

Effect of the pre-emergence bioherbicide *Phomopsis convolvulus* on seedling and established plant growth of *Convolvulus arvensis*

S. VOGELGSANG, A. K. WATSON,
A. DITOMMASO AND K. HURLE*

Department of Plant Science, Faculty of
Agricultural and Environmental Sciences,
McGill University, 21 111 Lakeshore Road,
Ste-Anne-de-Bellevue, Québec, Canada H9X
3V9, and *Institut für Phytomedizin, Universität
Hohenheim, D-70593 Stuttgart, Germany

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Summary

The effects of the fungal pathogen *Phomopsis convolvulus* Ormeno on seedling and established plant performance of *Convolvulus arvensis* L. were compared under both controlled and field conditions. Under a controlled environment, a granular barley formulation of the fungal inoculum that had been applied on to the soil surface of pots containing pre-germinated *C. arvensis* seeds resulted in above-ground biomass reductions of up to 87%. However, application of the fungus to established plants that had been cut to ground level produced biomass reductions (43%) that were nearly half of those obtained for seedlings. In a parallel field experiment conducted over two growing seasons, application of *P. convolvulus* resulted in dramatic above-ground biomass reductions for both seedlings and established plants. In one trial, biomass reductions of up to 100% and 98%, respectively, were obtained. *C. arvensis* coverage within field plots was closely correlated with above-ground biomass. Findings in this study indicated that *P. convolvulus* may provide effective control of *C. arvensis* when applied pre-emergence.

Introduction

Convolvulus arvensis L. (field bindweed) is a serious perennial weed in many crops and is prevalent in almost every agricultural area of the world (Holm *et al.*, 1977). Effective control of this weed using current methods, such as cultivation, crop rotation and chemical herbicides (Derscheid *et al.*, 1970), is often not possible because of its extensive root system, high competitiveness and variable susceptibility to several important herbicides (Whitworth & Muzik, 1967; DeGennaro & Weller, 1984; Kosinski & Weller, 1989; Yerkes & Weller, 1996). In addition, the move towards reduced cultivation or zero tillage has led to an increased prevalence of *C. arvensis* (Phillips *et al.*, 1980). The first reported incidence of *C. arvensis* being infected by the foliar pathogen *Phomopsis convolvulus* Ormeno was in 1988 (Ormeno-Núñez *et al.*, 1988a). Since then, studies on host specificity, conidia mass production, storage and efficacy of foliar treatments have been carried out (Ormeno-Núñez *et al.*, 1988b; Morin *et al.*, 1989a,b, 1990). One key characteristic of the fungus that has limited its efficacy is the requirement for a long dew period during the germination and infection phases for sufficient disease to develop. In an attempt to overcome this limitation, a granular pre-emergence application of *P. convolvulus* has been tested. Inoculum produced on pot barley grains and applied on to the soil surface showed high efficacy of control against *C. arvensis* seedlings under both controlled environment and field conditions (Vogelgsang *et al.*, 1994). However, a major obstacle for effective control of *C. arvensis* is the ability of this vigorous weed to regenerate vegetatively from established plants (Swan & Chancellor, 1976). This is especially important given that, within most cropping sys-

tems, the largest proportion of *C. arvensis* biomass develops from existing intact or fragmented roots and not from seedlings (Weaver & Riley, 1982). Hence, the objective of this study was to evaluate the pre-emergence activity of *P. convolvulus* on seedling and established plant growth of *C. arvensis* under both controlled environment and field conditions.

Materials and methods

Inoculum production of starter cultures

Single conidia isolates of *P. convolvulus* were maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 4 °C. From these stock cultures, small pieces of mycelia were placed on 9-cm-diameter Petri dishes with PDA and incubated in the dark at 24 ± 1 °C. After 4–5 days, several 1-cm-diameter mycelial plugs were transferred to PDA plates and incubated at room temperature (21 ± 2 °C) and 12 h day⁻¹ near-ultraviolet light (F40 BLAB Blacklight, General Electric Lighting, Cleveland, OH, USA). After 3 weeks, conidia were harvested by washing plates with 12 mL of sterile deionized water. Conidia density was adjusted to 1 × 10⁷ conidia mL⁻¹ with the aid of a haemocytometer.

