Identification and characterization of sources of resistance in *Avena sativa*, *A. byzantina* and *A. strigosa* germplasm against a pathotype of *Puccinia coronata* f.sp. *avenae* with virulence on *Pc94* resistance gene.

J. Sánchez-Martín¹, D. Rubiales¹, J.C. Sillero² and E. Prats¹*

¹Institute for Sustainable Agriculture, CSIC, Apdo. 4084, 14004 Córdoba, Spain;
²CIFA-IFAPA, Área de Mejora y Biotecnología, Apdo. 3092, E-14080 Córdoba, Spain.

*To whom correspondence should be addressed:
Elena Prats
Institute for Sustainable Agriculture, CSIC, Apdo. 4084, E-14080 Córdoba, Spain.

E-mail: elena.prats@ias.csic.es
Tel: +34 957499291
Fax: +34 957499252
Abstract

Oat crown rust resistance gene Pc94 is currently one of the most effective genes for resistance to Puccinia coronata f.sp. avenae. However, we identified a P. coronata isolate with virulence for this gene. In order to identify alternative sources of resistance to this virulent isolate we screened a collection of 159 Avena accessions including 107 A. sativa and 29 A. byzantina landraces together with 23 commercial A. sativa and A. strigosa varieties. Eight resistant landraces and 4 varieties were selected according to macroscopic assessment to further characterize the operative defense mechanisms. Histological studies showed a range of defense mechanisms, acting alone or combined, that impeded fungal development at different stages. Some accessions allowed a reduced fungal growth before mesophyll penetration. In others the fungus was arrested at penetration stage due to mesophyll cell wall strengthening and/or papilla deposition. Mesophyll cells of several accessions were penetrated by the fungus but then hypersensitive response (HR) leading to cell death hampered fungal development. In some cases cell death was very fast and colony aborted early whereas in other cases necrosis was observed later and associated to numerous secondary hyphae suggesting a slow HR. Characterization of defense mechanisms will be useful for breeding programs and for further cellular and molecular studies to unravel the bases of resistance. In addition, this work will discuss commonalities with the resistance on the same oat collection to another biotrophic fungus, the powdery mildew.

Additional Keywords: hypersensitive response; oat; papilla; posthaustorial resistance; penetration resistance; rust.
INTRODUCTION

Oats is a crop of Mediterranean origin used as feed grain, green or conserved fodder and, more recently, as a winter cover crop in no-till rotations (Stevens et al., 2004). *Avena sativa* L. and *A. byzantina* K. Koch sometimes known as the white oat and red oat respectively are the main cultivated oats. They are self pollinating hexaploids with 42 chromosomes (6n=42), and compatible with hybridizing techniques (Stevens et al., 2004).

Crown rust caused by *Puccinia coronata* f.sp. *avenae* Eriks is the most important disease of oat crops causing high yield and grain quality losses worldwide (Simons, 1985), and particularly in the Mediterranean basin (Hemmami, 2006) where populations are more virulent than in the center and north of Europe. The use of race-specific (*Pc*) genes for resistance has been the primary means of control. Presently, more than 90 genes for crown rust resistance have been assigned with permanent designations (Chong et al., 2000). However these genes are unfortunately rapidly defeated, due to selection pressure on the pathogen as a result of large-scale and long term cultivation practices, by new populations of the pathogen. The gene *Pc94* transferred from *A. strigosa* is currently regarded as the most effective gene for resistance to *P. coronata* (Chen et al., 2007), and has been incorporated into a number of oat cultivars recently released (Carson, 2008). However, virulence against this gene, albeit at low frequency, has already been detected in Canada and Europe (Chong et al., 2011, Jirakova & Hanzalova, 2008). Caution should thus be taken to monitor spread of this virulence, as it might rapidly increase and spread as soon as *Pc94* carrying cultivars are widely deployed.
The rust infection process starts with the germination of the urediospores on the leaf surface. When the germ tube contacts a stoma, an appressorium develops over the guard cells and then a penetration hypha penetrates through the stomata (Hoch & Staples, 1987, Prats et al., 2002). The penetration hypha develops a substomatal vesicle from which a secondary hypha and a haustorium mother cell, at its tip, forms. Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, that takes up nutrients for fungal growth (Parlevliet & Kievit, 1986). Attacked plants may trigger different host cell defense responses that can act before, during, or after cell penetration to arrest fungal development. However, when resistance mechanisms are lacking or they are insufficient to hamper fungal development, disease symptoms appear. These consist on yellow pustules containing masses of urediospores which are exposed after the rupture of the epidermis. Lesions are circular or oblong and occur in both surfaces of the foliar sheet and can reach other green parts of the plant, when the epidemic becomes more severe. After some weeks, the borders of the pustules can turn black, with teliospore formation. When the infected plants reach maturity, production of urediospores ceases and they are then replaced by teliospores (Simons, 1985).

