Silicon nutrition and resistance against *Pythium aphanidermatum* of *Lycopersicon esculentum* and *Mormodica charantia*

von der Naturwissenschaftlichen Fakultät der Universität Hannover
zur Erlangung des akademischen Grades eines Doktor des Gartenbaus
(Dr. rer. hort.)

genehmigte Dissertation

von
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geboren am 19. November 1971 in Bergisch Gladbach

2005

Tag der Prüfung: 12. Juni 2005

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Abstract

Pythium aphanidermatum is a mayor threat for vegetable production in the tropics. Silicon (Si) nutrition was reported to stimulate pathogen resistance of many crops and could be an environmental friendly alternative to the current, mainly chemical-based control strategies of *P. aphanidermatum*. However, it is still elusive whether beneficial Si effects on plant health are linked to the degree of Si uptake. Therefore, the ability of Si to increase resistance against *P. aphanidermatum* was studied using two vegetable species, tomato and bitter gourd, which were expected to differ in Si uptake.

An in-depth study on Si uptake showed that tomato discriminates Si from uptake leading to an accumulation of Si in the root water free-space (RWFS). In contrast, bitter gourd actively takes up Si, which was illustrated by a calculated and measured depletion of Si at the root surface and in the RWFS. A fractionated Si analysis of roots revealed that in tomato, root Si is almost completely located in the cell walls, whereas bitter gourd accumulates Si to a higher degree in the root symplast.

Under controlled conditions, inoculation with *P. aphanidermatum* of tomato but not bitter gourd caused damping-off even at moderate levels of inoculation. For tomato and bitter gourd, a sub-lethal infection reduced root length and shoot growth. Amendment of the substrate with Si did not have an effect on tomato or non-inoculated bitter gourd plants. However, growth of *P. aphanidermatum*-inoculated bitter gourd plants was stimulated by Si supply. The effect was linked to a lower degree of infection in the roots, as revealed by a *Pythium* spp specific ELISA.

P. aphanidermatum was also highly pathogenic for tomato under protected cultivation in Thailand. An inoculation of the substrate caused damping-off and the growth of surviving plants was reduced. In agreement with the experiments under controlled conditions, Si did not affect growth and fruit yield, regardless of the *P. aphanidermatum* inoculation. Sampling for *Pythium* spp. in the substrate and in roots after harvest indicated a decreasing virulence of the pathogen during the time course of the experiment.

The use of rhizotrons combined with an ELISA specific for *Pythium* spp. allowed the quantitative determination of pathogen colonization in specific root sections as affected by *P. aphanidermatum* inoculation and Si supply. Inoculation with zoospores of *P. aphanidermatum* caused a strong inhibition of root growth of tomato and bitter gourd, particularly when applied to the 1 cm root apex. Si supply did not alleviate this inhibition of root growth in either species. In tomato, no effect of Si supply was observed on the basipetal spread of the pathogen from the infected root apex. However, in bitter gourd the spread of *P. aphanidermatum* in the roots was inhibited when plants were continuously supplied with Si before and after the inoculation. Application of Si to the entire root system or to individual root zones of bitter gourd only during and after the infection did not affect the spread of the pathogen.

In conclusion, Si supply is not a suitable tool to enhance the resistance of the Si excluder tomato against *P. aphanidermatum*, whereas the resistance of the Si accumulator bitter gourd can be stimulated by Si application. The results of this study indicate that the beneficial effect of Si on plant resistance against *P. aphanidermatum* is linked to symplastic rather than apoplastic effects.

Keywords: Silicon/*Pythium* / tomato / bitter gourd / plant resistance / ELISA / protected cultivation

Table of contents

Abbreviations

det	determination number
dw	dry weight
CFU	colony forming unit
cv.	cultivar
DDW	double distilled water
ELISA	enzyme linked immuno sorbent assay
et al.	et alii
fw	fresh weight
IgG	Immunoglobulin G
M	molar concentration
MDH	malate dehydrogenase
mg	milligram
mM	milimolar
µM	micromolar
MIS	mycelium infested soil
MS	mycelium solution
N	nitrogen
n.d.	not determined
n.s.	not significant
PDA	potato dextrose agar
RWFS	root water free space
SDW	sterile distilled water
SD	standard deviation
Si	silicon
v	volume
w	weight
WFS	water free space

1. General Introduction

The development of sustainable production systems is a major challenge for agricultural research. One important component of sustainability in agriculture is the management of pests and diseases by exploiting internal regulating systems of the agro-ecosystem. In particular vegetable production often strongly depends on the application of pesticides. Strengthening the self-regulatory capacity, therefore, facilitates a reduction in the use of pesticides, thus avoiding environmental risks and preventing hazards for growers and consumers (Jacobsen, 1997; De Waard et al., 1993).

Mineral fertilizers are among the environmental factors influencing both, the tolerance (i.e. the ability to endure a disease) and the resistance (i.e. the ability to avoid the challenge of the activity of a pathogen) of plants against pathogens. Plants well equilibrated supplied with all nutrients have a higher fitness and thus can tolerate a pathogen infection to a higher degree than plants suffering from nutrient deficiency (Sieling, 1990). The resistance of plants against diseases also depends on the nutritional status of the plants and is generally improved when the nutrient supply is increased from deficiency to the optimum range (Graham, 1983). Depending on the mineral nutrient, different mechanism can account for the increase in resistance.

Calcium deficiency is widely known to increase the predisposition of plants to pathogens. Calcium supply to deficient plants intensifies the cross-linkage of cell wall polygalacturonic acids yielding calcium-pectate, which is more resistant to degradation by pathogen polygalcturonase (Conway et al., 1998; Bateman and Lumsden, 1995; Pagel and Heitefuss, 1989). In addition, free calcium ions inhibit the activity of pathogen pectolytic enzymes (Corden, 1965). In plants suffering from potassium deficiency the synthesis of proteins and carbohydrates is impaired which leads to an accumulation of low-molecular-weight organic compounds and to a higher predisposition to pathogens. Consequently, the supply of potassium to deficient plants can improve their pathogen resistance (Ollagnier and Renard, 1976). Both, the constitutive and the maximum inducible activity of the resistance marker-enzymes peroxidase and chitinase were higher in nitrogen-sufficient as compared to nitrogen-deficient *Arabidopsis thaliana* plants (Dietrich et al., 2004). Positive effects of copper on plant resistance were explained by their stimulating effect on lignifications making plants more resistant to fungal penetration (Graham, 1980).

These examples illustrate that plant health in most cases is increased when the nutrient supply is raised from deficiency to the optimum range. However, results of related studies are not always consistent and the plant response to nutrient supply may also depend on the type of pathogen (facultative or obligate; Kiraly, 1976), the degree of plant resistance (Shaner and Finney, 1977), the nutrient form (NO_3^- or NH_4^+; Toussoun, 1960), or even on an indirect effect to the pathogen by changing the soil pH (Trolldenier, 1981).

The effect of an increase in nutrient supply beyond the optimum for plant growth depends on the type of nutrient. Graham (1983) pointed out that micronutrient supply has the capability to exert positive effects on the health of plants even when applied at the supra-optimal level. Examples are the control of *Gaeumannomyces graminis* in wheat by copper (Reis et al., 1982) and manganese (Wilhelm et al., 1990), the suppressive effect of manganese application on potato scab caused by *Streptomyces scabies* (McGregor and Wilson, 1964), and the release of pathogenesis-related proteins in the apoplast of cowpea under manganese toxicity (Fecht-Christoffers et al., 2003). In contrast to micronutrients, supra-optimal supply with macronutrients was often reported to increase the susceptibility of plants to diseases. Especially many studies are reporting an unfavourable effect of high nitrogen supply on plant health (Conner et al., 1992; Shaner and Finney, 1977; Bainbridge, 1974; Krauß, 1970) but also of potassium (Drobny et al., 1983).

Silicon (Si) has a special status among the mineral elements with respect to plant nutrition. It is the most abundant mineral element in most soils and certain plants can contain Si in amounts comparable to those of macronutrients (Epstein, 1999). However, even when taken up in high amounts it is the only element that is not harmful for plants (Takahashi et al., 1990).

Members of the plant families *Equisitaceae* (Chen and Lewin, 1969) and *Chrysophyceae* (Lewin and Reimann, 1969) were shown to have an essential requirement for Si and some reports are speculating about Si as an essential element for certain species of the *Spermatophytae*. Miyake and Takahashi (1978) interpreted malformations of Si-free grown tomato plants as Si deficiency symptoms and postulate a nutritional role for Si in tomato. This conclusion was challenged by Marschner et al. (1990) who traced the malformations back to imbalances in the nutrient solution used causing P-induced Zn deficiency. By using highly purged nutrient solution, Wooly (1957) reduced Si contents in shoots of tomato plants to 0.0006% but did not observe a change in plant growth.

Epstein (1999) summarized that with regard to the stringent definitions of Arnon and Stout (1939) a nutritional role of Si for higher plants is not yet conclusively proven. However, for the production of rice, Si was designated as an "agronomical essential element" (Takahashi et al., 1990).

As mentioned above, Si is a ubiquitous element in the earth crust and most soils contain Si in considerable amounts. In solutions below pH 9 Si is almost entirely present in the monomeric form silicic acid $Si(OH)_4$ up to a saturated concentration of 2 mM (McKeague and Cline, 1963). In soil solutions the concentration of silicic acid is mainly controlled by adsorption to Fe and especially Al oxides/hydoxides in a pH-dependent manner with adsorptions decreasing on either side of a maximum of about 9.5 (Jones and Handreck, 1963). Accordingly, for solutions of a given soil the concentrations of silicic acid were reported to increase to either side of a minimum of pH 9 (McKeague and Cline, 1963). Jones and Handreck (1965) investigated a wide range of soils and reported values for silicic acid ranging from 0.12 to 1.33 mM. Factors that can contribute to low Si concentrations in the soil solution are the low solubility of Si minerals in the parent materials (Jones and Handreck, 1967) or leaching (Gérad et al, 2002). Highly weathered Oxisols and Ultisols and in particular peat substrates, which are used for greenhouse production of vegetables, are frequently associated with low concentrations of silicic acid (Epstein, 2001; Savant et al., 1997).

Plants take up Si from solutions below pH 9 in the form of silicic acid (Jones and Handreck, 1965; Raven, 2003). However, among plant species there are large differences in the magnitude of Si uptake, a fact that may contribute to the different perspectives among scientists regarding to the role of Si in plant nutrition. When grown under similar conditions for 72 h with initially 0.21 mM Si in the nutritional solution, Ma et al. (2001) found 4.5 and 0.2 mg Si per g dry weight in the shoots of rice and tomato, respectively. Differences in Si contents between plant species are related to differences in the Si uptake mechanism at the root plasma-membrane. Three modes of Si uptake can be derived from comparisons between the uptake rates of Si and water. Plant species, which take up Si faster than water have an active mechanism of Si uptake and are classified as Si accumulators. Plants belonging to the group of Si excluders have a rejective mode of Si uptake and consequently take up Si to a lower degree than water. Intermediate plants take

up Si at the same rate as water and thus a passive mode of Si uptake is assumed (Ma et al., 2001).

Raven (2001) pointed out that an active transport of silicic acid across membranes would depend on an exergonic driving reaction for energy generation and the process would require a membrane protein with a binding site for silicic acid. In fact, metabolic inhibitors repress the active uptake of silicic acid (Mitani and Ma, 2005). However, to date genes encoding a transporter for silicic acid were only identified for diatoms (Hildebrand et al., 1998) but not for plants. In rice, Tamai and Ma (2003) deduced the existence of a transporter with a low affinity for Si from kinetic studies and Ma et al. (2004) were able to detect the location of a gene for Si loading into the xylem by using molecular mapping. Within the important group of *Angiospermae* the ability to accumulate Si is limited to a few plant families. In an extensive study covering 147 species grown in the same soil, Takahashi and Miyake (1976) demonstrated that Si accumulators belong to the plant families *Gramineae* and *Cyperaceae*, intermediate plants are found within the orders *Cucurbitales* and *Urticales*, whereas species of all the other plant families were Si excluder. However, the classifications were merely based on Si contents in the plant dry mass whereas detailed studies involving the measurements of Si uptake rates, mass flow and transpiration are missing. Such a detailed measurement could be especially interesting for a more precise characterization of intermediate plants.

In Si research, so far, most studies were carried out with Si accumulators that are characterized by a high Si translocation to the shoots. Accordingly, comparatively many studies investigated Si in the aboveground plant organs, but there is still a great lack of knowledge with regard to Si in roots (Sangster and Hodson, 1992). For rice (Parry and Soni, 1972), sorghum (Lux et al., 2003) and wheat (Bennett, 1982), silica deposition incorporated in the inner tangential walls of the root endodermis was reported. The ability to form such silica aggregates in roots is exclusively present in members of the (Si accumulating) grasses, but there are exceptions since no silica depositions were found in the roots of maize (Benett and Sangster, 1982). The deposition starts with the initiation of secondary wall formation by selective accumulation and concentration of soluble Si in the protoplast and subsequent transport into the apoplast (Sangster and Parry, 1976). Sangster and Hodson (1992) proposed that the process is under metabolic control, e.g. by ionic forces of membrane surfaces, to which silica particles are attracted (Kaufman et al.,

1981). In root organs other than the endodermis, Si could be only detected in low amounts that were presumed to be silicic acid (Hodson and Sangster, 1989a, b). In general, the major proportion of Si taken up into the root symplasm is translocated to the shoots (Jarvis, 1987; Iwasaki, 2002) following the transpiration stream (Aston and Jones, 1976). In the xylem Si exists exclusively in the forms of mono- and disilicic acid. No organosilicate complexes were detected in the xylem and no studies reported the occurrence of Si in the phloem, indicating that Si cannot be retranslocated (Casey et al., 2003). Si deposition in leaves occurs primarily passive at sites of high water losses by transpiration due to polymerization of silicic acid-forming silica ($SiO_2 \cdot nH_2O$, also termed: opal, opaline silica, silica gel) (Jones and Handreck, 1967; Kaufman et al., 1981). Preferential sites of silica deposition are cell walls, the cell lumens, or intercellular spaces of epidermal tissue and guard and subsidiary cells of stomata (Sangster and Hodson, 1986). The degree of deposition usually increases with age and apical position of leaves (Jones and Handreck, 1967), whereas the amount of Si supply does not affect the distribution of Si between organs (Jones and Handreck, 1969). In addition to passive deposition, higher plants can also actively produce localized silicified structures (termed phytoliths by Piperno, 1988) like silica cells and silicifid trichomes by attracting silicic acid to modified organic cell wall matrixes and membrane surfaces (Kaufman et al., 1981; Sangster, 1970). A silica cuticle double layer, which is of interest with regard to disease resistance in rice, can be formed by excretion of silicic acid through ectodesmata of epidermal cells (Soni and Parry, 1973). Once polymerized, silicic acid is not longer available as a source of Si for any other part of the plant. It was estimated that only about 1% of the total plant Si is present in the form of silicic acid (Jones and Handreck, 1965).

Even though from a scientific point of view Si was not proven to be essential for higher plants it is well established that Si can promote plant growth especially under conditions of biotic and abiotic stress. Due to its manifold positive effects on growth Si was designated as a „beneficial element“ for plants (Asher, 1991; Marschner, 1995). Positive Si effects cover a wide range of abiotic stress factors, such as increased tissue tolerance of manganese toxicity (Horigushi and Morita, 1987; Horst and Marschner, 1978), aluminium resistance (Wang et al., 2004), resistance against salt stress (Liang et al., 2003; Zhu et al., 2004), P excess (Ma and Takahashi, 1990), drought (Lux et al., 2002; Hattori et al., 2001), and temperature extremes (Agarie et al., 1998; Larcher et al., 1991).

In addition, Si nutrition prevented lodging of rice (Idris et al., 1975) and wheat (Gartner and Paris-Pireyre, 1984) by strengthening culm walls and vascular bundles, and increased photosynthesis by promotion of an upright stature, especially under conditions of high nitrogen supply (Yoshida et al., 1969).

Si nutrition also bears potential as an environmental friendly method for the control of numerous diseases. Many researchers reported successful disease control for various combinations of plant pathosystems under practical conditions on a field scale. A reduction in disease incidence in rice caused by *Magnaporthe grisea* by Si application was first reported from Japanese researchers (Ishiguro, 2001) and subsequently confirmed by researchers in other countries (e.g. Datnoff et al., 1991; Kim and Lee, 1982; Seebold et al., 2000). Si nutrition also proved to be an effective means for the control of *Leptosphaeria sacchari* in sugarcane (Raid et al., 1992) and *Erysiphe graminis* in wheat (Bélanger et al., 2003; Leusch, 1986). Among the dicotyledons most results related to Si and disease resistance were reported from cucumber where Si amendment to the nutrition solution increased resistance against *Sphaerotheca fuliginea* (Adatia and Besford, 1986; Bélanger et al., 1995) and root rot caused by *Pythium ultimum* (Chérif and Bélanger, 1992) and *Pythium aphanidermatum* (Chérif et al., 1994). In field experiments with cucumber, Miyake and Takahashi (1983) found a reduction of *Fusarium* wilt by Si treatment.

Despite the wide range of examples for Si-enhanced disease resistance the mechanisms how Si interacts with plant health are still subject of scientific debate. Two sometimes overlapping mechanisms are generally discussed. The classical approach follows the initial ideas of Wagner (1940) and attributes the Si effect to a physical barrier impeding penetration of fungal germ tubes and hyphae. The reinforcement is brought about by Si depositions in cell walls and papillae, in particular in the epidermis. It was shown that Si application enhanced silification of rice epidermal cells, which leads to a higher mechanical resistance of Si-treated cells (Ishiguro, 2001). The Si deposition in cell walls can be constitutive (Volk et al., 1958) or induced as a reaction to fungal penetration as reported for cucumber (Samuels et al., 1991) and barley (Wiese et al., 2005; Sargent and Gay, 1977) and also for the non-host interaction between French bean and *Uromyces phaseoli* (Heath, 1979). Negative correlations between Si concentration in plants and leaf colonization as well as fitness of the pathogens support the mechanical barrier hypothesis (Leusch and Buchenauer, 1986; Menzies et al., 1991a). A mechanical barrier impeding

root pathogen infestation was also assumed as a function of the Si depositions in the root endodermis of grasses (Maiti et al., 1984).

A more complex approach of the physical barrier theory is based on the fact that Si can form insoluble complexes with phenolic compounds. These compounds are thought to be precursors for lignin (Weiss and Herzog, 1978), a material known to constitute a mechanical barrier for fungal growth (Vance et al., 1980; Nicholson, 1992). In fact, co-deposition of lignin and Si was frequently reported as a reaction to fungal attack. However, reports differ whether Si deposition is stimulated by lignin (Perumall and Heath, 1991), the lignin deposition is stimulated by Si (Menzies, 1991b) or both processes occur independently (Heath, 1979). Koga et al. (2004) proposed a model for Si-phenolic complexes in hypersensitive dead cells of barley leaves challenged by *Erysiphe graminis*. According to these authors, Si accumulation mediated by high transpiration rates of senescent cells coincides with phenolics released by lysing cells.

