Pyramiding QTLs for *Verticillium dahliae* resistance.

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- *Verticillium dahliae* (Kleb) is a soil-borne pathogen widely distributed which causes the premature death and stem broken of sunflower. In Argentina this disease is considered a major problem that produces up to 30 percent of yield reduction in susceptible commercial hybrids. In 2004, two main physiological Argentinean races (Varg1 and Varg2) were described. The objective of this work was to increase *V. dahliae* resistance by pyramiding of resistance QTLs to the main pathogenic races of Argentina.
- Sources of resistance were detected for each race and the quantitative trait loci (QTL) were located in different chromosomes by mapping populations. Near isogenic lines (NILs) with the resistance QTL for each race were obtained from backcross program with molecular markers and phenotypic selection. NILs (L1: Varg1 resistance, L2: Varg2 resistance), families F3 and F4 (from a cross between L1 and L2) and the susceptible line were evaluated under natural infection conditions and artificial inoculation in growth chamber, with each separately race.
- Typical disease symptoms were observed at flowering stage in the field. The susceptible line had 17-53 % of incidence, L1 showed 0-6 % and L2 had 5-27 % of diseased plants. Fifty percent of F3 families with both QTLs were totally resistant and the rest exhibited 6-19 % susceptible plants. When the isolines were artificially inoculated, they had the expected behavior according to the race used in each trial. On the other hand, F4 selected families with both QTLs were resistant to both races. No significant differences were observed between selected families and the original line with respect to oil % and yield, in healthy plants.
- These results show the presence of different races of this fungus in the field and reveal that the combined QTLs increase the level of resistance to the disease. Nevertheless the search of resistance sources to minor races is being investigated.
- This research shows the importance of pyramiding QTLs, with the assistance of molecular tools and phenotypic selection, in breeding programs to obtain a more durable resistance in sunflower materials without yield drag.

Keywords: sunflower, *Verticillium dahlie***,** races, quantitative trait loci.

INTRODUCTION

Sunflower is a worldwide crop affected by several diseases according climate conditions and production regions. In Argentine, one of them is Verticillium wilt that produce important losses in grain and oil yield (Creus *et al*, 2007). The fungus microesclerotia survive in the soil for many years (10 or more) and plant debris. Mycelia from microesclerotia germination infect the roots and penetrate the vascular system causing wilt and plant premature death (Schnatorst, 1981). Incidence and intensity of damage depend on inocula quantity and distribution in the soil, climate conditions and agronomic management practices (Quiroz, F. *et al*, 2008). Several isolates of *Verticillium dahliae* from different locations were studied since 1990 in Argentine by the Advanta research team. Trough the evaluation of a wide range of public and Advanta materials, a set of differential lines was established and two races were detected (Galella *et al*, 2004). Quantitative trait loci (QTL) were mapped for each race in different linkage groups (nonpublic results). One breeding strategy to obtain durable resistance would be the pyramiding of race resistance QTL. The objective of this study was to obtain Near Isogenic Lines (NILs) with single and combined QTL.

MATERIALS AND METHODS

Fungal Isolates

The Argentinean Verticillium races, Varg1 and Varg2, used in this study were maintained and increased by artificial inoculation of specific differential lines (Galella *et al*, 2004), trough root immersion technique (a modification of the Moser and Sackston method, 1973).

Parental and Near Isogenic lines

Two different donor lines (L1 and L2) were selected from cultivated germplasm to introgress resistance to both races into one susceptible line (L51). Resistance QTL were independently incorporated in L51 by backcross method and two Near Isogenic Lines (NIL) were obtained, L51R1 and L51R2 (Varg1 and Varg2 resistant respectively). From the cross between L51R1 and L51R2 were derived families F2, F3 and F4 with resistance to both races. The selection in each generation was made by molecular markers (SSR) and natural or artificial infection.

Field trial

Fifteen F2:3 families selected by DNA and phenotypic studies were sowed at Advanta field (2010-11) to be evaluated under natural infection conditions. Only one row of each family was sown due to seed availability. Each family was also characterized by its agronomic attributes.

The isolines with and without resistance QTL were used as checks across the trial.

Families were evaluated at flowering and physiological maturity. The percentage of plants with typical Verticillium symptoms (incidence) and the foliage affected proportion (severity) were evaluated. The severity score assigned was: 1=100% diseased leaves and 9= without wilt symptoms.

After harvesting, seed weight and oil content of each head were measured in selected progenies. Oil content was determined by nuclear magnetic resonance (NMR).

