European Journal of Plant Pathology **101**: 399–404, 1995.

Microscopical studies of the effect of metalaxyl on the interaction between sunflower, *Helianthus annuus* L. and downy mildew, *Plasmopara halstedii*

Saïd Mouzeyar[1], Felicity Vear[2] and Denis Tourvieille de Labrouhe[1]
[1]*INRA, Laboratoire de Pathologie Végétale, 12 avenue du Brezet 63039 Clermont-Ferrand, France;* [2]*INRA, Station d'Amélioration des Plantes. Domaine de Crouelle 63039 Clermont-Ferrand, France*

Accepted 21 December 1994

Key words: Helianthus annuus, hypersensitivity, metalaxyl, *Plasmopara halstedii*

Abstract

The mode of action of the fungicide metalaxyl against *Plasmopara halstedii*, the causal agent of sunflower downy mildew, was studied following its application before, during and after artificial contamination of seedlings. The development of the fungus within the treated seedlings was examined microscopically and compared to that occuring in untreated genetically susceptible or resistant genotypes. Hypersensitive-like reactions and necrotic zones leading to the inhibition of fungus growth within the hypocotyl were observed for the three modes of application. This suggests that sunflower defence mechanisms activated by genetical resistance are also involved in the control of downy mildew by metalaxyl.

Introduction

Downy mildew, *Plasmopara halstedii* (Berl.) Farl. et de Toni, of sunflower, *Helianthus annuus* L., can be effectively controlled either by the use of genetically resistant varieties, or by seed treatment with metalaxyl. This fungicide is a systemic acylalanine with an acropetalous circulation [Cohen and Coffey, 1986]. For the sunflower crop, it is used as a seed treatment. It may, however, also have a curative effect if sprayed onto leaves or roots [Melero-Vera *et al.*, 1982].

The obligate parasitic nature of *P. halstedii* makes it difficult to study the direct effect of fungicides on this fungus. However, Oros and Viranyi [1987] showed that *P. halstedii* displays differences in sensitivity to various fungicides depending on its developmental stages. Furthermore, Viranyi and Oros [1991] demonstrated that the host dependant growing mycelium is the most sensitive to metalaxyl. In contrast, host-independant events such as zoospore release or cystospore germination are less sensitive to this fungicide.

This paper describes histological modifications which occur when genetically susceptible sunflower seedlings, infected with downy mildew, are treated with metalaxyl, in comparison with infected but untreated genetically susceptible or resistant sunflower seedlings.

Materials and methods

Sunflower genotypes

Two genotypes, HA89 and RHA266 were used. HA89 is an inbred line, bred by USDA (INRA form coded CD), susceptible to all downy mildew races. RHA266 is an inbred line bred by USDA and containing the resistance gene Pl1, confering resistance to race 1 (European race) [Fick and Zimmer, 1974]. This genotype shows cotyledon limited infection [Vear, 1978] or type II resistance [Mouzeyar *et al.*, 1994]. These lines are maintained at INRA, Clermont-Ferrand by selfing under paper bags.

Downy mildew isolate
An isolate of race 1 was used. Only sunflower genotypes with no resistance genes are susceptible to this race. The fungus is maintained at INRA, Clermont-Ferrand on the sunflower line HA89.

Chemical product
Treatments were made with commercial quality Apron 35-SD containing 35% metalaxyl (Ciba, France).

Infection methods and metalaxyl treatments
Seedling infection. Untreated susceptible and resistant seedlings were prepared and infected as described previously [Mouzeyar *et al.*, 1993]. Two-day-old seedlings were soaked in a freshly prepared suspension of downy mildew zoosporangia for 4 h. The infected plants were sown in trays of soilless compost and placed in a growth chamber at 18 ± 1 °C. In all experiments, the concentration of zoosporangia suspension was adjusted to 40000 zoosporangia/ml.

Metalaxyl treatments
Three application methods were used and three replicates of 50 plants per treatment were studied.

Method A. Treatment before downy mildew infection: After 36 h germination, the seeds which had germinated were soaked for 12 h in an aqueous suspension of metalaxyl. The concentration was 70 mgl^{-1} (active ingredient: a.i.) They were then thoroughly rinsed under running water and infected in the same way as the untreated controls.

