PLANT RESEARCH INTERNATIONAL

Use of environmentally friendly substances to induce plant defence mechanisms

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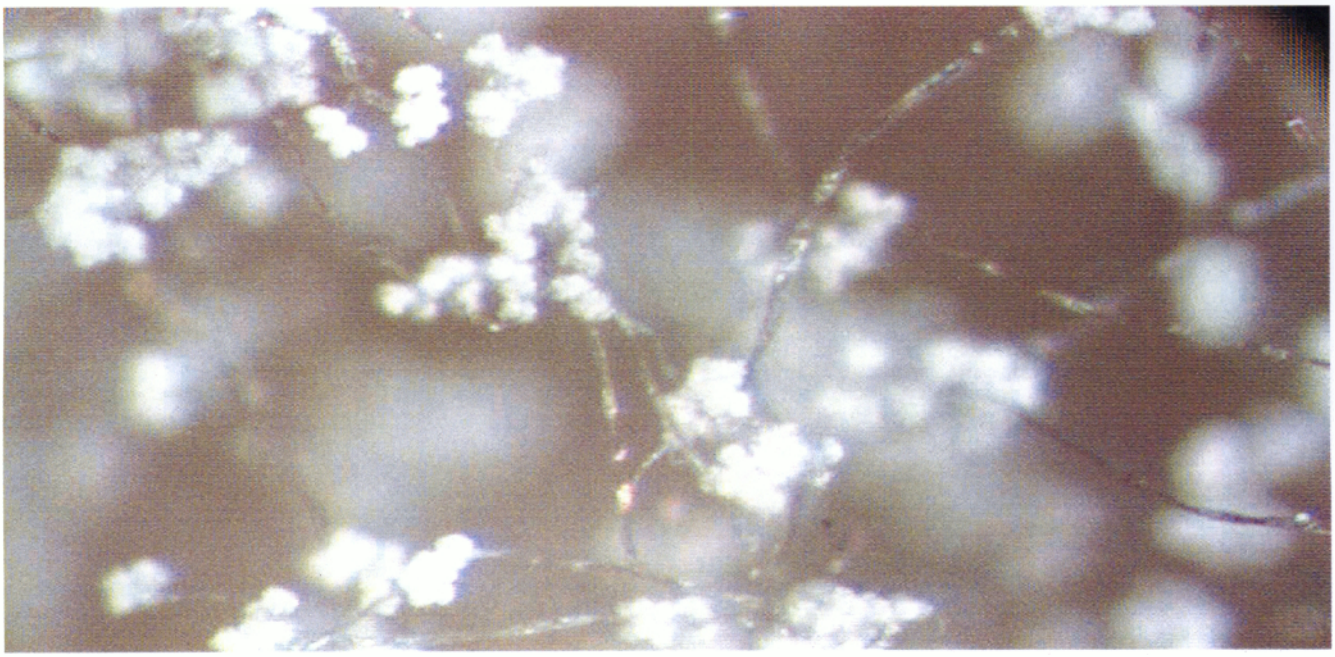

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WAGENINGEN UR

To my mother

who is guide in my life.

Thanks

After six months my final practical period has come to an end. I can look back on a nice and educational period. For me it was a great experience to work at a company like Plant Research International. I met here a lot of nice people who made my stay here in the Netherlands to a nice period, so nice that I will be back in October.

At this point I want to thank a few people who helped me during my stay here. I want to thank **Dr. Jordi Wilco** for giving me the opportunity to carry out my six-month training period in the cluster of Function and Diagnostic of Plant Development and Reproduction, a business unit in Plant Research International Institute.

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Thanks for everything!!!

Dr. Jordi Wilco

Dr. Wim van der Krieken

Jan Kodde

Christina Dimitriadou

PLANT RESEARCH INTERNATIONAL

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Plant Research International, together with the Department of Plant Sciences of Wageningen University and the Institute for Applied Plant Research, forms the Expertise Group Plant Sciences of Wageningen University and Research Center.

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The town of **Wageningen** is an excellent base for your stay in the Netherlands. Students and guests will find a pleasant study environment and opportunities to exchange of expertise in Wageningen. In addition to Wageningen's delightful surroundings, a wide range of accommodation, cultural and recreational facilities are available, Wageningen lies in the centre of the Netherlands and is an ideal base from which to explore the rest of the country.

The cities of Amsterdam, Rotterdam and The Hague are only and hour away. The surroundings of Wageningen are delightful: to the North lies the Veluwe (the largest nature reserve in the Netherlands) and to the South lie the Betuwe and the water meadows of the river Rhine, a typically, water rich, Dutch landscape.

Wageningen's 35000 or so inhabitants enjoy a thriving cultural life and the many international students, guest researchers and congress visitors from abroad create international atmosphere in the town. Wageningen is a town on 'bicycle scale'. All the student facilities, University buildings and the town center are within cycling distance from each other. The bicycle is the most popular and practical mode of transport. English is commonly spoken, which means it is easy to communicate with the locals.

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ABSTRACT

Plant diseases are responsible for annual crop loss order of 12 percent worldwide and, although pathologists have long known what makes plants sick, only recently have significant advances been made with respect to the genetics and biochemistry of disease resistance in plants. Plants respond to attack by fungi and other pathogens by mobilizing a complex network of active defenses. Localized defenses in cells immediately surrounding the side of infection include strengthening of the cell wall through callose deposition and lignification, synthesis of antimicrobial phytoalexins, and induction of lytic enzymes that attack the invading pathogen. This combination of responses can be effective in preventing the spread of infection, but there is also evidence that plants are capable of responding with a more broadly based defense mechanism known as **systemic acquired immunity (SAR).**

SAR involves the induction of a set of pathogenesis-related (PR) proteins that appear to help ward off secondary infections. In this sense, SAR appears to resemble the human response in principle. Once activated by an invading pathogen, the immune capacity is distributed throughout the plant. Both naturally occurring compounds like salicylic acid (SA) and synthetic compounds, like benzo (1,2,3) thiadiazole-7-carbothioic acid-S-methyl ester (BTH), can induce SAR.

In this report, a number of elicitors were used against *Botrytis cinerea* on tomato plants, of which Lignosulfonate (**LS**) proofed positive. LS proved in many experiments to be a powerful tool for controlling plant diseases not only when used before the infection, but also after. It proofed to enhanced resistance not only against *Botrytis cinerea* on tomato and maize plants, but also against *Phytophthora infestans* on tomato and potato plants. Combined with low concentrations of fungicide, LS proved to be as effective as the standard treatment. Therefore, LS can be used to decrease the amount of fungicide and also to induce the side effects caused by this fungicide. However, the other elicitors in this report did not induce resistance towards *Botrytis.* This does not mean that they are not important signaling molecules for defense pathways.

INTRODUCTION

➢ PLANT-FUNGAL INTERACTIONS

It is estimated that there are about 250,000 species of plants, but six times as many (1.5 million) species of fungi. Fungi are, ultimately, all dependent on plants for their carbon and energy source, like most other organisms that are not able to photosynthesize. Fortunately for plants, the relationship between them and fungi is usually a mutually beneficial one. The great majority of fungi are saprophytic, i.e. they live on dead plant material, breaking this down and so recycling the nutrients to become available again for living plants. During the course of evolution, some fungi have started to interact actively with living plants. Most of these interactions are advantageous to plant, e.g. for their growth and development, as in the case of **mycorrhizae** and **endophytes**. A small minority of fungal species has developed further and broken the fine balance of mutual benefit to become plant pathogens. However, in most plant populations there are individuals that are resistant to fungal infection.

The interaction between plants and their pathogens is complex and may be very specific to a given combination of the plant and the fungus. The defense strategies of plants against their pathogens are manifold and include the use of antifungal chemicals. On the other hand, pathogens have evolved mechanisms to evade these chemicals. The chemical aspects of the warfare between plants and fungi are discussed below.

➢ CONSTITUTIVE AND INDUCED ANTIFUNGAL COMPOUNDS

When a fungal spore comes in contact with a plant surface, the microclimate (temperature, humidity, light conditions, etc.) has to be right before it can germinate. Then it

has break several lines of defense set up by the plant before reaching a living cell. These include mechanical barriers such as a thick cuticle, and chemical ones such as exudate compounds, which inhibit spore germination and germ tube elongation. These constituents are part of the arsenal of constitutive (or preformed) antifungal compounds produced by plants, also called preinfectional metabolites, prohibitions or phytoanticipins. If all these plant weapons are not sufficient to stop germination of the fungal spores and penetration of the hyphae through the epidermis, the plant usually responds by blocking or delaying the advancement of the invader. **Reactive oxygen species (ROS)** are often generated as warning signals within the cell or to the neighboring cells, triggering off various reactions. These include the structural reinforcement of the cell wall, the **hypersensity response** (a programmed cell death), development of **systemic acquired resistance (SAR)** and the accumulation of newly produced antifungal chemicals, which are called **phytoalexins**. The term phytoalexin is usually restricted to antibiotic compounds that require de novo expression of the enzymes involved in their biosynthetic pathway. This is a very economical way to counteract pathogens, because the carbon and energy resources are diverted to phytoalexin synthesis only at the early period of infection, and only at its site. Unchallenged plants can use these resources for more basic process of life such as development of flowers and production of seeds or accumulation of reserve carbohydrates in their storage organs. Some plants do not produce phytoalexins when challenged by pathogens, but release toxins that are normally stored as less toxic glycosides in the vacuoles of their cells, e.g. phenolic and iridoid glycosides, glycosinolates and saponins. If the integrity of the cell is broken when penetrated by fungal hyphae, the glycoside comes into contact with hydrolysing enzymes present in other compartments of that cell, releasing the toxic aglycone. Although this aglycone released after fungal attack is not present in the intact plant and is newly produced, it is strictly speaking not

a phytoalexin, because the enzymes involved (glycosidases) were already present in the healthy plant and were not formed de novo.

➢ PHYTOALEXINS

As long ago as 1911 the French botanist Noel Bernard discovered that plants could produce antifungal substances, which are specifically, formed when, the plant is attacked by fungi. He found that the tubers of two orchid species, *Orchids morio* and *Loroglossum hircinum* (=*Himantoglossum hircinum*) became resistant to further fungal attack after they had been infected by the fungus *Rhizoctonia repens.* By placing infected tuber tissues on agar and introducing fungi into the medium, Bernard found that the fungus-infected tissue produced a diffusible inhibitor or fungal growth, but the compounds involved were not identified until many decades later. Müller and Börger (1940) observed the same phenomenon in potato tubers infected by *Phytophthora infestans,* and they called these induced substances **'phytoalexins'** (Greek φυτον=plant, αλεξειν=to defend). Müller and Börger defined phytoalexins as "chemical compounds produced as a result of invasion of living cells by a parasite". This definition has been modified frequently whenever new evidence revised earlier concepts. For instance, it soon became clear that phytoalexins were not only formed in plants after exposure to fungi, but also by various non-biological stress factors such as irradiation with short-wavelength UV light or treatment with heavy metal ions such as copper or mercury salts. For this reason Ingham (1973) redefined phytoalexins as "antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors". Others called compounds induced by environmental factors 'stress compounds'. Furthermore, the term phytoalexin is generally limited to secondary metabolites

of low molecular weight, usually below 1000, so that it does not apply to antifungal peptides and proteins produced by plants.

➢ SYSTEMIC ACQUIRED RESISTANCE (SAR)

Infection of plants, particularly by necrotizing pathogens, leads to enhanced resistance to subsequent attacks by the same or even unrelated pathogens. This phenomenon is referred to as **systemic acquired resistance (SAR)**. SAR is one such inducible defense response that is triggered in the plant by previous exposure to pathogens that cause cell death. SAR is long lasting and confers protection against a broad spectrum of pathogens (bacteria, fungi and viruses). Amore rapid defense response that precedes the onset of SAR is **the hypersensitive response (HR)**, which is localized at the site of attempted pathogen entry. HR is characterized by programmed death of host cells and is a consequence of the interplay of the products of the pathogen **avirulent genes (Avr)** and the **host disease resistance gene (R)**. Tightly correlated with the HR and the SAR is the production of antimicrobial compounds, the increased expression of a subset of the **pathogenesis-related (PR) proteins**, many of which possess antimicrobial activities, and the reinforcement of mechanical barriers such as cell walls.

➢ HYPERSENSITIVE RESPONSE (HR)

'Hypersensitive' was a term first applied by Stakman (1915) to describe the rapid and localized plant cell death induced by rust fungi in rust-resistant cereals. The subsequent realization that such death was a common expression of disease resistance in plants, regardless of the type of inducing pathogen, led to its designation as the hypersensitive

response, usually defined as 'the rapid death of plant cells is association with the restriction of the pathogen growth'. The HR is generally recognized by the presence of brown, dead cells at the infection site and, depending on the pathogen, their number may vary from one to many. The HR may or may not be restricted to cells physically invaded by, or having direct contact with, the pathogen. A visible brown lesion may develop if sufficient cells die.

Plant genotypes within an otherwise susceptible plant species may exhibit resistance, and the HR, against specific genotypes of a pathogen. This resistance is generally, but not always, controlled by single, **parasite-specific resistance (R) gene**. For biotrophic fungal pathogens in particular, the HR requires the pathogen to have an **avirulence (avr) gene** that 'matches' the R gene in a 'gene-for-gene' relationship. R and avr genes appear to have a more complex relationship for bacterial pathogens, with single R genes 'matching' more than one avr gene.

Whether the HR expressed in non-host plants has the same type of genetic control is controversial and conceptually most likely when plants are both hosts and non-hosts of related pathogens. The evolution of plant-bacteria interactions is further complicated by the ease of gene transfer between different bacteria.

➢ ELICITORS

As a result of host-pathogen convolution, plants have developed sophisticated mechanisms to protect themselves from disease. Besides performed physical and chemical barriers that hinder infection, a wide variety of defense responses in induced only after pathogen attack. When these induced responses are triggered rapidly and coordinately during a given plant-pathogen interaction, the plant is resistant to disease. A susceptible plant responds more slowly with an onset of defense mechanisms after infection. Thus, the timely

recognition of an invading microorganism and the rapid and effective induction of defense responses appear to make a key difference between resistance and susceptible plants.

The activation of resistance in plants is initiated by host recognition of molecules called elicitors, which are directly or indirectly released from an invading pathogen. Although elicitors vary widely in their chemical composition, and the mechanisms by which plants perceive them may differ, many pathogen elicitors appear to trigger a common network of signaling pathways that coordinate the overall defense responses of plants. The induced mechanisms frequently manifest themselves as a **hypersensitive response (HR)**, which is characterized by necrotic lesions resulting from localized host cell death at the size of infection. The HR prevents growth ant the spread of the pathogen into healthy tissues.

❖ Specific Elicitors

Avr gene products might be expected to trigger the HR *only* in plants that contain a matching R gene, but few such 'specific elicitors' have been isolated. For viruses, specific elicitors have been identified as coat proteins, the helicase domain of a replicate gene, or a movement protein. For fungi, specific elicitors are primarily peptides of unknown function that are known or assumed to be products of *avr* genes and are secreted only under specific conditions or stages of development.

Many more *avr* genes have been cloned from bacteria than from fungi, but the identification of their products has been hampered by the fact that they appear to be secreted directly into the plant cell via a type III secretion system, components of which are encoded by *hrp* (hypersensitive reaction and pathogenicity) genes. *Avr* genes products alone seem sufficient to cause cell death since the latter is induced when *avr* gene are expressed in transgenic plants containing the corresponding R gene. *Avr*D from *Pseudomonas syringae*

pathovars seems to direct the production of syringolides, glycolipid elicitors of *Rpg4*-mediated cell death in soybean.

