Abstract

Jung, M.C.V. 2003. *The role of selected plant and microbial metabolites in the nutrient solution of closed growing systems in greenhouses.*
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Recirculation of nutrient solution in greenhouse growing systems inhabits environmental and economic advantages regarding saving water and fertilizers. The disadvantages are a greater risk for spread of phytopathogens by the nutrient solution and for accumulation of organic compounds at phytotoxic levels. Organic compounds are excreted as root exudates by the crop as well as by microorganisms in the rhizosphere and are also being released by constituent devices in the growing system. In the present thesis, benzoic, caffeic, chlorogenic, ferulic, *p*-hydroxybenzoic, salicylic, vanillic acids, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin and 2,4-diacetyl phloroglucinol were studied with respect to occurrence in the nutrient solution of closed hydroponic growing systems, phytotoxic response, persistence and optimised formation.

Phytotoxicity levels were studied on young tomato plants exposed to initial concentrations of benzoic, caffeic, chlorogenic, ferulic, *p*-hydroxybenzoic, salicylic, vanillic acids in the fresh solution. Effects were seen primarily on roots at 200 and 400 µM for most of the compounds. Supported liquid membrane technique was adopted to extract several plant and microbial metabolites *in situ* in the greenhouse. The method was then used to study the nutrient solution in tomato, cucumber and gerbera crops, which were grown in closed growing systems including artificial infection of root pathogenic fungi and/or selected disinfestation methods, namely slow sand filtration and UV-radiation. They were investigated at different developmental stages and compounds were determined in the range of 10-200 nM in the nutrient solutions. Benzoic acid was found throughout all crops, sampling occasions and treatments. Furthermore, *p*-hydroxybenzoic acid was determined, as well as occasionally 2,4-diacetyl phloroglucinol, especially in systems with slow sand filter treatment. *In vitro,* synthetical organic compounds added to effluent nutrient solution disappeared rapidly, mostly within two days. The occurrence of plant and microbial metabolites should rather be regarded as an asset. Optimising the living conditions for the resident microflora in the nutrient solution might favour biocontrol of root pathogens. Increased metabolite production of the control strain was observed. However, low levels of selected microbial metabolites were found after enrichment of the nutrient solution microflora. Preconditions for future studies are discussed.

Keywords: antagonist, cucumber, disinfestation, phenolic acid, gerbera, recirculation, root pathogen, slow filtration, tomato, UV-radiation

Author's address: Victoria Jung, Department of Crop Science, Division of Root Biology, SLU, P.O. Box 44, SE-230 53 Alnarp, Sweden. Victoria.Jung@vv.slu.se

Populärvetenskaplig sammanfattning

I kommersiell växthusproduktion av både grönsaker och snittblommor använder man sig idag i norra Europa mestadels av odlingssystem där plantorna växer i stenullsmattor som bevattnas med näringslösning. För att undvika onödigt stor vatten- och näringsämnesförbrukning samlas bevattningsvattnet från odlingen upp och återanvänds (*slutna odlingssystem*). Man undgår utsläpp av näringsberikat vatten till kringliggande mark vilket kan leda till övergödning av sjöar och vattendrag.

Återanvändning av bevattningsvattnet kräver att näringsinnehållet justeras och oftast genomgår vattnet någon form av rening för att undvika eventuell spridning av växtsjukdomar som finns i odlingen. Förutom sjukdomsalstrare (*patogener*) skulle även organiska ämnen kunna anrikas i lösningen, t. ex. fenoliska syror som av olika anledningar utsöndrats från växtrötter och från mikroorganismer i rotmiljön. Under det sista decenniet har dessa fenoliska syror ofta fått skulden för att en odling där bevattningsvattnet återanvänts (*recirkulerats*) har misslyckats.

Mitt avhandlingsarbete har syftat till att undersöka dessa organiska syrors förekomst i näringslösningen. Arbetet har ingått i ett EU-projekt med titeln MIOPRODIS (”MIcrobial Optimization to Prevent ROot DISeases”), som har knutit ihop arbete i fem länder med tre universitet, två forskningsstationer och två näringsidkare som aktörer (http://europa.eu.int/comm/research/agro/fair/en/nl4309.html, http://www.imag.wageningen-ur.nl/PDF/mioprodis.pdf).

Vid vilken koncentration reagerar plantorna negativt på de olika syrorna? Unga tomatplantor utsattes för olika koncentrationer av bensoesyra, kaffesyra, klorogensyra, ferulasyra, *p*-hydroxybensoesyra, salicylsyra och vaniljsyra (artikel I). Koncentrationsnivåerna som skulle till för att ge tydliga tillväxthämningar låg för flera syror mellan 200 och 400 µM. Vilka nivåer förekommer då ”naturligt” i näringslösningen av slutna odlingssystem? Jag var ute efter att få en rättvisande bild av situationen i odlingssystemet. Därför anpassade jag en extraktionsteknik (*SLM*) som koncentrerar de oerhört små mängderna syror direkt i växthuset (artikel II). Vilka syror kan man hitta och i vilken mängd? Prover från flera odlingssystem med gerbera, gurka och tomat undersöktes (artikel IV). Två reningsåtgärder testades i sammanhanget: sandfilter och UV-behandling. Prover togs vid fem tidpunkter under två odlingssäsonger för att se huruvida syrorna ackumulerades i systemet. Näringslösningen i vissa led i försöket hade infekterats med en växtskadlig svamp. Jag fann en rad syror som t. ex. bensoesyra och *p*-hydroxybensoesyra. De förekom i mycket låga koncentrationer, 10-200 nM (artikel II, IV), dvs runt tusen gånger lägre än vad som är skadligt för växten (artikel I). Undersökningar av näringslösning i gerbera-, gurk- och tomatodlingen under säsongen och med olika reningssystem gav dock inget specifikt mönster och heller inte någon ackumulering av de fenoliska syrorna (artikel IV). Vad kan då dessa låga koncentrationerna bero på? Omsättningshastighet i näringslösningen studerades därför för valda organiska syror (artikel III) och det visade sig att den naturliga mikrofloran tog hand om de tillsatta ämnena inom loppet av två dygn.

Dessa undersökningar ger inget stöd åt teorin att fenoliska syror äventyrar kulturen i slutna odlingssystem. Diskussionen har kretsat kring de skadliga effekterna i odlingen, däremot har få undersökningar gjorts på eventuella positiva effekter av fenoliska ämnen. Vissa mikroorganismer kan bilda fenoliska ämnen som hämmar sjukdomsalstrande svampar, dvs. de fungerar som biologisk bekämpning. För att stimulera mängden av dessa substanser, har jag i artikel V undersökt möjligheten att öka deras produktion i näringslösningen från slutna odlingssystem. Dessa första resultat visar att möjligheten finns även om tillvägagångssättet måste förfinas i framtida studier.

Contents

Appendix

The present thesis is based on the following papers (I-V), which will be referred to by their Roman numerals:

I. Jung, V., Olsson, E., Asp, H., Jensén, P., Caspersen S. and Alsanius, B.W. 2002. Response of young hydroponically grown tomato plants to phenolic acids. *Scientia Horticulturae. In press.*

II. Jung, V., Chimuka, L., Jönsson, J.Å., Niedack, N., Bowens, P. and Alsanius, B.W. 2002. Supported liquid membrane extraction for identification of phenolic compounds in the nutrient solution of closed hydroponic growing systems for tomato. *Analytica Chimica Acta*. 474: 49-57.

III. Alsanius, B.W. and Jung, V. 2003. Potential of utilization of selected organic compounds by the microflora inhabiting the effluent nutrient solution of closed greenhouse systems. *Submitted to European Journal of Horticultural Science*.

IV. Jung, V., Jönsson, J.Å., Whipps, J., Pettit, T., Jackson, A., Wohanka, W., Seidel, R., van Os, E., Bruins, M., Postma, J., McPherson, M. and Alsanius, B.W. 2003. Occurrence of selected plant and microbial metabolites in nutrient solution at different developmental stages of three crops grown in closed systems as affected by disinfestation treatments. *Submitted to Horticultural Science and Biotechnology*.

V. Jung, V. and Alsanius, B.W. 2003. Prospects for enhanced phenolic metabolite formation by the microflora inhabiting nutrient solution of closed growing systems. *Manuscript*.

Introduction

Background

Sustainable production of greenhouse crops is an important topic in today's horticulture and emphasises many different aspects, ranging from energy use, climate control, plant morphology, pathogen and pest management, to nutrient and water use. The need for environmentally sound growing systems is not only set by legislation, but is also demanded by growers, who have become more concerned about health risks while working with chemicals, and last but not least by consumers, who have developed an increased awareness of buying products of environmentally sound origin. The use of soilless growing systems has become increasingly common, due to enhanced yield, easy management of water and nutrient supplies and the potential to avoid chemical soil fumigation such as methyl bromide (van Os and Benoit, 1999; Gieling *et al*., 1997). Commercial soilless growing systems most often adopt stone wool as a growing medium, but nutrient film technique is also used (Göhler and Molitor, 2002), as well as growing systems including pumice, vermiculite and perlite. In the European Union, soilless production of vegetables covered 4600 ha out of a total of 16600 ha (Göhler and Molitor, 2002) by the beginning of the 1990s and had grown to 8500 ha in 1996 (Steiner, 1996). In 1999, protected vegetable production in Sweden was 145 ha out of a total of 331 ha greenhouse production, with tomato and cucumber production representing over 80% of the greenhouse vegetable production (SCB, 2000).

Hydroponic growing systems

Jensen and Collins (1985) defined hydroponics to be a technology for growing plants in nutrient solution with or without an artificial medium such as sand, gravel, vermiculite, stone wool, peat moss and saw dust to provide mechanical support. Within the technology, solid and liquid hydroponic growing systems are differentiated.

Solid hydroponic growing systems

Mineral wool is the collective term for stone wool and glass wool products with rockwool being a commercial brand of stone wool (Henri Beekers, personal communication). Although mineral wool production requires high amounts of energy, the use of both glass wool and of stone wool slabs is common in greenhouse horticulture and also recommended from an environmental point of view if the system is used for more than one year (van Os, 1994). Mineral wool mats are inert and most often drip irrigated. Next to mineral wool mats, stone wool cubes can be used in cut flower production in the so-called WORM-system. In this growing system, pots are flushed at intervals with a defined nutrient solution. Mineral wool has several advantages: it is lightweight when dry, easily handled, simple to bottom heat and permits an accurate and uniform delivery of nutrient solution (Jensen and Collins, 1985). Drip irrigation, where capillary tubes reach

each plant at the stone wool cube top, is mainly adopted, since it is easy and cheap, although clogging sporadically may occur.