Preparation of granular barley inoculum

For controlled environment experiments, 20 mL of deionized water was added to 20 g of pot barley grains (*Hordeum vulgare* L.) in 250-mL Erlenmeyer flasks and autoclaved (18 min, 100 kPa, 120 °C). Flasks were cooled to room temperature and inoculated with 1 × 10⁷ conidia (1 mL of starter culture). Flasks were incubated at room temperature (21 ± 2 °C) and exposed to 12 h of near-ultraviolet light day⁻¹ and shaken every second day by hand in order to minimize the likelihood of the substrate clumping. Colonized barley grains were harvested after 3 weeks and milled using an electric coffee grinder (Braun KSM 2, Lynnfield, MA, USA) by milling each load continuously for 10–15 s. The granules produced were dried for 2 days and sieved, resulting in inoculum particles of < 710 µm diameter. In an earlier study, inoculum of this size was found to have a high pre-emergence activity and a shelf-life of at least 6 months (Vogelgsang *et al.*, 1994).

For field experiments, 1-L screw cap bottles containing 100 g of autoclaved barley grains and 80 mL of deionized water were inoculated with 5 × 10⁷ conidia (5 mL of starter culture) and incubated as described above. After 3 weeks' incubation and 2 days before application treatments, a portion of the granules was milled as described above. As sieving is quite time-consuming and often leads to substantial losses of inoculum, material was ground a second time using an electric meat grinder (Quaker City Mill, Model 4-E, Westinghouse, PA, USA), resulting in a mixture of large and small particles with ≈ 70% of the particles being < 710 µm diameter.

The inoculum to be used for both controlled environment and field studies was routinely tested for conidia levels and viability. One gram of granules was suspended in flasks with 100 mL of deionized water and placed on a rotary shaker at 150 rev. min⁻¹ for 30 min. Contents were poured through two layers of cheesecloth, and flasks were rinsed with 50 mL of deionized water in order to wash out any remaining inoculum particles. Conidia quantities were determined and then adjusted to 1 × 10⁶ conidia mL⁻¹. For testing of conidia viability, two PDA plugs (10 mm diameter) were placed on glass slides in a Petri dish, each receiving 30 µL of the conidia suspension, incubated in the dark at 24 ± 1 °C for 18 h, and conidia were killed and stained with 0.1% lactophenol-cotton blue (Tuite, 1969). The percentage germination was evaluated microscopically (500×) by counting 50 conidia per agar plug. Conidia were considered to have germinated when the germ tube length was greater than the width of the conidium.

Plant production

For controlled environment experiments, *C. arvensis* seeds (Valley Seed, Fresno, CA, USA) were washed under warm running tap water for 2 h and soaked overnight in deionized water. Imbibed seeds were incubated on moist paper towels in a glass Petri dish at 24 ± 1 °C for 24–36 h. Four germinated seeds having emerged radicles were sown at a depth of 3 cm into 13-cm-diameter plastic pots containing a mixture of sandy loam (Modugno-Hortibec, St-Laurent, Québec, USA), potting medium (Pro-Mix BX, Les Tourbières Premier Ltée, Rivière-du-Loup, Québec, Canada), vermiculite (Vil Vermiculite,

Montréal, Québec, Canada) and peat moss (**Les Tourbières Premier Ltée**) [3:3:2:1 (V/V/V/V)]. Pot size and soil mixture were selected to allow for substantial root development. Pots were placed in a growth chamber (Conviron, Model E-15, Controlled Environments, Winnipeg, Manitoba, Canada) at $23/18 \pm 1$ °C day/night temperature with a 15-h photoperiod ($350 \mu\text{E}^{-2}\text{m}^{-2}\text{s}^{-1}$). Plants were grown for 8 weeks and fertilized every second week with 200 mL of 20:20:20 (N:P:K, 3 g L⁻¹). After 8 weeks, the above-ground tissue in all pots was cut at the soil line and the remaining root matter was considered as the 'established' plant material. Two days after cutting 'established' plants, 'seedlings' were produced by sowing four pre-germinated seeds in 13-cm-diameter pots and placing in a growth chamber under the same conditions as described above.

