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in

Molina-Cano J.L. (ed.), Christou P. (ed.), Graner A. (ed.), Hammer K. (ed.), Jouve N. (ed.), Keller B. (ed.), Lasa J.M. (ed.), Powell W. (ed.), Royo C. (ed.), Shewry P. (ed.), Stanca A.M. (ed.).
Cereal science and technology for feeding ten billion people: genomics era and beyond

Zaragoza : CIHEAM / IRTA
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 81

2008
pages 159-162

The specific effets of the *pch1* resistance gene on the development of *Oculimacula yallundae*, the causing agent of wheat eyespot

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SUMMARY – The resistance gene *Pch1* is widely used in wheat breeding but neither its function nor its role in the plant defence reaction are known. Its effects on the pathogen development were previously studied but the host genotypes used did not allow to discriminate the influence of *Pch1* from the one of the genetic background. This is the first report highlighting the specific effects of the *Pch1* gene on *Oculimacula yallundae* development. We compared the development of eyespot spores on wheat near isogenic lines (NILs) of which genetic backgrounds only differ by the presence or the absence of *Pch1*. We collected data on the main developmental stages of the pathogen: spore germination, secondary hyphae formation, appressoria formation, development of a mycelial network, latency period, infection mat formation and penetration. The *Pch1* gene has no effect on the germination of spores. It rather seems to delay the development of the pathogen. In parallel to this work, we are also using the wheat NILs to carry out a functional analysis of the effects of *Pch1* in the plant defence reaction.

Introduction

Oculimacula yallundae (Crous *et al.,* 2003) is a cereal stem base parasitic ascomycete causing important damages to winter wheat crops (*Triticum aestivum* L.) in temperate countries (Ponchet, 1959). It survives on culture residues and, in the autumn and spring, produces asexual spores dispersed by rain splash. Infection process begins with colonisation of the coleoptile (Bateman and Taylor, 1976) and continues with penetration of successive leaf sheaths to the stem. Several reports were dedicated to the study of the relation between the level of resistance of the host and the development of the pathogen (Khan *et al.,* 1986; Murray and Ye, 1986; Soulié *et al.,* 1985). Khan *et al.* (1986) reported that there was a delay in the germination of spores on the resistant genotypes; Macer (1966) noted that the pathogen grew more slowly in tissues of resistant hosts; Murray and Ye (1986) observed that penetration of coleoptiles and leaf sheaths occurred sooner on susceptible hosts; Scott (1971) noticed that, after an initial lag phase, the rate of fungal growth through leaf sheaths was independent of the cultivar. Altogether, those results do not appear consistent because of the diversity of the resistance genes and of the genetic backgrounds used to compare several levels of resistance.

The present work focused on one resistance gene through the use of wheat near isogenic lines (NILs) only differing by the presence or the absence of the *Pch1* resistance gene. *Pch1* acts as a major resistance QTL ($25\%<R^2<70\%$ across years and genetic backgrounds, Muranty, pers. com.). It was introduced in wheat genome from *Aegilops ventricosa.* It is currently the most efficient resistance gene against eyespot, but neither its function nor its role in the plant defence reaction are known. Resistant genotypes carrying *Pch1* differentiate from susceptible genotypes by a smaller number of attacked leaf sheaths. The aim of our study was to determine how the *Pch1* gene acts on the pathogen life cycle to impede its progression in the plant. Based on previous data on the infection process of the pathogen (Daniels *et al.,* 1991; Defosse, 1966; Defosse and Dekegel, 1974), key stages of its development were chosen and used as markers to study the relation between the level of resistance conferred by *Pch1* and the progression of the pathogen.

Materials and methods

Plant material

Two couples of wheat NILs for the *Pch1* gene were used in our experiment. The first one was

obtained from a cross between 'Renan' (carrying *Pch1*) and 'Recital' (without *Pch1*). The second one was obtained from a cross between Re714 (carrying *Pch1*) and 'Hardi' (without *Pch1*). The NILs were derived from heterozygous recombinant inbred families obtained in F6. The susceptible and the resistant lines were fixed in F10 and F12 respectively.

Fungal isolate and inoculation

Isolates of *O. yallundae* were collected in different sites and their aggressiveness was determined on a range of hosts of the *Triticeae* family (wheat, rye, triticale). The most aggressive isolate on wheat was selected and maintained on sterilized oat grains. A fungal inoculum was obtained by incubating contaminated oat grains in Petri dishes containing humid sterile sand at 9°C under black U.V. light (370 nm) during 2 weeks. A suspension of spores was prepared by washing oat grains with water and by adjusting the concentration to 10^6 spores / ml. Seedlings were inoculated with 1ml of the prepared suspension of spores by means of a dispensing pipette.

