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Role of *Pythium aphanidermatum* (Edson) Fitzp. in tomato sudden death in the tropics with emphasis on integrated disease management

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ABSTRACT

Sudden death causes severe damage to tomato grown under field conditions following flooding at high soil temperatures. *P. aphanidermatum* plays a key role in sudden death. In the present study it was shown that tomato sudden death only occurred after flooding for 48 hrs or more at soil temperatures of 28 to 32°C when the soil was inoculated with *P. aphanidermatum*. The disease was reduced significantly at soil temperatures of 18 to 25°C. The fungus caused severe damage to the root cortex, caused wilting and plant death.

In a field experiment conducted in the hot growing season between June and September 2002 in Taiwan, 51, 72 and 93-day-old tomato plants were flooded and tested for susceptibility to sudden death. When the plants were 51 days old, they survived in significantly higher numbers than older plants. Flood duration plays a significant role in the death of tomato in infested soil. Flooding for 24 hrs did not have any effect on the disease whereas flooding for 48 and 72 hrs caused 100% of the plants to wilt.

Three isolates of *Trichoderma harzianum*, two of *Trichoderma virens* and one of *Streptomyces saraceticus* were evaluated for biological control of tomato sudden death due to *P. aphanidermatum* in the greenhouse and in the field. *T. harzianum* and *T. virens* isolates were used to treat the seed and the potting medium used for seedling production, and also incorporated into the soil before transplanting. *Streptomyces* was added to the potting medium and applied daily to the field soil or greenhouse plots until 40 days after transplanting. The results revealed that neither *Trichoderma* spp. nor *Streptomyces* gave significant biocontrol of tomato sudden death. The percentage of diseased tomato plants growing in soil treated with either *Trichoderma* isolates or *Streptomyces* after flooding was not significantly different when compared to the soil treated with *P. aphanidermatum* alone.

The soil amendments, SFMC, FNB-5A and S-H Mixture tested in this study also showed little promise for controlling tomato sudden death due to *P. aphanidermatum*. In both greenhouse and field experiments conducted in the summer of 2001 in Taiwan, the number of wilted and dead tomato plants was not significantly reduced over the control. The *Trichoderma* aureoviride added to the soil amendment treatments also gave no significant improvement in control of *P. aphanidermatum* with respect to tomato sudden death. Similar poor results were observed in treatments with the fungicide Mefenoxam.

Grafting tomato onto eggplant rootstocks successfully protected the plants against tomato sudden death. In greenhouse tests, the number of diseased plants was significantly reduced on grafted tomato. The result in the greenhouse and field experiments collaborated each other. The eggplant roots were colonized by *P. aphanidermatum*, but no extensive damage was caused to the plant.

The results of the present study indicate that, with exception of grafting with eggplant rootstocks on biocontrol or the use of organic amendments would not give adequate control in the field. More research on biocontrol and organic matter for control of tomato sudden death is thus required.

Die Rolle von *Pythium aphanidermatum* (Edson) Fitzp bei "tomato Sudden Death" in den Tropen mit Schwerpunktpunkt integriertes Pflanzenschutzmanagement

ZUSAMMENFASSUNG

"Sudden Death" ruft schwere Schäden an Tomaten hervor, die unter Gewächshaus- und Freilandbedingungen nach Überflutung und hohen Bodentemperaturen heranwachsen. In diesem Zusammenhang spielt *Pythium aphanidermatum* eine Schlüsselrolle. Die Erkrankung tritt nur auf, wenn hohe Bodentemperaturen (28-32°C) mit der Überflutung einhergehen und *Pythium aphanidermatum* im Boden vorliegt oder der Boden nicht sterilisiert wurde. Bei niedrigeren Temperaturen (18-25°C) wurde das Ausmaß des Schadens reduziert. Der Pilz rief eindeutige Schäden an der Wurzelrinde hervor, die zu Welkeerscheinungen und Absterben der Pflanzen führte, sowie das Wurzelfrischgewicht reduzierte.

Im Freilandversuch von Juni bis September 2002 wurden Tomatenpflanzen zu unterschiedlichen Zeitpunkten, 51, 72 und 93 Tage nach dem Auspflanzen geflutet. Pflanzen die 51 Tage nach dem Auspflanzen geflutet wurden überstanden besser als solche, die zu einem späteren Zeitpunkt geflutet wurden. Die Überflutungsdauer spielt eine Rolle beim "Sudden Death" an Tomaten auf infizierten Böden. Nach Flutungszeiten von 24 Stunden traten keine Symptome auf, aber es kam zum Totalausfall, wenn die Überflutung 48 oder 72 Stunden andauerte.

Drei Isolate von *Trichoderma harzianum*, zwei Isolate *Trichoderma virens* und ein Isolat *Streptomyces saraceticus* wurden auf ihre biologischen Kontrolleigenschaften gegen "tomato Sudden Death", welches durch *Pythium aphanidermatum* hervorgerufen wird, im Gewächshaus und unter Freilandbedingungen untersucht. *Trichoderma harzianum* und *Trichoderma virens* Isolate wurden sowohl ins Aussaatsubstrat als auch in das Kultursubstrat eingemischt. *Streptomyces* wurde erst ins Kultursubstrat und erneut nach 40 Tagen appliziert. Die Ergebnisse zeigen, dass weder *Trichoderma* noch *Streptomyces* wenig Aussichten haben als biologische Bekämpfungsmaßnahmen gegen "tomato Sudden Death" eingesetzt zu werden. Der Prozentsatz an gesunden Tomatenpflanzen die in, mit *Trichoderma* oder *Streptomyces* Isolaten, behandelten Böden herangezogen wurden, war nicht signifikant unterschiedlich, wenn man sie mit der unbehandelten Kontrolle verglich.

Keine Änderung der Bodenzusammensetzung, ob mit SFMC, FNB-5A oder S-H, die in dieser Studie getestet wurden lässt vermuten, dass man hierdurch einen Bekämpfungserfolg von *Pythium aphanidermatum* erreichen kann. In keinem der beiden Gewächshaus- und Freilandversuchen, die im Sommer 2001 durchgeführt wurden, konnten durch die Veränderungen der Bodenzusammensetzung signifikante Reduzierungen des Befalls erzielt werden. *Trichoderma aureoviride,* welches den veränderten Böden zugefügt wurde, zeigte keine Effektivität gegen *Pythium aphanidermatum*. Ähnliche Resultate wurden für die mit Mefenoxam behandelte Kontrolle beobachtet.

Veredeln der Pflanzen auf Auberginenunterlagen schützte die Tomaten erfolgreich gegen "Sudden Death". In Gewächshausversuchen wurde die Zahl der infizierten Pflanzen signifikant reduziert und dies konnte in Freilandversuchen bestätigt werden. Die Auberginenwurzeln wurden von *Pythium aphanidermatum* besiedelt ohne umfangreiche Schäden hervorzurufen.

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1 GENERAL INTRODUCTION

The importance of *Pythium aphanidermatum* in tomato sudden death disease

Tomato (*Lycopersicon esculentum* Mill.) production in lowland areas of tropical and subtropical countries during the hot, wet season faces a number of constraints. Yield is generally quite low compared to that in the cool season or from highland production areas (Abdelhafeez et al. 1969). High temperature reduces fruit set (Abdelhafeez et al. 1975), heavy rainfall directly damages aerial plant parts, soil water logging reduces plant vigor, and short periods of soil flooding often result in wilt and sudden death of the tomato plants (Kuo et al. 1982). Also contributing to the difficulties of growing tomatoes in the hot, wet season are diseases such as damping-off, bacterial wilt, southern blight, root-knot, target leaf spot, gray leaf spot, early blight, and bacterial spot.

The sudden death disease is a major and growing problem in tomato production during the hot, wet season (Villareal et al. 1978). Rapid wilting and death of tomato plants after a short period of flooding are usually observed under hot and humid conditions, which is likely due to the combination of high temperature with flooding (Kuo et al. 1982). Tomato has been shown to be one of the most flood-sensitive vegetables (Iden 1956). Flood resistant genotypes of tomato have been investigated and reported by Reid *et al*. (1969); Kuo et al. (1980), and Kuo et al. (1982). However, the flood-tolerant genotypes are not sustainable, since some accessions, which appeared flood tolerant in one trial, were not tolerant in another. In addition, plants did not die when flooded at low temperature or in sterilized soil in greenhouse experiments, but died in the field following flooding at high temperature (Kuo et al. 1982). It has been suggested that there might be a biological component responsible for plant wilt and sudden death in field soils, since the disease was eliminated by soil sterilization. Many fungal root pathogens have been recorded as causing soilborne diseases of tomato including *Phytophthora* spp., *Fusarium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium* spp. (Frank 1995; McCarter 1997).

Species of *Pythium*, including *P. aphanidermatum, P. ultimum* and *P. debrayan*, have worldwide distribution (Van Der Plaats-Niterink 1981) and have been recorded as the cause of damping-off and stem root of many crops such as tomato, chickpea, cucumber,

pepper, bean, spinach in both soil and soilless cultivations (Jenkins et al. 1983; Trapero-casa et al. 1990; Wulff et al. 1998). Among these, *Pythium aphanidermatum* (Edson) Fitzp. is one of the most serious pathogens, capable of causing catastrophic yield losses (Favrin et al. 1988). This fungus is known to have a high temperature optimum of 35-40°C and it is also known that temperature greatly influences infection and the subsequent damage it causes to its plant host (Thomson et al. 1971; Kammedahl et al. 1979). At lower temperatures, damage is much less or negligible (Bolton 1980).

Plant age and susceptibility to soilborne diseases

Plant susceptibility to disease varies with plant age and also changes with time of year (Populer 1978). Susceptibility of plants to soilborne diseases depends on plant age. Four general classes of change in susceptibility with plant age are recognized: (1) decreased susceptibility with the age of plant; (2) increased susceptibility with age and intermediate age susceptibility that is either greater or less than that of younger or older plants (Yarwood 1959; Populer 1978). Decreasing susceptibility to soilborne pathogens with plant age may be related to the structure and function of different types of roots and with their position in the overall root system (Zobel 1991). If the pathogen infects primarily first-older roots, the proportion of tissue susceptible to soilborne pathogens at different times or stages of host development will depend on the ratio of susceptible first-older roots to non-susceptible second roots (English et al. 1994). In addition, many other components of host development may contribute indirectly to changes in susceptibility to root disease such as pathogens, beneficial microorganisms, soil temperature, and soil pH (English et al. 1994).

The susceptibility of a plant to changing environmental factors varies depending on field and glasshouse conditions. The most important abiotic factors that influence changes in the susceptibility of hosts to root disease are light, soil moisture, temperature, aeration, mineral nutrition, hydrogen ion concentration, and bulk density (English et al. 1994). Excess water or flooded soil, for example, may increase disease severity by directly affecting the chemical and physiological structures of the plant root (Kou et al. 1980). It may also directly influence the pathogen by promoting the release of zoospores and

stimulating the processes of infection by species of *Phytophthora* and *Pythium* (Duniway 1983).

Cultivation practices may play important direct or indirect roles in changing host susceptibility (Rovira et al. 1990). Predisposition of the root to disease in monoculture or crop rotations under different cultivation practices may be a consequence of autotoxicity or allelopathy (Rovira et al. 1990). If root development is slowed or roots are damaged by compaction, susceptibility to disease may be altered (English et al. 1994). Timing of crop management practices can greatly influence host susceptibility (Palty et al. 1997). Some *Pythium* spp. affect plant roots at temperatures favorable for root growth but only cause disease at higher temperatures (Mitchell, 1975).

Biological control of soilborne diseases

Biological control has been known since 1874, when Roberts showed the suppressive activity of *Penicillium glaucum* against bacteria and regarded this phenomenon as antagonism. For more than 60 years, the term biological control, meaning control of one organism by another, has been used by both entomologists and plant pathologists. In biological control of plant disease, three antagonistic mechanisms are recognized: antibiosis, which means production of metabolites toxic or inhibitory to the plant pathogens; competition for nutrients and space; and parasitism, where the antagonist directly extracts nutrients from the pathogen (Baker et al. 1996). The biological control of plant pathogens has been defined by Cook et al. (1983) as the reduction of the amount of inoculum density or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man. According to this broad definition, biological control includes cultural practices that create an environment favorable to antagonists, host-plant resistance, mass introduction of antagonists, nonpathogenic strains, or other beneficial organisms or agents such as compost (Cook et al. 1983).

Biological control of soilborne pathogens has emphasized the use of antagonistic microorganisms and mediates through mechanisms including competition, antibiosis and mycoparasitism (Baker et al. 1974; Tronsmo 1996). These activities occur among fungi that are not injurious to the plants (Backer et al. 1974). Competition, for example, occurs among

microorganisms for the resources: water, oxygen, nutrients and space (Baker et al. 1974). Biocontrol manipulates the environment around the crop plant to favor organisms that contribute to plant health and vigor. It is less disruptive to the ecosystem than applying chemical pesticides (Harman et al. 1989). The term 'biological system management' defined as the outside-in and inside-out perspective has been coined to describe full integration of biocontrol in crop production (Sikora 1997).

Biological control of soilborne disease has increased in the past two decades. Beneficial fungi, especially species of *Trichoderma* spp., have been used extensively as a seed treatment in order to reduce diseases (Harman et al. 1980; Lifshitz et al. 1986; Harman et al. 1989). The species *Trichoderma harzianum* and *Trichoderma virens* are among the most intensively studied biocontrol agents. Treatment of seed or plants with *Trichoderma harzianum* has promoted plant health and has also effectively controlled *Pythium* damping-off (Liu et al. 1965; Harman et al. 1980; Marshal 1982; Lifshitz et al. 1986; Harman et al. 1989). In addition, *Trichoderma harzianum* has shown broad-spectrum action to other soilborne pathogens such as *Pythium ultimum*, *P. aphanidermatum*, *Rhizoctonia solani*, and species of *Phytophthora* in many crops (Chet et al. 1981; Elad et al. 1982; Sinva et al. 1984; Harman et al. 1989; Roiger et al. 1991).

Biological control of soilborne diseases by soil organic amendments

Organic amendments such as crop debris, green and animal manures and other residues have been used in practical crop management in old agriculture systems in order to improve soil fertility (Rodríguez-Kábana et al. 1987). Early attempts were also made to use soil microorganisms in practical biological control under field conditions (Backer et al. 1974). Organic amendments may alter soil physical and chemical conditions such as pH (Blacker et al. 1983), and produce toxic fungal inhibitors (Spencer et al. 1982; Huang et al. 1993) that have important consequences for plant growth and the occurrence of soilborne diseases (Huang et al. 1993).

Soil amendments have either simple or complex mechanisms that affect plant pathogens depending on the amendments and pathogens involved (Huang et al. 1993). It directly affects the pathogens by killing the pathogen propagules; indirectly through the

breakdown of chemicals, pesticides or fertilizers that have harmful side-effects on the pathogens, or by increasing dormancy of propagules (Baker et al. 1974; Huang et al. 1993). Soilborne pathogens are in some cases indirectly controlled through a combination of factors, which include improved vigor of the host plants combined with enhanced activity of antagonistic microbes (Baker et al. 1974; Huang et al. 1993). Adding amendments, for example, in the form of green legume crops, which are rich in available nitrogen and carbon compounds, promotes host plant growth (Baker et al. 1974; Palti 1981; Mandelbaum et al. 1990). Soil pH changes and mineral availability increases for both soil microorganisms and plants (Baker et al. 1974; Lin et al. 1990). In addition, organic amendments with chitinous materials favour the development of chitinolytic microflora in the soil or even under soil-less cultivation (Godoy et al. 1983). Many microorganisms in the soil microflora are antibiotic-producing bacteria, actinomycetes and fungi antagonistic to phytonematodes and other soilborne pathogens (Palti 1981; Sikora 1992; Huang et al. 1993; Rodríguez-Kábana et al. 1997).

Many composts used as soil amendment have been investigated as part of integrated biological control due to their ability to suppress soilborne plant pathogens. This phenomenon has been studied on a wide range of pathogens such as *Pythium aphanidermatum*, *P. ultimum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Phytophthora cinnamomi*, using compost originated from waste materials such as hardwood or pine bark, grape marc, cattle manure or other materials (Nelson et al. 1983; Hadar et al. 1986; Mandelbaum et al. 1990; Shiau et al. 1999). These compost amendments provide an improved environment to the susceptible plant in which disease incidence is reduced (Hadar et al. 1992). The amendment named S-H, for example, has a direct effect on *P. aphanidermatum* by inhibiting mycelial growth and zoospore germination. It greatly decreases disease incidence on cucumber under greenhouse and field conditions (Lin et al. 1988; Lin et al. 1990). Compost amendments provide potentially effective biological control of plant pathogens, but the biological and natural aspects of this phenomenon also are associated with practical problems of use in agriculture (Hadar et al. 1992). In addition, organic amendments are not a panacea, and in some cases the amendments do not work well for deep-rooted crops or for plants with a long growing period (Lin et al. 1990).

Grafting to reduce soilborne diseases

Using grafted plants is currently popular in horticultural production around the world. In Japan, for example, 93% of watermelons, 72% of cucumbers, 50% of eggplants, 32% of tomatoes and 30% of all types of other melons are produced using grafted plants (Oda 1993). The grafted plant was developed for multi purposes that protect the plant roots in adverse soil environments. Abdelhafeez et al. (1975) first tested tomato grafted onto tomato or eggplant rootstocks with the assumption that the tomato/eggplant combination would be of benefit for tomato yield under hot, arid conditions. The eggplant rootstock has an effect on vegetative growth only, whereas the production of trusses and fruits lagged behind resulting in a lower yield than that of the tomato/tomato graft. However, the tomato grafted onto eggplant was more beneficial than that of non-grafted plants or the tomato/tomato graft under hot and rainfall conditions (AVRDC 1999).

Another main use of grafted plants was to reduce the overall problem of soilborne pathogens. Grafting plants in most cases has been shown to protect plants against diseases due to nematodes, bacteria, and fungi (Oda et al. 1994; Oda 1995).

Grafting on resistant rootstocks is a new component of integrated control of soilborne plant pathogens (Katan 1996). Grafting tomato onto disease-resistant varieties or onto eggplant rootstocks has multiple benefits. Grafted plants were shown to be resistant to several soilborne diseases such as bacterial wilt, damping-off or nematodes (Peregrine et al. 1982; Chadha 1988; Matsuzoe et al. 1993). A disadvantage of tomato grafted onto resistant tomato rootstocks is that the plants are not protected against excess water or flooding conditions (AVRDC 1999). In this case, tomato grafted onto eggplant rootstock is superior over the combination of tomato/tomato (AVRDC 1999).

Scope of the study

The objectives of the study presented here were to:

1. Assess the role of *Pythium aphanidermatum* in sudden death of tomato following flooding at high soil temperatures as well as the effects of soil temperatures on disease development.
2. Study the level of resistance in the tomato plant at different plant ages to tomato sudden death due to *Pythium aphanidermatum*.
3. Investigate biological control efficacy of selected fungal antagonists to sudden death due to *Pythium aphanidermatum* following flooding at high temperatures.
4. Evaluate the effect of soil organic amendments on biological control of tomato sudden death due to *Pythium aphanidermatum*.
5. Determine whether tomato (*Lycopersicon esculentum* Mill.) grafted onto eggplant (*Solanum melongena* L.) rootstocks protects against tomato sudden death due to *Pythium aphanidermatum* following flooding in high temperature conditions.

2 ROLE OF *PYTHIUM APHANIDERMATUM* IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) SUDDEN DEATH AND EFFECT OF SOIL TEMPERATURE ON DISEASE DEVELOPMENT

2.1 Introduction

Pythium aphanidermatum is known to have a high virulence at temperatures between 35 and 40°C (Van Der Plaats-Niterink 1981). It is also known that temperature greatly influences infection and subsequent damage that *P. aphanidermatum* causes to its host plant (Thomson et al. 1971; Kammedahl et al. 1979). Optimum temperature for oospore germination is 30°C (Adams 1971). Temperatures over 30°C are considered to be most favorable for disease development, while at lower temperatures damage is much less or negligible (Thomson et al. 1971; Bolton 1980). Similarly, high soil moisture is usually favorable for root disease caused by *Pythium* spp. (Kammedahl et al. 1979). *P. aphanidermatum* has a very fast growth rate and is a prolific producer of zoospores.

P. aphanidermatum has been reported as causing several diseases on young tomato plants such as damping-off, root rot, and cottony blight (Wells et al. 1954; Bolton 1980; McCarter 1997). It is also a major pathogen causing a series of diseases on other crops including cucumber, sugarbeet, spinach, cabbage, hot chilli, and turf grass (Hine et al. 1969; Gold et al. 1984; Satija et al. 1987; Lo et al. 1997; Wulff et al. 1998; Shiau et al. 1999). The damping-off disease has been well documented from many regions in the world. The disease has been the main cause of crop losses in tomato, cucumber and pepper in England, America, Canada, China, India, and Thailand. It is often cited as the causal agent of root diseases and wilting of a number of vegetable crops grown not only in hydroponic systems, but also in the field (Walker 1952; Yu et al. 1989; Zhang et al. 1990; Paulitz et al. 1992).

Pathogenic mechanisms of *P. aphanidermatum* for attacking the plant have been investigated. Environmental factors such as soil temperature, soil moisture and soil pH have effects on the population densities of the fungus. High soil moisture has been shown to have a positive influence on population densities of *P. aphanidermatum*. In addition, soil pH between 5-8 and temperatures of 30°C and higher have been shown to positively affect the penetration of the host plant by *P. aphanidermatum* (Bolton 1980). However, the true

cause of sudden death of tomato under hot, wet conditions is not clearly understood. During summer trials in recent years at AVRDC (Taiwan), the occurrence of stem rot, wilting and plant death caused by *P. aphanidermatum* have been observed after heavy rainfall.

The aims of this study were to:

1. Assess the role of *Pythium aphanidermatum* in tomato sudden death following flooding under high temperatures.
2. Investigate the effect of soil temperature on tomato sudden death development due to *Pythium aphanidermatum* following flooding.

2.2 Materials and methods

Three different greenhouse experiments were conducted from summer 2001 to summer 2002 at AVRDC, Shanhua, Taiwan, in soil infested with *P. aphanidermatum*. The fungus was incorporated into the soil 10 days after transplanting. Plastic containers (tubs) were used as micro experimental plots.

2.2.1 Tomato plants

The tomato line CL5915-206D, which was determined by AVRDC to be heat tolerant and virus resistant, was used. The seedlings were planted in a peat moss substrate for 30 days in the greenhouse and then transplanted into the tubs for experimentation.

2.2.2 Preparation of *Pythium aphanidermatum* inoculum

P. aphanidermatum, strain number 4, isolated by the mycology unit at AVRDC, was cultured on V-8 agar for 3 days at 28°C before being inoculated onto rice seed for solid state fermentation. Each 400 ml beaker containing 150 ml of rice grain and 75 ml of distilled water was autoclaved twice prior to being used as the final growth substrate. Two blocks of agar of 3-day-old *P. aphanidermatum* cultures were put into the rice grain in each beaker and then incubated in an illuminated chamber at 28°C for 10 days. One beaker of

rice grain was incorporated into the upper 10 cm of soil of each tub, 10 days after transplanting.

2.2.3 Micro plot design

Plastic tubs (31 cm x 51 cm x 40 cm: height x width x length) were used as experimental units. The tubs were sterilized with a solution of hydrochloride (1%) before being filled with soil. Five kg of sterilized pebbles (1-2 cm diameter) were placed in the bottom of the tub. A plastic net was then laid over the pebbles in the bottom of the tub. A U-shaded siphon was attached to the tub so that water could be siphoned out from under the net (Figure 2.1). Fifty-five kg of heavy clay soil, which had been taken from AVRDC fields, (Taiwan) was steam pasteurized, and then filled into each tub. Soil pH was evaluated and found to be 6.5.

About 82.5 g of organic commercially available granular fertilizer (N:P:K=4:4:4) and 20 g chemical fertilizer (N:P:K:Mg=15:15:15:4) was incorporated into the soil of each tub before transplanting. In addition, 3.5 g of chemical fertilizer (N:P:K=20:20:20) per tub was added weekly until the plants were harvested.

A  B 

Figure 2.1: Greenhouse experiment at AVRDC, Shanhua, Taiwan (A) and plastic tub with siphon tube (arrowed) for drainage (B).

2.2.4 *Pythium aphanidermatum* recovery from soil, floodwater and plant roots

Isolation of *Pythium aphanidermatum* from the soil

Soil samples were taken randomly from the tubs at 12-hr intervals during flooding as well as 1 and 2 days after floodwater has been removed. The soil samples were placed on paper towels and allowed to dry at room temperature for 2 days. Four dilutions 1/5, 1/10, 1/20, and 1/40 of soil (g soil per ml 0.3 % water agar) were used to quantify the population of *P. aphanidermatum* (Burr et al. 1973). Each dilution was mixed on a Vortex stirrer for at least 15 min and a 1 ml aliquot was dispensed evenly across the surface of the selective medium for *P. aphanidermatum* developed by Burr et al. (1973). The plates were incubated at 35°C for time intervals ranging from 24 to 72 hrs, after which the soil was carefully washed from the agar surface and the *Pythium* colonies were recorded.

Isolation of *Pythium aphanidermatum* from floodwater

Floodwater sampling was used to assess the fluctuation of the population density of *P. aphanidermatum* during flooding. A sample of 50 ml of floodwater was taken from each tub every 12 hrs during the flooding period. The water samples were diluted to 1/1, 1/10, 1/100, 1/1000 (1 ml floodwater sample per 9 ml sterilized water) and spread over ABPDA agar medium (AVRDC selective medium). The plates were incubated at 28°C for time intervals ranging from 24 to 72 hrs and the *Pythium* colonies were recorded.

Isolation of *Pythium aphanidermatum* from the roots

The roots were collected from the tubs at 12-hr intervals during flooding as well as 1 and 2 days after the floodwater has been removed. The roots were washed with running tap water and cut into 1 cm length. Ten root pieces were placed on a plate of Mircetich medium (Mircetich 1971) for fungal colony production. The plates were incubated at 28°C and the colony-forming units (CFU) of *P. aphanidermatum* recovered from each root section were observed and recorded after 24 hrs incubation.

2.2.5 Root weight

Tomato roots were removed from the soil by carefully washing with tap water 7 days after floodwater drainage. The washed roots were placed on paper towels at room temperature for 30 min to remove excess water and then fresh root weight was determined. The washed root was dried in an oven at a temperature of 50°C for 48 hrs after which the root dry weight was determined.

2.2.6 Flooding

The plastic tubs were flooded 60 days after transplanting. The water level was maintained at 2-4 cm above the soil surface for 48 or 72 hrs, and then quickly removed through the siphon tube (Figure 2.1, B).

2.2.7 Experimental design

Role of *Pythium aphanidermatum* in tomato sudden death following flooding at high temperature in artificially inoculated soil

The experiment was conducted in August-November, 2001. The soil temperature was maintained at 30-32°C during the experiment. The soil and air temperatures as well as the humidity inside the greenhouse were recorded daily. Two treatments with 3 replications each were tested (Table 2.1).

