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Evaluation of inoculation methods for testing Fusarium head blight resistance of winter wheat on single plant basis

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(With 3 figures)

Summary

Two methods for artificial inoculation of single plants were investigated regarding their reliability in the estimation of Fusarium head blight (FHB) resistance of winter wheat. The inoculation methods used were the injection and the cotton method. Head blight was provoked by two *Fusarium graminearum* and one *F. culmorum* isolate. The test material consisted of nine winter wheat genotypes, which were investigated in their FHB resistance by five European research institutes since 1990. From this international trial a FHB index (% FHB) was evaluated for each genotype. The environmental dimension of this trial was made up of 59 year by isolate by location combinations. Therefore the FHB index represents the best available measure of the resistance level of the nine tested genotypes. The FHB index served as a standard of comparison for the single plant data. The most reliable assessment of the FHB resistance level was reached with the cotton method and an isolate of high virulence. In this case repeatabilities about $R = 0.7$ were obtained. The best parameters for a reliable assessment of the resistance level were visual scoring of bleached spikelets done on the 18th and/or 22nd day after artificial inoculation and the determination of the systemic growth of the fungus in the ear ($r_s = 0.62 - 0.65$; $R = 0.66 - 0.72$). The last mentioned parameter, however, is more labor-intensive to determine.

Key-words: *Fusarium* spp., head blight, *Triticum aestivum*, wheat, inoculation method.

Vergleich von Inokulationsmethoden zur Ermittlung der Ährenfusariose-Resistenz von Winterweizen auf der Basis von Einzelpflanzen

Zusammenfassung

Zwei Methoden zur Inokulation von Einzelpflanzen, die Injektions- und die Wattebauschmethode, wurden hinsichtlich ihrer Genauigkeit zur Ermittlung der Ährenfusariose-Resistenz von Winterweizen untersucht. Die Inokulation wurde mit zwei *Fusarium graminearum* und einem *F. culmorum* Isolat durchgeführt. Das Testmaterial bestand aus neun Winterweizengenotypen, die als Be-

standteil eines internationalen Testsortiments in den Jahren 1990 bis 1993 von fünf verschiedenen europäischen Forschungsinstituten auf ihre Ährenfusariose-Resistenz untersucht wurden. Aus diesem internationalen Versuch wurde für jeden Genotyp ein Ährenfusariose (FHB) Index (% FHB) über 59 verschiedene Umwelten (Jahr * Isolat * Standort Kombinationen) ermittelt. Dieser FHB Index repräsentiert das bestmögliche vorhandene Maß für die Ährenfusariose-Resistenz der untersuchten Genotypen und diente als Vergleichsbasis. Es wurde festgestellt, daß sich die bestmögliche Prognose der Ährenfusariose-Resistenz anhand von Einzelpflanzen bei Inokulation mit der Wattebauschmethode unter Verwendung eines Isolates mit hoher Virulenz ergibt. In diesem Falle konnte eine Wiederholbarkeit von R=0,7 errechnet werden. Die beste Erfassung des Resistenzniveaus wurde durch eine Bonitur der Ährenfusariose-Symptome am 18. und/oder 22. Tag nach der Inokulation, sowie durch Ermittlung des Pilzwachstums in der Ähre erreicht (r_s=0,62–0,65; R=0,66–0,72). Die Bestimmung des zuletztgenannten Parameters ist jedoch für die praktische Anwendung zu arbeitsaufwendig.

Schlüsselworte: *Fusarium* spp., Ährenfusariose, *Triticum aestivum*, Weizen, Inokulationsmethodik.

