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Τμήμα Φυτικής Παραγωγής

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«Έλεγχος της αποτελεσματικότητας φυτοπροστατευτικών ουσιών, σε προσβολή από Botrytis elliptica στα πλαίσια της αειφόρου αντιμετώπισής του σε φυτά Lilium vivaldy»

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Contents

Περίληψη

Η εξάπλωση σε όλο τον πλανήτη, της χρήσης των γεωργικών φαρμάκων προκάλεσε περιβαλλοντικά αλλά και οικονομικά προβλήματα. Η μόλυνση του περιβάλλοντος αλλά και των υδάτων από γεωργικά φάρμακα, σε ορισμένες περιπτώσεις έχει προσλάβει επικίνδυνες διαστάσεις και από αρκετά χρόνια αναζητούνται εναλλακτικές λύσεις για την αντιμετώπιση των εχθρών και των ασθενειών των καλλιεργούμενων φυτών. Σε πολλές ανεπτυγμένες χώρες έχει ήδη αρχίσει η έρευνα για την ανεύρεση αγροχημικών ανεκτών από το περιβάλλον, φυσικής ή μερικώς συνθετικής προέλευσης. Η παρούσα πειραματική μελέτη αφορά στην αποτελεσματικότητα τέτοιων ουσιών αλλά και συνδιασμών τους, ενάντια στην προσβολή του φυτού, *Lilium vivaldy* από το μύκητα *Botrytis elliptica*.

Η έρευνα αρχικά διεξήχθει *in vitro* και στην συνέχεια *in vivo* σε κομμένα φύλλα *Lilium vivaldy*. Κατά το πρώτο πειραματικό σκέλος, κονίδια του μύκητα επωάσθηκαν σε υδατικά διαλύματα των υπό εξέταση ουσιών, για 1, 3, 4 και 24 ώρες και στην συνέχεια μεταφέρθηκαν σε κατάλληλο θρεπτικό υπόστρωμα (malt extract agar και άγαρ εκχυλίσματος φύλλων). Μετά από 24 ώρες καλλιέργειας των κονιδίων στο άγαρ, κάτω από συνθήκες σκότους και σε θερμακρασία δωματίου, αξιολογήθηκε η επίδραση της κάθε ουσίας στην βλάστηση των κονιδίων. Ακολούθως, εκτελέσαμε την δοκιμή στα φύλλα όπου μελετήσαμε την αλληλεπίδραση του φυτού με τον μύκητα και την εφαρμοζόμενη σ'αυτό, φυτοπροστατευτική ουσία.

Κατά την διάρκεια του δεύτερου πειραματικού σκέλους, κορυφές φύλλων μήκους 5 cm αφαιρέθηκαν από τα φυτά και τοποθετήθηκαν σε πλαστικούς δίσκους των 15 θέσεων. Στην συνέχεια, 8 δίσκοι φύλλων μεταφέρθηκαν σε πλαστικό κουτί για εγκληματισμό σε συνθήκες υψηλής υγρασίας. Μετά από διάρκεια μιας ημέρας, τα φύλλα ψεκάσθηκαν με τις φυτοπροστατευτικές ουσίες και την επόμενη ημέρα μολύνθηκαν με τα σπόρια του μύκητα. Η διαδικασία της μόλυνσης πραγματοποιήθηκε με εναποθέτηση σταγόνας 2 μl από αιώρημα κονιδίων συγκέντρωσης 200 κονίδια / μl, στην κάτω επιφάνεια του φύλλου. Η αξιολόγηση των επεμβάσεων έγινε μετά από 3 ημέρες με βάση το ποσοστό των προσβεβλημένων φύλλων και το μέγεθος των κηλίδων.

Οι ουσίες που χρησιμοποιήσαμε ήταν οργανικά οξέα, δευτερογενείς μεταβολίτες φυτών, τασιενεργές ουσίες, χιτοζάνες και διάφορες μεταλλικές ενώσεις του θείου. Ερευνήσαμε την αποτελεσματικότητα τους ενάντια στην μόλυνση και την εξάπλωση του *Botyrtis elliptica*. Ακόμα, ελέγξαμε αν προκαλούν συμπτώματα φυτοτοξικότητας και ποιοτική υποβάθμιση του φυτικού ιστού, με την δημιουργία καταλοίπων.

Διαπιστώσαμε ότι κατά την in vitro δοκιμή, ορισμένες ουσίες ήταν δραστικές έναντι του μύκητα αλλά κατά την εφαρμογή τους στα φύλλα, αύξησαν την προσβολή. Συνεπώς, συμπεραίνουμε ότι ενώ αναστέλλουν την δράση του μύκητα, αναστέλλουν και του αμυντικούς μηχανισμούς του φυτού με αποτέλεσμα το φυτό να παρουσιάζει αυξημένη ευπάθεια. Επίσης, άλλες ουσίες ήταν αποτελεσματικές κατά του *Botrytis elliptica,* αλλά όταν εφαρμόστηκαν στα φύλλα προκάλεσαν έντονα συμπτώματα τοξικότητας.

Διαπιστώσαμε ότι ουσίες με μειωμένη αποτελεσματικότητα, αύξησαν την αποτελεσματικότητά τους όταν συνδιάστηκαν με άλλες ουσίες. Ακόμα, ορισμένες, αυτούσιες ουσίες ήταν ιδιαίτερα αποτελεσματικές χωρίς να προκαλούν κανένα σύμπτωμα αδυναμίας στα φυτά. Εδώ, θα μπορούσαμε να υποθέσουμε ότι ίσως, οι συγκεκριμένες ουσίες να ενίσχυσαν τους μηχανισμούς άμυνας του φυτού αλλά αυτό αποτελεί θέμα για περαιτέρω έρευνα.

Ανακεφαλαιώνοντας, πρέπει να αναφέρουμε ότι η πιο ενδιαφέρουσα κατηγορία φυτοπροστατευτικών ουσιών από εκείνες που ελέγχθηκαν, ήταν οι μεταβολίτες φυτών καθώς, σχεδόν όλες οι ουσίες της ομάδας αυτής ήταν αποτελεσματικές χωρίς να προκαλούν συμπτώματα στρες στο φυτό.

ABSTRACT

Yearly millions of tonnes of pesticides are worldwide used in agriculture to protect crops against plant pathogens. As a result residues of these agrochemicals are present in significant quantities in our food and environment (soil and water). This requires alternatives that exhibit a more acceptable eco-toxicological profile. These alternatives are defined here as sustainable crop protectants (SCP's). This study was performed as part of a larger project that aims at the development of SCP's based on compounds that are relatively friendly to the users and the environment. They may be of natural or of synthetic origin. A large variety of potential ingredients of the SCP's provisioned were screened for their anti-fungal activity. The plant / pathogen system used for this screening was *Lilium vivaldy* / *Botrytis elliptica*, which represents one of the major agricultural problems in The Netherlands.

The interaction between the active compounds, the plant and the fungus has many aspects and therefore was our main subject. Our project consisted of two experimental projects, the in vitro assay and the leaf top assay. By means of the vitro assay, compounds were tested directly on the fungus. The plant factor was excluded and we investigated the interaction between *Botrytis* and the agents. The conclusions were subsequently applied in the leaf top assay in order to include the missing factor of plant reaction to the treatments.

12 series of experiments were performed and several compounds were tested. The categories in which our compounds belonged were lignosulfonates, organic acids, formulation agents, plant metabolites, and the chitosan compounds. They were tested in several concentrations and combinations. We have confirmed the efficacy of agents as individual compounds that were equally or even more effective in combination with others. Agents of no significant action against the infection size turned into great defending treatments when they were combined. In some cases, agents provoked a relative increase of the disease's spread on the plant material. Some of them also caused toxic symptoms.

The plant metabolites group proved to be the one including the most agents with a positive effect against *Botrytis elliptica.* Out of them, PM4 was

the one that inhibited the infection more efficiently withough causing any toxic symptoms. Therefore, our suggestion is that this agent should be used as a lead for further research of equivalent or additionally effective compounds against the infection caused by *Botrytis elliptica* on lily plants.

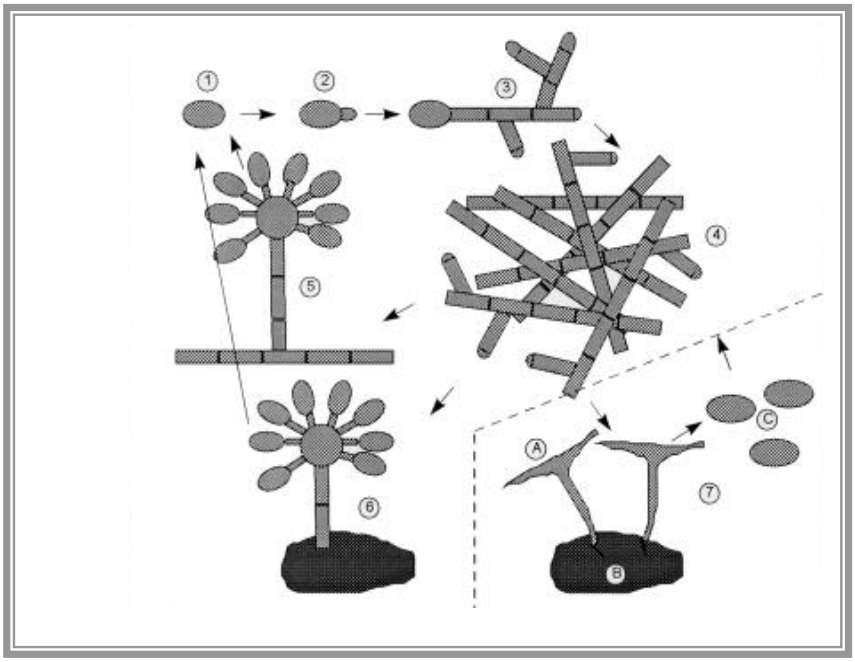
1. Introduction

1.1 Objective of the study

Yearly millions of tonnes of pesticides are worldwide used in agriculture to protect crops against plant pathogens. As a result residues of these agrochemicals are present in significant quantities in our food and environment (soil and water). It is therefore of major importance that the usage of these conventional pesticides is considerably reduced. This requires alternatives that exhibit a more acceptable eco-toxicological profile. These alternatives are defined here as sustainable crop protectants (SCP's). This study was performed as part of a larger project that aims at the development of SCP's based on compounds that are relatively friendly to the users and the environment. They may be of natural or of synthetic origin. In this study a large variety of potential ingredients of the SCP's provisioned were screened for their anti-fungal activity. The plant/pathogen system used for this screening was lily/*Botrytis elliptica*, which represents one of the major agricultural problems in The Netherlands.

