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Germination rate of *Erysiphe pisi* in the pea leaves after spray dechlorophylization

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ABSTRACT

Pea powdery mildew caused by the obligate biotrophic fungus *Erysiphe pisi* is an air-borne disease of worldwide distribution. Powdery mildew affects all green parts of pea plants. The first symptoms are small, diffuse spots on leaflets and stipules, usually first appearing on the lowest part of the plant. Its infection can be reduced in pea (*Pisum sativum*) by exogenous applications of chemicals, such as BTH and BABA. This protection is known to be related with the induction of the phenolic pathway but the particular metabolites involved have not been determined yet. BTH and BABA treatments changes in phyto-alexin content and development of the resistance to pea rust. These chemical treatments were effective against *E. pisi.* The enzyme activity PAL, PPO and CAT increased in leaves during infection by *E. pisi* and protected the plants from disease by SAR, detoxification of ROS by stabilizing sub cellular structures. Detailed analysis through high-performance liquid chromatography (HPLC) showed qualitative and quantitative differences in the content, as well as in the distribution of phytoalexins.

Key Words: *Enzyme activity, Pea Powdery Mildew, ROS, SAR, HPLC*

INTRODUCTION

Powdery mildew (*Erysiphe pisi*) is a adapted to pathogenesis on pea (*Pisum sativum* L.) only. It losses 10–65% of leaves caused by powdery mildew under warmer and drier conditions were reported in peas (Nagaraju and Pal 1990). Pea rust has become an important pathogen of dry pea since the mid-1980s and is mostly distributed in Europe, North and South America, India, China, Australia and New Zealand, particularly in regions with warm, humid weather. The pathogen usually appears during mid-spring when the crop is at flowering or podding stage. In years of epidemics, affected leaves dry up and fall off, and pods remain undeveloped, which consequently results in yield losses of higher than 30% (EPPO 2012). Chemical control of rust is possible (Singh *et al* 2004, Emeran *et al* 2011) but the use of host plant resistance is the most desired means of rust control (Rubiales *et al* 2011a). Pea rust has been reported to be caused either by the fungus Uromyces viciae-fabae (Kushwaha *et al* 2006) or by *U. Pisi* (Emeran *et al* 2005, Barilli *et al* 2009 a & b). These observations were confirmed by gathering several rust isolates from highly damaged pea crops from different geographical regions. The use of synthetic fungicides to reduce yield losses is the major practice by pea growers, which has serious implications for human health and a growing threat to environment.

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MATERIALS AND METHODS

Preparation of powdery mildew inoculum two highly virulent isolates of powdery mildew MUZ-1 and MUZ-2 (Azmat *et al* 2012a) were separately maintained on highly susceptible pea cultivar "Meteor Faisalabad". When 80-90% leaves were covered with white powdery mass of both the isolates, the leaves were excised and homogenized in 0.1% water–agar and 0.0025% Tween-20 solution

(Reeser *et al* 1983). The fresh inoculum used for inoculations had 4 × 10^4 conidia.mL−1 (Azmat *et al* 2012b).

Field Screening: The cultural practices suggested by Azmat *et al* (2011) were carried out for good crop. All the genotypes were inoculated with powdery mildew (*E.* pisi) inoculum at 8th node stage in water-agar and tween-20 solution (Reeser *et al* 1983). An inoculator calibrated to 3.5 × 104·m−2 was used for uniform and effective inoculation (Azmat *et al* 2012b). Control without powdery mildew inoculation was also maintained. The data on disease severity were recorded 15 days after inoculation (DAI) on five plants of each genotype from all replications. The disease severity of genotypes was recorded on a 0–9 scale, "0" as highly resistant and "9" as highly susceptible (Warkentin *et al* 1996). The disease severity scale is based on percentage of leaf area affected (% I): 0 = no infection, 1 = <1%, 2 = 1%–5%, 3 = 6%–10%, 4 = 11%–20%, 5 = 21%–40%, 6 = 41%–60%, 7 = 61%–80%, 8 = 81%–90%, 9 = >90%. The inoculated leaves were placed adaxial surface up in sealed petri plates. Control petri plates for each genotype without powdery mildew inoculation was maintained to check cross infectivity. The petri plates were placed in growth chamber at 22°C with a 14:10 h light: dark photoperiod with light intensity of 400 µmoL m-2s-1.

