Neth. J. Pl. Path. 99 (1993) 77–84

Induction of heat resistance in Fusarium oxysporum and Verticillium dahliae caused by exposure to sublethal heat treatments

MERCEDES CASTEJÓN-MUÑOZ[1] and G.J. BOLLEN[2]

[1] Centro de Investigación y Desarrollo Agrario, 41200 Alcalá del Rio, Spain
[2] Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, the Netherlands

Accepted 17 January 1993

Abstract

The effects of sublethal heat treatments on heat resistance were studied for *Fusarium oxysporum* f.sp. *dianthi* (Fod) and *Verticillium dahliae* (Vd), one isolate of each pathogen. Treatments of propagule suspensions of Fod at 55 °C and of Vd at 45 °C for 30 min were survived by less than 0.001% and 0.01% of the propagules, respectively. Pretreatment of suspensions of Fod at 45 °C increased survival of the 55 °C treatment up to 0.73% of the propagules and pretreatment of suspensions of Vd at 40 °C increased survival of the 45 °C treatment up to 0.40%. Induction of heat resistance was dependent on duration of the exposure to the sublethal temperature. With Fod, this duration was shorter for propagules from old cultures than for those from young cultures. Pretreatment at 45 °C of a suspension of an 1-week-old culture of Fod induced resistance when lasting 30 min or longer, but not when 20 min or shorter. With Vd, the duration of the pretreatment inducing heat resistance depended on type of culture – white or black – due to differences in microsclerotia formation. Implications of induced heat resistance for control of plant diseases by thermotherapy are discussed.

Additional keywords: Fusarium oxysporum f.sp. *dianthi*, heat shock response, heat treatment, soil disinfestation, thermal death, thermotherapy.

Introduction

Heat treatments are widely practised in plant disease control. Thermotherapy of soil and planting material is one of the oldest cultural measures for control of soil- and seed-borne pathogens. In recent years, new types of applications appeared, e.g. soil solarization (Katan, 1987; Katan and DeVay, 1991) and heat treatment of drain water in soilless cultures (Runia et al., 1988). Renewed interest in thermotherapy of soils and other substrates has arisen in particular because of restrictions imposed on chemical disinfestation.

Pathogen kill by heat treatment is commonly proportional to a temperature–time product. A linear relationship between the logarithm of the decimal reduction time (time required to reduce viable propagules by a factor 10) and temperature of heat treatment was found for four fungal pathogens by Pullman et al. (1981a). Similar results were mentioned by Roebroeck et al. (1991) for *Fusarium oxysporum* f.sp. *gladioli*; however, they did not present detailed data. The same relationship was suggested for thermokill of microorganisms in general (Van Asten and Dorpema, 1982). Pathogen kill is, however, not always proportional to a temperature–time product of the treatment. One of the exceptions to the rule comprises examples where heat resistance is induced by exposure of living cells to sublethal heat treatments. This phenomenon was reported for three non-pathogenic

fungi by Plesofsky-Vig and Brambl (1985a,b) and for one pathogen, *F. oxysporum* f.sp. *niveum*, by Freeman et al. (1989). In these fungi, induction of heat resistance was associated with formation of heat shock proteins.

The aim of the present study is to investigate whether heating at sublethal levels induces heat resistance in two pathogens that were known for their anomalous responses to heat treatments in previous trials on thermotherapy (Bollen, unpublished). The pathogens were *F. oxysporum* f.sp. *dianthi* (Fod) and *Verticillium dahliae* (Vd).

Materials and methods

Fungal material. Of each pathogen one fresh isolate was used. The isolate of Fod came from *Dianthus caryophyllus* cv. Medio and that of Vd was obtained from greenhouse soil under a plantation of roses. The suspensions used for heat treatments were prepared from shaking cultures or agar plate cultures.