Liquid hydroponic growing system

Liquid hydroponic growing systems are characterised as crop production without the use of growing medium. The most commonly used system is the nutrient film technique (NFT), where the nutrient solution floods the roots at an inclination of 1-2%. The seeds of for example tomato or cucumber are germinated in mineral wool cubes and then placed in a gutter coated with white-black plastic film, which is closed around the stem of the plant. The liquid growing system is sensitive to technical hazards due to the absence of growing medium buffering water and nutrients. However, it has the advantage as regards nutrient management and accommodates both economical as well as environmental demands well. For cucumber and tomato production, 10-15% increase in yield can be expected when using NFT systems as compared to drip irrigation of stone wool mats (Göhler and Molitor, 2002).

Open and closed irrigation systems

Open irrigation systems are also called run-to-waste systems because the nutrient solution used is dissipated. Ammerlaan (1994) defined "closed system" as closed emission routes in such a way that soil, air and water are not polluted and waste is removed in a controlled way. More common is a wider definition, which only concerns the recovery, replenishment and recycling of the nutrient solution in contrast to open systems where the nutrient solution is not reused (Jensen and Collins, 1985). This definition is adopted in the present thesis. Nutrients and water are not allowed to leach from the pot or bench to the ground. In 1999, closed crop production technology was adopted in 197 out of 1264 greenhouse market gardeners with an area of 79 ha out of 327 ha of the total greenhouse production area (SCB, 2000).

Eutrophication of rivers and lakes as a consequence of excess run-off of fertilizers originating from agricultural and horticultural production sites has become a major concern in environmental contexts. According to Göhler and Molitor (2002), water use in open soilless culture of cut flowers ranges from 800 to 1500 l m^{-2} $year^{-1}$ while closed soilless culture requires 600-1100 l m^{-2} $year^{-1}$. Ehret *et al*. (2001) estimated a loss of up to 40% of the nutrient solution in open irrigation systems. Trials on tomato (Krüger, 1990) grown in mineral wool in a open irrigation system during two consecutive years showed an annual nutrient loss per hectare of 620-690 kg of N, 520-610 kg of K, 60 kg of P, 25 kg of Mg and 900 kg of Ca based on a plant density of 2.3 plants m^{-2}. Certain nutrients accumulate in the nutrient solution (Graves, 1983). Several researchers (Hurd, 1978; Jensen and Aðalsteinsson, 1993; Yu and Matsui, 1993; Yu and Matsui, 1994; Evans and Vestergard, 1999) pointed to the fact that biological decomposition products may accumulate as well.

There is a higher risk of pathogen spread in closed compared to open ones (Jensen and Collins, 1985; Wohanka, 1992; McPherson *et al*., 1995; Vanachter,

1995; Göhler and Molitor, 2002). This is considered to be especially true for NFT systems, where the roots are freely exposed to the passing pathogen. Kegler *et al*. (1982) showed potential spread of tomato mosaic virus and *Clavibacter michiganense* pv. *michiganense. Phytophthora* and *Pythium* spp. in particular, but also *Chalara elegans*, *Colletotrichum coccodes*, *Phomopsis sclerotioides* and *Verticillium dahliae,* are spread in closed growing systems (McPherson *et al*., 1995). Contrasting information was given about the risk for spread of *Fusarium oxysporum* in the growing system (Kegler *et al*., 1982; Lundqvist and Svedelius, 1991; McPherson *et al*., 1995). Stanghellini and Rasmussen (1994) found that zoosporic fungi *Pythium*, *Phytophthora*, *Plasmopora* and *Olpidium* caused the most destructive root diseases in hydroponics, due to their production of motile zoospores, which are favoured by an aquatic environment. This was confirmed by Buysens *et al*. (1995) who suggested the spread of *Pythium* in NFT systems to be due to zoospores, which are smaller in size and more mobile than hyphal swellings, which tend to sink to the bottom of the container.

Disinfestation methods

Disinfestation is any activity to eliminate pests and vermin. The disinfestation of nutrient solution is demanding due to high amounts of recirculated nutrient solution (Vanachter, 1995). Ehret *et al*. (2001) distinguished five types of disinfestation methods, namely heat, filtration, chemical, radiation and biological control. Most disinfestation methods used in greenhouse growing today were once developed for preparation of drinking water. Ultra violet (UV) radiation is characterised by its destructive effect on the DNA as well as a heat effect (Alsanius and Brand, 2000), while slow filtration is a combination of mechanical, physico-chemical and biological factors (Brand, 2000), where the microflora is not completely eradicated, but some pathogens eliminated (Postma *et al*., 1999).

UV-radiation

UV-radiation destroys microorganisms by photochemical reaction (Runia, 1995) and is thereby not selective in terms of affecting only pathogens in the nutrient solution. The highest lethal effect is reached within the range of UV-C at 253.7 nm (Wallhäuser, 1984). Less efficiency has to be expected when organic material enters the device and the nutrient solution should therefore pass through a rapid sand filter before treatment (Ehret *et al.*, 2001). The recommended dose is 100 mJ cm^{-2} for elimination of pathogenic fungi and 250 mJ cm^{-2} for complete disinfection including viruses (Runia, 1994). These are general recommendations and as Alsanius and Brand (2000) stated, there is a need for specific adjustment for treatment of different organisms.

Slow filtration

Slow filtration is widely used, especially in Germany (Göhler and Molitor, 2002), but also in the Netherlands and other European countries. The main advantage of slow filtration is a remaining stabilizing microflora in the nutrient solution. Slow sand filtration is a non-chemical, cheap and robust method (Wohanka, 1989; Wohanka, 1992; Runia *et al*., 1996; van Os *et al.*, 1999). Besides the mechanical filtration, biological processes in the uppermost layer of the filter (the

"Schmutzdecke") affect pathogens both of a bacterial and fungal, as well as of viral nature to some extent (Brand, 2000). Parameters of importance for the filtration process are filter material and flow rate, but also filter depth, in the case using sand: effective grain size and uniformity coefficient. Sand or mineral wool are predominantly used as filter material. Granulated stone wool showed a higher efficacy as filter material compared to sand, pumice or anthracite (Wohanka *et al.*, 1999). The most suitable flow rate was defined to be 100 l m^{-2} h^{-1} (van Os *et al.*, 1999, Wohanka *et al.*, 1999), but may be increased to 300 l m^{-2} h^{-1} in the event of lack of space for the filter. The minimum filter depth should not fall below 60 cm (Wohanka *et al.*, 1999). The filter material did not quantitatively influence the remaining bacterial populations after filtration, but the potential activity of microbial communities was found to be lower the filter effluent nutrient solution than in the influent (Postma *et al.*, 1999). For sand as filter material, the effective grain size (d_{10}) should be 0.15-0.30 mm and the uniformity coefficient (UC) if possible lower than 3-5 (Wohanka, 1992).

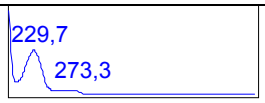
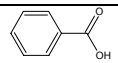
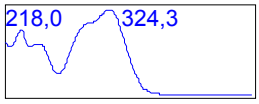
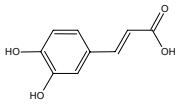
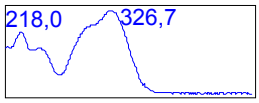
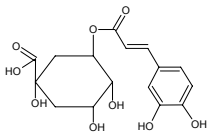
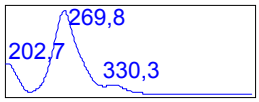
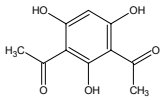
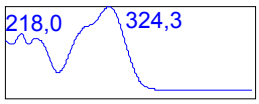
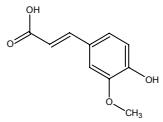
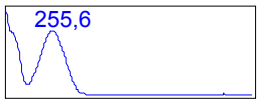
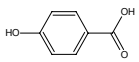
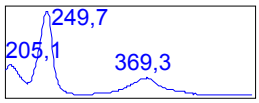
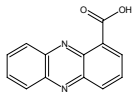
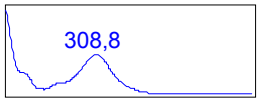
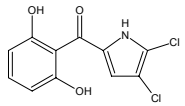
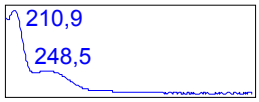
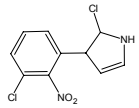
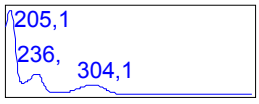
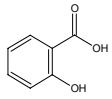
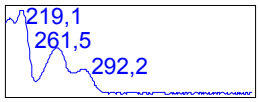
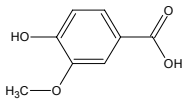
Phenolic and other organic acids in growing systems

Organic compounds are said to accumulate in the nutrient solution in closed systems (Jensén and Aðalsteinsson, 1992; Waechter-Kristensen *et al.*, 1997). As early as in 1964, McCalla and Haskins expressed concern about phytotoxic compounds accumulating in soil. In the case of failing crops in closed growing systems, there is a widely accepted consensus within the community of scientists and extension officers as well as growers to view phenolic acids as the main cause. Phenolic acids, as a group among organic acids, are released by plant roots (McCalla and Haskins, 1964; Yu and Matsui, 1997) as well as by microorganisms as metabolites or as products of biotransformation (Lynch, 1976; Lynch, 1990; Nowak-Thompson *et al.*, 1994) in the rhizosphere and in the nutrient solution. They may also derive from the substrate and growing devices (Waechter-Kristensen *et al.*, 1999). Phenolic acids are characterised by a benzene ring with a hydroxyl group attached. Selected organic compounds may be found in nutrient solutions are shown in Table 1.

Root exudates

The origin and nature of rhizodeposition have been summarised by several researchers (Caspersen, 1997; Hoagland *et al.*, 2000; Pinton *et al.*, 2001). Lynch and Whipps (1990) divided carbon loss from roots into four categories namely: exudates (sugars, amino acids, organic acids, hormones, vitamins), secretions (polymeric carbohydrates and enzymes), lysates (cells walls) and gases (ethylene, carbon dioxide). Root exudates have been found to differ between different plant species and ages (Rovira, 1959; Hoagland *et al.*, 2000) and external growing conditions as mentioned below. Many root exudates are allelopathic, which is defined by Rice (1984) as any direct or indirect beneficial or harmful effect of one plant (including microorganisms) on another through release of chemicals into the environment.

