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Comparison of five stem inoculation methods with respect to phytoalexin accumulation and Fusarium wilt development in carnation

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Abstract

Five methods of stem inoculation of carnations with conidial suspensions of *Fusarium oxysporum* f.sp. *dianthi* were compared for uptake of the suspension, induction of phytoalexin accumulation and wilt development. Inoculation was performed by incision of the stem across droplets of inoculum placed on leaves, or by injection of droplets into the stem. With both methods, higher inoculum dosages led to higher wilting rates and higher phytoalexin concentrations. Injection was more effective than incision since a lower inoculum dosage was required to obtain the same phytoalexin levels. Injection therefore appears the most promising technique for the development of routine screening methods for resistance based on phytoalexin accumulation.

Additional keywords: Dianthus caryophyllus, Fusarium oxysporum f.sp. *dianthi*, dianthramides, dianthalexin, resistance screening.

Introduction

The use of Fusarium wilt-resistant cultivars is a necessity in carnation production. Breeding resistant cultivars is a complex procedure, particularly because of the variable results obtained with the present screening technique in which rooted cuttings are infected via the soil, after which wilt development is monitored during several months. Efforts are currently being made to develop alternative techniques. One possibility is an HPLC assay for dianthramides which accumulate in carnation tissue after infection with *Fusarium oxysporum* f.sp. *dianthi*. The accumulation of these phytoalexins in the first two weeks after stem inoculation is positively correlated to the level of partial resistance of the cultivar as assessed using traditional methods after several months (Baayen and Niemann, 1989). Correlations up to $r = 0.9$ have been found although less consistent results are frequently encountered. Lower correlations are often associated with insufficient accumulation of phytoalexins resulting in inadequate discrimination between cultivars. A method of stem inoculation is therefore needed which consistently induces the accumulation of high amounts of phytoalexins. At the same time such a method must also be easy to apply. The influence of inoculum dosage and two inoculation methods on phytoalexin accumulation and disease development was compared in the present study.

Material and methods

Inoculation methods. Rooted cuttings of the susceptible cultivar Adelfie were obtained from M. Lek & Zonen B.V., planted in steamed soil and inoculated three weeks later via the stem with a conidial suspension of isolate WCS 816 of *F. oxysporum* Schlecht. emend. Snyder & Hansen f.sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen race 2. The suspension had been prepared by flooding culture slants with water and adjusting the resulting suspension to 1.25×10^7 conidia ml^{-1}. Prior to inoculation, the plants had been checked to be free of *F. oxysporum* by placing stem segments of randomly chosen cuttings on potato dextrose agar (PDA) and observing fungal development. Five inoculation methods were compared. In methods I and II, an artificial pair of leaves lacking the characteristic funnel-shaped base of normal leaves had been constructed by folding Parafilm around the stem. Droplets of 20 μl inoculum were placed on the Parafilm leaves against the stem, after which the stem was horizontally incised with a scalpel blade (Swann-Morton surgical blade no. 11) through the droplets. The inoculum was usually taken up within 1-3 min. after incision. The method was described and illustrated by Baayen and Elgersma (1985) and Niemann and Baayen (1988b). In method I, a 20 μl droplet was applied to both leaves while in method II only one droplet was applied on one leaf, resulting in inoculum dosages of 500×10^3 and 250×10^3 conidia, respectively. Method III differed from method II only in that the droplet was placed on a natural leaf; the leaves used were kept dry to prevent the droplet from disappearing into the funnel-shaped leaf base. In methods IV and V, a Hamilton microliter syringe (702 LT) with disposable needle was horizontally pierced through the stem, and 5 μl inoculum injected into the minute perforation during retraction of the needle. This was done twice in method IV, the second perforation being made 1-2 mm above the first one and at cross angles with it. In method V only a single perforation was made. Plants were thus inoculated with 125×10^3 (method IV) or 62.5×10^3 conidia (method V).

Analyses. Each method was applied to 55 plants. Five of these were sampled one day after inoculation to estimate the amount of inoculum that had been taken up by the plants. Fifty-millimeter-long stem pieces directly above the inoculation site were defoliated, surface-sterilized, and homogenized for 30 sec in 10 ml of sterilized tapwater by means of an Ultra-Turrax homogenizer. The homogenates were filtered through cheese-cloth and plated out in duplo in serial dilutions on PDA. After several days, the mean number of colony-forming units (cfu, not necessarily single conidia) per segment were determined and the inoculation methods compared in an analysis of variance (ANOVA).

