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The Biology and Biological Activity of *Pseudomonas syringae* pv. *tagetis*

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ABSTRACT

Pseudomonas syringae pv. *tagetis* (Pst) is a disease of plants in the family Asteraceae. A distinctive characteristic of this bacterial pathogen is the symptom of apical chlorosis in infected plants, caused by the phytotoxin tagetitoxin. Strains of Pst have been isolated from several plant species from a number of countries. One strain isolated from *Cirsium arvense* (Canada thistle) has been evaluated as a biological control agent for this invasive weed and other weeds in the family Asteraceae. Genetic analysis of the strains in this pathovar indicate that it is highly clonal. There is another strain of *P. syringae* (CT99) that was also isolated from Canada thistle and causes apical chlorosis that may produce tagetitoxin as well. However, multilocus sequence typing analysis indicates that it is not a Pst strain. The major impact of Pst on infected plants is stunting and the reduction in sexual reproductive structures, symptoms attributed to tagetitoxin. While initially considered for the control of Canada thistle, the utility of this pathogen as a biological control agent may be limited to controlling annual weeds. Alternatively, tagetitoxin may be of value as a natural herbicide because of its impact on chloroplasts.

Keywords: biological control, Canada thistle, *Cirsium arvense*, CT99016C, multilocus sequence typing, MLST, RNA polymerase inhibitor, tagetitoxin

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INTRODUCTION

Pseudomonas syringae pv. *tagetis* (*Pst*) was first described as a disease of marigolds (*Tagetes erecta* L.) (Helmers 1955). It has since been isolated from a number of plants in the family Asteraceae. Interest in utilizing *Pst* as a biological control agent for weeds in the family Asteraceae emerged shortly after it was isolated from the invasive weed Canada thistle (*Cirsium arvense* (L.) Scop.) (Johnson and Wyse 1991, 1992). This review covers the characteristics of the disease and pathogen, the phytotoxin, tagetitoxin, that the pathogen produces, and what is known about the use of *Pst* as a biological control agent.

NATURAL HOSTS, DISTRIBUTION, AND CHARACTERIZATION

Pseudomonas syringae pv. *tagetis* has a global distribution. It was first described as a disease of African marigold (*Tagetis erecta* L.) grown in Denmark (Hellmers 1955). A distinguishing feature of *Pst* diseased plants is the development of apical chlorosis, which is often accompanied by stunting (Trumboli *et al.* 1978; Gulya *et al.* 1982; Fucikovsky and Kennedy 1987). The pathogen can be transmitted from seed and overwintering vegetative structures (Trumboli *et al.* 1978; Gulya *et al.* 1982; Shane and Baumer 1984; Laberge and Sackston 1986; De Gotuzzo *et al.* 1988). Since its original description, *Pst* has been isolated from marigold species growing in Australia, Mexico, Morovia, Norway, the United Kingdom, the U.S., and Zimbabwe (from which the pathotype strain was isolated) (Trimboli *et al.* 1978; Styer *et al.* 1980; Young and Fletcher 1997; Kůdela and Zacha 1998). *Pseudomonas syringae* pv. *tagetis* is also a common pathogen of sunflower (*Helianthus annuus* L.), with strains reported from plants grown in the U.S., Argentina, and South Africa (Gulya *et al.* 1982; Styer and Durbin 1982a; De Gotuzzo *et al.* 1988; Fourie and Viljoen 1994; Kong *et al.* 2005). There are also three reports of *Pst* isolated from Jerusalem artichoke (*Helianthus tuberosus* L.) grown in the U.S., Canada, and New Zealand (Shane and Baumer 1984; Laberge and Sackston 1986; Kong *et al.* 2005), one report from globe artichoke (*Cynara scolymus* L.) grown in New Zealand (Kong *et al.* 2005), two reports from common ragweed (*Ambrosia artemissifolia* L.) (Styer and Durbin 1982b; Gronwald *et al.* 2004), one report from dandelion (Rhodehamel and Durbin 1985), and one report from compass plant (*Silphium perfoliatum* L.) and a willow-leaf sunflower (*H. salicifolius* A. Dietr) (Rhodehamel and

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Invited Review

Durbin 1989a). One strain (1-502a) isolated from Canada thistle (*Cirsium arvense* (L.) Scop.) growing in Minnesota (Johnson and Wyse 1992) has been evaluated by several researchers for its potential for the control of this and other weeds (see below).

To assess the host range of *Pst*, isolates from marigold, Jerusalem artichokes, ragweed, and dandelion were tested for pathogenicity on the same range of plant species. When stab-inoculated, all strains infected each of the hosts except dandelion, as determined by the development of apical chlorosis (Rhodehamel and Durbin 1985). When spray-inoculated, dandelion did not become infected and ragweed was only infected when sprayed with isolates from ragweed. The other hosts were all infected with all strains and there was no correlation between strain virulence and the hosts from which they were originally isolated (Rhodehamel and Durbin 1985). Gulya *et al.* (1982) tested the host range of *Pst* isolates from marigold and sunflower by injecting them into 32 species from eight plant families. Eight of the 10 Asteraceae plants tested exhibited strong apical chlorosis, while the remaining two Asteraceae species exhibited slight chlorosis. Three of the four Brassicaceae plants had one or two leaves displaying chlorosis. Tomato (Solanaceae) and cucumber (Cucurbitaceae) displayed very mild, ephermeral chlorosis. Plants of Chenopodiaceae, Fabaceae, Linaceae, Poaceae, and Polygonaceae were not affected by the treatment.

