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Reaction of Iranian Cereal Genotypes to Multiple Strains of *Xanthomonas translucens* pv. *cerealis*

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ABSTRACT

Bacterial leaf streak (BLS) caused by *Xanthomonas translucens* pv. *cerealis* (*Xtc*) is an important disease of wheat (*Triticum aestivum* L.) worldwide. The management methods presently in practice are insufficient to meet current safety and/or efficacy standards. Therefore, use of resistant genotypes is the best approach to manage BLS. The present study was undertaken to identify possible sources of resistance to *Xtc* in cereal cultivars and germplasm. Twelve strains of *Xtc* were isolated from symptomatic leaves in several regions in Kerman province. Out of twelve, nine strains produced the expected *Xtc*-specific 120 bp fragment using PCR and the primer pairs PABr/PBf. Six strains produced water-soaked streaks covered with exudates on wheat cultivars, whereas the three remaining strains incited only chlorotic streaks with no water-soaking on leaves. A highly virulent strain that caused conspicuous water-soaking and necrosis was used for inoculation of 645 winter and spring wheat, barley, and rye accessions to identify possible sources of resistance to BLS. The fourth leaves of test plants were infiltrated with bacterial suspension and scored after seven to ten days. Among all the accessions evaluated, only two rye accessions, namely, 4538 and 4794, were resistant to BLS. These two rye accessions can potentially be used in breeding rye and triticale cultivars for resistance to BLS.

Keywords: Bacterial leaf streak, Barley, Resistance, Rye, Wheat.

INTRODUCTION

Bacterial leaf streak (BLS), caused by *Xanthomonas translucens* pv. *undulosa* (*Xtu*) and *X. t.* pv. *cerealis* (*Xtc*), is an important disease of wheat (*Triticum aestivum* L.) worldwide (Akhtar and Aslam, 1985; Boosalis, 1952; Duveiller, 1992; El Attari *et al*., 1996; Milus and Mirlohi, 1994; Milus *et al*.,1996). BLS of wheat and barley, caused by *Xtc* and *Xanthomonas translucens* pv. *translucens* (*Xtt*), respectively, has been reported from different regions of Iran including Kerman (Alizadeh and Rahimian, 1989), Sistan-o-Baluchestan (Zakeri and Al-e-Agha, 1986), and Golestan provinces (Razi Nataj *et al*., 2010).

Xtc has a wide host range among cereals and grasses affecting wheat, barley (*Hordeum vulgare*), rye (*Secale cereale*), oat (*Avena* spp.), crested wheat grass (*Agropyron cristatum*), *Bromus* sp., *Dactylis glomerata*, *Elymus repens*, *Leymus mollis*, *L. angustus*, *Loliumarun dinaceum*, *L. perenne*, *Psathyrostachy sjuncea*, *Sclerochloadura*, and intermediate wheatgrass (*Thinopyrum intermedium*) (Wallin, 1946; Bragard *et al*., 1997; Mohan and Bijman, 2001).

Typical symptoms of leaf streak consist of elongated, light brown lesions, several centimeters long, which are initially distinct,

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but later coalesce to form larger necrotic solid areas (Mehta, 1993). Lesions are water-soaked and translucent and produce bacterial exudates under humid conditions (Mehta, 1993).

Losses attributed to BLS can reach up to 40% yield reduction in susceptible wheat cultivars (Schaad and Forster, 1985). The disease also affects grain quality (Mehta, 1990). The infected wheat plants are stunted and chlorotic (Cunfer, 1987).

Crop debris, alternative hosts, and soil appear to be the major sources of primary inoculum (Boosalis, 1952; Leben, 1981; Smith *et al*., 1919; Wiese, 1987). The bacterium can infect (Wallin, 1946) or infest seed (Smith *et al*., 1919) and, although a low transmission rate was observed, seed is considered an important source of primary inoculum (Tubajika *et al*., 1998). The pathogen can disseminate a short distance through dew, rain, and contact between plants (Boosalis, 1952), and long-distance or intercontinental dissemination can occur through wheat germplasm exchange (Maraite *et al*., 2007).

Recommended strategies for control of leaf streak include the use of certified pathogen-free seeds, seed disinfection by treating seeds with hot water or copper fungicides (Forster and Schaad, 1985, 1988; Fourest *et al*., 1990), and seed production in disease-free areas (Sands *et al*., 1986; Mehta, 1993). Control of BLS through spraying bactericides is inefficient, and cultivars with acceptable level of resistance have not been made available to growers (Mehta, 1993). Therefore, identifying genes that condition resistance and their use in breeding to develop resistant cultivars is the best approach to manage BLS (Adhikari *et al.,* 2012). The objective of the present research was to find new sources of resistance to BLS among wheat, barley, and rye cultivars and germplasm from Iran.

