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Tumours of Begonia and some other ornamentals, induced by Corynebacterium fascians

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Abstract

In 1975 many tumours were observed in plants of *Begonia* 'Schwabenland' grown in Aalsmeer. Submersion of the roots of *Nicotiana megalosiphon* seedlings in a homogenate of tumorous tissue, induced tumours after two weeks. Short periods of submergence yielded results similar to those obtained after longer periods. Tumour homogenates lost their infectivity after ten min at 50°C. Aphids transmitted the infectious agent.

Treatment with propylene oxide did not inhibit infectivity completely. Filtration through a 450 nm filter removed the infectious agent.

Tobacco tumor virus or a viroid could not be isolated. Cultures of *Corynebacterium fascians*, isolated from tumours of *N. megalosiphon* were highly infectious and induced tumours in healthy *N. megalosiphon* and *Begonia*. Tumorous tissue homogenates of *Pelargonium zonale*, *Dahlia* sp., *Gladiolus* sp., and *Lilium* sp. also caused tumours in *N. megalosiphon*, from which *C. fascians* was isolated. It was not possible to produce tumours in *N. megalosiphon* with homogenates from roses with symptoms of bud proliferation.

Additional keywords: Dahlia, Gladiolus, Lilium, Pelargonium, Nicotiana megalosiphon.

Introduction

In 1975 a mass appearance of organoid tumours on the root collars of *Begonia* 'Schwabenland' (Fig. 1) was noticed. Although a *Corynebacterium* sp. has been described already as the causal agent of tumours in Lorraine-Begonia in Germany by Stark (1964), and *C. fascians* has been described as the causal agent of similar tumours in *Dahlia* spp. (Beaumont, 1950), *Pelargonium zonale* (Maas Geesteranus et al., 1966) and *Gladiolus* spp. (Zacha and Moravčík, 1975), this bacterium could not be isolated from our *Begonia* material in initial trials. The causal agent, however, could easily be transmitted to *Nicotiana* spp. (Van Hoof, 1978) which then produced tumours identical in appearance to those obtained by Gliem et al. (1976) and Misra and Nienhaus (1976). These authors reported the isolation of tobacco tumor virus from such material. In this article we describe the characterization of the tumour-inducing agent isolated from *Begonia* and some other ornamentals.

Materials and methods

Most of the work was done with material from tumour-bearing plants of *Begonia* 'Schwabenland' originating from nurseries at Aalsmeer. In some later trials we used material from freeze-dried tumours of *N. christii*, kindly supplied by F. Nienhaus, Institut für Pflanzenkrankheiten, University of Bonn.

To transmit the tumour-inducing agent and to determine the infectivity of preparations, the method developed by Van Hoof (1978) was applied. Inocula were prepared by macerating tumorous tissue in an equal weight of water in a Waring blender or with a pestle and mortar. Roots of *N. megalosiphon* were submerged in the suspension to be tested for 20 h, rinsed under running tap water and then planted. Two weeks after inoculation the first tumours appeared. Final readings were after uprooting of all plants four weeks after inoculation.

This method was also used to test the infectivity of tumorous tissue from *P. zonale, Dahlia* sp., *Gladiolus* sp. and *Lilium* Mid Century Hybrid 'Enchantment'. Infected material from the latter three crops was kindly provided by W. Kamerman and others, Bulb Research Centre at Lisse. Roses with symptoms of bud proliferation were received from F.W. Perquin, Research Station for Arboriculture at Boskoop.

The susceptibility of *Pisum odoratum, Kalanchoë daigremontiana,* and *Melilotus officinalis* for the tumour-inducing agent under examination was determined because these species are known as test plants for *C. fascians, Agrobacterium tumefaciens,* and wound tumor virus, respectively (Lacey, 1939; Schilperoort et al., 1975; Black, 1970).

The persistence of infectivity in tumour homogenates was determined in the way customary for homogenates of virus-infected tissue.