1.2 Plant pathogenic *Botrytis* spp.

Necrotrophic *Botrytis* spp. cause important economic damage during the production phase as well as post-harvest. Field and greenhouse vegetables, small fruits, ornamentals, flower bulbs and forest tree seedlings are all attacked by *Botrytis* spp. The symptoms include rotting of above ground plant parts and the harvested product. Members of the genus *Botrytis* are commonly classified in two groups. 1) *B. cinerea*, a broad spectrum pathogen which attacks a wide range of host plants and 2) species morphologically distinct from *B. cinerea* which are more specialized in their parasitism e.g. *B. elliptica* on lilium spp. and *B. aclada* on onion. Hyphal cells of *Botrytis* spp. are multinucleate and frequently heterokaryotic. Sexual recombination and heterokaryosis control the heritable variation found in *Botrytis* spp. and generate the genetic potential of *Botrytis* spp. to adapt to changes in the environment.



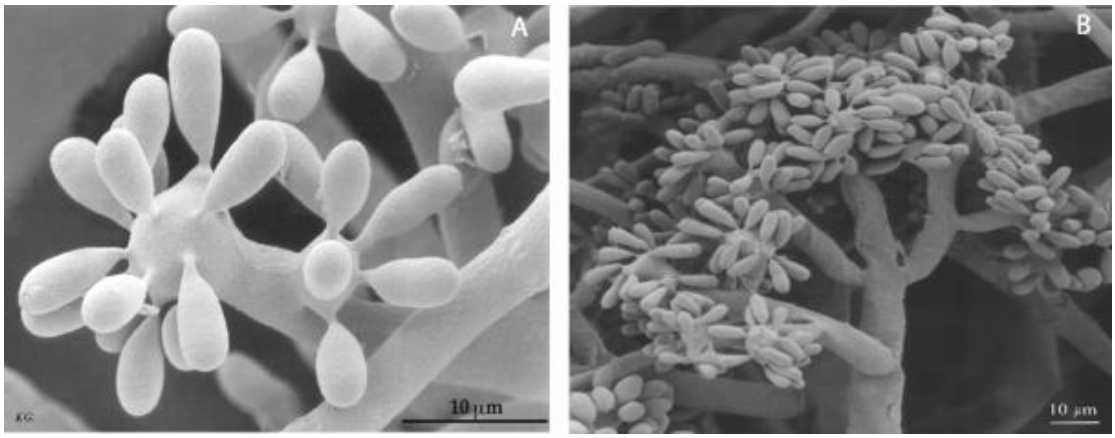
Pic.1: propagation organs of *Botrytis* spp.

1) Conidia in dormancy
2) Conidia in germination
3) Production of hyphens
4) Mycelium
5) Conidiophores carrying conidia
6) Conidiophores produced by sclerotia
7) Apothecia (A) producing ascospores (B) on sclerotia (C)

Infection of the host can arise from germinating conidia and ascospores, from mycelium growing in dead plant parts and from mycelium established in extraneous organic material. The presence of water and exogenous nutrients is known to enhance infection by *B. cinerea*. However, conidia can also be infective after dry inoculation and in the absence of exogenous nutrients. Conidia, ascospores, mycelia and sclerotia can be dispersed by wind, water splash or growers activities. Conidia are produced in large quantities in infected crops and can be dispersed over large distances (pic.2). Despite this dispersal potential, KÖhL et al (1995a) demonstrated in a field experiment that the majority of the conidial infections originated from conidia produced within a few meters from the infection site. Sclerotia are generally believed to be the primary survival structures of *Botrytis* spp (pic. 1). Sclerotia can germinate in three ways myceliogenic, sporogenic or carpogenic, giving rise to new dispersal propagules.

B. elliptica Berk. Cooke causes destructive leaf infections in lily plants (‘lily fire’) resulting in serious yield losses. The host plant range of *B. elliptica* is practically limited to lily (Lilium spp) although alternative hosts have been reported. Conidia or ascospores causing primary infections of *B. elliptica* were

reported to originate from sclerotia present in the soiL and on plant debris. *B elliptica* is spreading within and between lily plants through contact infections and splash or wind dispersal of conidia. *B elliptica* infection of lily leaves results in brown expanding lesions. Associated with the lesion, yellow streaks of senescing tissue can be formed, distally and proximally from the lesion. From the leaves *B. elliptica* migrates to the stem where it can block vascular transport and kill plant parts located above the location of stem infection.



Pic. 2: Conidia and conidiophores of *Botrytis* spp.

1.3 Control of plant diseases

Strategies for the control of plant diseases include planting resistant crop varieties, changing crop cultural practices or storage conditions to those less favorable for disease development, employing biological controls, applying chemical pesticides, and using integrated disease management (combining two or more of the above approaches). The ability to develop any of these strategies depends first on identifying the pathogen causing the disease and then learning how to interrupt its disease cycle. The more known about the genetic, biochemical, and physiological processes that operate in the host and pathogen as infection and disease progress, the more likely a control method can be devised. Understanding the ecology of pathogens is also important (that is, how they survive, are dispersed, and otherwise interact with their environment). In addition, an understanding of the epidemiology or

outbreak of disease and spread of pathogens is important for deciding which control actions are most effective.

As with all microorganisms, plant pathogens exhibit a remarkable ability to change and adapt. Newly discovered pathogens and more virulent strains of old pathogens continually arise and often overcome resistant crop varieties or can no longer be controlled by strategies and chemicals that were once effective. Continual research to develop new control methods is necessary. Furthermore, public concern has grown in recent years, regarding the use of pesticides to control diseases. This concern stems from the fear that such chemicals may contaminate food or accumulate in the soil and ground water (and so be introduced into the food chain). As a result, pressure has increased the need to develop environmentally friendly approaches to plant disease control.

1.4 Active resistance mechanisms in plant fungus interactions

Active or inducible defense mechanisms result from a confrontation between a host cell or group of host cells and the infection structures or metabolites (elicitors) produced by a fungus attempting infection. The resistance mechanisms lead to the development of a highly localized hostile environment that prevents further colonization by the fungus. It seems unlikely that a single factor is responsible for the arrest of the fungal growth, and it is more probable that a number of related or unrelated metabolites are involved. Central to the concept of active defense is the ability of host cells to perceive the presence of a foreign organism, and following this recognition process, to trigger the co-ordinated organization of resistance mechanisms.

1.4.1 The hypersensitive response (HR)

The rapid death of cells in response to infection is usually described as hypersensitivity. It first attracted attention as a typical response of plants resistant to obligate parasites. Here its significance is obvious because such parasites do not grow continuously on dead substrates. Death must also be accompanied by changes in dead cells, or in others around them that prevent growth of the parasite. For some diseases, it is now clear that tissue killed in

response to infection does come to contain sufficient of substances toxic to the pathogen to prevent its growth, substances called phytoalexins.

1.4.2 Phytoalexins (low molecular weight compounds)

Phytoalexins are secondary metabolites, mainly synthesized via three metabolic pathways: the acetate-malonate, the acetate mevalonate and the shikimate pathways, according to the compound and the plant species involved. In many cases phytoalexins accumulate in plants exposed to various types of stress in the absence of infection. This feature has led to the suggestion that phytoalexins synthesis may be part of a co-ordinated response to injury representing an integral part of a wound healing or cellular repair mechanisms following cell death or damage.

1.5 Experimental assays

All applied agents cause stressing conditions to the plant. The response of the plant to this situation is exhibited via enhance or restrain of its resistance mechanism. Therefore, the plant can defend itself and avoid the infection or become susceptible to it. In conclusion, induced stress is an important parameter of the infection size.

Antifungal activity of the compounds is another inhibiting mechanism against the pathogen infection. Furthermore, concentration of the applied compounds is a parameter which determines the size of antifungal activity. Thus the infection size is depending on the stress caused to the plant and the compound's action against the pathogen.

The interaction between the active compounds, the plant and the fungus has many aspects and therefore was our main subject. Our project consisted of two experimental projects, the in vitro assay and the leaf top assay. By means of the vitro assay, compounds were tested directly on the fungus. The plant factor was excluded and we investigated the interaction between *Botrytis* and the agents. The conclusions were subsequently applied in the leaf top assay in order to include the missing factor of plant reaction to the treatments.

2. Materials and methods

2.1 In Vitro assay

Conidia from *Botrytis elliptica* were incubated in different dilutions of new potential crop protectants or adjuvants, isolated after various incubation periods, washed and plated on malt extract agar to analyze their germination and mycelium development.

2.1.1 *Botrytis elliptica* culture

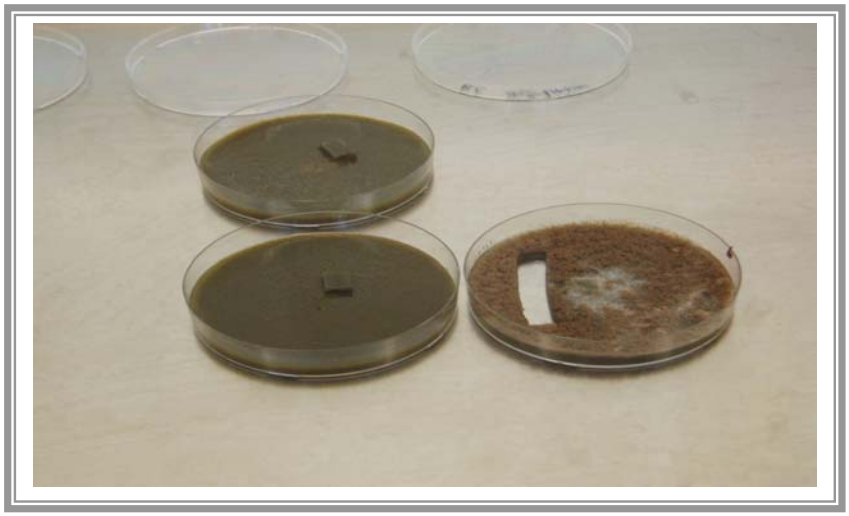
The fungal/pathogen material of this experiment were conidia of *Botrytis elliptica* which have been inoculated 2 weeks ago. They were cultured on various agar media, such as agar containing malt extract and agar containing Lilly leaves.

Preparation of 500ml of malt extract agar was done according to the manufacturing company's protocol (OXOID LTD, CM 159, malt extract agar). It was sterilized at 125ºC for 15 minutes using a pressure cooker and then cooled down at 55ºC in a water bath. Afterwards, 20 petri dishes were filled in a cross flow cabinet and left to solidify with their lids partly open. Finally, our agar dishes were packed in plastic bags and stored at room temperature in the dark.

For the preparation of the lily leaf - extract agar, we used 100 g of fresh/frozen leaves which were carefully washed and blended in 300 ml of demineralized water. The mixture was transferred into a bottle containing 1.2% purified agar (OXOID) and then sterilized the same way like the malt extract agar. 16-18 petri-dishes were prepared and stored exactly as described before.

