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BARD

FINAL REPORT

PROJECT NO. US-1740-89

**Basis for the Weakening of Propagules of Soilborne
Plant Pathogens by Sublethal Heat and Chemical
Disinfestation and Its Implications for Disease
Control**

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1994

DATE: April 6, 1994

BARD project No. US-1740-89

Title:

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disease control

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Project's starting date: October 1, 1990

Type of Report: 1st Annual ☐ 2nd Annual ☐ Final ☒

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ABSTRACT

Propagules of soilborne plant pathogens which survive sublethal treatments may be partially damaged and thus more vulnerable to biocontrol agents. Measuring the weakening of pathogen propagules is essential for the development of biorational management plans.

In the U. S., studies were carried out on i) combining sublethal heating with the biocontrol fungus Talaromyces flavus, ii) developing a method to quantify weakening in microsclerotia of Verticillium dahliae, and iii) combining T. flavus and Gliocladium roseum with sublethal metham sodium for control of V. dahliae. Sublethal heating of V. dahliae had significant effects on the growth rate of colonies produced from the heated microsclerotia. In addition, these colonies produced few new microsclerotia and these microsclerotia were slower than those from the control treatments to become melanized. There was a synergistic interaction between heating and T. flavus resulting in a delay in melanin deposition, and an additive effect on disease incidence. Methods developed for measuring weakening in other fungi were found not suitable for microsclerotia of V. dahliae because of the thick walls and melanin content. A method using sodium dodecyl sulfate polyacrylamide gel electrophoresis was developed. Several protein bands were associated with weakening by metham sodium. Sublethal rates of metham sodium significantly affected the growth rate of V. dahliae but not T. flavus. In a greenhouse test, neither the low rate of the metham sodium nor the two biocontrol fungi suppressed Verticillium wilt of eggplant. However, the combination of the low rate of the metham sodium with either or both biocontrol fungi resulted in significantly fewer diseased plants than the disease control.

In Israel, the weakening phenomenon was studied using ultrastructural, vital staining and survival parameters. Combining heating and Benlate, at sublethal doses resulted in a synergistic effect in the control of Macrophomina phaseolina, as tested in the laboratory, simulation and field conditions. A similar trend was obtained with other combinations of stress agents and pathogens. We further characterized the weakening phenomenon by studying the heat shock response in F. oxysporum by comparing pairs of closely related strains, which differ in their ability to survive elevated temperatures. The more thermotolerant strain within each pair had a higher capacity to synthesize proteins, and responded rapidly to heating with a faster repression of heat shock protein synthesis and by a fast resumption of protein synthesis.

Development of alternative control methods is increasingly important as methyl bromide and other prominent pesticides are withdrawn from the market and environmentally benign pest control strategies are sought. Results from studies in the U. S. and Israel indicate that combining methods of control, for reducing pesticide dosage, while improving disease control, can be feasible and attainable.

B. Objectives of the research

- a. To determine the effects of sublethal dosages of heat and chemical fumigants as incitants of propagule weakening for the plant pathogenic fungi Fusarium and Verticillium at the cellular and population levels;
- b. To determine the contribution of the propagule weakening effect to biocontrol of these plant pathogenic fungi; and
- c. To elucidate the physiological basis of the propagule weakening effect.

C. Methodology, data, results and discussion

I. STUDIES CARRIED OUT IN THE U. S.

1. Combining heating and the biocontrol fungus Talaromyces flavus to control V. dahliae
2. Alterations in proteins in microsclerotia of V. dahliae in response to sublethal metham sodium
3. Effects of sublethal metham sodium on V. dahliae, T. flavus and control of Verticillium wilt of eggplant through a combination of Gliocladium roseum, T. flavus and sublethal metham sodium

1. COMBINING HEATING AND THE BIOCONTROL FUNGUS T. FLAVUS

The thermophylic (Beauchat, 1988; T. Katan, 1985) fungus Talaromyces flavus has shown promise for control of Verticillium wilts (Davis *et al.*, 1986; Fravel *et al.*, 1986; Tjamos and Paplomatas, 1991; Tjamos *et al.*, 1991), but control is inconsistent and may not always be economically meaningful. Our hypothesis was that levels of heat lower than those usually generated during solarization might weaken V. dahliae, making it more susceptible to attack by T. flavus. Three main types of experiments were carried out: i) effects of various heating regimes on germination of V. dahliae microsclerotia and on formation and melanization of new microsclerotia formed from the heated, germinated microsclerotia, ii) survival and subsequent melanin deposition of heated V. dahliae microsclerotia in soil with and without T. flavus, and iii) pathogenicity of heated V. dahliae on eggplant and its interaction with T. flavus.

1.a. Effects of heating on germination and melanization.

Three heating regimes were selected to simulate temperatures generated by plastics during solarization (Katan *et al.*, 1976; Katan and DeVay, 1991). Regime 1 consisted of 31 C for 10 h followed by 35 C for 14 h; regime 2 was 33 C for 10 h then 36 C for 14 h; and regime 3 was 35 C for 10 h followed by 38 C for 14 h. Microsclerotia in aqueous suspension were not heated or heated in a water bath for 1 - 5 days with each regime. Microsclerotia were plated onto an agar medium and germination was recorded. The microsclerotia which germinated were observed for formation of new melanized microsclerotia.

All microsclerotia that were not heated had germinated 1 - 2 days after plating. Data in Fig. 1.a.1a¹ demonstrate that 90 % of the nonheated microsclerotia germinated within 24 h and 100 % within 48 h after plating. Regardless of the duration of heating, 90 % of the microsclerotia heated under regime 1 (31-35 C) had germinated 5 days after plating. Although additional days of heating with regime 1 did not affect the number of microsclerotia which had germinated after 5 days, it did affect the rate at which they germinated ($P \leq 0.0001$) (Fig. 1.a.1a). Additional days of heating with regime 2 or 3 affected both the final number of germinated microsclerotia ($P \leq 0.01$) and the rate at which they germinated ($P \leq 0.0001$) (Figs 1.a.1b and 1.a.1c). Under regime 2 (33 - 36 C), 90 % of microsclerotia heated for 1 day germinated in 5 days while 82, 62, 62, and 50 % of those heated for 2, 3, 4 and 5 days, respectively, had germinated in 5 days (Fig. 1.a.1b). Five days after plating, 87, 61, 14, 0.9 and 0.2 % of microsclerotia treated for 1 to 5 days with regime 3 (35 - 38 C), respectively, had germinated (Fig. 1.a.1c).

Of the microsclerotia which germinated (above), nearly 40 % of the colonies arising from microsclerotia in the nonheated control formed numerous, round and melanized microsclerotia by 3 days after plating and 75 % of the germinated microsclerotia had formed melanized microsclerotia by 7 days after plating (Fig. 1.a.2). In contrast, only 4 % of the colonies arising from microsclerotia which were heated for 1 day under regime 3 formed melanized 6 days after plating and 11 % at 9 days after plating. Compared to the control, there was at least a 3 day delay in melanin deposition in colonies arising from microsclerotia heated with regime 3. When treated with regime 2 for 1 day, 17 and 34 % of germinated microsclerotia produced new, melanized microsclerotia in 5 and 11 days after plating, respectively.

Heating regime (nonheated, regime 2 or regime 3) significantly affected both the final number of melanized microsclerotia ($P \leq 0.003$) and the rate at which they were produced (0.001). Additional days of heating with regime 3 significantly affected both the final number of melanized microsclerotia ($P \leq 0.01$) and the rate at which melanized

¹Figures and tables are placed at the end of each section. Literature cited for the first three sections is at the end of the U. S. section.

microsclerotia were produced ($P \leq 0.001$). Cells of newly formed microsclerotia from the heated treatment were initially formed in linear chains before becoming spherical as in the nonheated control.

1.b. Greenhouse test on effects of heating plus T. flavus on survival and melanin deposition by V. dahliae.

Nylon mesh envelopes (3 cm x 3 cm) were prepared by sewing mesh together with nylon thread. Microsclerotia of V. dahliae were heated as described previously and 10^3 microsclerotia were placed in each envelope using a syringe. The hole made by the syringe needle was sealed with glue. In preparation for transplanting 6-wk-old eggplants, 23 cm diam plastic pots were partially filled with nonsterile field soil. Three envelopes were placed on the soil surface. Envelopes were not treated or treated with T. flavus and 6-wk-old eggplants were transplanted on top of the envelopes. T. flavus was added as a drench or as prill. In the drenching treatment, 3 ml of 10^6 / ml ascospore suspension of T. flavus was placed on each envelope with an additional 1 ml in the center of the triangle formed by the envelopes. One week after transplanting, these plants received an additional 10 ml of 10^6 ascospores / ml. Alginate prill were prepared with or without ascospores of T. flavus as previously described (Fravel, *et al.*, 1985) using ground corn cobs as the carrier. For microsclerotia receiving prill, 0.1 g of prill was divided among the 3 envelopes in each pot. Prill were placed directly on the envelopes before transplanting the eggplants. Envelopes were destructively sampled at 4 and 8 weeks after transplanting by plating onto a medium semi-selective for *Verticillium* (ESA) (Ausher *et al.*, 1975).

With respect to survival of V. dahliae microsclerotia, there was a highly significant interaction ($P \leq 0.0075$) between heating and T. flavus reflecting a synergistic interaction between the heating and T. flavus. Results from the two sampling times were similar and data from the first sampling are shown (Fig. 1.a.3). In addition to the direct effect of the combination of heating and T. flavus on survival, the microsclerotia which did survive formed new melanized microsclerotia at a slower rate than the nontreated microsclerotia (Fig. 1.a.4).

1.c. Effect of heating and T. flavus on pathogenicity of V. dahliae on eggplant.

Eggplant seeds were planted in soilless potting mix in 7 cm-diam plastic pots. For plants receiving prill of T. flavus, 0.5 g of prill was incorporated into the potting mix at transplant. Plants receiving drenches were drenched with 10 ml of 10^6 ascospores / ml at 2, 3 and 5 weeks after seeding. Microsclerotia were not heated or were heated in a water bath for 1, 2 or 3 days under regime 3. Microsclerotia were mixed into a loamy sand soil to result in 40 microsclerotia / g of soil. Wilt incidence was recorded every 2 - 3 days for 92 days. Petioles were collected from plants with wilt symptoms and were plated onto ESA to confirm infection by V. dahliae. At the termination of the experiment, petiole isolations were made from all remaining plants.

At 49 days after planting (median wilt for many treatments), all but three treatments were similar to the healthy control (Fig. 1.4, Table 1.c.1). At the same time, the healthy controls and microsclerotia heated for three days were different from the disease control ($P \leq 0.02$). All other treatments were similar to the disease control although there was a trend ($P \leq 0.07$) for all treatments with T. flavus prill suppressing wilt as well as the combination of microsclerotia heated for 3 days and plants drenched with T. flavus. There was no overall interaction between heating and T. flavus ($P \leq 0.32$). Disease incidence in this experiment was low compared to other experiments. With higher disease incidence, it may be possible to distinguish more treatment differences.

