Eur J Plant Pathol (2011) 131:497–510
DOI 10.1007/s10658-011-9825-z

Epidemiological and histological components of crown rust resistance in oat genotypes

Felipe André Sganzerla Graichen · José Antônio Martinelli · Caroline de Lima Wesp · Luis Carlos Federizzi · Márcia Soares Chaves

Accepted: 20 June 2011 / Published online: 7 July 2011
© KNPV 2011

Abstract Crown rust, caused by *Puccinia coronata* f. sp. *avenae*, can cause significant damage in all regions where oats (*Avena sativa* L.) are cultivated. The primary means of controlling crown rust has been through genetic resistance, although in most cases resistance has been quickly overcome by the pathogen. More durable partial or non-specific resistance may possess different mechanisms from those underlying genes with specific effects. We studied the epidemiological and histological components of crown rust resistance with potential use in plant protection. Among the components evaluated, pustule density showed the clearest effect on resistance, while the latent period was not an important component. Cell death associated with the accumulation of autofluorescent and phenolic compounds was common in the resistant genotypes, but temporally distinct for the genotypes studied. Genotype Pc68/5*Starter, which has race-specific resistance, showed rapid cell death that prevented the development of pathogen colonies. Conversely, with cultivar URS 21 and genotypes 04B7113-1 and 04B7119-2, cell death and associated accumulation of autofluorescent and phenolic compounds was delayed until pathogen colonies were already established. Pathogen colonies developed normally in susceptible plants genotypes, and had usually produced sporogenic tissue by 5 days after inoculation. The data suggest that the resistance mechanisms, especially hypersensitivity and phenolic compound production, active in resistant plants are similar but may be differently expressed over time. The temporal variation in the expression of hypersensitivity and phenolic compound production reflects the level of field resistance in these genotypes.

Keywords Oat cell autofluorescence · Oat cell death · Partial resistance · Pustule density · *Pc68* gene · *Puccinia coronata* f. sp. *avenae*

F. A. S. Graichen · C. de Lima Wesp
Programa de Pós-Graduação em Fitotecnia, Universidade Federal do Rio Grande do Sul,
91540-000 Porto Alegre, RS, Brazil

J. A. Martinelli (✉)
Departamento de Fitossanidade, Universidade Federal do Rio Grande do Sul,
91540-000 Porto Alegre, RS, Brazil
e-mail: jamfito@ufrgs.br

L. C. Federizzi
Departamento de Plantas de Lavoura, Universidade Federal do Rio Grande do Sul,
91540-000 Porto Alegre, RS, Brazil

M. S. Chaves
Embrapa - Centro Nacional de Pesquisa de Trigo,
99001-970 Passo Fundo, RS, Brazil

Present Address:
F. A. S. Graichen
Universidade Estadual de Mato Grosso do Sul,
79200-000 Aquidauana, MS, Brazil

Introduction

Oat (*Avena sativa* L.) is an important small grain cereal crop in Australia, Europe, North and South America. In the Southern Hemisphere, where the climate is subtropical to temperate, oat is used as a winter cover crop, food for livestock and humans, and as a raw material for industrial uses. In southern Brazil it is the third most important winter crop (Leonard and Martinelli 2005).

Crown rust caused by the fungus *Puccinia coronata* f. sp. *avenae* is the main disease of oat crops, occurring in all environments where this cereal is cultivated. In years when environmental conditions are favourable to the disease, severities above 90% are observed and in these situations damage can be greater than 50% (Martinelli et al. 1994).

Resistance is by far the most important defence of plants against pathogens, and is considered durable when it remains effective for a considerable time despite wide exposure to a pathogen (Johnson 1984). In evolutionary terms, no resistance will last forever, but it is possible to discern three groups of predominantly durable resistances: quantitative resistance to generalist pathogens with a wide host range; quantitative resistance to specialist pathogens, this resistance being based on from one to several genes with additive effects; and non-hypersensitive monogenic resistance to specialist pathogens (Ribeiro do Vale et al. 2001). Durable resistance to cereal rusts is often due to genes producing small modifications to pathogen development, with such effects being greatly enhanced if they occur in combination with other, including race-specific, genes. The combination of different resistance mechanisms can be of great value in increasing the durability of resistance (Rubiales and Niks 2000).

The resistance of cereals to rusts can be classified as pre-haustorial and post-haustorial mechanisms (Heath 1981); pre-haustorial mechanisms being commonly involved in non-host reactions preventing rust fungi colonizing plant tissue, while post-haustorial mechanisms are expressed after the onset of host colonization by pathogens. These mechanisms may involve cell death, production of papillae and the accumulation of phenolic compounds, among others. The expression of resistance can be viewed as a reduction of the epidemiological components, slowing disease progress and, consequently, the epidemic in the field. The reductions of these epidemiological components are directly linked to cellular events that hinder the development of the fungus inside the plant, or cause the death of the fungus, impairing its normal development. Díaz-Lago et al. (2003) reported that the epidemiological components of the two unrelated oat genotypes that they studied were highly correlated. This is supported by Chaves et al. (2004a), who found that the expression of partial resistance to crown rust is not due to only one resistance component and is more expressed when various components act together so that the combined effect of these components reflects the level of observed resistance. In other rust pathosystems, but not crown rust, rusts that develop larger colonies have a shorter latent period and produce a higher density of lesions. However, for plant genotypes which have rust colonies associated with plant cell death, rusts have lower infection efficiency and produce smaller individual colonies (Sillero and Rubiales 2002; Barilli et al. 2009).

Despite much research regarding resistance to rust fungi, there are still a variety of unexploited resistance mechanisms and combining those acting at different stages of the infection process could provide multiple barriers to the pathogen (Rubiales and Niks 2000). Detailed germplasm screening may result in the discovery of alternative, more durable, defence mechanisms not associated with hypersensitivity (Niks and Rubiales 2002). In this sense, histological studies on interactions between plants and rusts can help both to distinguish the various resistance mechanisms and to combine them in a genotype which may result in increased durability (Rubiales and Niks 2000). Furthermore, correctly interpreted cytological data can provide valuable information to broaden the current knowledge-base on plant resistance and on which phenomena may be the most promising to investigate physiologically and biochemically (Heath 1981).

In southern Brazil, oat cultivars released by breeding programs show race-specific resistance to crown rust. Such resistance is often not very durable, but some genotypes remain resistant despite being widely grown, an example being the Brazilian cultivar URS 21, which has remained resistant to this disease since 2000. The main feature of URS 21 is the presence of a small amount of disease throughout the crop cycle. Although more than 100 major genes (*Pc* genes) are known to confer resistance to crown rust caused by *P. coronata*, none of these genes are

currently fully effective against this pathogen. In North America, the *Pc68* gene remained effective for about 10 years, and it seems promising to explore the effectiveness of this gene in the South America environment, where the frequency of *P. coronata* races virulent against oats containing the *Pc68* gene is low (Leonard and Martinelli 2005; Vieira et al. 2007).

Our study investigated some epidemiological and histological components of oat genotypes with different resistance types potentially helpful in achieving durable resistance to crown rust of oats in southern Brazil. We evaluated the area under the disease progress curve, latent period, pustule density, infection type, area of individual *P. coronata* colonies, autofluorescence and the presence of phenolic compounds in an oat genotype with *Pc68* gene race-specific resistance as well as in the race-non-specific resistance oat cultivar URS 21 and three oat genotypes carrying undetermined resistances.