Inoculation of agar dishes with *Botrytis* was a crucial procedure because in this way we provided our project with healthy fungal material free from any bacterial infection. To avoid contamination of the environment with *Botrytis* and to work antiseptic, all steps were carried out in a down flow cabinet. New dishes were inoculated using a small square piece of agar

originating from a sporulating culture of *B. elliptica* which has been inoculated 2 weeks ago (pic. 3).



Pic. 3: Inoculation of lily leaf extract agar dishes with *B.elliptica* spores

The petri-dishes with the fungal spores were incubated at 20ºC under constant normal light in combination with black light (UV), to induce sporulation (pic.4).

pic. 4: Incubation of *B. elliptica* cultures

2.1.2 Incubation of *B. elliptica* in the crop protectant solutions

■ Preparation of the agents

Also at this point, we worked under sterile conditions. All solutions, including stocks (highest concentration) and their dilution series were prepared in sterile demineralised water. Stocks were filter sterilized and 1.5 ml of them were transferred into sterile 2 ml aliquots. Dilutions were prepared in the same type of Eppendorf tubes. The incubations were also carried out in Eppendorf tubes.

■ Isolation of *B. elliptica* conidia

This experimental phase occurred one day after the preparation of agents. During the harvest of fungus spores, we worked under sterile conditions in a down flow cabinet and we extracted the conidia of a sporulating culture growing, which has been inoculated 2 weeks ago in agar petri dish. For this purpose, we added 20 ml of sterile tap water and we released the conidia from the mycelium by gently rubbing the agar surface using a glass triangle. We collected the suspension and filtered it through sterile cheese cloth in a 50 ml tube. Then, the filtered *Botrytis* spores were centrifuged for 5 min at 1000 rpm and the supernatant was carefully poured out. We mixed the conidia that were stacked to the bottom of the tube with the remaining water in order to dissociate the aggregates formed. Further on, the suspension's volume was adapted to the size of the pellet by addition of sterile tap water. This means that a small pellet was adapted into 3 ml and a big one into 10 ml. Finally 100 µl of the conidia suspension were transferred into a 2 ml aliquot which was used for the counting procedure.

■ Quantification of the fungal spores

The Bürker Türk heamocytometer and a microscope were our instruments to determine the amount of spores in our suspension. The heamocytometer was cleaned with demi water and then carefully filled out completely with the conidia suspension. Afterwards, it was mounted into the microscope where 8 areas of 16 squares were visible.

The amount of conidia per μl equals the average of spores that were counted at the 8 areas, divided by 0.064 μl (the volume of the 16 squares of each area). Finally the conidia suspension's concentration was adjusted to 250 conidia/μl with sterile tap water.

■ Incubations and culture on agar

12 μl of conidia suspension were added to the tubes containing the treatment series and incubated at room temperature for 1, 3, 4 and 24 hours. After each incubation period, 250 μl were taken out of each treatment tube and transferred to new ones of the same type. Then, centrifugation took place at 1000 rpm for 5 minutes and 225 μl of the supernatant were removed. The washing step followed, where 750 μl of sterile tap water were added and the suspensions were centrifuged again. Finally, the 750 μl of water were removed and everything that was left in the tubes (15 - 30 μl) was set on the 24 wells agar dish. We should specify that the content of each tube was placed to the equivalent agar well for each incubation period. To conclude, the fungal spores, after their treatment with several agents, were left to germinate on agar at room temperature, in the dark. On the following day, our compounds were evaluated by comparing the mycelium development of the treated spores with the ones contained in the controls.

List of apparatus required for:

► Isolation of conidia:

- sporulating culture of Botrytis elliptica
- sterile tap water
- sterile cheese cloth
- sterile pipettips (1 ml and 200 µl)
- sterile tubes (50 ml)
- pipettor
- sterile pipettes (10 ml)
- heamocytometer (Burker Turk)
- Sigma centrifuge with rotor for 50 ml tubes
- down flow cabinet

► Preparation of sterile crop protectant solutions, incubation and culture on agar:

- sterile demi-water
- sterile pipettips (1 ml and 200 µl)
- sterile tubes (1.5 ml, 2.0 ml, 10 ml and 50 ml)
- cross flow cabinet
- 0.2 µm filters
- 10 ml syringes
- 5 ml pipet tips non sterile
- 24 wells dishes (sterile)
- malt extract agar (Oxoid)
- pipettor and sterile 10 ml pipettes

2.2 Lilly leaf assay

2.2.1 Plant material

The research for new sustainable crop protectants, apart from the in - vitro assay, was carried out on leaf tissue of Lilium vivaldi (Asiatic lily) (pic. 5).

Bulbs of the above mentioned plants were planted in soil (leliegrond) of ph 5.8 in a greenhouse from May until August. Climate conditions of the greenhouse were not regulated and therefore they were variable and dependent on the weather of this time of season.

After 6-8 weeks, when our plants were reaching an early flowering stage (2-3 days before the first flower blossoms), leaf tops were taken from the whole plant, treated with several compounds and afterwards infected with *Botrytis.* Plant material was used at this specific stage of its development because it's then that lily plants are concerned to be most susceptible to *Botrytis* infection. The effectiveness of each compound was evaluated by monitoring the formation of lesions.

pic. 5: Lilium vivaldy

2.2.2 Leaf – top system

The lily leaf experiment was basically carried out on special plastic trays of 25 square sections (2 x 2 cm), out of which only 15 were used.

First, 13 sections were filled with 3 ml of tap water and 2 sections were covered with tape for the later placement of the untreated leaves (control) as described in fig.1. Afterwards, the leaf tops (top 5cm of the leaf) were cut off and placed backwards in the sections. Each tray contained randomly chosen leaves from the bottom, middle and top part of each plant. The back row of the tray contained bottom leaves, the middle middle ones and the front one contained leaves from the top part of each plant.

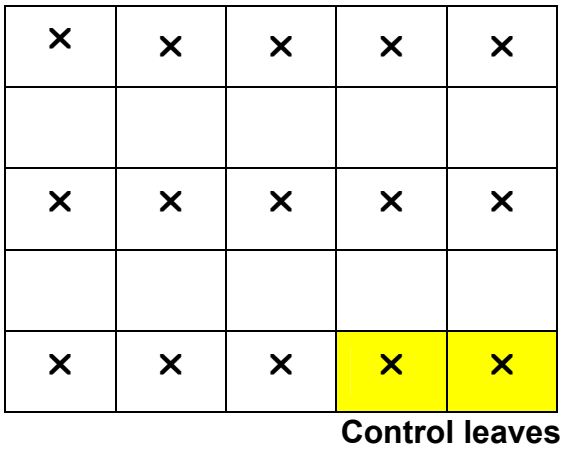


Figure 1: Set up of the leaves in the trays.

Plastic boxes with a carpet of wet filter paper were used for the achievement of high humidity, which is necessary for the growth of the fungus and the conservation of the leaf tops. The cover of the boxes was transparent, in order to let the sunlight pass through it, resulting in the rise of the temperature at the interior (pic.3). 8 trays were placed in each one of the plastic boxes and they were transferred into a greenhouse of 20°C day and 18°C night temperature ,as well (pic. 6). Added to this, in order to avoid direct sun radiation, the boxes were placed in the shade.

Pic. 6: set up of the trays in the plastic box

Since the conditions per box may slightly differ, each box was considered as a separate experimental unit.

2.2.3 Preparing and applying the crop protectant compounds

Several compounds and combinations of them were tested and evaluated by their effect on fungal development. The ones that were considered to be interesting, were tested again in different concentration and combinations.

Solutions of the compounds to be tested were prepared in the laboratory with demineralized water in total volume of 20 ml and applied to the leaf tops by spraying. During spraying, each tray containing the leaf tops was placed in a carton box with filter paper attached to its walls so that spraying vapours would be absorbed. All safety precautions were taken such as use of gloves, mouth mask, glasses and coat.

As far as the spraying technique that we used is concerned, we applied the treatments 3 times to each row of leaf tops in the trays, starting from the back and proceeding to the front one. Our goal was to apply the same volume of each compound to all the leaves by creating a fine, homogenous layer on them. Control leaves were sprayed separately with demi – water. The applied volume was 5 ml and we decided that it should be performed in the way that is described below (fig. 2).

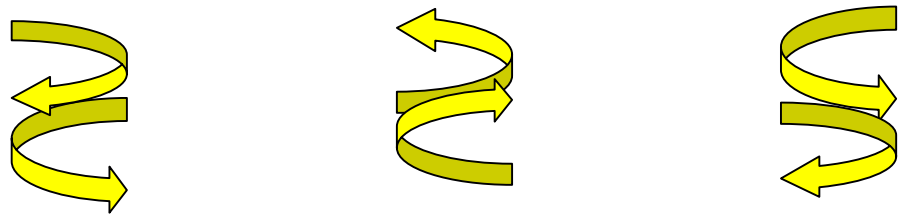


Figure 2: Spraying of the leaves over the trays.

Finally all leaves were left to dry for 30-40 min and then placed back to the boxes. At that experimental point, humidity was not needed, therefore the wet filter paper was removed from the bottom of the boxes and placed back again during the inoculation of the fungal spores.

2.2.4 Isolation of *B. elliptica* conidia

Apart from some minor modifications, the same isolating procedure was followed as described in the in vitro assay. The modifications that have occurred were related to the non - sterile character of this assay. This means that the isolation wasn't performed in a down flow cabinet and no sterile materials were used.

Another modification is that we poured 20 ml of 0.01% Tween 20 to the surface of the agar plate containing the *Botrytis* culture in order to achieve the detachment of the conidia from their mycelium threads. Furthermore, washing step with 10 ml of tap water had to occur before quantify and inoculate the spores.

2.2.5 Inoculation of the leaf tissue with *B. elliptica*

Once conidia were isolated from the sporulating cultures of *Botrytis* that we were preserving, they were applied onto the leaves in a droplet of 2 μl who contained 400 conidia. The droplet was placed at the top left side of the leaf, using a pipette. (pic. 7)

Conidia are relatively dense particles and have the tendency to sink quickly to the bottom. Therefore, mixing of the solution had to be quite frequent to ensure that each droplet applied, would contain 400 conidia.

Afterwards, wet filter paper was placed under the trays and the boxes were sealed with tape to help the humidity increase.

pic. 7: Leaf-tops inoculated with B.elliptica spores

Incubation boxes were transferred to the greenhouse, in the shade, covered with cheese cloth, as well. Monitoring of the infections and measurement of the lesions' length occurred 3 days later.