❖ Non-specific Elicitors

In addition to *avr* gene products, fungal and oomycete pathogens have a variety of components or secretory products, such as arachidonic acid, cell wall carbohydrates, glycoproteins and proteins, that can elicit plant defense responses and, in some cases, cell death. These 'non-specific elicitors' of cell death kill cells in a wide range of plants, often including those susceptible to the pathogen, and their binding-sites generally seem to be associated with the plant plasma membrane. Although proof of a role for these elicitors in the HR is generally lacking, an involvement in the HR of non-host plants seems likely. Direct evidence comes from the case of transformants of the potato pathogen, *Phytophthora ifestans*, in which the lack of INF1, a 10 kDa protein of the death-eliciting alicitin family, is associated with a loss of ability to trigger the HR in one of three non-host *Nicotiana* species. For this oomycete, and for *P. capsici*, it also has been suggested that pathogen wall components act as non-specific elicitors of cell death in host species, and that cell death is suppressed in susceptible host genotypes by cultivar-specific suppressors. If this is the case, then R genes against these oomycetes may be involved in interfering with these suppressors, rather than directly triggering the HR.

In comparison to fungi and oomycetes, few non-specific elicitors of cell death have been isolated from bacteria. The exception is a family of glycine-rich; bacteria secrete cysteine-lacking proteins known as harpins which when grown in minimal medium in which *hrp* genes are depressed. Harpins elicit an apparent HR when introduced in high concentrations into the intercellular spaces of plant leaves, and induce the putative HR marker

gene *HIM1* in tobacco. However, the membrane potential responses elicited by harpin differ from those elicited by bacteria. Recent evidence suggests that the site of action of harpins may be the cell wall and, overall, there is still some doubt as to their natural role in the HR.

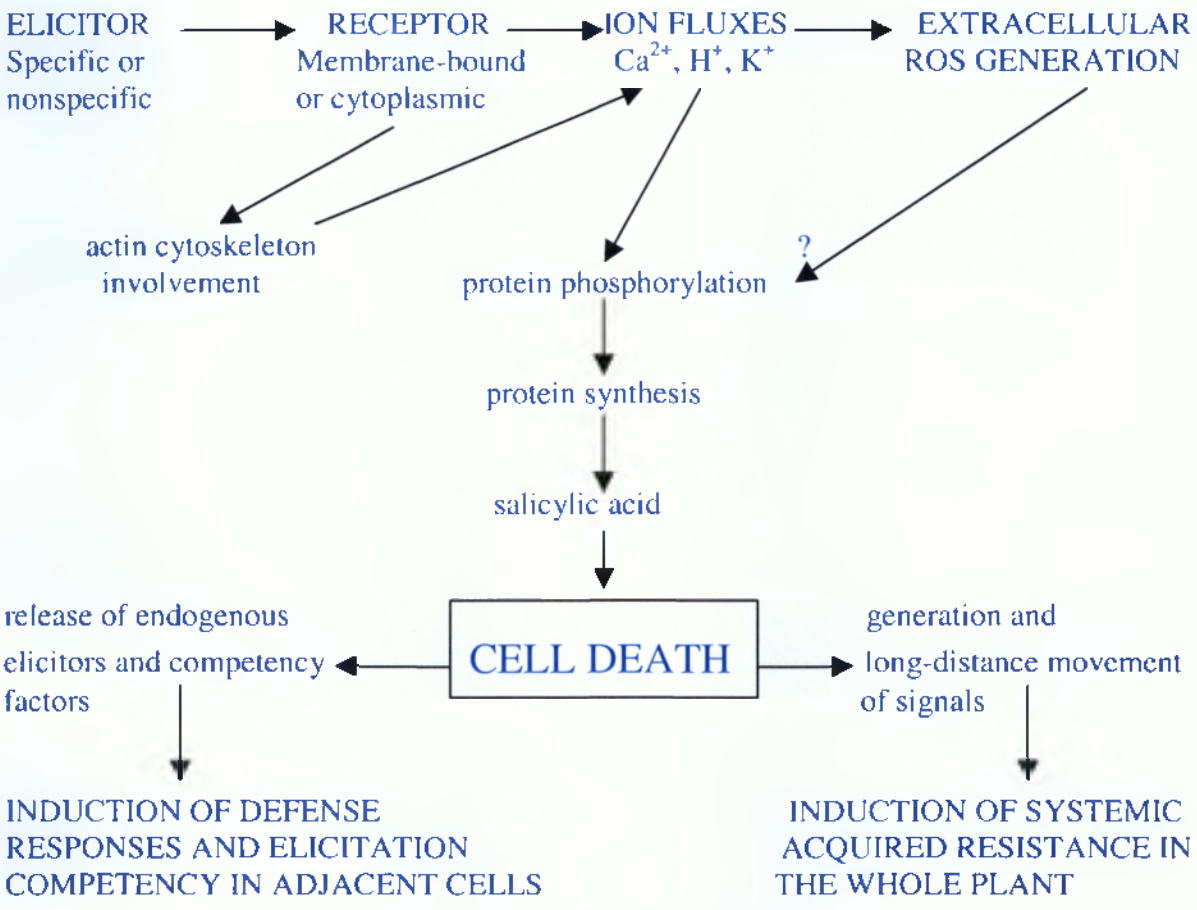


*Figure*1: Factors for which there is, in at least some plant-pathogen interactions, current evidence of involvement in the induction of hypersensitive cell death, or in the consequences of such death. Note that single arrows do not preclude the presence of multiple, parallel, pathways. ROS, reactive oxygen species.

➢ SALICYLIC ACID (SA)

During the past few years extensive studies from several laboratories have provided a mounting body of evidence that **Salicylic acid (SA)** is an important endogenous signal for activation of certain plant defense mechanisms associated with disease resistance. More than a decade ago, application of exogenous SA or its derivative, acetylSA (aspirin), was shown to induce **PR protein** synthesis and partial resistance to pathogens such as tobacco mosaic virus in tobacco. More recently, elevated levels of SA have been found to be associated with resistance of infected plants to the invading pathogens in an increasing number of plant species, including tobacco, *Arabidopsis* and cucumber.

Transgenic tobacco and *Arabidopsis* plants provide further evidence for the involvement of SA in the induction of defense responses that constitutively express the *nah*G gene encoding a salicylate hydroxylase from *Pseudomonas putida*. In these transgenic plants there is little or no accumulation of SA after pathogen infection, and their ability to restrict pathogen spread and to establish **SAR** is correspondingly impaired. Finally, number of *Arabidopsis* mutants with defective SA signal transduction show compromised defense responsiveness against pathogen infection.

➢ JASMONIC ACID

Evidence is accumulating that the wound response pathway might also play a role in defense against specific fungal pathogens. It has been known for some time that fungal elicitors can trigger this response. Jasmonates are proposed to be signaling molecules associated with the activation of an increasing number of plant genes. They have important role in the wound response as well as in the pathogen attack response in plants. Jasmonic acid

was first associated in the activation of genes encoding protease inhibitors following wounding. Jasmonate levels also increase rapidly but transiently in wounded plants and induce gene expression through a lipid-based signaling pathway called the octadecanoid pathway.

Jasmonic acid (JA) is indeed important signal in the induction of systemic defense responses. It is rapidly produced when the plant is attacked by pathogen, particularly during necrotizing infections where the rise in JA even extends to systemic tissues. Moreover, exogenous application of JA has been shown to enhance the expression of an array of stress-related genes such as thionin and defencins in *Arabidopsis* and proteinase inhibitors in tomato. In contrast, elevated levels of JA can down-regulate other genes such as those encoding proteins required for photosynthesis. In addition, the signaling molecules JA regulate the wound response genes, and the expression of some basic PR genes in tobacco.

➢ BION

BION [active component benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester: CGA-245 704, also known as **BTH**] has been shown to induce resistance in several plant species like *Arabidopsis*, been, cucumber, tobacco and wheat. **BTH** is a synthetic inducer of pathogen resistance and induced a set of **'SAR genes'**, including members of the pathogenesis-related (PR) proteins.

In tobacco and *Arabidopsis*, two model plants for studying SAR, BTH induced the same set of genes, as did biological inducers of SAR like tobacco mosaic virus or *P. syringae.* Therefore, the BTH was assumed to trigger the same SAR pathway as the biological inducers and to be functional analogues of the SAR-signaling compound salicylic acid (SA). Support

for this hypothesis came from studies with mutant *Arabidopsis* plants that BTH was impaired in their response to biological inducers of resistance as well as to SA.

BTH was reported to induce resistance in wheat against several pathogens, and to induce a set of so-called **'wheat chemically induced'** (WCI) genes.

➢ LIGNO B (LS)

Lignosulfonates (LS) are low-cost by-products from the paper industry and are already commercialized as fertilizers. LS produced from acid sulfite pulping process have similar characteristics to those of soil organic materials. Application of LS has shown to be beneficial to agriculture through increasing soil organic matter and improving the efficiency of fertilizers.

LS consist of the degradation product of lignin, an abundant organic polymer produced by vascular terrestrial plants. These lignin products contain a variety of impurities such as sugars, sugar acids, extractives, and inorganic. Use of lignosulfonates is based upon the ability of their components combination to act as binders, dispersants and to rebuild lignin-like complexes. They are environmentally friendly (non-toxic) and very cheap. Because of their important properties their application in a lot of fields in agriculture may be useful. Their possible mode of action in tissue cultures and not only has been investigated. Finally, lignosulfonates as cell wall breakdown products playing a role as elicitors.

➢ SUPPOSITION

During this project the potential role of many elicitors (like SA, JA, Bion, Bioalgeen and LS) were tested. The possible effective action of these compounds, by activating the defense

pathway was investigated on tomato, potato, maize, sweet pepper and *Arabidopsis* tissues. These elicitors were tested against different pathogens. Also synergetic or adaptive effect of these elicitors when combined with (low amounts) of fungicide was investigated.

To have reliable results the leaf top system were used. With maize and potato tissue, whole plants were used in some cases. During this project three kinds of fungal material were used: *Botrytis cinerea, Phytophthora infestans and Mildew.*

➢ *Botrytis cinerea*

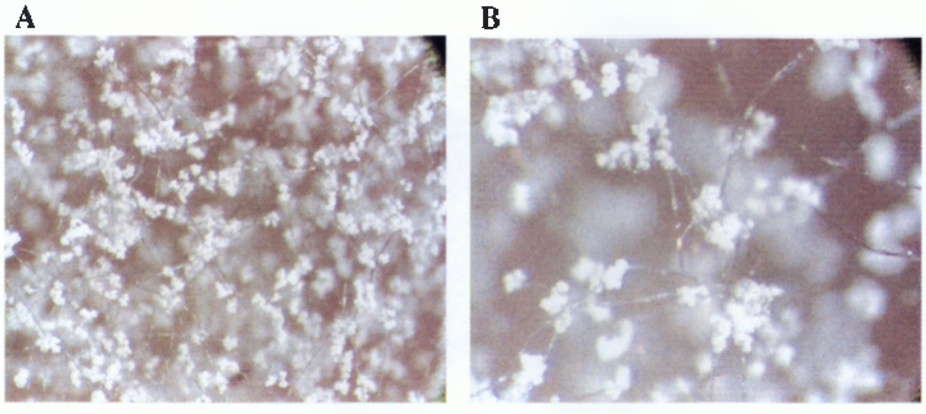
Botrytis cinerea is the most common and the most widely spread diseases of vegetables, ornamentals, fruits, and even some field crops throughout the world. *Botrytis cinerea* belong to family of *Moniliaceae* the order of *Moniliales* of the *Hyphomycetes* of the subdivision *Deuteromycetes*. *B. cinerea*, the causal agent of blight, rot, and gray mold in different plant species, secretes various endopolygalacturonases during all stages of infection. The pathogen, produces abundant gray mycelium and long, branched conidiophores that have rounded apical cells bearing clusters of colorless or gray, one-celled, ovoid conidia. The conidiophores and clusters of conidia (picture 1) resemble a grapelike cluster. The conidia are released readily in humid weather and are carried by air currents. The fungus frequently produces black, hard, flat, irregular sclerotia (figure 2).

In the field, blossom blights often precede and lead to fruits rots and stem rots. The fungus becomes established in flower petals, particularly when they begin to age, and there it produces abundant mycelium. In cool, humid weather the mycelium produces large number of conidia, which may cause further infections. The mycelium grows and invades the inflorescence, which becomes covered with a whitish-gray or light brown cobwebby mold. The fungus then spreads to the pedicel, which rots and lets the buds and flowers lop over. The

fungus later moves from the petals into the fruit and causes a blossom end rot of the fruit, which advances and may destroy part or all of the fruit. Infected fruit and succulent stems, become soft, watery, and light brown. As the tissue rots, the epidermis cracks open and the fungus fruits abundantly. Flat black sclerotia may appear on the surface or are sunken within the wrinkled, dry tissue.

Botrytis causes leaf spots on their hosts. The spots are small and yellowish at first but later become larger, whitish gray or tan, and sunken, coalesce, and frequently involve the entire leaf. Stem lesions usually appear on succulent stems or stalks. They may spread through the stalk and cause it to weaken and break over at the point of infection. In wet weather the diseased parts become covered with a grayish-brown coat of fungus spores. Sclerotia may also be produced on infected stems.

Infection of belowground parts, such as bulbs, corms, tubers, and roots, may begin while these organs are still in the ground or on harvest. Infected tissues usually appear soft and watery at first, but later they turn brown and become spongy or corky and light in weight. Black sclerotia are often found on the surface or intermingled with the rotted tissues and mycelium.



Picture 1: ***Scanning electron micrograph of a typical grape clusterlike conidiophore (B) and conidia (A) of Botrytis cinerea.***

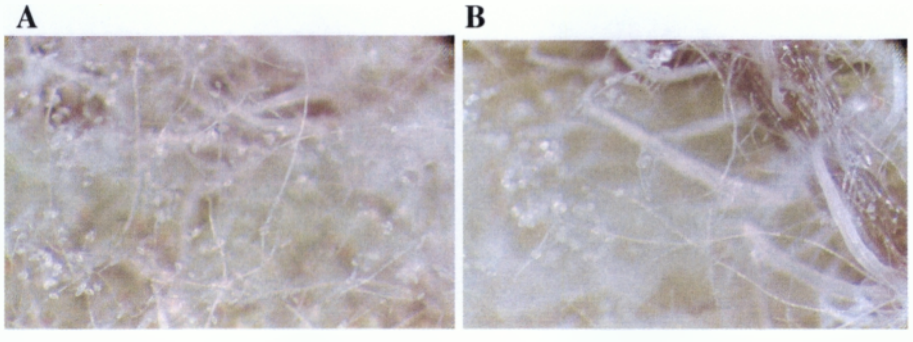
➢ *Phytophthora infestans*

Phytophthora infestans cause a variety of diseases on many different types of plants ranging from seedlings of annual vegetables or ornamentals to fully developed fruit and forest trees. It is the most important potato pathogen worldwide and the introduction of cultivars with resistance to *P. infestans* is one of the main potatoes breeding objectives. The pathogen *P. infestans* belong to family of the *Pythiaceae* the order of P*eronoslorales* of the *Oomycetes.* It is the best known species from the *Oomycetes,* the cause of late blight of potatoes and tomatoes. The mycelium of the pathogen produces branched sporangiophores that produce lemon-shaped sporangia at their tips. At the places where sporangia are produced, the sporangiophores (picture 2) form swellings that are characteristic for this fungus. Sporangia germinate almost entirely by releasing 3 to 8 zoospores at temperatures up to 12 or 15^0C, whereas above 15^0C sporangia may germinate directly by producing a germ tube (figure 3).

