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Symptomatological and Morphological Study of the Resistance of Wild Beet Species of the *Patellares* Section to *Cercospora beticola* Sacc.

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With 4 figures

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Abstract

The reactions of *Beta procumbens* C. Sm. and *Beta webbiana* Moq. were compared to those of *Beta vulgaris* L. with regard to an infection by *Cercospora beticola* Sacc. The fleck reaction observed in *B. webbiana* may be interpreted as hypersensitivity based on symptomatological, light microscopical, fluorescent microscopical and electron microscopical data. The *B. procumbens* clone was found to show resistance characteristics similar to those of *B. webbiana* and *B. vulgaris*, as it reacted both by flecks (*B. webbiana*) and leaf spots (*B. vulgaris*) to a *C. beticola* infection.

Zusammenfassung

Eine symptomatologische und morphologische Untersuchung der Resistenz von Wildrübenarten der Patellares-Sektion gegen *Cercospora beticola* Sacc.

Verglichen wurden die Reaktionen von *Beta procumbens* C. Sm. und *B. webbiana* Moq. mit denen von *B. vulgaris* L. nach einer Infektion mit *Cercospora beticola* Sacc. Die bei *B. webbiana* beobachtete Klecksreaktion kann als Hypersensitivität anhand von symptomatologischen, licht-, fluoreszenz- sowie elektronenmikroskopischen Daten angesehen werden. Beim *B. procumbens*-Klon wurden Resistenzmerkmale festgestellt, die denen von *B. webbiana* und *B. vulgaris* ähnelten: der Klon reagierte mit Klecksen (*B. webbiana*) sowie mit Blattflecken (*B. vulgaris*) nach einer *C. beticola*-Infektion.

The resistance of sugarbeet to *Cercospora beticola* Sacc. has been extensively studied. It appears to be a polygenic trait (SMITH and GASKILL 1970, LEWELLEN and WHITNEY 1976, RUSSEL 1978). The expression of defenses of resistant sugarbeet genotypes, with respect to the inhibition of the infection process of *C. beticola*, can occur at the level of spore germination and hyphal growth on the leaf surface (KOVACS 1955, HARRISON *et al.* 1970, FEINDT *et al.* 1981a, WHITNEY and MANN 1981), stomatal penetration (SOLEL and MINZ 1971, FEINDT *et al.* 1981a, LIEBER 1982), hyphal growth in the intercellular spaces (SOLEL and MINZ 1971, WHITNEY and MANN 1981, FEINDT *et al.* 1981b) and/or the size of the necrotic area (SOLEL and MINZ 1971, WHITNEY and MANN 1981, FEINDT *et al.* 1981b). The potential role of host molecules in the inhibition of hyphal growth of *C. beticola* in sugarbeet leaves has been described by TRZEBENSKI (1961), HARRISON *et al.* (1961, 1967), RAUTELA and PAYNE (1970), HECKER *et al.* (1975), JOHNSON *et al.* (1976) and MARTIN (1977). Attempts have also been made to correlate plant resistance to the fungus and to its metabolites (LIEBER 1982) and to understand which metabolites could be the primary determinants of pathogenesis (SCHLÖSSER 1969, BALIS and PAYNE 1971, LEPOIVRE and CAREL 1986).

In this paper, we will describe on the resistance to *C. beticola* as observed in species belonging to the *Patellares* section. COONS (1975) reported a very high resistance or even immunity of such species to *C. beticola*. OSINSKA (1970) reported leaf spot occurrence on wild beet hosts after *C. beticola* inoculation. However, no fungal sporulation at the infection site was observed. Finally, KOCH (1985) proposed the term "hypersensitivity" to describe resistant reactions encountered in species of the *Patellares* section.

Wild beet species of the genus *Beta* are potentially useful in breeding on account of their resistance to pests and diseases (review, see DE BOCK 1986), which includes successful introgression of resistance to the beet nematode *Heterodera schactii* Schm. (SAVITSKY 1975, LÖPTIEN 1984, YU 1982, HEIJBROEK *et al.* 1983). This result paved the way to genetic transfer from wild species of the *Patellares* section to *Beta vulgaris* L. Furthermore, developments in beet genetics, using isozymes and/or RFLPs, have led to a speed up in improvement schemes for beet selection (SMED *et al.* 1989). *In vitro* culture would contribute to this and permit still new progress in this field (TÉTU and SANGWAN-NORREEL 1987).

Materials and Methods

1. Plants

The *in vitro* Pr 3 clone of *B. vulgaris*, as well as seeds of *B. webbiana* and *B. procumbens* were obtained from Van Geyt (Vrije niversiteit Brussel, St. Genesius Rode, Belgium).