Table 1. *Chemical specimen of the studied organic compounds (structures: J.Å. Jönsson)*

Compound (pKa) isocratic mobile phase: acetonitrile/ 0.085% phosphoric acid, retention time (min)	**Absorbance spectrum 200 to 500 nm**	**Structure**
Benzoic acid (4.2) 35/65, 7.1		
Caffeic acid (4.5) 20/80, 6.7		
Chlorogenic acid (3.9) 15/85, 6.4		
2,4-diacetyl phloroglucinol (11.8, 9.2, 7.7)		
Ferulic acid (4.6) 25/75, 7.7		
p-hydroxybenzoic acid (4.6) 17/83, 7.9		
Phenazine-1-carboxylic acid (2.3, -0.42)		
Pyoluteorin (12.8, 8.1, 6.6)		
Pyrrolnitrin (not acidic)		
Salicylic acid (4.6) 40/60, 7.7		
Vanillic acid (4.5) 20/80, 6.9		

Microbial metabolites

The indigenous microflora is essential for both anabolic and catabolic processes of organic compounds in the rhizosphere (van Elsas *et al*., 1997). Secondary microbial metabolites may occur, for example antibiotics, encompassing a chemically heterogeneous group of compounds (Thomashow *et al*., 1997). Antibiotics are defined as a chemical agent produced by one organism that is harmful to other organisms (Madigan *et al.*, 1997). Numerous reports cover phytotoxic substances produced by plant pathogens. These will not be considered in the present thesis. Plant growth inhibiting effects of antibiotic compounds produced by antagonistic microorganisms have been observed on wheat seeds treated with *Pseudomonas fluorescens* 2-79 (Slininger *et al*., 1996). Lynch (1990) considered the possibilities of the accumulated organic acids to be of biocontrol value within horticultural crops. Different groups of microorganisms such as fluorescent pseudomonads excrete phenolic compounds with antibiotic effect, such as 2,4-diacetyl phloroglucinol, pyoluteorin and phenazine-1-carboxylic acid (Howell and Stipanovic, 1979; Howell and Stipanovic, 1980; Shanahan *et al*., 1993; Nowak-Thompson *et al*., 1994; Bonsall *et al.*, 1997; Ligon *et al*., 2000).

Considering the disappearance of phenolic compounds, Waechter-Kristensen *et al.* (1994) discussed the importance of rhizosphere microorganisms, which utilise organic compounds in nutrient solution. Patrick (1971) reported the importance of oxygen when considering the degradation rate of organic compounds such as phenolic acids. Regarding soil growing conditions, Patrick underlined the often rapid fluctuations between aerobic and anaerobic situations. Aerobic and anaerobic degradation involve different biosynthetic pathways (Brune, 1998).

Water quality

Other sources apart from plant and microorganisms also contribute to the content of phenolic acids in the nutrient solution. These include tubes, gutter material, the growing media and the incoming water. Water quality comprises a broad spectrum of parameters influencing the chemical and biological composition of the nutrient solution in the growing system, which affect plant and microorganisms and their interactions, and the release of organic acids (Rovira, 1969). Nutrient content and temperature influence the composition of plant and microbial metabolites in the nutrient solution (Rovira, 1959), but also pH (Gulati *et al.*, 1999) and electrical conductivity (EC). Measuring the total salt concentration, EC provides no indication of adequate nutrient ratios for plant uptake. Stress situations therefore easily occur, which influence the root exudation strongly. Brand (2000) observed a negative impact of EC on bacterial counts.

In NFT-systems, the oxygen supply to the roots may not be uniform. The growing root mat inhibits the nutrient solution from passing all the roots freely with stagnant water and eventual oxygen deficiency within the mat as a consequence (Graves, 1983). The oxygen level decreases rapidly along the gutter and aeration of the flowing nutrient solution is variable due to flow rate, temperature, root mat thickness and varies from day to day and with plant species (Gislerød and Kempton, 1983).

Detection of organic compounds in soil and organic growing media

Scientists have extracted phenolic and other organic compounds (Table 1) both from soil and in soilless growing systems. Vanillic, *p*-hydroxybenzoic and protocatechuic acid were detected in ploughed and subtilled soil (Guenzi and McCalla, 1966). Ortega *et al.* (1996) identified gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid and ferulic acid in bark substrate based on cork. Politycka *et al*. (1984) showed that prolonged cultivation of cucumber using a peat-bark growing medium could lead to accumulation of phenolic compounds, such as ferulic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic, salicylic, syringic and vanillic acid. Whitehead (1964) found several of the above-named phenolic acids when scrutinising four soil types, namely calcareous loam, sand, clay with flints and clay loam with different types of vegetation. All these observations indicate that phenolic acids are present under various growing conditions.

Determinations of organic compounds in nutrient solution

Few publications give a differentiated picture of the qualitative content of organic compounds in the nutrient solution. Sundin *et al*. (1996) found *p*-coumaric, *p*-hydroxybenzoic and vanillic acid in the nutrient solution of a commercial closed hydroponic greenhouse system with stone wool grown tomato. Vaughan and Ord (1991) showed the occurrence of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acid in the nutrient solution of axenically grown pea plants. Yu and Matsui detected among others benzoic, caffeic, ferulic, vanillic and *p*-hydroxybenzoic acids in the nutrient solution of a tomato crop (1993) and benzoic and *p*-hydroxybenzoic acids in the one of a cucumber crop (1994). Caffeic, chlorogenic, gallic and protocatechuic acids were described to be present in the nutrient solution from closed system with stone wool grown tomato (Johansson, 1999).

Phytotoxicity

Phytotoxicity is defined as toxic to plant (Agrios, 1997). Toxic effects on plant growth and development by aromatic acids have been discussed by several researchers (Blum *et al*., 1985a, b; Blum and Dalton, 1985; Vaughan and Ord, 1990; Yu and Matsui, 1997). Generally, phytotoxicity increases with rising carbon chain length. Aromatic acids like phenolic acids are more toxic than aliphatic (Lynch, 1980; Lynch, 1990). Evenari (1949) reported a decreasing inhibitory effect with increasing number of hydroxyl groups of the phenol and stated the inhibition to be an effect of the active hydroxyl group independent of the nature of the structural nucleus to which it is attached. The same was stated for aromatic carboxylic acids. Lee (1977) showed the undissociated forms of the compounds, as crucial for their impact on plant development, while their conjugate anions are inactive. Among fungicidal or fungistatic phenolic acids, effects of the compound itself and its oxidative products should be discriminated as exemplified by Zaprometov (1993) where enzymatic oxidation products of chlorogenic and caffeic acids in apples restricted germination of *Sclerotinia fructigena*.

Results about stimulatory effects at low concentrations while higher concentrations reveal inhibitory effects have been reported (Evenari, 1949; Rasmussen and Einhellig, 1977). The phytotoxic action of phenolic acids was by Feucht and Treutter (1989) associated with the regulation of indoleacetic acid oxidase. Compounds with one hydroxyl group associated to the benzene ring are growth inhibiting, while rising number decreased the effect. Chlorogenic acid with many hydroxyl groups was even regarded as growth stimulatory. Suppressed germination and emergence might be a result of restricted or inhibited water uptake and in most cases also inhibited respiration (Evenari, 1949). Blum *et al.* (1985a) observed stomatal closure of cucumber leaves after treatment with some phenolic acids. Some compounds such as benzoic and cinnamic acid severely limited inorganic ion uptake by the root (Glass, 1974; Yu and Matsui, 1997).

Other compounds of interest regarding phytotoxicity might be humic substances. Aliphatic acids such as acetic acid (Lee, 1977), propionic acid and butyric acid (Lynch, 1980), as well as hydrogen sulphide and alcohols, are discussed by Lynch (1985) to be candidate compounds for accumulation in soil. Aliphatic compounds accumulate preferentially under anaerobic circumstances and injure the cell membranes with leakage of cell contents as a consequence (Lee, 1977).

Extraction techniques used for determination of plant and microbial metabolites

In nutrient solution, as in soil (Patrick, 1971), a dynamic system with production, conversion and degradation of organic compounds is present, which requires suitable extraction methods. Phenolic acids occur in such small amounts in the nutrient solution that they have to be extracted prior to analysis, which most often is performed with high performance liquid chromatography (HPLC), or alternatively with gas chromatography (GC). Lynch (1985) underlined the importance of the extraction procedure in reflecting the *in situ* situation instead of producing artefacts due to fragmentation of the compound during extraction. Thus, extraction time and technique are decisive. The extract should only reflect the organic compounds available for plant roots and strong solvents should therefore be avoided.

Liquid-liquid extraction

Liquid-liquid extraction (LLE) is one of the oldest extraction techniques and involves distribution of samples between two immiscible liquid phases. The aqueous phase usually contains the sample and the organic phase is used to purify and extract the analyte from the sample. The organic extract can then be analysed by gas or liquid chromatography. Guenzi and McCalla (1966) extracted phenolic acids from soil with 0.1 M $Na_4P_2O_7$ and acetone. Fractionation was performed by Yu and Matsui (1993) with diethyl ether and ethyl acetate from an acidic aqueous solution prepared by concentration and acidification of the nutrient solution. Disadvantages with LLE include large amounts of waste organic solvent and the fact that the organic/aqueous volume phase ratio is limited to 0.01. LLE is also laborious, difficult to automate and that emulsion formation often occurs (Poole,

2002). On the other hand, it can be possible to obtain clean extracts and low detection limits.

Solid phase extraction

In solid phase extraction (SPE), the analyte in the sample adsorbs to a solid phase and is then recovered by elution, using a liquid or by thermal desorption into the gas phase. Yu and Matsui (1993) used activated charcoal, which was desorbed with different solvents such as NaOH and methanol. For extraction followed by gas-chromatographic analysis, Yu and Matsui (1994, 1997) used a column packed with Amberlite XAD-4 resin as an absorbent for hydrophobic compounds. Methanol was used as eluent and evaporated to dryness at reduced pressure. Disadvantages of the method include limited sorption capacity and analyte displacement by matrix components, which result in poor and variable extraction efficiency. In the case of the studies of Yu *et al*. (1993) the activated charcoal treatment had a significant influence on the growing crop. The SPE uses less organic solvent, but leads typically to extracts with a higher load of undesired compounds.

