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Full Length Article

Detached Leaf Assay Coupled with Microscopic Conidial Quantification: An Efficient Screening Method for Powdery Mildew Resistance in Pea

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Abstract

The genetic resistance in high yielding pea cultivars against powdery mildew provides a cost effective and reliable strategy to reduce the yield losses and save quality of the harvest. The dependable selection of powdery mildew resistant source(s) is the most crucial step in breeding for disease resistance. The efficiency of a simple, reliable and reproducible screening method on detached leaves of pea under controlled environmental condition was evaluated in comparison with field screening under natural conditions. The detached leaf assay coupled with microscopic quantification of susceptibility percentage (%S) appeared ten times more precise and reproducible than field screening based on visual observation of percentage of leaf area affected with disease. A new 0-5 microscopic disease scale proposed here is more robust and stringent for making selections for powdery mildew resistant parents and effectively challenging the segregating populations against different isolates of *Erysiphe pisi* simultaneously. The disease scale is based on susceptibility percentage (%S) as a function of percentage of germinated conidia with mycelial growth. For making more precise calculations non germinated conidia without germ tube were not taken in to account. The use of detached leaf assay and microscopic quantification of conidia has provided a simple and reliable screening method to clearly distinguish between escape and resistance mechanisms and the results are not prone to fluctuations in environmental conditions. The assay results were highly correlated (0.993) with the disease severity of whole plants under field conditions; moreover, dCAPS markers have also validated the assay results. Two pea genotypes (It-96 and No. 267) have been selected as highly resistant to powdery mildew, which could be used as a source for breeding disease resistant pea cultivars.

Keywords: Biotrophs; *Erysiphe pisi*; *In vitro*; Legumes; *Pisum sativum*; Susceptibility; Yield losses

Introduction

Powdery mildew (*Erysiphe pisi* Syd.) is the most troublesome foliar disease of pea (*Pisum sativum*) affecting all aerial portions of plants. The incidence of disease induces severe decline (25% to 50%) in green pod yield and produce objectionable odour (Gritton and Ebert, 1975; Dixon, 1978; Reiling, 1984; Agrios, 1988; Azmat *et al*., 2010). 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. The development of genetically resistant cultivars is a cost effective and desirable option to reduce yield losses caused by powdery mildew. The precise selection of powdery mildew resistant source(s) is a pre-requisite for the development of stable powdery mildew resistant and high yielding pea cultivars. Field screening based on natural epidemic of disease is usually employed for making selection of powdery mildew resistant plants in pea. Natural epidemics of powdery mildew usually don't occur every year evenly due to environmental variation leading to erroneous selection of disease resistant source(s). Moreover, *E. pisi* is an obligate biotroph (Hückelhoven, 2005), which grow and propagate through haustoria by redirecting the host's metabolism without causing the death of host (Perfect and Green, 2001; Bélanger *et al*., 2002; Mendgen and Hahn, 2002). Powdery mildew, downy mildew and rust fungi are phylogenetically unrelated biotrophs that are difficult to culture extensively *in vitro.* A significant range of variability for pathogenicity, virulence, disease severity and morphological parameters exist among different geographical isolates of the causal organism (Azmat *et al*., 2012a). Owing to the dietary importance of pea, as an alternative of animal protein, the yield losses incurred by powdery mildew; the shortcomings of field screening method; the presence of pathogenic variation and most importantly, the obligate biotrophic nature of *E. pisi*, there is a need of a reliable and reproducible method of screening for powdery mildew.

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The current research was undertaken on pea genotypes to check the efficiency and reliability of powdery mildew screening method using detach leaf assay under controlled conditions. A new precise microscopic scale is presented to quantify the disease severity of pea genotypes with more authenticity and reproducibility.