Field experiments were performed at the Horticulture Research Centre of Macdonald Campus, Ste-Anne-de-Bellevue, Québec, Canada. The soil type was a Chicot fine-sandy loam with 70% sand, 20% silt, 10% clay, a pH of 5.3 and 3% organic matter. To produce established plants, seeds were soaked for 20 s in near boiling water and incubated on moist paper towels for 24–36 h at 30 ± 1 °C. Two hundred imbibed seeds were sown in each 0.25-m² plot at a depth of ≈ 7 cm on 12 May 1995 and 16 May 1996. Plants were grown for 6 weeks before they were cut at the soil line, dried in paper bags at 60 °C for 4 days and weighed. The biomass obtained from this harvest was used as a relative value of plant vigour in each plot before cutting when compared with biomass data from the final harvest. On the same day as that on which established plants were cut, seedling material was produced by sowing 200 imbibed seeds in 0.25-m² plots not containing established plant material.

The air temperature and precipitation data were obtained from the McGill Meteorological Observation Centre (1.5 km away from field site). Soil surface temperatures were determined using thermocouple readings from a datapod reader (Model 217 DSM Reader, Omnidata International, Logan, UT, USA) that was placed at ground level within the field. During the 1995 field trial, no precipitation was received for nearly 1 month before and during the application of inoculum. Hence, plots were watered regularly during early plant establishment and once on day 2 of the application treatments

(≈ 1 L plot⁻¹). For the 1996 field trial, weather conditions were much wetter, and supplemental irrigation was carried out only during the first 2 weeks of initial established plant growth.

Inoculation procedure

All experiments were designed as two-factor experiments involving two *C. arvensis* growth stages and four treatments including three fungal application dates and one uninoculated control.

For controlled environment experiments, the amount of inoculum applied was based on earlier studies that made use of smaller pots (10-cm-diameter) and a dose of 1 g (Vogelgsang *et al.*, 1994). Consequently, a dose of 1.7 g of granules per pot was applied in this study, containing $3\text{--}5 \times 10^9$ conidia with $> 95\%$ germination. The material was manually spread on to the moistened soil surface and pots were immediately covered with plastic bags until all seedlings emerged (5–6 days). Inoculum applications were carried out 0, 1 and 2 days after sowing (DAS).

For field experiments, 30 g of fungal inoculum with $2\text{--}5 \times 10^{10}$ conidia and $> 80\%$ viability was uniformly spread by hand on the soil surface. Uninoculated plots served as controls. During the 1995 field trial, treatments were conducted 3, 4 and 5 DAS. However, in 1996 with cooler weather conditions prevalent, delayed emergence was expected and application dates were changed to 3, 5 and 7 DAS. In both trials, all applications were carried out late in the afternoon.

Assessment of efficacy

For controlled environment experiments, foliar necrosis was evaluated 11 DAS using the following rating system: 0 = no visible symptoms; 1 = 1–25% necrosis; 2 = 26–50% necrosis; 3 = 51–75% necrosis; and 4 = 76–100% necrosis (Ormeno-Nuñez *et al.*, 1988a). Disease rating was performed for each plant (if seedlings) or shoot (if established plants), and results were pooled and averaged for each pot. Above-ground and root biomass were determined 14 and 15 DAS respectively. Plants were cut at the soil line, roots were carefully removed from the potting medium, and living tissues were dried in paper bags for 4 days at 60 °C and weighed. The biomass was recorded as total biomass per pot. For the field experiment, above-ground biomass

per plot was determined 26 DAS. At harvest, the margins of each plot were delineated with white string, and plant coverage was determined with the aid of black and white photographic pictures. Contours of living plant material were traced on the photograph, and the percentage coverage of healthy tissues was assessed using an image analyser (Leco 2001 Image Analysis System, Instruments Ltée, Longueuil, Quebec, Canada).