Symptoms appear at first as water-soaked spots, usually at the edges of the lower leaves. In moist weather the spots enlarge rapidly and form brown, blighted areas with indefinite borders. A zone of white, downy fungus growth 3 to 5 mm wide appears at the border of the lesions on the undersides of the leaves (Material and Methods, picture 4). Soon entire leaves are infected, die, and become limp. Under continuously wet conditions all tender, aboveground parts of the plants blight and rot away, giving off a characteristic odor. In dry weather the activities of the fungus are checked. Existing lesions stop enlarging, turn black, curl, and wither, and no fungus appears on the underside of the leaves. When the weather becomes moist again the fungus resumes its activities, and the disease once again develops rapidly.

Affected tubers at first show purplish or brownish blotches consisting of water-soaked, dark, somewhat reddish brown tissue that extends 5 to 15 mm into the flesh of the tuber. Later

the affected areas become firm and dry and somewhat sunken. Such lesions may be small or may involve almost the entire surface of the tuber without spreading deeper into the tuber interior. The rot, however, continues to develop after the tubers are harvested. Secondary fungi and bacteria, causing soft rots and giving the rotting potatoes a putrid, offensive odor may subsequently invade infected tubers. Tomato fruit is attacked and may rot rapidly in the field or in storage.



Picture 2: ***Scanning electron micrograph (using microscope) of a typical grape clusterlike sporangia (A) and sporangiofhores (B) of Phytophthora infestans on potato leaf.***

➢ *Mildew*

Mildews are probably the most common, conspicuous, widespread, and easily recognizable plant diseases. *Mildew* diseases caused by *Ascomycetes* and Imperfect (asexual) fungi. These diseases of the various crops or other plants are caused by many species of fungi of the family Erysiphaceae grouped onto seven main genera. These genera are distinguished from one another by the number (one versus several) of asci per cleistothecium and by the morphology of hyphal appendages growing out of the wall of the cleistothecium (figure 4).

Mildews are characterized by the appearance of spots or patches of a white to graying, powdery, mildew growth on young plant tissues, or of entire leaves and other organs being completely covered by the white mildew. Tiny, pinhead-sized, spherical, at first white, later yellow-brown, and finally black cleistothecia may be present singly or in-groups on the white to grayish mildew in the older areas of infection. *Mildew* is most commonly observed on the upper side of the leaves, but it also affects the underside of leaves, young shoots and stems, buds, flowers, and young fruit.

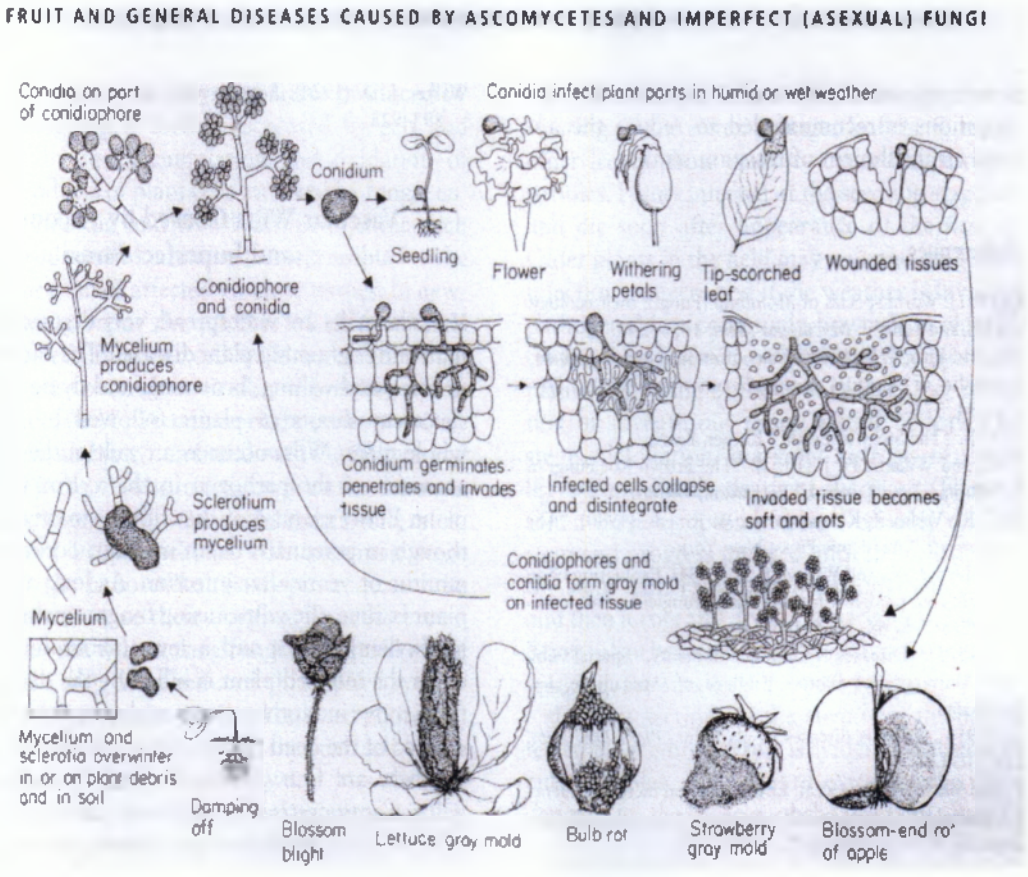


Figure 2: ***Development of Botrytis gray mold diseases.***

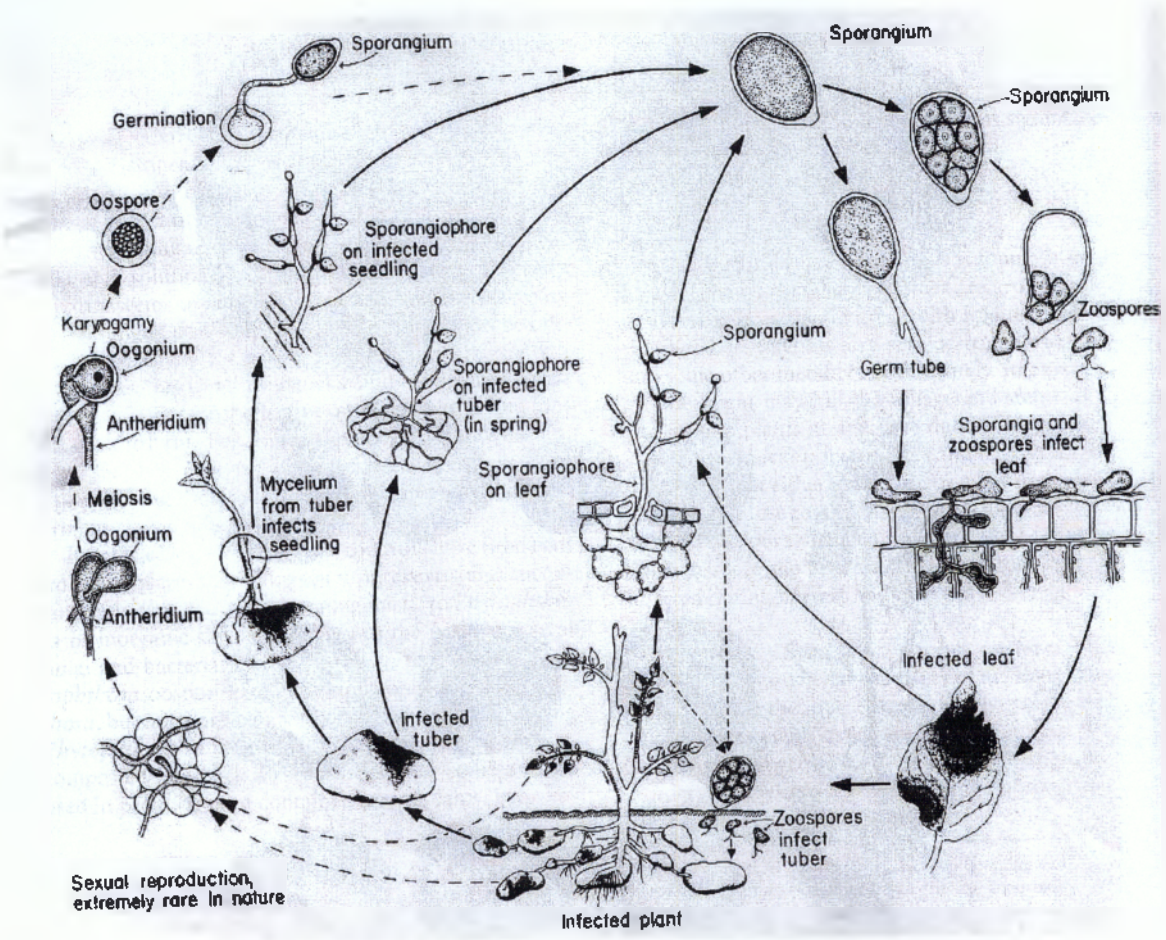


Figure 3: ***Disease cycle of late blight of potato and tomato caused by Phytophthora infestans.***

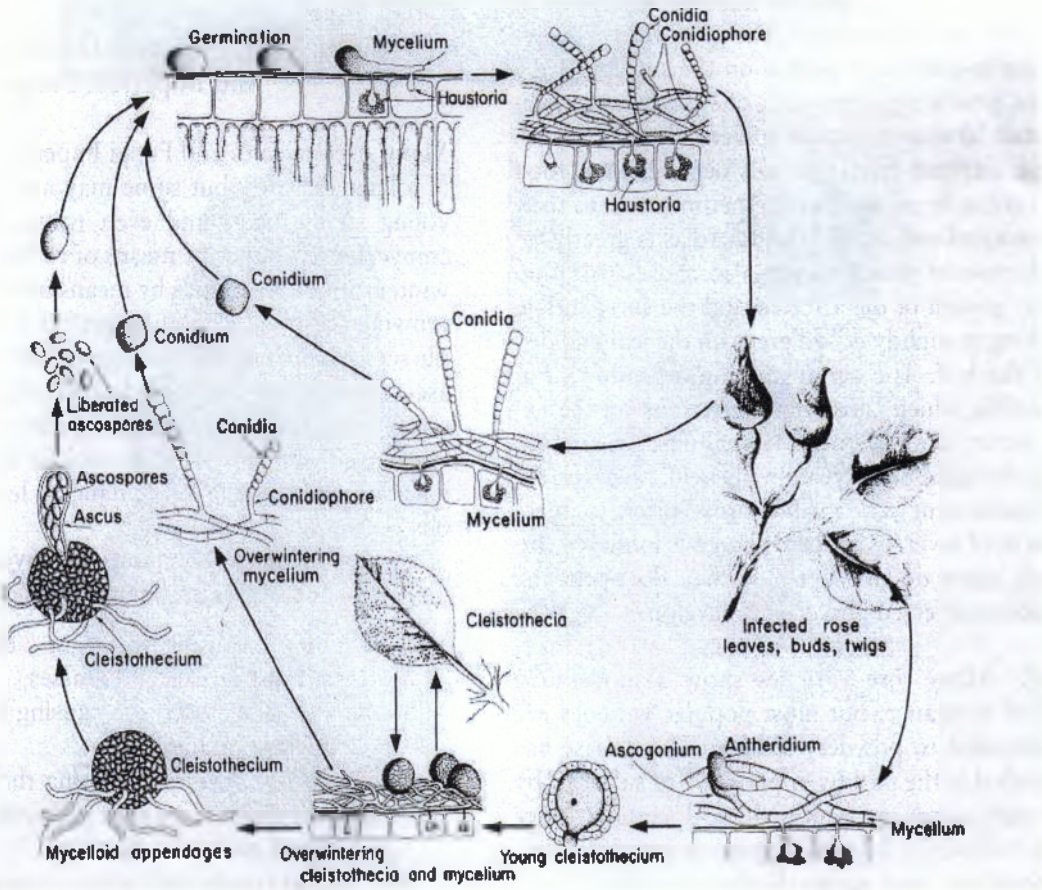


Figure 4: ***Disease cycle of Mildew of roses caused by Sphaerotheca pannosa f. sp. rosae.***

MATERIALS AND METHODS

AIM OF THE PROJECT

The aim of these experiments was to study the effects of contain elicitors on plant defense and to assess their perspectives for use in agriculture.

➢ PLANT MATERIAL

In this project, tomato *var. Moneymaker* plants (CAPITA) were used. Starting from seed the plants were grown in the greenhouse in potting soil (LENTSE GROND) under 12 hours of light, 20^0C, 70% humidity and 400-600 ppm of CO_2. The plants were irrigated with water and after the appearance of the 6^{th} leaf, irrigation continued only with STEINER that was provided to the roots. From 6 weeks old plant, unless described else, leaf tops were taken from the 3-8 leaf, counting from below for infection with pathogens, depending on the size and condition of the leaf.

Also, potato *var. Bintje* plants were used. The conditions of growing, starting from tubers, were the same as for tomato. Four weeks old shoots were taken from the tubers and placed in new potting soil. After 2-3 weeks these plants were used for infection pathogens.

We used maize *var. Geronimo* plants (supplied by Cebeco seeds). Maize plants grown under the same conditions as the tomato plants.

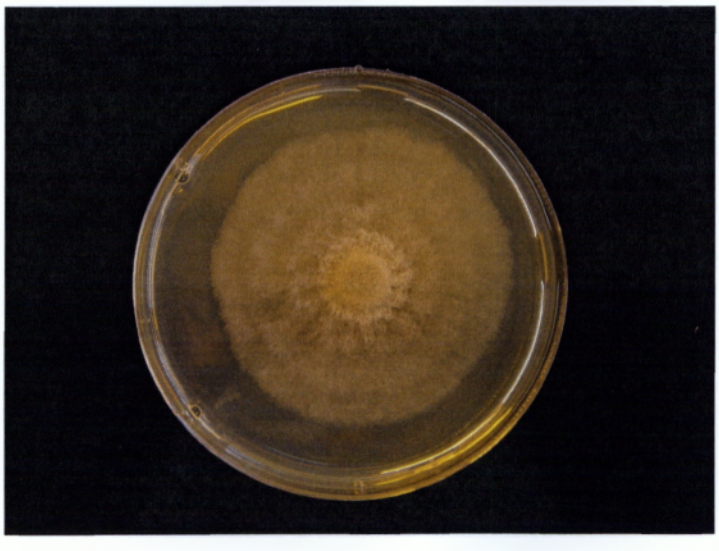
➢ FUNGAL (OR PATHOGEN) MATERIAL

• Preparation of pathogens

For *Botrytis* cultivation the fungi were grown on 25 ml sterile (20 min at 120^0C) solid medium, LB-agar (supplied by DUCHEFA). Therefore the petri-dishes containing the media were inoculated with fungi originating from another petri-dish or out of a spore suspension stored at - 80^0C. To provide even distribution of the fungi over the petri-dish in some cases (sterile) top-agar was used. This top-agar has the

same characteristic as LB-agar except for the agar concentration which is 0.7% instead of 1.5%. To prepare these top-agar petri-dishes, the spore suspension was mixed with the liquid top-agar (appr. 16.000 spores/ml, 5 ml/petri-dish) and this mixture was then pored on a petri-dish with 5 ml of solidified standard LB-agar. Care was taken the temperature of the top-agar-spore mixture was never higher then 45^0C. To avoid contamination of the environment with *Botrytis* and to work antiseptic all steps were carried out in a down flow cabinet.

