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Heat treatment and meristem culture for the production of virus-free plant material[1]

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Abstract

To obtain virus-free material from plant species or cultivars which are vegetatively propagated and totally infected with virus, three methods were developed. Heat treatment (with hot water or hot air) resulting in an inactivation or an inhibition of the multiplication of the virus has been successful in several cases (a.o. sugar-cane, fruit trees). However, there are viruses which are not inactivated by heat; to obtain virus-free material from plants infected with such viruses the small tip meristems are isolated and cultivated on nutrient media because in several cases the tip meristem of systemically infected plants appeared to be virus-free. The possibilities of this method and the difficulties in composing suitable nutrient media for further shoot development and rooting are treated in detail. Because the tip meristem is very small (0.1 mm) the percentage of growing meristems is not always satisfactory. To increase this percentage a combination of heat treatment and meristem culture has been applied. This gives the possibility of using somewhat larger stem tips (1 mm) which grow better on the nutrient media and may still be virus-free. It always remains necessary to test the treated material for presence of virus. A list is given of crop plants from which virus-free stocks are obtained in The Netherlands and other countries.

Introduction

In contrast to insects, mites, nematodes, fungi and bacteria, plant viruses cannot be controlled by applying chemicals to the crop as virus multiplication is too closely linked with normal metabolic processes in plants. Therefore all chemical virus inhibitors appear to be phytotoxic.
By killing the vectors (insects, nematodes, mites) with chemicals the spread of some virus diseases can be prevented. However, there are viruses which are transmitted mechanically or by insects immediately after the insect starts feeding and the spread of such viruses cannot be prevented by chemicals.

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Fortunately most viruses do not reach the seed of infected plants, so these seeds will in general develop into healthy plants. But this does not help us when dealing with valuable heterozygous species and varieties which have to be propagated vegetatively such as potato, sugar-cane, raspberry, strawberry, fruit trees and many ornamental plants, including flower bulbs. Here the progeny remains infected once systemic infection has taken place. This is especially so with latent viruses inducing no or only very weak symptoms.
Methods have been developed to cure vegetatively propagated varieties which in the course of the years became totally infected with one or more viruses. Such plants can be cured or virus-free progeny obtained by either a heat treatment, by meristem culture or by a combination of both.
The success of these methods depends on the virus to be eliminated and on the characteristics of the plant material to be treated. Results have shown that heat treatment is usually effective only against isometric ("spherical") viruses and not against rod-shaped viruses. In fact chlorotic leafspot virus, which can readily be eliminated by heat treatment, is the first instance in which a non-isometric virus has been removed (Campbell, 1961, Lister et al., 1965).
According to Kassanis and Posnette (1961) about half the viruses affecting vegetatively propagated plants can be eliminated by heat treatment. If this method is not successful, the more laborious method of apical meristem culture may be used. In some cases a combination of both methods gives the best results.

Heat treatment

Heat treatment of diseased plant material was already applied to sugar-cane before the cause of the disease involved was known. As early as 1889 Kobus, working in Java, observed that sugar-cane cuttings suffering from sereh disease (now known to be caused by a virus) grew better after having been kept in water of 50–52 °C for 30 minutes. Later other sugar-cane virus diseases, like ratoon stunt, could also be controlled in this way (2 hours at 50 °C, Hughes and Steindl, 1955). As a result in many parts of the world several thousands of tons of cuttings are treated annually in large water baths before they are planted (Kassanis, 1965). Thung (1952) got good results with Rubus stunt virus in raspberries when using the hot-water method at 45 and 50 °C, and Van der Meer (1967) with witches' broom in *Opuntia exaltata*. Kunkel found already in 1936 that peach trees could be cured from yellows virus by a hot-water treatment of dormant trees at 50 °C for 10 minutes. But he also found that results were better if growing trees were kept for 2 – 4 weeks in air at 34–35 °C.
The application of hot air, mostly at temperatures from 35–38 °C during 2–4 weeks, now is common practice in several crops (Kassanis and Posnette, 1961). Unlike the hot-water treatment of sugar-cane cuttings, this method is mainly used for building up a virus-free nuclear stock which then has to be propagated under conditions preventing re-infection.
It is not easy to explain the mechanism of heat treatment at temperatures of 35–38 °C. As Kassanis (1965) pointed out the ability of viruses to infect and multiply in plants at 36 °C is not correlated with their thermal inactivation points, i.e. the temperature at which they are inactivated in 10 minutes in vitro. Tomato bushy stunt virus for instance has a thermal inactivation point of 80 °C. However, it cannot infect plants at

