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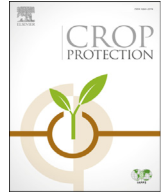

Characterization, geographic distribution and host range of *Curtobacterium flaccumfaciens*: An emerging bacterial pathogen in Iran

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ABSTRACT

Bacterial wilt of dry beans, caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*), is one of the most severe diseases affecting dry bean production worldwide. In Iran, the disease outbreak was first reported in 2012, which was limited only to some restricted areas of the country. Subsequently, *Cff* has rapidly spread and established itself across the major dry bean growing areas of Iran, and currently it represents a severe constraint for growers in terms of crop yield and quality. To better understand the spread of the disease and genetic and phenotypic diversity of the pathogen, including host range, we conducted a comprehensive survey across twelve Iranian provinces, where legume crops are widely grown. In addition, we performed isolations and characterizations of the causal agent. Our results showed that, besides previously reported disease outbreaks from East Azerbaijan and Markazi provinces, legumes cultivated across West Azerbaijan, Zanjan, Ghazvin, Alborz, Fars and Lorestan provinces were widely affected by the pathogen. *Cff* strains with two different colony variants (yellow and orange) were isolated from the infected tissues which were different in terms of pathogenicity and host range. Ten out of eleven different legume species tested were infected by the pathogen and only yellow sweet clover did not show any symptoms. The yellow variant of the pathogen was more aggressive than the orange one on a number of hosts including cowpea, lima bean, broad bean and pea. Unlike the orange variant, the yellow variant of the pathogen caused disease on wild hairy vetch, a major weed in dry bean growing areas of Iran, thereby suggesting the role of weeds as a potential reservoir of the pathogen. It is concluded that, given the seed-borne nature of the pathogen, frequent phenotypic diversity observed, and a wide host range of the strains, an effective management of the disease requires the development and adoption of a number of tools, still poorly available. In particular, there is an immediate need to develop more sensitive and reliable detection methods (e.g. molecular seed screening techniques) that allow detection of the pathogen from seeds, and help reduce the risk of pathogen introduction into a given area.

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1. Introduction

Bacterial wilt — caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) — is an economically important disease of dry beans worldwide (Huang et al., 2009). The pathogen is responsible for severe yield losses (Huang et al., 2007) and seed quality reduction (Camara et al., 2009). The disease was first identified from South Dakota (USA) in 1926 on *Phaseolus vulgaris* (Hedges, 1926). Since then, the pathogen has rapidly spread to several geographic regions, and to date, bacterial wilt disease has been reported from Mexico (Yerkes and Crispin, 1956), Australia (Wood and Easdown, 1990), Brazil (Maringoni and Rosa, 1997), Canada (Hsieh et al., 2002), South-eastern Spain (González et al., 2005), South America and Tunisia (EPPO, 2011), and recently from Germany (Sammer and Reiher, 2012). In Iran, the disease was first observed in 2012 on cowpea (*Vigna unguiculata*) within a restricted area of Marand county, East Azerbaijan, and subsequently in Markazi province in 2014 (Osdaghi and Lak, 2015a). *Cff* is included in

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the A2 quarantine list of the European and Mediterranean Plant Protection Organization (EPPO, 2011; Sammer and Reiher, 2012). To date, Iran is the only country in Asia where bacterial wilt occurrence has been described (Osdaghi et al., 2015a,b).

Based on the color of their colony, different phenotypic variants of *Cff* are described worldwide (Hedges, 1926; Conner et al., 2008; Harveson and Vidaver, 2008). While the yellow and orange variants are the most common phenotypes isolated from areas affected by the disease (Hedges, 1926; Schuster and Christiansen, 1957; Conner et al., 2008), there are several other colony variants of the pathogen. For example, the purple variant was reported on bean from western Nebraska (Schuster et al., 1968) and Canada (Huang et al., 2006). Likewise, the pink variant of the pathogen was described on soybean from Brazil (Soares et al., 2013), and on common bean from western Nebraska (Harveson and Vidaver, 2008). The aggressiveness of the strains often differs from one colony variant to another.

Host range of the pathogen varies among common bean (*P. vulgaris*) (Hedges, 1926; Urrea and Harveson, 2014), lima bean (*Phaseolus lunatus*) (Hedges, 1926; Schuster and Sayre, 1967; Maringoni and Camara, 2006), cowpea (*V. unguiculata*) (EPPO, 2011), mungbean (*Vigna radiata*) (Wood and Easdown, 1990), soybean (*Glycine max*) (Sammer and Reiher, 2012), and pea (*Pisum sativum*) (EPPO, 2011; Silva Júnior et al., 2012). Although leaves and stems of wheat (*Triticum aestivum*) were shown to be colonized by *Cff*, no leaf chlorosis/necrosis or wilting symptoms are reported on wheat to date (Silva Júnior et al., 2012).

