

Komplementerende forskning i FØJØ II
Control of scab in organic apple growing (StopScab)
Område 2. Fødevarer kvalitet, sikkerhed og sundhed, Projekt nr. VII.5

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Acronym: StopScab

Date: April 2002 – September 2004

Summary in Danish:

Æbleskurv forvoldt af *Venturia inaequalis*, forårsager store afgrødetab og kvalitetsforringelse af økologisk dyrkede æbler både i Danmark og i mange andre lande. I økologisk æbleavl er de fleste tilgængelige metoder til bekæmpelse af skurv ineffektive og skurv er tillige et stort problem i konventionel æbleavl. Nærværende projekt omhandler udviklingen af flere lovende metoder til at bekæmpe skurv. En række bekæmpelsesmuligheder vil blive screenet for deres effekt overfor skurvpatogenet i vækstkammer, væksthus og i plantage. Lovende bekæmpelsesmuligheder vil gennemgå histopatologiske undersøgelser for at karakterisere virkemekanismer. Detail-studier vil blive udført for at karakterisere effekten af udvalgte behandlinger på produktionen af sekundære metabolitter i æblefrugter, og molekylære analyser af forsvarsresponsen i blade vil blive gennemført. Det overordnede mål med dette projekt er at udpege og udvikle nogle nye bekæmpelsesmuligheder mod skurv, der respekterer den økologiske dyrknings principper.

1. Summary

Apple scab, caused by *Venturia inaequalis*, causes serious losses in quality and yield of organically grown apples in Denmark and elsewhere. Materials available to organic growers in Denmark for the control of scab are largely ineffective and scab also is a growing problem for conventional apple growers. This project is concerned with developing several promising approaches to the control of scab. A range of potential control materials are to be screened for efficacy against the scab pathogen in growth chamber, greenhouse and orchard experiments. Promising materials are to be further investigated histopathologically to establish the mechanism of control. More detailed studies will be made to characterise the effect of selected treatments on the secondary metabolites in apples and a molecular analysis of defence responses will be carried out. With respect for the principles of organic growing, the overriding aim of this proposal is to identify and begin to develop some new approaches and materials for the control of apple scab.

2. Research group

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3. Introduction

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint., is a limitation to apple production wherever apples are grown. Despite years of research and development it is still the most economically important disease of apple worldwide. During years of high disease pressure, apple scab may make apple production unprofitable. These statements have been applied to conventional apple growing, but are truer for organic growers, who have fewer effective control options available to them. Furthermore, much effort has been put into management packages based on the use of chemical fungicides, much less effort has gone into developing the several options that undoubtedly exist for exploiting biological, cultural and ecological methods (MacHardy 1996). There is thus a need for innovative research in the area of scab control, particularly in order to develop control systems that can be used by organic apple grower. It is our aim with this research proposal to make a contribution to the solving of some of the apple scab problems facing organic apple growers, using a combination of classical and modern methods.

4. State-of-the-art

The life cycle of the apple scab pathogen, *V. inaequalis*, is conveniently divided into two parts or phases: a “winter phase”, beginning at the end of the growing season continuing throughout the winter, and a “spring & summer phase”, that starts in early spring and continues for most of the growing season. Ecologically these two phases differ greatly because under its “winter phase”, *V. inaequalis* lives saprophytically, whereas under its “spring & summer phase” - when it causes disease to the apple tree - it lives parasitically.

Control of apple scab may be divided broadly into defensive and offensive strategies. Defensive strategies include the application of protective, curative and eradicate fungicides to protect the plant from primary infection and slow the build up of secondary scab. No eradicate or curative fungicides are available for use by organic growers, and while protective, copper-based fungicides are permitted in most European countries (Lindhard & Callesen, 1999), only sulphur is allowed in Denmark and sulphur is not very effective against scab (Zimmer, 1997).

A further group of defensive strategies are cultural practices that lessen environmental conditions favourable for infection and a number of such methods are currently under investigation in the project, FØJOII-DJF-2: Development of sustainable production systems for apples.