During past growing seasons (2004-2005 and 2005-2006) we found a severe crown rust virulence on our experimental oat fields at Córdoba not previously observed. In the present work we aimed 1) to determine the virulence spectrum of this *P. coronata* f. sp. *avenae* isolate 2) to find new sources of qualitative / quantitative resistance to this isolate in a germplasm collection of *A. sativa* and *A. byzantina* landraces and in a group of *A. sativa* and *A. strigosa* commercial varieties, 3) to characterize the resistance responses to ease breeding programs and as a starting point for cellular and molecular
studies that will reveal further the basis of resistance. In addition, since this collection
was screened for powdery mildew resistance (Sánchez-Martín et al., 2011) this work
will also discuss differences in the plant resistance response to both fungi.

MATERIALS AND METHODS

Pathogen and plant material

The used *P. coronata* f.sp. *avenae* (*Pca*) isolate Co-04 was derived from a bulk
population collected on oat crop at Córdoba in 2004. Urediospores were multiplied on
plants of oat cvs. Cory and Araceli which were highly susceptible. One day before
experimental inoculation, spores were collected and kept overnight in a desiccator.

The virulence spectrum of this isolate was determined from a monospore
derivate on a set of differential genotypes possessing different resistance genes (Table
1) kindly supplied by J. Chong (A. Agri-Food, Canada) and M. Leggett (IGER,
Aberystwyth, UK).

For the resistance screening we used a germplasm collection of landraces
containing 107 genotypes of *A. sativa* and 29 of *A. byzantina* kindly provided by the
“Centro de Recursos Fitogenéticos”, INIA, Madrid, Spain, and 23 commercial varieties
supplied by the Andalusian Network of Agriculture Experimentation (RAEA). For
easier comparison among genotypes and manuscript reading, germplasm bank codes
were substitutes for others codes easier to read (Sánchez-Martín et al., 2011). Oat
varieties studied were: Ac1, Aintree, Alcudia, Araceli, Caleche, Chambord, Chappline,
Charming, Cobeña, Cory, Edelprinz, Flega, Fringante, Hammel, Kankan, Kassandra,
Norly, Orblanche, Pallini, Prevision, Primula, Rappidena and Saia. Oat varieties Aracely and Cory were used as susceptible controls. Seedlings were grown in 5 L trays filled with peat:sand (3:1) in a growth chamber with 20°C, 65% relative humidity and under 12 h dark/12 h light with 150 μmol m⁻² sec⁻¹ photon flux density supplied by high-output white fluorescent tubes. All experiments used fully expanded first-formed leaves of 11 day-old plants (their second-formed leaf was unrolled).

Inoculation

When plants had the first leaf completely expanded they were inoculated with urediospores mixed with pure talcum (1:1, w/w) by dusting them over the plants to give approximately 30 spores mm⁻² (checked by counts made from glass slides laid adjacent to leaves). Homogeneous inoculation was ensured by placing the leaves attached to the plant horizontally with the help of metallic clips. After inoculation, plants were incubated for 12 hours in darkness at 100 % RH and 18°C, and thereafter at 20 °C under a 14 h photoperiod with 150 μmol m⁻² sec⁻¹ photon flux density.

Macroscopic observations

Latency period (LP), infection frequency (IF) and infection type (IT) were determined as macroscopic parameters of the disease as previously described (Prats et al., 2002). LP, the time elapsing between inoculation and appearance of 50% of the pustules, was determined by daily counting of the number of pustules visible in a 4 cm² marked area on the leaves. This was done by using a pocket lens (magnification 7 ×) until the number of pustules in the marked areas ceased to increase. The time at which 50% of the final number of pustules had appeared was estimated by interpolation. Infection frequency, given as the number of pustules per unit area, was calculated 15
days after inoculation, from the same leaf area in which LP was estimated. Infection
frequency scores were then converted into relative values, expressed as a percentage of
the reading on the susceptible check. Infection type was determined by visual inspection
of the leaf according to the 0-9 scale of McNeal et al. (Mc Neal et al., 1971).

Microscopic observations

For microscopic assessment of fungal development, middle segments of 1-3 cm²
from each inoculated leaf still attached to the plant (four leaves per treatment) were
excised at 84 hours after inoculation (h.a.i.) and stained with Uvitex (Ciba, Barcelona,
Spain), according to Niks and Rubiales (Niks & Rubiales, 1994). These were observed
by ultraviolet light incident fluorescent microscopy (330 nm excitation/380 nm
emission) using a Leica DM LS phase contrast microscope (Leica Microsystems,
Wetzlar, Germany; 100x objective).