MATERIALS AND METHODS

Bacterial Strains

Six strains of *Xtc* were isolated from infected samples collected from different geographic regions of Kerman province in March 2012 as described by Alizadeh and Rahimian (1989). Six strains previously isolated from translucent lesions accompanied by bacterial exudates were received from Iranian Research Institute of Plant Protection. A loop-full of bacterial suspension was streaked on GYC agar (2% glucose, 1% yeast extract, 2% $CaCO_3$ and 1.5% agar) and the plates were grown at 28°C. Strains were maintained at 4°C for routine use and in 40% (w/v) glycerol at –70°C for long-term storage.

DNA Isolation and Polymerase Chain Reaction (PCR) Analysis

To confirm the identification of the *Xtc* strains, genomic DNA was prepared using the alkaline lysis procedure (Arabi *et al.,* 2006). PCR was performed in a final volume of 20 µL containing 160 µM deoxynucleoside triphosphate (dNTPs), 2.5 mM $MgCl_2$, 10 pmol of each primers [(PBf: 5´-ACAGTCTAAGGGACCTGCG-3´) and (PABr 5´-TCACTGCTGGCGCATCTTA-3´))] (Marefat *et al*., 2006), 2 µL of 10X PCR buffer (100 mM Tris- HCl, 500 mM KCl (pH 8.4)), 1.5 U *Taq* polymerase (CinnaGen, Iran) and 2 µL of template DNA. The reaction was performed in a thermocycler (Master- Cycler®, Eppendorf, Germany).

DNA amplification was performed under the following conditions: initial denaturation at 95°C for 15 minutes, 30 cycles of denaturing at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 2 minutes. Final extension was performed at 72°C for 10 minutes and the reactions products were held at 4°C until used.

PCR products were separated by electrophoresis on 1.3% agarose gel in 1X TBE buffer (100 mM Tris, 500 mM boric acid and 1mM EDTA), at 85V for 45 minutes and the gel was stained with 0.05% ethidium bromide. A 1 Kb GeneRuler™

ladder (Fermentas, Lithuania) was used as a size marker.

Virulence of *Xanthomonas translucens* pv. *cerealis* Strains

Virulence of strains was confirmed by inoculating the susceptible wheat cultivar "Falat" through infiltration of leaves of plants at the four-leaf stage with bacterial suspensions from cultures grown on YDC for 48 hours at 28°C. Plants were grown in a greenhouse at 24/18°C, day/night temperatures, under a 16 hour photoperiod. The inoculum was prepared by suspending bacterial cells in sterile water at an optical density of 0.2 at 600 nm (approximately 10^7 CFU ml^{-1}). The adaxial surface of a fully expanded fourth leaf was infiltrated using a disposable syringe without a needle (Milus and Mirlohi, 1994). Approximately 10 to 15 µL of the bacterial cell suspension was gently infiltrated into the middle of the fourth leaf of each plant. One area per leaf was infiltrated with the bacterial suspension and the infiltrated areas were marked using a nontoxic permanent marker.

Disease reactions within the infiltrated areas were scored visually seven to 10 days after inoculation using a 0 to 6 rating scale (Milus and Chalkley, 1994), where 0= No visible symptoms; 1= Chlorosis without water-soaked lesions; 2= Water-soaking less than 10%; 3= Water-soaking 10 to 30%; 4= Water-soaking 31 to 70%; 5= Water-soaking 71 to 100% and 6= Water-soaking extending beyond the infiltrated area. Disease scores of 0 to 2 were considered as resistant (R) (Tillman *et al*., 1996), and scores of 2.1 to 6 were regarded as susceptible (S) reactions.

Plant Materials

A total of 610 bread (*T. vulgaris*, *T. aestivum*, *T. comfactum*, *T. turfidum*, *T. turgium*, and *T. persicum*) and durum (*Triticum durum*) wheat genotypes, including 64 cultivars and 546 accessions and landraces from different geographic regions of Iran, 25 barley cultivars, and 10 rye accessions were evaluated for their responses to BLS. These accessions were supplied by the National Plant Gene Bank, Cereal Research Department and Natural Resource of Khuzestan.