Offensive management strategies include practices that reduce the ascospore inoculum at source i.e. in over-wintering leaves on the orchard floor. Such practices include destruction of leaves by mechanical shredding, soil tillage, collection by vacuum cleaner, increasing the activity of earth worms, chicken and sheep grazing, and microbial strategies in which antagonists are applied alone (Bengtsson, 2001, Carisse

et al., 1999) or together with extra nitrogen (Bengtsson 2001, Burchill, 1968). In a similar pathosystem, cherry leaf spot, the mechanisms by which applications of urea effect pathogen survival are described by Green *et al.* (submitted). Reducing the over-wintering ascospore inoculum gives a smaller disease pressure the following spring making scab management possible with fewer applications of pesticides (Carisse, 2000). This was demonstrated for the first time by MacHardy *et al.*, (1993) who showed that it is possible to delay the first fungicide applications up to pink stage when the inoculum potential is very low.

A second offensive strategy is to make use of apple's natural resistance to scab. The infection process and stroma formation by *V. inaequalis* on apple leaves with different degrees of resistance to scab are described by Gessler & Stumm (1984). Resistance to scab is under constant threat from changes in the virulence pattern of the pathogen and many of the varieties in current use have resistances originating from *Malus floribunda*. Races new to Denmark have recently been registered at the race-screening orchard at Årslev (Bengtsson *et al.*, 1999), and also in commercial orchards (Korsgaard, 2001). Thus an important focus in apple breeding is resistance to apple scab and several breeding programmes in different parts of the world have scab resistance as a major goal. While genetic engineering strategies are increasingly being used in an attempt to improve disease resistance to scab (e.g. Norelli *et al.*, 2000), this may result in even fewer varieties with improved resistance to apple pests and diseases being available to organic growers in the future. In Denmark, utilisation of genetically engineered plant material (or micro-organisms) in organic farming is not permitted. Work to evaluate resistance of different apple varieties has been carried out over a number of years in Denmark and recommendations for organic production made (Bertelsen & Grauslund 1995). Evaluation of new accessions is also a part of the ongoing project, FØJOII-DJF-2: Development of sustainable production systems for apples. There is, however, only a slow adoption of resistant cultivars as they generally do not possess the required agronomic characteristics such as fruit quality, yield, fruit storability (MacHardy, 1996) and resistance to diseases.

Plant protection materials currently available for use on organic apples

Danish organic fruit growers have far fewer plant protection materials at their disposal than colleagues in other European countries. Thus the plant protection products permitted for use in organic apple growing in Europe (in accordance with EU directive 2092/91), compiled at the meeting of the European group of Researchers in Organic Fruit held 29-30th November, 2000, in Frick, Switzerland, consists of materials based on plant extracts, micro-organisms, pheromones and diverse materials such as copper and sulphur, various paraffin oils and clay minerals etc. Only a few insecticides, certain micro-organisms and sulphur are permitted (M. Korsgaard, *pers. comm.*). As mentioned, in Denmark copper products are not permitted, and while sulphur is widely used against apple scab, its efficacy is low (M. Korsgaard, *pers. comm.*).

New approaches to control of apple scab.

Plant extracts and disease control.

Plant extracts are potential sources of alternative pesticides (Cutler & Cutler 1999) and much of the plant kingdom still remains unexplored for possible exploitation against plant pathogens. To date more use has been made of plant extracts against pests than plant pathogens in organic apple growing (M. Korsgaard, *pers. comm.*). However, activity against plant pathogens has been demonstrated in extracts from algae and a range of plants including neem (*Azadirachta indica*) (Amadioha, 2000), and in essential oils from plants such as neem (Amadioha, 2000), thyme (*Thymus vulgaris*), and carrot (*Daucus carota*) (Dwivedi *et al.*, 1991, V. Leth, *pers. comm.*). While under experimental conditions, disease control efficacies may equal those of chemical fungicides (Amadioha, 2000, Dwivedi *et al.*, 1991), good field data in support of this is largely lacking. In addition, the mechanism of action of most natural products is generally poorly understood and their potential as inducers of resistance against plant pathogens deserves investigation. Work in this area is ongoing at CIAT, Columbia (John Loke, *pers. comm.*).

Biological control based on single-strains of biocontrol agents.