Percentages of germinated urediospores were determined from 100 random
urediospores per leaf segment. Percentages of germtubes forming an appressorium over
a stoma were determined from 100 germinated urediospores per leaf segment. In
addition, on each leaf segment, 100 infection units (growth arising from individual
urediospore) that successfully formed an appressorium on a stoma were scored and
classified according to their developmental stage i.e whether they formed the
substomatal vesicle, penetrate the mesophyll cell, and establish a colony. Accordingly,
early aborted infection attempts due to penetration resistance, were those that formed a
substomatal vesicle and one or more primary infection hyphae but forming less than six
haustorial mother cells (Fig 2C) and where colony growth had ceased. Infection units
with six or more haustorial mother cells were considered established (Fig 2F). The
presence of host cell death associated with early aborted or established colonies was recorded. Death of plant cells was recognized by yellow whole-cell fluorescence under violet incident light (420 nm excitation/490 nm emission).

The length (L) and width (W) of fifty colonies per leaf segment were measured by eyepiece graticule and colony size (CS) was calculated using the formula: $CS = \frac{1}{4} \pi LW$ (where $L =$ maximum length and $W =$ maximum width of a colony). Area values were transformed to square roots to obtain a linear value for colony area.

**Statistical analysis**

Four leaves (from four different plants) per genotype were studied in a complete randomized block design. For statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = $180/\pi \times \text{arcsine}\left(\sqrt{\left(\%\right)/100}\right)$) to normalize data and stabilize variances throughout the data range. Data were then subjected to analysis of variance using GenStat 7th Edition after which residual plots were inspected to confirm data conformed to normality. In addition Shapiro-Wilk test and Bartlett’s test were performed to test normality and homogeneity of variances respectively. Multiple comparison among all genotypes were carried out according to Tukey test ($p<0.05$).

**RESULTS**

The isolate Co-04 used in this study caused a compatible IT in most of the differential genotypes used in the present study including the one carrying the resistance gene $Pc94$ (Table 1). Indeed, it resulted a highly virulent isolate with avirulence only on $Pc52$ and $Pc59$. According to the nomenclature proposed by Chong et al., (2000) the
classification of this pathotype is TNTT, with virulence to all genes of subset 1 (Pc40, 45, 46 and 50), to all genes of subset 2 (Pc38, 39, 48, and 68), two genes of subset 3 (Pc 51 and 58) and all genes of subset 4 (Pc54, 56, 62 and 64).

The macroscopic assessment of the 159 A. sativa, A. byzantina and A. strigosa accessions including both the landraces and the commercial varieties showed that most of them were moderately to highly susceptible to the isolate used in the present study. Approximately 75% of accessions had a similar or even higher infection frequency than the susceptible control (Fig. 1A). Those accessions that showed some degree of resistance respect to the control (i.e. ranging lower than 80% in IF) were assessed to determine their latency period in order to detect accessions with mechanisms affecting slow colony growth and sporulation. In most of these accessions (90%) LP was similar to the control and pustules appeared during the 7th and the 8th day after inoculation (d.a.i). Only 2 genotypes, Kankan and Primula had a LP of more than 9 days (216h; Fig 1B). These two genotypes together with those considered highly resistant, resistant and moderately resistant according to their IF (i.e. ranging from 0 to 60%) were selected for histological studies in order to determine the underlying resistance mechanisms. Table 2 shows the values of the macroscopic parameters of the selected genotypes. These included 8 A. sativa landraces and 4 commercial varieties. No accessions belonging to the A. byzantina group fitted the selection criteria. All selected genotypes showed high infection type with well-formed pustules except Kankan which showed an infection type of 1 indicating visible necrosis associated with the infection sites (Table 2).