The pathotype strain of *Pst* is levan negative, oxidase positive, potato rot negative, arginine dehydrolase negative, and tobacco hypersensitivity positive, placing it in the LOPAT group Ib (Young and Fletcher 1997). Strains of *Pst* are generally unable to utilize sucrose as a carbon source (**Table 1**). For example, Laberge and Sackston (1986) reported that 11 out of 12 *Pst* isolates from Jerusalum artichoke that caused apical chlorosis were unable to utilize sucrose or l-lactate as a carbon source. Kůdela and Zacha (1998) reported that 12 out of 12 strains isolated from marigold growing in Moravia failed to utilize sucrose as a carbon source. However, eight *Pst* strains (which included the pathotype) tested by Young and Tiggs (1994) were reported to utilize sucrose (**Table 1**).

Based on the results of 36 nutritional and biochemical tests, Young and Tiggs (1994) used centroid linkage to generate similarity clusters in evaluating determinative tests for pathovars of *Pseudomonas syringae*. The analysis included 32 pathovars, with a total of 395 strains, seven of which were *Pst* (Young and Tiggs 1994). Most strains of 12 pathovars formed distinct clusters, one of which was composed of *P. syringae* pv. *heliathi* strains. *Pseudomonas syringae* pv. *tagetis* was one of eight *P. syringae* pathovars that did not form a distinguishable cluster.

In a comprehensive genetic analysis based on DNA/DNA hybridization and ribotyping (Gardan *et al.* 1999) that divided *P. syringae* into nine genomospecies, *Pst* was placed in genomospecies group 7, with the only other member of the group being *P. syringae* pv. *helianthi*. The *Pst* pathotype strain and *P. syringae* pv. *helianthi* also formed a group separate from other *P. syringae* pathovars based on 16S-23S intergenic DNA sequence analysis (Kong *et al*. 2005). Based on restriction fragment length polymorphism analysis, which included 18 *Pst* strains from seven hosts (Rhodehamel and Durbin 1989b) and 16S-23S intergenic DNA sequence analysis, which included 24 strains of *Pst* from 8 hosts (Kong *et al*. 2005), there is very little genetic difference between *Pst* strains.

A novel *P. syrinage* strain (CT99016C, henceforth referred to as CT99) that induces chlorotic symptoms identical to those induced by *Pst* was isolated from *C. arvense* growing in Canada (Zhang *et al.* 2002). The results of nutritional and biochemical tests were similar to those reported for *Pst* (see **Table 1**) except that CT99 was able to utilize trehalose as a carbon source (Zhang *et al.* 2004). Genetic fingerprints using amplified fragment length polymorphisms based on two genes required for tagetitoxin production (Kong *et al.* 2004) indicated that CT99 is genetically distinct from *Pst.* In a phylogenetic analysis based on multilocus sequence typing (MLST) utilizing four house-keeping genes and including 99 *P. syringae* pathovars, CT99 fell into a clade (I) mostly composed of *P. syringae* pv. *syringae* strains (**Fig. 1**). Strains of *Pst* fell into a separate clade (clade III) accompanied only by the pathotype of *P. syringae* pv. *helianthi*, which is consistent with the results of Gardan *et al.* (1999) and Kong *et al* (2005). Based on these results, CT99 does not appear to be a *Pst* strain. Whether there are differences in the biological activity of CT99 and 1-502a has not been determined.

Table 1 Nutritional and biochemical analysis of *Pseudomonas syringae* pv. *tagetis* strains. Y and N indicate that the carbon source did or did not, respectively, support growth or the characteristic was or was not, respectively, detected. A dash indicates that an observation for that carbon source or characteristic was not reported.

Carbon utilization	Trimboli *et al.* 1978† (10)	Styer *et al.* 1980‡ (9)	Young and Triggs 1994 (8)	Bowden and Percich 1983 (1)
Adonitol	N	N	N	-
β-Alanine	N	N	-	-
D-Arabinose	Y	Y	-	Y
Cellobiose	-	-	-	N
Citrate	-	-	-	Y
Ethanol	Y	N	-	-
Erythritol	N	N	6Y/2N	Y
D-Galctose	Y	Y	-	Y
Gluconate	Y	Y	Y	-
Glucose	Y	Y	-	Y
Glutarate	-	-	Y	-
Glycerate	-	-	Y	-
Glycerol	-	-	-	-
Fructose	-	-	-	Y
myo-Inositol	Y	Y	Y	Y
DL-β-hydrozybutyrate	-	Y	-	-
2-ketogluconate	N	N	-	-
Inulin	-	-	-	-
Lactate	N	N	N	N
D(+)Mannose	-	-	-	Y
Mannitol	Y	Y	Y	N
Quinate	Y	Y	Y	Y
Ribose	Y	Y	-	Y
Sorbitol	Y	Y	Y	N
Starch	N	N	-	-
Succinate	Y	Y	Y	Y
Sucrose	N	N	Y	N
Trehalose	N	N	-	N
Triacetin	N	Y	-	-
Trigonelline	-	-	Y	Y
L-Valine	N	N	-	-
D-Xylose	Y	Y	-	-
Other characteristics				
Arginine dihydrolase	N	N	-	-
Esculin hydrolysis	-	-	-	Y
Fluorescent pigment	9Y/1N	Y	-	-
Gelatin liquification	2Y/8N	N	3Y/5N	Y
Indole production	N	N	-	-
Oxidase	N	N	-	-
Tobacco hypersensitivity	Y	Y	-	Y

†Similar results were reported by Gulya *et al.* 1982, except the sunflower isolate was not able utilize ethanol as a carbon source.
‡Similar results were reported by Shane and Baumer 1984 except strains utilized betaine, citrate, and D(+)mannose and did not utilize cellobiose, inulin, L-homoserine, or fructose; and Styer *et al.* 1982.