About 80 biological control products against plant diseases are near to or on the market (Whipps and Davies, 2000) and almost all of these are based on a single strain of bacterium or fungus. Very few of these, however, are targeted for use in the phyllosphere (Whipps and Davies, 2000) and this is probably a reflection of the relatively severe conditions that prevail in the canopies of trees, which are characterized by fluctuating, generally low levels of available nutrients, frequent and often rapid changes in temperature and wetness and exposure to UV radiation, and intensive competition between micro-organisms for nutrients and space. Nutrient are important as microbial substrates and for the synthesis of antibiotics, and these nutrients are frequently limiting on the phylloplane. In this respect the potential antagonist must be able to survive and act in this environment. And in fact, whether biocontrol in the phyllosphere is an attainable goal has been questioned (Andrews, 1992).

The use of biological control agents against the apple scab pathogen is described by Burr *et al.*, 1996 and orchard trials have also recently been conducted with promising isolates (G. Auling, *pers. comm.*). The Canadian group working on biological control of apple scab evaluated fungal and yeast-like isolates recovered from apple leaf litter for their ability to inhibit vegetative growth of the inaequalis in vitro (Ouimet *et al.*, 1997a). An isolate of *Ophiostoma* sp. That was found to inhibit mycelia growth and conidial germination seemed, in further studies, not to be influenced by environmental and nutritional conditions in vitro and was found to be a promising candidate for biocontrol (Ouimet *et al.*, 1997b). More recently, Fiss *et al.*, (2000) isolated and screened epiphytic fungi from the apple phyllosphere for ability to inhibit mucelial growth and conidial germination in vitro and to reduce disease incidence an apple seedlings.

Use of communities of beneficial micro-organisms.

Application of communities of micro-organisms to plant surfaces is a second approach to achieving disease control with micro-organism. This has been attempted by applying mixtures of cultured micro-organisms e.g. "Effective microorganisms" (Sangakkara & Higa, 1995), and extracts from compost (Cronin, *et al.*, 1996) containing populations of micro-organisms and nutrients to sustain their growth. The problems encountered with compost extracts included low efficacies, poor repeatability and phytotoxicity. Systems have been developed subsequently in the USA and in Europe (Elaine Ingham, Soil Foodweb Inc. USA & Compara Co., The Netherlands, *pers. comm.*) that enable the microbial and nutritional make-up of the compost liquid (compost tea) to be controlled and standardised. Compost tea, which can be produced by the grower himself or by a company is currently used by a number of growers and disease control trials are currently in progress in the US on grapes, oak and tomato (E. Ingham, *pers. comm.* And Colombia, J. Loke, *pers. comm.*). The compost tea is diluted and applied to the foliage by sprayer. The dosage is regulated so that the surfaces are populated by a given number of micro-organisms per sq. cm. To maintain population levels, further applications are made (monitoring kits are available from these companies). In the case of apple scab control, application would be coupled to some form of scab infection-period warning system. Mechanisms of control would probably include antibiosis, parasitism and competition for nutrients and space between the applied micro-organisms and the pathogen. Induction of a resistance reaction in the leaves is also a distinct possibility.

Utilizing Induced disease resistance.

A modern topic in control of scab is based on induced resistance (Kloepper *et al.*, 1992). Treatments that stimulate the natural defence responses of plants offer a novel plant protection strategy against pathogens. Pathogens have developed an array of offensive strategies to parasitise plants and, in turn, plants have deployed a wide range of defence mechanisms. Induction of resistance in plants is based on the enhanced expression of defence response genes, which increase the natural responses of the host against infection. The aim of this method is thus to direct the metabolism of the plant in such a way that production of defence compounds is enforced. This is done by the application of so called inducers that activate or sensitise the plant leading to a more rapid or intense defence response, thus increasing the ability of the plant able to combat the infection successfully. Induction of resistance is possible by pre-inoculation with inducers of biological, microbial, chemical or physical origin, but the effect on the plant cell is similar to its defence responses during attack by a pathogen. The mode of action of

induced resistance is very complex, however. Disease resistance reactions result from changes in cell biochemistry and physiology, leading to e.g., with structural modifications including the formation of, e.g., callose-enriched wall appositions, the infiltration of phenolic substances at sites of potential pathogen penetration and hypersensitive reactions (Dean & Kûc, 1987). In induced resistance, a multitude of diverse defence reactions are activated and therefore this form of protection is not likely to be overcome by changes in the pathogen population (Ortega *et al.*, 1998a). Furthermore, induction of resistance may affect the whole life cycle of the pathogen, thus inhibiting the initial penetration, growth in the tissue as well as the final sporulation. The latter was, e.g., shown for induced resistance in barley against *Drechslera teres* (Jørgensen *et al.*, 1998). So far, development of resistance against disease control systems that act with induced resistance has not been reported (Ortega *et al.*, 1998a). Microscopic studies of induced resistance to apple scab has been undertaken by Ortega *et al.*, 1998b.