Method B. Treatment during downy mildew infection: the zoosporangia were suspended in an Apron 35-SD suspension in place of water, giving 40000 zoosporangia/ml and 20 mgl^{-1} (a.i.) of metalaxyl. The seeds were soaked for 8 h in this mixture and then sown as above.

Method C. Treatment after downy mildew infection: this treatment was primarily to study the effect of metalaxyl on downy mildew mycelium within the sunflower seedling. The germinated seeds were infected and sown as for the controls and then 3 days later, the emerging seedlings were drenched with a 200 mgl^{-1} suspension of metalaxyl (a.i.).

Symptom observations
Three weeks after planting, the trays were covered with plastic bags for 48 h to induce sporulation of the fungus. Plant stunting and leaf chlorosis were also recorded.

Microscopic observations
After planting, 5 infected susceptible seedlings, 5 infected resistant seedlings and 10 seedlings from each metalaxyl treatment were removed every day from the trays of compost, washed and fixed in FAA (Formalin-Acetic acid-Alcohol: 5:5:90) [Johansen, 1940]. Between 10 and 50 free hand cross sections were cut along the whole length of the seedlings, according to fungal development.

Observations of UV-autofluorescence were made as described previously [Mouzeyar *et al.*, 1993] using an Olympus BH2 microscope equipped with a 100 watt HBO mercury vapour lamp and a DMU dichroic mirror (Excitation filter 200–400nm, BP: 360, L420 filter). The microphotographs were taken using 160T Ektachrome film.

Results

Macroscopic observations
The susceptible control plants showed normal downy mildew symptoms such as stunting leaf chlorosis and fungal sporulation. In contrast, the resistant control plants showed only limited symptoms, with slight fungus sporulation on some cotyledons. Following metalaxyl treatments, the plants showed no downy mildew symptoms except for treatment C where plants had slightly stunted hypocotyls.

Microscopic observations
Susceptible control. The fungus penetrated the base of the hypocotyl and developed haustoria and intercellular mycelium (Fig. 1A). Fifteen days after infection, invasion appeared systemic with all plant parts colonized, including the epicotyl and the true leaves. In all plants, the only histological reaction was a limited UV-fluorescence in the intercellular spaces around the hyphae.

Resistant control. The fungus was found to penetrate the base of the hypocotyl as often as in the

