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PRODUCTION OF VIRUS-FREE CARNATIONS BY MEANS OF MERISTEM CULTURE[1]

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The carnations which are normally grown are practically all infected with various viruses, and therefore attempts were made to obtain virus-free plants of a number of varieties by means of heat treatment and meristem culture. Incubators were designed in which the plants could be subjected to a heat treatment at a temperature of 37°C and at a high degree of humidity of the air. The ultimate percentage of virus-free plants in 1960 amounted to an average of 1.3%. This is sufficient to obtain a virus-free clone, but not enough to build up a virus-free assortment within a short time. The factors which may affect the percentage of successful isolations are discussed, viz. the effect of the season, of varietal differences and of differences between the material supplied by different growers. When the virus-free plants are propagated in practice it is essential that the material should be closely inspected and tested at regular intervals. The new material was found to possess most favourable qualities. It is sold with a special certificate.

INTRODUCTION

It has been known for a long time that the carnations which are grown nowadays (mainly of the 'American Sim'-type) are practically all infected with various viruses. Amongst the symptoms caused by these viruses are mottling of the leaves and in some cases a slight discoloration of the flowers. The general spread of these viruses is caused by the fact that carnations are propagated vegetatively.

Two viruses are important in the Netherlands: carnation mottle virus, a mild virus which can be detected in all commercial varieties, even in symptomless plants, and carnation ringspot virus. We are still in doubt as to the occurrence of carnation vein-mottle virus, but it is a fact that all other viruses described in the literature have never been found in Dutch carnations.

In the Annual Reports of the Experimental Station of Floriculture in the Netherlands BELGRAVER (1950, 1951, 1952) published about the measure in which the joint carnation viruses effect flower production and quality. For several varieties well-selected symptomless plants were compared with slightly and seriously diseased plants. For a widely grown variety like 'William Sim' the following results were obtained: in the second year of cultivation the flower production of the slightly diseased plants was 14% and that of the seriously diseased plants 23½% lower than the production of the well-selected ones. Moreover it was clear that the quality of the flowers deteriorated according as the plants were less well-selected.

In France a method had been developed which made it possible to obtain virus-free plants by means of vegetative propagation. This so-called meristem culture is based on indications by LIMASSET & CORNUET (1949), who pointed out that the apical meristem of a virus-diseased plant may still be virus-free.

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Proceeding from this assumption, MOREL & MARTIN (1952) isolated extremely small meristems from virus-infected dahlia shoots, which they put on a nutrient agar. The plants grown from these meristems were indeed virus-free.

The experiences described by BELGRAVER led QUAK (1957, 1961) to investigate whether this method could be applied to carnations. She applied the method to the carnation varieties 'Harvest Moon' and 'Pink Sim'. She preceded meristem culture by a heat treatment, subjecting the plants to a temperature of 45°C for some weeks, before isolating the apical meristem. According to KASSANIS (1955), ringspot virus is inactivated by this heat treatment. Moreover it was to be expected that in general under these circumstances viruses would penetrate less quickly into the meristematic tissue, so that it was now possible to isolate not only the apical meristem, but to include the first leaf primordia. These slightly larger tips proved to grow much better than the mere meristems did. In this way QUAK indeed succeeded in obtaining a number of carnation plants which reacted negatively to repeated tests for the known carnation viruses. So the conclusion might be drawn that with the aid of meristem culture virus-free carnations had been obtained.

In view of this favourable result, and because of the interest carnation growers took in this material, the Institute of Phytopathological Research (I.P.O.), the Experimental Station of Floriculture in the Netherlands and the Plant Protection Service (P.D.) jointly decided to start the cultivation of virus-free carnation plants with the aid of meristem culture on a practical scale. With financial support from the Central Organization for Applied Scientific Research in the Netherlands (T.N.O.) this project was started at the Plant Protection Service at Wageningen in 1959. A number of interested growers were to provide carnation material, while the virus-free plants obtained were to be returned to the grower concerned, for propagation.

The following chapters contain a short description of the method applied, and its results.

METHOD

Heat treatment

In accordance with the method developed by QUAK, rooted cuttings were used, which were kept at a temperature of 37°C and a relative humidity of the air of about 80% for three weeks. It was found that this treatment did not always result in complete inactivation of ringspot virus. However, the plants will not stand a longer treatment or a higher temperature very well.