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

Seed of 30 pea cultivars belonging to the same maturity group (Azmat *et al.*, 2011) was surface sterilized using 2% Sodium hypochlorite. The seed were sown in the field in a randomized complete block design with two replications. The soil was a well drained silt loam, pH 7.6. The field was well prepared. Synthetic NPK fertilizer was applied by band incorporation at 40N-40P-25K kg ha^{-1} at bed formation. The N and P were from urea and diammonium phosphate; K was from muriate of potash. Each genotype was planted on 75 cm wide raised beds with 8 cm space between plants and 35 cm between rows. 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 $3.5 \times 10^4 \cdot m^{-2}$ 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%.

Detached Leaf Assay

For detach leaf assay 30 pea genotypes were grown separately in sterilized vermiculite containing approximately 200 mL of nutrient solution. The nutrient solution contained 1 mM$CaCl_2.2H_20$, 100 µM KCl, 800 µM $MgSO_4.7H_2O$, 10 µM Fe EDTA, 35 µM H_3BO_3, 9 µM $MnCl_2.4H_2O$, 0.8 µM $ZnCl_2$, 0.5 µM $Na_2MoO_4.2H_2O$, 0.3 µM $CuSO_4.5H_2O$, 800 µM KH_2PO_4, 700 µM Na_2HPO_4 and 1 mM NH_4NO_3. The pots were placed in a growth chamber under controlled conditions. A 14 h light period at 22^{o}C and a 10 h dark period at 15^{o}C were maintained in the growth chamber. The relative humidity was maintained at 60% and the light intensity was 400 µmol m^{-2} s^{-1}. There were two replications for each genotype.

At six to eight node stage six leaves from three healthy plants of individual genotypes from each replication were excised. The excised leaves were placed in Petri plates containing 1% agar, 6 mL 5% sucrose solution and 150 mg L^{-1} benzimidazole as a senescence inhibitor. To ensure even distribution of conidia, Petri plates were individually inoculated using a hand-held inoculator maintaining a conidial density of 20–50 spores/mm^2. 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^{-2} s^{-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 (Fig. 1a) were not included in data recording. The conidia that were just germinated having germ tube (Fig. 1b) on leaves 48 HAI were considered as "resistant" while germinated conidia having mycelia growth (Fig. 1c) 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:

$$\%\, S = \frac{Conidia\ with\ mycelial\ growth}{Total\ number of germinated conidia} \times 100$$

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%.

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).

dCAPS (derived Cleaved Amplified Polymorphic Sequences) Validation

The DNA from three highly resistant (It-96, No.267 and No. 20171) genotypes and one powdery mildew susceptible pea genotype (Meteor-Fsd) was extracted from the leaves using modified CTAB method (Azmat *et al*., 2012c). The genomic DNA was subjected to dCAPS validation of the assay results. The primers used for the validation included, dCAPS-HphI (AGGCACTTGAGCATGTGGGCTCGGT), dCAPS-Ple1 (AGGCACTTGAGCATGTGGGCTGAGT) and dCAPS-Tth111I (AGGCACTTGAGCATGTGGACTCAGT). The primer dCAPS- HphI and the corresponding restriction enzyme (HphI) cut the powdery mildew resistant genotypes only, while dCAPS-Ple1 and dCAPS-Tth111I were designed to cut corresponding DNA amplification of powdery mildew susceptible genotypes only.

Results

Field Screening

Significant variability for disease severity was observed among the genotypes. In response to field screening based on percentage of leaf area affected with powdery mildew, all the genotypes were broadly classified into six groups (Fig. 2). Of the 30 genotypes 16 were resistant of which only two (It-96 and No. 267) showed "immune" disease reaction and one was "highly resistant" (Acc. 20171). Among 14 susceptible genotypes, one was "highly susceptible" (Meteor-VRI) while 10 were ranked as "susceptible" (Table 1).

The SD (standard deviation) and SE (standard error) estimates for % I (Percent Infection on leaves) for individual experiment were recorded as 36.63 and 8.19; 35.48 and 7.94, respectively for field experiment 1 and 2. The difference in the infection percentage (Δ% I) among two independent experiments ranged from 0-14% with an average of 4.97%. The SD and SE estimates for Δ% I among two experiments were 3.34 and 0.61, respectively (Table 1).

