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Parallel response between gametophyte and sporophyte for *Fusarium* wilt resistance in the recombinant inbred lines of chickpea (*Cicer arietinum* L.)

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In the present communication, we study the possibility of screening of a large set of chickpea recombinant inbred lines (RILs) for wilt resistance through *in vitro* pollen tube growth inhibition in the presence of pathotoxin. Further, the number of alleles with resistance differs in their degree of resistance at both gametophytic and sporophytic phase. The RILs of cross JG-62 (highly susceptible) × WR-315 (resistant), segregate for both the loci of wilt resistance and molecular markers linked to the wilt resistance loci. The pollen grains of 43 randomly selected RILs were cultured in cavity slides in the laboratory. The addition of increased concentration of the pathotoxin to the pollen germination medium inhibited pollen tube growth of all the lines. The tube growth inhibition was not uniform across all the lines. It was highest in highly susceptible lines followed by susceptible lines. The degree of pollen resistance depends on the number of alleles/genes for resistance it carries and pollen resistance segregates with alleles for resistance. The study evidenced the parallel response between gametophytic and sporophytic wilt resistance. The toxin concentration required to inhibit 50% pollen tube growth was determined for all the lines. The resistant lines required significantly higher mean toxin concentration for 50% pollen tube growth inhibition, followed by susceptible and highly susceptible lines. The correlation between sporophytic wilt resistance and toxin concentration to inhibit pollen tube growth was highly significant. Higher the sporophytic resistance to wilt, higher was the concentration required to inhibit its pollen tube growth under *in vitro* conditions. Consequently, pollen response has a potential for rapid and inexpensive screening of a large set of genotypes for biotic stress tolerance.

Keywords: Chickpea, *Fusarium* wilt, gametophyte, *in vitro* pollen germination, sporophyte.

THE gametophyte has been the subject of intense studies not only for its importance as the male partner in plant reproduction, but also as a potential arena for selection in crop improvement programmes[1,2]. From pollen formation to successful fertilization, pollen grains experience vari-

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ous selection pressures and the genotype of pollen endows pollen selection with great potential[3]. In this respect the possibility of rapid screening of large sets of genotypes by means of pollen assay has been practised to achieve parallel response between gametophyte (pollen) and sporophyte for pathotoxin resistance[4–7], heavy metal resistance[8,9], moisture stress tolerance[10], temperature tolerance[11–13], herbicide resistance[14], salt tolerance[15] and oil quality[16].

Despite the above-mentioned potential for rapid and inexpensive screening of a large set of genotypes, particularly for biotic and abiotic stress tolerance, the gametic screening technique has not become popular in plant breeding. The reason for this lack of enthusiasm for gametophytic selection could be that so far the association between pollen and sporophyte was demonstrated in different plant species using only a limited number of genotypes. Besides, such associations were not demonstrated in segregating generations to provide genetic proof, casting a doubt on the validity of gametophytic screening for the sporophyte.

In this study, the main objective was to provide genetic evidence for *in vitro* response of the pollen grains to the *Fusarium* toxin which is parallel to sporophytic response to *Fusarium* wilt using recombinant inbred lines (RILs) in chickpea. Wilt caused by *Fusarium oxysporum* is one of the major diseases of chickpea in the Indian subcontinent, causing significant yield losses leading to low productivity. Resistant sources to *F. oxysporum* race 1, a widespread race in India, have been identified. Genetic studies[17–19] suggested that two to three independent loci govern resistance to race 1.

The present study was carried out in a number of chickpea RILs segregating for wilt resistance loci. The selected lines were phenotyped for wilt reaction in a wilt sick plot at the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, and genotyped for resistance loci using linked molecular markers. The basic question we asked was whether the pollen response to pathotoxin as determined by *in vitro* pollen bioassay is influenced by the genotype of the pollen grains?

One hundred thirty-nine F_7 generation RILs of the cross JG-62 × WR-315 were developed. The genetic and molecular marker analysis suggested that the parent JG-62 is highly susceptible and homozygous dominant at both H_1 and H_2 loci ($H_1H_1H_2H_2$). The male parent WR-315 is resistant with homozygous recessive at both the loci ($h_1h_1h_2h_2$). Accordingly, the RILs show a wide range for wilt reaction. Forty-three RILs were randomly selected for *in vitro* pollen bioassay, wilt reaction in the wilt sick plot and molecular marker analysis using linked markers.