Histological analysis of the two susceptible controls, Araceli and Cory, showed significant differences among then in several on the assessed parameters. Thus Araceli
had more established colonies without necrosis, colony size, early aborted colonies without necrosis and lower number of germlings not associated with substomatal vesicle and early aborted colonies associated with necrosis. Microscopy studies on the 8 selected accessions and 4 varieties revealed that resistance was due to different mechanisms/responses and in some cases to a combination of these responses. Several accessions showed impairment on fungal development prior to mesophyll cell penetration. A low but significant reduction in spore germination was observed on leaves of Gen15 with lower values than the two susceptible genotypes, Cory and Araceli (Table 3). Rates of germtubes successfully forming an appressorium over a stoma were on the range of c.a. 60-70%, except on accessions Gen81 on which appressorium formation was significantly reduced respect to Araceli and Cory and cvs. Alcudia, Kankan and Saia with a significant reduction respect to Cory (Table 3). This reduction on appressorium formation was because the sporelings elongate the germ tube without a pattern and did not find the stomata, passed over the stomata but did not recognize it or form an appressorium away from a stomata. The four genotypes showed all forms of abnormality in appressorium formation without significant differences among them. About 75-90% of the sporelings that succeeded in forming an appressorium over a stoma formed a substomatal vesicle into the substomatal cavity on leaves of the controls. Success in stomatal penetration and substomatal vesicle formation was significantly impaired by c.a. 50% in Gen7, and Gen64 (Table 3).

The percentage of early aborted colonies not associated with plant cell necrosis (EA-NoN) was high in Gen18 (30%) and in cvs. Alcudia (20%) and Saia (61%).
In several genotypes i.e. Gen3, Gen9, Gen15, Gen46, Gen64, Gen81, and Alcudia, early aborted colonies associated with mesophyll cell death, recognized by yellow whole-cell fluorescence under violet incident light (Fig 2D) were significantly higher than any of the controls. Furthermore, in cv. Alcudia, in addition to the rapid cell death, a late cell death, associated with established colonies could be observed (Table 3). This late cell death affected up to 44% of the infections units in Kankan and was the only microscopic resistance response observed in Primula (Table 3). In the latter, this late cell death affected several but not all of the mesophyll cells penetrated by the haustorial mother cells so the colony growth was delayed but not suppressed (Fig 2E). This would explain the high infection frequency together with the long latency period observed in this genotype.

Interestingly several accessions showed a combination of resistance responses. The landraces, Gen7 and Gen64 showed a limited fungal development before mesophyll cell penetration and also a moderate level of cell death. In Saia the appressorium formation was decreased and in addition it showed a high level of penetration resistance and a moderate level of rapid cell death lower than in Araceli. Kankan also reduced the rust appressorium formation rate and showed a high level of late cell death. Finally, in Alcudia it could be observed a reduced appressorium formation, a moderate level of penetration resistance and also moderate levels of early and late cell death. The several resistance responses associated with these genotypes explain the low level of infection frequency observed in them (Table 2).
DISCUSSION

In the present work virulence of *P. coronata* f.sp. *avenae* on the resistance *Pc94* gene is reported in an isolate collected in the field at Córdoba. The pathotype isolated in our fields was highly virulent as shown by its classification (TTNT) according to Chong et al. (2000) compared to the one detected in Czeck Republic (QLBB; Jiráková & Hanzalová, 2008). It is virulent to all resistance genes of subset 1, 2, and to all genes of subset 4. The origin of these isolates is not known although the simultaneous discovery of virulence in so distant areas, suggests sexual recombination and not the spreading from a near area. In the Mediterranean areas, where summer months are dry, the fungus may rely on sexual recombination to complete its annual cycle, thus resulting in a higher frequency of new physiological races (Reinhold & Sharp, 1982). Furthermore, several species of *Rhamnus*, the alternate *P. coronata* host, in which sexual recombination occurs are widespread in Andalusian area (Vadés et al., 1987). The appearance of this new pathotype emphasizes the need for the search for new sources of resistance not only for race-specific resistance rapidly overcome by new fungal isolates but also for quantitative resistance, since virulence to *Pc94* is expected to increase with the spreading of cultivars carrying the corresponding resistance gene.

Following detailed histological assessment of resistant genotypes selected, we observed impairment in fungal developmental stages prior to mesophyll cell penetration in several genotypes. It is well known than the physical structure and the chemical composition of the host surface may influence germination, germtube elongation and appressorium formation in rust fungi (Carver & Ingerson, 1987, Gniwotta et al., 2005). In addition, fungitoxic compounds constitutively excreted to the leaf surface may also interfere with germination and initial developmental stages including the substomatal
vesicle formation (Pasechnik et al., 1997; Prats et al., 2007b). Our observations of poor spore germination and appressoria formation in several genotypes support the hypothesis of mechanisms acting this early during the infection process. Prestomatal penetration mechanisms are frequent in nonhosts, but have seldom, if ever been reported in hosts against their appropriate rusts (Niks & Rubiales, 2002). Notable exceptions are the low appressorium formation by various leaf rusts of cereals in some genotypes of *Hordeum chilense* Roem. et Schult. (Rubiales & Niks, 1996) and of other wild barleys (Rubiales & Niks, 1996), the reduction in appressorium formation by *P. hordei* Otth in some barley cer-mutants (Rubiales et al., 2001) and in stomatal recognition by *P. striiformis* Westend. in some resistant wheat cultivars (Broers & Lopez-Atilano, 1996).