B. procumbens and *B. webbiana* shoot tips were surface sterilized according to VAN GEYT and JACOBS (1985) and transferred to *in vitro* culture for cloning. Subcultures of Pr 3 and the two wild beet species were grown on PGo medium (0.3 mg/l) and were rooted in PGo supplemented with NAA (1 mg/l). Micropropagation was carried out by axillary bud culture on PGo + Kin (0.3 mg/l). The *in vitro* plantlets were transferred to a glasshouse, where they were grown in "Jiffy-7" pots for at least 4 weeks, and fertilized as necessary. The plants were then compared for their resistance to *C. beticola*.

2. Fungus

Strain 4C of *C. beticola* was isolated from an infected field of sugar beet from France. *C. beticola* isolation from infected *B. vulgaris* and *B. webbiana* leaves was carried out in the following manner: leaf samples of 1 cm^2 were surface sterilized by immersion in ethanol 70% for 30 s and then for 1 min in $AgNO_3$ 1%, after which they were rinsed twice in distilled water for 15 min. The leaf samples were then transferred to PDA for colony development. Further, culture of the isolates for their sporulation was carried out on SBLA, as described by RUPPEL (1972).

3. Inoculations

A conidial suspension was prepared in a 0.1% aqueous Tween 20 solution and this was diluted to give 30,000 conidia/ml. Plants were then inoculated with this suspension by spraying the leaves to run-off. The plants were then allowed to dry before an estimation of the leaf inoculum was carried out by the sellotape technique of DHINGRA and SINCLAIR (1985). The plastic film carrying the leaf conidia from the samples (three leaves per plant) was stained with anilin blue (0.1%) in lactophenol and then washed with water prior to making conidial counts. The inoculated plants were then maintained in a humid chamber in a glasshouse, according to WHITNEY and LEWELLEN (1976). Temperature varied between 20°C and 30°C with a light intensity of at least 10,000 lux.

4. Symptom evaluation

Disease severity in the inoculated plants was measured by use of the attack index (i_A), 10 days after inoculation. In the case of sugar beet, i_A was defined by:

$$aV + bW + cX + dY + eZ$$

where V, W, X, Y and Z are the class indices which represent the percentage of necrotic leaf tissue. The values awarded to these indices are summarized below:

class	V	W	X	Y	Z
value	0.2	0.4	0.6	0.8	1
disease severity (% of necrotic leaf tissue)	< 20	40	60	80	100

Leaves showing no symptoms were not taken into account.

a, b, c, d, and e are the number of leaves belonging to a given class expressed as a percentage of each plant's total leaf number. As species of the *Patellares* section exhibit atypical flecks, i_A was defined by:

$$Av + Bi_{v1} + Ci_{v2}$$

In this expression:

A — is the class where only leaves showing the fleck reaction were considered;
V — is the leaf percentage in class A;
B — is the class where leaves showing both the fleck and the susceptible reactions were considered;
i_{v1} — is the attack index of leaves in class B, which was obtained in the same way as defined for *B. vulgaris;*
C — is the class for leaves showing only the susceptible reaction;
i_{v2} — is the same as i_{v1} but for class C.

5. Light microscopy

Red flecks on samples of wild beets were circled with paint (Humbrol Enamel 88) prior to the bleaching and staining treatments to allow their localization under microscope. These circled red fleck samples as well as areas of sugar beet necrosis of about 1 cm^2 were immersed in 5 M HCl and placed in a vacuum of −260 mm Hg for 10 to 20 min. The leaf samples were then immersed for at least 24 h in a mixture of acetic acid and ethanol (1/1). Staining was carried out by immersion of the bleached samples in trypan blue (0.1%) in lactophenol, which were then heated for 10 min at 110°C in an autoclave. Stained leaf samples were then destained in a 5 parts chloral hydrate: 2 parts water solution for at least 1 h until satisfactory contrast was obtained.

6. Fluorescent microscopy

In order to ensure effective dye penetration the dorsal epidermis was removed from *B. webbiana* leaves and pieces (+/− 1.6 mm²) were then treated with 0.01 % fluorescein diacetate solution (WILDHOLM 1972). After an incubation period of 4 min, the pealed sides of the leaf samples were observed under UV light by means of a Leitz microscope Fluovert equipped with block filter number 12/3.

7. Phase contrast microscopy

Leaf pieces of sugar beet or *B. webbiana* about 1 mm² including necrotic lesions of flecks, were observed 6, 14 and 26 days after inoculation. After treating the leaf samples for 48 h in a fixative solution of 2.5 % glutaraldehyde solubilized in 100 mM cacodylate buffer pH 7, they were treated for 2 h with a solution of 1 % osmiumtetroxide, 1 % potassium dichromate and 10 mM calcium chloride. After this, the samples were dehydrated using ethanol and anhydrous propylene and imbedded in Epon Spurr. Semi-thin (2.5 µm) sections were then cut with a glass knife and examined under a phase contrast microscope (Anoptral-Reichert).

