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Is *Xanthomonas campestris* pv. *begoniae* transmitted in meristem culture? Inoculation trials with *Begonia elatior* and testing for bacteria in meristem plants

Overføres Xanthomonas campestris *pv.* begoniae *ved meristemkultur? Infektionsforsøg i* Begonia elatior *og bakterietestning af meristemplanter*

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Summary

Inoculation trials with *Xanthomonas campestris* pv. *begoniae (Xcb)* in *Begonia elatior* showed a shorter period of latency during the spring and summer months (10-14 days) compared with the winter period (20-40 days).

Meristems, about 0.25 mm, were excised from heavily infected mother plants. Established meristem plants *in vitro* were tested twice for bacteria both by the indirect immunofluorescence method and by plating on agar 6 and 12 months after excision of the meristems. In this introductory investigation, no transmission of *Xcb* with the meristems was demonstrated.

Key words: *Xanthomonas campestris* pv. *begoniae, Begonia elatior,* latent period, meristem culture, transmission, bacteria testing, immunofluorescence.

Resumé

Infektionsforsøg med *Xanthomonas campestris* pv *begoniae (Xcb)* i *Begonia elatior* viser, at latenstiden er betydelig kortere i forårs- og sommermånederne, 10-14 dage, end i vinterperioden, 20-40 dage.

Fra kraftigt inficerede begoniemoderplanter er skåret meristemer på ca. 0,25 mm. Etablerede meristemplanter *in vitro* er testet to gange for bakterier ved den indirekte immunofluorescensmetode og pladespredning henholdsvis ca. 6 og 12 måneder efter skæring af meristemerne. I denne indledende undersøgelse er der ikke konstateret overførsel af *Xcb.*

Nøgleord: *Xanthomonas campestris* pv. *begoniae, Begonia elatior,* latenstid, meristemkultur, smitteoverførsel, bakterietestning, immunofluorescens.

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Fig. 1. Symptoms on *Begonia*-leaf after infection with *Xanthomonas campestris* pv. *begoniae*.
Begonieblad smittet med Xanthomonas campestris *pv.* begoniae. *(Photo: Karen Bech).*

Introduction

Begonia cultivation is made difficult by attacks by the bacterium *Xanthomonas campestris* pv. *begoniae (Xcb)* (Takimoto 1934, Dye, 1978), the cause of bacterial blight of begonia.

Bacterial blight of begonia is widespread wherever begonia are grown and, the disease can cause much damage, in particular following periods of poor control of the glasshouse climate (15). Sometimes the disease may have a long period of latency before symptoms (Fig. 1) suddenly appear (4, 21), e.g. after periods with high humidity and high temperatures (20).

The aim of this investigation was through inoculation trials, to learn about seasonal variations in the length of the latency period and following excision of meristems from infected plants, to assess the risk of transmission of *Xcb* via the meristems.

Methods

Begonia elatior hybrids of the cultivars 'Nixe' and 'Elfe' were grown under normal glasshouse conditions, i.e. 18-20°C during the winter period, and in summer the ventilators were opened at 25°C. To keep the plants vegetative supplementary light was given during the winter period so that the day length was just above 14 hours. The plants were watered from beneath with 0,1 per cent nutrient solution.

Inoculation trials

Inoculum

For the first inoculation, a 48-hour old pure culture of *Xcb* isolated from naturally infected begonia plants was prepared. Inoculum density was 2×10^8 cfu/ml.

On nutrient agar (NA), the Gram negative non-spore-forming bacteria form yellow colonies. Biochemical reactions were: Kovacs' oxidase negative (10), nitrate reduction negative, acid produced aerobically from glucose but not anaerobically (9), aesculine positive, soft rot of potato tissue negative. The bacteria culture was also tested with the indirect immunofluorescence (IF) method (18). A polyclonal antiserum was used against *Xcb* produced and tested at the Research Centre for Plant Protection. Dilution 1:200.

For subsequent inoculations leaves with typical symptoms of bacterial blight was used. The leaves were comminuted in sterile water and then shaken for 1 hour. A sample was then taken for the IF-test and plated onto NA. Shaking of the leaves

continued until the following morning when the liquid from the agitated sample was used for inoculation of healthy begonia plants.