In addition, we observed a reduced percentage of early aborted colonies not associated with host cell necrosis reducing the number of haustoria per colony in several accessions, particularly in Saia. Pre-haustorial resistance plays a major role in so-called partial resistance, (Niks & Rubiales, 2002) and have been reported in several plants/rust interactions such as barley/*Puccinia hordei* (Niks et al., 2000), garlic/*P. allii* (Fernández-Aparicio et al., 2011), *Medicago truncatula/Uromyces striatus* (Rubiales & Moral, 2004), faba bean/*U. viciae-fabae* (Sillero et al., 2000), pea/*U. pisi* (Barilli et al., 2009), *Lathyrus sativus/U. pisi* (Vaz-Patto & Rubiales, 2009) or chickpea/*U. ciceris-arietini* (Madrid et al., 2008). An important component of pre-haustorial resistance is the penetration resistance, which is associated with papillae, apoplastic cell wall appositions deposited by host cells acting as physical and/or chemical barriers to attempted penetration (Zeyen et al., 2002). However, A lower intercellular hyphal development has also been associated with pre-haustorial resistance as in *Lr34* in wheat (Rubiales & Niks, 1995). As we did not study papillae formation, we cannot conclude
on the mechanistic basis of this early abortion of colonies. In addition, genetic analysis would be needed to conclude on the inheritance of the identified resistance as there are examples of non-hypersensitive resistance based on single genes such as Lr34 (Rubiales & Niks, 1995) and Lr46 (Martínez et al., 2001) conferring resistance to P. triticina in wheat, or the resistance against U. ciceris-arietini in chickpea (Madrid et al., 2008) or against U. pisi in pea (Barilli et al., 2010). The fact that several accessions showed pre-haustorial resistance offers opportunities for breeding for this trait. This is of high importance since pre-haustorial resistance is non-race dependent and based on multiple and quantitative genes and therefore it is more difficult to be overcome by new races of pathogens than other resistance mechanisms based on single or qualitative genes such as the hypersensitive response (Niks & Rubiales, 2002).

When penetration resistance fails and haustoria develop within host cells, another defense mechanism, a programmed cell death named hypersensitive response (HR), can be triggered. In the present work, some of the studied accessions had a high level of HR. In several genotypes this response could be observed very early whereas in others a slow or late cell death associated with established colonies was observed. This HR, associated with late-acting programmed cell death has also been reported in other plant–biotrophic fungi interactions such as during faba bean-U. viciae-fabae or barley-Blumeria graminis f.sp. hordei interactions (Prats et al., 2010, Rojas-Molina et al., 2007). According to our macroscopic assessment the combination of both early and late cell death was the most efficient response reducing the infection frequency whereas the late cell death observed in Primula, which affected only some of the mesophyll cells penetrated by the fungi delayed the time of sporulation but did not highly reduced the IF. This limited effect of the late-acting cell death was also observed in barley and faba
bean attacked by powdery mildew and rust respectively (Prats et al., 2010, Rojas-Molina et al., 2007). As the isolate used was avirulent on \textit{Pc52} and \textit{Pc59}, this effect could be due to the presence of any of these genes, or to the presence of unidentified ones. Additional studies such as inoculations with \textit{Pc52} and \textit{Pc59} virulent isolates and allelic tests would be required to confirm if new genes different from \textit{Pc52} or \textit{Pc59} are present in the accessions displaying HR resistance.

In Saia, a significant reduction of the area of the established colonies could be also observed. Resistance mechanisms acting after haustorium formation, which lead to a restriction of nutrients flow to the pathogen restricting their growth and delaying or impeding sporulation might be the cause of the reduced area of the established colonies. However, since this variety also showed a high level of penetration resistance we cannot rule out that the reduce area of the colonies was due to this penetration resistance, not strong enough to abort the colony but able to reduce the size.

