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Axenic culture and influence of wetness period and inoculum concentration on infection and development of cercospora blight of *Heliotropium europaeum*

S. Hasan[1], M. Jourdan[1], L. Brun[1] and A.W. Sheppard[2]
[1]*CSIRO Biological Control Unit, Campus International de Baillarguet, 34982 Montferrier-sur-Lez CEDEX, France;* [2]*CSIRO Division of Entomology, GPO Box 1700, Canberra ACT 2601, Australia*

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Abstract

Cercospora heliotropii-bocconii is a fungal pathogen of the ephemeral annual weed *Heliotropium europaeum*. The effects of wetness period and inoculum concentration on disease severity were studied under controlled conditions. The fungus was grown on different artificial culture media and carrot juice agar with 5 g l^{-1} yeast extract was found to be the most suitable medium for conidial production under artificial conditions. Abundant disease symptoms only occurred after 8 h of wetness at 20 °C. The minimum incubation period before disease symptoms appeared was 8 days following a wetness period of at least 40 h. Inoculum concentration of 1×10^4 conidia per ml killed plants in less than one month and reduced seed production by two thirds. These results suggest that this pathogen has the potential to reduce plant survival and seed bank replenishment of this annual weed species.

Introduction

Cercospora blight caused by *Cercospora heliotropii-bocconii* Scalia is a fungal disease of common heliotrope, *Heliotropium europaeum* L. in the Mediterranean region. This plant, of largely Mediterranean and Middle Eastern origin is a summer annual which has become a serious weed in Australia where it may cause mortality of grazing animals [Bull *et al.*, 1956, Culvenor, 1985; Howell *et al.*, 1991]. The disease attacks all green parts of the plant at any stage of development and causes characteristic necrotic lesions. On leaves, these lesions vary from small spots to those that may cover the entire surface. The lesions are light to dark brown with internal mycelium and dark coloured conidiophores which emerge through stomata. The conidiophores bear conidia which are hyaline, filiform, truncate, multiseptate and straight or slightly curved. Conidia measure 29 mμ to 324 mμ (based on 920 conidial measurements). They germinate in contact with water or under high humidity and give rise to several germ-tubes; one per cell of the conidia. The germ-tube penetrates the leaves through stomata thereby initiating infection.

Blights caused by other species of *Cercospora* [Chupp, 1953] are important diseases of a number of crops throughout the world including carrot [Carisse and Kushalappa, 1992], lettuce [Savary, 1983], soybean [Franca-Neto and West, 1989], peanut [Alderman *et al.*, 1987], sugar beet [Pundir and Mukhopadhyay, 1987] and asparagus [Cooperman *et al.*, 1986]. Their destructive capacity, apparent high specificity and their simple asexual life cycle has led *Cercospora* species to be considered as potential agents for the biological control of weeds [Charudattan *et al.*, 1985, Hasan and Ayres, 1990].

This paper presents the results of studies on the

influence of climatic factors on infection of common heliotrope by *C. heliotropii-bocconii*. These studies were aimed to understand the conditions of development of the disease and its potential for spread when introduced to Australia for the biological control of common heliotrope. Another fungus, the rust *Uromyces heliotropii* Sred., has already been released against this weed in Australia [Hasan, 1985; Hasan and Aracil, 1991; Hasan *et al.*, 1992; Sheppard *et al.*, 1993].

Materials and methods

Isolate. C. heliotropii-bocconii collected near Montpellier, S. France (IS157) was isolated on acidified PDA (Potato-Dextrose-Agar-Difco) with the addition of 5g l^{-1} yeast extract. Although no sporulation was obtained on this medium, when the fragmented mycelium produced was suspended in water and sprayed on common heliotrope, typical brown lesions with conidia were produced. Fragments of mycelium germinated giving rise to several germ-tubes, often one from each cell, as produced by conidia under similar conditions.

Culture media. A search was made to identify suitable mycological media on which *C. heliotropii-bocconii* would sporulate including PDA, PDA plus yeast extract, PDA plus plant (common heliotrope) extract, V8 juice agar, V8 juice agar plus CaCO3, V8 juice agar plus CaNO3, V8 juice agar plus plant extract, Knop solution agar, Knop solution agar plus plant extract. Carrot juice agar plus yeast extract was also tested under similar conditions but not during the initial comparison. This was because information about the potential value of this medium for culturing this type of fungus was not available at the start of the study (G. Defago, pers. comm.). The diameters of the cultures on the different media were measured after 11 and 14 days and the number of conidia produced per petri dish was scored in three categories, zero conidia (–), < 10^4 conidia per dish (+) and > 10^4 conidia per dish (++) after 14 days. Those media that produced conidia in the greatest numbers (category ++) were compared in a further experiment in which spore counts were made in greater detail after 12 and 17 days.