Taking into account the difficulty to manage the disease with chemical and biological control agents (Hsieh et al., 2005; Martins et al., 2013), prevention represents the best way to avoid disease occurrence. In particular, the use of certified seeds is the best way to prevent the introduction of the pathogen into a new area (Huang et al., 2009). In addition, breeding for resistance for the development of resistant cultivars is highly recommended (Huang et al., 2007; Conner et al., 2008; Silva Júnior et al., 2012; Urrea and Harveson, 2014) although such an approach is lengthy and resource-intensive.

The main objectives of this paper were to: i) investigate the geographic distribution of the disease across the areas where leguminous crops are widely grown, and ii) evaluate any variability occurring among *Cff* strains, including their host range, colony phenotypes, and their 16S rDNA sequences. This knowledge is of great importance in order to develop measures that help reduce the risks of bacterial wilt epidemics in Iran.

2. Materials and methods

2.1. Study sites and sampling

Twelve provinces, accounting for nearly 70% production of dry beans in Iran (Anonymous, 2014) — including northwestern, northern, central, and southwestern areas — were surveyed for the presence of bacterial wilt pathogen (Fig. 1). The number of fields from each province, their geographic coordinates, long-term precipitation data (mm), and temperature (°C) of the surveyed areas are described in Table S1.

Following the first disease outbreak in 2012 — which had affected leguminous crops grown on a small scale — growers reported extended disease epidemics in the year 2013. This has led to the hypothesis that the disease was rapidly spreading across regions where dry bean is grown. Consequently, during the summer of 2014, surveys were conducted in dry bean fields of East Azerbaijan, West Azerbaijan, Zanjan, Ghazvin, Alborz, Tehran, Gilan, Mazandaran, Markazi, Lorestan, Fars, and Khuzestan provinces (Fig. 1). A number of fields were considered where common bean, cowpea, lima bean, mungbean, and soybean were grown. Ten to fifteen fields from each region and for each type of legume species described above were surveyed. Plant parts affected by visible disease symptoms were sampled and brought to the laboratory for further analysis.

2.2. Isolation of the pathogen

Infected leaves were surface sterilized in 0.5% sodium hypochlorite for 30 s and rinsed twice using sterile distilled water. The margin of the leaf lesion was cut and ground in sterile distilled water. A loopful of the resulting suspension was spread onto yeast peptone glucose agar (YPGA) medium as previously described (EPPO, 2011). Plates were incubated at 27–29 °C under dark conditions (Tegli et al., 2002; EPPO, 2011). In addition to leaves, seeds with discoloration symptoms were used for isolation of the pathogen. Ten to fifteen seeds were put in 25 mL of sterile distilled water and macerated for 24 h at 5 °C. A loopful of the resulting suspension was streaked onto YPGA medium (Lelliot and Stead, 1987; EPPO, 2011). All purified bacterial isolates were grown on yeast extract dextrose calcium carbonate (YDC) medium, resuspended in sterile distilled water, and stored at 4 °C for further use. For the long-term storage, bacterial isolates were maintained in 15% glycerol at −70 °C.

2.3. Biochemical characterization of the isolates

Biochemical tests were carried out as described previously (Schaad et al., 2001; EPPO, 2011). Forty-six isolates from different geographic areas and various host plants were used (Table 1). The putative isolates of *Cff* were subjected to standard biochemical and physiological tests. All isolates were tested for gram reaction, catalase, oxidase, urease, potato soft rot, indole production, growth on triphenyl tetrazolium chloride (TTC) and YDC media, growth at 37 °C, acid production from sorbitol, mannose, erythritol, maltose, inositol, inulin, mannitol, and hydrolysis of gelatin, casein, and aesculin. All the biochemical tests were repeated twice.

2.4. Plant growth and host range determination of the isolates

Host range of the pathogen was determined on mungbean, lima bean, soybean, pea, lentil, chickpea, broad bean, yellow sweet clover, and hairy vetch (Table 2). Five bacterial isolates obtained in this study and one reference strain of *Cff* were used (Table 2). The procedure followed for the selection of the representative bacterial strains used for this test was described previously (Osdaghi and Lak, 2015b). Seeds were sown in 20-cm-diameter plastic pots containing a mixture of perlite, soil and peat (ratio 1:1:1) and all pots were maintained in a glasshouse at ambient temperature (22–28 °C and 14 h natural light). For each plant species, five seeds per pot for a total of three pots per plant species (15 plants/species) were sown. After germination, the seedlings were thinned to three in each pot.

Pathogenicity test was conducted on 5 to 7-day-old common bean and cowpea seedlings grown in the glasshouse. The bacterial suspension, which consisted of 1×10^8 CFU/mL, was prepared from a 48 h old culture grown on YPGA (Osdaghi and Lak, 2015b). Node infiltration method (Diatloff and Imrie, 2000; Urrea and Harveson, 2014) was used for inoculation at the V3 stage (Schoonhoven and Pastor-Corrales, 1987) of plant development (Urrea and Harveson, 2014). The inoculation was performed by inserting a sterile dissecting needle, previously immersed in the bacterial suspension, into the whole diameter of the internode between the first and the second node of each plant (Urrea and Harveson, 2014). A reference strain of *Cff* (UTMC00162) — provided by the University of Tehran Microorganisms Collection (UTMC) — and sterile distilled water

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