The Production of Clean Plants in the Laboratory

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INTRODUCTION

Tissue-culture techniques complement conventional methods for the production, storage and propagation of disease-free plants. In the propagation of pathogen-free and contaminant-free plants, there are four important elements:

- The problem of detecting microorganisms in the stock plants.
- The requirement to develop an appropriate protocol to eliminate potentially harmful microorganisms.
- The problem of confirming the elimination of microorganisms.
- The requirement to maintain a good health status during multiplication and storage of the clean or elite stock.

It is increasingly the situation that elements of both the in vivo and in vitro approaches are combined in modern practice. The risks in both in vitro and in vivo techniques for the production of clean planting material will be considered here in the context of good working practice.

CONVENTIONAL STRATEGY FOR THE PRODUCTION OF CERTIFIED PLANTING MATERIAL

Potato and fruit crops, e.g. strawberry, provide models to illustrate good working practice in the production of clean planting material as both are subject to governmental certification schemes in many countries (for details of the potato scheme in The Netherlands see de Bokx and van der Went, 1987). In these crops individual plants are indexed for known crop pathogens (viruses and, where appropriate, viroids, bacterial, and bacteria-like pathogens) using proven methodology (for potato see de Bokx and van der Went (1987), Rowe, 1993; for strawberry see Mass, 1984). It is important, to emphasize that the phytopathology of these crops is relatively very well understood. Historically, test plants were used to index for pathogenic viruses but now ELISA is routinely used, especially for mass screening during field multiplication (Hill and Jackson, 1984; Fox, 1993).

Symptoms are a valuable indicator of the health status of a crop and the selection of symptomless individuals, i.e. "disease escapes", has long been the foundation of clean stock production schemes. Where disease escapes have not been available, thermotherapy has been widely used and is still used with a high level of success (Hollings and Stone, 1968). Where symptomless escapes, or symptomless individuals have been obtained after thermotherapy, these are tested as the parental material for pathogens. It is recognized, in woody species for example, that virus concentration may be low in heat-treated material and, consequently, early virus testing made give false negative results (Leonhardt et al., 1997). From the latter, it can be appreciated that confirmation of freedom from known pathogens can depend on the developmental stage of the host plant.

The multiplication of disease-free individuals by conventional vegetative propagation is slow and there is always the risk of pathogen re-entry into the crop. The risk

of re-infection is less with a protected crop such as strawberry, multiplied in vector-proof greenhouses, than in potato, a field crop. The elite stock of the latter is either multiplied in vector-free areas or vector populations are carefully monitored to minimise the risk of infection (de Bokx and van der Went, 1987). In potato certification schemes, contamination of the elite stock during successive years of field multiplication is recognised. The crop is monitored both in the field and laboratory at the end of each season and graded according to the levels of specific disease present. A mandatory down-grading of the health status occurs in each successive field generation and, depending on the extent of re-contamination, the seed may be down-graded by more than one grade.

PRODUCTION AND MULTIPLICATION OF CLEAN PLANTS IN THE LABORATORY

Meristem culture is an effective way of eliminating most microbial contaminants, whether pathogenic or not, from plant material (George, 1993). A caution is that the tissue excised from the tip of the plant should not contain any of the vascular system. Meristem culture, however, while eliminating xylem-restricted and phloem-restricted organisms (bacteria and the larger phloem-restricted viruses), cannot be guaranteed to eliminate smaller viruses and viroids that may extend into the apical region (Matthews, 1991). In the latter case, escapes can be sought or thermotherapy applied to the donor plant in vivo (Walkey, 1985), prior to further attempts at meristem culture.

Alternatively, thermotherapy may be applied in vitro, or the plant tissue may be cultured in vitro in the presence of antimicrobial compounds (Cassells, 1983; Barrett and Cassells, 1994). Thermotherapy in vitro operates on the same principle as in vivo, namely, on culturing the tissue at temperatures that are nonpermissive for virus replication and that may enhance the breakdown on pre-formed virus particles. Under these conditions virus may be eliminated or new tissue growth may be virus-free.