The petri-dishes with the *Botrytis* spores were incubated at 20^0C in a IKS incubator, with 16 hours dark and 8 hours (black) light to induce sporulation (picture 1) unless mentioned else.



Picture 1: ***Appearance of the vegetative body (mycelium) of Botrytis cinerea in culture with petri-dish containing LB-agar.***

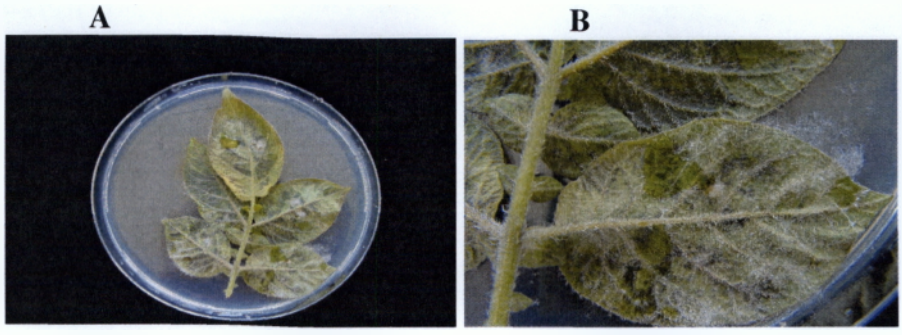
• Harvest of pathogens

During the *Botrytis* harvest, 10 ml of a solution containing 0.05% Tween 80 was added with a pipette (0.5-5 ml) on top of the petri-dish and the spores were brought in suspension using a Trichalski organ. This suspension was collected and if many spores were still on the petri dish the procedure was repeated. The combined factions containing the spores were filtered over glass wool to remove fungal threads etc.

The filtered Botrytis spores were centrifuged (10 min, 120 xg). The pellet was washed in half of the original volume of tap water and centrifuged for second time (10 min, 120 xg). The pellet was washed again in 1/4 of the original a volume of tap water and centrifuged (10min, 120 xg) for third time.

Finally, the pellet was washed in 1/10 of the original a volume of Gamborg B5 medium (Gamborg 3.16 g/l, Na-phosphate 10 mM pH=6.5, sucrose 10 mM).

During *Phytophthora* harvest, 20 ml tap water were used. The leaves with the *Phytophthora* spores were placed in the tap water. After few seconds, the spores were brought in suspension from the leaf, by moving the leaf inside the water. The collected suspension was set for 10-15 minutes to precipitate the spores on the bottom of the tube. Afterwards, most water removed carefully without disturbing the spore pellet leaving 1 ml of suspension in the tube.



Picture 2: ***Late blight symptoms of Phytophthora infestans on leaf of potato tissue. The whitish zone surrounding the necrotic area consists of sporangiophores and sporangis of Phytophthora infestans. Almost all the part of the leaf has been destroyed.***

The harvest of *Mildew* was followed the process of *Phytophthora*. The only exception was that the spores were centrifuged before counting for 10 minutes at 120 xg.

• Quantification of the fungi spores

The spores were counted using the Bürker-Türk counting chamber and a microscope. For this purpose a small quantity (10 µl) is diluted up to 100 times and 7 µl of this was applied to the counting chamber. The amount of spores counted in square E multiplied with 250 equals the number of spores per µl. The *Botrytis* spore suspension was adjusted to a final concentration of appr. 10.000 spores/ µl. The *Phytophthora* spore suspension was adjusted to appr. 7.000 spores/ µl and *Mildew* to appr. 4.000 spores/ µl.

Finally, the *Botrytis* spore suspension was divided in 0.5 ml portions in ependorf vails (containing 10% glycerol) and stored at – 80 ^{0}C until use. For keeping the *Botrytis* spore suspension in good condition in ependorf vails placed 10% glycerol (Appendix G, figure 9). For successfully infection on the leaf tissue, in contrast with *Botrytis*, the *Phytophthora* and *Mildew* spore suspension must be use immediately after the harvest.

➢ ELICITORS AND CHEMICALS

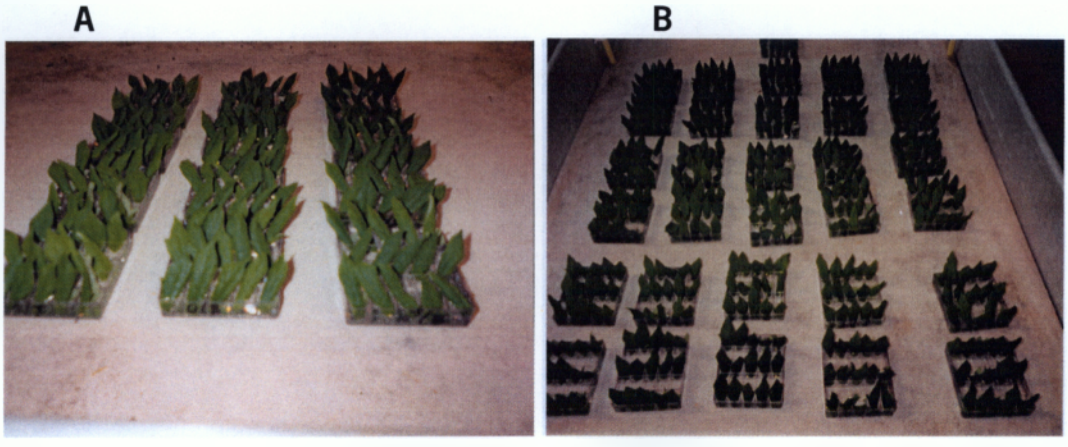
The elicitors tested were:

- Bioalgeen extract of Sea Algae supplied by Schulze & Hermsen GmbH
- Bion supplied by MTA Novartis
- Ligno B supplied by J&W WEGMAN.CO with an average MW=10.000
- Salicylic acid supplied by SIGMA with a MW=138.1
- Jasmonic acid supplied by DUCHEFA.CO with a MW=210.3

Unless described else the plant material was treated before infection by spraying with dilutions of the different test compounds or elicitors. As a negative control the dissolvent (water or 0.1% Tween 20 solution) was taken. Adding 1 µl of the spore suspension (appr. 10.000 spores) of the appropriate fungi infected plant material. After, the plant tissue was incubated under high humidity conditions to enable the growth of the fungi. With a sub-optimal Eupareen concentration of 10% or 20%, different concentrations of elicitors were combined.

➢ TOMATO/BOTRYTIS

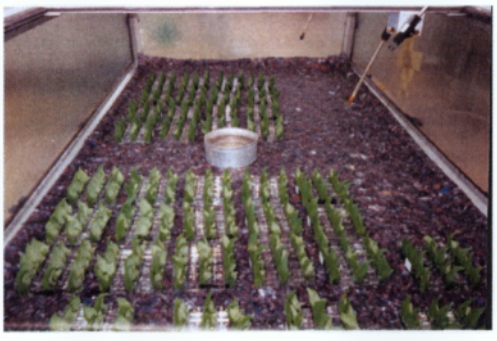
To investigate the role of inducible plant defense systems against *Botrytis cinerea* the leaf top system was used. In this system parts of leaf tops of tomato were cut off of 3rd, 4th or 5th leaf and placed into special square plastic tray (10 cm 10 cm x 2 cm) which was divided in 25 small sections of 2 by 2 (pictures 3). At the start of the experiments first the trays were filled with water (4 ml per small incubator). Then the leaf tops were placed in the trays and finally the leaf tops were sprayed with the compound of interest. As a positive control standard treatment against *Botrytis* (0.23 gr/lt of Eupareen 100% BAYER) was used. Eupareen containing tolyfluanide as an active compound was always diluted in water because of secondary effects (necrosis) when diluted in 0.1% Tween 20. All tested elicitors were dissolved in water or Tween 20 (0.1%).



Pictures 3: ***Defense Resistance of elicitors to Botrytis infection with the leaf top system. The tray is separated in 25 small sections of 2 by 2.***

After treatment of the leaf tops, the water was removed carefully out of the sections, paying attention not to injure the issues. The reason of this is that the effect of the compound via spray application is studied and not the effect of uptake via the vascular system. Finally, trays were filled again with 4 ml of water.

The leaf tops in the trays were infected with 1 µl of *Botrytis* spores (appr. 10.000 spores/ml) 24 hours after treatment, unless mentioned else and put in the glass container (picture 4) or smaller plastic container (picture 5) on a carpet, soaked with water to provide the high humidity necessary for the growth of the fungi. In the middle of glass container we put an extra pot (picture 4) with water for keeping high humidity. After the infection the trays were placed in a glass container to provide the necessary high humidity conditions. The glass container was placed in a temperature and humidity controlled Greenhouse (12 hours of light, 20^0C, 70%, humidity and 400-600 ppm of CO_2) after the infection.



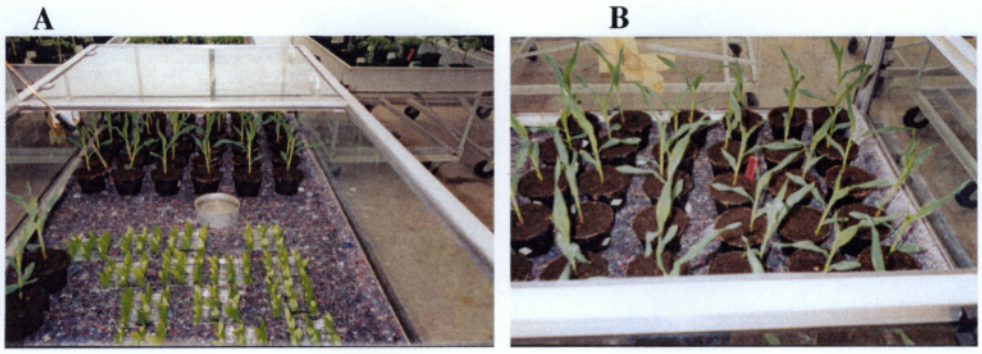
Picture 4: ***Glass container. The glass container containing the trays and the soaked with water carpet. Also, the extra pot with water for keeping the humidity high.***

Each tray contained 15 leaf tops and all experiments were performed in triplicate. Solutions were made one day before the experiment and stored at 4°C until usage. For good comparison of the results, all the leaf tops of a particular plant were randomly distributed over the treatments. The results were measured 5 days after the infection.

Picture 5: ***Small plastic container. The small plastic container consist of two parts, the upper part (transparent) and lower part (with dark gray color). The previously described trays were incubated in the plastic container on a carpet soaked with water.***

➢ MAIZE/BOTRYTIS

During experiments with maize plants, young plants (no leaf tops) were used (picture 6). For attachment of the water droplet containing the *Botrytis* spores to the maize leaf 1 µl of Tween 20 (detergent) was added to 1 ml of spores. After the treatment and the infection (like describing above) the plants were placed in a glass container to provide the necessary high humidity conditions.

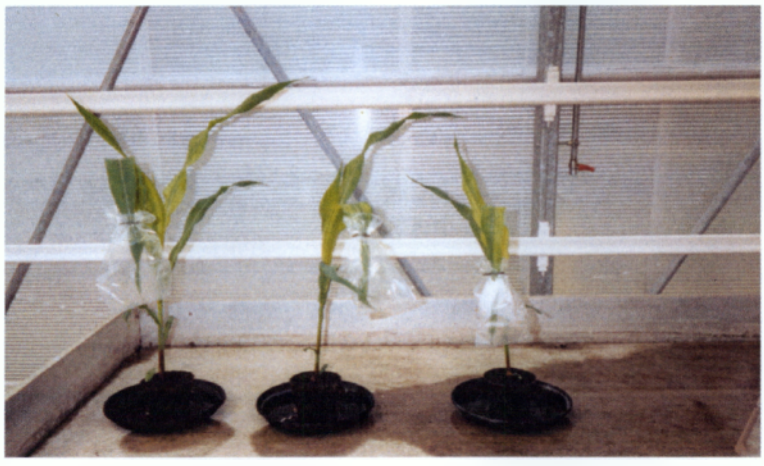


Picture 6: ***Whole maize plant. 10 maize plants were used. 24 hours after treatment with the test compounds the leaves were infected with 1 µl of Botrytis spores. The infected plants are going to be kept in the glass container for 5 days and after they measured.***

In experiments with mature maize plants specific plastic bags were used, which were fixed over leaves that were infected. Inside the bag we put a small paper soaked with water for realizing a high humidity (picture 7).

Picture 7: ***Whole maize plant. The infected leaves were covered with specific plastic bags to increase the necessary humidity. A small paper soaked with water placed into the plastic bag.***

During experimentation we found that the paper soaked with water was not necessary for good infection. Therefore, it was omitted in later experiments (picture 8).



Picture 8: ***Whole maize plant. Infected leaves covered with plastic bags without to containing a small paper soaked with water.***

➢ POTATO/PHYTOPHTHORA

51 young potato plants infected with 1 µl *Phytophthora infestans* (7.000 spores/ µl) after the treatments and were placed into the glass container (like describing above). 3 weeks old tissue was used and the results were measured 6 days after the infection.

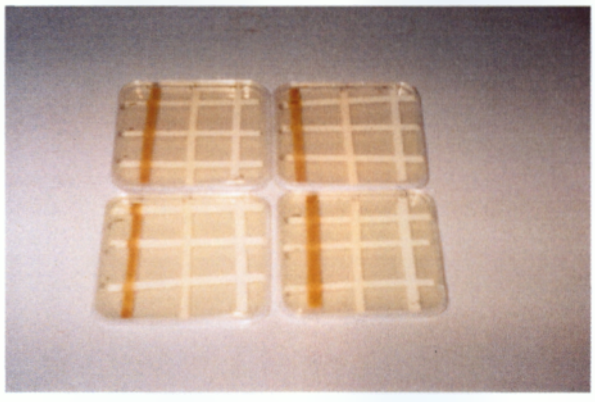
➢ PETRI-DISH TEST SYSTEM

To study the direct effect of LS on Botrytis growth, top-agar petri-dishes were used (see above).

Thin (5 mm) strokes of sterilized paper soaked with different concentrations of LS (0.1 mM, 1 mM and 10 mM) or different concentrations of Eupareen (1%, 10% and 100%) were placed on the Botrytis containing petri dishes. The LS strokes were parallel to each other and the Eupareen strokes were placed rectangular over the LS strokes (picture 9). On the points where the LS and Eupareen strokes cross, the combined effect on Botrytis development can be studied.

For incubation conditions see above. Before the experiments, all the solutions were filter (0.2 µm filter) or heat (20 minutes at 120^0C) sterilized. If the paper is sterilized it remained at least 4 hours at 180^0C in an oven.

In some experiments small wholes (5 mm diameter) were made in the top-agar, the LS and Eupareen solutions (100 µl) was pored these wholes. All the other steps were as described for the paper stroke experiments.