Discussion

Freeman and Katan (1988) demonstrated that Fusarium oxysporum f. sp. vasinfectum propagules are weakened by sublethal heating. Lifshitz et al. (1983) also showed effect of sublethal heating on sclerotia of Sclerotium rolfsii. The levels of heat used in our experiments were sufficient to cause some loss of viability. It is plausible to envisage that the remaining, viable microsclerotia were weakened and that the weakening was expressed as reduced melanin deposition and pathogenicity, and increased susceptibility to T. flavus.

Sublethal heating of microsclerotia of V. dahliae had detrimental effects on germination of microsclerotia as well as on the rate of melanization of new microsclerotia and the final percentage of new microsclerotia which became melanized. A key enzyme(s) involved in the production of melanin in V. dahliae are inactivated by heating at 40 C for 35 minutes (Wheeler, 1982). Possibly sublethal heating inactivates this enzyme or disrupts its activity. Melanin functions in the defense of microsclerotia from microbial attack (Bell and Wheeler, 1986). Thus, reductions in the percentage of microsclerotia which become melanized and reduced rates of melanin deposition would be expected to result in increased susceptibility to attack by biocontrol agents.

The synergistic interaction of sublethal heating with the biocontrol fungus T. flavus in melanin deposition offers new opportunities for integrated disease control. For solarization, soil moisture and plastic type are optimized to generate heat sufficient to kill pathogen propagules. Because of the levels of heat generated, plants cannot be grown during solarization. Further, the duration of the treatment means that the land is out of production for 1 - 2 months. By combining sublethal solarization with biocontrol, it may be possible to keep the land in production by using a plastic mulch which generates lower temperatures that are usually used in solarization. This may have particular importance for perennial crops such as olive. Alternatively, it may be possible to reduce the length of time that the soil is covered by conventional solarization plastics if the solarization is combined with thermotolerant biocontrol agents.

A manuscript on this work has been prepared for Phytopathology (Tjamos and Fravel). Portions of this work will be presented at the Sixth International Verticillium Symposium and the annual meeting of the American Phytopathological Society.

Figure 1.a.1.

Effect of heating microsclerotia of *V. dahliae* for 1 - 5 days with three heating regimes. Aqueous suspensions of microsclerotia were not heated or heated in a water bath for 1 - 5 days with each of three different heating regimes. Regime 1 (Fig. 1.a.1.A) consisted of 10 h at 31 C then 14 h at 35 C. Regime 2 (Fig. 1.a.1.B) was 33 C for 10 h then 36 C for 14 h and regime 3 (Fig. 1.a.1.C) was 35 C for 10 h then 38 C for 14 h.

Figure 1.a.2.

Effect of heating on melanization of new microsclerotia formed from germinated microsclerotia which had been heated. Regime 2 was 33 C for 10 h then 35 C for 14 h. Regime 3 was 35 C for 10 h then 38 C for 14 h.

Figure 1.a.3.

Effect of heating and *T. flavus* on melanin deposition in newly formed microsclerotia of *V. dahliae*. Microsclerotia were not heated or heated 1 or 2 days using regime 3 (35 C for 10 h then 38 C for 14 h). Microsclerotia were placed in nylon packets and buried in soil with or without *T. flavus*. Microsclerotia were recovered after 4 weeks and plated onto agar medium. This figure indicates melanin deposition in new microsclerotia formed from the recovered microsclerotia.

Figure 1.a.4.

Effect of heating of microsclerotia of *V. dahliae* on Verticillium wilt of eggplant. Eggplants seedlings received prill of *T. flavus* (Fig. 1.a.4.A), drenches of *T. flavus* (Fig. 1.a.4.B), or water (Fig. 1.a.4.C). Microsclerotia were heated for 0 - 3 days with regime 3 (35 C for 10 h then 38 C for 14 h). Eggplant seedlings were transplanted into soil into which the heated microsclerotia had been mixed. Data indicate disease incidence.

Table 1.c.1. Effect of sublethal heating and *T. flavus* on Verticillium wilt of eggplant.
Columns 1 and 2 are for 49 days after transplant. Column three considers the entire disease progress curve. P value is the probability that These results are due to chance alone. Asterisks indicate significant differences ($P \leq 0.05$) and plus marks indicate a trend toward significant differences ($P \leq 0.10$).

	P value for treatment compared to healthy check	P value for treatment compared to disease control	P value for survival curve compared to disease curve
No Vd - No Tf	-	0.02*	0.09 ⁺
Vd - No heat - No Tf	0.02*	-	-
Vd - heat 1 day - No Tf	0.15	0.20	0.11
Vd - heat 2 days - No Tf	0.04*	0.70	0.98
Vd - heat 3 days - No Tf	1.0	0.02*	0.05*
No Vd - Tf drench	1.0	0.02*	0.10 ⁺
No Vd - Tf prill	0.32	0.07	0.10 ⁺
Vd - No heat - Tf drench	0.15	0.20	0.60
Vd - Heat 1 day - Tf drench	0.04*	0.70	0.76
Vd - Heat 2 days - Tf drench	0.15	0.20	0.11
Vd - Heat 3 days - Tf drench	0.32	0.07	0.06 ⁺
Vd - No heat - Tf prill	0.32	0.07*	0.76
Vd - Heat 1 day - Tf prill	0.32	0.07*	0.20
Vd - Heat 2 days - Tf prill	0.32	0.07*	0.34
Vd - Heat 3 days - Tf prill	0.32	0.07*	0.10 ⁺

2. ALTERATIONS IN PROTEINS IN MICROSCLEROTIA OF V. DAHLIAE IN RESPONSE TO SUBLETHAL METHAM SODIUM

One of the central difficulties in studying the interaction between sublethal fumigants and biocontrol fungi is the lack of sensitive methods to quantify "weakening" of pathogen propagules. In our original proposal we indicated that we would use methods developed for other fungi and for heat stress to study weakening in V. dahliae by sublethal metham sodium (Vapam®). These methods include intensity of vital staining (Freeman and Katan, 1988) and measurement of heat shock proteins (Freeman and Katan, 1989). Heat shock proteins are highly conserved throughout evolution and, in spite of their name, these proteins are formed in response to a variety of stresses, not just heat stress. We hypothesized that heat shock proteins, or similar proteins, might be formed in response to sublethal fumigation with metham sodium. We found vital stains not suitable for microsclerotia of V. dahliae. Microsclerotia did not take up the stains and/or the melanin in the microsclerotia made it impossible to see if they had incorporated the stain. This was true even when microsclerotia were sectioned on a freezing microtome immediately either before or after staining.

When fungal are heated, they can be immediately disrupted and their proteins examined electrophorically. Since metham sodium converts to its active form, methylisothiocyanate, only in soil, treating V. dahliae microsclerotia had to be done in soil and the microsclerotia could not be recovered immediately after treatment. Because heat shock proteins are somewhat ephemeral, these proteins were not useful indicators of stress from sublethal metham sodium. Hence, we developed a method to look at other protein changes that occur in response to stress from metham sodium. The greatest difficulty in developing the method was finding an appropriate way of disrupting the microsclerotia. Many of the methods we tried resulted in excessive melanin in the preparations, making it difficult to detect protein differences due to background staining. The following method was developed by C. A. Engelkes using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

V. dahliae was grown for 1 - 9 wk in Czapek-Dox broth (CDB) with shaking. The amount of total microsclerotial protein decreased with increasing culture age, averaging 54 to 20 µg protein/g fresh weight nontreated microsclerotia for 1 and 5-wk-old cultures, respectively (Table 2.1). Cultures were comminuted and microsclerotia (0.5 g) were collected on a 7.5 cm-diam 100 µm-pore size nylon mesh fabric circle with a vacuum pump and rinsed repeatedly with distilled water to remove CDB and mycelial fragments. A packet was created by covering the microsclerotia with an additional sterile mesh fabric circle (7.5 cm-diam), then stitching the two circles together with a hand-held sewing machine. The microsclerotia were kept moist with distilled water.

To treat the *V. dahliae* microsclerotia with metham sodium (Vapam®), 130 g of screened, moist Galestown gravely loamy sand (GGLS) soil was placed in a 9 cm wide x 6 cm tall plastic-coated paper cup. Dosages of 0, 0.045 or 0.45 μl metham sodium/g soil (approximately equal to 0, 93.5 or 935 l/ha) were pipetted into 30 ml sterile distilled water (SDW), vortexed for 5 s, and poured over the soil. A mesh fabric packet with microsclerotia was then placed in the paper cup over the soil and covered with an additional 70 g of nontreated soil and an inverted petri dish bottom.

Packets of microsclerotia were retrieved after 2 days. To assess sterility and effectiveness of the metham sodium treatment, microsclerotia were transferred to Czapek-Dox agar (CDA) plates and inspected for growth for 4 wk. Microsclerotia from soils treated with 0.45 μl metham sodium/g soil did not germinate, while those from soils treated with 0.045 μl metham sodium/g soil germinated later and grew more slowly than nontreated controls.

Because sublethal dosages of metham sodium affect only a small number of the treated microsclerotia and/or cells of an injured microsclerotium, it was imperative to use a small sample. Freeze-fracturing the microsclerotia resulted in a finely ground preparation which diluted metham-sodium affected cells with unaffected cells. A mini-beadbeater BX-B cell disrupter was used for grinding microsclerotia because it ruptured primarily the surface cells most affected by the metham sodium treatment.

For grinding, a 2-ml vial was layered with 0.8 g of 0.5 mm-diam zirconium beads, 0.5 g *V. dahliae* microsclerotia (fresh weight), and 1.0 g zirconium beads on top. A vial was inserted into the mini-beadbeater and shaken for two 50-s bursts on medium speed alternating with a 60-s rest period. After the dry grinding, 750 μl of ice cold SDW or 10 mM Tris-HCl buffer (pH 8.0) with 1 mM EDTA was added to each vial and vials were again shaken for two 50-s bursts on medium speed alternating with a 60-s rest period. We found 0.05 M Tris-HCl (pH 6.8) with 0.5% Triton X-100 and 0.05 M NaCl to be an unsuitable extraction buffer (Bandla et al. 1992). Total grinding times of 250-900 s increased release of melanin from *V. dahliae* microsclerotia which caused background discoloration on the gels, but did not significantly increase recoverable protein concentrations.

Microsclerotial suspensions (1.4 ml) were centrifuged for 75 min at 18 000 x g. Particulates > 0.20 μm were removed from suspensions with Micro-Spin series 8 000 filter units with cellulose acetate membranes for 40 - 80 min at 2,000 x g. Filtrates with 10-50 μg protein / g microsclerotia (fresh weight) were centrifuged at 25° C in a 10,000 MWCO Micron concentrator for 30 min at 18,000 x g. A final volume of 10 μL per sample was spun for 3 min at 2,000 x g and stored in a freezer (-15° C) until the microsclerotial proteins were separated electrophoretically.

