Chapter 5

Genetic Improvement of Lentil for Fusarium wilt Resistance

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SYNOPSIS

Lentil is one of the major components of low-input agriculture in the sub-tropics including parts of Indian sub-continent. It plays an important role in alleviating protein malnutrition for millions of vegetarian peoples of these regions. The production and productivity of lentil is severely constrained by Fusarium wilt (FW). FW has been reported to cause 100% yield losses, if it affects the crop in the seedling stage. However, recent advances in the identification of races of the pathogen, screening techniques to identify resistant genotypes, genetic dissection and mapping and tagging of resistance genes through molecular markers have resulted in the release of several FW resistant varieties the world over. This has not only narrowed the gaps between potential and realized yields, but also minimized yearly fluctuations in production and productivity of lentil.

Keywords: Fusarium wilt, screening techniques, gene tagging, wilt resistance, molecular markers, gene pyramiding.

Introduction

Pulses form an important component of typically low input agriculture in sub-tropical regions. Lentil (*Lens culinaris* Medikus subsp. *culinaris*) is one of the major pulse crops in these areas. Resource poor small and marginal farmers hardly provide better-than-average-management practices to this crop. The crop is also exposed to harsh, erratic and unpredictable climatic conditions of these regions in addition to biotic stresses imposed by several pathogens and insect pests. This has not only slowed down its productivity growth, but also caused yield instability leading to shifts into other crops mainly cereals. Among the biotic stresses, Fusarium wilt (FW) disease is

considered as the most severe yield limiting factor in lentil. The FW affects the lentil from seedling to the stage of maturity, causing heavy losses both in quantity and quality. Chemical and cultural control measures are not very effective; however, resistance breeding has been very efficient, and many wilt rust resistant varieties in lentil have been released and adopted by farmers in sub-tropical and semi-arid (SAT) regions (Choudhary *et al*., 2013).

Importance of wilt disease in lentil

Among several biotic stresses affecting stability of production, FW is the most important. This vascular wilt, which is caused by *Fusarium oxysporum* f.sp. *lentis*, is a widespread disease of lentil with its report of occurrence from as many as 26 countries in South Asia, Sub-Saharan Africa and West Asia and North Africa (WANA) regions. It was first reported from Hungary, and later on from many countries including India, USA, Czechoslovakia, USSR, France, Turkey, Syria, Myanmar and Pakistan, Nepal, Ethiopia and Egypt. The disease is known to cause economic yield losses in parts of WANA region, Sub-Saharan Africa and South Asia (Erskine *et al*., 1994). In India, FW is the major factor limiting lentil production in the states of Uttar Pradesh, Madhya Pradesh, Himachal Pradesh, Bihar, West Bengal, Assam, Rajasthan, Haryana and Punjab (Agrawal *et al*., 1993; Chaudhary *et al*., 2009; 2010). Yield losses due to FW in lentil depend on the crop stage at the time of infection (Khare *et al*., 1979), environment and crop variety (Table 5.1). Wilt incidence at seedling stage can lead to a complete crop failure whereas at adult stage (flowering and podding) infection, the plants are able to produce some grain yield that could be shriveled. Wilt incidence as high as 50-78% has been reported in some fields of Madhya Pradesh (Khare *et al*., 1979; Agrawal *et al*., 1993). In west Asian countries like Syria, the yield losses range from 5-72% (Bayaa *et al*., 1986). There is a strong correlation between wilt incidence and grain yield, estimating 8.8% yield loss for every 10% wilt incidence (Erskine and Bayaa, 1996).

Table 5.1 Economic losses due to Fusarium wilt in lentil

Stage of infection	Economic loss (yield/value)	Country	Reference
Seedling to pod filling stage	50-78%	India	Khare *et al.* (1979), Agrawal *et al.* (1993)
Seedling stage	100%	India	Khare (1981)
Stage not specified	29.98%	India	Kumar and Bourai (2012)
Stage not specified	5-72%	Syria	Bayaa *et al.* (1986)
Stage not specified	8.8% (for 10% wilted plants)	Syria	Erskine and Bayaa (1996)

