A Greenhouse Screening Assay for *Botrytis tulipae* Resistance in Tulips

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Abstract

As a leaf pathogen, *Botrytis tulipae* severely affects tulip bulb production. Chemical control is not desired for environmental reasons. Thus, resistant cultivars can play an important role in the control of this disease. To select genotypes resistant to *B. tulipae*, a reliable screening assay is needed. A greenhouse test was developed to establish resistance at clonal level. Removal of the wax layer by soft rubbing of the leaves was essential to obtain a homogeneous distribution of the spore suspension on the leaves. Small lesions were found within 24 hr after inoculation. Continuous leaf wetness was obtained by regular water evaporation. This was essential to obtain rapidly spreading lesions. After eight days, leaves of susceptible genotypes were completely destroyed. Large differences in resistance were found within tulip genotypes. Feeding additions to the inoculum suspension enhanced the infection process, but were unnecessary. Plants inoculated before flowering were less sensitive to *B. tulipae* than plants inoculated at flowering. Plants inoculated after flowering were most sensitive to infection. Ranking of resistance for the tested genotypes before or after flowering however remained nearly the same. The influence of factors involved in the screening test and the implications for resistance breeding are discussed.

INTRODUCTION

Worldwide, the tulip (*Tulipa* L.) is one of the most important flower bulb crops. In The Netherlands, there are 10,000 hectares producting 1 billion flowers and an export of 2 billion bulbs. Most of the cultivars belong to *T. gesneriana* and are widely used for cut flower production. Tulips are affected by the fungus *Botrytis tulipae* (Lib.) Lind. which can infect the bulbs, leaves and flowers. Infection of the leaves, particularly early in the season, can significant reduce bulb yield. Because of the rapid development and the destructive habit of the so called 'fire', fungicides are sprayed weekly on a preventive base. However, resistance to fungicides can occur within *Botrytis* (Migheli et al., 1989) and effective fungicides can become outlawed.

Cultivation of leaf resistant cultivars is an environmental friendly alternative. Breeding for resistance to *B. tulipae* requires a reliable screening test and genetic resources of resistance. Field trials as a screening assay often are variable because of weather conditions and differences in the natural flowering time of the cultivars. A detached leaf or leaf punch assay to screen for resistance would be practical. Limited space and plant material would be required and it provides the possibility to screen in seedling stage of the breeding programme. All attempts to develop a reliable detached leaf or leaf punch assay have failed. An assay to screen for *Botrytis* flower resistance of tulips has been developed at the Bulb Research Centre in the Netherlands (Van der Lans et al., 1998).

On this report, we describe the development of a greenhouse test to determine *Botrytis* leaf resistance of tulip cultivars.

MATERIALS AND METHODS

Plant Materials

Flowering-sized bulbs of 20 *T. gesneriana* cultivars and *Tulipa* accessions (Table 1) were obtained from growers. Cultivars were chosen so that variation in resistance level could be expected. Bulbs were stored dry at 20-17°C. Bulbs were planted individually in 0.3 litre pots. They were cooled for several weeks (Table 1) at 9-2°C and forced in the greenhouse at 16-20°C. They flowered in 3 to 4 weeks.

Inoculum

Two highly aggressive isolates of *B. tulipae* (Bt-Z1 and Bt960402) were used. These isolates were provided by the Bulb Research Centre (LBO), Lisse, The Netherlands. Bt-Z1 was isolated from a tulip sprout of 'Apeldoorn' grown in a greenhouse and Bt960402 was isolated from a dead lily stem. Isolates were stored on Protect Bacterial Preservers (Technical Service Consultants LTD, Lancs, UK) at -80°C for long term preservation. Before each experiment, fresh cultures were obtained by plating this stock on a Tulip Leaf Agar medium. This medium was made by grinding fresh green leaves (250 gram) in a blender with addition of 150 ml of tap water. The pulp was stored in a freezer at -20°C. Pulp was thawed in a microwave, grinded again and filled to a liter with tap water. Twelve grams of Agar no. 3 (Oxoid LTD, Hampshire, UK) were added per litre and subsequently autoclaved at 121°C for 15 minutes. The fungus was grown at 17°C with continuous black light (Philips TLD 18W/08) for two weeks. Conidia were transferred to new medium and grown for another two weeks. A conidia suspension was obtained by collecting conidia in tape water with one drop of Tween 20 per litre. Suspension of each isolate was diluted to 2.10^5 spores/ml and mixed equally.