Table 2.1: Treatments for evaluating the role of *Pythium aphanidermatum* in tomato sudden death in artificially inoculated soil

Treatment	***Pythium aphanidermatum* inoculation**	**Flood duration**
Inoculated	10 days after transplanting	48 hrs
Non-inoculated control (ck)	None	48 hrs

Role of *Pythium aphanidermatum* in tomato sudden death following flooding at high temperature in naturally and artificially infested soil

The experiment was conducted between January and April 2002 in soils both naturally and artificially infested with *P. aphanidermatum* to assess the role of the fungus in tomato sudden death. The soil was taken from AVRDC fields where tomato sudden death had previously occurred. Half of the field soil was used as the treatment with *P. aphanidermatum* infested soil, and the other half was steam-sterilized for the treatments 'sterilized soil-inoculated' and 'sterilized non-inoculated' control. Both treatments with infestation were compared to the non-inoculated control. The tubs were flooded 60 days after transplanting for 48 hrs. During crop development, the temperature ranged between 15 and 20°C due to natural cool winter conditions. Seven days before flooding, the temperature was increased to 28-32°C and stayed at this level until 7 days after floodwater removal. The following treatments with three replications each were tested (Table 2.2).

Table 2.2: Treatments to evaluate the role of *Pythium aphanidermatum* in causing tomato sudden death in naturally and artificially infested soil

Treatments	***Pythium aphanidermatum* inoculation**	**Flood duration**
Field soil + natural infestation	None	48 hrs
Sterilized soil + inoculated	10 days after transplanting	48 hrs
Sterilized soil + non-inoculated (ck)	None	48 hrs

Role of *Pythium aphanidermatum* in tomato sudden death following flooding in two soil temperature regimes

The experiment was conducted between January and April 2002 to investigate two temperature regimes in soil artificially infested with *P. aphanidermatum*. Temperatures were maintained at 18-25°C (Set I) and 28-32°C (Set II), respectively. The experiment was conducted in the cool season to maintain the greenhouse at 18-25°C. Higher soil temperatures of 28-32°C were maintained through a heating system 7 days prior to flooding

and 7 days after flooding. All tubs of Set I were flooded 45 days after transplanting and of Set II after 60 days. The water level was maintained at 2-4 cm above the soil surface for 48 hrs and then quickly removed by the siphon system. The following treatments with three replications each were tested (Table 2.3).

Table 2.3: Treatments to evaluate the role of *Pythium aphanidermatum* in causing tomato sudden death following flooding depending on soil temperature

	Treatments	**Flood duration**
Set I 18-25°C	Non-inoculated control (ck)	48 hrs
	P. aphanidermatum inoculated (Pa)	48 hrs
Set II 28-32°C	Non-inoculated control (ck)	48 hrs
	P. aphanidermatum inoculated (Pa)	48 hrs

2.2.8 Data and statistical analysis

The number of wilted plants from all treatments was recorded 2, 4 and 7 days after floodwater was removed. Seven days after flooding, plants were harvested and root dry weights determined. The data on incidence of permanent wilt of tomato were analyzed with the SAS (SAS Institute Inc. 1989) program, using the general linear model procedure, including analysis of variance, Duncan's multiple range test, least significant difference, (LSD) and/or orthogonal contrasts.

2.3 Results

2.3.1 Role of *Pythium aphanidermatum* in tomato sudden death following flooding at high temperature in artificially inoculated soil

Incidence of permanent wilt: No infected tomato plants were observed after transplanting to soil inoculated with *P. aphanidermatum* or in the non-inoculated control. However, after flooding for 48 hrs, a few plants wilted in the non-inoculated controls. In the infested soil, 65% of the tomato plants showed wilt symptoms 4 days after flooding. Seven days after

drainage, over 70% of the plants showed permanent wilt symptoms. In the non-inoculated control, 3% of the plants showed wilt symptoms, which was significantly (P≤0.01) lower when compared to the inoculated treatment (Figure 2.2).

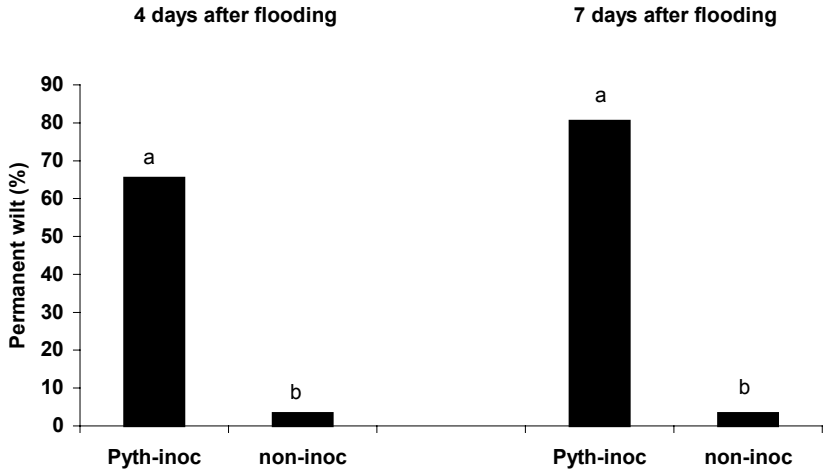

Figure 2.2: Effect of *Pythium aphanidermatum* on incidence of permanent wilt of tomato plants 4 and 7 days after floodwater was removed in a greenhouse experiment between August and November 2001 at AVRDC, Shanhua, Taiwan. Different letters show statistically significant differences between two treatments according to Duncan's multiple range test (P≤0.01) n=6.

Root damage and root dry weight: Seven days after the floodwater was removed, severe damage to the root system by *P. aphanidermatum* was observed. The root cortexes were sloughed-off or missing in all plants grown in infested soil. No injured roots were observed on tomato plants grown in non-inoculated soil (Figure 2.3).

Root damage by *P. aphanidermatum* resulted in reduced root biomass. Both fresh and dry weights of the roots of tomato plants grown in soil infested with *P. aphanidermatum* were significantly (P≤0.05) lower when compared to the root weight of plants grown in the non-inoculated soil (Figure 2.4).

A

B

Figure 2.3: Healthy roots and roots damaged by *Pythium aphanidermatum* following 48 hrs flooding at 28-32°C soil temperature in a greenhouse experiment between August and November 2001 at AVRDC, Shanhua, Taiwan.

A(1): Root of plant grown in soil infested with *Pythium aphanidermatum*

A (2): Root of plant grown in non-inoculated soil

B(1): Root cortexes missing on the plant grown in soil infested with *Pythium aphanidermatum*

B (2): Root of plant grown in non-inoculated control

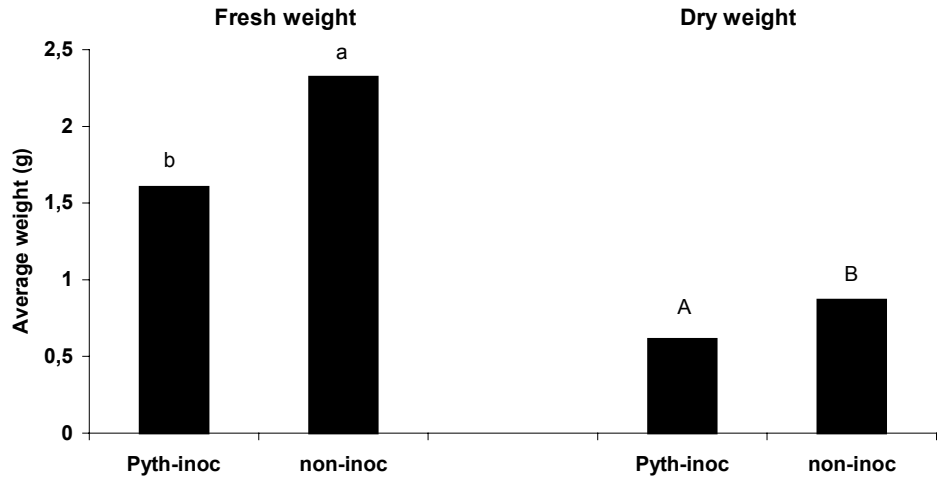

Figure 2.4: Effect of *Pythium aphanidermatum* on fresh and dry weight of tomato roots, in greenhouse experiment between August and November 2001 at AVRDC, Shanhua, Taiwan. Different letters show statistically significant differences between two treatments according to Duncan's multiple range test ($P \leq 0.05$) n=6.

P. aphanidermatum recovery from soil, water and root: The population density of *P. aphanidermatum* in the soil, floodwater and roots, was determined every 12 hrs during the flooding period. The soil and roots were also sampled 1 and 2 days after floodwater was removed. Due to a high variation in the colony-forming units (CFU) of the pathogen in the samples, statistical analysis was not possible. The results, however, are presented as the means of the samples at the different sampling periods.

P. aphanidermatum population densities in the soil dropped slightly between 12 and 48 hrs during flooding. However, after floodwater removal, *P. aphanidermatum* was recovered in higher densities in the soil 1 and 2 days after floodwater has been drained (Figure 2.5). *P. aphanidermatum* zoospores isolated from the floodwater were highest 12 hrs after flooding and then dropped 24, 36 and 48 hrs thereafter (Figure 2.5). The percentage of *P. aphanidermatum*-infected roots remained more or less stable from 12 to 48 hrs after flooding. However, one day after floodwater removal, the number of CFU increased and resulted in a 3-fold higher number after 2 days compared to the initial CFU number at 12 hrs (Figure 2.5).

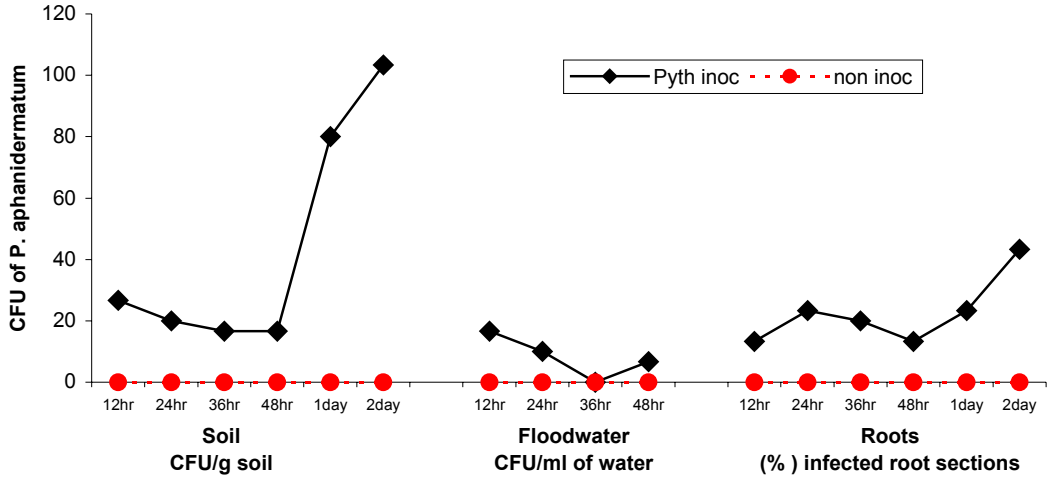

Figure 2.5: *Pythium aphanidermatum* recovery from soil (CFU/g soil), floodwater (CFU/ml of flood water), and roots (% infected root sections) in a greenhouse experiment to test the effect of artificial inoculation on tomato sudden death.

2.3.2 Role of *Pythium aphanidermatum* in tomato sudden death following flooding at high temperatures in naturally and artificially inoculated soil

Permanent wilt: No significant difference was observed 4 days after flooding in the wilting rate of tomatoes grown in the treatments of field soil naturally infested (20%) and sterilized soil inoculated with *P. aphanidermatum* (27%). Four days after flooding the control showed 10% wilted plants. However, 7 days after flooding, 3.3 % of the plants in the control had wilted. The number of wilted plants observed in the control was significantly lower when compared to both naturally infested and artificially inoculated soil. There was also no obvious difference in wilt between the 4- and 7-day periods (Figure 2.6).

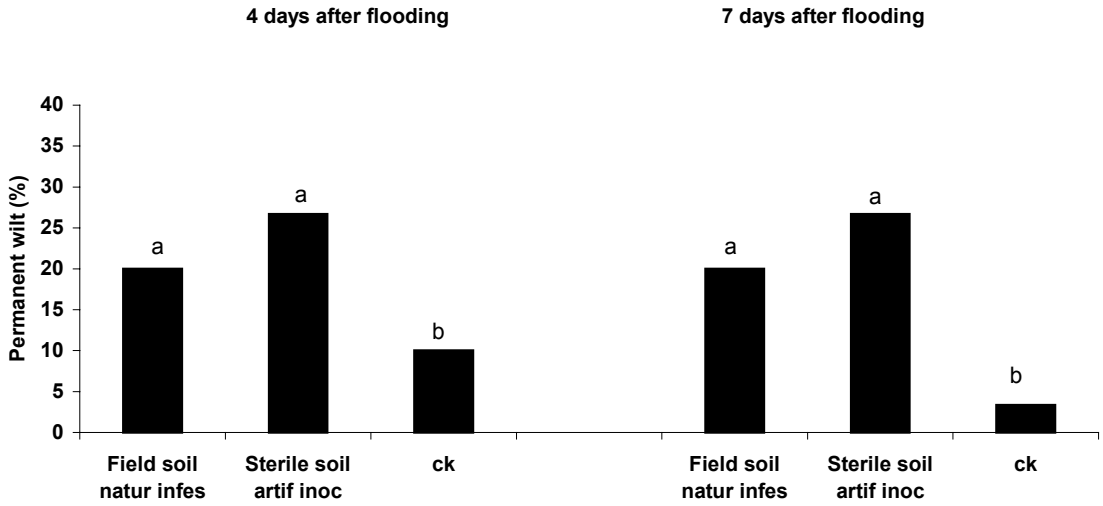

Figure 2.6: Occurrence of sudden death of tomatoes grown in field soil with infested and sterilized soil inoculated with *Pythium aphanidermatum*, 4 and 7 days after floodwater removal in a greenhouse experiment between January and April 2002 at AVRDC, Shanhua, Taiwan. Different letters show statistically significant differences between two treatments according to Duncan's multiple range test ($P \leq 0.05$) n=9.

Root damage and root dry weight: Seven days after flooding, the damaged to the root cortex by *P. aphanidermatum* at high temperatures was evaluated. The root cortex sloughed off or was missing in all plants grown in naturally infested as well as in artificially inoculated soil. No damaged roots were observed on plants grown in non-inoculated, sterilized soil (Figure 2.7).

Figure 2.7: Healthy roots (2) and roots damaged (1 and 3) by *Pythium aphanidermatum* following 48 hrs flooding at 28-32°C soil temperature in a greenhouse experiment to test the effect of natural and artificial inoculum on tomato sudden death.
1: Roots of plant grown in field soil naturally infested
2: Roots of plant grown in sterilized soil non-inoculated control
3: Roots of plant grown in sterilized soil artificially inoculated with *Pythium aphanidermatum*

The root damaged by *P. aphanidermatum* led to the reduction of root biomass. Both the root fresh and dry weight of plants grown in either field soil or inoculated soil with *P. aphanidermatum* were significantly lower compared to that of plants grown in sterilized, non-inoculated soil (Figure 2.8).

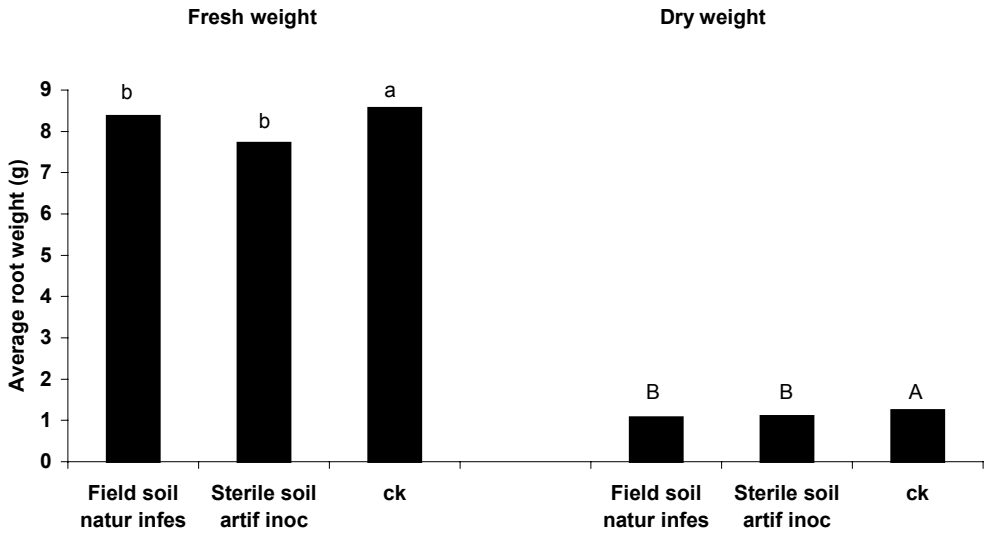

Figure 2.8: Effect of *Pythium aphanidermatum* on root fresh and dry weight of tomato plants in a greenhouse experiment between January and April 2002 at AVRDC, Shanhua, Taiwan. Different letters show statistical significant differences among the treatments according to Duncan's multiple range test (P≤0.05) n=9.

P. aphanidermatum recovery from soil, floodwater and roots: The population density of *P. aphanidermatum* in soil, floodwater and roots was determined every 12 hrs during the flooding period. In addition, the density of the fungus in the soil and roots was quantified 1 and 2 days after floodwater removal. Because of the large variation in colony-forming units (CFU) of the pathogen in the samples, statistical analysis was not possible. The results are presented as the means of the samples at the different sampling times.

The population density of *P. aphanidermatum* isolated from soil samples, floodwater and roots showed a fluctuation over time in both naturally infested and artificially inoculated soil. The number of *P. aphanidermatum* in the soil remained more or less the same in the time period 12 to 48 hrs during the flooding period. There was also no change 1 and 2 days after floodwater has been drained (Figure 2.9). In floodwater samples, the density of *P. aphanidermatum* also remained stable during the sampling period in naturally infested and artificially inoculated soil (Figure 2.9). The percentage of roots colonized by *P. aphanidermatum* increased during flooding both in naturally infested and artificially inoculated soil. A maximum of 80% infected roots was observed 48 hrs after

flooding when the soil has been artificially inoculated. However, 1 and 2 days after drainage, the percentage of colonized roots decreased to about 40% (Figure 2.9). When the soil was naturally infested with *P. aphanidermatum*, a maximum of 20% infected roots was observed 1 day after drainage, and dropped slightly 2 days after floodwater removal. In the artificially inoculated soil, 30% of the roots were infested at 1 day after drainage, and after 2 days the level of infection rose to 40% (Figure 2.9).

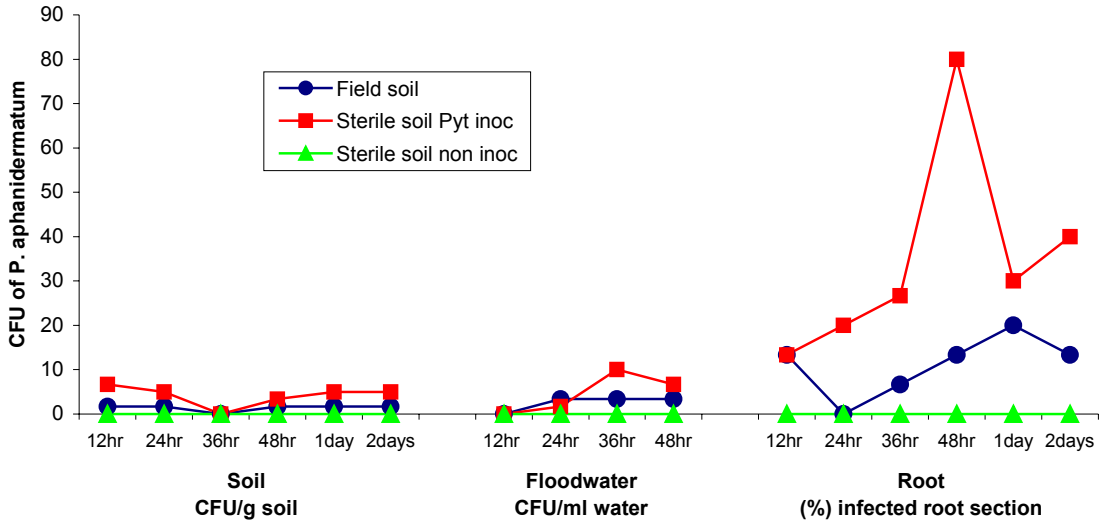

Figure 2.9: *Pythium aphanidermatum* recovery from soil, floodwater and roots during 48 hrs flooding, 1 and 2 days after drainage in a greenhouse experiment comparing naturally infested and artificially inoculated soil.

2.3.3 Role of *Pythium aphanidermatum* in tomato sudden death following flooding in two soil temperature regimes

Permanent wilt: Temperature showed a clear effect on pathogenicity of *P. aphanidermatum* on tomato plants. No wilted plants were observed at low soil temperatures of 18-25°C (Figure 2.10). At higher soil temperatures (28-32°C), 28% wilted plants were observed in soil is that actually infested with *P. aphanidermatum*, 4 days after flooding. Seven days after flooding, about 25% of plants grown in infested soil showed wilt symptoms. As shown in Figure 2.10, the percentage of wilted plants in the control was significantly lower 4 and 7 days after flooding.

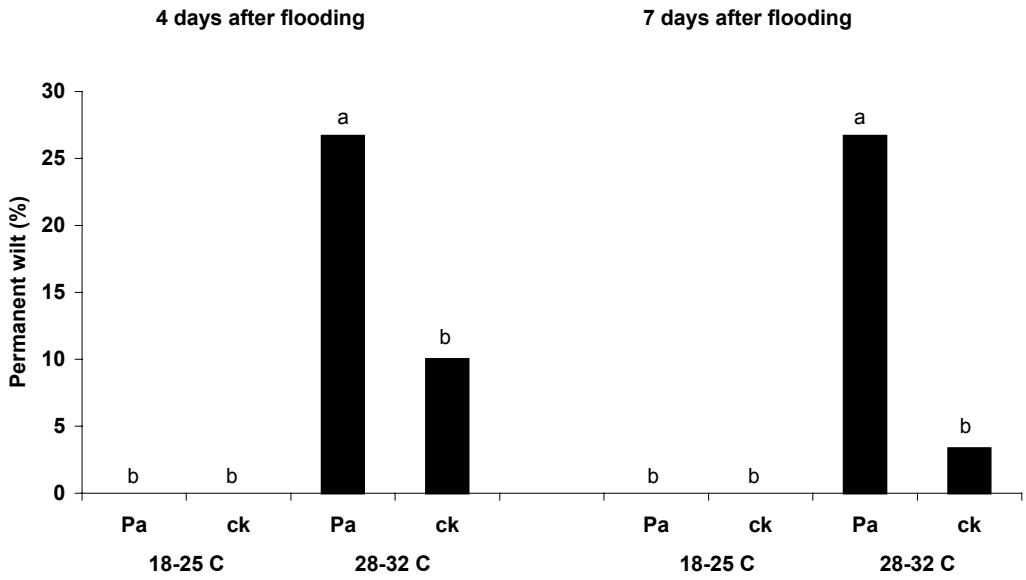

Figure 2.10: Effect of temperature at the time of flooding on development of sudden death of tomato plants due to *Pythium aphanidermatum* in a greenhouse between January and April 2002 at AVRDC, Shanhua, Taiwan. Pa = *Pythium aphanidermatum* inoculated; ck = non-inoculated control. Different letters show statistically significant differences among the treatments according to Duncan's multiple range test (P≤0.05) n=12.

P. aphanidermatum recovery from soil, floodwater and roots: *P. aphanidermatum* was isolated from soil, floodwater and plant roots (Figure 2.11). The soil temperature showed no major effect on the development of the fungus in the soil. Independent of the soil temperatures, the population density of *P. aphanidermatum* decreased during flooding and then recovered and remained constant after floodwater was removed. In floodwater samples, the *P. aphanidermatum* number increased at soil temperatures of 28-32°C. The highest CFU of the fungus was isolated at 28-32°C, 36 hrs after flooding. Similar results were observed with root samples in the two soil temperature regimes. The percentage of roots infested with *P. aphanidermatum* at 18-25°C and 28-32°C were 66% and 80%, 36 and 48 hrs during flooding, respectively. Although the percentage of infected root sections had increased during flooding at 36 and 48 hrs at low and high temperatures, respectively, drainage resulted in a reduction of infection. However, levels of infection were still elevated compared to the initial percentage found at 12 to 24 hrs.

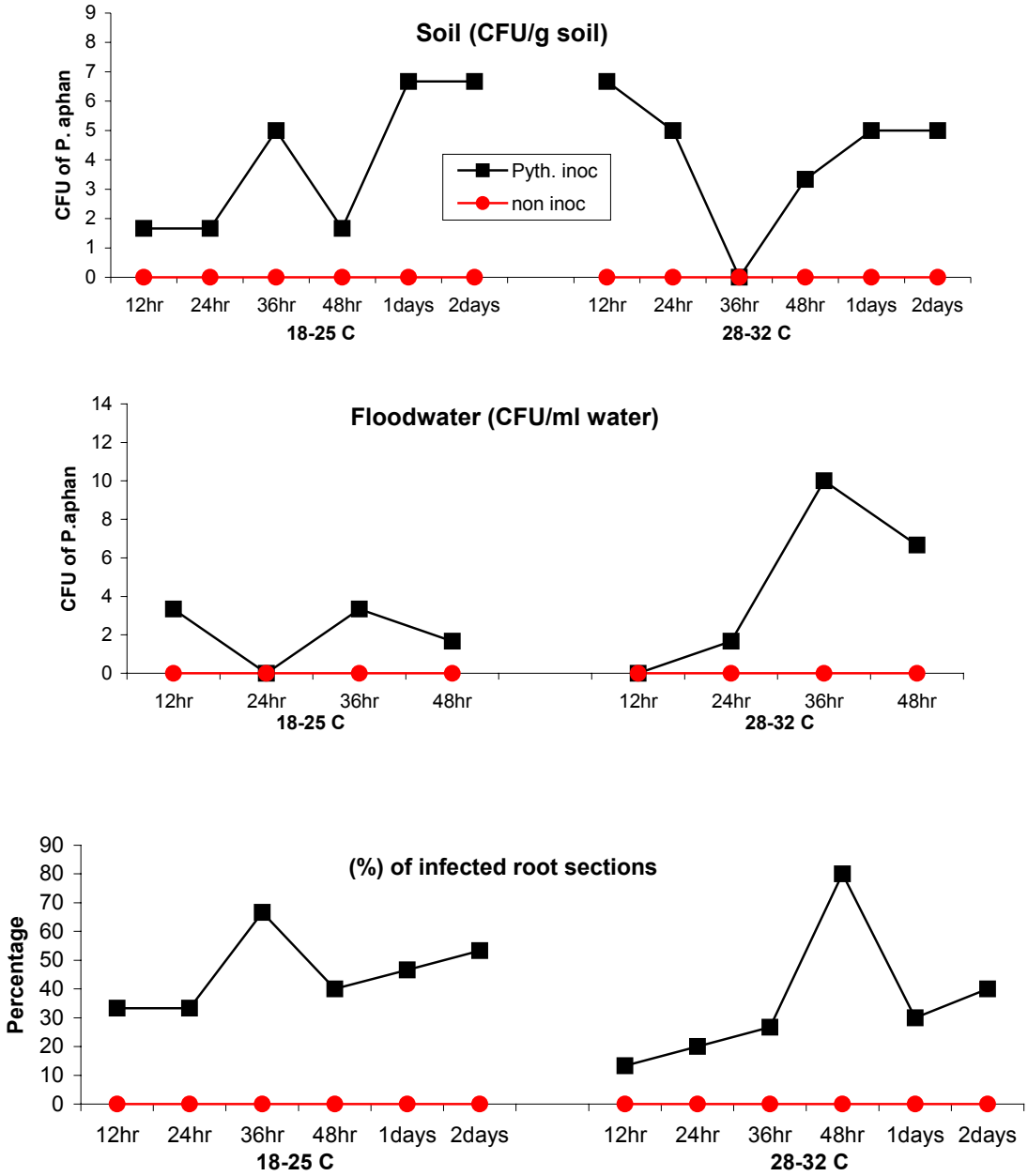

Figure 2.11: *Pythium aphanidermatum* recovery from soil (CFU/g soil), floodwater (CFU/ml of flood water) and roots (% infected root sections) in a greenhouse experiment between January and April 2002. Plants in both soil temperature regimes of 18-25°C and 28-32°C, were flooded for 48 hrs. n=12.

