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Genetics of resistance to the fungus *Helminthosporium sativum* in wheat: use of culture filtrates in tissue culture

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ABSTRACT

Six wheat genotypes and their F_1 and F_2 generations were exposed to the action of *Helminthosporium sativum* culture filtrates to examine the genetics of hexaploid wheat resistance. The objective was to improve the efficiency of breeding programs by identifying the action and number of genes involved in the resistance. The varied response of the tested genotypes to the culture filtrates allowed division of the genotypes into four groups: resistant, moderately resistant, moderately susceptible and susceptible. This variability was detected in the progeny, suggesting that the parents have distinct genetic constitutions. Additive gene action predominated and genetic gain was shown to be possible through selection. The genetic control of the resistance trait seems to be complex because of the presence of gene interaction and the difficulty of eliminating the environmental effects. The inheritance seems to be oligogenic.

INTRODUCTION

Before man's intervention, fungus/plant associations were confined to restricted communities. These associations have been rapidly disseminated over the continents because of man's inadvertent interference since pre-historic times through agriculture and plant exploitation and introduction, leading to the subsequent evolution of pathogenic communities (Dick, 1988). Although the majority of plants are resistant to pathogens, because they have an ample array of defense constitutive components and/or physically block the entrance of microorganisms, many cultivated plants are susceptible to a certain number of pathogens which can cause enormous losses in yield (Chasan, 1994). Diseases caused by fungi are still the main limiting factors to productivity in cultivated plants today (Lamb *et al.*, 1992).

Wheat yields in regions with short hot and humid springs are limited by the fungus *Helminthosporium sativum* Pam. King and Bakke (synonyms *Bipolaris sorokiniana* (Sacc. in Sorok) Schoem. and *Drechslera sorokiniana* Sacc. ex Sorok., where *Cochliobolus sativus* (Ito & Kurib. ex Kurib.) Drechs. ex Dast. is its perfect form). Ludwing (1957) showed that *H. sativum* produces a toxin that is essential to the development of the disease in the attacked plants. This toxin, called helminthosporal, is chemically a sesquiterpenoid dialdehyde (De Mayo *et al.*, 1961). It interferes directly in cellular respiration, inhibiting electron transfer and oxidative phosphorylation processes in the mitochondria. Its site of action is located between flavoprotein dehydrogenase and cytochrome c (Taniguchi and White, 1967). The helminthosporal toxin has effects similar to non-purified filtered fungus

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culture in tests with plant tissues, reproducing many of the symptoms of the disease caused by *H. sativum* (Stoessl, 1981).

Technological advances in the use of fungicides have not contributed greatly to the control of this disease. The use of resistant cultivar genotypes may be an efficient means of control. Knowledge of the genetic control of resistance is necessary to obtain resistant cultivars quickly and efficiently.

The analysis of the pathogen-host interactions is difficult because of the presence of multiple variables, such as the fungus, the host wheat, and the environment. Cristaldo (1993) studied the response of wheat genotypes to *in vitro* treatment with culture filtrates of *H. sativum* and found a significant correlation between *in vitro* and greenhouse responses of plantlets when the same genotypes were scored for disease development.

MATERIAL AND METHODS

One fungal isolate was obtained from contaminated seeds of the wheat cultivar BR 35. Contaminated seeds have a black point, a characteristic symptom of the disease caused by *H. sativum*.

Spores of the *H. sativum* from this isolate were put onto Petri dishes with PDA (potato-dextrose-agar) culture medium. After seven days at a temperature of 24 ± 2°C and a photoperiod of 12 h, small blocks of the culture medium with fungus mycelia were transferred to 250 ml Erlenmeyer flasks with 25 ml of modified liquid Fries medium (Luke and Wheeler, 1955) or 1000 ml Erlenmeyer flasks with 200 ml of modified Fries medium and small balls of glass. The fungus culture was incubated for 21 days in an orbital incubator at a temperature of 24 ± 2°C. After incubation, the fungus mycelium was separated from the liquid phase by filtering through Whatman No. 1 filter paper. The filtrate was concentrated at 45°C in a steam bath in a vertical flux chamber to 10% of its original volume. The product was diluted in two volumes of ethanol (70% v/v) and maintained at 4°C overnight to precipitate salts in the culture medium. The precipitate was removed by filtering through Whatman No. 1 filter paper. With the discarding of the solid phase, the liquid phase was again concentrated at 40°C in a steam bath to 10% of the initial volume, eliminating the added ethanol.