Supported liquid membrane extraction

For the present task, one difficulty lay within the goal of detecting the compound of interest during the interval between production and disappearance (Patrick, 1971). A reliable tool was therefore needed to extract the organic acids directly from the nutrient solution and to give an *in situ* picture of the composition and concentrations. For this purpose, the supported liquid membrane (SLM) extraction, which may be viewed as a modern variant of LLE, was considered as highly promising. In comparison with LLE, SLM extraction involves less risk of contamination, minimal use of organic solvents and easier automation (Jönsson and Mathiasson, 2000; Jönsson, 2003). In comparison with SPE, SLM extraction primarily reduces overloading and risk for breakthrough. In brief, the SLM extraction an extraction system, where the sample solution (donor) is acidified and passes a membrane soaked in an organic solution and analytes of interest are caught in the alkaline buffer solution on the other side of the membrane (acceptor) over a range of time. The solution on the acceptor side (Paper II, Figure 1) can easily be collected and after neutralisation, the content analysed most straightforwardly by HPLC. The pH in the donor and acceptor is adjusted to values lying respectively well beneath and above the pKa value of the organic compounds to be analysed. Analyses with GC have also been developed, but for the actual compounds they are complex and difficult to operate. Generally stated (Snyder and Kirkland, 1979), GC is only able to handle 20% of known organic compounds, whereas LC is ideally suited for the separation of for example plant and animal metabolites.

The extraction efficiency is usually between 20 and 80%. This does not influence the accuracy as it is stable and determined by calibration (Jönsson and Mathiasson, 2000). To increase extraction efficiency, a carrier such as trioctylphosphine oxide (TOPO) can be used in the membrane solution; this is called carrier mediated transport (Paper II, Figure 1). Johansson (1999) showed

that the efficiency reached its maximum at an addition of 5% TOPO when different phenolic acids such as chlorogenic, vanillic and *p*-hydroxybenzoic acid were extracted. Knutsson *et al*. (1996) observed that detection limits for phenolic acids could be lowered (by approximately a factor of four) when the flow rate was increased from 1.3 ml min^{-1} to 6.5 ml min^{-1} using the same extraction time, 30 min.

European Union project MIOPRODIS

The work of this thesis lies within the framework of the EU-project MIOPRODIS (Fair 6 CT98-4309, http://europa.eu.int/comm/research/agro/fair/en/nl4309.html, http://www.imag.wageningen-ur.nl/PDF/mioprodis.pdf). The aim of the MIOPRODIS project (MIcrobial Optimisation to Prevent ROot DISeases) was to develop a sustainable system for the prevention of root diseases in closed soilless growing systems by optimising microbial suppression. The system had to be robust, with low technique input and inexpensive, in order to replace the soil grown system in which the soil fumigant methyl bromide is used in Southern Europe and the open soilless growing systems in Northern Europe. The project was coordinated by Erik van Os, IMAG B.V, Wageningen, NL and incorporated research of seven additional partners, namely Plant Research International, Wageningen, NL, State Research Station Geisenheim, Geisenheim, DE, University of Turin, Grugliasco, IT, Horticultural Research International, Warwick, UK, Rockwool Grodan B.V., Roermond, NL, Saint-Gobain Cutilène, Tilburg, NL and the Swedish University of Agricultural Sciences, Alnarp, SE. In this context, it has been the task of researchers at SLU in cooperation with Analytical Chemistry, Lund University, Lund, SE to evaluate the role of some plant and microbial metabolites in the recirculated nutrient solution of greenhouse growing systems including tomato, cucumber and gerbera. Besides determination, phytotoxicity studies were performed on small tomato plants in a phytotron environment and utilisation and enrichment studies of the indigenous microflora were carried out in the recirculated nutrient solution.

Objective

The objective of this thesis has been to study the occurrence and effect of selected plant and microbial metabolites in the nutrient solution of closed growing systems in greenhouses.

Several hypotheses have determined the work of the past four years. The selected plants and microbial metabolites:

- are harmful to tomato plants
- are present in closed hydroponic growing systems
- accumulate during the growing season
- are degraded by microorganisms in the nutrient solution
- vary in quantity and quality between different crops
- are influenced in composition by the disinfestation method used, and

- environmental conditions can be optimised in order to enhance formation of antimicrobial compounds

Materials and methods

Growing systems

Phytotron experiments

Studies of phytotoxic effects on small tomato plants were performed under optimised conditions in phytotron chambers. Tomato seeds (cultivar 'Aromata') were germinated in petri dishes for seven days. The seedlings were transferred to 1-l beakers at a density of one plant per beaker and grown in static aerated culture for two weeks. The plants were kept in place by black polyethylene foam (Figure 1), which also prevented algal growth. The nutrient solution was replaced every fourth day. The tomato plants were exposed to 0, 50, 100, 150, 200 and 400 µM of synthetically produced benzoic, caffeic, chlorogenic, ferulic, *p*-hydroxybenzoic, salicylic and vanillic acid. Nutrient solution composition is shown in Table 2. Detailed information about climatic conditions is found in Paper I. The nutrient solution was prepared from stock solutions (double strength nutrient solution/double strength organic compound in water, 1:1) due to unwanted microbial growth when dissolved in a solvent such as alcohol or acetonitrile. Each time the nutrient solution was replaced, the pH was set to 5.50 ± 0.05 in each treatment and adjusted to 5.5 ± 0.1 every day. Each trial lasted two weeks. The experiment was performed twice and evaluation of persistence of the added compound and counting of microorganisms was carried out in the second run of the experiment.

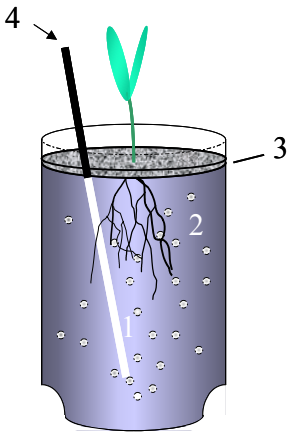


Figure 1. Cultivation system using static aeration technique; 1: aeration tube, 2: nutrient solution, 3: black polyethylene foam, 4: air supply (Illustration: B.W. Alsanius).

Fresh and freeze-dried weights of leaves, stems and roots were measured. Stem length was measured with 0.5 cm accuracy and leaves bigger than 1 cm counted, as well as visual assessment conducted twice by the same person according to Table 3. Photosynthetic activity was measured by assessing the quantum yield of photosystem II (Genty *et al*., 1989) and was performed twice on the oldest leaves of all replicates on the day before trial termination. A representative part of the tip of the leaf was chosen.

Table 2. *Initial nutrient solution composition (mM) in the different growing systems: phytotron (Sonneveld and Straver, 1989) and greenhouse experiments at experiments at SLU (SLU, Alnarp, SE), IMAG (IMAG, Wageningen, NL), State Research Station Geisenheim (FG, Geisenheim, DE) and Horticulture Research International (HRI, Stockbridge (2000) and Efford (2001), UK). * micronutrient supplemented as chelates not defined for the user*

Ion	**Phytotron**	**Greenhouse**			
		SLU	**IMAG**	**FG**	**HRI**
crop	tomato	tomato	cucumber	gerbera	tomato
SO_4	3.75	2.0	1.25		
H_2PO_4	1.25	2.3	1.25		
P				1.4	1.3
NO_3	13.75	20.0	11.75	added if pH < 6	3.2
NH_4	1.25		1.0	added if pH > 7	low
N				10	
K	8.75	10.3	6.5	5.6	12.8
Mg	2.0	2.0	1.0	0.9	3.5
Fe	0.015	0.4	0.015	*	0.2
Ca	4.25	6.0	2.75		6.2
Mn	0.01	0.01	0.01	*	0.01
Zn	0.005	0.005	0.005	*	0.015
B	0.03	0.03	0.03	0.01	0.05
Cu	0.0008	0.0008	0.0008	*	0.0016
Mo	0.0005	0.0005	0.0005	0.0004	0.0005
EC mS cm^{-1} (25 ºC)	2.3	3.2	1.7	0.9	2.4-2.8
pH	5.5 ± 0.1	5.8 ± 0.1	4.1 – 5.0	6.0 – 7.0	5.2-5.4

Table 3. *Visual assessment parameters and definitions for discrete scale of assessment*

Character	1	2	3	4	5
Root tips	No discolouration	A few discoloured root tips	25% discoloured tips	50% discoloured tips	All tips discoloured
Root colour	White	White-yellow	Yellow, blackish covering, slimy	Yellowish-brown, with clear black covering	Brown, rotten, with clear black covering
Canopy performance	Well balanced, healthy, fully developed leaves, green	Stress symptoms, short/long internodes	Stressed, bleached, yellow spots	Poorly developed leaves, mostly yellow	Yellow, only cotyledons developed, dying, stunted

Greenhouse experiments

SLM-extraction was used and the method had to be adapted to on-line extraction of the compounds of interest (benzoic, chlorogenic, ferulic, *p*-hydroxybenzoic, phenazine-1-carboxylic, salicylic, vanillic acids, pyoluteorin and 2,4-diacetyl phloroglucinol) in the greenhouse (Paper II). This was executed in greenhouse cultivation with tomato cultivar 'Gitana' at the Department of Crop Science, SLU (Alnarp, SE), as well as with tomato cultivar 'Elegance' at Rockwool/Grodan B.V. (Roermond, NL). After adaptation of the method, nutrient solutions from closed growing systems with tomato (cv. 'Espero') at Horticulture Research International (Stockbridge House Efford/Wellesbourne, Warwick, UK), cucumber (cv. 'Sabrina') at IMAG B. V. (Wageningen, NL) and gerbera (cv. 'Shimony' and 'Kaliki') at State Research Station Geisenheim (Geisenheim, DE) were extracted for qualitative and quantitative determination of the chosen compounds at selected developmental stages during the growing season (Paper IV). Stone wool based growing systems were drip irrigated. Nutrient solution composition and growing conditions for the individual growing system are presented in Tables 2 and 4.

In addition to comparison between developmental stages and crops, samples were taken from nutrient solutions treated with different disinfestation methods (UV-radiation and slow sand filtration), as well as from control treatments without disinfestation (Paper IV). The growing system was inoculated with *Phytophthora cryptogea* in tomato and cucumber, as well as *Pythium aphanidermatum* in cucumber. For inoculation with the pathogen, three infected plants with severe wilting symptoms were introduced per row to the gerbera growing system, while the pathogen was added on top of the stone wool blocks as a suspension in the cucumber and tomato growing systems. In some treatments, the antagonists *Lysobacter enzymogenes* and *Trichoderma harzianum* were added (Paper IV, Table 2). The antagonist was introduced three times by drenching the gerbera pots in 0.2% *Trichoderma harzianum* suspension, while *Lysobacter enzymogenes* was introduced into the nutrient solution by drip irrigation.

