DISEASE-FREE PLANTS VIA TISSUE CULTURE PROPAGATION

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The potentials of tissue culturing for plant propagation and plant breeding have been described by numerous authors and reviewed by Murashige (30) and Nickell (31).. Unfortunately, the popularity and excitement of this field have caused more words than action and the apparent cookbook approach to tissue culture propagation has given the horticulturist the impression it is rather simple in execution and success. To a degree the execution is simple, but a successful propagation system is not. Tissue culturing is not doing all the things we know it is capable of doing, i.e. mass propagation, storage of germplasm, fusion of protoplasts, production of disease-free plants, etc., because the practical details have not yet been worked out.

It appears to us the field of horticulture will be one of the major benefactors of tissue culturing. Many of our commercially grown plants are vegetatively propagated and tissue culturing shows tremendous potential for propagation. Tissue culturing shows a great potential for use as a plant breeding tool. We in horticulture are interested in maximizing production, which involves optimal environmental conditions, water, nutrition, plant selection, as well as disease and insect freedom. The horticultural tissue culturist must, therefore, be concerned with plant selection and freedom from diseases and insects. The genetic problems have already been discussed in this symposium. The purpose of this paper is to review the responsibilities of the tissue culturist and the role and significance of disease in such programs.

Unforunately, there is an apparent conception among horticulturists that tissue culturing and disease-freedom are synonymous. The same misconception was true of the so-called meristem cultured plants. One of the objectives of this paper will be to show this is not true.

A classic example of this misconception can be seen in the orchid industry where "Mericloning," which resulted from the pioneering work of Morel (27,28), made a tremendous impact. The standard method of propagation involved hybridization, seedling production and a very limited division of desirable plants. Tissue culture propagation has meant hundreds of plants can be rapidly derived from a desirable plant. This has allowed detailed selection of plants for flower color, vigor, size, time of flowering, keeping quality, etc. and, once a desirable plant has been selected, a very rapid multiplication. It is fair to say this method of propagation revolutionized the industry. Before "mericloning," orchid viruses were a minor problem. However, orchid viruses are now common, wide-spread, and costly. "Mericloning" of orchids serves as an excellent system for virus transmission (24,25). We are not faulting a person, but rather the system. Growers and others who propagate via the "mericlone" system were not cautioned enough about the virus transmission hazards. In fact the system was not complete for it did not involve pathological tests to reduce the chance of a virus and/or other pathogens from contaminating all the plant material being propagated. If we refer to Morel's work (27) we find he used very small (0.1 mm) tips. In commercial "mericloning," a tip that size was found difficult to keep alive and/or slow to grow; therefore, larger tips are used. As we will show later, the larger the tip the greater the percentage of tissue infected with virus.

Much of our experience has been with tissue culture propagation of the chrysanthemum (4,11,12,23). We found tissue cultured plants infected with chrysanthemum stunt (ChSV), aspermy (ChAV), mosaic (ChMV), and chlorotic mottle (ChCMV) through the leafy callus stage and back to plantlets; 100% of the plantlets still contained ChSV, ChMV and ChCMV. Approximately 33% of the plantlets contained ChAV as determined by the inoculations made to *Nicotiana tabacum* used as a test plant. The explants were shoot tips 0.3 to 0.6 mm in height, which is about as small as practical. Survival of tips smaller than 0.3 mm is low, whereas it is 90% or better in the size we used. It appeared from this study that host plant callus tissue would be a good place to store chrysanthemum viruses rather than in greenhouse grown stock host plants as is necessary today.

It would be unfair to imply that it is impossible to obtain virus free chrysanthemum plants. Workers such as Quak in Holland (12,20,33), Hollings and Stone in England (15,16,17,18) and Brierley, Dimock and Horst in the U.S. (7,10,19) have identified and devised systems to obtain virus free plants.

Hollings and Stone (17) attempted to obtain chrysanthemum plants free of ChSV by combining shoot tip culture with heat treatment of the stock plants. They grew the plants at 35°C for a period up to 37 weeks and at various intervals removed a total of 337 shoot tips. Just 2 stunt-free plants were obtained; these 2 plants were called "escapes." This is the approach and range of percentages that commercial chrysanthemum propagators use to obtain tissue free of some viral type infectious agents. Heat treatment of the stock will help reduce ChMV and ChAV but not ChSV or ChCMV. It appears that ChSV and ChCMV, which are known to be viroids (9,10,19), move readily into meristematic tissue and are heat stable.

Carnation viruses are somewhat easier to cope with, as a combination of heat treatment and small tips will produce some plants free of the described viruses affecting carnation (19). Stone (36) in 1968 reported obtaining plants free of carnation mottle (CarMV), carnation ring spot (CarRSV), carnation vein mottle (CarVMV), and carnation latent virus (CarLV). The percentage infected varied from 10 to 88%. In other words, some of the plantlets produced were not virus-free. In no case was she 100% effective in producing virus-free plants from infected plants.