Infection Assay

Plants were inoculated at the moment of flowering. The wax layer of the first (bottom) and second (middle) leaf of each plant was slightly damaged by hand rubbing. Each leaf was sprayed with the conidia suspension until drops were equally distributed on the leaf (approximately 4 ml per leaf). Plant material was incubated on benches in a greenhouse at 18°C and 100% humidity (under plastic). Water vaporisers were used to obtain leaf wetness. The vaporisers were turned on 12 hours after inoculation for 15 min followed by an other 15 minutes two to three times a day. Spreading lesions on the inoculated leaves were rated on a scale of 1-5 (1 = 0%, 2 = 1-5%, 3 = 5-15%, 4 = 15-50% and 5 = 50-100% affected area). Per plant an average disease rating (ADR) was calculated. The experimental design had three randomised blocks, with each block containing the 20 cultivars. Four plants in a row were used per block for all cultivars. All experiments were at least conducted twice in the same year or repeated over two years. Data were subjected to analysis of variance.

RESULTS AND DISCUSSION

The wax layer of the tulip leaves creates difficulty in obtaining an equal distribution of water droplets on the leaves. The conidia suspension flowed easily except from places were the wax layer was damaged. Therefore, it was essential to damage the wax layer artificially to obtain a homogenous distribution of the conidia suspension. This provided reproducible infections. We feel that damaging the wax layer did not remove an important resistance factor from the plant which could have influenced the outcome of the resistance levels. In nature, wax layers are damaged by wind, sand, and night frost. Inoculation of the leaves at the upper side did not show differences compared to inoculation of the bottom, indicating that stomata are probably not the main port of entrance supporting the observations of Mansfield and Hutson (1980).

The first small lesions were observed within 24 hr after inoculation. In all cultivars primary 'non-aggressive' (Price, 1969; 1970) restricted lesions were found. However, these are not informative for leaf resistance. A leaf wetness for several days stimulated the formation of 'aggressive' spreading lesions (Price, 1969; 1970) which seriously affect the plants. Price (1970) found that the humid period should extent 5 days. In our conditions high humidity was not found effective, in contrast creating a leafwet situation was very effective. The long periods of leaf wetness were achieved by regular water evaporation in a with plastic foil closed tunnel with very high humidity. In this way, the disease developed rapidly in susceptible cultivars. The

water evaporations simulated the natural morning mist, which probably is an important factor in sporulation and infection of the plant in its natural environment.

The 20 tulip cultivars were inoculated at the flowering stage. The flowers were removed to prevent that they are acting as a second source of inoculum. The disease symptoms were scored eight days after inoculation and the ratings showed a highly significant cultivar effect (Table 2). Absolute resistance was found in *T. tarda* (Table 3), which showed some necrotic lesions but no spreading lesions. This resistance unfortunately can not be used for improving the cut flower tulips because of crossings barriers (Van Eijk et al., 1991). Partial resistance was found in several genotypes, e.a. 'Flair' (probably a *T. gesneriana* x *T. greigii* hybrid) and *T. kaufmanniana* 'Johann Strauss'. Hybrids between these genotypes and *T. gesneriana* cultivars are obtained. The resistance level of 'Bellona' (ARD of 2.6) was considered to be the minimal resistance level which should be used for breeding purposes. 'Renown' and 'Christmas Marvel' were highly susceptible and after eight days the leaves were completely destroyed. Repeated screening tests over two successive years showed very similar results ($R^2 = 0.93$; Figure 1). A higher inoculum concentration enhanced the infection process (data not shown).

Addition of nutrients, i.e. pollen, to the inoculum was expected to enhance the infection process. To simulate this effect, conidia were suspended in 0.067 M KH_2PO_4 and 0.11 M glucose at a pH of 5.0 (Van den Heuvel and Waterreus, 1985). The additions to the inoculum significantly enhanced the infection process (Figure 2; Table 4). A high correlation was found between the disease ratings, five days after inoculation with additions, and the disease ratings, eight days after inoculation without additions ($R^2 = 0.92$; Figure 3). Small experiments with pollen added to the inoculum suspension produced the same enhancing effect. Although feeding additions to the inoculum suspension enhanced the infection process, they were unnecessary. Therefore, they were discontinued.