Table 4. *Growing conditions for the greenhouse experiments at experiments at SLU (SLU, Alnarp, SE), Rockwool/Grodan B.V., (Grodan, Roermond, NL), IMAG (IMAG, Wageningen, NL), State Research Station Geisenheim (FG, Geisenheim, DE) and Horticulture Research International (HRI, Stockbridge (2000) and Efford (2001), UK). For details see relevant paper*

	SLU	**Grodan**	**IMAG**	**FG**	**HRI**
Paper	II, III, V	II	IV	IV	IV
Crop	tomato	tomato	cucumber	gerbera	tomato
Growing media	no	stone wool	stone wool	stone wool cubes in pots (WORM-system)	stone wool
Solution volume	50 l	135[1] and 180[2] l	180 l	120 l	160 l
No. of plants	15	40	12	15	15/16
Disinfestation	slow stone wool filter		UV, slow sand filter, without	UV, slow sand filter, without	UV, slow sand filter, without
Pathogen	no	no	*Pythium aphani-dermatum*	*Phytophthora cryptogea*	*Phytophthora cryptogea*
Antagonist	no	no	*Lysobacter enzymogenes*	*Trichoderma harzianum*	no

[1] 60% water content in the stone wool slab

[2] 80% water content in the stone wool slab

Extractions and Analyses

Table 1 shows the structure, the absorbance spectrum and isocratic mobile phase conditions for the phenolic acids: caffeic, chlorogenic, ferulic, *p*-hydroxybenzoic, salicylic and vanillic acid, pyoluteorin and 2,4-diacetyl phloroglucinol as well as the carboxylic acids: benzoic and phenazine-1-carboxylic acid, and pyrrolnitrin. All of these were used as reference compounds throughout the experiments.

Extractions of plant and microbial metabolites with SLM technique

The extraction was based on the method elaborated by Knutsson *et al*. (1996). For the adaptation to on-line extraction of the chosen compounds in the greenhouse, extraction times, flow rates and organic solutes were varied (Paper II). The nutrient solution was extracted at the end of the gutter in all systems except for the gerbera WORM-system, where nutrient solution had to be collected after an irrigation flush prior to extraction. The adapted method was then used for all phenolic acid extraction in further experiments. However, the extraction time of 3 hours at 0.3 ml min^{-1} in Paper II was changed to 1 hour at 1 ml min^{-1} for the comparative experiments in Paper IV.

Analyses of plant and microbial metabolites

All analyses were performed with HPLC. In Paper II, no diode array detector (DAD) was available, and compounds were identified according to coincident retention times between sample and synthetic standard. All analyses in Papers I and III-V were carried out on HPLC-DAD. Thus, determination could be assured by both retention time and match with spectra of the synthetic standards. Injections of 80 µl (100 µl in Paper II) were analysed with gradient elution using acetonitrile/0.085% phosphoric acid (acetonitrile/0.4% acetic acid in Paper I) and separation of all compounds was reached after 45 min at a flow rate of 0.7 ml h^{-1}. Quantifications were calculated based on peak area at 254 nm in paper II, while quantification in Papers I, IV and V was performed at the optimum wavelength for each compound. Single compound analyses were performed in isocratic runs according to Table 1.

Analyses of microorganisms and of microbially mediated processes

Analyses of microorganisms and of microbially mediated processes within the different papers is seen in Table 5.

Table 5. *Analyses of microorganisms and of microbial mediated processes*

	Paper I	**Paper III**	**Paper IV**	**Paper V**
Viable count				
Total bacterial flora	x	x	x	x
Total fungal flora		x	x	
Fluorescent pseudomonads		x	x	
Trichoderma		x	x	
Actinomycetes		x		
Sole carbon source utilisation		x		
HPLC				
Persistence	x	x		
Enhancement				x

Viable counts

Viable counts were enumerated as colony-forming units (CFU ml^{-1}) using R2A (Difco 1826-17-1) for the general bacterial flora (3 days, 20 ºC), for fluorescent pseudomonads King B agar (King B agar, King *et al*., 1954, supplemented with cycloheximide 100 µg ml^{-1}) for 2 days at 25 ºC, for the general fungal flora (4 days, 20 ºC) and *Trichoderma* (7 days, 20 ºC) 0.25 potato dextrose agar (Difco 013-01-4) (Papers I, III-V). Viable counts were enumerated during the growing season synchronically with extraction of plant and microbial metabolites in Paper IV.

Viable count enumerations within the phytotoxicity trials (Paper I) were carried out before the first and the last change of nutrient solution. Samples were taken

from three beakers each with concentration 0, 100, 200 and 400 µM. To mimic the environment in the nutrient solution as closely as possible, the pH of R2A was adjusted to 5.50 ± 0.05. CFU ml^{-1} were enumerated after two days of incubation at room temperature.

Sole carbon source utilisation

In Paper III, the microbial utilisation of organic compounds was also studied using Microlog GN-panel (Biolog, Haywood, CA, USA). The colonised nutrient solution from three tomato growing systems was inoculated in microtitre plates. The 95 wells of the plate contained a low concentration, buffered nutrient medium, a single carbon source and a redox dye, tetrazolium. The sole carbon sources present on the panel may be divided into eleven classes (alcohols, amides, amines, amino acids, aromatic compounds, brominated compounds, carbohydrates, carboxylic acids, esters, phosphorylated compounds and polymers) according to Garland and Mills (1991). Utilisation was analysed spectrophotometrically (590 nm; DigiScan, Asys, Linz, Austria; software: Digiwin) as a result of microbial respiration by the colour change of tetrazolium to formazan directly after inoculation, as well as after 4, 18, 24, 30, 36, 48, 60, 72 and 92 h of incubation at 20 ºC.

Persistence of organic compounds

Persistence of the synthetically added organic compounds during phytotoxicity studies (Paper I) was measured by means of HPLC at the first and last nutrient solution change. One ml was sampled at 0, 2, 4, 17, 24 and 48 h from beakers containing nutrient solution with initial concentrations of 0, 100, 200 and 400 µM (Table 1).

Disappearance of synthetic standards added to the nutrient solution from a closed irrigation system with tomato (Paper III) was studied on three sampling occasions by filtering the solution (0.22 µm) after 7, 31 and 54 hours, freezing the samples and analysing by HPLC as described above. Benzoic acid, ferulic acid, *p*-hydroxybenzoic acid, salicylic acid and vanillic acid were added to 10 ml nutrient solution to a final concentration of 30 µM. Non-sterile tap water was used as a control. Samples were kept at 4 ºC while waiting for injection to minimise abiotic and biotic degradation.

Enhanced metabolite formation

Enhanced production (Paper V) of selected antibiotic compounds (2,4-diacetyl phloroglucinol, pyoluteorin, pyrrolnitrin) by the microflora inhabiting the effluent nutrient solution of a closed growing system with tomato was achieved under *in vitro* conditions by mixing the nutrient solution with double strength nutrient broth (1:1). Yeast malt broth (YM, Bangera and Thomashow, 1996); King B broth (KBB, Proteose Peptone No 3 (Difco 0122-17-4) 20 g, glycerol 10 ml, $MgSO_4$ * 7 H_2O 1.5 g, K_2HPO_4 1.5 g; per 1000 ml deionised water, modified King *et al.,* 1954); mineral nutrient broth supplemented with 2% glycerol (NBglyc, peptone 5.0 g; meat extract 3.0 g, deionised water 1000 ml, 2% glycerol) were chosen in accordance with results from a screening for suitable liquid nutrient media for

enhancing formation of selected antibiotic compounds. A wild type and a mutant strain of *Pseudomonas fluorescens* were used as the control for metabolite production: Pf-5 which overproduces 2,4-diacetyl phloroglucinol, pyoluteorin and pyrrolnitrin (Corbell and Loper, 1995) and 5-2/4, a mutant strain of 5.014, which overproduces 2,4-diacetyl phloroglucinol (Alsanius *et al*., 1998). The strains were added as 10 µl of a 8 log CFU ml^{-1} suspension to 16 ml of the specific nutrient solution. Aliquots of 1 ml were membrane filtered (0.22 µm) 4-10 days after inoculation (see Paper V for detailed description) and samples frozen prior to gradient HPLC analysis as described previously.

Statistics

Experiments were repeated, in the case of greenhouse growings in Papers III and IV, over two growing seasons. HPLC analyses were carried out according to ASTM (standard D 3856) recommendations for water samples 11.12 (1-5, 7-10). Results from viable counts are expressed as mean ± standard deviation after log transformation (Angle *et al.*, 1996). All statistical analyses were performed using Minitab version 13 (Minitab Inc., Philadelphia, USA). For detailed information the reader is referred to the individual papers.

Results and discussion

The widely accepted opinion of organic compounds, especially phenolic acids being the cause of failing crops due to accumulation in the recirculated nutrient solution and thereby causing phytotoxic effects on the growing crop could not be verified in the experiments covering this thesis. The organic acids studied (Paper II, IV) appeared in concentrations 100-1000 times lower than phytotoxic levels of the single compounds on small tomato plants (Paper I) and were utilised within two days by the microflora inhabiting the nutrient solution (Paper III).

Phytotoxicity

Plants exposed to ferulic and salicylic acid showed the most prominent effects on young tomato plants among the compounds studied. These effects were significant with respect to the total plant fresh weight (Paper I, Figure 1) already at a concentration of 200 µM, compared to the untreated control. Plants subjected to benzoic and caffeic acid revealed differences between the 400 µM treatment and the untreated control, while plants exposed to chlorogenic and *p*-hydroxybenzoic acid did not differ between the chosen concentrations. Plants treated with vanillic acid differed significantly between 50 µM and 400 µM. Fresh and dry weight shoot:root ratio (paper I, Figure 2) declined with increasing concentration for plants exposed to ferulic, *p*-hydroxybenzoic and vanillic acid, while it increased for plants subjected to salicylic acid.

Considering the conclusions of Evenari (1949) about the effects of an active hydroxyl group on the phenolic structure, salicylic acid including one hydroxyl

group next to the carboxylic group (Table 1) confirmed the difference in phytotoxicity between salicylic and benzoic acid obtained in the present studies. However, the position of the hydroxyl group seemed to be crucial, since plants exposed to *p*-hydroxybenzoic acid did not reveal severe phytotoxic symptoms. Additional hydroxyl groups seemed to decrease phytotoxicity and chlorogenic acid is even considered as growth promoting by Feucht and Treutter (1989). The latter could not be confirmed in this study, although no fresh or dry weight decreases were assessed. However, not only the effect of single compounds should be considered. Synergistic inhibitory effects of several phenolic acids were shown by Rasmussen and Einhellig (1977) and (Einhellig and Rasmussen, 1978).

