Comparison of the effectiveness of three *pyrenophora tritici-repentis* field inoculation methods on wheat

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Abstract

Field inoculation technique adapted to target environment is a necessity for breeding wheat cultivars resistant to tan spot (Pyrenophora tritici-repentis). The objectives of this study were to evaluate early spray, late-spray, and straw field tan spot inoculation methods in two different environments. Eighty wheat genotypes were tested at Sidi-El-Aydi and Annoceur experiment stations in Morocco. In the early and late spray inoculation methods, conidial inoculum in aqueous suspensions was sprayed before and after heading respectively. Inoculated plots were covered with polyethylene plastic and intermittently sprayed with water for one night. In the third method, inoculation with infested straw was applied at the 2 to 3 leaf growth stage. The wheat genotypes were also tested at the late-boot growth stage for reaction to tan spot spray inoculation in the greenhouse, and results were compared with those of field inoculations. The results showed that the three field inoculation methods effectively created significant differences in disease reaction among wheat genotypes in both environments. The effect of location, genotype and inoculation method as well as the interaction between inoculation method and site were significant to highly significant. The ranges of disease reactions, genetic variances, and heritability values indicated that early spray inoculation was most effective in showing genotype-related differences in the cooler and more humid northern location. In the southern location, where conditions were drier, straw mulching performed better than either early or late-spray inoculation. Phenotypic correlations between tan spot expression and plant height and earliness to maturity were shown to be potentially restrictive.

Key-words: Durum wheat, *Pyrenophora tritici-repentis*, Tan spot, Inoculation methods.

مقارنة ثلاثة طرق حقلية لتعفين القمح بفطر (Phyrenophora tritici repentis)

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ملخص:

دراسة طرق التعفين بمرض اللفحة الورقية المسببة من طرف الفطر (Pyrenophora tritici repentis) مهمة لمنتخبي أصناف القمح الصلب. الغرض من هذه الدراسة هو مقارنة الرش المبكر والرش المتأخر ووضع التبن المتعفن بالفطر إزاء ظهور فوارق في اصناف القمح.

استعملت 80 سلالة متقدمة في محطتي التجارب االعنوصرب واسيدي العيديب. إستعملت عصارة اكونيدياب للرش والتبن المتعفن بالفطر وتمت تغطية التجارب ببيوت بلاستيكية لمدة ليلة واحدة. تمت دراسة تعفن النباتات في البيوت الزجاجية حيث قورنت نتائجها مع نتائج الحقل. أظهرت النتائج أن للطرق التعفين الثلاثة وفاعل محطة التجارب، والسلالة، والتفاعل بين طريقة التعفين والمحطة لها معنوية إحصائية عالية. كما أظهرت أن للرش المبكر مفعولية أحسن في المحطة ذات الجو البارد في حين كان وضع التبن المتعفن ذا مفعولية أكبر في المحطة ذات المناخ الحار نسبيا. ظهر إرتباط بين بعض الخاصيات النباتية (الطول الساقي وطول فصل الازهار) مع قابلية الاصناف للمرض.

الكلمات المفتاحية: القمح الصلب ، اللفحة الورقية ، طرق التعفين ، Pyrenophora tritici repentis

Comparaison de l'efficacité de trois méthodes d'inoculation au champs du blé par Pyrenophora tritici repentis.

Résumé

Les techniques d'inoculation au champ par la maladie de la tache bronzée (*Pyrenophora tritici-repentis*) sont nécessaires pour la sélection. Les objectifs de cette étude étaient d'évaluer et de comparer les techniques de pulvérisation d'inoculum aux stades précoce, et tardif et de l'inoculation par la paille. Quatre vingt génotypes de blé dur ont été ainsi inoculés dans les stations expérimentales de 'Sidi-El-Aydi' et 'Annoceur'. Les suspensions conidiales ont été pulvérisées aux stades montaison et floraison. Après inoculation, les essais ont été recouverts par des tentes de polyéthylène pour une nuit. La paille infestée a été appliquée au stade 2-3 feuilles. Les gén otypes ont été testés sous serre, les résultats de champ et de serre ont été comparés. Les résultats ont montrés que l'effet du génotype, du site et de la méthode d'inoculation, ainsi que celui de l'interaction méthode*site étaient significatifs. L'étude de la variabilité de l'infestation, a montré que la pulvérisation précoce était plus effective en station à climat relativement froid (Annoceur). Dans la station de Sidi El Aydi, l'infestation par la paille était plus efficace. Des corrélations entre les infestations, la hauteur et la précocité ont été montrées et devraient être prises en compte.