Experimental design and data analysis

All experiments were performed twice. Controlled environment studies were set up in a completely randomized design with four replicates per treatment. For field trials, a randomized complete block design with five blocks was used. Blocks were arranged so that their length was perpendicular to a slight slope within the field site. In total, 40 plots, 0.25 m² in size, were established in each year. In 1995, the distance between plots and experimental blocks was 0.3 m and 0.8 m respectively. In 1996, spacing between plots was increased to 0.5 m and to 1 m between blocks in an attempt to reduce rain splash dispersal of inoculum. Before analysis of variance, coverage and biomass data were arcsin or log₁₀ (x + 1) transformed respectively. Treatment means for all parameters were compared using orthogonal contrasts (Steel & Torrie, 1980). Owing to significant interactions between the two plant stages, data for seedlings and established plants were analysed separately.

In both controlled environment and field studies, results for the two trials were not pooled because of heterogeneity of variances as determined by Levene's test (Dufner *et al.*, 1992). For controlled environment experiments, data from only one trial are presented as both trials showed similar trends.

Results

Controlled environment

Seedlings typically emerged 2–3 days after sowing, whereas regrowth of established plants occurred during a longer time period, ranging from 3 days to 1 week after cutting. Disease symptoms were visible for all inoculum application dates on both seedlings and established plants. However, control of *C. arvensis* was greatest for delayed application times and seedlings (Table 1). All pre-emergence treatments on seedlings resulted in significant reductions in above-ground and root biomass ($P < 0.05$). Above-ground biomass of seedlings was reduced between 41% (0 DAS) and 87% (2 DAS). Disease incidence for established plants was less severe than for seedlings, with only one treatment (2 DAS) leading to a significant reduction ($P < 0.05$) in shoot biomass (43%) compared with the uninoculated control (Table 1). Root biomass of established plants was variable with no significant differences between control and inoculation treatments being observed.

Table 1. Effect of pre-emergence application of *Phomopsis convolvulus* on disease severity and *Convolvulus arvensis* seedling and established plant growth under controlled environment conditions*

Plant stage	Treatment†	Above-ground biomass (g per pot)	Root biomass (g per pot)	Disease rating‡
Seedlings	Control	0.095 (0.008)	0.066 (0.008)	0.06 (0.06)
	0 DAS	0.056 (0.009)	0.031 (0.006)	1.38 (0.22)
	1 DAS	0.030 (0.004)	0.023 (0.006)	2.25 (0.14)
	2 DAS	0.012 (0.002)	0.010 (0.006)	3.25 (0.31)
Established plants	Control	1.185 (0.117)	2.524 (0.313)	0.05 (0.02)
	0 DAS	0.991 (0.032)	2.328 (0.148)	0.72 (0.06)
	1 DAS	0.845 (0.056)	2.156 (0.161)	0.62 (0.07)
	2 DAS	0.674 (0.076)	2.512 (0.272)	1.07 (0.24)

*Observations from one trial are presented. Trials were not combined because variances were not homogeneous. Numbers in parentheses are the SEM. Biomass data were log₁₀ (x + 1) transformed before analysis but were back-transformed before tabular presentation.

†0 DAS, 1 DAS, 2 DAS: application of 1 g *P. convolvulus* granules plot⁻¹ at 0, 1, 2 days after sowing respectively.

‡Disease rating scale is 0, no visible foliar symptoms; 1 = 1–25% necrosis; 2 = 26–50% necrosis; 3 = 51–75% necrosis; 4 = 76–100% necrosis.