Two days after inoculation, another 10 plants per treatment were sampled to estimate the translocation distance of the fungal conidia in the plants shortly after inoculation. These plants were cut off, defoliated and surface-sterilized. Two-millimeter-thick slices were cut from the stem aseptically at 5, 10, 15, 20, 25, 30, 40, 50, 75 and 100 mm height above the inoculation site, placed on PDA, and scored for colony development of *F. oxysporum*. The maximum translocation distance of the inoculated pathogen was recorded and the inoculation methods were compared using and ANOVA.

Eight days after inoculation, the accumulation of phytoalexins was assayed for 20 plants per method, in four samples of five plants. Fifty-millimeter-long segments directly above the inoculation site were cut off, defoliated, weighed, homogenized in acetone

and analyzed with HPLC as described by Niemann and Baayen (1988a). The quantities of dianthalexin (DX), hydroxydianthramide B (HDB), methoxydianthramide R and B (MDR/MDB; not separated under the conditions used) and methoxydianthramide S (MDS) were determined and expressed in absorption units per gram fresh weight (1000 units = 3 μg compound). The quantities found with the various methods were compared in an ANOVA.

The development of wilt symptoms was monitored at weekly intervals for the remaining 20 plants over a two month period. Wilt symptoms were scored using the 0-5 scale described by Baayen and Niemann (1989) in which 0 = healthy and 5 = complete death. The Kruskal-Wallis test followed by Wilcoxon's test (Sokal and Rohlf, 1969) was applied to compare the distributions of diseased plants over the six symptom classes for the different inoculation methods and to construct a classification of disease levels.

Results and discussion

With all five inoculation methods, the conidial suspensions appeared to have been taken up reasonably well. One day after inoculation, higher numbers of colony-forming units were recovered with the incision methods in which higher inoculum dosages had been applied than with the injection methods (Table 1), although the cfu numbers were quite variable (CV 70-170%). The ANOVA indicated that the inoculation methods had a significant effect on the cfu number, while the LSD test revealed three significantly different levels of cfu numbers among the five methods. The maximal height reached by the fungus in the first two days after inoculation (mainly conidial transport) was 50 mm. Only minor differences were found between the methods in the translocation distance from the inoculation site; these differences were significant ($P = 0.048$) in the ANOVA but not ($P = 0.056$) in a Kruskal-Wallis test.

All inoculated plants developed wilt symptoms, but wilting rates differed between the treatments. Higher inoculum dosages led to higher phytoalexin concentrations and higher wilting rates both for incision and injection treatments (Table 2). However, the injection methods appeared to be more effective in eliciting phytoalexin accumulation and disease development than the incision methods, since a fourfold higher inoculum

Table 1. Effects of inoculation method and inoculum dosage on the recovery and translocation of propagules of *F. oxysporum* f.sp. *dianthi* in carnation cultivar Adelfie.

Inoculation method		Inoculated conidia	Recovered cfu	Maximal translocation distance (mm)	
				range	mean
I	(incision)	500×10^3	2370 a[1]	5-40	20.5 ab[1]
II	(incision)	250×10^3	420 bc	10-50	24.5 a
III	(incision)	250×10^3	1720 ab	5-50	24.0 a
IV	(injection)	125×10^3	30 c	0-20	14.0 ab
V	(injection)	62.5×10^3	170 c	0-30	10.5 b

[1] Values followed by different letters were significantly different in an LSD test at $\alpha = 0.05$ (columnwise comparison only).

Table 2. Effects of inoculation method and inoculum dosage of *F. oxysporum* f.sp. *dianthi* on the amounts of phytoalexins DX, HDB, MDB/MDR and MDS (in absorption units per gram fresh weight) accumulated after eight days in carnation cultivar Adelfie, and on the numbers of plants with disease symptoms in classes 0 to 5 after 7 weeks.

Inoculation method	Inoculated conidia	Accumulated amounts of phytoalexins				Number of plants in disease class						Disease classification
		DX	HDB	MDR/MDB	MDS	0	1	2	3	4	5	
I (incision)	500×10^3	17.6 b[1]	51.1 a	32.2 a	9.6 a	0	0	0	2	4	14	a[2]
II (incision)	250×10^3	13.2 bc	27.2 b	18.0 b	4.6 b	0	0	0	11	3	6	b
III (incision)	250×10^3	12.8 bc	30.2 b	17.1 b	4.7 b	0	0	1	9	5	5	b
IV (injection)	125×10^3	24.7 a	66.0 a	26.0 a	9.8 a	0	0	0	4	6	10	a
V (injection)	62.5×10^3	11.0 c	30.2 b	11.5 b	5.3 b	0	0	3	17	0	0	c
Standard deviation		3.6	11.1	5.2	2.1							

[1] Values followed by different letters were significantly different in an LSD test at $\alpha = 0.05$ (columnwise comparison only).