2.4 Discussion

2.4.1 Assessment of the role of *Pythium aphanidermatum* in tomato sudden death following flooding at high soil temperatures

Incidence of the disease: The aim of this part of the study was to assess the role of *P. aphanidermatum* in tomato sudden death following flooding at high soil temperatures. *P. aphanidermatum* and other fungi, including *Rhizoctonia solani* Kühn, *Fusarium solani* and *Phytophthora* spp., are well known pathogens, which cause damping-off on vegetables (Stanghellini et al. 1982; Menzies et al. 1996; McCarter 1997). In this study, the plants that were grown in the soil treated with *P. aphanidermatum* showed wilting symptoms only when the experimental plots were flooded for 48 hrs. The percentage of wilted plants observed in inoculated treatments was significantly higher than that of the untreated control. The results are in agreement with other previous studies on the role of *P. aphanidermatum* in wilting and death of vegetables. Zhang et al. (1990) and Gabor (1997) found that *P. aphanidermatum* is a common pathogen of pre-emergence damping-off of vegetables that tends to be most severe under conditions of high soil moisture. Hine et al. (1969) reported that the highest incidence of disease in sugarbeets due to *P. aphanidermatum* occurred during July-August when soil temperatures were highest, together with maximum rainfall. In addition, *P. aphanidermatum* has been known as the most aggressive pathogen in the hydroponic system (Stanghelline et al. 1996; Wulff et al. 1998).

Pythium species are well known as poorly competitive pathogens in the soil relative to other root-colonizing organisms, and often act only as primary colonizers (Kammedahl et al. 1979). Among these, *P. aphanidermatum* has been reported as less effective in causing damping-off than the other species, including *P. debaryanum* and *P. ultimum* (Thomson et al. 1971). It seems to be the major pathogen, however, in the high temperatures during the summer season. Under these conditions, the fungus was the predominant species isolated from infested plants (Gold et al. 1984). In the present study, no significant differences in the percentage of wilted plants grown in field soil naturally infested or soil sterilized and artificially inoculated with *P. aphanidermatum* was observed. This may be due to the fact that the environmental factors, including high soil temperatures

and soil moisture, enhance the activities *P. aphanidermatum* (Stanghellini et al. 1975). Damage may also occur whenever a minimum amount of inoculum is present in the soil. Favrin et al. (1988) also found that *P. aphanidermatum* showed higher pathogenicity to cucumber than other *Pythium* species, such as *P. irregulare*, at high soil temperatures and moisture levels.

Root damage: In the present study, the roots of tomato plants grown in the soil infested with *P. aphanidermatum* showed severe damage after the experimental plot has been flooded. The cortical root tissues were injured and sloughed-off. This was probably due to the fact that a high number of zoospores of *P. aphanidermatum* had penetrated into the cell and then created an appressorium to ceasing the cyclosis of the cell (Kraft et al. 1967). Zoospores aggregate primarily in the region of maturation on uninjured roots and only rarely in the differentiation and elongation regions, except when the roots are injured in these regions (Kraft et al. 1967). The growth and reproduction process of the fungus after penetration is based on the mucilage of the root (Zheng et al. 2000). However, the root mucilage in the nutrient solution did not correlate significantly with the colony density of *Pythium* in young tagged roots, while in the regression analysis, root growth was related negatively to colony density (Zheng et al. 2000). Shorter root elongation was observed in cucumber plants treated with *P. aphanidermatum* when compared to the untreated control (Wulff et al. 1998).

In the present study, the root dry weight of tomato plants in soil infested with *P. aphanidermatum* was significantly lower than in non-inoculated controls. The results are in agreement with other research assessing the role of *P. aphanidermatum* in the wilting of vegetables. Zhou et al. (1994) found that *P. aphanidermatum* caused reduced root volume and root dry weight of cucumber. It showed stronger effects on the host than other *Pythium* species, which was indicated by lower fresh weight of the host plant 6 days after inoculation. In addition, plant growth was severely suppressed (Wulff et al. 1998).

2.4.2 The role of soil temperature in tomato sudden death due to *Pythium aphanidermatum* following flooding conditions

In the present study, the soil temperature played an important role with respect to the pathogenicity of *P. aphanidermatum*. Tomato plants did not show wilting symptoms at soil temperatures of 18-25°C. The occurrence of wilted plants was observed at higher temperatures (28-32°C) after the experimental plots were flooded. The findings are in agreement with previous research on the relationship between temperature and pathogenicity done by Hine et al. (1969); Gold et al. (1984) and Yu et al. (1989). *P. aphanidermatum* is known to be virulent in a temperature range of 24-36°C and shows little effect below 20°C (Thomson et al. 1971). The temperature range for fungus growth is between 10 and 40°C and the optimum is 35 to 40°C (Van Der Plaats-Niterink 1981).

Another environmental factor, which was not directly investigated in this experiment, was soil moisture. It has been reported as a major factor for pathogenicity of soilborne pathogens. For diseases caused by *Pythium* spp., high soil temperature and abundant moisture have been identified as the two most important factors (Hendrix et al. 1973). *P. aphanidermatum* was responsible for killing seedlings as well as mature tomato and cucumber plants that were irrigated 3-4 times daily at soil temperatures above 30°C (Stanghellini et al. 1975). In hydroponic systems, *P. aphanidermatum* causes severe stunting and death of spinach plants within 3-4 days after inoculation at 21 and 27°C but not at low temperature (17°C) (Gold et al. 1984). In the present study, the incidence of the disease due to *P. aphanidermatum* occurred in the plants only after the experimental plots were flooded for 48 hrs.

Isolation from soil, water and root samples showed that *P. aphanidermatum* was recovered independent from soil temperatures ranging from 18 to 32°C. The temperature, however, has a direct effect on the fungus virulence. Although the fungus was widely distributed in the soil, floodwater and roots at low temperatures of 18 to 25°C, it was less aggressive to the host plant. The results are in agreement with other previous studies done by Hine et al. (1969) and Gold et al. (1984). They reported that at 17°C soil temperature, the development of *P. aphanidermatum* was limited.

The role of *P. aphanidermatum* on tomato sudden death following flooding in the hot season was demonstrated. However, the roles of secondary pathogens including bacteria and other soilborne fungi involved in the death of host plants was considered. Studies relating to the effect of bacteria on disease symptoms are rare in the soil. More information is needed to understand the complex mechanisms affecting disease severity caused by rhizobacteria in the presence and absence of *P. aphanidermatum*.

3 PLANT AGE AMD FLOOD DURATION EFFECTS ON TOMATO SUDDEN DEATH CAUSED BY *PYTHIUM APHANIDERMATUM*

3.1 Introduction

Studies concerning the effect of plant age on susceptibility to soilborne diseases have been conducted for many years. These studies revealed that the date of planting or transplanting can be used to control diseases. Populer (1978) demonstrated that plants vary in their susceptibility to disease with age. Several reports have shown that older plants are more resistant to *Pythium* spp. than younger ones (Mellano et al. 1970; Populer 1978; Nelson 1984). Tomato seedlings of different ages differ in susceptibility to attack by *P. aphanidermatum* and 5-week-old tomato plants were shown to be resistant to *P. aphanidermatum* (Black 2000, unpublished data). In hydroponic systems, older tomato plants were also not as susceptible to damage by *Pythium* spp. as younger plants. Damage can be quite severe on older plants (Jenkins et al. 1983).

The most important abiotic factors that influence changes in the susceptibility of the host plant to soilborne pathogens include soil moisture and temperature (English et al. 1994). It has been reported that flooding of tomato plants for 24 hrs does not have any significant effects on the plants, whereas flooding for over 72 hrs seriously damages tomato crops (Kuo et al. 1980; Kent et al. 1981).

P. aphanidermatum is a well known pathogen of tomato that has been shown to cause damping-off, stem rot, root disease, and fruit rot. In summer tomato production in tropical growing areas, the stem rot caused by *P. aphanidermatum* occurred in scattered plants following heavy rains that did not result in soil flooding. The pathogen has a very fast growth rate and is a prolific producer of zoospores, which are released under high moisture conditions. *P. aphanidermatum* is also the causal agent of root diseases and wilting of a number of vegetable crops grown in hydroponics systems (McCarter 1997; Postma et al. 2000; Stanghellini et al. 2000).

The objectives of the present field study were to:

1. Study the pattern of population development of *Pythium aphanidermatum* during the cropping season.
2. Determine the effect of tomato plant age on susceptibility to tomato sudden death.
3. Examine the effect of flood duration on severity of tomato sudden death.

3.2 Materials and methods

3.2.1 Plant materials

The tomato variety PT4723 F1, which was developed by AVRDC as heat tolerant, bacteria and virus resistant, was used. The seeds were sown at intervals of approximately 2 weeks in the greenhouse for the different transplanting schedules in the field. The first sowing was on May 1st, followed by May 16th and June 2nd 2002.

3.2.2 *Pythium aphanidermatum* recovery from the soil

Changes in pathogen population density in the field soil were measured during the crop production period from June to August 2002. *P. aphanidermatum* was detected and quantified based on the method of Burr et al. (1973). Five soil samples were taken randomly from the upper 20 cm along the diagonal of the field by a cylinder (5 cm diameter). The first sample was taken shortly before transplanting and samples were then taken on a monthly basis. In the flooded fields, the quantitative change of *P. aphanidermatum* populations due to flooding was also checked at 24, 48 and 72 hrs after flooding. In order to quantify the number of spores of *P. aphanidermatum* in the soil, 3 soil samples were taken randomly from the upper 20 cm between 2 rows of plants of each treatment plot. The soil samples were left to dry at room temperature for 2 days and then used to quantify population densities of *P. aphanidermatum.*

Soil dilutions of 1/5, 1/10, 1/20, and 1/40 g soil per ml 0.3 % water agar were used to quantify the population of *P. aphanidermatum* (Burr et al. 1973). Each dilution was mixed on a Vortex stirrer for at least 15 min and a 1 ml aliquot was dispensed evenly across

the surface of the selective medium for *P. aphanidermatum* developed by Burr et al. (1973). The plates were incubated at 35°C for time intervals ranging from 24 hrs to 72 hrs after which the soil was carefully washed from the agar surface and the *Pythium* colonies were recorded.

3.2.3 Field experiment design

This experiment was set up in the summer season from June to August 2002 in the experimental field of AVRDC in Shanhua, Taiwan. The field was divided into 12 plots with 3 replications of 4 flood-duration treatments. The treatments are listed in Table 3.1.

Table 3.1: Treatments to assess the effects of plant age and flood duration on tomato sudden death caused by *Pythium aphanidermatum*

Flood duration	**Time between transplanting and flooding**		
	51 days	**72 days**	**93 days**
Non-flooded	+	+	+
24 hrs	+	+	+
48 hrs	+	+	+
72 hrs	+	+	+

A field covering 1400 m^2 was weeded, ploughed and fertilized 15 days prior to transplanting. Twelve rectangular blocks (15 m x 3 m = length x width) were prepared. A distance of 5 m between the blocks was established with 1-m-deep trenches, which were used to regulate the water level during and after the flood period. In each block, 3 sub-blocks (1 m x 5 m = width x length) were laid out for the 51, 72 and 93 day-old plants. Each flood duration (0, 24, 48 and 72 hrs) treatment was replicated 3 times (Figure 3.1).

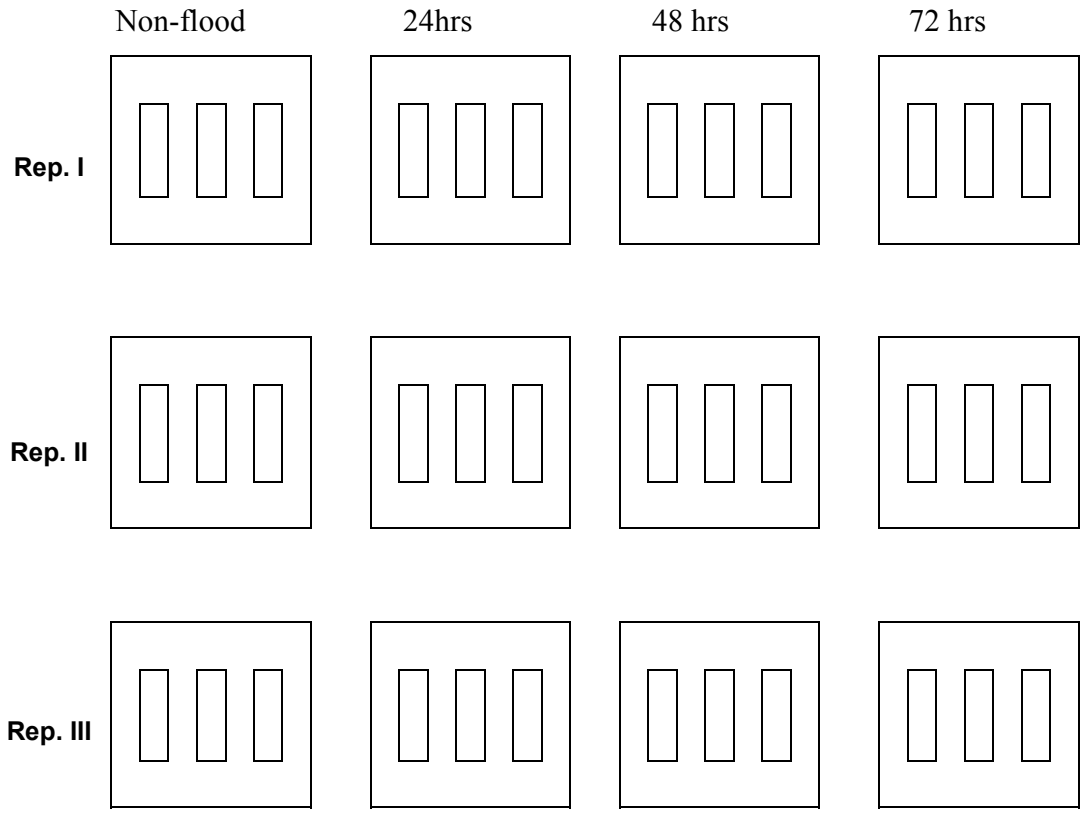

Figure 3.1: Experimental layout in field experiment between June and August 2002 at AVRDC, Shanhua, Taiwan.

Twenty-four tomato seedlings (30 days old) were planted into the field for the 93-day-old plants trials. The plants were tied to bamboo sticks to avoid damage through heavy rain and strong wind. Three weeks later, another 24 tomato seedlings were transplanted into the bed of the 72-day-old plants trials. Six weeks after the first transplanting, the trial of 51 day-old plants was transplanted.

The field experiment was flooded 3 weeks after the last transplanting (9 weeks after the first transplanting) using a controlled irrigation system. The water level was maintained at 10 cm above the upper soil surface for 0, 24, 48 or 72 hrs and then removed by natural drainage through a canal system.

3.2.4 Data collection and statistical analysis

The number of wilted plants was recorded at 2, 4 and 7 days after the floodwater has been removed. The data on the incidence of permanent wilt of tomato plants were analyzed by SAS (SAS Institute Inc. 1989), using the general linear model procedure, including analysis of variance, Duncan's multiple range test, least significant difference (LSD) test, and orthogonal contrast.

3.3 Results

3.3.1 Effect of plant age and flood duration on tomato sudden death

The wilting symptoms were first observed on the upper leaves two days after the floodwater has been drained. Wilting then steadily progressed to the lower leaves of the plant. The percentage of wilted plants increased sharply from 1 and 2 days to 4 days after flooding. The incidence of tomato permanent wilt observed for all treatments and plant ages was significantly lower 7 days after floodwater drainage than after 4 days (Figures 3.2 and 3.3).

Plant age clearly affected susceptibility to tomato sudden death caused by *P. aphanidermatum*. The percentage of plants with permanent wilt was significantly ($P \leq 0.05$) lower in younger 51-day-old plants than in the older 72- and 93-day-old plants in the same flood regime (Figure 3.2). Hundred percent wilted plants were observed in the 72-day-old treatment at 4 days and 7 days after flooding (Figures 3.2 and 3.3). In the 93-day-old plant treatment, the rate of wilting was slightly lower 7 days after flooding than after 4 days (Figure 3.2). However, the differences were not significant when 72-day-old plants were compared with 93-day-old plants in the same flooding regime (Figures 3.2 and 3.4).

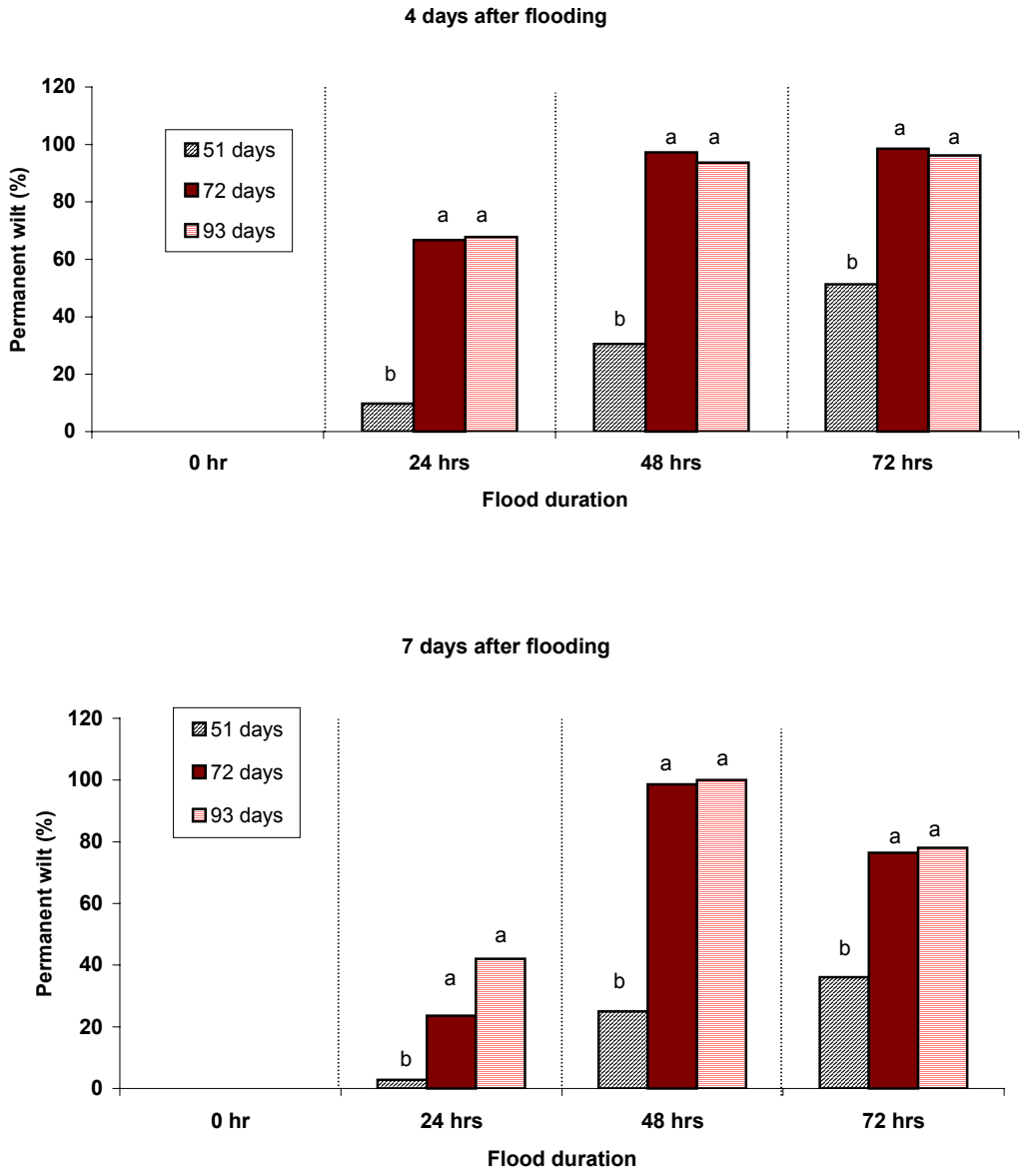

Figure 3.2: Effect of plant age (51, 72 and 93 days) and duration of flooding (0, 24, 48, 72 hrs) on incidence of permanent wilt caused by *Pythium aphanidermatum* on tomato 4 and 7 days after flooding in a field experiment between June and September, 2002 at AVRDC, Shanhua, Taiwan. Different letters in a group of columns indicate statistically significant differences between two treatments according to Duncan's multiple range test ($P \leq 0.05$). n=48.

The results clearly show that tomato sudden death caused by *P. aphanidermatum* increased in response to flooding under hot conditions. Flooding for 24 hrs caused less damage than flood regimes of 48 and 72 hrs, regardless of plant age. The level of wilting recorded after 24 hrs flooding on day 4 was significantly ($P \leq 0.05$) lower compared with treatments of 48 and 72 hrs flooding (Figure 3.3). There were no significant differences between the flood regimes of 48 and 72 hrs for any of the plant age treatments. Wilting exceeded 90% in both flood regimes 4 days after floodwater was removed. Seven days after flooding, 80% of the older plants wilted after 72 hrs flooding. The level of wilting here was not significantly different to the 48 hrs treatment.

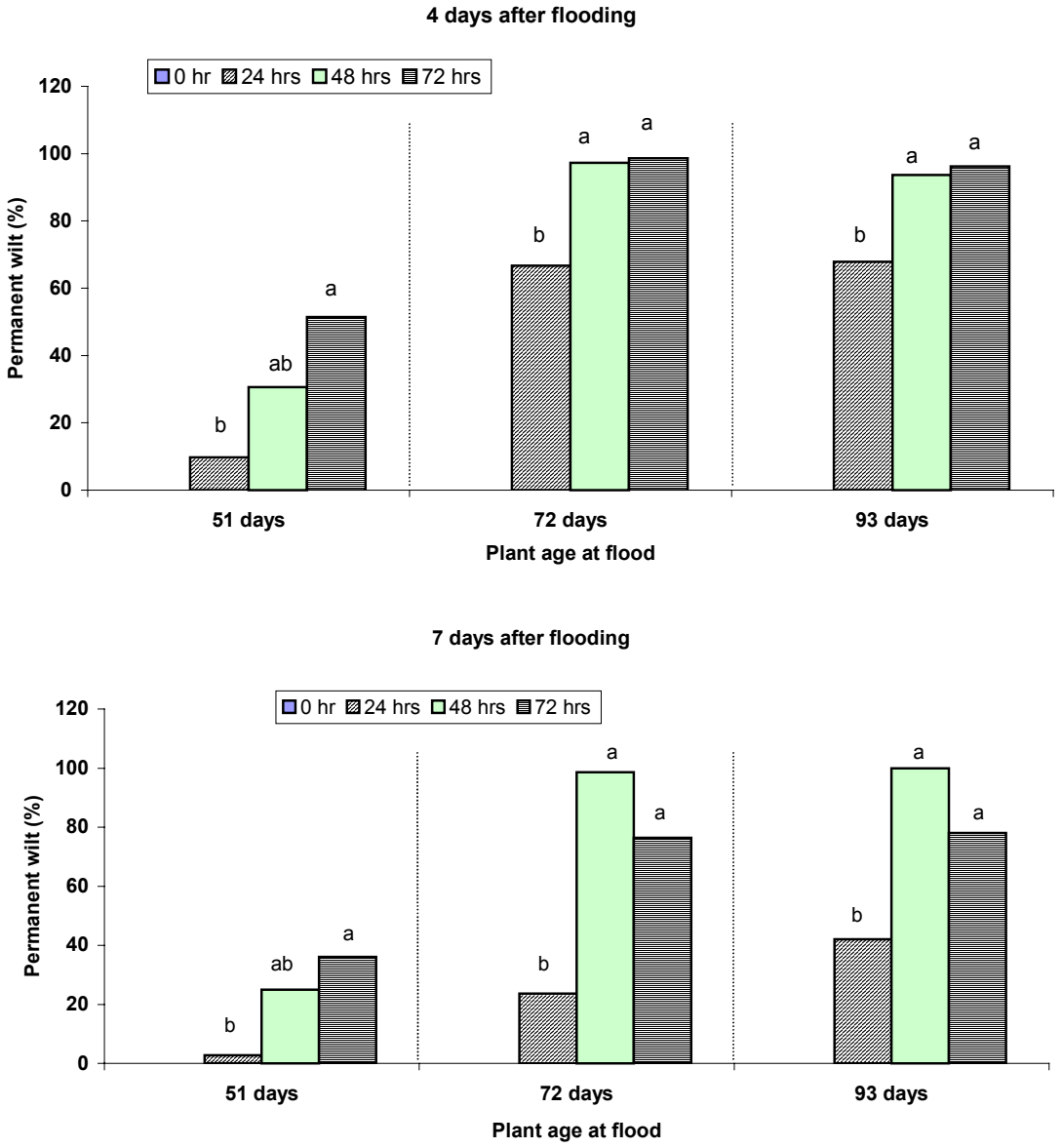

Figure 3.3: Effect flood duration (0, 24, 48, 72 hrs) and plant age (51, 72 and 93 days) on incidence of permanent wilt caused by *Pythium aphanidermatum* in tomato at 4 and 7 days after floodwater removal in a field experiment between June and September, 2002 at AVRDC, Shanhua, Taiwan. Different letters in a group of columns indicate statistically significant differences between two treatments according to Duncan's multiple range test ($P \leq 0.05$). n=48.