Field trials

1995

Most seedlings and some shoots from established plants emerged 4–5 DAS; however, shoot emergence of the latter continued until the termination of the experiment. During the inoculum application period, weather conditions were hot and dry, with air temperatures reaching 31 °C. However, based on field thermocouple readings, granules on the soil surface were exposed to temperatures between 27 and 40 °C (Fig. 1A).

Significant reductions in above-ground biomass were achieved for both inoculated seedlings and established plants (Fig. 2A). Biomass reductions under field conditions were similar for all inoculum application times. Although observed disease incidence for established plants was lower than for seedlings, above-ground

biomass reductions for established plants in the field increased compared with results obtained under a controlled environment. Above-ground biomass reductions for seedlings ranged from 94% to 99%, whereas regrowth from established plants was reduced by 53% to 80% compared with uninoculated controls. A significant difference in biomass of control and inoculated established plants was only observed for the second application day ($P < 0.05$). A possible explanation is that several uninoculated control plots became contaminated with *P. convolvulus*, probably because of conidia dispersal from rain-splash or run-off along a slight slope within the field. Nonetheless, when final harvest biomass data are compared with biomass data from the first harvest after initial establishment (in which plants had been grown for 6 weeks), all inoculum applications resulted in established plants having significantly lower biomass

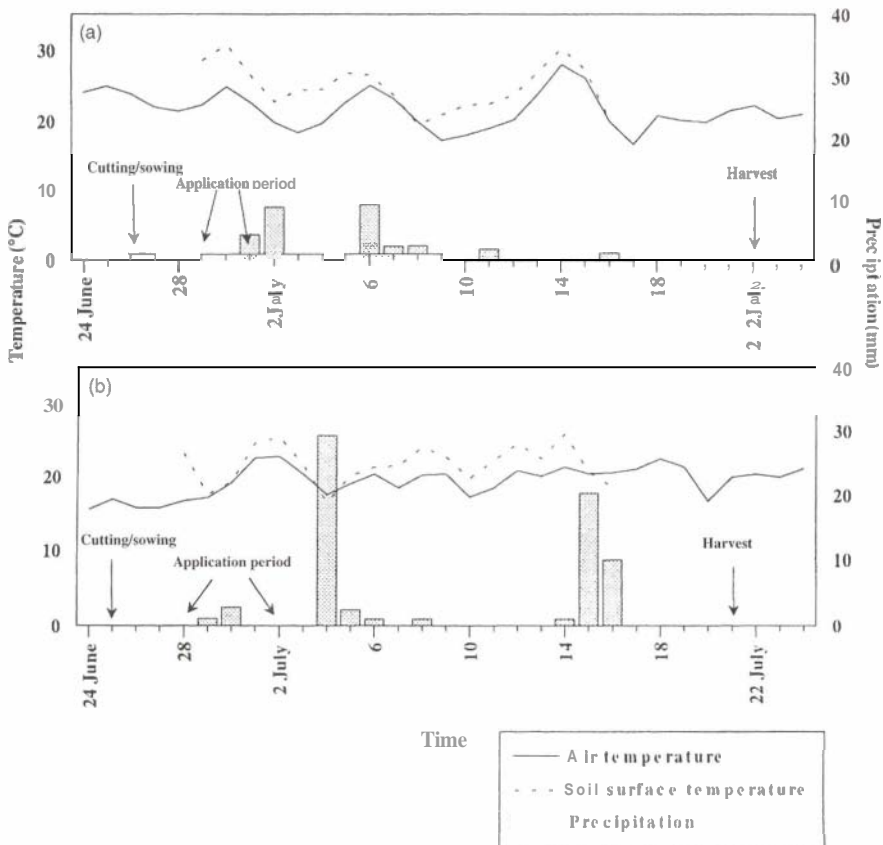


Fig. 1. Precipitation, air and soil temperatures during (A) 1995, (B) 1996 field trials. Temperatures are daily means.