Plant production and maintenance. Seeds of *H. europaeum* received from Australia were grown in petri dishes with moist filter paper maintained at 30 °C with 14 h photoperiod. The germinated seedlings were transferred to plastic pots holding steam-sterilised soil with equal volumes of sand, peat and loam. These were placed on greenhouse benches at night temperatures of 15–20 °C and day temperatures of 15–35 °C. Sunlight was supplemented with 1000-W high pressure sodium lamps (for 14 h per day). Plants were grown in the greenhouse for 6 weeks before being used in experiments.

Inoculation. The isolate IS157 of *C. heliotropii-bocconii* was maintained on V8 juice agar at 20 °C. Numerous conidia developed on the periphery of the colony. Mature conidia were collected by flooding cultures with 10 ml of distilled water containing 0.05% Tween 80 (polyoxyethylensorbitannemonooleate; Sigma Chemical, St Quantin Flavier, France) to give a concentration of 10^5 conidia ml^{-1}. Conidia were dislodged by gentle agitation with a camel hair brush. Mature conidia were also obtained by incubating infected leaves in petri dishes provided with moist filter paper for 48 h at 20 °C. These leaves were washed in distilled water to obtain a conidial suspension. Conidial concentration was determined with a haemacytometer (Nageotte).

Inoculations were made on 6 wk old potted plants by spraying the conidial suspension using a household hand-held spray bottle. Each plant was sprayed with the required conidial suspension five times and each time the plant was rotated 1/5 turn maintaining the sprayer at a distance of 30 cm. In this way each plant received an equal quantity of conidia. Inoculated plants were then incubated in incubation chambers with a water saturated atmosphere (c 1 m × 1 m × 0.5 m high plastic, light proof, each fitted with a 1 m × 1 m × 3 mm thick air-tight glass top) at 19–20 °C for required periods. This allowed the plants to maintain droplets of sprayed conidial suspension on the surface for required duration of wetness.

Effect of wetness period on infection. Eight batches of five plants were inoculated with the conidial suspension of *C. heliotropii-bocconii* at a concentration of 10^5 conidia ml^{-1}. Each batch of

five inoculated plants was incubated in an incubation chamber, as described above, for 0, 8, 16, 24, 40, 48, 64 or 72 h of wetness. At the end of each wetness period the plants were taken to a greenhouse set at 15–20 °C (night) and 15–35 °C (day) temperatures and RH 30–65. Observations were made for disease symptoms 8, 10, 13, 17, 26 and 28 days after inoculation. Fifty leaves from each batch were examined for the percentage of infection and graded according to the following 0–5 rating scale of disease severity.

0	0% leaf surface
1	1–25% leaf surface
2	26%–50% leaf surface
3	51%–75% leaf surface
4	76%–100% leaf surface
5	100% leaf surface and petiole

At each observation day, the leaves were examined directly on the plants and approximate infected surface areas were recorded.

The disease severity, i, on the leaves all together and for each batch and wetness period was assessed according to the formula published by Rossi and Battilani [1989];

$$\sum_{i=1}^{n} \text{severity}/n$$

where n is the number of leaves.

Effect of inoculum concentration on infection and disease severity. Conidial suspensions at two concentrations, 2×10^2 (A) and 1×10^4 (B), were used for inoculation. Conidia were obtained from infected leaves incubated in moist petri dishes as described above. Nine plants were inoculated with the suspension A and 9 others with the suspension B while still 9 others were used as controls. The inoculated plants were incubated in moist chamber for 48 h and then transferred to the greenhouse set as above. Disease severity was recorded at 5, 12, 20 and 25 days after inoculation using the 0–5 rating scale as above. The number of fruits produced by each plant were counted at the same time as the observations on disease severity. Mature seeds were collected and pooled, one lot for each treatment. 100 seeds from each lot were germinated on moist filter paper in Petri dishes soaked in 0.06% gibberellic acid to promote germination.

Results and discussion

Culture media. C. heliotropii-bocconii produced a mycelial mat on most media used, but only a few of these produced conidia (Table 1). V8 agar and carrot juice agar with yeast extract were the most suitable media on which abundant conidia were produced. A separate comparison between these two showed that carrot juice agar with yeast extract was found to produce more conidia (Table 2). The ability to culture *C. heliotropii-bocconii* on this artificial medium will be a great advantage to the practicalities of biological control, should this pathogen prove specific enough for release in Australia [Hasan, 1992].