Shaking cultures were prepared with Czapek Dox solution (pH 6.8). They were incubated at 22–24 °C in a gyratory shaker (120 times up and down per min). Morphological features were recorded once in 6 days and the cultures were used when spores or chlamydospores (Fod) and spores or microsclerotia (Vd) had been formed. Mycelium and spores were removed by filtration (Millipore 0.8 μm) and were resuspended in a sterilized mineral solution and blended at 2100 rpm during 15 min. The mineral solution was the same as that being used as the nutrient drain water in soilless cultures of glasshouse crops. It contains 0.8 g Nutriflora-T (NPK and micro-elements, EC 1.8 dS m^{-1} at 25 °C; Windmill BV) and 1 g $Ca(NO_3)_2$ l^{-1}; pH 5.2. The suspensions contained mycelium fragments, conidia, chlamydospores or microsclerotia. Propagule densities were $>10^6$ ml^{-1}.

Potato dextrose agar (PDA) plates (pH 5.6; oxytetracycline 50 μg ml^{-1}) were inoculated with Fod or Vd and incubated at 22–24 °C. In order to promote sporulation of Fod, the plates were exposed to diffuse daylight from a north window. Plates with Vd were incubated at 22 °C for 1 week in diffuse daylight and cultures to be used for preparing suspensions with conidia only were subsequently placed under black light (General Electric F40BL) in order to suppress formation of microsclerotia (Brandt and Reese, 1964). Spores were harvested by flooding the plates with 10 ml of sterilized mineral solution and loosing the spores with a sterile bent glass rod. Spore densities of the suspensions were $>10^6$ ml^{-1} with Fod and $>10^7$ ml^{-1} with Vd.

Heat treatment. Samples of 3 ml of suspension were heated in culture tubes (18 mm diameter, wall 1.5 mm) that were placed in a thermostatically controlled stirring water bath (± 0.2 °C). In order to avoid contamination of the suspension by placing thermocouples, temperatures were measured with thermocouples placed in replicate tubes treated in the same way.

The first series of treatments was performed to find the temperature required to kill most propagules of the populations of both species during a 30-min treatment. The ranges of temperatures were chosen on the basis of literature data (Pullman et al., 1981a; Bollen, 1985). The suspensions of Fod were treated at 50, 55 and 60 °C and those of Vd at 40, 45, and 47 °C. Survival was assessed with dilution platings of the suspensions where 0.1 ml was added to each of four PDA plates for each dilution. The experiment was repeated three times with different shaking cultures.

The second series of treatments was conducted to study the effect of preheating at a sublethal temperature on survival of treatment at a level that was shown to be nearly lethal to the fungal population in the first series (without pretreatment). Duration of the pretreatments varied from 5 to 90 min. Temperatures of pretreatments were 45 °C for Fod and

40 °C for Vd. Immediately after pretreatment, the tubes with suspensions were transferred to the water baths with 55 °C for Fod and 45 °C for Vd. Time needed for warming up to these temperatures was less than 2 min. Survival was assessed with four PDA plates for each dilution. The experiments were repeated three times for both pathogens, except for the experiment with different culture types of Vd (Table 5), which was done twice.

Statistical analysis. The results of each experiment were expressed as percentages of viable counts of propagules that survived as compared with the viable counts of the untreated suspensions. The data were transformed by $\sqrt{x + \frac{1}{2}}$ and subsequently processed by analysis of variance (ANOVA), using the method of LSD 95% intervals.

Results

Heat resistance of populations of Fod and Vd without pretreatment
Survival of propagules in suspensions prepared from shaking cultures was below detection level after treatment at 60 °C for Fod and at 47 °C for Vd (Table 1). Treatments at 55 °C for Fod and 45 °C for Vd were appropriate for evaluation of induced heat resistance in the isolates of the two pathogens. Besides conidia and mycelium fragments, the suspensions of Fod contained chlamydospores and those of Vd microsclerotia.