The development stage of the plant has been found to influence disease sensitivity in many plant species. To determine whether this is also true for *B. tulipae* resistance in tulips, we tested the 20 cultivars at three different plant stages: (1) before flowering, (2) during flowering and (3) after flowering. A highly significant plant stage effect was found (Table 5). Plants inoculated before flowering were less sensitive to *B. tulipae* than plants inoculated at the moment of flowering. Plants inoculated after flowering were most sensitive to infection (Figure 4). A small but significant cultivar x plant stage effect was found. Some of the cultivars did not rank identically in susceptibility when the different plant stages were compared. Inoculation at the moment of flowering is recommended, because this physiological stage can be determined. Selected resistant genotypes have to be retested during the pre- and post- flowering period.

Screening for *B. tulipae* resistance at an early stage is preferable in a tulip breeding programme. Since a leaf punch test is not available, several plants per genotype are required for reliable testing. The plant stage has to be standardized since it can only be performed 8-10 years after the crossings. Therefore, indirect selection with molecular markers linked to *B. tulipae* resistance is desired.

The simplicity of the here developed screening assay makes it possible to continue our work on the *B. tulipae* – tulip interaction in the direction of induced resistance using chemicals that interfere with plant signaling and elicitors and microorganisms that stimulate the natural defense of the plant.

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Literature Cited

Migheli, Q., Aloi C., and Gullino, M.L. 1989. Resistance of *Botrytis elliptica* to fungicides. Acta Hortic. 266:429-436.

Mansfield, J.W. and Hutson, R.A.. 1980. Microscopical studies on fungal development and host responses in broad bean and tulip leaves inoculated with five species of *Botrytis*. Physiol. Plant Path. 17:131-144.

Price, D. 1969. Tulip fire caused by *Botrytis tulipae*. Rep. Glasshouse Crops Res. Inst.1966. pp144-149.

Price, D. 1970. Tulip fire caused by *Botrytis tulipae* (Li.) Lind.; the leaf spotting phase. J. Hort. Sci. 45:233-238.

Van Eijk J.P., Van Raamsdonk L.W.D., Eikelboom W. and Bino R.J. 1991. Interspecific crosses between *Tulipa gesneriana* cultivars and wild *Tulipa* species: a survey. Sexual Plant Reproduction 4:1-5.

Van den Heuvel, J. and Waterreus L.P. 1985. Pectic enzymes associated with phosphate-stimulated infection of French bean leaves by *Botrytis cinerea*. Neth. J. Plant Path. 91:253-264.

Van der Lans, A.M., Paterkamp, H.P. and Koot, T.W. 1998. *Botrytis* in tulp: Pokkentoets bepaalt gevoeligheid van de bloem. Bloembollencultuur 109:23-24.

Tables

Table 1: Description of *Tulipa* cultivars used in the *Botrytis tulipae* experiments.

Cultivar	Code used	Species	Group	Flower colour(s)	Cold treatment (weeks)
Bellona	Be	*T. gesneriana*	Single Early	Yellow	16
Bonanza	Bo	*T. gesneriana*	Double Late	Red, edged yellow	20
Brilliant Star	BS	*T. gesneriana*	Single Early	Scarlet	15
Christmas Marvel	CM	*T. gesneriana*	Single Early	Cherry-pink	15
Couleur Cardinal	CC	*T. gesneriana*	Triumph	Scarlet	19
Early Yellow	EY	*T. gesneriana*	Triumph	Yellow	15
Flair	Fl	*T. gesneriana*[1]	Single Early	Red on yellow ground	14
Gander	Ga	*T. gesneriana*	Triumph	Magenta	15
Generaal de Wet	GW	*T. gesneriana*	Single Early	Orange	18
Ile de France	IF	*T. gesneriana*	Triumph	Red	17
Leen v/d Mark	LM	*T. gesneriana*	Triumph	Red, edged white	16
Monte Carlo	MC	*T. gesneriana*	Double Early	Yellow	16
Pax	Px	*T. gesneriana*	Triumph	White	15
Renown (3x)[2]	Re	*T. gesneriana*	Single Late	Red	20
Thule	Th	*T. gesneriana*	Triumph	Yellow, flushed red	15
Ad Rem (3x)	AR	Hybrid	Darwin hybrid	Scarlet, edged yellow	19
Parade (3x)	Pa	Hybrid	Darwin hybrid	Red	20
Princeps	Pr	*T. fosteriana*	Fosteriana	Red	18
Johann Strauss	JS	*T.kaufmanniana*	Kaufmanniana	Red on white ground	14
Tarda	Ta	*T. tarda*	Miscellaneous	White, flushed green	19