Pathogenic variability

Though *F. oxysporum* f. sp. *lentis* (FOL) is host specific, presence of genetic variability has been reported on the basis of reactions in host genotypes and pathogen morphology and cultural characters (Kannaiyan and Nene, 1978; Belabid *et al.,* 2004; Taheri *et al.,* 2010). Khare *et al.* (1975) reported eight strains of FOL, whereas Kannaiyan and Nene (1978) established seven strains. However, no variation in virulence/aggressiveness was detected among these Indian strains that could play a major role in breaking the resistance of existing genotypes of lentil. Belabid *et al.* (2004) studied virulence and vegetative compatibility of 32 Algerian isolates of FOL, and grouped them as a single race (Table 5.2). However, these isolates differed in their aggressiveness on susceptible lines. Study on 333 isolates from different states of India revealed 43 cultural and morphological groups (Chaudhary, 2008). On the basis of disease reactions against 7 lentil differentials, these isolates were grouped into three clusters. Similarly, variability analysis of 24 isolates collected from north eastern Indo-Gangetic plains using 40 RAPD and 12 SSR primer pairs revealed two sub-populations with little genetic variations (Datta *et al.,* 2009).

Table 5.2 Races/variants of *F. oxysporum* f. sp. *lentis* (FOL)

Race/variant	Reported from	References
Eight strains	India	Khare et al. (1975)
Seven strains	India	Kannaiyan and Nene (1978)
Single race	Algeria	Belabid et al. (2004)
Three classes (based on 43 cultural and morphological groups)	India	Chaudhary (2008)
Two groups	India	Datta et al. (2011)

Screening techniques to identify wilt resistant genotypes

The initial step to utilize host plant resistance (HPR) relates to the development of reliable and reproducible disease screening techniques to evaluate large numbers of germplasm accessions and breeding materials. Effective and efficient screening for resistance to soil borne pathogens such as *Fusarium* spp. calls for simulation of natural soil and environmental conditions and uniform inoculum load across all the plants of test genotypes to discriminate between resistant and susceptible genotypes. In general, screening under field and controlled conditions (green house and laboratory conditions) has been suggested to identify resistant genotypes for FW resistance in lentil (Kraft *et al.,* 1994; Alessandro *et al.,* 2006).

Field screening

The most common and widely used method for screening of FW resistant genotypes has been the wilt sick plot (WSP) method. The main advantage of WSP method is that it allows screening of a large number of genetic materials under field conditions (Infantino *et al.,* 2006). Typical disease symptoms are the main criteria for evaluating breeding lines and establishment of WSPs, while the re-isolation of the causal organism is a confirmatory test. In WSP method, inoculum load needed to get the typical wilt symptoms can vary with race/ variant, environmental conditions, crop and its maturity groups and ecotypes (e.g., *Macrosperma* and *Microsperma* types of lentil) type. The procedures of the "field screening" for WR are almost similar in all the pulses including lentil. The details of field screening have been described in lentil (Kumar *et al.,* 2010). It involves planting of test genotypes along with a susceptible cultivar, which serves as an

indicator line or the susceptible check after every 2-4 test entries to monitor uniformity of the inoculum in the plot (Fig. 5.1). The widely used susceptible checks are 'ILL 4605'. In addition, resistant genotype(s) (e.g., ILL 5588) should also be planted after every 10 rows to monitor if there are other pathogens that can confound the wilt reaction. For screening a large number of gemplasm lines against FW, WSPs have been developed at ICARDA and NARS systems of countries where lentil is a major crop. In India, WSPs have been created for lentil at IIPR, Kanpur, and at selected major centres of the AICRP on MULLaP. Field screening of germplasm and cultivars for wilt resistance has been carried out widely for so many years, which has resulted in the identification of a number of wilt resistant genotypes and cultivars.

Screening under controlled conditions

Field screening, although widely used, has been criticized because many edaphic and climate factors are not under control, and involvement of other soil borne fungal pathogens and nematodes has also been noticed. Screening under controlled conditions in glasshouse is suggested to confirm the results of WSP method. This is particularly important for inheritance and molecular mapping and tagging studies using a well characterized race of the pathogen. Moreover, pathogenic diversity studies can be done under the controlled conditions that support genotypic information.

Greenhouse and laboratory screening

The greenhouse screening technique (multiplication of inoculum, raising of seedlings of pigeonpea in autoclaved soil, root dipping in inoculum and transplanting in pots filled with autoclaved soil and assessing disease incidence) as developed for pigeonpea can also be applied for screening FW resistant accessions of lentil (Haware and Nene, 1994). The sick pot screening technique (Nene and Kannaiyan, 1982) can also be refined and utilized for scoring wilt reaction in lentil (Choudhary *et al*., 2013).