[2] Classification of the frequency distributions of the plants over the disease classes by means of the tests of Kruskal-Wallis and Wilcoxon. Different letters indicate significantly different frequency distributions at $\alpha = 0.05$.

dosage was needed in the latter to obtain similar phytoalexin levels. It was apparent that the means by which conidia are brought in contact with the xylem strongly influences the development of disease and, as a reflection of the intensity of the interaction, the accumulation of phytoalexins. The relative inefficiency of incision compared with injection was not due to inadequate absorption of the inoculum (Table 1). It is suggested that the comparatively large amount of damage caused by incision compared with injection may result in more intense wound-healing responses which may have an adverse effect on disease development.

Similar results were obtained with the moderately susceptible cultivar Silvery Pink, cuttings of which were obtained from M. Lek & Zonen B.V., grown as above and inoculated with a conidial suspension (0.75×10^7 conidia ml^{-1}) of isolate WCS 816 using methods I (20 plants), IV and V (10 plants each). Seven days after inoculation, extracts were made of stem pieces of the individual plants and analyzed for the presence of phytoalexins as described above. The ANOVA showed that, as in previous experiments, similar phytoalexin levels were obtained with methods I and IV, while method V resulted in lower levels (Table 3).

With both cultivars, the residual variance for accumulation of the phytoalexins DX, HDB, MDR/MDB and MDS was found to be homogeneous, irrespective of the various inoculation methods and dosages used. It follows that the higher phytoalexin concentrations found with higher inoculum dosages and with injection compared with incision directly improve the discriminating ability of these methods. Lower sample sizes will therefore suffice for discrimination between cultivars. The twofold higher phytoalexin concentrations found with double inoculum dosages, for example, would allow for fourfold lower sample sizes.

The laborious construction of Parafilm leaf pairs around the stem, which was designed

Table 3. Effects of inoculation method and inoculum dosage of *F. oxysporum* f.sp. *dianthi* on the amounts of phytoalexins DX, HDB, MDB/MDR and MDS (in absorption units per gram fresh weight) accumulated after seven days in carnation cultivar Silvery Pink.

Inoculation method	Inoculated conidia	Accumulated amounts of phytoalexins			
		DX	HDB	MDR/MDB	MDS
I (incision)	300×10^3	24.3 a[1]	55.8 a	21.4 a	11.7 a
IV (injection)	75×10^3	27.9 a	59.9 a	20.4 a	12.8 a
V (injection)	37.5×10^3	11.8 b	30.2 b	9.2 b	4.2 b
Standard deviation		9.1	13.3	5.7	4.2

[1] Values followed by different letters were significantly different in an LSD test at $\alpha = 0.05$ (columnwise comparison only).

to ensure the uptake of the inoculum droplets, appeared to be redundant since no significant differences were found between methods II and III (Table 2). Injection of inoculum appears a promising alternative to the currently employed technique. Injection also offers better potential for automated application, which would be of great advantage if phytoalexin accumulation is to be employed as a routine screening method for resistance to Fusarium wilt.

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Samenvatting

Vergelijking van vijf stengelinoculatiemethoden ten aanzien van de accumulatie van fytoalexinen en het verloop van Fusarium-verwelkingsziekte bij anjer

Vijf methoden om anjers via de stengel te inoculeren met een sporensuspensie van *F. oxysporum* f.sp. *dianthi* werden vergeleken wat betreft de opname van de suspensie, de accumulatie van fytoalexinen en het ziekteverloop. Inoculatie vond plaats door de stengel aan te snijden dwars door een druppel inoculum die op een blad was gelegd, of door druppeltjes inoculum te injecteren. Bij beide methoden leidden hogere doses inoculum tot heviger ziektesymptomen en accumulatie van grotere hoeveelheden fytoalexinen. Injectie van inoculum was effectiever dan aansnijden daar er minder inoculum nodig was voor eenzelfde resultaat. Injectie biedt daarom meer perspectief voor de ontwikkeling van routinematige resistentietoetsen op basis van de accumulatie van fytoalexinen.

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