A 

B 

Figure 3.4: Wilted tomatoes 3 days after flooding (A) and dead plants at 42 days after flooding in treatment of 72 and 93 day-old plants (B) in a field experiment from June to August 2002 at AVRDC, Shanhua, Taiwan.

3.3.2 *Pythium aphanidermatum* recovery from the soil

Fluctuations in the populations of *P. aphanidermatum* in the soil were found to be correlated to soil moisture and soil temperature. About 0.51 CFU of *P. aphanidermatum* were isolated per gram of soil before transplanting compared to up to 1 CFU/g of soil and 2 CFU/g 30 and 60 days after transplanting, respectively. There were no differences in the number of *P. aphanidermatum* CFUs recovered from the soil between treatments of different flood regimes in August 2002 (Figure 3.5). The number of *P. aphanidermatum* CFU isolated 7 days after flooding was similar in the non-flooded control compared to the 72 hrs flood treatment. During the pre-experiment phase from June to August, 1-2 CFU/ g soil was detected (Figure 3.5). The highest air and soil temperature was reached during May-July (Figure 3.6).

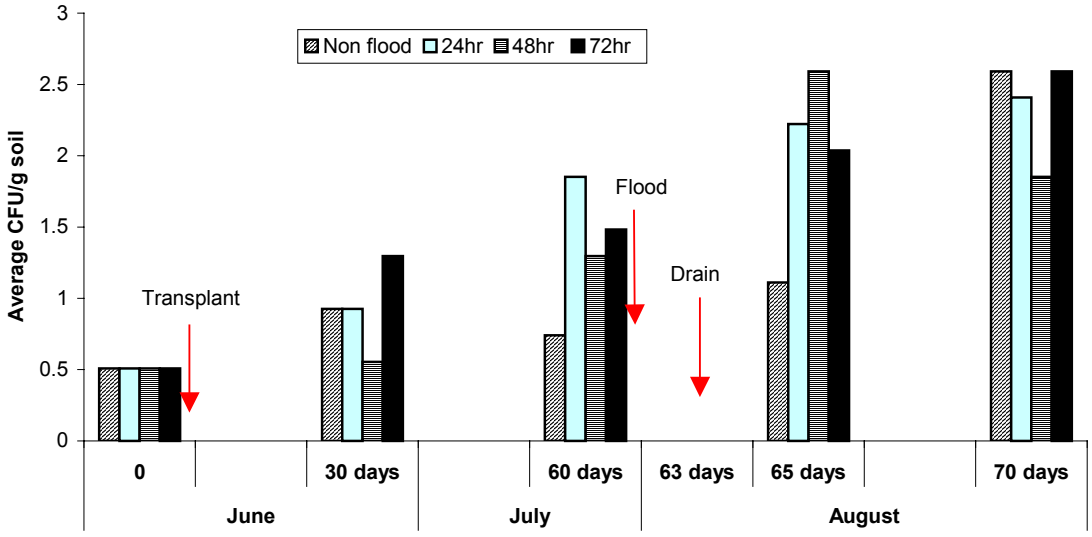

Figure 3.5: Number of CFU/g soil of *Pythium aphanidermatum* in the soil before flooding and 7 days after flooding under different flood conditions in a field experiment between June and August 2002 at AVRDC, Shanhua, Taiwan (Results not statistically analyzed).

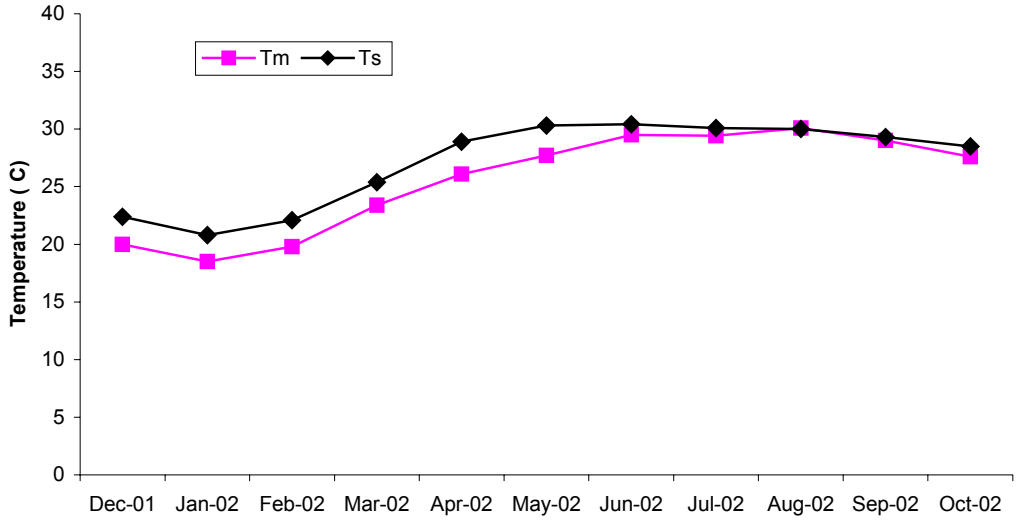

Figure 3.6: Daily mean air temperature (Tm), mean soil temperature (Ts) in the experimental field between June and September 2002 at AVRDC, Shanhua, Taiwan.

3.4 Discussion

The main goal of this study was to determine how plant age affects susceptibly to *P. aphanidermatum* following flooding at high soil temperatures. The susceptibility of plants to soilborne diseases is known to decrease with the age of the plant (Yarwood 1959; Populer 1978; Seem 1988). Maximum susceptibility of host plants to pathogens has been shown to coincide with the period of maximum growth of the plant (Populer 1978). However, in this study, the highest percentage of surviving plants was observed in the treatment with young plants. Here, the level of survival was significantly higher than in the treatment with older plants. This conflicts with previous results. The susceptibility of plants to *Pythium* spp. in the nursery or early seedling stage differs greatly from that of older plants grown under field conditions. Mellano et al. (1970) tested the susceptibility of *Antirrhinum majus* seedlings to *P. ultimum* and found that the fungus penetrated the elongation and maturation regions of the root tips of *Antirrhinum majus*, but rarely the mature root portions and never the meristem or the differentiation region just behind it. Seedlings inoculated after 15 days or earlier were killed, and growth of the pathogen was

rapid in the root, stem and leaves. On seedlings that were inoculated after 20 days, the fungus easily colonized tertiary and quaternary roots, but was unable to colonized the mature portions of secondary and primary roots, and the plants survived.

The reason for decreasing severity of wilt with plant age at the time of infection is due to restricted fungal growth in the plant vessels rather than to decreased infectibility of roots (Nyvall et al. 1976). In the present study, all plants in the field experiment at the time of flooding were over 50 days old and therefore beyond the susceptible nursery or seedling stage. The results obtained are due to the fact that often-older tomato plants are more susceptible to *Pythium* spp. than younger ones (Jenkins et al. 1983). Another explanation could be that biotic and abiotic environmental factors affect susceptibility through changes in the host prior to infection or in the host-pathogen interaction after infection has occurred (Levitt 1972; Schoeneweiss 1975; Burdon 1987). Furthermore, under field conditions where complex interactions between soil microflora and micro macrofauna occur, both within and around the plants, sudden changes in plant-microbe densities and interactions due to flooding may result in increased attack by specific soilborne pathogens favoured by high moisture (Duniway 1983).

The root system also plays an important role in the susceptibility of host plants to soil pathogens. Damage to the root system due to excess water as a result of physiological and chemical processes are often associated with the infection process (Matheron et al. 1985; Duniway 1983; Schaffer et al. 1992). For example, oxygen deficiency is induced by flooding and may inhibit water and nutrient uptake, this in turn may result in chlorosis, wilting and subsequent predisposition to attack (Palti 1981; Kawase 1981). In the present study, the entire root system of the tomato plants was damaged by *P. aphanidermatum* 7 days after flooding. This was indicated by encysted zoospores occurring inside all of the injured roots. Damage also may be due to direct influences on *P. aphanidermatum* by flooding, such as promotion of zoospore release and mediation of the infection process (Duniway 1983). The appearance of new adventitious roots reduced the level of permanent wilt in the 51-day-old plants, which tended to recover (Figure 3.6). However, plants 72 and 93 days old at flooding did not recover after drainage. This may be due to the fact that younger plants were better able to recover from flooding by producing new roots to supply

their comparatively smaller shoots with water and nutrients. Older plants had already invested energy in shoots now too large to be supplied with water and nutrients by the damaged root system. The result was permanent wilting.

Flooding as an environmental factor is of major importance concerning changes in susceptibility to root disease (Burdon 1987). It may increase disease severity by predisposing plants to infection through adverse effects on host physiology (Fraedrich et al. 1989; Schaffer et al. 1992). Depletion of soil oxygen which restricts tissue growth can also enhance disease regeneration from necrotic roots (Stolzy et al. 1984; Schaffer et al. 1992). In the present study, tomato plants flooded for 24 hrs were not affected, even though tomato is considered one of the vegetables most sensitive to flooding (Kuo et al. 1982). This may be due to the fact that the tomato plant is capacity to transport sufficient O_2 from the shoot to sustain the root respiratory system during shorter periods of flooding (McNamara et al. 1989). The growth of the roots and stem of tomato plants is inhibited when the plant is flooded for 48 or 72 hrs (Kent et al. 1981; Kuo et al. 1982; McNamara et al. 1989). Flooding for this duration causes collapse and death of the plant. Another factor greatly contributing to sudden death is the fact that the flooding increases disease severity by direct influence on the pathogens. Flooding promotes the release of zoospores and mediates the infection processes by species of *Phytophthora* and *Pythium* (Duniway 1979; 1983). Under the multiple side effects caused by flooding listed above, the survival ability of tomato plants, even when adventitious roots develop, is greatly limited. Therefore, even though 51-day-old plants survived, they were very small and probably poor yielding.

Fluctuations in the population density of *P. aphanidermatum* in the soil were related to soil temperature and soil moisture. The number of *P. aphanidermatum* recovered from the soil was lower in the sample recovered 30 days after transplanting than in the one recovered after 60 days. Even higher densities were recovered in the soil at the end of the cropping season (August-October) when the field was fallowed. This may be due to *P. aphanidermatum* moving into the roots of the host plants, reducing the density of the fungus in the soil (Zhang et al. 1990). Changes in the population density of this fungus were dramatic. At times, large numbers of propagules were detected in the soil but at other times could not be detected at all. The population of *P. aphanidermatum* was highest in

October, when soil temperatures were low, and disease infested plants were not present. In contrast, lower population densities of the fungus were found in the soil during June-July, when high numbers of wilted and dead plants were observed. This variation could be due to the fact that *P. aphanidermatum* is a typical plant pathogen of warm regions (Van der Plaats-Niterink 1981) and reproduces to higher levels in hot plant tissue than in soil.

The response of tomato plants of different ages to tomato sudden death due to *P. aphanidermatum* under flooding at high temperature may be useful for cultural management practices. Timing the planting of the crop can greatly influence host susceptibility (English et al. 1994). Planting dates should thus be selected to avoid exposing susceptible tomato plants to the disease during times when seasonal environmental influences such as high soil temperatures and flooding increase the disease attack potential. Predictive models using long-term weather data could help in selecting planting dates less prone to early flooding. Future research should be conducted in pasteurized soil to assess the response of differently aged tomato plants to *P. aphanidermatum*. More information is needed to understand the complex microbial mechanisms involved in increasing the susceptibility of older plants to *P. aphanidermatum* following flooding at high temperatures under field conditions.

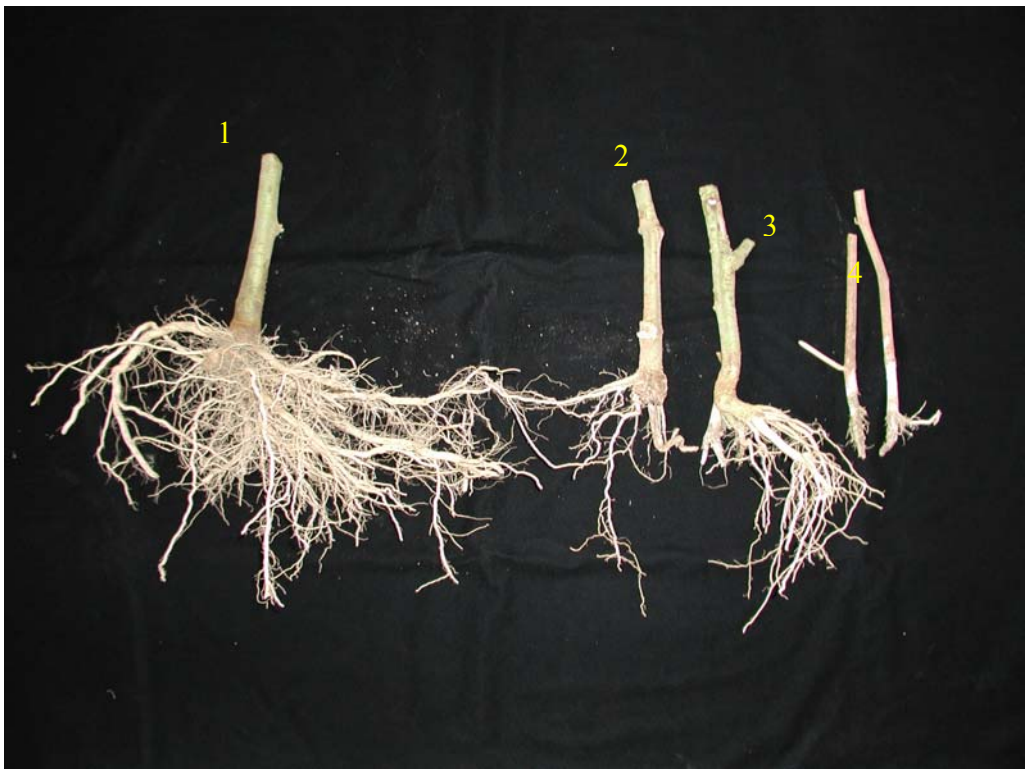

Figure 3.6: Root system of plants 42 days after 48 hrs flooding in a field experiment between June and September 2002.
1: Root of the 51-day-old plant, non-flood control
2: Root of the 51-day-old plant, flooded for 48 hrs
3: Root of the 72-day-old plant, flooded for 48 hrs
4: Root of the 93-day-old plant, flooded for 48 hrs

4 EVALUATION OF BIOLOGICAL CONTROL AGENTS FOR MANAGEMENT OF TOMATO SUDDEN DEATH CAUSED BY *PYTHIUM APHANIDERMATUM* FOLLOWING FLOODING UNDER HIGH TEMPERATURE CONDITIONS

4.1 Introduction

Biological control is a promising strategy for managing soilborne and other disease in a wide range of crops (Baker 1987; Cook 1993). Biocontrol through beneficial microbes introduced into the environment around a crop plant to enhance the presence and/or beneficial effects of these organisms can contribute significantly to plant health. This method is less disruptive to ecosystems than the use of chemical pesticides (Cook et al. 1983). Several beneficial organisms, which parasitize or are antagonistic to a number of soilborne fungi, have been detected. Well known beneficial fungi that have been used for the control of *Pythium aphanidermatum* include *Trichoderma harzianum*, *Trichoderma virens*, and *Trichoderma hamatum* (Hadar et al. 1979; Harman et al. 1980; Sivan et al. 1984; Lo et al. 1997). Previous studies have shown that *T. harzianum* reduced the incidence of damping-off disease on bean due to *P. aphanidermatum*, *Rhizoctonia solani* and other pathogens such as *Sclerotium rolfsi* (Elad et al. 1980; Sivan et al. 1984; Lo et al. 1997).

Despite the success of *Trichoderma* spp. in the control of soilborne pathogens causing pre-emergence damping-off, these beneficial fungi have not been used to control sudden death disease due to *Pythium aphanidermatum*, which occurs at a later stage in plant development following flooding at high temperature.

The objective of the experiment described in this chapter was to evaluate isolates of *Trichoderma harzianum*, *Trichoderma viren*, and *Streptomyces saraceticus* with respect to biological control of tomato sudden death due to *Pythium aphanidermatum* following flooding at high temperatures under greenhouse micro-plot and field conditions.

4.2 Materials and methods

4.2.1 Preparations

Preparation of *Pythium aphanidermatum* inoculation

P. aphanidermatum strain number 4 was cultured on V8 agar in Petri dishes for 3 days at 28°C before being inoculated onto rice seed for solid state fermentation. Each 400 ml beaker containing 150 ml of rice grain and 75 ml distilled water was autoclaved twince before being used as the final growth substrate. Two blocks of agar of a 3-day-old culture of *P. aphanidermatum* were placed into the rice grain in each beaker and then incubated in an illuminated chamber at 28°C for 10 days. One beaker of rice grain was incorporated into the upper 10 cm of soil of each micro-plot container 10 days after transplanting.

Tomato plant

The tomato line CL5915-206D, which was determined by AVRDC to be heat tolerant and virus resistant, was used in both greenhouse and field tests. The seedlings were planted in a peat moss substrate for 30 days in the greenhouse and then transplanted into the micro-plot container or into the field plots for experimentation.

4.2.2 Inoculum preparation and inoculation techniques

Two isolates each of *Trichoderma harzianum* and *Trichoderma virens* from the Taiwan Agricultural Research Institute (TARI) and one strain of *Trichoderma harzianum* isolated from an AVRDC field were tested. An isolate of *Streptomyces saraceticus,* which had been introduced into the market as a commercial product by National Chunghsing University (Taiwan), was also tested. The isolates tested were:

Trichoderma harzianum: Th-Gl-6 and Th-R1-6
Trichoderma harzianum: AVRDC (Th-3)
Trichoderma viren: Tv-Y3-7 and Tv-R4-2
Streptomyces saraceticus

Seed inoculation

Trichoderma isolates were grown on PDA medium for conidia development. They were incubated for 5 days at 28°C, except Th-G1-6, which was incubated for 8 days. The conidia, after being removed from the medium by adding 20 ml distilled sterilized water with additional scraping by a glass rod, were used for seed and substrate inoculation. The conidial suspension, adjusted to 5 x 10^9 conidia/ml tap water, was supplemented with 1 ml of CMC 1% (Carboxymethylcellulose) as sticker and spreader. Tomato seeds were surface sterilized with 0.1% hydrochloride before being treated with the biocontrol agents. Three milliliter of a suspension of each test organism was used to coat 10 g of tomato seeds. The inoculated seeds were then placed inside a sterile transfer hood to dry prior to sowing (Lo et al. 1997). All experiments were carried out with untreated surface-sterilized seeds as controls.

Potting soil inoculation

Five hundred ml of a substrate mixture of peat moss and wheat bran (1:1=v/v) at 40 % (w/w) moisture was put into a beaker (1000 ml) before being autoclaved for 1 hr at 121^0C (Sivan et al. 1984). The substrate mixture was inoculated separately with 10 ml conidial suspensions containing 2 x 10^4 of each of the five isolates of *Trichoderma* and incubated in an illuminated chamber for 14 days at 28°C. The culture media of the five *Trichoderma* isolates were then mixed with peat moss (5g/kg) and filled into 5 cm diameter pots (Sivan et al. 1984). Seeds treated with the *Trichoderma* isolates were then sown into these pots, which contained peat moss pre-inoculated with the same isolate. These seedlings were used for the field and greenhouse tub experiments.

Field soil and greenhouse micro-plot inoculation

The culture media (5g/plant) from each of the five *Trichoderma* isolates as described above were placed in the planting hole 2 days before the seedlings were transplanted into the field. The plants seed-treated with the isolates of *Trichoderma* and were growing in inoculated potting soil were placed into similar holes at transplanting.

The commercial *Streptomyces* product was diluted in water (1/1000) and watered daily onto the potted plants in pure peat moss after the seedlings had emerged, and treatment continued with 1/500 dilution until 40 days after transplanting.

4.2.3 Field experiment to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* for controlling tomato sudden death due to *Pythium aphanidermatum*

General methods

The field experiment to test the efficacy of *Trichoderma* species for controlling tomato sudden death was established in a field in AVRDC, Taiwan. The experiment was conducted from July 15th to October 15th 2001, in a paddy rice field (clay soil), where damping-off disease of tomato due to *P. aphanidermatum* had been prevalent in previous years. The field was left fallow for one month for drying and the rice straw was burned prior to ploughing. Twenty-eight plots of 5 m^2 (1m x 5 m) were established to host seven treatments of each isolate of *Trichoderma* and the control. The treatments were set up in a random manner along the length of the field and repeated in four blocks along the width. The treatments were randomly distributed to the plots and plots labeled one week before transplanting. Empty bands were left between plots to separate these from each other and to avoid cross contamination between treatments. The plots were covered by plastic to control weeds and limit damage by insect pests. A total of 210 kg (N:P:K=4:4:4) organic fertilizer was broadcast over the field prior to the establishment of the plots. In addition, a total of 65 kg (N:P:K:Mg=15:15:15:4) of a chemical fertilizer was used as top dressing.

Five soil samples were taken randomly from the upper 20 cm along the diagonal of the field with a 5-cm-diameter cylinder before transplanting. In order to measure pH and quantify the population of *P. aphanidermatum* (see 2.3.2), the soil samples were mixed and placed on paper towel at room temperature for two days until dry. Twenty grams of this dried soil was dissolved in 0.01M calcium chloride ($CaCl_2$) on a rotary shaker for one hour to check the pH. The soil pH was measured as an average of three sub-samples and found to be 6.47.

Isolation of *Pythium aphanidermatum* from the soil

The soil samples were taken randomly from the field prior to transplanting, as mentioned above. They were taken 30 and 60 days after transplanting and again 7 days after floodwater removal. The soil samples were placed on paper towels and allowed to dry at room temperature for 2 days. Four dilutions of the soil of 1/5, 1/10, 1/20, and 1/40 g soil/ml 0.3 % water agar were used to quantify the population of *P. aphanidermatum* (Burr et al. 1973). Each dilution was mixed on a Vortex stirrer for at least 15 min and a 1 ml aliquot was dispensed evenly across the surface of the selective medium developed for *P. aphanidermatum* developed by Burr et al. (1973) (see 2.1.3). The plates were incubated at 35°C for time intervals ranging from 24 hrs to 72 hrs, after which the soil was carefully washed from the agar surface and the *Pythium* colonies were recorded.

Experimental design

The aim of this experiment was to test the efficacy of 3 isolates of *T. harzianum*, 2 isolates of *T. virens* and 1 isolate of *Streptomyces* to control tomato sudden death in the field. The following treatments, each with four replicates are listed in Table 1.

Twenty 30-day-old seedlings were transplanted into each plot in 2 rows of 10 plants each and attached to bamboo sticks to avoid damage from strong wind and heavy rainfall. The experiment was flooded 2 months after transplanting, when the soil temperature reached 30-32°C. The water level was maintained 10 cm above the upper soil surface for 48 hrs and then drained by a canal system around the blocks.

Table 4.1: Treatments to assess the efficacy of *Trichoderma* spp. and *Streptomyces saraceticus* with respect to biological control of tomato sudden death due to *Pythium aphanidermatum*

Isolate	**Treatment**
Th-G1-6	- treated seed - treated seedling potting medium in the nursery - incorporated into field soil at time of transplanting
Th-R1-6	- treated seed - treated seedling potting medium in the nursery - incorporated into field soil at time of transplanting
Th-3	- treated seed - treated seedling potting medium in the nursery - incorporated into field soil at time of transplanting
Tv-Y3-7	- treated seed - treated seedling potting medium in the nursery - incorporated into field soil at time of transplanting
Tv-R4-2	- treated seed - treated seedling potting medium in the nursery - incorporated into field soil at time of transplanting
Streptomyces saraceticus	- treated seedling potting medium in the nursery - drench treatment of plants until 40 days after transplanting in the field
Control	Non-treated

4.2.4 Greenhouse experiments to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on control of tomato sudden death due to *Pythium aphanidermatum* under flooding different durations

General methods

Plastic containers (tub) of size 31 cm x 51 cm x 40 cm (height x width x length) were used as experimental units. The tubs were sterilized with a solution of 1% hydrochloride before being filled with soil. Five kg of sterilized pebbles (1-2 cm diameter) were placed in the bottom of the tub and covered by a plastic netting. A U-shaded plastic siphon was attached to the tub so that water could be siphoned out from under the net (see Figure 1, Chapter 2). Fifty-five kg of heavy clay soil, which had been taken from AVRDC fields, was steam-pasteurized, and then filled into each tub. Soil pH was evaluated prior to filling the soil into the tubs and found to be 6.5.

About 82.5 g of organic commercially available granular fertilizer (N:P:K=4:4:4) and 20 g chemical fertilizer (N:P:K:Mg=15:15:15:4) was incorporated into the soil of each tub before transplanting. In addition, 3.5 g of chemical fertilizer (N:P:K=20:20:20) were added to each tub weekly until the plants were harvested.

Flooding and temperature control

The plastic tubs were flooded 60 days after transplanting. The water level was maintained at 2-4 cm above the soil surface for 48 or 72 hrs, respectively, and then removed rapidly through the drainpipe system. The temperature was maintained at 28-32°C for the entire period of each experiment.

Root weight

Tomato roots were removed from the soil by careful washing with tap water 7 days after floodwater was removed. The washed roots were placed on paper towels at room temperature for 30 min to remove excess water and then dried in an oven at a temperature of 50°C for 48 hrs followed by determination of root dry weight.

Experiments design

Two different greenhouse experiments were conducted in the summer seasons of 2001 and 2002. In the first experiment, all 6 isolates of the biocontrol agents were used. Flooding lasted done for 48 hrs. The second experiment was conducted with 3 biocontrol agents and a extended flooding time of 72 hrs. The soil was inoculated with isolates of *T. harzianum* (Th-R1-6) and *T. virens* (Tv-Y3-7) at the time of transplanting (see 2.2.3) and inoculated with *P. aphanidermatum* 10 days after transplanting (see 2.1.1). Ten treated tomato plants per treatment were transplanted in two rows in each micro-plot.

Greenhouse experiment to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on control of tomato sudden death due to *Pythium aphanidermatum* after 48 hrs of flooding

The experiment was conducted between August and November 2001. Twenty-four plastic containers were used for 3 replicates of the 5 treatments of each isolate of *Trichoderma* spp., *Streptomyces saraceticus* and two controls. Tomatoes were transplanted on August 5th, 2001. *P. aphanidermatum* was grown on rice grain and incorporated into the soil 10 days after transplanting. The containers were flooded for 48 hrs, 60 days after transplanting.

Table 4.2: Treatments to assess the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on biological control tomato sudden death due to *Pythium aphanidermatum*.