Culture conditions and preparation of plant samples

Seeds of each line were pre-germinated in Petri dishes 4 d before being planted in vessels containing decontaminated soil. One day after planting, the seedlings were inoculated at the coleoptile stage. Experiment was conducted in a growth chamber at 9°C with a 8 hours photoperiod during 45 d. At the end of the experiment, the plants were at the beginning of the tillering stage. A second independent experiment is currently in progress for the couple obtained from 'Renan' x 'Récital'.

Three plants per NIL were randomly collected at 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 5 d, 8 d, 15 d, 21 d, 30 d, 35 d, 40 d and 45 d after inoculation (ai). Samples were fixed as described in Murray and Ye (1986). The coleoptile and the first leaf sheath were removed and vacuum-infiltrated with a solution of 0,05% aniline blue in water. The samples were observed using a light microscope (Nikon Optishot 2, Nikon Europe B.V., Badhoeverdorp, The Netherlands) and photographed at magnification x 250 to x 630 using a digital camera (Nikon CoolPix 950, Nikon Europe B.V., Badhoeverdorp, The Netherlands).

Observation of events during the infection process

The times of appearance of the following events were recorded: germination of spores, formation of appressoria, formation of secondary hyphae, formation of a mycelial network, formation of infection mats, secondary sporulation indicated by the presence of newly formed spores produced by hyphae fragmentation, maturation of infection mats indicated by a brown coloration, penetration indicated by the presence of hyphae in epidermal cells and appearance of mycelium on the first leaf sheath. Between 12 hai and 96 hai, the rate of spore germination, calculated from the first 50 spores starting from the base of the coleoptile, was recorded for each sample.

ANOVA analysis was performed using R software to determine significant (P-value < 0,05) effect of the *Pch1* gene on the germination rate.

Results

Effect of pch1 on the rate of spore germination

Data were collected for both couples of NILs in the first experiment (Table 1) and for the couple obtained from 'Renan' x 'Récital' in the second experiment (data not shown). Spore germination began within 12 hai (Fig. 1A) and by 3 dai, most spores had germinated. There were no differences in the rate of spore germination (P-value > 0,05) whatever the genotype or the genetic background.

Table 1. Average rate of germinated spores over 3 replicates at each sample time and for the two couples of NILs studied in the first experiment.

	'Renan' x 'Récital'				Re714 x 'Hardi'			
	Resistant		Susceptible		Resistant		Susceptible	
Time after inoculation	No. of spores	% germinated spores	No. of spores	% germinated spores	No. of spores	% germinated spores	No. of spores	% germinated spores
12 h	164	88.6 +/-6.5	148	89.5 +/-7	33	64.5 +/-21.3	93	82.9 +/-1.2
24 h	172	96.4 +/-2	159	90.0 +/-3.7	88	93.2 +/-7.2	55	91.2 +/-3.2
36 h	190	92.3 +/-11	105	98.6 +/-1.3	32	88.3 +/-5.7	117	92.6 +/-1.1
48 h	162	94.0 +/-8.8	147	95.9 +/-3.2	152	98.8 +/-2.1	72	89.1 +/-12.3
72 h	59	96.4 +/-6.2	82	100 +/-0	65	100 +/-0	75	100 +/-0
96 h	123	90.0 +/-13	59	99.1 +/-0.9	120	100 +/-0	121	98.1 +/-1.7

Effect of pch1 on the events during the infection process

Data were collected in the first experiment for the couple obtained from 'Renan' x 'Récital'. Appressoria appeared between 48 hai and 72 hai in both the resistant and the susceptible lines. They formed on a hyphae from a germinating spore and were generally located above the cell wall separating two cells. Secondary hyphaes were detected within 72 hai in the susceptible line and 96 hai in the resistant line. They formed on a hyphae from a germinated spore (Fig. 1B). A mycelial network was clearly identified by 5 dai and 8 dai in the susceptible and the resistant lines respectively. Infection mats began to form from 8 dai in the susceptible line and after 8 dai in the resistant line. They turned brown, sign of their maturity, at 15 dai in the susceptible line and at 21 dai in the resistant line. Secondary sporulation (Fig. 1C) occurred by 21 dai and 30 dai in the susceptible and the resistant lines respectively. Discrete penetration sites were detected at 30 dai in the susceptible line and 21 dai in the resistant line. By 40 dai, the intracellular mycelium had invaded the epidermal cells. Mycelium appeared on the first leaf sheath by 40 dai in the susceptible line and by 35 dai in the resistant line.