The treatment takes place in a completely closed incubator with artificial illumination. Both temperature and illumination are adjustable. The outside measurements of the incubator are $1{,}50 \times 1 \times 1$ m, and it has double walls, except at the top and the door. It consists of an outer box and an inner box. The outer box consists of a wooden frame covered with asbestos sheets, a glass roof and a wooden floor. The inner box is supported by four legs resting on the floor of the outer box. The wooden floor of the inner box is lined with zinc sheet. Its side walls are lined with 1 mm aluminium sheet. There is no top. The free space between the sidewalls of the two boxes amounts to about 10 cm. Near the door this free space is shut off by transverse walls. The space between

the two floors amount to about 15 cm, and the distance between the upper edge of the side-walls of the inner box and the glass top of the outer one is about 2 cm. The carnations are put in boxes, standing on a rack which is mounted about 30 cm above the floor. In this way the whole of the inner floor-surface (130×75 cm) can be used. A ventilator is fitted in the middle of the floor.

The temperature in the incubator can be raised with the aid of a 1200 Watt tubular heater, which is 10 m in length and is fixed in two coils along the walls of the outer box, on a level with the free space at the bottom. The temperature can be adjusted with the aid of a contact thermometer, measuring from 0° to 50°C, to an accuracy of half a degree. This metal-jacketed contact thermometer is suspended from the roof of the box, in such a way that its mercury container hangs about halfway the inner box. The connection is effected via a simple 2000 Watt mercury relay.

For extra security a DANFOSS thermostat has been connected in series with the contact thermometer. This thermostat is always adjusted a few degrees higher than the contact thermometer. If the thermometer should fail, the temperature can never rise too high. The thermostat is mounted high against one of the side-walls. Moreover there are signal-lamps which indicate whether or not the heater is operating.

The air in the incubator is circulated by a low-capacity ventilator (Indola type MFH 435 c/15), which is fitted in a 20 cm hole in the bottom of the inner box. It is covered by a zinc lid in order to prevent water from entering it. The ventilator blows into the space between the two floors, the air then passes along the heater and the heated air rises between the walls. It then enters the inner box via the 2 cm chink under the glass, and is then exhausted again by the ventilator. The circulating air dries quickly, but is moistened by water in a zinc container under the ventilator. This container is replenished regularly. Moreover the level of humidity in the incubator is maintained by watering plants and soil daily. The temperature of the water used for watering is only slightly lower than the temperature in the incubator.

Light is supplied by six 65 Watt fluorescent tubes with inner reflection (Philips TLF 65 Watt, colour 33), mounted outside the incubator, over the glass. In one case six 80 Watt tubes without inner reflection were applied. In both cases an aluminium reflector is mounted over the tubes, which covers the whole top of the incubator. So the available energy amounts to 260 and 300 Watt to the square metre respectively. The 80 Watt tubes produce more heat, so in order to prevent the temperature inside the incubator from being affected, the reflector is perforated and mounted somewhat higher. The lamps are connected via a clock, and burn for 16 out of every 24 hours.

Disinfection

Before isolating the meristem, the top-shoots harvested from the incubator should be thoroughly disinfected. For that purpose the shoot is first defoliated. Then the bare shoots are shaken in 96% alcohol, and subsequently submerged in a 5%-solution of calcium hypochloride. After being rinsed in water, the shoots should be left in clean water for half an hour, before the meristem can be isolated.

The nutrient medium

The composition of the nutrient medium is almost identical to the one developed by MOREL (personal communication). Addition of 1 ppm α-NAA is necessary in order to promote root-formation.

KNOP's solution, half strength	1000 ml
solution of BERTHELOT[3]	0.5 ml
vitamin solution A	1 ml
vitamin solution B	1 ml
cystein	1 mg
adenin	5 mg
casein hydrolysate	200 mg
saccharose	20 mg
α-naphtyl-acetic acid	1 mg (1 ppm)
agar (DIFCO bacto-agar)	

The vitamin solutions consist of:

A:	Ca-panthotenate	– 1 mg	
	inositol	– 100 mg	per 100 ml of distilled water
	biotin	– 10 mg	
B:	niacin	– 1 mg	
	pyridoxin	– 1 mg	per 100 ml of distilled water

This nutrient solution is poured into Pyrex glass tubes (length 7 cm, diameter 15 mm). After filling the tubes are closed with a pad of cottonwool, and before use they are autoclaved at 110°C for half an hour.

