Genetic evidence for resistance to *Fusarium* wilt of pollen grains in chickpea (*Cicer arietinum* L.)

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In the present study, we have investigated the possibility that genes conferring wilt resistance in chickpea determine the resistance at both sporophyte and gametophyte phases. The wilt resistance of 16 diverse genotypes was determined using an *in vitro* screening technique. The presence of resistance genes in these genotypes was confirmed using molecular markers linked to resistance loci. Increased concentrations of the pathotoxin, fusaric acid, in the pollen germination medium inhibited pollen tube growth of chickpea under *in vitro* conditions. The inhibition was more in susceptible early wilting genotypes compared to the susceptible late wilting. The tube growth inhibition was least in resistant genotypes. The degree of pollen tube growth inhibition was influenced by the number of resistance alleles that it contained. The resistant pollen grains had resistance alleles at both h_1 and h_2 loci, while the susceptible pollen grains had none as that of sporophytic resistance. The evidence of pollen performance on the number of resistance allele(s) in the pollen indicates the potentiality of pollen selection for wilt resistance in chickpea. The study demonstrated that pollen bioassay can be used to select resistance of pollen grains, and consequently the genotype of the sporophyte producing pollen grains.

Keywords: Biotic stress, gametophyte, *in vitro* pollen germination, sporophyte, wilt resistance.

THE gametophyte derived from the diploid sporophyte generation 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]. The male gametophyte of flowering plants represented by pollen grains contains all the genetic information required to function as a simple autonomous organism and to unite with the female gamete and form a new sporophyte. Studies on several plant species have indicated that the bulk of mRNA accumulates in pollen grains before germination[3,4]. Further, transcriptome analysis of haploid male gametophyte is characterized by large-scale gene expression in pollen grains[5-7]. Hybridization of pollen cDNA libraries also suggested that the majority of the pollen expressed mRNAs showed gametophytic overlap[8,9]. Since copies of genes are presumably present in pollen DNA, it seemed possible that exposing the pollen to stresses under *in vitro*

conditions could induce expression. 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 gametophytes (pollen) and sporophytes for pathotoxin resistance[10-12], heavy-metal resistance[13], moisture stress tolerance[14], temperature tolerance[15], herbicide resistance[16], salt tolerance[17] and oil quality[18].

In this study, the main objective was to provide the molecular marker/genetic evidence for *in vitro* response of pollen grains to the *Fusarium* toxin, fusaric acid. Wilt caused by *Fusarium oxysporum* is one of the major diseases of the chickpea in Indian sub-continent causing significant yield losses every year[19]. The disease is difficult to control through chemical applications; in-built resistance would be the most economic means to reduce yield losses due to wilt. Resistant sources to *Fusarium oxysporum* race 1 – a widespread race in India – have been identified. Genetic studies[20,21] suggested that two to three independent loci govern resistance to race 1. The dominant alleles at both h_1 and h_2 loci ($H_{1-} H_{2-}$) result in early wilting, while recessive alleles at either one locus ($h_1h_1H_{2-}$; $H_{1-}h_2h_2$) produce late wilting and recessive alleles at both loci ($h_1h_1h_2h_2$) result in complete resistance[22]. The major limitation in wilt resistance breeding is the difficulty in the development and maintenance of uniform wilt sick plot for selection of resistance. The maintenance of uniform virulence of given *F. oxysporum* race, elimination of other soil-borne pathogens like Rizoctonia and non-pathogenic biocontrol agents like *Bacillus* species, *Pseudomonas fluorescence* is difficult at field level[23]. Alternatively, male gametophytic selection is expected to be extremely efficient.

The study was carried out in a number of chickpea genotypes differing in wilt resistance. The selected lines were phenotyped for wilt reaction and genotyped using molecular markers linked to wilt resistance loci. The basic question we asked was whether the pollen response to pathotoxin as determined by *in vitro* pollen bioassay is also influenced by the genotype of the pollen parent, and consequently the pollen grains?