None of the genotypes with high resistance to rust showed also high resistance to powdery mildew (Sánchez-Martín et al., 2011). This could be expected for those genotypes showing a resistance response based on hypersensitivity, since this kind of resistance implies a specific gene-for-gene interaction and resistance responses greatly differ even between different isolates of the same fungal species (race-specific resistance). Interestingly, those genotypes displaying a broad spectrum basal resistance (i.e. penetration resistance) to rust neither showed the same response against powdery mildew. This might be due to a lack of the necessary microbial-associated molecular patterns (MAMPs) receptor-mediated recognition for the rust and powdery mildew,
which have been reported to be crucial for displaying the basal defense machinery
(Jones & Dangl, 2006)

The characterization of resistance operating in oat presented here is likely to be
of high practical importance, since it can be an alternative to the \textit{Pc94} resistance so
widely used nowadays. Attempts to breed oat cultivars resistant to crown rust have been
frustrated in most countries by the rapid appearance of new virulent races of \textit{P. coronata}, often within a few years of the release of cultivars with new types of race-
specific resistance. The use of a plant genotypes able to stop the pathogen at different
developmental stages may prove more durable and difficult to be overcome by evolving
pathogenic races than the use of genotypes with a single defense response or
mechanisms governed monogenetically as occur in the HR (Rubiales & Niks, 2000). In
the oat collection studied we found several genotypes with resistance acting at several
fungal developmental stages which will allow breeding of cultivars with more durable
resistance (Niks & Rubiales, 2002). On the other hand knowledge of the mechanisms
underlying resistance is important for breeding programs since the different resistance
mechanisms have different effects in plant physiology and may affect plant performance
and fitness in combination with other stresses (Prats et al., 2007a). As far as we know,
there is little information on the genetic background of the selected genotypes or
pedigree of the selected varieties (http://avena.agr.gc.ca). Our preliminary genetic
studies on the oat collection shows that the selected genotypes belong to the 5 different
clusters in which collection is grouped, indicating a very different genetic background,
which is interesting from the breeding point of view (manuscript in preparation).
Selected genotypes might be crossed with the elite varieties to increase resistance
responses or used in crosses with already described resistant oats for other virulent
isolates. The fact that one of the selected genotypes correspond to the diploid black oat, *A. strigosa*, difficult introgression of resistance into hexaploids, *A. sativa*, oats due to differences in ploidy levels and of homology between chromosomes. Nevertheless, introgression is still possible and in fact, the first source of resistance containing the *Pc94*, the cv Leggett, released by the AAFC Winnipeg program come from the *A. strigosa* accession RL1607 (Carson, 2008).

ACKNOWLEDGEMENTS

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LITERATURE CITED


Figure Legends

Figure 1. Macroscopic assessment of *P. coronata* f.sp. *avenae* infection on *A. sativa* ( ) and *A. byzantina* ( ) landraces and in commercial oat varieties ( ). A, Frequency distribution of the *P. coronata* IF (number of pustules per cm² of leaf) in the studied oat genotypes. Data based on four plants. Accessions from first to fourth range were selected for assessment of latency period. B, Frequency distribution of LP in the studied oat genotypes. LP was determined by daily counting the number of pustules and then grouping the genotypes in which 50% of the final number of pustules had appeared during the 168-192 hours (7th day), 192-216 hours (8th day) or after 9 days. Accessions within the first three ranges of IF and/or a latency period of more than 216 hours were selected for microscopic assessment.

Figure 2. Light micrographs showing examples of *P. coronata* f.sp. *avenae* development and leaf mesophyll cell responses of oat accessions, viewed by incident fluorescence microscopy. A, Germinated uredospore that did not form an appressorium. B, The uredospore germ tube forms an appressorium over an stomata but not the substomatal vesicle. C, Early aborted colony not associated with cell death, the substomatal vesicle differentiates two primary infection hyphae and a haustoria mother cell, but mesophyll cell are not penetrated and infection process stops. D, Early aborted colony associated with cell death, the haustorial mother cell penetrates the mesophyll cell but the penetrated and surrounding cells collapse very fast impeding further haustorial and hyphal development. E, Late cell death, haustorial mother cell penetrated the mesophyll cell, which die but not before haustoria develop allowing the growth of several secondary hyphae. F, Established colony, numerous secondary hyphae not associated with necrosis indicate haustorial functionality. GT = germ tube; U = uredospore; A = appressorium; SSV = Substomatal vesicle; DC = Dead cell (indicated
by whole-cell autofluorescence); H = haustorium; Arrow heads indicate the haustorial mother cells. Bars = 25µm
Table 1. Reaction of *P. coronata* f.sp. *avenae* Co-04 isolate on a set of differential lines carrying different *Pc* resistant genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Original source</th>
<th>Infection Type</th>
<th>Reaction Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>Pc38</td>
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<td>R</td>
</tr>
<tr>
<td>Pc62</td>
<td><em>A. sterilis</em> PI 295919</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc64</td>
<td><em>A. sterilis</em> PI 296244</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc68</td>
<td><em>A. sterilis</em> PI 287211</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc94</td>
<td><em>A. sterilis</em> PI 287211</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc96</td>
<td><em>A. sterilis</em> CAV 4274</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc1</td>
<td><em>A. sterilis</em> CAV 4248</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc5</td>
<td><em>A. sterilis</em> CAV 4656</td>
<td>9</td>
<td>S</td>
</tr>
<tr>
<td>Pc6,7,8,9c,21</td>
<td><em>A. sterilis</em> CAV 4904</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Pc6d</td>
<td><em>A. strigosa</em> RL1697</td>
<td>9</td>
<td>S</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reaction phenotypes are R= resistant, S= susceptible
Table 2. Macroscopic components of resistance to *P. coronata* f.sp. *avenae* assessed on accessions selected for microscopic analysis