Heat resistance of populations of Fod and Vd after pretreatments at sublethal temperatures
F. oxysporum f.sp. dianthi. Pretreatment at 45 °C of propagule suspensions of a 23-day-old shaking culture considerably increased heat resistance (Fig. 1). This behaviour was consistent as it was found in all three sequential replications. Induction of heat resistance was dependent on duration of the pretreatment. This effect was more pronounced with suspensions of microconidia from 1-week-old plate cultures (Table 2) than with suspensions of different propagules from older shaking cultures (Table 3). With the microconidial suspensions, a pretreatment lasting longer than 20 min was required to induce resistance to a level that was significantly higher than in the control without pretreatment (Table 2). Induction of resistance was restricted to only a small fraction of the population (max. 0.73%; Table 3). Response to pretreatment decreased with ageing of the cultures (Table 3).

Table 1. Survival (viable counts per ml) of heat treatments by propagules of *Fusarium oxysporum* f.sp. *dianthi* and *Verticillium dahliae* from 3-week-old shaking cultures.

Pathogen[a]	Temperature (°C) of treatment[b] and dilution factor						
	22–24 10^5	40 10^5	45 10^2	47 10^1	50 10^5	55 10^1	60 10^1
F. oxysporum f.sp. *dianthi*	22 ± 2[c]	–	–	–	13 ± 2	1 ± 0	0
V. dahliae	57 ± 3	39 ± 2	3 ± 1	0	–	–	–

[a] For each pathogen, the experiment was repeated three times with corresponding results ($\alpha = 0.05$).
[b] Treatment was for 30 min; warming up to temperature of treatment took 2–3 min.
[c] Mean numbers and standard deviation of the means in four replicate plates.

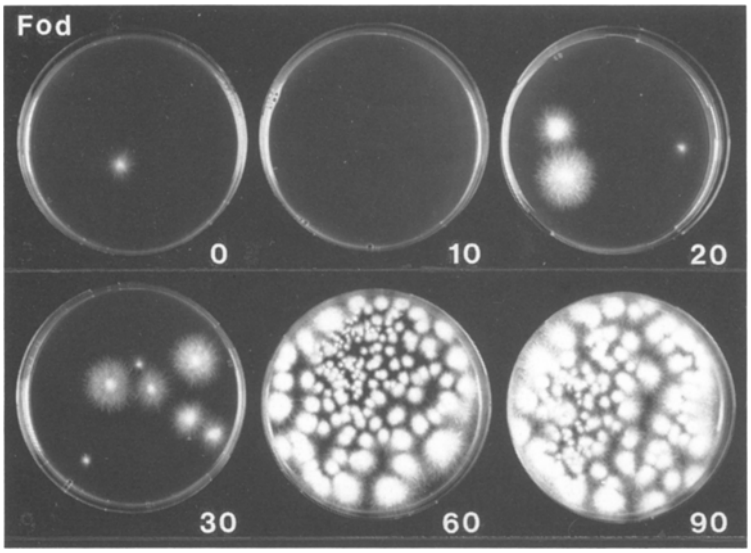

Fig. 1. Induced resistance in *Fusarium oxysporum* f.sp. *dianthi* to heat treatment at 55 °C (30 min) by pretreatments at 45 °C. Each plate received 0.1 ml of a tenfold dilution of a treated suspension that originally contained 13×10^4 viable propagules per ml of a 23-day-old culture. Figures below the plates refer to duration (min) of pretreatment.

V. dahliae. Pretreatment of 5 min at 40 °C already induced resistance (Table 4). An anomalous pattern was found in the effect of the duration of pretreatment of suspensions from 23-day-old shaking cultures, where heat resistance was less increased after a 30-min treatment than after 20- or 60-min treatments (Table 4). The results suggest two peaks in the effect of preheating on heat resistance, one with exposure times between 5 and 20 min and a second one at a duration of longer than 30 min.