36 °C and it is eliminated from fully infected plants by keeping these at this temperature. Therefore in this and similar cases virus inactivation by prolonged exposure of infected plants to temperatures around 37 °C must be attributed to some host metabolic system, or to failure of the virus to multiply at this temperature.
In some instances the entire plant is freed from its virus: Rubus stunt and other viruses in raspberry (Thung 1952; Chambers, 1954), mottle virus in strawberry (Posnette, 1953), and mosaic in apple (Posnette and Cropley, 1956).
More often only the youngest parts are free from virus as is the case in fruit trees, probably due to the failure of the virus to multiply at high temperature. Then 1 cm tips of young shoots, produced while the trees were in the cabinet at 37–38 °C, are grafted onto young seedlings of the same diameter (Ellenberger, 1960, Campbell, 1962).
Testing of treated plants is of the utmost importance. However, a detailed description of the procedure of testing fruit trees on the absence of viruses cannot be given here. Grafting onto a range of indicator varieties of fruit and related tree species is necessary (Posnette and Cropley, 1961).
A simplification of the testing is possible by first screening the treated material on the herbacious test plant *Chenopodium quinoa* which shows necrotic leaf spots if chlorotic leafspot virus and/or some other latent viruses of apple and pear are present (Pfaeltzer, 1962, 1968). By omitting positively reacting apple and pear clones from further indexing much time, material and space can be saved.
In general for successful heat therapy the physiological condition of the material to

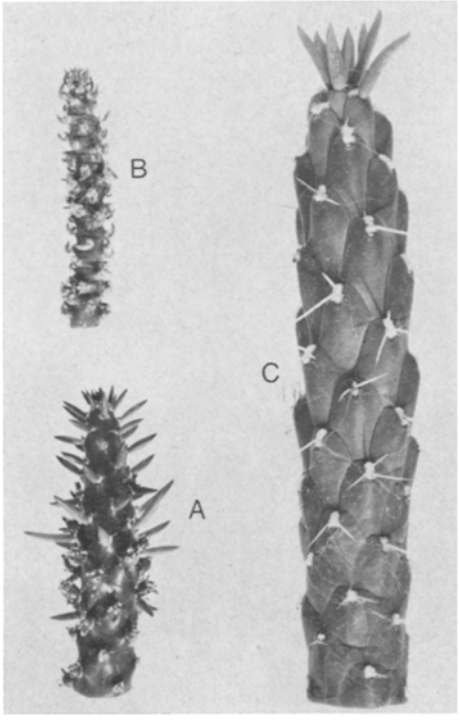

Fig. 1. *Opuntia exaltata*
A. Actively growing plant infected with witches' broom virus. The plant does not survive 2 h at 50 °C.
B. Virus-infected plant, air-dried for 6 weeks. The plant survives 2 h at 50 °C, which suffices to eliminate the virus.
C. Plant cured by heat treatment.
Fig. 1. Opuntia exaltata
A. Groeiende plant, geïnfecteerd met een virus dat heksenbezem veroorzaakt. De plant verdraagt geen warmwaterbehandeling van 2 uur bij 50 °C.
B. Sterk uitgedroogde plant die zo'n behandeling wel kan overleven.
C. Door warmtebehandeling genezen plant.

be treated is important. Baker (1962) emphasizes that for a hot-water treatment (at ± 50 °C) a physiologically dry condition is favourable for survival of the plants. In practice this means that the chance of obtaining virus-free material increases. Results obtained with *Opuntia exaltata* confirm this (van der Meer, 1967) (Fig. 1).
Hot-air treatment of apples can best be started with material which has terminated growth in autumn, and thus is physiologically drier than it would be in spring. When placed in the cabinet at 37–38 °C the dormancy will be broken and during the treatment growth will be resumed. Material treated in this way has a very high survival rate, also after prolonged heat treatment (van der Meer, 1967b).
Heat treatment and particularly the testing of heat-treated fruit tree material are too complicated to be left in the hands of the average grower. In The Netherlands heat therapy is carried out at the Plant Protection Service and in the Institute of Phytopathological Research to produce fruit trees without the known viruses, to be used for the production of budwood. Promising material of several apple and pear varieties as well as of some rootstocks is already available.
In general the method of heat treatment varies in details from crop to crop and from virus to virus. If heat therapy is not possible, success may be obtained by using the method of apical meristem culture.