Mots clés : Blé dur, *Pyrenophora tritici repentis*, tache bronzée, méthode inoculation.

Introduction

Tan spot of wheat, caused by *Pyrenophora tritici-repentis* (Died.) Drechs (PTR), is important in many major wheat growing regions (Hosford, 1982; Rees and Platz, 1979). This disease is characterized by foliar symptoms consisting of tan, diamond-shaped lesions with a dark center surrounded by a yellow border (Hosford, 1976). Tan spot lesions may have different amounts of necrosis and chlorosis depending on the genotypes of the host and the pathogen (Lamari and Bernier, 1989). Genetic resistance is the best strategy for reducing losses due to the disease.

Greenhouse inoculation, consisting of spraying a suspension of PTR spores and incubating the inoculated plants in a mist chamber for approximately 30 h gives suitable data for consistent plant resistance scoring (Hosford, 1982). Toxin culture filtrates also have been used for screening tan spot resistance (Tomas and Bockus, 1987). Greenhouse inoculation is not practical, however, for screening large numbers of breeding lines in fields. Field inoculation methods, where the inoculum is introduced either by spraying a suspension of infectious spores or by spreading infected straw, have been reported in a number of variations (Elias et al., 1989; Lamari and Bernier, 1989). In these experiments, the leaf wetness at the time of inoculation was enhanced by covering the plots with a clear polyethylene cover for one or two nights. The length of time free moisture is available to pathogen spores on the leaf is important in producing the disease. In greenhouse experiments, host resistance apparently breaks down the longer the leaf is wet (Hosford, 1982). Ideal conditions of moisture and temperature and timing of inoculum application leading to homogeneous infection can be achieved in small greenhouse experiments more easily than in larger field-planted nurseries. Lesion size, disease severity, and yield loss caused by tan spot depend on the initial inoculum and plant growth stages (Cox and Hosford, 1987, Hosford et al., 1987, Shabeer and Bockus, 1988).

The objectives of this study were to evaluate three inoculation methods for PTR that could routinely be used by wheat breeders in the field. These methods are early (before mean heading time) spray, late spray (past heading time) and early straw mulch with infested straw.

Material and methods

The Hosts: Eighty spring-type durum wheat lines and cultivars were used in this study and comprised advanced lines from the Moroccan durum wheat breeding program and Checks including three moderately susceptible durum wheat cultivars 'Monroe', 'Lloyd', 'Vic', and the resistant cultivar 'Wells', as well as two resistant durum wheat accessions, PI184526, PI166308; and two moderately resistant bread wheat genotypes BH1146, and 'Chris' and the highly susceptible experimental line ND495.

The Lines were planted in Annoceur experiment station, (North central, higher elevation, wet climate) and in Sidi-El-Aydi experiment station (Central west, dryer climate) of Morocco. Experiments were planted in hill plots, using a randomized complete block design with six replicates. The distance between hill plots was 40 cm. At Sidi El Aydi site, the experiments were irrigated (25 mm) after mulching with straw and otherwise when necessary. The experiments were weeded manually as necessary. One experiment was planted for each of the three tested field inoculation methods (early spray, late spray and straw mulch) in each site and year.

The Pathogens: Two PTR isolates 'Oulmes' and 'SEA2' virulent on all cultivars grown in Morocco were used. The 'SEA2' isolate was obtained in 1990 from wheat leaves from the Sidi-El-Aidi Experiment Station. The 'Oulmes' isolate was obtained from leaves sampled in the central northern zone in Morocco (Nsarellah, 1991). Spore production was conducted according to the method outlined by Odvody and Boosalis, (1978) and Gilchrist, (1982). Spore collection was made by flooding the plates with sterile distilled water and dislodging the conidia with a small plastic knife. The suspension obtained was blended for two minutes at low speed, and the spore concentration was adjusted to 2.0 to 3.0 x 104 propagules/ml-1 with sterile distilled water. Three drops of the surfactant 'Tween-20' (polyoxyethylene sorbitan monolaurate) were added per liter of prepared suspension.