Since there was uneven protein recovery from grinding *V. dahliae* microsclerotia (Table 2.1), protein quantities were standardized before electrophoretic analysis. Total

protein concentrations ($\mu\text{g}/\text{m}$) of the filtrates, ranging from 0 - 290 μg protein/g fresh weight microsclerotia of 1- and 5-wk-old cultures of V. dahliae, were determined with the Bradford protein assay method (Bradford 1976). Absorbances were read with a spectrophotometer at a wavelength of 595 and plotted against standard protein concentrations of bovine serum albumen.

Concentrations of recoverable protein from metham sodium-treated V. dahliae microsclerotia were significantly ($P < 0.01$) affected by an interaction between the dosages of metham sodium and culture age (Table 2.1). As metham sodium dosage was increased from 0.045 to 0.45 $\mu\text{l}/\text{g}$ soil, there was a decrease in the amount of recoverable protein from 90 to 37 $\mu\text{g}/\text{g}$ microsclerotia (fresh weight), respectively, when data were averaged across culture age. Protein concentrations were higher for 1-wk-old microsclerotia treated with 0.045 μl metham sodium/g soil compared with the nontreated controls.

An 8 μl sample of microsclerotial proteins was denatured with 2 μl containing both 5 % sodium dodecyl sulfate (SDS) and 10 % β -mercaptoethanol at 100° C for 5 min. Bromophenol blue (0.02 %) was used as the tracking dye. Recommended percent concentrations of these chemicals were doubled to reduce the amount of liquid added to the samples (Phastsystem, 1986). After cooling to room temperature, 0.5 μl of sample per lane was applied to gels. Protein bands were more distinct when using eight 0.5 μL samples than with twelve 0.3 μl samples per gel. Microsclerotial proteins were separated on PhastGel Gradient 8-25 in 30 min using the Phastsystem SDS-PAGE (Phastsystem Separation Technique File No. 110, Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Protein profiles were detected with a combination of Coomassie blue and silver stains (de Moreno et al., 1985). Immediately after separation, gels were developed with a 30 min Coomassie blue stain and no preserving solution (Phastsystem Development Technique File No. 200). Only 36 and 70 kDa - bands which contained $\geq$ 5-100 ng protein were visible after the Coomassie stain. Gels were soaked in 30 % ethanol for at least 45 min with agitation to remove excess Coomassie stain (Heukeshoven and Dernick 1985). Bands which contained $\geq$ 0.05 - 0.1 ng protein were then detected with a 60 min high sensitivity silver stain (Heukeshoven and Dernick 1988; PhastGel Silver Kit Instruction Manual). The background reducer step was increased from 2 to 6 min.

The molecular weight range of V. dahliae microsclerotia proteins was between 43 and 135 kDa when separated on a PhastGel Gradient 8-25 and compared with bands of the low molecular weight calibration kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) (Fig. 2.1). PhastGel homogeneous 12.5 medium has the highest selectivity for this molecular weight range and was used for further protein separations. PhastGel SDS buffer strips were used for continuous buffer separations with a pre-run for 60 volthours (Vh) and 131 accumulated Vh.

Electrophoretic protein profiles of V. dahliae microsclerotia are consistently produced with this procedure. Several differences among treatments were noted (Fig. 2.1). In

particular, bands at 34.2, 38, 48.5 and 58 kDa increase in intensity with increasing levels of metham sodium. The protein near 135 kDa appears in the weakened 1-wk-old microsclerotia but not in the nontreated or dead microsclerotia. Although the 135 kDa protein did not appear in weakened 5-wk-old microsclerotia, it may be a useful indicator of weakening in young microsclerotia. The large size of this protein would facilitate purification of the protein so that it could be quantified.

This procedure will be used to quantify the effects of dosages of metham sodium and possibly other stressing agents on microsclerotial proteins of V. dahliae. A manuscript on this method has been prepared for the Canadian Journal of Microbiology (Engelkes and Fravel). A portion of this work was presented at the 1993 annual meeting of the American Phytopathological Society (Engelkes and Fravel, 1993).

Table 2.1. Effect of metham sodium rate and age of microsclerotia (MS) of *Verticillium dahliae* on recoverable protein

Metham sodium ($\mu\text{L/g}$ soil)	<u>Average recoverable MS proteins¹ ($\mu\text{g/g}$ fresh weight MS)</u>			
	1-wk-old MS	SD ²	5-wk-old MS	SD
0.45	66	49	42	35
0.045	140	77	39	36
0	54	35	20	14

¹Total protein concentrations determined with Bradford protein assay method (Bradford 1976). Values based on 20 microsclerotial samples ground for two 50 s bursts then for two 50 s bursts in buffer.

²P<0.01. Standard deviation of the mean.

Fig. 2.1. Proteins of metham sodium-treated 1-wk-old microsclerotia of Verticillium dahliae in SDS-PAGE. Low molecular weight standards (lane 4); 10 μ g protein/ml sample (lanes 1 - 3) of microsclerotia treated with 0.45 (lane 1), 0.045 (lane 2), and 0 (lane 3) μ L metham sodium/g soil.

3. EFFECTS OF SUBLETHAL METHAM SODIUM ON T. FLAVUS AND V. DAHLIAE, AND COMBINING G. ROSEUM, T. FLAVUS AND SUBLETHAL METHAM SODIUM TO CONTROL V. DAHLIAE.

Many soil-inhabiting fungi demonstrate some ability to suppress plant diseases. In many cases, the level of control is not sufficient to be economically meaningful and control varies with the influence of abiotic and biotic environmental parameters. One way to augment performance of biocontrol fungi and to improve their consistency may be to combine the antagonists with sublethal chemical or heat disinfestation. "Sublethal" indicates that the treatments are not lethal to the organisms of interest although the treatments may be lethal to other microbes. Thus, sublethal treatments may reduce the number of competitive interactions facilitating establishment and proliferation of the biocontrol agent. In addition, sublethal treatments may weaken pathogens making them less able to defend themselves from attack by biocontrol agents (Freeman and Katan, Lifshitz, Tjamos and Fravel). This research was undertaken to determine the effects of sublethal levels of metham sodium on the pathogen Verticillium dahliae and the biocontrol fungi Gliocladium roseum and Talaromyces flavus.

3.a. Effects of sublethal metham sodium on viability and growth of V. dahliae and T. flavus.

Microsclerotia of V. dahliae were embedded in 0.5-cm square pieces of nylon mesh (0.42 μ pore size) resulting in approximately 15 - 20 microsclerotia / mesh square (Fravel *et al.*, 1987). A similar experiment was performed where mesh packets were prepared as in the protein extractions (above, section 2). GGSL soil was collected and passed through a 0.3 cm - diam opening screen to remove rocks and debris. Plastic coated paper cups (6 cm tall x 9 cm wide) were filled with 200 g (fresh weight) of soil and mesh squares or packets containing microsclerotia were placed on the soil surface. For the mesh squares, 0, 0.09, 2.25, 4.5, 9, or 90 μ l of metham sodium made with water to a total volume of 30 ml was applied. For the envelopes, 0, 18 or 90 μ l metham sodium was mixed with 30 ml water and poured over the entire surface including the mesh packets (approximately equivalent to 0, 93.5 and 935 l/ha). An additional 50 g of soil was used to cover the surface and the bottom half of a 9 - cm diam plastic petri plate was inverted over the cup and pressed into the cup to seal the cup. Cups were incubated under ambient conditions in a fume hood. After 48 hours, the petri plate bottoms were removed and the cups incubated an additional 6 days under the fume hood. Mesh squares or envelopes were retrieved and washed for 2 min under running tap water. Mesh squares were plated onto ESA (1 / plate) and colony diameter was recorded every 2 - 3 days for 24 - 28 days. Mesh envelopes were opened and individual microsclerotia were plated onto ESA. Percent viable microsclerotia was recorded and individual microsclerotia that had germinated were transferred to fresh CDA. Colony diameter was recorded every 2 - 3 days and growth rate was calculated. Differences in growth rate were determined by comparing slopes of growth curves using analysis of variance. Microsclerotia of V. dahliae were killed by the high rate of metham sodium. Compared to the nontreated control, all of the reduced rates of metham sodium significantly

($P \leq 0.001$) slowed the growth rate of V. dahliae when mesh squares were plated (Fig. 3.a.1). However, the low rate of metham did not affect the growth rate of V. dahliae ($P \leq 0.05$) compared to the nontreated control when individual microsclerotia were plated (Fig. 3.a.1). The discrepancy in growth rates between the two methods may be due to the fact that in plating individual microsclerotia, the largest microsclerotia were chosen for plating, while in plating the mesh squares, the growth rate reflects a population of microsclerotia of all sizes. Further, microsclerotia embedded in the mesh may be more exposed to the metham sodium than those in the envelopes. Additional information is needed on the responses of different size classes of microsclerotia to sublethal metham sodium.

Similar experiments were conducted in the paper cups with T. flavus. Ascospores of T. flavus were produced on potato dextrose agar at 27 C for 4 to 8 weeks. For some experiments, ascospores of T. flavus were formulated in alginate prill with pyrophyllite as the carrier to result in 10^5 propagules / g prill (Fravel et al., 1985). One g of prill or 10 ml of a 10^5 aqueous suspension of ascospores were mixed with the 200 g of soil. Metham sodium was applied at the rates used for V. dahliae and the soil packets were covered with an additional 50 g of soil and a petri plate bottom. Cups were incubated, uncovered and incubated as described previously. Individual prill were recovered and plated onto a medium semi-selective for T. flavus (TFA) (Marois et al., 1984). Colony diameter was recorded every 2- 3 days for 14 days. Metham sodium did not effect viability of T. flavus in prill ($P \leq 0.43$). There were no differences in growth rate from T. flavus in prill treated or not treated with metham sodium (Fig. 3.a.2a). Viability of naked ascospores was inconsistent ranging from 0 - 100% viable. Growth rate of germinated ascospores was not affected by the reduced rate of metham sodium (Fig 3.a.2b).

3.b. Greenhouse tests on the effect of sublethal metham sodium on pathogenicity of V. dahliae and interactions with biocontrol fungi.

Eggplant seeds (Solanum melongena cv Black Beauty) were planted in Promix® in 7.5 cm - diam plastic pots. For eggplants receiving G. roseum, 10 % (weight) of vermiculite culture (Keinath et al., 1991) was mixed into the potting mix prior to seeding. G. roseum was maintained on PDA at 27 C. Additional pots received sterile vermiculite. Other plants were drenched with 10 ml of SDW or an aqueous suspension of 10^5 ascospores / ml of T. flavus at 0, 3 and 6 wks after seeding.