1. Introduction

In Austria an increase of cereal diseases caused by *Fusarium* spp. was observed in the last few years (Zwatz 1987). So far thirteen different *Fusarium* species have been isolated from contaminated Austrian wheat material. These species are *F. graminearum, F. avenaceum, F. culmorum, F. poae, F. sacchari var. subglutinans* (syn. *F. subglutinans*), *F. verticillioides* (syn. *F. moniliforme*), *F. crookwellense* (syn. *F. cerealis*), *F. tricinctum, F. equiseti, F. oxysporum, F. sporotrichoides, F. sambucinum* and *Gerlachia nivalis* (syn. *F. nivale*) (Adler 1993, Lew 1993). The damage caused by an infestation with *Fusarium* spp. is particularly serious in the case of head blight. The kernels on infected spikelets are usually shriveled (which results in yield losses) and nonviable. Moreover diseased grain may be contaminated with mycotoxins (above all with deoxynivalenol (DON)) that are a risk to human and animal health (Snijders 1990a). The main pathogens of FHB of winter wheat in Austria are *F. graminearum, F. avenaceum, F. poae* (Adler 1993) and *F. culmorum* (Zwatz 1987). So far it is impossible to control FHB by a fungicide and control by crop rotation does not lead, in most cases, to a decrease of the disease. Therefore the breeding of resistant cultivars is probably the best solution. Genetic resistance is expressed by several resistance mechanisms. Schroeder and Christensen (1963) observed resistance to initial infection (penetration) and resistance to systemic spread within the spike (hyphal spread, colonization of plant tissue). Miller and Arnison (1986) described the existence of an additional resistance mechanism in the cultivar *Frontana* which degrades the non-host specific *Fusarium* toxin DON. The results of research activities on the inheritance of FHB resistance in wheat give rather contradictory information. Bai and Xiao (1989) report on two to three dominant genes with some minor modifiers, whereas Snijders (1990b), Yu (1982, 1991), and Liao and Yu (1985) found one to six segregating genes. Li and Yu (1988) report as many as nine resistance genes.

If a reliable assessment of the resistance level were feasible on single plant basis it would be possible to test FHB resistance in e.g. F_2 populations. In this contribution two single plant inoculation methods, the injection method and

the cotton method, were compared. A set of winter wheat genotypes, the resistance of which has been meticulously determined, was inoculated with *F. graminearum* and *F. culmorum* isolates with widely varying virulence. Correlation coefficients and repeatabilities were calculated.

2. Materials and methods

2.1 Winter wheat genotypes

Nine European winter wheat genotypes (see table 1), taken from an international winter wheat nursery, were used as test material. Their FHB resistance level has been investigated since 1990 in five European countries (Austria, France, Germany, Hungary and The Netherlands). As a measure for their resistance level a FHB index (% FHB) was computed from the international trial data for each genotype (VAN EEUWIJK et al. 1995). This FHB index served as standard of comparison for the data evaluated on single plant basis. The winter wheat genotypes were sown by hand as single seed in 10 m^2 plots at the experimental station in Großenzersdorf near Vienna. For artificial inoculation only plants with at least two normally developed ears were selected. A determined number of plants were not inoculated to serve as control plants. The number of inoculated plants and control plants varied from 112 to 192 per genotype.

Table 1

Winter wheat genotypes

genotype/variety	origin	%FHB
Justus	Austria	53
Rescler	France	55
SVP 75059–32	The Netherlands	56
HOH 77/82/1	Germany	62
Renan	France	41
HOH 47/83/2	Germany	54
85–92	Hungary	48
81-F3–79	France	35
Bence	Hungary	47

2.2 Production of inoculum

Fusarium spp. were isolated as described by NELSON et al. (1983) from infected wheat kernels harvested in Austria in 1990. The species were identified according to NELSON et al. (1983). The identity of the species was confirmed by A. MESTERHAZY (Cereal Research Institute, Szeged, Hungary). The long term preservation is done by the soil storage method (DHINGRA and SINCLAIR 1985). The number of colony forming units per ml was determined (NELSON et al. 1983). The required amount of inoculum for the field inoculations was produced by the "bubble-breeding" method (MESTERHAZY 1977). The pathogenicities and virulences of the isolates were determined by means of the Petri-dish test before and after the field inoculations (see table 2, LEMMENS et al. 1993).