8. Electron microscopy

The material was prepared as described for phase contrast microscopy. Ultrathin sections (50 nm) were cut with a diamond knife and examined using a transmission electron microscope (TEM) Siemens.

Sugar beet necrosis and *B. webbiana* flecks, which had been induced to sporulate, were examined by scanning electron microscopy (SEM). Samples were fixed with osmiumtetroxide vapour and dried according to TIEDT *et al.* (1987) prior to be fixed to stubs with cyanamid glue and coated with gold.

Results

1. Symptomatology

B. webbiana showed a fleck reaction (Plate 2 C) within 4 days after inoculation, whereas necrotic lesions appeared on *B. vulgaris* 12 days after inoculation (Plate 1 A). *B. webbiana* flecks were red and generally smaller than 1 mm in diameter at onset and some reached 2 mm at 30 days after inoculation. The fleck reaction was observed on the upper or on the lower side of the leaf, but was rarely translaminar.

B. procumbens was less resistant than *B. webbiana* (Table 1).

Plate 1: (A) Characteristic leaf spot disease in sugar beet 12 days after *C. beticola* conidia inoculation — scale bar (5 cm). (B) Mycelial germination on infection site originated conidia near red ring. Notice the appressorium on a stomatal opening on one of the germinating tubes — scale bar (68 µm). (C) Infection peg arising from appressorium (plate 1 B) invading intercellular space. Conidia can be seen as a shadow (arrows) — scale bar (34 µm). (D) Mycelium invading the intercellular spaces of parenchymatous tissue 6 days after inoculation — scale bar (34 µm). (E) Interface of invaded blue ring (BR) and healthy tissues (HT) — scale bar (136 µm). (F) Conidia (arrows) formed on conidiophores in the necrotic center (NC), 20 days after inoculation — scale bar (136 µm). (G) Necrotic aspect at infection site 16 days after inoculation showing a cellular reaction in invaded previously healthy tissue corresponding to the HT area of plate 1 E. This reaction was linked with cellular red pigment accumulation and the apparition of a red ring (RR) — see arrows — 16 days after inoculation — scale bar (700 µm). (H) Characteristic droplet-like material (arrows) on invaded red ring cell walls, 20 days after inoculation — scale bar (68 µm)

