PROPAGATION OF VIRUS-SYMPTOMLESS MATERIAL

MICHAEL G. MULLINS

Professor of Horticulture, University of Sydney
New South Wales

The subject I have today is one in which I have considerable interest, but must admit, is one in which I have no great personal experience. I would not like it to be thought that I was an authority in the field of plant virology at the University of Sydney. It belongs more properly to my collegue, Professor Brian Deveral, Professor of Plant Pathology, a micrologist.

I hope to show in this lecture that the fields of virology and physiology and biochemistry have many affinities and it will be certain that new research programmes will be forthcoming which will be of some interest, I hope, to the nursery industry and plant propagators in general. So if we can just turn to the subject at hand, and that is virus-free plant propagation material. Many people are not too keen on the use of the term "virus-free material." That is because it implies that the plant is free of all known viruses and that it has been tested and shown to be free. The "in" term is *virus symptom-free material*, but we needn't be too purist. Virus-free is well entrenched and we know what it means and I use it in the sense that, if something is virus-free it is free from debilitating virus diseases.

The first point to bear in mind about virus diseases is, there are no practical cures for these conditions; once plants are infected and are set out in the field or the nursery, that's it. They can't be cured; the control of virus diseases is possible primarily through the maintenance of clean stock programmes. As you know, a multitude of virus diseases are of importance in horticulture and these are of particular significance in long-lived woody perennial crops. It is in the woody perennial crops, the ones that take a long time to come into bearing, ones which involve considerable capital investment to get them started, that the effects of the virus diseases are particularly serious. There is a long waiting period before you can get an economic return and the investment is a long term one and the grower here is particularly dependent on the integrity and competence of nurserymen for his livelihood and also for his long-term profitability. With all crops, the strategy of disease control through clean stock or virus-free material is the same.

The strategy of the clean stock programme works in the following way:

(a) First of all we hope to dilute the infection in the population of plants by the continuous introduction of virus-free, or virus-symptomless, material.

(b) Then we wish to replace or destroy the infected material and establish vigorous clean stock.
(c) The third part is the isolation of the foundation of the mother stock to produce propagating material for further populations.

That's the strategy, and it depends on, firstly, the recognition of the disease and then reliable indexing methods, then therapy or treatment to establish virus-free, or virus-symptomless clones, followed by measures to maintain the health of the foundation stocks once we have cleaned them.

Recognition: In developing clean stock programmes or recognising virus diseases in the field in general is not always particularly easy. Many diseases are extremely obvious, like fan leaf, or leaf roll in the grapevine; anyone who has seen them once does not forget them. Other virus diseases are much less easy to identify from symptoms, e.g. the grapevine virus, yellow speckle. This is wide spread in commercial grape cultivation and it is rather difficult to recognise unless you know what you are looking for. In addition, there are variegations due to gymerism. These variegations look like virus diseases and they are easy to confuse with it, so with recognition, we need reliable indexing methods; that is, tests that can be applied to the material which will tell us whether or not it is infected.

Of the six methods that I will describe here, the first one is by far the most important in practical terms; that is *infectivity tests*, where parts of an infected plant or a plant, which is suspected to be infected, is grafted onto a host — and if the virus is in the scion, then it will produce symptoms in the host.

Similarly, *Sap-rubbing inoculation* is another commonly and widely used method of indexing, quite often using plants in differing species, differing genera, differing families. Plants of the family Canopodeacea can be used as indicator plants because these produce leaf symptoms which are recognisable.

The serological tests involves the antigen-antibody reaction where virus particles are injected into rabbits and then tests are done of the serum afterwards.

Electronmicroscopy is using the electron microscope to examine, not usually parts of tissues, but plant extracts, to see if the virus particles can be seen in the extract. This, of course, requires a high concentration of the virus to be visible.

Density gradient centrifugation. This is a technique where the plant is homogenised, an extract is made of it and this extract is placed in a centrifuge tube with a solution of sucrose or dextran, or some other compound, and it is then subjected to very very high speed centrifugation of the various subcellular particles which have been subtracted from the plant. They sediment out in

differing positions in the centrifuge tube; it is just a way of concentrating the virus so that one can separate the virus from the other particles which is found in the plant tissues.