Monitoring the effect of treatments against apple scab by molecular approaches

As outlined above, the defence response of plants comprises the coordinated expression of a plethora of functionally unrelated genes (Collinge *et al.*, 2001). Essentially the same defence response is induced in a particular plant species by all pathogens irrespective of their taxonomic relationships. These defences are individually effective against a few (often) taxonomically related pathogens but collectively are effective in repulsing the vast majority of taxonomically diverse pathogens to which the plant is exposed. The individual components include antimicrobial proteins and antimicrobial metabolites. The demonstration that a particular treatment induces these defence response thus indicates that the treatment is likely to be effective against the majority of pathogens to which the plant is exposed. The extent to which a particular treatment induces the defence response will be reflected in the level of protection afforded. Molecular tools are available to monitor the degree to which different treatments induce the defence response.

The gene sequence databases currently list in excess of 800 sequences from *Malus × domestica*, and many more have yet to be submitted (Y. Hatsuyama, *pers. comm.*). These include a number of representatives of the defence response (various PR proteins, components of secondary metabolism, and regulatory components and, as the a number of these have been published (e.g. Komjanc *et al.*, 1999; Yao *et al.*, 1999); they will be available for public research (i.e. the sequences are in the data bases). We will therefore be able to make dot-blot arrays (macroarrays) for these well characterised gene sequences and probe the arrays with probe prepared from apples treated in different ways (Gregersen *et al.*, 1997). The data bases also include a large number of unpublished EST's (expression sequence tags) from both shoot and fruit cDNA libraries, prepared by Japanese and Korean researchers, respectively. We are on contact with both these groups. The Japanese group has embarked on a microarray study and we are negotiating with them as to the possibility of collaborating as this would clearly be the most effective way to make these kind of studies.

Human health and aspects related to plant resistance reactions.

An advantage that has been related to induced resistance is the production of defensive compounds, which can increase the quality of foodstuffs (Treutter, 2000). One important group are the phenolics, which may possess human disease- preventive properties (King & Young 1999.) Phenolic acids occur in most vegetable products and can be found either as the free acid, as glucosides and bound to polymeric materials (e.g. cellulose, lignin). In the free form, they exhibit broad-spectrum antimicrobial properties. When bound, they assist in the stabilisation of texture, especially as dimeric phenolic acids, and as such affect the digestibility and fibre content and properties. Flavonoids in most plants are primarily involved in UV-defence but also against bacteria, fungi and insects.. These compounds all have high antioxidant capacity and many also have interesting pharmacological properties, e.g. effects related to prevention of cancer (Sangwan *et al.*, 1998). Plant foods contain other components with effects on health than the well-known nutrients, for example many biologically active secondary metabolites. None of them are known to be absolutely necessary for a long and healthy life (per definition - in the

past, as soon as a specific physiological role was shown for a secondary metabolite, it was redefined as a vitamin). Secondary metabolites in plants have a variety of functions in the plant. However, the biologically active ones, which are most likely to influence human health, are primarily involved in defence against pests, herbivores and diseases, some of them as anti-nutrients, making the protein or other essential components less available to plant-eating animals or pests (Mawson *et al.*, 1994, Waghorn 1990). Differences in the aroma profile of apples will affect taste and might thus influence consumer preference. Some volatiles might also be important from a health perspective (Sangwan *et al.*, 1998). The level and composition of volatiles might change during induction of resistance as plants use these compounds to attract or deter insects and animals.