The randomly selected 43 RILs were used for *in vitro* pollen bioassay study. At blooming time the flowers with anthers, which were about to dehisce pollen grains, were collected in the morning (8–10 am) in petri plates and brought to the laboratory. The flowers of each RIL were collected separately. The pollen grains of each sampled individual were collected in cavity slides after dehiscence and incubated at 80–90% relative humidity (by keeping a moist filter paper on top and below the cavity slides in the petri plates) for 20 min at room temperature (22–25°C).

The sitting drop method in cavity slides was used for pollen grain germination[7]. The pre-conditioned pollen grains were sprinkled onto the pollen germination medium[20] containing 0 (control), 50, 75 and 100 μg fusaric acid (Sigma cat F-6513) per ml medium. Then the cavity slides were incubated in humid chamber at 80–90% relative humidity (by keeping a moist filter paper on top and below the cavity slides in the petri plates) at room temperature for 45 min and tube growth was measured. Four replications for each concentration with two cavities per replication were prepared for each sampled individual. From each cavity five fields were randomly chosen and for each field, five random pollen grains (5 × 5 = 25 pollen grains in total) were selected for recording observation on pollen tube growth (in mm) on the screen of a projection microscope (SPICON, SP-585/SP-585A).

The 43 selected RILs were raised in pots in the greenhouse for DNA extraction. The DNA was extracted following the protocol described by Edwards *et al.*[21]. The DNA samples were diluted to a working concentration of 20–25 ng/μl and stored at 4°C for further PCR amplification.

DNA from each sampled individual was amplified by PCR using the allele-specific associated primer (ASAP) CS-27F/CS-27R (AGCTGGTCGCGGGTCAGAGGAAGA/AGTGGTCGCGATGGGGCCATGGTG) following the protocol of Mayer *et al.*[22], and A07C primer (GAAACGGGTGC) following the protocol of Soregaon *et al.*[23]. The PCR products were separated on 1.2% (w/v) agarose gel stained with ethidium bromide and all the sampled individuals were scored for presence or absence of specific amplicons. The ASAP amplifies a specific band $CS\text{-}27_{700bp}$ linked to H_1 locus and A07C produces $A07C_{417bp}$ linked to H_2 locus of wilt susceptibility.

The F_8 generation RILs were tested for wilt reaction in the wilt sick plot located at ICRISAT, during post-rainy season in 2007. The wilt sick plot maintained at ICRISAT has been considered as a standard for the evaluation of chickpea wilt[24]. Each genotype was grown in a single row of 5 m/replication in two replications. The susceptible check JG-62 and resistant check WR-315 were sown after every 20 rows. Observations on the number of plants showing wilting in each row were recorded on the 30th day after sowing. In each row the dead plants were uprooted and examined for characteristic vascular wilt. The wilt percentage of each line was calculated as (number of wilted plants in each row/total number of plants) × 100. In general the genotypes are classified into resistant

(0.00–20.00% wilting), susceptible (21.00–50.00% wilting) and highly susceptible (> 50.00% wilting) in chickpea.

Data on pollen tube growth of the 43 RILs at four different concentrations were analysed following two factorial completely randomized design with genotypes as the first and toxin concentration as the second factor.

Further for each genotype and toxin concentration, the relative pollen tube growth was calculated as: percentage relative pollen tube growth = [(tube growth in control – tube growth at a given toxin level)/tube growth in control] × 100. The relative tube growth values were used to estimate the fusaric acid concentration (µg/ml PGM) required to inhibit 50% pollen tube growth (ID_{50}) for each genotype separately using probit analysis[25]. The replication-wise ID_{50} values were determined. Data on ID_{50} values of RILs were analysed following completely randomized design.

The correlation between wilt score and toxin concentration required to inhibit 50% pollen tube growth inhibition was determined.

In pollen bioassay, it was observed that the increased concentration of fusaric acid inhibited the pollen tube growth of all RILs. The mean wilt score, molecular markers amplification and their toxin ID_{50} values of selected RILs are presented in Table 1. Factorial analysis suggested a significant effect of fusaric acid levels, RILs and their interaction on pollen tube growth. The inhibition of pollen tube growth with the addition of increased concentrations of fusaric acid was not uniform across RILs (Table 2). Further, the ID_{50} value for each line was determined which showed significant variation among RILs. The ID_{50} values ranged from 54.04 µg/ml in RIL 49 to 127.10 µg/ml in RIL 27.