Histological and systological tests. This is cutting plant sections, treating them with various chemical compounds to determine if differences in reactions between infected and clean plants can be seen,. Chemical tests, either to extracts or to the plants themselves, were at one time thought to hold a lot of promise, but really they do not seem to have lived up to expectations.

So we can recognise the presence of a virus condition by visual symptoms or indexing techniques and, having discovered that the material is infected, we then want to see what we can do about it. We now proceed to consider some of the techniques which can be used for eliminating debilitating viruses from horticultural crops.

1. *Natural escape.* This works because some viruses are not fully systemic; that is, their occurrence within any one plant is rather random and if bits and pieces are taken from an intact plant, *some of them will be infected and some will not.* An example is in apple-mosaic. Buds from a mosaic infected tree when grafted onto healthy trees will give a proportion of infected plants and a proportion of clean plants. Another example is carnation-mosaic in gladioli. Some cormlets from the mother corm escape infection so this is random natural escape.
2. *Shoot tips.* This is a zone of the plant which can sometimes escape virus infection. In rapidly growing plants the shoot tips may be free from virus particles which are systemic throughout other parts of the plant; e.g. in dahlia, which can be freed from tomato spotted-wilt virus by just growing rapidly and taking a shoot tip cutting; similarly, pelargonium can be freed of leaf-curl virus in this way.
3. *Apomixis* is another very useful way of getting virus-symptomless material, related strictly to the sexual behaviour of plants. In plants the embryo sac is surrounded by a tissue called the nucellus and when the pollen tube grows into the embryo sac during the fertilization process, resulting in the embryo in the seed, this signals certain cell divisions to take place in the nucellus — and these cell divisions lead to the formation of other embryos. This is particularly important in citrus where we get nucellar embryos; these are usually virus free and this is thought to be due to the fact that there are no direct vascular connections between the nucellus and the other parts of the plant.

Apomixis, or nucellar embryony is of great commercial importance in citrus and mango, but no great use has been

made of this phenomenon in other species. Some of the research that we are doing is looking at plants which have a well developed nucellus (a good example of this is the grape) to see if we can, by using tissue culture techniques, artificially induce the formation of embryos in nucellar tissues. This work is still in its early stages.

4. *Chemotherapy* for cleaning plants. There are very few instances indeed where this has been successful. What happens is by applying chemicals to plants in order to cure them of a virus, the symptoms are suppressed for a while. Compounds like cytovirin 2 and thiouracil work by becoming incorporated into the nucleic acid of the virus. The problem is that these compounds are also incorporated into the nucleic acid, the DNA, of the plant. These techniques, which showed fairly early promise, are not particularly practical.
5. *Heat treatment* is the most practical way of all for cleaning plant stock of virus conditions; this is very successful and has been widely used for many years. Hot water treatment of sugar cane sets is an example of this. The techniques of heat treatment have been refined and expanded considerably in recent years. There are two main methods — use of hot water, and hot air.

   Hot water treatments are usually used on dormant material, sugar cane sets, budwood, dormant trees, potato tubers, and the like.

The effects of subjecting plant tissues to high temperatures when they are virus infected is supposed to work this way: At high temperature, there is a destruction of the virus which takes place at a greater rate than its replication. Storing of these tissues at 35° to 45°C is done for periods of a few minutes up to a few hours, or a few months.

I suppose, in the case of *hot air treatment,* it is sufficient to clean the viruses from at least a large proportion of them by bringing about destruction of the virus particles.

The Hot Box Methods using growing material. Plants are grown at a high temperature, around 35° to 45°C, depending on the cultivar, and in many cases viruses can be eliminated from the very top parts of these plants. When cuttings are made from these tips of heat-treated plants, the progeny are more often than not virus symptomless.