In lentil, inoculum density of 10^6 conidia ml^{-1} is generally used to inoculate seedlings. Different inoculation methods such as seeding surface of disinfected lentil seeds in the soil infested with the pathogen grown on autoclaved millet or other grains (10% w/w) and inoculating by pouring spores grown on PDA near the roots of 15-day old seedlings in pots are used (Riccioni *et al.,* 2003). Roots of 10-

day old seedlings grown on sterilized sand can be dipped in a spore suspension with concentration of 10^5 conidia ml^{-1}. The wilt reaction in terms of severity of incidence can be evaluated after 7-10 days of inoculation. Fusaric acid (FA), one of the toxins produced by the *Fusarium*, is used as the selective agent to screen chickpea genotypes in laboratory. Concentration of FA to inhibit 50% pollen tube growth differs for resistant, late wilting and susceptible types, which has also been validated by molecular markers. This selective agent can be revalidated for lentil as well. However, despite many limitations, field screening is still a widely used technique to discriminate between resistant and susceptible genotypes of lentil for FW owing to operational simplicity and economy of labour. Nonetheless, it should be used for preliminary screening only. Resistant genotypes must be confirmed for resistant reaction under controlled screening condition. Rapid discrimination between resistant and susceptible genotypes may be performed *in vitro* by using selective agents such as FA, which has already been used in tissue culture studies to select the wilt resistant variants in banana (Matsumoto *et al.,* 1995) and pigeonpea (Pandey *et al.*, 1995). However species/race specificity of FA produced by the pathogen needs to be investigated further. With the availability of diagnostic PCR based molecular markers, resistance to FW can be established without subjecting germplasm and segregating generations for phenotyping in wilt-sick plot. For this purpose, molecular markers that are closely linked with WR genes are required.

Fig. 5.1 Screening of lentil germplasm in wilt sick plot at Tel Hadya, Syria. The picture shows highly susceptible check 'ILL 4605' along with resistant lines.

Genetics of wilt resistance

Development of wilt-resistant varieties is the major objective in the pulse breeding program to ensure stability in production and productivity (Choudhary, 2010). The accomplishment of the objective more often becomes difficult due to evolution of new races and co-existence of more than one pathotype at any one location. The transfer of FW resistant genes from the donors to an otherwise high-yielding genotype requires knowledge about the inheritance and genetics of wilt resistance. Only limited inheritance studies have been carried out to know the genetics and inheritance pattern of WR in lentil. Five independent genes have been reported to confer resistance to FW in lentil (Kamboj *et al.,* 1990). Based on allelism test, two duplicate genes and two complementary genes have been identified, imparting WR in the variety 'PL 234' and in 'JL 446' and 'PL 286', respectively. However, only a single dominant gene has been reported to control WR in the crosses made at ICARDA (Abbas, 1995). Eujayl *et al.* (1998) also recorded monogenic inheritance for WR in 'ILL 5588' and designated the gene as *Fw* (Table 5.3).

Tagging of resistance gene(s) through molecular markers

Though simply inherited, the transfer of WR to locally adapted cultivars has been difficult due to linkage drag and difficulty in accurate phenotyping under field screening because of uneven concentration of inoculum and presence of different races/pathotypes of *Fusarium* spp. (Choudhary *et al*., 2013). Therefore, tagging of WR gene(s) through molecular markers is highly desirable. Only limited progress has been achieved towards tagging of resistance gene(s) in lentil (Table 5.4).

Table 5.3 Inheritance of FW resistance in lentil

Fusarium race/ variant	Number and nature of WR gene	Gene symbol	Remarks	Reference
Strain not specified	Five genes	-	Independent genes	Kamboj *et al.* (1990)
Strain not specified	Duplicate genes	-	Resistance in PL 234	Kamboj *et al.* (1990)
Strain not specified	Two complementary genes	-	Resistance in JL 446 and PL 286	Kamboj *et al.* (1990)
Strain not specified	Monogenic dominant gene	-	ICARDA experiments	Abbas (1995)
Strain not specified	Monogenic dominant gene	*Fw*	Resistance in ILL 5588	Eujayl *et al.* (1998)