TAGETITOXIN

A distinguishing feature of *Pst* is the development of apical tissue that is chlorotic or white (**Fig. 2**) in infected plants of the Asteraceae family. Trimboli *et al.* (1978) were the first to demonstrate that the chlorosis-inducing constituent ac-

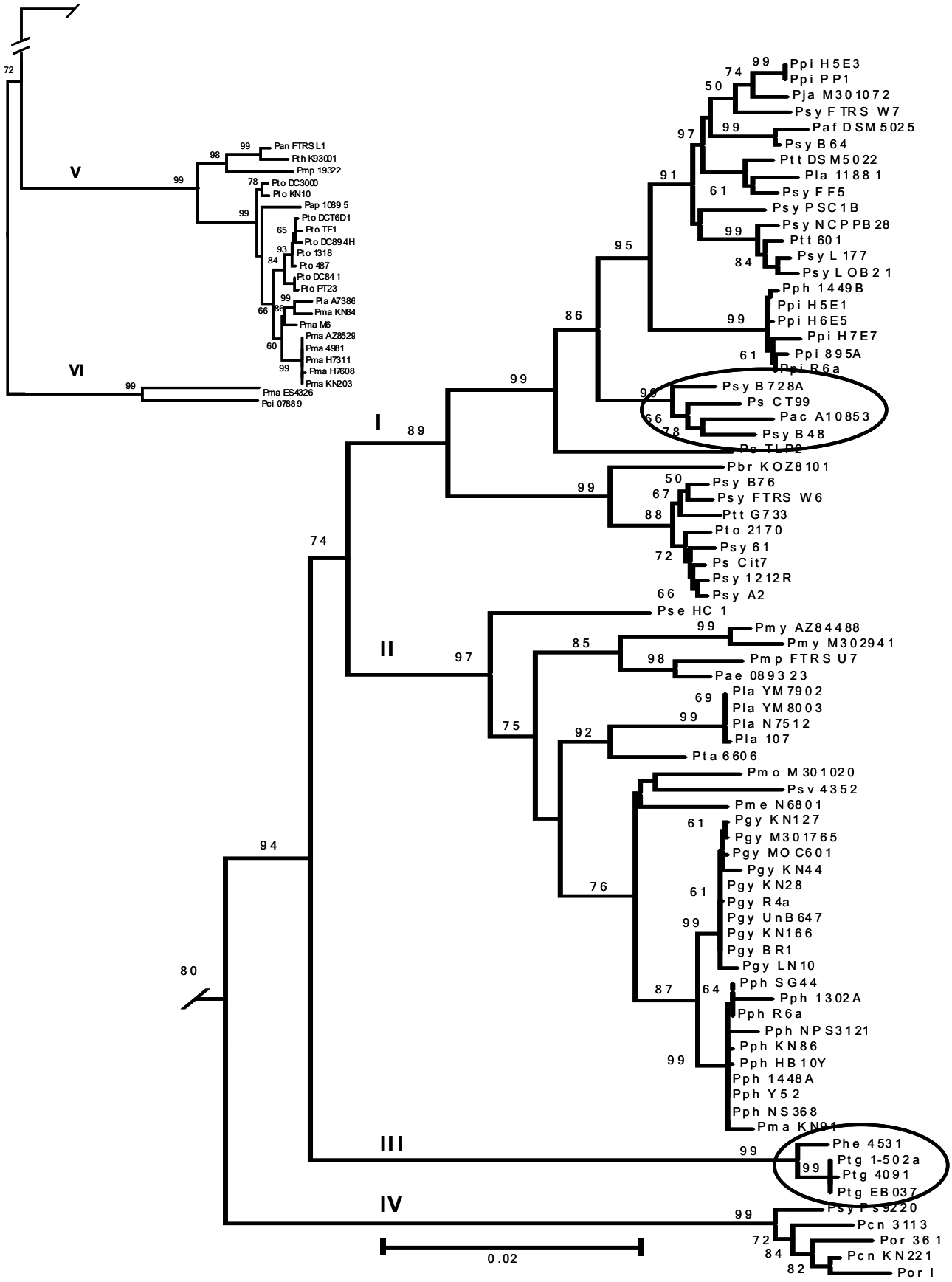

Fig. 1 Neighbor-joining tree of the concatenated data set consisting of sequence data from 94 *P.* syringae strains (Hwang *et al.* 2005), sequence data from *Pst* strains ICMP4091 (pathotype), 1-502a, and EB037, *P. syringae* pv. *helianthi* ICMP4531 (pathotype), and CT99. The four housekeeping genes sequenced were rpoD, encoding sigma factor 70; gyrB, encoding DNA gyrase B; gltA (also known as cts), encoding citrate synthase; and gapA, encoding glyceraldehyde-3-phosphate dehydrogenase were amplified from the *Pst* strains, *P. syringae* as previously described (Hwang *et al.* 2005). Sequences from each locus were aligned by using CLUSTAL W and were trimmed to their minimal shared length in GeneDoc. Neighbor-joining and maximum-likelihood phylogenetic analyses were performed on the individual and combined datasets by using MEGA version 4.1. Although not presented, the tree was rooted with orthologous sequences from *Pseudomonas fluorescens* Pf0-1 (U.S. Department of Energy, Joint Genome Institute. Analyses were performed as described by Sarkar and Guttman (2004). Bootstrap scores greater than 60 are given at each node. The section of the tree showing clades V and VI was separated from the remainder of the tree because of space limitations.