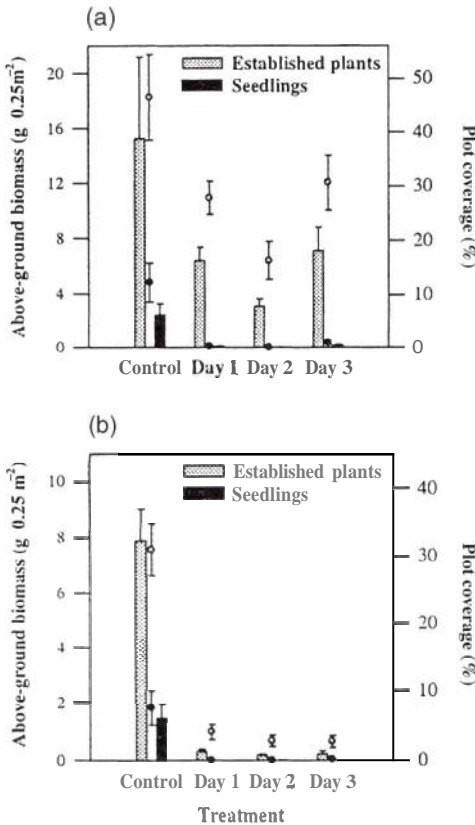


Fig. 2. Field experiment – effect of pre-emergence field application of *Phomopsis convolvulus* on *Convolvulus arvensis* above-ground biomass (bars) and plot coverage (circles) for seedlings and established plants in (A) 1995 and (B) 1996. Day 1, 2, 3 treatments refer to 30 g *P. convolvulus* granules 0.25 m² plot⁻¹ applied 3, 4, 5 (1995) or 3, 5, 7 (1996) days after sowing respectively. Plants were harvested 26 days after sowing. Each vertical bar is the SEM. Before analyses of variance, biomass and coverage data were log₁₀ (x + 1) and arcsin transformed, respectively, but were back-transformed before plotting.

compared with uninoculated controls (data not shown). In general, plot coverage data closely paralleled above-ground biomass results (Fig. 2A).

1996

The emergence pattern in 1996 was approximately 1 day behind that in 1995. Throughout the 1996 application period, weather conditions were warm and moist with heavy precipitation 2 days after the last granular application (Fig. 1B). Air and soil temperatures reached 28 and 34 °C respectively. Above-ground regrowth

from established plants was drastically reduced by all fungal applications [i.e. 9.698% compared with uninoculated controls (Fig. 2B)]. In contrast to the results for 1995, the treatment application for all dates produced significant biomass reductions compared with uninoculated controls. This occurred despite substantial cross-infection of control plots even after spacing between experimental plots was increased.

Discussion

In this study, the pre-emergence potential of the fungal pathogen, *P. convolvulus*, to suppress *C. arvensis* at two growth stages was evaluated. Both seedlings and established plants of *C. arvensis* developed disease symptoms after fungal inoculation. However, under a controlled environment, damage of regrowth tissue from established plants was substantially lower than for seedlings. This confirms results from earlier studies that used aqueous conidial suspensions of *P. convolvulus* as a post-emergence inoculum source (Morin *et al.*, 1989b). The possible development of a thicker cuticle by physiologically more mature plants could result in altered defence mechanisms (Martin, 1965), and could explain why tissue from established plants was less susceptible to fungal damage. Similarly, a greater amount of constitutive antibiotics and/or phytoalexins in the host tissue from established plants could have led to increased resistance compared with seedlings (Bell, 1980). In addition, regrowth from root stocks occurred in a staggered manner and was not completed by the time all seedlings had emerged. Thus, a considerable amount of inoculum could have lost viability by lying on the soil surface. Likewise, timing of fungal application was crucial for disease severity. For both growth stages, the level of control declined with increasing time interval between fungal application and actual emergence.

