ROMANIAN AGRICULTURAL RESEARCH, NO. 39, 2022
First Online: November, 2021. DII 2067-5720 RAR 2022-130

EXPERIMENTAL TREATMENT OF BIOPREPARATION BASED ON *Pseudomonas syringae* pv. TAGETIS FOR WEEDS CONTROL

Marga Grădilă[1*], Sorina Dinu[1], Daniel Jalobă[1], Valentin-Marius Ciontu[1], Szilárd Bartha[2]

[1]Research and Development Institute for Plant Protection Bucharest, 8 Ion Ionescu de la Brad Blvd, District 1, Bucharest, Romania

[2]Department of Forestry and Forest Engineering, University of Oradea, 26 Magheru Blvd, Oradea, Bihor County, Romania

[*]Corresponding author. E-mail: marga_gradila@icdpp.ro

ABSTRACT

***Cirsium arvense* is one of the worst weeds in agriculture. As actual herbicides are not very effective and not accepted by organic farming or in order to reduce the cantity of herbicides used, possible biocontrol agents have been investigated since many decades. The work carried out aimed at obtaining and assessing the herbicidal potential of bacterial preparation based on *Pseudomonas syringae* pv. tagetis (PST) for biological control of *Cirsium arvense* (L.) Scop. Silibase 2848P, an organosilicone surfactant, was required to facilitate PST penetration into creeping thistle leaves. The experiments were conducted under controlled conditions (growth chamber) to determine the concentration of Silibase 2848P required for the maximum penetration of PST into creeping thistle leaves. As well as to evaluate disease incidence in emerging leaves, and to quantify the effects of inundative foliar application on fresh and dry weights of shoots. In field conditions, efficacy of PST applications was assessed in terms of disease incidence and effect on shoot height, number of flower buds and survival of *C. arvense*. Maximum disease severity were recorded when PST was applied at a concentration of 10 x 10^8 cfu/ml + Silibase 0.3%.**

Keywords: *Pseudomonas syringae*, bioherbicide, organosilicone surfactant, tagetitoxin, creeping thistle.

INTRODUCTION

Within world science and agricultural practices, creeping thistle [*Cirsium arvense* (L.) Scop] is considered a true phenomenon because of its biology, vitality and ecological adaptability. It is one of 2.000 species of weeds that cause annually damage worldwide. At the same time, it is one of the 708 segetal species existing in all agricultural crops in Romania. Its extensive root system produces adventitious root buds that give rise to new shoots in the spring and summer (Donald, 1994; Moore, 1975; Tiley, 2010). *C. arvense* causes yield losses ranking from 15 to 60%, depending on weed density. In cereal crops, densities of 6 to 20 creeping thistle plants per square metre result in 18 to 30% loss in grain yield and can cause annual yield losses of $320 millions. This weed is difficult to be controlled because the root system lies well below the depth reached by tillage and herbicide applications are difficult to be timed as new shoots are continually emerging. Biological control agents that attack either the root system or can be translocated throughout the plant may provide an alternate way of weed control. It may also be possible to enhance biological control of the weed through synergy with existing chemical herbicides (Christy et al., 1994).

P. syringae pv. tagetis (PST) causes leaf spot and apical chlorosis in various *Asteraceae*, including the weeds *C. arvense* and common ragweed *Ambrosia artemisiifolia* L. (Gronwald et al., 2002; Gulya et al., 1982; Johnson and Wyse, 1991, 1992; Rhodehamel and Durbin, 1985; Styer and Durbin, 1982). Apical chlorosis elicited by PST is caused by tagetitoxin, a non-host-specific toxin (Durbin, 1990; Lukens and Durbin, 1985). Tagetitoxin is translocated to emerging leaves, where it inhibits plastidic RNA polymerase III, which in turn prevents chloroplast biogenesis (Mathews and Durbin, 1990, 1994; Steinberg et al., 1990).

Received 25 October 2021; accepted 15 November 2021.

On the basis of scientific data and because of highly infested crops by *C. arvense* in Romania, we studied some biological methods of weed control.

