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Two *in vitro* assays to evaluate resistance in *Linum usitatissimum* to *Fusarium* wilt disease

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Abstract

Two types of *in vitro* seedling tests were developed to evaluate resistance in flax (*Linum usitatissimum*) against *Fusarium oxysporum* f.sp. *lini.* In the first test a solid medium was used. The second test was based on a liquid medium. Disease severity was assessed after three weeks, using the observed reduction of plant length as a scale. Both methods proved to be useful for screening for resistance, for evaluating race specificity of resistance and for studying symptomatology. The solid medium method proved to be the more accurate for the screening, but the liquid medium method was much less time- and labour-consuming. The results of the tests were significantly correlated with observations under field conditions.

Introduction

In plant breeding, efficient selection for disease resistance depends on the availability of representative tests such as trials in field plots, greenhouse tests and *in vitro* tests. The *Fusarium* wilt disease, caused by *Fusarium oxysporum* f.sp. *lini* (Bolley) Snyd. & Hans., results in economic damage in flax and linseed (*Linum usitatissimum* L.) (Beaudoin, 1988). Within the species *Linum usitatissimum*, flax and linseed are two distinguishable groups. Flax is grown for fibres while linseed is grown for oil. The two groups differ much in agronomic characters. Linseed has reduced height, more branching and a later harvest time compared with flax. Field observations in a flax wilt nursery in Normandy, France, gave rise to speculation that there might be a difference between the two groups in *Fusarium* resistance. Very little is known about the interaction between *Fusarium* and either flax or linseed. Nor is much known about the resistance mechanisms in flax and linseed or about the infection process and the way the fungus develops in the flax and linseed tissues. This lack of knowledge is largely because of the lack of reliable *in vitro* screening methods, which would allow histological studies of the plant-pathogen interaction.

All over the world, screening for *Fusarium* resistance is included in breeding programmes for flax and linseed (Liu et al., 1993; Ondrej, 1993; Kenaschuk and Rashid, 1993; 1994; Li et al., 1994; Popescu et al., 1994; Gent, 1995). Chlamydospores of the fungus are difficult to destroy by agrochemicals and resistant cultivars are the only effective way to control the disease. Conventional methods for screening of resistance in flax and linseed consist of field trials at infested sites or flax wilt nurseries, with visual assessment of wilt development. Such trials give highly variable results and therefore require large numbers of replications, both in space and time. Most soil types contain different species of *Fusarium*, and the infestation patterns of *Fusarium oxysporum* in the soil may be variable (Tamietti and Pramotton, 1987). Because of interactions between pathogenic and non-pathogenic *Fusaria* (Davis, 1966) and because the severity of the disease is influenced by soil type (Alabouvette et al., 1982), it is difficult to determine how different flax and linseed cultivars react at different locations. Also the existence of races of *Fusarium oxysporum* f.sp. *lini*

has been reported, based on field, pot and greenhouse tests (Broadfoot, 1926; Borlaug, 1945; Kulkarni et al., 1969), but these reports are not clear cut. Using a greenhouse test, Fouilloux and Chaboche (1996) found no indication of race specificity.

The genetic system of *Fusarium* resistance in flax and linseed is complex (Popescu, 1995) and a simple and reliable screening test would be of great help for detecting the genes involved. Davis (1966) pointed out that microfloral contaminants competing with fusarial wilt pathogens and cross contamination among *formae speciales* cannot be excluded using greenhouse tests, so *in vitro* screening methods are highly desirable. In the present study, two recently developed *in vitro* tests were evaluated for use in resistance breeding. To be able to compare the *in vitro* tests with existing screening methods, a comparison was made with data, obtained from observations in 1991 of a flax-wilt nursery in Normandy (Beaudoin, 1991), whereby a scoring method was used as described by Rashid and Kenaschuk (1993). In short, the score is based on symptoms such as percentage of wilted and dead plants, and can vary between 1 (resistant) and 9 (fully susceptible). This nursery serves as a guideline for determining *Fusarium*-resistance in cultivars listed in the French descriptive list for new cultivars (Anon., 1992). Furthermore an evaluation is made of the use of the two tests in research on more fundamental aspects of the *Fusarium*-flax interaction, such as the study of race specificity and infection and colonisation patterns.