Inoculation of plants
Inoculation was made by injecting the bacterial suspension into stems or leaf veins or by atomizing the bacterial suspension over the entire plant surface. Then the plants were grown under high humidity for 48 hours to encourage infection. Control plants were inoculated by injecting or atomizing with sterile water and then placed under humid conditions.

Registration of symptoms
The plants were visually assessed for symptoms twice a week from inoculation until appearance of symptoms.

Meristem culture
Meristems were excised from bacteria-inoculated plants showing symptoms and from water-inoculated begonia control plants, respectively.

The meristem culture started with the excision of explants measuring approx. 0.25 mm and consisting of the meristem dome and 1-2 leaf primordia. Explants were excised from both apical and axillary buds.

The excised plant material was not disinfected.

For culturing Murashige and Skoogs medium (12) was modified to contain 50 per cent concentration of the macronutrients. To the medium was added sterile filtered growth regulators comprising furfurylaminopurine (FAP) and indolyl acetic acid (IAA) in varying combinations and concentrations. 0.5-1 mg/l FAP and 1 mg/l IAA was used for the establishment of the cultures. As the explants developed they were transfered (approx. every 1½-2 months) to a new medium with lower concentrations of 0.2 FAB and 0.2 IAA mg/l and later, to stimulate root development, to 0.1 ml/l IAA. 7 g/l of agar was added to all the media.

The ability of the media to support the growth of *Xcb* was tested for two inoculation methods: streaking bacteria on the surface of the medium and by stabbing with an inoculation loop dipped in bacteria suspension (Fig. 2).

Meristem cultures were grown *in vitro* in a growth room kept at a day and night temperature of 20-18°C and with 16 h illumination from a Philips flouorescent lamp 'cool white' TLF 40 W/33 (10 Wm^{-2} PAR).

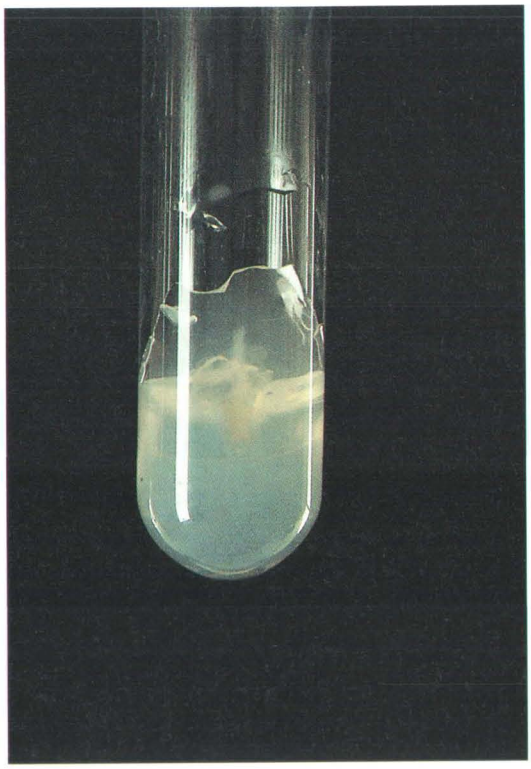

Fig. 2. Growth of *Xanthomonas campestris* pv. *begoniae* on Murashige & Skoogs medium after inoculation.
Vækst af Xanthomonas campestris *pv.* begonia *på Murashige & Skoogs medium efter podning.*
(Photo: Jens Begtrup).

Several contaminated cultures were analysed for possible attacks by fungi or bacteria.

Bacterial testing
When explants had been excised from mother plants infected with bacterial blight the tissue immediately below the explant or the leaf nearest to the explant was tested for *Xcb*.

The meristem plants were tested for the first time after about 6 months of *in vitro* culture, where the base of the established plant was tested. From the apical part of the plants new *in vitro* cultures were established and these were tested again about 6 months later.

The indirect immunofluorescence method (IF) was used for all the tests. The plant tissue for the IF-test was comminuted and shaken in sterile

water for 16 hours (7). Samples for IF staining were taken from the liquid, concentrated and diluted 1:50, before and after centrifugation. To test that the IF-staining was correctly carried out, a positive sample of *Xcb* pure culture, and a negative sample, from a healthy plant, were included. Nikon Epi-fluorescence equipment was used for microscope examination (1000 X).

The samples were furthermore streaked out on NA to check for bacterial growth.

An outline of the trials is shown in Fig. 3.