Apical meristem culture

Investigations of Limasset and Cornuet (1949) on the variation in virus contents of tobacco leaves of different age, infected with tobacco mosaic virus, showed that the amount of virus diminished the closer the leaves are to the vegetation point (= apical meristem). In the vegetation point itself no virus was detectable in half of the cases.
This result brought Morel and Martin (1952) to the hypothesis that it might be possible to isolate, under sterile conditions, the apical meristem of virus diseased

Fig. 2. Meristem culture of potato

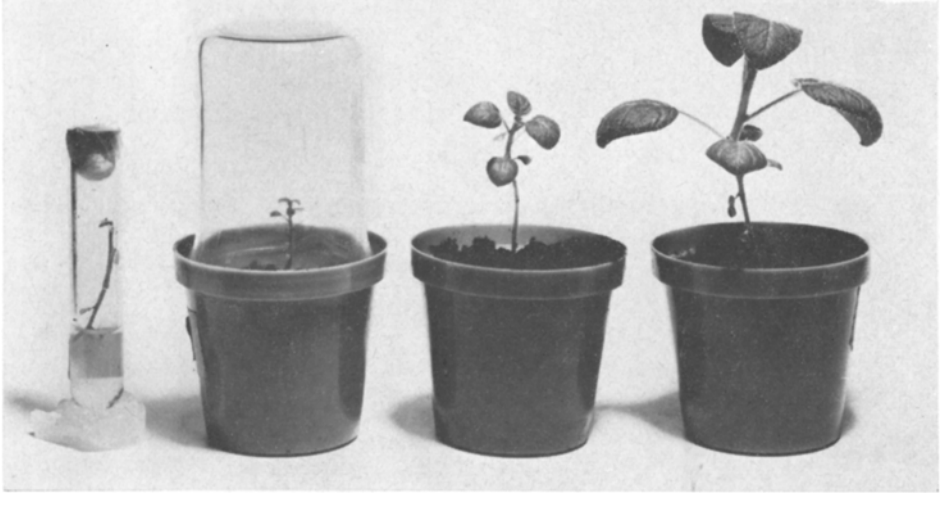

Fig. 2. Meristeemcultuur van aardappel

dahlia and cultivate this meristem aseptically in vitro in order to get healthy plants. They succeeded indeed, after using various nutrient media, in obtaining from meristems some plantlets of 1 or 2 cm length, although without roots. By grafting them on young virus-free seedlings they finally got healthy plants.

Ever since the method has been used by several research workers in order to free totally infected valuable varieties of a number of crops from virus (Fig. 2). The many difficulties they encountered are clearly described by Morel (1964) and Kassanis (1965). Not only is the apical meristem, a dome of actively dividing cells, extremely small, measuring at most 0.1 mm in width and 0.25 mm in length, but all the work has to be carried out under sterile or semi-sterile conditions. Moreover, success depends on the composition of the nutrient medium which may vary from species to species and sometimes even from variety to variety. Either a liquid or a solid agar medium containing all necessary minerals, sugar, vitamins and growth promoting substances is used.

In the case of carnations gibberellic acid stimulates a quick differential growth of an aerial shoot but mostly no roots develop. By transferring such shoots to another sterile medium, without gibberellic acid but with a trace of α-naphthalene acetic acid, roots are induced to start growing (Quak, 1966). It has been found that normal growth is strongly stimulated when the concentration of salts containing potassium and ammonium ions is increased in the medium (Morel, 1964).

Some viruses can more readily be eliminated than others. For instance meristems from tuber sprouts of virus infected potato varieties gave plantlets which were all free from leafroll virus, but only 80% did not contain the viruses Y and A, whereas only very few plantlets were found to be free of virus X.

With the early variety 'Eersteling' this method gave no plants free from viruses X and S. Only after a previous treatment with 2-thiouracil some plantlets were free of X, but they still contained virus S. However, meristem isolations from special selections of 'Eersteling' produced plants free from both viruses.

The case of 'Eersteling' particularly demonstrates that different viruses may occur at different levels in the tip of the host plant. Application of chemicals having an inhibitory effect on virus multiplication may increase the size of the virus-free zone (Quak, 1961). In The Netherlands all seed potatoes of 'Eersteling' used to be free of leafroll virus, and the viruses Y^O, Y^N, A and M, but they were until recently fully infected with viruses X and S. Thanks to meristem culture the potato selectionists are now propagating 'Eersteling' free from X and S as well; these will be used exclusively in the near future. Although up to now no increase in tuber production has been observed, using virus-free 'Eersteling' means eliminating an important source of infection for later varieties.

In England a virus-free stock of the variety 'King Edward', previously fully infected with paracrinkle (a strain of virus S), was propagated in seed growing areas and has been used for yield experiments. The new virus-free stock was compared with seven of the best commercial stocks in nine different centres for four consecutive years. The average yield of the new stock was 10 per cent more than that of the commercial stocks and the tubers were more uniform in size (Kassanis, 1965).

In The Netherlands virus-free stocks of the following crop plants were obtained by using apical meristem culture: carnation (Quak, 1957), iris (Baruch and Quak, 1966), freesia (Brants and Vermeulen, 1965), hycacinth (van Slogteren, 1965), potato (Quak,

1966), strawberry (Quak, 1965). In other countries the method was successfully applied amongst others for dahlia (Morel and Martin, 1952), potato (Kassanis, 1957; Morel and Muller, 1964), carnation (Hollings and Stone, 1965), strawberry (Belkengren and Miller, 1962), orchids (Morel, 1960).