GGLS soil was collected and passed through a screen as before. Zero or 50 ms/g of V. dahliae were added to the soil and mixed thoroughly. Soil was divided into 30 kg quantities were placed in plastic bags in metal tubs. Zero, 1.36, 2.73 ml, or 13.65 ml of metham sodium with water to a total volume of 1.33 L of water was added to each bag and bags were sealed. Metham sodium rates are approximately equivalent to 0, 93.5, 187, or 935 l/ha. Temperature during soil incubation was 21 C. After 48 h, bags were opened and allowed to air for an additional 8 days before planting. Soil was placed in 15.7 diam plastic pots and 6 wk old eggplants which had been not treated or treated with G. roseum or T. flavus were transplanted into this.

Plants were examined every 2 - 3 days for wilt symptoms. Plants were individually tagged for the date of first symptom appearance. Petioles of plants with symptoms were plated onto ESA to confirm infection by V. dahliae. At the termination of the experiment, petioles from all plants were plated onto ESA to determine if there were infected, symptomless plants. Data were analyzed using proc lifetest SAS. Proc lifetest was designed to determine if differences exist in binary data (wilted or not wilted) over time. The analysis gives greater weight to earlier wilting. Proc phreg was used to calculate a risk ratio indicating which disease progress curves were different from the disease control treatment.

In the first experiment at 49 days after transplant, incidence in neither the low rates of metham sodium nor the biocontrol fungi alone were different from the disease control (Table 3.b.1, Fig. 3.b.1). However, the combined treatments of the two low rates of the metham sodium-with the biocontrol agents had significantly less disease than the disease control. Contrasts of disease survival curves indicated that T. flavus ($P \leq 0.01$) and metham sodium ($P \leq 0.001$) had survival curves with significantly lower disease incidence than that of the disease control. There were no interactions.

A similar greenhouse test was conducted using only one low rate (2.7 ml / 30 kg) of metham sodium (approximately equivalent to 187 l/ha). Comparing disease progress curves indicated that the low rate of metham sodium or the two biocontrol fungi alone did not suppress wilt (Table 3.b.2, Fig. 3.b.2). The combination of either G. roseum, T. flavus, or both biocontrol fungi with the low rate of metham sodium resulted in suppression of wilt.

3.c. Field tests on the effects of sublethal metham sodium, G. roseum and T. flavus on Verticillium wilt of eggplant

Identical field tests were set out at Beltsville, MD and Bridgeton, NJ. The Bridgeton test was set out by S. A. Johnston, J. Lashomb and C. G. Hamilton with inoculum of G. roseum and T. flavus prepared in Beltsville. The field in Beltsville was artificially infested with V. dahliae while the field in Bridgeton was naturally infested. Metham sodium was applied to plots at 0, 140 or 701 l/ha using shanks spaced 18 cm apart. For each level of the fumigant, four replicate areas 3 m x 9.1 m were fumigated. In Bridgeton, a pipe was dragged behind the fumigator to seal the soil surface while Beltsville plots were lightly irrigated for this purpose.

Plants receiving T. flavus were drenched with 10 ml of 10^6 ascospores / ml at 0, 4 and 8 wks after seeding. G. roseum was formulated in alginate prill using pyrophyllite as the carrier. Furrows 9.1 m long were opened and 180 g of G. roseum prill was put in the furrow for plants receiving this treatment. Furrows were closed and 10 plants were transplanted into each 9.1 m long plot (0.86 m plant spacing) and rows were 1.5 m apart. The experiment had 12 treatments: 0, 140 or 701 l/ha metham sodium and no biocontrol agent, G. roseum, or T. flavus in all combinations. Weekly throughout the summer the number of flowers and fruit / plant, weight of fruit / replicate, and Verticillium wilt incidence and severity were recorded.

Disease incidence in Beltsville was low and only the data for the Bridgeton test are given. Analysis of variance was done for each parameter for each sampling date. Tables 3.c.1 - 3.c.4 give P values for the parameters for each date. For clarity, only P values < 0.05 (or near this value) are given. For most sampling dates, there was a strong block effect in the field which is most noticeable in the weight and number of fruit, and in the disease rating. For example, at 47 - 96 days after transplanting the block effect can be seen in the wilt rating (Table 5). The effect from the metham sodium was most noticeable on these same three parameters and for later sampling times. For example, 54 - 89 days after transplanting the effect of metham sodium on wilt rating can be seen. T. flavus affect wilt rating at 47, 61, 75 and 82 days after transplant while G. roseum affect wilt rating only at 75 days after transplanting. In general, interactions between metham sodium and the two biocontrol agents were not observed.

Discussion

Our hypothesis was that there would be a synergistic interaction between sublethal metham sodium and biocontrol fungi resulting in destruction of V. dahliae microsclerotia. In most cases, the effect of sublethal metham sodium and the biocontrol fungi was additive, rather than synergistic. Thus, sublethal heating may be a better choice than sublethal metham sodium for combining with biocontrol fungi, although combining sublethal fumigation with biocontrol fungi merits further investigation. Since neither viability nor growth rate of T. flavus from prill was affected by the reduced rate of metham sodium, T. flavus prill could be added prior to fumigation and thus be well placed to colonize niches left vacant when some microflora are killed by the low rate of metham sodium.

A manuscript is being prepared on the effects of sublethal metham sodium on G. roseum, T. flavus and V. dahliae, and on the greenhouse studies. The field work is being repeated this summer.

Figure 3.a.1.a

Effect of metham sodium on the growth rate of colonies from *V. dahliae* microsclerotia embedded in nylon mesh. Microsclerotia were embedded in 0.5 cm -square pieces of nylon mesh. The mesh squares were buried in soil and metham sodium was applied. Meshes were recovered and the mesh, containing approximately 15 - 20 microsclerotia, were plated onto agar medium.

Figure 3.a.1.b

Effect of metham sodium on the growth rate of colonies from individual microsclerotia of *V. dahliae*. Microsclerotia were placed in nylon mesh envelopes. The envelopes were buried in soil and metham sodium was applied. The envelopes were recovered and individual microsclerotia were plated onto an agar medium.

Figure 3.a.2. Effect of metham sodium on naked ascospores of *T. flavus* and ascospores of *T. flavus* in alginate prill.

Figure 3.b.1. Effect of two reduced rates of metham sodium, *G. roseum* and *T. flavus* on incidence of Verticillium wilt of eggplant.

- healthy control ○—○ *V. dahliae* + *G. roseum* + 1:10 rate metham sodium
 ▲—▲ disease control ◇—◇ *V. dahliae* + *G. roseum* + 1:5 rate metham sodium
 △—△ *V. dahliae* + *G. roseum*
 □—□ *V. dahliae* + 1:10 rate metham sodium
 ▽—▽ *V. dahliae* + 1:5 rate metham sodium

- healthy control ○—○ *V. dahliae* + *T. flavus* + 1:10 rate metham sodium
 ▲—▲ disease control ◇—◇ *V. dahliae* + *T. flavus* + 1:5 rate metham sodium
 △—△ *V. dahliae* + *T. flavus*
 □—□ *V. dahliae* + 1:10 rate metham sodium
 ▽—▽ *V. dahliae* + 1:5 rate metham sodium

Figure 3.b.2. Effect of reduced rate of metham sodium, *G. roseum* and *T. flavus* on Verticillium wilt of eggplant.

- — healthy control
- ▲ — disease control
- △ — *V. dahliae* + *G. roseum*
- — *V. dahliae* + 1:5 rate metham sodium
- ◇ — *V. dahliae* + *G. roseum* + *T. flavus* + 1:5 rate metham sodium
- ▽ — *V. dahliae* + *G. roseum* + 1:5 rate metham sodium

Table 3.b.1. Effect of reduced rates of metham sodium, *G. roseum* and *T. flavus* on Verticillium wilt of eggplant (Experiment 1).

	Compared to Healthy Control ¹	Compared to Disease Control	Survival Curve Compared to Disease Control ²
Healthy Control	-	0.001*	>0.0001*
1/10 Metham sodium	1.0	0.001*	-
1/5 Metham sodium	1.0	0.001*	-
Metham sodium	1.0	0.001*	-
Vd	>0.0001*	-	-
Vd + 1/10 Metham sodium	>0.0001*	0.067	0.003*
Vd + 1/5 Metham sodium	>0.0001*	0.067	0.008*
Vd + Metham sodium	1.0	0.001*	-
Vd + Gr	0.001*	0.317	0.70
Vd + 1/10 Metham sodium + Gr	0.012*	0.012*	0.0007*
Vd + 1/5 Metham sodium + Gr	0.012*	0.012*	0.002*
Vd + Metham sodium + Gr	1.0	0.001*	-
Vd + Tf	>0.0001*	0.146	0.03
Vd + 1/10 Metham sodium + Tf	0.03*	0.004*	0.0002*
Vd + 1/5 Metham sodium + Tf	0.067	0.001*	>0.0001*
Vd + Metham sodium + Tf	1.0	0.001*	-

¹Comparison at 49 days after transplant.

²Entire disease progress curve (DPC) compared to DPC of pathogen control.

Table 3.b.2. Effect of reduced rate of metham sodium, G. roseum and T. flavus on Verticillium wilt of eggplant (Experiment 2).

	Risk Ratio ¹
No Vd	— ²
No Vd + Gr + Tf	—
Vd	0.99
Vd + Vermiculite	1.00
Vd + Low Metham sodium ³	0.46
Vd + Gr	0.54
Vd + Tf	0.50
Vd + Gr + Tf	0.44
Vd + Gr + Low Metham sodium	0.14*
Vd + Tf + Low Metham sodium	0.25*
Vd + Gr + Tf + Low Metham sodium	0.09*

¹Risk ratio compares the entire disease progress curve and represents the probability that the treatment is different from the disease check. The smaller the number, the more likely it is that the treatment is different from the disease check.. Asterisks indicate that this treatment is significantly different from the disease check ($P \leq 0.05$).

²There were no diseased plants in this treatment. Risk ratio cannot be calculated for zero.

³Approximately equivalent to 1/5 the recommended rate of metham sodium.

Table 3.c.1.

Field test on the effect of G. roseum, T. flavus and a reduced rate of metham sodium on Verticillium wilt rating. Eggplants were grown in a field naturally infested with V. dahliae.

	Days After Planting											
	26	33	40	47	54	61	68	75	82	89	96	103
Tf ¹				0.0116		0.0180		0.0002	0.0228			
Gr ²								0.0001				
Tf*Gr												
Vapam					0.0009	0.0001	0.0001	0.0001	0.0001	0.0048		
Vapam*Tf				0.0024						0.0204		
Vapam*Gr												
Vapam*Tf*Gr												
Block				0.0001	0.0010	0.0001	0.0001	0.0001	0.0010	0.0198	0.0012	
Tf*Block								0.0001		0.0517	0.0037	
Gr*Block				0.0012		0.0076		0.0001				
Tf*Gr*Block				0.0468				0.0007				
Vapam*Block					0.0754			0.0001	0.0061		0.0593	
Vapam*Tr*Block					0.0748		0.0247	0.0048				
Vapam*Gr*Block				0.0076		0.0019		0.0044				
Vapam*Tf*Gr*Block					0.0008	0.0001	0.0019	0.0001				

¹Talaromyces flavus

²Gliocladium roseum

³P value

Table 3.c.2.