Six wheat genotypes were tested in this study (Mitacoré, CNT 1, LD 7831, 289, 290 and 293) with different responses to the pathogen action, varying from resistance to susceptibility. The F_1 and F_2 generations obtained from a half diallel cross between these genotypes were also used.

Immature wheat embryos collected 10 to 12 days after anthesis were used as explants cultured in callus-inducing medium MS (Murashige and Skoog, 1962) with 2.0 mg/l of 2,4-D (dichlorophenoxyacetic acid), 3% sucrose and 0.8% carrageenin. They were kept in this culture for four weeks, in the dark and at a temperature of 25 ± 1°C.

The calli obtained after four weeks were cut in portions approximately 1.0 mm in diameter and transferred to Petri dishes containing callus maintenance and growth culture medium MS with 0.5 mg/l of 2,4-D, 3% sucrose and 0.8% carrageenin. Filtered toxins were added in order to obtain a final ratio of 1:16 (v:v) of filtrates to medium. Each callus was cut into 14 pieces, 12 of which were put in the culture filtrates and two in culture medium without the filtrates as controls. After four weeks the callus were measured again to determine their growth rate during the period (final measurement - initial measurement = growth). Approximately 12 embryos from each parent and from each F_1 were assessed. Between 100 and 200 F_2 generation embryos were used because of the segregation observed in this generation.

Statistical analysis

The callus growth in the culture with the filtrates was compared with the growth of the controls. Each wheat genotype has distinct genetically determined callus growth potentials (Lange *et al.*, 1995), therefore, the growth proportion (%) was estimated using the mean growth of the callus stemming from a single embryo divided by the mean growth of the control and multiplying the result by 100. These percentage values were analyzed.

The diallel cross system of Griffing (1956), fixed model, method 2, where the parents and a set of F_1 are included, was used to estimate the genetic effects attributed to each genotype.

The phenotypic, genetic and environmental variances and heritability of the resistance trait were estimated according to the methods of Allard (1960) and Falconer (1970), where:

1. Environmental variance: $\sigma^2_E = (\sigma^2_{P1} + \sigma^2_{P2} + \sigma^2_{F1})/3$

2. Phenotypic variance: $\sigma^2_P = \sigma^2_{F2}$

3. Genetic variance: $\sigma^2_G = \sigma^2_P - \sigma^2_E$

4. Heritability: $h^2 = \sigma^2_G / \sigma^2_P$

where σ^2_{P1} = variance of the maternal parent; σ^2_{P2} = variance of the paternal parent; σ^2_{F1} = variance of the F_1; σ^2_{F2} = variance of the F_2.

The values were also analyzed according to the generation mean model, described by Mather and Jinks (1982) to estimate gene action. This model consists of estimating the mean (m), additive (a) and dominance (d) parameters from the means of all the available generations in each cross followed by the chi-square test (χ^2) among the observed and estimated means.

RESULTS

Differences among means

In the presence of the culture filtrates of *H. sativum*, the six parents showed different reactions.

Table I - Differences between the means of the tested parents.

Comparison	Differences between means
290 - 289	7.60
290 - Mitacoré	16.23*
290 - 293	18.27*
290 - CNT 1	28.39*
290 - LD 7831	40.01*
289 - Mitacoré	8.62
289 - 293	10.67
289 - CNT 1	20.78*
289 - LD 7831	32.41*
Mitacoré - 293	2.04
Mitacoré - CNT	12.16
Mitacoré - LD 7831	23.78*
293 - CNT 1	10.12
293 - LD 7831	21.74*
CNT 1 - LD 7831	11.62

*Significant differences at the 5% level of probability.