The strategy to eliminate bacteria from tissue cultures is usually based on incorporation of antibiotics into the medium. These may be bacteriostatic, rather than bactericidal and so new tissue is excised and subcultured. Antiviral chemotherapy of whole plants is very difficult, in principle, due to difficulties in maintaining an inhibitory concentration of the few, mainly virus-static, chemicals that have any efficacy (Cassells, 1983). The problem, as is the case with antibiotics, is reduced by their use in in vitro cultures. The most widely used plant antiviral chemical has been Ribavirin, which appears to have broad spectrum activity (Cassells, 1983; 1997a). It has been used alone or in combination with thermotherapy (Cohen, 1986).

Regardless of the method used to eliminate microbial contaminants, it remains to be confirmed that de-contamination has been achieved. While viruses have been detected in in vitro cultures, tissue-printing has shown that distribution may be uneven, leading to problems in confirming elimination at the in vitro stage without destructive sampling (Knapp et al., 1995). Plant hormones are known to influence virus replication in plants, and the hormones in in vitro culture, may suppress plant virus replication, leading to false negative results (Cassells, 1983).

A positive aspect of in vitro methods in clean plant production is that pathogen-free material is at low risk of re-infection with pathogens and thus can be stored and multiplied safely. Contamination during in vitro multiplication by cultivable envi-

ronmental microorganisms, such as fungi (including yeasts) and bacteria, is a risk but the problems are well understood and good laboratory management can minimise losses (Leifert and Waites, 1994).

INTEGRATED STRATEGIES FOR THE PRODUCTION OF CERTIFIED PLANTS

Potato and fruit crops provide good models for the integration of in vivo and in vitro methods for the production of clean planting material.

In potato and strawberry, micropropagation plays an important role in the multiplication of disease-free plants even though: (a) the starting material is certified in vivo as virus-free and (b) the progeny plants are further multiplied in the field; the certification is based on field and post harvest inspection of the crops.

In certified potato multiplication, multiplication in vitro reduces the time taken to introduce new stock by four seasons and, as a consequence, the certified seed material is likely to be less contaminated during field multiplication than that obtained from conventional (not in vitro) seed production. Similarly, micropropagation is used to produce strawberry microplants for the production of certified runners.

HEALTH STATUS OF MICROPROPAGATED PLANTS

The presence or absence of disease symptoms in the parental plant material may be the only indication to the propagator that the starting material is diseased. In many cases, symptom expression may be seasonal and latent contamination a consequence (Matthews, 1991). Even if symptoms are present, the causal agent may not be economic to detect and/or identify, especially in lesser or exotic crops where the knowledge base is limited or fragmented and diagnostics are not commercially available. Under these circumstances, micropropagators depend heavily on fortuitous elimination of pathogens in establishing their cultures. Here meristem culture, with the proviso that the minimum size explant is excised, offers a broad spectrum solution. The risk, especially where explants other than the apical tips are used, is that pathogens will be transmitted vertically and that clonally infected cultures will be multiplied. Symptom suppression in tissue cultures is common in the cases of fastidious organisms and viruses and viroids.

A further complication is the lack of broad spectrum diagnostics although this problem is being recognised and solutions developed (Bariana et al., 1994). Maintenance of test plants is expensive and modern diagnosis may be too strain-specific. Furthermore, there is uncertainty regarding both the concentration and distribution of viruses in in vitro tissue, which may result in false negative results (Knapp et al., 1995).

The strategy followed in potato and strawberry, of growing on the crop in disease-monitored fields or greenhouses is not practical for the producer who sells in vitro cultures or established microplants and where there is no legal requirement for certification. However, it can be followed by propagators who grow on microplants as mother plants for the production of cuttings (Jones, 1986).

CONCLUSIONS

A great deal is known about diseases of cultivated plants and for the major crops this information has been published in compendia (e.g., the series published by the American Phytopathological Society). In the case of minor crops, the development of computer databases makes this information readily available to the micropropagator via the Internet. In spite of this potential for greater information about crop diseases, problems of latency and the lack of availability or high cost of diagnostics, mean that in most commercial micropropagtion laboratories working practice is based on the establishment and maintenance of cultures free of cultivable bacteria. Where specified by legislation or the client, tests, usually ELISA-based, for specific pathogens of the crop may also be carried out. A scheme outlining the categories of health status of microplants is shown in Fig. 1.

The production of higher health status material via micropropagation can be achieved in the same ways as in potatoes, if the starting material is certified disease-free based on conventional procedures and if the health status of the progeny is confirmed by inspection and testing of the field progeny.