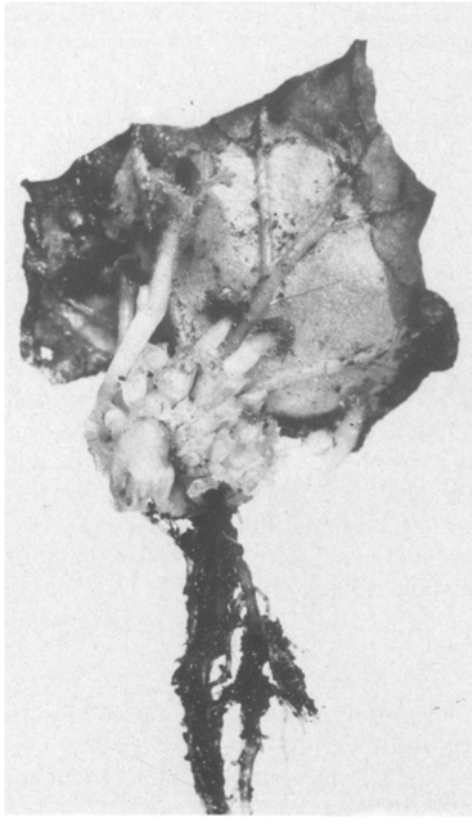

Fig. 1. Leaf cutting of *Begonia* 'Schwabenland' showing a great number of deformed stems.

Fig. 1. Bladstek van Begonia 'Schwabenland' met een groot aantal misvormde scheuten.

Fig. 2. Seedlings of *Nicotiana megalosiphon* showing different degrees of infection one month after being dipped into tumour juice.

Fig. 2. Zaailingen van Nicotiana megalosiphon die, een maand na te zijn gedompeld in tumorsap, verschillende maten van aantasting vertonen.

Attempts to purify a tumour-inducing virus were performed with tumours from *N. christii, N. clevelandii, N. megalosiphon,* and *N. rustica* using differential and density-gradient centrifugation. Attempts to detect the occurrence of viroids were made according to the methods of Morris and Smith (1977) and Mosch et al. (1978).

Bacteria were isolated on 'special peptone' medium (Oxoid) at 22°C.

Tests with *N. megalosiphon* tumours for the presence of octopine and nopaline, unusual amino acid derivatives found in all tumours induced by *A. tumefaciens* (Schilperoort et al., 1975), were kindly carried out by R.A. Schilperoort, Department of Biochemistry, University of Leiden.

Results

Symptoms. Begonia is vegetatively propagated by leaf cuttings. At the basis of the midrib shoots and roots are formed. In diseased material some of the shoots were odd-shaped: extremely long or short and thickened (Fig. 1). Sometimes only abnormal growth occurred.

Using homogenates of *Begonia* tumours as inoculum tumours were induced in several tobacco species. In *N. megalosiphon* abnormal branching occured (Fig. 2) starting with leaf-like outgrowths which later disappeared. This was followed by the development of large tumours. The main stem often remained unaffected and

Fig. 3. *Melilotus officinalis*; infected plant (left) showing abnormal outgrowths at the first node. Healthy plant on the right.

Fig. 3. Melilotus officinalis; geïnfecteerde plant (links) met veel misvormde zijstengels ter hoogte van het eerste internodium. Rechts gezonde plant.

developed in a normal way. Symptoms in *N. christii, N. clevelandii,* and *N. rustica* were similar to those in *N. megalosiphon.* Tumours of *N. tabacum 'White Burley'* usually had small thick leaves.

M. officinalis and *P. odoratum* infected with a homogenate of *N. megalosiphon* tumours showed abundant stem formation in the regions of the cotyledons; these stems being rigid and thickened (Fig. 3 and 4). The symptoms on *M. officinalis* differed markedly from those described for wound tumor virus by Black (1970) because no stem tumours developed and the leaves did not show depressed veins. *K. daigemontiana,* either inoculated as a young or as a full-grown plant, could not be infected with the tumour-inducing agent of *Begonia*.

An identical type of tumours was obtained by inoculation of *N. megalosiphon* with homogenates of tumours from *P. zonale, Dahlia* (Fig. 5), *Gladiolus* (Fig. 6) or *Lilium* (Fig. 7). The symptoms of bud proliferation of roses (Bos and Perquin, 1975) showed a great similarity to the symptoms of the disease of *Begonia* studied. The *N. megalosiphon* test, however, was negative with this material.

Inoculation experiments. Initially the roots of *N. megalosiphon* seedlings were submerged for 20 h in the inocula (Van Hoof, 1978). However, much shorter periods were highly effective too (Table 1). Even if the roots were submerged for 1 s and immediately afterwards rinsed by running tap water 25 out of 55 plants became infected. In routine experiments we submerged the roots of *N. megalosiphon* seedlings for 1 h.

Fig. 4. *Pisum odoratum* with thickened and rigid outgrowths in the region of the cotyledons and at the first node.