<table>
<thead>
<tr>
<th>Accession</th>
<th>Infection Frequency(^d) (%Relative to Control)</th>
<th>Latency Period (^d) (%Relative to Control)</th>
<th>Infection Type(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARACELI</td>
<td>100 (48.7)(^{eb,c})</td>
<td>100.0 (209.2)(^{abc})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 3</td>
<td>37.4 (18.3)(^{bc})</td>
<td>91.8 (192.1)(^{ab})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 7</td>
<td>59.5 (29)(^{cd})</td>
<td>83.9 (175.6)(^{a})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 9</td>
<td>60.5 (29.5)(^{cd})</td>
<td>90.9 (190.4)(^{ab})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 15</td>
<td>32.8 (16.0)(^{abc})</td>
<td>92.5 (193.7)(^{ab})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 18</td>
<td>50.3 (24.5)(^{cd})</td>
<td>92.2 (193.1)(^{ab})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 46</td>
<td>49.2 (24.0)(^{bc})</td>
<td>93.8 (196.4)(^{abc})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 64</td>
<td>51.3 (25.0)(^{cd})</td>
<td>96.6 (202.3)(^{abc})</td>
<td>9</td>
</tr>
<tr>
<td>GEN 81</td>
<td>54.9 (26.7)(^{cd})</td>
<td>91.7 (192.1)(^{ab})</td>
<td>9</td>
</tr>
<tr>
<td>ALCUDIA</td>
<td>2.0 (1.0)(^{a})</td>
<td>89.8 (188.0)(^{ab})</td>
<td>9</td>
</tr>
<tr>
<td>KANKAN</td>
<td>4.7 (2.3)(^{ab})</td>
<td>150.7 (315.5)(^{d})</td>
<td>1</td>
</tr>
<tr>
<td>PRIMULA</td>
<td>76.9 (37.5)(^{de})</td>
<td>111.6 (233.6)(^{c})</td>
<td>9</td>
</tr>
<tr>
<td>SAIA</td>
<td>11.8 (5.7)(^{ab})</td>
<td>102.7 (215.0)(^{bc})</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\) Those with and IF similar or less than 60% and/or more than 216 hours of LP

\(^b\) Different letters for each assessed parameter indicate significant differences at *P* < 0.05 according to Tukey test.

\(^c\) Numbers in brackets indicate raw data: pustules cm\(^{-2}\) for infection frequency and hours for latency period

\(^d\) IF and IT were determined 15 days after inoculation
Table 3: Microscopic assessment of *P. coronata* f.sp. *avenae* development and leaf epidermal cell responses of *Avena* accessions.a.

