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Effect of High Temperature on Inhibtion of the Growth of Bacterial Wilt Pathogen (*Ralstonia solanacearum*) in Soil

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ABSTRACT

This study was carried out to examine the efficacy of heat treatment on soil for the control of bacterial wilt (*Ralstonia solanacearum*). The viability of *Ralstonia solanacearum* was tested in both wet and dry soils under simulated elevated temperatures in either constant, cyclic or short temperature regimes. In wet soil, viability was reduced to zero by a constant temperature of 45°C and above for 2 day or a temperature of 60°C for 2 h. Viability of *Ralstonia solanacearum* was reduced to zero by a 2 h temperature cycle for 2 days at 50°C or 3 days at 45°C. In dry soil at 55°C, a 1-day constant temperature or at 45°C a 2 days 2- h temperature cycle reduced *Ralstonia solanacearum* viability by 87.5 and 37.5%, respectively. Data on time and temperature relationships on loss in viability of *Ralstonia solanacearum* in soil could be used to predict efficacy of soil solarization.

Key words: *Ralstonia*, soil, temperature, time

INTRODUCTION

Bacterial wilt is widely distributed in tropical, subtropical and some temperate regions of the world. The disease ranks as one of the most important disease of bacterial origin in the world, causing sometimes total losses in tomato crops (Ram-Kishun and Kishun, 1987). Causal agent is *Ralstonia solanacearum* (Smith) (Yabuuchi *et al*., 1995) a highly diverse and adaptive bacterium, that differs in host range, geographical distribution, pathogenicity, epidemiological interactions and physiological properties (Buddenhagen *et al*., 1962).

Ralstonia solanacearum is the causal agent of potato brown rot and bacterial wilt in many crops (Hayward, 1994), and it is responsible for losses of up to 75% of the potato crop in several countries (Cook and Sequeira, 1994). Most of the outbreaks seem to be related to the irrigation of crops with *Ralstonia solanacearum*-contaminated water. However, little is known about its distribution and persistence in natural reservoirs such as water or the molecular or physiological bases of survival strategies for this bacterium (Van Elsas *et al*., 2000).

On the basis of host range, *Ralstonia solanacearum* strains have been traditionally divided into races (Hayward, 1964), while physiological and genetic characterization resulted in the formation of biovars and divisions. The bacterium invades the plant vascular tissues through wounded roots or natural openings, which occur after the emergence of secondary roots. It progresses through intercellular spaces into the xylem. Temperature strongly influenced the

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survival of *Ralstonia solanacearum*, as optimal survival, in particular in water systems, was demonstrated at physiologically favorable temperatures (Van Elsas *et al*., 2001) and reduction of *Ralstonia solanacearum* populations was observed at low temperatures. This study presented the experiments designed to test the efficacy of either continuous, 2-h cycle or short exposures to a variety of high temperatures and a range of time periods in reducing the viability of *Ralstonia solanacearum*in wet or dry soils under laboratory conditions.

MATERIALS AND METHODS

Inoculations

Inoculum of *Ralstonia solanacearum* (from Bacteriology group, Plant Disease Research and Development Department of Agriculture) was prepared from overnight shaken cultures incubated at 30°C. Cultures were centrifuged at 6000g for 20 min at room temperature, then the pellet was washed twice in distilled water. After the final wash the pellet was suspended in sterile distilled water (SDW) and the number of cells in the suspension was determined from optical density measurements at OD_{600} (Creach *et al*., 2003). The suspension was diluted to give the required number of cells ml^{-1} and samples were mixed into a sandy loam soil used for plant cultivation.

Soil treatment

One milliliter bacterial suspension containing 1×10^8 cfu prepared from an overnight culture of *Ralstonia solanacearum* strains were mixed thoroughly with each gram of sterile soil. For wet soil treatment, the containers were kept in glass dishes containing 100 ml water so that the soil was maintained at 100% MHC (Moisture Holding Capacity). In the dry soil treatments, air dry soil of 2-3% MHC containing *Ralstonia solanacearum* was not watered.

Continual temperature

The unit consisting of soil mixed with 1×10^8 cfu/g culture of *Ralstonia solanacearum* strains and Petri dishes were covered in 40 μm thick polyethylene bags, and kept for 10 days in an incubator adjusted to a range of temperature between 30 and 60°C.