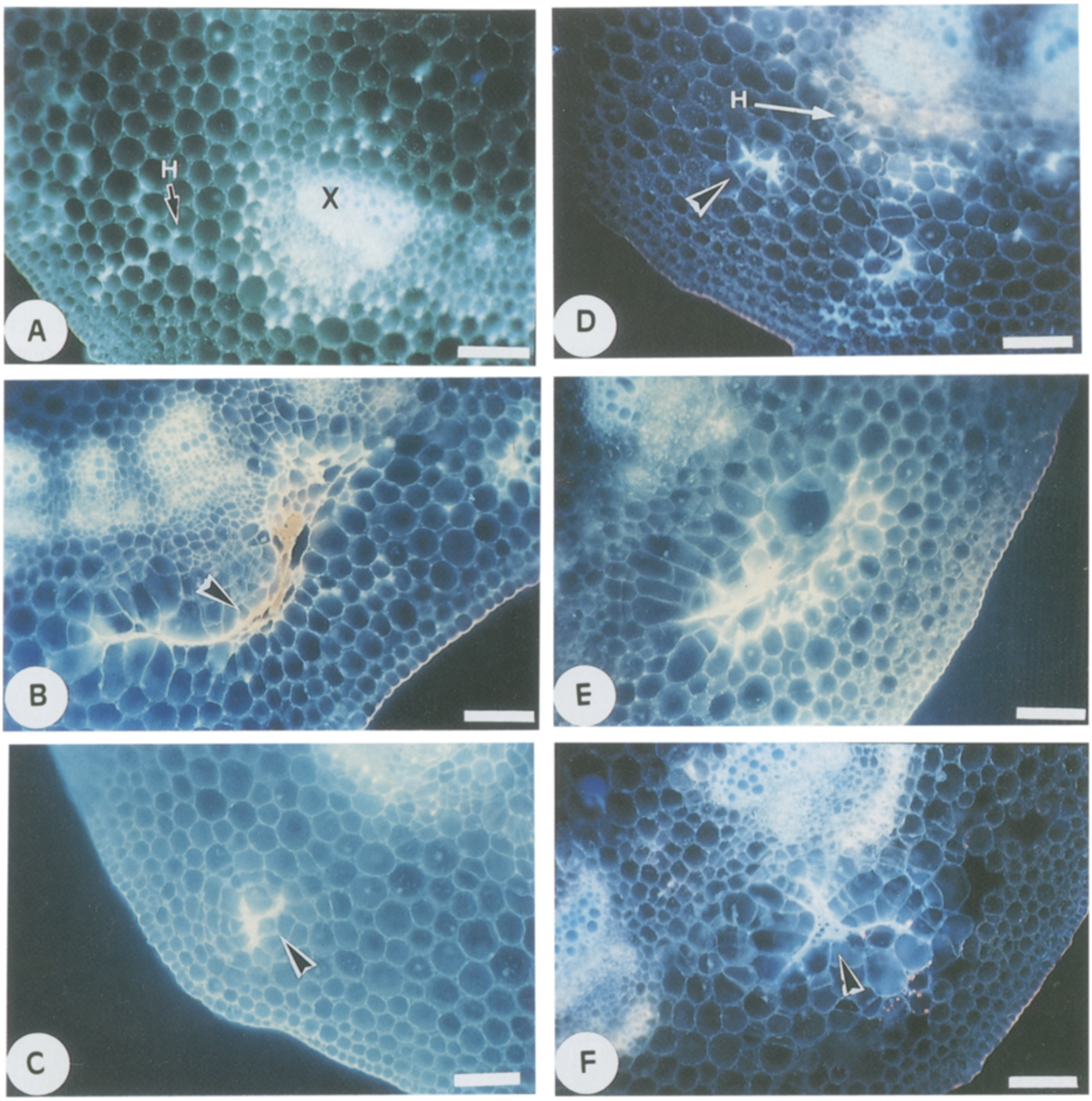

Fig. 1. UV-fluorescence micrographs of transverse hypocotyl section of untreated sunflower seedlings (A, B, C) or treated with 70 mgl^{-1} of metalaxyl (D, E, F) and then infected with *P. halstedii.* (A) Fifteen-day-old susceptible seedling; the UV-fluorescence is localized around the intercellular hyphae. (H) = Haustorium; X = Xylem (scale bar = 160 μm); (B) Ten-day-old resistant seedling; a strong hypersensitive-like reaction develops within the parenchyma (arrow) (scale bar = 160 μm); (C) Fifteen-day-old resistant seedling with a 'spider's web' necrosis in the upper part of the hypocotyl (arrow) (scale bar = 160 μm); (D) Cell divisions in the basal part of hypocotyl of treated susceptible seedling (arrow); some haustoria appear normal; (H) = haustorium (scale bar = 160 μm); (E) Ten-day-old treated and infected susceptible seedling; a hypersensitive-like reaction develops within the hypocotyl (scale bar = 160 μm); (F) Fifteen-day-old treated and infected susceptible seedling; showing a 'spider's web' necrosis (arrow) (scale bar = 160 μm).

susceptible, forming haustoria and intercellular hyphae. However, 5 days after infection, hypocotyl cells close to the hyphae became brown and granular and fluoresced under UV light. They showed a hypersensitive-like reaction leading to formation of necroses in the cortical parenchyma (Fig. 1B). Cell divisions occured around these necroses and the general aspect of these zones gave a 'spider's web' effect (Fig. 1C). Fifteen days after infection, the fungus was limited to the hypocotyl, the epicotyl and leaves were free of the parasite.

Treatment A. Downy mildew mycelium was found at the base of the hypocotyl, but less frequently than in the controls. As in genetically resistant plants, hyphae and haustoria were observed, with sunflower cells close to the fungus often appearing to have divided at least once (Fig. 1D). From 8 days after infection, the cytoplasm of these cells became granular and fluoresced strongly under UV light (Fig. 1E). Necroses were observed in the cortical parenchyma (Fig. 1F) and fungal development appeared to be stopped.