Growth chamber trial

Some F3:4 selected families were inoculated by root immersion technique with each race independently. The growth chamber conditions were 25 ºC ± 2 ºC and 16h/8h, light/dark, photoperiod.

A completely randomized block design was conducted with two replicates (8 plants / rep.).

Inoculated plants were evaluated at 21 and 35 days after inoculation. As in the field trial, incidence and severity were evaluated.

RESULTS

Field trial

Incidence and severity data for each row from the checks and F2:3 families are shown in the following table:

Table 3: Field trial results

Materials	Total plants	Diseased plants	Incidence	Disease score	Agronomic selection
L51	18	3	16.7	5	
L51	18	4	22.2	4	

L51	19	6	31.6	5	
L51	17	9	52.9	4	
L51R1	17	0	0.0		
L51R1	16	1	6.3	5	
L51R2	20	1	5.0	3	
L51R2	15	4	26.7	5	
F2:3-1	17	0	0.0		**Selected**
F2:3-2	15	0	0.0		
F2:3-3	17	1	5.9	6	
F2:3-4	19	1	5.3	6	**Selected**
F2:3-5	19	2	10.5	6	
F2:3-6	19	0	0.0		
F2:3-7	17	0	0.0		**Selected**
F2:3-8	16	1	6.3	4	
F2:3-9	21	2	9.5	5	**Selected**
F2:3-10	16	3	18.8	6	**Selected**
F2:3-11	15	0	0.0		**Selected**
F2:3-12	16	2	12.5	6	
F2:3-13	13	0	0.0		
F2:3-14	17	1	5.9		
F2:3-15	19	0	0.0		

Three F2:3 families were selected by their agronomic attributes and absence of diseased plants.

Variance analysis of oil content and seed weight of selected families and checks can be observed in Tables 4 and 5

Table 4: Oil content

Analysis of variance					
Variable	N	R2	R2 Adj	CV	
% OIL	44	0.4	0.32	6.21	

Analysis of variance table (Partial SS)					
SV	SS	df	MS	F	p-value
Model	194.10	5	38.82	5.11	0.0011
Materials	194.10	5	38.82	5.11	0.0011
Error	288.53	38	7.59		
Total	482.62	43			

Test Tukey Alpha: =0.05 LSD:=4.8317					
Error 7.59 df :38					
Materials	Means	N			
L51R2	41.11	3	A		
F3:4-7	41.52	9	A		
L51R1	41.99	3	A	B	
F3:4-1	45.02	11	A	B	
L51	45.72	3	A	B	
F3:4-11	46.40	15		B	

Table 5: Seeds weight

Analysis of variance					
Variable	N	R2	R2 Adj	CV	

Weight	44	0.43	0.36	29.03	

Analysis of variance table (Partial SS)					
SV	SS	df	MS	F	p-value
Model	1199.09	5	239.82	5.78	0.0005
Materials	1199.09	5	239.82	5.78	0.0005
Error	1575.46	38	41.46		
Total	2774.55	43			

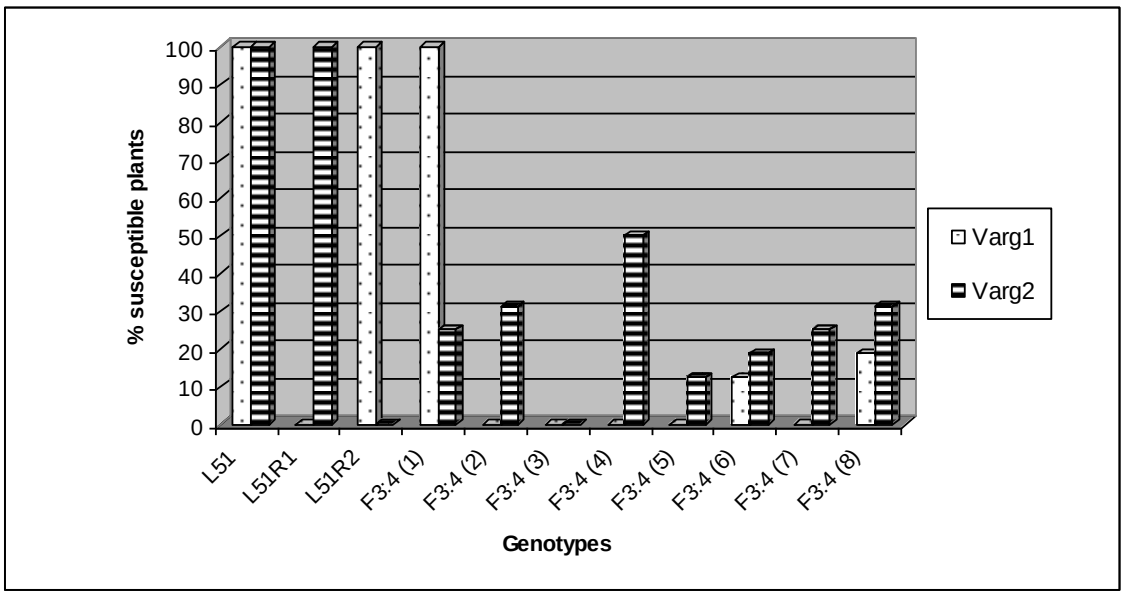
Test Tukey Alpha: =0.05 LSD:=11.3018					
Error 41.46 df :38					
Materials	Means	N			
L51R2	9.67	3	A		
L51	17.00	3	A	B	
F3:4-7	17.78	9	A	B	
L51R1	21.67	3		B	
F3:4-1	23.18	11		B	
F3:4-11	27.73	15		B	