The second mode of action that is proposed with regard to the role of Si in disease resistance emphasizes a metabolic role of Si. For wheat (Bélanger et al., 2003), rice (Rodriguez et al., 2003), and cucumber (Menzies et al., 1991b) affected by leaf pathogens, Si nutrition stimulated the accumulation of phenolic compounds in infected cells, resulting in decreased fitness and a lower colonization of the plant tissue by the pathogens. Interestingly, in no case the incidence of primary infection was decreased by the Si treatment ruling out the possibility of a mechanical barrier during the infection step. Fawe et al. (1998) found out that the pattern of phenolic compounds in cucumber plants inoculated with *Sphaerotheca fuligena* is changed when plants are supplied with Si. They identified a phytoalexin from the group of flavonols that displayed antifungal activity against *Cladosporium cucumerinum* in *in-vitro* experiments. Another example for an active role of Si in disease resistance is the pathosystem cucumber/*Pythium* spp. The initiation time of the defense reaction related to the enzymes chitinase, peroxidase and polyphenoloxidase after inoculation with *Pythium ultimum* or *Pythium aphanidermatum* were considerably faster in Si supplied plants (Chérif et al., 1994a). In addition, in Si-treated plants phenolic-like material accumulated rapidly in the proximity of invading hyphae and in stellar cells, but no Si accumulations were found in the roots of infected plants (Chérif et al., 1992a and b). The phenolic compounds were highly toxic to the pathogens *in vitro* (Chérif et al., 1994a) and *in situ* as indicated by destroyed fungal

hyphae (Chérif et al., 1992a). As a consequence, the pathogen often failed to colonize the stele tissue and the mortality of inoculated plants was significantly decreased (Chérif et al., 1994b).

It is noteworthy that the two theories are based on two different forms of Si. In the mechanical barrier theory rather inert depositions of polymerized solid silica hemper fungal colonisation whereas in the metabolic theory the soluble form silicic acid is decisive for the antipathogenic effect.

It is not known whether all plants can benefit from elevated levels of Si supply and little is known about the relation between expression of disease resistance and Si uptake. Although there are quite a few examples showing that the disease resistance of plants was enhanced by Si supply, at least for foliar diseases that, the mechanisms involved remain mostly elusive. With respect to soil-borne diseases like *Pythium* spp., the information on Si effects on disease resistance is even more fragmentary than the knowledge about Si in roots.

The genus *Pythium* belongs to the class of oomycetes, which are pseudofungi of the kingdom Chromista. Important characteristics of this group are hyphae composed of cellulose and glucans and a (initial) lack of septa (Van der Plaats- Niterink, 1981).

Of the worldwide 87 *Pythium* species recognized by Waterhouse (1968), *Pythium aphanidermatum* (Edson) belongs to the species most frequently associated with root diseases. Due to the high cardinal temperatures (minimum 10°C, optimum 30-40 °C, maximum > 40°C), the species is a typical plant pathogen of warm regions (Van der Plaats- Niterink, 1981). Therefore, the occurrence in temperate climates is confined to greenhouses (Raffin and Tirilly, 1995). *P. aphanidermatum* can grow saprophytically in the soil, but *Pythium* species in general are sensitive to competition from other soil microorganism (Martin and Loper, 1999). The consequence is a limited survival of the mycelium in the soil (Agnihotri and Vaartaja, 1967). To overcome periods of extensive competition by other microorganism or unfavourable environmental conditions *P. aphanidermatum* forms thick-walled oospores for long-term survival. The oospores remain dormant in the soil until germination is triggered by external stimuli like moisture (Hoppe, 1966) or root exudates (Kraft and Erwin, 1968). Short-term survival is mediated by asexually formed sporangia that can germinate either directly or indirectly by the formation of zoospores. The zoospores, which are initially wall-less and mobile in

water, are responsible for dispersion in moist environments. Zoospores are attracted to the root hair zone of hosts by chemotaxis where they encyst and germinate (Jones et al., 1991).

Each of the described stages in the life cycle of *P. aphanidermatum* is capable of infecting susceptible, primarily young and juvenile root tissue, by direct penetration (Agrios, 1997). Factors influencing the infection are mainly temperature (Gold and Stanghellini, 1985), moisture (Stanghellini and Bur, 1973), and inoculum density (Mitchell, 1978). Subsequent to infection, *P. aphanidermatum* spreads intra- and intercellular and the pathogen releases proteolytic enzymes that macerate pectin in the host cell walls (Van der Plaats-Niterink, 1981). When the invasion reaches the stem base, damping-off may occur. In contrast to young tissue, well- thickened and lignified cells cannot be broken down by *P. aphanidermatum*, which restricts propagation of the pathogen in older plant tissue (Agrios, 1997; Hendrix and Campbell, 1973). However, root tips can be attacked at any stage of plant growth, causing retardation of plant growth and yield reduction (Martin and Loper, 1999).

Like soil-borne pathogens in general, *P. aphanidermatum* is difficult to control (Runia, 1995). Chemical treatment is possible and genotypic differences in plant tolerance against *Pythium* spp. were observed (Higginbothan et al., 2004). Widely used management practices are soil sterilization by chemicals and fumigation (MacNab and Sherf, 1986), but these approaches have disadvantages in terms of cost or environmental concerns (Jacobsen, 1997). In addition, they are directed against the pathogen in the soil in order to avoid detrimental damping-off but do not ensure protection when *P. aphanidermatum* is introduced later into the system.

Other more environmental friendly means for the control of *P. aphanidermatum* are treatments with antagonistic fungi (Punja and Yip, 2003) and bacteria (Chen et al., 1998) or breeding for resistance. However, biological protection against *P. aphanidermatum* is not always effective and so far no commercial varieties with resistance to *Pythium* spp. are available (Agrios, 1997; Martin and Loper, 1999).

Therefore, enhancing the plant natural resistance by optimising the mineral fertilization could be a useful contribution to a sustainable control of *P. aphanidermatum*.

Si supply might be a tool to archive this goal since positive effects of Si on plant health have been widely demonstrated. However, when reviewing the available literature

dealing with Si and plant health it is conspicuous that studies are almost exclusively focused on plants that belong to the Si accumulators. In contrast, information on Si-discriminating plants appears to be unavailable.

The plant species tomato (*Lycopersicon esculentum*, *Solanaceae*) and bitter gourd (*Mormodica charantia*, *Cucurbitaceae*) represent vegetables that are characterized by low and rather high Si uptake, respectively. However, precise information about the status of Si in the roots is lacking. It is also unclear how and to which degree Si can stimulate defense mechanism in both species. A comparison of tomato and bitter gourd with respect to the cellular distribution of Si in the roots on the one hand, and the effectiveness of Si supply in enhancing the resistance against *P. aphanidermatum* on the other hand would, therefore, contribute to a better understanding of the role of Si in defense against pathogens. Knowledge about the effectiveness of Si application could be a key to the exploration of the plants natural resistance and, therefore, of major relevance for the development of sustainable vegetable-crop production-systems.

The objective of the present study, which focuses on tomato and bitter gourd, is to explore the potential of Si in sustainable vegetable production. In addition, it aims at a better understanding of the mechanism involved in Si-induced resistance against *P. aphanidermatum*. The experimental part of the thesis is divided into four chapters each organized as independent publication:

- In-depth understanding of the dynamics of Si uptake and compartmentation in the root systems of tomato and bitter gourd (Chapter 1).
- Effect of Si on the response of tomato and bitter gourd to an infection with *P. aphanidermatum* under controlled conditions (Chapter 2).
- Evaluation of Si as a means to enhance the disease resistance of tomato under conditions of protected cultivation in Thailand (Chapter 3).
- Quantitative assessment of the infection success and the spread of *P. aphanidermatum* along individual roots of tomato and bitter gourd as affected by Si application (Chapter 4).

2. Silicon nutrition of tomato and bitter gourd with special emphasis on silicon distribution in root fractions

This chapter was published as:

Heine, G., Tikum, G., Horst, W. J. (2005): Silicon nutrition of tomato and bitter gourd with special emphasis on silicon distribution in root fractions. J. Plant Nutr. Soil Sci. 167, 600-606.

3. Silicon enhances the resistance of bitter gourd but not of tomato against *Pythium aphanidermatum* under controlled conditions.

3.1. Abstract

Pythium species are a worldwide threat for vegetable production causing damping-off as well as poor growth and low yields of surviving plants. Soil amendments with the beneficial element silicon (Si) have been shown to improve the resistance of many crops against pathogens. Interestingly, most studies regarding Si nutrition and plant health were conducted with Si accumulator plants that are characterized by a high Si uptake and a high translocation rate to the shoots. In contrast, Si excluder plants discriminate Si at the plasma membrane leading to an accumulation of Si in the root apoplast.

In experiments under controlled conditions the effectiveness of Si supply to increase resistance against *Pythium aphanidermatum* was tested using the plant species tomato and bitter gourd, which belong to the group of Si excluder and Si accumulator plants, respectively. *P. aphanidermatum* caused rapid death of tomato plants already at low levels of inoculation and the incidence of damping-off gradually increased with inoculation level. Si supply did not affect the incidence of damping-off on tomato. For bitter gourd a suitable inoculation method and high inoculation levels were required for a successful infection, but no damping-off was observed.

As a result of a non-lethal infection, growth of both species was strongly decreased by *P. aphanidermatum*. The presence of *Pythium* spp. in the roots was confirmed by a specific ELISA test. Si nutrition had no positive effect on growth parameters or infection levels of inoculated tomato plants. In contrast, shoot and root dry weight of inoculated bitter gourd plants was strongly positively affected by Si application. In agreement with the improved growth of Si-treated bitter gourd plants the ELISA measurement revealed lower root colonization with *Pythium* spp. It is concluded that the capability of Si to induced resistance is linked to a symplastic rather than an apoplastic effect of Si.

3.2. Introduction

Tomato (*Lycopersicon esculentum*) and bitter gourd (*Mormodica charantia*) are important vegetables in South East Asia, but a high input of pesticides is employed for the control of various diseases. A major threat for production is the infection by soil borne pathogens such as *Pythium aphanidermatum* that can originate from contaminated irrigation water (Gold and Stanghellini, 1985) or peat-based growth media (Favrin et al., 1988). An infection with *P. aphanidermatum* destroys mainly the root tips, thus decreasing the uptake of nutrients causing poor plant growth and reduction in fruit yield (Hendrix and Campell, 1973; Grosch and Schwarz, 2003). In case of a severe infection and under favorable environmental conditions, *P. aphanidermatum* can spread into the hypocotyl and cause stem rot and post-emergence damping-off (Domsch et al., 1980; Favrin et al., 1988).

Pesticide application is often related to environmental concerns and soil-borne pathogens are generally difficult to control by chemicals. An indirect approach for disease control could be the optimization of the mineral nutritional status of the plants in order to strengthen the disease resistance of the crops (Marschner, 1995; Sieling, 1990; Graham, 1983).

Numerous reports on beneficial effects of silicon (Si) on plant health are available for several plant pathogen combinations. Many studies report the effect of Si application to the nutrient solution against powdery mildew for several plants such as vine (Bowen et al., 1992), barley (Koga et al., 1988), wheat (Leusch and Buchenauer, 1988), and for cucumber (Adatia and Besford, 1986). Foliar applications of Si to grape (Bowen et al., 1992), cucumber, muskmelon, and zucchini squash (Menzies et al., 1992) caused a leaf coating with Si leading to a decrease in colonization with powdery mildew. In upland rice, the application of Si to the soil in the form of wollastonite ($CaSiO_3$) reduced the level of leaf blast, caused by the fungus *Magnaporthe grisea*, in sensitive and moderately resistant rice varieties to the level of resistant varieties (Seebold et al., 2000). The disease severity was thereby negatively correlated with the Si content in the leaf tissue. Due to the fact that Si depositions in rice leaves occurred predominantly in epidermal cell walls, Kim et al. (2002) emphasised that these cell wall fortifications are related to resistance against *M. grisea*. Savant et al. (1999) postulated a mechanical mode of Si action for sugarcane. They explained results of

Raid et al. (1992), in which the severity of ringspot was reduced by 67% with the addition of silicate slag, by employing a theory of Yoshida et al. (1969) for rice that silicic acid is polymerizing between cuticle and epidermal cells. The resulting Si accumulation could protect plants from certain diseases.

On the other hand, Rodrigues at al. (2003) also working with *M. grisea* did not found a difference in the incidence of primary infection between rice plants supplied or not supplied with Si. However, Si nutrition restricted the further development of the disease by activating phenolic substances that enclosed the fungal hyphae in the epidermal layer of Si-supplied plants. Fungal hyphae were subsequently reduced to empty shells indicating a toxic effect of the phenols. In cucumber, up to 72 h after infection with *Sphaerotheca fuliginea*, a decreased parasitic fitness of the fungus was correlated with a Si accumulation at the sites of penetration (Menzies et al., 1991a; Samuels et al., 1994). The presence of Si was shown to initiate the accumulation of phenolic-like compounds in infected cells (Menzies et al., 1991b). Moreover, Fawe et al. (1998) reported that cucumber plants supplied with Si but not without Si were able to counteract an infection of *S. fuligenea* by the formation of phytoalexins. These findings lead to the conclusion that Si actively stimulates plant disease-resistance mechanisms rather than impairing fungal penetration as a physical barrier. An active role of Si in disease resistance of cucumber was also proposed for root rot caused by *Pythium ultimum*. Si amendment in the nutrient solution of hydroponically grown cucumber triggered a rapid activation of peroxidase and chitinase after infection with *P. ultimum* (Cherif et al., 1994). Root cells of Si supplied plants were filled with phenolic-like materials that displayed strong antifungal properties and prevented the vascular tissue from fungal colonization (Cherif et al., 1992b). Interestingly, in no instance, Si was detected at the sites of fungal penetration by the means of SEM or X-ray analysis.According to Ma et al. (2001) plants can be classified as Si accumulators (Si content in the dry weight ≥0.5%) or as Si excluder (Si content in the dry weight <0.5%). With a few exceptions, most studies on the beneficial effect of Si nutrition on plant health were carried out with leaf diseases. In leaves Si accumulators clearly have higher Si concentrations than excluders. Furthermore, almost exclusively Si accumulating plants were used for the experiments. Since reports describing positive Si effects with respect to fungal pathogens in Si excluders are missing, the ability of Si to

mediate a defense response after pathogen infection could be linked to the distribution of Si in the root.

Tomato and bitter gourd greatly differ in Si uptake. Studies on Si uptake revealed that tomato belongs to the group of Si excluders whereas bitter gourd is a Si accumulator (Heine et al., 2005a). By using a sequential extraction method for Si it was clearly shown that in the roots of tomato Si primarily accumulates in the cell walls. In contrast, a high proportion of the total root Si was found in the symplast of bitter gourd roots. Irrespective of the Si nutritional state, the Si concentrations in the roots were higher in tomato than in bitter gourd (Heine et al., 2005a).

In the present study, the effect of Si on the plant response upon infection with the root pathogen *P. aphanidermatum* was therefore compared between the Si excluder tomato and the Si accumulator bitter gourd.

3.3. Material and methods

3.3.1. Plant cultivation

Bitter gourd (local variety from Lion seeds, Thailand) and tomato (KingKong II, Known-YouSeed Co., Ltd, Taiwan) were germinated in peat that was limed to a pH value of six. Seedlings were supplied with or without Si in the form of silicic acid. Six days old seedlings of bitter gourd and two weeks old seedlings of tomato were transferred to pots containing limed peat.

The experiment was set up as a 2 x 2 factorial combination of treatments (Si, *P. aphanidermatum*) and experimental units were distributed in a complete randomized design with 5 replicates. Plants were kept in a growth chamber under the following conditions: photoperiod 16/8; 32/28°C day/night; 75 % relative humidity, 200 µmol m^{-2} s^{-1} light intensity. All pots were daily fertigated with a nutrient solution of the following composition (µM): $Ca(NO_3)_2$ 4000; K_2SO_4 1875; $MgSO_4$ 1625; KH_2PO_4 500; H_3BO_3 40; $CuSO_4$ 1; $ZnSO_4$ 1; $MnSO_4$ 10; Na_2MOO_4 0.5; NaCl 10; and NH_4NO_3 1000.

During the experiment some of the inoculated plants died, which was attributed to *Pythium* spp. by symptoms on the plant as well as by using a plating technique on selective media as described by Martin (1992).

3.3.2. Si treatment

Peat was supplied with 0.6% Aerosil (Degussa AG, Frankfurt, Germany). In addition, plants received a daily irrigation with silicic acid (1 mM) whereas the same volume of deionized water was applied to the -Si controls.

3.3.3. P. aphanidermatum *inoculation*

The isolate used for the experiments originated from vegetable production sites in Thailand and was confirmed as *P. aphanidermatum* by the Centraalbureau voor Schimmelcultures, Netherlands (det 273-2002).

For each plant species, two different inoculation methods were employed. To obtain a comparable growth inhibition, for both methods, the level of inoculation with *P. aphanidermatum* had to be higher for bitter gourd than for tomato.

For the first inoculation method the growing substrate was mixed with mycelium-infested soil (MIS) that was produced according to a modified method of Chaengchaiyasakulthai and Chamswarng (1986). A mixture of sand, soil, and maize flour (3/1/1 w/w) was inoculated with mycelia plugs from four days old cultures of *P. aphanidermatum* grown on PDA and incubated for two weeks at 35°C. A 5% (w/w) mixture of the stock culture was then made with peat and 1.5% (w/w) maize flower was added. The mixture was then incubated overnight at 25°C prior to inoculation.

For the second inoculation method, seven days old cultures of *P. aphanidermatum* grown on PDA were homogenized in 100 ml SDW. Subsequent to planting 5 ml and 50 ml mycelium-solution (MS) was dispensed to the pots of tomato and bitter gourd, respectively.

For all experiments, control plants received a similar amount of sterilized inoculation material. The level of *P. aphanidermatum* inoculum for both methods was quantified subsequent to inoculation by diluting the inoculation material in 0.1 % (w/w) agar solution and spreading 0.5 ml of the well-mixed solution on selective media (Martin, 1992). Colony forming units (CFU) were counted after 24 hours.

3.3.4. Effect of Si on mycelia growth

P. aphanidermatum was transferred to Petri dishes containing 0.1 % agar solution amended or not amended with 2 mM of silicic acid. The pH of the media was adjusted to pH 6.6 prior to autoclaving. Growth of colonies was determined after 24 h and 48 h by averaging the spread of hyphae in four directions.

3.3.5. Plant growth parameters

Tomato and bitter gourd plants were harvested 10 days after transplanting. Shoots were removed and roots were carefully washed out of the substrate. The total root system of tomato and sub-samples of bitter gourd roots were then cut into 1-2 cm segments and root length was measured with a root scanner (Win Rhizo 2002, Regent Instruments, Canada). Total root length of bitter gourd plants was calculated on the basis of the relation between weight of the total root and weight of the sub-sample. Plant tissue was subsequently dried at 65°C, and dry weight of roots and shoots was determined.

3.3.6. Si determination

The determination of the total Si content in the plant tissue was performed using the colorimetric method of Novozamsky et al. (1984) modified according to Iwasaki et al. (2001).