Treatment B. The number of infection sites observed at the base of the hypocotyl was considerably reduced compared with treatment A and the controls. Apparently abnormal haustoria were sometimes visible within cells close to the epidermis. As in treatment A and the resistant control, these cells were highly fluorescent with granular cytoplasm (Fig. 2A). However, in contrast with the above treatment, necroses with cell divisions

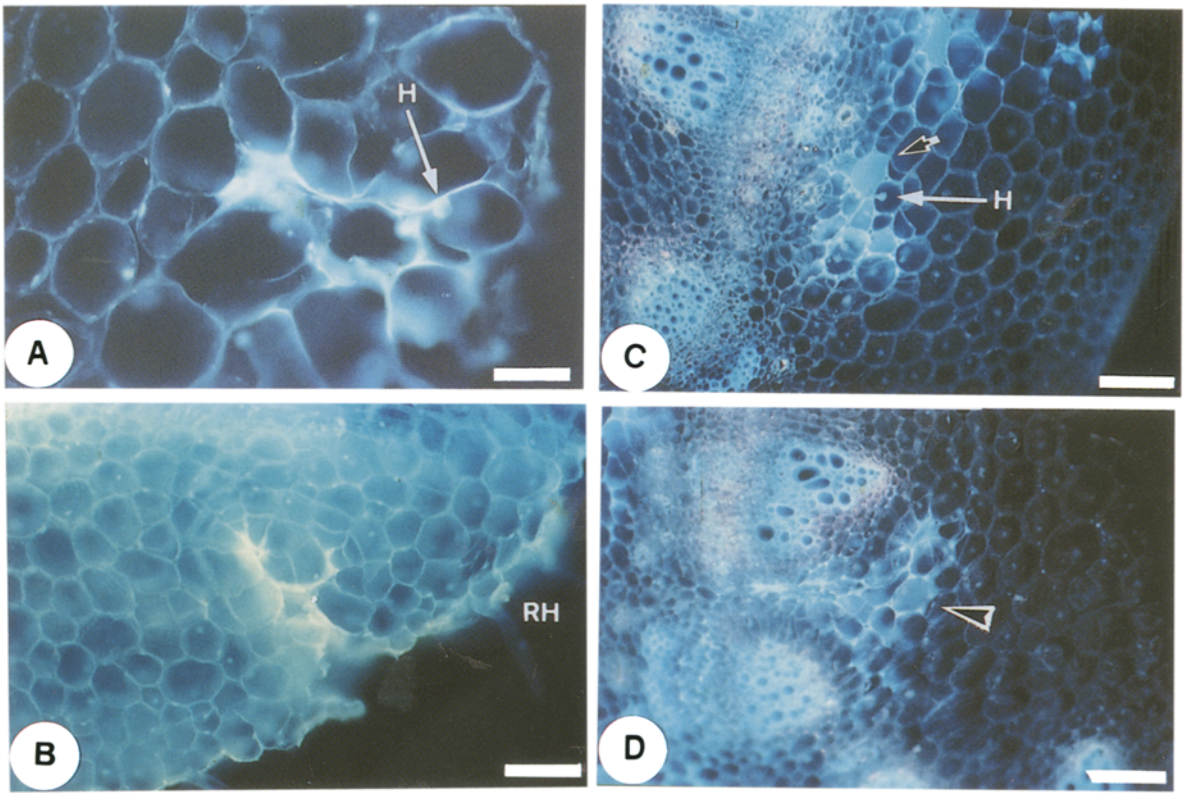

Fig. 2. UV-fluorescence micrographs of sunflower susceptible seedlings treated with metalaxyl and infected with *P. halstedii* (A, B) treated with 20 mgl^{-1} of metalaxyl during infection. (A) Ten-day-old seedling; penetrated cells at the base of the hypocotyl, note the intense fluorescence of the cell walls. (H) = haustorium (scale bar = 64 μm); (B) Fifteen-day-old seedling; necrosis and cell divisions at a penetration site (RH) = root hair (scale bar = 160 μm). (C, D) drenched with 200 mgl^{-1} of metalaxyl 3 days after infection; (C) Thirteen-day-old seedling, cells reacting hypersensitively at the base of the hypocotyl (arrow). (H) = haustorium (scale bar = 160 μm); (D) Eighteen-day-old seedling; with a necrosis (arrow) between vascular bundles at the cotyledonary node (scale bar = 160 μm).