Treatments	***Pythium aphanidermatum* inoculation**	**Flood period**
T. harzianum Th-G1-6	10 days after transplanting	48 hrs
T. harzianum Th-R1-6	10 days after transplanting	48 hrs
T. harzianum Th-3	10 days after transplanting	48 hrs
T. virens Tv-Y3.7	10 days after transplanting	48 hrs
T. virens Tv-R4-2	10 days after transplanting	48 hrs
Streptomyces saraceticus	10 days after transplanting	48 hrs
Pythium aphanidermatum alone (Pa)	10 days after transplanting	48 hrs
Non-inoculate control (ck)	None	48 hrs

Greenhouse experiment to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* to control tomato sudden death due to *Pythium aphanidermatum* after 72 hrs of flooding

The experiment was conducted between May and August 2002. Twenty-four tubs for 8 treatments each with 3 replicates were used. Tomato plants were planted into the tubs on May 1st 2002. Biocontrol agents were inoculated both in the soil infested with *P. aphanidermatum* and in the non-infested soil. The tubs were flooded for 72 hrs 60 days after transplanting.

Table 4.3: Treatments to assess the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on biological control of tomato sudden death due to *Pythium aphanidermatum*

Treatment	***Pythium aphanidermatum* inoculation**	**Flood period**
T. harzianum Th-R1-6	None	72 hrs
T. virens Tv-Y3-7	None	72 hrs
Streptomyces saraceticus	None	72 hrs
T. harzianum Th-R1-6	10 days after transplanting	72 hrs
T. virens Tv-Y3-7	10 days after transplanting	72 hrs
Streptomyces saraceticus	10 days after transplanting	72 hrs
Pythium aphanidermatum alone (Pa)	10 days after transplanting	72 hrs
Control (ck)	None	72 hrs

4.2.5 Data collection and statistical analysis

The number of wilted plants in all treatments were recorded at 2, 4 and 7 days after the floodwater was removed. Seven days after flooding, the plants were harvested and root dry weights measured and recorded. The data on incidence of permanent wilt of tomato were analyzed with the SAS (SAS Institute Inc. 1989) program, using the general linear model procedure, including analysis of variance, Duncan's multiple range test, least significant difference (LSD) and /or test orthogonal contrast.

4.3 Results

4.3.1 Field experiment to evaluate *Trichoderma* spp. and *Streptomyces saraceticus* control of tomato sudden death due to *Pythium aphanidermatum* after 48 hrs of flooding

Pythium aphanidermatum recovery from the soil: The detection of 1.6 CFU/g soil of *Pythium aphanidermatum* prior to transplanting confirmed that the field soil was naturally infested by the fungus. The population density of *P. aphanidermatum* changed during the cropping season and achieved the highest density a month after tomato transplanting. There was no difference in density directly before or after flooding (Figure 1).

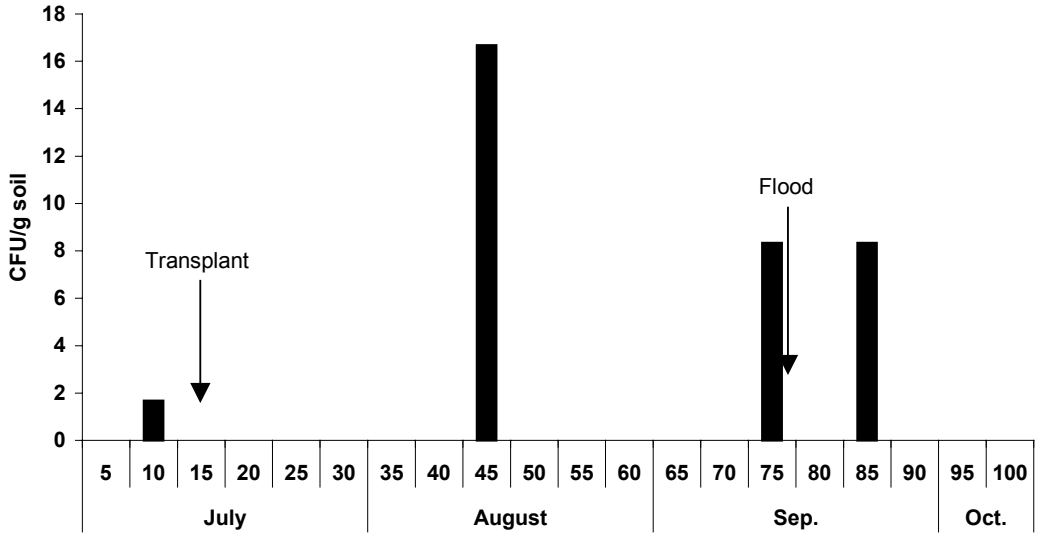

Figure 4.1: Population density of *Pythium aphanidermatum* in soil in a field experiment between July and October 2001 at AVRDC, Shanhua, Taiwan. The soil was sampled before, 30 and 60 days after transplanting and 7 days after flooding.

Permanent wilt: Four days after flooding, the percentage of wilted tomato plants in the soil treated with *T. virens* Tv-Y3-7 was significantly ($P \leq 0.05$) lower than the control treatment. There were no significant differences between percentage of wilted plants observed in treatments with the antagonists tested (Figure 2). Seven days after flooding, Tv-Y3-7 did not have a significant effect on tomato sudden death. Similar results were observed in the treatments with *Streptomyces* and the other isolates of *T. harzianum*: Th-G1-6, Th-R1-6, Th-3; and *T. virens*: Tv-R4-2 (Figure 2).

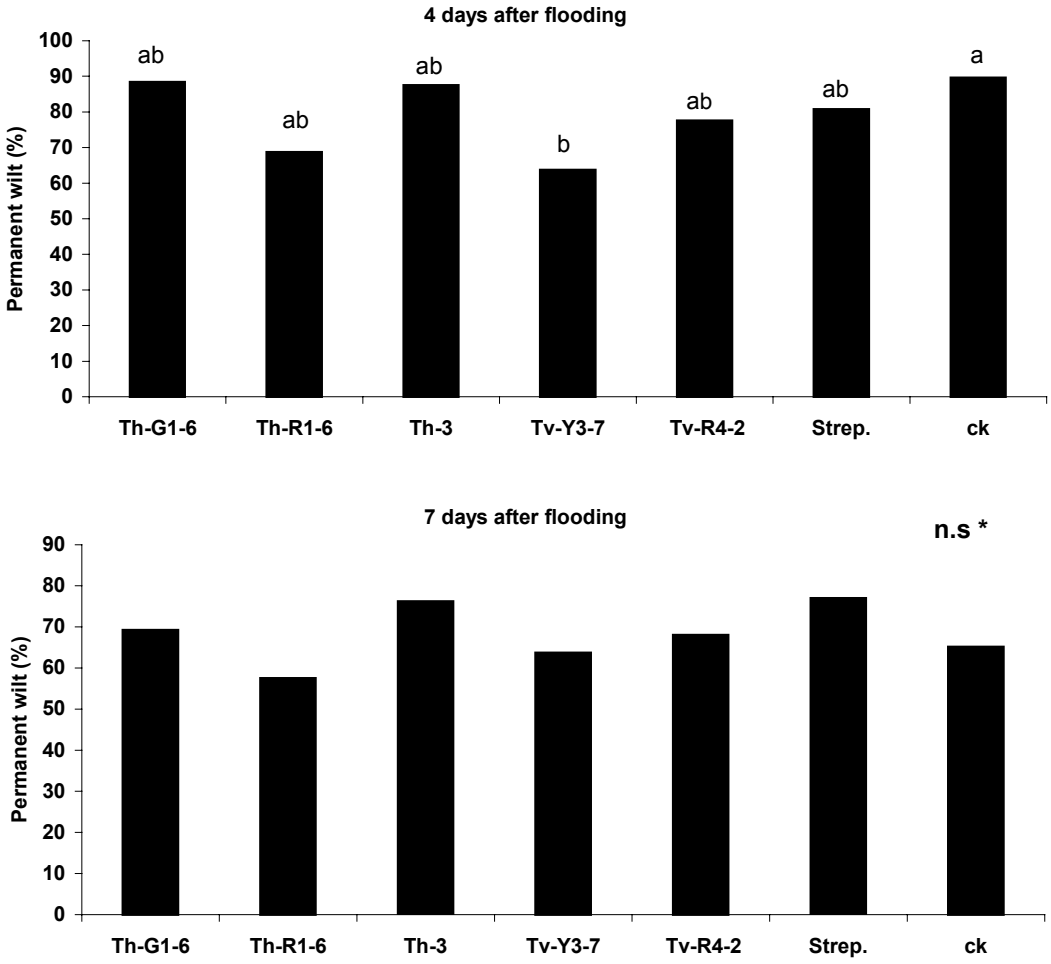

Figure 4.2: Effect of biocontrol agents on permanent wilt caused by *Pythium aphanidermatum* on tomato plants 4 and 7 days after 48 hrs flooding in a field experiment between July and October, 2001 at AVRDC, Shanhua, Taiwan. Th-G1-6, Th-R1-6 and Th-3 = isolates of *Trichoderma harzianum*; Tv-Y3-7 and Tv-R4-2 = isolates of *Trichoderma virens*; Strep. = *Streptomyces saraceticus*; ck = control. Treatment indicated by the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$), n.s* = not significant. n=28.

4.3.2 Greenhouse experiment to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on the control of tomato sudden death due to *Pythium aphanidermatum* after 48 hrs of flooding

Permanent wilt: Most tomato plants had wilted after 48 hrs flooding at 30°C soil temperature in the greenhouse test. The percentage of wilted tomatoes grown in the soil inoculated with the biocontrol agents was significantly higher compared to that of plants grown in untreated soil (Figure 3). Seven days after flooding, the percentage of wilted tomatoes observed in the treatment with *P. aphanidermatum* alone was not different from the treatments of the *T. harzianum* isolates, *T. virens* isolates or *Streptomyces saraceticus*. *T. harzianum* (Th-R1-6) reduced sudden death symptoms by only 10% compared to the *P. aphanidermatum* control and, therefore, was not effective in reducing *P. aphanidermatum* on tomato to economic levels. The percentage of wilted plants observed in all the treatments with biocontrol agents was significantly higher than the untreated control (Figure 4.3).

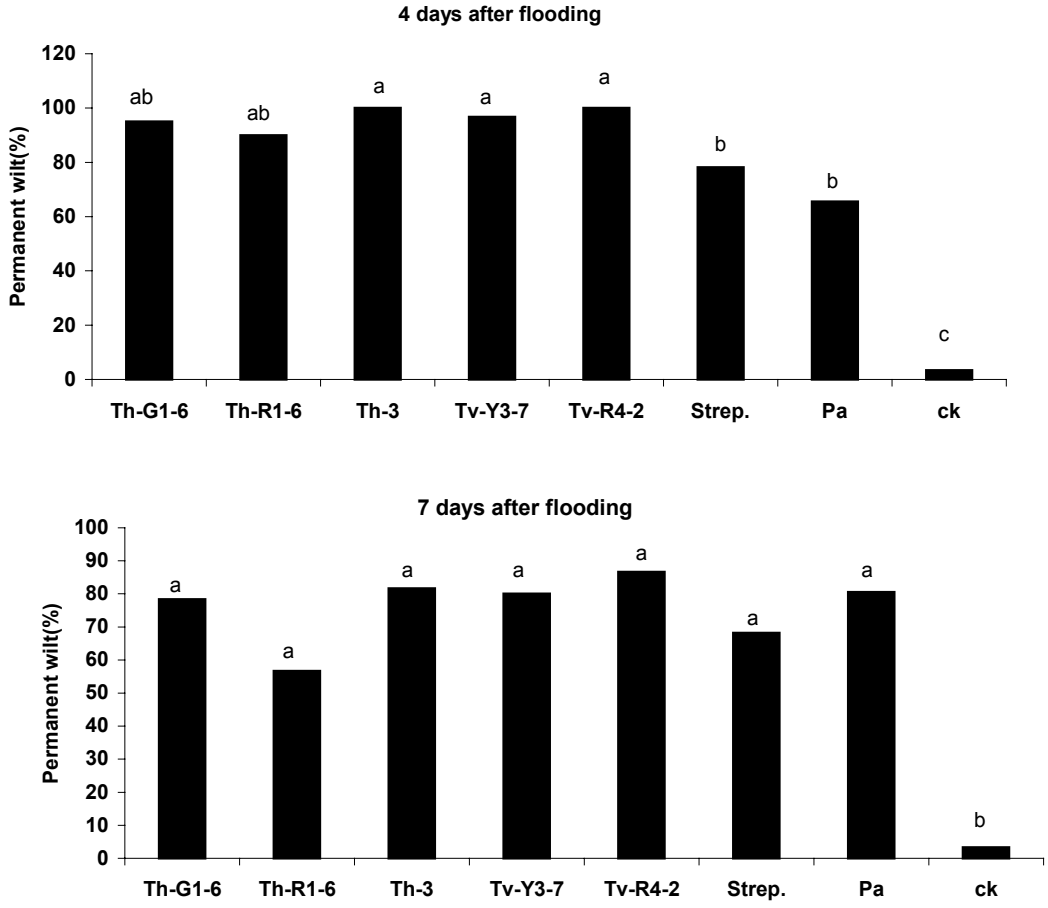

Figure 4.3: Effect of different biocontrol agents on permanent wilt of tomato plant caused by *Pythium aphanidermatum* 4 and 7 days after 48 hrs flooding in a greenhouse experiment conducted between August and November 2001 at AVRDC, Shanhua, Taiwan. Th-G1-6, Th-R1-6 and Th-3 = isolates of *Trichoderma harzianum*; Tv-Y3-7 and Tv-R4-2 = isolates of *Trichoderma virens*; Strep. = *Streptomyces saraceticus*; Pa = *Pythium aphanidermatum* inoculated; ck = non-inoculated control. Treatments indicated by the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$). n=24.

Root dry weight: Root dry weight of tomatoes grown in soil infested with *P. aphanidermatum* alone was significantly lower compared to the non-inoculated (Figure 4). The root weight of plants treated with Th-G1-6 and Th-R1-6 in the soil infested by *P. aphanidermatum* were significantly higher. The isolates Tv-Y3-7, Tv-R4-2, and Th-3 caused a non-significant increase in root weight compared to the *P. aphanidermatum*

inoculated control (Figure 4). Generally, none of the *Trichoderma* isolates affected root weight compared to the untreated control. There was also no significant difference in root dry weight between the *Streptomyces saraceticus* treatment and the *P. aphanidermatum* inoculated control (Figure 4). However, root weight was significantly lower in this treatment compared to that with *Trichoderma* isolates.

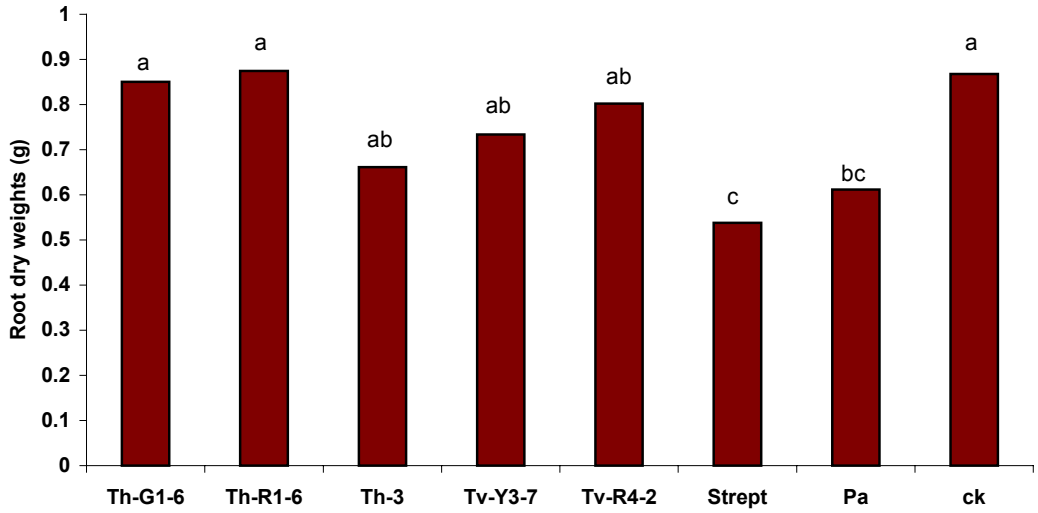

Figure 4.4: Effect of biocontrol agents and *Pythium aphanidermatum* on root dry weight of tomato in a greenhouse experiment on August-November, 2001 in AVRDC, Shanhua, Taiwan. Th-G1-6, Th-R1-6 and Th-3 = isolates of *Trichoderma harzianum*; Tv-Y3-7 and Tv-R4-2 = isolates of *Trichoderma virens*; Strep. = *Streptomyces saraceticus*; Pa = *Pythium aphanidermatum*; ck = non-inoculated control. Different letters show statistically significant differences among treatments according to Duncan's multiple range test ($P \leq 0.05$). n=24.

4.3.3 Greenhouse experiment to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on the control of tomato sudden death due to *Pythium aphanidermatum* following 72 hrs of flooding

Permanent wilt: There were no significant differences in the percentage of wilted plants among treatments with biocontrol agents in *P. aphanidermatum* infested soil compared to the non-infested control 4 or 7 days after flooding (Figure 5). Permanent wilt of tomatoes grown in the soil treated with Th-R1-6, Tv-Y3-7 or *Streptomyces* was slightly lower than in the untreated control but not significantly different. High levels of wilting were also observed in the *Pythium*-free soil (Figure 5).

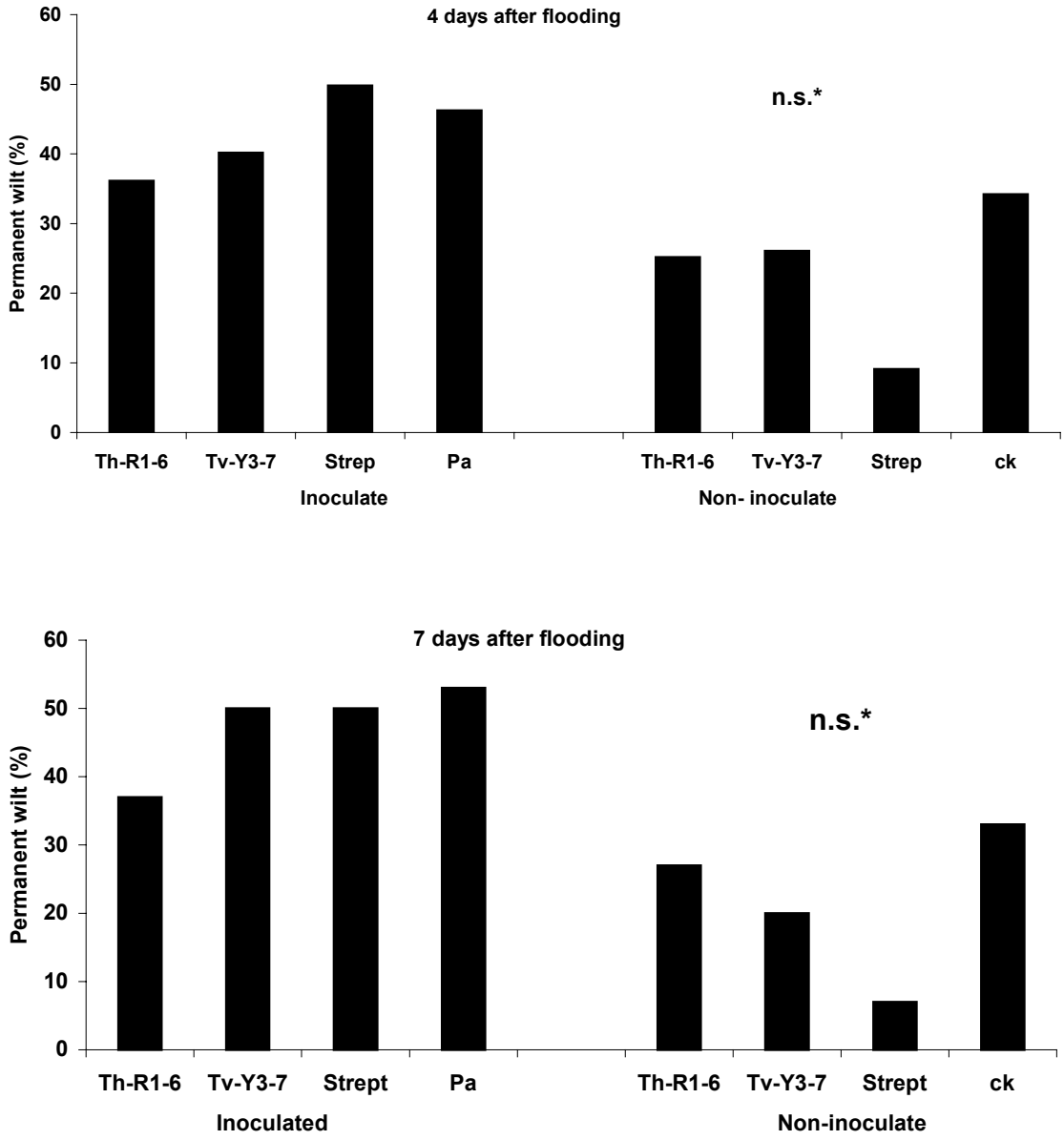

Figure 5: Effect of biocontrol agents on permanent wilt of tomato plants caused by *Pythium aphanidermatum* 4 and 7 days after 72 hrs flooding in a greenhouse experiment conducted between May and August 2002 at AVRDC, Shanhua, Taiwan. Th-R1-6 = *Trichoderma harzianum*; Tv-Y3-7 = *Trichoderma virens*; Strep. = *Streptomyces saraceticus*; Pa = *Pythium aphanidermatum* inoculated control; ck = non-inoculated control. n.s.* = not significant, n=24.

Root dry weight: The biocontrol agents slightly increased root dry weight in *P. aphanidermatum* infested soils compared to the control. Th-R1-6 increased root dry weight in soil both infested and not infested with *P. aphanidermatum*, but not significantly. Inoculation with *P. aphanidermatum* did not significantly affect root dry weight compared to the untreated control (Figure 6).

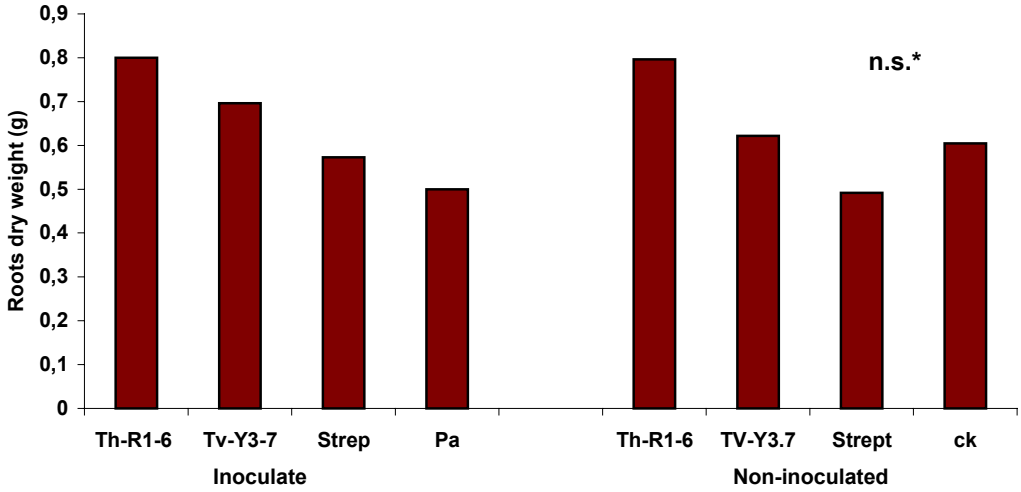

Figure 6: Effect of biocontrol agents and inoculation with *Pythium aphanidermatum* on root dry weight of tomato in a greenhouse experiment conducted between May and August 2002 at AVRDC, Shanhua, Taiwan. Th-R1-6 = *Trichoderma harzianum*; Tv-Y3-7 = *Trichoderma virens*; Strep. = *Streptomyces saraceticus*; Pa = *Pythium aphanidermatum* inoculated control; ck = non-inoculated control. n.s.* = not significant, n=24.

4.4 Discussion

Field experiment to evaluate *Trichoderma* spp. and *Streptomyces saraceticus* for the control of tomato sudden death due to *Pythium aphanidermatum*

Field experiments give a true picture of the biocontrol potential of fungal antagonists as influenced by the abiotic and biotic conditions existing in the ecosystem (Merrian et al. 1990; Pusey 1990). It is also known that results of biocontrol experiments are often reproducible in *in-vitro* or in greenhouse experiments, but not under field conditions (Mahaffee et al. 1993). In the present study, a field experiment was conducted in soil naturally infested with *P. aphanidermatum*. The soil analysis demonstrated that *P. aphanidermatum* was ubiquitous and the population density fluctuated during the cropping season. The highest population density was detected one month after transplanting of tomatoes and was affected by soil temperature (30-32°C) and high soil moisture.

Of the biocontrol agents tested here, only *T. virens* isolate Tv-Y3-7 showed some effect on tomato sudden death due to *P. aphanidermatum* under high temperature following flooding conditions after 4 days. None of the tested isolates protected tomato effectively against *P. aphanidermatum* infection after flooding. This may be because *Trichoderma* spp. mycoparasitism does not occur until 24 hrs after contact with the pathogen and then at low frequency (Chet et al. 1981). Thus, mycoparasitism was not initiated soon enough to protect against *P. aphanidermatum* under flooding at high temperatures, conditions that favor the release of *P. aphanidermatum* zoospores and host attack (Van der Plaats-Niterink 1981). So far, effectiveness of *Trichoderma* spp. for control of *P. aphanidermatum* was shown in non-flooding or cool conditions. Lo et al. (1997) found that *T. harzianum* treatment significantly reduced disease incidence up to 83 percent caused by *P. aphanidermatum* in turfgrass in non-flooding conditions. Sivan et al. (1984) reported that an isolate of *T. harzianum* reduced the incidence of damping-off disease due to *P. aphanidermatum* up to 87% on tomato and 90% on pepper seedlings in non-flooded conditions.

In this study, there was no evidence to support reports of *Streptomyces saraceticus* biocontrol of tomato sudden death due to *P. aphanidermatum*. The reason for the poor results with *Streptomyces* on tomato sudden death is unknown. It may be due to the direct effect of flooding on the population density of *Streptomyces* in the soil, which leads to

reduced antibiotic activity. Bolton (1980) found that *Streptomyces* spp. had little affected on *P. aphanidermatum* in hydroponic culture when the concentration dropped below 10^7 propagules/ml.

The poor results obtained in the present study may be due to the influence of abiotic factors, such as soil moisture and soil temperature, on the biocontrol agents. Soil microorganisms may also play a direct role in limiting the potential level of biocontrol over long periods of time during the growing season. In the present study, treatment with the antagonists was followed by flooding the soil 2 months after transplanting for 48 or 72 hrs. It is well known that *P. aphanidermatum* survives as oospores in soil and on plant debris and as mycelium in infected roots where the biocontrol agents may not be active. Wet or flooded soil favors the production and release of zoospores and infection of roots or stems of the host. In addition, high soil temperature meets the virulence requirements of the pathogen for development of the disease (Hine et al. 1969; Van der Plaats-Niterink 1981). The high density of infective zoospores of the pathogen in the soil released during flooding may have led to unsucessful biocontrol because of the massive release of infective zoospores. It is also possible that the agents used are not suitable for effective control of *P. aphanidermatum* attacking tomato as the plants grow out of the seedling stage. New isolates that come from fields where flooding does not lead to sudden death of the tomato need to be tested.