The selected 43 RILs showed significant variation for sporophytic wilt reaction at both stages of wilt scoring (Table 3). The wilt score ranged from 1.43% in RIL 65 to 100.00% in RIL 55. The parental lines JG-62 ($H_1H_1H_2H_2$) and WR-315 ($h_1h_1h_2h_2$) differ at both H_1 and H_2 loci of wilt resistance. The susceptible parent JG-62 produces bands for both the markers – CS-27_{700bp} and A07C_{417bp} – linked to H_1 and H_2 respectively. On the other hand, resistant parent WR-315 did not produce any amplicon. The markers segregate independently in the RILs. Among the 43 RILs selected for pollen bioassay, 13 did not produce specific amplicons for both ASAP and A07C primers and were considered as resistant with alleles for resistance at both h_1 and h_2 (Table 4). On the contrary, ten genotypes showed amplifications for both markers, suggesting the

Table 1. Wilt score, molecular marker amplification and ID_{50} values of selected recombinant inbred lines (RILs) in chickpea

RIL no.	Fusaric acid (µg/ml)	Marker		Wilt score (%)
		CS-27_{700}	A07C_{417}	
27	127.10	–	–	21.95
59	123.40	–	–	14.29
34	102.90	–	–	5.84
50	119.70	–	–	27.09
66	118.70	–	–	1.67
10	117.70	–	–	11.59
60	115.80	–	–	4.17
36	115.40	–	–	12.78
38	114.40	–	–	43.55
64	108.20	–	–	7.15
65	107.10	–	–	1.43
20	106.70	–	–	16.99
43	105.90	–	–	38.29
78	105.80	–	+	50.74
52	121.00	–	+	46.80
4	90.73	–	+	34.52
68	89.84	+	–	23.12
83	86.44	+	–	39.47
24	84.31	–	+	20.93
70	84.09	–	+	46.43
73	83.11	+	–	96.29
16	83.03	+	–	12.56
86	82.86	–	+	1.43
40	82.35	–	+	61.28
6	81.08	+	–	10.00
35	80.62	–	+	7.38
77	78.23	–	+	22.15
39	77.12	–	+	21.18
2	76.76	+	–	45.19
96	76.70	–	+	42.62
58	75.11	+	–	27.85
7	74.67	+	–	25.24
32	73.65	–	+	27.67
47	73.52	+	+	56.25
33	73.52	+	+	79.43
3	71.94	+	+	74.55
14	71.92	+	+	40.72
26	69.26	+	+	62.26
5	66.00	+	+	37.50
46	63.20	+	+	71.67
56	59.98	+	+	68.60
49	54.04	+	+	21.11
55	80.67	+	+	100.00

Table 2. Analysis of variance for *in vitro* pollen tube growth in chickpea RILs at different toxin levels

Source of variation	Mean sum of squares
RILs	62565.19*
Toxin levels	1532525.46*
Interaction	2881.14*

*$P = 0.01$.

Table 3. Analysis of variance for ID_{50} values and wilt score in RILs

Source	Mean sum of squares
ID_{50} values	1011.154*
Wilt score (Ist stage)	1316.134*

*$P = 0.01$. ID_{50} = Fusaric acid (µg/ml) for 50% inhibition of pollen tube growth.

Table 4. Mean ID_{50} (fusaric acid, μg/ml) values and wilt score of resistant, susceptible and highly susceptible RILs

Marker reaction (genotype)	No. of RILs	Mean toxin (LD_{50})	Wilt score (%)
Without both DNA markers ($h_1h_1h_2h_2$)	13	114.07[a]	15.90[a]
With any one marker ($h_1h_1H_2H_2$ or $H_1H_1h_2h_2$)	20	84.25[b]	33.14[b]
With both DNA markers ($H_1H_1H_2H_2$)	10	68.41[c]	62.21[c]

Values with the same superscript do not differ. ID_{50} = Fusaric acid (μg/ml) for 50% inhibition of pollen tube growth.

presence of alleles for susceptibility at both H_1 and H_2 loci and were considered as highly susceptible lines. The remaining 20, considered as susceptible, produced amplification either for ASAP or A07C alone, implying the presence of dominant alleles for susceptibility at H_1 or H_2 locus only.