Fig. 2 Canada thistle plant inoculated with *Pst* displaying apical chlorosis. The plant was grown under growth chamber conditions and inoculated as described by Robinson *et al.* 2004.

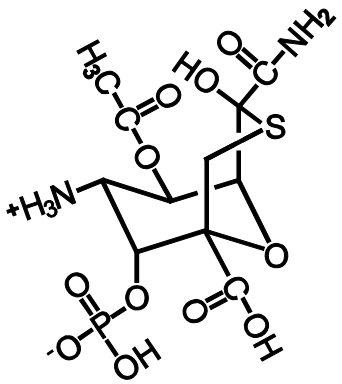

Fig. 3 The structure of tagetitoxin (Mitchell *et al.* 1989).

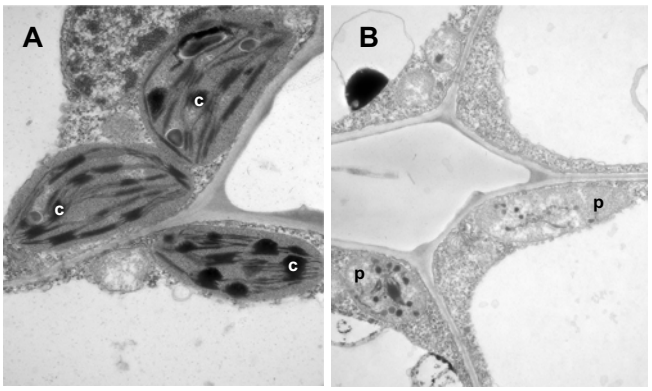

Fig. 4 Electron micrographs of sunflower leaf tissue from sunflower plants either uninoculated (A) or inoculated with *Pseudomonas syringae* pv. *tagetis* EB037 (B). Plants were grown and inoculated and electron micrographs were prepared as described by Robinson *et al.* 2004. c = chloroplast and p = plastid.

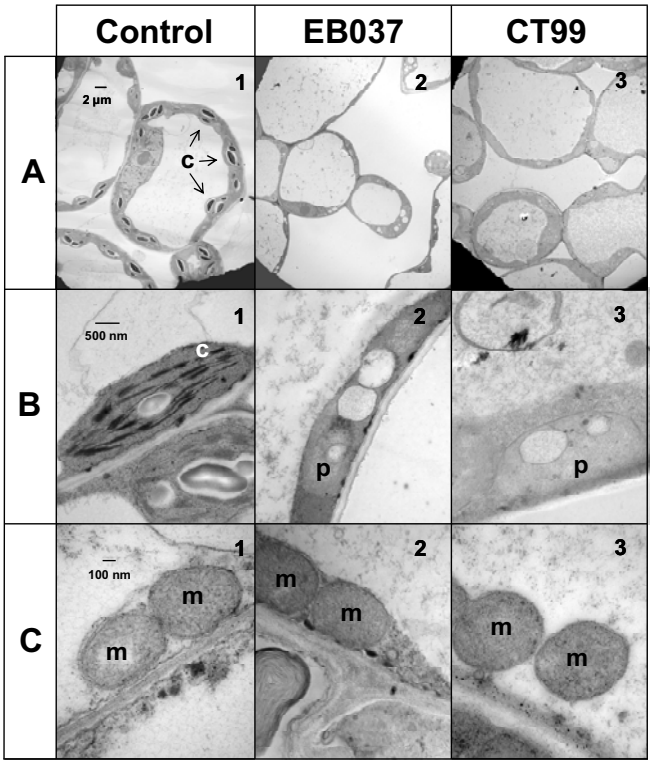

Fig. 5 Electron micrographs of sunflower leaf tissue from plants either uninoculated (Control) or inoculated with *Pseudomonas syringae* pv. *tagetis* (EB037) or bacterial strain CT99B016C (CT99). A. cells, B. chloroplasts and plastids, and C. mitochondria. The scale bars on the first image in a row represents the scale for all images in that row. Plants were grown and inoculated and electron micrographs were prepared as described by Kong *et al.* 2006. c = chloroplast, p = plastid, and m = mitochondria.

cumulated in cell free culture filtrates recovered from broth cultures of *Pst* grown in Woolley's medium (WM) (Woolley *et al.* 1952). The active constituent was later isolated from WM broth cultures of *Pst* strain DAR 26807 (an African marigold isolate) and named tagetitoxin (Mitchell and Durbin 1981). Tagetitoxin was determined to have a molecular weight of 416 and a proposed structure of a substituted 9-oxa-3-thiabicyclo[3.3.1]nonane (Mitchell and Hart 1983; Mitchell *et al.* 1989) (**Fig. 3**). Recently, a chlorosis-inducing toxin with the same molecular weight and mass spectral characteristics reported by Mitchell *et al.* (1989), was isolated from *Pst* strain EB037 (Kong *et al.* 2006). This strain was isolated from common ragweed (Kong *et al.* 2004), and so it was concluded that *Pst* strain EB037 produces tagetitoxin.