2 h temperature cycle

Since at least 3 h elapsed for soil to attain the temperature of the incubator, the experimental units consisting of *Ralstonia solanacearum* in soil was therefore incubated for 5 h which included 3 h gradual rise in temperature plus 2 h to obtain a constant temperature. The dishes were then transferred to 30°C for 19 h when at least 6 h elapsed to reach the ambient temperature. The effect of 2 h temperature cycle was tested every day for 10 days.

Short time exposure

To determine the minimum time required for the inactivation of *Ralstonia solanacearum*, soils containing *Ralstonia solanacearum* were exposed to temperatures in the ranges of 30-60°C for 30, 60, 120, 180, 240, 300 and 360 h.

Viability analysis

The effect of the heat treatments was evaluated on the total population of soil *Ralstonia solanacearum* The homologous samples from the same plot were mixed together and three sub-samples (1 g each) were analysed by the plate dilution method on potato synthesis agar (PSA). After heat treatment, the soils were counted for *Ralstonia solanacearum* growing on PSA. Bacteria growing on PSA were counted. The results were expressed as cfu g^{-1} of dry soil. *Ralstonia solanacearum* population was estimated by plate dilution in 1 g soil samples in triplicate. At the end of the heat treatments, samples were recovered and populations determined by plate dilution on PSA medium. The results were

expressed as cfu g^{-1} of dry soil.

RESULTS AND DISCUSSION

Effect of continuous temperature

Effects of continuous temperature on viability of *Ralstonia solanacearum* in dry and wet soils are shown in Figure 1. In dry and wet soil (Figure 1(a) and Figure 1(b),respectively), continuous exposure for 48 h at temperatures of 45°C and above was sufficient to destroy *Ralstonia solanacearum* viability, but temperatures of 35°C and below had no effect. In dry soil (Figure 1(a)) held at 55°C, *Ralstonia solanacearum* viability was destroyed after 2 d with 45% of *Ralstonia solanacearum* surviving 1 d. Reduction of viability was less at lower temperatures in both dry and wet soils (Figure 1(a) and Figure 1 (b)).

Effect of a daily 2 h temperature cycle

Effect of daily 2 h high temperature exposure on the viability of *Ralstonia solanacearum* in dry or wet soils are shown in Figure 2. In the effect of a daily 2-h high temperature exposure of dry soil (Figure 2(a)), a minimum daily 2-h exposure of *Ralstonia solanacearum* to 60 °C resulted in complete loss of viability after one day exposure. In dry soil, daily exposure of *Ralstonia solanacearum* to 40°C for 2 h had no effect by 4 days. However, at 35°C and below 5 days of exposure to a high temperature for 2 h day^{-1} this caused no marked reduction in viability of *Ralstonia solanacearum* in dry soil (Figure 2(a)). In wet soil, treatments lasting 1, 2, 3 and 4 days, to a daily 2-h exposure to 60, 55, 50 and 45°C, respectively, were necessary to destroy *Ralstonia* viability. Daily exposure of wet soil to 45°C for 2 h reduced the viability to 50% after 1 days and to zero after 3 days in wet soil (Figure 2b).

Effect of short time exposures

In the study of short temperature exposure to determine the minimum time required to inactivate *Ralstonia solanacearum*, the units consisting of *Ralstonia solanacearum* in wet soils were exposed to temperatures in the ranges of 30 to 60°C for 30, 60, 120, 180, 240, 300 and 360 minutes as shown in Figure 3. From ambient (28°C) soil temperature gradually increased and the required temperature was attained after 3 h. At 50°C the viability of *Ralstonia solanacearum* was reduced by 75% after 3 h and to zero after 4 h whereas at 55°C and 60°C, the viability were reduced to zero after 3 h (Figure 3).