Picture 9: ***Petri-dish test system.***

➢ LIST OF APPARATUS REQUIRED FOR:

A) BOTRYTIS CULTUVATION

1. Down-flow system
2. Pressure cooker or Autoclave
3. Water bath
4. Petri-dishes (square, circle)
5. Tubes (50ml)
6. Pipette (200 µl)
7. Tips (with filter)
8. Parafilm "M" (laboratory film)
9. Black-Light lamp

B) BOTRYTIS HARVEST

1. Down-flow system
2. Petri-dishes (square, circle)
3. Tubes (50ml)
4. 2 Pipettes (0.5-5 ml)
5. Tips (with filter)
6. Sterilized (Pasteur) pipette
7. Syringe (60 ml)
8. Cotton

C) PHYTOPHTHORA AND MILDEW HARVEST

1. Tubes (50 ml)
2. Forceps

3. Syringe (60 ml)
4. Cotton

D) MEASURING THE SPORES

1. Centrifuge machine
2. Tubes (50 ml)
3. Balance
4. A Bürker-Türk counting chamber
5. Pipette (200 µl)
6. Pipette (1000 µl)
7. Tips (with filter)
8. Microscope
9. Counter

E) LEAF TOPS SYSTEM

1. Glasshouse or plastic box
2. Trays
3. Sprayers (AIR-BOY)
4. Sucking machine
5. Pipette (1-10 µl)
6. Pipette (1-50 ml)
7. Tips (white)
8. Tips (blue)
9. Flasks (500 ml)
10. Carpet
11. Pot

RESULTS

In the effort to study the effect of the different elicitors on *Botrytis* infection, of tomato the leaf tops system was used. During these experiments, plant tissue was treated with different concentrations of these elicitors one-day before infection.

The effect of LS on resistance of tomato leaf tops against *Botrytis cinerea* was investigated. Therefore, the leaf tops were treated (spraying) with different concentration of LS in combination with/without Eupareen 10% (= 1/10 of the concentration used in the standard treatment). LS had shown to have a negative effect on the infection size (figure 1). LS also had an additive effect on the action of Eupareen when applied at low concentration (≤ 0.5 mM). This effect of LS was measured in a number of experiments (appendix).

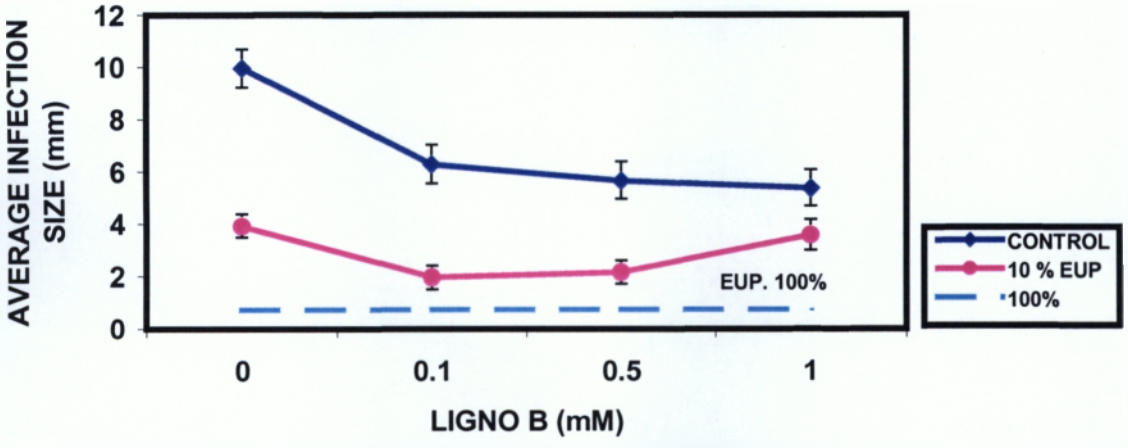


Figure 1: ***Effect of Combination LS and Eupareen 10% on tomato leaves tops.*** *The leaf tops were cut from 3rd and 4th leaf, counting from below from 5-week-old tomato plants. The experiment was done in 45 fold (15 leaf per tray).*

Values are average ± SE.

The second figure shows the effect of Salicylic acid with and without 10% Eupareen on the infection of tomato with *Botrytis* in the leaf tops system. Leaf tops were cut from 6 and $8^{1/2}$ weeks old tomato plant (cultivated in the Greenhouse). As a result of these experiments Salicylic acid and Jasmonic acid proofed to have no effect on the infection size (figure 2 and 3).

Bioalgeen and Bion even stimulated the fungi growth (figure 4 and 5).

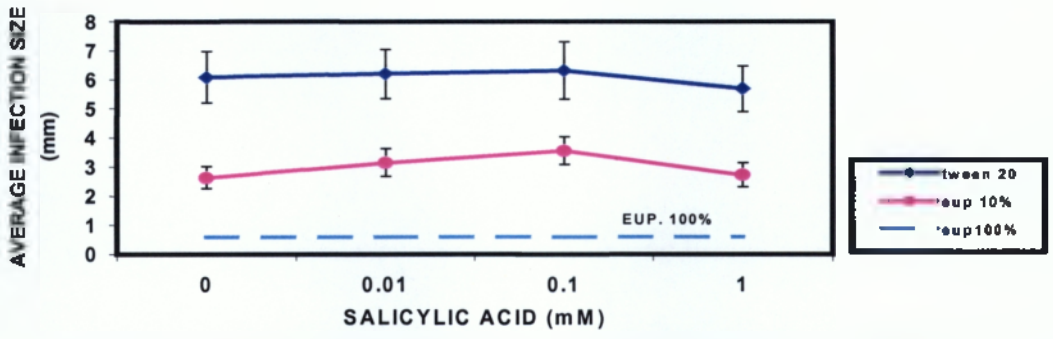


Figure 2: ***Effect of Salicylic acid and Eupareen when combined against Botrytis infection on tomato leaf tops.*** *The experiment was performed in 45 fold divided over three trays (15 leaf per tray). The leaf tops were cut from the 7th and 8th leaf from $8^{1/2}$-weeks old tomato plant and from the 3rd and 4th leaf from 6-weeks old tomato plant (counting from below). Dilutions of the compounds were done in 0.1% Tween 20.*

Values are average ± SE.

During all the experiments, leaf tops were used from 3rd until 8th level depending on the condition of the leaves. If the leaves were starting to senescence (yellow color) leaves from on higher lever were taken. The highest level was determined by the size of the leaves.

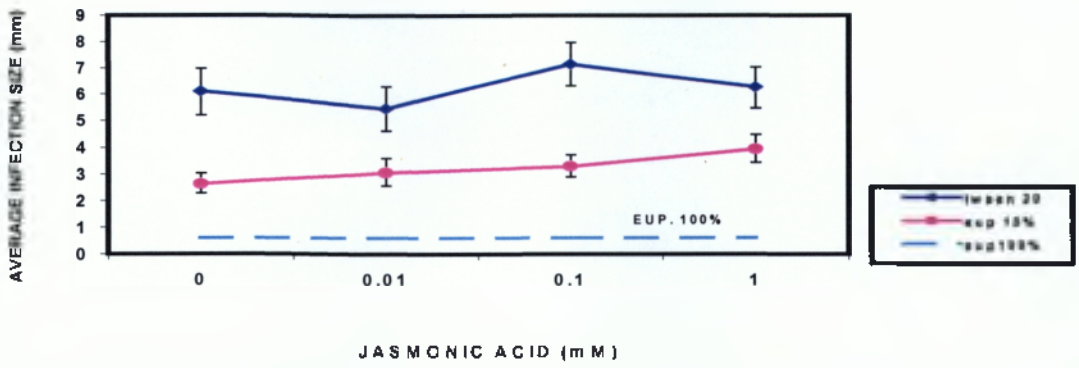


Figure 3: ***Effect of Jasmonic acid and Eupareen when combined against Botrytis infection on tomato leaf tops.*** *The experiment was performed in 45 fold divided over three trays (15 leaf per tray). The leaf tops were cut from the 7th and 8th leaf from 8½-weeks old tomato plant and from the 3rd and 4th leaf from 6-weeks old tomato plant (counting from below). Dilutions of the compounds were done in 0.1% Tween 20.*

Values are average ± SE.

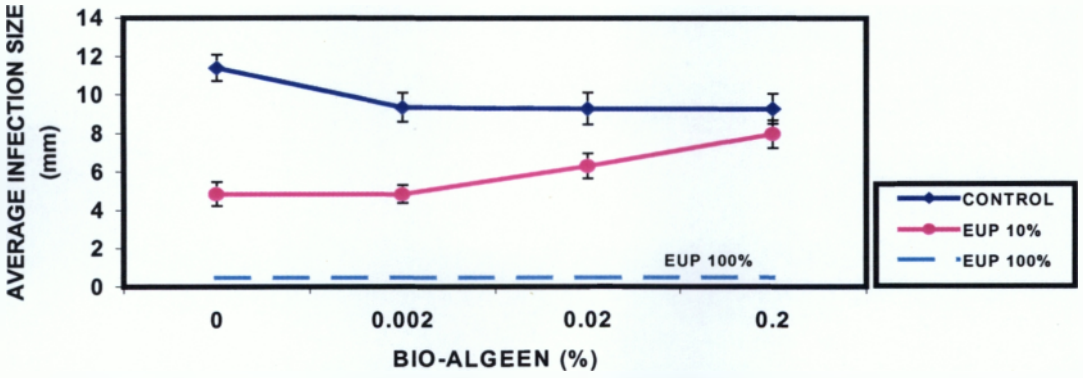


Figure 4: ***Effect of Bioalgeen and Eupareen when combined against Botrytis infection on tomato leaf tops.*** The experiment was performed in 45 fold divided over three trays (15 leaf per tray). The leaf tops were cut from the 5th and 6th leaf, counting from below from 6-weeks old tomato plants. Dilutions of the compounds were done in 0.1% Tween 20.

Values are average ± SE.

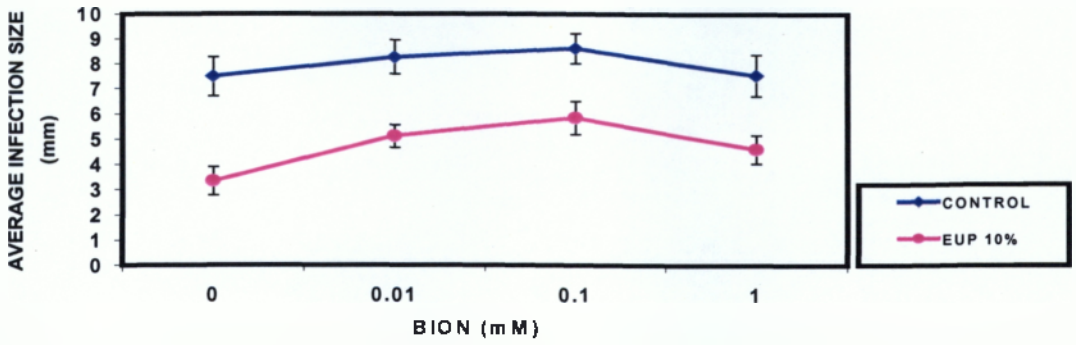


Figure 5: ***Effect of Bion and Eupareen when combined against Botrytis infection on tomato leaf tops.*** *The experiment was performed in 45 fold divided over three trays (15 leaf per tray). The leaf tops were cut from the 7th and 8th leaf, counting from below from 7-weeks old tomato plants. Dilutions of the compounds were done in 0.1% Tween 20.*

Values are average $\pm$ SE.

To elucidate if LS acts only preventive or also as a curative against *Botrytis* leaf tips were treated at different time points, before and after the infection. It appears that LS inhibit the size of infection not only when applied before, but also when applied after the infection (figure 6).

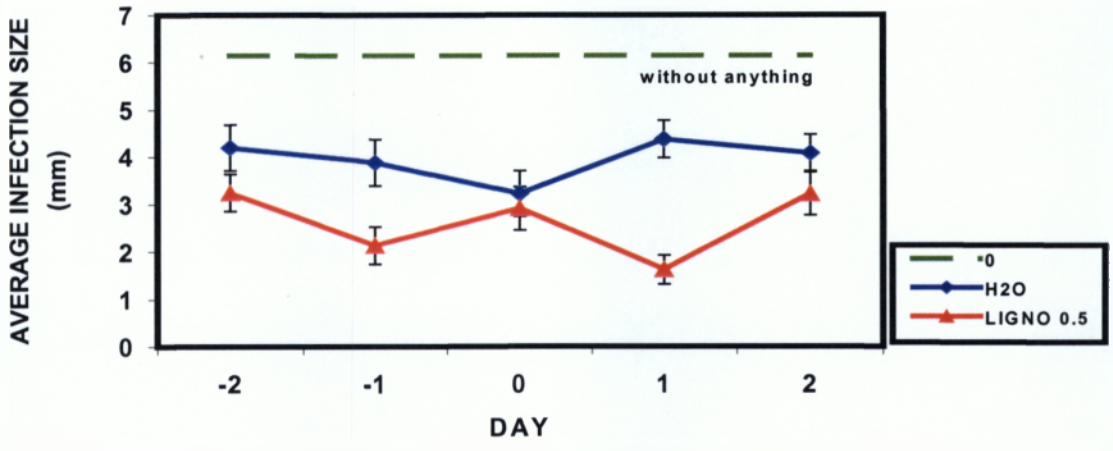


Figure 6: ***Time experiment No 1 - Effect of LS against Botrytis infection on tomato leaves tops.*** *The leaf tops were cut from 3rd, 4th and 5th leaf, counting from below from 6-week-old tomato plants. The experiment was done in 45 fold (15 leaf per tray).*

Values are average ± SE.

Treatment of the tissue three days after the infection had no effect on the growth of *Botrytis*. This was the case when LS was used but also for the standard treatment with Eupareen 100% (figure 7).

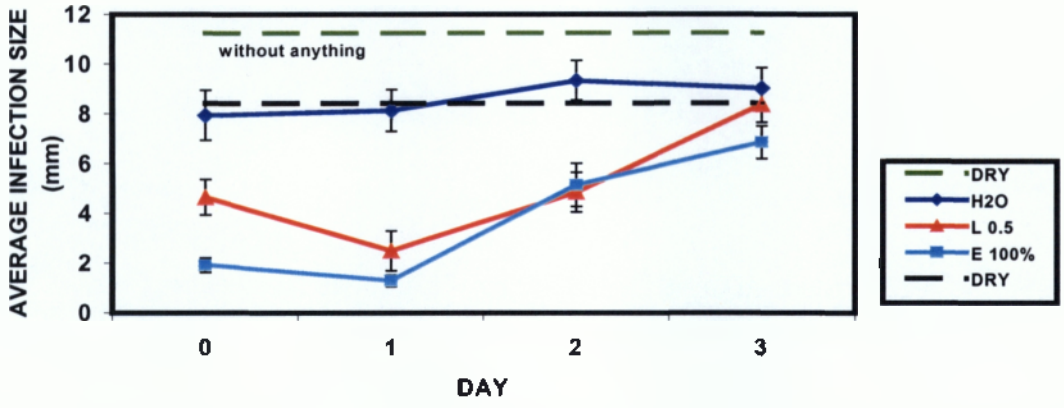


Figure 7: ***Time experiment No 3 - Effect of LS and Eupareen 100% against Botrytis infection on tomato leaves tops.*** *The leaf tops were cut from 6th, 7th and 8th leaf, counting from below from 8-week-old tomato plants. The experiment was done in 45 fold (15 leaf per tray).*

Values are average ± SE.

In a separate experiment the combination of LS with Eupareen 10% was even more effective against *Botrytis*. In other words, the combination is more close to the standard treatment with Eupareen 100% than LS alone. Even more good results gave the combination of LS with Eupareen 20%, specially, the combination with 0.1-0.5 mM LS (figure 8).