Planting Wheat Accessions and Experimental Designs

Four seeds of each accession were planted in each of three cones. Wheat cultivar Falat (Beiki *et al*., 2004) served as the susceptible control. Wheat accessions were arranged in a randomized complete block design (RCBD) with three replications. Each treatment consisted of four plants per replication. Each cone was regarded as an experimental unit, and the fourth leaf of each plant was treated as a sampling unit. The greenhouse temperature was maintained at 24 to 28°C with a 16 hour photoperiod. The experiment was repeated at least twice.

Retesting a Subset of Accessions against Three Additional Bacterial Strains

A subset of the 17 accessions that exhibited disease scores of less than 4 to strain *Xtc*4 in the preliminary screening were selected for further evaluation. From this group, three accessions of wheat i.e. 3157, 3889 and 5155, and two accessions of rye i.e. 4538 and 4794, again produced disease scores of four or lower and were chosen for further evaluation against *Xtc*4 and three additional strains of the bacterium. These test stains included two strains (*Xtc*1 and *Xtc*8) collected from Kerman and *X. campestris* pv. *cerealis* strain ICMP11055, the representative Pathotype strain from the "International Collection of Microorganisms from Plants" (ICMP).

Plants growing conditions, inoculum preparation, inoculation, and disease scoring were as described in the preliminary evaluation trials. The plants were arranged

in a RCBD with three replications. Each cone was considered as an experimental unit. The fourth leaf from the soil level was rated for each strain in each replication (a total of 12 leaves per accession) for each strain.

Statistical Analysis

The average disease reaction for the four plants within each experimental unit was calculated. Data analysis was performed using a nonparametric procedure and were conducted using the statistical package SPSS version 21. Significant differences among the reactions of wheat accessions to *Xtc* were used to categorize genotypes reactions.

RESULTS

Identification of *Xtc* Strains

Xtc strains were identified based on their phenotypic features including; negative for Gram staining, oxidase, fermentative metabolism of glucose, urease, arginine dehydrolase, and pigment production on King's medium B and were positive for presence of catalase and H_2S production from cysteine. Nine of the 12 strains tested produced the expected 120 bp fragment from PCR product using the PABr and PBf primer set for *Xtc* (Marefat *et al*., 2006). These strains were used in pathogenicity and relative susceptibility tests. No PCR products were obtained from the other bacterial species and pathovars tested (data not shown).

Pathogenicity of *Xanthomonas translucens* pv. *cerealis* Strains

A significant difference in pathogenicity was observed among *Xtc* strains on cv. Falat. Two types of symptoms (Table 1, Figure 1) were produced on the inoculated leaves depending on the strain. The first type, induced by strains *Xtc*1, *Xtc*2, *Xtc*3, *Xtc*4, *Xtc*7 and *Xtc*8, consisted of water-soaked streaks. These water-soaked areas developed into necrotic

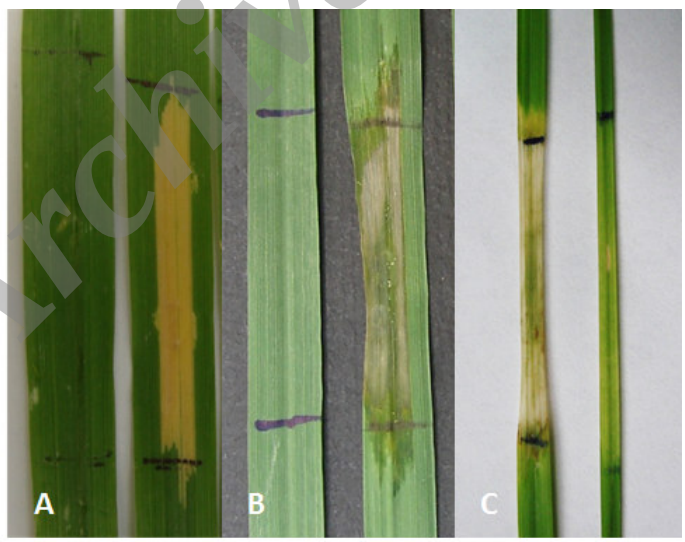

Figure 1. Symptoms produced nine days after inoculation by different strains of *Xanthomonas translucens* pv. *cerealis* on *Triticum aestivum* cv. Falat: (A) Chlorotic streaks without water-soaked lesions or yellowish exudates produced by *Xtc*40 (right) and control infiltrated with sterile water (left); (B) Water-soaked streaks accompanied by bacterial exudates (susceptible reaction) produced by strains *Xtc*4 (right) and control infiltrated with sterile water (left), and (C) On a susceptible (left) and a resistance accession (right) *Secale cereale* accession streak symptoms produced by the virulent strain *Xtc*4.