The Field inoculation methods: Three field inoculation methods were studied: 1) early and 2) late-spray as well as 3) straw mulch field inoculations.

Table 1. Description of the field inoculation methods evaluated.

Inoculation method	Name	Main features
I	Early spray	Aqueous spore suspension applied at late jointing stage. Covering and humidifying of experiment thereafter
II	Late spray	Aqueous spore suspension applied past mean heading time. Covering and humidifying of experiment thereafter
III	Straw mulch	Mulch of infected straw at the 2 to 3 leaves growth stage. Watering of straw after mulch.

In the early spray inoculation experiment, plants were inoculated at Zadoks' growth stage (ZGS) 39 to 51 (Zadoks et al., 1974) (at average jointing stage). In the late-spray experiment, inoculation was performed at ZGS 55 to 65 (past the mean heading time). For the straw inoculation, the experiments were mulched with weathered, naturally infected straw at 2-3 leaf growth stage (Elias et al., 1989; Lamari and Bernier, 1989) was used. A uniform (15-cm high) layer of infested straw was mulched on the plots at the 2 to 3 leaf stage.

Inoculation Procedures : Spray inoculations were performed at night time. The experiments were first watered using sprinkle irrigation, experimental plot was covered with a clear polyethylene cover suspended on a wooden frame. Inoculum suspension was applied to each experiment (approximately 3.4 ml per plant) and the plants were subsequently sprayed with backpack water sprayers at 3 hours intervals during the night to maintain high relative humidity and moisture on the leaves. The plastic cover was removed in the morning.

Greenhouse inoculation : All wheat genotypes were also planted in the greenhouse and inoculated at ZGS 47 to 59 with each of the 'SEA2' and 'Oulmes' PTR isolates using the inoculation and rating protocols described by Luz and Hosford (1980). Plants were incubated in a mist chamber for 30-h following inoculation.

Data Collection and Analysis: Field data for both early and late-applied spray inoculations were collected 15 to 25 days after inoculation when leaf spotting differences among genotypes were apparent. Mean lesion size was rated on a scale of 0 to 5 (Cox and Hosford, 1987) and was evaluated for each plant level and hill plot. Heading date (number of days from seeding to approximately 50% of the plants with their spikes completely emerged) and plant height (awns excluded) were recorded. In the greenhouse, experiment disease reaction, based on lesion size was evaluated 8 days after inoculation on a scale of one to five (Cox and Hosford , 1987). Lesion size was assessed on separate leaves of three plants per entry and averages per plant were used for each entry.

Combined analyses of variance across locations were computed after using the Bartlett test for the homogeneity of variances (Steel and Torrie, 1980). The range, standard deviation (both of cultivar/line mean disease reaction), genetic variance, and heritabilities (from variance components analysis as described by Fehr, 1987) were computed for each field inoculation method. Comparative evaluation is based on the ability of the three field inoculation methods to differentiate among genotypes. The range and standard deviations of cultivar/line mean disease reactions are used to rate variations. Genetic variance and heritability were used to compare genetic variability detected with each inoculation method. Correlation study of field with greenhouse disease reactions is used to evaluate the repeatability of each field inoculation method.

Results and discussion

Success of inoculation: The early spray inoculation was applied during cool and humid weather conditions in both sites. The late spray coincided with slightly higher temperatures and lower air relative humidity in Sidi El Aydi site. Tan spot development was slow at first and picked up in both the early and late- spray experiments. At Annoceur, both spray inoculations were followed by mild weather and by good rainfall and or humidity. In Sidi El Aydi site, post inoculation temperatures were higher and relative air humidity was lower. These condition could have had an effect on the success of inoculation as will be discussed below.