Detached Leaf Assay

The disease score data based on susceptibility percentage (%S) have classified 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 cv. 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 (Fig. 3). Following %S 13 genotypes (9057, 9370, 9375, 10609, 10612, 18412, 19598, 19611, 19727, 19782, 20152, 19616 and No. 380) that were ranked as resistant on the basis of field screening have emerged as susceptible to powdery mildew (Table 1 and 2). Among all the genotype "% S" ranged from 0.83-97.58 with an average of 46.94. A negligible difference in "%S" (0.02-1.15) was observed among independent observations of each experiment with an average difference of 0.47. The estimates for SD and SE for each experiment were the same to the average values of both experiments *i.e.*, the values of SD and SE were 36.7 and 6.71; 36.65 and 6.7, respectively for lab experiment 1 and 2. The values of SD and SE For "Δ% S" among the observation of two experiments were 0.35 and 0.06, respectively (Table 2).

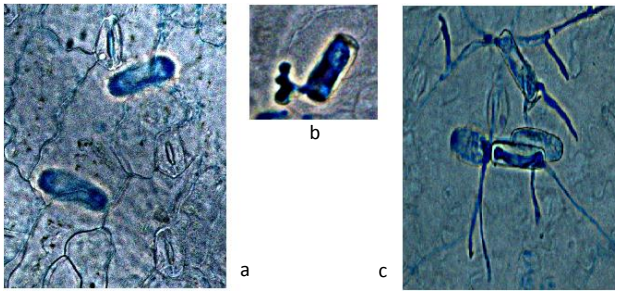

Fig. 1: Stages of *Erysiphe pisi* conidia stained with coomassie blue 48 HAI under dissecting microscope using 40×10 magnifying lenses; (a) Non-germinated conidia (b) Germinated conidia with germ tube without mycelial growth (c) Germinated conidia with mycelial growth

The SE estimates between the values of two field experiments conducted under natural conditions were higher (2.54) than those conducted under controlled conditions (0.24) in growth chamber (Table 1 and 2). Moreover, the results of detached leaf assay coupled with microscopic disease quantification were highly correlated (0.993) with the disease response of whole plants in the field screening under natural conditions. The dCAPS markers have also validated the results of detached leaf assay. As expected dCAPS- HphI and the corresponding restriction enzyme digested the amplification products of powdery mildew resistant genotypes only (Fig. 4). The amplification products of powdery mildew resistant genotypes remained un-digested by using dCAPS-Ple1 and dCAPS-Tth111I (along with corresponding restriction enzymes), hence confirming the results of detached leaf assay (Fig. 4).

Table 1: Response of 30 pea genotypes to *Erysiphe pisi* under field conditions, 15 DAI using 0-9 scale of Warkentin *et al.* (1996) in two independent experiments