Material and methods

Crown rust resistance mechanisms were investigated using a set of eight oat genotypes, previously selected from a larger number of genotypes according to the severity of the disease and the type of infection visible on the leaves of the plants. This genotype set was evaluated under field conditions from 2006 to 2008. The genotypes were also assessed under controlled conditions in 2007 for a number of epidemiological components and for some other histological components related to resistance in 2008. Genotype 04B7119-2 (see below) could not be evaluated in 2007 because it produced only a small amount of seeds which was then increased in that year. In 2007, cultivar URS Guapa (see below) was added to the genotype set due to the sudden and complete breakdown of resistance in this genotype during that year, which could provide evidence of susceptibility to the new *P. coronata* races present. During 2007 URS Guapa was assessed under controlled conditions for epidemiological components and in 2008 for field evaluation and histological assessment under controlled conditions.

Field experiments

We used field experiments to evaluate *P. coronata* crown rust resistance in the resistant oat cultivar URS 21 and the susceptible cultivars URS 22, URS Guapa and UFRGS 17, all developed by the Oat Breeding Program of the Federal University of Rio Grande do Sul (UFRGS) in the southern Brazilian state of Rio Grande do Sul (RS). Cultivars URS 21, URS 22 and URS Guapa were used because they are currently the most widely grown in southern Brazil, while cultivar UFRGS 17 is an older susceptible cultivar no longer recommended for cultivation but included in this study for comparison. Three genotypes (UFRGS 04B7107-2, UFRGS 04B7113-1 and UFRGS 04B7119-2) were also evaluated because previous assessments had shown a low intensity of crown rust disease associated with the presence of chlorotic or necrotic halo and reduced pustule size. We also evaluated the resistant genotype Pc68/5*Starter, a near isogenic line of the North American cultivar "Starter" with *Pc68*, which has also shown resistance in southern Brazil. All seeds were produced 'in house' at the Agronomic Experimental Station of the Universidade Federal do Rio Grande do Sul, Eldorado do Sul, RS, Brazil (AES-UFRGS).

Field experiments were carried out during June to early November (winter/spring) of 2006, 2007 and 2008 using 3 m×1 m plots spaced 40 cm apart and arranged in a randomised block design, with three replications in 2006 and four replications in the subsequent years. About 950 seeds were sown in each plot, the seeds having been treated with 36 g per 100 kg seed of the insecticide imidacloprid (Bayer CropScience, Kansas City, USA) and 37.5 g per 100 kg seed of the fungicide triadimenol (BASF SA, Bonn, Germany). Genotype 07B7119-2 was not evaluated in 2007 and cultivar URS Guapa was assessed only in 2008. The plants were cultivated in a no-till system, always after soybean as a summer crop, using the recommended fertilizer inputs and allowed to become naturally inoculated with uredospores of *P. coronata*. The experiments were conducted at AES-UFRGS.

Crown rust severity was assessed weekly after the appearance of the first pustules, according to a modified Cobb's diagrammatic scale, and the normalized and corrected area under the disease progress curve (AUDPCc) calculated using the following formula:

$$\mathrm{AUDPCc} = \frac{\sum_{i=1}^{n}\left[\frac{Y_i+Y_{i+1}}{2}\times(t_{i+1}-t_i)\right]}{n}\times c$$

where: y_i = % leaf area affected by crown rust (severity at the i^{th} observation; t_i = time in days after sowing at the i^{th} observation, n = period of days from the onset and latest assessment of the disease, and c = the longest epidemic period among the genotypes tested (from Graichen et al. 2010).

Evaluation of oat seedling epidemiological components of resistance under controlled conditions

To evaluate some seedling epidemiological components of resistance under controlled conditions, we sowed seeds of oat genotypes URS 21, URS 22, URS Guapa, UFRGS 17, 04B7107-2, 04B7113-1 and Pc68/5*Starter in 25-ml styrofoam pots arranged in rows with 10 replicates. Each pot was sown with three seeds of a genotype, and thinned after emergence to one seedling per pot. The pots were placed in a growth chamber at 22°C±2°C under 200 μmol/m^2/s illumination provided by two 40 W Grolux plus two daylight type fluorescent tubes (model T-12, Sylvania, Danvers, USA) placed 30 cm above the top of the pot and with a 16 h light and 8 h dark photoperiod.

Ten days after sowing, when the seedlings had the first fully developed leaf, the leaves were fixed horizontally, with the adaxial blade side up, sprayed with 0.1 ml Tween 20® (Synth) in 1 l of distilled water, put at the bottom of a cylindrical aluminium settling tower (Wesp et al. 2008) and then inoculated with 0.025 g of dry *P. coronata* uredospores (~500 cm^{-2}), which were blown into the settling tower through an aperture near the top. These spores were bulk collected in 2006 in the field from various oat genotypes and multiplied under controlled conditions on the susceptible oat cultivar URS 22. After inoculation the seedlings were placed in the dark for 14 h at 20°C in a moist chamber (100% RH).

Two epidemiological components of resistance were evaluated, the latent period in days and the pustule density in pustules cm^{-2}. The latent period, defined as the number of days between inoculation and the appearance of the first pustule spores (Chaves et al. 2004a; Wesp et al. 2008), was determined by examining the seedlings each day with a 20x magnification hand lens until they all presented symptoms, the latent period for each genotype being calculated as the mean value for the ten replicates. The pustule density, calculated as the number of pustules developing at the midpoint of the prophyl (Wesp et al. 2008), was assessed 10 days after inoculation by examining them under a Leica MZ 12 stereoscope microscope (Leica Microsystems, Wetzlar, Germany) at 40x magnification and counting the number of pustules occurring up to 2.5 cm from either side of the longitudinal midpoint of each leaf.

The infection type (IT) produced was assessed employing the same leaves as were used for calculating the pustule density value. The IT was based on a qualitative 0 to 4 scale (Chong et al. 2000), plus some additional symbols to represent the following infection classes: no uredinia or other macroscopic signs of infection (0); no uredinia, but necrotic or chlorotic flecks (;); small uredinia surrounded by chlorosis or necrosis (1); small to medium-size uredinia in chlorotic areas (2); medium-size uredinia in chlorotic areas (3); and large uredinia without necrosis or chlorosis (4). Smaller than usual pustules (−) and larger than usual pustules (+) were also noted along with strong necrosis (N) and chlorotic halo (Cl) associated with the type of infection indicated (Bonnett et al. 2002). A heterogeneous IT was indicated by a combination of the IT classes, with a IT value equal to or greater than 3 being considered as a susceptible reaction.

Evaluation of the histological components of resistance to established *P. Coronata* colonies

This study considered a rust colony to be a discrete or diffuse collection of hyphae that grow from one plant cell, or a group of cells, inside the plant tissue and is generally larger in area than the pustule that it may, or may not, produce. A pustule is a small blister-like elevation of epidermis created as spores form from the sporogenic tissue. Colonies of *P. coronata* established in the eight oat seedlings genotypes URS 21, URS 22, URS Guapa, UFRGS 17, 04B7107-2, 04B7113-1, 04B7119-2 and Pc68/5*Starter were microscopically evaluated for histological resistance components using the same inoculum, inoculation conditions and seedling incubation conditions as those used for the determination of the epidemiological components of resistance in seedlings. Ten days after sowing a spore settling tower was used to inoculate leaves with dry *P. coronata* uredospores, 0.010 g (~100 spores cm^{-2}) for bright field and phase contrast microscopy and the determination of lignin-like compounds or 0.030 g (~500 spores cm^{-2}) for blue-light epifluorescence microscopy and the determination of phenolic compounds.