List of apparatus required for:

► Setting the leaf – top system

- trays with 25 square sections of 2x2 cm
- pipette (25 ml)
- knife with surgical blade
- tape
- plastic box (30x35 cm)
- tissue paper
- cheese cloth

► Preparing and applying the crop protectant solutions

- 50 ml tubes
- pipettes (0,01 – 5 ml)
- tips
- balance
- ultrasonic bath
- roller mixer
- ph meter
- carton box
- sprayers (AIR BOY)
- demineralized water

► Isolating, counting and inoculating the conidia

- a 2 weeks old sporulating culture of Botrytis on malt-extract or lily leaf agar medium
- 20 ml of 0.01% Tween 20
- pipettes (0.2 – 5 ml)
- tips
- glass spatula
- cheese cloth
- 50 ml tubes
- Sigma centrifuge with rotor for 50 ml tubes

- alcohol 70 %
- heamocytometer (Bürker-Turk)
- microscope
- 2 ml eppendorf micro test tubes
- 2 µl pipette and pipette-tip
- tape

► Botrytis cultivation

- malt-extract agar medium plates
- Lilly – leaf agar medium plates
- down flow cabinet
- a sporulating Botrytis culture of 2 weeks old
- incubator of 18°C with constant UV and normal light

► Preparing the agar plates for Botrtytis cultivation

- cross flow cabinet
- 9 cm petri-dishes (circle)
- alcohol 70%
- demineralized water
- 4.8 g purified agar (OXOID 1.2%)
- 25 g malt extract agar (OXOID)
- 100 g fresh/frozen Lilly leaves
- blender
- heat sterilization system using pressure cooker
- water-bath system
- plastic storage bags

3. RESULTS

Reliable results can only obtained with reliable test system. Therefore much effort was put in this. Setting up the bio – systems was of crucial importance. We had to ensure that all conditions were controlled and every procedure was functioning by having the best-expected result. Only then our results would be comparable and we would be able to draw reliable conclusions from them. Therefore several factors were tested.

During the in vitro assay, all the treatments were used in very small volumes. Added to this, the amount of conidia used was also low and therefore even after the centrifugation process no visible pellet was obtained. This made our work more complicated and we had to make a lot of estimations about their place. Loss of conidia during their transfer from one aliquot to another had to be avoided. For that reason, after each centrifugation the suspension was left on the table for some time to let the conidia sink to the bottom of the tube. We had to make sure that conidia were not floating in the suspension.

For the lily leaf - top assay, we had to check more factors because this assay was more complicated and included more steps sensitive to external factors. The spraying technique, the amount of spores contained in the inoculation droplet (fig. 3) and whether we should add nutritional solution in it, the fungal developing conditions, the fungal activity depending on its origin, the susceptibility of the plant material and the incubation conditions were tested and optimized.

3.1 IN VITRO ASSAY

Conidia of *Botrytis elliptica* were incubated in different dilutions of various potential crop protectants or adjuvants. The incubation periods were 1, 3, 4 and 24 hours. Subsequently, the conidia were isolated, washed and plated on malt extract agar to analyze their germination and mycelium development. The incubation periods were 1, 3, 4 and 24 hours.

2 experiments were performed. In the first experiment the treatments performed were 1.1 – 6.2. In the second experiment, we repeated the treatments of experiment 1 and we added some extra combinations (7.1 – 9.2). Table 1 shows all the compounds and combinations of compounds tested. Their concentration is also listed.

The experiment included 4 water treatments as controls i.e. 2 of tap water (OT1, OT2) and 2 of demineralised (OD1, OD2). They have been held in the beginning of the treatment series and in the end, in order to check the possibility of variation in conidia's germination capacity during the processing time. No change of germination capacity in time, was observed.

The treatment solutions containing the compounds to be tested, were prepared the day before of incubations. The effect of each treatment was analyzed by judging the spore's development of mycelium (fig. 3,4), one day after the end of last incubation period. The results were expressed in comparison to the controls. We used values 0, 1, 2, 3 and 4 that stand for no, small, intermediate and heavy mycelium growth respectively.

Table 3, figure 3 and 4 show the growth of mycelium after each incubation period for every treatment of the first experiment and the second experiment, respectively. As expected, the increase of incubation time and concentration resulted in the inhibition of the mycelium growth for most of the treatments. Specifically, the combination of FA1 together with OA2/chitosan (7.1), OA1/chitosan (8.1, 8.2, 8.3) or M2 (9.1, 9.2) was very effective and inhibited totally the germination and mycelium development of *Botrytis* even after 1 hour of incubation. FA1 applied by itself was also effective but not at the lowest concentration (2.3). Formic and OA2, as individual compounds, were also proofed to be effective but not in their lowest concentration. Especially for the case of OA2, any spores germinated after 3 hours of incubation. Unfortunately, it wasn't possible to draw any conclusions about treatments 7.2 and 7.3 (0.1% OA2/chitosan + 0.5 μl/l FA1, 0.02% OA 2/chitosan + 0.5 μl/l FA1) because they were heavily contaminated with bacteria. Also, during the second experiment, no conclusions could be drawn for the effect of treatment 1.2 (2.5 % mono-propylene glycol + 2% Tween 20) because there weren't any conidia incubated in this solution. The most likely explanation for this is mistaken pipetting.

After 3 hours of incubation, M1, used in its higher concentration stimulated an inhibiting effect to the spore's germination and mycelium growth. The compound of 5 % mono-propylene glycol + 4% Tween 20 didn't exhibit a significant antifungal action. This was also obvious in the lower concentration.

Conidia's capacity to develop was limited after 4 hours of incubation in solution of M2 1g/l but after 24 hours there were still some that have germinated and developed mycelium.

During the second experiment, there was done another type of observation especially for the incubation period of 24 hours. 500 µl of the incubated conidia suspension was analyzed and germination of the spores was observed microscopically. Figure 5 shows the amount of conidia contained in the analyzed sample and how many of them have germinated. This observation was done separately for each treatment. The results were expressed in comparison to the controls. We used values 0, 1, 2, 3 and 4 that stand for no, low, intermediate and high conidia amount and germination, respectively.

Compounds FA1, M1, OA 2/chitosan, OA1/chitosan used in their highest concentration prevented the germination totally. The combinations of FA1 together with OA2/chitosan, OA1/chitosan or M2 had the same result but in all their tested concentrations.

The agar plates were also observed 2 days after the end of the incubations to see whether the above mentioned compounds had a long-term result. Table 2 shows in which treatments the mycelium had covered completely the surface of the agar wells. By doing this observation, we concluded that the above mentioned treatments had a long - term efficient result.

Table 1: Treatments of the in vitro assay

Treatments
1.1) solvant 1 **1.2) solvant 2**
2.1) 5 µl/L **FA1** **2.2)** 0,5 µl/L **FA1** **2.3)** 0,05 µl/L **FA1**
3.1) 0,5 % **M1** **3.2)** 0,1 % **M1** **3.3)** 0,02 % **M1**
4.1) 0,1 **% OA 2/chitosan** **4.2)** 0,02 % **OA 2/chitosan** **4.3)** 0,004 % **OA 2/chitosan**
5.1) 0,1 % **OA 1/chitosan** **5.2)** 0,02 % **OA 1/chitosan** **5.3)** 0,004 % **OA 1/chitosan**
6.1) 1 g/L **M 2** **6.2)** 0,25 g/L **M 2**
7.1) 0,1 **% OA 2/chitosan** + 0,5 µl/L **FA1** **7.2)** 0,02 % **OA 2/chitosan** + 0,5 µl/L **FA1** **7.3)** 0,004 % **OA 2/chitosan** + 0,5 µl/L **FA1**
8.1) 0,1 % **OA 1/chitosan** + 0,5 µl/l **FA1** **8.2)** 0,02 % **OA 1/chitosan** + 0,5 µl/l **FA1** **8.3)** 0,004 % **OA 1/chitosan** + 0,5 µl/l **FA1**
9.1) 1 g/L **M 2** + 0,5 µl/L **FA1** **9.2)** 0,25 g/L **M 2** + 0,5 µl/L **FA1**