For the determination of the total Si content a 1:2 mixture of hydrochloric acid (1 M) and hydrofluoric acid (2.3 M) was added to the dried and grinded tissue and incubated on a shaker. After 24 h the suspension was centrifuged at 10,000xg and 20 µl of the supernatant was added to 250 µl of boric acid (3.2%). Following another incubation period of 24 h, 250 µl of a 1:1 mixture of sulfuric acid (0.08 M) and ammonium molybdate tetrahydrate solution (20 g/l) was added. Thirty minutes later, 250 µl of each, tartaric acid (33 g/l) and ascorbic acid (4 g/l) were added and the absorption was read spectrometrically at 811 nm.

3.3.7. Re-isolation of Pythium *spp.*

Small pieces were cut from the upper root part prior to surface sterilization with sodium hypochloride (0.5 %) and ethanol (70 %). They were then placed on a medium selective for *Pythium* spp. (Martin, 1992) and incubated for 2 days at 25°C. Growth of a colony was noted as successful re-isolation.

Since the method is specific only on the level of the genus *Pythium*, a successful re-isolation is termed as *Pythium* spp. isolation.

3.3.8. Quantification of infection level in roots

Prior to the measurement of root length, ten small root sections with a combined fresh weight of 25 mg were cut from randomly selected parts of the root system of each plant and pooled as one sample. This sample was then homogenized in extraction buffer using a ball mill (MM200, Retsch, Germany). The degree of infection with *Pythium* spp. was quantified in the homogenate by using a double sandwich ELISA system (Löwe, Sauerlach, Germany) with Anti-Pythium IgG as primary antibody and Anti-Pythium IgG coupled with acid phosphatase as secondary antibody.

Since the test is specific only on the level of the genus *Pythium*, regarding results are termed as *Pythium* spp. infection.

3.3.9. Data analysis

Statistical analysis was only based on surviving plants. The GLM procedure of SAS version 8.1 was used for analysis of variance (SAS, 2001) by employing a nonparametric method (Brunner and Puri, 2001).

3.4. Results

Irrespective of the employed method, the inoculation with *P. aphanidermatum* did not affect the Si concentration in the roots and shoots of both, tomato and bitter gourd. Thus, Si concentrations in the plant tissue were combined for both *P. aphanidermatum* infected and control plants and for both methods of inoculation (Tab. 1).

Substantial amounts of Si were found even in the treatments not amended with Si. However, additional Si nutrition increased the Si concentrations in roots and shoots

of both species. In tomato, a higher Si concentration was found in the roots than in the shoots whereas in bitter gourd, shoot Si concentrations exceeded those in the root. Si concentrations in the roots were higher in tomato, but Si concentrations in the shoots were higher in bitter gourd.

Table 1: Si concentration in roots and shoots of tomato and bitter gourd as affected by Si supply. Means between plant species or Si treatments followed by the same small or major letter, respectively, are not significantly different at $p < 0.05$ (Tukey test). $n = 5$.

	Si concentration [mg (g dw)$^{-1}$]			
	Root		Shoot	
Species	Si-	Si+	Si-	Si+
Tomato	0.74 ± 0.12 bB	1.54 ± 0.09 bA	0.07 ± 0.05 aB	0.53 ± 0.21 bA
Bitter gourd	0.44 ± 0.24 aB	1.19 ± 0.32 aA	2.69 ± 0.10 bB	4.15 ± 0.14 aA

Tomato plants inoculated with MIS were affected by damping-off already at the lowest level of inoculation (Fig. 1). The incidence of dead plants increased when the inoculation levels were higher. From all dead tomato plants *Pythium* spp. was re-isolated from diseased tissue. No systematic difference in the occurrence of damping-off was observed between the Si treatments.

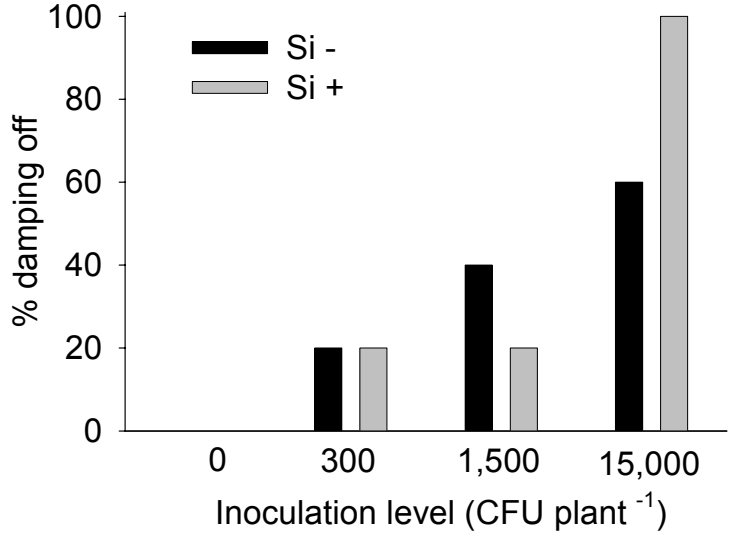

Figure 1: Effect of the level of P. aphanidermatum *inoculation and Si application on percent mortality through damping-off 18 days after transplanting 12 days old tomato seedlings in mycelium-infested soil (MIS). $n = 5$.*

A quantitative evaluation of *P. aphanidermatum* infection on plant growth and disease development requires sub-lethal infection. When using the MS inoculation method for tomato the incidence of damping-off was below 10 %. However, growth of the surviving plants, as illustrated by root length and shoot dry weight, was significantly reduced as a result of *P. aphanidermatum* inoculation (Fig. 2). The presence of *Pythium* spp. in the roots was confirmed by the ELISA readings, which were within the range of the background value for non-inoculated plants but significantly higher in roots of plants inoculated with *P. aphanidermatum*.

Si application to tomato did not affect root length or shoot dry weight of tomato regardless of *P. aphanidermatum* inoculation. In addition, no differences in ELISA readings were found between Si treatments.

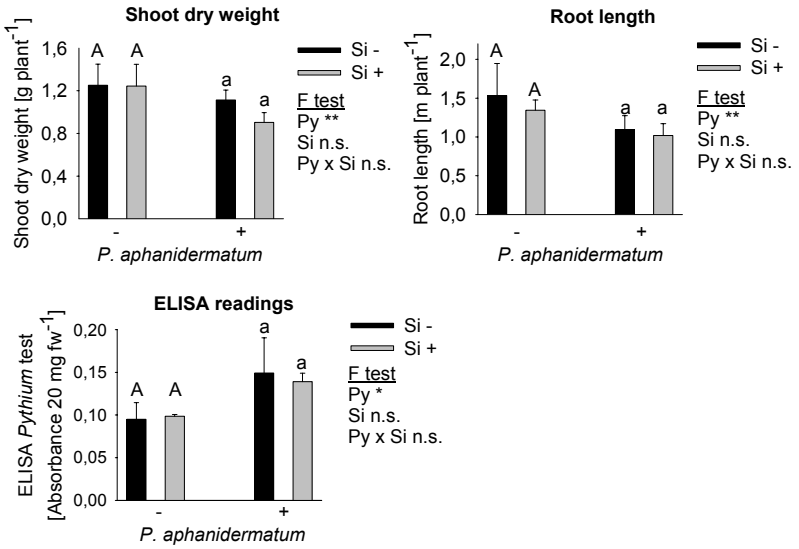

Figure 2: Effect of P. aphanidermatum *inoculation (350 CFU plant[1]) on root and shoot growth of tomato plants 10 days after transplanting to substrate inoculated with mycelium-solution (MS), and root infection as revealed by a* Phythium*-specific ELISA test. Bars are means ± SD of five replicates. *, **, *** indicate significance at P > 0.05, 0.01, and 0.001, respectively (F test). Capital and small letters stand for comparison of Si at* P. aphanidermatum *- and +, respectively.*

No decrease in root length or shoot growth was observed for bitter gourd when inoculated with the MS method although the level of inoculation was ten times higher as compared to tomato. Sampling roots with the ELISA test after harvest confirmed the failure of causing an infection with this method. Neither root length nor shoot dry weight differed when comparing the Si treatments (data not shown).

In contrast, the MIS inoculation of bitter gourd significantly decreased both, root length and shoot weight which was related to a much higher level of CFU (Fig. 3) as compared to the MS method. No differences between Si treatments were observed for root length or shoot dry weights in plants grown in *P. aphanidermatum* free substrate. In contrast to the results for the MS inoculation of tomato (Fig. 3), Si nutrition proved to have an effect on growth of inoculated bitter gourd plants. Shoot dry weights and root length of *P. aphanidermatum* inoculated plants supplied with Si were significantly higher than of plants not supplied with Si.

The ameliorating effect of Si on *P. aphanidermatum*- inoculated bitter gourd plants was supported by the results of the *Pythium*-specific ELISA test. The ELISA readings revealed that the level of *Pythium* infection in the roots of inoculated bitter gourd plants was reduced by the application of Si.

A direct effect of Si on the pathogen is not likely because the supply of Si did not influence the growth rate of *P. aphanidermatum* on agar medium (data not shown). The spread of hyphae was identical on medium with or without Si at both dates of sampling.

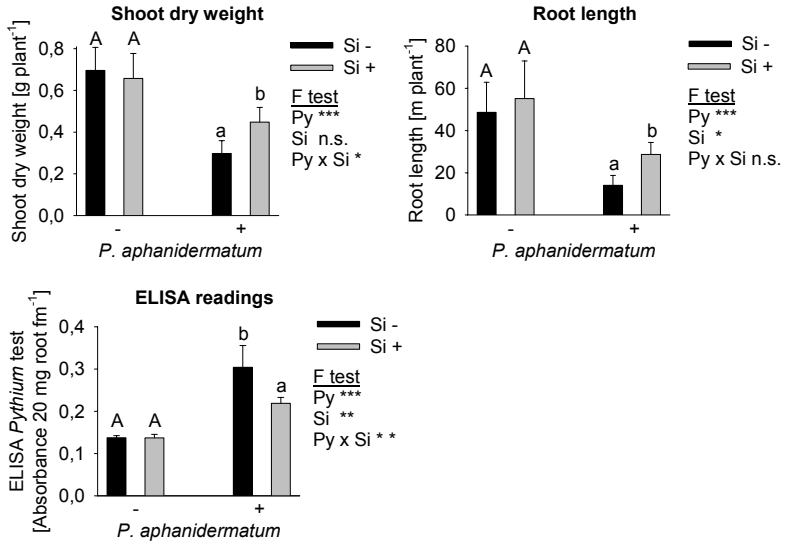

Figure 3: Effect of P. aphanidermatum *inoculation (10,500 CFU plant[1]) on root and shoot growth of bitter gourd plants 10 days after transplanting to substrate inoculated with mycelium-infested soil (MIS), and root infection as revealed by a* Phythium*-specific ELISA test. Bars are means ± SD of five replicates. *, **, *** indicate significance at P > 0.05, 0.01, and 0.001, respectively (F- test). Capital and small letters stand for comparison of Si at* P. aphanidermatum *- and +, respectively.*

3.5. Discussion

P. aphanidermatum for the most part is known as a pathogen causing pre- and post-emergence damping-off (Jones et al., 1991; Martin, 1992). Rapid death of seedlings after inoculation with *P. aphanidermatum* can be confirmed from the experiments for tomato (Fig. 1) but not for bitter gourd. For tomato the incidence of damping-off increased with increasing level of inoculation, but the ratio of damping-off to infection level decreased as the inoculation level increased (Fig. 1). Mitchell (1978) reported decreasing ratios between infection rate and inoculation height as a general pattern for several combinations of plants and *Pythium*- as well as *Phytophthora* species. The phenomena can be attributed to competition between spores for susceptible sites at the host (Van der Plank, 1975). On the basis of inoculation experiments of tomato with *P. aphanidermatum*, Mitchell (1978) calculated that 250 and 1,500 oospores per plant were required for 50% and 100% infection, respectively. In our experiments the inoculation of tomato with 350, 1,500, and 15,000 CFU of *P. aphanidermatum* per plant resulted in at least 20, 30, and 80% infection, respectively, as indicated by dead plants. Despite some differences in the experimental setup, these values are in a comparable range to the results of Mitchell (1978). When comparing different studies, it has also to be considered that environmental factors like soil moisture and soil temperature have a major impact on the infestation of plants with *Pythium* species (Xi et al., 1995; Gold and Stanghellini, 1985; Hendrix and Campbell, 1973).

Resuming the three different levels of *P. aphanidermatum* inoculation (Fig. 1), no relationship between Si supply and the incidence of damping-off could be discerned, indicating that Si is not a means to control damping-off. Suppression of damping-off was often associated with a lower biological activity of the pathogen in the substrate (Martin and Loper, 1999). However, in-vitro experiments clearly showed that Si did not influence the growth of *P. aphanidermatum*. There is evidence from other studies that Si is not directly toxic to pathogens (Menzies et al., 1992).

For bitter gourd, no information related to inoculation with *P. aphanidermatum* and disease incidence is available. However, the amount of inoculum required for infection with a given pathogen can be highly variable between plant species (Banihashemi and Mitchell, 1975).

In order to study the effects of Si on disease resistance for longer periods, it was necessary to modify the inoculation methods in a way that allowed a non-lethal but nevertheless uniform infection of plants. For tomato, the inoculation with MS turned out to be the most suitable method due to the higher survival rate of plants. For bitter gourd the more aggressive MIS method was used since low inoculation levels failed to ensure successful infection.

Within the 10 day experimental period, Si supply alone also did not influence growth of non-inoculated tomato or bitter gourd plants (Fig 2 and 3). This is in agreement with observations of Chérif and Bélanger (1992) who also did not find an influence of Si on root or shoot dry weights of non-inoculated cucumber plants. These findings are supporting the idea that Si is not an essential nutrient for these plant species but rather a beneficial element as proposed by Epstein (1999) and Marschner (1995).

Inoculation of the substrate with *P. aphanidermatum* caused a reduction of root length and shoot dry weight of treated tomato and bitter gourd plants. Even a non-lethal infection with *P. aphanidermatum* can be detrimental for root formation, leading to reduced plant growth. This was frequently confirmed for different plant species, for example for tomato by Rafin and Tirilly (1983) and for cucumber by Moulin et al (1994). Martin (1992) reported that several *Pythium* species could infect taproots, root tips and feeder roots of mature plants resulting in limited plant vigor and yield. A reduction of root length is also possible without visible symptoms in the shoot (Grosch and Scharz, 1998). *Pythium* species can be even present in symptom-less roots (Chérif and Bélanger, 1992; Rafin and Tirilly, 1983).

Si amendment to the peat combined with Si supply through the nutrient solution resulted in a better root growth and a higher shoot dry weight of bitter gourd compared to inoculated plants not supplied with Si (Fig. 3). The negative relation between root Si content and *Pythium* colonization of roots suggest that the resistance of plants was increased. In contrast, Si nutrition had no effect on *P. aphanidermatum*-inoculated tomato (Fig. 2).

Two different mechanisms were proposed regarding the role of Si nutrition in disease resistance. The first approach is a barrier theory in which polymerized Si mechanically impedes the growth of fungal hyphae in the plant tissue. The other

theory is emphasizing a metabolic role of Si on stimulating plant defense mechanism (Fawe et al., 2001).

The Si nutrition and the distribution of Si among root fractions of the two species, tomato and bitter gourd, were previously studied by Heine et al. (2005a). In this nutrient solution study it was observed that bitter gourd accumulates high amounts of Si in the root symplasm whereas tomato contains Si almost exclusively in the root cell walls. As a result of the higher Si uptake, shoot Si concentrations of bitter gourd exceeded those of tomato. Interestingly, the total root Si concentrations and especially the Si concentrations in the root cell-wall fraction were higher in tomato than in bitter gourd. Comparable differences in Si distribution between roots and shoots of the two plant species were found in the present experiments in peat (Tab. 1), and it can be assumed that the Si distribution between root cellular compartments (cell wall/ symplast) is similar as described by Heine et al. (2005a).

In the light of the these results, the ability of Si to induce resistance against the root disease *P. aphanidermatum* seems to be linked to the site of Si accumulation in the root: not the Si in the cell walls, but the Si taken up into the root symplast is responsible for the enhanced resistance of the plant against *P. aphanidermatum*. This assumption is supported by findings of Chérif et al. (1992a), who studied the positive effect of Si on *P. ultimum* infected cucumber roots by using energy dispersive X-ray microanalysis (EDX). These authors were not able to detect Si deposition in the root tissue and concluded that enhanced protection does not result from accumulation of insoluble Si in the cell walls. In another study with the same pathosystem Chérif et al. (1992b) found that the presence of Si at the sites of fungal penetration was not essential for the induction of enhanced resistance, indicating that Si accumulation and deposition in the root tissue is not essential for the Si effect on disease resistance.

Our results additionally argue against the barrier theory: *Pythium* spp. could be re-isolated from the roots of all inoculated plants, irrespective of the Si treatment. In case of a mechanical impedance of fungal growth by Si deposition it should not have been possible to isolate *Pythium* spp. from hypocotyl tissue. However, in bitter gourd the ELISA revealed a significant reduction of root colonization by *P. aphanidermatum* due to Si treatment. Sampling the roots of tomato with the ELISA showed that, despite the

high accumulation in the root cell walls, Si supply did not reduce the root colonization by *P. aphanidermatum*.

Since a mechanical barrier through polymerized Si is not a likely cause for the observed reduction of root colonization in bitter gourd, a stimulation of the plant defense mechanism by Si could be involved. Chérif et al. (1994a) proposed that Si could activate "a cascade of biochemical changes" in cucumber when attacked by *Pythium* spp. and Chérif and Bélanger (1992) emphasized a systemic action of Si in enhancing disease resistance. However, on the basis of this study, such an active role of Si in the disease resistance of bitter gourd cannot be shown.

Further studies with Si application to an individual root could more conclusively characterize the role of Si in preventing root colonization by *P. aphanidermatum*.

4. Silicon fails to enhance the resistance of tomato against *Pythium aphanidermatum* under conditions of protected cultivations in Thailand.

4.1. Abstract

Vegetable production in Thailand is limited by soil-borne pathogens, among them *Pythium aphanidermatum*. So far, the control is based on chemical means, but the demand for sustainability calls for alternative options in disease control. Previous studies for cucumber emphasized silicon (Si) nutrition as a means to stimulate resistance against root diseases. It was also postulated that Si could counteract negative effects of high nitrogen supply. However, none of these studies dealt with tomato.

In the current experiment carried out under the conditions of protected cultivation in Thailand it was investigated whether Si supply to peat substrate low in plant available Si can improve the resistance of tomato plants against *P. aphanidermatum* under two levels of nitrogen (N) supply.

Results clearly showed that under suitable conditions *P. aphanidermatum* is highly pathogenic to tomato. Plants rapidly died when challenged with high inoculation levels whereas moderate levels caused a long-term reduction in plant vigor. However, no difference was found in yield. Regardless of the *P. aphanidermatum* inoculation, Si supply did not improve the growth and fruit yield of tomato under our experimental conditions. Different possibilities for the failure of Si to induce resistance in tomato are discussed.

4.2. Introduction

Protected cultivation in net houses is an approach used for the production of vegetables in the humid tropics of South East Asia because plants can be protected from pests and from environmental damages, especially during periods of heavy rainfall. However, due to the resulting higher temperature and humidity in such net-houses plants are more affected by fungal diseases, which are commonly controlled by chemical means, causing hazards for the environment as well as for growers and consumers. Strengthening the resistance of crops against diseases would allow a

reduction in pesticide application and would thus represent a key factor in meeting the demand for sustainability (Jacobsen, 1997).