Previous research indicated that PST might have potential as a biological agent for controlling *C. arvese* in soybean. According to studies, multiple inundative foliar applications of Pst (10^8 to 10^9 colony-forming units [cfu]/ml) and adding of the organosilicone surfactant Silwet L-77 (0.05 to 0.2%, v/v) elicited disease incidence (apical chlorosis) and reduced creeping thistle survival, height, and seed production (Johnson and Wyse, 1991, 1992). Single inundative foliar applications of PST appear to be less effective.

So far in Romania only the chemical control is practiced within the integrated weeds management of *C. arvense*, besides other ordinary agrotechnics.

This study reports results on the evaluation of the bacterial agent, *Pseudomonas syringae* pv. tagetis (PST) for biological control of *Cirsium arvense* weed.

MATERIAL AND METHODS

The experiments were carried out in the Biological Testing Laboratory of Research-Development Institute for Plant Protection Bucharest, in Romania, during 2018-2020.

Growth chamber experiments - PST culture

To obtain a rich bacterial biomass, a strain of *P. syringae* pv. tagetis, of the Belgian collection of micro-organisms (LMG), conditioned as lyophilized, stored at +4°C, was brought to a viable state by aseptical prelevation of small amounts of lyophilisate in a stream of sterile air.

Then, they were added in 10 ml of King B broth and distributed in 50 ml Erlenmeyer flasks. The Erlenmeyer flasks were incubated by horizontal shaking (150 rpm) at 28°C until high cell density was obvious. A 5 ml aliquot of each microbial culture flask was used as a starter inocula for the inoculation of 50 ml of sterile KB growth medium, distributed in 250 ml Erlenmeyer flasks. To determine the titer of the microbial suspension, the bacterial culture was centrifuged for 20 minutes at 3750 rpm, followed by removal of the supernatant and the resulting cell pellet was resuspended in sterile distilled water. For mass multiplication of the bacterial strain, 3 liquid culture medium were tested (Table 1).

The PST bacterial strain was refreshed on Luria Bertani culture medium agarized, by the loop depletion technique, to obtain isolated colonies.

Table 1. The growth medium used in the experiment

Variant	Composition (g/L)
King B (KB)	20 g proteose-peptone; 10 g glycerin; 1.5 g K_2HPO_4; 1.5 g $MgSO_47H_2O$; 1000 ml distilled water.
Luria Bertani (LB)	10 g bactotriptone; 5 g yeast extract; 10 g Na Cl; 1000 ml distilled water.
Nutrient-Broth (NB)	5 g peptone; 3 g meat extract; 1000 ml distilled water.

The inoculated Petri dishes were incubated at 28°C for 24 hours. The bacterial growth obtained on the solid medium was washed with sterile distilled water for inoculation with 10% of 50 ml of each liquid culture mediums (KB, LB, NB) previously distributed in 250 ml Erlenmayer flasks. The flasks were incubated in a thermostated environment (28°C) with horizontal stirring (150 rpm) for 48 hours, making 3 flasks/growth medium, corresponding to the 3 replications. Subsequently, the liquid cultures for each experimental sample were stored in 50 ml Falcon tubes and centrifuged for 20 minutes at 3750 rpm. The bacterial inoculum obtained in laboratory conditions, having a concentration of 10^8 cfu/ml (colony forming unit/ml) determined spectrophotometrically, and this was used as a biological control agent in laboratory and field experiments.

For all experiments, except those to determine optimum surfactant concentration, Silibase 2848P was added to PST spray suspensions at 0.3%.

Experiments under controlled conditions

Growth chamber experiments were conducted to (1) determine the concentration of Silibase 2848P required for the maximum penetration of PST into creeping thistle leaves, to (2) evaluate disease incidence (apical chlorosis) in emerging leaves of creeping thistle depending on PST concentration applied, and to (3) quantify the effects of inundative foliar application on fresh and dry weights.