Field test on the effect of G. roseum, T. flavus and a reduced rate of metham sodium on the number of eggplant fruit. Eggplants were grown in a field naturally infested with V. dahliae.

	Days After Planting											
	26	33	40	47	54	61	68	75	82	89	96	103
Tf ¹				0.0021					0.0565		0.0385	
Gr ²						0.0145				0.0116		
Tf*Gr						0.0591						
Vapam								0.0001	0.0001	0.0001	0.0003	0.0006
Vapam*Tf									0.0111			
Vapam*Gr									0.0538			
Vapam*Tf*Gr										0.0162	0.0095	
Block				0.0434			0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Tf*Block										0.0006	0.0024	0.0106
Gr*Block									0.0254			
Tf*Gr*Block				0.0252		0.0186				0.0001		
Vapam*Block								0.0094			0.0060	0.0064
Vapam*Tr*Block							0.0131	0.0189		0.0046	0.0037	
Vapam*Gr*Block								0.0565	0.0021			
Vapam*Tf*Gr*Block								0.0058	0.0091	0.0019	0.0004	

¹Talaromyces flavus

²Gliocladium roseum

³P value

Table 3.c.3.

Field test on the effect of G. roseum, T. flavus and a reduced rate of metham sodium on the weight of eggplant fruit. Eggplants were grown in a field naturally infested with V. dahliae.

	Days After Planting											
	26	33	40	47	54	61	68	75	82	89	96	103
Tf ¹					0.0045							
Gr ²						0.0137						
Tf*Gr						0.0319						
Vapam				0.0002				0.0002	0.0011	0.0383	0.0074	0.0055
Vapam*Tf												
Vapam*Gr						0.0414						
Vapam*Tf*Gr												
Block				0.0001		0.0535	0.0001	0.0001	0.0001		0.0002	0.0150
Tf*Block												
Gr*Block												
Tf*Gr*Block												
Vapam*Block												0.0280
Vapam*Tr*Block							0.0659					
Vapam*Gr*Block												
Vapam*Tf*Gr*Block								0.0150				

¹Talaromyces flavus

²Gliocladium roseum

³P value

Table 3.c.4. Field test on the effect of G. roseum, T. flavus and a reduced rate of metham sodium on the number of eggplant flowers. Eggplants were grown in a field naturally infested with V. dahliae.

	Days After Transplanting											
	26	33	40	47	54	61	68	75	82	89	96	103
Tf ¹	0.0403 ³				0.0324					0.0484	0.0023	0.0172
Gr ²					0.0345			0.0031				
Tf*Gr	0.0403										0.0415	
Vapam					0.0038		0.0551	0.0010				0.0063
Vapam*Tf								0.0084				0.0291
Vapam*Gr						0.0313						
Vapam*Tf*Gr						0.0520						
Block						0.0028	0.0006				0.0065	0.0542
Tf*Block												0.0282
Gr*Block						0.0455						0.0134
Tf*Gr*Block												
Vapam*Block				0.0190				0.0066				0.0101
Vapam*Tr*Block							0.0157	0.0075	0.0107	0.0211	0.0548	
Vapam*Gr*Block								0.0192		0.0468	0.0304	0.0001
Vapam*Tf*Gr*Block												0.0001

¹*Talaromyces flavus*

²*Gliocladium roseum*

³P value

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II. Studies carried out in Israel

1. Weakening pathogen propagules by heating pesticides or other stress agents
2. Combining heating or solarization with pesticides at reduced dosages, or organic amendments, for improved control
3. The heat shock response of heat-sensitive and tolerant strains of *Fusarium oxysporum*

1. WEAKENING PATHOGEN PROPAGULES BY HEATING, PESTICIDES, OR OTHER STRESS AGENTS

1.1 Characterization of the Weakening Phenomenon

Dead fungal propagules placed in natural soil are degraded quickly by microbes. Living propagules, especially if they are dormant, are resistant to microbial degradation. For example, microsclerotia of *Verticillium* or chlamydospores of *Fusarium* can survive in a dormant state for up to 30 years in agricultural fields. Under some conditions, propagules are not killed, but instead are weakened or stressed by sublethal heat or chemical treatment. These weakened propagules exhibit an impaired ability to defend themselves from attack by other microbes and therefore become more sensitive to the action of soil microorganisms or other stress agents. Characterization and quantitative assessment of the weakening phenomenon is essential for the determination of the most effective combinations of control agents. We characterized the weakening of the propagules of *F. oxysporum* of sp. *niveum* subjected to sublethal heating treatments. *F. o. niveum* causes a wilt disease on watermelon.

In previous studies we used microscopic vital fluorescent staining for defining those propagules of *F.o. niveum* which were weakened upon sublethal heating. We further improved this approach by developing quantitative spectrophotometric methods to measure the reduced viability. For this purpose we used fluorescein diacetate (FDA). We standardized this method by adjusting inoculum concentration, length of incubation period, duration of germination and other factors. Typical results in Fig. 1.1** show that sublethal heating treatments reduced FDA activity. Further studies showed reduced activity at 36°C. We also studied the possibility of using radiolabel incorporation as a tool to define the weakening. Figure 1.2 depicts such a comparison between three

**Figures and tables are presented at the end of each text section.

methods of assessment. Radiolabel incorporation was found to be the most sensitive method. We carried out ultrastructural studies as a means for characterization of the damage inflicted to sublethally treated propagules. Conidia of *F.o. niveum* exposed to sublethal heating were examined by scanning electron microscopy (Plate 1).

Plate 1. Effect of heating at 40C for 30 minutes (B), 50C for 30 minutes (C) or at 40C for 30 minutes and incubated with soil bacteria for 3 days (D) on conidia of Fusarium oxysporum f. sp. niveum. Nonheated conidia shown in (A) (SEM).

These studies showed shrinking and distortions in the surface of treated conidia. Upon extended incubation in the soil, the conidia became colonized by bacteria. Thus, any change in a vital process in the treated pathogen related to the growth, reproduction, survival or capacity of the pathogen to induce disease may be considered as a parameter of weakening. Such parameters include morphological (e.g. germination) and ultrastructural changes, vital staining, radiolabel incorporation, microbial colonization and breakdown of propagules, decline of population, diminished production of reproductive and resting structures, and decreased disease incidence. Our ability to detect the damage also depends on the considered parameter (Fig. 1.2).

1.2 Interaction between stress factors and the effect on weakening

Our aim in combining methods of control was to achieve an improved control of a pathogen (or another pest) which could not be controlled by a single method. The pathogen *F.o f. sp. niveum* served as a model organism. The stress factors examined were: heating, the fungicide benomyl (Benlate) and starvation. The stress factors were examined (at various combinations) at different sublethal levels or exposure time. Selected results presented in Fig. 1.3 are summarized as follows:

- a. Benomyl at concentrations of up to 250 ppm and heating at 36-40°C had only a partial effect on the pathogen (0-20% reduction in population) when each factor was tested separately.
2. Combining heating with benomyl (a simultaneous treatment) considerably increased the effectiveness of the control, to almost complete reduction. Moreover, combining heating with benomyl enabled a reduction of the dosage of benomyl. The interactions obtained were significant and are in agreement with the results of previous studies. A significant interaction between benomyl and heating was also demonstrated with *Verticillium dahliae* (Fig. 1.4).

Starvation is a significant stress factor for many soilborne organisms and competition for nutrients is an important mechanism in biological control. Propagules of *F.o f. sp. niveum* were produced on Czapek medium (CM) containing 30 g/l, 6 g/l, 1.2 g/l or 0.25 g/l sucrose. They were subjected to sublethal heating at 42°C for 1 hr. The respective percent survival on these media were 73, 48, 13 and 13. The respective figures at 45°C were 30, 16, 1 and 0. Thus, similar to the heating-benomyl interaction, the starvation-heating interaction was harmful to the pathogen. The topic of the combined treatment is further discussed in Section 2.

Fig 1.1. Fluorescein diacetate hydrolysis by *Fusarium oxysporum* f. sp. *niveum* as affected by temperature. Except for one case (H₂O), acetone was used to stop the reaction.

Fig. 1.2. Weakening of conidia of *Fusarium oxysporum* f. sp. *niveum* by heat treatment. FDA hydrolysis - vital staining using fluorescein diacetate. Radiolabel incorporation was evaluated by using L-[³⁵S]methionine.

Fig. 1.3. Effect of Benlate at various concentrations at 25°C or heating at 36°C for 0-6 hrs or at 40°C for 1 hr, with or without Benlate, on viability of *Fusarium oxysporum* f. sp. *niveum*.

Fig. 1.4. Interaction of heating and fungicide on viability of sclerotia of *Verticillium dahliae*. MS = microsclerotia.

2. COMBINING HEATING OR SOLARIZATION WITH PESTICIDES AT REDUCED DOSAGES, OR ORGANIC AMENDMENTS, FOR IMPROVED CONTROL

The use of appropriate combinations of control methods may enable achieving the following goals:

- a. Improving the control of certain pathogens, or widening the spectrum of control of pests, when it cannot be achieved by a single method. *Macrophomina phaseolina*, a soilborne heat-tolerant pathogen which is normally not controlled by solarization, is such an example.
- b. - Reducing the dosage of pesticides.
- c. To overcome some of the difficulties encountered with non-chemical methods of control. For example, one of the drawbacks of soil solarization is the need to mulch (cover) the soil with polyethylene for 4-6 weeks, thus the soil remains out of production during this period. Therefore, improving solarization performance may enable to shorten the solarization period or extend its use to a cooler season. Another example is the use of organic amendments for the biocontrol of soilborne pathogens. Organic amendments are normally used at large amounts (20-50 tons/ha), which may render their use non-economic. Increasing their effectiveness (upon combining with other methods) may enable their use at lower rates.
- d. Certain pesticides are activated (and become more potent) upon heating. Therefore, combining heating or solarization with pesticides may enable their use at reduced dosages without decreasing their effectiveness.

In the following experiments we examined various combinations of methods of control, under laboratory, simulation systems and field conditions in an attempt to achieve some of the above mentioned goals.

2.1 Combining heating or solarization with pesticides for the control of *Macrophomina phaseolina*

2.1.1 Preliminary studies

- a. *M. phaseolina* is a heat-tolerant fungus. Its viability is not affected by heating at 47°C for 24 hrs or at 42°C for 10 days or at simulated solarization for 12 days. Such heat tolerance is far above that of other pathogens, as shown, for example, with *F. oxysporum* f. sp. *niveum* (see in Section 1).
- b. In all studies carried out in the soil, we used sclerotia of *M. phaseolina* produced on bean stems as inoculum. They were incorporated in the soil for the desired

period, then removed and washed on sieves and plated on a selective medium (Mihail and Alcorn, 1982) to determine their viability. Results were expressed as percent control (killing), by comparison with the viability of untreated sclerotia incubated under the same conditions.