A major group of diseases limiting the sustainable production of tomato under protected cultivation are root pathogens such as *Pythium* spp. Contamination with *Pythium* spp. can occur through the nutrient solution (Jenkins and Averre, 1983) or peat based propagation substrates (Favrin, 1988). After infection the fungus easily spreads in the root tissue (Wulf et al., 1998). The most devastating effect caused by *Pythium* spp. is pre- or post-emergence damping-off (Jones, 1991), but *Pythium* spp. can also infect older plants leading to a reduction of root length, shoot dry matter, and fruit yield (Grosch et al., 1999; Moulin et al., 1994). Among the *Pythium* species isolated from diseased plants in commercial vegetable production sites, *Pythium aphanidermatum* was frequently reported to be the most aggressive (Favrin et al., 1988; Jenkins and Averre, 1983; Raffin and Tirilly, 1995). *P. aphanidermatum* is especially virulent at high temperatures (Gold and Stanghellini, 1985) and high humidity (Feng et al., 2002), conditions that frequently occur in the humid tropics during the rainy season.

The resistance of plants against pests and diseases is not only genetically controlled but also depends on the nutritional status of the plants (Graham, 1983). It is generally accepted that both, tolerance and resistance is higher in a plant well and balanced supplied with nutrients than in a plant deficient in one or more nutrients (Sieling, 1990; Marschner, 1995). Increased susceptibility to diseases is well established for potassium (Ollagnier and Renard, 1976), calcium (Berry et al., 1988), manganese (Graham and Rovira, 1984), copper (Graham, 1980), and boron deficiencies (Graham and Webb, 1991). For these elements the most spectacular positive effects on plant health, therefore, occur when the nutritional status of the plant is increased from the deficiency to the optimum range for growth. In contrast, an increase in the supply of macronutrients to supra-optimal concentrations does not further increase the resistance of plants but may rather result in increased susceptibility to pests and diseases. Numerous studies report that luxury consumption of N predisposes crops to diseases (Conner et al., 1992; Krauß, 1970; Shanner and Finney, 1977). The increased susceptibility is related to a higher concentration of low molecular weight nitrogen compounds in the plant as well as to a decreased activity of enzymes of phenolic

synthesis such as phenylalanin ammonia-lyase and tyrosin ammonia-lyase leading to lower lignin contents (Matsuyama and Diamond, 1973).

The role of the beneficial element Si in plant resistance against leaf pathogens is well established since Wagner (1940), and has been subsequently confirmed for many pathosystems. Major Si effects were often found in monocotyledones plant species that accumulate large amounts of this element. Due to the suppressing effect on the foliar pathogens *Magnapoethe grisea* and *Leptosphaeria sacchari* a routine application of Si was recommended for rice (Datnoff et al., 1997) as well as for sugarcane (Savant et al., 1999). Among the dicots, cucumber is the plant most extensively studied for Si–pathogen interactions. Si was reported to stimulate resistance against *Sphaerotheca fuliginea*, which was associated with a faster accumulation of phenolics and lignin at the side of fungal penetration (Menzies et al., 1991b). Furthermore, Si conferred protection of cucumber against *Pythium ultimum* (Chérif and Bélanger, 1992) and *P. aphanidermatum* (Chérif et al., 1994) by a Si-induced production of phytoalexins (Fawe et al., 1998).

In spite of its ubiquitous occurrence in the environment, Si is not always readily available for most plants. The reason is that the concentration of monomeric silicic acid, which is the only Si form available for uptake by plants, is controlled by the low solubility of Si minerals, polymerization reactions and leaching processes (Jones and Handreck, 1967). Particularly in peat substrates, which are used for greenhouse production of vegetables, the Si supply is low due to the naturally low Si contents. However, the availability of Si can be enhanced by the application of soluble Si sources to the substrates (Voogt and Sonneveld, 2001).

The objective of the present study was to test the effect of different nutrient management regimes on the diseases resistance of tomato grown under conditions of protected cultivation in the humid tropics. Of special interest was the question if enhanced Si nutrition could contribute to growth and prevent fruit yield losses of tomato caused by an infection with *P. aphanidermatum*.

4.3. Material and methods

4.3.1. Site characterization

The experiments were carried out in net houses located at the campus of the Asian Institute of Technology (AIT) in Pathumthani, Thailand. Pore size of the nets was 0.18 mm (Econet M, Ludvig Swensson, Netherlands). The net house was equipped with an automatic fertigation system and the basic fertigation solution was made up from 2 stock solutions, Hakaphos basis 3-15-36-4 (Mg) (COMPO Austria, GmbH) and Calcinit 15.5-0-0-19 (Ca) (Yara, Germany). The stock solutions were diluted with tap water to give the following concentrations of macronutrients: 166 mg/l Ca, 146 mg/l nitrate-N, 48 mg/l P, 225 mg/l K, and 40 mg/l Mg. The volume of solution applied depended on global radiation and was gradually increased with increasing plant age. Soil and air temperatures and humidity were monitored by a psychrometer (ITG, Germany) and values were recorded with a data logging system.

The climatic conditions from May to December 2003 are displayed in Fig. 1. The average daily air temperatures were almost constantly higher than 30°C and fell below 30°C only occasionally. During the entire growing period the average daily soil temperature was higher than the average daily air temperature, especially in the period between the middle of July and the middle of September. The average daily relative humidity was fluctuating to a higher degree than the daily temperatures. It was constantly higher than 60%, but frequently reached up to 90%.

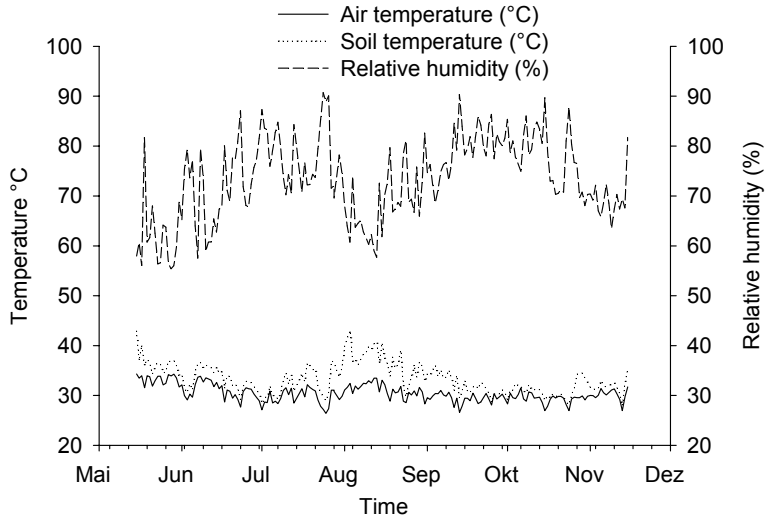

Figure 1: Climatic conditions at the experimental site during May to December 2003

4.3.2. Pre-cultivation of tomato plants

Seeds of the tomato variety KingKong II (Known-YouSeed Co., Ltd, Taiwan) were sown in peat substrate (Pindstrup substrate No. 1, Pindstrup, Mosebrug, Denmark) and grown under disease and pest-free conditions in a nursery. For all experiments plants were used two weeks after seeding.

4.3.3. Preparation of inoculum

Mycelium infested substrate (MIS) was prepared according to a modified method of Chaengchaiyasakulthai and Chamswarng (1986). Mycelia pieces of a pathogenic isolate of *P. aphanidermatum* grown for 4 days on Potato Dextrose Agar were added to flasks containing sand, soil, and grounded maize flower in the proportion 3:1:1. The mixture was incubated for two weeks at 35°C prior to dilution to 5% by adding peat substrate. Maize powder (1.5%) was added and the stock culture was incubated overnight at 25°C previous to inoculation. In all experiments, a similar stock-culture mixture but without *P. aphanidermatum* was used for control plants.

The number of colony forming units (CFU) were quantified by diluting 1g of stock culture with 100 ml agar solution (0.1 %). 0.5 ml of the mixture was spread on a selective media for *Pythium* spp. (Tsao, 1970) and the CFU of 6 independent replications were counted after 24h.

4.3.4. Incidence of damping-off

On July 18th 2 l pots containing peat substrate supplemented with various amounts of MIS were placed in a net house in a completely randomized design. Tomato plants were transferred to the pots and incidence of damping-off was recorded for 6 days. Dead plants were sampled for *Pythium* spp. by re-isolating on selective media (Tsao, 1970).

4.3.5. Interaction of mineral nutrition and plant health

Two experiments were carried out. The first main experiment started on May 20th and was terminated on July 30th. The second main experiment was carried out between the August 1st and the November 1st. Both main experiments were arranged as factorial

combination of treatments, having two levels of Si (minus and plus), two levels of *P. aphanidermatum* (minus and plus inoculation), and two levels of nitrogen (optimal and supra-optimal). Experimental units were arranged in a randomized complete block design with 4 replicates. Each replicate consisted of 3 plants and all measurements were averaged for the plants within a replicate.

Peat substrate was prepared according to the individual treatments by using a concrete mixer. 3 g/l Agrosil (Compo, Germany) as a source of Si was added as Si treatment, and MIS was applied as *P. aphanidermatum* inoculation. Si application resulted in a Si concentration of 0.69 mM in the 0.01 M $CaCl_2$ substrate extract (Haysom and Chapman, 1975), as compared to a Si concentration of 0.37 mM in the substrate without Si application.

The amended substrate was then filled to 10 l pots, and pots were transferred to the net house. Tomato seedlings were planted in the evening of the same day. For the supra-optimal nitrogen treatment, KNO_3 was hand applied during the experiment to give the double nitrogen supply in comparison to the optimal nitrogen treatment. The greenhouse was kept free of pests by application of the insecticides Cypermethrin and Benomyl three times a week. Tomato plants were pruned and layered weekly.

Ripe tomato fruits were harvested daily and grouped into marketable and non-marketable fruits. Fruit weight of either group was taken. At the end of all experiments the fresh and dry weights of shoots were recorded. Roots were washed free of substrate before recording root fresh- and dry weight. In the first experiment leaf area was measured by using a leaf area meter (LI-3100, Li cop, inc., Lincoln, Nebraska, USA).

4.3.6. Pythium *re-isolation*

In order to investigate the activity of the pathogen during the experiment the potato bait method of Stanghellini and Kronland (1985) was used for re- isolation of *Pythium* spp. from the substrate. 5 g of substrate was taken from the top 10 cm of every pot and filled into 9 cm petri-dishes. Three potato tuber slices (0.5 cm) bearing a water-agar slice were placed on the top of each substrate sample, and deionized sterile water was added up to saturation level. Petri dishes were closed and incubated at 25°C in the dark. After 48 h agar slices were removed and placed on a selective medium for

Pythium spp. (Tsao, 1970). Plates were incubated at 35 °C in the dark and monitored after 24 h. Samples with mycelia growths were assessed as successful re-isolation.

Since the method is specific only on the level of the genus a successful re-isolation is termed as *Pythium* spp. isolation.

4.3.7. Quantification of root infection

Ten randomly selected root sections with a combined fresh weight of 1 g were cut from the root system and homogenized in 10 ml extraction buffer in a blender. The degree of *Pythium* infection was quantified in the homogenate by using a double sandwich ELISA system (Löwe, Sauerlach, Germany) with anti-Pythium IgG as primary antibody and anti-Pythium IgG coupled with acid phosphatase as secondary antibody. Absorption was measured spectrometrically 1 h after addition of the substrate at 405 nm.

Since the test is specific only on the level of the genus, regarding results are termed as *Pythium* spp. infection.

4.3.8. Si quantification

The total Si content in the plant tissue was measured using the colorimetric method of Novozamsky et al. (1984) modified according to Iwasaki et al. (2001).

For the determination of the total Si content 500 µl of a 1:2 mixture of hydrochloric acid (1 mol/l) and hydrofluoric acid (2.3 mol/l) were added to 10 mg of dried and grinded tissue and incubated on a shaker. After 24 h, the suspension was centrifuged at 10,000 x g and 20 µl of the supernatant was added to 250 µl of boric acid (3.2%). Following another incubation period of 24 h, 250 µl of a 1:1 (v/v) mixture of sulfuric acid (0.08 M) and ammonium molybdate tetrahydrate solution (20 g/l) was added. Thirty minutes later, 250 µl of each, tartaric acid (33 g/l) and ascorbic acid (4 g/l) were added and the absorption was measured spectrometrically at 811 nm.

4.3.9. Data analysis

For statistics only surviving plants were used. The GLM procedure of SAS version 8.1 was used for the analysis of variance (SAS, 2001).

4.4. Results

In the experiment on incidence of damping-off no tomato plants of the non-inoculated control treatment died. However, the first *P. aphanidermatum*-inoculated plants died after two days (Fig. 2). Subsequently, in the treatments T-2 to T-4 the incidence of dead tomato plants rapidly reached 80% or more. Only at the lowest inoculation level (T-1) 75% of the plants survived longer than 6 days. The result clearly showed that the inoculation level should not go much beyond 24 CFU/l under the prevailing experimental conditions. Based on this results 0.02 g/l stock culture equal to 36 CFU/l substrate was given to the first main experiment. To the second main experiment 0.05 g/l stock culture equal to 40 CFU/l substrate was applied. Incidence of damping-off was below 10 % for both main experiments and no treatment effects on damping-off were observed.

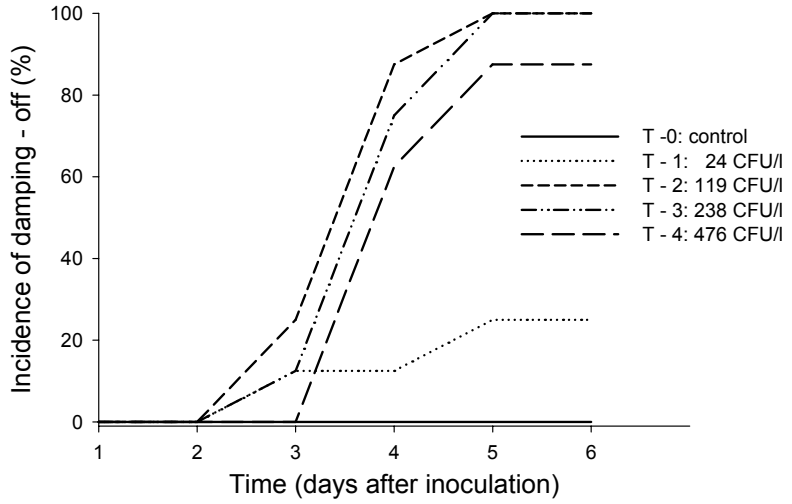

Figure 2: Damping-off incidence of tomato plants as affected by the inoculation level with P. aphanidermatum.

In the first main experiment leaf area and shoot fresh weight were negatively influenced by *P. aphanidermatum* inoculation, but no significant effect was observed on fruit yield (Tab. 1). Leaf area was positively affected by the Si treatment and Si-treated leaves appeared more rigid than leaves of non-treated plants. The supra-optimal nitrogen supply had a significant negative effect on shoot fresh weight. Root fresh weight and shoot dry weight were not affected by any treatment.

Table 1: Effect of Si and nitrogen supply on growth, fruit yield, and Si content in roots and shoots of tomato plants grown in peat substrate with or without P. aphanidermatum *(Py) inoculation at transplanting. Fruits were harvested for a period of 2 weeks.*

Treatments			Leaf area	Root fw	Shoot fw	Shoot dw	Yield	Si concentration	
Py	Si	N	[m^2]	[g]	[kg]	[g]	[g/plant]	[mg(g dw)$^{-1}$]	
								Root	Shoot
-	-	+	0.98±0.06	46.9±5.4	1.46±0.08	178± 6	133± 82	0.22±0.14	0.73±0.16
+	-	+	0.93±0.05	48.6±3.9	1.38±0.10	179±19	108± 58	0.28±0.07	0.82±0.22
-	+	+	1.04±0.05	52.4±6.5	1.45±0.09	186± 9	143± 15	0.24±0.06	0.88±0.24
+	+	+	0.97±0.09	41.5±6.7	1.33±0.10	165±18	117± 97	0.39±0.07	0.85±0.11
-	-	++	0.96±0.05	43.7±2.3	1.28±0.08	165±19	98± 58	0.18±0.07	0.78±0.23
+	-	++	0.88±0.09	48.6±5.3	1.29±0.18	168±25	167±144	0.24±0.07	0.76±0.16
-	+	++	1.04±0.08	51.5±5.1	1.42±0.09	173± 4	105± 97	0.24±0.10	0.71±0.10
+	+	++	0.98±1.00	42.7±6.3	1.26±0.09	159±13	201± 27	0.31±0.09	0.93±0.40
Py			(N)*	n.s.	(N)*	n.s.	n.s.	n.s.	n.s.
Si			(P)*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N			n.s.	n.s.	(N)*	n.s.	n.s.	n.s.	n.s.

* Significant at α=0.05
n.s. not significant
(P), (N) positive, negative effect of a factor

The negative effect of inoculation with *P. aphanidermatum* on the shoot fresh matter production of tomato plants was confirmed in the second main experiment (Tab. 2). In addition, the shoot dry matter of inoculated plants was also significantly lower. As in the first main experiment, inoculated plants tended to have lower fruit yields than non- inoculated plants but this failed to be significant. Neither Si application nor additional nitrogen nutrition influenced any parameter in the second experiment. Again, root fresh weight was not affected by any treatment in the second experiment.

In the first experiment the Si analysis revealed an increase in the Si concentrations of both leaf and shoots of Agrosil-supplied plants (Tab. 1). However, the differences between the Si contents were not significant. The leaf Si concentrations were generally much higher in the second than in the first experiment. Again, the Si concentrations in the leaves of Si-amended plants were slightly higher.

Additional nitrogen treatment or inoculation with *P. aphanidermatum* did not affect Si concentrations in roots or leaves.

Table 2: Effect of Si and nitrogen supply on growth and fruit yield and shoot Si content of tomato plants grown in peat substrate with or without P. aphanidermatum *(Py) inoculation at transplanting. Fruits were harvested for a period of 4 weeks.*

Treatments			Root fresh wt	Shoot fw	Shoot dw	Yield	Si concentration
Py	Si	N	[g]	[kg]	[g]	[g/plant]	[mg/g leaf dw $^{-1}$]
-	-	+	59.3±11.0	1.40±0.59	253± 92	591±266	1.50±0.19
+	-	+	62.0±15.9	1.24±0.25	224± 42	498±194	1.63±0.06
-	+	+	68.2±18.8	1.66±0.48	317±103	562±163	1.87±0.14
+	+	+	50.4±10.3	1.47±0.32	281± 63	517±209	1.71±0.20
-	-	++	77.1±17.2	1.62±0.28	314± 55	679±250	1.62±0.55
+	-	++	64.7±17.2	1.16±0.18	212± 46	405± 79	1.82±0.22
-	+	++	58.1±12.1	1.53±0.31	305±144	476±373	1.73±0.22
+	+	++	62.5±18.1	1.00±0.33	179± 30	455±179	1.67±0.14
	Py		n.s.	(N)*	(N)*	n.s.	n.s.
	Si		n.s.	n.s.	n.s.	n.s.	n.s.
	N		n.s.	n.s.	n.s.	n.s.	n.s.