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>Species</th>
<th>Germ</th>
<th>No App</th>
<th>No Sub Ves</th>
<th>EA-No N</th>
<th>EA-N</th>
<th>Est-N</th>
<th>Est-No N</th>
<th>Colony size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARACELI</td>
<td><em>A. sativa</em></td>
<td>95,75cd</td>
<td>31,00abc</td>
<td>9,00a</td>
<td>7,25b</td>
<td>0,00a</td>
<td>0,00a</td>
<td>83,75g</td>
<td>8,79d</td>
</tr>
<tr>
<td>CORY</td>
<td><em>A. sativa</em></td>
<td>92,50bcd</td>
<td>21,00a</td>
<td>24,88bcd</td>
<td>0,00a</td>
<td>9,46b</td>
<td>0,00a</td>
<td>65,67f</td>
<td>6,71abc</td>
</tr>
<tr>
<td>GEN 3</td>
<td><em>A. sativa</em></td>
<td>92,25bcd</td>
<td>20,75a</td>
<td>27,39bcd</td>
<td>0,48a</td>
<td>23,32e</td>
<td>0,95a</td>
<td>37,87d</td>
<td>6,61abc</td>
</tr>
<tr>
<td>GEN 7</td>
<td><em>A. sativa</em></td>
<td>93,50bcd</td>
<td>28,75ab</td>
<td>48,87e</td>
<td>0,00a</td>
<td>20,44bcde</td>
<td>0,00a</td>
<td>30,69d</td>
<td>6,41abc</td>
</tr>
<tr>
<td>GEN 9</td>
<td><em>A. sativa</em></td>
<td>90,00abc</td>
<td>34,25abcd</td>
<td>38,72de</td>
<td>0,00a</td>
<td>23,73de</td>
<td>0,00a</td>
<td>37,55d</td>
<td>7,38bcd</td>
</tr>
<tr>
<td>GEN 15</td>
<td><em>A. sativa</em></td>
<td>84,00a</td>
<td>23,25a</td>
<td>31,33cde</td>
<td>0,00a</td>
<td>33,06e</td>
<td>0,00a</td>
<td>35,61d</td>
<td>6,25abc</td>
</tr>
<tr>
<td>GEN 18</td>
<td><em>A. sativa</em></td>
<td>92,50bcd</td>
<td>27,00ab</td>
<td>40,39de</td>
<td>29,94c</td>
<td>12,22bcd</td>
<td>0,75a</td>
<td>16,70bc</td>
<td>5,90ab</td>
</tr>
<tr>
<td>GEN 46</td>
<td><em>A. sativa</em></td>
<td>90,75abcd</td>
<td>23,75a</td>
<td>33,61cde</td>
<td>0,00a</td>
<td>27,28e</td>
<td>0,00a</td>
<td>39,11de</td>
<td>7,25abcd</td>
</tr>
<tr>
<td>GEN 64</td>
<td><em>A. sativa</em></td>
<td>88,25ab</td>
<td>32,00abc</td>
<td>51,45e</td>
<td>0,00a</td>
<td>21,39cde</td>
<td>0,00a</td>
<td>27,16cd</td>
<td>6,56abc</td>
</tr>
<tr>
<td>GEN 81</td>
<td><em>A. sativa</em></td>
<td>94,00bcd</td>
<td>50,67d</td>
<td>30,08cde</td>
<td>0,00a</td>
<td>36,50e</td>
<td>0,00a</td>
<td>37,00d</td>
<td>7,82cd</td>
</tr>
<tr>
<td>ALCUDIA</td>
<td><em>A. sativa</em></td>
<td>96,00cd</td>
<td>44,00bcd</td>
<td>17,00abc</td>
<td>20,33c</td>
<td>28,00e</td>
<td>28,33b</td>
<td>6,33a</td>
<td>7,38bcd</td>
</tr>
<tr>
<td>KANKAN</td>
<td><em>A. sativa</em></td>
<td>95,25cd</td>
<td>47,75cd</td>
<td>7,25a</td>
<td>7,50b</td>
<td>11,00bc</td>
<td>44,00c</td>
<td>30,25cd</td>
<td>8,82d</td>
</tr>
<tr>
<td>PRIMULA</td>
<td><em>A. sativa</em></td>
<td>96,33d</td>
<td>34,00abcd</td>
<td>11,33ab</td>
<td>8,00b</td>
<td>0,00a</td>
<td>25,67b</td>
<td>55,00ef</td>
<td>7,84cd</td>
</tr>
<tr>
<td>SAIA</td>
<td><em>A. strigosa</em></td>
<td>93,25bcd</td>
<td>43,25bcd</td>
<td>12,25ab</td>
<td>61,00d</td>
<td>13,50bcd</td>
<td>0,00a</td>
<td>13,25ab</td>
<td>5,52a</td>
</tr>
</tbody>
</table>

a Data obtained at 84 h.a.i. Data corresponding to percentage of germination (Germ) were calculated from 100 spores. Percentages of germtubes that did not form an appressorium over a stoma (No App) were determined from 100 germinated spores. The percentage of germlings that reached different developmental stages (passing from one stage to the next), a) formed an appressorium but not the substomatal vesicle (No Sub Ves), b) formed the substomatal vesicle and one or more primary infection hyphae but due to mesophyll penetration resistance form less than six haustorial mother cells (early abortion; EA) c) early aborted due to cell death (EA-N), d) penetrated the mesophyll cell and establish a colony (Est), e) establish a colony but late cell death limited colony growth (Est-N), were scored from 100 infection units (growth arising from individual spore) that successfully formed an appressorium on a stoma. Analysis of variance was applied to transformed replicate data. Different letters for each assessed parameter indicate significant differences at $P < 0.05$ according to Tukey test.
Figure 1.
Figure 2