Materials and methods

Host

Flax seeds from 'Ariane', 'Belinka', 'Laura', 'Marina', 'Regina', 'Saskia' and 'Viking' (CPRO-DLO stock collection) and 'Hermes' (Landbouwbureau Wiersum, Dronten, the Netherlands) and linseed seeds from 'Atalante', 'Barbara' and 'Linda' (CPRO-DLO stock collection) were used. Directly before use, the seeds were sterilised for 15 s in 70% ethanol, followed by 15 min in 1% hypochlorite.

Pathogen

Single-spore cultures of *Fusarium oxysporum* f.sp. *lini*, originating from wilted flax straw of 'Regina' grown in a flax wilt nursery in Ingelmunster, Belgium (B2) and from wilted straw from a flax wilt nursery in Normandy, (F1) were provided by Dr. G. Marshall, The West of Scotland College, UK. Stock cultures were kept for long time preservation at -80 ° C on Protect Bacterial preservers (Technical Service Consultants Ltd, UK). Before use, the stock cultures were grown on potata dextrose agar (PDA) in the dark at 24 °C for 14 days.

In vitro *screening*

Method 1

Test tubes of 15 mm in diameter and 250 mm high were filled with 0.65 g vermiculite and 7.0 ml of a 10% Murashige Skoog-solution (MS, Murashige and Skoog, 1962). The pH was adjusted to 5.8. The tubes were covered with glass caps and autoclaved for 20 min. One seed per tube was sown, and 20 tubes for each cultivar per isolate treatment (10 cultivars and two isolates) were prepared. The tubes were placed in a climate chamber at 22 °C with 16 h light (Philips 84 HF, 1100 lux) per day. After six days, when the cotyledons had just opened, ten seedlings were selected on the basis of least variation in length from each set of 20 tubes. In this test, ten cultivars were exposed to two isolates while in addition ten replicates per cultivar were treated with sterile water as a control, so the test consisted of ten inoculated replicates per cultivar/isolate treatment. The selected seedlings were inoculated by adding 1 ml spore suspension of one of the two isolates, containing 10^5 spores ml^{-1}, to the vermiculite. For the controls, 1 ml sterile water was used. After treatment, the seedlings were placed back in the climate chamber, in a randomised block design of ten blocks. To determine the best parameters for screening, disease symptoms as well as shoot lengths were measured.

The following disease symptoms were observed: yellowing of leaves, brown spots on leaves, wilting of the apex, necrosis of the apex and necrosis of the stem. Because of cultivar-dependent re-growth after damage, which influenced the scores, re-growth as a parameter for disease symptoms was also taken into account. The observations of the different disease symptoms and the occurrence of growth after damage were done at day seventeen. Length of the shoot was measured at 2, 4, 6, 8, 11 and 17 days after inoculation.

Method 2

A 5 cm high strip of filter paper was placed on the inside of the wall of two-litre preserving jars and 100 ml of a 10% MS-solution was poured into the jar. The jars were autoclaved for 20 min. In this test, eight cul-