Hollings and Stone (15) studied shoot tip size and its relationship to CarMV freedom. They excised 5 sizes of shoot tips, 0.1, 0.25, 0.50, 0.75, and 1.0 mm. The percentage of shoot tips infected with virus were 33, 60, 87, 89, and 100% respecitively. Clearly, the smaller the shoot tip the less the chance of virus infection. The inability to free completely the meristems from virus should not be a surprise, for Sheffield (34) in 1942 reported tobacco mosaic virus (TMV) in the meristems of tomato. There are apparently no tissues in an infected plant that can be considered absolutely virus-free.

The same relationships of heat therapy, meristem and viruses can be found with potato (22,26,35), strawberry (1), sweet potato (2), citrus (29), freesias (6), iris (3), rhubarb (38), gooseberry (21), hops (37), gladiolus (8), and geranium (32). It is possible to produce some virus-free plants, but indexing for viruses *must* be done to assure virus freedom.

Viruses are the major concern, but bacteria can also present a difficult detection problem. Hannings (14) reported systemic bacterial contamination in begonia. Apparently some species of bacteria could pass through the tissue culture propagation without detection.

The solution to the problem is to include an indexing system as part of the tissue culture propagation system. We have shown it is possible to produce 10^9 chrysanthemum plantlets in 1 year from 1 shoot tip. If this initial tip contained virus, the problem would be serious.

Virus indexing for the chrysanthemum tissue culture system includes removal of plugs of tissue from the callus culture with a sterile surgical cannula (5). One tissue plug is ground and rubbed on tobacco leaves (*N. tabacum*) to test for ChAV and other callus plugs are placed in a hole made in the stem of an indicator chrysanthemum cultivar (like a graft) to test for ChSV, ChMV and ChCMV. In a few weeks the test plant will show virus symptoms if the callus was infected; if not, the flask and callus can be certified free of that virus and the propagation system continued. Another virus inspection should be made on the first group of plants grown from that culture.

We suggest the following steps be incorporated in a tissue culture plant propagation system to insure disease freedom and horticultural trueness.

1. *Know the plant material.* A background on the plant species and cultivars to be used will give information on a) how the plant is being propagated and the ease of propagation and b) what systemic pathogens (fungi, bacteria and viruses) have been reported for these plants. These pathogens may then be tested for in an indexing system. For example, with the chrysanthemum we should look for the fungus *Verticillium*, the bacterium *Erwinia chrysanthemi* and the viruses ChSV, ChAV, ChMV, and ChCMV.
2. *Determine the type of explant.* This would include the tissue that gave rise to the most rapid growth and the greatest potential for pathogen freedom. In our example of the chrysanthemum, the shoot tip would be best. This tissue starts growth rapidly in vitro and generally is fungus and bacteria-free. It

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appears these pathogens are present in the vascular tissues and the 0.3-0.6 mm shoot tip is above organized vascular tissues.

3. *Determine aseptic technique.* This includes the steps necessary to have tissue without surface contamination. It may be necessary to surface sterilize the tissue and it may be necessary to grow the stock plant material in a protected area. It does include techniques that avoid contamination in handling. In our example of the chrysanthemum, we find the stock plants must be grown in a greenhouse without overhead watering to avoid surface bacterial contamination. We do not have to surface sterilize the tips as the surrounding leaves keep the tip sterile.
4. *Determine the optimum media, hormones and environmental conditions.* This includes a determination of the type of tissue culture propagation desired. In our example of the chrysanthemum we have described 2 pathways - multiple shoots or leafy callus (11,12). Each has its own special requirements.
5. *Determine disease-freedom.* This should be done at an early stage. The tissue should be sampled and tested for the known possible pathogens. In our example of the chrysanthemum, we found fungi and bacteria grow quite rapidly on our media and hormone combinations. Thus, fungal or bacterial contamination could be readily seen and discarded. To determine virus-freedom we indexed using the cannula implant system (5). If the sample index is negative for the pathogens indexed in our tests the propagation system is continued.
6. *Determine horticulture trueness and recheck for disease.* A few plantlets should be removed early in the propagation system to be grown to the flowering stage. Since tissue culture propagation systems generate such large numbers from one small piece of tissue a check should be made to insure against "sporting." At the same time another index can be made to recheck for viruses.

The above tissue culture propagation system will insure the plantlets are free of specified pathogens and true to type. It is the only way. We suspect the loose use of the terms tissue cultured, meristemmed, mericloned, callused, etc. has lead to misuse, misunderstanding, and misinterpretation by both producers and consumers. A plant can be tissue cultured and still contain disease causing agents. The plant is only disease-free when it has been pathologically checked and then it is only known to be free of the disease for which it was checked. Once the plant is exposed to unprotected conditions it can become infected with fungi, bacteria or viruses. It is important to recognize the plants are not immune. We feel that investigators should come to some agreement about terminology in both the tissue culture and the pathological testing systems. If not, federal and/or state regulations may be instituted which require these procedures be used in tissue culture propagation.

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