Fig. 4. Pisum odoratum met verdikte en stugge zijstengels ter hoogte van de cotylen of het eerste internodium.

When seedlings are uprooted for inoculation tests the roots are usually damaged. To see whether this damaging is essential for infection we planted seedlings in a tray and grew them for seven days before the soil around each plant was drenched with 2.5 ml of tumour homogenate. Forty out of 50 plants developed tumours showing that undamaged roots can successfully be inoculated. When the homogenate was poured on the soil one week before planting, the *N. megalosiphon* seedlings became

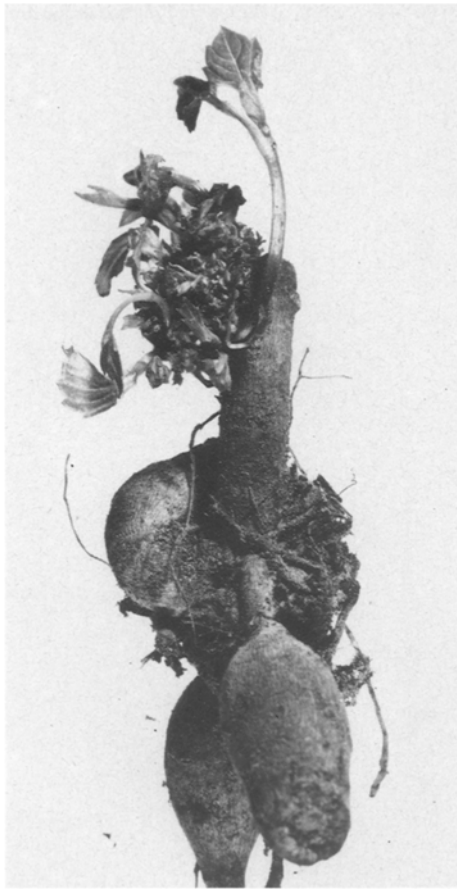

Fig. 5. *Dahlia* with witches' broom-like outgrowth at the stem base. Some normal looking shoots occur between many deformed ones.

Fig. 5. Dahlia met heksenbezemachtige vergroeiing aan de stengelbasis. Enkele normaal uitziende scheuten bevinden zich tussen vele abnormale.

Table 1. Reaction of *N. megalosiphon* seedlings after submersion of their roots in tumour homogenates for various periods. Numerator: number of plants with tumours after four weeks; denominator: number of plants tested.

Submersion time (min.)	Score	Submersion time (h)	Score
1	33/55	1	39/51
2	11/60	2	31/51
3	32/51	3	23/51
4	21/54	4	44/55
5	36/48	8	43/47
15	22/50	16	34/42
30	41/45		

Tabel 1. Reactie van N. megalosiphon zaailingen na dompelen van hun wortels in homogenaten van tumoren gedurende verschillende tijden. Teller: aantal planten met tumoren na vier weken; noemer: aantal getoetste planten.

Fig. 6. *Gladiolus* corm showing outgrowth of the flesh at the basis.

Fig. 6. Gladioleknol met abnormale uitgroei van het knolweefsel aan de basis.

Tabel 2. Reaction of *N. megalosiphon* seedlings after submersion of their roots in homogenates of tumorous tissue, homogenates of healthy tissue of infected plants, or homogenates of seedlings from plants with tumours. Numerator: number of plants with tumours after four weeks; denominator: number of plants tested.

Inoculum prepared from	Score from		
	N. clevelandii	*N. megalosiphon*	*N. rustica*
Healthy stem part of plant with tumours	0/98	4/60	3/27
Stem parts with tumours	78/118	60/60	14/34
Seedlings grown from seeds of plants with tumours	2/17	1/200	0/200

Tabel 2. Reactie van zaailingen van N. megalosiphon na dompeling van hun wortels in homogenaten van tumorweefsel, homogenaten van gezond weefsel van aangetaste planten, of homogenaten van zaailingen opgekweekt uit zaad van planten met tumoren. Teller: aantal planten met tumoren na vier weken; noemer: aantal getoetste planten.

Fig. 7. Young bulbs of *Lilium* Mid Century Hybrid 'Enchantment' formed on the underground part of the stem of the mother plant. Bulb at upper left is healthy, the others show deformed, elongated bulb scales and excessive clustering.