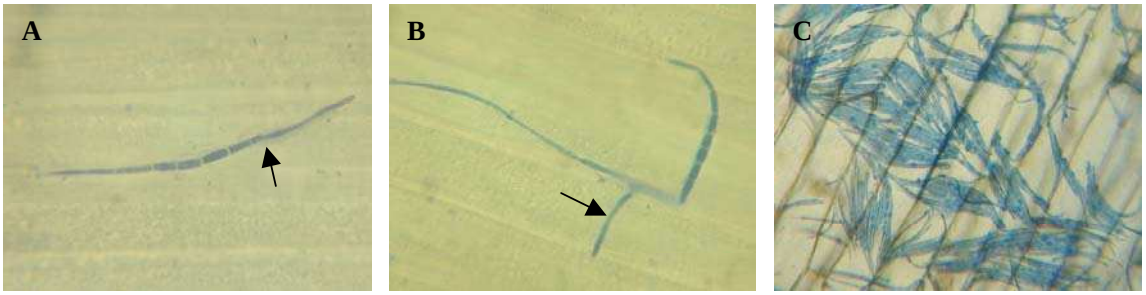

Fig. 1. Three stages of the infection process of *O. yallundae*. **A.** Primary hyphae (arrow) emerging from a germinating spore. **B.** Secondary hyphae (arrow) forming on the primary hyphae of a germinated spore. **C.** Heap of agglutinated spores from secondary sporulation.
A, B: x 630, C: x 250.

Discussion

The kinetics of the infection process observed in our study varies from those described by other authors (Daniels *et al.,* 1991; Khan *et al.,* 1986; Murray and Ye, 1986). These differences were likely due to the heterogeneity in the genetic backgrounds, to the developmental stage of plants and, in a lesser extend, to the tissues observed (coleoptile or leaf sheath) and to the process of inoculation. The use of NILs is a great advantage to focus the study on the genetic factor *Pch1*.

Khan *et al.* (1986) observed that the delay between inoculation and spore germination on the

resistant cultivar 'Roazon' (containing *Pch1*) was twice than on the susceptible species *T. speltoides* and *T. monococcum.* Nevertheless, a study carried out on a range of wheat cultivars containing either *Pch1*, *Pch2* (another major QTL of resistance) or no resistance gene showed that there were no differences between resistant and susceptible genotypes at this stage of fungal development (Murray and Ye, 1986). The results obtained in our study, in agreement with Murray and Ye (1986), confirm that, within wheat, there is no effect of the *Pch1*gene on the germination of spores.

The appearance of secondary hyphaes seems to be a hinge in the pathogen development since, from their formation, the infection process was delayed on the resistant line: formation and maturation of mycelial mats and secondary sporulation occurred later on the resistant line than on the susceptible line. The mechanical pressure exercised by the extension of new hyphaes could be the factor responsible for the release of the plant defence mechanisms. Nevertheless, several studies reported the production of extracellular cellulolytic and pectolytic enzymes by *O. yallundae* (Defosse 1966; Hänssler *et al.,* 1971; Soulié *et al.,* 1985). The release of these enzymes from secondary hyphae could be another hypothesis. Surprisingly, penetration of mycelium in epidermal cells and appearance of mycelium on the first leaf sheath occurred sooner on the resistant line than on the susceptible line. It could be due to an observation bias as the first penetrations were very sober and infrequent. The data currently collected in the second experiment should confirm those results.

The secondary sporulation is poorly documented. It is mentioned by Daniels *et al.* (1991) as giving rise to large numbers of closely associated parallel whorled arrays corresponding to our observations (see Fig. 1C). Soulié *et al.* (1985) detected secondary spores at 4 dai only in the necrosis of the susceptible tissues but as they did not make observations later than 4 dai, it is possible that secondary sporulation occurred later on the resistant genotype. This would be consistent with our result showing that secondary sporulation occurs later on the resistant line.

From our results, we may infer that the *Pch1* gene could delay the development of the pathogen so as to lengthen its life cycle and reduce the number of subsequent contaminations. Higgins and Fitt (1984) observed that lesions could be initiated by a few conidia but developed much more rapidly from a larger inoculum dose. This result raises another hypothesis: *Pch1* could act negatively on the quantity of conidia produced across the life cycle of the pathogen so that the subsequent contaminations are less and less efficient. To validate these two hypothesis, we are planning to count the secondary spores produced on the resistant and on the susceptible lines. In parallel to this work, we are also using the wheat NILs to carry out a functional analysis of the effects of the *Pch1* gene in the plant defence reaction through a candidate gene strategy.

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