[1]Probably a *T. gesneriana* x *T. greigii* hybrid.

[2]3x = triploid and sterile.

Table 2. Analysis of variance calculated from disease ratings in tulip after *B. tulipae* infection.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	2	2.3	1.2	4.1	
Cultivar	19	342.9	18.0	64.0	<0.001
Residual	218	61.4	0.28		
Total	239	406.7			

Table 3. Average disease ratings (ADR) of various tulip cultivars tested for leaf resistance eight days after inoculation with *B. tulipae*.

Code[1]	**ADR**	**Code**[1]	**ADR**	**Code**[1]	**ADR**	**Code**[1]	**ADR**
Ta	1.0	Pr	1.8	EY	3.4	Ga	4.0
Fl	1.2	Be	2.6	CC	3.7	MC	4.1
JS	1.3	IF	3.0	LM	3.8	Pa	4.3
BS	1.3	Px	3.3	Th	3.8	Re	4.5
GW	1.5	Bo	3.4	AR	3.8	CM	4.7

[1]See table 1 for abbreviations.
Average ADR = 3.0; standard error of differences = 0.22

Table 4. Analysis of variance calculated from disease ratings in tulip inoculated with and without additions to the *B. tulipae* inoculum medium.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block	2	0.7	0.3	1.6	
Cultivar	19	727.4	38.3	178.5	<0.001
Additions	1	124.0	124.0	578.2	<0.001
Cultivar x Additions	19	31.6	1.7	7.8	<0.001
Residual	438	94.0	0.21		
Total	479	977.6			

Table 5. Analysis of variance calculated from disease ratings in tulip inoculated with *B. tulipae* at three stages of plant growth and development.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block	1	0.1	0.1	0.6	
Cultivar	19	558.2	29.4	131.0	<0.001
Stage	2	198.3	99.2	442.4	<0.001
Cultivar x Stage	38	73.7	1.9	8.7	<0.001
Residual	419	93.9	0.22		
Total	479	924.3			

Figures

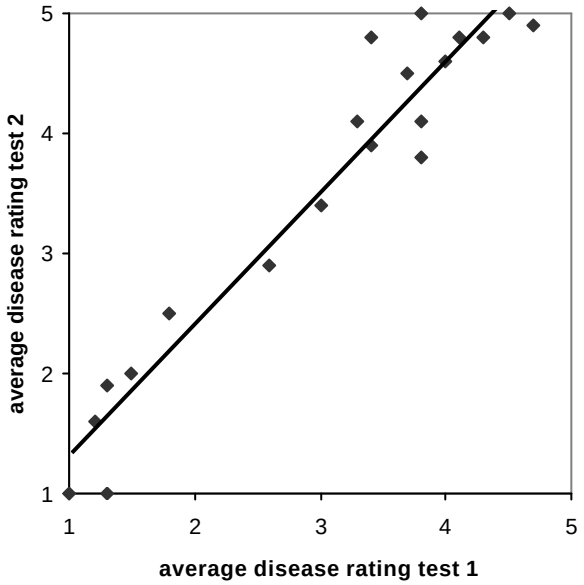


Fig 1. Correlation between two *B. tulipae* greenhouse tests with 20 tulip cultivars.