Although incubated under the same conditions, two types of colonies appeared on PDA

Table 2. Effect of pretreatment at 45 °C on survival of heat treatment at 55 °C for 30 min by conidia of *Fusarium oxysporum* f.sp. *dianthi* from 1-week-old plate cultures.

Experiment	Untreated control (numbers)	Viable counts (% of numbers in untreated control)					
		Duration of pretreatment at 45 °C (min)					
		0	10	20	30	60	90
1 X[a]	34×10^4	0	0	0	0.13	0.16	0.10
2 X	41×10^4	0	0	0	0.21	0.29	0.08
3 X	51×10^4	0	0	0	0.28	0.52	0.15
		a[a]	a	a	bc	c	ab

[a] Data followed by different letters are different at $\alpha = 0.05$. Data were compared for the response in three sequential replicate experiments (capitals) and as a group for duration of pretreatment (lowercase).

Table 3. Effect of pretreatment at 45 °C on survival of heat treatment at 55 °C for 30 min by propagules (spores and mycelium fragments) of *Fusarium oxysporum* f.sp. *dianthi* from shaking cultures of different ages.

Age (days)	Untreated control (numbers)	Viable counts (% of numbers in untreated control)[a]					
		Duration of pretreatment at 45 °C (min)					
		0	10	20	30	60	90
23 X[b]	13×10^4	0.01 a[b]	0.02 a	0.03 a	0.14 ab	0.73 c	0.58 bc
75 Y	30×10^4	0.01 a	0.03 b	0.22 e	0.12 c	0.15 d	0.14 cd

[a] Data of three sequential experiments.
[b] Data followed by different letters are different at $\alpha = 0.05$. Data were compared for the age response (capitals) and for the effect of duration of pretreatment with each age (lowercase). Interaction between effects of age and of duration of pretreatment was significant at $\alpha = 0.01$.

plates with subcultures of the same Vd isolate as had been used for the experiment mentioned in Table 4. One type was white with only few microsclerotia and an abundant sporulation with conidia. The other type was black, due to an abundant formation of microsclerotia, and with less conidia. The effect of preheating of propagule suspensions originating from both types of plates differed insofar that maximum induction of resistance was attained at a shorter duration with the white (conidial) type than with the black (microsclerotial) type (Table 5).

Discussion

Heat resistance of micro-organisms is dependent on conditions prevailing in the environment prior to the treatment. In a recent study on effects of heat treatment on bacteria, it was shown that starvation increased their resistance against the treatments (Jouper-Jaan et al., 1992). For fungal pathogens, Baker (1962) pointed to the influence of factors pre-

Table 4. Effect of pretreatment at 40 °C on survival of heat treatment at 45 °C for 30 min by propagules (conidia, mycelium fragments and microsclerotia) of *Verticillium dahliae* from 23-day-old shaking cultures.

Experiment	Untreated control (numbers)	Viable counts (% of numbers in untreated control)					
		Duration of pretreatment at 40 °C (min)					
		0	5	10	20	30	60
1 A[a]	42×10^5	0.00	0.23	0.31	0.25	0.06	0.40
2 A	26×10^5	0.00	0.19	0.24	0.24	0.04	0.16
3 A	32×10^5	0.01	0.19	0.37	0.31	0.18	0.25
		a[a]	b	b	b	a	b

[a] Data followed by different letters are different at $\alpha = 0.05$. Data were compared for the response in three sequential replicate experiments (capitals) and as a group for duration of pretreatment (lowercase).

Table 5. Effect of pretreatment at 40 °C on survival of heat treatment at 45 °C for 30 min by conidia and microsclerotia of Verticillium dahliae from 2-week-old plate cultures with different types of colonies.