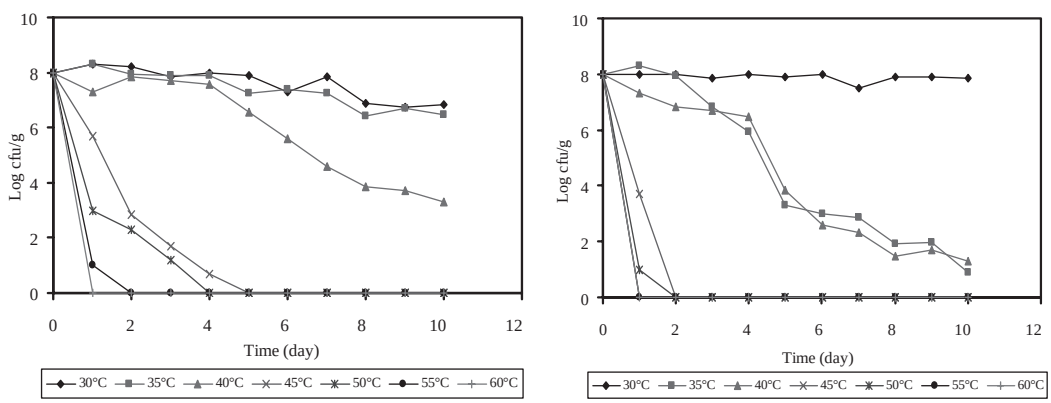

Figure 1 Effect of continuous temperatures on viability of *Ralstonia solanacearum* in dry soil (a, moisture holding capacity (MHC) = 2-3%) and wet soil (b, MHC = 96-100%).

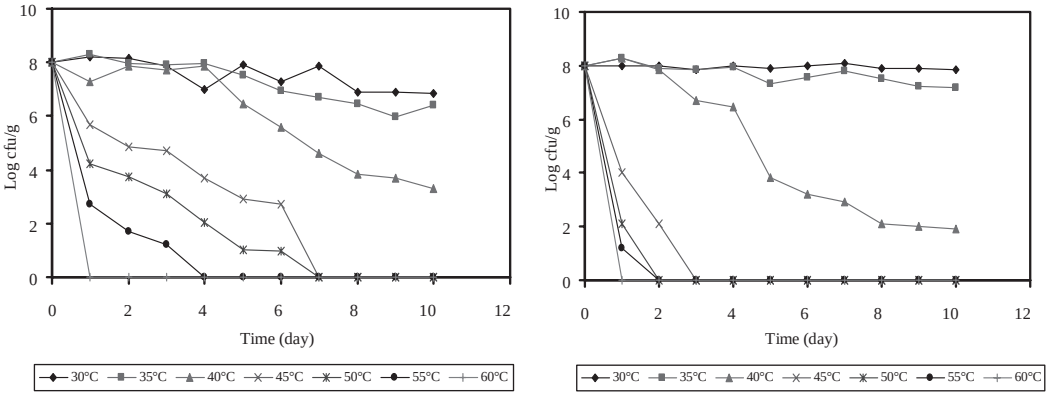

Figure 2 Effect of daily 2 h high temperature exposure on the viability of *Ralstonia solanacearum* in dry soil (a, moisture holding capacity (MHC) = 2-3%) and wet soil (b, MHC = 96-100%).