Microscopic Quantification of Disease Severity: Forty eight HAI (Hours after Inoculation) the inoculated leaves were placed in de-staining solution (1 lactic acid: 2 glycerol: 1 d $2H_2O$) for 48 h and then stained with, Coomassie blue. The stained samples were observed under dissecting microscope using 40X × 10X magnifying lenses. The slides were prepared by placing the adaxial surface of stained leaves upward in mounting medium (50% glycerin) on microscopic slides. The cover slip was placed over the leaves after adding few drops of mounting medium. For the quantification of disease severity a scale was devised on the basis of susceptibility percentage (%S). The susceptibility percentage was calculated on the basis of successful germination and growth (mycelia development) of *E. pisi* conidia on pea leaves. Non germinated conidia (Figure 1a) were not included in data recording. The conidia that were just germinated having germ tub on leaves 48 HAI were considered as "resistant" while germinated conidia having mycelia growth showed "susceptible" disease reaction. The minimum number of conidia (standard) was taken as 190 for each observation. The susceptibility percentage (% S) was calculated using following formula:

% S = Conidia with Mycelia Growth X100
Total Number of Germinated Conidia A 0-5 scale based on susceptibility

percentage (% S) is elaborated as under: 0 (Immune) = zero susceptibility, 1 (Highly resistant) = <1–5%, 2 (resistant) = 6–10%, 3 (Moderately susceptible) = 11–40%, 4(Susceptible) = 41– 70%, 5(Highly susceptible) = 71–100%.

Fungal Growth Bioassay: Two leaves at the 2nd node per plant and genotype were used, with a total of six plants per treatment. Plants were treated with phyto-alexins at concentrations previously found in the leaves (i.e. 5.0, 7.2 and 1.2 lg per leaf of scopoletin, pisatin, and medicarpin, respectively), by applying the solution with a pipette on the leaf surface and ensuring their complete absorption. As a control, BTH and BABA solutions were also applied to both genotypes (as reported above). Twenty-four hours after the treatments, plants were inoculated with *U. pisias*. Two days after inoculation (Dai), leaves were harvested and stained (Sillero and Rubiales *et al* 2002). The different stages of the infection process were assessed using a phase contrast Leica DM LS microscope at X400 magnification (Leica Microsystems Wetzlar Germany).

Statistical analysis: The experiments were repeated twice and the data recorded for each experiment were pooled due to significant homogeneity. All the values given here are the arithmetic means of two replications. Statistical analyses were carried out using Microsoft Excel (QI Macros) and MVSP 3.1 (Kovach Computing Services, Anglesey, Wales). The correlation observed between DS values measured under field conditions during three growing seasons, and between DS and AUDPC was high (Barilli *et al* 2009b) suggesting that the final DS estimation on pea provides a feasible estimation of partial resistance. DS estimation needs less computation and is less time-consuming than assessing AUDPC, epidemic growth rate (r) or the first pustule appearance (t0). The epidemic growth rate and the first pustule appearance were poor estimators of *U. pisi* partial resistance as they were less discriminating than the other parameters and showed a low correlation within experimental designs. These can include limited germination or germling adhesion to the leaf surface (Mendgen 1978).

RESULTS AND DISCUSSION

The development of conidia of *Erisiphe pisi* on pea leaves during *Rabi* season appeared after treatments of dechlorophylization and histochemical staining were done. The pathogen inoculated leaves of pea plant which were previously treated with BTH. In BTH treated leaf to development of secondary from mycelium increased as the concentration increased 50-150 ppm after 72 hrs. $ZnSO_4$ and SA were collected from pots at different time of intervels during 24, 48 and 72 h. After given time leaves were kept on slide containing blotting paper with decolorizing solution (ethyl alcohol: acetic acid) till decolorization. After decolorization leaves were kept in lacto phenol solution for softing the leaves. Germination percentage of conidia under microscope was measured after softing of leaves.