For bright field and phase contrast microscopy, 120 h after inoculation leaves were collected from two plants per genotype in each of the three replicates and 1.5 cm samples excised from the mid-region of each leaf. The samples were fixed for 24 h in 3:1 (v:v) ethanol:dichloromethane containing 0.15% (w/v) trichloroacetic acid, stained by boiling for 5 min in 1:2 (v:v) lactophenol:ethanol containing 0.05% (w/v) trypan blue and cleared in a 5:2 (w:v) chloral hydrate:water mixture. After clearing, the stained samples were dehydrated with ethanol (80% (v:v) for 30 min; 90% for 30 min; and 100% for 2×30 min) and stained for 5 min with a saturated solution of picric acid in methyl salicylate, excess picric acid being removed by 15 min clearing in methyl salicylate (MacBryde 1936). The stained samples were mounted in methyl salicylate with their adaxial sides up under cover slips sealed with nail varnish, bright field or phase contrast microscopy, using an Olympus BX 41 microscope (Olympus Corporation, Tokyo, Japan), was employed to detect fungal/plant cell interactions, three replicates being assessed for each genotype. The proportion of *P. coronata* colonies associated with cell death was calculated as the number of *P. coronata* colonies associated with dead oat cells, i.e. those intensely stained with trypan blue, divided by the total number of *P. coronata* colonies evaluated. Apposition (papillae) on the host cell wall was detected by the presence of intensely bright marks on the cell wall when *P. coronata* hyphae were present (Bender et al. 2000). The area of each *P. coronata* colony was estimated using a calibrated eyepiece micrometer to measure the colony length parallel to the leaf veins and the colony width perpendicular to the leaf veins, coalesced colonies and those located on the edges of the samples were excluded. Individual colony area was calculated, assuming elliptical colonies, by applying the equation $area = \pi \times \frac{length \times width}{4}$ (Bender et al. 2000). The proportion of *P. coronata* colonies with sporogenic tissue was estimated by dividing the number of colonies with sporogenic cells or uredospores by the total number of colonies evaluated.

To detect host autofluorescent compounds associated with established *P. coronata* colonies showing at least an appressorium, a substomatal vesicle and an infective hypha, three plants per genotype were inoculated and sampled 48 h and 120 h after inoculation using the method described for brightfield microscopy. After collection, samples were fixed for 24 h in 3:1 (v:v) ethanol:dichloromethane containing 0.15% (w/v) trichloroacetic acid and then stored in 50% glycerol until analysis. To detect autofluorescence, the fixed samples were cleared in 5:2 (w:v) chloral hydrate:water, mounted in 50% glycerol and examined using blue-light epifluorescence microscopy with Olympus BX 41 microscope fitted with a U-MWB2 excitation filter set comprising a 460 to 490 nm dichroic beamsplitter 500 nm BA520 barrier filter (De Vleesschauwer et al. 2009). Leaf samples collected 120 h after inoculation were also assessed for the formation of *P. coronata* sporogenic tissue. Phenolic compounds were detected by toluidine blue staining (O'Brien et al. 1964; with minor modifications) of leaf samples from two plants per genotype. The fixed samples (described above) were cleared in 5:2 chloral hydrate:water, incubated twice for 30 min at 22°C in 50 mM citrate buffer (pH 3.5) to stabilise the pH, stained for 20 min with 0.05% (w/v) toluidine blue in the same buffer, washed in the same buffer and mounted in 1:1 (v:v) glycerol:citrate buffer. The presence or absence of *P. coronata* colonies associated with plant cells stained with toluidine blue was noted but the proportion of colonies associated with stained plant cells was not recorded due to the difficulty in identifying the association/reaction in some colonies, particularly when a reaction was deeper inside the leaf mesophyll.

The presence of lignin-like compounds associated with *P. coronata* colonies was investigated in samples collected 48 h and 120 h after inoculation using Weisner staining and Cross-Bevan staining (Deon 1997). In brief, for Weisner staining the samples were fixed after collection, stored in 50% glycerol (v:v) and then immersed in 50% ethanol (v:v) followed by incubation for 30–60 min at 22°C in an ethanolic solution of 2% phloroglucinol (w:v) in 12 N HCl (2:1, v:v) and then mounted in 50% glycerol (v:v) and microscopically examined immediately afterwards. For the Cross-Bevan reaction, samples were immersed in 50% ethanol for 1 min then transferred to 1% aqueous sodium hypochlorite (w:v) for 5 min and then incubated for 30 min in 5% sodium hypochlorite (w:v) acidified to pH 1 with HCl before being washed in deionized distilled water and vacuum infiltrated with 2% aqueous $NaSO_4$ (w:v) at 4°C for 10 to 15 min. The samples were mounted in $NaSO_4$ (20%, w:v) and immediately observed microscopically.

Three replicates were evaluated for each reaction, genotype and collection time.

Unless otherwise stated, all reagents were at least analytical grade and were purchased from the Sigma Chemical Corporation.

When assessing the seedling epidemiological and histological resistance components we also assessed the type of infection produced 13 to 15 days after inoculation.

Data analysis

The AUDPCc data obtained in 2006 and 2008 showed heterogeneous variances, which were corrected using the $Y_{ij}^{*} = \log(Y_{ij} + 10)$ transformation and subsequent analysis of variance (ANOVA). The latent period data were analysed using descriptive statistics, considering the mean and standard deviation of the mean. The pustule density data showed no homogeneity of variances and were transformed as $Y_{ij}^{*} = \log(Y_{ij})$ following ANOVA in a randomised complete block design. The data on individual *P. coronata* colony size were subjected to ANOVA using a completely randomised design with three replicates. The size of individual *P. coronata* colonies in each replicate was obtained by evaluation of individual colony size on two plants of each genotype. The discrimination of AUDPCc means, pustule density and lesion size was performed using Duncan's test at the 5% probability level.

Data on the proportion of *P. coronata* colonies with sporogenic tissue or autofluorescence, or associated with plant cell death, were analysed using the chi-squared (χ^2) test with a 2×8 contingency table. Discrimination between proportions was performed using a Tukey-type multiple comparison test (Zar 2009) at the 5% probability level.

Spearman correlation coefficients (r_s) were used to investigate any association between pustule density, pustule area, the proportion of *P. coronata* colonies associated with sporogenic tissue, autofluorescence or plant cell death at 48 h and 120 h after inoculation. Each r_s value was based on eight pairs of data, except for the pustule density data, which used only seven pairs of data since there was no pustule density data for the 04B7119-2 genotype. The r_s values were considered significant when $P \leq 0.05$. All statistical analyses were performed using the statistical analysis system (SAS) software version 8.0 (SAS Institute Inc. Cary, NC, USA).