Evenari (1949) suggested organic acids to be phytotoxic by property of their intrinsic nature and not due to a pH effect. However, pH contributes to the inhibition as shown by Blum *et al*. (1985b) in that stronger inhibition of cucumber leaf area expansion occurred of ferulic and *p*-coumaric acid at pH 5.5 than at pH 7.0. Therefore, the daily adjustments of pH in the phytotron trial (Paper I) were of the utmost importance to maintain the objective of the experiment. According to Table 2, both EC and pH values varied widely between the different greenhouse crops and are expected to vary even more within different micro-sites in the growing system creating different preconditions for phytotoxic damages.

Observed increases in dry weight fraction, especially for plants treated with chlorogenic and ferulic acid (Paper I, Table 1) might be explained by increased lignin formation with the phenolic acids as precursors and thereby decreasing cell wall extensibility with smaller cells as a consequence (Tan *et al*., 1992). This is not necessarily a negative process for the plant, since it is more protected against physical stress such as pests. Lehman and Blum (1999) suggested that acclimatisation to one form of environmental stress such as ferulic acid exposure, nutrient stress or drought may result in acclimatisation to others. If this is due to direct plant effects or indirect through a change in the microflora associated to the roots remains unknown. It implies, however, that plants such as those studied here would be especially vulnerable since they were grown under optimised climatic conditions. These aspects would be of special interest under greenhouse conditions constituting a range of stress factors to the grown crop.

Greenhouse experiments all resulted in well developed crops. On the basis of assessed parameters it was not possible to conclude, whether quantities of especially benzoic acid (Paper IV) found in the growing system affected plant growth. Growing systems including inoculation of pathogens showed surprisingly few dead plants (data not shown), except for the gerbera crop in 2000, which had to be terminated earlier than planned due to collapsing crop.

Visual assessment of the roots showed clear effects of the added compounds (Figure 2). Roots of plants exposed to chlorogenic as low as 50 μM were soot-blackened. Roots of plants subjected to ferulic acid revealed yellowish mucilage and salicylic acid blackish mucilage. Vanillic acid treated plants showed roots with evenly distributed brown colouring. Patrick (1971) observed inhibition of the primary root as well as browning of roots treated with phytotoxic decomposition products. The black coating found on roots subjected to caffeic acid and chlorogenic acid was also found on pea roots treated with caffeic acid under axenic conditions described by Vaughan and Ord (1990). Since only few phytotoxic effects were assessed by means of fresh and dry weights on plants detected with coatings, it would be interesting to study the origin and role of the coatings for development of phytotoxic responses.

Canopy health was surprisingly good considering the effects on the roots (Paper I), although in many cases, plant size decreased with rising concentration of the added compound. Only plants exposed to salicylic acid showed an increasing amount of chlorotic spots with increasing concentration. One should exercise care in drawing conclusions about the phytotoxic effects on the evidence of root discolourations alone. Exposure to the studied compounds during a longer period of time such as a growing season under less optimised conditions might have more devastating consequences.

Extractions of plant and microbial metabolites with SLM technique

Method adaptation

The achievement in Paper II was the adaptation of the SLM-technique to on-line extraction in the greenhouse. An *in situ* picture could thereby be given of the content in the nutrient solution of the growing system, without metabolisation of the small amounts of organic compounds on the way to the laboratory. Three-hour extractions proved to give acceptable extraction efficiencies at a flow rate of 0.3 ml min^{-1} (Paper II). In later extractions (Paper IV), one-hour extractions at a flow rate of 1 ml min^{-1} were preferred due to time concerns when many samples and replicates needed to be extracted one day. Table 6 shows the extraction efficiency loss that had to be tolerated to save time. Chlorogenic acid and pyoluteorin were unsatisfactorily extracted. The extraction efficiency was approximately 0.10 and considering the high standard deviations, quantifications could not been made. Most probably, the SLM-extraction method can be optimised to extract both pyoluteorin and chlorogenic acid to a satisfactory degree, if one focuses on one compound at a time, instead of ten compounds together. Johansson (1999) report an extraction efficiency of 0.20 for chlorogenic acid with SLM-extraction. Knutsson *et al*. (1996) obtained extraction efficiencies between 0.22-0.33 at 1.3 ml min^{-1} in 30 min for caffeic, ferulic, *p*-hydroxybenzoic and vanillic acid. They made the observation that high flow rates up to 6.5 ml min^{-1} gave lower detection limits while the extraction efficiency decreased, which is explained by higher accumulation rate. As presented in Table 6, detection limits in my studies were lowered significantly during the one hour extractions and thereby confirmed the

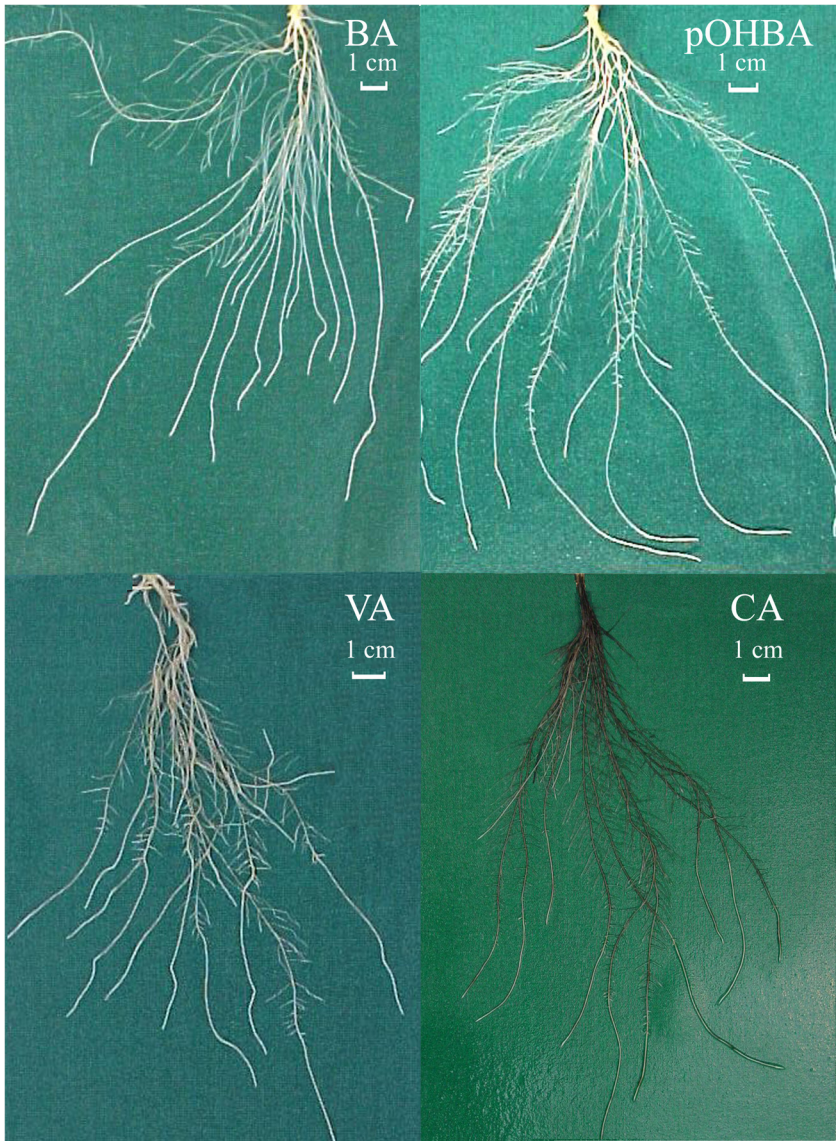
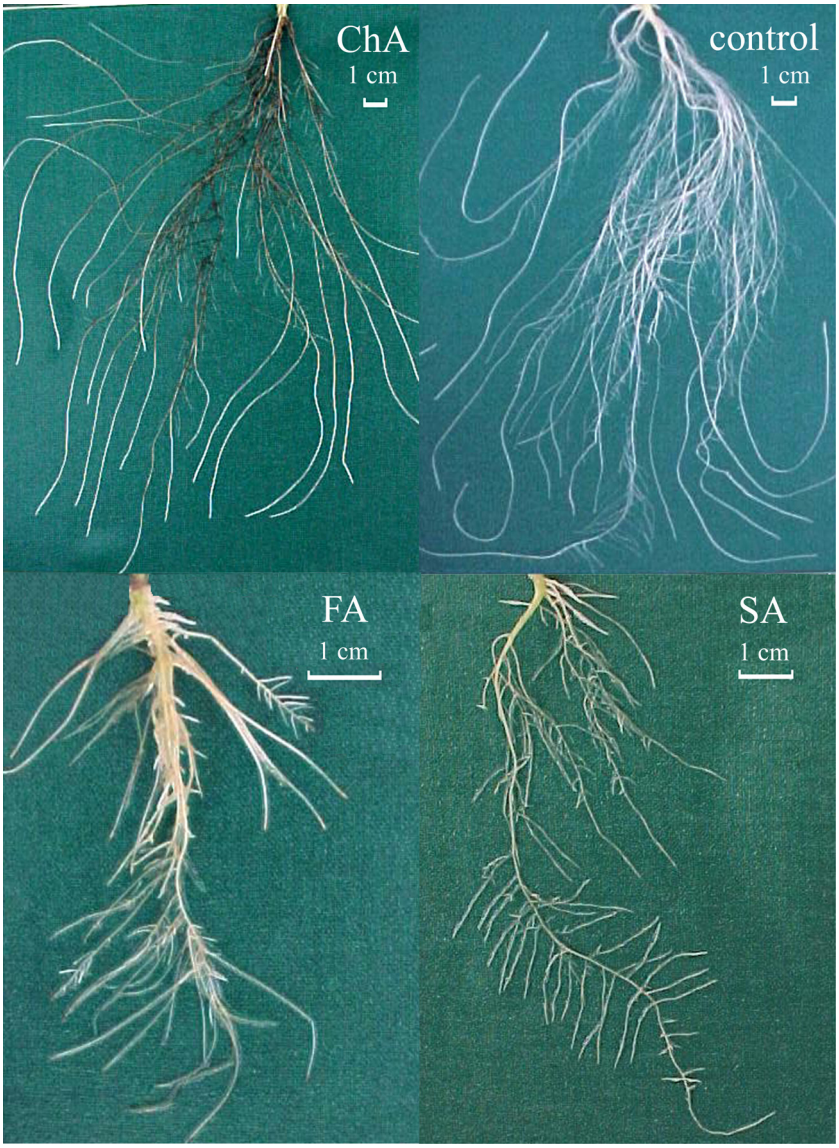


Figure 2. Roots of three-week old tomato plants treated with benzoic (BA), caffeic (CA), chlorogenic (ChA), ferulic (FA), *p*-hydroxybenzoic (pOHBA), salicylic (SA) and vanillic (VA) acids for two weeks, as well as untreated control. (Pictures on opposite page reprinted from Paper I with permission by Elsevier).