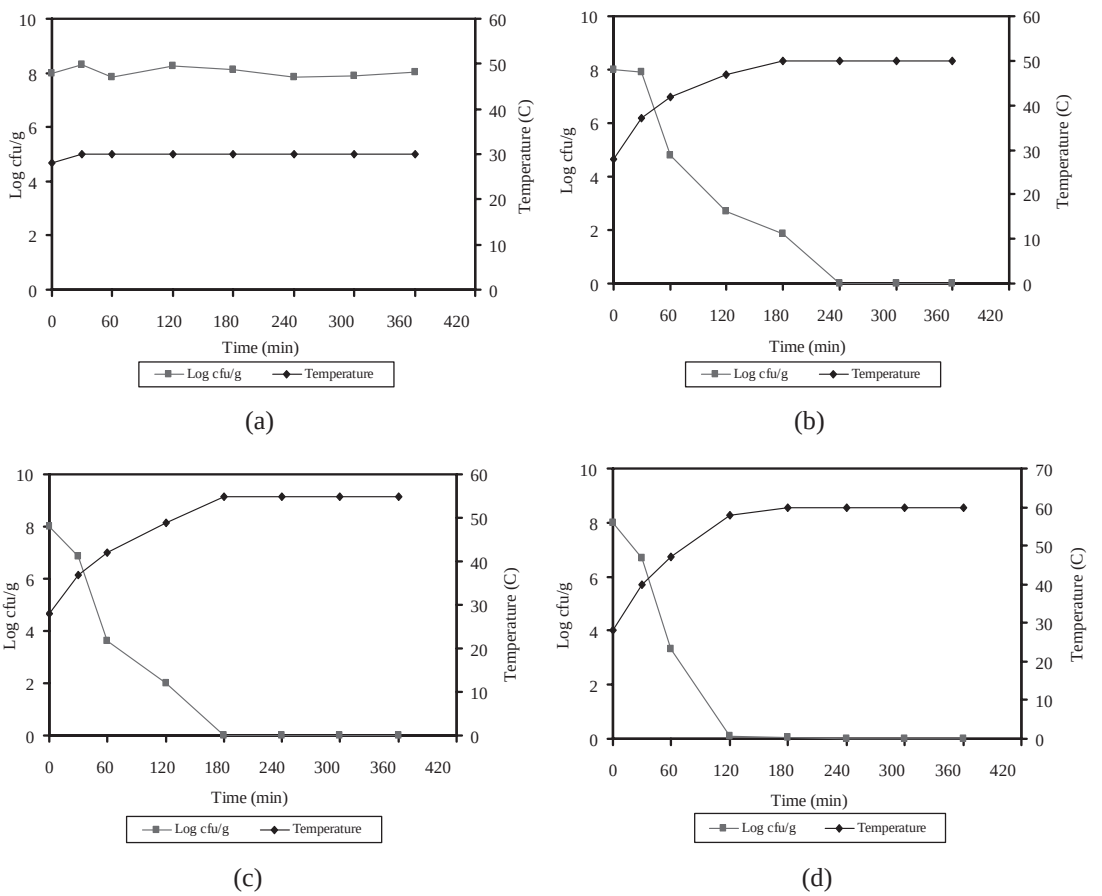

Figure 3 Effect of short exposure to high temperatures (a)30°C, (b) 50°C, (c)55°C and (d) 60°C on the inactivation of *Ralstonia solanacearum* in wet soil. Moisture holding capacity (MHC) of soil: 96-100%.

The results indicated that *Ralstonia solanacearum* in wet soil exposed to a constant temperature of 45°C and above had lost their viability after 24 h (data not shown). Such constant temperatures did not prevail when soil was tarped under field conditions. In study of Usmani and Ghaffar (1982), the maximum temperature attained in wet soil was 48°C for a duration of 2 h. At 60°C a 2-h exposure or a daily 2-h temperature exposure at 50°C for 3 days was necessary for complete loss in sclerotial viability. In a similar study 6-h high temperature exposures, the number of viable propagules of *Verticillium dahliae, Sclerotium cepivorum, Sclerotinia minor* were reduced by temperatures of 45°C and above after 2 weeks (Porter and Merriman, 1983).

Simulation of elevated temperatures in a cyclic regime to study the survival of *Ralstonia solanacearum* is, therefore, of prime importance to predict the efficacy of mulching treatments. Environmental factors such as soil moisture and temperature interact with each other in their effects upon soilborne pathogens. In experiments where dry soil was used, a uniform temperature for 2 days at 55°C or exposure to 60°C for 2 h for wet soil was necessary to kill all *Ralstonia solanacearum*. According to the thermal death times, *Ralstonia solanacearum* is not among the temperature resistant bacteria. Therefore, it is possible that pathogen inoculum can be reduced with an increase soil temperature and therefore controlled with the low dose of fumigant treatment. It has been reported that *Sclerotium rolfsii* lost its viability in 4-6 h at 50°C, and it population is reduced at 15 cm depth by means of solarization treatment (Pullman *et al.,* 1981).

The resulting high soil temperatures inactivate some of the soil microbes and therefore partially disinfest the soil. Heat treatment has been used to control *Fusarium oxysporum* f.sp. *conglutinans* (Ramirez-Villapudua and Munnecke, 1988); *Verticillium dahliae*; *Phytophthora cinnamomi* (Pinkerton *et al*., 2000) and *Macrophomina phaseolina* (Lodha *et al*., 1997).

This study demonstrated the potential of high different temperature and condition for control of bacterial wilt disease caused by *Ralstonia solanacearum*. According to Katan (1981), the effectiveness of the method depended on the temperatures and soil properties. In addition to this, maintaining adequate moisture levels, prolonging the treatment time gave rise to higher efficiency to decrease the population of the bacteria.

The viability of the pathogen inoculum was weak in the wet soil but the treatment had lesser effects in the dry soil.