Results

Field experiments

Long-lasting crown rust epidemics, ranging from 50 days to 67 days, occurred during the three years of trials from 2006 to 2008. The genotypes differed in the intensity of crown rust, with severities varying from traces of the disease on genotypes 04B7119-2 and Pc68/5*Starter to more than 80% on genotype URS 22 during all the years and UFRGS 17 in 2008. Cultivars URS 22 and UFRGS 17 showed the highest AUDPCc values, and were therefore considered susceptible to disease (Table 1). Cultivar URS Guapa also showed a high AUDPCc value of 470 in the only year it was assessed, and was considered susceptible to disease. The remaining genotypes URS 21, 04B7107-2, 04B7113-1, 04B7119-2 and Pc68/5*Starter showed low disease severity and therefore were classified as crown rust resistant.

Evaluation of oat seedling epidemiological components under controlled conditions

There were no significant differences in latent period among the six genotypes that developed pustules in the seedling test. The average latent period for the three resistant genotypes was only 0.2 days longer than the average for the three susceptible ones (Table 1). Pustules were not observed in any seedling of the Pc68/5*Starter genotype.

Analysis of variance of the pustule density values gave significant effects for genotypes ($P<0.0001$) and between inoculations ($P<0.0001$), with the pustule density value ranging from 13.3 pustules cm^{-2} on cultivar URS 21 to 135.3 pustules cm^{-2} on cultivar UFRGS 17 (Table 1). Cultivars URS 22, URS Guapa and UFRGS 17 and genotype 04B7107-2 had the highest pustule density values, but there was no significant difference between these genotypes. Cultivar URS 21 had the lowest pustule density value, differing from all the other genotypes except genotype 04B7113-1. Since genotype Pc68/5*Starter presented no pustules and only a few chlorotic flecks, this genotype was not included in the statistical analysis.

The infection type occurring in the URS 22, UFRGS 17 and URS Guapa cultivars was type 3 or 3+ (Table 2), indicating susceptibility in all inoculations with the *P. coronata* bulk races used. All other genotypes showed infection types characteristic of resistance, with IT<3.

Table 1 Epidemiological components of resistance to oat crown rust evaluated on field-grown adult plants and seedlings grown under controlled conditions in a growth chamber

Oat genotypes	Adult plants (field-grown)			Seedlings (growth chamber)	
	Area under disease progress curve normalized and corrected (AUDPCc)[b]			Pustule density (pustules cm^{-2}) [d]	Latent period
	Year				
	2006	2007	2008		
URS 22	907a[a]	826a[a]	1561a[a]	115.7a[a]	6.8[NS]±0.101[f]
UFRGS 17	265B	259b	1089a	135.3a	7.0±0.053
URS Guapa	–[c]	–[c]	470b	94.2a	6.9±0.118
URS 21	43 C	112c	46c	13.3c	7.5±0.125
04B7113-1	7	5e	7 d	36.5bc	7.0±0.000
Pc68/5*Starter	6D	4e	4 d	–[d]	–[d]
04B7107-2	5D	35 d	69c	83.2ab	6.9±0.143
04B7119-2	4D	–[c]	5 d	–[c]	–[c]
CV%	11	8%	8	17	NA

[a] Values with the same letter in a column are not significantly different by Duncan's test at the 5% probability level;

[b] Data transformed using $y^*_{ij}=log\ (y_{ij}+10)$;

[c] Unevaluated genotypes;

[d] Data transformed using $y^*_{ij}=log(y_{ij})$;

[e] Not included in the statistical analysis because no pustules were formed;

[f] Standard deviation of the mean;

CV Coefficient of variation

NA not applicable, data not included in the analysis of variance.

Genotypes 04B113-1 and 04B7119-2 showed from small to large pustules, often surrounded by necrotic or chlorotic halos, while 04B7107-2 had fewer pustules with these characteristics. Cultivar URS 21 showed predominantly small pustules associated with a yellow, sometimes necrotic, halo. Genotype Pc68/5*Starter showed no pustules and flecks only rarely, corresponding to IT. Flecks were more commonly observed on cultivar URS 21 and genotype 04B7119-2 as well as, more rarely, on genotype 04B7113-1. There were slight variations amongst the types of infection between inoculations, especially with genotype 04B7113-1, where at the third inoculation the IT was 3, indicating susceptibility.

Evaluation of the histological components of resistance to established *P. Coronata* colonies

The *P. coronata* colonies observed at 120 h (5 days) after inoculation showed intense development of intercellular mycelium, haustorium mother cells and haustoria. The longest axis of the *P. coronata* colonies was in the direction parallel to the vascular bundles of the leaves, generally having the lateral developmental of colonies limited by the presence of the bundles. The average length and width of the colonies ranged from 0.27 mm long by 0.12 mm wide for genotype Pc68/5*Starter to 1.71 mm long by 0.81 mm wide for genotype 04B7107-2 (data not shown). The mean area of individual *P. coronata* colonies ranged from 0.027 to 1.124 mm^2 for the different oat genotypes tested, with an overall mean of 0.84 mm^2. Analysis of variance indicated no effect of repetitions (P=0.3885) but significant differences between genotypes (P= 0.0001) for the area of colonies. However, when the averages were compared by Duncan's test at the 5% probability level, only genotype Pc68/5*Starter differed significantly from the other genotypes (Table 2).

Cells colonized and killed by *P. coronata* stained intensely with trypan blue, unlike live cells, whether

Table 2 Histological components of resistance to *Puccinia coronata* f. sp. *avenae* colonies on oat genotypes as assessed 120 h after inoculation with *P. coronata*. The type of infection was assessed in three laboratory experiments, inoculation 1 (2007) and inoculations 2 and 3 (both in 2008)

Oat genotype	*P. coronata* colony area (mm^2)	Oat cell death (%)[b]	*P. coronata* sporogenic tissue (%)[b]	Infection type[d]			Oat phenotype
				Inoculation 1	Inoculation 2	Inoculation 3	
04B7107-2	1.124a[a]	13.6c[c]	74.2a[c]	3−3	3 3−	3 3−	Susceptible
04B7113-1	0.940a	67.9b	25.0def	2N 2+ 3	2N 2+ 3	3−3	Resistant
UFRGS 17	0.930a	6.5c	64.6ab	3 3+	3 3+	3 3+	Susceptible
04B7119-2	0.912a	63.0b	31.6cde	2 + N 3−	2 + N 1;	2N 1 3−	Resistant
URS 22	0.901a	2.2c	54.6abc	3 3+	3 3+	3 3+	Susceptible
URS 21	0.857a	60.5b	7.9ef	2 3−;	1;	2 1;	Resistant
URS Guapa	0.823a	10.4c	45.4bcd	3 3+	3 3+	3 3+	Susceptible
Pc68/5*Starter	0.027b	100.0a	0.0 g	0	0	0;	Resistant
CV	21.73	–	–				

[a] Values with the same letter in a column are not significantly different at the 5% probability level by Duncan's test;

[b] Percentage of fungal colonies associated with plant cell death or the presence of sporogenic tissue. Cell Death, chi-squared (χ^2) value=175.29 ($P<0.0001$). Presence of sporogenic tissue, χ^2 =94.67 ($P<0.0001$);

[c] Values with the same letter in the same column are not significantly different at the 5% probability level by a multiple comparison for proportions Tukey-type test (Zar 2009);

[d] Infection classes: 0 = no uredinia or other macroscopic signs of infection;; no uredinia, but necrotic or chlorotic flecks; 1 = small uredinia surrounded by chlorosis or necrosis; 2 = small to medium-size uredinia in chlorotic areas; 3 = medium-size uredinia in chlorotic areas; and 4 = large uredinia without necrosis or chlorosis; - = smaller and + = larger than usual pustules; N = strong necrosis; Cl = chlorotic halo. Heterogeneous IT is indicated by a combination of the classes, a value equal to or greater than 3 being considered a susceptible reaction;

CV Coefficient of variation;

or not colonized, which were only slightly blue. The different oat genotypes showed varying proportions of *P. coronata* colonies associated with cell death (Table 2), and were divided into three distinct groups: genotype Pc68/5*Starter only, for which all the colonies were associated with oat cell death; cultivar URS 21 plus genotypes 04B7113-2 and 04B7119-2, for which 60% of colonies were associated with oat cell death; and all the other genotypes tested, all of which showed less than 15% of colonies associated with oat cell death (Table 2).