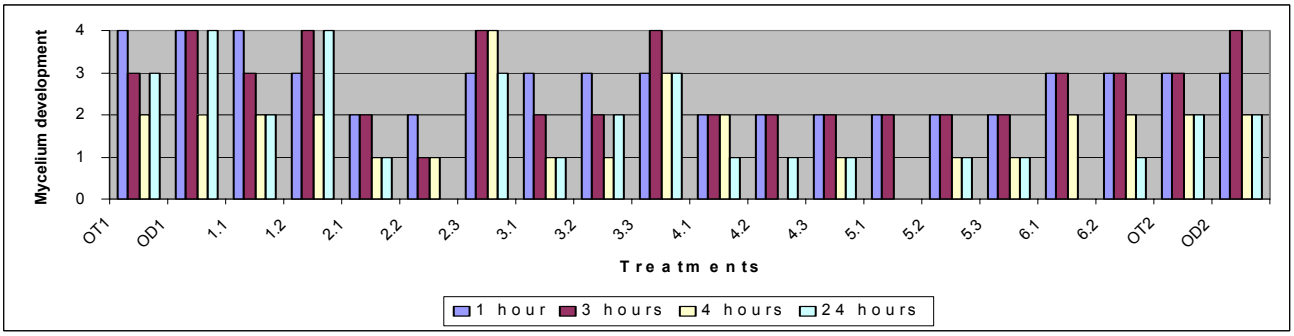


Figure 3: Effect of the treatments on the first experiment

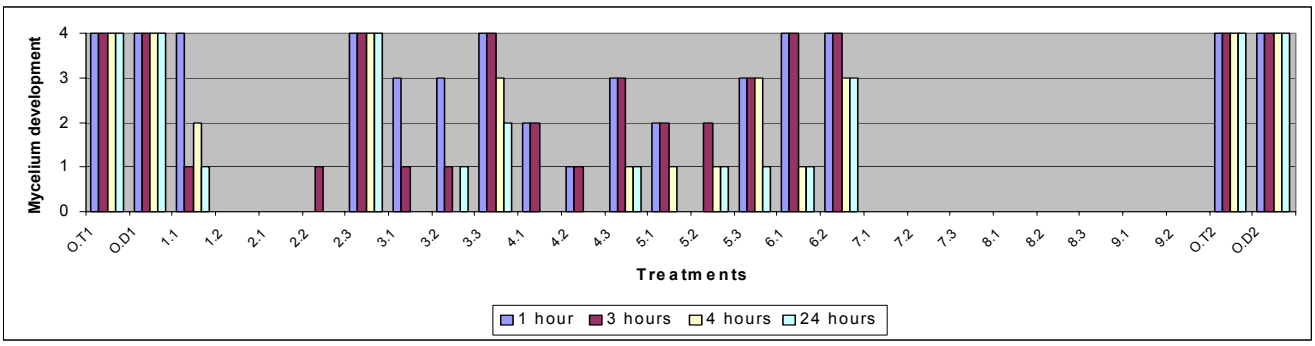


Figure 4: Effect of the treatments on the second experiment

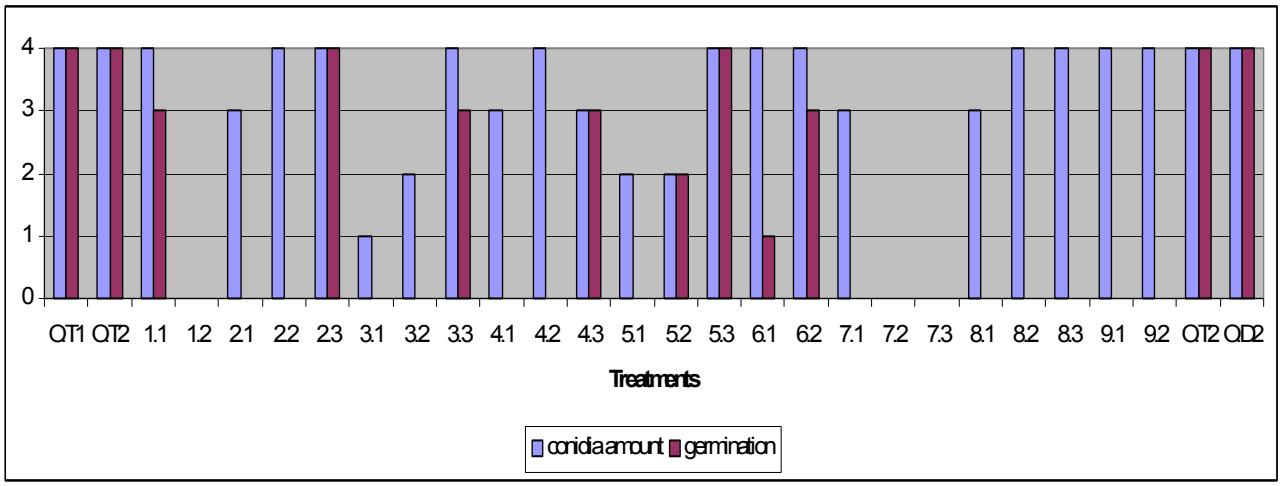


Figure 5: Germination and amount of the conidia contained in the 24 hour incubation agar wells.

Table 2: Evaluation of mycelium's growth 2 days later.

Treatments	Fully grown mycelium	Treatments	Fully grown mycelium
O.T1	√	**5,2**	√
O.D1	√	**5,3**	√
1,1	√	**6,1**	√
1,2	-	**6,2**	√
2,1	×	**7,1**	×
2,2	√	**7,2**	-
2,3	√	**7,3**	-
3,1	×	**8,1**	×
3,2	√	**8,2**	×
3,3	√	**8,3**	×
4,1	√	**9,1**	×
4,2	√	**9,2**	×
4,3	√	**O.T2**	√
5,1	√	**O.D2**	√

Symbols explanation	
√	yes
×	no
-	inconclusive

Table 3: Overview of the efficacy of the treatments.

		Effect (+,-)			
		Time periods			
Compounds	Concentrations	1h	3h	4h	24h
M 2	1 g/l	-	-	+	+
	0.25 g/l	-	-	-	-
FA1	5 µl/l	+	+	+	+
	0.5 µl/l	+	+	+	+
	0.05 µl/l	-	-	-	-
M 1	0.50 %	-	+	+	+
	0.10 %	-	-	+	-
	0.02 %	-	-	-	-
OA 1-chitosan	0.10 %	+	+	+	+
	0.02 %	+	+	+	+
	0.004 %	-	-	-	+
OA 2-chitosan	0.10 %	+	+	+	+
	0.02 %	+	+	+	+
	0.004 %	-	-	+	+
Combinations					
solvant 1 Tween 20	5 % 4 %	-	-	-	+
solvant 1 Tween 20	2.5 % 2 %	?	?	?	?
FA1 OA 1-chitosan	150 µl/l				
	0.10 %	+	+	+	+
	0.02 %	+	+	+	+
	0.004 %	+	+	+	+
FA1 OA 2-chitosan	150 µl/l				
	0.10 %	+	+	+	+
	0.02 %	?	?	?	?
	0.004 %	?	?	?	?
FA1 M 2	150 µl/l				
	1 g/l	+	+	+	+
	0.25 g/l	+	+	+	+

Symbols explanation	
+	Effective
-	Not effective
?	Inconclusive

3.2 LILY LEAF ASSAY

3.2.1 Pilot tests

Leaf tops, removed from lily plants, were treated by spraying with the agents to be tested. One day after, they were inoculated with spores of *Botrytis elliptica*. Monitoring of the infections and evaluation of any inhibiting action against fungal development was performed 3 days later.

Pilot tests were performed in order to optimize the system by adjusting various parameters. Achievement of good infections was one of them. We tested the effect of inoculation with 1) 400 conidia / droplet, 2) 400 conidia / droplet combined with nutrient buffer solution (9.1g/l KH_2PO_4 + 21.8 g/l glucose), 3) 1200 conidia / droplet, 4) 1200 conidia / droplet combined with the nutrient buffer solution, 5) 400 conidia / droplet at the front side of the leaf, 6) 400 conidia / droplet and incubation at 22 °C greenhouse in the sunlight.

Leaves that have been inoculated on their front side were not infected. Leaves who have been incubated at 22 °C and were in direct sunlight, have turned yellow and wick. This is not wanted because should reflect the natural conditions under which the disease in normally spread. Leaves that were inoculated in combination with the buffer solution were heavily infected and the lesions had covered the whole leaf area (pic. 8). This was also not wanted in our research for the same reason mentioned above.

Pic. 8: **Leaves inoculated with conidia suspension in nutrient solution.**

From previous tests it has been estimated that maximal infection was achieved with an inoculum of more than Ca. 400 conidia. Therefore, in our experiment we used 400 conidia per inoculum. Figure 6 illustrates the effect of the inoculum size to the infection

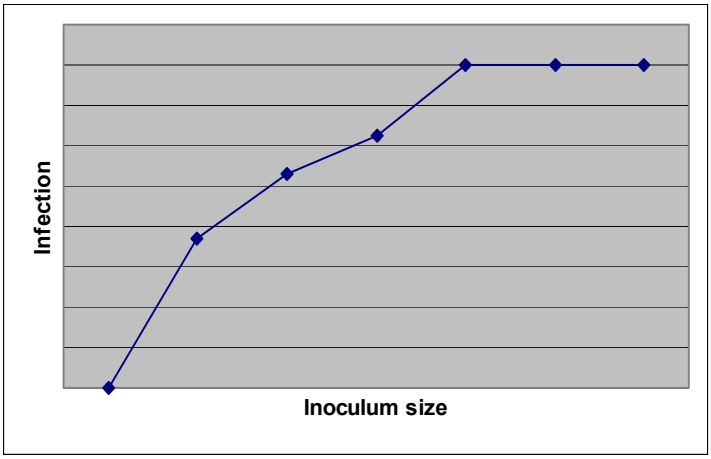


Figure 6 : Effect of the inoculum size to the infection

On the following pilot test we investigated whether the infecting capacity of the spores is depending on their origin. We used spores derived from malt-extract agar and lily leaf-extract agar plates. The effect of light conditions on them was also examined.

It was observed that covered boxes with aluminum foil (dark boxes) contained less infected leaves than the uncovered ones, but their lesion length was bigger. In the dark, spores cultivated on lily leaf-extract agar were more active than the malt extract ones. Both kinds of spores developed the same action when they were incubated in the light.

3.2.2 The experiment

During this research project, 12 series of experiments were performed and several compounds were tested. The categories in which our compounds belonged were lignosulfonates, organic acids, formulation agents, plant metabolites, the chitosan compounds and some compounds that are assigned to the category of miscellaneous. They were tested in several concentrations and combinations. Their effectiveness was evaluated in comparison to the control leaves and the untreated ones. The treatments were applied to experimental units of 13 units. The effect was expressed by the average

lesion length (mm), the number of the lesions occurred among the 13 treated leaves and the relative lesion length (mm) which is defined as lesion length per number of infected leaves.

Table 4 shows the tested compounds listed in categories, their effect on the infection development and their characteristics, such as phytotoxic action and creation of residues on the leaves.

Table 4 : Overview of the tested compounds and their effectiveness.

1. Lignosulfonates (LS)			Remarks	
	Concentrations	Effect (+,/,-)	Phytotixicity	Residue
LS 1	0.05 g/l	-	High	No
LS 2	0.05 g/l	-	High	No
LS 3	0.05 g/l	/	Small	No
LS 4	2.5 g/l	+	No	No
	0.5 g/l	+	No	No
	0.25 g/l	/	No	No
	0.1 g/l	+	No	No
	0.05 g/l	-	No	No
LS 5	4 ml/l	-	Small	No
	1 ml/l	-	Small	No
	0.5 ml/l	-	Small	No
LS 6	10 ml/l	/	High	No
	4 ml/l	+	Small	No
	1 ml/l	+	No	No
	0.5 ml/l	+	No	No
	0.25 ml/l	+	No	No
LS 7	5 g/l	+ +	No	Many
	1 g/l	+	No	Many
	0.5 g/l	+	No	Few
	0.2g/l	/	No	Few
2. Chitosan compounds(CC)				
CC 1	0.50%	+	Medium	No
	0.10%	/	No	No
CC 2	0.50%	-	Medium	No
	0.10%	-	No	No
CC 3	0.50%	/	No	No

	0.10%	/	No	No

3. Organic acids (OA)

OA 1	0.10%	/	No	No
OA 2	0.05%	-	No	No
OA 3	2.5 ml/l	-	No	No
	1 ml/l	-	No	No
	0.5 ml/l	/	No	No
OA 4	2.5mM	/	No	No
	0.5 mM	/	No	No

4. Formulation agents (FA)

FA 1	5 µl/l	+	No	No
FA 2	100 µl/l	+	No	No

5. Plant metabolites (PM)

			Remarks	
	Concentrations	Effect (+,/,-)	Phytotoxicity	Residue
PM 1	5 mM	/	No	No
	1 mM	/	No	No
	0.2 mM	/	No	No
PM 2	5 mM	/	No	No
	1 mM	/	No	No
	0.2 mM	/	No	No
PM 3	5 mM	/	No	No
	1 mM	/	No	No
	0.2 mM	/	No	No
PM 4	5 mM	+ +	No	No
	2.5 mM	+ +	No	No
	1 mM	+ +	No	No
	0.5 mM	+	No	No
	0.2 mM	/	No	No
PM 5	5 mM	+	No	No
	2.5 mM	+	No	No
	1 mM	-	No	No
	0.2 mM	-	No	No
PM 6	5 mM	+	No	Many
	2.5 mM	+	No	Few
	1 mM	-	No	Few
	0.2 mM	-	No	Few
PM 7	5 mM	+	No	No
	1 mM	/	No	No
	0.2 mM	/	No	No
PM 8	5 mM	/	No	No
	1 mM	/	No	No
	0.2 mM	-	No	No

6. Miscellaneous (M)

M 1	0.50%	-	No	No
	0.10%	/	No	No
	0.02%	/	No	No
M 2	2.5 g/l	+	High	No
	1 g/l	+	High	No
	0.5 g/l	/	Small	No

	0.1 g/l	-	No	No
M 3	1 ml/l	+	No	No
	0.2 ml/l	/	No	No
M 4	5%	?	Very high	?
	0.40%	?	Very high	?
	0.04%	+	No	No
	0.01%	+	No	No
M 5	10 mM	+	No	No
	2 mM	+	No	No

Symbol's explanation	
+	Positive effect
-	Negative effect
?	Inconclusive
/	No effect

Table 5 shows the tested combinations, their effect on the fungal development and their characteristics, such as phytotoxic action and creation of residues on the leaves.