Initial studies on the mode of action of tagetitoxin demonstrated that the biogenesis of chloroplast development was disrupted in developing leaves of plants either treated with cell suspension of *Pst* or preparations of tagetitoxin (Jutte and Durbin 1979; Freeman *et al.* 1985; Lukens and Durbin 1985). It was later demonstrated that the tagetitoxin inhibits bacterial RNA polymerase (RNAP III), whereas nuclear RNAP I and II are much less affected (Steinberg *et al.* 1990; Mathews and Durbin 1990). Based on transcription assays, Mathews and Durbin (1994) proposed that tagetitoxin does not affect nucleotide substrate binding or phophodiester bond formation, but instead appears to affect the stability of binding of nascent oligonucleotides to the enzyme-template complex or translocation of the catalytic center. The inhibition was proposed to be related to a pausing process in the elongation step (Mathews and Durbin 1994). Recent studies indicate that tagetitoxin interferes with the binding of a Mg^{2+} ion required for catalysis, which leads to the stabilization of an inactive transcription intermediate (Vassylyev *et al.* 2005).

Normal mesophyll cells have an abundance of well developed chloroplasts containing organized thylakoid membranes and large starch granules (**Fig. 4A**). The genes required for the development of the photosynthetic apparatus reside in the chloroplast and are transcribed by a chloroplast RNA polymerase, commonly referred to as plastid-encoded RNA polymerase (Sakai *et al.* 2004). Tagetitoxin inhibits chloroplast RNA polymerase resulting in plastids that have no, or poorly developed, thylakoid membranes (Mitchell and Durbin 1981; Freeman *et al.* 1985; Lukens and Durbin 1985; Mathews and Durbin 1990) (**Fig. 4B**). The toxin, however, does not alter mitochondrial ultrastructure (Freeman *et al.* 1985; Lukens and Durbin 1985).

Sunflower leaf cells affected by tagetitoxin (**Fig. 5A 2**)

or the toxin produced by strain CT99 (**Fig. 5A 3**) had fewer chloroplasts than cells in control leaves (**Fig. 5A 1**). The plastids present in the leaf cells affected by tagetitoxin (**Fig. 5B 2**) or the toxin produced by CT99 (**Fig. 5B 3**) failed to develop properly, being devoid of organized thylakoid membranes or starch grains, but instead contained large vacuoles as compared to cells in control leaves (**Fig. 5B 1**). The mitochondria were unaffected by the treatments (**Fig. 5C**). These conditions, including the vacuoles which were previously described as vesiculated thylakoid membranes, were present in cells of sunflower leaves from plants either inoculated with *Pst* or treated with tagetitoxin (Freeman *et al.* 1985). Thus the effect of toxin produced by strain CT99 on leaf organelles is the same as that caused by tagetitoxin. Preliminary studies on the isolation of the toxin produced by CT99 showed that, like tagetitoxin, it was not retained on a C18 chromatography column but was retained on an anion exchange column (data not shown). Therefore, the biological activity and chemical characteristics of the toxin produced by CT99 are similar to those of tagetitoxin. A more definitive characterization of the CT99 toxin remains to be done.

A commercial preparation of tagetitoxin (Tagetin®, EPICENTRE® Biotechnologies, Madison, WI) (2010) is now a common inhibitor used in RNAP studies. Electron density maps of Tagetin (molecular weight 416) bound to RNAP verified the structure proposed by Mitchell *et al.* (1989) (Vassylyev *et al.* 2005). Nevertheless, Gronwald *et al.* (2005) reported that the chlorosis-inducing, RNAP-inhibiting compound from *Pst* EB037 has a molecular weight of 678. Furthermore, they reported a MS/MS ion of *m/z* 417 was obtained from the parent ion (*m/z* = 679) and a molecular ion (MH^+) of *m/z* = 679.5 from the only biologically active TLC spot obtained from Tagetin. However, no structure for this higher molecular weight compound was proposed. These results suggest that the phytotoxin produced by *Pst* may be a 678 molecular weight protoxin that during culture and/or isolation is readily converted to the active constituent, tagetitoxin. Other examples of protoxins that are metabolized or degraded to phytotoxins are hydantocidin, phaseolotoxin, and bialaphos, which upon degradation or metabolism release the phytotoxins PSorn and phosphinothricin, respectively (Bayer *et al.* 1972; Ogawa *et al.* 1973; Templeton *et al.* 1985; Heim *et al*. 1995).