Sporogenic tissue was identified by thickening of *P. coronata* mycelium and the presence of sporogenic cells, with immature or mature uredospores or without either. All these structures were formed within host oat tissue before rupture of the epidermis and release of the uredospores. The oat genotypes tested showed different proportions of colonies that developed sporogenic tissue (Table 2), with sporogenic tissue being absent for genotype Pc68/5*Starter, low for cultivar URS 21 and genotypes 04B113-1 and 04B7119-2, and high for genotype 04B7107-2 and cultivars UFRGS 17 and URS 22. Repeat experiments using the same methodology produced similar results (data not shown). For most of the oat genotypes, only a small number of *P. coronata* colonies showed oat cell autofluorescence when evaluated 48 h after inoculation. The exception to this was genotype Pc68/5*Starter, for which 79% of colonies were autofluorescent (Fig. 1). At 120 h after inoculation, cultivar URS 21 and genotypes 04B7119-2 and Pc68/5*Starter showed high levels of autofluorescence, with the other genotypes showing no more than 25%.

Most of the *P. coronata* colonies present on genotypes Pc68/5*Starter and URS 21 showed no sporogenic tissue but did show oat cell autofluorescence (Table 3). In *P. coronata* colonies associated with genotype 04B7119-2, autofluorescence occurred with or without sporogenic tissue formation, but in this genotype a large number of colonies developed

sporogenic tissue associated with autofluorescence compounds (Fig. 1). In cultivars UFRGS 17 and URS Guapa plus genotypes 04B7113-1 and 04B7107-2, a small proportion of colonies with sporogenic tissue showed autofluorescence, although a few colonies showed autofluorescence without sporogenic tissue. About 25% of the colonies on genotype URS 22 showed no sporogenic tissue or autofluorescence (Table 3).

The presence of phenolic compounds was indicated by toluidine blue staining, which produced a turquoise-blue colouration of mesophyll cells in contact with *P. coronata* hyphae whether or not associated with disruption of the mesophyll cells. In general, no toluidine blue staining was observed for epidermal cells, the exception being the stomata guard cells which, when penetrated by *P. coronata*, stained more intensely than the other plant cells. The vegetative fungal structures were not stained by toluidine blue but uredospores were stained blue. All the plant genotypes tested showed some level of phenolic compound production as indicated by toluidine blue staining of leaf samples taken 48 and 120 h after inoculation, and this was particularly apparent for genotypes Pc68/5*Starter and 04B7119-2 and cultivar URS 21, where there was a high frequency of staining for plant cells associated with *P. coronata* colonies. The evaluation of phenolic compounds with toluidine blue produced similar results to the autofluorescence results.

Microscopic examination of the samples before staining to visualise lignin-like compounds showed yellow disrupted cells with no browning for samples collected 120 h after inoculation only for genotypes URS 21, Pc68/5*Starter, 04B7113-1 and 04B7119-2. The other genotypes did not exhibit disrupted plant cells. The Cross-Bevan reaction was positive, showing red colouration of vascular bundles, stomatal guard cells and trichomes. Weisner staining showed positive stained intensely red purple vascular bundles, while guard cells and trichomes were negative. These two methods did not identify lignin-like compounds at the infection sites.

The individual area of the *P. coronata* colonies was not significantly correlated with any of the components evaluated. The presence of plant cell death was negatively correlated with the presence of sporogenic tissue (r_s=−0.76) and with pustule density (r_s=−0.93) (Table 4). However, plant cell death was positively correlated with autofluorescence in the samples collected 48 h (r_s=0.73) and 120 h (r_s=0.93) after inoculation. The pustule density values were positively correlated with the presence of sporogenic tissue (r_s=0.79) but negatively correlated with autofluorescence in the samples collected 120 h after inoculation (r_s=−0.96). Correlation analysis was not performed for the AUDPCc with the components of resistance data due to the different conditions in which the data were obtained.

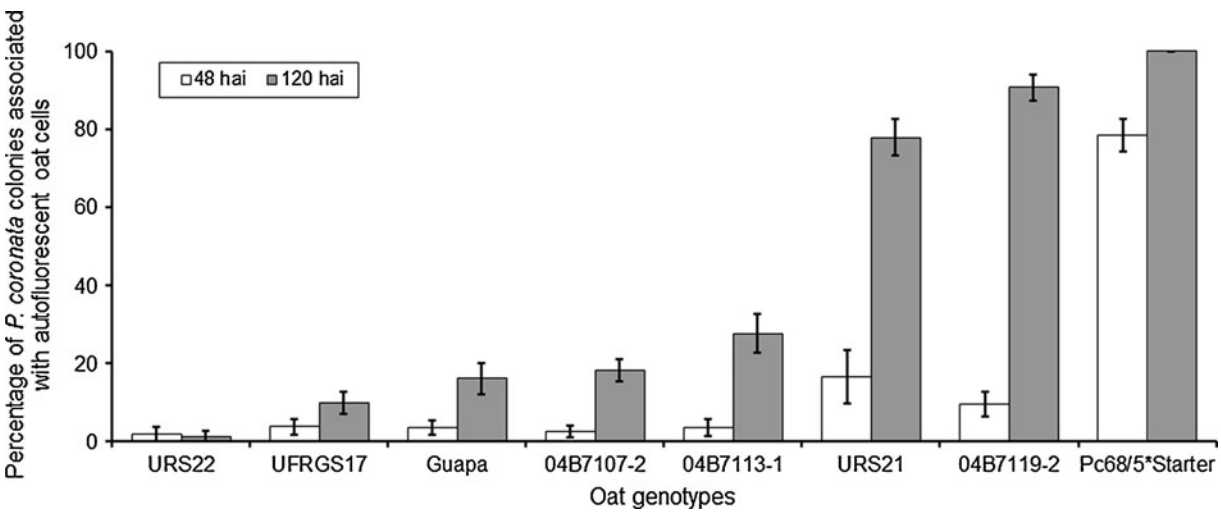

Fig. 1 Percentage of *Puccinia coronata* f. sp. *avenae* colonies associated with the presence of autofluorescence in cells of different oat genotypes. Bars indicate the standard error and hai = hours after inoculation

Table 3 Percentage of *Puccinia coronata* f. sp. *avenae* colonies with and without sporogenic tissue and associated or unassociated with autofluorescence at an excitation wavelength of 450–480 nm