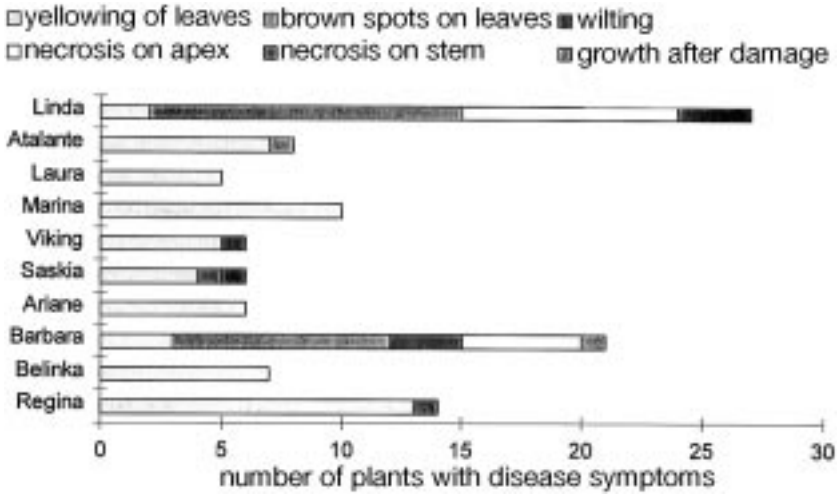

Figure 1. Number of plants of a range of cultivars showing a number of disease symptoms, seventeen days after inoculation using method 1. Cultivars are ranked according to susceptibility as defined by Beaudoin (1991) with the most resistant at the top.

tivars were exposed to two isolates. Sixteen sterilised seeds per jar were placed in a randomised block design in 45 jars, i.e. two replicates per cultivar per jar. The seeds were placed on the upper edge of the paper, so that the young roots could develop between glass and paper. The outsides of the jars were covered with aluminium foil to protect the developing young roots from direct light. The jars were placed in a climate chamber at 22 °C with 16 h light (Philips 84 HF, 1100 lux) per day. After six days, the seedlings were inoculated by adding 1 ml spore suspension of 10^5 spores ml^{-1} close to each seedling, between the paper and the glass. Sterile water (1 ml) was added to the controls. Fifteen jars were inoculated with the isolate B2, 15 jars with the isolate F1 and 15 jars with sterile water as a control. The test thus consisted of 30 replicates per cultivar/isolate combination. The shoot lengths of the seedlings were measured 21 days after inoculation.

Statistical procedures

Data of the shoot length measurements from both methods at the final days of the respective tests were subjected to an analysis of variance (ANOVA) and rating of disease severity per cultivar was determined subsequently as% length reduction compared to the average values of the control tests. Disease rating per experiment was calculated as a grand mean over the results with the isolates used. Normal correlations and rank correlations were calculated between the results of the different data sets, for the *in vitro* experiments and the field data.

Results

Disease symptoms

Disease symptoms from the test tube experiment (method 1) varied with the different cultivars (Figure 1). With all cultivars the disease was sometimes expressed by a yellowing of the leaves, but not all diseased plants showed this symptom. Several plants of some cultivars showed development of necrotic spots on the leaves (‘Barbara’, ‘Saskia’, ‘Atalante’ and ‘Linda’), or reacted with death of the apex (‘Barbara’ and ‘Linda’). Cultivars ‘Barbara’ and ‘Viking’ started to wilt first and died shortly afterwards, whereas in other cases stem necrosis occurred, followed by death of the seedling or by re-growth from the plant parts located beneath the necrotic portion (CVS ‘Barbara’ and ‘Linda’). Because the disease symptoms differed so much between cultivars, no overall disease scale could be used. The different symptoms were noted separately and were compared with field data of the flax wilt nursery in Normandy. As can be seen in Figure 1, neither single disease symptoms nor cumulative symptoms gave good correlations with field data. Compared to the other observed disease symptoms, the most common symptom ‘yellowing of leaves’ had the highest correlations with the field data, but even when the data from both isolates B2 and F1 were summarized the correlation of yellowing of leaves with the field data was not significant, (r=0.38, see Figure 1 and Table 3).