Genotype	Field Experiment 1		Field Experiment 2		Mean % I	Mean Score	Δ% I	SE[b]
	% I	Score	% I	Score				
Climax**	91	9	83	8	87	8	8	4.0
It-96*	0	0	0	0	0	0	0	0
KQP-6121	81	8	75	7	78	7	6	3.0
KQP-6173	83	8	82	8	82.5	8	1	0.5
KQP-6185	93	9	86	8	89.5	8	7	3.5
Meteor-VRI	92	9	93	9	92.5	9	1	0.5
No. 267	0	0	0	0	0	0	0	0
No. 380	9	3	11	4	10	3	4	1.0
P1	83	8	87	8	85	8	4	2.0
PF-400	92	9	86	8	89	8	6	3.0
Premium	83	8	78	7	80.5	7	5	2.5
9057	11	4	3	2	7	3	8	4.0
9370	14	4	5	2	9.5	3	9	4.5
9375	9	3	13	4	11	4	4	2.0
10609	11	4	9	3	10	3	2	1.0
10612	17	4	9	3	13	4	8	4.0
10649	86	8	72	7	79	7	14	7.0
18293	17	4	29	5	23	5	12	6.0
18412	15	4	11	4	13	4	4	2.0
19598	13	4	19	4	16	4	6	3.0
19611	11	4	13	4	12	4	2	1.0
19616	43	6	39	5	41	6	4	2.0
19727	19	4	15	4	17	4	4	2.0
19750	14	4	21	5	17.5	4	7	3.5
19782	11	4	7	3	9	3	4	2.0
20126	33	5	41	6	37	5	8	4.0
20152	11	4	9	3	10	3	2	1.0
20171	7	3	4	2	5.5	2	3	1.5
9800-10	83	8	87	8	85	8	4	2.0
9800-5	87	8	85	8	86	8	2	1.0
Min	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Max	*93*	*9*	*93*	*9*	*92.5*	*9*	*14*	7.02
Ave	*40.63*	*5.33*	*39.07*	*4.97*	*39.85*	*5*	*4.97*	2.54
SD	*36.62*	*2.62*	*35.48*	*2.57*	*35.94*	*2.51*	*3.34*	1.69
SE[a]	*8.19*	*0.59*	*7.94*	*0.57*	*8.04*	*0.56*	*0.61*	0.31

* =The values for resistant cultivars are given in bold font, **=The values for susceptible cultivars are given in normal font. The abbreviations used are % I = Percentage of leaf area affected with powdery mildew, DAI= Days after infection, SE[a]= Standard Error of values within experiment, SE[b]= Standard Error of values between experiments

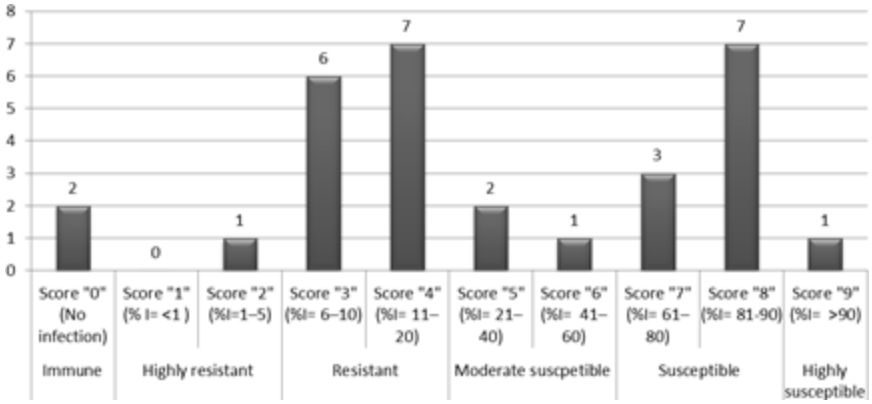

Fig. 2: Disease response groups of pea genotypes on the basis of average percentage of leaf area affected (%I) under field conditions 15 DAI

Discussion

Regardless the nature and number of gene(s) governing powdery mildew resistance in pea, the development of high yielding and disease resistant cultivars is a lengthy procedure. Therefore, a simple, reliable and reproducible

Table 2: Response of pea genotypes to *Erysiphe pisi*48 HAI using detached leaf assay coupled with microscopic quantification of disease on a 0-5 scale under controlled condition in two independent experiments