Plant material

Cirsium arvense plants (2-3 cm height) harvested from non-herbicide maize crop, were transplanted into pots and kept for 15 days for acclimatization under identical conditions of temperature and humidity. Creeping thistle were grown in a growth chamber at 25/20°C with 16-h photoperiod. PST was applied on creeping thistle in the eighth-to ninth-leaf stage (approximately 10-12 cm height), which occured 2 to 3 weeks after emergence. Plants were watered daily with 0.13% Hoagland's solution (Hoagland and Arnon, 1950). During this period, creeping thistle plants were carefully monitored to identify any symptoms that could have influenced the results (wilting, stunting, chlorosis, yellowing, browning, discoloration, insect attack, etc.). Plants showing these symptoms were removed.

PST application

The treatments with experimental bacterial preparation was applied to creeping thistle at a pressure of 242 kPa in a ventilated fume hood using a handheld paint sprayer. Four plant pots were placed in a tray (25 by 52 cm), and two sprayings of approximately 3 seconds each were made with the sprayer positioned approximately 25 cm above the tray at a 458 angle. The tray was turned 180° between sprayings. After spraying, the treated plants were immediately placed back in the growth chamber. Aproximatively fifteen-days old creeping thistle plants (10-12 cm height) were inoculated with PST at 3 concentration (1×10^8, 5×10^8 and 10×10^8 cfu/ml) with 4 different treatments (PST, PST + Silibase (10^8 cfu/ml + 0.3%), Silibase and only water.

Efficacy of PST on disease incidence in leaves

For creeping thistle, two mature leaves (fifth or sixth leaf of plants in the eighth - or ninth-leaf stage at the time of treatment) were evaluated at 3 and 5 weeks after PST application. Disease severity were estimated visual as follows: 0 = healthy; 1 = stunting with no chlorosis; 2 = detectable chlorosis; 3 = moderate chlorosis; 4 = severe chlorosis with some necrosis; 5 = severe chlorosis and necrosis. The percentage of two leaf infection of each plant was estimated using the following scale: 0 - no disease; between 0-0.2 (20% of leaf area affected), between 0.2-0.4 (40% of leaf area affected), between 0.4-0.6 (60% of leaf area affected), between 0.6-0.8 (80% of leaf area affected) and between 0.8-1.0 (100% of leaf area affected).

Effect of PST on fresh and dry weights

The effects of foliar application of 1, 5 and 10×10^8 cfu/ml PST+Silibase (0.3%) on fresh and dry weights of creeping thistle plants were examined at 5 weeks after application of PST. Shoots were harvested 5 weeks after treatment, and dry weights were obtained after drying plant material in oven for 72 h at 95°C.

Experiments under field trials

Experiments to evaluate the effects of inundative PST applications on controlling *C. arvense* growing within the rows of maize were conducted on Didactic Farm Moara Domnească, Ilfov County. Based on the results of the growth chamber experiments described earlier, field trials were conducted using foliar applications of 1×10^8 cfu/ml PST + Silibase 0.3%. Apical chlorosis of treated plants was used to evaluate disease incidence. The trial field soil is brown reddish with a clay loam texture and good fertility that led to a high natural infestation by *C. arvense*. The planting density of maize crop was 60.000 plants per hectare. DKC

4670 was the variety sown in the experimental field. The previous crop was wheat. Sowing was performed on 12th April 2019 and on 15th April 2020, with planting rate of 20 kg/ha. The following agro-technical measures have been applied: systematic crop rotation, the adequate previous crop, deep plowing up to 30 cm depth in summer, seedbed tillage by 2 passes with disc harrow followed by milling and high quality hybrids. Weeds were controlled between rows by two mechanical cultivations. In each plot ten *C. arvense* plants within the maize rows were randomly selected and marked with a plastic tag around the base of the stem. Treatments were applied with backpack sprayer at 276 kPa. Two treatments were applied at an interval of 14 days. The first PST application was made when *C. arvense* plants were approximately 10 cm height. In 2019 the first PST application was made on May 15th and the second was made on May 30th. For both dates, air temperature at the time of application was approximately 25°C. In 2020 the first PST application was made on May 20th and the second was made on June 4th. Air temperatures at the time of the first and second PST applications were approximately 24 and 26°C, respectively. For the selected plants in each plot, disease incidence (measured as % plants exhibiting chlorosis) was recorded twice at 4 and 8 weeks after initial treatment. Plant height, number of flower buds per plant, and plant survival measurements were assessed.