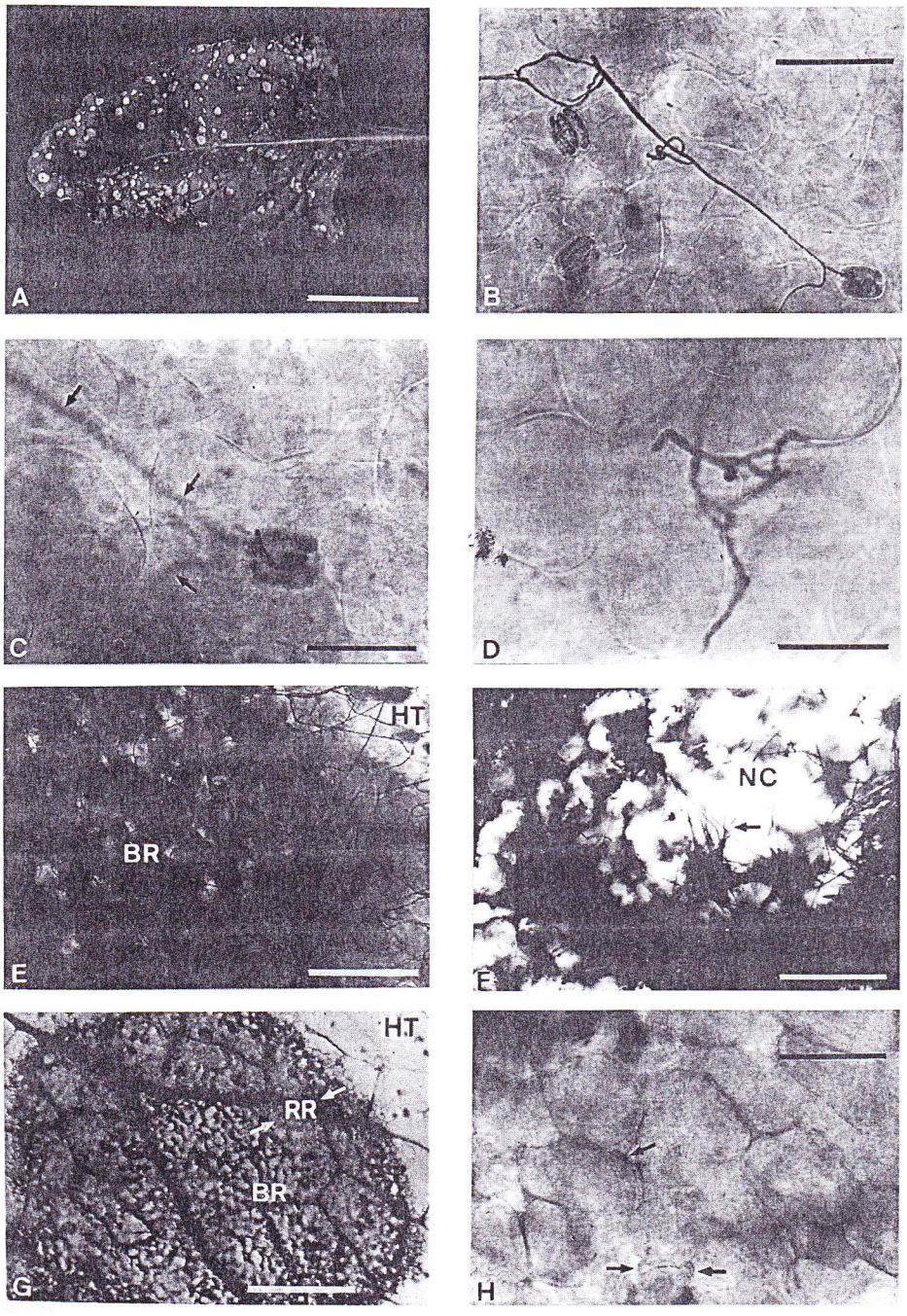

A
B
C
D
HT
BR
E
NC
F
HT
RR
BR
G
H

Table 1
Comparison between the resistance of *B. vulgaris, B. procumbens* and B. webbiana

Species	n	N	s	i_A	s_{iA}
B. vulgaris	20	297	15	53	11.5
B. procumbens	20	343	50	7.6	6.4
B. webbiana	20	463	39	0.1	—

n: amount of plants tested;
N: mean number of conidia per cm^2 of inoculated leaf, evaluated by examination of stained plastic film under the microscope;
s: standard deviation of N;
i_A: mean attack index;
s_{iA}: standard deviation of the attack index i_A.

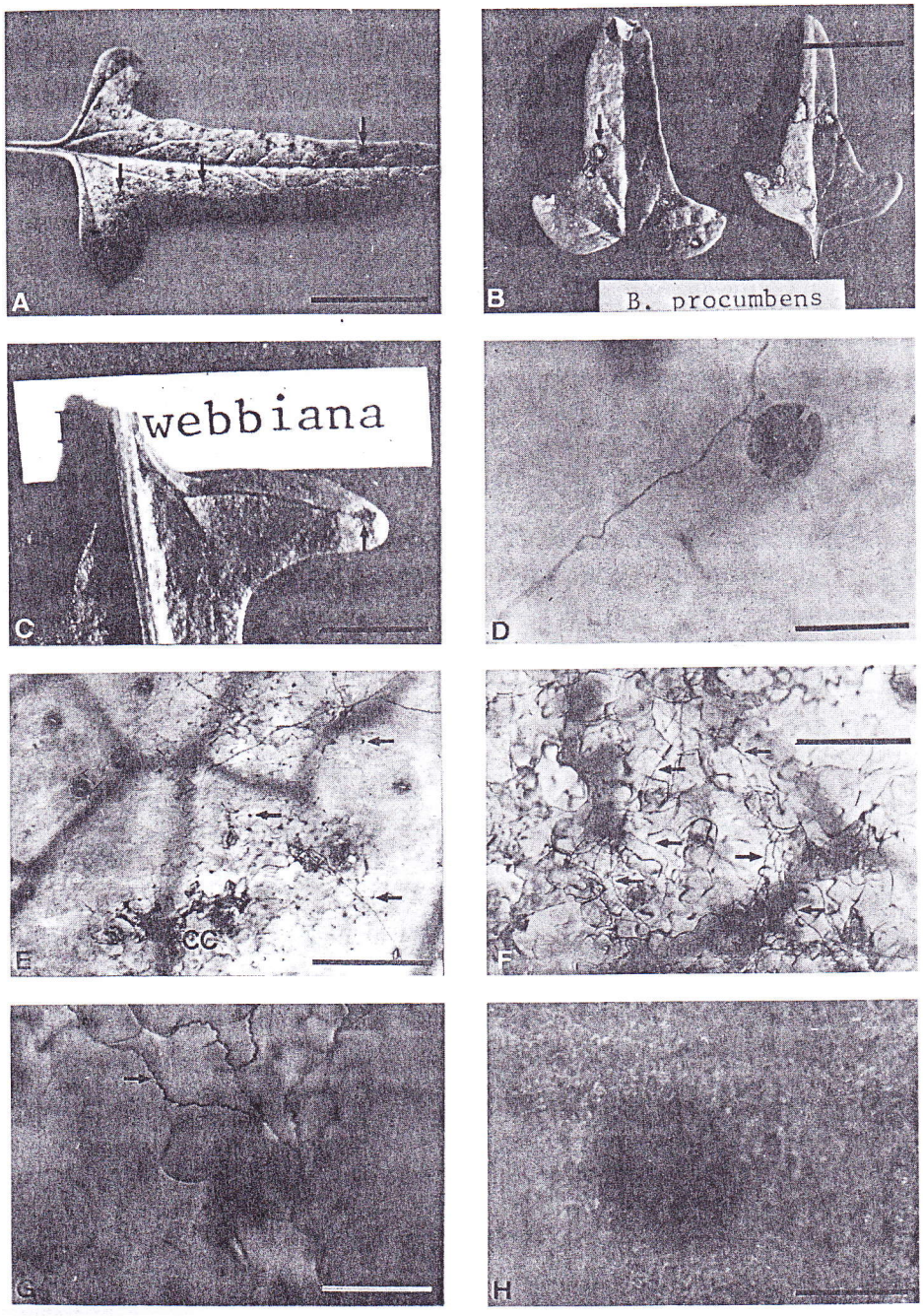
B. procumbens showed a fleck reaction (Plate 2A) 7 days after inoculation, whereas typical red ringed spot necrosis appeared to some extent 10 days after inoculation (Plate 2B).