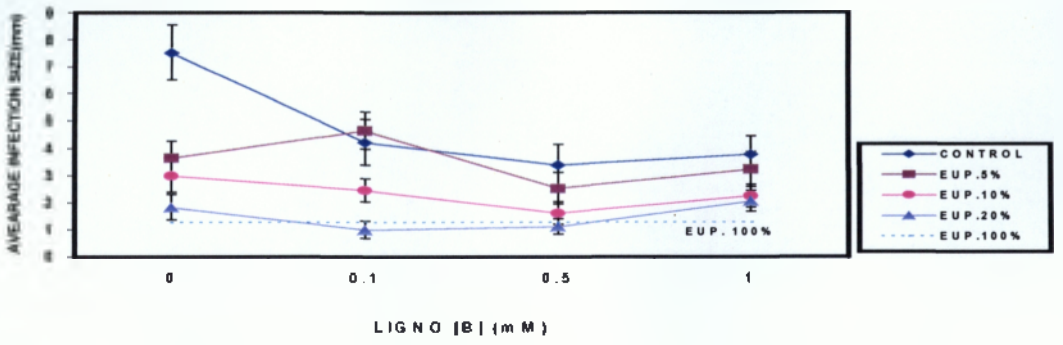


Figure 8: ***Effect of LS and Eupareen against Botrytis infection on tomato leaf tops.*** *The leaf tops were cut from 5th, 6th, 7th and 8th leaf, counting from below from 7-week-old tomato plants. The experiment was done in 45 fold (15 leaf per tray).*

Values are average ± SE.

0.5 mM LS was combined with Salicylic acid in different concentrations (0.01 mM, 0.1 mM and 1mM) with and without Eupareen 10%. As the figure 9 show, none of these combinations had any effect on the Botrytis infection.

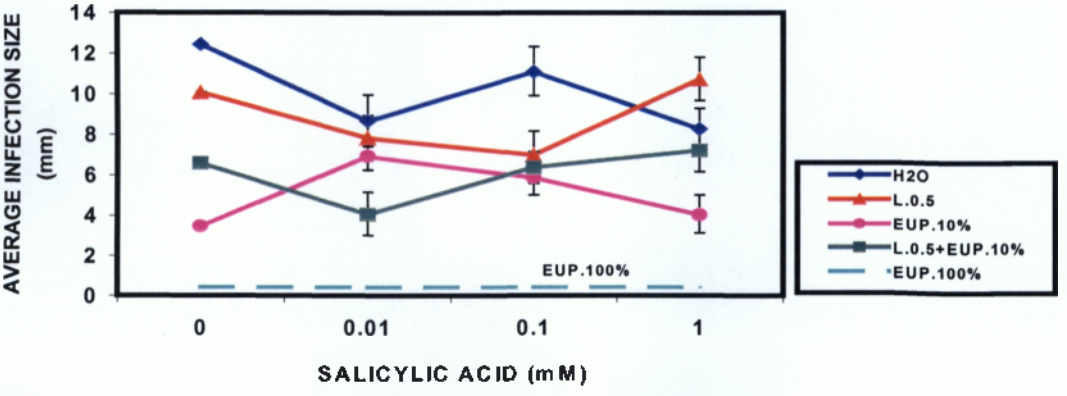


Figure 9: ***Effect of LS, Salicylic acid and Eupareen 10% against Botrytis infection on Tomato leaf tops.*** *The leaf tops were cut from 4th, 5th and 6th leaf, counting from below from 4 1/2-week-old tomato plants. The experiment was done in 45 fold (15 leaf per tray).*

Values are average ± SE.

In addition the effect of LS when applied on the soil was studied. Therefore, tomato and maize were infected with *Botrytis*. Also in this case, LS has a comparative effect on the growth of the fungi on the both tissues (Figure 10 and 11). When applied in low concentration (0.01 mM), LS stimulated the infection, but when applied in high concentration (0.1 mM) it inhibited the infection.

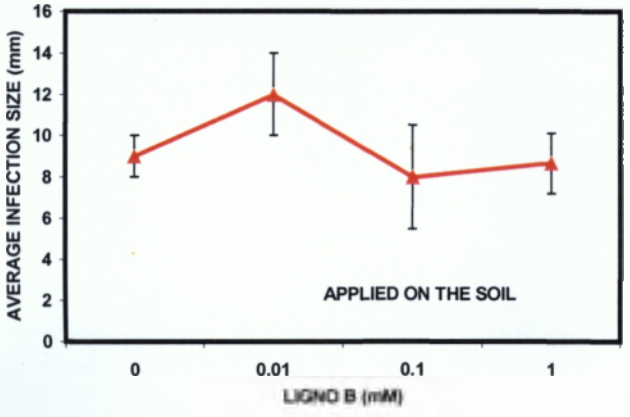


Figure 10:

Effect of LS on maize plant when applied on the soil.

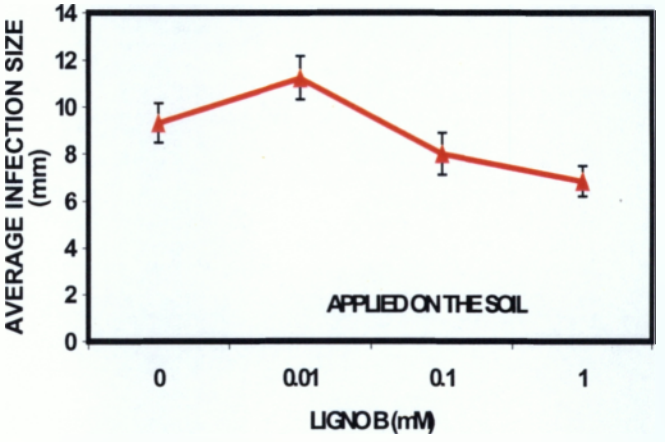


Figure 11:

Effect of LS on tomato plant when applied on the soil.

In many experiments Tween 20 was used as a formulation for the potential induction of resistance. In, an experiment with different elicitors alone and/or combined with Tween 20 the effect of Tween 20 on *Botrytis* infection was tested. Tween 20 stimulated the *Botrytis* infection. Probable, it helped *Botrytis* to invade faster (figure 12). In the same experiment, black spots appeared on the leaf when Tween 20 combined with 20% Eupareen and the leaves became more yellow than without Tween 20 (picture 1, A until E). Also, LS combined with 20% Eupareen slowed the *Botrytis* growth more than the other combinations, except for the standard treatment (100% Eupareen).

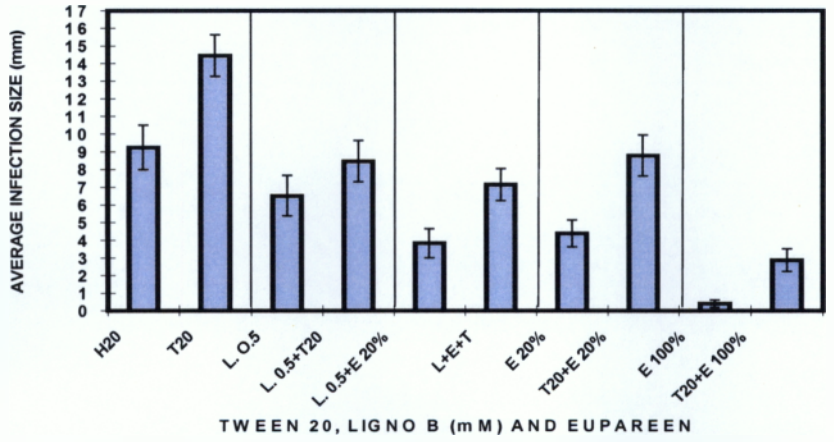
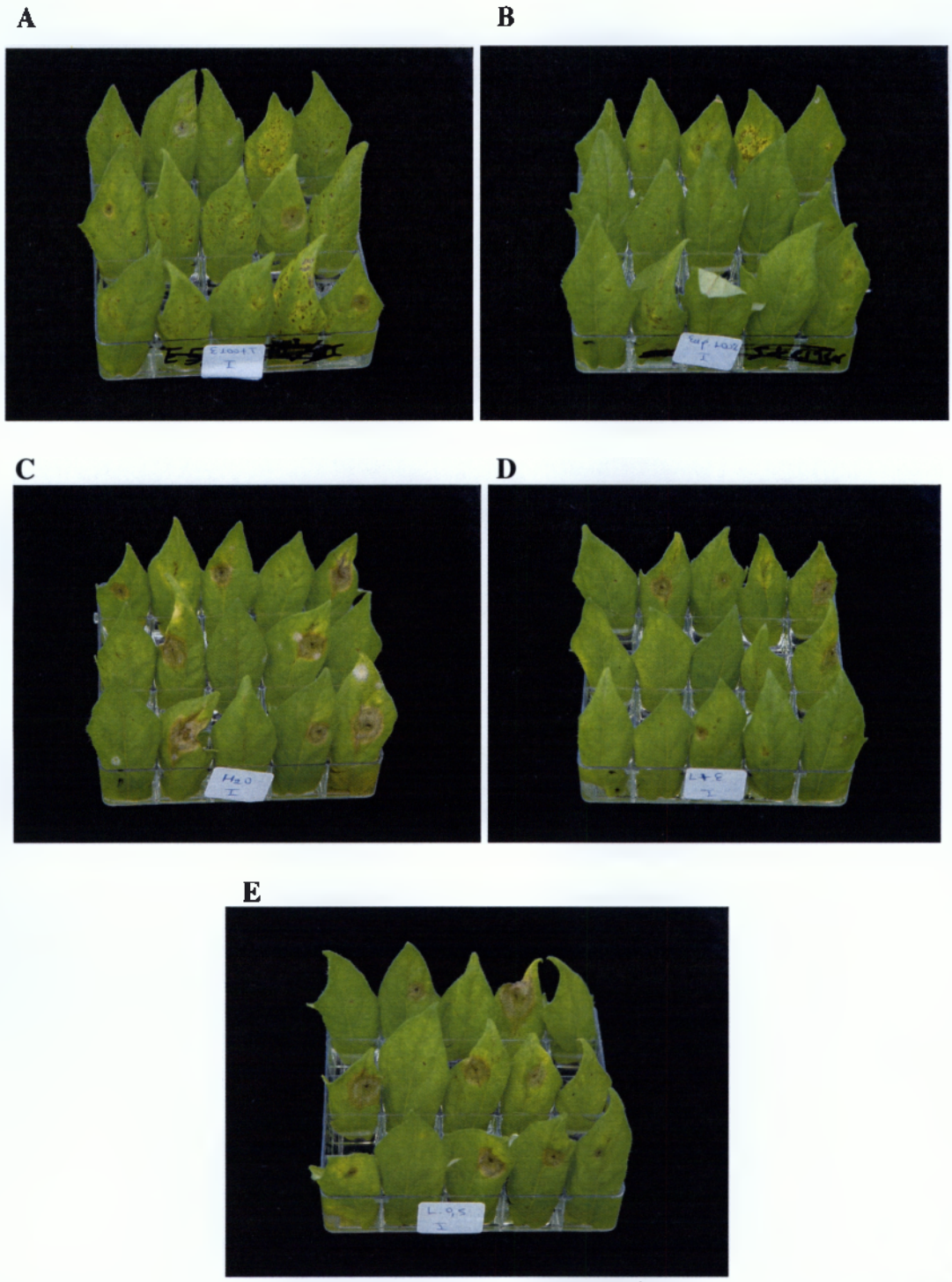


Figure 12: ***Effect of Tween 20 and LS against Botrytis infection on tomato leaf tops.*** *The leaf tops were cut from 5th and 6th leaf, counting from below from 6-weeks old tomato plants. The experiment was done in 45 fold (15 leaf per tray). Values are average ± SE.*



Picture 1: *Used LS against Botrytis cinerea infection in tomato tissues. A) Combination of 20% Eupareen and Tween 20, B) 100% Eupareen, C) Control, D) Combination of 0.5 mM Ligno and 20% Eupareen, and E) 0.5 mM Ligno.*

LS do not has only effect against *Botrytis cinerea*. On potato infected with *Phytophthora infestans*, LS inhibited the growth of the pathogen. During these experiments young potato plants used because the leaf tops system was not useful due to callus formation (figure 13).

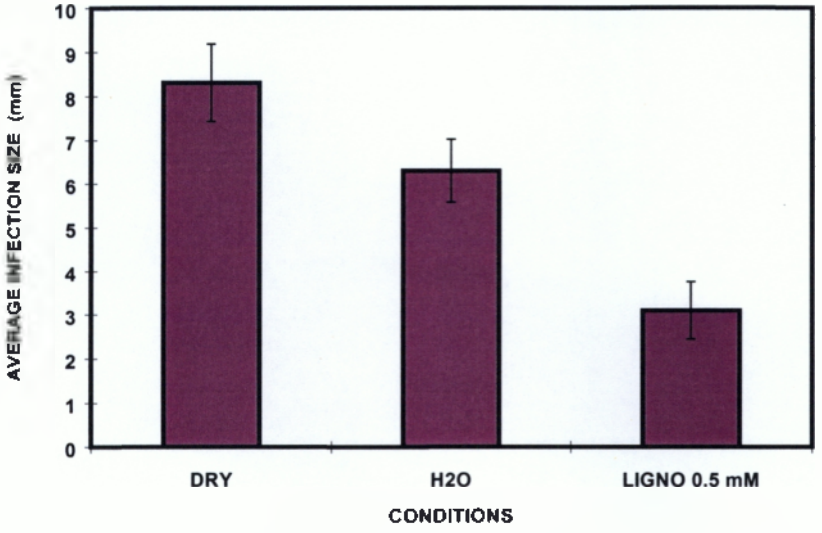


Figure 13: ***Effect of LS against Phytophthora infestans on potato plants.*** *3-weeks old potato plants were used. The experiment was done in 17 folds. Values are average ± SE.*

The effect of *Botrytis cinerea* was tested also in other plants, like *Arabidopsis*, maize and sweet pepper. In these experiments only the infection of maize plants was successful. This infection was reduced when plants were treated with LS, but these results were not reproducible (Appendix I, figure 11).

In addition to investigate the effect of LS directly to *Botrytis*, the petri-dish system with top-agar was used. In all experiments, LS showed not to have any effect on the growth rate of the pathogen. We noted only a small negative effect of Eupareen on the growth of *Botrytis*.

CONCLUTIONS / DISCUSSION

Pathogen components that act as elicitors of recognition by the host plant and subsequent mobilization of plant defenses are still poorly understood. Elicitor molecules may be released from attacking pathogens before or during entry into the host. They may be components of the cell surface of the pathogen that are released by the action of host enzymes, or they may be synthesized and released by the pathogen after it enters the host in response to host signal. The latter elicitors include the hairpin proteins of bacteria that induce development of the hypersensitive response, certain hydroxylipids, and certain peptides and carbohydrates that induce specific host defense responses such as production of phytoalexins. Elicitors are considered as determinants of pathogen avirulence since by their presence they elicit the hypersensitive response and initiation of transcription of the plant genes that encode the various components of the defense response. These in turn result in the pathogen appearing as avirulent. When the initial recognition signal received by the pathogen favors growth and development, disease may be induced, if the signal suppresses pathogen growth and activity, disease may be aborted. On the other hand, if the initial recognition elicitor received by the host triggers a defense reaction, pathogen growth and activity may be slowed or stopped and disease may not develop, if the elicitor either suppresses or bypasses the defense reaction of the host, disease may develop.

The ability to respond rapidly and effectively to environmental signals and pathogens is essential of the survival of all organisms. This report has focused mainly on LS and its potential role in inducing plant disease resistance, but this compound and its signaling pathways are only one aspect of the many responses activation by pathogen attack.