* = significant at α=0.05
n.s. = not significant
(P)/ (N) = positive/ negative effect of a factor

In the first experiment the long-term activity of the pathogen was investigated by two different methods. The substrate was sampled for *Pythium* spp. by using potato baits during the experiment and washed roots were analyzed with a *Pythium* specific ELISA after the experiment. Isolation of *Pythium* spp. from the substrate was possible only from two experimental units, both of them belonging to the *P. aphanidermatum* treatment.

Although there was a significant effect of *P. aphanidermatum* on shoot fresh weight in the first main experiment (Tab. 1), ELISA readings higher than the controls were detected in the roots of only three inoculated plants. Noteworthy, two of these plants originated from the same pots where *Pythium* spp. had been re-isolated. The small number of positive samples in both tests indicates a generally low long-term survival of *Pythium* spp. under the experimental conditions.

In both main experiments, no interaction was found for any of the possible combinations (Py x Si; Py x N; Si x N; Py x Si x N).

4.5. Discussion

Results clearly show that *P. aphanidermatum* is highly pathogenic to tomato under the climatic conditions of South Thailand. However, they also demonstrate the difficulty to choose an inoculation level causing a permanent infection of tomato plants with *P. aphanidermatum* without killing the plants. Even a low level of inoculation resulted in the death of some plants and the mortality quickly reached 100 % when the degree of inoculation was higher (Fig. 2). On the other hand, at the moment of inoculation the exact number of CFU applied is unknown because the quantification of CFU takes two days.

A higher mortality of plants at higher levels of infection was expected and is described by Bhatti and Kraft (1992) for root rot of chickpea, where disease incidence was enhanced with increasing level of *P. ultimum* inoculation. For tomato, Mitchell (1978) described a gradual increase in the percentage of diseased seedlings with increasing level of inoculation with *P. aphanidermatum*. However, the total number of CFU in our experiment was much lower than the number of oospores used by Mitchell (1978), indicating that the inoculation method used in our experiments was highly effective.

For both main experiments, the amount of inoculation material used resulted in a non-lethal infection that caused a long-term reduction of plant growth. Shoot fresh weight of inoculated plants was significantly reduced in both experiments (Tab. 1+2). In addition, a reduction in leaf area and shoot dry weight of *P. aphanidermatum* inoculated plants was observed for the first and second main experiment, respectively. The reduction in plant vigor in both experiments could be explained by a decreased

ability of the plants to acquire nutrients and water due to partial destruction of the root system by *P. aphanidermatum* and decreased root activity in the early phase of plant growth (van Noordwijk and de Willingen, 1997). Stress during the early development of tomato is correlated with the subsequent growth of the plant (Calvert, 1957). Moorman (1986) obtained similar results working with poinsettia. Plants that survived an inoculation with *Pythium ultimum* were retarded in growth as compared to non-inoculated plants.

At harvest, however, differences in root fresh weight between inoculated and non-inoculated plants were no longer observed (Tab. 1+2). Inoculated plants may have compensated the early root damage caused by *P. aphanidermatum* by increasing root growth of healthy roots. In addition, during the course of the experiments, roots of all treatments might have reached the maximum capacity of the pots. It is a well-known problem of pot experiments that an insufficient space for root growth in non-inoculated control roots can mask any treatment effects on root growth, because uninfected healthy roots are slowed down in growth to the level of infected roots. (Hendrix and Campbell, 1973).

Results of root fresh weights indicate that *P. aphanidermatum* was not able to constantly destroy roots during the complete experimental period. The low activity of *Pythium* spp. in the substrate is supported by the unsuccessful attempt to re-isolate *Pythium* spp. from the pots eight weeks after inoculation. As pointed out by Hendrix and Campbell (1973), *Pythium* species are weak competitors for food sources with other microorganisms. Therefore, it seems more likely that *P. aphanidermatum* survived in the substrate by the formation of persistent resting structures like oospores rather than through saprophytic growth. However, oospores are hard to detect by a plating method because germination is often low due to fungistasis (Lumsden, 1980). In addition, at harvest *Pythium* spp. was hardly detectable in the roots by using a specific ELISA test, confirming a decreasing virulence of the pathogen during the time course of the experiment.

Under our experimental conditions, additional Si nutrition failed to improve growth parameters of both inoculated and non-inoculated plants. A Si effect to non-inoculated plants was not expected because Si is not considered as a nutrient essential for tomato (Epstein, 1999; Marschner, 1995). Adatia and Besford (1986) as well as

Stamatakis et al. (2003) found no influence of Si on the yield of hydroponically grown tomato plants when grown under stress-free conditions. However, Heckman et al. (2003) reported that Si application increased yield of pumpkin up to 60%. For cucumber, Adatia and Besford (1986) found a higher fresh and dry weight per unit leaf area of Si-supplied cucumber plants, but leaf area was not affected by Si supply. It seems that cucurbitaceous species are more responsive to Si amendment than tomato, which may be related to their higher ability to take up Si. The only Si effect found in our experiments was an increase in leaf area in the first experiment, which is in contrast to the results of Adatia and Besford (1986) for cucumber. The reasons for this difference are unknown.

The lack of a positive effect of additional Si application on growth parameters in *P. aphanidermatum*-inoculated tomato plants is not in agreement with results described for cucumber. Chérif et al. (1994) reported that the application of Si to inoculated cucumber plants increased survival, dry weight, and yield as compared to plants not supplied with Si. These contradictory results may have different reasons: (i) The control plants were already sufficiently supplied with Si. The Si concentration in both roots and shoots of the control plants was only slightly and non-significantly lower than of the Si-treated plants. This indicates that other Si sources from the substrate and/or the fertigation water have been available for the control plants. Water samples analyzed had Si concentrations as high as 0.26 mM. (ii) Si failed to enhance resistance of tomato against *P. aphanidermatum* at high ambient temperature during the experiment (Fig. 1). Schuerger and Hammer (2003) found that the resistance mechanism of cucumber against *S. fuliginea* triggered by Si failed at temperatures exceeding 30°C. (iii) The ability of Si to stimulate disease resistance could differ among plant species. Ma (2004) stated that beneficial Si effects are mainly to be expected in Si accumulating plants and the bulk of positive Si effects described in the literature are related to this group. The prime example is rice, for which Si was even termed as an "agronomically essential element" (Takahashi et al., 1990), because of, among other beneficial effects, the ability to stimulate resistance against diseases. Even though Si uptake of cucumber is much lower than of rice it is still considerably higher than in tomato (Mitani and Ma, 2005). Tomato is known to discriminate Si from uptake and, therefore, belongs to the group of Si excluders. In the light of these results

it could be possible that the ability of Si to stimulate disease resistance is linked to the degree of Si uptake by the plant. However, this conclusion is challenged by results of Dannon and Wydra (2004), who found an increase in tolerance of tomato against the bacterial pathogen *Ralstonia solanacearum* when plants were supplied with Si. It appears that beneficial Si effects with respect to plant health not only depend on the plant species studied but also on the pathogen.

The only effect of the supra-optimal nitrogen nutrition observed was a reduction in shoot fresh matter in the first main experiment. Nitrogen belongs to the nutrients with the greatest impact on plant growth. In the deficiency range, nitrogen supply highly stimulates plant growth, but supply exceeding the optimum for plant growth can cause growth reduction (Jones et al., 1991). In the first main experiment the N threshold value in terms of biomass production was apparently exceeded. However, the excess N supply influenced inoculated and non-inoculated plants to a similar degree, indicating that there was no influence of N on disease severity of *P. aphanidermatum*. This is not in agreement with results by Huber and Watson (1974) who reported a stimulating effect of nitrate on diseases caused by *Pythium* spp.

In conclusion, the results of our study indicate that under the experimental conditions described, infection of the growing substrate with *P. aphanidermatum* at an infection level not leading to damping-off did not negatively affect tomato fruit yield. The application of Si to the substrate seems not to be suitable means to improve the growth of tomato infected with *P. aphanidermatum*.

5. Spatial sensitivity of tomato and bitter gourd roots towards *Pythium aphanidermatum* infection as affected by silicon nutrition.

5.1. Abstract

The objective of the present study was to quantitatively assess the infection success and the spread of *Pythium aphanidermatum* along individual roots of the silicon (Si) excluder tomato and the Si accumulator bitter gourd as affected by Si supply. Individual roots of intact plants were mounted into PVC boxes, which allowed the application of Si and zoospores to defined root zones.

At harvest, root growth was recorded and roots were cut into segments. A *Pythium* sensitive ELISA was developed to quantify the pathogen colonization in individual root segments. Si contents of root segments were also determined.

Inoculation with zoospores to the apical and sub-apical root zones revealed that in tomato as well as in bitter gourd the root tip is the most sensitive root section to *P. aphanidermatum* infection leading to cessation of root growth. Application of Si did not affect the inhibition of root growth in both species, and no effect of Si supply was observed on primary infection and spread of *P. aphanidermatum* in the roots of tomato. However, in bitter gourd the infection levels were increased in the root tips but decreased in basipetal root segments when plants were supplied with Si both during pre-treatment and during *P. aphanidermatum* infection. Si application to an individual root zone of tomato or bitter gourd failed to inhibit the spread of *P. aphanidermatum* even though Si concentrations of the root sections were more than doubled. In-vitro tests revealed that Si did not inhibit the germination of zoospores.

We conclude that accumulation of Si in the root cell-walls does not represent a physical barrier to the growth of *P. aphanidermatum*. However, Si nutrition induced resistance mechanism against *P. aphanidermatum* in the Si accumulator bitter gourd but not in the Si excluder tomato. In the roots of bitter gourd the effect of Si requires both the presence and continued uptake of Si into the root symplasm.

5.2. Introduction

Root rot, associated with *Pythium* spp., is a major threat for many agricultural crops (Hendrix and Campbell, 1973). *Pythium aphanidermatum* (Edson) has a mainly tropical

distribution and is pathogenic to a wide host range (Domsch et al., 1980). It infects mainly roots of seedlings or the root tips of older plants (Hendrix and Campbell, 1973) and is known to be a good root colonizer that consistently inhibits root growth (Wulff et al., 1998). After root infection, *P. aphanidermatum* can also spread through the roots of seedlings into the hypocotyl, causing stem rot and eventually post-emergence damping-off (Jones et al., 1991b).

A typical feature of *P. aphanidermatum* is the ability to infect plants grown in solution culture (Moulin et al., 1994) and its spread in solution is facilitated by asexual produced zoospores (Stanghellini and Rasmussen, 1994). The zoospores are attracted by root diffusates and then attach to the root mainly in the root hair zone (Grosch and Schwarz, 1998; Wulff et al., 1998; Jones et al., 1991a) where the spore adhesion and germination is then stimulated by uronic acid (Donaldson and Deacon, 1993) and Ca^{2+} but not by other cations (e.g. Na^{2+}, K^{+}, Mn^{2+}) (Donaldson and Deacon, 1992).

Many studies report a good control of *Pythium ultimum* and *P. aphanidermatum* by amending the nutrient solution of greenhouse-grown cucumber with silicon (Si) (Bélanger et al., 1995; Chérif et al. 1994b; Chérif and Bélanger, 1992). Whether Si decreases root infection by modifying root surface characteristics and/or spore germination is not known. Despite the considerable number of studies on the interaction between Si and several species of *Pythium*, quantitative measurements on the spread of *Pythium* species on a root level as affected by Si are lacking. Si deposition in cell walls may represent a physical barrier for fungal growth as originally proposed for powdery mildew by Wagner (1940). However, based on the studies mentioned above it appears more likely that Si nutrition enhances plant resistance mechanisms against *Pythium* spp. (Fawe et al., 2001). Heine et al. (2005) previously studied the Si status of the two plant species tomato and bitter gourd. They demonstrated that the total root Si concentration was higher in the Si excluder tomato than in the Si accumulator bitter gourd. This was particularly true for the cell-wall fraction. Therefore, the comparison of these plant species appeared especially suitable for studies concerning the mechanisms of Si-enhanced resistance against root diseases (Heine et al., 2005). If Si accumulation in the root cell-walls represent a mechanical barrier against the spread of *Pythium* spp. in the root a high Si content should lead to

a lower spread. Thus, spread of *Pythium* in roots of tomato should be slower than in bitter gourd because of the higher Si contents of the root apoplast of tomato.

The objectives of the present study were to establish an experimental protocol, which allowed the application of Si and zoospores of *P. aphanidermatum* to defined apical root sections of tomato and bitter gourd plants, and to develop a methodology that allowed the quantitative assessment of the infection success and the spread of the fungus along individual roots as affected by Si supply. Of special interest was the question whether root zones with high Si status represent a barrier against the spread of the fungus.

5.3. Material and Methods

5.3.1. Cultivation of plants

Tomato (*Lycopersicon esculentum*) seeds of the variety King Kong II were germinated at 25°C on water soaked filter paper in the dark. After three days, the germinated seeds were placed in a "sandwich system" between two layers of foam over-layered with filter paper and supported by PVC plates. The sandwich was placed in a plastic box containing 10 mM $CaSO_4$ and 5 µM boric-acid solution and kept in a growth chamber under the following controlled conditions: photoperiod 16/8; temperature 30/25°C day/night; 70% relative humidity, 150 µmol m^{-2} s^{-1} light intensity at plant canopy level.

After one week, tomato seedlings were transferred to a complete nutrient solution with the following composition (µM): $Ca(NO_3)_2$ 800; K_2SO_4 375; $MgSO_4$ 325; KH_2PO_4 100; H_3BO_3 8; $CuSO_4$ 0.2; $ZnSO_4$ 0.2; $MnSO_4$ 2; Na_2MOO_4 0.1; NaCl 10; NH_4NO_3 200; and FeEDTA 40. Depending on the experimental approach, 1.4 mM silicic acid freshly prepared by passing potassium silicate (BDH chemicals, England) through a H^+ cation-exchange column was added. After another week taproots were cut off to induce adventitious root formation. When adventitious roots were several centimetres long, plants were used for the experiment.

Bitter gourd (*Mormodica charantia*) seeds of a local variety from Lion seeds, Thailand, were heat-treated at 65°C overnight to break dormancy and subsequently germinated in limed peat (pH 6.0) in a growth chamber under the conditions

mentioned above. After 12 days, roots of bitter gourd plants were carefully washed free of substrate before transfer to a nutrient solution as described above. Bitter gourd was grown for four days prior to experimental use.

5.3.2. Inoculum production and inoculation

All used isolates were tested by the Centraalbureau voor Schimmelcultures, Netherlands (det 273-202), and specified as *P. aphanidermatum*. Zoospores were produced by a modified method of Rahimian and Banihashemi (1979). One week-old V8 agar plates with *P. aphanidermatum* were cut in small stripes, transferred to two Petri dishes, and flooded with 20 ml of double distilled water (DDW). The solution was replaced after 30 minutes and plates were incubated at 34 °C under continuous light (100 μmol m^{-2} s^{-1} light intensity). After four days, the solution was replaced again, and incubating the plates for 16 hours at 18 °C triggered zoospore release. Spores were counted using a Fuchs- Rosenthal chamber and adjusted to a concentration of 10,000 spores/ml by using DDW. Roots were inoculated in compartment boxes (see below) for two hours renewing the solution after one hour. Except for the first experiment (see below) zoospores were applied to the 1 cm root apex, only.

5.3.3. Influence of Si on spore germination

The influence of Si on the fungal germination was studied based on a method of Bowen et al. (1992), which was modified in order to study zoospore germination. Zoospores were produced as described above and immobilized by shaking on a vortex mixer for 20 sec. After centrifugation for 5 min with 420 g at 4° C, the supernatant was removed and nutrient solution amended or not amended with 1.4 mM silicic acid was added to the pellet. Percent germination was recorded after 24 h. A minimum length of the germination tube with at least the diameter of the cyst was required for a positive score.

5.3.4. Experimental setup for spatial sensitivity experiments

The roots of intact plants were spread in plastic trays and covered with nutrient solution. One single root per plant was carefully inserted in a compartment box made

from PVC (Fig. 1). Compartments were sealed with agarose (1%). This experimental approach allowed the application of specific solutions to different root zones along an individual root. When not mentioned otherwise, the compartments were filled with nutrient solution. After application of the solutions, the compartment boxes and the remaining root system spread in the plastic tray were covered with tin foil and sealed with parafilm to keep the roots in the dark and to avoid evaporation. After two days, (except for the first experiment) root growth of single roots was recorded and the roots were then cut into root sections as indicated. The level of *Pythium* colonization of individual root sections was analyzed by using the double sandwich ELISA system described below. Three different types of experiments were carried out:

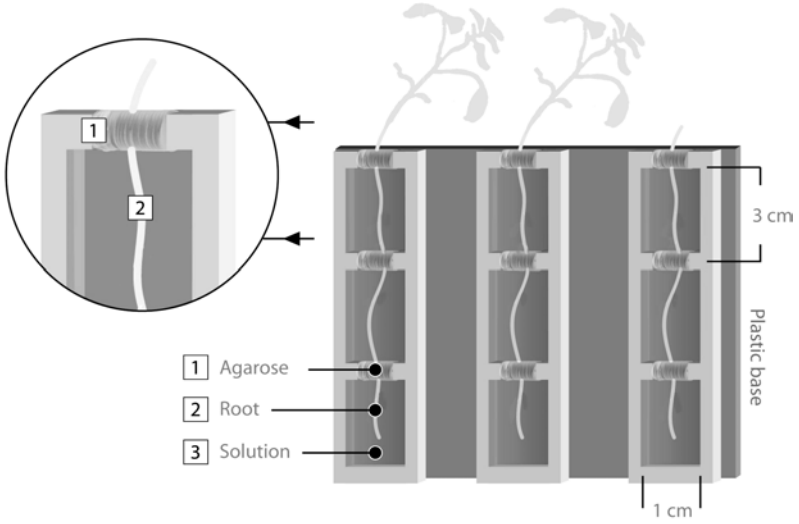

Figure 1: Model of a rhizotron used for the spatial inoculation of roots

(1) Detection of the most sensitive root zone for infection

Plants grown in nutrient solution not amended with Si were used and no Si was used during the experiment. Zoospores were applied to the 1 cm root apex or to the 1-2 cm root zone. After 24 h, root growth was monitored, and 1 cm root sections were harvested for the determination of *P. aphanidermatum* colonization.

(2) Si application to the whole root

Plants were grown in nutrient solution containing or not containing Si. Depending on the experiment, Si treatment with Si-containing nutrient solution (1.4 mM) was continued or discontinued after the transfer to the compartments or started directly after inoculation. Zoospores were applied to the 1 cm root apex and roots were harvested after 2 dpi.

(3) Si application only to the second root section

After pre-cultivating in Si-free nutrient solution Si supply was started together with the *P. aphanidermatum* inoculation (transfer to compartments). Si in the form of silicic acid (2mM) was applied only to the root section 2-5 cm. Zoospores were applied to the 1 cm root tip, and roots were harvested after 48 h.