Oat genotypes	*P. coronata* colonies with sporogenic tissue		*P. coronata* colonies without sporogenic tissue	
	Autofluorescent (%)	Not autofluorescent (%)	Autofluorescent (%)	Not autofluorescent (%)
Pc68/5*starter	0.0	0.0	100.0	0.0
URS 21	18.2	10.4	59.7	11.7
04B7119-2	65.3	2.7	25.3	6.7
URS Guapa	7.0	76.7	9.3	7.0
04B7113-1	21.7	65.1	6.0	7.2
04B7107-2	17.2	74.4	1.1	7.2
UFRGS 17	9.0	81.1	0.9	9.0
URS 22	0.0	72.2	1.4	26.4

Discussion

The intensity of the disease assessed as AUDPCc for the crown rust susceptible cultivars URS 22, URS Guapa and UFRGS 17 reflected the high potential for disease of these cultivars, such susceptibility reflecting the favourable environment found in southern Brazil. Under these conditions, it is possible to identify oat genotypes with different levels of resistance. However, in our study, the absence of completely immune oat genotypes in the field, with sporulating pustules found in all entries, suggests that all the major resistance genes are ineffective or will soon become ineffective against *P. coronata* if widely used commercially in southern Brazil.

The virulence of *P. coronata* isolates from southern Brazil towards the *Pc68* gene conferring resistance to *P. coronata* in oat genotype Pc68/5*Starter has previously been reported by Leonard and Martinelli (2005) and Graichen et al. (2010). This oat genotype shows no disease in the early stages of development but pustules are present after flowering at the end of the plant growth cycle. This genotype could have a different resistance mechanism, which would be effective in the earlier stages of plant development even under high disease pressure and the presence of virulent *P. coronata* races; or, alternatively, the frequency of virulence to *Pc68* gene in the prevailing pathogen population is low, thus taking longer for an epidemic to develop over the course of the season

Table 4 Spearman's correlation coefficients among histological components of resistance to *Puccinia coronata* f. sp. *avenae* crown rust in oat seedlings and between the histological components and pustule density in seedlings

Component of resistance	Oat cell death	*P. coronata* sporogenic tissue	Autofluorescent oat cells (48 h after inoculation)	Autofluorescent oat cells (120 h after inoculation)	*P. coronata* pustule density
P. coronata colony area	−0.14 (0.736)[a]	0.62 (0.102)	−0.51 (0.194)	−0.29 (0.493)	0.36 (0.432)
Oat cell death		−0.76 (0.028)	0.73 (0.039)	0.93 (<0.001)	−0.93 (0.002)
P. coronata sporogenic tissue			−0.83 (0.011)	−0.76 (0.028)	0.79 (0.036)
Autofluorescent oat cells (48 h after inoculation)				0.86 (0.007)	−0.70 (0.077)
Autofluorescent oat cells (120 h after inoculation)					−0.96 (0.002)

[a] Values between parenthesises correspond to the probability that $\rho=0$

(Table 1). Cultivar URS 21 showed partial crown rust resistance and proved to be resistant throughout the three years of evaluation, with only a small amount of disease apparent during all stages of development, indicating that resistance is still effective in this cultivar. Two genotypes 04B7113-1 and 04B7119-2 from more recent crosses were significantly more resistant in the field than cultivar URS 21 (Table 1). The genotype 04B7107-2 showed a similar level of field resistance to that of URS 21, although at seedling stage tests it was susceptible (Table 2). This may indicate the presence of effective adult plant resistance to crown rust that is not operative in 10-day old seedlings but in advanced stages, as observed in some wheat genotypes resistant to stripe rust (Jagger et al. 2011).

Traditionally, small grain cultivars with partial resistance to rust have shown some increase in latent period and a reduced rate of infection or infectious period (Chaves et al. 2004b; Kloppers and Pretorius 1997; Parlevliet 1975). In our study, however, no differences were observed in the latent period (Table 1) nor in the area of individual *P. coronata* colonies (Table 2), clearly indicating that the resistance mechanisms of the resistant oat genotypes studied by us did not prevent colony development but instead acted later by preventing, or limiting, pustule development resulting in the development of a lower pustule density on resistant genotypes (Table 1). Our data may also indicate a lack of relationship between the level of host field resistance and individual colony area (Table 2). Considered separately from the field experiments, the histological and epidemiological components measured under controlled conditions showed some significant negative and positive correlations (Table 4). Other authors have reported a lack of correlation between pustule size and other epidemiological components (Chaves et al. 2004b; Wesp et al. 2008). In our study, the area of individual *P. coronata* colonies was similar for the various oat genotypes but the infection type differed (Table 2), suggesting that early development of *P. coronata* was very similar on all the genotypes, because there was no mechanism to retard the rate of fungal development during the first 120 h after inoculation. Similarly, a late resistance mechanism was reported in wheat x *P. striiformis* (Moldenhauer et al. 2006; Jagger et al. 2011) and in wheat x *P. graminis* f. sp. *tritici* (Fehser et al. 2010).

Most of the *P. coronata* colonies growing on the resistant genotypes were associated with the death of the host plant cells (Table 2). In genotype Pc68/5*Starter, the plant cells surrounding the substomatal cavity, where the fungal structures developed, died, resulting in small fungal colonies limited to this area. This type of reaction is characteristic of the hypersensitivity resistance conferred by major genes (Heath 1981; Niks and Dekens 1991; Lin et al. 1998). Cell death in cultivars URS 21 plus genotypes 04B7113-1 and 04B7119-2 showed a different pattern, with dead plant cells scattered throughout the area colonized by *P. coronata*. The greater fungal colony development in these genotypes indicated that cell death occurred later as compared to genotype Pc68/5*Starter.

Although resistance may be linked to cell death it can be triggered by different physiological events in the different genotypes. When plant cells die in the early stages of fungal development the fungus stops growing and also dies, this type of plant reaction often occurring in highly resistant plant genotypes (Lin et al. 1998). In genotypes with intermediate resistance, cell death occurs relatively late in the advanced stages of infection; the fungus is not killed quickly and can continue to grow slowly, such a reaction cannot be regarded as a hypersensitivity reaction *stricto sensu* (Bushnell 1982; Richael and Gilchrist 1999; Barilli et al. 2009). In our experiments, the late (i.e. around 120 h after inoculation) death of groups of dispersed plant cells in cultivars URS 21 and genotypes 04B7113-1 and 04B7119-2 suggests the occurrence of a distinct resistance mechanism, leading first to the death of the fungus and later to death of the plant cells. Such a mechanism has been described by Jiang and Kang (2010) for the fungus *P. striiformis* growing in wheat. This 'late death' mechanism of may also suggest the presence of resistance genes common to these three oat genotypes. When a biotrophic fungus dies by a non-hypersensitivity resistance mechanism after it is established in the host, the production of elicitors that maintain host-pathogen compatibility can be restricted, leading to cell death in the host due to damage caused during cell penetration by the pathogen and the development of fungal structures inside the plant cell (Schulze-Lefert and Panstruga 2003).