Under conditions which were appropriate to *C. beticola* sporulation on necrotic lesions of *B. vulgaris,* conidia formation was not observed on *B. webbiana* flecks, but it was on the typical *B. procumbens* red ringed necrosis. Cultures of *C. beticola* isolates obtained from *B. webbiana* flecks were induced to sporulate *in vitro,* and conidia were then sprayed on *B. vulgaris.* After 10 days, typical lesions were formed.

2. Light microscopy

Conidial germination, stomatal penetration and intercellular proliferation of *C. beticola* was observed in *B. vulgaris* during the first 8 days after inoculation (Plates 1B, C, and D). After about 10 days, lesions appeared with a necrotic center occupied by sporulating mycelium (Plate 1F), which was surrounded by a blue ring (Plate 1E) due to dye accumulation in the intercellular spaces. This blue ring was invaded by mycelium as well as the outer tissues (Plate 1E). After about 10 days, a red ring developed in the outer blue ring tissues (Plate 1G). This red ring was surrounded by cells accumulating the dye and forming a thin blue ring. The droplet-like material supposed to be callose by STEINKAMP *et al.* (1979) and FEINDT *et al.* (1981b) was observed on walls of the red ring cells about 20 days

Plate 2: (A) *B. procumbens* leaf symptoms 7 days after inoculation with *C. beticola* conidia showing an intensive fleck reaction (arrows) — scale bar (1.5 cm). (B) *B. procumbens* leaf symptoms 12 days after inoculation with *C. beticola* conidia showing subsequent necrosis — scale bar (2 cm). (C) *B. webbiana* leaf symptoms 4 days after inoculation with *C. beticola* conidia showing fleck reaction (arrow) — scale bar (0.8 cm). (D) *B. webbiana* stomatal penetration by *C. beticola* — scale bar (34 μm). (E) Infection site of *B. webbiana* 8 days after inoculation with *C. beticola* showing epidermis wall thickening (arrows) and collapsed brown parenchymatous cells (CC) along the mycelium pathway — scale bar (136 μm). (F) Infection site of *B. webbiana* 18 days after inoculation with *C. beticola* showed poor mycelial ramifications (arrows) — scale bar (68 μm). (G) Details of plate 2E showing the gnarled aspect of the mycelium (arrows) — scale bar (34 μm). (H) The cells from the red flecks (RF) of the *B. webbiana* leaves colored by fluorescein diacetate and observed under UV light did not fluoresce and appeared as dark spots in a background of green light — scale bar (600 μm)


A
B
B. procumbens
webbiana
C
D
E
cc
F
G
H

after inoculation (Plate 1H). In *B. webbiana*, *C. beticola* was found in red fleck tissues (Plate 2E) about 4 days after inoculation. Brown collapsed cells (Plate 2E) were sometimes found along the mycelium pathway. A thickening of the epidermal cell walls (Plate 2E) was always noticed around the infection site. Mycelium was scarce and slow growing (Plates 2F and 2G) compared to that in *B. vulgaris*. No droplet-material deposition, characteristic of *B. vulgaris*, was found.

With *B. procumbens*, stomatal penetration was followed either by a fleck reaction (Plate 2A) or a susceptible reaction (Plate 2B) which led to necrotic lesions and fungal sporulation under suitable conditions. The necrotic tissue was surrounded by a red ring, but without the characteristic droplet-like depositions seen in the *B. vulgaris* reaction.