5.3.5. Quantification of Pythium *colonization by ELISA*

The assay was carried out following a standard protocol for the "double antibody sandwich technique". Briefly, samples were homogenized in sample buffer and 200 µl of the obtained homogenate was transferred to wells of microplates, previously coated with a *Pythium*-specific polyclonal antibody (IgG). After incubation at 4 °C overnight, 200 µl of a solution containing the *Pythium*-specific antibody coupled to an alkaline phosphatase (IgG-AP) was added and plates were then incubated for 4 hours at 37 °C. The colorimetric reaction was induced by applying 200 µl of 4-nitrophenyl phosphate (1 mg/ml). The absorbance was measured after one hour with a photometer (Biotech, USA). When analyzing sections of non-inoculated roots an absorbance of about 0.12 representing a background value was usually measured.

The ELISA was calibrated by measuring a dilution series of *P. aphanidermatum* mycelia that was obtained from cultures grown on PDA plates (Fig. 2). The relationship between ELISA adsorption and mycelia mass was best described by an exponential function. The detection limit was lower than 1 µg mycelium/ml corresponding to 0.2 µg mycelium per sample.

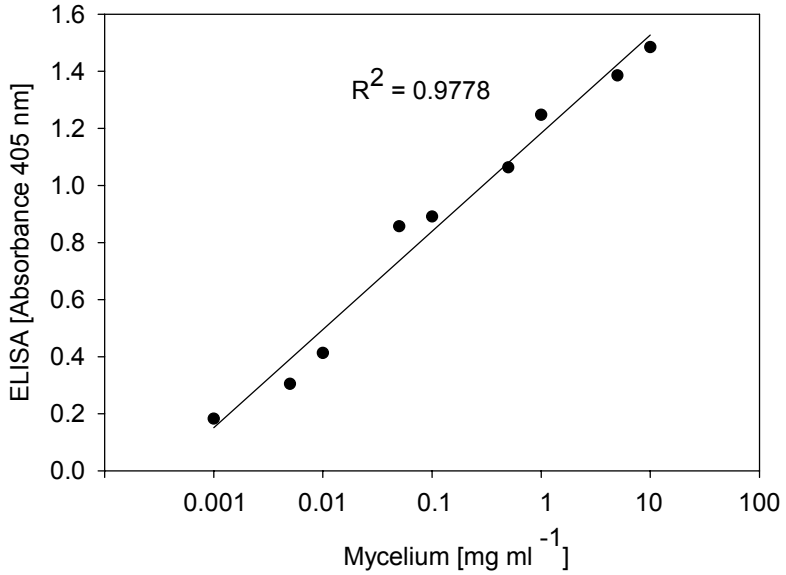

Figure 2: Calibration curve of the Pythium *ELISA.*

5.3.6. Si determination in plant tissue

Root sections were collected in Eppendorf vials, shock-frozen in liquid nitrogen, and homogenized at a speed of 30^{-1} using a ball mill (MM200, Retsch, Germany). Si was analyzed by using a modified colorimetric method of Novozamsky et al. (1984). Briefly, a 1:2 (v/v) mixture of hydrochloric acid (1 M) and hydrofluoric acid (2.3 M) was added and the samples were incubated for 24 hours on a horizontal shaker. Subsequently, the suspension was centrifuged at 10,000 g and 20 µl of the supernatant was added to 250 µl boric acid (3.2 %). Following a further incubation period of 24 hours, 250 µl of a colour reagent, made up of a 1:1 mixture (v/v) of sulfuric acid (0.08 M) and ammonium molybdate tetrahydrate solution (20 g l^{-1}) was added. 30 minutes later, 250 µl of each, tartaric acid (33 g l^{-1}) and ascorbic acid (4 g l^{-1}) were added, and the absorption was immediately measured spectrometrically at 811 nm.

5.3.7. Statistical analysis

All experiments were conducted using a completely randomised design. If not otherwise stated the results of one representative out of at least three independent experiments are shown. Data analysis was performed using the GLM procedure of SAS Version 8.1 (SAS 2001).

5.4. Results

The average length of germ tubes of zoospores was not negatively affected by addition of Si to the incubation medium. There was even a slight stimulation of zoospore germination in Si-amended medium in both experiments (Tab. 1).

Table 1: Influence of Si supply on germination rate and germ-tube length of zoospores of P. aphanidermatum*. n = 324 and 210 for experiment 1 and 2, respectively.*

	Germinated zoospores [%]		Length of germ tubes [□m]	
	Si -	Si +	Si -	Si +
Experiment 1	36	48	51	49
Experiment 2	20	27	43	50

In tomato as well as in bitter gourd, root growth was inhibited to a greater extent when zoospores were applied to the root tip as compared to inoculation of the subapical root zone (Tab. 2). Also, for both species ELISA readings of the root tips were significantly higher than for the section 1-2 cm after inoculation of the respective root segment (Fig. 3). This indicates that the root tip is the most sensitive root section to infection of *P. aphanidermatum* in both plant species. Whereas in tomato, a significantly enhanced infection was not detected when the subapical section was inoculated, significantly higher ELISA readings were found for this section in bitter gourd. Possibly, *P. aphanidermatum* infection can also occur through the developing lateral roots, which were more frequently observed in subapical root sections in bitter gourd than in tomato.

Table 2: Root growth of tomato and bitter gourd as affected by the zone of inoculation with P. aphanidermatum. *Means (n = 8) followed by different letters within a row are significantly different at $p < 0.05$ (Tukey test).*

	Root growth [mm d^{-1}]		
	Control	Zone of inoculation	
		0 – 1 cm	1 – 2 cm
Tomato	11.2 ± 3.8 c	0.3 ± 1.1 a	4.9 ± 0.39 b
Bitter gourd	25.0 ± 1.0 c	2.6 ± 1.8 a	12.3 ± 4.5 b

In both species, the inoculation of the root tip resulted in fungal growth in the basipetal direction, as indicated by significantly higher ELISA readings of the root section 1-2 cm compared to the root tips of non-inoculated plants. In contrast, when zoospores were applied to the root section 1-2 cm the apical spread of the fungus was low.

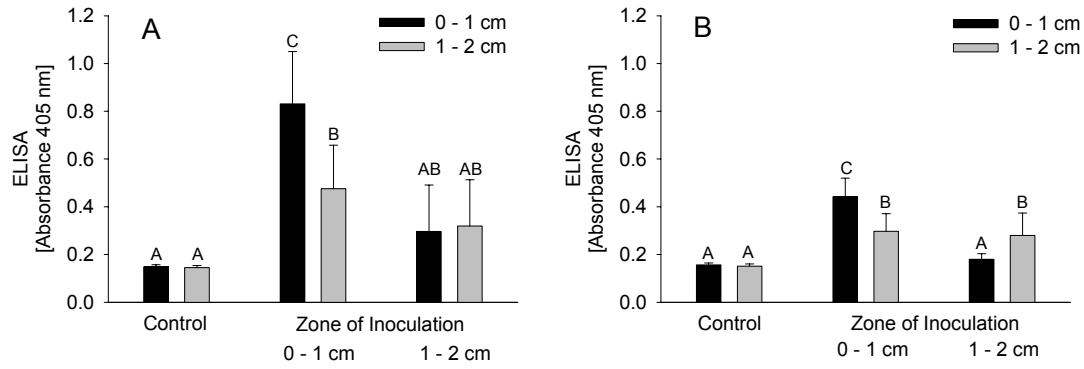

Figure 3: Infection levels of 1 cm root sections 1 dpi as affected by the zone of inoculation with P. aphanidermatum. *(A) Tomato (B) Bitter gourd. Means (n = 8) with different letters are significantly different within species at $p < 0.05$ (Tukey test).*

In a next approach, the basipetal spread of *P. aphanidermatum* from the inoculated root apex as affected by Si supply was studied. Again, the application of zoospores to the root apex lead to heavy infection of the root apex and a complete cessation of root growth within 48 h in both plant species independent of the Si supply (Fig 4). The higher ELISA readings in *P. aphanidermatum*-infected roots demonstrated the successful infection and the basipetal rapid spread of *P. aphanidermatum* along the

roots. ELISA readings declined from the inoculated root apex to more basipetal root zones. In tomato, Si treatment did affect neither the infection (ELISA reading of the apical root section) nor the spread of the fungus. However, in bitter gourd Si treatment significantly increased the ELISA reading of the apical root section, whereas the basipetal spread of the fungus was reduced by Si as indicated by significant lower ELISA readings of the Si treated roots in the more basipetal root zones.

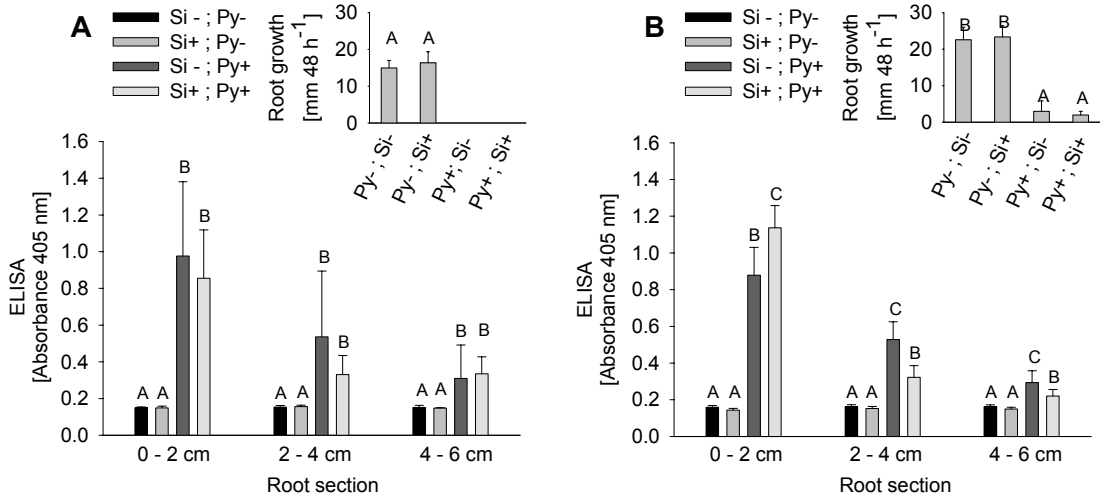

Figure 4: Effect of continuous Si supply to the whole root on root growth (inserts) and the infection by P. aphanidermatum *and its spread along individual roots 2 dpi of (A) tomato and (B) bitter gourd plants, inoculated with zoospores at the 1 cm root apex. Means (n = 6) with different letters within root sections are significantly different at p < 0.05 (Tukey test).*

The corresponding Si concentrations for the single root segments of bitter gourd are shown in Tab. 3. Inoculation with *P. aphanidermatum* did not change the Si concentration in any of the sampled root segments. However, Si application to the whole root system or only to the 2-5 cm root section resulted in significantly higher Si concentrations in the Si-treated root segments.

Table 3: Si concentrations in root sections of bitter gourd as affected by Si supply and P. aphanidermatum *(Py) inoculation 2 dpi. Means (n = 8) followed by different letters within a row are significantly different at p < 0.05 (Tukey test).*

	Silicon concentration [mg Si (g dw)$^{-1}$]			
	Si -		Si +	
Root section	Py-	Py+	Py-	Py+
0 – 2 cm*	0.42 a	0.30 a	1.14 b	1.24 b
2 – 4 cm*	0.72 a	n.d.	1.36 b	n.d.
4 – 6 cm*	0.74 a	0.53 a	1.54 b	1.61 b
2 – 5 cm**	0.86 a	0.80 a	2.01 b	1.76 b

* Si supply (1.4 mM) to the whole root system
** Si supply (1.4 mM) to the section 2 – 5 cm
n.d. = not determined

In contrast to continuous Si supply, no effect of Si on fungal spread in the roots of bitter gourd was observed when the Si supply was not continuous (Fig. 5). Neither Si supply to the complete root only during the pre-treatment nor Si supply only after transfer to the compartment boxes influenced the *P. aphanidermatum* colonisation in any of the sampled root sections. Again, in both experiments the highest ELISA readings were detected in the root tips followed by a gradual decrease in the basipetal direction. As could be expected from the experiment with continuous supply, Si had also no effect on infection and spread of *P. aphanidermatum* when applied only before or after the inoculation in tomato (data not shown).

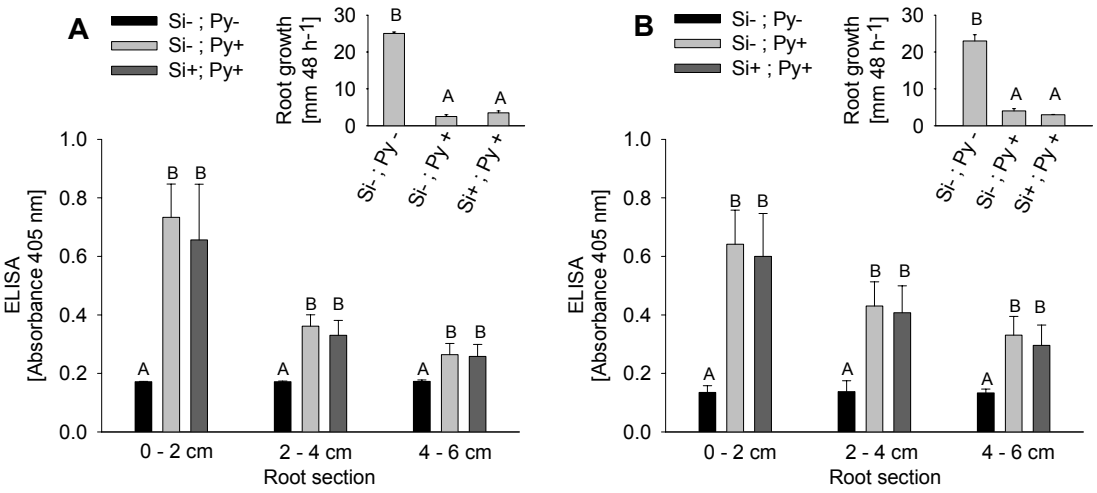

Figure 5: Effect of Si supply on the infection and spread of P. aphanidermatum *along individual roots of bitter gourd plants inoculated with zoospores at the 1 cm root apex 2 dpi. (A) Si supply during pre-treatment only (B) Si supply starting directly after inoculation. Means (n = 6) with different letters within root sections are significantly different at p < 0.05 (Tukey-test).*

A similar result was observed when bitter gourd plants were pre-grown in Si-free medium and Si supply only to the 2-5 cm root zone started simultaneously with the *P. aphanidermatum* inoculation to the root apex (Fig. 6). ELISA readings revealed that Si supply did not influence the growth of *P. aphanidermatum* within or through the Si-treated root zone although the Si concentration of this zone was more than doubled by the application of Si (Tab. 3).

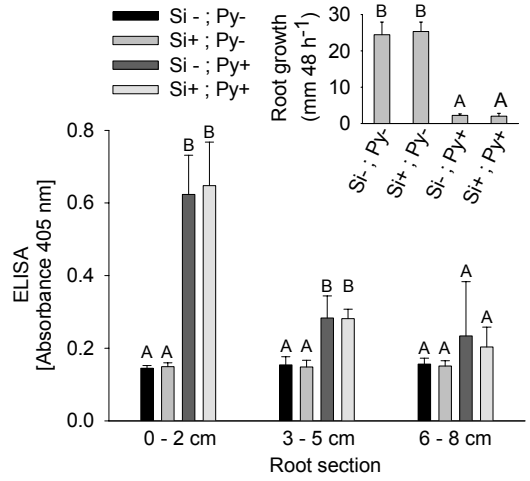

Figure 6: Effect of Si supply to the root zone 2-5 cm simultaneously with the inoculation of the root apex on the spread of P. aphanidermatum *along individual roots of bitter gourd plants 2 dpi. Means (n = 6) with different letters within root sections are significantly different at p < 0.05 (Tukey-test).*

5.5. Discussion

The results of this study clearly revealed that Si supply to Si pre-treated plants inhibits the spread of *P. aphanidermatum* in individual roots of bitter gourd (Fig. 4). In contrast, no effect was observed in identical experiments with tomato. The different effectiveness of Si as a controlling agent for *Pythium* species between tomato and other *Cucubitaceae* is confirmed by the available literature. Several studies describe the ability of Si to enhance the resistance of *Cucurbitaceae* against root rot caused by *P. aphanidermatum* and *P. ultimum* (Bélanger et al., 1995; Chérif et al., 1994b; Chérif and Bélanger, 1992). Comparable results with regard to tomato are missing, indicating that Si failed to confer resistance to this species.

The *Pythium*-specific double sandwich ELISA proved to be a suitable tool for the quantitative detection of *P. aphanidermatum* in the roots of tomato and bitter gourd. A reliable detection was possible down to 1 µg fungal mycelium per ml corresponding to 0.2 µg mycelium per sample. Using a similar ELISA, Yuen et al. (1998) found a good correlation between infection levels of roots of sugar beet and beans with *P. ultimum*. The amount of antigen was best displayed on a $\log_{10}$ scale, which was also found in our study (Fig. 1).

For both species, inoculating the 1 cm root tip resulted in significantly higher infection levels as compared to inoculation of the section 1 to 2 cm. In line with this result, root growth was almost totally inhibited when the 1 cm tips were inoculated but was only reduced to 44 % for tomato and 49 % for bitter gourd upon inoculation of the adjacent basipetal root section (Tab. 2). A cessation of root growth following a successful infection of *P. aphanidermatum* was reported for cucumber (Wulf et al., 1998) and tomato (Grosch and Schwarz, 1998). Our results clearly demonstrate that the infection by zoospores of *P. aphanidermatum* primarily took place in the root tip (Tab. 2, Fig. 3). Working with the same pathogen, Wulf et al. (1998) and Jones et al. (1991a) showed that zoospores accumulated primarily in the root-hair zone of cucumber and tomato. Since no root hairs were formed in nutrient solution in our experiments, the root apex could have been the only infection pathway in our system. This confirms that fully differentiated root tissue is less susceptible to infection by *P. aphanidermatum* (Martin and Loper, 1999).

Once established in the roots, the spread of *Pythium* spp. is affected by several factors, among them temperature (Grosch and Schwarz, 1998) and the nutritional status of the host (Hendrix and Campbell, 1973). In the case of bitter gourd, Si supply proved to be an important factor influencing the disease spread of *P. aphanidermatum*. After infection of the root apex, infection levels were significantly lower compared to –Si plants in the basipetal root sections 2-4 cm and 4-6 cm when plants were continuously supplied with Si (Fig. 3). Surprisingly, in the root tip infection levels were found to be higher in bitter gourd supplied with Si. This could have two reasons: (i) the growth of the fungus was restricted to this section due to the inability to spread and colonize basipetal sections. The consequence would be an increased growth in the root tip where the infection threat was too high for Si to be effective in suppressing fungal growth. (ii) Si enhanced zoospore germination leading to a higher number of penetration pegs. This is supported by the slight stimulation of zoospores in the presence of Si (Tab. 1). On the other hand, an increased infection level in the apex of Si treated roots was not found in the experiment with tomato. However, the Si effect on zoospore germination might have been masked by the high variability in infection of the root tips. Information on the effects of mineral nutrients on spore germination is rare. Bowen et al. (1992) observed a stimulation of Si treatment on in-vitro conidial germination of *Uncimala necata*, whereas Menzies et al. (1992) did not find an influence of Si on the germination of *Sphaerotheca fuliginea*. Zoospore germination of *P. aphanidermatum* was stimulated by high concentrations of Ca^{2+}, Mg^{2+}, and Sr^{2+} (Donaldson and Deacon, 1992).