Results

Inoculation trials

Atomization of the bacterial suspension over the plant surface caused characteristic symptoms on the plants. This method of inoculation was therefore preferred to injection of the bacterial suspension into stems and leaf veins where symptoms did not always appear. The control plants showed no symptoms.

By shaking comminuted leaf material with symptoms a more virulent bacterial inoculum was

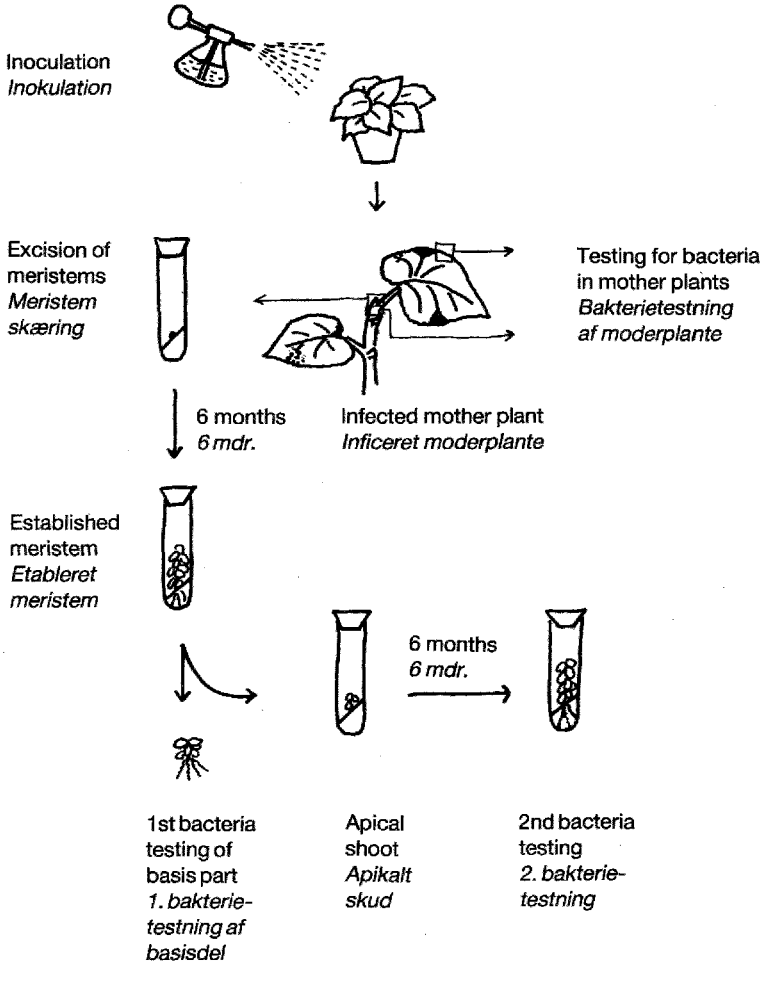

Fig. 3. Outline of inoculation and bacteria testings.
Oversigt over inokulation og testning for bakterier.

Table 1. Periodes of latency at different inoculation times.
Latensperioder på forskellige årstider.

Seasonal infection *Årstid for smitte*	Water-soaked leaf spots visible *Vanddrukne bladpletter synlige*	Yellowing of leaves and symptoms of wilting *Gulfarvning af bladvæv og begyndende visning*
March	20-28 days/*dage*	
April	11-13 –	25-30 days/*dage*
July	10-14 –	
August	14 –	
September	35 –	
November	35-40 –	60 –

obtained compared to a suspension of bacteria obtained from NA.

The seasonal variation of the period of latency from inoculation until the appearance of symptoms is given in Table 1.

Meristem culture

Of the 516 explants excised from begonia plants infected with bacteria, 141 gave rise to meristem plants (27 per cent), 336 plants were contaminated with fungi (65 per cent) and 39 explants failed to grow (8 per cent).

The results from the establishment of the meristem cultures are given in Table 2.

A high degree of contamination of the *in vitro* cultures occurred. The degree of contamination of the meristem cultures from the bacteria inoculated plant material was much higher than from the control group.

The contamination percentage of explants from 'Nixe' plants infected by bacteria was the same whether or not *Xcb* was found in the stem of the mother plant. In infected 'Elfe' plants most contaminations were from stems where *Xcb* was not found.