Type of colony	Untreated control (numbers)	Viable counts (% of numbers in untreated control)[a]					
		Duration of pretreatment at 40 °C (min)					
		0	5	10	20	30	60
White X[b]	186×10^5	0.028 a[b]	0.126 b	0.066 b	0.064 ab	0.039 a	0.038 a
Black Y	105×10^5	0.024 a	0.043 ab	0.038 ab	0.044 ab	0.144 c	0.112 bc

[a] Data of two repetitions, each with four plates for each dilution.
[b] Data followed by different letters are different at $\alpha = 0.05$. Data were compared for the total response of each colony type (capitals) and separately for the effect of duration of pretreatment with each type of colony (lowercase). Interaction between effects of type of colony and duration of pretreatment was significant at $\alpha = 0.01$.

vailing in the substrate on the efficacy of thermotherapy of infested soil or substrates. The results of this study demonstrate that, at least with Fod and Vd, substrate temperature prior to treatment is one of the factors that can influence this efficacy by induction of resistance. Disinfestation by heat is practised for the control of both pathogens. Vd is effectively controlled by soil solarization (Pullman et al., 1981b; Katan, 1987; Jimenez-Diaz et al., 1991). However, Porter and Merriman (1983) found that part of its population survived in the lower zones (at a depth of 14 to 21 cm) of the tillage layer. As yet it is unknown whether induced resistance actually contributes to survival in deeper layers of solarized soil. In these layers, pathogens are exposed to gradually increasing temperatures, including those within the range where heat resistance can be acquired.

It is tempting to speculate that induced heat resistance is involved in the anomalous response to serial heat treatments with increasing dosages, as was found for *F. oxysporum* f.sp. *melongenae* in drain water of soilless cultures (Runia et al., 1988). With treatments in the range between 69.5 and 90.0 °C, survival was considerably higher when spore suspensions were exposed to these temperatures for 2.0 min than for 0.5 min. During the early phase of heat treatment, products might have been formed that are associated with induction of resistance. As such, formation of heat shock proteins in cells exposed to sublethal treatments was established for *Neurospora crassa* (Plesofsky-Vig and Brambl, 1985b) and *F. oxysporum* f.sp. *niveum* (Freeman et al., 1989). These proteins contribute to a basic defense against stressful environmental conditions like exposure to high temperatures (Plesofsky-Vig and Brambl, 1985a). If these products are involved in the resistance of *F. oxysporum* f.sp. *melongenae* mentioned above, spores might die when the duration of resistance-inducing treatment is too short for completing the process of heat shock protein formation. Most records on thermokill in the literature show linear or curvilinear regression lines for survival and temperatures of heat treatment, like those of the pathogens studied by Pullman et al. (1981a). When heat resistance is induced already within the time needed for warming up of the spore suspensions and when a given dosis is needed for this induction, regression lines for survival with increasing temperatures of treatment will differ from true linear or exponential ones. Responses like that observed for *F. oxysporum* f.sp. *melongenae* by Runia et al. (1988) probably occur under specific physiological conditions of the pathogen that have not been well identified so far.

Induction of heat resistance in Fod was more pronounced with young than with aged

cultures (Tables 2 and 3). For a very early stage in fungal development, i.e. that of germlings, Freeman et al. (1989) observed an even more pronounced response to pretreatment of *F. oxysporum* f.sp. *niveum*.

The different response to pretreatment by propagules taken from white and black colonies originating from the same Vd isolate is most likely due to the presence of microsclerotia in suspensions taken from the latter ones. Obviously, microsclerotia need a longer treatment to become resistant (Table 5). As has been demonstrated by Brandt- already in 1964, colony morphology of Vd shows a non-hereditary variation and is determined by a substance produced by the fungus itself. Its production is dependent on light and trivial external factors such as shape of the plates and shallow conditions of the medium.

Resistance to treatments at 55 °C with Fod or 45 °C with Vd was acquired by only a small fraction (max. 0.73 or 0.40%, respectively) of the propagule populations of these pathogens. However, these percentages are still too high for an effective disinfestation of soils or other substrates. The impact of the phenomenon of induced resistance for thermotherapy under commercial conditions is not clear. It might play a role where thermotherapy is practised at marginal temperatures.