Isolation of the meristem

The meristem is isolated under a stereomicroscope, under as sterile conditions as possible. The instruments used are dissecting needles and blades. Of course these instruments are continually disinfected with alcohol, while moreover the last cut with which the apical meristem is excised, is always performed with a clean blade. With this blade the meristem is placed in an upright position in the nutrient medium.

As was described by QUAK, some leaf primordia are included when the meristem is excised. If they are omitted, there is little chance of the meristem growing successfully. However, if too many leaf primordia are included, there is a great chance of virus-infection. The size of the isolations varies from 0.6 mm to a little over 1 mm.

If a flower-bud is developing in the meristem, such a meristem is not fit for our purpose. However, it is usually not easy to tell whether or not a flower-bud is developing, so probably many failures should be put down to the fact that the meristem was in the wrong stage.

[3] The solution of BERTHELOT consists of: Mn SO_4 2.0 g, Ni SO_4 0.06 g, Ti O_2 0.14 g, H_2SO_4 (66° Bé) 1 ml, $CoSO_4$ 0.06 g, $ZnSO_4$ 0,1 g, $CuSO_4$ 0.05 g, $BeSO_4$ 0.1 g, H_3BO_3 0.05 g, $Fe_2(SO_4)_3$ 50.0 g and K J 0.5 g to 1000 ml of distilled water.

After the meristem has been placed on the nutrient agar, the pad of cotton-wool is flamed, and the tube (which is still warm) is sealed with Parafilm, a special plastic foil which prevents loss of moisture and yet allows some gas exchange.

The growth

The meristems grow at room temperature, under artificial illumination. The tubes are put in racks, under two 65 Watt fluorescent tubes. These tubes burn continuously, because light duration was not found to affect the growth of the meristem in any way. No reliable differences could be demonstrated in experiments in which the meristems, after isolation, were first kept in the dark fore some days, and were subsequently exposed to different photoperiods.

After some days the two tiny leaves enclosing the meristem, expand. This stage may last for some weeks or even for some months. Many meristems perish during this stage, perhaps partly because they had already reached the generative stage. Now callus tissue may develop, which will later produce the roots. However, in some cases this callus formation is so abundant that it suffocates the meristem, without developing roots.

A typical feature of carnations is the fact that most of the roots grow upwards from the nutrient solution, so a kind of negative geotropy. This could not be avoided by changing the composition of the nutrient medium in various ways, e.g. as regards the quantity of growth regulating substances added. Possibly lack of oxygen in the nutrient solution is responsible for this tendency, but it was found to be practically impossible to eliminate this phenomenon.

Contamination with fungi or bacteria in the tubes is hardly possible under the prevailing sterile conditions.

As soon as the plants have begun growing again, they are gradually hardened plantlets are carefully taken out of the tubes, with part of the nutrient agar, and put in small boxes filled with sterile sand or a very light sterile soil, and covered with glass. Especially plantlets with upward growing roots may suffer much from being transplanted. Accordingly some plantlets perish even in this stage.

As soon as a sufficient number of roots and a shoot have developed, these off, after which they are potted in a light soil mixture, and are further grown in an aphid-free glass-house.

Method of testing for virus-infection

The plantlets are tested for the presence of virus as soon as they have reached a height of 10–15 cm, at which stage they can easily do without two leaves. These leaves are rubbed in a mortar, or crushed with a pair of Dutch tongs (hand-press by SALM & KIPP). The sap is rubbed on young leaves of *Chenopodium amaranticolor* L., which have previously been dusted with carborundum powder. We chose this test plant because it produces a quick, clear and reliable reaction to the three carnation viruses we are interested in.

If the plant is not virus-free the inoculated leaf of the test plant displays after 10–15 days many sharply outlined, sometimes red-edged necrotic lesions, or slightly larger yellow spots with vaguer outlines. The reaction more or less depends on the character of the virus present, although it is not possible to diagnose the virus by the type of reaction. However, in our case proof of the fact

that any virus whatsoever is present, is sufficient. The plantlet in question is then destroyed at once.