Table 1. Disease reaction nine days post inoculation of *Triticum aestivum* cultivar "Falat" to *Xanthomonas translucens* pv. *cerealis* strains *Xtc*1; *Xtc*2; *Xtc*3; *Xtc*4; *Xtc*7; *Xtc*8; *Xtc*40, *Xtc*188 and *Xtc*902.[a]

Xanthomonas translucens pv. *cerealis* strains								
*Xtc*1	*Xtc*2	*Xtc*3	*Xtc*4	*Xtc*7	*Xtc*8	*Xtc*40	*Xtc*188	*Xtc*902
5.7[a]	5.3 [a]	5.1[a]	5.8[a]	5.0[a]	5.50[a]	1.0[b]	1.0[b]	1.0[b]

[a] Means with the same letters are not significantly different (P≤ 0.05) based on Duncan multiple range test.

streaks after 7 to 9 days and were covered by droplets of bacterial exudate. The streaks kept a translucent appearance and the exudates usually turned into loose yellow granules. The other strains i.e. *Xtc*40, *Xtc*188 and *Xtc*902, produced chlorotic streaks without water-soaking and were devoid of leaf exudates. All strains caused water-soaked lesions on barley that developed into translucent streaks in 7 to 9 days after inoculation. Leaves of wheat, barley, and rye plants which were infiltrated with sterile distilled water were symptomless. Based on the severity of the symptoms produced, *Xtc*4 was highly virulent and selected for use in inoculation trials aimed at assessing the *Xtc* resistance level of the assembled genotypes (CVs and accessions) of wheat, barley, and rye.

Reactions of Cereal Accessions to *Xtc* Strains

Among 645 wheat, barley, and rye genotypes evaluated, the BLS scores ranged from one to six, two rye accessions i.e. 4538 and 4794, showed scores of less than 2 and were rated as resistant. Among the susceptible accessions, 631 genotypes had water-soaking with 71 to 100% coverage of the area and water-soaking extending beyond the infiltrated area and were given scores of 4.1 and 6. The remaining 12 accessions were rated 2.1 to 4 (data not shown).

Disease reactions within the infiltrated areas on the fourth leaves were scored visually 7, 9, and 10 days after infiltration. The reaction at day 9 post inoculation was the most reliable overall response of the seedlings under the conditions of this study.

Inoculation of a subset of five wheat and rye genotypes with three additional strains of *Xtc* showed that only the two rye accessions i.e. 4538 and 4794, were resistant to all of the strains with disease ratings of 0.40 to 1.86 (Table2).

Table 2. Disease reactions of six wheat and rye accessions to four strains of *Xanthomonas translucens* pv. *cerealis* (*Xtc*1, *Xtc*4, *Xtc*8 representative of the strains isolated from wheat in Iran and *Xtc* ICMP11055 the *Xtc* reference strain from the International Collection of Microorganisms from Plants). The reactions were scored nine days post inoculation.[a]

	Reaction to *Xanthomonas translucens* pv. *cerealis* strains			
Accession	*Xtc*1	*Xtc*4	*Xtc*8	*Xtc* ICMP11055
3157 (wheat)	6.00[a]	6.00[a]	5.93 [a]	6.00 [a]
3889 (wheat)	5.06[a]	4.60 [a]	5.00 [a]	5.13 [a]
4538 (rye)	0.40[b]	1.46 [b]	1.33 [b]	0.46 [b]
4794 (rye)	1.86 [b]	0.80 [b]	1.13 [b]	0.86 [b]
5155 (wheat)	5.00 [a]	5.00 [a]	6.00 [a]	6.00 [a]
Control (cv Falat)	6.00 [a]	6.00 [a]	6.00 [a]	6.00 [a]

[a] Means with the same letters are not significantly different (P≤ 0.05) according to Duncan multiple range test.

DISCUSSION

Xtc can reduce grain yield and quality in cereals, especially in areas with warm and humid climates, due mainly to lack of effective disease management strategies. Current control measures consist of seed treatment with hot water or chemicals (Forster and Schaad, 1985; Forster and Schaad, 1988; Fourest *et al*., 1990) and planting resistant cultivars (Maraite *et al*., 2007). The use of antibiotics for the control of BLS is not recommended due to the cost and the risk of emergence of resistant strains in bacterial populations (Levy, 1998).