RESULTS AND DISCUSSION

Obtaining PST biomass in the Growth chamber experiments

The experiment conducted in order to establish the growth medium that determines the highest accumulations of bacterial biomass led to the selection of King B (KB) medium, as on this medium was obtained the highest amount of biomass after 48 h from inoculation (Table 2). The results highlighted a biomass accumulation that ranged between 0.0028 and 0.0058 g/ml on NB and LB media, while the KB media provided the highest biomass growth for the analyzed bacteria, respectively 0.019 g/ml culture.

Table 2. Bacterial biomass determination at 48 hours

Experimental sample	Bacterial biomass (g/l)
Pseudomonas syringae (PST)/KB	19.00
Pseudomonas syringae (PST)/LB	5.80
Pseudomonas syringae (PST)/NB	2.80

Efficacy of PST on disease incidence in growth chamber experiments

The effect of PST concentration on disease incidence in *Cirsium arvense* was determined by estimation the disease severity (Figure 1).

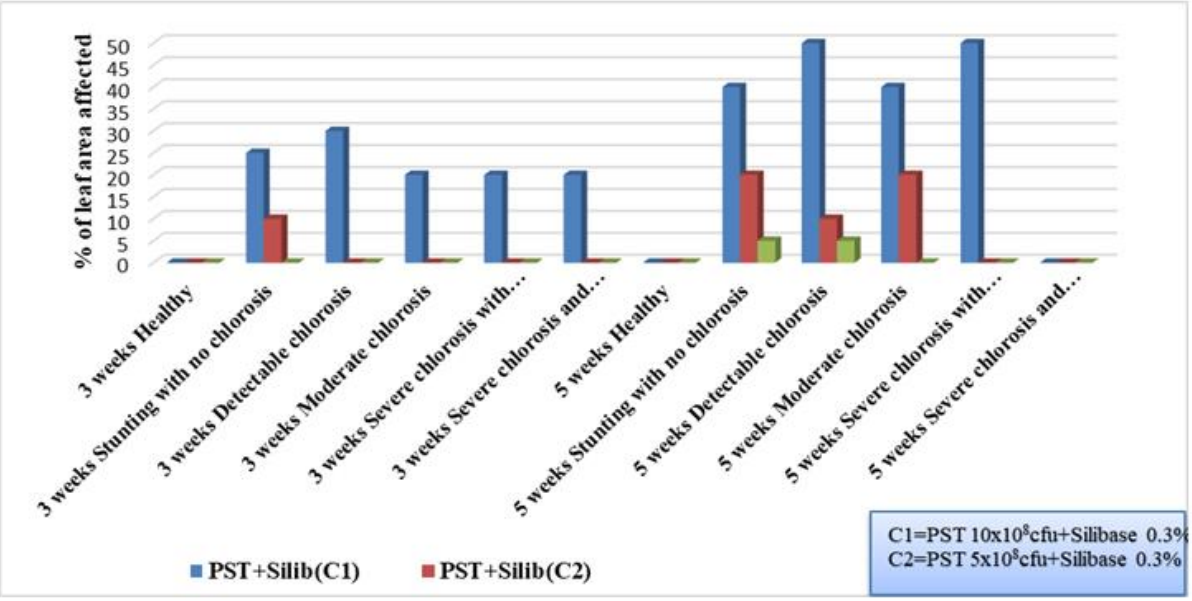

Figure 1. Efficacy of PST. Disease severity on *C. arvense*

Maximum disease severity were recorded when PST was applied at a concentration of 10 x 10^8 cfu/ml + Silibase 0.3%. Without the addition of Silibase to the application medium, very low disease incidence were found in leaves. Previous studies demonstrated the necessity of adding of an organosilicone surfactant to the application suspension to facilitate the entry of bacteria into leaves (Zidack and Backman, 1996; Zidack et al., 1992). Our results are similar in the previous report where a organosilicone surfactant concentration of 0.2% (v/v) or greater was required for maximum penetration of *P. syringae* pv. phaseolicola into bean (*Phaseolus vulgaris* L.) leaves (Zidack et al., 1992). Thus, the application of 5 x 10^8 cfu/ml + Silibase 0.3% determined symptoms of chlorosis in a proportion of only 10% at 5 weeks after treatment. The concentration of 10 x 10^8 cfu/ml + Silibase 0.3% PST determined severe clorosis with necrosis in proportion of 50% of leaf area affected.