Heat treatment followed by apical meristem culture

As the percentage of successfully grown virus-free meristems is not always satisfactory, due to the small size of the apical meristem, a combination of heat treatment and meristem culture has been applied by some workers.
The main advantage of the combined treatment is that bigger tips can be used, comprising meristem and some leaf primordia. Such tips are about 1 mm in length instead of the usual 0.1–0.25 mm. The result is a better growth on the nutrient media.
This combined treatment was successfully applied in The Netherlands for carnation (Quak, 1957), chrysanthemum (Hakkaart and Quak, 1964) and strawberry (Quak, 1965).
Close cooperation between the Institute of Phytopathological Research, the Plant Protection Service, The Netherlands Inspection Service and some growers, who built aphid-free greenhouses for this purpose, has led to the commercial production of virus-free carnations of some 30 varieties. They are tested regularly on *Chenopodium amaranticolor* for the presence of the ringspot, mottle and vein mottle viruses and for etched ring virus by grafting onto the susceptible carnation variety 'Joker'.
An additional advantage of having a large amount of virus-free carnation plants available appeared to be that it was possible to study the symptoms of each separate virus and to evaluate its influence on production and quality on a semi-commercial scale (Hakkaart, 1964). This investigation has convinced the Dutch growers of the importance of using virus-free cuttings. As a result ringspot virus has been fully eliminated.

Conclusion

In conclusion it can be said that for vegetatively propagated agricultural and horticultural crops both heat treatment and apical meristem culture are of great importance for the production of virus-free planting material. It should be kept in mind that such plants are not immune, so re-infection may occur, particularly when the viruses concerned are insect-borne. In some cases, like for instance with fruit trees, re-infection with most viruses is not very probably if both rootstock and scion are virus-free, as the majority of the viruses involved have no vectors.
It is expected that these methods wil have an ever increasing application.

Samenvatting

Warmtebehandeling en meristeemcultuur voor het verkrijgen van virusvrij plantenmateriaal

Directe chemische bestrijding van virussen in de plant is niet mogelijk zonder de gastheer te schaden. De verspreiding van virussen, die op andere wijze dan door vectoren

worden overgebracht, kan evenmin door chemische middelen voorkomen worden. Daardoor kunnen soorten en cultivars, die uitsluitend vegetatief vermeerderd worden, volledig met virus besmet raken. Voor deze plantesoorten of cultivars zijn methoden ontwikkeld door middel waarvan virusvrij plantenmateriaal verkregen kan worden, nl. toepassing van warmte, van meristeemcultuur en van een combinatie van beide. Warmte kan worden toegepast op twee manieren nl. als warm water en als warme lucht. Bij de eerste wordt het zieke gewas behandeld en bij de tweede uit het behandelde gewas virusvrij vermeerderingsmateriaal geïsoleerd en opgekweekt. De eerste methode werd met succes toegepast op sereh-zieke suikerrietstekken; verscheidene fruitgewassen zijn door middel van warme lucht virusvrij gemaakt.

Wanneer warmtebehandeling niet tot het gewenste resultaat leidt, kan men door middel van meristeemcultuur proberen virusvrije planten te verkrijgen. Het meristematisch weefsel aan de top blijkt nl. in vele gevallen geen virus te bevatten. Door deze topjes te isoleren en op een voedingsbodem over te brengen is het mogelijk een plant op te kweken, die geen virus bevat. Deze cultuur stelt hoge eisen aan de voedingsbodems; deze zijn voor de scheutvorming en de beworteling zeer verschillend en dikwijls voor elke plantesoort en cultivar ook. Door de plant vóór de isolatie van het topmeristeem een warmtebehandeling te laten ondergaan kan men de stengeltopjes iets groter nemen d.w.z. met twee bladprimordia; daardoor wordt de kans dat het geïsoleerde weefsel zich ook verder tot een plantje ontwikkelt groter. Het blijft altijd noodzakelijk om het behandelde of het opgekweekte materiaal op aanwezigheid van virus te toetsen. Van verscheidene gewassen worden met behulp van meristeemcultuur – al of niet in combinatie met warmtebehandeling – reeds grote hoeveelheden virusvrij materiaal gekweekt. Behalve praktisch belang heeft dit laatste ook grote betekenis voor het onderzoek naar de invloed van een bepaald virus op de produktie en de kwaliteit van een gewas. Plantesoorten en cultivars waarvan in Nederland en andere landen virusvrij materiaal verkregen is worden opgenoemd.

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