Genotype	Lab Experiment 1			Lab Experiment 2			Mean % S	Mean Score	Δ% S	SE[b]
	Total conidia	Conidia with Mycelia	% S	Total conidia	Conidia with mycelia	% S				
Climax**	267	261	97.75	193	188	97.41	97.58	5	0.34	0.17
It-96*	293	2	0.68	225	3	1.33	1.01	1	0.65	0.33
KQP-6121	314	278	88.54	311	274	88.10	88.32	5	0.43	0.22
KQP-6173	219	198	90.41	217	198	91.24	90.83	5	0.83	0.42
KQP-6185	242	229	94.63	253	242	95.65	95.14	5	1.02	0.51
Meteor-VRI	301	291	96.68	267	258	96.63	96.65	5	0.05	0.02
No. 267	307	3	0.98	291	2	0.69	0.83	1	0.29	0.15
No. 380	248	47	18.95	261	47	18.01	18.48	3	0.94	0.47
P1	270	253	93.70	253	237	93.68	93.69	5	0.03	0.01
PF-400	198	189	95.45	237	224	94.51	94.98	5	0.94	0.47
Premium	213	190	89.20	241	216	89.63	89.41	5	0.42	0.21
9057	221	35	15.84	197	31	15.74	15.79	3	0.10	0.05
9370	224	39	17.41	257	45	17.51	17.46	3	0.10	0.05
9375	231	56	24.24	305	73	23.93	24.09	3	0.31	0.15
10609	228	43	18.86	316	59	18.67	18.77	3	0.19	0.09
10612	211	47	22.27	207	47	22.71	22.49	3	0.43	0.22
10649	273	237	86.81	279	239	85.66	86.24	5	1.15	0.58
18293	245	67	27.35	281	77	27.40	27.37	3	0.06	0.03
18412	312	44	14.10	274	41	14.96	14.53	3	0.86	0.43
19598	231	65	28.14	209	60	28.71	28.42	3	0.57	0.29
19611	253	37	14.62	239	35	14.64	14.63	3	0.02	0.01
19616	306	147	48.04	287	140	48.78	48.41	4	0.74	0.37
19727	287	49	17.07	313	57	18.21	17.64	3	1.14	0.57
19750	255	50	19.61	272	54	19.85	19.73	3	0.25	0.12
19782	243	43	17.70	261	46	17.62	17.66	3	0.07	0.04
20126	251	132	52.59	254	134	52.76	52.67	4	0.17	0.08
20152	271	72	26.57	249	65	26.10	26.34	3	0.46	0.23
20171	235	14	5.96	283	18	6.4	6.16	2	0.40	0.20
9800-10	287	253	88.15	291	258	88.66	88.41	5	0.51	0.25
9800-5	254	239	94.09	221	209	94.57	94.33	5	0.48	0.24
Min	*198*	*2*	*0.68*	*193*	*2*	*0.69*	*0.83*	*1*	*0.02*	0.01
Max	*314*	*291*	*97.75*	*316*	*274*	*97.41*	*97.58*	*5*	*1.15*	0.58
Ave	*256.33*	*120.33*	*46.88*	*258.13*	*119.23*	*46.99*	*46.94*	*3.63*	*0.47*	0.24
SD	*33.04*	*97.52*	*36.70*	*34.69*	*92.41*	*36.65*	*36.67*	*1.22*	*0.35*	0.18
SE[a]	*6.04*	*17.83*	*6.71*	*6.34*	*16.89*	*6.70*	*6.70*	*0.22*	*0.06*	0.03

* =The values for resistant cultivars are given in bold font, **=The values for susceptible cultivars are given in normal font. The abbreviations used are % S = Susceptibility percentage, HAI= Hours after infection, SE[a]= Standard Error of values within experiment, SE[b]= Standard Error of values between experiments

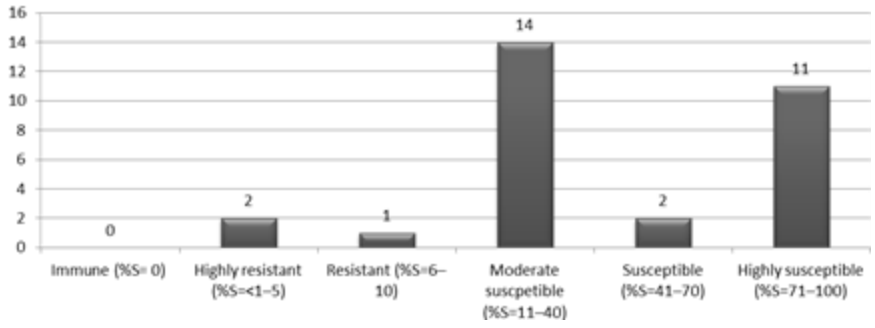

Fig. 3: Six groups of pea genotypes on the basis of susceptibility percentage (%S) against powdery mildew using detached leaf assay and quantitative disease scale