Effect of wetness period on infection. Both percentage of diseased leaves (Fig. 1A) and disease severity (Fig. 1B) increased with the length of wetness period from 8 to 72 h. At 8 h and below few disease symptoms were observed. The incubation period required for the appearance of the first symptoms of disease decreased to 8 days at wetness periods above 40 h. These results agree with field observations. First symptoms of disease and the characteristic bursts of disease spread, as an epidemic develops in the field, occurred roughly 8 days after rain showers at sites near Montpellier [L. Brun, unpubl.]. The requirement for rain showers may indicate that only under such conditions will the dew period be long enough (> 8 h) to initiate infection. In host specificity tests

Table 1. Growth of *Cercospora heliotropii-bocconii* on different culture media (see text for details)

medium	Diameter of culture (cm) after 11 days	14 days	conidia
PDA	4.5	6.2	–
PDA + Yeast	4.6	6.5	–
PDA + *H. europaeum*	4.8	7.3	+
V8	4	6.1	++
V8 + CaCO3	4.3	6.3	+
V8 + CaNO3	4.8	6.8	–
V8 + *H. europaeum*	4.5	6.5	+
Knop	3	4.5	–
Knop + *H. europaeum*	3.6	4.6	+
Carrot juice + Yeast*	4.5	6.8	++

* not tested at the same time.

Table 2. Production of conidia of *Cercospora heliotropii-bocconii* on different culture media (see text for details)

Medium	Number of conidia/ petri dish after*	
	12 Days	17 Days
V8	$3.213 \times 10^4 \pm 8.442 \times 10^3$	$1.115 \times 10^5 \pm 3.637 \times 10^4$
Carrot juice + Yeast	$2.7 \times 10^5 \pm 2.409 \times 10^4$	$1.355 \times 10^6 \pm 1.707 \times 10^5$

* Values are means ± standard errors. Student *t*-test on spores/ml after 12 days on two media $t = 9.26$, df = 4, $p = 0.0008$; after 17 days $t = 7.12$, df = 4, $p = 0.0021$.

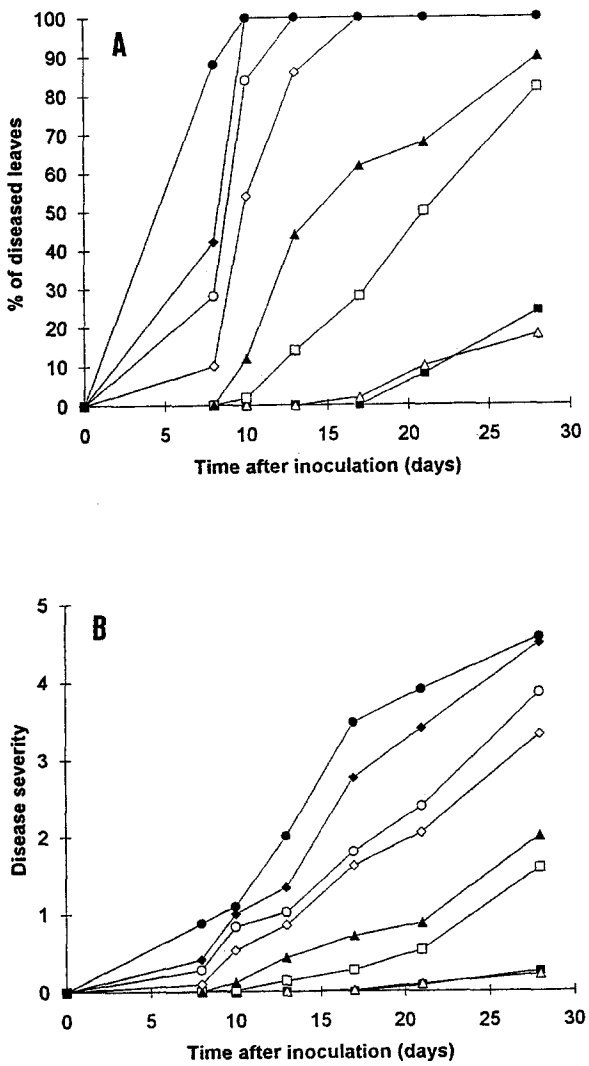

Fig. 1. Effect of wetness periods on the disease incidence (A) and severity (B) of *Cercospora heliotropii-bocconii* on *Heliotropium europaeum*; 0 (—■—), 8 (—△—), 16 (—□—), 24 (—▲—), 40(—◇—), 48 (—○—), 64 (—◆—) and 72 (—●—) h of wetness.

and other infection studies an average wetness period of 48 h was used.