Efficacy of PST on fresh and dry weights in growth chamber experiments

The effects of foliar application of 1, 5 and 10 x 10^8 cfu/ml + Silibase (0.3%) on fresh and dry weights of creeping thistle plants were examined (Figure 2). Plants sprayed with PST + Silibase showed low fresh weight compared to PST and water, while some phytotoxicity was observed on creeping thistle with application of Silibase alone (Figure 2). No significant differences in dry weight amongst treatments were observed. The best results on fresh and dry weights of creeping thistle plants were obtained when the PST were applied at 10 x 10^8 ufc/ml + Silibase 0.3%. Silibase (0.3%) applied alone caused small water-soaked spots on leaves that became necrotic after 24 h. However, this injury was relatively minor and did not have a significant effect on shoot dry weight. None of the creeping thistle plants treated with PST (10^8 cfu/ml) plus Silibase (0.3%) died.

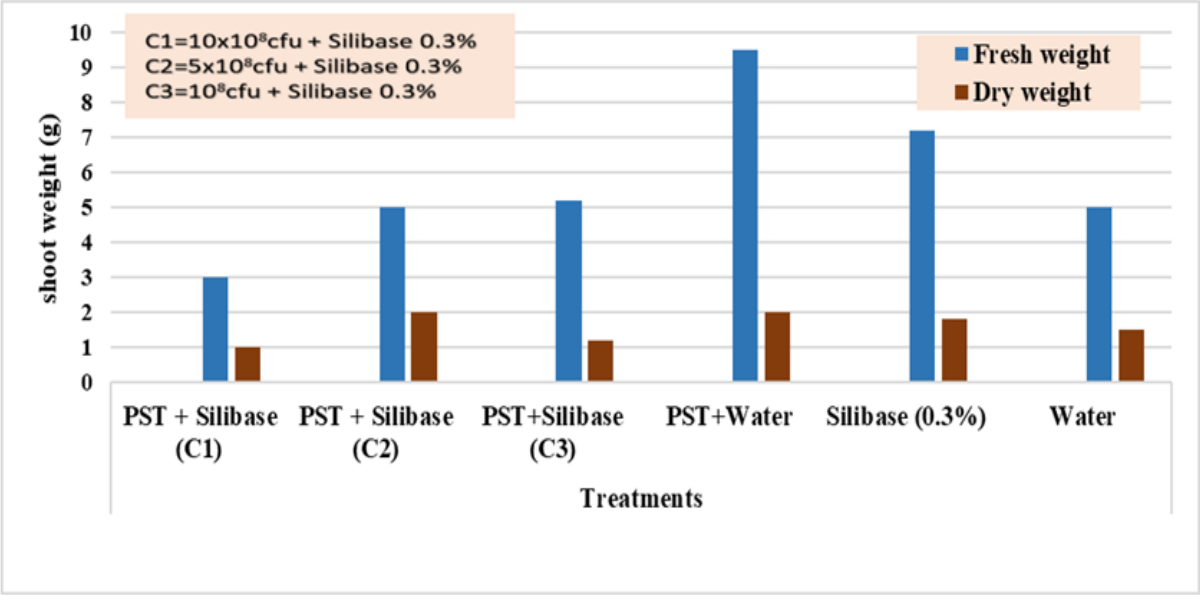

Figure 2. Efficacy of PST. Fresh and dry weights of *C. arvense* plants

Field Trials

For both 2019 and 2020, disease incidence was greater at 4 weeks after treatments than at 8 weeks (Table 3). In 2019 the highest levels of disease incidence measured at 4 weeks were obtained with two applications: 90% when the PST were applied at 10 x 10^8 ufc/ml + Silibase 0.3% and 42% of treated plants with PST 5 x 10^8 ufc/ml + Silibase 0.3%. In 2020 one or two applications with a backpack sprayer resulted in maximum disease incidence at 4 weeks with 64 and 40% of the treated plants exhibiting chlorosis, respectively. For both 2019 and 2020, a