Carnation mottle virus and carnation ringspot virus can also be detected serologically, with the aid of the gel-diffusion method (VAN SLOGTEREN, 1954). We used an antiserum against mottle virus and a mixed serum against both viruses mentioned above[4]. However, as the serological test is less reliable than the test with *Chenopodium,* the quicker serological method was only employed for testing samples from commercial stocks of virus-free carnations.

RESULTS

The percentage of the isolations which is successful, is low, and has retarded the building-up of a virus-free assortment for commercial growth. When judging the percentage of successful isolations, we should distinguish between the number of plants developed from meristem isolations and the number of these plants which were actually found to be virus-free. The latter group makes the ultimate percentage of successful isolations, which for the year 1960 amounted to an average of 1.3%, with monthly variations from 0.5 to 2.8%.

Our further discussion only refers to the results of 1960, because in the spring of 1961 the work was taken over by a new technician. In view of the special character of this work, it is very well possible that this fact affected the results, and the figures for 1961 prove this assumption. When this factor is eliminated, there are still a number of other factors which may effect the ultimate result. Some of these can be demonstrated by figures.

Season

In order to obtain an impression of the way in which the month of isolation affects the result, we examined the percentage of successful isolations per month. Only the month in which the isolation was made is taken into account, because with some meristems growth started pretty soon after isolation, whereas others took a long time before beginning to grow again.

In Fig. 1 the results per month are shown, as a percentage of the number of isolations made. If we compare the isolations made in the months of November, December and January with the ones made in the summer months, we are struck by the fact that the former have produced more plants than the latter. However, in winter the ratio between the number of successful isolations and the number which were found to be virus-free, is much more unfavourable. The small number of plantlets obtained from the isolations in July and August were all virus-free, but the 78 plantlets obtained from the December isolations only yielded 23 virus-free ones. These figures lead to the hypothesis that the shoots used in winter root more easily than the summer ones. This hypothesis indeed agrees with the experience obtained when taking normal carnation cuttings. Moreover there are indications that the virus is less active in summer than it is in winter. This may be due to the higher temperatures in summer.

[4] Prepared at the Laboratory for Flower Bulb Research at Lisse. The author wishes to render thanks to Ir. D. H. M. VAN SLOGTEREN and to Miss N. DE VOS for their co-operation in the serological investigation.

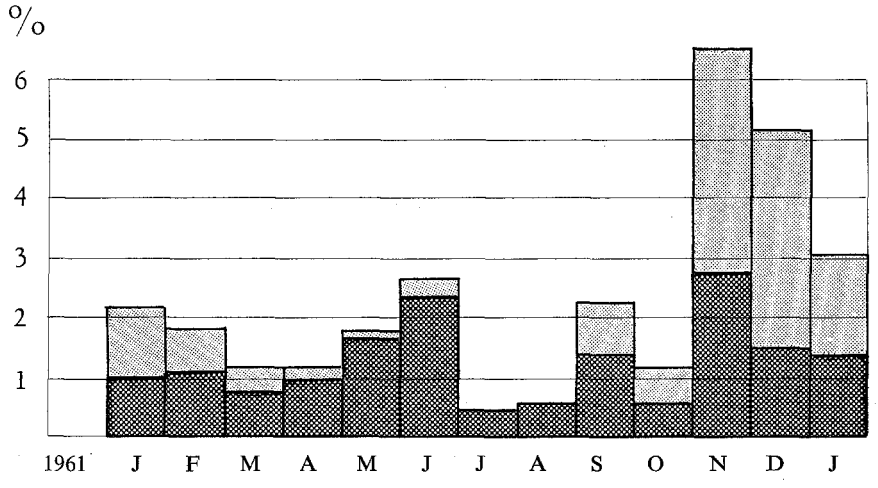

FIG. 1. Number of growing meristems (whole columns) and number of these which were virus-free (dark columns), expressed as percentages of the total number of isolated carnation meristems per month.

TABLE 1. Number of successful isolations and number of virus-free plants per variety, expressed as a percentage of the number of plants treated.