method of identifying resistant plants is imperative for a successful breeding program. Field screening method is preferred, because of its ease to execute, although it has many serious limitations. Detached leaf assay has been used for disease resistance screening in different crops including barley (Edwards, 1983), tobacco (Rufty *et al.,* 1987), peanuts (Foster *et al.,* 1980) and beans (Tu, 1986). There are many advantages of detach leaf assay as it provides a reliable method of screening for foliar diseases under controlled atmospheric conditions.

Two different disease screening and scoring methods for powdery mildew resistance in pea were evaluated.

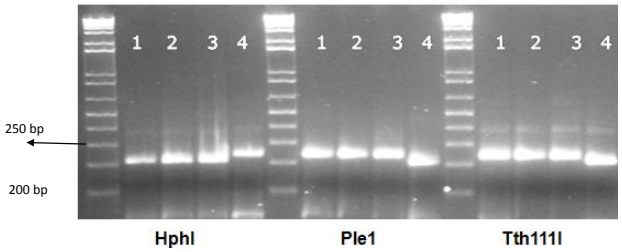

Fig. 4: dCAPS markers validating the results of detached leaf assay, Where 1= It-96, 2= No.267, 3= No. 20171 and 4= Meteor-Fsd

Sixteen of 30 pea genotypes were ranked as resistant in response to field screening on the basis of disease scale reported by Warkentin *et al.* (1996).When these genotypes were challenged by the same isolates of powdery mildew under controlled atmospheric conditions using detached leaf assay and were classified using microscopic disease quantification scale,13 (9057, 9370, 9375, 10609, 10612, 18412, 19598, 19611, 19727, 19782, 20152, 19616 and No. 380) of 16 resistant genotypes were placed in different classes of susceptibility. The detached leaf assay coupled with microscopic quantification of conidia is an effective method of screening for resistance against powdery mildew in pea. The statistical attributes of data have shown that results obtained by counting germinated conidia with/without mycelial growth on detached leaves are 10 times more precise and reproducible as compared to just visual assessment of percentage of leaf area affected with powdery mildew as in case of field screening. Different explanations for such results can be speculated *viz.*,(i) by employing field screening it is difficult to distinguish between the escape and resistance mechanism (Azmat *et al.*, 2012a); while no such risk is involved in detached leaf assay as it is based on precise counting of germinated conidia only. (ii) Chances of human error are more in visual assessment of disease scoring which is not an issue in detached leaf assay, as precise counting of conidia with mycelial growth is done in this method, (iii) Under field conditions, the results of powdery mildew screening can be misleading even if uniform application of inoculum had been ensured due to unpredictable fluctuations in weather. While in case of detached leaf assay atmospheric conditions were optimized for having maximum disease severity, (iv) For precise selection of powdery mildew resistant pea genotypes a more robust and strict scale was devised. On the basis of the scale reported here, only the genotypes having 0-10% susceptibility were considered as resistant, while the scale provided by Warkentin *et al.* (1996) was more relaxed where genotypes with 0-20% leaf area affected with disease were selected as resistant.

Two genotypes It-96 and No. 267 have shown highly resistant diseases response to *E. pisi* using detached leaf assay based on microscopic quantification of disease susceptibility. The resistant genotypes selected on the basis of newly devised more robust scale can be effectively used in breeding programs to incorporate powdery mildew resistance in well adapted high yielding pea cultivars. Detached leaf assay can also be used effectively for the screening of individual plants from segregating populations against different isolates of *E. pisi* simultaneously. The use of detached leaf assay along with microscopic quantification of disease has the potential to reduce the erroneous selection of resistant plants due to escape mechanism.

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