Greenhouse experiments to evaluate the effect of *Trichoderma* spp. and *Streptomyces saraceticus* on the control of tomato sudden death due to *Pythium aphanidermatum* after 48 and 72 hrs of flooding

Wilting: In the greenhouse experiment conducted in the hot season between August and November 2001, three isolates of *T. harzianum*, two isolates of *T. virens*, and *Streptomyces saraceticus* were tested. There was no evidence of biocontrol on tomato sudden death due to *P. aphanidermatum*.

The greenhouse test was repeated between May and August 2002 with 72 hrs of flooding to evaluated the antagonistic potential of *Trichoderma* and *Streptomyces* toward *P. aphanidermatum* and their effects on tomato growth. The biocontrol agents had no

significant effect on disease incidence compared to the infested control. There were also no significant differences in dry root weight among treatments or in the treatments compared to the untreated control. However, *T. harzianum* and *T. virens* had a slightly positive effect on plant growth compared to the treatment *Streptomyces saraceticus* alone.

The results seem to be in conflict with those of previous studies (Sivan et al. 1984). They found that *Trichoderma* spp. reduced the disease incidence of damping-off due to several pathogens including *P. aphanidermatum* and *Rhizoctonia solani*. Plants treated with *T. harzianum* reduced the level of damping-off by 87% in tomato seedlings in soils artificially infested with *P. aphanidermatum*. Bolton (1980) found that *Streptomyces* delayed infection of *P. aphanidermatum*, which caused root rot and sudden wilt of poinsettia, in soilless culture. The poor results observed in the present study could be due to the failure of the *Trichoderma* spp. mycoparasitism of *P. aphanidermatum*. Under non-flooded conditions, *Trichoderma* spp. can parasitize the hyphae of *Pythium* spp. (Lifshitz et al. 1986). Elad et al. (1982) also reported that the ability of isolates of *T. harzianum* to control damping-off caused by *P. aphanidermatum* was correlated to the level of hydrolytic enzyme production in the soil. Hydrolytic enzymes were the sole carbon source when the biological agent was grown on fungal cell wall components (Elad et al. 1982). This enzyme may be negatively affected by flooding or even disappear during flooding periods, leading to ineffective biocontrol of *P. aphanidermatum*.

The poor results recorded from both greenhouse experiments regardless of duration of flooding may be due to the effect of flooding on host plant susceptibility to *P. aphanidermatum*. It is well known that the tomato plant is the most flood-sensitive vegetable (Kuo et al. 1982). Flooding of the tomato plants for 48 hrs or longer damages the root system leading to a breakdown of the plant's disease resistance (Palti 1981). In addition, flooding soil favors the production and release of zoospores and increases infection of roots or stems of the host. Wilting may result from injury to the root system caused by flooding and the aggressive pathogen.

The failure of *Trichoderma* and *Streptomyces* to control *P. aphanidermatum* after flooding may be related to their population density in the environment around the host

plant. Bolton (1980) found that in soil-less culture *Streptomyces* spp. and *T. viride* had little impact on *P. aphanidermatum* when the concentration was below 10^7 propagules/ml.

Biomass: In the experiment in 2001, the plant treated with the *T. harzianum* isolates Th-G1-6 and Th-R1-6 had a significantly larger dry root weight than infested controls. The dry root weights of plants treated with *T. virens* in infested soil were slightly higher but were not significantly different from the infested control.

In the May-August 2002 experiment, the biocontrol agents caused limited growth promotion to tomato plant. However, there were not significant differences between the root dry weights of tomato plant grown in the soil treated with *Trichoderma* spp. or *Streptomyces* compared to the control.

The results are in agreement with research on the use of *T. harzianum* and *T. virens* for control of other plant pathogenic diseases. Windham et al. (1986) found that tomato root and shoot dry weights grown in soil treated with *Trichoderma* spp. increased 213-275% and 259-318% respectively, over control. There could be several reasons for this. Conversely, they found that treatments with *Streptomyces* resulted in root weights lower than the infested control. The dry shoot weights of tomato plants grown in the *P. aphanidermatum* infested soil treated with *Streptomyces* spp. was also significantly lower than in the untreated control and that of the treatments with *Trichoderma* spp.

The increase in root dry weight may be due to two mechanisms that explain increased growth response induced by soil microflora (Windham et al. 1986). The enhanced growth of plants induced by *Pseudomonads* is predominantly due to biological control of minor pathogens (Salt 1978; Suslow et al. 1979; Kloepper et al. 1981). The other reason could be that a microbial agent produces growth-regulating metabolites that affect plant growth (Lindsey et al. 1967; Windham et al. 1986).

Despite intensive research on biological control of soil born diseases, the effect of flooding on biological control of soilborne pathogens is not well understood. Future research to increase the efficacy of fungal antagonists should concentrate on the effects of flooding, soil temperature and root colonization on the biocontrol agents before, during and after flooding. Because of the nature of this interaction, biocontrol with soil-based

antagonists may not be effective. The isolates used here, for example, were developed for damping-off control and not for long season effects. There is also a need to look at fungal and bacterial endophytes for control. They can be applied to the seedlings and live inside the root tissue; they would thus not be affected by flooding (Sikora 1992, Hallman 2001).

5 EFFECT OF SOIL AMENDMENTS TO COTNROL TOAMTO SUDDEN DEATH DUE TO *PYTHIUM APHANIDERMATUM* FOLLOWING FLOODING IN HOT SEASON

5.l Introduction

Pythium aphanidermatum (Edson) Fitzp. is a serious soilborne pathogen in high temperature regions affecting many high value crops worldwide. It causes damping-off, root rot and fruit rot in tomato, cucumber, pepper, lettuce, spinach, and bean in both field-planted and hydroponic cultures (Sumner et al. 1976; Jenkins et al. 1983; Bates et al. 1984; Paulitz et al. 1992; McCarter 1997). *P. aphanidermatum* has been detected during tomato production in hot summer in the tropics after heavy rainfall, and it is considered the main causal agent of the occurrence of stem rot, wilting, and dead plants (Bolton 1980; McCarter 1997).

Many control methods have been suggested and tested to solve the disease problem. The use of soil amendments, resistant varieties, application of fungicides, as well as other methods have been tested (Palti 1981; Bates et al. 1984; Palti et al. 1997). New products based on organic amendments have been developed in Taiwan in attempts to control *P. aphanidermatum*. Previous reports indicate that adding organic matter to the soil is effective in reducing the incidence of certain soilborne pathogens and in promoting healthy growth of the host plants (Huang et al. 1993). Lin et al. (1988) found that the soil amendments called S-H mixture reduced the incidence of cucumber damping-off due to *P. aphanidermatum*. Other soil amendments composed of mushroom compost (SFMC) have been shown to reduce damping-off disease caused by *P. aphanidermatum* on cucumber (Wang et al. 2000). Complex mixtures (FNB-5A) have shown control of damping-off on cabbage seedlings due to *Rhizoctonia solani* (Shiau et al. 1999). Organic matter has been used in general to improve soil fertility, increase plant growth and stimulate soil microbial densities, thereby improving plant health (Palti 1981).

The goal of the investigation reported here was to test the efficacy of soil organic amendments and *Trichoderma* spp. enhanced amendments against tomato sudden death caused by *P. aphanidermatum* under flooded and high soil temperature conditions in the field and in greenhouse micro-plot experiments.

5.2 Materials and methods

5.2.1 Preparations

Preparation of *Pythium aphanidermatum* inoculation

Pythium aphanidermatum strain number 4 was cultured on V8 agar in Petri dishes for 3 days at 28°C before incorporation into rice seed for solid state fermentation. Each 400 ml beaker containing 150 ml of rice grain and 75 ml distilled water was autoclaved twice prior to being used as the final growth substrate. Two blocks of agar of a 3-day-old culture of *P. aphanidermatum* were inserted into the rice grain in each beaker and then incubated under illuminated light at 28°C for 10 days. One beaker of rice grain was incorporated into the upper 10 cm of each tub 10 days after transplanting.

Culture media

The media used in these studies were autoclaved for 20 minutes at a temperature of 121°C. All the antibiotics were added to the respective medium after autoclaving and after cooling to 50°C. The composition of the media is given below:

Burr and Stanghellini medium (Burr *et al.* 1973)

17g	Corn Meal Agar (Difco)
100mg	Pimaricin
200mg	Streptomycin sulfate
150mg	Rose Bengal
5mg	Benomyl
1000ml	distilled H_2O

Potato Dextrose Broth Agar (PDA)

37g	PDA (Difco)
1000ml	distilled H_2O

Vegetable broth (V-8)

200ml	V-8 vegetable juice (albi)
3g	$CaCO_3$
15g	Agar
1000ml	distilled H_2O

Tomato plant

The tomato variety CL5915-206D, which was determined by AVRDC to be heat tolerant and virus resistant, was used in both greenhouse and field tests. The seedlings were grown in a peat moss substrate for 30 days in the greenhouse and then transplanted into tubs or into field plots.

Soil amendment preparation and inoculation with *Trichoderma*

Trichoderma aureoviride, which was isolated by a scientist from the Plant Pathology Department of Chung Hsing University, Taiwan, was added to the composts to enhance control. The fungus was grown on PDA medium and incubated for 5 days at 28°C for conidia development. The conidia, after being removed from the medium by adding 20 ml distilled sterilized water with additional scraping by a glass rod, were used to inoculate the SFMC and FBN-5A composts.

Soil amendments developed by scientists in the Plant Pathology Department of Chung Hsing University, Taiwan, were also tested. The organic amendments used were:

1. Spent forest mushroom compost (SFMC)
2. Fish meal, blood meal, NH_4N0_3, lime, SFMC, allyl alcohol (FBN-5A)
3. Bagasse, rice husks, oyster shell, urea, $KN0_3$, $CaSO_4$, mineral ash (S-H Mixture)

Seedling substrate treatment: The soil amendment SFMC was mixed with peat moss No.4 (Bas Van Burren) and water at the rate of 2:1:1 (v/v). FBN-5A was mixed with peat moss at the rate of 2:1000 (v/v) (Shiau et al. 1999). Both mixtures were inoculated with conidia of *Trichoderma aureoviride* (2×10^5 conidia/g medium) 7 days prior to being filled

into trays (Recommendation from Plant Pathology Department of Chung Hsing University, Taiwan). Tomato seeds were surface-sterilized with sodium hydrochloride (1%) for 3 minutes and then sown into the trays. One month later, they were transplanted into the greenhouse tubs or into field plots for experimentation.

The S-H mixture without *Trichoderma aureoviride* was applied at transplanting. The dose, 1kg/m^2 (Lin et al. 1988), was incorporated into the upper 10 cm of the soil in the greenhouse tubs or field experimental plots by hand, 7 days prior to transplanting. Tomato plants that had grown in standard peat moss potting medium were transplanted to these plots.

5.2.2 Field experiment on effect of soil amendments on the control of tomato sudden death due to *Pythium aphanidermatum*

General method

The experiment was conducted from July 15th to October 15th 2001 in a paddy rice field at AVRDC, Shanhua, Taiwan, with a clay soil, where damping-off caused by *P. aphanidermatum* had been prevalent in previous years. Rice straw was burned and the field left to fallow for one month for drying prior to ploughing. Sixteen plots of 5 m^2 (1m x 5m) were established and labeled to host the treatments. Four plots for each of the treatments were set up in a random manner along the length of the field and repeated in four blocks along the width. Empty bands were left between plots to separate them from each other and to avoid cross contamination between treatments. The plots were covered with plastic to control weeds and limit insect damage. A total of 210 kg (N:P:K=4:4:4) organic fertilizer was broadcasted over the field prior to the establishment of the plots. In addition, a total of 65 kg (N:P:K:Mg=15:15:15:4) of chemical fertilizer was used as top dressing. The treatments were randomly distributed within the plots.

Five soil samples were taken randomly from the upper 20 cm along the diagonal of the field with a 5-cm-diameter cylinder before transplanting, in order to measure soil pH and to quantify the population of *P. aphanidermatum* (see 2.2.2). The soil samples were mixed and placed on paper towel at the room temperature for two days until they became dry. Twenty gram of this dried soil was dissolved in 0.01M calcium chloride ($CaCl_2$) on a

rotary shaker for one hour to check the pH. The soil pH was measured as an average of three sub-samples and found to be 6.47.

Twenty 30-day-old seedlings were transplanted into each plot in 2 rows of 10 plants each and attached to bamboo sticks to avoid damage from strong wind and heavy rain. The experiment was flooded 2 months after transplanting when the soil temperature reached 30-32°C. The water level was maintained 10 cm above the upper soil surface for 48 hrs and then drained by a canal system between and around the 4 blocks.

Isolation of *Pythium aphanidermatum* from the soil

The soil samples were taken randomly from the field prior to transplanting, as mentioned above. In addition, the soil was sampled 30 and 60 days after transplanting as well as 7 days after floodwater removal. The soil samples were placed on paper towel and allowed to dry at room temperature for 2 days. Four dilutions of the soil of 1/5, 1/10, 1/20, and 1/40 g soil per ml 0.3 % water agar were used to quantify the population of *P. aphanidermatum* (Burr et al. 1973). Each dilution was mixed on a Vortex stirrer for at least 15 min and a 1 ml aliquot was dispensed evenly across the surface of the selective medium for *P. aphanidermatum* developed by Burr et al. (1973). The plates were incubated at 35°C for time intervals ranging from 24 to 72 hrs after which the soil was carefully washed from the agar surface and the *Pythium* colonies recorded.

Experimental design

The following treatments with four replicates were used:

Table 5.1: Treatments to study the effect of soil organic amendments with and without *Trichoderma* on tomato sudden death due to *Pythium aphanidermatum* under flooding and hot conditions

Amendment	Treatment description
SFMC + *Trichoderma*	- Treated seedling potting medium in the nursery - Incorporated into the field soil at the time of transplanting
FBN-5A + *Trichoderma*	treated seedling potting medium in the nursery
S-H mixture	- Treated seedling potting medium in the nursery - Incorporated into the field soil at the time of transplanting
Control	- Standard seedling substrate without *Trichoderma* - No field incorporated

5.2.3 Greenhouse experiment on effect of soil amendments enriched with and without *Trichoderma* spp. on tomato sudden death due to *Pythium aphanidermatum*

General methods

The experiment was conducted from August to November 2001 in a greenhouse micro-plot test at AVRDC, Shanhua, Taiwan. Plastic tubs 31 cm x 51 cm x 40 cm (height x width x length) were used as experimental units. The tubs were sterilized with a 1% solution of hydrochloride before being filled with soil. Five kg of sterilized pebbles (1-2 cm diameter) were placed in the bottom of the tub and covered by plastic netting. An inverted U-shaded siphon was attached to the tub so that water could be siphoned out from under the net (see Figure 2.1, Chapter 2). Fifty-five kg of a heavy clay soil, which had been taken from AVRDC fields at Shanhua, Taiwan, was steam-pasteurized, and then filled into each tub. Soil pH was evaluated and found to be 6.5.

About 82.5 g of organic commercially available granular fertilizer (N:P:K=4:4:4) and 20 g chemical fertilizer (N:P:K:Mg=15:15:15:4) was incorporated into the soil of each tub before transplanting. In addition, 3.5 g of chemical fertilizer (N:P:K=20:20:20) was added weekly to each tub until the plant harvest.

Root weight

Tomato roots were removed from the soil by carefully washing with tap water 7 days after floodwater drainage. The washed roots were placed on paper towels at room temperature for 30 min to remove excess water and then dried in an oven at a temperature of 50°C for 48 hrs after which root dry weight was determined.

Experimental design

The experiment included 3 soil amendments and 3 control treatments with Mefenoxam + *P. aphanidermatum*, *P. aphanidermatum* alone and untreated control. Mefenoxam was incorporated at 2 g/m^2 at the time of transplanting and again 57 days after planting. The treatments with three replications are described in Table 5.2.

Table 5.2: The treatments to test the effect of soil amendments enhance with and without *Trichoderma* spp. on tomato sudden death due to *P. aphanidermatum* under flooding and hot conditions.

Treatments	**Flood period**	***Pythium aphanidermatum* inoculation**
SFMC + *Trichoderma*	48 hrs	10 days after transplanting
FBN-5A + *Trichoderma*	48 hrs	10 days after transplanting
S-H mixture	48 hrs	10 days after transplanting
Mefenoxam	48 hrs	10 days after transplanting
Pythium aphanidermatum alone (Pa)	48 hrs	10 days after transplanting
Non-inoculate control (ck)	48 hrs	None

Ten tomato seedlings, pre-treated with soil amendment, were transplanted into the tub in the greenhouse. Tubs were inoculated with *P. aphanidermatum* 10 days after transplanting.

Sixty days after transplanting, the tubs were flooded. The water level was maintained at 2-4 cm above the soil surface for 48 hrs and then removed rapidly through the siphon tube. The temperature in the greenhouse was maintained at 28-32°C for the duration of the experiment.

5.2.4 Data collection and statistical analysis

The number of wilted plants from all treatments was recorded at 2, 4 and 7 days after floodwater removal. In addition, 7 days after flooding, plants were harvested and root dry weight determined. The data on incidence of permanent wilt of tomato were analyzed with the SAS (SAS Institute Inc. 1989) program, using the general linear model procedure, including analysis of variance, Duncan's multiple range test, least significant difference (LSD) and /or orthogonal contrasts.

5.3 Results

5.3.1 Field experiment on effect of soil amendment and Trichoderma enhancement on the biological control of tomato sudden death due to *Pythium aphanidermatum*

Pythium aphanidermatum recovery from the soil: The detection of 1.6 CFU/g soil of *P. aphanidermatum* prior to transplanting confirmed that the field soil was naturally infested by the fungus. The population density of *P. aphanidermatum* changed during the cropping season and reached the highest density one month after tomato transplanting (Figure 5.1).

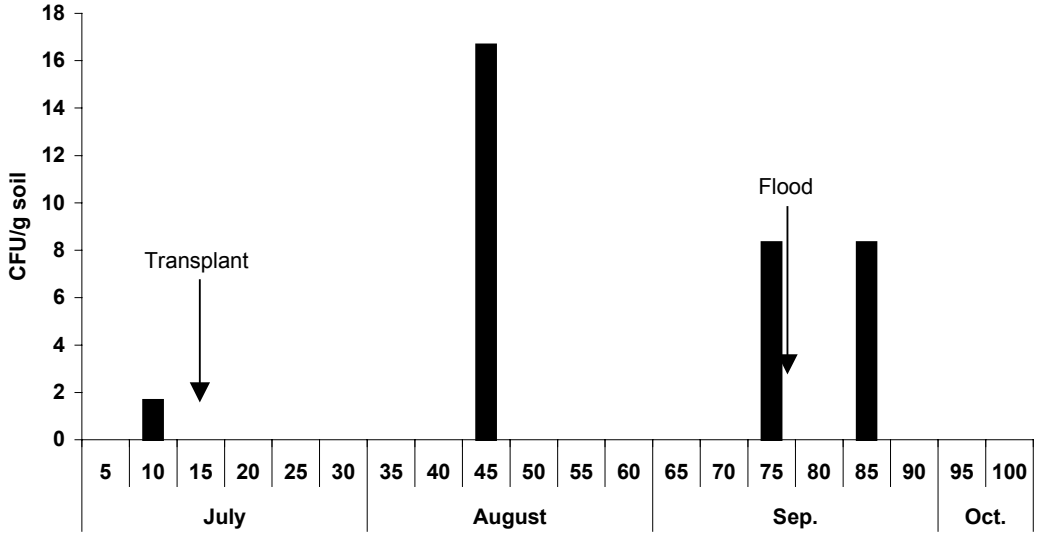

Figure 5.1: Density of *Pythium aphanidermatum* (CFU/g soil) in soil in a field experiment between July and October 2001 at AVRDC, Shanhua, Taiwan. The soil was sampled before transplanting, 30 and 60 days after transplanting as well as 7 days after flooding.

Permanent wilt: The soil amendments SFMC, FBN-5A or S-H Mixture had no effect on tomato sudden death due to *P. aphanidermatum*. Permanent wilt of tomato plants grown in the treated soil was not significantly different compared to the control (Figure 5.2). Furthermore, organic matter enhanced with *Trichoderma aureoviride* did not increase the level of control.

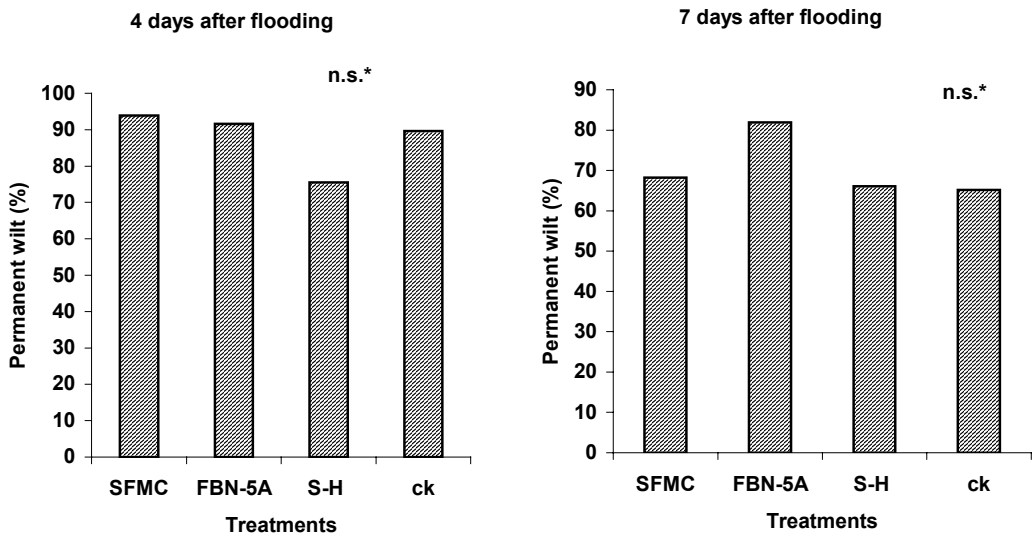

Figure 5.2: Effect of the organic soil amendments SFMC, FBN-5A, and S-H Mixture on permanent wilt of tomato caused by *Pythium aphanidermatum* 4 and 7 days after 48 hrs flooding in a field experiment between July and October 2001. ck = non-treated control. n.s.* = not significant, n=16.

5.3.2 Greenhouse experiment on effect of soil amendments and Mefenoxam on tomato sudden death due to *Pythium aphanidermatum*

Permanent wilt: None of the soil amendments reduced the rate of wilting determined 4 and 7 days after flooding. Enhancement of the soil with *Trichoderma aureoviride* did not have any additional effect, although a slight increase was seen in FBN-5A over S-H and the control. The percentage of wilted tomatoes grown in treated, infested soil was not significantly different in comparison to the untreated, infested control (Figure 3). In addition, Mefenoxam, which was used as a standard chemical control treatment, did not reduce sudden death in infested soil (Figure 5.3).

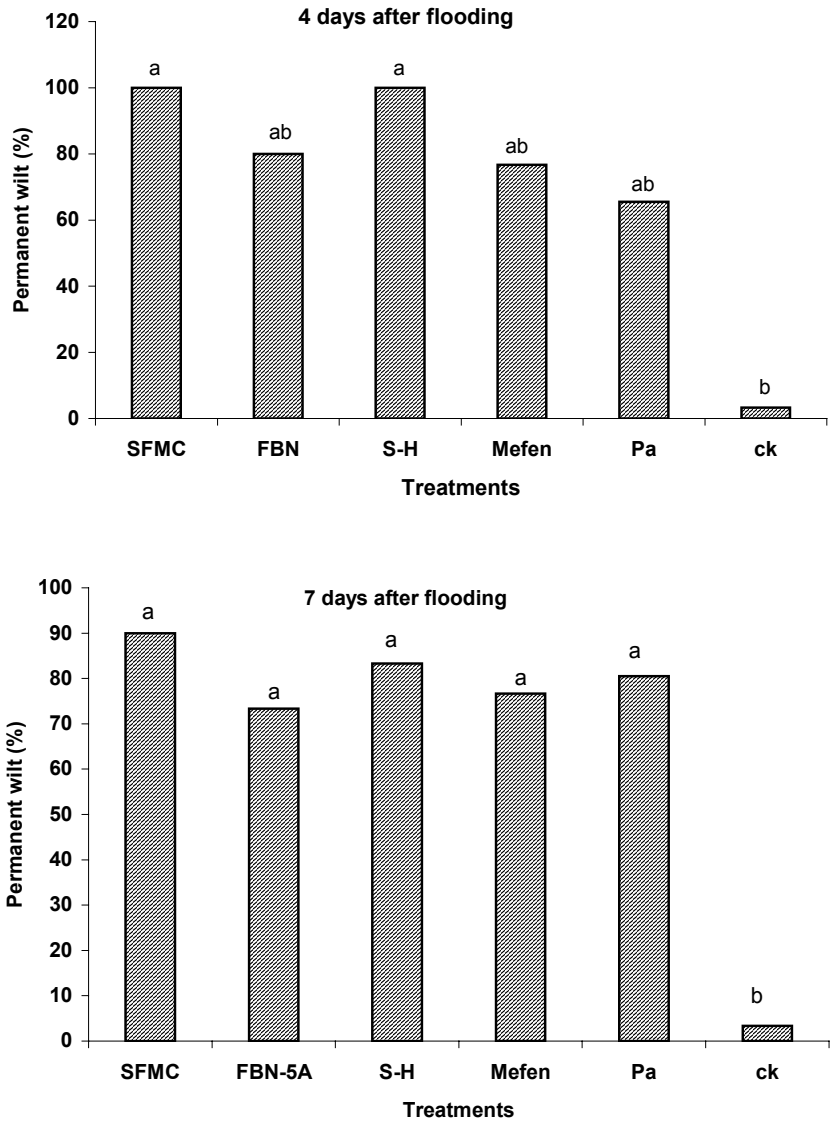

Figure 5.3: Effect of the organic amendments, SFMC, FNB-5A and S-H Mixture, on permanent wilt of tomato caused by *Pythium aphanidermatum* 4 and 7 days after 48 hrs flooding in a greenhouse experiment between August and November 2001 at AVRDC, Shanhua, Taiwan. Mefen = Mefenoxam; Pa = *Pythium aphanidermatum* inoculated; ck = non-inoculated control. Different letters show statistically significant differences among treatments according to Duncan's multiple range test ($P \leq 0.05$). n=18.

Root dry weight: Root dry weight of tomato plants grown in the soil treated with soil amendments or Mefenoxam was slightly but not significantly increased compared to the

untreated control. However, significant increases in root dry weight due to SFMC, FBN-5A, and Mefenoxam treatments were observed when compared to *P. aphanidermatum* inoculated control (Figure 5.4). In the treatment with the S-H Mixture, the root dry weight of tomato plants was not higher than that in the infested control.