- c. Sclerotia of *M. phaseolina* were incorporated in soil treated with pesticides at 0, 2 and 5 ppm, incubated for 120 hrs. Inhibition percentages at each temperature were calculated in order to examine the effect of heating on fungicidal activity. A total of 18 fungicides belonging to various groups and one herbicide (Eptam) were tested. The pesticides were tested at relatively low concentrations and short exposure in order to select the more promising ones. On the basis of the results obtained, the fungicide Benlate (benomyl, 50% a.i.) and the fumigant Formalin (formaldehyde, 37 a.i.) were chosen for further studies. The soil used the studies (mentioned in Section 2.1) was sandy (95% sand, 4% clay, 0.5% organic matter, pH=7.9).

2.1.2 Studies with Benlate (summary of results)

- a. Sclerotia of *M. phaseolina* were exposed to various concentrations of Benlate in culture or in the soil and incubated for 7 days, after which viability was assessed. ED₅₀ values were 0.5 and 3.0 ppm, respectively.
- b. Sclerotia of *M. phaseolina* were incorporated in soil and incubated for various periods at different concentrations of Benlate either with or without heating at constant temperatures, using a water bath. Results from six experiments (Table 2.1) show that, with one exception, all tested concentrations of Benlate in nonheated soil were sublethal to the pathogen. Heating decreased viability of sclerotia in all cases. A significant interaction between Benlate treatment and heating was obtained in three out of six experiments, indicating a synergistic effect. For example, in experiment 4, Benlate at 2 ppm had no effect on viability, while heating decreased the effect by 23%, but the combined effect resulted in a pronounced reduction of 83%. A variation between the experiments was evident but the same trend was obtained.
- c. Sclerotia of *M. phaseolina* were incorporated in soil treated with Benlate at various concentrations and incubated either at 27°C or in a system simulating the heating obtained by soil solarization under Israeli conditions at 10 cm soil depth (Fig. 2.1), as described by Yarden *et al.* (1989). Results (Table 2.2) show a trend similar to that obtained at constant temperatures (Table 2.1). Thus, increasing Benlate concentrations, or extending the exposure to the fungicide decreased sclerotia viability. Simulated solarization by itself did not affect sclerotia survival. Certain combinations of simulated solarization and Benlate drastically reduced the survival rate.

- d. The effect of Benlate and soil solarization on viability of sclerotia of *M. phaseolina* were tested under field conditions. Results (Table 2.3) essentially confirm those obtained under constant heating or simulation system and are summarized as follows:
1. After 10 days of incubation in the unsolarized plot under field condition, Benlate significantly decreased viability of sclerotia at 10 cm depth, but not at 20 cm depth. This can be attributed to soil temperature; at 10 cm depth soil temperature is approximately 5°C higher. This further confirms the observation that Benlate is more effective at higher temperatures.
 2. Soil solarization did not affect viability of sclerotia; in certain cases it even improved their survival (see below).
 3. When Benlate effect was mild (at 20 cm depth, 1 ppm concentration, 10 days exposure), a synergistic interaction between solarization and Benlate treatment was obtained. In this case, the combined treatment enabled pathogen control at low Benlate concentration and during short solarization. With the other combinations where Benlate alone was highly effective, such an interaction could not be demonstrated.
- e. Benlate is degraded in soil to carbendazin (MB). Previous studies (Yarden *et al.*, 1989) had shown that solarization enhances degradation in soil. We therefore assessed degradation of Benlate in both solarized and unsolarized soil. MBC was assessed by means of a bioassay technique which was developed in our laboratory, using *Penicillium digitalum* (Yarden *et al.*, 1985). Results (Fig. 2.1) show that soil solarization enhanced fungicide degradation. A similar trend was obtained in the simulated solarized system. Thus, after 15 days of treatment, percentages of MBC remaining in the soil, at initial rate of 2 ppm, were 70 and 12 in the untreated and heated soil, respectively. The respective figures at initial concentration of 5 ppm were 75 and 10.

In conclusion, combining heating with Benlate application has the potential to improve pathogen control, reducing dosage of applied pesticide, shortening solarization period **while enhancing dissipation** of the fungicide from soil, i.e. fast soil decontamination. The latter phenomenon is very desirable from the environmental point of view.

2.1.3 Studies with Formalin (summary of results)

The design of the following experiments was similar to that described for Benlate in the previous section.

- a. The effect of Formalin on *M. phaseolina* in the soil, with or without heating at

constant temperatures was studied. Results (Table 2.4) clearly show an interaction between Formalin and heating. Increasing Formalin concentrations increased mortality of sclerotia. For example, in experiment 3, Formalin or heating at the tested levels had no significant affect on sclerotia survival but combining them decreased sclerotia germination by 84%. Additional experiments yielded a similar trend. Results with Formalin were less variable as compared to studies with Benlate. Apparently, this was due to a more even application of Formalin (a volatile liquid) than Benlate (a wettable powder).

The interaction between Formalin and heating was tested in additional experiments using various combinations of Formalin concentrations and duration of treatments, with or without heating at various temperatures. Results (Figs. 2.3, 2.4) showed again the improved control of the pathogen upon heating, or increasing Formalin concentration. The synergistic effect of the combined treatment is evident. At increasing temperatures, the effectiveness of Formalin in pathogen control increased (Fig. 2.5).

- b. The effect of Formalin on *M. phaseolina* was tested in a simulated solarization system. Results (Table 2.5) show a significant interaction between Formalin and simulated solarization, confirming results obtained at constant heating (Table 2.4). The combined treatment resulted in a complete destruction of the sclerotia. In an additional experiment, the effect of simulated solarization was tested with various concentrations of Formalin. Results (Fig. 2.6) demonstrate the increased effectiveness of Formalin at elevated temperatures.
- c. The significant interaction of heating and Formalin may be due to:
 1. Activation of the pesticide at elevated temperatures.
 2. Weakening of the propagules by sublethal temperatures and increasing their vulnerability to fungicide action.
 3. Combination of a and b.

In order to study the above mechanisms, the sclerotia were subjected to Formalin (at 50-75 ppm) or heating (at 45°C for 24 hrs), at various sequences. The results showed that, again, each treatment alone was not effective (0-5% killing) compared to 100% killing when both treatments were combined. When the two treatments were given separately (first heating then Formalin treatment, or *vice versa*) effectiveness was reduced. Thus, the simultaneous treatment is essential to obtain improved control. It also appears that moistening the sclerotia itself for 24 hrs has a weakening effect.

- d. Incubating sclerotia of *M. phaseolina* in soil results in a certain decline in their

population in time. It was observed that this decline in population was occasionally somewhat inhibited by heating (for example in Table 2.4). This might be due to dormancy breaking. This possibility was excluded in a series of experiments in which sclerotia (of which germination was about 50%) was exposed to heating. In additional studies this phenomenon was found to be due to a decreased activity of soil microorganisms in the heated soil.

- e. The above experiments were carried out with sclerotia produced on and attached to bean stems, simulating a natural inoculum incorporated in the soil. In some experiments we compared the sensitivity to heating and Formalin of detached sclerotia to sensitivity of sclerotia attached to the stem. Similar results regarding the effects of heating or Formalin treatment were obtained.
- f. Formalin solution normally contains 10% methanol in order to stabilize this substance. We tested the effect of methanol on sclerotia of *M. phaseolina*, under conditions similar to those prevailing in the Formalin tests, at concentrations up to 5 times higher than those of the Formalin solution. No effect on sclerotia viability was observed. We concluded that the above described effects are attributed to Formalin.

In conclusion, combining Formalin with heating significantly improved the control of *M. phaseolina* and its effect was more pronounced than that of Benlate. This combination enables a significant reduction in Formalin dosage; at higher temperatures lower dosages of Formalin are required. Recently, there is a renewed interest in Formalin. In Israel, it is in the process of registration for controlling potato scab and soilborne bacteria and in the UK for various pathogens in greenhouses. Lowering Formalin dosages will reduce the side-effects of this substance. Our results are in accordance with those of a previous study with metham, a water soluble fumigant which has a vapor phase (as does Formalin). Combining solarization with metham resulted in a synergistic effect on the control of rots in peanuts (Frank *et al.*, 1986).

2.2 Combining organic amendments, with heating or solarization for the control of soilborne pathogens

Organic amendments incorporated into soil were often reported to control soilborne fungi, either directly upon release of antifungal compounds or indirectly upon stimulation of antagonists in the soil. The use of organic amendments for disease control is environmentally desirable. We examined the possibility to improve their effectiveness and reduce the needed amount of organic amendments by combining them with other stress agents such as heating or solarization. We tested three types of organic amendments: leaf residues of *Majorana* (spice plant), oil extract from the leaf residues and poultry manure. Fifteen experiments were carried out under a variety of conditions: laboratory, simulated solarization and

solarization in the field. Three experiments are described below:

Under laboratory conditions, leaf residues of *Majorana*, at the tested rates, had no inhibiting effect at 25°C on growth of *F. oxysporum* f. sp. *melonis* (Table 2.5.a).

At elevated temperatures, however, growth inhibition was evident.

The effect of poultry manure on viability of sclerotia of *Sclerotium rolfsii* was examined in a simulated solarization system as described in section 2.1, at maximal temperatures of 40°C. Nonheated poultry manure had no detrimental effect on viability of the sclerotia after 7-21 days of incubation (Table 2.6).

Heating for 7 or 14 days under simulated solarization had only a partial effect.

Combining the two treatments significantly increased sclerotia control rate. After 14 or 21 days of the combined treatment, 100% mortality was obtained.

A solarization experiment was carried out in September (1992) which is not suitable for solarization (under Israeli conditions) on purpose, to test the possibility of improving solarization under marginal conditions. Under these conditions, solarization for 26 days, application of either oil extract or poultry manure had a slight effect on viability of sclerotia (Table 2.7). The combined treatments improved the control up to 100% killing of the sclerotia. Additional experiments essentially confirmed these results with other pathogen, e.g. *Pythium* and *Rhizoctonia solani*. However, the effect of combined control methods was less pronounced when one of the control agents was used at a high level, e.g. - solarization in the hotter months.

2.3 Additional experiments

Field experiments with tomatoes were carried out in order to test the effectiveness of combining solarization and 50% dosage of methyl bromide in controlling the crown root rot caused by *F. oxysporum* f. sp. *radicis lycopersici*. Methyl bromide treatment is applied in strips which is less expensive but also less effective in disease control. Preliminary studies carried out in 1991 and 1992 showed that the above combined treatment was more effective than each treatment alone and was comparable to methyl bromide at a 100% recommended dosage (75 g/m²). A more detailed experiment has been started in September 1993 and is now at its last stages. At this stage, results are similar to those obtained in the previous years, namely the combined treatment (solarization plus reduced dosage of methyl bromide) was highly effective in disease control and so far gave a high yield.