The mean toxin required for inhibition of 50% pollen tube growth of the three groups of genotypes, resistance, susceptible and highly susceptible, differed significantly. The resistance lines required a mean toxin concentration of 114.07 μg/ml followed by the susceptible lines (84.25 μg/ml) and the highly susceptible lines (68.41 μg/ml). The mean sporophytic wilt reaction in the wilt sick plot of resistant and susceptible groups was compared. The resistant genotypes recorded significantly lower sporophytic wilt score and higher toxin concentration for pollen tube inhibition compared to the susceptible and highly susceptible genotypes. Similarly, the susceptible genotypes recorded significantly lower mean toxin for *in vitro* pollen tube growth inhibition and lower sporophytic wilt score compared to highly susceptible genotypes (Table 4).

The correlation between toxin concentration required to inhibit pollen tube growth and sporophytic wilt reaction was significant and negative (–0.430). The genotypes requiring higher toxin concentration for pollen tube growth inhibition were resistant to wilt in the sick plot.

In the present study, we provide a strong foundation to the *in vitro* pollen bioassay for biotic stress tolerance in chickpea through molecular genetic evidence. In the presence of pathotoxin, resistance could be selected for during pollen development[26], germination and tube growth[27,28]. Evidence for pollen expression of resistance was found in the association of sporophyte and pollen resistance in contrasting genotypes[29]. The work reported here shows the dependence of pollen response on the alleles/genes for resistance. In addition, it was evidenced that the pollen resistance segregates with alleles for resistance.

The chickpea pollen grains germinate readily in the liquid medium and produce good tube growth[30]. The addition of toxin (fusaric acid) to the pollen germination medium inhibited pollen tube growth. Fusaric acid is a well-known phytotoxin produced by several pathogenic strains of *F. oxysporum*, the causal organism of wilt in a great variety of plants[31]. Also, a positive correlation was reported between virulence of *F. oxysporum* and production of fusaric acid[32]. The increased concentration of fusaric acid in the pollen germination medium reduced tube growth in all the genotypes. Such dose-dependent effect of toxin on pollen tube growth has been reported in *Brassica*[5], maize[4], chickpea[30] and sunflower[7]. However, the reduction was not uniform across genotypes. The reduction was more in highly susceptible genotypes compared to susceptible and resistant genotypes. The sporophytic resistance significantly influenced the tolerance of pollen grains in the medium. The correlation between sporophytic wilt resistance of the RILs in the wilt sick plot and gametophytic resistance to pathotoxin under *in vitro* conditions was significant. There is a parallel response between sporophyte and gametophyte for resistance to wilt. The positive correlation between sporophyte and gametophyte response to several biotic and abiotic factors has been reported in many plants[29,33]. The resistant genotypes produced pollen grains which show resistance to pathotoxin. Therefore, resistant/susceptible reaction of the pollen grains depends on the sporophyte producing them, implying the alleles present in the pollen grains.

Molecular evidence with linked molecular markers could validate the role of alleles in the performance of pollen grains under selection. The linked DNA marker analysis of genotypes suggested that the genotypes differ for the resistance alleles. The resistant genotypes had homozygous recessive alleles at h_1 and h_2 loci, and consequently produced pollen grains with alleles for resistance. The pollen grains with alleles for resistance showed resistance in pollen bioassay and the resistance was regardless of gametophytic and sporophytic control. Sensitive alleles decreased the pollen tube growth and vigour when they were grown under toxin stress. The expression of these genes resulted in selective growth of pollen grains as the toxin concentration increased. Accordingly, when a pollen grain carried genes for wilt resistance, the probability of its growth increased with increasing toxin concentration; the growth was also quantitatively influenced by the number of resistance alleles it carried. The significant association between marker loci, sporophytic resistance and *in vitro* pollen response suggests the efficiency of pollen bioassay in detecting the genes for resistance in pollen grains. The technique is also useful to identify the genotypes with homozygous resistance and the number of resistant genes.