2.3 Field inoculation

2.3.1 Time of inoculation

Inoculation was carried out at the time of flowering. At this time the wheat plant is most susceptible and the consequences of an infection are the most seri-

Table 2

Characteristics of the isolates

isolate	*Fusarium* species	source	CFU/ml	(1) virulence	(2)
2	*F. graminearum*	*T. durum*	45×10^4	0.55	0.56
3	*F. graminearum*	*T. durum*	51×10^4	0.13	0.11
4	*F. culmorum*	*T. aestivum*	48×10^4	0.77	0.77

(1) = virulence before
(2) = virulence after field inoculation (the lower the value, the higher the virulence [LEMMENS et al. 1993])

ous. Good indicators for the right time of inoculation are intensive yellow or yellow-reddish coloured anthers, which are filled to bursting. Beginning of flowering can also be recognized by the protrusion of anthers in the middle part of the ear.

2.3.2 Inoculation methods

Injection method

By means of a small syringe (10 ml) one droplet (about 14 µl) of a *Fusarium* suspension was injected into one floret. Injection of the spore suspension was carried out without injuring the plant tissue. For easier use the needle of the syringe was shortened to about one centimeter. After the injection the glumes of the floret must be closed with care, otherwise the suspension can drip and contaminate other parts of the spike. For inoculation usually a spikelet from the middle of the ear is used (BEKELE 1987).

Cotton method

With sharppointed tweezers a little piece of cotton soaked with *Fusarium* suspension was placed between the glumes of a spikelet in contact with the anthers and the stigma of a floret (BEKELE 1987). The size of the cotton was about one fifth of a glume and the amount of the soaked inoculum about 14 µl.

2.4 Evaluation of the disease

Visual scoring started the 10th day after inoculation and was repeated the 14th, 18th and 22nd day after inoculation. The number of bleached, typically "water soaked" spikelets was determined. In addition the spikes were assessed regarding "wilting" (white) heads. "Wilting" heads (the upper part of the ear fades) is a conspicuous symptom of extremely susceptible genotypes. After ripening and harvesting the relative ear weight of the inoculated ears was estimated. The data of visual scoring was plotted against the day after inoculation and connected by lines. The resulting curve reflects the development of the disease. The area under the disease progressing curve is a measure for the resistance, the greater value represents a more susceptible genotype. As a certain measure for the resistance against colonization of the plant tissue, the systemic growth of the fungus was computed from the date of inoculation to the end of the visual scoring period according to formula (1):

$$SGF = \{(BS22\text{–}1) \times \text{mm ear length} / \Sigma \text{ spikelets per ear}\} \quad (1)$$

in which BS22 stands for the bleached spikelets determined the 22nd day after inoculation.

2.5 Statistical analyses

All statistical analyses were done with the single plant data, which means that for each single plant a mean of the inoculated ears was computed for the several estimated parameters.

2.5.1 Analysis of variance

The analysis of variance was computed by using the GLM (general linear models) procedure, a procedure especially for analysis of variance with unbalanced datasets (SAS Institute Inc. 1988a). The model used in the analysis of variance is summarized in equation (2):

$$Y_{ijk} = \mu + G_i + M_j + I_k + (G \times M)_{ij} + (G \times I)_{ik} + (M \times I)_{jk} + (G \times M \times I)_{ijk} + \varepsilon_{ijk} \quad (2)$$

in which G stands for genotype, M for method and I for isolate.

2.5.2 Spearman rank correlation

The correlation between the single plant data and the "real" resistance level expressed by the FHB index was computed by a Spearman rank correlation (SAS Institute Inc. 1988b). The parameters best correlated with the FHB index were calculated. The correlation between the FHB index and the two inoculation methods was computed with means over all three isolates for each genotype.