Fig. 7. Jonge bolletjes van Lilium Mid Century Hybrid 'Enchantment' die op het ondergrondse gedeelte van de moederplant worden gevormd. Het bolletje bovenaan links is gezond, de andere zijn misvormd. Ze hebben verlengde bolschubben en worden in abnormaal grote aantallen gevormd.

infected too. This method was very suitable to test homogenates of tumours of *Gladiolus*, because the submersion test could not be used due to the phytotoxic substances present in *Gladiolus*.

Occurrence of a tumour-inducing agent in the plant. Both tumorous tissue and healthy-looking parts of *N. clevelandii, N. megalosiphon* and *N. rustica* plants infected with the tumour-inducing agent of *Begonia,* were tested for infectivity using *N. megalosiphon* seedlings. Also seedlings originating from plants with tumours were tested (Table 2). A high infectivity was found for the inocula prepared from tumorous tissue. Inocula from plant parts showing no symptoms had a low degree of infectivity. Transmission via seed occurred sporadically.

To locate the tumour-inducing agent a big *N. clevelandii* tumour was divided into three equal parts. One part was homogenized without further treatment, the second and the third were shaken in a detergent solution and then washed in water. The second part was then homogenized, the third one was peeled and subsequently homogenized. All three homogenates were tested on *N. megalosiphon* seedlings. The results were: 44 out of 59, 5 out of 43, and 20 out of 49 seedlings developed tumours after treatment 1, 2 and 3, respectively. This indicated that the tumour-inducing agent was present on the surface and inside the tumour.

Persistence of infectivity in homogenates of N. megalosiphon tumours. Dilution end-point in homogenates was between 10^3 and 10^4. Thermal inactivation started at 48°C and was complete after 10 min at 50°C.

The infectivity was hardly affected after 10 days storage at room temperature, then it decreased slowly but was still present at a low level after 100 days.

Changing the pH of the tumour homogenates with acetic acid or KOH from the original 5.5 to values between 4.5 and 9.8 did not affect their infectivity.

Transmission of infectivity by aphids. Aphids that had been starved for 4 h were placed on *N. tabacum* 'White Burley' tumours for 3 to 10 min. Batches of five aphids were then transferred to young plants of *N. megalosiphon*. Thirty of 100 plants produced tumours when using *Myzus persicae*, 7 out of 104 with *M. ascalonicus*, and 5 out of 105 with *M. ornatus*. If *M. persicae* after an acquisition feeding on *N. tabacum* 'White Burley' was kept for 18 h on a healthy *N. tabacum* 'White Burley', before being transferred to *N. megalosiphon*, only one out of 67 plants became infected. In a similar experiment in which the acquisition feeding was 18 h, none of the test plants produced tumours.

Filtration experiments. Filtration of tumour homogenates through cotton wool or filter paper hardly reduced the infectivity in some experiments. In others, however, it reduced the infectivity drastically. After filtration through a 450 nm Millipore filter the homogenate was not infectious any more.

Inactivation by propylene oxide. Propylene oxide is efficient in killing microorganisms (Tuite, 1969). By mixing a tumour homogenate with propylene oxide in the ratio 3 : 1, bacteria possibly present might be eliminated. The mixture was well shaken in a closed vessel and kept overnight at room temperature. Before testing, the propylene oxide was allowed to evaporate. Five out of 43 *N. megalosiphon* seedlings developed tumours compared with 44 out of 51 when the untreated homogenate was used.

The presence of octopine and nopaline. These substances could not be detected in tumorous tissue from *N. megalosiphon*. Furthermore arginine and γ-guanidine-butyric acid were present in normal quantities.

Isolation of bacteria. Initial experiments to isolate a bacterium failed. However, in another series of isolations we used highly diluted inocula and long growing periods, because tumour-inducing bacteria like *C. fascians* grow slowly on agar media and

their colonies are often overgrown by saprophytic bacteria. In doing so we succeeded in isolating a Gram-positive bacterium from tumorous tissue of *N. megalosiphon* infected with inocula from diseased *Begonia, P. zonale, Dahlia,* or *Lilium*. This bacterium was identified as *Corynebacterium fascians* (Tilford) Dowson. Seedlings of *N. megalosiphon* and leaf cuttings of *Begonia* dipped into a suspension of the isolated bacterium developed tumours after 14 and 60 days, respectively. From these tumours *C. fascians* could be re-isolated.

The question remained whether the tumorous tissue with which Misra and Nienhaus (1976,1977) and Gliem et al. (1976) had experimented also contained *C. fascians*. From freeze-dried tumours of *N. christii* from Nienhaus' laboratory we were able to isolate *C. fascians*. A suspension of this bacterium induced the characteristic tumours in *N. megalosiphon*.