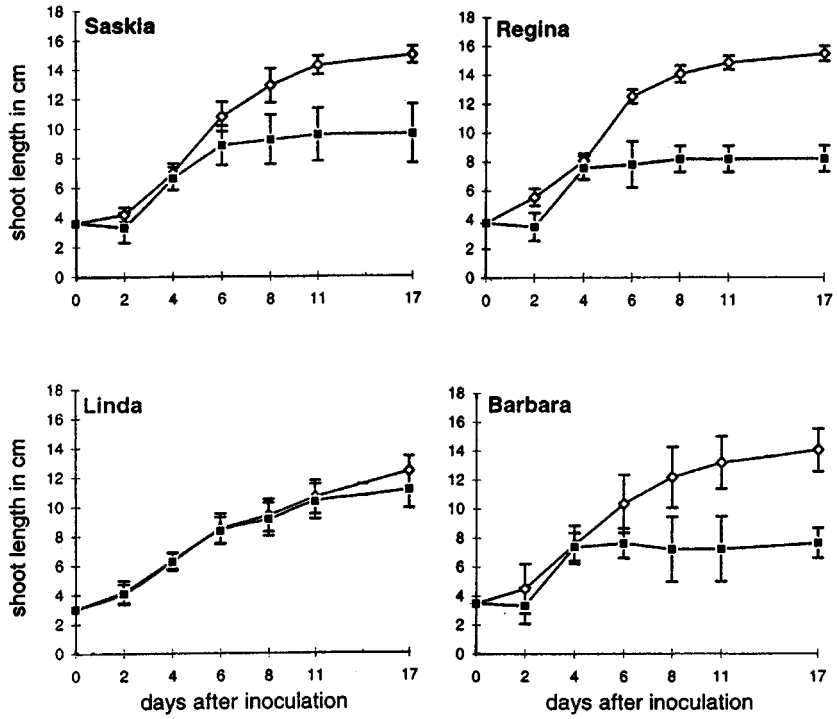

Figure 2. Shoot length of two cultivars of resistant and susceptible flax ('Saskia' and 'Regina') and two of resistant and susceptible linseed ('Linda' and 'Barbara'), following inoculation with *Fusarium* isolate Fof-B2 (♦), or with sterile water (◊), using method 1. Standard deviations shown by error bars.

Length measurements

Method 1

A large variation in shoot length was found for all cultivars. The average length at the end of the experiment was 12.2 cm. The development of one of the the less affected and the most affected flax cultivars ('Saskia' and 'Regina' respectively) and the least affected and the most affected linseed cultivars ('Linda' and 'Barbara' respectively), inoculated with the isolate B2 and compared with the controls is shown in Figure 2. ANOVA (Table 1) showed that differences between isolates as well as cultivars were important. The isolate × cultivar interaction was significant, but the contribution to the total variance was relatively small. In Table 2 the average lengths as% of the controls for ten cultivars and two isolates are presented.

Method 2

A large variation in shoot length was found for all cultivars and for the two isolates used in method 2. The average total shoot length at the end of the experiment was 8.3 cm, which was considerably less than the average length from method 1, 12.2 cm. ANOVA showed again that the differences between isolates as well as the differences between cultivars were major factors (Table 1), while the cultivar × isolate interaction variance, though significant, was again small. The mean shoot lengths at the end of the experiment, as% of the controls are listed in Table 2.

Correlations between screening methods

The most significant correlations with the field data were found using method 1 and isolate B2, the more aggressive isolate (Table 3). The correlation coefficient for the only qualitative parameter which might possibly be useful, namely 'yellowing of leaves', was correlated only on a significant level ($P<0.05$) with length reduction as caused by isolate B2 using method 1, and the average disease rating (ADR) determined by method 1. Method 1 (isolate B2 and ADR) correlated well with the French field data, but method 2 did not correlate at a significant level with the field data, although the regression coefficient with B2 was similar to that in method 1. Comparable correlation coefficients were found when using rank correlations. The only differences were that no significant correla-

Table 1. Analyses of Variance (ANOVA) of shoot length measurements of flax and linseed seedlings inoculated with *Fusarium oxysporum* f.sp. *lini* isolate B2, isolate F1 or with sterile water using method 1 and method 2