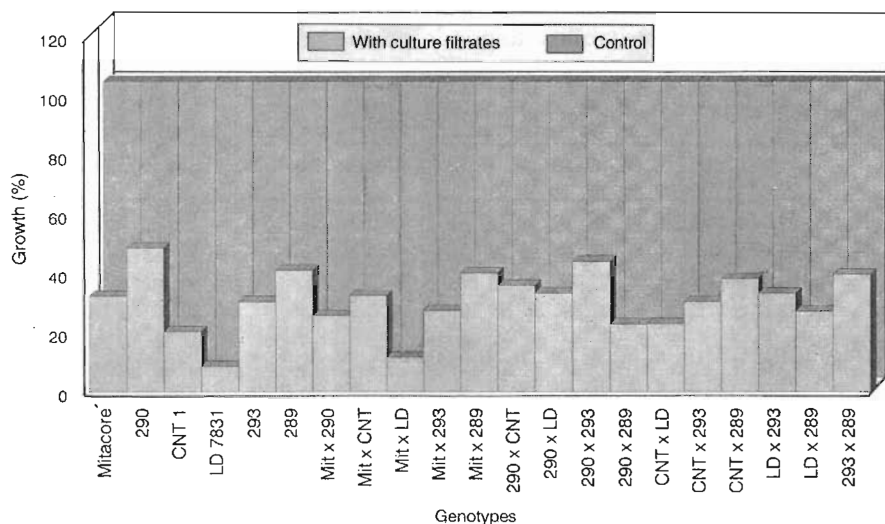

Figure 1 - Growth of callus in medium with culture filtrates as a % of the control.

Parental 290 had the greatest growth compared with the control and 289 also showed excellent growth. CNT 1 and LD 7831 had less growth, characterizing distinct classes of reaction to the presence of the disease (Figure 1).

The responses to the culture filtrates of *H. sativum* were assessed by the least significant difference (LSD) among the means of the genotypes used as a parent (Table I). It was possible to group the genotypes into four distinct classes of responses according to the significance of these differences: 290 - resistant (R); 289, Mitacoré and 293 - moderately resistant (MR); CNT 1 - moderately susceptible (MS); and LD 7831 - susceptible (S).

Diallel

Parental 290 showed the greatest effect of general combining ability (GCA) and was responsible for the increase of the resistance trait in the progeny. LD 7831 had the lowest (negative) GCA value (Table II). The presence of genes from Mitacoré and CNT 1 also determined a greater susceptibility to the culture filtrates, as shown by the results and negative GCA values.

The effects of the specific combining ability (SCA) in some crosses were greater than those of the GCA in both parents, indicating that there are specific genotype combinations that result in a progeny with greater resistance to the action of the *H. sativum* culture filtrates. This fact was noticeable in the cross between LD 7831 and 293. The opposite also occurred, especially in cross 290 x 289, whose SCA was greatly inferior to the GCA of the two parents.

Variances and heritabilities

The variances and heritablities were estimated only for the crosses which had an F_2 segregant generation (Table III). The environmental variance in the phenotype was much larger than the genetic variance.

The cross Mitacoré x CNT 1 showed a very large environmental and a small genetic variance. Heritability was low, lower than those of the other crosses.

Generation mean

The estimated means of the generations were greater in the

crosses which involved Mitacoré (Table IV). Similarly, additive and dominance effects showed greater values in crosses where Mitacoré was present. Dominance showed negative values, but was positive in the crosses CNT 1 x 289 and LD 7831 x 289. The estimated additive values were greater than the estimated dominance in all the crosses.