Table 5 : Overview of the tested combinations and their efficacy.

Combinations

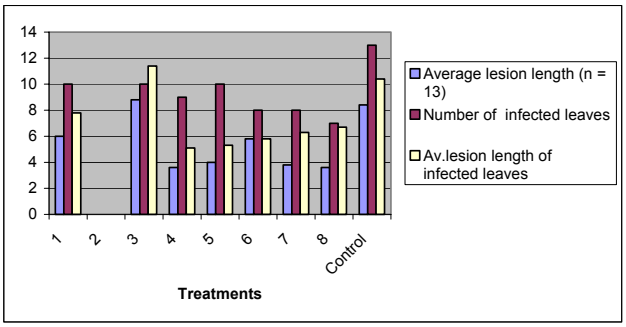
			Remarks	
Compounds	Concentrations	Effect (+,-)	Phytotoxicity	Residue
LS 7 M 3 M 5	1 g/l 1 ml/l 10mM	+ +	No	Many
PM 4 PM 6 / PM 5	2.5 mM 2.5 mM	+ +	No	Few
PM 4 LS 7 M 3	0.2 mM 0.1 g/l 1 ml/l	+ +	No	No
PM 4 M 3 LS 4	0.5 mM 1 ml/l 0.25 g/l	+	No	No
LS 7 M 3 M 5 PM 4	1 g/l 1 ml/l 10mM 1mM	+ +	No	Many

LS 7 M 3 M 5 PM6 / PM 5	1 g/l 1 ml/l 10mM 1 mM	+	No	Many
M 2 PM6 / PM5 / PM4	1 g/l 2.5 mM	+	High	No
LS 6 M 2 PM6 / PM5 / PM4 / PM3	4 ml/l 1 g/l 1 mM	+	High but less In combination With PM3	No
FA 2 PM5 / PM4 A29	100 µl/l 5 mM	+	No	No
LS 7 M 3 M 5 FA 2 PM 7	1 g/l 1 ml/l 10mM 100 µl/l 1 mM	+ +	No	No
CC3 FA 1	0.1% 1µl	-	No	No
LS 7 M 3 OA 3	0.2 g/l 0.2 ml/l 0.5 ml/l	-	No No	Few
OA 3 M 3 OA 4	2.5 ml/l 0.2 ml/l 0.5 mM	-	No	No

As we can see in table 4, LS4 was the only compound from the group of metal LS that had a positive effect against the infecting capacity and growth of *Botrytis*. Furthermore, its combination together with M3 and PM4 was even more effective. Interestingly, at relative low concentration, LS4 resulted in a negative effect. Possibly, LS4 also weaken the plant resistance which at low concentrations dominates the antifungal effect. The same negative effect also had LS1 and LS2, in addition to the intense phytotoxic symptoms they caused. LS3 didn't prove to be a compound of any interest.

The two acid LS tested, LS5 and LS6, at the high concentrations tested had opposite effect. LS5 stimulated the infection size while LS6 developed an inhibiting action against it. Specifically for LS5, when its concentration was reduced the relative lesion length was also reduced while the lesion number

was remaining high (fig.7). LS6, in all tested concentrations reduced drastically the average and relative lesion length as well as their number. This compound also stimulated an interesting action while used together with M2 and PM6, or PM5, or PM4 or PM3, equivalently (appendix fig:1,2,3,4). At that case, phytotoxicity was intense but less in the combination with PM3. In conclusion, PM3 may be an interesting protective agent.

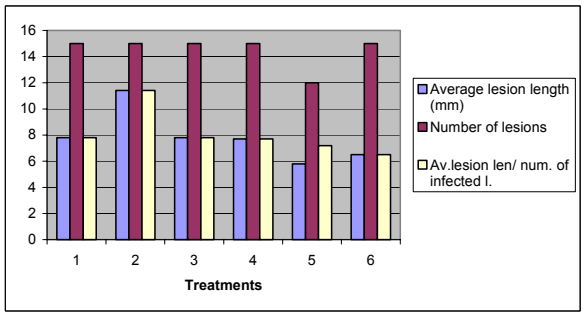


Tray	Treatment	*Concentration*
1	Untreated	
2	LS7	(5 g/l)
3	LS5	4 ml/l
4	LS5	1 ml/l
5	LS5	0.5 ml/l
6	LS6	4 ml/l
7	LS6	1 ml/l
8	LS6	0.5 ml/l

Fig. 7: Effect of LS5 and LS6

LS7 was a significantly effective treatment in most of its tested concentrations and therefore it was used in many assays, as our positive control. Its negative characteristic was the big amount of residue left on the leaf tissue, which was of course proportional to the concentration applied.

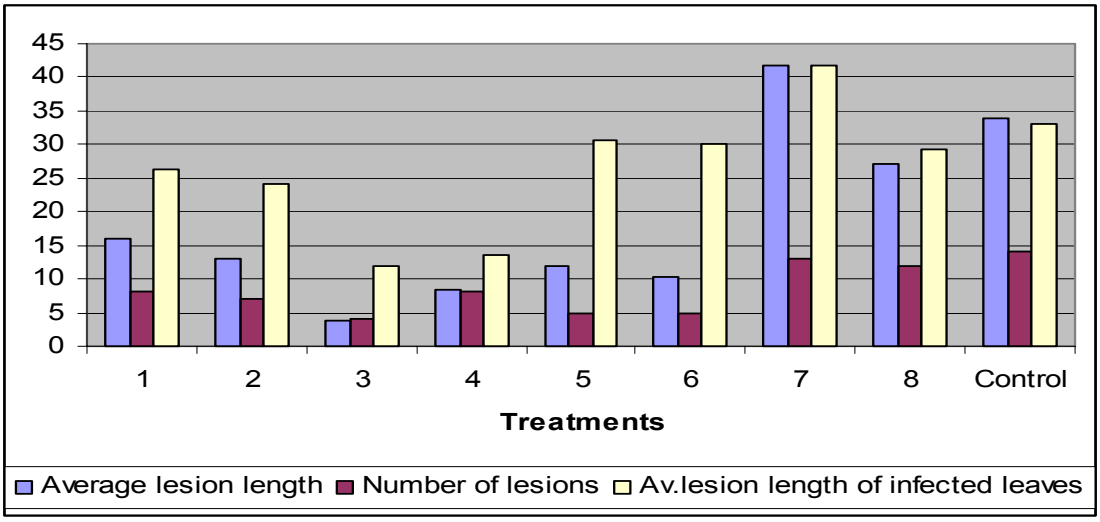
CC2 in both of its tested concentrations increased the lesion length of the infected leaves and made no effect to their number, which was equal to the controls. Therefore, it is considered to have a negative effect. When CC3 (0.1 %) was tested as an individual compound, it was observed that it is not active. On the contrary, its combination together with the wetting agent, FA1 (fig. 8) had a negative effect. Some antifungal activity was exhibited by CC1.



	Treatments	Acid conc.
1	Untreated (control)	
2	CC3+1µl/l FA1	0.10%
3	CC3+1µl/l FA1	0.50%
4	CC3	0.10%
5	CC3	0.50%
6	OA1	0.05%

Figure 8: Effect of CC3 (0.1 %) + FA1

None of the tested organic acids, acquired an inhibition to the development of the disease. Indeed, OA2 and OA3 increased the lesion length. Combinations of the above mentioned acids with compounds like LS7 and M3 were active against the pathogen but increased but the relative lesion length. Probably, this specific combination had a restraining effect to the defense of the plant (fig. 9).



Tray	Treatment		
1	LS7 0.2 g/l	M3 0.2 ml/l	OA3 0.1 ml/l
2	LS7 1.0 g/l	M3 0.2 ml/l	OA3 0.1 ml/l
3	LS7 1.0 g/l	M3 1.0 ml/l	OA3 0.1 ml/l
4	LS7 1.0 g/l	M3 1.0 ml/l	OA3 0.5 ml/l
5	LS7 0.2 g/l	M3 1.0 ml/l	OA3 0.1 ml/l
6	LS7 0.2 g/l	M3 1.0 ml/l	OA3 0.5 ml/l
7	LS7 0.2 g/l	M3 0.2 ml/l	OA3 0.5 ml/l
8	LS7 1.0 g/l	M3 0.2 ml/l	OA3 0.5 ml/l

Figure 9: Effect of LS7 (0.2 g/l) + M3 (0.2 ml/l) + OA3 (0.5 ml/l)

The Formulation agents that we tested were FA1 and FA2. The second one, reduced the infection number but increased the lesion size (fig. 10). Effective combination were those together with LS7, M3, PM7 and M5 as well as the one together with PM5 or PM4 (fig. 5 - appendix). FA1, in comparison with the control, wasn't active against the fungal infection (fig. 11) but exhibited a relative reduction of the lesion length. For that reason, FA2 was used more often in our project.

From the group of Plant metabolites PM1, PM2 and PM3 had no interesting effect in any of their tested concentrations.