Method	Source of variation	D.f.	S.s.	M.s.	V.r.	F pr.
1	Isolate (or water)	2	1482.7	741.4	435.1	<.001
	Residual	27	46.0	1.7		
	Cultivar	9	438.4	48.7	32.7	<.001
	Cultivar × Isolate	18	186.6	10.4	7.0	<.001
	Residual	243	669.5	1.5		
	Total	299	3565.3			
2	Block	14	122.8	8.8	1.7	
	Cultivar	7	425.6	60.8	11.6	<.001
	Isolate (or water)	2	287.3	143.6	27.3	<.001
	Cultivar × Isolate	14	138.8	9.9	1.9	0.003
	Residual	322	1520.7	5.2	1.7	
	Block × Isolate × Cultivar × Plants	360	179.9	3.2		
	Total	719	2624.2			

Table 2. Disease rating of flax (F) and linseed (L), given as the% lengths of the cultivars compared with controls of the same cultivars, for the two wilt isolates B2 and F1 from method 1 or method 2, and average disease rating per cultivar (ADR). Also field data from a flax wilt nursery in Normandy, France (Beaudoin, 1991), scored according to Rashid and Kenaschuk (1993). The overall aggressiveness of the isolates for the two methods is given as the average isolate aggressiveness (AIA)

Cultivar	Type	Method 1			Method 2			Field data
		B2	F1	ADR	B2	F1	ADR	
Linda	L	89.9	93.4	91.7	96.6	108.9	102.7	1.3
Atalante	L	71.8	96.1	84.0	82.7	85.8	84.3	1.5
Laura	F	69.1	86.5	77.8	–	–	–	1.8
Marina	F	67.4	91.3	79.4	81.0	80.4	82.8	1.8
Viking	F	55.8	81.6	68.7	79.7	85.9	82.8	2.3
Hermes	F	–	–	–	98.7	100.9	99.8	2.3
Saskia	F	64.4	92.4	78.4	–	–	–	2.8
Ariane	F	51.5	80.8	66.2	88.3	88.9	88.6	5.0
Barbara	L	54.3	70.9	62.6	73.9	85.0	79.6	7.5
Belinka	F	58.3	91.4	74.9	–	–	–	8.0
Regina	F	53.0	87.7	70.4	74.3	84.8	79.4	9.0
AIA		63.6	87.2		84.4		90.1	

tions were found for the parameter for 'yellowing of leaves'. However, many significant correlations were found between the *in vitro* methods and the French flax wilt nursery, but not between the two *in vitro* methods.

Discussion

Disease symptoms

Because of the diversity of the disease symptoms between and within cultivars (Figure 1) using method 1, and the lack of correlation with the field data, these disease symptoms do not seem suitable for disease rating and resistance scoring. Jouan and Sailly (1991) developed an *in vitro* seedling test for *Fusarium*-

Table 3. Correlation coefficients (r), calculated for the disease-parameter 'yellowing of leaves' averaged over both isolates (YL), for the two isolates B2 (B2) and F1 (F1) separately using method 1 (M1) and method 2 (M2), the average disease rating for the two methods (ADR), and the results from the field data obtained from a flax wilt nursery in Normandy, France (FR)

	M1 YL	M1 B2	M1 F1	M1 ADR	M2 B2	M2 F1	M2 ADR	FR
M1YL	1.00							
M1B2	−0.69*	1.00						
M1F1	−0.42	0.62	1.00					
M1ADR	−0.65*	0.94**	0.85**	1.00				
M2B2	−0.63	0.72*	0.46	0.67*	1.00			
M2F1	−0.33	0.74*	0.29	0.61	0.84**	1.00		
M2ADR	−0.49	0.76**	0.39	0.66*	0.95**	0.96**	1.00	
FR	0.38	−0.69*	−0.42	−0.65*	−0.63	−0.33	−0.49	1.00

* Significant correlations at 5% level.
** Significant correlations at 1% level.