Sixteen diverse genotypes representing germplasm accessions, popular varieties and local collections were selected for this study (Table 1). The selected genotypes were grown in the field during the 2005 chickpea-growing season at the Main Agricultural Research Station, University of Agricultural Sciences, Dharwad (15°27′19″N, 75°0′27″E), for collection of flowers for *in vitro* pollen bioassay.

The 16 selected accessions were phenotyped for wilt reaction following the root feeding technique[24]. The number of days taken for wilting was recorded for each accession and was classified as early wilting, late wilting and resistant type.

The 16 selected genotypes were raised in pots in the greenhouse for DNA extraction. The DNA of all geno-

Table 1. Grouping of selected genotypes based on seedling reaction to fusaric acid and molecular markers linked to resistance loci

| Genotype | Days to wilting at 15 ppm fusaric acid | Markers | | Grouping |
		$CS-27_{700}$	$A07C_{417}$	
ICC-12249	13	–	–	R
WR-315	13	–	–	
A-1	9	–	+	SLW
ICCV-2	9	–	+	
ICC-96030	9	–	+	
ICC-4958	9	–	+	
ICC-96029	9	–	+	
BG-256	9	–	+	
K-850	9	–	+	
ICC-12237	9	–	+	
ICC-12252	9	–	+	
ICC-12236	9	+	–	
Karikadle	6	+	+	SEW
ICC-12434	6	+	+	
JG-62	6	+	+	
ICC-12429	6	+	+	

R, Resistant; SLW, Susceptible late wilting; SEW, Susceptible early wilting; +, Presence of DNA marker; –, Absence of DNA marker.

types was extracted following the protocol described by Edwards et al.[25]. 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 (AGCTGGTCGCGGGTCAGAGGAA-GA/AGTGGTCGCGATGGGGCCATGGTG), following the protocol of Mayer et al.[26] and A07C primer (GAAA-CGGGTGC) following the protocol of Soregaon et al.[27]. The PCR products were separated on 1.2% (w/v) agarose gel stained with ethidium bromide and all the sampled individuals were scored for the presence or absence of specific amplicons. The ASAP primer amplifies a specific band $CS-27_{700\ bp}$ linked to the H_1 locus and A07C produces $A07C_{417\ bp}$ linked to the H_2 locus of wilt susceptibility.

The 16 genotypes were used for *in vitro* pollen bioassay study. At the time of blooming, 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 accession 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 petri plates) for 20 min at room temperature (22–25°C).

The sitting drop method in the cavity slides was used for pollen grain germination[12]. The pollen grains were sprinkled onto the pollen germination medium[28] containing 0 (control), 50, 75 and 100 μg of fusaric acid (Sigma

cat F-6513) per ml medium. Then the cavity slides were incubated in a humid chamber at 80–90% relative humidity (by keeping a moist filter paper on top and below the cavity slides in petri plates) at room temperature for 45 min and then 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 ($5 \times 5 = 25$ pollen grains in total) were selected for recording observation on pollen tube growth (in micrometres) on the screen of a projection microscope (SPICON, SP-585/SP-585A).

Two Factorial Completely Randomized Design with genotypes as the first and toxin concentrations as the second factor was followed. Further, for each genotype and toxin concentration, the relative pollen tube growth was calculated as: % 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 pollen germination medium (PGM)) required to inhibit 50% pollen tube growth (ID_{50}) for each genotype separately using probit analysis[29]. The replication-wise ID_{50} values were determined for all the accessions. Data on ID_{50} values of 16 genotypes were analysed separately following the Completely Randomized Design.

Four genotypes, Karikadle, ICC-12434, JG-62 and ICC-12429, took 6 days for wilting and were grouped as early wilting. Ten genotypes were grouped as late wilting as they showed wilting on the ninth day. The remaining two genotypes, ICC-12249 and WR-315, survived for more

than 12 days and were considered as resistant (Table 1). JG-62 and Karikadle are being used as susceptible checks and WR-315 as a resistant check in all the wilt nurseries.

