protocorms appeared free from virus when analysed by the ELISA test. After six subcultures, ORSV was no more detected in any protocorm by either ELISA test, inoculation to test plants, or electron microscopy.

A detailed understanding of the interactions between medium and plant cell physiology would be useful for the improved production of virus-free plants, on account of the interferences between host-cell metabolism and virus multiplication. For example, a high auxin content in the meristems might inhibit virus multiplication through interference with nucleic acid metabolism [HOLLINGS, 1965].

3. Tissue culture as a source of variability

Genetic variations within plants regenerated from protoplasts, cells, or callus cultures (i.e. somaclonal variation) may be a useful source of potentially valuable germplasm of asexually or sexually propagated crops [SCOWCROFT and LARKIN, 1982 ; SCOWCROFT *et al.*, 1983 ; SHEPARD, 1982]. Desirable traits, such as resistance to fungal or viral diseases, have been obtained by this method [EVANS and SHARP, 1986].

A limitation in using tissue culture technology for plant improvement is the lack of regeneration technique. This situation, however, can be overcome. VISEUR (unpublished results) developed a method using *Agrobacterium tumefaciens* as regeneration-promotor agent with pear. Some tumors formed upon *in vitro* inoculation with specific strains of *A. tumefaciens* regenerated plantlets. For example, strain Ti 3839 induced plantlet regeneration from cv. «Conférence», without going through a callus phase, and formed apparently untransformed plants ; it may be that in this system, transformed cells synthesize growth regulators which stimulate morphogenesis from the neighbouring untransformed cells.

With cvs. «Doyenné du Comice» and «Durondeau», DNA analysis showed integration of bacterial T-DNA into the genome of some regenerated plantlets (VISEUR and BALL, unpublished results).

4. Tissue culture as an *in vitro* screening system

Field-selection of vitroculture variants, under conditions of natural infection, does not require a detailed knowledge of host-parasite interactions.

Sugar-cane somaclones resistant to virus or fungal diseases were among the earliest examples of useful variation reported after field selection [HAMMERSCHLAG, 1984]. But space for field experiments is scarce, and valuable *in vitro* screening would be of great interest. Indeed, a major advantage of *in vitro* selection resides in the reduction of the number of plants to be field-tested. Another advantage is to screen new phenotypes from a large population of cells, under defined conditions, within a limited space. Thus, the impact of somaclonal variation on plant-breeding will depend on the efficiency of screening plants from tissue and cell cultures for new traits. A number of recent reviews cover the problem of *in vitro* selection of disease resistant genotypes [DAUB, 1986 ; HAMMERSCHLAG, 1984].

Schemes of *in vitro* selection, developed for microbial screening, have been tentatively applied to phytopathological problems.

The most common *in vitro* method to screen plant material for disease resistance rely on the use of toxins of the pathogen as selection agent. When such toxins are involved in disease development, screened material is expected to yield plants with a significant increase of disease resistance. This approach, however, is inadequate when toxins are not involved in the infection or pathogenesis processes. YODER [1980] outlined some of the criteria to be used in this respect.

GENGENBACH *et al.* [1977] used the toxin produced by *Helminthosporium maydis* (HmT) to select calli from immature maize embryos. Plants regenerated from resistant calli were resistant to HmT toxin in a leaf bioassay and were resistant to *H. maydis* infection.

Crude extracts of pathogen cultures have been commonly used as screening agent. BEHNKE [1979] investigated the *in vitro* selection of potato tissue for resistance to *Phytophthora infestans* by adding crude culture filtrates of the pathogen in a callus-induction medium. Upon inoculation, regenerated clones developed smaller local lesions than the original cultivar, but in field experiments under natural infection pressure [FOROUGHI-WEHR *et al.*, 1986], the variants were inferior to the variety for many characters.

LEPOIVRE *et al.* [1986] developed an adaptation of the double-layer technique, classically used in microbiology, to select sugar-beet calli for

their resistance to toxic compounds produced by *Cercospora beticola*. Fungal cultures were covered with medium containing a fungistatic concentration of mycostatin or Benlate. After diffusion of metabolites from the fungus layer, calli died rapidly when transplanted on top of the upper medium layer ; the toxins produced in the double layer technique were unrelated to cercosporin or *C. beticola* toxin (CBT) [LEPOIVRE and CARELS, 1986].

Similarly, wheat calli (cv. «Odéon») were grown on a double-layer medium containing the toxic compounds of *Septoria nodorum*. Regenerated plantlets were propagated by selfing, and tested for possible resistance to infection by the fungus. Differences in symptoms were noticed in the greenhouse, and field evaluation of the material is currently underway.

The direct use of a pathogen as selective agent *in vitro*, although feasible in some systems, is seldom useful because microorganisms overgrow plant tissue and culture medium. This technique, however, allowed SACRISTAN [1982] to select calli of *Brassica* species resistant to *Phoma lingam*.

The limited results obtained so far by using selective pressure of toxic compounds of *P. infestans* prompted TEGERA and MEULEMANS [1985] to develop an *in vitro* selection method, by spraying live inoculum of the fungus in dual cultures of micropropagated potato plantlets and the parasite. After adjustment for age of plantlets and environmental conditions (such as photoperiod), a good correlation was observed between *in vitro* resistance to *P. infestans* and known resistance of the cultivars in field tests.