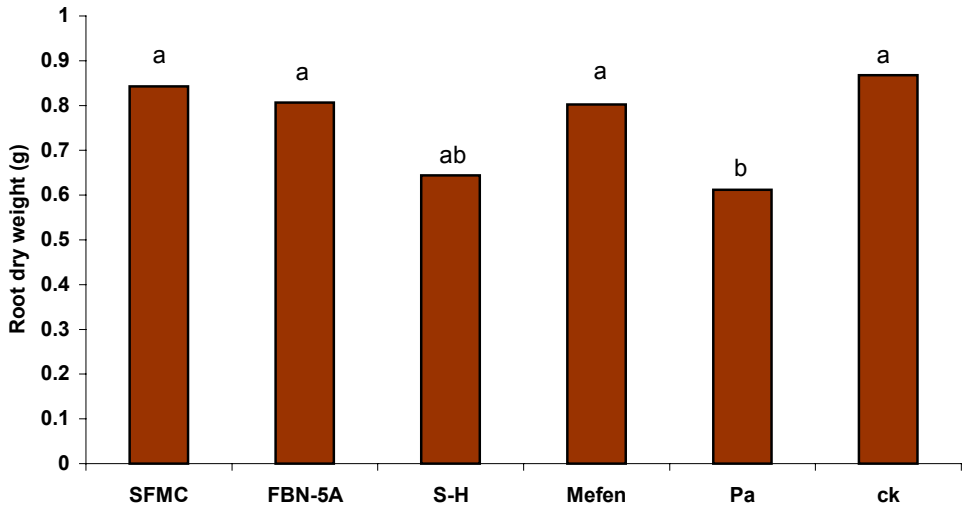

Figure 5.4: Effects of the soil amendments, SFMC, FNB-5A, S-H Mixture, and *Pythium aphanidermatum* on root dry weight of tomato in a greenhouse experiment between August and November 2001 at AVRDC, Shanhua, Taiwan. Mefen = Mefenoxam; Pa = *Pythium aphanidermatum* inoculated; ck = non-inoculated control. Different letters show statistically significant differences among treatments according to Duncan's multiple range test ($P \leq 0.05$). n=18.

5.4 Discussion

Field experiment on effect of soil amendments for biological control of tomato sudden death due to *Pythium aphanidermatum*

The goal of the present study was to assess whether different soil amendments are capable of controlling or limiting tomato sudden death under high temperature conditions following flooding. Soil amendments are known to modify the soil environment, shifting the ecological balance to the disadvantage of crop pathogens (Huang et al. 1993). The mechanisms by which soil amendments affect plant pathogens can be simple or complex depending on the amendments and the pathogens. In some cases, control is achieved by

direct killing of the pathogen propagules, in other cases, disease suppression is the result of a combination of multiple factors, including direct toxicity to the pathogen or indirect effects due to enhanced microbial activity or improved vigor of plants (Huang et al. 1993). Sun et al. (1985) reported that the S-H Mixture soil amendment has indirect effects on *P. aphanidermatum* by increasing the population of antagonistic fungi from 2 to 25 times in watermelon field soil. In addition, it greatly reduced the incidence of damping-off disease on cucumber due to *P. aphanidermatum* in the field (Lin et al. 1988). However, in the present study, S-H Mixture did not significantly reduce disease incidence of tomato sudden death caused by *P. aphanidermatum*. Similarly, the soil amendments SMFC and FBN-5A plus *Trichoderma aureoviride* showed no significant impact on *P. aphanidermatum*.

The lack of effectiveness of these soil amendments to control tomato sudden death could be related to effects of flooding combined with the soil amendments on the microbial environment in the soil. Under non-flooded conditions, the S-H mixture has been shown to improve plant vigor against pathogens (Huang et al. 1993) by producing ammonia in the soil, which inhibits mycelia growth and zoospore germination of *P. aphanidermatum* (Lin et al. 1988). SFMC and FBN-5A also enrich and enhance the activity of antagonistic microbes in the soil against soilborne pathogens (Shiau et al. 1999; Wang et al. 2000). However, their effectiveness may have been reduced or lessened due to the anaerobic conditions resulting from the 48 hrs flooding. The interaction between flooding and soil amendments and their influence on soilborne pathogens causing sudden death by a quick release of infective zoospores needs to be studied in future research. The exponential increase in number of infective propagules and the weakening of plants defence mechanisms seems to limit the amendments to control this disease.

Greenhouse experiment on effects of soil amendments and Mefenoxam on tomato sudden death due to *Pythium aphanidermatum*

The soil amendments SFMC, FBN-5A, and S-H mixture did not significantly inhibit *P. aphanidermatum* on tomato. In fact, higher levels of disease incidence following treatment of SFMC, FBN-5A, and S-H Mixture compared to the *P. aphanidermatum* infested control were observed. The results are in contrast to other studies related to the use of soil

amendments and control of damping-off diseases due to soilborne pathogens including *P. aphanidermatum* and *Fusarium* spp. Shiau et al. (1999) and Liu et al. (2000) studied the effect of FBN-5A alone on *Fusarium oxysporum* and *Rhizoctonia solani*. They concluded that treating a potting unit with FBN-5A at a rate of 1000:1 (Peat moss: FBN-5A = v:v) significantly reduced disease incidence on radish and cabbage seedlings. Wang *et al*. (2000) reported that SFMC alone was more effective in suppressing damping-off of cucumber seedlings caused by *P. aphanidermatum* compared to other potting substrates like BVB peat moss. SFMC, however, was ineffective in the control of damping-off of cabbage caused by *Rhizoctonia solani* (Huang et al. 2000). Lin et al. (1988) found that under greenhouse conditions, S-H at 2% or 5% (S-H Mixture: soil=w:w) completely inhibited damping-off disease on cucumber by *P. aphanidermatum*. It is important to have in mind that control in the above-mentioned studies occurred at the seedling stage and not on older plants growing under field conditions that are affected in the present experiments by sudden death.

The poor results achieved after flooding could be related to the negative effect of flooding on the predeposition of the plants to disease (Gold et al. 1984). In the present study, the rate of wilting of tomato plants was not significantly reduced by the amendments when compared to the infested control when plants were flooded 2 months after transplanting. The lack of control is probably due to several factors related to interactions between soil moisture, environment and the population dynamics of the pathogen in the soil. Soil amendments probably only provide short- to medium-term protection against *P. aphanidermatum* under aerobic or normal growing conditions. Lin et al. (1990) reported that S-H mixture is not a panacea. It does not work well for deep-rooted crops or for plants with a long growing period, because the period of disease suppression lasts about 40 days. Under the long-term growing conditions in the present tests, the positive short-term effects of organic amendments were not able to cope with disease pressure. Large numbers of wilted and dead tomatoes were observed in all treatments after flooding, whereas only a few or no dead plants were found in the period before flooding.

Lack of effective control by soil amendments could also be due to the level of *Pythium* inoculum used, which may have been too high for effective control. Similar tests

at lower inoculum densities may have been more effective, since organic amendments are often not effective at high disease pressure. Plants flooded at soil temperature 30-32°C are more susceptible to *P. aphanidermatum* (Hine et al. 1969; Van Der Plaats-Niterink 1981; Yu et al. 1989). Flooding probably triggers a massive release of zoospores in the field soil. This massive release coupled with root tissue injury may cancel any positive effects organic matter may have on *P. aphanidermatum* infection earlier in the season.

Therefore, despite the fact that the soil amendments SFMC, FBN-5A, and S-H Mixture have been reported to be highly successful as a means of protecting vegetable seedlings, they were ineffective toward tomato sudden death due to *P. aphanidermatum* following flooding at high temperatures condition in the present tests.

The *Trichoderma aureoviride* added with the soil amendment SFMC and FBN-5A did not enhance control of tomato sudden death due to *P. aphanidermatum*. This could be due to the fact that in previous studies, *T. aureoviride* was only effective on *Pythium* seedling disease. The fungus may also be unable to grow in clay soil over long periods of time to levels effective in control. Hoitink et al. (1996) reported that excessively stabilized organic amendments such as highly decomposed peats in the soil do not support the activity of biological control agents. Biological control agents inoculated into these types of organic matter decline in population density and are not able to induce sustained biological control of P*ythium* root rot (Hoitink et al. 1996). This may have occurred in the present test over the long growing season till sudden death took place.

There is further need to study the control of *P. aphanidermatum* at high soil temperatures and under short-term anaerobic conditions. Future research should concentrate on other types of soil amendments in order to enhance control of tomato sudden death. The application rate and application time of organic amendments to soil can also be modified in the future to achieve a better control of *P. aphanidermatum* sudden death which occurs in mature plants and not in seedlings.

6 RESPONSE OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.); EGGPLANT (*SOLANUM MELONGENA* L.) AND TOMATO GRAFTED ONTO EGGPLANT ROOTSTOCK TO TOMATO SUDDEN DEATH DUE TO *PYTHIUM APHANIDERMATUM* (EDSON) FITZP.

6.1 Introduction

In tomato production in lowland areas of tropical and subtropical countries during the hot, wet season, yield is generally quite low compared to that obtained in the cool season or from highland production areas. High temperatures reduce fruit set, heavy rainfall directly damages aerial plant parts, soil water logging reduces plant vigor, and short periods of soil flooding often result in wilt and sudden death of tomato plants. *Pythium aphanidermatum* is responsible for the death of seedlings as well as of matured tomato and cucumber plants that were irrigated 3-4 times daily at soil temperatures above 30°C (Stanghellini et al. 1975). The pathogen has a very fast growth rate and is a prolific producer of zoospores. It is often cited as the causal agent of root diseases and wilting for a number of vegetable crops grown in hydroponic systems (Paulitz et al. 1992). *P. aphanidermatum* is a well known pathogen of tomato that has been shown to cause damping-off, stem rot, root disease, and fruit rot (Bolton 1980; McCarter 1997).

Grafting techniques have been widely applied in many crops such as tomato, eggplant, cucumber, and watermelon (Carlsson 1963; Tubbs 1974; Zijlstra et al. 1987; Chadha 1988; Cohen et al. 2002). Grafting has been used effectively as a strategy to control a number of soilborne diseases and nematodes affecting vegetables including those attacking cucumber, tomato, eggplant and melons (Peregrine et al. 1982; Chadha 1988; Matsuzoe et al. 1993; Edelstein et al. 1999). In Japan, 93% of the watermelons, 72% of the cucumbers, 50% of the eggplants, 30% of the tomatoes, and 30% of all types of melon have been improved using grafted plants (Oda 1993).

Grafting of the tomato (*Lycopersicon esculentum* Mill.) onto tomato rootstocks, which are resistant to soilborne diseases such as bacterial wilt or root-knot nematodes, provides protection against the disease, but it does not give any protection against sudden death caused by soil flooding (AVRDC 1999). When the tomato scion is grafted onto eggplant rootstocks (*Solanum melongena* L.), positive effects on tomato production against

bacterial wilt under high temperature summer conditions in tropical areas were demonstrated (Peregrine et al. 1982; Matsuzoe et al. 1993). The strong eggplant root system seemed to stimulate plant growth at high air and soil temperatures (Abdelhafeez et al. 1975). In addition, eggplant roots have shown a high level of survival under water logged or flooding conditions (AVRDC 1999).

The aims of the present study were to:

1. Test the ability of tomato plants grafted onto eggplant rootstocks to limit tomato sudden death due to *Pythium aphanidermatum* following flooding under high temperatures under field conditions.
2. Study the susceptibility of tomato, eggplant and tomato grafted onto eggplant rootstocks to sudden death due to *Pythium aphanidermatum* under controlled greenhouse conditions.

6.2 Materials and methods

6.2.1 Plant material

Tomato plants of the line CL5915-206D, which was determined by AVRDC to be heat tolerant and virus resistant, were used in both non-grafted and grafted tests in the greenhouse and field. Eggplants of the variety EG203, which were reported by AVRDC to be flood resistant, were also used. The tomato and eggplant seeds were sown in a peat-moss substrate and kept in the greenhouse for 25-30 days, after which they were transplanted into the experimental plastic tub (see 6.2.3) or field (see 6.2.2).

To produce grafted tomato, the scion of the tomato line CL5915-206D was grafted onto a rootstock of the eggplant variety line EG203 13-15 days after sowing (Figure 6.1). The grafted tomatoes were placed in a growth chamber with temperatures ranging from 25-32°C and over 85% humidity. Five days later, the grafted plants were moved to a net house for 7 days prior to being transplanted into the field or greenhouse tubs.

A 

B 

C 

Figure 6.1: Tomato scion (A) grafted onto eggplant rootstock (B and C) in AVRDC, Shanhua, Taiwan (Source: Dr. Black LL; AVRDC, Shanhua, Taiwan, 2002).

6.2.2 Field experiment on response of tomato grafted onto eggplant rootstock to tomato sudden death due to *Pythium aphanidermatum*

General methods

The field experiment was conducted between July and October 2001 in an AVRDC field at Shanhua, Taiwan, naturally infested with *P. aphanidermatum*. The experiment was set up in the same field as the experiment on soil amendments and biocontrol agent (see Chapter 5). The field was left fallow for one month to dry prior to ploughing. Rice straw was burned in the field. Eight plots of 5 m^2 (1m x 5m) were established and labeled to host the treatments with grafted and non-grafted tomatoes. The treatments were set up in a random manner along the length of each block and repeated in four blocks along the width of the field. The treatments were randomly distributed to the blocks. Empty boarder rows were left between plots to separate them from each other and avoid cross contamination between treatments. The plots were covered by plastic to control weeds and limit damage by insect

pests. A total of 210 kg (N:P:K=4:4:4) organic fertilizer and 65 kg (N:P:K:Mg=15:15:15:4) chemical fertilizer were broadcasted over the entire field prior to the establishment of the plots.

Five soil samples were taken randomly from the upper 20 cm along the diagonal of the field with a 5 cm diameter cylinder before transplanting. In order to measure soil pH and quantify the population of *P. aphanidermatum* (see 2.2.2), the soil samples were mixed and stored at room temperature for two days until they became dry. Twenty grams of this dried soil was dissolved in 0.01M of Calcium chloride ($CaCl_2$) on a rotary shaker for one hour to check the pH. The pH was measured as an average of three sub-samples and was found to be 6.47.

Twenty plants (30 days old) of either tomato or tomato grafted onto eggplant rootstocks were transplanted into each plot and tixed to bamboo sticks to avoid damage from strong wind and heavy rain. The experiment was flooded for 48 hrs 2 months after transplanting, when soil temperatures were between 30 and 32°C.

Isolation of *Pythium aphanidermatum* from the soil

Soil samples were taken randomly from the field prior to transplanting, as mentioned in section 2.2.1, 30 and 60 days after transplanting as well as 7 days after floodwater removal. Four dilutions 1/5, 1/10, 1/20, and 1/40 of soil (g soil per ml 0.3 % water agar) were used to quantify the population of *P. aphanidermatum* (Burr et al. 1973). Each dilution was mixed on a Vortex stirrer for approximately 15 min and a 1 ml aliquot of mixed soil and 0.3% water agar was dispensed evenly across the surface of the *P. aphanidermatum* selective medium developed by Burr et al. (1973). The plates were incubated at 35°C from 24 to 72 hrs after which the soil was carefully washed from the agar surface and the *Pythium* colonies were counted.

6.2.3 Greenhouse experiments on the response of tomato, eggplant and tomato grafted onto eggplant rootstock to tomato sudden death caused by *Pythium aphanidermatum*

Two experiments were conducted in the greenhouse to study the susceptibility of tomatoes grafted onto eggplant rootstocks to *P. aphanidermatum*. The experiments were conducted between August and November 2001 and again between August and November 2002 at the AVRDC experimental site in Shanhua, Taiwan.

Preparation of fungus substrate and application

P. aphanidermatum strain number 4, isolated by the mycology unit at AVRDC, was cultured on V8 agar for 3 days before incorporation into rice seed for solid state fermentation. Each 400 ml beaker containing 150 ml of rice grain and 75 ml distilled water was autoclaved twice prior to being used as the final growth substrate. Two blocks of agar of a 3-day-old culture of *P. aphanidermatum* were placed into the rice grain in each beaker and then incubated in an illuminated chamber at 28°C for 10 days. One beaker of rice grain was incorporated into the upper 10 cm of soil of each tub 10 days after transplanting.

Experimental design

Plastic containers (tub) of size 31 cm x 51 cm x 40 cm (height x width x length) were used as experimental micro-plot units. The tubs were sterilized with a solution of 1% hydrochloride before being filled. Five kg of sterilized pebbles (1-2 cm diameter) were placed in the bottom of the tub and covered by a perforated plastic net. A U-shaped plastic siphon tube was then attached for draining (Figure 6.2). Fifty-five kg of a heavy clay soil taken from the AVRDC field at Shanhua, Taiwan, was steam pasteurized and then filled into the tub. Soil pH was evaluated and found to be 6.5.

About 83 g of organic commercially available granular fertilizer (N:P:K=4:4:4) and 20 g chemical fertilizer (N:P:K:Mg=15:15:15:4) were incorporated into the soil of each tub before transplanting. In addition, 3.5 g inorganic fertilizer (N:P:K=20:20:20) was added weekly to each tub until the plants were harvested.

A  B 

Figure 6.2: The experiment in the greenhouse in AVRDC, Shanhua, Taiwan:
(A) Greenhouse experiment
(B) Plastic tub with siphon tube (arrowed) for drainage

Flooding and temperature control

The plants were flooded 60 days after transplanting. The water level was maintained at 2-4 cm above the soil surface for 48 or 72 hrs and then removed rapidly through the siphon tube. The temperature was maintained at 28-32°C for the entire duration of each experiment.

Greenhouse experiment I: Response of non-grafted tomato and tomato grafted onto eggplant rootstock to *Pythium aphanidermatum* infection after 48 hrs of flooding

The experiment was conducted between August and November 2001 to test the susceptibility of non-grafted tomato and tomato grafted onto eggplant rootstock to *Pythium aphanidermatum* following flooding at high soil temperature. Each treatment was tested with three replications (Table 6.1).

Table 6.1: Treatments to test response of non-grafted tomato and tomato grafted onto eggplant rootstock to tomato sudden death due to *Pythium aphanidermatum* under flooding and hot conditions

Treatment	***P. aphanidermatum* inoculation**	**Flood period**
Non-grafted tomato	10 days after transplanting	48 hrs
Non-grafted tomato	None	48 hrs
Grafted tomato	10 days after transplanting	48 hrs

Greenhouse experiment II: Response of non-grafted tomato, tomato grafted onto eggplant rootstock, and eggplant to *Pythium aphanidermatum* after 72 hrs of flooding

Experimental design

The aim of this experiment conducted between August and November 2002 was to study the response of tomato, tomato grafted onto eggplant rootstock, and eggplant to *P. aphanidermatum* after 72 hrs flooding at high temperature. The treatments, each with three replications were tested (Table 6.2).

Table 6.2: Treatments to test response of tomato, eggplant, and tomato grafted onto eggplant rootstock to *Pythium aphanidermatum* under flooding and hot conditions

Treatments	***P. aphanidermatum* inoculation**	**Flood duration**
Eggplant, inoculated	10 days after transplanting	72 hrs
Eggplant, non-inoculated	None	72 hrs
Eggplant, non-inoculated	None	None
Tomato, inoculated	10 days after transplanting	72 hrs
Tomato, non-inoculated	None	72 hrs
Tomato, non-inoculated	None	None
Grafted, tomato inoculated	10 days after transplanting	72 hrs
Grafted, tomato non-inoculated	None	72 hrs
Grafted, tomato non-inoculated	None	None

Isolation of *Pythium aphanidermatum* from the roots

The roots of tomato and eggplant collected from the tubs treated with *P. aphanidermatum* 2 days before and 1 day after flooding were washed in running tap water and cut into 1 cm lengths. Ten root pieces were placed on a plate of Mircetich medium for fungal detection (Mircetich, 1971) and then incubated at 28°C for 24 hrs. The colony-forming units (CFU)

of *P. aphanidermatum* growing out of each root section were recorded after 24 hrs incubation.

Root weight

Roots of tomato, eggplant, and grafted tomato plants from tubs inoculated or not inoculated with *P. aphanidermatum* were washed with tap water. The washed root were placed on paper towels at room temperature for 30 min to remove excess water and then dried in an oven at 50°C for 48 hrs after which the root dry weight was determined.

6.2.4 Data collection

The number of wilted plants in both field and greenhouse micro-plot trials were recorded at 2, 4 and 7 days after floodwater removal. Plants in greenhouse experiment II were also harvested to measure the root dry weight. The data on incidence of permanent wilt of tomato, eggplant, and grafted tomato were analyzed with the SAS (SAS Institute Inc., 1989) program, using the general linear model procedure, including analysis of variance, Duncan's multiple range test, least significant difference (LSD) and /or orthogonal contrasts.

6.3 Results

6.3.1 Field experiment to assess the response of tomato and tomato grafted onto eggplant rootstock to sudden death *Pythium aphanidermatum*

Permanent wilting: No wilted plants due to *P. aphanidermatum* of neither tomato grafted onto eggplant rootstock nor non-grafted were observed prior to flooding. Four days after 48 hrs flooding, over 90% of the non-grafted tomato as showed wilt symptoms. After 7 days, 70% of the non-grafted tomatoes showed severe wilting. The wilting of non-grafted tomato plants was significantly higher compared to grafted tomato plants during both observation periods (Figure 6.3). Only 2% of the tomato plants grafted onto eggplant exhibited wilt symptoms in both periods.

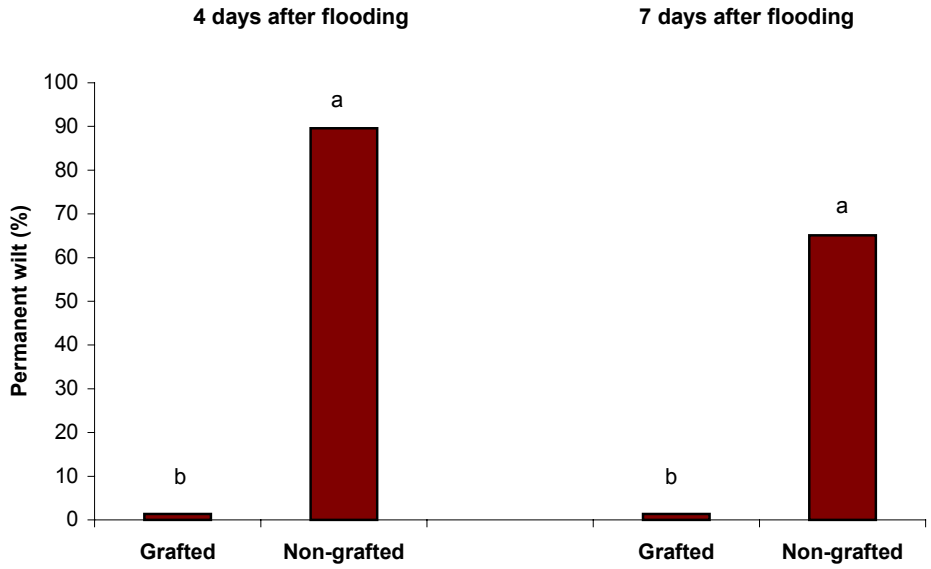

Figure 6.3: Permanent wilt of tomato CL5915 and tomato grafted onto eggplant (E203) rootstock 4 and 7 days after 48 hrs flooding in a field experiment during July-October 2001 in AVRDC, Shanhua, Taiwan. Different letters show statistical differences between the treatments according Duncan's multiple range test ($P \leq 0.05$), n=8.

Pythium aphanidermatum recovery from the soil: The population density of 1.6 CFU/g soil of *P. aphanidermatum* measured prior to transplanting confirmed that the field soil was naturally infested by the fungus. The population density of *P. aphanidermatum* fluctuated during the cropping season and reached the highest density one month after transplanting (Figure 6.4).

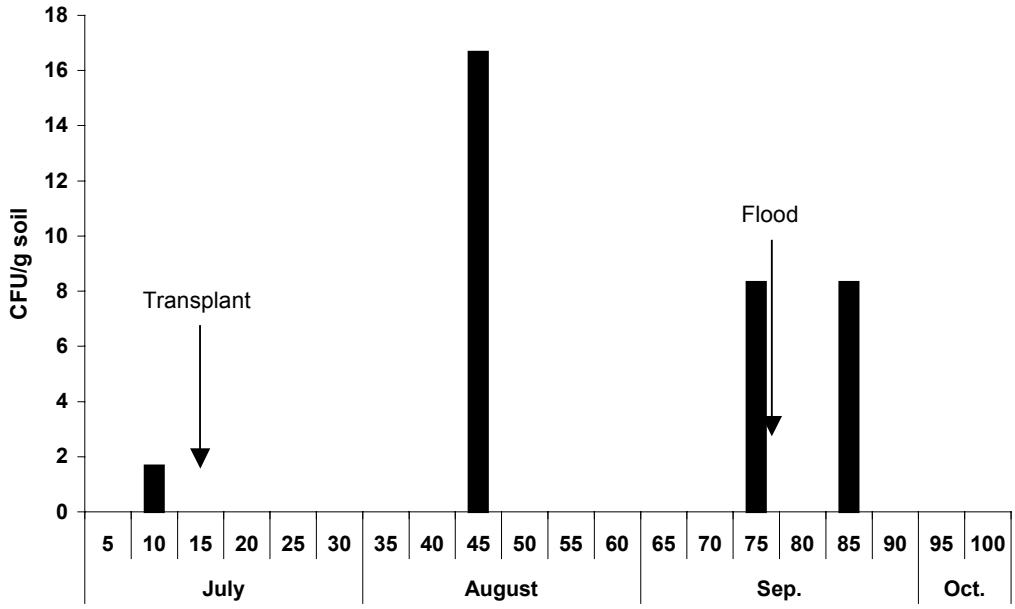

Figure 6.4: Population density (CFU/g soil) of *Pythium aphanidermatum* in soil in a field experiment between July and October 2001 at AVRDC, Shanhua, Taiwan. Soil was sampled before, 30 and 60 days after transplanting and 7 days after flooding.

6.3.2. Greenhouse experiment I: response of non-grafted tomato and tomato grafted onto eggplant rootstock to sudden death tomato due to *Pythium aphanidermatum* after 48 hrs flooding

A high number of non-grafted tomato plants exhibited wilt symptoms in soil infested with *P. aphanidermatum* compared to the non-infested control. The number of plants infected with *P. aphanidermatum* in the treatment with non-grafted tomatoes observed 4 days after flooding was 65% and increased to 80% after 7 days (Figure 6.5). The grafted plants grown in the soil infested with *P. aphanidermatum* were as healthy as the non-grafted and non-inoculated controls, 4 and 7 days after flooding.