It is thought to work in this way — in the apical and subapical meristematic dome, the vascular elements are not well developed and, this being so, virus particles which are allegedly translocated mainly in the woody tissues, are unable to get into

this area. When this tip is chopped off, the plant which will grow from it is virus-symptomless.

Heat treatment is more often than not these days combined with organ culture — the techniques of sterile culture of plant tissues. In fact, one can produce virus-symptomless plants by just chopping off the shoot tip and growing it in tissue culture without previously subjecting the plant to heat treatment but in many species, particularly in carnation, it has been shown that a combination of heat treatment and tissue culture is extremely advantageous in cleaning up infected tissue; it is thought that the mechanism of this treatment is that under the conditions of tissue culture or heat treatment the rate of replication of the virus is less than its rate of synthesis.

We should, perhaps, tell you something about the techniques of plant tissue culture. *Apical meristems* is a term often used for the micro cuttings. The very tip, the very growing point of a plant, is a structure called the apical dome. It is here that the cell divisions take place that lead to the formation of the tissues of the stem. Apices on either side are the leaf primordia in their very early stages; additional leaf primordia occur further down the stem. They occur in a sort of spiral way in the same way that leaves do since they will ultimately produce the leaves. Culture of apices involves dissection under a microscope in which a piece of tissue, usually 10 to 20 microns in size — extremely small — is chopped out of the apex of a plant. This is maintained by aseptic culture methods, including treatments with plant hormones such as auxins. Usually included in the mixture is coconut milk, which is a source of one of the families of plant hormones involved in regeneration. By the use of this mixture of coconut milk and auxin it is possible to generate a new plant from these tissues which will be virus symptomless.

Apical meristem culture is a term which can be used to describe growing the very apex plus a few leaf primordia. This involves chopping off only a few millimeters below the top. *Shoot tip* or micro cuttings involves a bigger section again.

We know that heat treatment works and Dr. Posnett, a well known virologist at East Malling Research Station, England, who was out here some time ago — and I know many of you met him — has calculated that about half of the virus diseases affecting horticultural plants could be eliminated by heat treatment. This means that if we could develop plant tissue culture techniques to a fair level of sophistication, we could do a lot more in eliminating virus diseases.

One of the problems, however, is that there are relatively few species which can be handled in this manner. Many of the highly desirable species, like apples, pears, plums, avocados and such

nature cannot be regenerated from apices. We are taking particular interest in looking at biochemical differences between tissues which are easily regenerated in aseptic culture and those which are not. Some of the main problems which we encounter in growing shoot tips and apices in tissue culture is that we may remove virus from them, giving foundation material for a propagation programme, but at the same time many other problems arise; we may get cytological abnormalities, changes in chromosome number, giving polyploids; many of you are familiar with this.

We have found in our asparagus programme that we get a very high incidence of fasciation. There are many techniques available to clean up material for foundation mother stock but the techniques I have been describing are only half the story. Once we have cleaned the material or taken it through these various steps, then we have to go back and re-index the material to make sure the treatments have worked. This is a particularly long job in plants such as the grapevine.

I am not going to say too much about the organisational procedural or legislative measures that are obvious companions with any technical work on the cleaning of propagation material, i.e. the measures to prevent the dissemination of "dirty" materials, and such things as the development of repositories for virus-free mother stock. I am aware that much is being done in this country along these lines with pome and stone fruits, with grapevines and strawberries.

I did say that this sphere is not my own so I am not too certain about what is being done with the ornamental species. I am aware of work going on in Britain — the "Emlar" scheme; that is, a joint venture between the East Malling Research Station and the Long Ashton Research for the production of high quality material. This has been used for pome fruits and stone fruits for some time. Now the latest annual report from Long Ashton informs us that they are using these techniques to clean up ornamental *Prunus* species.

From the brief account I have given, you can see that the production of virus-free material is in some ways a big enterprise. It has a high capital cost, a high running cost, a high labour requirement and it requires well-qualified labour. I think you would agree that it would not be a job for the general nurseryman. It would be a specialist operation undertaken by the State Department of Agriculture, perhaps, or by co-operative ventures such as nucleus stock associations financed and controlled by participant members. Anyone who has suffered the economic consequences of infected material knows that the cleaning up of this material is well worth while.