Analysis of variance : Highly significant differences among wheat genotypes, Locations, and inoculation methods were observed for disease reactions (Table 2). Interaction of wheat genotype by location was significant, but produced lower magnitude mean square errors than did the main effects. In addition, mean wheat genotype disease reactions were ranked similarly at all locations. Spearman rank correlation coefficients of cultivar mean disease reactions between locations within sites were positive and highly significant (0.4 to 0.8; P=0.01), indicating that genotype by environment interaction was due to magnitude rather than the ranking of cultivars. These correlations values indicate that comparisons of wheat genotypes disease reactions may be based on the means across locations. A significant effect of the interaction between Location and method of inoculation was also observed. These results indicate that the choice of location and of the inoculation method are very important factors for screening genotypes for tan spot resistance. The crucial importance of the choice of a specific Location and method of inoculation is demonstrated by the level of significance and the magnitude of the Mean square error generated by these factors (Table 2).

Table 2: Analysis of variance of disease reaction

Factor of variation	df	MSE	S/NS
Location	1	101.99	HS
Block (Location)	10	29.07	S
Genotype	79	39.67	HS
Genotype * Location	79	71.27	S
Inoculation method	2	198.63	HS
Location * Inoculation method	2	29.59	HS

Df: degrees of freedom, MSE: mean square error, S/NS/HS: significant, non significant, highly significant .

Range of variation in disease expression of check varieties: The review of check genotypes reaction to tan spot is herein used to verify the similarity of obtained check reaction to previously documented ones and, to assess the range of variation of disease expression. In this study, significant differences were observed among check cultivars (Table 3). Also, the previously known resistant checks PI184526 and PI166308 were rated as resistant and the previously known susceptible check was rated as the most susceptible among the genotypes studied. In Annoceur, lesion size ranged from 1.4 to 4.7, 2.3 to 4.7, and 2.7 to 4.7 in early-spray, late-spray, and straw mulch inoculation methods, respectively. In Sidi El Aydi lesion size ranged from 3.1 to 5.0, 3.3 to 4.8, and 2.6 to 4.8 in the early-spray, late-spray, and straw mulching inoculation methods, respectively. These results indicate that the three inoculation methods were effective in producing important epidemic of tan spot. And that disease expression in checks of different documented levels of resistance was maintained across all sites and inoculation methods.

Table 3. Average disease reaction on checks in early spray, late spray, and straw mulch inoculation methods with *Pyrenophora tritici-repentis*.

Genotypes	Early spray	Late spray	Straw	Early spray	Late spray	Straw
Lloyd	2.4	3.1	4.2	4.9	3.7	3.3
Monroe	2.1	2.8	3.2	4.6	3.7	3.7
Vic	2.1	3.3	3.3	5.0	4.6	4.7
Wells	1.4	2.3	2.5	3.6	3.5	2.6
PI166308	1.8	3.5	3.6	3.1	3.3	2.7
PI184526	1.8	2.7	2.7	3.8	3.7	2.7
Chris	2.1	3.4	3.3	4.3	4.2	4.1
BH1146	2.8	3.1	2.9	4.1	4.1	4.2
ND495	4.7	4.7	4.8	4.7	4.8	4.8
LSD (0.05)	**2.3**	**1.8**	**1.8**	**1.1**	**1.1**	**1.2**

Lesion size (scale: 0-5).

Variation in disease expression of breeding lines : From the breeder standpoint, a successful inoculation methodology should lead to maximal variation among cultivar/line reactions rather that maximal development of the disease on all tested material. In Annoceur, lesion size in early spray inoculation had a wider range and higher variance (mean standard deviations of wheat line disease reaction) than those of straw and late-spray inoculation (Table 4). In Sidi El Aydi, the range of variation for lesion size was greatest for early-spray inoculation, but the standard deviation of cultivar/line mean was greatest for straw mulching. These data indicate that in addition to the variation in check disease expression, variation in disease expression of undocumented genotypes are showing the same levels of performances. The mean disease expression was higher in Sidi El Aydi but the range of variation in disease expression was wider in Annoceur. This means that the effect of the experiment site is more important than the method of inoculation (i.e. Annoceur better than Sidi El Aydi). But that within site one method may be better than an other.

Table 4. Variation in disease reaction of 80 wheat genotypes inoculated with *Pyrenophora tritici-repentis* in Annoceur and in Sidi El Aydi experiment stations.

Inoculation Method	Annoceur site				Sidi E l Aydi site		
	Min	Max	Mean	SD	Min	Max	Mean
Early spray	0.7	4.9	2.7	1.4	2.1	5.0	4.1
Late spray	1.2	5.0	3.4	1.2	2.4	4.9	4.2
Mulch	1.3	5.0	3.5	1.2	2.4	5.0	4.1

Lesion size (scale: 0-5).