ChA
1 cm
control
1 cm
FA
1 cm
SA
1 cm

findings of Knutsson *et al*. (1996) However, HPLC analysis was carried out on two different pieces of equipment, which might also influence the detection limit.

Table 6. *Comparison of detection limits of compounds studied in the nutrient solution, as well as extraction efficiency of the SLM extraction method 3 h at 0.3 ml min^{-1} and 1 h at 1 ml min^{-1}. Efficiency calculations are means of ten replicates with duplicate HPLC-injections of each sample ± standard deviation*

Compound	**3 h at 0.3 ml min^{-1}**		**1 h at 1 ml min^{-1}**	
	Extraction efficiency	**Detection limit (nM)**	**Extraction efficiency**	**Detection limit (nM)**
Benzoic acid	0.64 ± 0.03	200	0.44 ± 0.11	<1
Chlorogenic acid	0.04 ± 0.02	-	0.04 ± 0.02	-
2,4-diacetyl phloroglucinol	0.013 ± 0.006	-	0.24 ± 0.12	<1
Ferulic acid	0.46 ± 0.09	30	0.33 ± 0.11	<1
Phenazine-1-carboxylic acid	0.53 ± 0.07	5	0.71 ± 0.20	<1
p-hydroxybenzoic acid	0.63 ± 0.09	20	0.38 ± 0.11	<1
Pyoluteorin	0.013 ± 0.008	-	0.18 ± 0.27	-
Salicylic acid	0.70 ± 0.08	300	0.47 ± 0.13	<1
Vanillic acid	0.57 ± 0.07	30	0.31 ± 0.07	<1

Analyses of plant and microbial metabolites

To allow all compounds to be separated within one analysis, a gradient was developed in Paper II, Table 1. Acetic acid, used as the mobile phase was replaced by phosphoric acid, since the first one was not congruent with the DAD system (gradient in Paper IV, Table 3). The chromatographic system used in later analysis had a lower base line noise level, which is probably a result of the use of a fully automated and modern equipment.

Identifications and quantifications of plant and microbial metabolites

When the extraction method was developed in Paper II, a number of compounds were detected, primarily vanillic acid, but also ferulic acid, *p*-hydroxybenzoic acid and phenazine-1-carboxylic acid. Amounts varied between 5 and 90 nM. Quantifications made in the later study including tomato, cucumber and gerbera crops were similar with respect to quantities, in some cases up to 200 nM, but the composition differed qualitatively. Benzoic acid was most prominent as well as frequent occurrence of *p*-hydroxybenzoic acid, whereas vanillic acid was not re-verified in the extractions made in Paper IV.

Table 7 shows results from different studies of detections in nutrient solution. Yu and Matsui (1993) found benzoic acid concentrations of 1.1 µM in fractionated nutrient solution samples of tomato and 42.6 µmol benzoic acid in 300 g residual activated charcoal in the same nutrient solution extracted by means of desorption

Table 7. *Organic acid findings (nM) in nutrient solution in horticultural crops. + indicates occurrence*

Compound	**Tomato**	**Tomato**	**Cucumber**	**Gerbera**	**Tomato**	**Tomato**	**Cucumber**	**Tomato**	**Tomato**	**Pea**
Benzoic acid			10-70	40-200	10-80	1100	+			
Caffeic acid						+				+
Chlorogenic acid									500	
Ferulic acid	80					+				+
Phenazine-1-caboxylic acid	7									
p-hydroxybenzoic acid		18	30-80	50-80		+	+	+		+
Pyoluteorin			+							
Vanillic acid	50-70	50				+		+		+
2,4-diacetyl phloroglucinol			110-170	140-150						
Comments	NFT 99 Table 4	Grodan Table 4	IMAG Table 4	FG Table 4	HRI Table 4	residual activated charcoal extraction or fractionation	Amberlite XAD-4 extraction	SLM-extraction	SLM-extraction	axenic conditions, Amberlite XAD-4 extraction
References	Paper II	Paper II	Paper IV	Paper IV	Paper IV	Yu and Matsui (1993)	Yu and Matsui (1994)	Sundin *et al*. (1996)	Johansson (1999)	Vaughan and Ord (1991)

from residual activated charcoal. Quantities were far higher than those presented in Papers II and IV, although these vary with extraction technique. The difference in quantities in comparison with results in Papers II and IV could have several reasons, the most likely being the use of strong solvents by Yu and Matsui (1993). Additionally, they found caffeic, ferulic, *p*-hydroxybenzoic and vanillic acids in the range 0.8–16.1 µM. Johansson (1999) found quantities of chlorogenic acid of 0.5 µM and caffeic acid of 1.9 µM.

To minimise the risk of producing artefacts, duration of the process from sampling to analysis should be kept as short as possible and nutrient solution counteract microbial metabolisation, enzymatic activity, light and high temperatures. Oxidation of organic compounds was prevented by supplementing ascorbic acid by Johansson (1999) and by Sundin *et al.* (1996) additionally adjusted to pH 2 to inhibit microbial activity.

Determination of antimicrobial metabolites

Williams (1982) asked whether antibiotic compounds might be produced in soil. For a long time this question was difficult to answer and Williams considered evidence for it to be poor. Lynch (1985) summarised four reasons for failure to detect antibiotics in soil: a) instability of antibiotics in soil conditions; b) adsorption of antibiotics by soil colloids; c) insufficient sensitivity of detection methods; d) insufficient nutrients for widespread, frequent growth of producer microbes. However, Thomashow *et al.* (1997) reviewed *in situ* findings in soil and rhizosphere of phenazine-1-carboxylic acid, pyoluteorin, 2,4-diacetyl phloroglucinol and pyrrolnitrin in the range of ng - µg $root^{-1}$ or $seed^{-1}$ by LLE. Although this discussion has its background in soil, it is assumable to be appropriate for closed growing systems, too. With the *in situ* extraction in the greenhouse, the SLM-technique offers the possibility to study antibiotic production in the growing system and has here been confirmed for 2,4-diacetyl phloroglucinol in recirculated nutrient solution. The presence of phenazine-1-carboxylic acid and pyoluteorin was also confirmed, but not at the same level of certainty. Phenazine-1-carboxylic acid was detected only by retention time.

Differences in compound composition in the nutrient solution could not be distinguished between the different disinfestations methods for most compounds. However, it is tempting to conclude an effect of the slow sand filtration on the formation of 2,4-diacetyl phloroglucinol, since this compound was primarily found in slow sand filters regardless of the addition of an antagonist.

Nutritional considerations

In the performed greenhouse experiments, no attempts were made to optimise nutrient conditions with respect to the crops' demands along the growing season. Amounts in Table 3 are initial concentrations, which are expected to change along the gutter. In order to compare the data, it is necessary to adopt supply to the uptake and demand of the crop. Differences in plant and microbial metabolites might depend on differences in pH, climate conditions and nutrients. Different volumes of nutrient solution recirculated per plant and differences in flow rate

affect the dilution of the metabolites as well as the aeration in the nutrient solution. Oxygen availability is crucial in the context of metabolite formation; Bencini *et al.* (1983) report pyoluteorin to be favoured by decreased oxygen levels.

Hence, special attention might be given to pH since it varied between 4 and 7 in the different crops and growing systems (Table 4). Regarding the potential of antifungal metabolites produced by the microorganisms as discussed further below, the production varies strongly with pH (Gulati *et al.,* 1999). *Pseudomonas chlororaphis* strain I-112 was shown to produce antifungal zones – pyrrolnitrin was identified – only at initial KBB pH values of 6.5 and 7.0, while the others between 6.0 and 8.5 did not exhibit inhibition zones.

Analyses of microorganisms and of microbially mediated processes

Viable count (Paper IV) on the general bacterial flora, fluorescent pseudomonads and *Trichoderma* in the nutrient solution of the closed tomato, cucumber and gerbera system during the two growing seasons resulted in only small differences between sampling dates and disinfestation treatment. The general fungal flora increases in the nutrient solution of all crops towards the end of the season. It seemed that the microflora was much more influenced by the development of the crop itself than the individual disinfestation technique. However, both UV-radiation and slow sand filter eliminated propagules of *Pythium aphanidermatum* in cucumber and *Phytophthora cryptogea* in tomato and gerbera throughout the duration of the trial (van Os *et al*., 2001).

Persistence of organic compounds

Monthly fluctuations of water soluble phenolics could be observed in soil under the stands with *Fagus sylvatica*, with lowest values in spring and early summer (Shen *et al*., 1996). In the present studies, there was no accumulation of the selected compounds over time in the nutrient solution for tomato, gerbera or cucumber crop (Paper IV, Figure 1).

A precondition for accumulation is non-metabolisation of organic compounds. Mineralization in turn is mediated by microorganisms. The metabolisation pace of the microflora inhabiting the effluent nutrient solution of the added organic acids was rapid (Paper III, Figure 2), however no conclusion regarding degradation or conversion can be made. The potential of nutrient solution inhabiting microflora degrading phenolic acids has been shown before (Waechter-Kristensen *et al*., 1994), which should be compared with axenically performed trials (Vaughan and Ord, 1990; Caspersen *et al.*, 1999; Caspersen *et al.*, 2003), where the phenolic acid in the nutrient solution persisted significantly longer than in non-axenic studies (Sundin *et al*., 1995; Caspersen, 2000; de Kreij *et al*., 2003). This underlines the rapid metabolisation, which was also seen in the present study, considering both the potential of the indigenous microflora (Paper III) as well as the rapid disappearance of the compounds exposed to small tomato plants (Paper I). In general, water-solubility of the target compound, local match between

metabolising microflora and target compound as well as suitable enzyme production by the microflora vary, but seem in this case have favoured metabolisation.

Utilisation by the effluent nutrient solution microflora of the eleven substrate groups of the GN-panel resulted in five groups which were used above the threshold level (amines, amino acids, carbohydrates, carboxylic acids and polymers). Carbohydrates showed the shortest lag-phase (Paper III, Figure 3), which might indicate that the microflora to be well acclimatised to this group of compounds.

Several studies confirmed the theory of accumulation in the nutrient solution to be unlikely: observations on soil microorganisms capable of using phenolic acids as single carbon source (Schmidt, 1988), enrichment of the classes of organisms able to metabolise phenolic acids in microflora exposed to phenolic acids (Blum and Shafer, 1988; de Kreij *et al.*, 2003) and increase of fast-growing bacteria (Shafer and Blum, 1991). However, other degradation processes such as auto-oxidative have been discussed for caffeic acid (Sundin *et al*., 1995).