Variety	Percentage of successful isolations	Percentage of virus-free plants
'William Sim'	2.4	1.6
'Crowley's Sim'	2.0	1.2
'White Sim'	3.8	1.7

Differences between the varieties

In order to judge possible differences between the varieties treated, the results per variety were counted. If we ignore seasonal influences, a reliable comparison is possible between the varieties 'William Sim', 'Crowley's Sim' and 'White Sim'. Of the other varieties the number of plants treated was too small proportionately. The figures are shown in Table 1. No important differences are found in this case.

Growers

Experience has taught us that there were marked differences in quality between the material provided by the different growers. Accordingly, differences in the percentages of successful isolations per grower were to be expected. In order to be able to make a more or less reliable comparison, we chose five growers who had provided about equal numbers of plants. The results of the varieties 'William Sim', 'Crowley's Sim' and 'White Sim' were counted and the percentages were calculated (Table 2).

TABLE 2. Percentage of successful isolations and percentage of virus-free plants of the varieties 'William Sim', 'Crowley's Sim' and 'White Sim' collectively, calculated for five different growers.

Grower	Percentage of successful isolations	Percentage of virus-free plants
A	3.2	1.6
B	8.4	3.1
C	2.4	1.8
D	0.6	0.2
E	0.6	0.4

There are striking differences, which cannot be directly accounted for. The various circumstances existing while a cutting is growing and roots are developing, and differences between the selection of one variety, may all exert a certain influence.

It was not possible to make an investigation into all these factors and into the degree to which they affect the result.

THE VIRUS-FREE CARNATIONS IN PRACTICE

The plants whose sap do not cause virus reaction on the test plants, are available to the grower who supplied the initial material. His code number and that of the variety have accompanied the plants throughout the treatment. Now it is up to the grower to propagate the material as quickly as possible, but at the same time as carefully as possible. It is desirable that each virus-free meristem plant should be propagated as a separate clone. In this way a possible reinfection can be checked efficiently. Moreover clone selection as to quality remains necessary for the virus-free material too.

Practice has demonstrated that virus-free cuttings root easily and grow quickly. Comparative experiments are being made.

During the propagation various growers test the plants at regular intervals by inoculation on *Chenopodium amaranticolor*. Moreover the crop is checked regularly by inspectors of the Netherlands Inspection Service for Ornamentals. This service takes random samples of all stocks. At Wageningen these samples are tested for possible virus-infection (either serologically or by means of sap-inoculation).

Growers are required to receive and propagate the virus-free plants in insect-free glass-houses. At first this requirement caused quite a few difficulties and restricted participation in this project. At the moment there are specially built glass-houses at three Aalsmeer nurseries, while at five other nurseries temporary equipment is being used. Various systems are applied with satisfactory results.

A number of growers, possibly disappointed by the low percentage of successful meristems, failed to provide a suitable accomodation. Consequently building up a virus-free assortment required more time than had otherwise been necessary. The value of the few virus-free plantlets was not realized sufficiently, so that some of the laboriously obtained plants perished. By the end of 1961 26 varieties had been treated. Material of 17 varieties had been re-

turned to the growers. Meanwhile virus-free cuttings of some of these varieties are already on the market. On the certificate granted to approved carnation cuttings by the Inspection Service, the virus-free cuttings are given a special mark. Cuttings originating from mother plants which have been grown under aphid-free conditions and have regularly been tested for virus-infection, are indicated by the mark EE on the certificate. If the cuttings come from virus-free mother-plants grown under normal conditions, they are given the mark E.

CONCLUSION

To carnations as to any crop it is important that a healthy product should be yielded. Therefore we persevered in our efforts to produce virus-free carnations, in spite of the low percentage of succesful isolations, and in spite of the fact that at first we were not sure whether these virus-free plants would yield better results than well-selected but slightly diseased ones. However, our perseverance has already been proved justified by experiences with the virus-free cuttings and by the preliminary results of current experiments at the Experimental Station of Floriculture, published by HAKKAART (1963).

Objections have been raised to the term *virus-free* carnations. Indeed, no proof has been furnished as to the absence of some viruses which have so far not been found in this country and for which no satisfactory test method exists. However, it does seem extremely improbable that these viruses should be present, considering that it was not possible to detect any virus at all in these plants with the aid of the general test methods. From a scientific point of view it might be preferable to refer to these plants as *virus-tested* carnations. However, for practical purpose the term *virus-free* carnations does not appear to present any difficulties at all.

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