In the longer term, the prospect is that the development of highly sensitive, broad-spectrum nucleic-acid-based diagnostics will enable micropropagators to produce high health status material with confidence. In the interim, it may be in the interest of micropropagators and growers to develop nuclear stock associations through strategic links with governmental institutions and to combine the best practice elements of the potato certification scheme in the production of high health status plants.

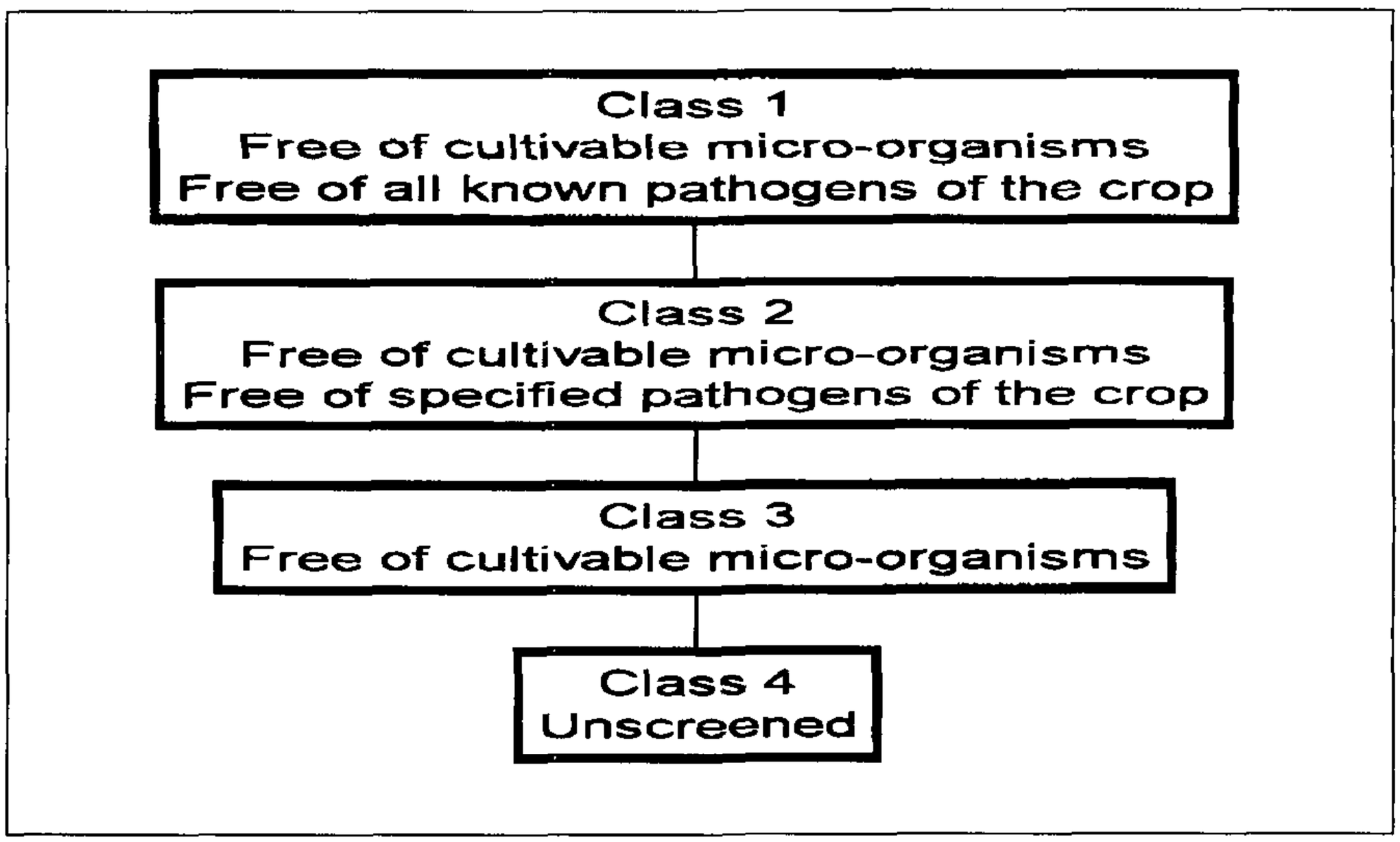

Figure 1. Catagories to describe the health status of micropropagated plants.

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