Caspersen *et al.* (2000) found several *Pseudomonas* spp. strains in a commercial lettuce growing system being able to degrade ferulic acid, one of which even showed growth-stimulatory characteristics. It is tempting to conclude, that the addition of ferulic acid selects for a microflora stimulatory to crop growth. Bacterial utilisation needs further investigation with respect to metabolic pathways and micro-site for metabolisation.

Enhanced metabolite formation

Disinfestation of the nutrient solution minimises the risk of spread of pathogens (McPherson *et al.*, 1995; Tu *et al.*, 1999; Ehret *et al*., 2001), however, some commercial growers using closed systems do not experience greater disease problems and the development of a suppressive microflora seems likely (McPherson *et al*., 1995; Postma *et al*., 2000). One biotic parameter in this context could be the excretion of microbial metabolites.

Several attempts have been made (van Peer and Schippers, 1989; Buysens *et al*., 1995; van Os *et al*., 2002; Alsanius and Gertsson, 2003) to introduce different antagonists such as strains of *Pseudomonas* species into the nutrient solution and rhizosphere of greenhouse grown crops. Both *Trichoderma harzianum* (Lorito *et al*., 1996) and *Lysobacter enzymogenes* (Zhou *et al*., 2002; Folman *et al*., 2003) , which are used in paper IV, are primarily known for their antifungal activities through excretion of extra-cellular enzymes. In the present studies, *Lysobacter enzymogenes* 3.1T8 showed formation of a compound with high similarity with 2,4-diacetyl phloroglucinol (Folman, 2003). This result together with the occurrence of 2,4-diacetyl phloroglucinol preferentially in nutrient solution treated with slow sand filtration and treatments amended with an antagonist, leads to the suggestion of general mechanisms of biocontrol to play a role in the growing system.

There are several advantages of supporting the resident microflora to produce antibiotic compounds (Paper V) instead of introducing a specific antagonist. The indigenous microflora is well adapted to its surroundings and has a competitive advantage in contrast to an introduced antagonist as well as a pathogen. Stimulating a group or even several groups of bacteria to produce antagonistic metabolites would decrease vulnerability of the biocontrol system than if only one organism or multiple strains are introduced.

A prospect for such an approach is given in Paper V, where fluorescent pseudomonads were in focus. They are fast to grow and are able to produce a range of organic compounds (Whipps, 2001), however, other groups could also be relevant. It was shown that production of antibiotic compounds such as 2,4-diacetyl phloroglucinol in nutrient solution amended with yeast malt broth was possible (Figure 3) although further studies are needed before results can be transformed into application in greenhouse growing systems. Organic amendments to the nutrient solution gave increased enzyme activity and inhibition of *Fusarium oxysporum* f.sp. *cyclaminis* (Brandt and Alsanius, 2003). Next to organic amendments, inorganic nutrients such as copper and zinc can be key factors in metabolite production (Duffy and Défago, 1997). The spatial allocation (Bazin *et al*., 1990) of different types of microorganisms according to their carbon metabolisation has to be considered in this context. Moreover, the conditions for survival and development are different for a single cell, for a specific strain and for a whole population. Interactions between the antagonist and the pathogen are of importance (Rattink and Postma, 1996). Besides competition for space and nutrients, chemical and physical characteristics such as oxygen availability, pH, EC and temperature are decisive at the specific micro-site. In present studies the number of fluorescent pseudomonads was low (2.6 log CFU ml^{-1}), which could be due to high EC values. Many mechanisms are involved in the process of biocontrol and *in vivo* studies for production of specific antibiotic compounds are needed to include these factors affecting the biocontrol process.

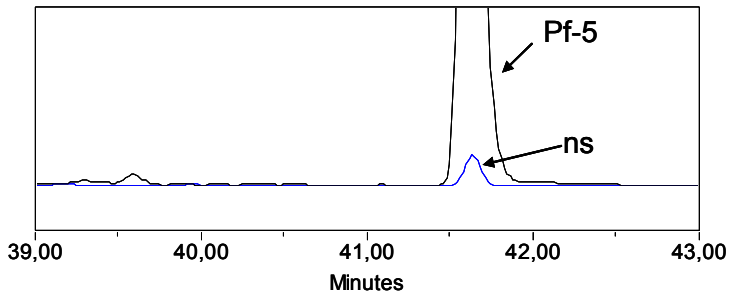


Figure 3. Chromatogram of 2,4-diacetyl phloroglucinol produced by *Pseudomonas fluorescens* strain Pf-5 (45 µM) and the microflora inhabiting the nutrient solution (ns, 1µM) of a closed hydroponic NFT-system with tomato under optimised conditions. Metabolite formation was optimised in an enriched culture (yeast malt) at room temperature. Enrichment was performed for 9 days on a rotary shaker (200 rpm) before HPLC-analysis.

The role of benzoic acid

Benzoic acid was the most frequently determined compound. Therefore, I would like to give a summarised picture on the impact of benzoic acid in nutrient solution of closed growing systems.

Benzoic acid was not found at all in the NFT-growing system when the extraction method was adapted (Paper II), but was verified (10-200 nM) throughout the whole growing season within all crops and disinfestation treatments followed in Paper IV (Paper IV, Figure 1). As Inderjit (1996) underlined, it is important to demonstrate bioactive concentrations substantial and persistent enough to affect plants. In the present study, high concentrations of benzoic acid were required to decrease plant growth (Paper I) and persistence of the compound was low (Figure 4; Paper I and III). Acclimatisation of the microflora to benzoic acid is suggested. Sundin *et al*. (1995) observed that colony-forming units increased parallel to the disappearance of phenolic compounds. As discussed previously, the rhizosphere microflora is of importance in this context but conclusions by means of nutrient solution inhabiting microflora, dissipated every fourth day, are limited. A microbial population able to utilise benzoic acid was probably built up during the experiment, as suggested in soil studies of ferulic acid (Shafer and Blum, 1991). The potential in tap water was significantly lower than that in the nutrient solution, which points to a utilisation potential even by the microflora not acclimatised to metabolites such as phenolic acids. However, this potential was assessed *in vitro* under favourable growing conditions with respect to oxygen consumption. Local anaerobiosis must be expected in the root mat, which would change preconditions for utilisation. In the growing system there is a continuous excretion of root exudates and C-sources which influence the microflora.

Benzoic acid concentrations in the mineral wool slabs was initially about ten times higher than the concentration measured in the nutrient solution from the growing crop. As concentration of benzoic acid was constant over the observation period and disappeared rapidly in the laboratory experiment (Figure 4), a release from the stone wool might be possible, however nothing can be said about release rate, concentrations or duration. Furthermore, continuous excretion by the plants and/or production by the microorganisms in the nutrient solution and rhizosphere should not be excluded. The concentration of benzoic acid in the nutrient solution could be neither correlated to fluorescent pseudomonads nor total number of bacteria. Commercial formulations of benzoic acid are used in horticultural production have suppressed plant pathogens (Wohanka and Woelk, 1998; Büttner and Bandte, 1999; Wohanka and von Dömming, 1999). Excessive doses enhanced yield and resulted in increased viable counts of fluorescent pseudomonads (Wohanka and Lindemann, 2003).

Benzoic acid is known to be an intermediate in many microbial metabolic processes. Benzoic acid is for example transformed to *p*-hydroxybenzoic acid by *Penicillium* sp. (Hofrichter and Scheibner, 1993). It should be further studied, if

there is a metabolic link between benzoic acid and 2,4-diacetyl phloroglucinol, since benzoic acid was also found in preliminary studies optimising the growing conditions for the resident microflora in the nutrient solution. Phenolic acids change the microbial ecology in the rhizosphere (Shafer and Blum, 1991) and thereby the preconditions for metabolisation.

Further studies should focus on the role of benzoic acid in the nutrient solution and rhizosphere of hydroponically grown crops in order to assess its potential for biocontrol mediating mechanisms.

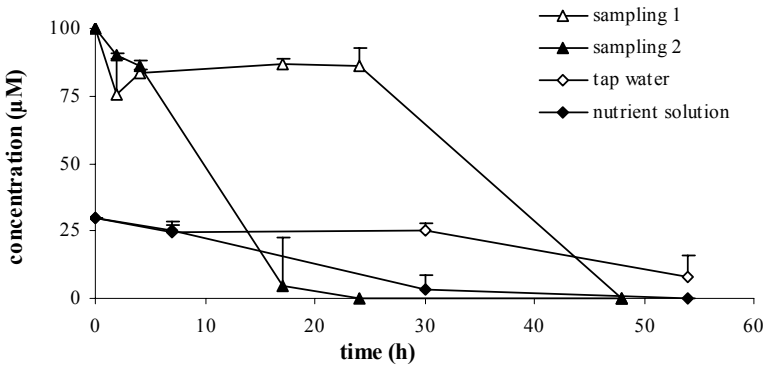


Figure 4. Disappearance of benzoic acid (100 µM; △, ▲) in the nutrient solution of static aerated culture with young tomato plants (Paper I) and *in vitro* in the effluent nutrient solution from a closed NFT system (◇) or tap water (◆) supplemented to final concentrations of 30 µM (Paper III). Samplings 1 and 2 represent sampling occasions in the beginning (1; △) and at the end (2; ▲) of the phytotoxicity trial.

Conclusions and outlook

The obtained results can counter the concerns of commercial growers about plant and microbial metabolites accumulating in the recirculated nutrient solution. Risk for damaging levels of the studied compounds on tomato small plants appears low as concentrations of phytotoxicity are elevated whereas found concentrations were low under prevailing conditions. Aromatic compounds disappear rapidly under optimised conditions, thus the risk for accumulation appears low. Qualitative and quantitative variations occur between crops and disinfestation treatments but no pattern was identified. Future studies should include thorough optimisation of nutritional and climatic conditions in order to draw consistent conclusions. The adapted SLM extraction method for greenhouse use proved to be an easy tool for evaluating the actual situation in the growing crop and can easily be adapted to compounds others than those studied within this thesis work. Accumulation of other toxic compounds is still possible, but root exudates and microbial metabolites such as the selected compounds studied here, should be

regarded as an asset instead of a problem. Production of antimicrobial compounds may be favoured by appropriate nutrient supply. *In vivo* investigations, where growing conditions are non-optimised are decisive in future studies.

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Personal communications:

Henri Beekers, Cultilène, Saint-Gobain, Postbus 10190, 5000 JD Tilburg, NL.