Events other than the lack of pathogen-produced elicitors can also cause death of host cells. Our data indicate a high correlation between plant cell death and the accumulation of autofluorescent (Table 4) and phenolic compounds as indicated by toluidine blue staining, a similar correlation having been

reported by other workers (Mayama et al. 1982; Tiburzy and Reisener 1990; Stadnik and Buchenauer 1999; Moldenhauer et al. 2008). Our observation that in the resistant oat genotypes mesophyll cells were stained by toluidine blue supports the phenolic nature of the fluorescent compounds. The phenolic nature of the fluorescent compounds was further tested in which tissues from all genotypes were stained with the lignin stain phloroglucinol, to detect guaiacyl lignin groups and with chlorine-sulphite for the detection of syringyl and guaiacyl lignin groups. The results of these procedures were negative, thus excluding the presence of lignin-like compounds or lignification as a resistance mechanism. Mesophyll cells that have accumulated avenanthramide phytoalexins (produced in oat genotypes resistant to crown rust) have been reported by Mayama and Tani (1982) to produce a lemon-yellow autofluorescence at the same excitation wavelength (500 nm) used in our study. Mayama et al. (1982) also reported the accumulation of avenanthramides in oat genotypes highly resistant to *P. coronata* (IT=0), the accumulation occurring between 24 h and 48 h after inoculation. These authors further stated that when avenanthramide accumulation was delayed there was a reduction in the level of resistance of the genotypes. In our study, genotypes Pc68/5*Starter and 04B7119-2 along with cultivar URS 21 showed a similar pattern to that described by Mayama et al. (1982), with a high proportion of colonies with phenolic compounds (Fig. 1). However, without further tests, it is not possible to affirm that the autofluorescence seen in the genotypes studied by us was due to avenanthramides. The early detection of autofluorescence in genotype Pc68/5*Starter 48 h after infection was related to IT=0 and small *P. coronata* colonies, while in cultivar URS 21 and genotype 04B7119-2 the development of autofluorescence at 120 h after inoculation produced larger initial colonies and a different infection type, higher than IT=0 (Table 2).

The reduced production of *P. coronata* sporogenic tissue in the resistant oat genotypes studied was correlated with lower pustule density, suggesting that the resistance mechanism prevents differentiation of sporogenic tissue (Table 4). There was also a negative correlation between host cell death and sporogenic tissue formation. The presence of autofluorescence, indication the presence of fluorescent compounds, appeared to be associated with reduced differentiation of sporogenic tissue, particularly for genotypes Pc68/5*Starter and 04B7119-2 and cultivar URS 21 (Table 3 and Fig. 1). With the exception of genotype Pc68/5*Starter, where resistance is attributable to a hypersensitivity reaction, our data showed a higher proportion of autofluorescent *P. coronata* colonies without the differentiation of sporogenic tissue in the cultivar URS 21. In this case, pustules that managed to develop were smaller later (IT=2) and were present at a lower density per cm^2. When autofluorescence occurred concomitant with the development of sporogenic tissue, the IT included necrosis of tissue around the pustules (IT=2 N), as was detected in genotype 04B7119-2 (Tables 2 and 3). These data indicate that the presence of fluorescent compounds in cultivar URS 21 prevented the development of sporogenic tissue, while in genotype 04B7119-2 spore or pustule production was prevented.

One of the major difficulties faced by plant breeders is obtaining oat cultivars with durable resistance to crown rust. New cultivars have their resistance overcome by the pathogen one or two years after their release. Since being released in 2000, oat cultivar URS 21 has proved to be highly resistant to *P. coronata* and has maintained a significant cropping area (15%) among the different oat varieties grown in Brazil. Despite virulent *P. coronata* races having already been reported for this cultivar (Vieira et al. 2006), the amount of disease produced by these races has been consistently low, independently of the environmental variation occurring over the years. Thus cultivar URS 21 has durable resistance *sensu* Johnson (1984), and is a valuable source of resistance to crown rust for use in breeding in disease-conducive environments. Paradoxically, the mechanism of resistance is associated with cell death, which in itself is not sufficient to predict the durability of a determined resistance. Possibly, cell death does not occur in cultivar URS 21 due to a hypersensitivity reaction but as a consequence of the late accumulation of phenolic compounds. Although the inheritance of resistance in cultivar URS 21 is still under investigation, a few race-specific resistance genes have been identified (Vieira et al. 2006), although these have later been overcome by *P. coronata*. Nevertheless, cultivar URS 21 has consistently shown “slow rusting” over several years of cultivation (Table 1), demonstrating the existence of partial resistance in this cultivar. Taken as a whole, such traits fall into the type-2 durable resistance class described by Ribeiro do Vale et al.

(2001) and Rubiales and Niks (2000). The moment at which cell death occurs and phenolic compounds accumulate seems to have a role in the specificity and durability of resistance conferred to a specific oat genotype. Cell death in cultivar URS 21 occurred 120 h after inoculation, much later compared to genes with large, qualitative effects, which cause cell death about 30 h after inoculation. The later also produce only ephemeral resistance when deployed in South Brazil. The late production of high levels of phenolic compounds in URS 21 may be associated with cell death, especially in terms of preventing future development of fungal colonies.

These findings may be useful as tools to use for selecting genotypes more likely to show durable resistance, using a rapid and simple test. Field tests over several seasons would be useful in validating the association of these traits with durable resistance.

Acknowledgements The authors thank CNPq for granting a PhD scholarship to the first author of this work.

References

Barilli, E., Sillero, J. C., Serrano, A., & Rubiales, D. (2009). Differential response of pea (*Pisum sativum*) to rusts incited by *Uromyces viciae-fabae* and *U. pisi*. *Crop Protection, 28*, 980–986.

Bender, C. M., Pretorius, Z. A., Kloppers, F. J., & Spies, J. J. (2000). Histopathology of leaf rust infection and development in wheat genotypes containing *Lr12* and *Lr13*. *Journal of Phytopathology, 148*, 65–76.

Bonnett, D. G., Park, R. F., Mcintosh, R. A., & Oates, J. D. (2002). The effects of temperature and light on interactions between *Puccinia coronata* f. sp. *avenae* and *Avena* spp. *Australasian Plant Pathology, 31*, 185–192.

Bushnell, W. R. (1982). Hypersensitivity in rusts and powdery mildews. In Y. Asada, W. R. Bushnell, S. Ouchi, & C. P. Vance (Eds.), *Plant infection: The physiological and biochemical basis* (pp. 97–116). Tokyo: Japan Scientic Press.

Chaves, M. S., Martinelli, J. A., & Federizzi, L. C. (2004a). Quantitative resistance to crown rust in white oat genotypes: II—Evaluation of resistance components. *Fitopatologia Brasileira, 29*, 47–55.

Chaves, M. S., Martinelli, J. A., & Federizzi, L. C. (2004b). Quantitative resistance to crown rust in white oat genotypes: III—Correlation of its resistance components with each other and with the amount of disease in the field. *Fitopatologia Brasileira, 29*, 197–200.

Chong, J., Leonard, K. J., & Salmeron, J. J. (2000). A North American system of nomenclature for *Puccinia coronata* f. sp. *avenae*. *Plant Disease, 84*, 580–585.

De Vleesschauwer, D., Chernin, L., & Höfte, M. M. (2009). Differential effectiveness of *Serratia plymuthica* IC1270-induced systemic resistance against hemibiotrophic and necrotrophic leaf pathogens in rice. *BMC Plant Biology, 9*, Article 9, Retrieved December, 5, 2010, from http://www.biomedcentral.com/content/pdf/1471-2229-9-9.pdf.