Table 5.4 WR genes tagged in lentil

Fusarium race	Gene tagged	Marker identified	Distance (cM)	Linkage group	Reference
Strain not specified	*Fw*	RAPD marker OPK-15_{900} OP-BH_{800} and OP-$D15_{500}$OP-C0465o	10.8	Coupling phase Repulsion phase	Eujayl *et al.* (1998)
Strain not specified	WR gene	SSR59-2B	8.0		Hamwieh *et al.* (2005)
Strain not specified	WR gene	AFLP p17m30710	3.5		Hamwieh *et al.* (2005)

Eujayl *et al.* (1998) identified RAPD marker OPK-15_{900} linked with *Fw* gene at a distance of 10.8 cM and established its linkage with the RAPD markers OP-$B17_{800}$ and OP-$D15_{500}$ in coupling and OP-$C04_{650}$ in repulsion phase. These arbitrary markers can be made more useful by converting them into locus-specific sequence characterized amplified region (SCAR) markers for marker-assisted screening and selection. Subsequent study identified one SSR and AFLP markers that were linked with *Fw* gene at 8.0 and 3.5 cM, respectively

(Hamwieh *et al.,* 2005). However, WR genes present in the Indian germplasm are yet to be mapped. Efforts are underway to develop mapping populations involving 'Precoz' and 'Sehore 74-3' as the susceptible and 'PL2' and 'IPL406' as the resistant parents. For developing mapping populations without any segregation distortion, molecular markers have been very useful in establishing hybridity of F_1 plants (Solanki *et al.,* 2010). New RILs have been developed at ICARDA involving parents from different geographical regions for mapping race-specific resistance genes.

Conventional and molecular breeding for wilt resistance in lentil

Lentil is a highly self-pollinated crop. As FW resistance appears to be simply inherited, conventional breeding methods used in autogamous crops such as backcross and recombination breeding should be equally effective for breeding wilt resistant varieties. Simple field screening in WSPs and selection has resulted in the identification and release of a number of FW resistant donors and varieties, respectively in lentil (Table 5.2). Recombination breeding, a selection-crossing-selection cycle which consists of controlled crossing between agronomically superior genotype(s) and wilt resistant donor(s) followed by pedigree selection or its various modifications in the segregating generations has been the most utilized breeding approach for incorporating WR in these three pulse crops. The bulk pedigree method has been the preferred method at ICARDA in which targeted crosses are advanced under disease-free conditions as bulks up to F_4 generation, and the selected single plant progenies (F_5) are grown in the wilt-sick plot. Plant progenies with resistant reaction are further evaluated in WSP and in normal field as preliminary screening nursery (F_6), preliminary yield trial (F_7), and advanced yield trial (F_8). Finally, the elite lines with WR, high yield and other desirable traits in different genetic backgrounds are included in Lentil International Fusarium Wilt Nursery (LIFWN) and other yield nurseries for multi-location testing in the targeted countries. In addition to genetically fixed elite lines and germplasm, segregating populations are also made available to the national programs for selection in the local wilt-sick plot and agro-climatic conditions. Systematic utilization of resistant sources such as 'ILL 5883', 'ILL 5588', 'ILL 4400' and 'ILL 590' at ICARDA has resulted in the development of a wide spectrum of FW resistant varieties for cultivation in different countries (Table 5.5). Some of the prominent wilt resistant varieties are 'Idleb 2', 'Idleb 3', 'Idleb 4' and

'Ebla 1' in Syria; 'Talya 2', 'Rachayya' and 'Hala' in Lebanon; 'Firat 87' and 'Syran 96' in Turkey; 'Ada', 'Alemaya', 'Assano', 'Alemtina' and 'Teshale' in Ethiopia; 'Kimiya' in Iran and 'IPA 98' in Iraq. In India, national program has released several wilt resistant varieties, and prominent among them are 'L 4147', 'Pant L 406', 'Pant L 4', 'Pant L 639', 'Priya', 'Seri', 'JL 3', 'Noori', and 'VL 507' (Pandya *et al.,* 1980; Singh *et al.,* 1994; Rahman *et al.,* 2009).