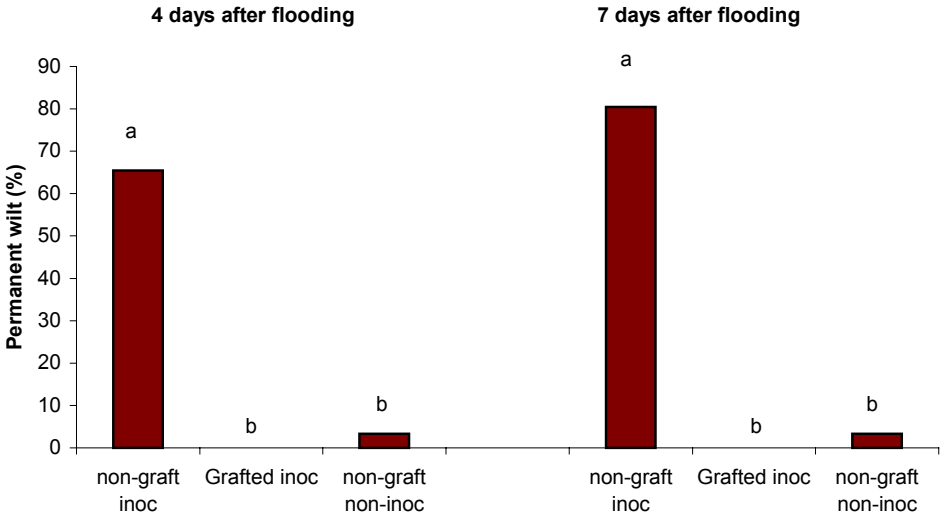

Figure 6.5: Permanent wilt caused by *Pythium aphanidermatum* of non-grafted tomato and tomato grafted onto eggplant rootstocks 4 and 7 days after 48 hrs flooding in a greenhouse experiment between August and November 2001 at AVRDC, Shanhua, Taiwan. Different letters show statistical difference among treatments according to Duncan's multiple range test (P≤0.05), n=9.

Figure 6.6: Plant wilt due to *Pythium aphanidermatum* 7 days after 48 hrs flooding in a greenhouse experiment conducted between August and November 2001 at AVRDC, Shanhua, Taiwan. From left to right: tomato + *Pythium aphanidermatum*; tomato + non-inoculated control; tomato grafted onto eggplant rootstock + *Pythium aphanidermatum*.

6.3.3 Greenhouse experiment II: Response of non-grafted tomato, tomato grafted onto eggplant rootstock, and eggplant to *Pythium aphanidermatum* after 72 hrs of flooding

The response of tomato, tomato grafted onto eggplant rootstock, and eggplant to *P. aphanidermatum* is shown in Figure 7. After flooding for 72 hrs in the absence of *P. aphanidermatum* tomato, eggplant, and tomato grafted onto eggplant rootstock showed no wilt symptoms. However, in soil infested with *P. aphanidermatum*, the non-grafted tomato plants were severely damaged by sudden death. Eggplant and tomato grafted onto eggplant rootstock showed a significantly lower percentage of wilted plants with 3% and 20%, respectively (Figure 6.7).

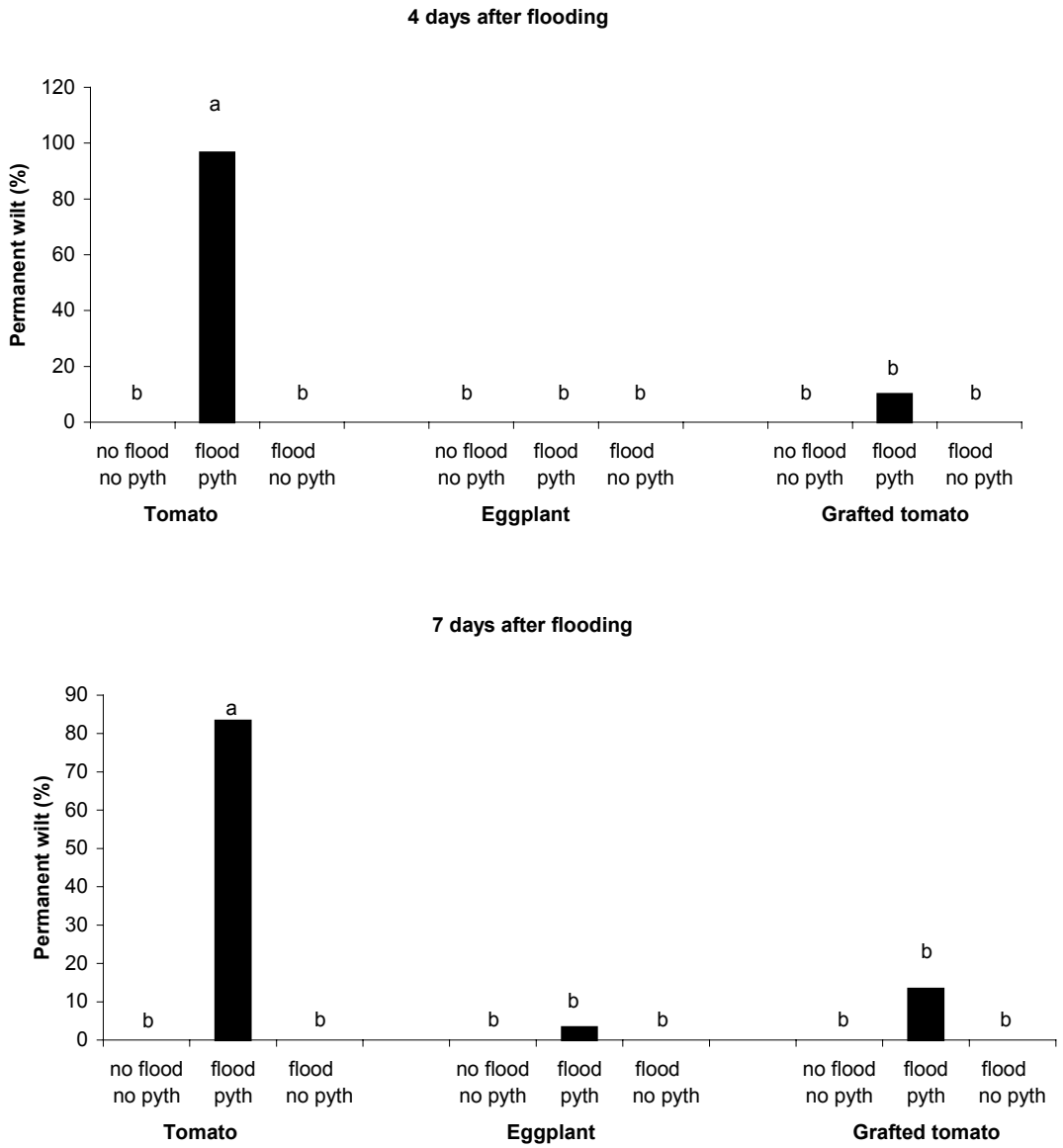

Figure 6.7: Effect of *Pythium aphanidermatum* on permanent wilting of tomato, eggplant, and tomato grafted onto eggplant 4 and 7 days after 72 hrs flooding in a greenhouse experiment between August and October 2002 at AVRDC, Shanhua, Taiwan. Different letters show statistical difference among treatments according Duncan's multiple range test ($P \leq 0.05$), n=18.

Root dry weight: Without flooding, the root dry weight of eggplants was 2- to 3-fold higher than that of tomato. Tomato grafted onto eggplant rootstock had the lowest root dry weight,

about 1/6 of the eggplant. Flooding in the absence of *P. aphanidermatum* led to a non-significant reduction in tomato root growth of 40%. Eggplant root weight was significantly reduced by 44%. The root weight of the tomato on eggplant rootstock was not affected. After inoculation with *P. aphanidermatum* the root systems of all three groups was not significantly different compared to those in the treatment flooding without inoculation. Flooding combined with *P. aphanidermatum*-infestation did not affect the grafted tomatoes (Figure 6.8).

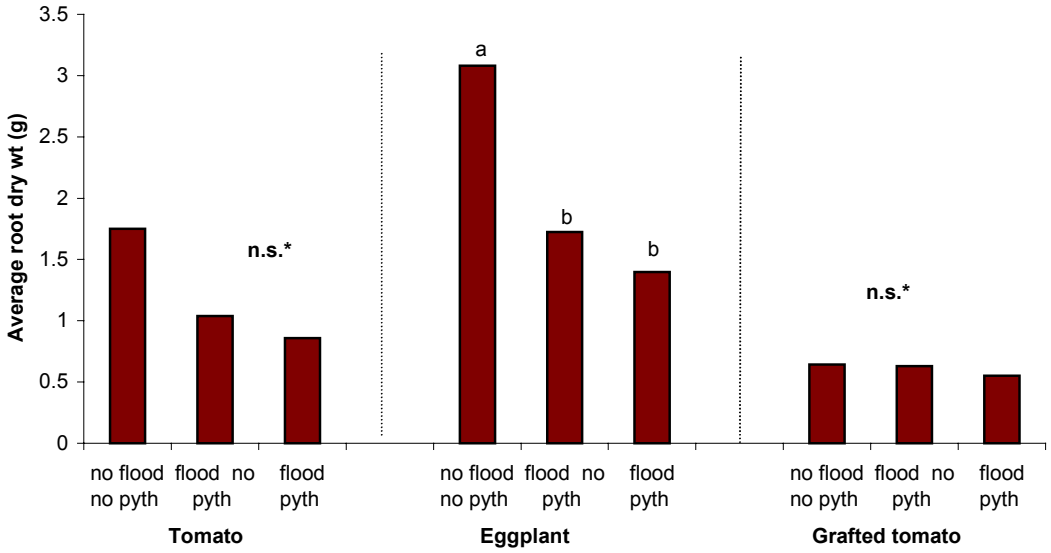

Figure 6.8: Effect of flooding and *Pythium aphanidermatum* on root dry weight of tomato, eggplant, and eggplant rootstock 7 days after 72 hrs flooding in a greenhouse experiment between August and November 2002 at AVRDC, Shanhua, Taiwan. Different letters in a column group show statistical differences among treatments according Duncan's multiple range test (P≤0.05), n.s.* = no significant, n=18.

Pythium aphanidermatum recovery from the root: *P. aphanidermatum* was detected in the root samples of both tomato and eggplant 2 days prior to flooding and 1 day after flooding. The percentage of infected root sections of eggplant and tomato were not different 2 days before or 1 day after 72 hrs of flooding. The number of infected eggplant roots pieces was slightly lower than that of tomato roots under both flooded and non-flooded, conditions (Figure 6.9).

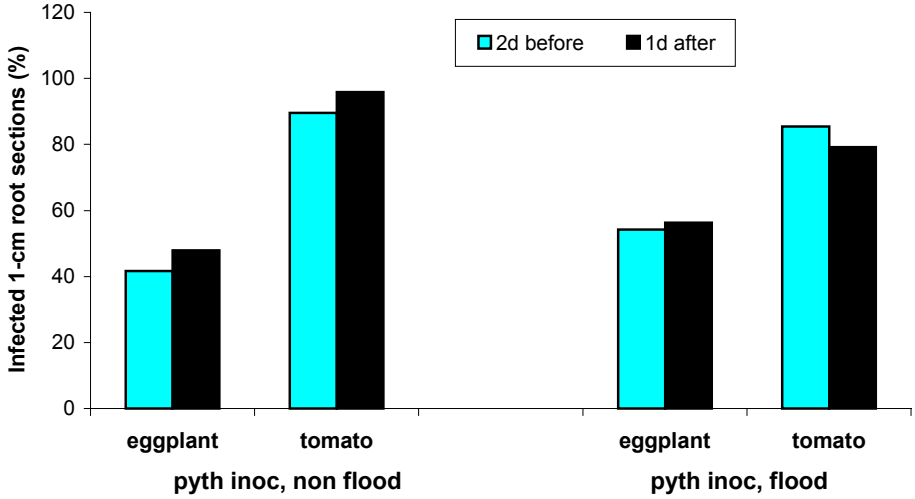

Figure 6.9: *Pythium aphanidermatum* recovery from root tissue 2 days before and 1 day after 72 hrs of flooding in a greenhouse experiment between August and October 2002 at AVRDC, Shanhua, Taiwan.

6.4 Discussion

Field experiment to assess the response of tomato and tomato grafted onto eggplant rootstock to tomato sudden death due to *Pythium aphanidermatum*

The aim of this study was to assess the response of tomato and eggplant rootstocks to *P. aphanidermatum* sudden death following flooding at high soil temperature conditions. The results from the field experiment gave a true picture of the response of tomato and eggplant rootstocks to *P. aphanidermatum*. Tomatoes grafted onto eggplant rootstocks were highly resistant to *P. aphanidermatum* with only 3% of the plants wilting 7 days after flooding. These results are in agreement with several previous studies designed to assess the response of tomato grafted onto eggplant rootstock to soilborne diseases. Black et al. (1999) reported that grafting tomato onto eggplant rootstocks gave protection against bacterial wilt disease caused by *Ralstonia solani* and damage by summer flooding. In the above mentioned study, approximately 62% of the non-grafted tomato plants wilted after 24 hrs of flooding as compared to 4% of the tomato plants grafted onto eggplant rootstocks. Matsuzoe et al. (1993) found that tomatoes grafted onto eggplant rootstocks (*Solanum toxicurium*) were

completely resistant to bacterial wilt due to *Pseudomonas solanacearum* and root-knot nematodes (*Meloidogyne incognita* Chitwood). Peregrine et al. (1982) reported that grafting of tomato on *Solanum torvum* was a satisfactory technique for overcoming bacterial wilt (*Pseudomonas solanacearum*) even under conditions ideal for the disease in the hot wet tropics.

In the present study, soil analysis demonstrated that *P. aphanidermatum* was ubiquitous and that the population density fluctuated during the cropping season. However, the highest population occurred one month after tomato transplanting into the field. This seems to be related to the high temperature (30-32°C) in association with high soil moisture. Similar observations were made by Zhang et al. (1990), who found that the population density of *P. aphanidermatum* in the field soil was highest in the hot summer between July and August when rainfall and temperature achieved the highest peaks.

Greenhouse experiment I and II: response of tomato, eggplant, and tomato grafted onto eggplant rootstock to sudden death of tomato due to *Pythium aphanidermatum* after 48 hrs or 72 hrs of flooding

Tomato grafted onto eggplant was highly resistant to *P. aphanidermatum* compared to non-grafted tomato. These results confirm those from field experiments and re-confirm that grafted tomato is highly resistant to this disease. The results are also in agreement with previous studies demonstrating that *Solanum melongena* provides successful control to a number of soilborne diseases ranging from bacterial wilt to root knot nematodes (Peregrine et al. 1982; Chadha 1988; Chadha et al. 1992; Matsuzoe et al. 1993).

In the greenhouse study, where flooding was maintained over 72 hrs, the response of tomato, eggplant, and tomato grafted onto eggplant rootstock to *P. aphanidermatum* was evaluated. The results are in agreement with the results of the 2001 trials in both the field and greenhouse. Eggplant roots again demonstrated higher resistant to *P. aphanidermatum* following flooding compared to tomato roots. In earlier tests, Black *et al*. (unpublished data) found that 3-week-old eggplant seedlings were highly resistant to *Pythium aphanidermatum* and completely resistant 5 weeks after sowing. Tomato plants on the other hand, were susceptible to the fungus when the plants were less than 6 weeks old. Some

tomato lines were resistant to soilborne diseases such as bacterial wilt, and these lines were also selected as rootstocks for grafting tomatoes. However, grafting onto tomato rootstocks resistant bacterial wilt gave no protection against damage due to soil flooding (AVRDC 1999) or to sudden death caused by *P. aphanidermatum*. Grafting tomato scions onto eggplant rootstocks has been shown to greatly increase yield of tomatoes grown in soils that often become waterlogged or where bacterial wilt is likely to occur (AVRDC 1999). Surprisingly, in the present test, 20% of the plants wilted in the treatment with tomato grafted onto eggplant grown in *P. aphanidermatum* infested soil. This occurred because the flood water level rose above the upper grafted union. This leads to infection of the tomato stem by zoospore of *P. aphanidermatum*.

The decrease in root dry weight of eggplant due to flooding is not clearly understood. It may be that excess water leads to necrotization of the feeder roots. In addition, induced oxygen deficiency may indirectly weaken the host in general (Shoeneweiss 1975). A significant reduction in root weight of eggplants was observed in flooded plants grown in both inoculated and non-inoculated soils. Similar observations were made by McNamara et al. (1989). He found that the roots of the plant become succulent after flooding and, therefore, lose higher amounts of water during the drying process. This results in a lower root dry weight of the plants. *P. aphanidermatum* has been shown by others to cause lower root weight in tomato (Wulff et al. 1998). Severe loss in root weight was, therefore, probably caused by the effects of flooding alone. In this study, neither flooding nor *P. aphanidermatum* affected the eggplant rootstock of grafted tomato. The root weight of eggplant rootstock from the grafted plants, however, was lower when compared to the roots of intact eggplant. This may be due to the fact that the rootstock of eggplant has an incomplete union with the tomato scion, thus leading to poor root system development (Oda et al. 1996).

Severe wilting due to *P. aphanidermatum* after flooding appeared only in the treatment with un-grafted tomato plants, although the percentage of root sections of eggplant penetrated by *P. aphanidermatum* was high. The reason for this is unknown. Eggplant has been reported to be highly resistant to soilborne pathogens in general (Peregrine et al. 1982; Matsuzoe et al. 1993; AVRDC 2001). This resistance may be due to

the fact that the root texture of eggplant is stronger and the pathogens are not able to penetrate the cortex. This results in less damage to the root system. In the present study, some resistance of tomato to *P. aphanidermatum* has been observed. Wilting was not observed before flooding even though 80% of the root sections were colonized by *P. aphanidermatum*. This may be due to the fact that older tomato plants under normal conditions are resistant to *P. aphanidermatum*. Its known that the susceptibility of plants to soilborne diseases decreases with the age of the plant (Yarwood 1958; Populer 1978; Seem 1988). Black et al. (unpublished data) found that 6-week-old tomato seedlings are resistant to *P. aphanidermatum*.

The high level of pathogenicity of *P. aphanidermatum* to tomato plants under hot tropical conditions is clearly related to environmental factors including flooding and high temperatures. In the present study, 80% of the tomato plants wilted only in the period after flooding, even though 80% of the root sections were infected prior to flooding. Eggplant resistance to sudden death caused by *P. aphanidermatum* may also be due to greater tolerance to flooding than tomato. AVRDC (1999) reported that eggplant EG203 is a flood-resistant variety line, while the tomato plant has been reported to be the most flood-sensitive vegetable (Kuo et al. 1982). McNamara et al. (1989) reported that tomato roots are damaged by flooding lasting 120 hrs or longer. However, the flooded plants are able to produce adventitious roots to avoid wilting (Kuo et al. 1980). This does not occur with plants damaged by both flooding and *P. aphanidermatum*. It seems that the effect of flooding predisposes tomato roots to *P. aphanidermatum* attack.

The grafting of tomatoes onto eggplant rootstocks has been shown to be highly successful as a means of protecting tomato from sudden death in the hot wet seasons in the tropics. However, tomatoes grafted onto eggplant rootstocks show a reduced vegetative growth of the tomato scion and a lower fruit yield (Oda et al. 1996). The benefit of grafting in protecting tomato against soilborne diseases such as bacterial wilt is not evident in non-flooded conditions (AVRDC 2001). Furthermore, the incidence of blossom end rot of the tomato fruits increased when the tomato was grafted (Oda et al. 1996). Grafting tomatoes is a strategy for tomato production in the summer season in the lowland tropics. Future research is needed to determine the resistance mechanism in eggplant roots to *P.*

aphanidermatum as well as the reasons for flood tolerance. A reduction in the incidence of blossom end rot of fruit in grafted tomatoes may be achieved in the future by improved cultural practice. Furthermore, research on the factors affecting the development of the eggplant rootstock of tomato grafted onto eggplant should be conducted.

7 SUMMARY

Sudden death following flooding at high soil temperatures causes severe damage to tomato plants grown in the field. *P. aphanidermatum* plays a key role in sudden death disease development on this crop. The disease caused by this fungus was observed in both naturally and artificially infested soils. *P. aphanidermatum* is a high temperature virulence pathogen. The fungus was isolated from the soil, floodwater, and roots even at low temperatures of 18-25°C, but no wilted plants were observed in the experimental plots when flooded for 48 hrs. The tomato plants showed disease symptom only under high soil temperatures of 28-32°C when flooded.

Sudden death of tomatoes is caused by rapid death of the root cortical tissue after flooding probably due to massive zoospore penetration. Flooding is essential for development of sudden death, but its role is still not fully known. There is a need to better understand the role of flooding in enhancing the *P. aphanidermatum* infection process of the host plant.

The importance of tomato age to tomato sudden death was tested in a field experiment conducted between June and September 2002, where the soil was naturally infested with *P. aphanidermatum*. Tomato plants, 51, 72 and 93 days old when the field was flooded, showed susceptible to the disease. The young 51 day-old plants were more likely to survive than older plants. A 4-fold higher survival rate of the 51 day-old plants than that of the 72- or 93 day-old plants was observed after 48 hrs flooding. A 50% lower wilting rate was also recorded in the treatment of 51 day-old plants in comparison to 72- or 93-day-old plants when the field was flooded for 72 hrs. Tomato susceptibility to *P. aphanidermatum* was also clearly related to flood duration. Higher percentages of wilted plants were observed in the experiment in which flooding lasted for 48 and 72 hrs as compared to 24 hrs flooding. No disease incidence was observed in the non-flood treatment in 51-, 72- or 93 day-old plants.

The isolates of *Trichoderma harzianum* (Th-G1-6, Th-R1-6 and Th-3) and *Trichoderma virens* (Tv-Y3-7 and Tv-R4-2) as well as *Streptomyces saraceticus* showed little promise for control of tomato sudden death due to *P. aphanidermatum*. Under field

conditions 4 days after flooding, *T. virens* Tv-Y3-7 reduced disease incidence by 20% on tomato plants compared to the control or other isolates. However, 7 days after flooding, the isolate *T. virens* Tv-Y3-7 and the other biocontrol agents showed no effect on the disease. This isolate also did not show any effect on the disease in greenhouse tests. Similar results were observed in the treatments with the isolates of *T. harzianum*, *T.virens* Tv-R4-2, and *Streptomyces saraceticus*. Although the biocontrol agents did not successfully control the disease, they did promote plant and root development. The root dry weight of tomato plants grown in the soil treated with isolates of *Trichoderma* spp. was significantly higher than that in non-treated control. No effect of *Streptomyces saraceticus* on root dry weight was observed.

All soil amendments (SFMC, FNB-5A and S-H Mixture) tested in this study have been shown to have no effect regarding the control of tomato sudden death due to *P. aphanidermatum*. The plants showed disease symptoms only after the experiments were flooded at high temperatures. The number of infected tomato plants was not significantly reduced when the seedlings were treated with soil amendments in both greenhouse and field experiments. The organic soil amendments SFMC and FNB-5A enhanced with *Trichoderma aureoviride* showed no effect on *P. aphanidermatum*. The fungicide Mefenoxam used as the control treatment in the greenhouse test was also not successful in reducing the incidence of the disease. The poor results achieved in the present study with soil amendments indicate that long-term effects with these substrates cannot be anticipated.

Experiments were conducted to investigate whether tomato grafted onto eggplant rootstocks successfully protected against tomato sudden death due to *P. aphanidermatum*. In the greenhouse tests, the numbers of infected tomato plants grafted onto eggplant rootstocks were significantly reduced in comparison with the non-grafted tomato. The results in repeated greenhouse tests as well as in the field experiments verified effective control. In the field 7 days after flooding, only about 2% of tomato grafted onto eggplant rootstock showed wilting symptoms that were significantly lower than in 70% of the tomato plants wilted in the non-grafted tomato. The effects of either flooding or *P. aphanidermatum* on eggplant rootstock in non-grafted and grafted plants were also investigated. Results show that flooding caused a reduction in the root dry weight of

eggplant on non-grafted plants, but did not show any effect on the rootstock of grafted plants. The eggplant roots were colonized by *P. aphanidermatum* but without extensive damage to the plant even if it was flooded for 48 hrs. The tomato roots were also colonized by *P. aphanidermatum* before flooding, but they were not damaged. The appearance of infected tomato plants only occurred when the experimental plots in the field or greenhouse were flooded for 48 or 72 hrs.

8 FUTURE PROSPECTS

The results of this study show that the occurrence of tomato sudden death is due to a combination of *P. aphanidermatum*, flooding and high temperature (>28°C) conditions. The susceptibility of tomato plants differs with the age of the plant. In the field tests, the resistance of younger plants to *P. aphanidermatum* at high temperatures following flooding conditions was demonstrated. The development of tomato production in areas exposed to flooding in the hot seasons will require targeted disease management systems.

Plants treated with *Trichoderma* isolates and *Streptomyces* did not show acceptable effects with respect to the biological control of *P. aphanidermatum*. This ineffectiveness is probably due to the source of the antagonists, which were originally isolated for damping-off control. Future research on biological control of tomato sudden death should be directed at evaluating the effect of flooding on biocontrol agents. Research to detect potential biocontrol candidates for high soil temperatures following flooding should also be considered. The importance of antagonists that grow endophytically should also be studied.

Evidence was obtained in the study that soil amendments cannot limit damage caused by *P. aphanidermatum* sudden death. There is a need to find other effective soil amendments for combating sudden death. The amount and timing of application should also be examined. In addition, the screening of effective fungicides for controlling this disease should be considered.

The results clearly show that tomato grafted onto eggplant rootstocks were highly resistant to sudden death disease. The mechanism of disease resistant in eggplant is not known. Future research on grafting tomato onto eggplant rootstock for combating *P. aphanidermatum* and other soilborne pathogens should focus on the resistance mechanism of eggplant rootstock to the pathogens. There is a need to understand the effect of flooding on the growth of eggplant roots. Furthermore, a study on the factors affecting the development of eggplant rootstocks in tomato/eggplant grafts should be conducted.

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10 APPENDIX

Culture media

AVRDC isolation medium (Mycology protocol, AVRDC, Shanhua, Tainan Taiwan)

20 g	Potato Dextrose Agar (PDA)
10 g	Agar
100 mg	Ampicillin
100 mg	Nystatin
50 mg	Rifampicin
1000 ml	distilled H_2O

Burr and Stanghellini medium (Burr *et al*., 1973)

17g	Corn Meal Agar (Difco)
100mg	Pimaricin
200mg	Streptomycin sulfate
150mg	Rose Bengal
5mg	Benomyl
1000ml	distilled H_2O

Mircetich medium (Mircetich, 1971)

17 g	Corn Meal Agar (Difco)	1 mg	$ZnCl_2$
23 g	Agar	0.02 mg	$CuSO_4$
20 g	Sucrose	0.02 mg	MoO
5 mg	Pimaricin	0.02 mg	$MnCl_2$
300 mg	Vancomycin	0.02 mg	$FeSO_4.7H_2O$
100 mg	PCNB	10 mg	$MgSO_4.7H_2O$
100 mg	Rose Bengal	10 mg	$CaCl_2$
1000 ml	distilled H_2O	1 g	K_2HPO_4

Potato Dextrose Broth Agar (PDA)

37g	Potato Dextrose Agar (PDA)
1000ml	distilled H_2O

Vegetable broth (V-8)

200ml	V-8 vegetable juice (albi)
3g	$CaCO_3$
15g	Agar
1000ml	distilled H_2O

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