Deon, J. F. D. (1997). Lignin analysis. In W. V. Dashek (Ed.), *Methods in plant biochemistry and molecular biology* (pp. 199–215). Boca Raton: CCR Press.

Díaz-Lago, J. E., Stuthman, D. D., & Leonard, K. L. (2003). Evaluation of components of partial resistance to oat crown rust using digital image analysis. *Plant Disease, 87*, 667–674.

Fehser, S., Beike, U., Stöveken, J., Pretorius, Z. A., van der Westhuizen, A. J., & Moerschbacher, B. M. (2010). Histological and initial molecular analysis of UG99, the *Sr31*-breaking race of the wheat stem rust fungus. *Journal of Plant Pathology, 92*, 709–720.

Graichen, F. A. S., Martinelli, J. A., Federizzi, L. C., Pacheco, M. T., Chaves, M. S., & Wesp, C. (2010). Inheritance of resistance to oat crown rust in recombinant inbred lines. *Scientia Agricola, 67*, 435–440.

Heath, M. C. (1981). Resistance of plants to rust infection. *Phytopathology, 71*, 971–974.

Jagger, L. J., Newell, C., Berry, S. T., MacCormack, R., & Boyd, L. A. (2011). Histopathology provides a phenotype by which to characterize stripe rust resistance genes in wheat. *Plant Pathology, 60*, (in press).

Jiang, X., & Kang, Z. (2010). Ultrastructural changes in the interaction between *Puccinia striiformis* and wheat cultivar with slow-rusting resistance. *Agricultural Sciences in China, 9*, 64–70.

Johnson, R. (1984). A critical analysis of durable resistance. *Annual Review of Phytopathology, 22*, 309–330.

Kloppers, F. J., & Pretorius, Z. A. (1997). Effects of combination amongst genes *Lr13, Lr34* and *Lr37* on components of resistance in wheat to leaf rust. *Plant Pathology, 46*, 737–750.

Leonard, K. J., & Martinelli, J. A. (2005). Virulence of oat crown rust in Brazil and Uruguay. *Plant Disease, 89*, 802–808.

Lin, K. C., Bushnell, W. R., Smith, A. G., & Szabo, L. J. (1998). Temporal accumulation patterns of defense response gene transcripts in relation to resistant reactions in oat inoculated with *Puccinia graminis*. *Physiological and Molecular Plant Pathology, 52*, 95–114.

MacBryde, M. C. (1936). A method of demonstrating rust hyphae and haustoria in unsectioned leaf tissue. *American Journal of Botany, 23*, 686–688.

Martinelli, J. A., Federizzi, L. C., & Bennedetti, A. C. (1994). Yield reductions of oat grains due leaf rust severity. *Summa Phytopathologica, 20*, 116–118.

Mayama, S., & Tani, T. (1982). Microspectrophotometric analysis of the location of avenalumin accumulation in oat leaves in response to fungal infection. *Physiological Plant Pathology, 21*, 141–149.

Mayama, S., Matsuura, Y., Iida, H., & Tani, T. (1982). The role of avenalumin in the resistance of oat crown rust, *Puccinia coronata* f. sp. *avenae*. *Physiological Plant Pathology, 20*, 189–199.

Moldenhauer, J., Moerschbacher, B. M., & van der Westhuizen, A. J. (2006). Histological investigation of stripe rust (*Puccinia striiformis* f. sp. *tritici*) development in resistant and susceptible wheat cultivars. *Plant Pathology, 55*, 469–474.

Moldenhauer, J., Pretorius, Z. A., Moerschbacher, B. M., Prins, R., & van der Westhuizen, A. J. (2008). Histopathology and PR-protein markers provide insight into adult plant resistance to stripe rust of wheat. *Molecular Plant Pathology, 9*, 137–145.

Niks, R. E., & Dekens, R. G. (1991). Prehaustorial and posthaustorial resistance to wheat leaf rust in diploid wheat seedling. *Phytopathology, 81*, 847–851.

Niks, R. E., & Rubiales, D. (2002). Potentially durable resistance mechanisms in plant to specialized fungal pathogens. *Euphytica, 124*, 201–216.

O'Brien, T. P., Feder, N., & McCully, M. (1964). Polychromatic staining of plant cell walls by toluidine blue *o*. *Protoplama, 59*, 368–373.

Parlevliet, J. E. (1975). Partial resistance of barley to leaf rust. I. Effect of cultivar and development stage on latent period. *Euphytica, 24*, 21–27.

Ribeiro do Vale, F. X., Parlevliet, J. E., & Zambolim, L. (2001). Concepts in plant disease resistance. *Fitopatologia Brasileira, 26*, 577–589.

Richael, C., & Gilchrist, D. (1999). The hypersensitive response: a case of hold or fold? *Physiologycal and Molecular Plant Pathology, 55*, 5–12.

Rubiales, D., & Niks, R. E. (2000). Combination of mechanisms of resistance to rust fungi as a strategy to increase durability. In C. Royo, M. M. Nachit, N. Di Fonzo, & J.L. Araus (Eds), Durum wheat improvement in Mediterraneous region: new challenges. *Options Mediterraneennes*, Series A, *40*, 333–339.

Schulze-Lefert, P., & Panstruga, R. (2003). Establishment of biotrophy by parasitic fungi and reprogramming of host cells for disease resistance. *Annual Review of Phytopathology, 41*, 641–667.

Sillero, J. C., & Rubiales, D. (2002). Histological characterization of resistance to *Uromyces viciae-fabae* in faba bean. *Phytopathology, 92*, 294–299.

Stadnik, M. J., & Buchenauer, H. (1999). Accumulation of autofluorogenic compounds at the penetration site of *Blumeria graminis* f. sp. *tritici* is associated with both benzothiadiazole-induced and quantitative resistance of wheat. *Journal of Phytopathology, 147*, 615–622.

Tiburzy, R., & Reisener, H. J. (1990). Resistance of wheat to *Puccinia graminis* f. sp. *tritici*: association of the hypersensitive reaction with the cellular accumulation of lignin-like material and callose. *Physiological and Molecular Plant Pathology, 36*, 109–120.

Vieira, E. A., Carvalho, F. I. F., Chaves, M. S., Costa de Oliveira, A., Bertan, I., Martins, A. F., et al. (2006). Resistance pattern of white oat genotypes to crown rust in the definition of crosses. *Pesquisa Agropecuária Brasileira, 41*, 607–614.

Vieira, E. A., Carvalho, F. I. F., Chaves, M. S., Costa de Oliveira, A., Benin, G., Hartwig, I., et al. (2007). Virulence variability of *Puccinia coronata* f. sp. *avenae* isolates collected in three counties from Rio Grande do Sul State, Brazil. *Plant Disease, 91*, 66–70.

Wesp, C. L., Martinelli, J. A., Chaves, M. S., Graichen, F. A. S., & Federizzi, L. C. (2008). Inheritance of quantitative resistance to crown rust in recombinant lines of white oat. *Tropical Plant Pathology, 33*, 138–147.

Zar, J. H. (2009). *Biostatistical analysis* (5th ed.). Upper Saddle River: Prentice-Hall.