1	Untreated	-	-
2	LS7(1 g/l) CAQ (1 ml/l) M5 (10 mM)	-	-
3	LS7(1 g/l) CAQ (1 ml/l) M5 (10 mM)	FA2(100 µl/l)	-
4	LS7(1 g/l) CAQ (1 ml/l) M5 (10 mM)	FA2(100 µl/l)	PM7(1 mM)
5	-	FA2(100 µl/l)	-
6	-	FA2(100 µl/l)	PM7(1 mM)
7	-	-	PM7(1 mM)
8	LS7(1 g/l) CAQ (1 ml/l) M5 (10 mM)	-	PM7(1 mM)

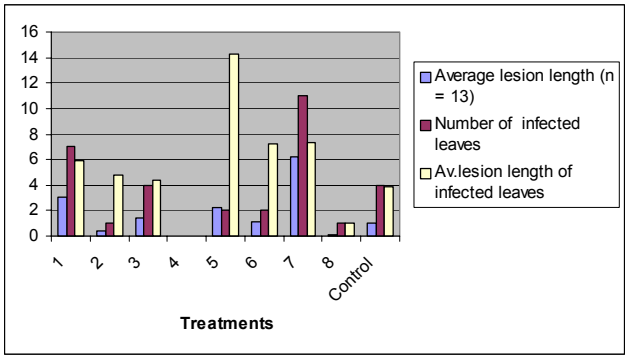
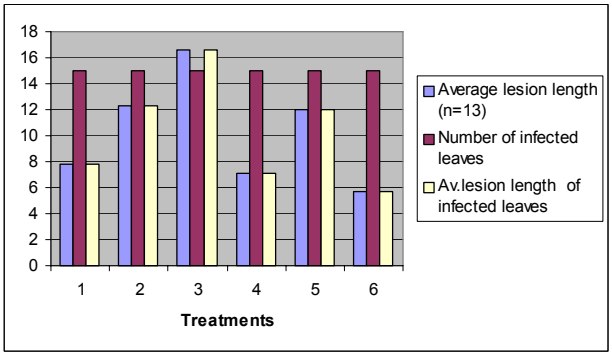


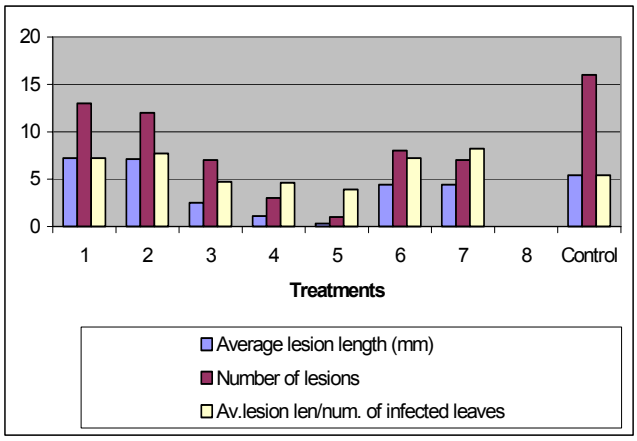
Figure 10 : Effect of FA2 100 µl/l



1	Untreated (control)	
2	LS 1	50 mg/l (0.79 mM)
3	CuSO4.5H2O	50 mg/l (0.79 mM)
4	LS2	50 mg/l (0.85 mM)
5	CoCl2.6H2O	50 mg/l (0.85 mM)
6	FA1	5 µl/l

Figure 11 : Effect of FA1 (5 µl/l)

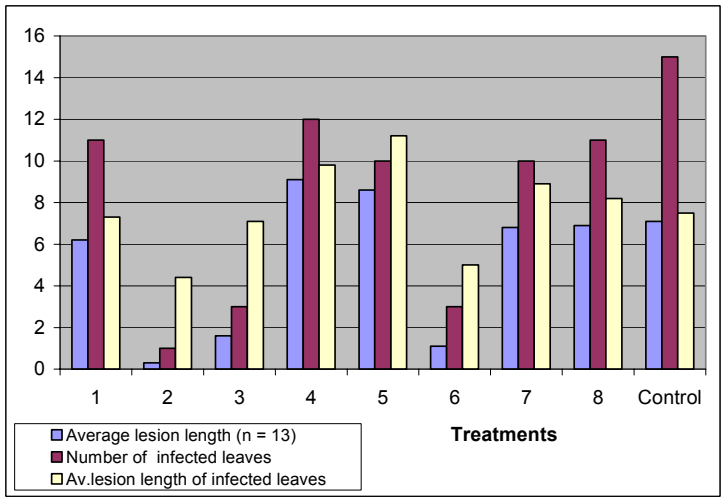
PM4 is a compound on which we have focused a lot in our project. Every time that it has been applied, regardless its concentration or the combinations, the results were very satisfactory. Its effect on the infection size is obvious. (fig. 12, appendix – fig. 3,5,6). Very promising combinations have been done with other plant metabolites like PM5 or PM6, with M3, LS7, M5, M2 and also with FA2 (fig. 12 /appendix : 3,5,6).



1	untreated	
2	M2 (1 g/l)	
3	M2 (1 g/l)	PM6(2.5 mM)
4	M2 (1 g/l)	PM5(2.5 mM)
5	M2 (1 g/l)	PM4(2.5 mM)
6		PM6(2.5 mM)
7		PM5(2.5 mM)
8		PM4(2.5 mM)

Figure 12 : Effect of PM4 2.5 mM

PM5 5 mM and 2.5 mM elaborated a reduction to the infection size but in lower concentrations showed no effect to the lesion number and an increase to their length was observed (fig. 13). The action of PM6 was similar to that of PM 5. In contrast to the other plant metabolites, PM6 5 mM and 1 mM formed residues.

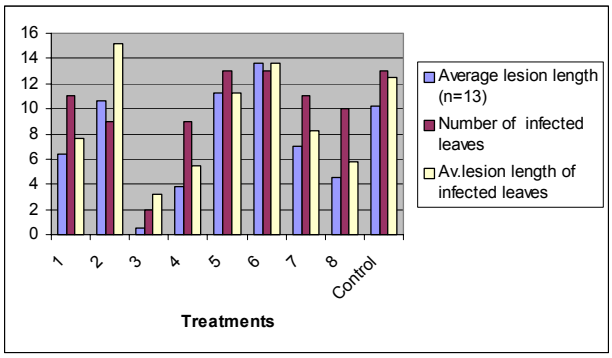


1	Untreated	
2	LS7	5 g/l
3	PM6	5 mM
4	PM6	1 mM
5	PM6	0.2 mM
6	PM5	5 mM
7	PM5	1 mM
8	PM5	0.2 mM

Figure 13 : Effect of PM5 and PM6

PM8 5 mM and 1mM didn't rise any action against *Botrytis* infection. Application of the compound in lower concentrations showed that the lesion length is increased. PM 7 tested on its higher concentrations had a positive effect which was increased when the compound was used with the combination of LS7, M3 and M5.

M2 was a very phytotoxic compound that in high concentrations (2.5 g/l) inhibited fungal activity but in low concentrations (0.1 g/l) was beneficial for it. Its combination together with PM6 or PM5 or PM4 exhibited a satisfactory effect against the fungal development but caused intense phytotoxicity.



1	Untreated
2	Demi water
3	M2 2.5 g/l
4	M2 0.5 g/l
5	M2 0.1 g/l
6	M1 0.5% (5 µl per ml)
7	M1 0.1%
8	M1 0.02%

Figure 14 : Effect of M2 and M1

M1 in high concentration had an interesting negative result, by increasing the infection size (fig. 14). M4 in high concentrations caused severe toxic damage to the leaves but it didn't in low concentrations (0.04 %, 0.01%). Added to this, it exhibited a positive effect.

M5 tested by itself had no effect to the number of infections but reduced sufficiently the length of the lesions that have appeared. Its combination together with LS7 and M3 proved to be very effective. These 3 compounds together may form an interesting agent against *Botrytis s*pread (fig. 15). Therefore, this powerful combination was sometimes used to raise the action of other compounds.

1	untreated	
2	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	
3	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	PM6 (5 mM)
4	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	PM6 (1 mM)
5	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	PM5 (5 mM)
6	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	PM5 (1 mM)
7	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	PM4 (5 mM)
8	LS7 (1 g/l) M3 (1 ml/l) M5 (10 mM)	PM4 (1 mM)

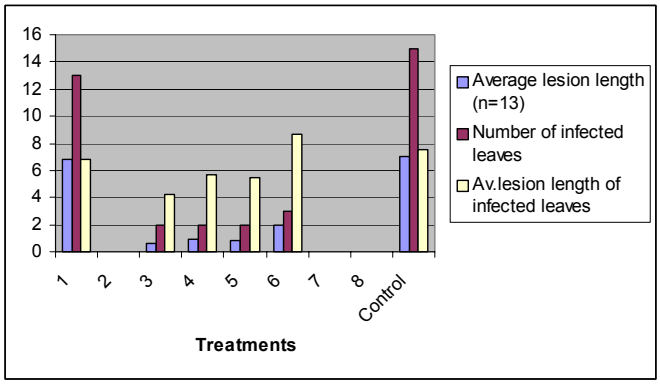


Figure 15 : Effect of LS7 + M3 + M5

4. Discussion

Botrytis elliptica, the casual agent of “lily fire” has been responsible for economically significant losses in lily production of greenhouse as well as field crops. At present, the control of *Botrytis* diseases is based on the frequent use of fungicides. However, *Botrytis spp*. have shown great potential to develop fungicide resistance. In addition, an increasing concern about the effects of pesticide residues on the environment and human health leads to an increasing number of restrictions on the use of pesticides.

Subsequently, this is a strong motivation for research and future improvement of the already existing methods. In this project, we focused in the research for sustainable, environmentally friendly, crop protectant agents of natural origin.

All applied agents cause stress condition to the plant. The response of the plant to this situation has two aspects. These are inducement or restrain of its resistance mechanism. Therefore the plant can defend itself and avoid the infection or become susceptible to it. In conclusion, stress condition is an defining parameter of the infection size.

Antifungal activity of the compounds is another restricting mechanism against the development of infection. Furthermore, concentration of the applied compounds is a parameter which determines the size of antifungal activity. Thus the infection size is relevant to the stress caused to the plant and the compound’s action against the pathogen.

The interaction between the active compounds, the plant and the fungus has many aspects and therefore was our main subject. Our project consisted of two experimental parts, the in vitro assay and the leaf top assay. By means of the vitro assay, compounds were tested directly on the fungus. The plant factor was excluded and we investigated the interaction between *Botrytis* and the agents. The conclusions were subsequently applied in the leaf top assay in order to include the missing factor of plant reaction to the treatments.

During the first research part, the increase of incubation time and concentration resulted in the inhibition of the mycelium growth for most of the treatments. Thus, the biggest inhibition of the spores' activity was observed in the treatment solutions with the highest concentration after 24 hours of incubation. At that point, in order to see more clearly the efficacy of these treatments, germination of the spores was checked microscopically.

Compounds FA1, M1, OA2/chitosan, OA1/chitosan used in their highest concentration prevented the germination totally. The combinations OA 2/chitosan + FA1, OA 1/chitosan + FA1, M2 + FA1 had the same result in but in all their tested concentrations. The effect of the above mentioned treatments was permanent. In all other cases (lower concentrations) and also in the tested concentrations of M2 germination was inhibited partly and after some time mycelium has developed normally.

During the next experimental phase, these treatments were tested on the leaf tops in order to investigate their interaction with the plants. OA1/chitosan did not have the expected effect to the infection size caused by *Botrytis* because it didn't act against it. In combination with FA1 its effect was negative because the infected leaves appeared big lesions. The explanation for this result may be that the compound is active against the fungus but it immobilizes plant's resistance mechanism. Therefore, the pathogen is in dominance of the plant and leaves appeared lesions of big length. For the case of M1, our conclusion is the same.