To make sure that the tumorous tissue of our material was not contaminated by a viroid or tobacco tumor virus we tried to isolate these. Using polyacrylamide-gel electrophoresis methods which can successfully detect potato spindle tuber and chrysanthemum stunt viroids (Morris and Smith, 1977; Mosch et al., 1978) no viroid could be found in tumours. Virus purifications using differential and density-gradient centrifugation, including the method described for tobacco tumor virus (Gliem et al., 1976), failed.

Discussion

Our experiments in which *K. daigremontiana* could not be infected with inocula prepared from *N. megalosiphon* tumours, and the negative test for the presence of octopine and nopaline, indicated that the *Begonia* tumours studied were not induced by *A. tumefaciens*. Inoculation experiments using *M. officinalis* as a specific host proved that wound tumor virus was not the causal agent either.

The evidence of aphid transmission prompted us to the assumption of a virus-induced phenomenon, as did the persistance of some infectivity after propylene oxide treatment. Furthermore Gliem et al. (1976) isolated a so-called tobacco tumor virus from identical tumours of *N. christii*. We could not confirm their results. All attempts to isolate a virus or a viroid failed.

From the facts that homogenates of tumorous tissue from *Begonia, Pelargonium, Dahlia, Gladiolus* or *Lilium* induced only one type of tumours in *N. megalosiphon*, and that from these tumours *C. fascians* was isolated and *C. fascians* suspensions induced typical tumours in *N. megalosiphon* and *Begonia,* we conclude that in all cases there is only one tumour-inducing agent: *C. fascians*.

Our isolation of *C. fascians* from freeze-dried tumour material obtained from Nienhaus' laboratory may explain why Misra and Nienhaus (1977) could repress tumour development with antibiotics.

Aphids transmission of *C. fascians* may be explained by assuming the transfer of the bacterium on the legs of the aphids or with the brush used to transfer the aphids.

The very useful test to ascertain the presence of *C. fascians* developed by Van Hoof (1978) and the possibility of the inactivation of the bacterium in homogenates of tumours after 10 min at 50°C offer ways to free *Begonia* material from *C. fascians*.

Although we proved that there is a direct causal relationship between *C. fascians* and tumour development, it is not unequivocally clear what really induces the

tumours. As with *A. tumefaciens* a plasmid carried by the bacterium (Schilperoort et al., 1975) may be the tumour-inducing agent.

Samenvatting

Tumoren in Begonia en enkele andere sierplanten veroorzaakt door Corynebacterium fascians

In 1975 werden vele tumoren waargenomen in *Begonia* 'Schwabenland' op Aalsmeerse bedrijven (Fig. 1). De infectiositeit van tumorweefsel kon goed en snel worden vastgesteld door de wortels van zaailingen van *Nicotiana megalosiphon* in een homogenaat van tumorweefsel te dompelen. Tumoren ontstonden na twee weken, de eindbeoordeling geschiedde na een maand (Fig. 2). Ook verschillende andere *Nicotiana* spp., *Melilotus officinalis* (Fig. 3) en *Pisum odoratum* (Fig. 4) werden aangetast.

Bij de infectiositeitstoets gaven zeer korte dompeltijden even goede resultaten als langere (Tabel 1). Infectieus sap verloor zijn infectievermogen na 10 min verhitting bij 50°C. Bladluizen brachten de smetstof over. Propyleenoxide verminderde de infectiositeit wel, doch onderdrukte deze niet totaal. Bij filtratie door een 450 nm filter bleef het infectieuse agens op het filter achter. Het tumor-inducerende agens was ook aanwezig in die delen van planten met tumoren welke gezond leken en het ging voor een gering deel over met zaad (Tabel 2).

Uit tumoren konden wij geen tabakstumorvirus of een viroïde isoleren. Culturen van *Corynebacterium fascians*, geïsoleerd uit tumoren van *N. megalosiphon* bleken zeer infectieus en veroorzaakten tumoren in *N. megalosiphon* en *Begonia*. Homogenaten van tumorweefsel van *Pelargonium zonale,* dahlia (Fig. 5), gladiool (Fig. 6) en *Lilium* Mid Century Hybrid 'Enchantment' (Fig. 7) veroorzaakten ook tumoren op *N. megalosiphon*, waaruit *C. fascians* werd geïsoleerd. Met sap van kroeskopzieke rozen konden wij *N. megalosiphon* niet besmetten.

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