It was proposed for leaves that Si deposition in the cell walls constitute barriers that impede the penetration of fungal hyphae (Heath and Stumpf, 1986; Heath, 1979) and it would be possible that Si in the root tissue affects plant resistance against *P. aphanidermatum* primarily through mechanical strengthening of the tissue by polymerised Si. The results of bitter gourd clearly disprove this hypothesis for a root infection of *P. aphanidermatum* since no influence of Si on the fungal spread in bitter gourd was observed when Si was applied exclusively to the root section 2-5 cm (Fig. 6), even though Si concentrations were greatly increased in the Si-treated sections (Tab. 3). The same conclusion can be drawn from the experiments in which bitter gourd plants were pre-treated with Si, but Si application was discontinued at the moment of

inoculation (Fig. 3a). Even though Si was present in the roots of pre-treated plants, Si failed to decrease fungal spread. Recently, Kauss et al. (2003) identified a gene encoding a protein that polymerises silicic acid to insoluble silica at the infection site of *Colletotrichum lagenarium* in the leaves of cucumber, thus creating a barrier to fungal spread. However, in the roots of bitter gourd, an infection with *P. aphanidermatum* did not affect the Si content (Tab. 3) so that a similar mechanism seems unlikely.

The mechanical barrier theory with regard to Si in roots was already ruled out as a disease mechanism of cucumber against *P. ultimum* (Chérif et al., 1992a, b). These authors rather emphasised a metabolic role of Si in disease resistance of cucumber by stimulating plant natural defence mechanisms. They observed a more rapid activation and higher expression of peroxidases, polyphenoloxidases, (Chérif et al., 1994a) and fungitoxic phenolic compounds (Chérif et al., 1992a) after infection with *P. ultimum* when plants were supplied with Si. It was concluded that Si is capable to induce resistance (Fawe et al., 2001). Though the mechanism how Si interferes with the growth of *P. aphanidermatum* in the roots of bitter gourd were not investigated in our experiments, an induction of resistance against *P. aphanidermatum* by Si seems possible. It was demonstrated that protein elicitors from *P. aphanidermatum* are capable to induce defence mechanisms in dicots (Veit et al., 2001), and it is possible that Si accelerates the expression of such mechanisms. As in the case of well-known activators of systemic resistance like DL-3-aminobutryric acid (Juen et al., 2000) or potassium phosphate (Mucharromah and Kuc, 1991), Si-induced defence mechanisms are activated after a lag phase: no Si effect was observed when Si supply started immediately after inoculation with *P. aphanidermatum* (Fig. 5b). However, in contrast to other activators of resistance the effect of Si was rapidly lost after plants were transferred to Si free nutrient solution (Fig. 5a). Kauss et al. (1993) demonstrated that the efficacy of a compound to induce resistance depends on its resistance to degradation. Our results suggests that the observed Si-enhanced resistance of bitter gourd against *P. aphanidermatum* is linked to cytosolic Si, which is rapidly depleted owing to polymerisation reactions and translocation to the shoot (Heine et al., 2005).

A rapid decline of Si-induced resistance after transfer to Si-free solution was also reported for the pathosystem *S. fuligena*/cucumber (Samuels et al., 1991). These authors postulated that the soluble silicic acid is the stimulating substance for the Si-induced

defence mechanisms, whereas residual polymerised Si had no effect on the pathogen. For the Si-triggered resistance of cucumber against *P. ultimum*, it was concluded that protection is not related to total Si in the root tissue but rather to the availability of mobile silicic acid at the time of infection (Chérif and Bélanger, 1992). However, a systematic study concerning the precise timing of the initiation of Si-mediated defence mechanism with regard to Si supply is missing. In addition, it is not known whether a signal for resistance can be transmitted systemically from one root to another, as demonstrated for the resistance against *P. aphanidermatum* induced by the biological control agent *Pseudomonas corrugata* in cucumber (Zhou and Paulitz 1994).

No effect of Si on the spread of *P. aphanidermatum* was observed in the roots of tomato, regardless of the experimental approach used (Fig. 4a). Interestingly, previous experiments had shown that the application of Si increases the Si concentrations in roots of tomato more than in bitter gourd. The discrepancy of Si concentrations and effectiveness with regard to disease control between the two species further supports the idea that the beneficial effect of Si on plant health is not a function primarily of Si concentration. The ability of Si to induce resistance could be rather linked to its compartmentation within the roots. By using a fractionating method for root Si, Heine et al. (2005) demonstrated that tomato is characterized by a Si accumulation in the cell-wall fraction. Since no difference in infection level between –Si and +Si-treated tomato plants was observed, it can be concluded that the cell wall Si does not influence fungal growth. In spite of the lower total concentrations in the root, Si concentrations in the symplast were higher in bitter gourd than in tomato. In the light of the presented results it therefore appears that the ability of Si to inhibit fungal spread depends on the uptake into the root symplast, whereas Si accumulation in the cell wall seems to have no influence on the disease progress. However, the way in which Si interacts with the signal transducting pathway and the molecular basis of the Si-induced resistance are still unknown (Fawe et al., 2001).

6. General Discussion

Root pathogens of the genus *Pythium* cause worldwide losses in a great variety of economic important crops (Hendrix and Campbell, 1973; Martin and Loper, 1999). In the present study, growth of both, tomato and bitter gourd was significantly reduced when plants were inoculated with *Pythium aphanidermatum*. Although pesticides for chemical control of *Pythium* species are available, recent studies give account on pathogen resistance to pesticides (Taylor et al., 2002; Moorman and Kim, 2004). Soil fumigation by chemical agents and heat are widely used practices for the control of soilborne disease under protected cultivation but entail major drawbacks, such as hazards for the environment and high costs (van Os et al., 2004). In fact, in the last years many governments have banned chemical agents used for soil fumigation. As pointed out by Katan (2004), in some cases for economical and ecological reasons it might be more reasonable to reduce a disease rather than to totally extinct it.

In line with the emergence of integrated pest and disease management, researchers started to focus on alternative means for the control of root diseases caused by *Pythium* spp. Tolerance and resistance are important components of sustainable agriculture. Even though some reports show genotypic differences in tolerance of (Higginbothan et al., 2004) or resistance (Lucas and Griffiths, 2004) against species of *Pythium*, crop varietal differences are not very marked (Martin and Loper, 1999; Stanghellini and Rasmussen, 1994; Desilets and Bélanger, 1991). An alternative to chemical control of diseases caused by *Pythium* spp. is the application of biological control agents. The spectrum of such natural antagonist includes non-pathogenic *Pythium* species that require the same ecological niche as pathogenic species (Martin and Hancock, 1987), antagonistic fungi like *Trichoderma* spp. that are widely used to control damping-off (Rose et al., 2004), as well as bacterial agents such as *Pseudomonas* fluorescens (Ramamoorthy et al., 2002; Chen et al., 1998). Together with chemical and physical factors the antagonistic microflora is considered as the reason for the reduction of pathogenic *Pythium* species in compost substrates (Ben-Yephet and Nelson, 1999) or suppressive soils (Whipps and Lumsden, 1991).

However, under practical conditions the use of natural antagonists is restricted for different reasons. Most antagonists are not registered for use in hydroponics (Stanghellini and Rasmussen, 1994). Furthermore, antagonists applied prior to

planting might not control pathogens that are introduced during the growing period (Martin and Looper, 1999). Since a particular method of disease control often does not give a sufficient protection, it is advisable to combine several methods.

The maintenance of plant health via mineral nutrition is an approach that fits perfectly in the concept of sustainable agriculture (Jacobsen, 1997). Especially the application of Si fertilizer proved to be an effective means to increase the resistance of plants for many combinations of plants and pathogens (Ma, 2004; Fawe et al. 2001; Graham, 1983) and recently, Qin and Tian (2005) demonstrated that Si and antagonists can interact in a synergistic way. In the present study it was shown that bitter gourd benefit from Si supply when inoculated with the root pathogen *P. aphanidermatum*. In pot experiments under controlled conditions both, root length and shoot weight of inoculated plants were higher when supplied with Si as compared to plants grown without the addition of Si. However, the ability to benefit from Si is not an universal characteristic for all plant species since under the same experimental conditions, no difference between the Si treatments were observed for *P. aphanidermatum* inoculated tomato plants. Also under conditions of protected cultivation in Thailand, additional Si supply to tomato plants failed to enhance the resistance against *P. aphanidermatum*.

The spatial sensitivity study revealed that a continuous supply of silicic acid slows down the spread of *P. aphanidermatum* in an individual root of bitter gourd. Thus, not only the progress of the pathogen in the direction of the shoot but also the ramification within the root system is reduced when plants are supplied with Si. Consequently, the apical root parts are to a lower degree subject to internal colonisation. This explains the positive Si effects on the growth of inoculated bitter gourd plants observed in the experiments under controlled conditions since *P. aphanidermatum* is known to inhibit plant growth in particular by the destruction of young root parts.

The reasons for the different effectiveness of Si supply among plant species in enhancing the resistance against *P. aphanidermatum* are not yet understood. In the first chapter of this study it was demonstrated that bitter gourd belongs to the Si accumulating plant species whereas tomato proved to be a Si excluder. Thus, species difference regarding Si enhanced resistance against *P. aphanidermatum* is in agreement

with the view of Ma (2004) who postulated in a recent review that beneficial effects of Si in alleviating biotic stresses are mainly expressed in plants that accumulate high amounts of Si in their shoots (i.e. Si accumulators).

This general statement is easily to understand for diseases that affect the plant shoots because Si accumulators are characterized particularly by higher Si concentrations in shoot organs. However, for root diseases such as *P. aphanidermatum* a higher effectiveness of Si-mediated disease resistance cannot be explained with higher Si root-tissue contents, because Si concentrations in roots are not known to be higher in Si accumulators as compared to Si excluders. In fact, in the present study Si concentrations in the roots were lower in bitter gourd than tomato, irrespective of whether plants were supplied or not supplied with Si. However, a fractionated Si extraction of roots revealed that in contrast to tomato, bitter gourd accumulates Si to a higher degree in the root symplast. These findings indicate that the beneficial effect of Si on plant resistance against P. aphanidermatum is linked to symplastic rather than to apoplastic effects. This hypothesis might also hold true for positive Si effects in the roots of other Si accumulators since high Si concentrations in the root symplast seem to be characteristic for Si accumulating plants (Mitani and Ma 2005; Wang et al., 2004). On the other hand, a recent example of Si effects in the roots of the Si excluder tomato (Dannon and Wydra, 2004) indicates that the uptake of Si into the root symplast is not a prerequisite for the expression of beneficial effects.

The mechanisms how Si confers resistance to pathogens are still under debate among scientists. A mechanical barrier theory ascribes the effect of Si to insoluble Si depositions that impede fungal growth. Si depositions could be constitutive (Volk et al., 1958) or induced as reaction to pathogen attack (Wiese et al., 2005). Kunoh and Ishizaki (1975) generalized that in contrast to monocotyledons in dicotyledons the deposition of Si at the penetration side of pathogens does not confirm resistance to pathogens. This view is, however, challenged by other workers. Blaich und Grundhöfer (1998) reported that a wide range of wild plants including dicots accumulate Si in the walls of cells infected with their respective powdery mildew pathogens. Heath (1979) noticed that French bean plants accumulate Si at the site of haustoria of *Uromyces phaseoli* and pointed out that the depositions are under metabolic control, since metabolic inhibitors prevented their formation. Recently, Kauss et al. (2003) identified

a gene of cucumber encoding a protein that polymerises silicic acid to insoluble silica at the infection site of *Colletotrichum lagenarium* in the leaves of plants expressing systemically induced resistance.

Information about the mechanisms by which Si interacts with plant health was predominantly obtained from studies carried out with pathogens that affect aerial parts of plants. It can be assumed that the mechanisms of Si enhanced resistance against root diseases are different due to differences in the morphology of aerial and subterranean plant organs. For example, structures like trichomes and cuticles that are frequently associated with the formation of mechanical barriers by Si in leaves are missing in roots. However, papillae can also be formed in roots and indeed wall coating with an osmiophilic, amorphous materials was reported as a Si-induced resistance mechanism of cucumber against *Pythium ultimum* (Chérif et al., 1992a). In contrast to papillae formed in leaves of cucumber as a reaction to infection by *Sphaerotheca fuliginea* (Samuels et al., 1994), no Si was detected in the amorphous material in roots. Chérif et al. (1992b) detected no Si at sites of penetration, which caused the authors to conclude that in the roots of cucumber metabolic controlled deposition of Si to form a barrier is not a mechanism of Si conferred *Pythium* resistance. This conclusion is supported by the results of the Si measurement in the current study. Neither for the complete root system nor for 2 cm segments of single roots the inoculation with *P. aphanidermatum* lead to increased Si contents. In addition, Si application to a defined root segment increased the Si concentration of this segment but did not prevent the spread of the pathogen.

Based on the results of this study and on the literature a constitutive physical barrier formed by Si depositions is unlikely as the major mechanism to prevent the spread of *P. aphanidermatum* in roots. Barriers impeding fungal spread are generally associated with lignin depositions in the plant cell walls (Nicholson, 1992; Vance et al., 1980) and the cell wall is also the main site of barrier formation by Si (Heath, 1979). Accordingly, Si depositions in roots are frequently associated with lignin depositions (Sangster and Hodson, 1992; Williams, 1986). Thus, it could be possible that Si impedes the intracellular spread of *P. aphanidermatum* by stabilizing the root cell walls. However, such an effect would be expected in tomato rather than in bitter gourd due to the higher Si contents in the cell walls of tomato. On the other hand, it could still

be possible that due to differences in the chemical composition of cell walls (e.g. amount and composition of phenolic substances) Si is capable to form a barrier against the pathogen in the cell walls of bitter gourds but not in tomato. Nevertheless, even when considering the results for bitter gourd separately, the possibility of a mechanical barrier formed by insoluble Si appears unlikely. A stabilizing effect of Si should have an impact already on the infection by the pathogen and initial penetration into the root. On the contrary, in the spatial sensitivity experiments no differences in the colonization of the 2 cm root tip was observed between Si treatments. In addition, it was shown that a root segment of bitter gourd with a high Si status did not inhibit the spread of *P. aphanidermatum*. Another factor that raises questions about the role of Si as a physical barrier impeding the spread of *P. aphanidermatum* is the fact that the pathogen is capable of producing pectolytic and cellulolytic enzymes (Van der Plaats, 1981). Whether Si can stabilize the cell wall when challenged with cell wall macerating enzymes is not known.

The physical barrier was also ruled out for the related pathosystem cucumber/ *P. ultimum* (Cherif et al., 1991a and b). Based on the finding that the rapidity and extent of fungitoxic phenolic depositions in the roots of infected plants was stimulated by Si supply, Chérif et al. (1994) put forward a theory that Si activates natural defence mechanisms of cucumber against *Pythium* spp. It was emphasised that the soluble form of Si, silicic acid, is the compound responsible for the elicitation of resistance mechanisms (Fawe et al., 2001). Recently, Veit et al. (2001) purified a protein elicitor from *P. aphanidermatum* that triggers a wide range of dicotyledones plants to induce defence mechanisms. It is possible that silicic acid somehow accelerates the expression of these naturally occurring mechanisms, thus conferring to the plant a temporal defence advantage against the pathogen. The spatial sensitivity experiments revealed that the effect of Si in impeding the spread of *P. aphanidermatum* in the roots of bitter gourd depends on a continuous supply of silicic acid to the whole root system, and the protection ceased when the supply with silicic acid is disconnected. This behaviour that is in contrast to other resistance activators can be explained by the low stability of silicic acid in the roots of bitter gourd due to the rapid translocation to the shoot (Heine et al., 2005a). A similar finding was made for cucumber by

Samuels et al. (1991) who observed a drop in Si-induced resistance against *S. fuligenea* when plants were transferred to Si-free solution.

Even though there is good evidence that Si can stimulate plant natural defence mechanism, Si interactions with the signal pathways of plants are elusive. The key problem for an improved understanding of metabolic functions of Si is that almost nothing is known about the involvement of Si in biochemistry (Epstein, 1999). An affinity of silicic acid to hydroxyl groups is likely (Williams, 1986) and ester-like bounds of silicic acid as bridge between polyuronides (Jones, 1978) are possible from a chemical point of view. Inanaga et al. (1995), by using UV absorption spectroscopy, found that Si deficiency decreased the level of a lignin-carbohydrate complex in the cell walls of rice. They proposed that in the cell walls of rice Si is involved in the formation of cross-links between lignin and carbohydrates. However, no organo-Si complexes have been detected under physiological conditions up to now (Peggs and Bowen, 1984; Knight and Kinrade, 2001), which demonstrates that a direct involvement of Si in plant metabolic functions is not yet proven. On the other hand, due to its chemical properties it appears likely that silicic acid has a high affinity of to o-diphenols. Weiss and Herzog (1978) and Williams (1986) indicated that organic hydroxyl-groups of phenols could be condensed with silicic acid in biological systems. Even though phenolic compounds can be precursors for phytoalexins (Siegrist et al., 1994) it is still elusive how Si can give rise to the production of phytoalexin from the group of flavonols, which was reported by Fawe et al. (1998) as a Si-induced defense reaction of cucumber against *S. fuliginea*. Jones et al. (1978) reported that in the roots of Si-deficient wheat the concentrations of phenolic compounds were enhanced at the expense of the lignin content.

Similar mechanisms could explain reports about increased plant resistance under Si deficiency that are not in agreement with the widely available literature on positive Si effects. Heath and Stumpf (1986) associated the finding that penetration of *U. phaseoli* in French bean stopped earlier in plants grown under Si-free conditions with the higher level of wall-associated phenolic compounds in Si-deprived plants. Carver et al. (1988) reported that in oat plants attacked by *Blumeria graminis* the activity of phenylalanine ammonia lyase and the production of phenols was stimulated by Si deprivation.

However, even under controlled conditions Si deficiency is difficult to achieve, and Si research in general is hampered by the fact that it is technical impossible to create a zero Si treatment. Highly purified water still contains between 10 nM Si (Werner and Roth, 1983) and 20 nM Si (Takahashi et al., 1990) and these minute amounts might be sufficient for the maintenance of all Si-related processes in plants, especially in species with a low Si uptake. A further development in water purification technology and analytical chemistry thus decreasing Si concentration in nutritional solutions near to zero would probably be the key for further progress in Si research.