The ability to produce tagetitoxin varies from strain to strain. For example, using an assay of chlorosis in zinnia seedlings (*Zinnia elegans* Jacq.) (Mitchell and Durbin 1981), 23 strains of *Pst* were evaluated for tagetitoxin production. Only five of 23 strains tested induced chlorosis when bacterial cells were injected into the plants as well as produced detectable levels of tagetitoxin *in vitro* (Rhodehamel and Durbin 1989b). The remaining 18 strains failed to produce detectable levels of tagetitoxin *in vitro*, although 15 of those did induce chlorosis when bacterial cells were injected into zinnia seedlings. Some chlorosis was produced from culture filtrates from four of these 15 strains when concentrated 25 fold (Rhodehamel and Durbin 1989b). The authors noted a correlation between the amount of toxin produced by a strain and its colony morphology. Colonies from WM that were mucoid produced up to 8x more toxin than colonies with normal morphology and retained the ability to produce high levels of tagetitoxin. Interestingly, the majority of colonies that lost the mucoid trait following several generations from serial cultures also lost the ability to produce tagetitoxin. Durbin *et al.* (1989) patented a protocol for the production of tagetitoxin that utilized a mucoid, high tagetitoxin-producing strain of *Pst* (C42mr2+, ATCC 53534), a derivative of *T. erecta* strain DARA26816 originating from Australia (Rhodehamel and Durbin 1989). This may be the protocol and strain used by EPICENTRE Biotechnologies in the production of Tagetin.

Gene clusters for the production of tabtoxin (Kinscherf *et al.* 1991), phaseolotoxin (Hatziloukas *et al.* 1995), coronatine (Alarcón-Chaidez *et al.* 1999), syringomycin and syringpeptin (Scholz-Schroeder *et al.* 2001) have been isolated from the strains of *P. syringae* that produce these phytotoxins and range in size from 32–80 kb. The gene cluster for tagetitoxin has yet to be isolated. Early studies (Mathews and Durbin 1990) indicated that tagetitoxin inhibits the RNAP of *Pst*. Kong *et al.* (2006) demonstrated that a gene that encodes for a protein that is part of a TonB membrane-bound transport system is required for tagetitoxin production. It appears that to prevent autotoxicity, internal cell levels of tagetitoxin must be tightly regulated through the combined action of the toxin efflux pump and a system of negative feedback inhibition. The only other gene that has been reported to be required for tagetitoxin production is a putative asparagine synthase (*asnB*) gene (Kong *et al.* 2004). Although yet to be determined, *asnB* may be involved in the synthesis of intermediates that lead to the incorporation of one or more of the nitrogen moieties present in tagetitoxin (**Fig. 3**).

USE IN BIOLOGICAL CONTROL

Interest in the use of *Pst* as a biological control agent of weeds developed after it was isolated from Canada thistle growing in Minnesota (Johnson and Wyse 1991, 1992). These early field trials by Johnson and Wyse demonstrated that *Pst* from Canada thistle was more effective at controlling annual weeds, such as common ragweed (*Ambrosia artemisifolia* (L.), horseweed (*Conyza canadensis*), prickley lettuce (*Lactuca serriola* L.), and common cocklebur (*Xanthium strumariaum* L.) than Canada thistle, whereas it did not injure common lambsquarters (*Cenopodium album* L.), redroot pigweed (*Amaranthus retroflexus* L.), quackgrass (*Elytrigia repens* (L.) Nevski), corn (*Zea mays* L.) or soybean (*Glycine max* L.). In instances where the treatment caused severe apical chlorosis but not death to Canada thistle, seed production was significantly reduced. However, where *Pst* was affective in impacting weed growth and/or seed production, multiple applications of the bacterial formulation were required (Johnson and Wyse 1991, 1992; Johnson *et al.* 1996). When Canada thistle was treated once and assessed five weeks after treatment, there was no significant difference in fresh or dry weight of Canada thistle plants treated with one application of *Pst* at 5×10^8 cfu ml^{-1} and Silwet L-77 (1,1,1,3,5,5,5,-heptamethyltrisiloxyanyl-proyl-methoxypoyl[ethylene oxide]) at 0.3% as compared to Silwet L-77 or water only treatments (Bailey *et al.* 2000a). The *Pst* with surfactant treatment did, however, result in an inhibition of flowering. Canada thistle fresh and dry weights were reduced approximately 50% with the same formulation of *Pst* when glyphosate at 0.125 L ha^{-1} was applied to the plants 1 day before treating with the bioherbicide (Bailey *et al.* 2000a). The latter study demonstrated the possible utility of *Pst* in an integrated crop management approach.

In a field study conducted over two years to assess the effects of *Pst* on Canada thistle in soybeans, Canada thistle plants treated with three applications of *Pst* (700 L ha^{-1} containing 10^9 cfu ml^{-1} and 0.2% Silwet L-77) were stunted and produced less seed than control plants (Hoeft *et al.* 2001). The effect of *Pst* on plant height was maintained up to when the plants were harvested at 70 days after the last treatment. Nevertheless, there was no significant difference in biomass or plant density of Canada thistle between the *Pst* treated and non-treated control plots in either year, nor were there treatment differences in soybean yield.

Several studies have demonstrated the need for a surfactant like Silwet L-77 for *Pst* to infect Canada thistle when applied as a foliar spray (Johnson *et al.* 1996; Bailey *et al.* 2000; Gronwald *et al.* 2002). Silwet L-77 facilitates the movement of bacteria into stomata and hydathodes by lowering surface tension of application droplets (Field and Bishop 1988; Zidack *et al.* 1992). In a study to determine the optimal concentration of Silwet L-77 to include in the application of *Pst*, Gronwald *et al.* (2002) demonstrated that, under growth chamber conditions, maximum internal populations of *Pst* in the leaves of Canada thistle 5 min. after treatment with *Pst* at 10^9 cfu ml^{-1} were achieved when the

formulation was brought to 0.3% Silwet L-77 (v/v) just prior to spraying the plants. Gronwald *et al.* (2002) also demonstrated that when Canada thistle leaves were sprayed with Silwet L-77 and *Pst* at 10^7-10^9 cfu ml^{-1} internal leaf populations of *Pst* reached levels of 10^8 cfu g fresh wt^{-1} within 2 days of treatment and maintained that density or greater up to 10 d after treatment. Similar population dynamics of *Pst* were observed in soybean leaves when treated at 10^8-10^9 cfu ml^{-1}.