WR-315 and ICC-12249 among the 16 genotypes tested did not produce amplicons for both primers and hence were recessive at both loci ($h_1h_1h_2h_2$). Karikadle, ICC-12434, JG-62 and ICC-12429 produced specific amplification for both the primers, suggesting they were dominant at both the loci ($H_1H_1H_2H_2$). Among the remaining genotypes, ICC-12236 was positive for amplification of CS-27$_{700}$ marker alone, while the remaining nine were positive for only A07C$_{417\,bp}$ (Table 1), suggesting the genotypes were late wilting. The grouping of genotypes – resistant, susceptible late wilting and susceptible early wilting – based on *in vitro* seedling reaction to fusaric acid and molecular markers linked to resistant loci was the same.

In the absence of fusaric acid in the control PGM, pollen tube growth of all the 16 genotypes was highest and ranged from 459.25 to 241.13 μm, with a mean tube length of 333.55 μm. The addition of toxin reduced the tube length in all the genotypes. The mean pollen tube length decreased from 333.55 μm in the control to 139.63 μm in 100 μg fusaric acid/ml PGM (data not shown). The analysis of variance (ANOVA) indicated the significant effect of toxins on pollen tube growth (Table 2). Further, the ANOVA also suggested that there is a significant genotype and genotype–toxin treatment interaction. The chickpea genotypes differ in their response to the fusaric acid concentration in the pollen germination medium. The degree of reduction in pollen tube growth with the addition of increased concentrations of fusaric acid in PGM was not constant in all the genotypes. It was more in Karikadle, ICC-12434, JG-62 and ICC-12429 and low in WR-315 and ICC-12249 (data not shown). To compare the effect of toxins on pollen tube growth of different genotypes, the concentration of fusaric acid required to inhibit 50% pollen tube growth (ID$_{50}$) was determined. The ID$_{50}$ values of genotypes differed significantly (Table 3). ICC-12249 and WR-315 had the highest values of fusaric acid for 50% inhibition of pollen tube growth, while Karikadle, ICC-12434, JG-62 and ICC-12429 had low values (Table 4).

The combined analysis of *in vitro* screening of genotypes for wilt resistance, molecular markers and *in vitro* pollen bioassay indicated that the grouping of geno-

types – susceptible early wilting, susceptible late wilting and resistant – was similar in all the three techniques (Table 4). The wilt-resistant genotypes did not produce amplification for any of the susceptibility-linked DNA markers and also required the highest concentration of fusaric acid for 50% pollen tube growth inhibition. The susceptible early wilting genotypes were positive for both the markers and had low ID$_{50}$ values. The susceptible late wilting genotypes showed amplification for either ASAP or A07C alone, and the ID$_{50}$ values of the genotypes were less than the resistant genotypes and more than the susceptible early wilting genotypes.

The mean ID$_{50}$ values for early wilting, late wilting and resistant groups were computed. The mean value of early wilting (66.41 μg/ml) genotypes was significantly lower than that of the late wilting genotypes (84.71 μg/ml). Similarly, the mean value of late wilting genotypes was significantly lower than that of the resistant genotypes (115.75 μg/ml). The Spearman's rank correlation[30] between the number of days to wilting and ID$_{50}$ values of genotypes showed highly significant positive association (r value = 0.805), suggesting that the resistant genotypes require higher toxin for pollen tube inhibition.

In the present study, we have provided 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 during pollen development[31], germination and tube growth[32]. Evidence of pollen expression of resistance was found in the association of sporophytes and pollen resistance in contrasting genotypes[33]. The work reported here shows the dependence of pollen response on the alleles/ genes for resistance.

The addition of toxin (fusaric acid) to the PGM inhibited pollen tube growth. Fusaric acid is a well-known phytotoxin produced by several pathogenic strains of *F. oxysporum*, the causal organism of wilt resistance in a variety of plants[34]. Also, a positive correlation was reported between virulence of *F. oxysporum* and production of fusaric acid[35]. The increased concentration of fusaric acid in the PGM reduced tube growth in all the genotypes. Such dose-dependent effect of toxins on pollen tube growth has been reported in *Brassica*[10], maize[11] and sunflower[12]. The resistant genotypes produced pollen grains which required higher concentration of pathotoxin in the PGM for 50% inhibition of pollen tube growth, compared to late wilting and early wilting susceptible genotypes.