SD: Standard deviation of cultivar/line mean disease reaction.

Correlation is between field and greenhouse for reaction: Results of the field disease ratings for the three inoculation methods were positively correlated to greenhouse disease rating results of all wheat genotypes (Table 5). These data indicate that genotype response was consistent across locations and inoculation methods in their relation to greenhouse testing results. However, it is to be mentioned that the effect of the location is stronger than the method of inoculation: Annoceur site showed stronger correlations than Sidi El Aydi. And straw mulch was better correlated to green house data in Sidi El Aydi. While late spray was the most correlated method to greenhouse data in Annoceur site. If one considers the data of greenhouse experiment which are better controlled and more precise, then the field tan spot expression data were highly correlated to tan spot created by the three inoculation methods. With a strong effect of experiment site and a less strong effect of the inoculation methodology. These findings are in agreement with those of the analysis of variance and the relative magnitude of mean square errors.

Table 5. Correlation coefficients (r) between wheat genotype mean tan spot reaction in the field and in the greenhouse for three field inoculation methods (n=8).NS,

Field inoculation Method	'Oulmes' isolate With Annoceur site	'SEA1' isolate With Sidi El Aydi site
Early Spray	0.69**	0.44**
Late Spray	0.74**	0.37**
Straw Mulch	0.72**	0.57**

**: Non significant, significant at the 0.01 levels of probability, respectively.

Lesion size (scale: 0-5).

Early spray = Spray inoculation before heading.

Late spray = Spray inoculation after heading.

Straw mulch = Straw mulch inoculation at the 2-3 leaf stage.

Genetic variances : Genetic variances are computed from variance component analysis and aim to separate the effect of genotype from unwanted interactions. Annoceur showed much higher genetic variance values than Sidi El Aydi. A less pronounced effect of the inoculation method was observed. In Annoceur, genetic variance for lesion size were highest for early-spray inoculation followed by straw and late-spray inoculations. In Sid El Aydi experiment site, genetic variance for lesion size was highest for straw inoculation (Table 6). Genetic variances showed trends similar to those found for the ranges of variation and standard mean deviations. The comparison between the two sites is explained by the favorable climate of Annoceur: under conditions favorable to disease, early spray inoculation will probably provide more time for disease development and be equivalent to straw inoculation. These results also show the predominance of the site effect and the importance of methodology choice.

Table 6. Genetic variance estimates for disease reaction in durum wheat in response to three field inoculation methods with *Pyrenophora tritici-repentis* .

Inoculation method	Annoceur site	Sidi El Aydi site
Early spray	1.3	0.2
Late spray	0.9	0.2
Straw mulch	1.0	0.4

Heritability values : Broad-sense heritability computed from variance components analysis should reflect the probability of success of selection based on environmental variances associated with each inoculation method and provide a more meaningful comparison for the breeders' purpose. Inoculation methods with high heritability values should be more suitable for screening wheat germplasm in breeding programs. In this study, the broad sense heritability values showed trends similar to those of the genetic variances for the three field inoculation methods in Annoceur (Table 7). Heritability values in Annoceur were higher than those in Sidi El Aydi. The three methods also showed similar values for the three methods in Annoceur. In Sidi El Aydi site, heritabilities were greatest for straw mulching (Table 7). This part of the analysis showed the importance of the site prior to the inoculation method.

Table 7. Heritability estimates of disease reaction in durum wheat in response to three field inoculation methods of *Pyrenophora tritici-repentis* in Annoceur and Sidi El Aydi

Inoculation method	**Annoceur site**	**Sidi El Aydi site**
Early spray	0.89	0.66
Late spray	0.88	0.70
Straw mulch	0.89	0.86