3. Fluorescent microscopy

Red flecks were treated with fluorescein diacetate as they appeared on *B. webbiana*. It was found that cells from such treated flecks did not fluoresce under UV light, whereas cells from surrounding healthy tissues did. *B. webbiana* red flecks thus appeared as black spots under UV light when stained by fluorescein diacetate (Plate 2H).

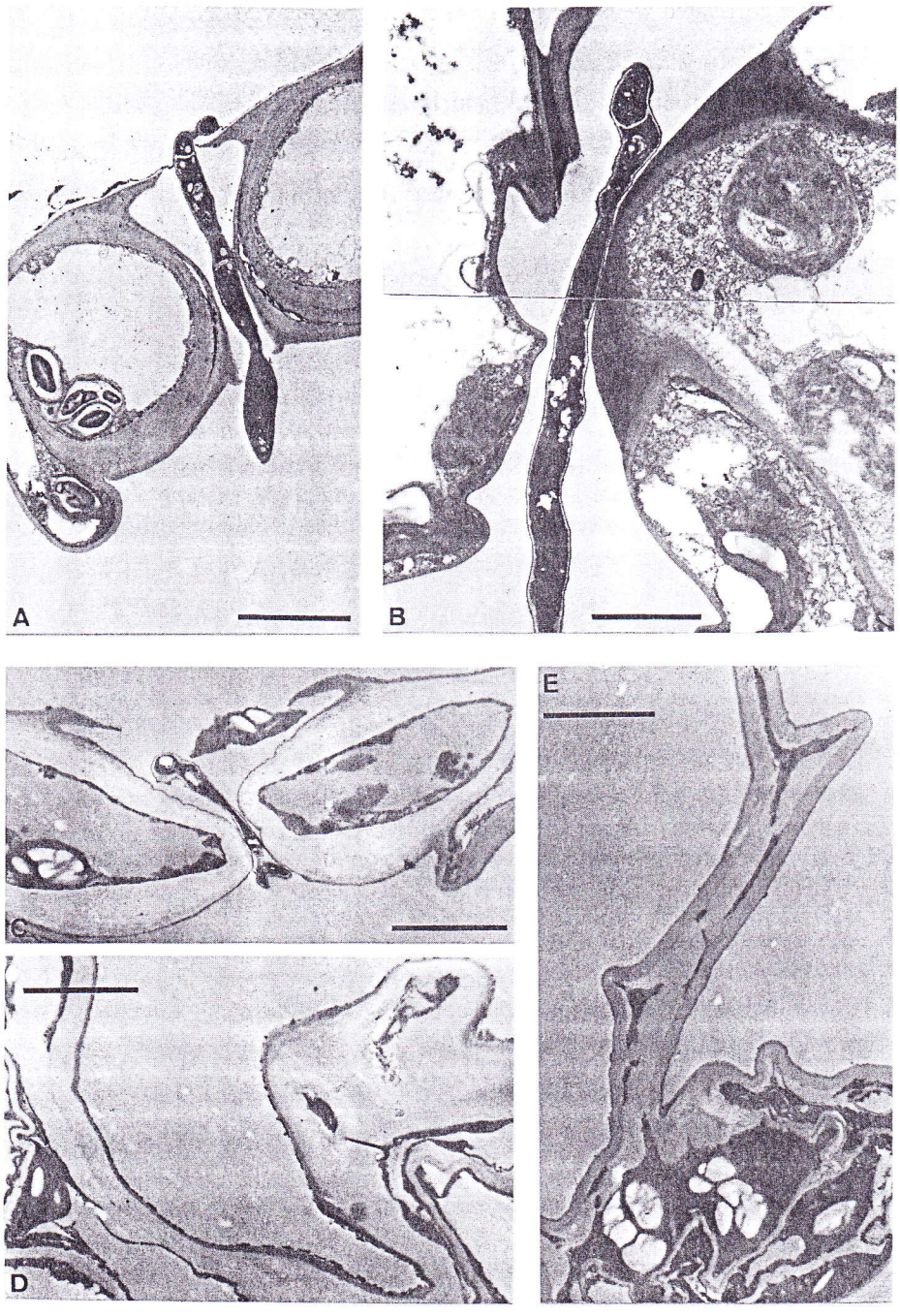
4. Phase contrast microscopy

Cell collapse was not observed by phase contrast microscopy in 2 day-old flecks on *B. webbiana*. However, cell collapse was obvious in 20 day-old flecks of *B. webbiana* as it extended to several cell layers.