Table 2.1 Effect of heating (heat) and Benlate (B) on survival of *Macrophomina phaseolina* in soil.

Experiment No.	Exposure (hr)	B Concentration $\mu\text{g/g}$	% survival at		Interaction ⁽¹⁾ B x Heat	Main effect ⁽¹⁾	
			25°C	42°C ⁽²⁾		B	Heat
1	48	0.	100	86	-	A	+
		0.5	90	65		A	
		1.5	92	65		A	
		5.0	79	15		B	
2	48	0.	98	92	-	A	+
		2.0	100	96		A	
		5.0	96	87		A	
3	120	0.	100	77	-	A	+
		2.0	100	88		A	
		5.0	86	67		B	
4	120	0.	100 Aa	77 Ab	+		
		2.0	100 Aa	17 Bb			
		5.0	100 Aa	23 Bb			
5	120	0.	97 Aa	97 Aa	+		
		2.0	86 Aa	28 Bb			
6	216	0.	100 Aa	65 Ab	+		
		2.0	92 Ba	19 Bb			
		5.0	9 Ca	6 Ba			

⁽¹⁾Factorial analysis: + = significant; - = insignificant ($P = 0.05$). When interaction was not significant, comparisons were made between the main effects. When interaction was significant, comparisons were made for each level. Uppercase letter designate differences between B levels; Lowercase letters designate difference between temperatures.

⁽²⁾Heating at constant temperatures.

Table 2.2 Effect of Benlate (B) with or without heating in a simulated solarization system (Sim) on survival of sclerotia of *Macrophomina phaseolina* in soil.

Experiment No.	Treatment (days)	B Concentration (ppm)	% survival at		Interaction ⁽¹⁾ B x Sim	Main effect ⁽¹⁾	
			27°C	Simul.		B	Simul
1	10	0	71 Aa	81 Aa	+		
		2	71 Aa	8 Bb			
		5	81 Aa	4 Bb			
	15	0	44	69	-	A	-
		2	6	0		B	
		5	11	2		B	
	21	0	31	79	-	A	
		2	2	0		B	
		5	8	0		B	
2	25	0	94		-	A	+
		0.25	50	69		B	
		0.50		0		B	
	40		4		-		
		0		0		A	
		0.25	92			B	
		0.50	4	98		B	
			0	4			
				0			
				0			

⁽¹⁾Statistical analysis described in Table 2.1.

Table 2.3 Effect of Benlate (B) with (sol) or without (Untr.) solarization on survival of *Macrophomina phaseolina* in soil under field conditions.

Soil depth (cm)	Treatment (days)	B Concentration (ppm)	% survival at		Interaction ⁽¹⁾ B x Sol	Main effect ⁽¹⁾	
			Untr. ⁽²⁾	Sol.		B	Sol.
10	10	0	85	86	-	A	-
		1	19	52		B	
		5	15	6		B	
10	22	0	86	88	-	A	-
		1	6	0		B	
		5	6	0		B	
10	30	0	38	94	-	A	+
		1	17	0		B	
		5	8	0		B	
20	10	0	84 Aa	90 Aa	+		-
		1	84 Aa	17 Bb			
		5	4 Ba	2 Ba			
20	22	0	75	86	-	A	-
		1	8	2		B	
		5	15	0		B	
20	30	0	56	88	-	A	
		1	8	4		B	
		5	0	0		B	

⁽¹⁾Statistical analysis described in Table 2.1.

⁽²⁾Untreated plot, covered with a plastic sheet but shaded to prevent heating.

Table 2.4 Effect of Formalin (F), with or without heating at constant temperature, on survival of sclerotia of *Macrophomina phaseolina* in soil.

Experiment No.	Treatment (days)	F Concentration (ppm)	% survival at		Interaction ⁽¹⁾ F x heating
			25°C	42°C	
1	5	0	81 Aa	80 Aa	+
		20	92 Aa	22 Bb	
		100	0 Ba	0 Ba	
2	5	0	43 Ba	67 Aa	+
		20	89 Aa	22 BCb	
		100	0 Ca	0 Ca	
3	3	0	92 Aa	92 Aa	+
		40	95 Aa	8 Bb	

⁽¹⁾Statistical analysis described in Table 2.1. Uppercase letters designate differences between F levels.

Table 2.5 Effect of Formalin (F), without or with heating via simulated solarization system (Simul.), on survival of sclerotia of *Macrophomina phaseolina*.

Experiment No.	Treatment (days)	F Concentration (ppm)	% survival at		Interaction ⁽¹⁾ F x Simul.
			25°C	Simul.	
1	12	0	71 aA	71 Aa	+
		40	75 Aa	0 Bb	
2	12	0	73 Aa	92 Aa	+
		40	90 Aa	0 Bb	

⁽¹⁾Statistical analysis described in Table 2.1.

Table 2.5.a Effect of temperature and leaf residues of *Majorana* in culture on growth of *Fusarium oxysporum* f. sp. *melonis*.

Temperature (°C)	Residues mg/plate	Inhibition ⁽¹⁾ (%)
25	60	8 e ⁽²⁾
33	60	29 b
37	60	48 a
25	120	8 e
33	120	25 c
37	120	50 a

⁽¹⁾Calculated upon comparison with untreated control.

⁽²⁾Different letters indicate a significant difference ($P = 0.05$).

Table 2.6 Effect of poultry manure (PM) and duration of heating treatment in a solarization simulation system (SH), on mortality of sclerotia of *Sclerotium rolfsii*.

Duration of SH (days)	Heating	MN g/container	killing (%)
7	-(⁽¹⁾)	0	0 e ⁽²⁾
7	-	50	0 e
7	+	0	38 b
7	+	50	53 cd
14	-	0	0 e
14	-	50	30 de
14	+	0	61 c
14	+	50	100 a
21	-	0	0 e
21	-	50	13 e
21	+	0	95 a
21	+	50	100 a

⁽¹⁾- = no heating; + = heating.

⁽²⁾Different letters indicate a significant difference ($P = 0.05$).

Table 2.7 Effect of poultry manure (PM), *Majorana* oil extract (OE) and solarization (SH) for 26 days under field conditions on the control of *Pythium*.

SH	OE (ppm)	PM g/m ²	Killing (%)
-(1)	0	0	0
+	0	0	35
-	5,000	0	0
+	5,000	0	100
-	0	210	6
+	0	210	41
-	0	735	0
+	0	735	53
-	0	2,500	23
+	0	2,500	82

(1)- = nonsolarized; + = solarized.

Fig. 2.1. Soil temperature at 10 cm depth during solarization (field solarization) or in a simulation system.

Fig. 2.2. Degradation of Benlate in nonsolarized and solarized soil (expressed as MBC remaining) at two initial concentrations of Benlate applied: 1 and 5 ppm.

Fig. 2.3 Effect of combining Formalin at various concentrations at 25°C or 42°C for 5 or 10 days on viability of *Macrophomina phaseolina*.

Fig. 2.4 Effect of Formalin at various temperatures on viability of *Macrophomina phaseolina*.

Fig. 2.5 Effect of temperature (exposure for 24 hrs) on viability of *Macrophomina phaseolina*, expressed as ED₉₉ (ppm Formalin).

Fig. 2.6 Effect of heating by simulated solarization for 12 days and Formalin at various concentrations on viability of *Macrophomina phaseolina*.

3. THE HEAT SHOCK RESPONSE OF HEAT-SENSITIVE AND TOLERANT STRAINS OF *Fusarium oxysporum*

A wide variety of organisms respond to elevated temperature stress by enhanced synthesis of a specific set of heat shock proteins (hsps) and by suppression of the synthesis of many regular proteins. Some stress treatments condition living cells to withstand the subsequent effect of severe heat that would otherwise be lethal treatments. Treatments which induce thermotolerance also induce the synthesis of hsps, and it is therefore assumed that hsps are involved in the protection of cells from heat. The relationships between thermotolerance and the capacity of cells to synthesize hsps at specific developmental stages or metabolic states have also been documented.

In earlier studies we have characterized the heat-shock response and acquired thermotolerance in *Fusarium oxysporum* f. sp. *niveum* (Freeman *et al.*, 1989). Strains of *F. oxysporum* may differ in their heat-sensitivity, while exposure to sublethal heating affects their germination, growth and survival. In the present studies, we have used pairs of closely related heat-sensitive and heat-tolerant strains of *F. oxysporum* f. sp. *niveum* and *F. oxysporum* f. sp. *dianthi* to compare the changes in protein synthesis during heat-stress. The differences between the strain pairs and the kinetics of hsp synthesis were examined in an attempt to relate these parameters to the differences in inherent heat-tolerance. These studies may lead to a better understanding of the weakening phenomenon induced by heating. Experimental details were described in previous publications (Freeman and Katan, 1988; Freeman *et al.*, 1989).

3.1 Heat-shock protein synthesis and incorporation of [³⁵S]methionine into protein in heat-sensitive and heat-tolerant strains of *F. oxysporum* f. sp. *niveum*

Germlings of heat-sensitive and heat-tolerant strains *F. oxysporum* f. sp. *niveum* and *F. oxysporum* f. sp. *dianthi* were radiolabeled with L-[³⁵S]methionine at various temperatures. Germlings of the heat-tolerant strains incorporated 3.7-7.7 times as much radioactivity as those of the respective heat-sensitive ones, depending on the temperature, indicating that the rate of protein synthesis in the heat-tolerant strain was higher under both regular and heat-stress conditions. Protein profiles of both *F. oxysporum* f. sp. *niveum* strains were similar, as assessed by SDS-PAGE (Fig. 3.1). The major hsps produced under heat-stress conditions corresponded to molecular masses of 95, 83, 74 and 35 kilodaltons (kDa). At 45°C only a few proteins were faintly detected in the heat-sensitive strain, whereas the heat-tolerant strain was still able to maintain the synthesis of all hsps and many of the regular cellular proteins.

3.2 Synthesis of heat-shock proteins during recovery from heating

Germlings of the heat-sensitive and heat-tolerant strains of *F. oxysporum* f. sp. *niveum* were heat-treated at 36-42°C for 30 min and then transferred to 25°C for recovery. Qualitative and quantitative changes in the protein synthesis

profiles were followed.