With increasing information on host-pathogen interaction, genetic variation in the pathogen and temporal variation in pathogenicity, more efficient screening and breeding methods would be required for improving WR in lentil (Fig. 5.2). For example, early and late wilt reactions are noticeable in genotypes. These host reactions may be under the control of different genetic systems. Combining them together through marker assisted selection (MAS) may be essential for stable resistance. Similarly, resistant sources identified in wild species require allelism test to establish their genetic relationship with resistance gene already identified in the cultivated germplasm and, if found alien, these should be introgressed in the cultivated germplasm for durable resistance. However, transfer of desirable alleles is not so simple because of difficulty in efficient tracking for desired and non-desired alleles in breeding lines. This problem can be overcome by advanced-backcross QTL based breeding (AB-breeding) as it is the most suitable for introducing novel alleles from wild relatives to the cultivated species cultivars or varieties in a controlled manner (Tanksley and Nelson, 1996). Furthermore, establishment of pathogenic races in FOL will require search for race-specific resistance genes and their pyramiding in superior genotypes. However, it is difficult through recombination breeding approach by selecting desirable plants on the basis of phenotype. Marker-assisted gene pyramiding can be used to combine in a single genotype the desirable WR genes as well-established tight association between markers and target traits has already been reported in chickpea and lentil (Kumar *et al.,* 2011). Recently, gametophytic selection for WR has been reported to be effective in chickpea for developing wilt resistant genotypes in a short period (Ravikumar *et al.,* 2013). They have demonstrated the effectiveness of gametophytic selection in two populations segregating for wilt resistance using molecular markers linked to H1 and H2 locus for WR in chickpea. The same may also be tried for lentil.

Table 5.5 Important varieties/donors of lentil for FW resistance

Resistant variety/donor	Country	Reference
Pant L 406	India	Pandya *et al.* (1980)
Pant L 4	India	Singh *et al.* (1994)
Pant L 639, Priya, Seri, JL 3, Noori, VL 507 L 4147	India	Rahman *et al.* (2009)
IPL 306	India	IIPR (2012)
IPA 98	Iraq	Rahman *et al.* (2009)
Adaa, Alemaya	Ethiopia	Sarker and Erskine (2002)
Firat 87, Syran 96	Turkey	Rahman *et al.* (2009)
Talya 2, Rachayya, Hala	Lebanon	Rahman et al. (2009)
ILL 5883, ILL 5588,ILL 4400, ILL 590	Syria	Erskine *et al.* (1994)
Idleb 2, Idleb 3, Idleb 4, Ebla 1	Syria	El-Ashkar *et al.* (2003; 2004a; 2004b)
ILL 6256	Nepal	Joshi and Maharjan (2003)

Therefore, identification and incorporation of new WR genes in breeding programs, and development of genotypes with multiple combinations of WR genes will remain a continuous activity for sustainable production of lentil. Molecular markers offer a viable option to accelerate breeding progress through indirect selection for WR in segregating generations without actual phenotyping in the wilt-sick plot. Marker-assisted introgression of WR gene(s) is possible only when locus specific co-dominant markers tightly linked with the WR gene(s) are identified. Presently, the most tightly linked marker with WR gene '*Fw*' in lentil is AFLP marker p17m30710 (3.5 cM) followed by SSR marker SSR59-2M (8 cM) and RAPD marker OPK-15_{900} (10.8 cM). However, their distance from the gene of interest '*Fw*' does not provide confidence for use in marker-assisted screening and selection. Therefore, there is a need to develop more locus-specific co-dominant markers such as SSR, ESTs, CAPS, and SNPs in the map of lentil at the closer proximity (<1 cM) with WR gene(s). It will make MAS an essential component in resistance breeding to develop FW resistant varieties in lentil.