Therefore, it was possible that the pathogen inoculum could be reduced with an increased soil temperature. It has been reported that *Sclerotium rolfsii* lost its viability in 4-6 h at 50°C and *Macrophomina plhaseolina* required 48 h at 50°C to be destroyed (Mihail and Alcorn 1984).

The high humidity and relatively high temperatures could play a key role in decreasing pathogen within the soil. Sufficient soil moisture is necessary to increase the heat conductivity of soil and to lower the ability of the pathogen to survive such high temperatures (Katan *et al*., 1976). In an experiment, Hilderbrand (1985) reported a 74% reduction in the population of *Fusurium* spp. after solar treatment. These findings showed that heat treatment brought about a decline in the population of bacterial wilt pathogens.

Environmental factors such as soil moisture and temperature interact with each other in their effects upon soilborne pathogens (Papavizas, 1977). A synergistic effect of these two factors in reducing the inoculum levels of *Macrophomina phaseolina* (Papavizas, 1977), *Verticillium alboatrum* (Nelson and Wilhem, 1958), *V. dahliae* (Pullman *et al.,* 1981), *Sclerotium rolfsii, S. cepivorum, Sclerotinia minor V. dahliae* (Porter and Merriman, 1983) and *Sclerotinia sclerotiorum* (Dueck *et al.,* 1981) have been

reported. In experiments where wet and dry soils were used to reduce the population of the *Ralstonia solanacearum*. Crowe and Hall (1980) found that sclerotia of *S. cepivorum* decayed due to increasing temperatures and soil moistures. The mechanism of loss in viability of microbes can be due to a single factor or a combination of factors: (a) The microbial proteins are more readily denatured by moist than by dry heat (Crisan, 1973); (b) At high temperatures microbes exude substances to such an extent that rejuvenation of the microbes is not possible (Papavizas, 1977); (c) Exudation at high temperatures followed by colonization of antagonists (Coley-Smith *et al.,* 1974); (d) High temperature neutralizes soil bacteria stasis resulting in lysis of microbes (Papavizas and Lumsden, 1980); or (f) High temperatures shift the microbial population in favour of antagonists. Evidence to support this last mechanism was obtained when it was found that a greater number of bacteria and actinomycetes inhibitory to *S. oryzae* were present in soil incubated at higher temperatures. This would suggest that addition of antagonists to soil during heat treatment may further increase the efficacy of the treatment (Elad *et al.,* 1980).

The basic principle is to heat the soil to a temperature that will effectively control all existing soil-borne pests, pathogens, weeds and arthropods. Soil heating for disinfestation has two basic requirements: (a) The temperature has to be equal to or above lethal for the most heat-tolerant pest existing in the soil. (b) The temperature cannot be too high, because temperatures above 70°C, and particularly approaching 100°C, become detrimental to most soil biota. High temperatures may result in eradication of beneficial microorganisms, e.g. rhizobia and mycorrhizae and microbial antagonists. Thus, a reduction in soil microbial activity may result in reinfestation of the heated soil by a contaminating inoculum, ultimately leading to disease incidence which can be even higher than that in the non-treated soil, due to 'biological vacuum' (Baker, 1962). Achieving an even distribution of temperature in the soil profile is, therefore, crucial to ensuring pest control with minimal disturbance of soil microbial activity. The achievement of this goal involves technical and economic problems.

Heat treatment can be improved in various ways. A very promising approach is its combination with other pest management methods, such as organic amendments producing volatiles. Soil heating is a more acceptable method of enhancing efficacy of antagonistic strains of *Trichoderma* spp. against *Armillaria* spp. than the use of fumigants (Davis, 1991).

Where the effects of the heat treatment are marginal in relation to disease control, the factors which improve its efficiency, such as maintaining adequate moisture levels (Mahrer, 1979), increasing the treatment time (Pullman *et al.,* 1979) assumes more importance.

This situation, now the potential of non-chemical methods for pest control, is being reassessed in an effort to include them in pest management programs. This study, therefore, showed that the high temperature treatment alone might be recommended as an alternative method to control of *Ralstonia solanacearum* in view of environmental and economical reasons.

CONCLUSIONS

Ralstonia solanacearum, is responsible for severe losses to many important crops, mainly solanaceous plants in tropical and subtropical regions where several outbreaks of the disease were recently reported, the disease represents a serious threat. In this study, data on time and temperature relationships on loss in viability of *Ralstonia solanacearum* in soil could thus be used to predict efficacy of soil solarization.

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