2.5.3 Repeatability

The repeatability R is a relative measure for the reproducibility of the test results in the next generation. The repeatability is in this case no genetic measure, but a tool to allow the comparison between the different parameters and the several method-isolate combinations. High R values mean, that the variance of the genotypes is great in relation with the variance of error. In other words, the mean variation between several plants of one genotype is minimal compared with the mean variation between the several genotypes. The repeatability was calculated by the variance components method according to equation (3):

$$R = \{Var_{(G)} / (Var_{(G)} + Var_{(error)})\} \quad (3)$$

in which $Var_{(G)}$ stands for the variance component of the genotypes and $Var_{(error)}$ stands for the variance component of the error (SAS Institute Inc. 1988a).

List of abbreviations

AUDPC: area under the disease progressing curve
BS_x: bleached spikelets determined the x^{th} day after inoculation (x = 10, 14, 18 or 22)
CFU: colony forming units
DF: degrees of freedom
DON: deoxynivalenol
FHB: Fusarium head blight
G: genotype
I: isolate
M: inoculation method
MS: mean square

R: repeatability
REW: relative ear weight
r_s: Spearman rank correlation coefficient
SGF: systemic growth of the fungus
WILT: evaluation of "wilting" heads

3. Results

3.1 Analysis of variance

Both inoculation methods led to a successful infection of the ears. The resulting visual scoring was generally higher if the cotton method was used (see figure 1), even though the amount of inoculum was the same for both methods.

Table 3

Analysis of variance for the parameters BS18 and SGF

Source	DF	BS18 MS	BS18 F	SGF MS	SGF F
genotype	9	292.29	122.9 ***	14067.7	141.7 ***
method	1	165.96	69.8 ***	6161.8	62.1 ***
isolate	2	246.04	103.5 ***	11385.4	114.7 ***
genotype*isolate	18	6.91	2.9 ***	286.2	2.9 ***
genotype*method	9	6.64	2.8 **	159.6	1.6 n.s.
method*isolate	2	2.42	1.0 n.s.	45.8	0.5 n.s.
genotype*method*isolate	18	2.78	1.2 n.s.	92.9	0.9 n.s.
error	743	2.38		99.3	

n.s. = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

The analyses of variance for all estimated parameters show significant differences between the genotypes, the methods and the isolates (see table 3). MS values of all significant interactions were small as compared to the MS values of the main effects G, M and I.

The cultivars differ significantly in the severity of symptom development and "wilting" head formation, but total resistance has not been observed. In the case of the resistant genotype *81-F3-79* the infestation was restricted to the

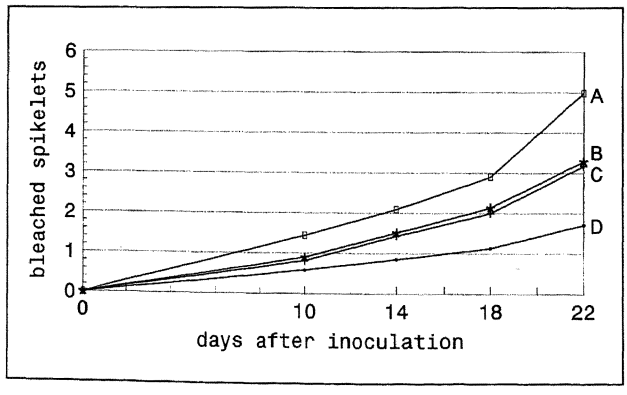

Fig. 1: Disease progressing curve (bleached spikelets) of several method – isolate combinations: (A) cotton-iso 3; (B) cotton-iso 2; (C) injection-iso 3; (D) injection-iso 2, demonstrated for the genotype Renan (iso 4 not demonstrated)