Correlation between disease expression and some agronomic traits : Disease expression data are dependant on the plant growth stage. Tan spot expression has been shown to be correlated to plant height and growth stage. Simple statistical analysis may show genotype differences, part of this variation may be due to plant height and growth stage. In this study, disease expression data were significantly and negatively correlated with the number of days to heading and to plant height for all three field inoculation methods (Table 8). Late maturing and tall genotypes exhibited lower disease severity and lesion size than earlier and shorter genotypes. In Annoceur, the magnitude of these correlation values did not change with the inoculation method while in Sidi El Aydi site, lesion size was less often correlated with plant height and with the number of days to heading in the early and late-spray inoculation methods (Table 8). Similar correlation values between tan spot reactions and plant growth characteristics were reported in other studies (Elias et al., 1989). This study indicated that correlation values are consistent regardless of the circumstances in which the disease is created. The magnitude of the correlations shown in table indicate that 16 to 25% of the total variation may be due to the effect of plant phenology and morphology rather than to true genetic resistance. The consequences of these findings are important to the breeder: Late plants and tall plants will appear more resistant than early and short plants. Height and earliness effects should be taken in to account. Authors have prescribed several ways to avoid this confounding of factors: a) Using parents of targeted range in phenology and morphology, b) taking in account the growth stage and plant height in evaluating the disease and correcting for it, c) taking several rating along the growth of the plants, d) using checks of different phonologies regularly in the screening nurseries, etc.

Table 8. Correlation coefficients between disease reaction and plant growth traits under three field inoculation methods with *Pyrenophora tritici-repentis* (n = 80).

	Annoceur site		Sidi El Aydi site	
Inoculation Method	**Lesion size with plant height**	**Lesion size with days to heading**	**Lesion size with Plant height**	**Lesion size with days to heading**
Early spray	-0.47**	-0.45**	-0.46**	-0.42**
Late spray	-0.45**	-0.50**	-0.47**	-0.44**
Mulch	-0.48**	-0.51**	-0.54**	-0.48**

**: significant at the 0.01 level of probability.

Lesion size (scale: 0-5).

Height: Plant height (cm) from ground to tip of spikes.

Days to heading: Number of days from seeding to 50% of spikes completely emerged.

The effectiveness of a field inoculation method depends on the level of primary inoculum and the environmental conditions (temperatures, humidity and / or free moisture) that prevail during inoculation and disease development (Adee and Pfender, 1989). Two of the field inoculation methods applied in this study provided primary inoculum in aqueous suspension and about 8 to 10 hours of favorable environmental conditions for infection. Incubation, disease development, and secondary spread were largely affected by the weather conditions following initial infection (Larez, 1985; Luz and Bergstrom 1986). In addition to unfavorable environmental conditions that may hinder further disease development, naturally present inoculum may affect the quality of the data by randomly affecting severity levels and possibly by introducing new virulence. Natural infection appeared to be moderate in Annoceur, and relatively high in Sidi El Aydi. All these factors may contribute to the potential usefulness of an experiment site regarding its suitability for selecting resistant genotypes.

Under Annoceur (wetter) conditions, early spray inoculation likely showed greater genetic variance because the pathogen had then more time to develop and enhance genetic differences. And in the generally less favorable conditions of Sidi El Aydi site (high temperatures and periods of relative drought), straw mulching showed consistently higher genetic variances, heritability values, and correlation to greenhouse results than did spray inoculations.

Conclusions

The evaluation of early spray (jointing / heading), delayed spray (heading flowering), and straw mulch (2 – 3 leaves stage), field inoculation with PTR on durum wheat indicated that these methods showed acceptable and comparable performances in the two widely differing environments tested. The checks reactions were conform to previously documented values. And all field reactions were similar to greenhouse data. However, Site effect, inoculation method and their interactions were highly significant. Under wetter and cooler condition, disease development in early spray inoculation showed more differences in host reaction than late-spray inoculation. Under Sidi El Aydi dryer conditions, this study indicated that straw mulching was more effective than either spray inoculations. straw mulching had the probable advantage of releasing inoculum whenever conditions are favorable for infection. The increased success and the simplicity of the straw inoculation method probably overcame the disadvantage of not using a specific isolate for inoculation. In addition to considering local environmental conditions, breeders should be able to choose an appropriate inoculation method based on additional factors, such as cost/effectiveness, and the goals of their programs. Plant height and earliness could be problematic in developing resistant wheat to tan spot. The breeder could use parents that have the correct range of phenology and use appropriate disease scoring methods. Also, statistical methods could be improved for replicated experiments in order to isolate the effect of phenology from tan spot expression.

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