The χ^2 values were significant, except for the CNT 1 x LD 7831 cross. The significance of the three parameters test indicated that the model was not appropriate to explain the resistance to culture filtrates *in vitro*, and that other genetic components besides additive dominance must be taken into account in the determination of this trait.

Table II - General and specific combining abilities according to Griffing (1956), fixed model, method 2.

Parents	Mitacoré	290	CNT 1	LD 7831	293	289	
Mitacoré	4.98[1]	-8.90[1]	5.23[1]	-8.77[1]	-4.17[1]	6.66[1]	**-1.67[2]**
290		6.97[1]	1.59[1]	5.85[1]	5.28[1]	-17.75[1]	**5.45[2]**
CNT 1			-6.79[1]	2.73[1]	-0.98[1]	4.99[1]	**-1.87[2]**
LD 7831				-4.64[1]	8.71[1]	0.82[1]	**-8.74[2]**
293					-5.43[1]	2.02[1]	**2.51[2]**
289						1.62[1]	**4.32[2]**

1 - Specific combining abilities; 2 - general combining abilities.

Table III - Estimates of the variances and heritabilities of the percentual proportion of callus growth in medium with culture filtrates and control.

	Mit x CNT	Mit x LD	Mit x 289	CNT x LD	CNT x 289	LD x 289
σ^2_E	287.36	523.59	341.41	161.54	238.34	286.50
σ^2_P	311.18	449.02	524.87	322.20	423.95	359.10
σ^2_G	24.52	-74.57	183.46	160.66	185.61	72.60
h^2_a	0.08	zero	0.35	0.50	0.44	0.20

Mit - Mitacoré; CNT - CNT 1; LD - LD 7831.

σ^2_E, σ^2_P, σ^2_G, h^2_a, Environmental, phenotypic and genetic variances and heritability of the resistance trait, respectively.

DISCUSSION

When the growth (%) of the genotypes used as parentals were compared with the F_1 generation (Figure 1) it was found that the three parents which showed the smallest means produced F_1 with higher means, and that the three parents which showed the largest means of growth produced F_1 with lower means. This happened because the genotypes with greater growth, when crossed with those with less growth, underwent a reduction in the mean and vice-versa, showing the action of the additive effects on the trait, and also a certain degree of heterosis. However, the analysis of the F_1 mean of each cross showed distinct behaviors. There were cases in which a strong negative heterosis was present, such as in cross 290 x 289, where the relationship of the F_1 and parental means was also corroborated by a high negative value of the SCA (Table II). An intense positive heterosis happened in other crosses, as in LD 7831 x 293, detected also by its means and by the highly positive SCA value. Additive effects were present in other cases, such as in cross CNT 1 x 293, whose F_1 mean was intermediate to the means of the two parents.

The heritability values for the genotypes along the generations analyzed showed that the environment is responsible for 50% or more of the plant response to the action of the culture filtrates (Table III). As heritability depends on the magnitude of the genetic

Table IV - Generation mean and three parameters gene action according to Mather and Jinks (1982).

	Mit x CNT	Mit x LD	Mit x 289	CNT x LD	CNT x 289	LD x 289
P_1	32.34 ± 24.54	32.34 ± 24.54	32.34 ± 24.54	20.18 ± 12.45	20.18 ± 12.45	8.56 ± 8.58
P_2	20.18 ± 12.45	8.56 ± 8.58	40.97 ± 14.32	8.56 ± 8.58	40.97 ± 14.32	40.97 ± 14.32
F_1	32.40 ± 10.24	11.52 ± 9.40	40.01 ± 14.73	22.83 ± 16.00	38.17 ± 18.84	27.10 ± 24.10
F_2	23.58 ± 17.66	23.14 ± 21.19	32.17 ± 22.91	20.79 ± 17.95	33.16 ± 20.59	24.38 ± 18.95
m	52.72 ± 12.79	53.09 ± 12.36	70.39 ± 13.13	20.24 ± 7.44	27.89 ± 9.29	13.47 ± 8.19
a	53.32 ± 13.36	52.84 ± 12.54	72.87 ± 13.86	19.56 ± 7.56	25.48 ± 9.49	10.81 ± 8.30
d	-25.09 ± 16.94	-43.77 ± 15.35	-38.05 ± 20.22	-1.26 ± 17.14	4.49 ± 20.33	8.94 ± 22.99
χ^2	13.10*	10.38*	31.14*	3.370	14.45*	10.71