The use of resistant cultivars is the most desirable and economically feasible strategy for management of leaf streak and black chaff of wheat and other small grains. In the present study, two rye accessions with high levels of resistance to multiple strains of *Xtc* were discovered. These accessions are valuable sources of resistance against a pathogen and can potentially be used in breeding programs to develop new resistant cultivars. Although the susceptibility tests were performed under greenhouse conditions, reactions of mature cereal plants have been shown to mimic those of the seedlings (Akhtar and Aslam, 1986; Milus and Mirlohi, 1994). Despite the susceptibility of the 610 wheat genotypes evaluated, any BLS-resistant and more materials should be evaluated to identify sources of resistance to BLS for use in wheat breeding programs.

Wheat cultivars Arvand and Deyhim have been reported in a previous study to be resistant to BLS (Alizadeh *et al*., 1995), but in the current study they were susceptible as were all other wheat cultivars and accessions tested. This difference could be due to differences in *Xtc* strains and inoculation methods between the studies.

Disease reactions (0 to 6 rating scale), an indicator of the level of susceptibility within the infiltrated areas on the fourth leaves, used to assess resistance in wheat germplasm and to determine variation in virulence of the bacterial strains, gave the clearest results at 9 days after infiltration as suggested by other workers (Milus and Mirlohi, 1994; Milus and Chalkley, 1994).

There was no difference between the responses of the resistant rye accessions to different strains of the bacterium. These accessions are potentially very useful sources of resistance to BLS for breeding purposes.

All wheat and barley cultivars evaluated were susceptible to *Xtc* and cultivation of these cultivars enables reproduction and maintenance of the pathogen population, which would lead to both the perpetuation of *Xtc* and increasing heterogenicity of its population due to mutation. The presence of a large and possibly genetically diverse pathogen population may be one reason for the scarcity of resistance among wheat and barley accessions. Existence of several wheat accessions resistant to *Xtu* (Adhikari *et al*., 2012), which has a narrow host range, would support such assumptions.

The present study used intense disease evaluation methods i.e. high inoculum dose of a highly virulent strain, use of multiple strains collected from different geographic areas, and conditions conducive to BLS development in the greenhouse and identified two BLS-resistant rye accessions. These accessions can potentially be used in breeding rye and triticale cultivars for resistance to BLS.

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واکنش ژنوتیپ‌های ایرانی غلات نسبت به استرین‌های *Xanthomonas translucens* pv. *cerealis*

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چکیده

بیماری نواری باکتریایی (BLS) که به‌وسیله باکتری (*Xtc*) *Xanthomonas translucens* pv. *cerealis* ایجاد می‌شود، یکی از بیماری‌های مهم گندم در سراسر دنیاست. روش‌های کنونی مدیریت بیماری کارایی و ایمنی لازم را ندارند؛ از این‌رو استفاده از ژنوتیپ‌های مقاوم بهترین راهکار برای مدیریت این بیماری محسوب می‌شود. مطالعه حاضر به منظور شناسایی منابع مقاومت به *Xtc* در ارقام و ژنوتیپ‌های موجود غلات دانه‌ریز انجام شد. دوازده استرین باکتری از برگ‌های دارای علائم بیماری که از مناطق مختلف استان کرمان جمع‌آوری‌شده بود، جداسازی شد. از بین ۱۲ استرین، در نه استرین قطعه دی. ان. ای مورد انتظار به اندازه ۱۲۰ جفت باز با استفاده از آغازگرهای PBf و PABr تکثیرشد. در آزمون بیماری‌زایی، شش استرین آبسوختگی ایجاد کردند و ترشحات باکتری بر سطح ناحیه مایه زنی شده، مشاهده‌شد، درحالی‌که سه استرین در ناحیه مایه‌زنی شده کلروز ایجاد کردند. به‌منظور شناسایی منابع مقاومت به BLS، یک استرین با قدرت بیماری‌زایی بالا که سبب ایجاد آبسوختگی پیوسته و نکروز شد، برای مایه‌زنی ۶۴۵ ژنوتیپ گندم بهاره و پاییزه، جو و چاودار انتخاب‌شد. واکنش برگ چهارم گیاهچه‌های مایه‌زنی‌شده با تزریق سوسپانسیون باکتری، هفت تا ده روز پس از مایه‌زنی امتیازدهی شد. آزمایش در قالب طرح بلوک کامل تصادفی و در سه تکرار انجام شد. از میان ژنوتیپ‌های بررسی شده، دو ژنوتیپ چاودار، ۴۵۳۸ و ۴۷۹۴، به عنوان ژنوتیپ‌های مقاوم شناسایی شد. این ژنوتیپ‌ها برای ایجاد مقاومت چاودار و تریتیکال به BLS قابل استفاده است.