In many experiments, LS has proved to be a powerful tool for controlling plant disease not only when used before the infection, but also when used after (Results,

figure 6). LS enhancing disease resistance of plants and decreasing the effect of pathogens. This phenomenon is clearer when LS is combined with low concentration (10% of standard) of Eupareen (Results, figure 8) a fungicide against *Botrytis cinerea.* The combination of this low concentration of Eupareen with LS (from 0.1 to 1 mM) inhibits *Botrytis* infection on tomato in the same extends as the standard treatment of Eupareen 100% (Results, figure 1).

LS also slowed or stopped the *Botrytis* growth and activity in tomato and maize plants (Results, figures 10 and 11) when applied on the soil. It is worthwhile to note (in literature) that a number of experiments are mentioned in which LS as cell wall breakdown product plays a role as elicitors. It is now clear that LS is a useful signal molecule in development of defense resistance in several plant species (e.g. tomato, potato, maize etc.). However, many unanswered questions remain. Does LS play a role in the initial restriction of the pathogens? If so, is a similar mechanism involved as in the induction of SAR or wounding pathway? It will be interesting to see whether SAR or another pathway mediates all of the action of LS in plant defense resistance or whether there are additional undiscovered modes of action.

LS was also used to induce resistance in potato. There it proofed to enhanced resistance not only against *Botrytis cinerea* but also against *Phytophthora infestans* (Results, figure 13). The fact that LS not only inhibits the infection of *Botrytis* but also of *Phytophthora* is on indicates that LS works via the plant system. Also the fact that LS had no negative effect on *Botrytis* growth when applied on top-agar culture indicates that LS is not inhibiting the growth of the pathogen of such.

Recent advances in plant defense signaling pathway research have shown that plants are capable of differentially activating distinct defense pathways. Depending on the type of invader encountered, the plant appears to be capable of switching on

the appropriate pathway or combination of pathways. The plant signaling molecules SA and JA play an important role in this signaling network: blocking the response to either of these signals can render plants more susceptible to pathogens and even insects. Resistance conferred by the SA-dependent pathway might be directed more against certain types of pathogens, whereas resistance conferred by SA-independent pathways might operate more effectively against other types of pathogens. Recently, evidence supporting this notion was obtained using *Arabidopsis* genotypes that are blocked in either the JA or SA response. The JA response mutant, ***coil***, lost some of its basal resistance against the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea*, whereas basal resistance against the biotrophic fungus *Peronospora parasitica* was unchanged.

In our experiments however JA and SA when applied did not induce resistance towards *Botrytis*. The JA response in tomato plants, became a bit less resistant against the necrotrophic fungal *Botrytis cinerea* (Results, figure 3). Also, SA response in tomato plants shows a lower level of resistance against *Botrytis cinerea* (Results, figure 2). So, a challenging questions for the future will be: are there different defense pathways and how are plants adapted to switch on the right combination of defense pathways after encountering a certain pathogen? Therefore, research on the interplay between the pathways that are activated by these signaling molecules (SA and JA) will provide important information.

Also, when LS was combined with SA with and without 10% Eupareen, the SA didn't have any effect against *Botrytis* (Results, figure 9). This means that SA not only is unable to induce any defense by itself, but also does not have any additive effect in the defense induced by LS.

It is very important to note that in many experiments with high concentration of Eupareen, small black spots appeared on the leaf tops (Results, picture 1), especially, the combination of Eupareen with Tween 20. The tissues also appeared more yellow. This effect was not seen when low concentrations of Eupareen and/or LS were used. This means that LS not induces resistance, but does this with less side effects than the standard treatment with 100"% Eupareen. In the first phase of this project Tween 20 was added to the compounds tested. This was done to ease the uptake by weakening the cuticula. This addition however proofed by itself to induce *Botrytis* infection (Results, figure 12). It is possible that Tween 20 had damaged and opened pathway for the pathogen after destruction of upper or lower epidermis or even the stoma of the leaf. For this reason Tween 20 was not used in latter experiments.

Several elicitors that drive defense expression in response to pathogen attack has been described else, but LS confers pathogen-inducible production, to tomato and potato plants without damaging the plants. So, if it will be combined with low concentrations of Eupareen may be will have the decider result. However, to be surer about the effect of LS, much more and more specific experiments must be done. Also, a combination between LS and other elicitors or compounds it will be interesting or at least their effects separate each elicitor. Below following a list with diverse compounds that can test them in the future:

Compound	concentrations
Xylanase	
Arachiodonic acid	25 µg/ml
Chytosan	>5 g/l
5-chlorosalicylic acid	0.05-5 mM
Salicylhydroxaminic acid	0.05-5 mM
L-Serine	?
Saccharin	1-100 mM
Potasium phosphate	10-250 mM
Aluminium chloride	5-150 mM
Methyl jasmonade 95% (in 0.1% Ethanol)	1 µM –100 µM
Rose bengal	20 mM
BIOCIT	
Milsana	
Synermix (AICI + trichoderma)	
Ferimzone	
Probenazole (Oryzemate)	
N-cyano-methyl-2-chloroisonicotinamide	
BABA-B-amino-butyric acid	
INA	

APPENDIX

❖ **A**

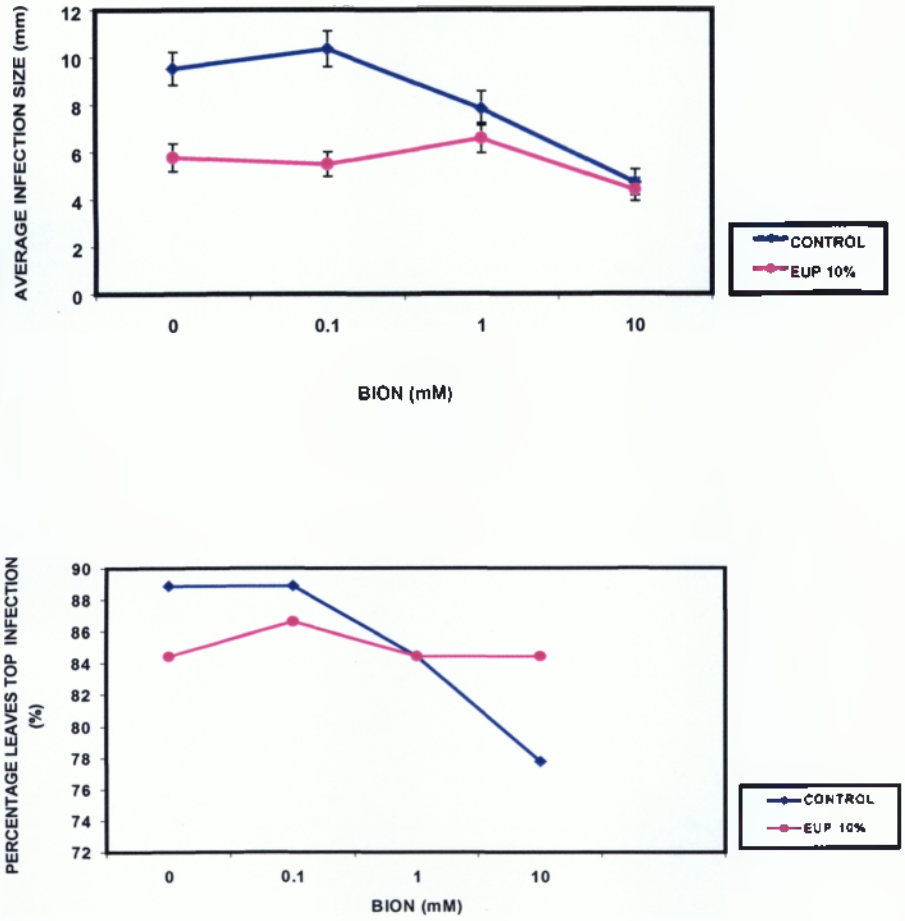


Figure 1: ***Effect of Bion and Eupareen on Botrytis infection tested with the leaf tops system.***

The experiment was performed in 45 fold, divided over three trays. The tomato tissue was 7 weeks old and was taken from the 6^{th} and 7^{th} leaf. Dilutions of the compounds were done in 0.1% Tween 20.

Values are averages

❖ **B**

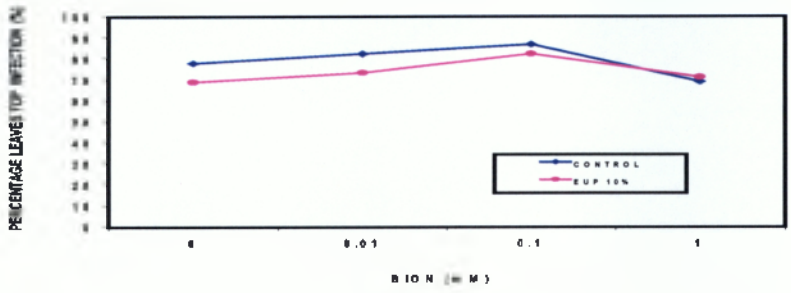


Figure 2: ***Effect of Bion and Eupareen on Botrytis infection tested with the leaf tops system (infection size in results, figure 5).***

The experiment was performed in 45 fold. The tomato tissue was 7 weeks old and was taken from the 7th and 8th leaf. Dilutions of the compounds were done in 0.1% Tween 20.

Values are averages ± SE.

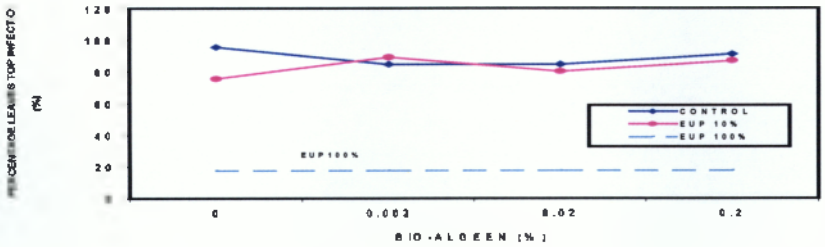


Figure 3: ***Effect of Bioalgeen and Eupareen on Botrytis infection tested with the leaf tops system (infection size in results, figure 4).***

The experiment was performed in 45 fold. The tomato tissue was 6 weeks old and was taken from the 5th and 6th leaf. Dilutions of the compounds were done in 0.1% Tween 20.

Values are averages ± SE.

❖ C

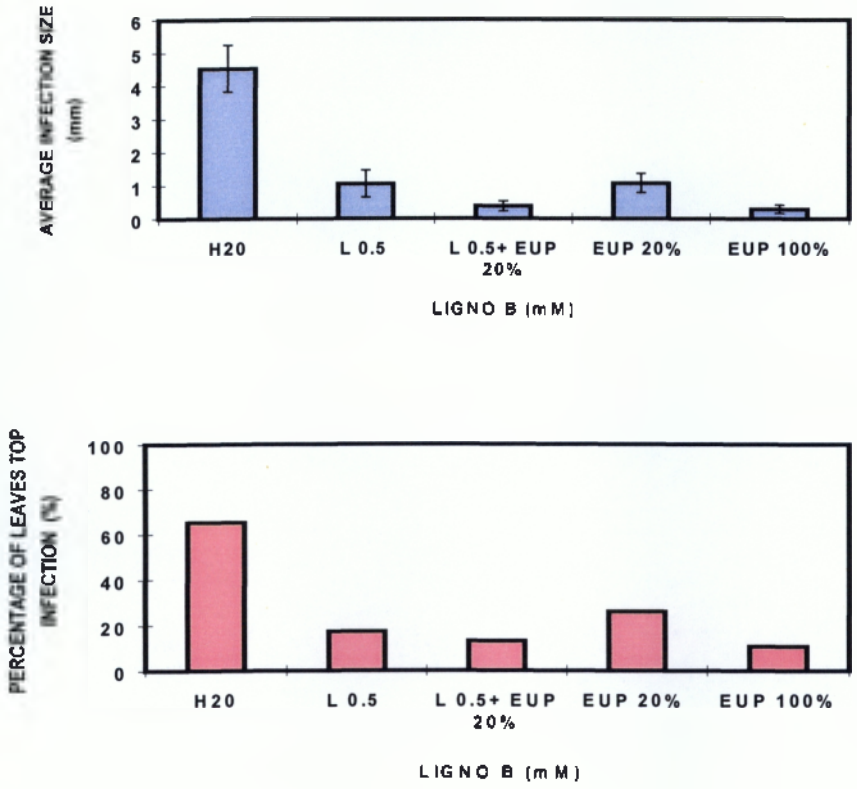
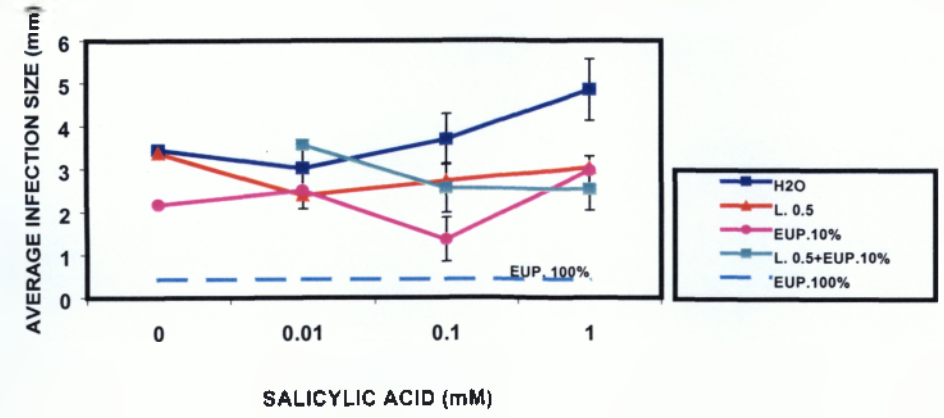


Figure 4: ***Effect of LS and Eupareen against Botrytis infection on tomato leaf tops.***

The experiment was performed in 45 fold. The plant tissue was 6 weeks old and was taken from the 4th and 5th leaf, counting from below. The solutions were made the same day with the experiment.

Values are averages $\pm$ SE.

❖ **D**



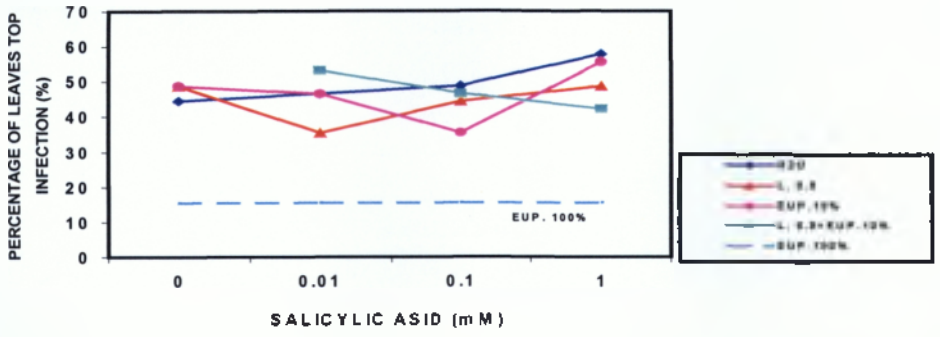


Figure 5: ***Effect of Salicylic acid alone and in combination against Botrytis infection on tomato leaf tops.***

The experiment was performed in 45 fold. The plant tissue was 7 weeks old and was taken from the 6^{th}, 7^{th} and 8^{th} leaf. Eupareen 100% was diluted in water as always.

Values are averages $\pm$ SE.