developed only at the penetration sites (Fig. 2B) and the interior of the hypocotyl was free of *P. halstedii.*

Treatment C. Since chemical treatment was applied only after infection, first mycelial development was as in the susceptible control and ten days after treatment, mycelium was observed in the basal part of the hypocotyl and haustoria had developed. In the external part of the parenchyma, cells close to or containing the parasite, showed characteristic signs of resistance, granular brown cytoplasm which fluoresced strongly under UV light (Fig. 2C). However, close to the vascular bundles, cells had only just begun to react. Fifteen days after treatment, most of the hyphae in the outer part of the cortex appeared to have been inhibited and none had reached the upper part of the hypocotyl. In contrast, necroses were visible around hyphae that were close to the vascular bundles at the cotyledonary node (Fig. 2D), but the epicotyl and leaves were not contaminated.

Discussion

P. halstedii development in susceptible sunflower seedlings was described in detail by several authors [Allard, 1978; Gray et Sackston, 1985; Wehtje *et al.*, 1978]. Mouzeyar *et al.* [1993] observed that colonization was systemic and that plants reacted only by a weak production of callose and lignin. In contrast, with genetically resistant seedlings, an unusual hypersensitive-like reaction developed in the hypocotyl. Cell divisions occured around the parasite, followed by lignification and development of necrotic zones which inhibited the mycelium. These reactions were observed in all incompatible combinations tested [Mouzeyar *et al.*, 1994]. Thus, they constitute a clear distinction between susceptible and resistant phenotypes.

Macroscopic observations showed that the concentration of metalaxyl used for each mode of application gave full protection against *P. halstedii.* They were determined empirically as maximum doses with no toxic effect on plants and then used for the microscopic studies.

The results described here indicate that metalaxyl treatment causes histological changes in genetically susceptible plants infected with downy mildew. The hypersensitive-like reactions, cell divisions and formation of necroses that were always visible, closely resemble the reactions observed in genetically incompatible combinations. However, the time of appearance and the location of the necroses differed according to time of metalaxyl application. This is probably due to absorption and circulation dynamics of metalaxyl and/or to the state of parasitic growth.

This is the first report of involvement of sunflower defence mechanisms in the control of *P. halstedii* by metalaxyl. This finding could explain why the host-dependant stages of *P. halstedii* are more susceptible to metalaxyl than the host-independant stages [Viranyi and Oros, 1991]. The primary site of action of metalaxyl in *P. halstedii* is unknown. It is thought to inhibit RNA synthesis [Davidse *et al.*, 1981], the target being an α-amanitin-insensitive RNA polymerase [Davidse *et al.*, 1983]. Nevertheless, the results described above suggest, as proposed by Viranyi and Oros [1991], that metalaxyl may affect some processes in the fungus that are essential for establishment of susceptibility.

Activation of host defence mechanims by metalaxyl has been reported for several plant-fungus interactions. This activation includes histological changes such as haustoria encapsulation by callose deposits [Hickey and Coffey, 1980] or development of restricted hypersensitive-like lesions [Lazarovits and Ward, 1982; Stössel *et al.*, 1982; Ward *et al.*, 1980] and biochemical changes such as phytoalexin accumulation. Cahill and Ward [1989] and Cahill *et al.* [1993] demonstrated that metalaxyl induces resistance in otherwise susceptible soybean plants infected with *Phytophthora megasperma*. Furthermore, they showed that the glyceollin accumulation and the decrease in abscissic acid induced were similar to those observed in incompatible combinations. However, it remains unclear whether this fungicide directly affects the fungus or indirectly by activating host defence reactions.