From *Xcb* infected mother plants 141 *in vitro* meristem plants were established. None of these showed any form of contamination or turbidity of the growth medium that could be attributed to bacteria.

In all the inoculation trials with *Xcb* (5 tubes per media) the media used showed conspicuous cloudlike turbidities.

Bacterial testing

At the time of excision of the explants all the infected mother plants had typical leaf symptoms characteristic for bacterial blight. In the cultivar 'Nixe', the leaves nearest to the explants from the 14 mother plants were tested and these were all found to be *Xcb* positive.

Grouped samples of stem tissue, taken 0-3 mm below the explants, were examined for *Xcb* immediately after the excision of the meristems. In 10 out of 11 samples from the cultivar 'Nixe' and 6 out of 10 samples from infected 'Elfe' mother

Table 2. Establishment of meristem cultures.
Etablering af meristemkulturer.

Mother plant *Moderplante*	Meristems *Meristemer*					Bacteria testing *Bakterietest*	
	Excised *Skåret*	Contaminated *Forurenet*		Growing *Ivækst*		No. of months after excision *Antal mdr. efter skæring*	
Cultivar *Sort*	No. *Antal*	No. *Antal*	% *pct.*	No. *Antal*	% *pct.*	1st testing *1. testning*	2nd testing *2. testning*
Xcb infected/*inficeret*							
'Nixe'	316	273	(86)	33	(11)	6	12
'Elfe'	200	63	(32)[1)]	108	(54)	5	–[2)]
Control/*kontrol*							
'Nixe'	50	7	(14)	33	(66)	10	16
'Elfe'	50	1	(2)	48	(96)	11	17

1) 50 tubes examined for fungi and bacterial growth.
50 rørglas analyseret for svampe og bakterieangreb.
2) Not made. *Ikke udført.*

Table 3. Testing for *Xanthomonas campestris* pv. *begoniae* in meristem plants excised from infected *Begonia elatior*. *Testning for* Xanthomonas campestris *pv.* begoniae *i meristemplanter skåret fra inficerede* Begonia elatior.

Mother plant *Moderplante*		Xcb established in No. of totally tested samples *Xcb påvist i antal af de i alt testede prøver*					
		Mother plants *Moderplanter*		Meristem plants / *Meristem planter*			
				1st testing *1. testning*		2nd testing *2. testning*	
Cultivar *Sort*	No. *Antal*	IF[1] leaves *blade*	IF[1] stem[3] *stængel*[3]	IF[1]	NA[2]	IF[1]	NA[2]
Xcb infected/*inficeret*							
'Nixe'	14	14/14	10/11	0/33	0/33	0/33	0/33
'Elfe'	10	–[4]	6/10	0/108	0/108	–[5]	–[5]
Control/*kontrol*							
'Nixe'	2	0/2	0/2	0/16	0/16	0/15	0/15
'Elfe'	2	0/2	0/2	0/19	0/19	0/14	0/14

1) Indirect method, immunofluorescence. *Indirekte metode, immunofluorescens.*
2) Nutrient agar. *KPA-agar.*
3) Stem tissue just below meristem. *Stængel lige under meristem.*
4) Not tested, symptoms on the plants. *Ikke undersøgt, men planterne viste symptomer.*
5) Not tested. *Ikke undersøgt.*

plants *Xcb* was found. The control plants were all *Xcb* negative (Table 3).

All the samples from both 1st and 2nd bacterial testing of the meristem plants were *Xcb* negative (Table 3). On some of the NA plates, whitish and sometimes yellow bacteria colonies developed. Representative bacteria colonies were grown in a pure culture and tested, partly by a biochemical test (1, 2, 21) and partly by IF on the bacteria pure cultures. All the sampled colonies were *Xcb* negative.

Discussion

Inoculation trials

Results from the inoculation trials show that the latency period is considerably shorter in the spring and summer months (10-14 days), than in winter (20-40 days), which is probably due to the climate. The optimum temperature for *Xcb* is approximately 28°C (1, 21) and high humidity will also favour bacterial growth (5, 20).