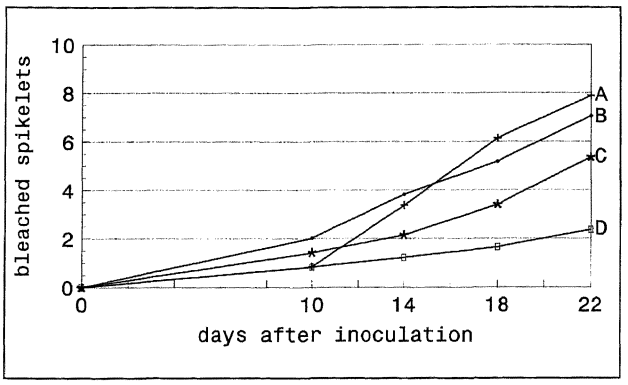

Fig. 2: Disease progressing curve (bleached spikelets) of four selected genotypes: (A) HOH 77/82/1; (B) Justus; (C) Bence; (D) 81-F3-79. The values represent means over both methods and all three isolates

inoculated spikelet and the spikelets adjacent to the infection point (see figure 2).

3.2 Spearman rank correlation

In table 4 the Spearman rank correlation coefficients r_s for the different parameters with the FHB index are demonstrated. The parameters with the highest rank correlation coefficients were WILT and SGF for both inoculation methods and BS18 and BS22 for the cotton method.

Table 4

Spearman rank correlation of the several parameters with % FHB

parameter	injection method r_s	P	cotton method r_s	P
BS10	-0.04	0.915	0.05	0.898
BS14	0.62	0.074	0.53	0.139
BS18	0.60	0.088	**0.65**	0.058
BS22	0.60	0.088	**0.62**	0.077
AUDPC	0.55	0.125	0.53	0.139
WILT	**0.70**	0.036	**0.67**	0.050
SGF	**0.65**	0.058	**0.62**	0.077
REW	-0.45	0.222	-0.25	0.517

The values for probability P of r_s are in most cases not below the significance level of 0.05. The reason can be seen in figure 3, which demonstrates the distribution of the genotypes plotted against the FHB index and the parameter SGF. Figure 3 demonstrates that there exists quite a trend in the distribution, but at least the genotypes *85–92* and *HOH 47/83/2* form outliers.

3.3 Repeatabilities

The computed values of the repeatabilities of the best rank correlating parameters are presented in table 5.

High R values were computed for the parameters BS18, BS22 and SGF, especially if inoculation was carried out with isolate 3. This isolate showed the highest virulence determined by the Petri-dish test (see table 2).

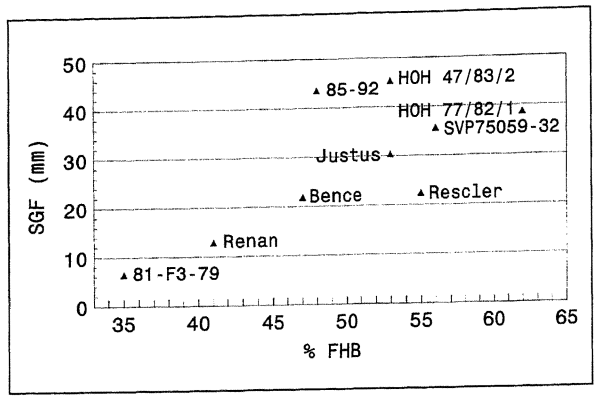

Fig. 3: Distribution of the tested genotypes considering the parameters SGF and % FHB

Table 5

Repeatabilities R of the injection and the cotton method

parameter	isolate	R injection	R cotton
BS18	2	0.593	0.572
	3	**0.666**	**0.713**
	4	**0.664**	0.530
BS22	2	0.640	0.628
	3	0.629	**0.674**
	4	**0.681**	0.633
WIL T	2	0.266	0.232
	3	0.442	0.263
	4	0.508	0.410
SGF	2	0.645	0.614
	3	**0.694**	**0.658**
	4	**0.716**	0.646