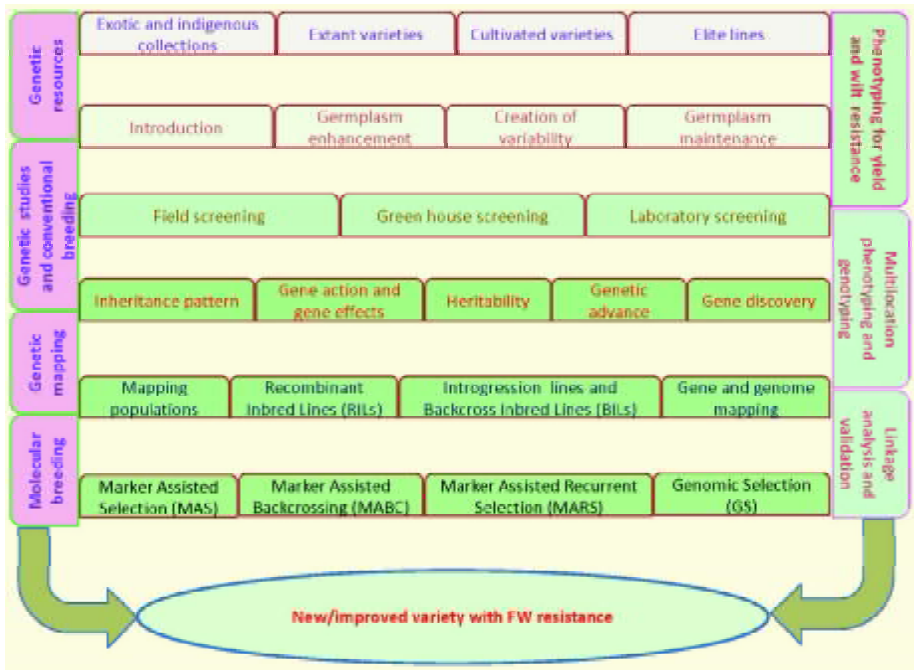

Fig. 5.2 Integration of conventional and molecular breeding for FW resistance in lentil

SUMMARY

Considerable progress has been made during the last three decades in characterizing pathogenic variability of FOL, identifying resistant sources for FW, establishing genetics of wilt resistance and incorporating WR gene(s) into the improved cultivars of lentil. The rapid adoption of these resistant varieties may prevent yield losses due to FW, reduce the gaps between potential and realized yield and bring about stability in production. However, many milestones have still to be achieved. For example, there is need to establish distinct race specificity. Further, there are many discrepancies regarding set of differentials used to classify the races. Since WR in lentil is governed by major resistance genes, there is a need to develop an improved differential set for lentil wilt to reduce ambiguity in variant/race determination. The major factor that has lead to discrepancy in molecular marker studies relates to the application of different phenotyping methods and disease scoring scales (Tullu, 1996). Therefore, it seems appropriate to standardize uniformly applicable phenotyping method and disease scoring scale along with permanent

mapping. Similarly, it seems imperative to combine in a single genotype multiple WR genes through MAS to make it resistant to multiple FOL races. The use of FA as a selective agent for scoring wilt reaction needs further investigation as it (fusaric acid) may not be the sole factor resulting in the development of wilt disease in pulse crops including lentil. In lentil, lack of precise knowledge on the existence of pathogen race is the major hindrance to develop durable resistant cultivars for different regions. In spite of good progress in breeding wilt resistant varieties, its impact could not be demonstrated in farmers' fields due to their susceptibility to other soil borne pathogens causing root rot diseases (e.g., collar rot and dry and wet root rots in lentil). Due to lack of efficient screening techniques, stable resistance for these related pathogens could not be identified, and thus remain the major breeding goals in Asia and Africa. Therefore, there is an urgent need to identify resistance genes for these soil-borne pathogens and incorporate them in wilt susceptible cultivars for visible impact in farmers' fields. Studies are also needed to ascertain that incorporation of WR should not accompany susceptible reaction for other diseases such as rust or *Ascochyta* blight. Besides, further studies are required especially in lentil to establish pathogenic races using an international differential set which is not yet available. Efforts are underway in lentil to develop a common differential set for pathogenicity test which can distinguish different FOL isolates into pathogenic races. This is the pre-requisite for generating information on geographical distribution of races and efficient deployment of race specific resistance genes in lentil cultivars for durable resistance. However, many gaps still exist in our knowledge on the influence of environmental parameters on disease progression which is very crucial for controlling the disease by cultural practices. Preliminary studies indicate that morphological and anatomical characters as well as biochemical constituents of roots of lentil do play an important role in disease reactions, and thus influencing the wilt incidence. However, there is no information on the underlying mechanism of wilt resistance. Marker-assisted breeding for FW resistance in lentil is very limited, partly because WR can be easily identified in field and laboratory. However, these markers can be strategically used to avoid combined effect of other soil-borne pathogens and genotype x environment interactions, and in identification of race-specific resistance genes and their pyramiding in a single cultivar.

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