Mit - Mitacoré; CNT - CNT 1; LD - LD 7831; P_1 - Maternal parent; P_2 - paternal parent; m - mean; a - additive effect; d - dominance effect; *χ^2 significant at the 5% level of probability.

variance (heritable) in the population and also of the non-inheritable portion, it could be increased by the introduction of more genetic variation in the population, and through a stabilization of the environment in which the individuals develop (Mather and Jinks, 1982). It is possible that the small size of the population was a factor responsible for the non-significant χ^2 of cross CNT 1 x LD 7831 (Tabela IV), for the extremely low heritability shown by cross Mitacoré x CNT 1 and for the zero heritability in the cross Mitacoré x LD 7831 (Table III). The fact that the environmental variance was high deserves attention (Table III), as one of the objectives of working with tissue culture and with culture filtrates was to try to control the effect of the environmental variable. This suggests that the environment must exercise a very intense action on the resistance to the fungus *H. sativum*, especially when the great variability of the fungus in its natural environment is taken into account. So, the way to reduce environmental variance would be to increase parcel size and also replication number.

The generation mean test (Table IV) confirmed the importance of the gene interaction, along with the additive and dominance effects, in the determination of the trait, suggesting that its inheritance is not controlled by a single gene. Additive effects were more important that those of dominance. As the χ^2 was significant, the test should have been carried out again involving a greater number of parameters (Mather and Jinks, 1982) to estimate the effect of the gene interactions on the trait. However, the absence of backcrosses in the experiment made it impossible to fit further parameters due to a lack of degrees of freedom. The high values of standard deviation of parents and F_1 showed the intense action of the environment on resistance (Table IV).

It is probable that there are a few major and minor genes responsible for the trait manifestation. These genes probably interact causing the detected heterosis, having complementary action, which is expressed by the additive effects. Because the gene interactions exercise considerable effect on the degree of resistance to the *H. sativum* fungus and because the environmental influence is greater or equal to the genetic influence, as reflected by the low heritability values, the trait is not very easy to modify in the plant breeding programs. While developing a breeding program for resistance to *H. sativum* in the field, it is of fundamental importance to have an efficient environmental control through suitable plot size and large number of replications in various years and sites to allow an increase in the trait heritability.

In the field, the fungus shows extreme variability, which added to the variability of the cultivated wheat genotypes sets up a complex pattern of pathogen-host interactions, making selection markedly more difficult. The present study showed that the genetics that governs the response of wheat to *H. sativum* involves, besides additive and dominance effects, interaction among the genes, which is shown in the form of positive and negative overdominance.

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RESUMO

Foram testados seis genótipos de trigo e suas gerações F_1 e F_2 com o objetivo de compreender a genética da resistência do trigo hexaplóide à ação de filtrados tóxicos de *Helminthosporium sativum*, através da identificação da ação gênica e do número de genes envolvidos, de modo a tornar possível uma maior eficiência nos programas de seleção. Os genótipos testados apresentaram variabilidade na resposta aos filtrados tóxicos, possibilitando sua caracterização em quatro grupos: resistente, moderadamente resistente, moderadamente susceptível e susceptível. Esta variabilidade foi detectada na progênie, sugerindo que os genitores possuíam constituições genéticas distintas. Foi evidenciado ganho genético através da análise das gerações, onde a ação gênica de maior importância foi a de aditividade. A herança do caráter testado parece ser complexa, devido à presença de interações gênicas e à dificuldade de evitar a participação do ambiente na manifestação do caráter. A herança parece ser oligogênica.

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