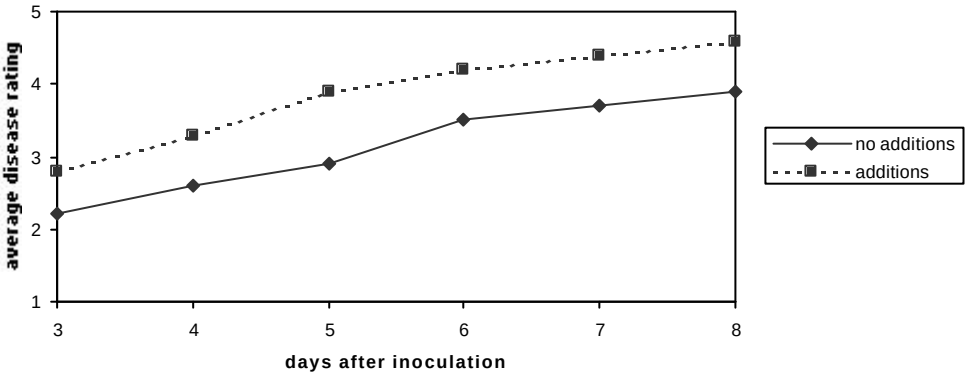


Fig 2. Average disease development over time of 20 tulip cultivar after inoculation with *B. tulipae* with and without medium additions.

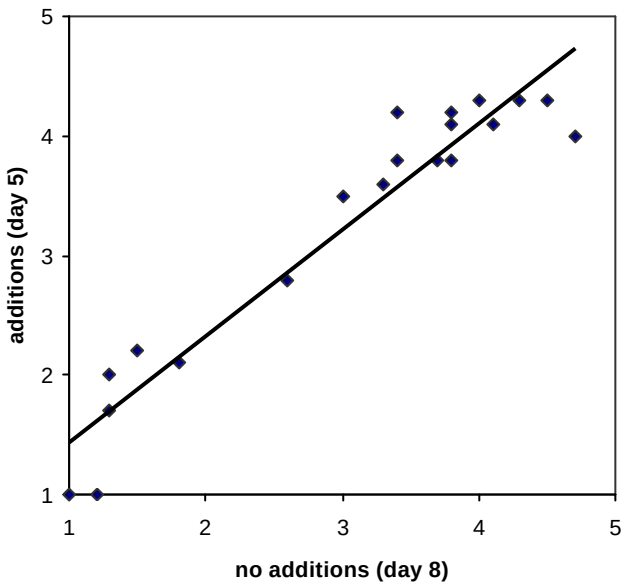
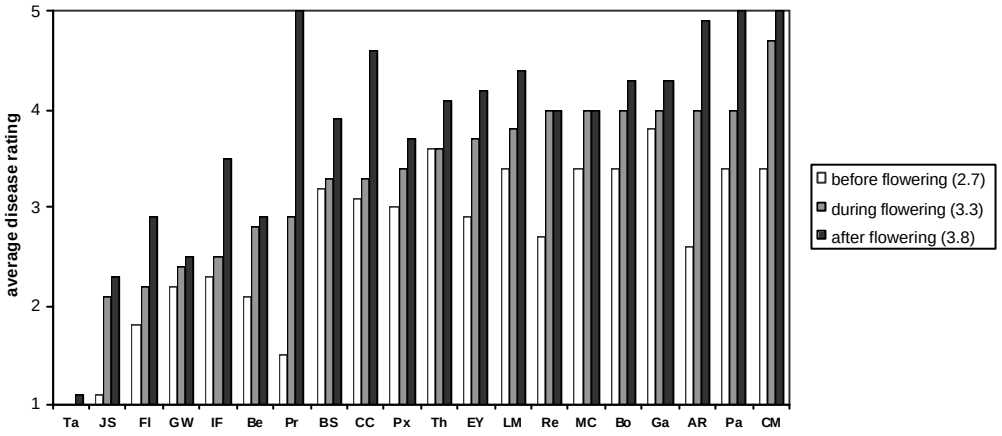


Fig 3. Correlation between a *B. tulipae* test without additions in the inoculum rated after eight days and with additions in the inoculum rated after five days.

Fig 4. Average disease rating of 20 tulip cultivars after inoculation with *B. tulipae* before, during,



or after flowering (see Table 1 for code abbreviations).