In Dutch experiments carried out in April in growth chambers with optimum conditions for bacterial growth the time of latency was 1 week, when an inoculum with high bacteria density (10^8 cfu/ml) was applied. When 10^2 cfu/ml was applied the first weak, symptoms could be observed after 3 weeks (5). In spring, *Strider* (20) found a latency period for leaves with and without lesions, after inoculation with 2×10^7 bacteria/ml of 8 and 12 days respectively. The time of latency in these experiments correspond with the results from the Danish infection trials carried out in April, when unwounded plants were inoculated with solutions with high bacteria densities. *Strider* (20) also found that the longer the plants were in the mist chamber (0-96 hours, temperature above 20°C) the more lesions developed. Wilting of plants was evident 4-6 weeks after inoculation.

Digat (4) found that large concentrations of *Xcb* could live on the leaf surface of begonia plants for 50 days at 20°C ± 5°C before symptoms developed. A bacteria density of a little less than 10^4 caused no symptoms after 76 days. In this experiment *Xcb* lived latently on the leaf surfaces for 40 days in winter and this observation can explain why disease problems may suddenly arise in apparently healthy begonia cultures after a period of – for the plant – unfavourable climatic conditions.

Meristem culture

One of the most important objectives of using meristem culture is the elimination of pathogens because attacks by vascular pathogenic bacteria and fungi as well as virus can cause great losses in glasshouse crops.

Only by excising very small meristems is it possible to isolate noninfected tissue suitable for the establishment of healthy plants.

The genetic stability of the established plant material is also of great importance. This is partly dependent on the method of tissue culture used as well as on the genetic stability of the plant material itself. Here meristem culture is often the most reliable method as, normally, the meristem contains all the genetic properties of the mother plant (16).

With regard to growth, no difference was observed between established meristem plants *in vitro* from bacteria infected and bacteria-free mother plants, respectively, because all the meristem plants were bacteria-free.

The very high percentage of contaminated explants from infected mother plants was probably due to the fact that the inoculated mother plants were very weak and had shoots near the soil surface. The material was not surface disinfected as earlier experience had shown that contamination rarely occurred. Furthermore, it was our intention not to affect the trial results by disinfection, which would kill possible *Xcb* on the plant surface.

Bacterial testing

The indirect immunofluorescence method was used to test for *Xcb*. This is the most sensitive of the serological methods (5, 11, 13, 17, 18).

Rattink et al. (15) compared isolation and plating with the IF method and found that IF is the best and most dependable method for detecting *Xcb* in symptom-free begonia plants. In the current investigation IF was also found to be very reliable and quick. Furthermore, the IF-method has the advantage that possible bacteria may propagate during the 16-hour long shaking period which can make detection more reliable.

Digat (3) observed *Xcb* more often in or on the leaves than in the petiole although the bacterium is spread systemically in *Begonia* (8, 20, 21). In this experiment, *Xcb* was also detected in all the leaf samples from infected plants but only in 76 per cent of the stem samples from the same plants.

Several investigators have shown that repeated testing over a period of time is necessary in order to detect latent infections (6, 14, 15, 19). In the present case the explants were tested twice over a period of 12 months. Approximately 6 months after excision of the explants, the 1st bacterial test of the meristem plants was made. No *Xcb* was found in the samples. In order to allow undetected bacteria time to propagate a second bacterial test was performed on 62 meristem plants after approximately 12 months after excision of the explants. In this second test, the meristem plants were also *Xcb*-free.

An additional indication that the meristems were *Xcb*-free was that no bacteria growth was observed on the media with *in vitro* plants. In inoculation trials, the MS-62 growth media proved to be suitable for the growth of *Xcb*.

German tests of pelargonium plants have shown that if shoot tips are longer than 1 mm there is a risk of transmission and propagation of endogenic bacteria during *in vitro* culture. The bacteria which were found to be latent in the meristem plant, did not grow on the growth medium used (17).

It was found that although apical meristems, from the top of the plant are less likely to be infected, they may nevertheless contain endogenic bacteria (17). It is recommended to excise explants immediately after the shoots have been harvested in order to avoid possible bacteria propagation and spread to the meristems (19).

The apical and axillary begonia explants in the present experiment were all very small, about 0.25 mm, and this probably explains why all the meristem plants were free from *Xcb*.

Conclusion

The latency period (from inoculation with *Xanthomonas campestris* pv. *begoniae* until visible symptoms appear) varies with the time of the year and is shortest in spring and summer. High humidity encourages infection.

Transmission of the bacterium *Xanthomonas campestris* pv. *begoniae* was not observed when explants about 0.25 mm long were excised from severely infected mother plants.

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