second PST application resulted in no significant increase in disease incidence measured 4 weeks, with one exception. The exception occurred in 2019 where a second PST application approximately doubled the number of plants exhibiting chlorosis. When disease incidence was measured at the time of flower bud formation (8 weeks), few, if any, plants exhibited chlorosis for any treatments (Table 3). In most cases the chlorotic leaf tissue observed at 4 weeks has re-greened, and new growth did not exhibit chlorosis. For both years, the mean height of nontreated thistle at the time of flower bud formation was approximately 60 cm (Table 3). Two applications of Silibase 0.3% caused minor leaf injury but did not have a significant effect on shoot height. Generally, inundative foliar application of PST (10^8 cfu/ml) + Silibase (0.3%) had relatively small effects or no effect on plant height. In 2020, two applications of PST applied at 10 x 10^8 ufc/ml + Silibase 0.3% reduced plant height by approximately 33%. Other treatments did not cause a significant reduction in plant height. In 2019 none of the treatments caused a significant reduction in plant height compared to the untreated control. For both years, a second PST application did not result in a significant reduction in plant height in contrast to that caused by a single application.

For both 2019 and 2020, nontreated creeping thistle plants exhibited an average of 18 flower buds/plant (Table 3).

Table 3. Efficacy of PST applications on disease incidence (chlorosis), shoot height, number of flower buds, and survival

Effects of foliar application of PST + Silibase (0.3%)	Nr. appl.	Treatments															
		PST + Silibase 10x10^8 ufc/ml+0.3%				PST + Silibase 5x10^8 ufc/ml+0.3%				Silibase 0.3%				Water			
		2019		2020		2019		2020		2019		2020		2019		2020	
		Weeks															
		4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8
% Plants exhibiting chlorosis	one	62	6	55	7	35	3	46	3	0	0	0	0	0	0	0	0
	two	90	8	64	5	42	4	40	3	0	0	0	0	0	0	0	0
Shoot height (cm)	one	-	48	-	38	-	55	-	58	-	64	-	63	-	58	-	60
	two		50	-	20	-	56	-	52	-	66	-	70	-	65	-	68
Number of flower buds/plant	one	-	4	-	5	-	8	-	10	-	16	-	18	-	18	-	18
	two		5	-	3	-	6	-	8	-	15	-	16	-	15	-	16
Percentage survival of shoots	one	-	38	-	40	-	75	-	70	-	88	-	88	-	90	-	86
	two	-	25	-	45	-	66	-	66	-	92	-	90	-	95	-	94

Two applications of Silibase (0.3%) had no effect on the number of flower buds. In 2019 and 2020 all treatments caused a significant reduction in the number of flower buds. For both 1999 and 2000, a second PST application resulted in no additional reduction in the number of flower buds. For both 2019 and 2020, approximately 10% of the untreated creeping thistle plants did not survive until the time of flower bud formation (Table 3).

Applying Silibase (0.3%) without PST had no effect on *Cirsium arvense* shoot survival. Except of two applications in 2019, which reduced survival by an additional 25% compared with the untreated control, none of the PST treatments caused a significant reduction in survival.

CONCLUSIONS

The obtained results showed a biomass accumulation that ranged between 0.0028 and 0.0058 g/ml, on NB and LB culture medium, while the KB medium ensured the highest biomass increase of *P. syringae* bacterium, 0.019 g/ml culture, respectively.

The bacterial inoculum obtained in laboratory conditions, having a concentration of 10^8 cfu/ml determined spectrophotometrically, was used as a biological control agent in laboratory and field experiments.

Application of PST with the surfactant, Silibase (0.3%) is required to facilitate entry of the PST into the stomata of the plants. Disease symptoms and fresh and dry weight reductions were most pronounced when a PST were applied at 10 x 10^8 ufc/ml + Silibase 0.3%.