In another field study of Canada thistle growing in soybean conducted over two years, plants were treated with 700 L ha^{-1} of a *Pst* formulation containing 10^9 cfu ml^{-1} at 0.3% Silwet L-77 in late June and again approximately 2 weeks later and the treatment effects on plant height, number of flower buds per plant, and plant survival assessed at 8 weeks after the initial treatment (which was at the time of flower bud formation) (Gronwald *et al.* 2002). The general conclusion from the study was that inundative foliar application of *Pst* as formulated on Canada thistle had relatively small effects on plant height, and what effects that were manifested were not altered with a second application. Treatment effects on plant survival were minimal as well. However what was significant, and consistent with previous studies (Johnson *et al.* 1996; Bailey *et al.* 2000; Hoeft *et al.* 2001), was the effect of *Pst* on the sexual reproductive capacity of Canada thistle plants as compared to controls. For example, a single Pst application with a backpack sprayer reduced the number of flower buds per plant by approximately 80% (Gronwald *et al.* 2002).

Sheikh *et al.* (2001) evaluated *Pst* for the control of wooleyleaf bursage (*Ambrosia grayi* (A. Nelson) Shinners), a perennial weed in the family Asteraceae. In their two-year field study, plants received 1,400 L ha^{-1} of *Pst* at 10^4-10^8 cfu ml^{-1} and Silwet L-77 at 0.25%, applied 4 to 13 times over 8 to 13 weeks. One result was that the level of disease that developed in wooleyleaf bursage treated with *Pst* was not significantly different across the concentrations of bacterium used. Also, weed densities were not significantly different between *Pst* treatments and the Silwet L-77 treatment alone. In assessing the effect of time of day on the efficacy of the application, results from treating plants once at various times of day with one application of *Pst* at 10^6 cfu ml^{-1} applied at 12:00 PM induced incidence of disease levels at 7 to 28 days after treatment were significantly greater than those induced in plants treated at any 3, 6, or 9 hr time period before or after 12:00 PM. The interpretation of these later results was that the optimum time of application reflected when the stomata of wooleyleaf bursage were most open, allowing the entry of the bacterium.

Studies have shown that the biological activity of biological control agents can be enhanced by herbicides or phytotoxins (Brooker *et al.* 1996; Bailey *et al.* 2000a, 2000b). Consequently, Gronwald *et al.* (2004) examined whether the biological activity of *Pst* could be enhanced with Nep1, a 24-kDa extra-cellular protein produced by *Fusarium oxysporum* f. sp. *erythoxyli* (Bailey *et al.* 1997), on three weeds known to harbor *Pst*, Canada thistle, common ragweed, and common dandelion. The use of Nep1 as a synergist for *Pst* seems counterintuitive, however, because to be effective, *Pst* must enter into healthy leaves in order to increase in population size and/or move from the point of entry to other portions of the plant. Nep1 causes rapid desiccation and leaf necrosis in dicotyledonous plants, a response that is similar to the hypersensitive reaction, a protective response induced in plants by invading plant pathogens that is thought to confine and reduce the spread of the invader. In fact, co-applications of Nep1 at 5 µg ml^{-1} (208 nM) + *Pst* at 10^9 cfu ml^{-1} + Silwet L-77 at 0.3% resulted in two orders of magnitude fewer cells per gm fresh leaf weight of *Pst* in Canada thistle leaves from 1 to 7 days after treatment as compared to the *Pst* + Silwet L-77 treatment (Gronwald *et al.* 2004). Interestingly, the shoot dry weight, level of leaf chlorosis, and total leaf area of Canada thistle at 2 weeks after treatment was the same for the *Pst* + Silwet L-77 and Nep 1 + *Pst* + Silwet L-77 treatments. This suggests that either the internal leaf populations of *Pst* dramatically increased in the Nep1 + *Pst* + Silwet L-77 treatment or dramatically decreased in the *Pst* + Silwet L-77 treatment shortly after the day 7 observation, or that the internal leaf populations of *Pst* can vary dramatically without having a big effect on the impact of the disease on the plant. Similar to Canada thistle, shoot dry weights for common ragweed and common dandelion were not significantly different between the *Pst* + Silwet L-77 and Nep1 + *Pst* + Silwet L-77 treatments. However, although not significantly different, there were trends towards lower shoot dry weight, lower leaf area, and greater chlorosis when Nep1 was added to the *Pst* formulations applied to common dandelion, suggesting that there may be some enhancement of *Pst* activity by Nep1 with this species.