Turning from lab-experiments to more practical application of Si supply, it appears that the potential of Si in agriculture has not yet been widely investigated. Voogt and Sonneveld (2001) summarized the effect of Si application to greenhouse crops in the Netherlands. They reported Si-increased yields of cucumber, zucchini, and roses whereas no yield difference was found for French bean and strawberry. However, Miyake and Takahashi (1986) achieved a yield increase of 29% by supplying strawberries with Si. Adatia and Besford (1986) found no effect of Si nutrition on the yield of cucumber and tomato. On the other hand Si deficiency was reported to reduce fruit yield of tomato (Miyake and Takahashi, 1978) and cucumber (Miayke and Takahashi, 1983). In most cases, the beneficial effect of Si nutrition seems to be related to the alleviation of some kind of biotic or abiotic stress whereas Si treatments usually have no effect under stress-free conditions (Ma, 2004; Epstein, 1999; Marschner, 1997). This is supported by the results of the present study. No effect of Si was ever observed on tomato or bitter gourd when plants were not exposed to a stress in the form of *P. aphanidermatum* inoculation. Stamatakis et al. (2003) reported that Si supply to tomato grown in soil culture positively affected yields under conditions of salt stress but not under stress-free conditions. An interesting example for the sometimes unpredictable effects of Si under field conditions is the results of Heckman et al. (2003). Whereas Si supply increased yield of pumpkin as much as by 60% in one year no effect on yield was found in the following year. However, it is not clear whether this difference was related to differences in powdery mildew epidemics caused by *Podosphaera xanthii*.

Even though the results of this and other studies demonstrate the potential of Si to enhance the resistance of plants against diseases, it is not self-evident that the positive effects of a Si supply can be observed under field conditions. Jones and Handreck

(1963) reported that the Si concentrations in soil solutions are commonly between 0.5 and 0.65 mM, and many soils (especially in the temperate regions) are capable of maintaining a steady state of silicic acid in the soil solution despite Si uptake of plants (Jones and Handreck, 1969). These concentrations might be already high enough for a good protection of plants. For example, Leusch and Buchenauer (1988) found a good control of *Erysiphe graminis* in wheat already at plant-available Si concentrations of 0.33 mM. As stressed by Epstein (1999), under natural conditions the situation of low Si supply is the exception whereas the state of Si supply can be regarded as control. The situation might be different in highly weathered tropical soils that are often characterized by low concentrations of plant-available Si. For an Oxisol and an Inceptidol in Colombia, Seebold et al. (2000) reported concentrations of 0.035 and 0.14 mM plant-available Si, respectively. Under such conditions, Si supply to crops with a high Si demand like rice or sugarcane can be highly efficient in suppressing diseases (Savant et al. 1999; Datnoff et al., 1997).

Due to the direct exposure to the environment, field grown plants are exposed to a higher level of stress than plants grown under laboratory conditions. Many abiotic stresses including osmotic stress and proton stress (Wiese et al., 2004), phosphate application (Gottstein and Kuc, 1989) and manganese toxicity (Fecht-Christofferes et al., 2003) were reported to induce resistance against pathogens. Thus, it can be speculated that under practical conditions natural inherent resistance mechanisms are often elicited by other stresses, thus masking potential Si effects.

When comparing with field growth, plants grown under protected cultivation like greenhouses are better protected from abiotic stresses and consequently Si effects can be expected to be more stable. In addition, under protected cultivation Si supply is often limited due to use of peat-based substrates, rockwool, or deionised water. Accordingly, the most spectacular and reproducible results of Si nutrition on disease control were obtained from cucumber grown under protected cultivation in greenhouses (Bélanger et al., 1995).

In contrast to the experiments described in the review of Bélanger et al. (1995), the Si level in the control treatment was rather high in the experiments with tomato under controlled conditions in Thailand presented in this study. This could explain that no effect of additional Si supply was observed on *P. aphanidermatum* inoculated plants in

addition to the fact that, even under controlled condition, Si failed to stimulate the disease resistance of tomato against the pathogen.

Even when assuming that Si could enhance the disease resistance of tomato against *P. aphanidermatum* there are concerns from a pathological point of view whether such an effect could be observed in the system that was used in the experiments in Thailand. It has been proven by *in vitro*-experiments that Si does not have a direct detrimental effect on the pathogen. Since the exposure of the plants to Si and the inoculation with the pathogen occurred simultaneously it is, therefore, unlikely that the Si treatment could control damping-off. Support for this conclusion is provided by the finding that the Si-induced disease mechanism was not expressed immediately after the beginning of the Si supply but required a certain time of pre-treatment (chapter 4) and by the well known fact that damping-off occurs predominantly within a short time after infection.

Once introduced into the substrate, the pathogenic activity of *P. aphanidermatum* seems to decline rapidly, which is caused by poor competition with other microorganism and the ephemeral nature of its mycelia (Martin and Hancock, 1986; Stanghellini and Bur, 1973). In agreement with this, after an initial growth inhibition of inoculated tomato plants no further difference in plant growth between treatments was observed. Though an infection of tomato plants by oospores would still be possible, oospore germination is known to be suppressed by several factors, among them constant high soil moisture (Mondal et al., 1995) as present under the existing experimental conditions. It is, therefore, doubtful whether a continuous new infection of tomato roots by *P. aphanidermatum* took place during the experimental period. Since on older plants other effects of *P. aphanidermatum* than the destruction of root tips are not considered as relevant (Martin and Loper, 1999), no effect of Si supply on plant health could be observed.

However, long-term effects of Si in increasing plant resistance against *Pythium* spp. were observed in hydroponics (Bélanger et al., 1995). In contrast to the peat substrate-based system used in the current study, the suppression of *Pythium* spp. by other microorganisms is much lower due to a biological vacuum in hydroponics (Os et al., 2004). In addition, hydroponics facilitates the spread of certain *Pythium* species within the system by the means of zoospores (Stanghellini and Rasmussen, 1994; Sanogo and

Moorman, 1993; Jenkins and Averre, 1983) thus giving rise to a continued new infection of young roots. Therefore, although Si nutrition failed to increase the resistance of tomato against *P. aphanidermatum* in this study, it might still bear the potential to enhance plant health under other experimental conditions or against other, more persistent pathogens.

7. Outlook

From the results of this study and from the literature it becomes clear that Si nutrition can contribute to disease control in sustainable agriculture. However, plant species differ in their ability to benefit from Si supply. In the current study, Si supply stimulated the resistance against the root pathogen *P. aphanidermatum* of bitter gourd but not of tomato. It appears, that the capability of plants to benefit from Si supply is related to the uptake of Si into the root symplast.

In order to confirm this hypothesis, it would be necessary to conduct similar studies with other crops exhibiting diverse degrees of Si uptake and by using different root pathogens. It would be of advantage to select pathogens or strains that cause a sub-lethal infection because long-term effects of Si would be probably better visible. These experiments, which should be conducted under both growth-chamber and practical conditions, could give rise to a targeted use of Si in the control of root diseases.

In the current study, the Si-induced resistance in bitter gourd was reflected by a slower spread of *P. aphanidermatum* in roots. Interestingly, the resistance was rapidly lost after transferring the plants to Si-free nutrient solution thus contrasting the mode of action of other compounds capable to induce resistance. A detailed kinetic study about the exact period of Si pretreatment required for the induction of resistance mechanism would be necessary to fully understand the interactions between Si, plant, and pathogen. Since it is not known whether the degree of resistance increases linearly with Si concentration or rather starts at definite threshold concentrations, it would be also of interest to use different concentrations of Si in the experiments.

There is strong evidence that the Si-induced resistance of cucurbitaceous species like bitter gourd against *Pythium* spp. is based on the rapid activation of enzymes related to plant defense against pathogens (Chérif et al., 1994) and to the formation of phenolic compounds with fungitoxic properties (Fawe et al, 2001). However, little is known about the physiological background of Si-induced resistance. It is also unknown whether Si induces metabolic changes already prior to inoculation thus preparing the plant to pathogen attack. Consequently, the next step in research on Si-induced resistance should focus on the interactions between Si and plant metabolic functions. In a basic approach, gene activation due to Si could be investigated by using a substractive cDNA library in order to identify genes that are responsive to Si. The influence of Si on the plant

proteom could be studied by using 2D-gel-electrophoreses, which would reveal whether Si induces the synthesis of higher levels of specific proteins.

It would be also interesting to clarify whether the Si-induced resistance is a local event confined to zones of elevated concentrations of silicic acid or whether the resistance can rather spread systemically within the root system as reported by Zhou and Paulitz (1994) for resistance against *P. aphanidermatum* induced by *Pseudomonas corrugata*. This question could be addressed by using split-root systems. In case of systemically induced resistance, it would by tempting to search for signals (e.g. salicylic acid) that are involved in the transmission of resistance.

To my knowledge, the role of Si as a physical barrier to pathogens was always discussed with regard to insoluble Si accumulations impeding the growth of hyphae. Rarely has been taken into account the possibility that Si could stabilize cell walls from degradation by cell-wall macerating enzymes, such as pectolytic enzymes released by *P. ultimum* and *P. aphanidermatum* (Chérif et al., 1991; Van der Plaats, 1981). On the other hand, it was speculated that Si is capable to stabilize pectin by acting as bridges in the structural organization of polyuronides (Jones, 1978). In order to investigate whether Si stabilizes pectin thus reducing cell-wall degradation by the pathogen, it would, therefore, be interesting to correlate the pectin content in roots with the progress of *Pythium* colonization. If the available (spectrometric/ fluorometric) methods for pectin quantification are not sensitive enough, the use of specific antibodies could be an interesting approach, especially since antibodies for different pectin epitopes and for *Pythium* spp. are available. The distribution of pectin and *Pythium* could then be localized using a confocal laser-scanning microscope.

It was emphasized that the ability to benefit from Si in root-disease resistance is related to the uptake of Si into the roots symplast. Accordingly, an approach to enhance the stress resistance of plants with low Si uptake would be the increase of Si uptake. Recently, Mitani and Ma (2005) concluded from kinetic studies that even the Si excluder tomato possesses transporters for active uptake of silicic acid into the root-cell symplast, and that the affinities of transporters is comparable between different plant species. These results indicate the possibility of increasing Si uptake of plants by classical breeding. The Si content in the root symplast could be used as a marker. This approach opens the possibility to enhance the resistance of Si excluders against root diseases.

8. Reference

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9. Silicon nutrition and resistance against Pythium aphanidermatum of Lycopersicon esculentum and Mormodica charantia – Summary

The soil-borne pathogen *Pythium aphanidermatum* limits the production of a wide range of economic important crops. Current control strategies are mainly based on chemical agents with inherent hazards for the environment and human health.

Silicon (Si) supply is an environmental friendly strategy to enhance disease resistance in many pathosystems. However, it is still elusive whether the ability to benefit from Si is a universal characteristic of all plants species or rather linked to their pattern of Si uptake.

For this study, which aimed at contributing to sustainable vegetable production in the humid tropics, two important horticultural crops, *Lycopersicon esculentum* (tomato) and *Mormodica charantia* (bitter gourd), were selected that are characterized by low and high Si uptake, respectively.

An in-depth study on Si uptake showed that tomato discriminates Si from uptake leading to an accumulation of Si in the root water free space (RWFS). In contrast, bitter gourd actively takes up Si, which was illustrated by a calculated and measured depletion of Si at the root surface and the RWFS. A fractionated Si analysis of roots revealed that in tomato, root Si is almost completely located in the cell walls whereas bitter gourd accumulates Si to a higher degree in the root symplast.

Under controlled conditions even a moderate inoculation of tomato plants with *P. aphanidermatum* resulted in damping-off and seedling mortality was enhanced with increasing levels of inoculation. For tomato and bitter gourd, a sub-lethal infection caused a reduction of root length and shoot growth. Amendment of the substrate with Si did not have an effect on tomato or non-inoculated bitter gourd plants. However, growth of *P. aphanidermatum* inoculated bitter gourd plants was stimulated by Si supply. The effect was linked to a lower degree of infection in the roots, as revealed by an ELISA specific for *Pythium* spp.

P. aphanidermatum was also highly pathogenic for tomato under practical growing conditions of protected cultivation in Thailand. An inoculation of the substrate with the pathogen caused damping-off and the growth of surviving plants was reduced. Attempts to re-isolate the pathogen from the substrate as well as a quantification of the infection level in washed roots after harvest indicated that the pathogenic potential

of *P. aphanidermatum* declined during the growing period under the experimental conditions. In agreement with the experiments under controlled conditions, Si did not affect growth and fruit yield of tomato, regardless of the *P. aphanidermatum* inoculation.

The effect of Si supply on the spread of *P. aphanidermatum* in the roots of tomato and bitter gourd was further studied on a single root level using rhizotrons. The *Pythium* spp.-specific ELISA allowed a quantitative assessment of *P. aphanidermatum* colonization in 1-2 cm root segments. Inoculation of individual apical root zones with zoospores of *P. aphanidermatum* revealed that particularly the inoculation of the 1 cm root apex led to a strong inhibition of root growth. Si supply did not alleviate the inhibition of root growth by *P. aphanidermatum* applied to the root apex in either species. In tomato no effect of Si supply was observed on the basipetal spread of *P. aphanidermatum* from the infected root apex. However, in bitter gourd the spread of *P. aphanidermatum* in the roots was inhibited when plants were continuously supplied with Si before and after the inoculation. Only Si pretreatment of the plants and Si application to the whole root system or individual root zones together with the root inoculation did not affect the spread of the pathogen in the roots.

In conclusion, Si does not enhance the resistance against *P. aphanidermatum* of the Si excluder tomato despite high Si contents in the root cell wall. In contrast, the pathogen resistance of the Si accumulator bitter gourd can be stimulated by Si supply. The Si effect depends on a continuous supply of Si to the whole root system. The results of this study indicate that the beneficial effect of Si on plant resistance against *P. aphanidermatum* is linked to symplastic rather than apoplastic effects.

Keywords: Silicon/*Pythium* / tomato / bitter gourd / plant resistance / ELISA / protected cultivation

10. Siliziumernährung und Resistenz gegen *Pythium aphanidermatum* bei *Lycopersicon esculentum* und *Mormodica charantia* - Zusammenfassung

Das bodenbürtige Pathogen *Pythium aphanidermatum* (*P. aphanidermatum*) gefährdet die Produktion einer großen Anzahl von Nutzpflanzen. Die gegenwärtigen Strategien zur Bekämpfung des Pathogens beruhen vor allem auf chemischem Pflanzenschutz, was Risiken für die Umwelt und die menschliche Gesundheit mit sich bringt.

Der Einsatz von Silizium (Si) ist eine umweltfreundliche Strategie zur Erhöhung der pflanzeneigenen Resistenz in vielen Pathosystemen. Es ist aber nicht bekannt, ob Si bei allen Pflanzenarten positive Wirkungen hervorruft oder ob diese Eigenschaft an einem besonders effektiven Si Aufnahmemechanismus gebunden ist. Für die vorliegende Arbeit, die ein Beitrag zum nachhaltigen Gemüseanbau in den Tropen ist, wurden mit *Lycopersicon esculentum* (Tomate) and *Mormodica charantia* (Bitter gourd) zwei wichtige gemüsebauliche Nutzpflanzen gewählt, die durch eine niedrige bzw. hohe Si Aufnahme charakterisiert sind.

Eine detaillierte Studie der Si Aufnahme zeigte, dass Tomate Si von der Aufnahme diskriminiert, wodurch Si im Wasser Freien Raum der Wurzel (RWFS) akkumuliert. Im Gegensatz dazu nimmt Bitter gourd Si aktiv auf, was sowohl durch eine kalkulierte als auch gemessene Si-Verarmung im RWSF bestätigt wurde. Eine fraktionierte Si Extraktion aus Wurzeln ergab, dass Si in den Wurzeln von Tomaten fast ausschließlich in den Zellwänden gebunden vorliegt, wohingegen Bitter gourd Si in einem höheren Maße im Symplasten akkumuliert.

Unter kontrollierten Bedingungen verursachte *P. aphanidermatum* schon bei geringen Inokulationshöhen das Absterben von Tomatenpflanzen, und die Mortalität stieg mit steigenden Inokulationshöhen an. Sowohl bei Tomate als auch bei Bitter gourd führte eine sub-letale Infektion zu einer Reduzierung von Wurzellänge und Sprosswachstum. Si-Gaben über das Substrat hatten keinen Einfluss auf nicht-inokolierte Pflanzen. Auch mit *P. aphanidermatum* inokulierte Tomatenpflanzen reagierten nicht auf Si-Gaben. Im Unterschied dazu war das Wachstum von inokulierten Bitter gourd Pflanzen durch Si Gaben stimuliert. Gleichzeitig wurde mittels eines für *Pythium* spp. sensitiven ELISA festgestellt, dass die Infektion der Wurzeln Si-Versorgter Pflanzen verringert war.

Auch unter praktischen Anbaubedingungen in Gewächshäusern in Thailand war *P. aphanidermatum* pathogen an Tomate. Eine Inokulation des Substrates führte zum Absterben von Pflanzen und das Wachstum überlebender Pflanzen war verringert.

Versuche, das Pathogen aus dem Substrat zu reisolieren, sowie die Quantifizierung der Infektionshöhe in ausgewaschenen Wurzeln nach Beendigung des Versuches deuteten an, dass sich das pathogene Potential von *P. aphanidermatum* unter den vorhandenen Bedingungen während des Experimentes verringerte. Übereinstimmend mit den Versuchen unter kontrollierten Bedingungen hatte Si-Gabe sowohl bei der inokulierten- als auch bei der nicht-inokulierten Variante keinen Einfluss auf Wachstum und Fruchtertrag der Tomatenpflanzen.

Die Wirkung von Si-Gaben auf die Ausbreitung von *P. aphanidermatum* in den Wurzeln von Tomate und Bitter gourd wurde mit Hilfe von Rhizotronen an einzelnen Wurzeln untersucht. Durch den *Pythium* spp. spezifischen ELISA war es möglich, in 1-2 cm Wurzelsegmenten die Kolonisation mit *P. aphanidermatum* quantitativ zu erfassen. Die Inokulation von definierten apikalen Wurzelsegmenten mit Zoosporen von *P. aphanidermatum* zeigte, dass besonders die Inokulation der 1 cm Wurzelspitze zu einer starken Hemmung des Wurzelwachstums führte. Bei keiner der beiden Pflanzenarten führten Si-Gaben zu einer Aufhebung der Hemmung des Wurzelwachstums. Si-Gaben zu Tomate beeinflussten nicht die basipetale Ausbreitung von *P. aphanidermatum* aus der infizierten Wurzelspitze. Dagegen verringerte bei Bitter gourd eine kontinuierliche Si-Gabe sowohl vor als auch nach der Inokulation die Ausbreitung von *P. aphanidermatum* in die Wurzeln. Wenn die Gesamtwurzel hingegen entweder nur während der Vorbehandlung oder nur nach der Infektion mit Si versorgt wurden, oder wenn nur einzelne basipetale Wurzelzonen mit Si behandelt wurden, führte dies nicht zu einer verminderten Ausbreitung des Pathogens.

Es wird geschlussfolgert, dass bei dem Si-Exkluder Tomate eine Si-Gabe trotz der hohen Si-Gehalte in der Zellwand nicht die Resistenz gegen *P. aphanidermatum* erhöht. Im Gegensatz dazu kann die Resistenz des Si-Akkumulators Bitter gourd durch Si stimuliert werden. Der Effekt hängt von einer durchgehenden Si-Zufuhr zum gesamten Wurzelsystem ab. Die Ergebnisse dieser Studie implizieren, dass die positiven Effekte von Si auf die Resistenz gegen *P. aphanidermatum* eher auf einem symplastischen als auf einem apoplastischen Effekt beruhen.

Schlagwörter: Silizium/*Pythium* / Tomate / Bitter gourd / Resistenz / ELISA / kontrollierter Anbau