In field trials, in 2019 the highest levels of disease incidence measured 4 weeks were obtained with two applications: 90% when the PST were applied at 10 x 10^8 ufc/ml + Silibase 0.3% and 42% of treated plants with PST 5 x 10^8 ufc/ml + Silibase 0.3%. In 2020 one or two applications with a backpack sprayer resulted in maximum disease incidence at 4 weeks with 64 and 40% of the treated plants exhibiting chlorosis, respectively.

This results open new opportunities for use bacterial agents for biological control of *Cirsium arvense* weed.

REFERENCES

Christy, A.L., Herbst, K.A., Kostka, S.J., Mullen, J.P., Carlson, P.S., 1994. *Synergizing weed biocontrol agents with chemical herbicides*. Pest Control with Enhanced Environmental Safety, 524: 87-100.

Donald, W.W., 1994. *The biology of Canada thistle (Cirsium arvense)*. Rev. Weed Sci., 6: 77-101.

Durbin, R.D., 1990. *Biochemistry of non-host-selective phytotoxins*. In Hoagland, R.E. (eds.) Microbes and Microbial Products as Herbicides. ACS Symposium 439, Washington DC, American Chemical Society: 63-71.

Gronwald, J.W., Plaisance, K.L., Ide, D.A., Wyse, D.L., 2002. *Assessment of Pseudomonas syringae pv. tagetis as a biocontrol agent for Canada thistle*. Weed Sci., 50: 397-404.

Gulya, T.J., Urs, R., Banttari, E.E., 1982. *Apical chlorosis of sunflower caused by Pseudomonas syringae pv. tagetis*. Plant Dis., 66: 598-600.

Hoagland, D.R., and Arnon, D.I., 1950. *The water-culture method for growing plants without soil*. University of California Agricultural Experiment Station Circ., 347: 32.

Johnson, D.R., and Wyse, D.L., 1991. *Use of Pseudomonas syringae pv. tagetis for control of Canada thistle (Cirsium arvense)*. Proc. N. Cent. Weed Sci. Soc., 46: 14.

Johnson, D.R., and Wyse, D.L., 1992. *Biological control of weeds with Pseudomonas syringae pv. tagetis*. Proc. N. Cent. Weed Sci. Soc., 47: 16.

Lukens, J.H., and Durbin, R.D., 1985. *Tagetitoxin affects plastid development in seedling leaves of wheat*. Planta, 165: 311-321.

Mathews, D.E., and Durbin, R.D., 1990. *Tagetitoxin inhibits RNA synthesis directed by RNA polymerases from chloroplasts and Escherichia coli*. J. Biol. Chem., 265: 493-498.

Mathews, D.E., and Durbin, R.D., 1994. *Mechanistic aspects of tagetitoxin inhibition of RNA polymerase from Escherichia coli*. Biochemistry, 33: 11987-11992.

Moore, R.J., 1975. *The biology of Canadian weeds: Cirsium arvense (L.) Scop*. Can. J. Plant Sci., 55: 1033-1048.

Rhodehamel, N.H., and Durbin, R.D., 1985. *Host range of strains of Pseudomonas syringae pv. tagetis*. Plant Dis., 69: 589-591.

Steinberg, T.H., Mathews, D.E., Durbin, R.D., Burgess, R.R., 1990. *Tagetitoxin: a new inhibitor of eukaryotic transcription by RNA polymerase III*. J. Biol. Chem., 265: 499-505.

Styer, D.J., and Durbin, R.D., 1982. *Common ragweed: a new host of Pseudomonas syringae pv. tagetis*. Plant Dis., 66: 71.

Tiley, G.E.D., 2010. *Biological flora of the British Isles: Cirsium arvense (L.) Scop*. J. Ecol., 98: 938-983.

Zidack, N.K., and Backman, P.A., 1996. *Biological control of kudzu (Pueraria lobata) with the plant pathogen Pseudomonas syringae pv. phaseolicola*. Weed Sci., 44: 645-649.

Zidack, N.K., Backman, P.A., Shaw, J.J., 1992. *Promotion of bacterial infection of leaves by an organosilicone surfactant: implications for biological weed control*. Biol. Control., 2: 111-117.