The F3:4-11 family had higher seed weight and oil content than the original line (L51). L51R1 (Varg1) had a similar behavior than L51, while L51R2 had the lowest seed yield and oil content in this experiment.

Growth chamber trial

Eight F3:4-11 progenies were inoculated with each race independently. One of them was totally resistant to both (Figure1).

Figure1: Varg1and Varg2 inoculation results of NILs and F3-4-11 progenies



DISCUSSION

Previous studies of *Verticillium dahliae* isolates revealed the presence of two Argentinean races (Varg1 and Varg2), which has different behavior patterns than other described races (Fick and Zimmer, 1974; Bertero de Romano and Vázquez, 1982 and Gulya, 1997). In 2004, Varg2 was not an important concern due to its low proportion (Galella, 2004). Nowadays, incidence field results with specific

differential lines could be indicating the increase of this race. On the other hand a new *V. dahliae* race has also been cited in the USA (Gulya, 2007), indicating the increase of *V. dahliae* variability.

Traditional breeding for disease resistance has used R genes "one at time" exerting strong selection for mutation and increasing the host vulnerability to new emergent pathogenic races (McDowell *et al*, 2003). The pyramiding of major genes into a genotype could give adequate resistance to prevalent races (Fehr, 1987). Application of this breeding strategy in commercial hybrids would allow the control of a broad spectrum of pathogen population and will provide a more durable resistance. This is possible through conventional breeding but it is very difficult or impossible at early generations. Extensive testing with the different races is required to ensure the presence of desirable alleles (Fehr, 1987).

Marker assisted selection is especially valuable due to greater efficiency in the detection of plants carrying QTL for both races. Methodological mistakes during the inoculation ("escapes") and probably crossing-overs between the QTL and the molecular markers (MM) could be the reasons of some unexpected results. Although this result could be avoided by the use of more MM linked to the QTL and with "flanking markers", the selection strategy was chosen to obtain a major number of F3 families and select the best ones based on others agronomic attributes beside the disease resistance. Field trials do not only include efficacy tests of the incorporated genes but also rigorous tests of agronomic characteristics and yield (Rommens and Kishore, 2000).

In the field experiment, all the checks had some plants with Verticillium symptoms indicating the presence of both races as it was expected. L51 was more susceptible than the other materials and the differences between isolines with resistance QTL would indicate that Varg1 is still the most abundant race as previous studies. Incidence results of each check show variability across the trial, this could be attributed to unequal distribution of pathogen in the soil. Nagtzaam *et al* (1997) found a linear relationship between inoculum density in soil and population density of *V. dahliae* in stem sap in eggplant, based on inoculum added and controlled conditions. Similar conclusions were obtained in studies with cauliflower (Xiao and Subbarao, 1998) and artichoke under natural infection conditions (Berbegal *et al*, 2007).

Some F2:3 families did not show symptoms as expected but other families had diseased plants with a lower score than the checks. The possible reasons are the use of only one MM for each QTL (previously exposed) or the presence of other minor races; one of them could be Varg3, which was detected at Balcarce in 2005 (nonpublic results).

Progeny test results in growth chamber confirmed the first hypothesis while the second one is not discarded. From pathologic test was selected one family homozygous for both races with similar agronomical attributes than the original line. Although favorable differences were observed in terms of oil content and seed weight, more replicates in future assays will be required to confirm this.

The necessity to ensure disease resistance into commercial hybrids for a long time was the basis of this research. According to the experimental results, the objective was achieved however the dynamic of pathogen population requires testing across different environments.

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