The first detectable changes in the pattern of protein synthesis after heat-treatment were steep increases in the incorporation of label into hsp74, hsp 35 and a parallel decline in label incorporation into some of the prominent regular proteins, such as protein bands of apparent M_r of 80, 70, 67, 60, 45 and 30 kDa (Fig. 3.2). Some other regular proteins were less affected. Hsp83 was synthesized at a low basal level under non-stress conditions, and increased more slowly than the other hsps upon exposure to heat-stress. Two proteins of M_r 77 and 78 kDa were induced at temperatures higher than 38°C at an intensity lower than hsp74, which may be members of the hsp70 family. Synthesis of hsp74 in the heat-tolerant strain reached its maximal level earlier than in the heat-sensitive strain (Fig. 3.3). Thus, maximal values of hsp74 were recorded for the tolerant strain 30 min after germlings treated at 36 or 38°C were returned to 25°C, and after 1-2 hrs at 40 or 42°C treatments. In the heat-sensitive strain maximal values of hsp74 were attained between 1 hr after exposure to 36°C and 3 hrs after exposure to 42°C. With increasing severity of heat-stress, there was a greater increase in incorporation of label into hsp74 in the tolerant than in the sensitive strain. The extent of hsp74 synthesis was commensurate with the severity of stress. After reaching a stress-specific level, the synthesis of hsps was reduced (Fig. 3.3) while the synthesis of regular proteins was gradually restored (Fig. 3.2). The four-fold difference in the rate of L-[³⁵S]methionine incorporation between the tolerant and sensitive strains, is sufficient to account for the more rapid kinetics of hsp synthesis in the tolerant strain. The kinetics of hsp83 synthesis were similar to those of hsp74 in both the heat-sensitive and heat-tolerant strains (Fig. 3.2). The quantitative pattern of hsp83 synthesis and recovery after heat-treatment, determined by densitometry, was also close to the of hsp74 (data not shown).

3.3 Radiolabel incorporation during exposure and recovery from heat-shock

In both strain incorporation of radioisotope-label into protein in germlings declined significantly during the heat-treatments (Fig. 3.4). The decline was dependent on the severity of heat-stress. Incorporation was further reduced during the first 30 min of the recovery period after transfer of germlings to 25°C. In the heat-tolerant strain, the rate of incorporation increased rapidly during recovery. This was paralleled by a decline in hsp synthesis and consumption of normal protein synthesis (Fig. 3.2). In contrast, the recovery rate of the heat-sensitive germlings, as expressed in incorporation rate was much slower than of the tolerant ones.

3.4 The heat-shock response of *F. oxysporum* f. sp. *dianthi*

Germlings of the heat-sensitive and heat-tolerant strains of this pathogen were treated at 40°C and then transferred to 25°C for recovery. Protein profiles and incorporation of radiolabel were assessed as in *F. oxysporum* f. sp. *niveum*. Very similar results were observed (Fig. 3.5). The two *F. oxysporum* f. sp.

dianthi strains, originating from different populations, had a different protein pattern when compared at 25°C, in contrast to the *F. oxysporum* f. sp. *niveum* pair which originated from the same population. Recovery of the heat-tolerant strain FOD 1547 from heat-shock was more rapid than the heat-sensitive one. Hsp74 as well as other hsps were synthesized more rapidly and then repressed faster in the heat-tolerant strain, in parallel with the restoration of synthesis of normal proteins.

Incorporation of radiolabel in germlings of *F. oxysporum* f. sp. *dianthi* was 5.1-7.7 times higher in the tolerant strain than in the sensitive one, depending on the temperature, in a way parallel to the respective strains of *F. oxysporum* f. sp. *niveum* (Table 2). Incorporation of label in the heat-tolerant strain FOD 1547 increased rapidly during recovery from heat-shock (Fig. 3.6). A slower heat-shock response was observed in the heat-sensitive strain FOD 99, similar to that of the heat-sensitive strain of *F. oxysporum* f. sp. *niveum*.

The present studies were aimed at defining differences between heat-sensitive and heat-tolerant strains of the soilborne fungus *F. oxysporum* which could serve as the basis for their differential response to heat-stress. Such a difference between closely related natural strains could result from variations in the efficiency of some elements of their heat-shock response system. This system is most likely comprised of heat-sensing and signal transducing mechanisms, the heat-shock proteins, peripheral mechanisms which affect and regulate hsp synthesis, and possibly other independent mechanisms.

Differences in heat tolerance between natural populations must be based on mechanisms other than disruption of the activity of essential genes.

The resumption of regular protein synthesis is an essential feature of recovery from stress. Comparing two independent pairs of strains of *F. oxysporum*, we found that in each pair the strain which could withstand more extreme stress conditions had a higher rate of protein synthesis and could restore the regular pattern of protein synthesis after a shorter period. In both pairs, heat tolerance was correlated with a more rapid heat-shock response.

Exposure of microorganisms to sublethal temperatures may render them more vulnerable to other stress agents. Understanding the mechanisms of both acquired and inherent heat tolerance of pathogens and the related changes in stress proteins is therefore important for the determination of heat dosages required for effective control of pathogens.

Fig. 3.1 Effect of heating on the pattern of protein synthesis in germlings of the heat-sensitive (FON 7A) and heat-tolerant (FON 10A) strains of *Fusarium oxysporum* f. sp. *niveum*. The autoradiogram shows L-[³⁵S]methionine-labeled proteins separated by SDS-PAGE. Germlings of the sensitive (S) and tolerant (T) strains were labeled during exposure to 25, 36, 42 and 45°C for a period of 30 min, as indicated. Aliquots of 5 µl of protein were applied in each lane. Molecular weight markers in kilodaltons are indicated on the left, and the apparent molecular weights of the major hsp appear on the right.

Fig. 3.2. Pattern of protein synthesis in germlings of the heat-sensitive (FON 7A) and heat-tolerant (FON 10A) strains of *Fusarium oxysporum* f. sp. *niveum* during recovery from heat-stress. The autoradiograms show L-[³⁵S]methionine-labeled proteins separated by SDS-PAGE. The germlings were exposed for 30 min to temperatures of 36°C (A), 38°C (B), 40°C (C) and 42°C (D). Label was applied to the sensitive (S) and tolerant (T) germlings 30 min before protein extraction at the end of each period of recovery (30, 60, 90 and 120 min; 1, 2, 3, 4, 5, 6 and 8 hrs), as indicated in the appropriate lanes. Lanes C and O show the protein profiles of germlings labeled at 25°C and at the respective heat-shock, for 30 min each. Equal counts of radioisotope-labeled protein were applied in each lane in A and B. Aliquots of 5 µl of protein were applied in each lane in C and D. Molecular weight markers in kilodaltons are indicated on the left, and the apparent molecular weights of the heat-shock and other proteins appear on the right.

Fig. 3.3 Kinetics of synthesis of hsp74 in germlings of the heat-sensitive (FON 7A) and heat-sensitive (FON 10A) strains of *Fusarium oxysporum* f. sp. *niveum* during recovery from heat-shock (H.S.) at 36, 38, 40 and 42°C each. Relative amounts of hsp74 (corresponding to experiments appearing in Fig 3.2) were quantified by densitometry. Pixel units (PU) multiplied by an incorporation factor were plotted for each period of labeling. Heating began at -0.5 hr and was terminated at 0 hr, with recovery taking place thereafter at 25°C.

Fig. 3.4 Rate of incorporation of L-[³⁵S]methionine into protein of germlings in the heat-sensitive (FON 7A) and heat-sensitive (FON 10A) strains of *Fusarium oxysporum* f. sp. *niveum* during recovery from heat-shock (H.S.) at 36, 38, 40 and 42°C for 30 min each. Label was applied 30 min before extraction of protein, at the end of each period of time. Incorporation (cpm, counts per min) into protein extracts was measured and calculated as percent of control at 25°C, in the sensitive and tolerant strains. Heating began at -0.5 hr and was terminated at 0 hr, with recovery taking place therefore at 25°C.

Fig. 3.5 Pattern of protein synthesis in germings of the heat-sensitive (FOD 99) and heat-tolerant (FOD 1547) strains of *Fusarium oxysporum* f. sp. *dianthi* during recovery from heating at 40°C for 30 min. The autoradiogram shows L-[³⁵S]methionine-labeled proteins separated by SDS-PAGE. Label was applied to the sensitive (S) and tolerant (T) germings 30 min before protein extraction at the end of each period of recovery (30 and 90 min; 3 and 5 hr), as indicated in the appropriate lanes. Lanes C and O show the protein profiles of germings labeled at 25 and 40°C, respectively. Equal counts of radioisotope-labeled protein were applied to each lane. Molecular weight markers in kilodaltons are indicated on the left and the apparent molecular weights of the major hsps appear on the right.

Fig. 3.6 Rate of incorporation of L-[³⁵S]methionine into protein in germings of the heat-sensitive (FOD 99) and heat-tolerant (FOD 1547) strains of *Fusarium oxysporum* f. sp. *dianthi* during recovery from heat-shock (H.S.) at 40°C for 30 min. Label was applied 30 min before extraction of protein, at the end of each period of time. Incorporation (cpm, counts per min) into protein was measured and calculated as percent of control at 25°C, in the sensitive and tolerant strains. Heating began at -0.5 hr and was terminated at 0 hr, with recovery taking place thereafter at 25°C.

Fig 1

Fig. 6

Fig. 5

Fig 3

Fig 4

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D. Description of cooperation

The three investigators involved were in continuous contact. C. Ginzburg visited D. Fravel in April 1991 to discuss the project and present a seminar. D. Fravel and J. Katan attended a NATO workshop on biological control in May 1991 where they both presented invited papers. They also discussed the research project. D. Fravel and Y. Katan met again and discussed the project in August 1991 at the annual meeting of the American Phytopathological Society. J. Katan visited D. Fravel in 1993. D. Fravel will visit J. Katan and C. Ginzburg in 1994. We are certain that the links among the three researchers will continue beyond the termination of the project.

E. Research achievements and benefit to agriculture

Weakening of propagules of soilborne pathogens, induced by sublethal treatments under a variety of conditions, was characterized. Ultrastructural, microscopic, physiological and ecological aspects of weakening were investigated. The use of heat tolerant mutants enabled a better understanding of the heat shock response which in turn determines the effectiveness of heat treatments. The achievements in both countries are in accordance with the objectives of the research. Studies were carried out under laboratory, field-simulated and field conditions. Weakening of propagules of various soilborne pathogens was studied as related to sublethal treatment by a variety of control or stressing agents. Combining methods of control, (usually at reduced dosages) was both a fundamental and an applicative goal in our studies. We believe that the results of our work will provide tools for reducing pesticide dosage, or reducing pesticides hazard, while improving disease control. Combining control measures is increasingly important as methyl bromide and other pesticides are removed from use. The concepts developed are also relevant (with certain modifications) to the control of arthropods and weeds.

F. List of publications

1. C. Ginzburg, S. Freeman and J. Katan. 1991. The heat shock response of sensitive and tolerant strains of Fusarium oxysporum Abstr. Meeting on: Stress Proteins and the Heat Shock Response, Cold Spring Harbor, N. Y. p. 60.
2. J. Katan, C. Ginzburg and S. Freeman. 1992. The weakening effect as a trigger for biological control. Pages 55-61 in: E. C. Tjamos, G. C. Papavizas and R. J. Cook, eds. Biological Control of Plant Diseases, Plenum Press, N. Y.
3. C. A. Engelkes and D. R. Fravel. 1993. Use of SDS-PAGE to analyze proteins of metham-treated microsclerotia of Verticillium dahliae. Abstr. Phytopathology 83:1397.