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

Source	df	Mean sum of squares
Genotype	15	70433.31*
Toxin level	3	448365.30*
Interaction	45	3002.72*
Error	192	20.13

*Significant at P = 0.01.

Table 3. Analysis of variance for ID$_{50}$ values in chickpea genotypes

Source	df	Mean sum of squares
Genotype	15	999.27*
Error	48	3.69

*Significant at P = 0.01.

ID$_{50}$, Fusaric acid (μg/ml) for 50% inhibition of pollen tube growth.

Table 4. Wilt reaction of selected chickpea genotypes and their ID_{50} values

Genotype	ID_{50} (fusaric acid μg/ml)	Wilt reaction based on *in vitro* seedling reaction	Wilt reaction based on molecular markers
ICC-12249	122.4	R	R
WR-315	109.1	R	R
A-1	92.41	SLW	SLW
ICCV-2	90.39	SLW	SLW
ICC-96030	88.98	SLW	SLW
ICC-4958	88.33	SLW	SLW
ICC-96029	87.89	SLW	SLW
BG-256	85.21	SLW	SLW
K-850	84.03	SLW	SLW
ICC-12237	81.15	SLW	SLW
ICC-12252	76.34	SLW	SLW
ICC-12236	72.04	SLW	SLW
Karikadle	67.68	SEW	SEW
ICC-12434	66.71	SEW	SEW
JG-62	66.53	SEW	SEW
ICC-12429	64.30	SEW	SEW
LSD ($P = 0.01$)	3.64		

The rank correlation values for sporophytic resistance and gametophytic tolerance to fusaric acid were positive and high, validating several earlier findings[32–35]. The technique can be a potential tool for rapid and inexpensive screening of a large set of chickpea genotypes for wilt resistance.

The linked DNA marker analysis of genotypes suggested that they differ for the resistance alleles, and consequently the pollen grains. Pollen grains with alleles for resistance showed resistance in pollen bioassay. The susceptibility 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 the pollen grains as the toxin concentration increases. Accordingly, when a pollen grain carries genes for wilt resistance, the probability of its growth increases with increasing toxin concentration; the growth was also quantitatively influenced by the number of resistance alleles that it carries. The differences in pollen performance would suggest the alleles present in the sporophyte producing the gametophyte. The significant association between the marker loci, sporophytic resistance and *in vitro* pollen response suggests the efficiency of pollen bioassay in detecting the genes for resistance in pollen grains. These results add to the body of evidence of expression of sporophytic genes in the male gametophyte[8] and reinforce the view extolling the utility of pollen bioassay and pollen selection as tools in plant breeding[36]. The technique is also useful to identify the genotypes with homozygous resistance and the number of resistant genes.

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Hybrid progenies in *Jatropha* – a new development

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The existing *Jatropha curcas* in the country exhibit varying degrees of success in terms of seed oil yield and susceptiblity to pest and diseases. Hence, an intensive hybridization programme has been attempted between *Jatropha curcas* and other *Jatropha* species to develop new hybrids with higher yield potential and resistance to diseases. Among the interspecific crosses, the cross between *J. curcas* and *J. integerrima* produced successful hybrids with more seed set, while the other crosses failed to produce seeds due to existence of crossability barriers. The F1 hybrids exhibited vigorous growth, but the fruit was small in size indicating *J. integerrima* characters. Hence backcross was attempted and the progenies showed unique characteristics of fruit, seed and oil yield.

Keywords: Interspecific hybrids, *Jatropha*, oil yield, pollen and pistil interaction.

THE suitability of vegetable oils for the production of biodiesel is gaining national and international importance. Tree-borne oilseeds are the best and potential alternative to mitigate the current and future energy crisis and also to transform the vast stretches of wasteland into green oil fields. The potential sources identified so far include *Jatropha curcas*, *Pongamia pinnata*, *Madhuca latifolia*, *Azadirachta indica*, *Calophyllum inophyllum* and *Simarouba glauca*. Among these, *J. curcas* emerges as the most promising tree-borne oilseed on the basis of its

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