The present results suggest that it should be possible to compare the genes activated in genetic resistance and those triggered by metalaxyl treatment. In the long run, this should help to determine the molecular basis of sunflower defence mechanisms against *P. halstedii.*

Acknowledgements

We wish to thank Dr. G. Bompeix for critically reading the manuscript and Ciba (France), Prograin-Génétique and CETIOM for their financial support.

References

Allard C (1978) Invasion et colonisation systémique de la plantule de tournesol (*Helianthus annus* L.) par le *Plasmopara halstedii* (Farl.) Berl. et de Toni. Annales de Phytopathologie 10: 197–217

Cahill DM and Ward EWB (1989) Effect of metalaxyl on elicitor activity, stimulation of glyceollin production and growth of sensitive and tolerant isolates of *Phytophthora megasperma* f.sp. *glycinea*. Physiological and Molecular Plant Pathology 35: 97–112

Cahill DM, Morris PF and Ward EWB (1993) Influence of metalaxyl on abscissic acid levels in soybean hypocotyls infected with *Phytophthora sojae*. Physiological and Molecular Plant Pathology 42: 109–121

Cohen Y and Coffey MD (1986) Systemic fungicides and the control of oomycetes. Annual Revue of Phytopathology 24: 311–338

Davidse LC, Gerritsma OCM and Hofman AE (1981) Mode d'action du métalaxyl. Phytiatrie-Phytopharmacie 30: 235–244

Davidse LC, Hofman AE and Velthuis GCM (1983) Specific interference of metalaxyl with endogenous RNA polymerase activity in isolated nuclei from *Phytophthora megasperma* f. sp. *medicaginis*. Experimental Mycology 7: 344–361

Fick GN and Zimmer DE (1974) RHA271, RHA273 and RHA274 sunflower parental lines for producing downy mildew resistant hybrids. Farm Research 32: 7–9

Gray AB and Sackston WE (1985) Early stages of infection of resistant and susceptible sunflower seedlings by three races of *Plasmopara halstedii*. Canadian Journal of Botany 63: 1725–1729

Hickey EL and Coffey MD (1980) The effects of Ridomil on *Peronospora pisi* parasitizing *Pisum sativum*: an ultrastructural investigation. Physiological Plant Pathology 17: 199–204

Johansen DA (1940) Plant Microtechniques. McGraw-Hill Book Co Inc. New York.

Lazarovits G and Ward EWB (1982) Relationship between localized glyceollin accumulation and metalaxyl treatment in the control of Phytophthora rot in soybean hypocotyls. Phytopathology 72: 1217–1221

Melero-Vara JM, Garcia-Baudin CL, Herrera C and Jimenez-Diaz RM (1982) Control of sunflower downy mildew by metalaxyl. Plant Disease 66: 132–135

Mouzeyar S, Tourvieille De Labrouhe D and Vear F (1993) Histopathological studies of resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii*). Journal of Phytopathology 139: 289–297

Mouzeyar S, Tourvieille De Labrouhe D and Vear F (1994) Effect of host-race combination on resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii*). Journal of Phytopathology 141, 249–258

Oros G and Viranyi F (1987) Glasshouse evaluation of fungicides for the control of sunflower downy mildew (*Plasmopara halstedii*). Annals of Applied Biology 110: 53–63

Stössel P, Lazarovits G and Ward EWB (1982) Light and electron microscopy of Phytophthora rot in soybeans treated with metalaxyl. Phytopathology 72: 106–111

Vear F (1978) Réaction de certains génotypes de tournesol résistants au Mildiou (*Plasmopara helianthi*) au test de résistance sur plante. Annales de l'Amélioration des Plantes 28: 327–332

Viranyi F and Oros G (1991) Developmental stage response to fungicides of *Plasmopara halstedii* (sunflower downy mildew). Mycological Research 95: 199–205

Ward EWB, Lazarovits G, Stössel P, Barrie SD and Unwin C H (1980) Glyceollin production associated with control of Phythophthora rot of soybeans by the systemic fungicide, metalaxyl. Phytopathology 70: 738–740

Wehtje G, Littelfield L and Zimmer DE (1979) Ultrastructure of compatible and incompatible reactions of sunflower to *Plasmopara halstedii*. Canadian Journal of Botany 57: 315–323