Detached leaf assay: The disease score data based on susceptibility percentage (%S) was used to classify 30 pea genotypes into six main groups with susceptibility score ranging from 0-5. Only two genotypes (It-96 and No.267) were highly resistant as minimum number of germinated conidia without mycelia (1% and 0.83%) were quantified microscopically on their leaves, respectively. The maximum conidia with mycelia growth were observed on the leaves of the genotypes *viz*, Climax (97.6%), Meteor-VRI (96.6%), KQP-6185 (95.1%) and PF-400 (95%). The "highly susceptible" genotypes with 71-100 %S made the 2nd largest group of genotypes followed by "moderately susceptible" genotypes with 11-40 %S. Thus, penetration resistance is an important mechanism to prevent the full development of *U. pisi* infection structures. This resistance is initially expressed with the arrest of the infection by early abortion, and continued by hampering subsequent haustoria formation.

Table 1 Effect of BTH, $ZnS0_4$ and Salicylic acid on total protein content in pea leaves infected by *Erysiphe pisi*.

Chemical treatments		Hg Protein/gm sample		
Chemicals	Concentration	24hrs	48hrs	72hrs
BTH	50ppm	190	270	250
	100ppm	195	230	235
	150ppm	185	210	220
ZnSo4	10-3	170	240	240
	10-4	185	210	225
	10-5	150	230	230
Salicylic acid	4mM	150	230	210
	8mM	185	225	240
	12mM	150	240	215
Control		140	180	220

The fact that the resistant accessions showed pre-penetration resistance which offers breeding opportunities for this trait. This is important since penetration resistance is usually non-race dependent and based on multiple genes. Thus, such resistance is expected to be more durable than single gene controlled by race-specific resistance, although easily manipulated in plant breeding, is also easily overcome by new races of pathogens.

Pea rust is a serious disease of pea with worldwide distribution. Although no completely effective source of resistance has been found, considerable progress has been made in identifying germplasm with moderate levels of resistance. The effectiveness of these incomplete levels of resistance in reducing *U. pisi* infection remains to be quantified, but might represent a major progress when compared to the lack of any means for the control of this rust one or two decades ago. Peas can be protected now by combining this resistance with cultural management options, selective fungicides and by biocontrol agents representing opportunities that did not exist before. The current focus in applied breeding is taking advantage of biotechnological tools to develop more and better markers to allow marker-assisted selection with the hope that this will accelerate the delivery of improved cultivars to the farmer. Our understanding of the genetics of resistance to pea rust in the available germplasm has improved considerably, but progress in marker development and delivery of useful markers is still limited. We are currently facing an accelerated progress in genomic and biotechnological research, which should soon provide important understandings on pathogen-host interactions and will provide candidate genes for resistance to pea rust.

Table 2 Germination percentage of *Erysiphe pisi* in pea leaves after inoculation in different duration.

Chemical treatments		**Time duration**								
		24hrs			**48hrs**			**72hrs**		
BTH		**G%**	**NG%**	**NSM**	**G%**	**NG%**	**NSM**	**G%**	**NG%**	**NSM**
	50ppm	18.67	81.33	-	21.33	78.67	8	22.33	77.67	9
	100ppm	28.33	71.67	-	29.33	70.67	12	30.13	69.87	11
	150ppm	28.00	72.00	-	26.33	73.67	10	27.20	72.80	12
ZnSo4	10-3	22.67	77.33	1	20.67	79	5	23	77	7
	10-4	28.00	72.00	-	21.00	79.33	3	22.33	77.67	5
	10-5	19.00	81.00	-	22.00	78	10	25	75	9
Salicylic	4mM	32.67	67.33	2	40.00	60.00	25	43.	57	29
acid	8mM	15.67	84.33	-	56.00	44.00	15	57.33	42.67	21
	12mM	14.00	86.00	3	62.67	37.33	26	63.67	36.33	37
Control		56.33	43.67	6	75.00	25.00	40	96.33	3.67	43

G= Germinated, **WCT**= Without Chemical Treatment, **NG** = Non gradient, **NSM** =N0 of sec. mycelium

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