5. Electron microscopy

No remarkable alterations were found neither in host cells surrounding the intercellular mycelium nor in the mycelium itself (Plates 3A and 3B) in red flecks of *B. webbiana* observed by electron microscopy (TEM) 10 days after inoculation. In comparison, *B. vulgaris* necrosis, 10 days after inoculation, was already established and leaf tissues were totally collapsed even destroyed at the necrotic center. At 26 days after inoculation, leaf tissue collapse in *B. webbiana* flecks was obvious, as seen stomatal shape (Plate 3C), cell flattening (Plates 3D and E) and epidermal protuberances (Plate 3E). Cell wall thickening was often observed, especially in the epidermis layer (Plates 3D and E). In spite of the collapsed state, cell wall breakage was never observed. At 20 days after inoculation by *C. beticola*, *B. webbiana* and sugar beet plants were submitted for two days to conditions suitable to *C. beticola* sporulation on sugar beet leaf necrotic area. By SEM, no sporulation could be observed on *B. webbiana* flecks (Plates 4E, F), whereas heavy sporulation was found on *B. vulgaris* necrotic lesions (Plates 4A, B, C and D).

Plate 3: (A) 10 days old red flecks of *B. webbiana* infected leaves observed by TEM showing stomatal penetration by *C. beticola* — scale bar (5 μm). (B) In the leaves of *B. webbiana* the mycelium was only observed in the intercellular spaces. No enzymatic activity against host cell walls was recorded — scale bar (5 μm). (C) In 26 days old leaves of *B. webbiana*, the tissues structure observed by TEM was still good enough to enable visualization of stomatal penetration by *C. beticola* — scale bar (5 μm). (D, E) Extensive cell collapse and wall thickening were observed in 26 days old leaves of *B. webbiana*, but even if the cells were completely flattened, their walls were still intact — scale bar (5 μm)