4. Discussion

A reliable assessment of the resistance level on single plant basis is possible. Both inoculation methods led to a successful infection and significant differences between the genotypes. The cotton method turned out to be more precise. The most probable cause for the higher visual scoring data in the case of the cotton method is a slower drying up of the inoculum in relation with the injection method. In an experiment with several replications a single drop of inoculum showed after 30 minutes a weight loss of 35–40 % compared with a drop of inoculum soaked up by the cotton. Consequently the cotton seems to guarantee best conditions for an infection over a longer period of time. BEKELE (1987) describes the cotton method as the most precise method for estimation of the resistance level as well. In using the injection method, skillness is necessary in order to inject consistently the same amount of inoculum into the floret. If the amount of inoculum is too large the spore suspension can drip and contaminate other parts of the spike. Moreover the injection method causes problems when a conidia-mycelium suspension is used as inoculum. In this case the mycelium can plug the needle of the syringe. Therefore a mere conidia suspension as inoculum can be recommended.

As the correlation between the single isolates was very high (r = 0.94-0.97) and the genotype * isolate interaction in the analysis of variance was very low, it can be concluded that using only one isolate in the field inoculations would be enough for a reliable assessment of the resistance level. Moreover this would be less laborious. If only one isolate is used it is advisable to use an isolate of high virulence, since in this experiment isolate 3 showed the best reproducibility. SNIJDERS and VAN EEUWIJK (1991), and MESTERHAZY (1989) also propose for large scale screening for FHB resistance highly virulent strains for experimental inoculation.

More than the half of the tested genotypes were extremely or relatively susceptible. Only the genotype *81-F3–79* can be classified as resistent. However the resistance is not absolute, but results according to the definition of horizontal resistance in a reduced infestation of the ear.

The best estimation of the resistance level by visual scoring is reached, when the scoring is carried out 18 or 22 days after inoculation. The "wilting" of the upper parts of the ears must be considered during visual scoring of the symptoms. The "wilting" is the result of premature ripening caused by an interruption in the supply of nutrients, water and assimilates to the distal spikelets of the ear (SNIJDERS and KRECHTING 1992, MIELKE 1988, WANG and MILLER 1987, TOMASOVIC 1981), as a result of an invasion of the rachis and the xylem vessels by the fungus. The exact form of breakdown of the vascular bundles has not yet been investigated. An interruption of the xylem vessels can be caused by physical barriers, for instance the hyphae of the fungus, deviation of the mass flow or by a chemical effect, e.g. toxins (PARRY 1990). XU et al. (1990) demonstrated that the non-host specific toxins of *F. graminearum* were responsible for the destruction of the plasmalemma. The collapse of the cells resulted in plugging of the vascular bundles. As the computed Spearman rank correlation coefficients between the parameter WILT and the FHB index were high, it would be quite advisable to integrate this parameter in an assessment scale of the disease symptoms. To use the parameter WILT as the only indicator of the disease can not be recommended, as the reproducibility of this parameter is not very good. By using only data from visual scoring for the estimation of the resistance level it must be considered that some genotypes may show tolerance. Nevertheless SNIJDERS (1990 c) favors visually assessed head blight as better selection criterion than yield reduction. Moreover it is more practical and less laborious.

Both the Spearman rank correlation coefficients and repeatabilities were high in the case of SGF. This means that SGF allows a reliable estimation of the resistance level. Moreover it confirms the importance of a resistance mechanism against the systemic growth of the fungus in the plant tissue. Maybe the gradient of the disease progressing curve could also be an indicator for colonization resistance. In this connection the type of ear (compact or loose) could be of importance and breeding varieties with loose ears may be one little step in the search for resistant genotypes. MESTERHAZY (1989) also argued that compact ears are more predisposed to FHB than loose ones. However the assessment of SGF is much more labor-intensive than visual scoring and can therefore not be recommended if a large amount of genotypes is tested.

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