Acknowledgements

We would like to thank the Agricultural Research Institute (INIA) in Spain for providing a postdoctoral research grant to M.C.-M. for a study leave at the Department of Phytopathology of the Wageningen University. We are grateful to Mrs Dine Volker for valuable technical assistance.

References

Baker, K.F., 1962. Thermotherapy of planting material. Phytopathology 52: 1244–1255.

Bollen, G.J., 1985. Lethal temperatures of soil fungi. In: Parker, C.A., Rovira, A.D., Moore, K.J. & Wong, P.T.W. (Eds), Ecology and management of soilborne plant pathogens. APS, St.Paul, Minnesota. p. 191–193.

Brandt, W.H., 1964. Morphogenesis in *Verticillium*: A self-induced, non-hereditary variation in colony form. American Journal of Botany 51: 820–824.

Brandt, W.H. & Reese J.E., 1964. Morphogenesis in *Verticillium*: a self-produced, diffusible morphogenetic factor. American Journal of Botany 51: 922–927.

Freeman, S., Ginzburg, C. & Katan, J., 1989. Heat shock protein synthesis in propagules of *Fusarium oxysporum* f. sp. *niveum*. Phytopathology 79: 1054–1058.

Jimenez-Diaz, R.M. Bejarano, M.A., Blanco, M.A., Gomez, J., Gonzalez, R. & Melero, J.M., 1991. Control of verticillium wilt and fusarium wilt diseases by solarization in Southern Spain. In: DeVay, J.J. Stapleton, J.J. & Elmore, C.L. (Eds), Soil solarization. FAO Plant Protection Papers 109. p. 94–107.

Jouper-Jaan, A., Goodman, A.E. & Kjelleberg, S., 1992. Bacteria starved for prolonged periods develop increased protection against lethal temperatures. FEMS Microbiology Ecology 101: 229–236.

Katan, J., 1987. Soil solarization. In: Chet, I. (Ed.), Innovative approaches to plant disease control. J. Wiley, New York. p. 77–105.

Katan, J. & DeVay, J.E. (Eds), 1991. Soil solarization. CRC Press, Boca Raton, Florida.

Plesofsky-Vig, N. & Brambl, R., 1985a. The heat shock response of fungi. Experimental Mycology 9: 187–194.

Plesofsky-Vig, N. & Brambl, R., 1985b. Heat shock response of *Neurospora crassa*: protein synthesis and induced thermotolerance. Journal of Bacteriology 162: 1083–1091.

Porter, I.J. & Merriman, P.R., 1983. Effects of solarization of soil on nematode and fungal pathogens at two sites in Victoria. Soil Biology and Biochemistry 15: 39–44.
Pullman, G.S., DeVay, J.E. and Garber, R.H., 1981a. Soil solarization and thermal death: a logarithmic relationship between time and temperature for four soilborne plant pathogens. Phytopathology 71: 959–964.
Pullman, G.S., DeVay, J.E., Garber, R.H. & Weinhold, A.R., 1981b. Soil solarization : effects on verticillium wilt of cotton and soilborne populations of *Verticillium dahliae*, *Pythium* spp., *Rhizoctonia solani*, and *Thielaviopsis basicola*. Phytopathology 71: 954–958.
Roebroeck, E.J.A., Jansen, M.J.W. & Mes, J.J., 1991. A mathematical model describing the combined effect of exposure time and temperature of hot-water treatments on survival of gladiolus corms. Annals of Applied Biology 119: 89–96.
Runia, W.Th., Van Os, E.A. & Bollen, G.J., 1988. Disinfection of drain water from soilless cultures by heat treatment. Netherlands Journal of Agricultural Science 36: 231–238.
Van Asten, J. & Dorpema, J.W., 1982. A new approach to sterilization conditions: the IMO concept. Pharmaceutisch Weekblad Scientific Edition 4: 49–56.