A
B
C
D
E

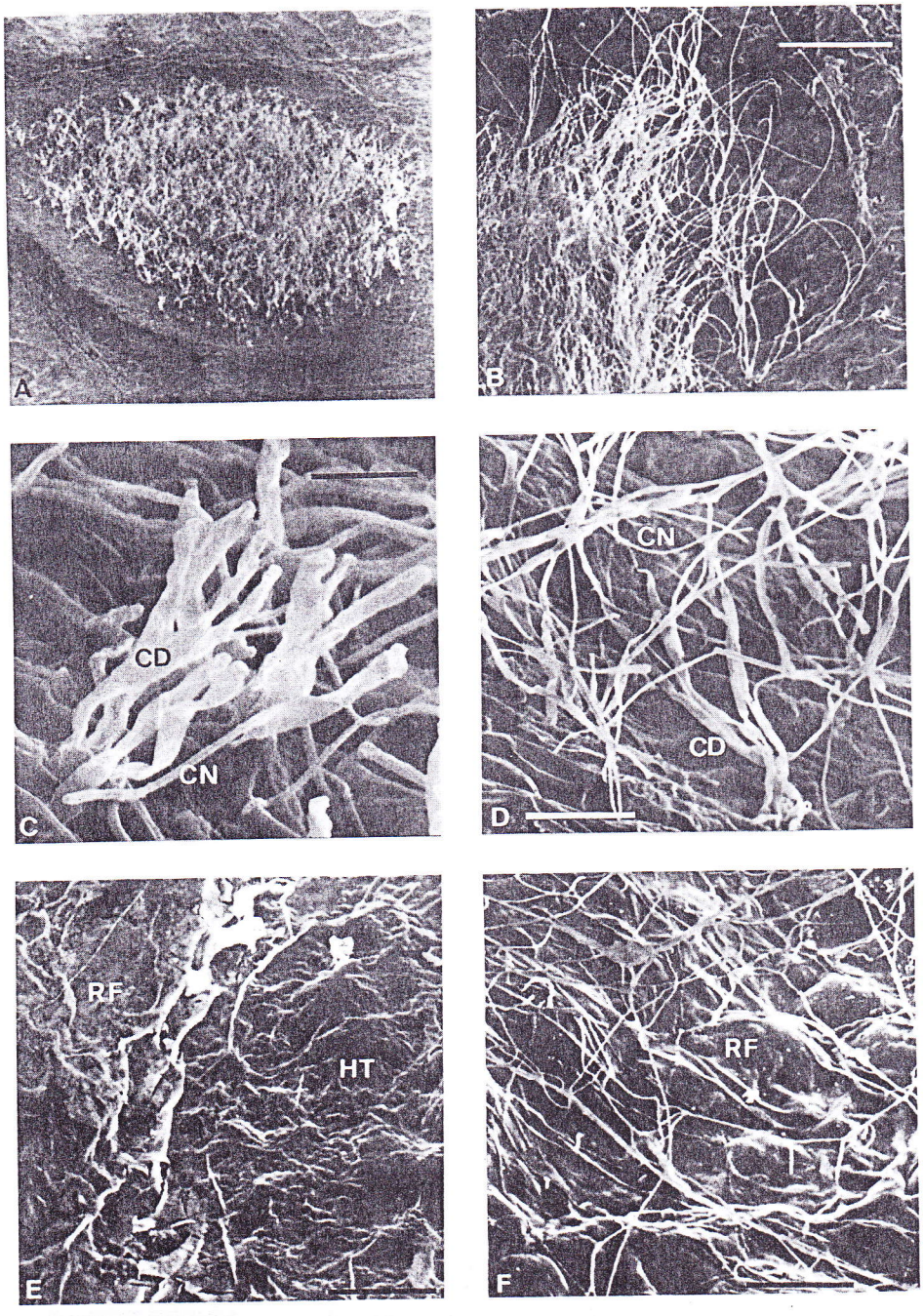
Discussion

Our light microscopy study of the infection process of *B. vulgaris* by *C. beticola* correlated well with the TEM studies made by STEINKAMP *et al.* (1979) and FEINDT *et al.* (1981 b). The first steps of disease development did not show any differences in terms of resistance between sugar beet and wild beets. Only after the development of necrotic lesions, which resulted from the collapse of leaf cells around the infection site, were resistance reactions apparent. In *B. vulgaris*, about 12 days after inoculation, ring-like reddening developed around the necrotic zone in the living tissues invaded by the fungus, together with a reduction in hyphal growth.

As pointed out by STEINKAMP *et al.* (1979), hyphae did not develop in the healthy tissues external to the red ring. The most resistant clone observed in our study belonged to the species *B. webbiana*. This clone showed an atypical red fleck reaction in response to a *C. beticola* infection, which appeared about two days after penetration of host tissue by the fungus. The fleck reaction may be linked to an active defence process (INGRAM 1982) of the host against the parasite, which would explain the poor fungal growth in flecks of *B. webbiana*. This is exemplified by the slow rise in hyphal density in host infected tissues, as observed by light microscopy. Some host cells may be found to be collapsed and brown along the mycelium pathway, which suggests a rapidly occurring cell death. This is consistent with the lack of fluorescence by the fleck cells after treatment by fluorescein diacetate. The lack of fluorescence of fleck cells as early as 5 days after inoculation is apparently not due to a lack of diffusion of fluorescein diacetate into the tissue, as no abnormal structures, such as wall thickening, were observed by TEM. The early occurring breakdown of the esterase system (WIDHOLM 1972) and the partial collapse of the fleck cells which was linked to the inhibition of hyphal growth, permits the use of the concept of hypersensitivity to describe the resistance of *B. webbiana* to *C. beticola* (INGRAM 1982, HOLLYDAY *et al.* 1981). The fleck reaction, however, does not kill the fungus, as it may be isolated from such flecks in axenic culture. The slowing down of hyphal growth in *B. webbiana* flecks is correlated with a lack of sporulation of the parasite on this host. *B. procumbens* showed a resistance intermediate between *B. webbiana* and *B. vulgaris*. Leaves from the upper part of the plant exhibited a fleck reaction identical to that of *B. webbiana*, whereas leaves from the lower part exhibited a susceptible reaction of typical red-ringed sporulating necrotic spots.

As previously mentioned, microscopic studies of *B. vulgaris* leaves showed that no successful resistant mechanism occurred up to 10 days after inoculation and fungal growth inhibition arised only as red-ring developed. Red-ring matura-

Plate 4: (A) Shows the necrosis of a *C. beticola* infected sugar beet leaf sample treated to induce sporulation. Extensive conidiophor formation was observed — scale bar (200 μm). (B) Details of the edge of a sporulating necrosis on sugar beet showing conidia still attached to conidiophores — scale bar (58 μm). (C) Details of some conidiophores (CD) cleared of their conidia — scale bar (5 μm). (D) Details of some conidiophores still carrying conidia (CN) — scale bar (17 μm). (E, F) Red fleck (RF) appearing after *B. webbiana* inoculation by *C. beticola*, induced to sporulation showed no conidia formation even when mycelial development could be observed on their surface — scale bar (17 μm)


A
B
CD
CN
C
CN
CD
D
RF
HT
E
RF
F

tion progressed from about 12 days up to 25 days after inoculation. This fungal growth inhibition has been tentatively explained by phytoalexin production, which was found in detectable amounts in *B. vulgaris* infected leaves, 3 weeks after inoculation (JOHNSON *et al.* 1976, MARTIN 1977).

As the red fleck reaction in *B. webbiana* leaves inhibit *C. beticola* fungal growth, the question arises as to whether the same phytoalexins as those described for *B. vulgaris* are implied in the *B. webbiana* resistance to *C. beticola*. Furthermore, an efficient cell recognition system must exist in *B. webbiana*, as the red fleck reaction appeared as early as two days after leaf penetration by the *C. beticola* mycelium. This recognition mechanism could be linked with PR-protein synthesis (SHINSHI *et al.* 1988).

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