When populations of potato plantlets (cv. Judith) regenerated from protoplasts internode calli, or leaflets, were sprayed under axenic conditions with a sporangia suspension of *P. infestans* (race 0), a few plantlets showed an increased *in vitro* resistance to the disease [MEULEMANS, 1984]. These plantlets were micropropagated and the resulting population was submitted to another *in vitro* inoculation. The resistant clones thus obtained were again micropropagated, and tested in the field. Under natural infection pressure of the late blight fungus, they appeared as susceptible to infection by *P. infestans* as the original cultivar. These results were corroborated by controlled inoculation of excised leaves from field-grown plants, with race 0 of *P. infestans* [MEULEMANS and FOUARGE, 1986].

Erwinia amylovora was used successfully as *in vitro* selection agent, for selecting pear plantlets for their resistance to fire blight. Culture media and inoculation parameters were adjusted as to permit *in vitro* screening for resistance ; this direct inoculation method gave a good correlation between *in vitro* resistance to *E. amylovora*, and known field resistance to fire blight [VISEUR and TAPIA y FIGUEROA, 1987]. Upon inoculation of

E. amylovora to a pear clone originating from root callus, symptoms on micropropagated plantlets were limited, and healthy axillary buds developed. This clone behaved similarly upon inoculation in the greenhouse and is currently evaluated under natural field conditions.

Whatever the screening agent, the trait selected *in vitro* is not always expressed in whole plants. Indeed, sugar-beet plantlets regenerated from calli grown in the presence of cercosporin (a toxin of *C. beticola*) were as susceptible to this toxin as plantlets regenerated from unselected calli (CARELS and LEPOIVRE, unpublished results). Conversely, induction of disease resistance may depend on organized tissue (such as floral structures, cuticle, etc.), and thus not be expressed at the cellular level.

5. Conclusions and perspectives

Plant tissue culture techniques are expected to be increasingly used to obtain pathogen-free plant clones, to enhance genetic variability, or to select for new traits. In this respect, it is hoped that embryoculture, *in vitro* fecondation, and haploid culture will accelerate classical breeding programs.

Selection for herbicide tolerance, tolerance to salt or other stresses, or amino acid content of the product, is rather simple to perform and to explain in molecular terms. The selection for resistance to a disease, however, reflects usually more complex situations, which require further investigations.

To be valid, an *in vitro* screening procedure should express resistance at the level of cultured tissue, the biosynthetic activities of *in vitro* cultures reflecting those of whole plants [HELGESON, 1983]. The use of selection agents other than the pathogen itself (or its metabolites) could be considered in this respect, but requires a sufficient understanding of host-parasite interactions. Molecular interactions of surface components are of uppermost importance in this field. Affinity chromatography allows to fractionate cell populations that do not otherwise significantly differ with respect to their physical properties (such as size, density or charge). Passage through a column containing beads with specific binding affinity for cell surface receptors, was used to separate mature T lymphocytes from peripheral blood [HERTZ *et al.*, 1985]. Surface components have also been traced by fluorochromes, to detect different reactions of isolated cells of resistant or susceptible peach cultivars to culture filtrates of *Xanthomonas campestris* pv. *pruni* [HAMMERSCHLAG, 1984].

Also, regulatory aspect of the *in vitro* biosynthesis of secondary metabolites involved in resistance (phenolic compounds, lignin) may be specifically associated with differentiation processes in the whole plant, or controlled by complex systems. For example, the extent of metabolite induction in cultured parsley cells (*Petroselinum crispum*) by an elicitor preparation from *Phytophthora megasperma* f. sp. *glycinea*, varied with the growth stage of the cell suspension [KOMBRINK and HALBROCK, 1985].

The composition of the culture medium is also of importance. Enzymes involved in lignin biosynthesis (phenylalanin ammonia-lyase, cinnamic acid 4-hydroxylase, *o*-methyltransferase, peroxidase), showed a maximum activity 2 days after transfer of tobacco cells into fresh liquid medium containing 2,4 D (2,4-dichlorophenoxyacetic acid) and BAP (6-benzylaminopurine) as phytohormones. In a cytokinin-free medium, cells did not form lignin and so could not be screened for their ability to respond to lignin elicitors [BOHM, 1980]. Chitinase, another enzyme related to pathogenesis in plants, is also inhibited by auxin and cytokinin in cultured tobacco tissues [SHINSKI *et al.*, 1987].

It is expected that further studies will clarify many problems related to the *in vitro* selection of traits for disease resistance in plants.

Résumé

La culture in vitro *pour la production de plantes saines et la sélection de génotypes résistants aux maladies*

Les cultures *in vitro* d'explants végétaux ont un impact important dans le domaine de la phytopathologie, notamment dans le cadre de la production de plants exempts d'agents pathogènes. Divers facteurs conditionnent la capacité d'obtention de plantes saines, notamment les conditions de culture et l'addition d'inhibiteurs de la réplication de l'agent pathogène. Les techniques de culture *in vitro* sont également utilisées dans le cadre de l'amélioration des végétaux, pour la production et la sélection de nouveaux génotypes résistants aux maladies. Dans ce domaine spécifique, l'exploitation des potentialités des cultures *in vitro* postule l'utilisation de techniques de sélection adéquates : application de composés toxiques produits par l'agent pathogène, co-culture de l'agent pathogène avec les tissus végétaux, ou sélection au champ des plantes régénérées.

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