M2 proofed to be very toxic for the plant and also very effective against *Botrytis,* even though during the in vitro assay its effect was moderate. FA1 was another active compound against the pathogen that didn't prevent plant's infection. OA2/chitosan in high concentration had a negative effect which was also in contradiction to its in vitro test.

Several compounds were tested only in the leaf top assay. We have confirmed the efficacy of agents as individual compounds that were equally or even more effective in combination with others. Agents of no significant action against the infection size turned into great defending treatments when they were combined. In some cases, agents provoked a relative increase of the disease's spread on the plant material. Some of them also caused toxic symptoms.

For specific agents like LS1, LS2, LS5, OA2 and OA3 we have observed that their low protective effect was remaining the same whether they were applied in high or low concentrations. On the contrary, the lesions appeared after the high concentrated treatments were bigger. Therefore, we conclude that these agents are not active and they also weaken the plant's defense. This is also found for the combination of OA3 with LS7 and M3 and also for the combination of OA3 with M3 and OA4.

Another interesting observation was done for agents LS4, PM5 and PM6. In that case, their high protective action was reduced when they were applied in lower concentrations,. Added to this, which is to be expected the increasingly infected leaves had bigger lesions than the control. This leads us to the conclusion that the specific compounds were active and they caused stress to the plant which was obvious only when their antifungal action was reduced. Therefore, the plant became more susceptible.

Agents FA1 and FA2 were used in order to obtain a homogenous layer of the sprayed treatment on the leaf tops. Apart from this faculty they have also stimulated an inhibiting result against *Botrytis* action. Specifically, FA2 prevented the infection of the plant successfully but had a negative result to the lesion length. FA1 had no significant effect against the infection but succeeded in controlling the length of the lesions. This discrimination to their action was the criterion that made FA2 preferable for use.

Compounds LS3, OA4, PM3, OA1, PM8, PM1 and PM2 didn't prevent the infection of the plant and in addition they didn't bring any consequences to its resistance mechanism. Therefore these compounds are not concerned to have any effect.

We came to the conclusion that PM4, LS7 and their combination together with M3 and M5 are very active against the pathogen without causing symptoms of weakness to the plant. Therefore, apart from their antifungal action these agents may also enhance plant's resistance to *Botrytis.*

M3 and M5 have not been tested individually but only in combination with other compounds. Therefore, our conclusion refers to their efficacy during the increase of their concentration. Specifically their effect was mostly observed in the control of the lesion's length, by reducing it. Therefore, it's possible that these compounds induce plant's resistance to the infection.

LS7 was also very effective compound against *Botyrtis* and therefore was used as our positive control. The negative point that was observed in its use, was the formation of residues on the leaf tissue. This is not preferable for the appearance of the plant and moreover for the appearance its flowers. Use of less concentrated LS7 was equally effective and low residue was present.

A relative negative effect to the infection was observed in the treatments of demineralised water but this is an estimation that should be tested further.

PM7 was a compound of moderate result, in contrast with PM 6 that had a positive result. PM6 is formed by the hydrolysis of PM7. Therefore, it's possible that the moderate effect of PM7 was caused by the slow release of PM6.

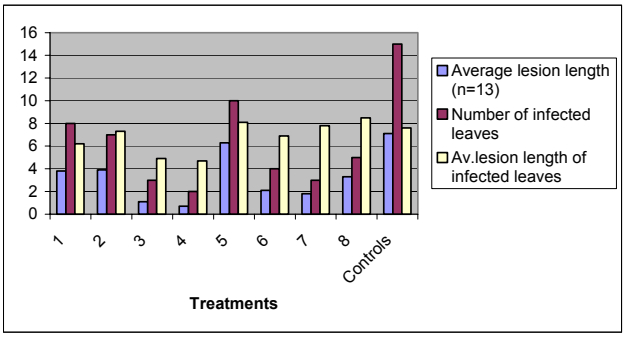
PM3 seems to have a protective effect against the phytotoxicity caused by M2 and LS6.

During our research project, the plant metabolites group proved to be the one including the most agents with a positive effect against *Botrytis elliptica.* Out of them, PM4 was the one that inhibited the infection more efficiently. Therefore, our suggestion is that this agent should be used as a lead for further research of equivalent or additionally effective compounds against the infection caused by *Botrytis elliptica* on lily plants.

Another factor that would probably influence the course of action of these experiments, is plant growing conditions. During our six months testing, the climate conditions were variable and effected the development of the plants. Plants were growing faster when the weather was warm and therefore our experiments were not performed at the same stage of growing. There are indications that older plants were more resistant to the infection.

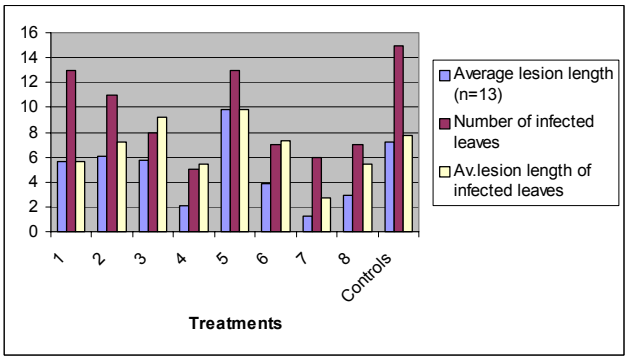
In vitro test provided us with valuable information about the antifungal action of the compounds. In addition to the information derived from the leaf top assay, we observed the interaction between the plant, the pathogen and the agent. All compounds should be tested in this way and therefore more in vitro tests should have been performed.

Appendix



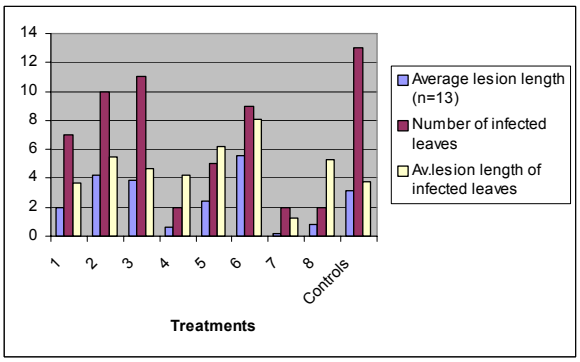
Tray	Treatment		
1	-	-	
2	LS6 (4 ml/l)	-	-
3	LS6 (4 ml/l)	M2 (1 g/l)	-
4	LS6 (4 ml/l)	M2 (1 g/l)	PM6 (1 mM)
5	-	M2 (1 g/l)	-
6	-	M2 (1 g/l)	PM6 (1 mM)
7	-	-	PM6 (1 mM)
8	LS6 (4 ml/l)	-	PM6 (1 mM)

Figure 1 : Effect of LS6 in combination with M2 and PM6



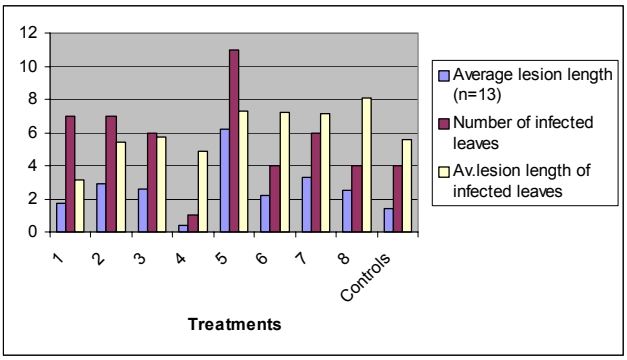
Tray	Treatment		
1	-	-	-
2	LS6 (4 ml/l)	-	-
3	LS6 (4 ml/l)	M2 (1 g/l)	-
4	LS6 (4 ml/l)	M2 (1 g/l)	PM5 (1 mM)
5	-	M2 (1 g/l)	-
6	-	M2 (1 g/l)	PM5 (1 mM)
7	-	-	PM5 (1 mM)
8	LS6 (4 ml/l)	-	PM5 (1 mM)

Figure 2 : Effect of LS6 in combination with M 2 and PM 5



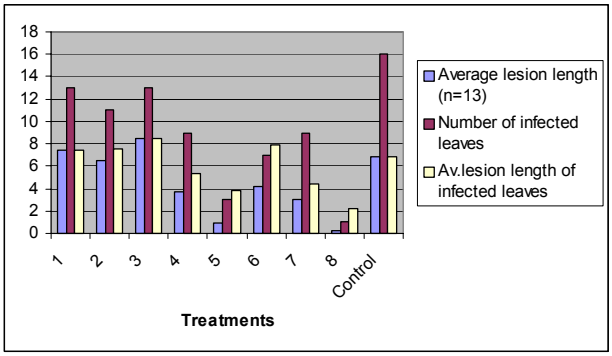
Tray	Treatment		
1	-	-	-
2	LS6 (4 ml/l)	-	-
3	LS6 (4 ml/l)	M2 (1 g/l)	-
4	LS6 (4 ml/l)	M2 (1 g/l)	PM4 (1 mM)
5	-	M2 (1 g/l)	-
6	-	M2 (1 g/l)	PM4 (1 mM)
7	-	-	PM4 (1 mM)
8	LS6 (4 ml/l)	-	PM4 (1 mM)

Figure 3 : Effect of LS6 in combination with M2 and PM4



Tray	**Treatment**		
1	-	-	-
2	LS6(4 ml/l)	-	-
3	LS6(4 ml/l)	M2 (1 g/l)	-
4	LS6(4 ml/l)	M2 (1 g/l)	PM3 (1 mM)
5	-	M2 (1 g/l)	-
6	-	M2 (1 g/l)	PM3 (1 mM)
7	-	-	PM3 (1 mM)
8	LS6(4 ml/l)	-	PM3 (1 mM)

Figure 4 : Effect of LS 6 in combination with M2 and PM3



1	untreated	
2	FA2 (100 µl/l)	
3	FA2 (100 µl/l)	PM6(5 mM)
4	FA2 (100 µl/l)	PM5 (5 mM)
5	FA2 (100 µl/l)	PM4 (5 mM)
6		PM6 (5 mM)
7		PM5 (5 mM)
8		PM4 (5 mM)

Figure 5 : Effect of FA2 and PM4

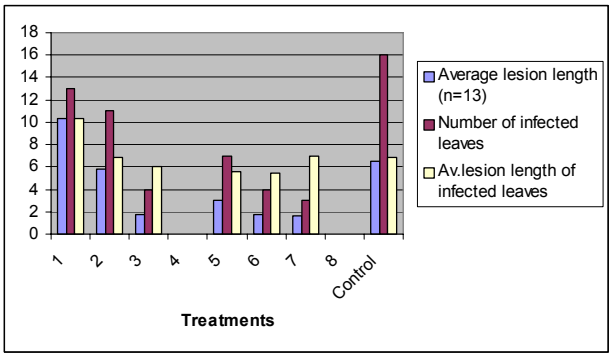


Figure 6 : Effect of PM4 with PM5 or PM6

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