The pollen bioassay has also been widely investigated; the inheritance of pollen response in segregating genera-

tions after selection of parental genotypes has not been demonstrated. We have clearly demonstrated in the selected RILs that the pollen grains with alleles for resistance express resistance to pathotoxin. The pollen resistance to pathotoxin segregates with alleles for sporophytic resistance. RILs were grouped into resistant, susceptible and highly susceptible depending on the presence or absence of markers. The mean ID_{50} toxin for the resistant group was significantly more than the highly susceptible and susceptible genotypes. The mean ID_{50} toxin for the susceptible group was significantly more than the highly susceptible genotypes.

The resistant RILs were homozygous recessive at both h_1 and h_2 loci as suggested by molecular markers. Consequently, the pollen grains possessed resistance alleles. It was vice versa for RILs in the highly susceptible group. Interestingly, the susceptible RILs had recessive allele either at h_1 or h_2 only. The pollen grains produced by these genotypes possessed recessive allele at h_1 or h_2 only. Our findings are most likely the result of heritable variations in pollen performance and genetic effect due to allele present in the pollen grains[15].

The present study demonstrates the efficiency of pollen bioassay to select pollen genotype through pollen performance at least for biotic stress tolerance. Consequently, the differences in pollen performance would suggest the alleles present in the sporophyte producing the gametophyte. These results add to the body of evidence of expression of sporophytic genes in the male gametophyte[34] and reinforce the view extolling the utility of pollen bioassay and pollen selection as tools in plant breeding[33]. Thus providing an important tool for rapid and inexpensive screening of a large number of genotypes in germplasm or segregating generation.

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Some observations on the determination of platinum group elements and gold in black shales

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The concentration levels and distribution features of the platinum group elements (PGE) in black shales with high carbon content from the Bureya Massif, Far East Russia are discussed. This study compares PGE and gold (Au) values in black shale ores from the eastern Bureya Massif. Mineralogical studies using SEM–EDS on PGE-bearing inclusions in black shales reveal that they always contain O and C along with Pt, Pd, Ir and Os. It is believed that PGE are present in the form of organometallic compounds which are extremely resistant to any exposure, including acid dissolution and fire assay procedures. There is a lot of variation in the concentration values obtained using different analytical methods. Observations, problems and possible causes for the erratic and low recoveries of Au and PGE in the highly carbonaceous black shales are discussed.

Keywords: Black shales, gold, organometallic compounds, platinum group elements, volcanic complexes.

THE average concentration of platinum group elements (PGE) and gold (Au) in rocks is extremely low, less than 3 ng/g (ref. 1). Because of the low concentration levels and inhomogeneous distribution of the elements in different rocks, large amounts of sample (usually > 20 g) are commonly taken for the pre-concentration steps such as fire assay before instrumental determination. It is well known that most of the PGE deposits are hosted by mafic–ultramafic plutons in association with chromite and sulphides. However, several studies in recent times have shown that PGE also exist in the sedimentary marine environment such as black shales[2,3]. Black shale-hosted PGE deposits have recently become promising sources of PGE and many other metals, and several unconventional PGE accumulations and new data on PGE distribution have been reported from marine black shales of China, Canada, USA, Czech Republic, Finland, Poland and Russia. In this context, the problems of the platinum metal potential in black shale sequences have attracted the attention of geologists[4–8] because highly carbonaceous rocks of different origins are considered to represent the most probable nontraditional natural source of Au and PGE in the future. Black shales with high carbon content are widespread in the world in general and in the Bureya Massif, Far East Russia in particular. In general, Au and PGE are collected by nickel sulphide fire assay (NiS–FA) into a NiS button as a separation and pre-concentration step before the determination of these elements by inductively coupled plasma mass spectrometry (ICP–MS)[9,10].

Some chemical analyses showed the presence of elevated concentrations of PGE ranging from 0.1 to 40 g/t in the black shales[11]. However, reproducibility of these analyses was low, and the forms of platinoids occurring in these rocks remained unknown. There is need to understand the factors which are hampering their recovery and development of technologies for their extraction.

Using scanning electron microscope with energy dispersive spectrometry (SEM–EDS), PGE-bearing inclusions in black shales of the Sutyr and Kimkan sequences in the eastern Bureya Massif have been studied (Figure 1). These sequences represent constituents of the Upper Riphean–Lower Cambrian Khingan Group. They are sub-

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