[1]What we have done is to take ovules from one of the immature seeds of the grapevine and have cleared it so that it is transparent, then when we look at it through a microscope we can get some idea of the internal structure. We see the integments, the outer parts of the immature ovule, and the hole where the pollen tube enters for fertilization; the embryo sac is here but cannot be seen. The nucellus can also be seen.

We can induce the nucellar tissue to start growing. That happens normally in citrus and mango after fertilization, but does occur in the grape unless we remove the ovules under sterile conditions at a certain stage of growth, then grow them under tissue culture, giving treatments such as coconut milk or some other constituants.

What we have managed to do is to produce an embryo; this is the apical dome that I told you about and it will, in fact, develop cotyledons. It is an embryo of the grape cultivar *Cabernet Savignon*. That is quite an interesting point.

One of the aspects of our undergraduate programme is that every fourth-year student becomes involved with his own research project; one aspect of the work that interested us was to take shoot tips — not apices but shoot tips — of carnations and grow them in tissue culture and see if from one shoot tip we could produce large numbers of plants.

Normally when shoot tips are grown in tissue culture one plant grows from the apex but, as you know, in the axils of leaves there are buds; well, in the axils of leaf primordia there are small pockets of tissue designed to grow into buds and my student, Mr. Batten, by taking these tips was able to get several carnation plants with one shoot tip.

One of the major programmes we have is on the rapid multiplication of asparagus by tissue culture. Asparagus in commerce is grown from seed and since there are no sure cultivars it is highly variable; it would be a very good scheme if we could produce a clone of asparagus, especially from a male plant of good commercial characteristics. It is possible to grow plants from single cells and this is what we are doing in this project.

Cells have been planted out in Petri dishes, and the new asparagus plants will grow from them. Plant tissues have the capability to produce plants from cells but you can see that in a slice of tissue one millimetre thick with several hundred thousand cells there is a capacity to produce a vast number of plants and techniques such as this, if we can overcome the problems of polyploiding, this will make the propagation of virus-free or virus-symptomless material a much less expensive and a much

more practical proposition mainly because there are several million cells in a plant.

We start with a male plant grown from a single cell and we are able to produce these by the hundreds of thousands. The main technical difficulties are not in the plant physiology, but the horticulture and nursery practice because the real skill comes in hardening them for transfer to the glasshouse.

In finalising, we go back to the point I made of the close relationship between work in plant physiology and work in virology. This gives the procedure we use for growing asparagus. We have a couple of possibilities here; we can produce a callus, we can then disperse it into free cells; the free cells will then produce embryos and then form plants. Take them in solid medium into the glasshouse or nursery field. Our first clone of asparagus is being grown at our new field station at Camden. On the other hand we can treat the callus rather like a cutting, i.e. induce roots to form and then induce buds to form and so forth but the yield of plants in this way is not so great.

PROPAGATION OF CONTAINER-GROWN ROSES

ROY RUMSEY

Rumsey's Rose Nursery,

Dural, New South Wales

Off and on, over the years, we had grown miniature roses from cuttings, but it was not until about 1968 that we stopped budding miniature roses, with the exception of "Standards", and grew them entirely from cuttings. It is quite satisfactory to take cuttings from either softwood or hardwood material and, according to the time of the year, a salable plant can be produced in quite a short time. We root the cuttings under constant daytime mist, harden them off outside, then pot them up as needed.

None of this is really news, as everyone knows by now, that miniature roses from rooted cuttings are, by far, better than budded plants; sales also have increased greatly since they were available for the public propagated in this manner.

However, this started us wondering if it would be possible to produce the larger type roses as budded plants, completely container-grown, with the rootstock placed in the soil mix and, at no time would the plants be field-grown. Of course, we had already seen the advantages of container-grown nursery stock amply demonstrated by growers of other plants, using steam-treated soil, etc. but, in spite of our queries all over the globe, we could not find anyone who had actually done this on a commer-