While the above studies relied on laboratory cultures as a source of inoculum, others have examined the possibility of utilizing natural sources for inocula. To accomplish this, Tichich and Doll (2006) made homogenates of the upper portion of infected Canada thistle plants and used this, combined with 0.3% Silwet L-77, to infect non-diseased plots of Canada thistle. They used the homogenates to address questions about when in the growing season would be best to apply the homogenates and what application frequency, homogenate concentration, and application volume would be most effective. The success of the applications were assessed based on the percentage of chlorotic plants (disease incidence) and the severity to which the plants were chlorotic (disease severity) per plot. Using this approach, it was determined that disease incidence and severity in plots treated with 187 L ha^{-1} of a homogenate of 65 g fresh infected Canada thistle tissue L^{-1} and 0.3% Silwet L-77 was greatest for plants treated in mid-July and lowest for plants treated in mid-June. The authors attributed the seasonal differences to differences in rainfall following the application. Increasing the spray volume 4.5 × ha^{-1} or doubling the concentration of plant tissue in the homogenate had no significant affect on disease incidence or severity at 56 days after treatment. Inconsistencies in the data obtained in different years were attributed, as in the month of application study, to differences in rainfall following application during the years tested. No consideration was given to the possibility that microbial populations in the source material used to prepare the homogenates might differ by month or year. Also, the authors assumed that all chlorotic tissue contained equal numbers of *Pst* cells. However, chlorosis is an indication of the presence of tagetitoxin and not necessarily the bacterium. Furthermore, it was assumed that chlorotic tissue indicated that the plants were infected with Pst. It is possible, however, that the disease was not caused by a bacterium but by a fungus, as chlorotic symptoms in Canada thistle identical to that induced by *Pst* can be induced by *Phoma macrostoma*, a fungal pathogen previously isolated from leaves of Canada thistle (Zhou *et al.* 2004). What this study did demonstrate, however, was that homogenates of leaf/stem tissue from diseased Canada thistle plants can be used to transfer the disease to healthy plants when combined with Silwet L-77. This technique could be used to isolate strains of *Pst*, or other chlorosis-inducing pathogens, from Canada thistle, a process that has been reported by many to be very difficult. Such homogenates could be sprayed on seedlings of a sensitive annual of the Asteraceae, from which obtaining the causal agent may be more successful.

Conducted currently with the study described above was one in which the effects of the frequency of rain events on leaf populations of *Pst* following application of known concentrations of the bacterium were assessed (Tichich *et al.* 2006). In that study, sets of plots at each of two locations were sprayed 11 days apart. The basic treatment difference was the weather that followed the application. More rain events occurred during the11 days between treatment of the first and second set of plots as compared to after the second set of plots were treated. Based on this difference, the plots were termed “wet” and “dry”, respectively. Precipitation and age of plant were not the only differences between the

wet and dry plots (which the authors referred to as the wet and dry environments), as the dry plots received 10-fold higher concentrations of *Pst* than the wet plots. Nevertheless, differences in rainfall during the first 14 days after treatment did not significantly affect the total leaf population of *Pst*, which was significantly lower at that time compared to 24 h after application for all treatments. While the authors pointed out that results of their study provided evidence that rain events lead to overall *Pst* population increases composed largely of *Pst* cells internal to the leaf, that observation was based on one time period which differed from results obtained from leaf analyses following rain events at later dates. Consequently, how leaf populations of *Pst* internal and external to the leaf are affected by rain has not been fully clarified. An interesting observation from the study, however, was that Pst was recovered from leaves of plants in both sets of plots at both field sites up to 76 days after treatment.

Excluding the study of Tichich and Doll (2006) in which the causal agent was not described, all of the above studies on the biological activity of *Pst* were conducted with strain 1-502a, which was isolated from Canada thistle by Johnson and Wyse (1992). How representative the biological activity of 1-502a is with respect to the many other *Pst* strains previously described remains unknown.

With an interest in developing *Pst* 1-502a as a commercial product for the control of Canada thistle, Mycogen Corp. (Davis, CA) obtained a permit from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service in 1994 for the release of *Pst* 1-502a in small field trials in California, Idaho, Maryland, North Dakota, Ohio, and South Dakota. The results of any trials conducted by Mycogen with *Pst* are not readily available. The company went through several changes and is now essentially a seed company (Mycogen Seeds, as subsidiary of Dow AgroSciences). Encore Technologies (Minnetonka, MN) also had a program to develop *Pst* 1-502a as a biological control agent of weeds in the Asteraceae family. This company is no longer in existence.

SUMMARY AND ANALYSIS

The future of *Pst* as a biological control agent remains questionable. The failure of two companies to develop a strain of *Pst* as a biological control agent may not necessarily indicate that such an effort is not worthwhile, however. Mycogen and Encore Technologies may have limited their likelihood of success by staying with the one *Pst* strain that was isolated from Canada thistle. While the genetic analysis of *Pst* indicates that this pathovar is highly clonal, a comparison of the biological activity of the various *Pst* strains may reveal that there is a better biological control candidate than 1-502a. Furthermore, the data from efficacy studies suggests that a *Pst* biological control agent might be best developed for the control of annual instead of perennial weeds. Perennial plants, such as Canada thistle, have carbohydrate reserves that allow them to sustain the impact of *Pst*. In addition, reductions in flower production and seed set, two commonly observed symptoms related to *Pst*, could severely limit the impact of an annual weed in subsequent years. Consequently, annual weeds with little carbohydrate reserve and a dependence on sexual reproduction make more appropriate targets for a *Pst* biological control agent. Lastly, because of its effects on chloroplast development, tagetitoxin could be of value as a natural herbicide. However, more knowledge of the genes involved in the biosynthesis of the toxin and their manipulation, as well as the selection of high producing strains, would be needed in order to maximize toxin production.

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