flax, using test tubes with vermiculite, and rating based on yellowing of leaves, wilting and death of plants. *Fusarium* spores were added immediately at the sowing date. To get a reliable disease screening the incubation time was 70 days, while three different levels of spore concentration were needed. However, the authors stated that this *in vitro* test was not an effective way to screen a large number of accessions because the test was too laborious. In the *in vitro* test for *Fusarium* disease resistance in flax developed by Van Westrhenen et al. (1995), resistance rating was also based on disease symptoms. It is not clear what kind of disease symptoms were used. The results from method 1 in the present study showed that screening using disease symptoms (Figure 1) is not very reliable.

Length measurements

Bos and Parlevliet (1995) stated that restriction of growth can be a significant disease symptom. In assessments of damage caused by *Fusarium* spp., length reduction measurements have been used successfully as a parameter for disease rating (Löffler et al., 1997). Our figures based on a percentage, compared with a healthy control, gave good correlations with resistance data obtained from the French trial using both methods in the present study.

In a pilot experiment, in which both isolates were exposed to a resistant, a moderately resistant and a susceptible flax cultivar, using both method 1 and method 2, the isolates were characterized as aggressive (B2) and moderately aggressive (F1) (Kroes, unpublished results). In the present experiment, the isolate F1 was moderately aggressive and gave a measure of discrimination between cultivars. Better results were obtained using the more aggressive isolate B2. For both *in vitro* methods, inoculation with the isolate B2 distinguished between resistant, moderately resistant and susceptible cultivars. The correlation with the French field data was better for the aggressive isolate B2 than for the less aggressive isolate F1. The French field data correspond well with the Dutch and French descriptive lists of cultivars (Anon., 1986; 1988; 1990; 1992; 1994; Ebskamp and Bonthuis, 1993, 1997) The use of the more aggressive isolate is thus recommended. Table 2 shows that there were no clear differences between linseed and flax in *Fusarium* resistance, either in the *in vitro* tests or in the field trial.

Application of in vitro *methods*

Method 1 gave the best correlation with the field data, and gave a better discrimination between the cultivars, compared with method 2. This can be explained by the fact that in method 1 a selection for even shoot length took place on the day of inoculation, removing a source of variation. This procedure was not feasible in method 2 because of the lack of separate units. However, method 2 consisted of units which were easier to work with and for that reason this method was much less time- and labour-consuming. With method 2, one person can test about three times more accessions in the same time as by using method 1. *Fusarium* resistance in flax and linseed is a very important selection criterion and it is desirable to select for this trait in a very early stage in a breeding programme. In the two *in vitro* methods 20 or 30 seeds per cultivar-isolate combination were required, while in field trials about

1000 seeds are normally used. The multiplication rate of flax and linseed is so low that screening for *Fusarium* resistance in field trials can only be performed after several multiplication steps, late in the breeding programme. Using one of the *in vitro* methods, a test for *Fusarium* resistance can be carried out at a very early stage of the breeding programme. While field trials have to be repeated for at least two years, the *in* vitro tests can be performed within the time span of one month and, provided standard cultivars and isolates are included for comparision, do not need to be repeated.

Cultivar × isolate interaction was observed but the analysis of variance showed that this interaction was of little practical importance compared with both the cultivar and the isolate effects. However, both *in vitro* methods proved to be useful in tracing even minor interaction patterns, which could be of value in obtaining a better understanding of the plant-pathogen interaction. In the experiments reported here, only external symptoms have been observed. To improve the understanding of the plant-pathogen interaction more knowledge is needed about the development of the fungus inside the plant. In particular method 2 could be used for such studies. Method 2 was based on a liquid medium and both the shoots and the roots stayed intact during the development of the disease. This is in contrast with method 1, where the roots nearly completely damped off in a very short time. Method 2 thus gives the additional possibility of performing more fundamental studies for infection and development processes of the disease both in the root system and in the shoot.

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