Effect of inoculum concentration on infection and disease severity. Once inoculated plants were moved from the incubation chamber to the glasshouse new infections were no longer observed. This was due to the fact that the plants were side watered and thus no free water was allowed on their surface which could cause the germination of conidia. Therefore all disease development on experimental plants was related to the initial infection. Experimental plants that received a higher inoculum concentration (2×10^2 vs 1×10^4 conidia ml^{-1}) developed more severe disease symptoms over time (Fig. 2). Thus most plants died within 4 weeks after inoculation. The inoculum concentration also appeared to alter the shape of the disease progression curve. At low concentrations increase in disease severity was slow and constant, while at high concentrations it was fast initially and then became slower to spread throughout the rest of the available leaf tissue. This difference may have been an artifact of the disease ranking system used. Also, while the weaker inoculum had 50 times fewer conidia per ml than the stronger inoculum, diseased plants took only four times as long to achieve the same severity of disease. Therefore inoculum concentration may not prove to be that important in allowing the disease to spread and multiply. Apparently the severity of disease was determined not only by the number of lesions, but by the growth of individual lesions as well.

The disease caused a significant reduction in fruit production (1 fruit = 4 seeds), which was more marked on the severely diseased plants in the higher inoculation treatment. The higher concentration inoculum reduced the capacity of the weed

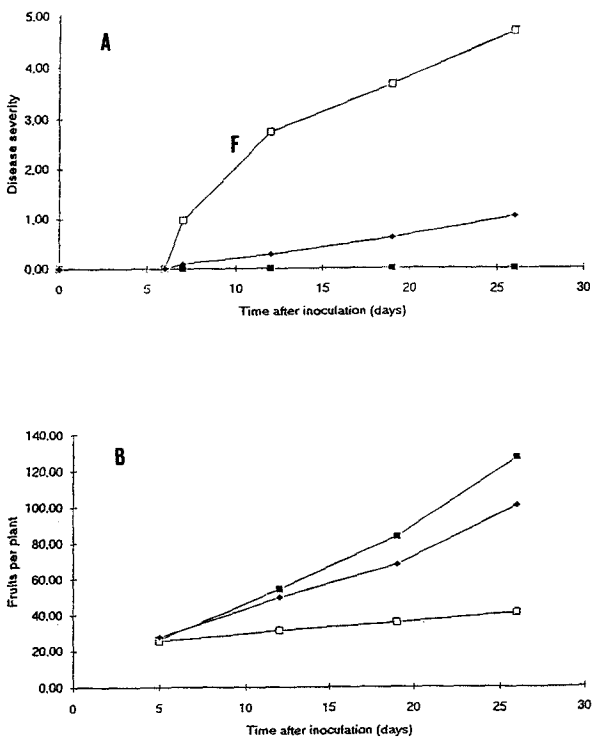

Fig. 2. Effect of inoculum concentration of *Cercospora heliotropii-bocconii* on disease severity (A) and fruit production (B) of *Heliotropium europaeum* for 1×10^4 conidia ml^{-1} (—□—), 2×10^2 conidia ml^{-1} (—◆—) and uninoculated control plants (—■—).

to produce seeds by two thirds compared to the controls. This suggests that this pathogen has good potential as a biological control agent, where the main target for control is to reduce seed bank replenishment. However a similar concentration of inoculum reduced seeding by only 15% in a field trial [L. Brun, unpubl.]. The 48 h dew period and glasshouse conditions used in our experiments clearly favoured the effectiveness of the pathogen to reduce the reproductive capacity of its host.

Seeds from inoculated plants were found to have sporulating mycelium on the seed coat. Infected seeds germinated well (Table 3) and some of these when transferred to the greenhouse gave rise to normal seedlings. In another field experiment to show the effectiveness of *C. heliotropii-bocconii* the infected seeds gave rise to weak seedlings most of which did not survive [Hasan, unpubl.]. Colonization of host seeds by other species of *Cercospora* and their effect on the

Table 3. Effect of inoculum concentration on the germination of seeds of *Heliotropium europaeum* inoculated with *Cercospora heliotropii-bocconii*

Seeds	% Germination	% showing *Cercospora**
Control	53	0
1×10^4 conidia/ml	83	80
2×10^2 conidia/ml	73	0

* In this column 0 means seeds with no apparent sign of infection.

reduction of germination and emergence of infected seeds have previously been reported [Wilcox and Abney, 1973; Yeh and Sinclair, 1982, Singh and Sinclair, 1985; Schuh, 1992].

Conclusion

In contrast to the rust, *U. heliotropii*, already released in Australia, *C. heliotropii-bocconii* requires a longer dew period for disease incidence. Advancement of any disease epidemic will depend on at least 8 hrs of wetness duration which will normally only be associated with rain showers. As such, Cercospora blight may only be an effective agent in areas with regular summer rains or in wet years. Our experiments were conducted at 20 °C except when test plants were moved to the glasshouse. We do not yet have sufficient information to understand how disease severity is affected by temperature. This is one necessary line of future research to predict disease epidemiology if this pathogen is allowed to be released under natural Australian conditions.

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