❖ **E**

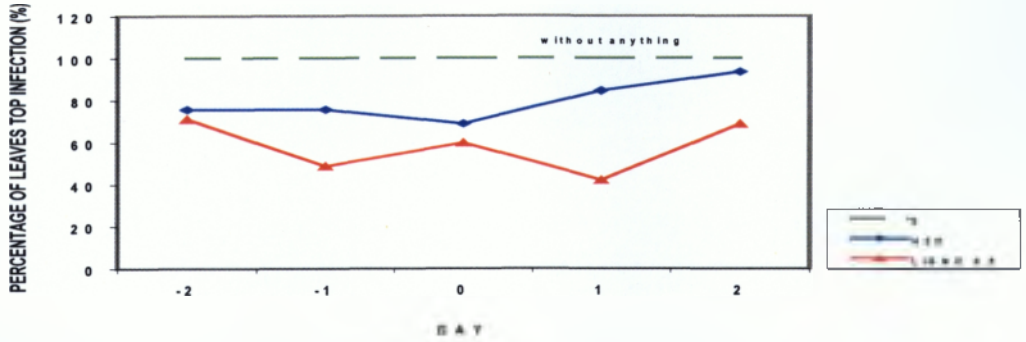


Figure 6: ***Time experiment 1 - Effect of LS against Botrytis infection on tomato leaf tops (infection size in results, figure 6).***

The experiment was performed in 45 fold. The leaf tops were cut from the 3rd, 4th and 5th leaf, counting from below from 6 weeks old plant tissue.

Values are averages ± SE.

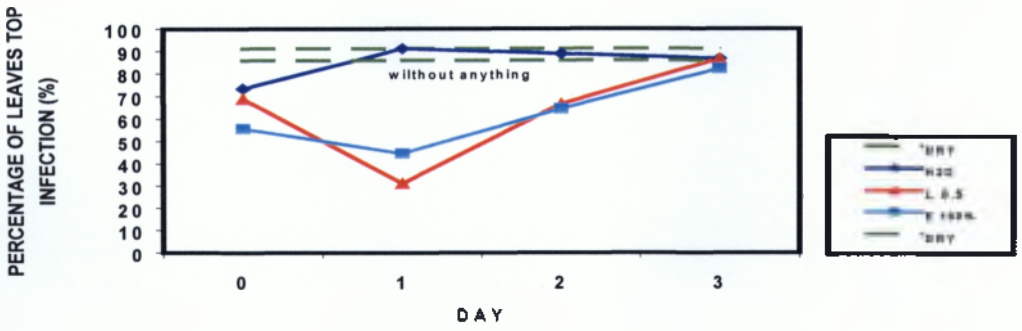


Figure 7: ***Time experiment 3 - Effect of LS against Botrytis infection on tomato leaf tops (infection size in results, figure 7).***

The experiment was performed in 45 fold on 8 weeks old plant tissue. The leaf tops were cut from the 6th, 7th and 8th leaf, counting from below. The infections took place before treatment, simultaneously or after treatment.

Values are averages ± SE.

❖ **F**

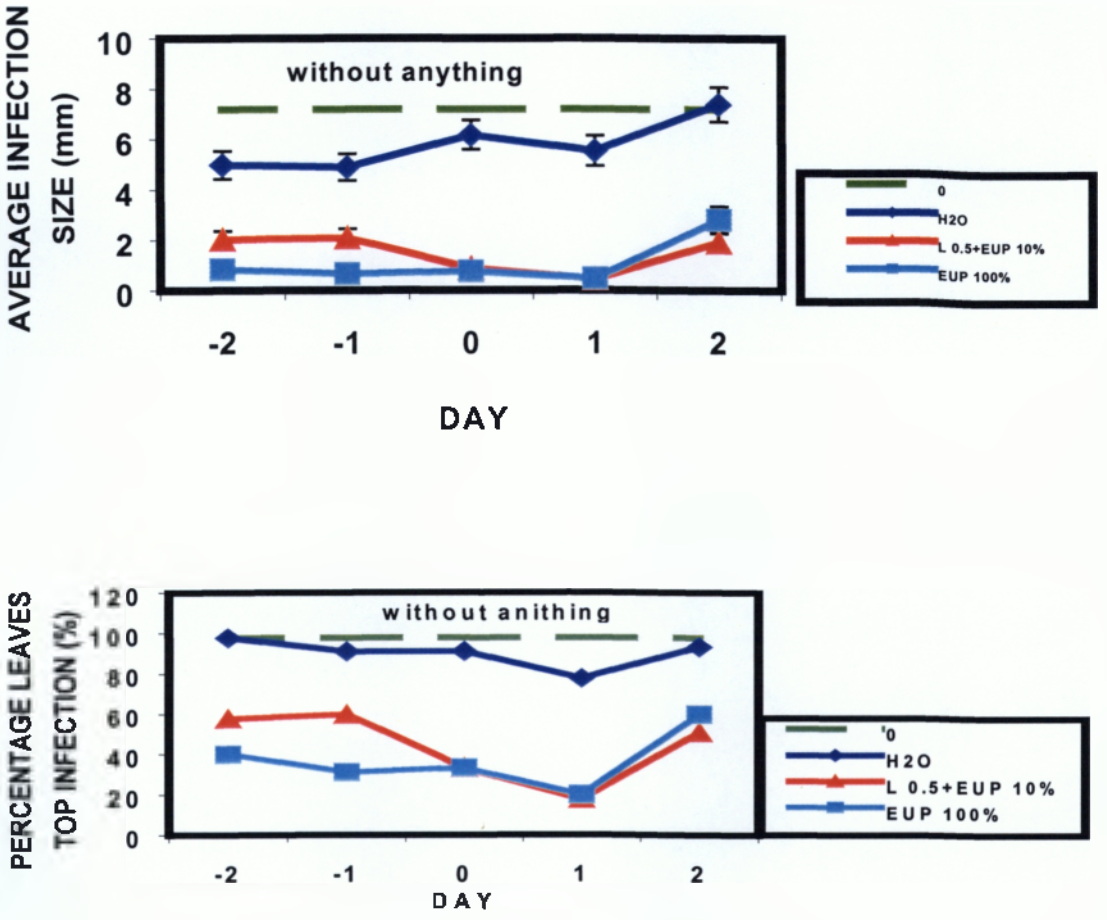
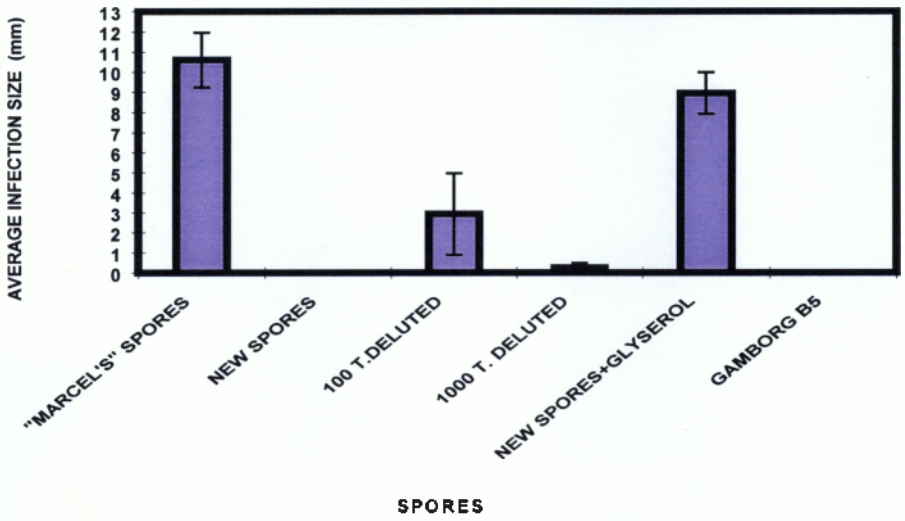


Figure 8: ***Time experiment 2 - Effect of LS against Botrytis infection on tomato leaf tops.***

The experiment was performed in 45 fold on 7 weeks old plant tissue. The leaf tops were cut from the 3rd, 4th and 5th leaf, counting from below. After the treatment the water in the trays was changed with fresh.

Values are averages ± SE.

❖ G



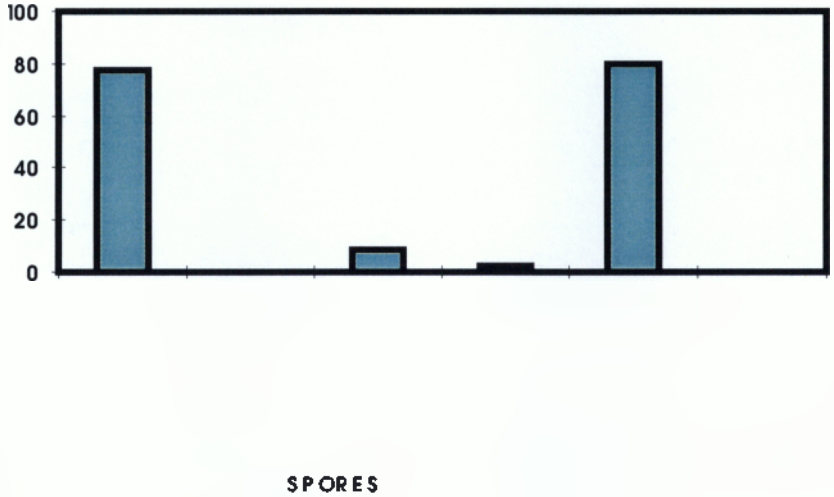


Figure 9: ***Botrytis harvest experiment.***

The experiment was performed in 45 fold and the leaf tops were cut from the 3rd and 4th level. We used $5^{1/2}$ weeks old tomato tissue. Best results showed with 10% glycerol.

Values are averages ± SE.

❖ **H**

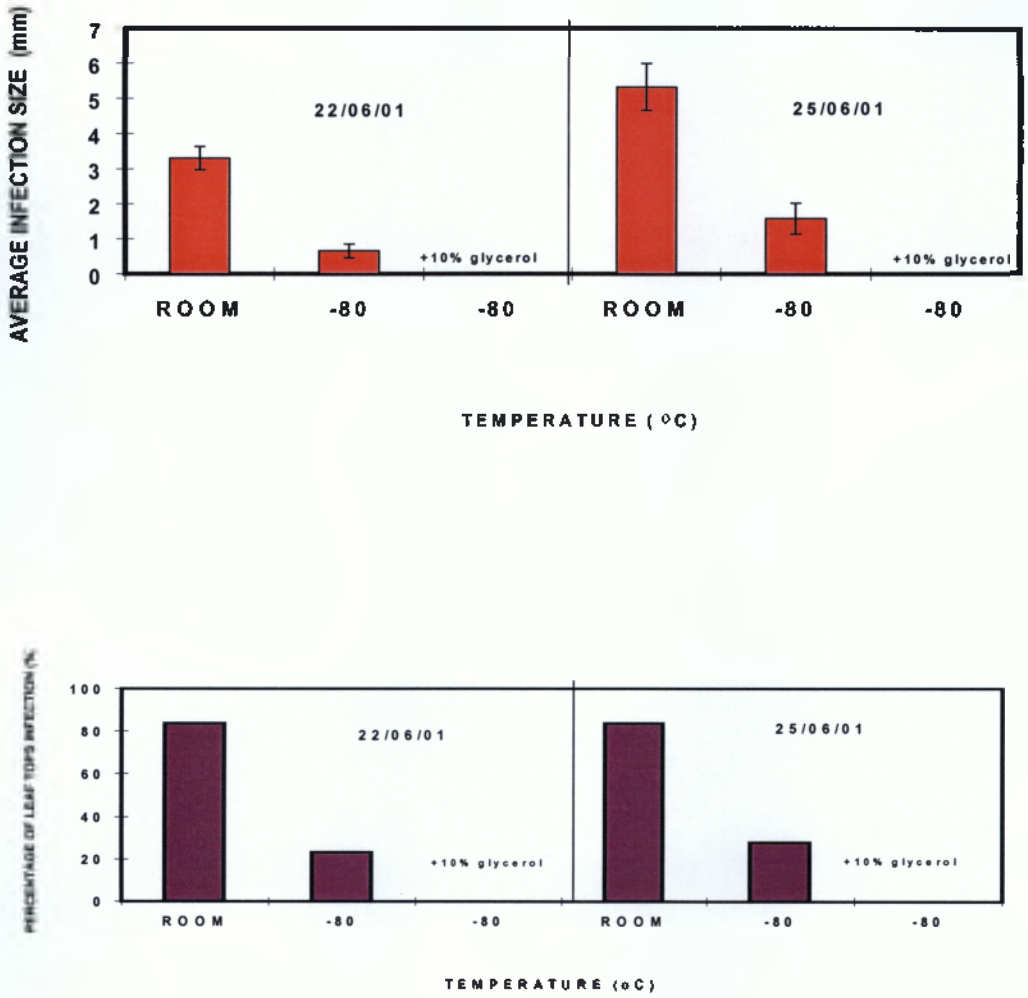


Figure 10: ***Phytophthora harvest experiment.***

The experiment was performed in 45 fold. From the 3rd and 4th leaf, 3 weeks old tomato tissue was used. The *Phytophthora infestans* had number F 95573.

Values are averages ± SE.

❖ I

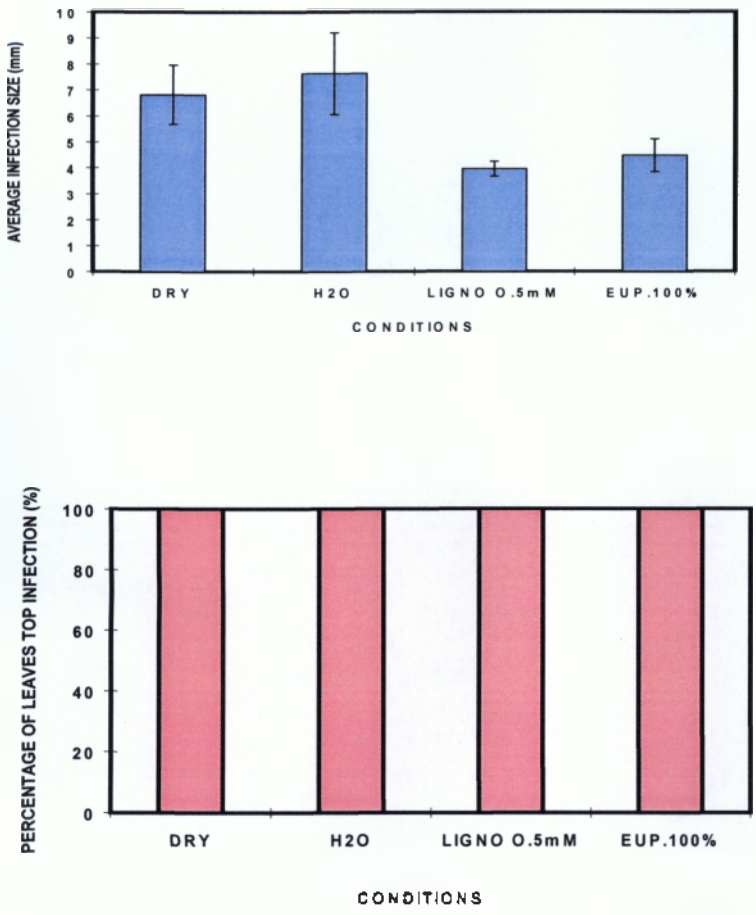


Figure 11: ***Effect of 0.5 mM LS against Botrytis infection on maize plants.***

The experiment was performed in 10 fold on 3 weeks old whole plants. The leaves were treated at the 2nd, 3rd and 4th leaf, counting from below. The infections took place after treatment.

Values are averages ± SE.

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