In field trials, biomass reductions were not dependent upon the day of application, as was observed for the controlled environment trials. Moreover, efficacy of control for established *C. arvensis* plants was significantly increased. In these experiments, the fungus was capable of attacking and suppressing both growth stages of the weed under a wide range of environmental conditions. Furthermore, there were indications

that the pathogen was more virulent in the field than under controlled growth chamber conditions. This is an unusual observation in bioherbicide research. Frequently, the high levels of disease obtained under laboratory conditions are difficult to reproduce in the field. The narrow environmental range of conditions that is often required to attain high levels of infection has commonly been cited to explain the disparity of results between laboratory and field trials (Watson & Wymore, 1990). The precise reasons for the observed outcome in this study are not clear. However, several abiotic and biotic, as well as methodological, factors could have affected the results. Soil type may have had a strong influence on the performance of the fungus. Factors such as particle size, pH and available nutrients have been shown to favour or inhibit the survival and activity of fungal pathogens (Stotzky, 1974; Paulitz & Baker, 1987; Hopner & Alabouvette, 1996). Furthermore, the presence of numerous micro-organisms under field conditions could have led to intense competition for resources (Waksman, 1952), thus preventing *P. convolvulus* conidia from germinating rapidly in the absence of host tissue. The inoculum production method used in this study may also have affected the results. Within the larger incubation bottles used for field trials, the greater amounts of conidial matrix observed could have protected the fungus to a greater extent from desiccation (Sparace *et al.*, 1991). In addition, granules prepared for field inoculations were less homogeneous in size. That is, smaller particles that could serve as immediate infection sources were mixed with larger particles, possibly being more persistent and having slower inoculum release rates. Finally, the development of a disease epidemic is also dependent upon an adequate distribution of the pathogen near its host. In the field, rain-splash, wind dispersal, as well as run-off, are common events that may have contributed to the dissemination of the fungal inoculum (Fitt *et al.*, 1989; Madden, 1992).

In both years of the field experiment, the control of established plants was greater compared with results from growth chamber studies. However, in the first field trial, only one *P. convolvulus* application (2 DAS) resulted in significant reductions in *C. arvensis* above-ground biomass compared with uninoculated controls. This finding was possibly due to the high temperatures and drought conditions present during

the 1995 fungal application period. Also, cross-infection of uninoculated control plots, presumably caused by rain-splash dispersal, might have led to less accentuated differences between treatments. In 1996, the control of established *C. arvensis* plants was even more apparent, with all fungal treatments resulting in substantial declines in resprouting biomass. During the 1996 application period, weather conditions were more favourable to disease development (i.e. lower temperatures and adequate rainfall). Hence, this increase in the availability of free water could have enhanced *P. convolvulus* germination and infection.

The granular formulation of *P. convolvulus* used in this study improved the capability of the fungus to withstand unfavourable weather conditions. Similar findings were observed by Boyette & Walker (1985) when granular pre-emergence field applications of *Fusarium lateritium* Nees ex Fr. were superior to post-emergence foliar sprays in suppressing *Abufilea theophrasti* Medic. Likewise, when *Fusarium solani* (Mart.) Appel & Wr. f. sp. *cucurbitae* was applied pre-emergence, sodium alginate granules provided greater and longer-lasting control of *Cucurbita texana* A. Gray compared with conidial applications (Weidemann & Templeton, 1988). Another granular preparation consisting of vermiculite, spores and mycelia of *Alternaria macrospora* Zimm. effectively controlled *Anoda cristata* Schlecht in both greenhouse and field tests (Walker, 1981). This method of spore production and formulation was later modified for use with other fungi including a pycnidia-forming fungus, *Phyllosticta* spp. (Walker & Connick, 1983).

Plant coverage determined from photographs of field plots closely reflected biomass data. Hence, this method may be more precise than evaluating disease by visual estimates of necrotic leaf area because computer-generated surface scans of plant cover or necrotic area are less subjective and therefore more accurate and reliable.

The results of our study indicate that pre-emergence applications of *P. convolvulus* are effective in controlling different growth stages of *C. arvensis*. Further research on soil incorporation of granules, fungal persistence in soil and effect under competitive cropping situations are in progress and should provide additional information about the potential of *P. convolvulus*

to be an effective biological agent against *C. arvensis*.

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