5. Objectives and expected achievements

The proposed research has the following objectives

- Following artificial inoculation of potted apple plants in the greenhouse, screen candidate materials for activity against the apple scab pathogen.(WP1)
- Where control of scab is found in the greenhouse screen, determine by means of observation under the microscope, whether the controlling effect appears to be direct (fungicidal or fungistatic) or whether it appears to involve an activation of host plant resistance mechanisms.(WP2). All materials showing activity will be considered for inclusion in further tests.
- Test the most promising anti-fungal materials under orchard conditions firstly on single trees and next, for the most promising materials, on blocks of trees. (WP3)
- For a candidate material acting by induced resistance, characterise the gene expression induced by the treatment in relation to known defence responses. (WP4)
- Investigate whether treatment with anti-fungal materials results in differences in secondary metabolite profiles (phenols and volatiles) in apple material harvested from treated orchard trees. (WP3)

6. Description of work packages including methods

Table 1: Workpackage list

WP No	WP title	Responsible participant	Budget	Start	End	Deliverable, No
1	Screening of candidate materials for scab control	JH (MVB)	1 330 100	1	30	D1.1, D1.2, D1.3, D1.4, D1.5, D1.6, D1.7, D1.8, D1.9 D1.10
2	Histopathological investigation of host-pathogen interactions	HJLJ (MVB)	336 900	5	24	D2.1 D2.2 D2.3
3	Orchard testing of selected control compounds and secondary metabolite characterisation	MB (EL, KP)	446 000	13	30	D3.1, D3.2, D3.3, D3.4
4	Molecular analysis of defence responses in apple	DBC (NN)	60 000	17	30	D4.1, D4.2, - D4.3
Total			2 173 000			

Table 2: Description of work packages

WP1: Development of scab control strategies: screening of candidate materials for scab control.

Workpackage number: **1**
Start date or starting event: **(1)April 1st 2002**
Responsible person: **JH**
Contributing persons: **MVB, technician**
Person-months: **Scientist 24, Technician 9**

Description of work:

Candidate scab control materials are to be collected. These are expected to include materials on National and EU lists that have registered effect on the scab fungus. Also other materials including plant extracts, compost tea, Effective Microorganisms, and biocontrol agents (BCAs) such as *Clonostachys rosea* and *Bacillus subtilis* will be obtained.

Reference materials will include sulphur and Cu-fungicides, Binab (BCA), and Bion (resistance inducer)

Strains of *Venturia inaequalis* are available from the collection at KVL and methods for inoculum preparation, leaf disc inoculation, plant inoculation and incubation etc are described (Benaouf & Parisi, 1998; Parisi & Lespinasse, 1996).

All screening will be carried out on apple leaves of susceptible varieties (compatibility with the selected strain of *V. inaequalis* will be checked by pathogenicity testing and microscopy –WP2). Test plants will be purchased from nurseries and grown in pots at KVL-Taastrup where suitable greenhouse and other plant-growing facilities are available.

A “workshop” area will be established where preliminary experiments to determine such matters as concentration range and inoculation methods to suit individual materials are to be determined. From this “workshop”, materials will pass on to the first screening by using an *in vitro* leaf disc assay. Candidate materials will be applied to disinfected apple leaf discs on agar before inoculation with the pathogen. Scab symptoms and sporulation will be assessed after three week’s incubation under controlled conditions using the scale of Parisi *et al.*, (1993).

In the second screening selected materials will be applied to apple seedlings before and after inoculation of the pathogen to potted apple seedlings. Inoculation and incubation methods for seedling plants has been described e.g. by Parisi & Lespinasse, 1996. Inoculated plants will be observed for development of symptoms for a period of 3-4 weeks using a grading scale for describing the class of symptoms (Chevalier *et al.*, 1991) and the percentage of leaf area with sporulating lesions.

Samples of leaf material from the second screening will be investigated in WP2 for indications of induced resistance.

Materials for this work may be investigated fresh or following storage after fixation of the leaf material (Carver *et al.*, 1991, Lyngkjær, 1995).

Leaf material will be supplied to WP4 from a treatment showing a clear induced resistance response (together with WP2).

Best-case anti-fungal materials of both categories will be tested on orchard trees (WP3).

Deliverables:

- D1.1 (month 2) Collection of materials for batch 1
- D1.2 (month 2) Growing of strains of *V. inaequalis* and establishment of inoculation procedures
- D1.3 (month 4) First screening of batch 1 of scab control materials in growth chambers. Material Supplied to WP2 for microscopy
- D1.4 (month 7) Second screening of promising materials from batch 1.
- D.1.5 (month 10) Collection of materials for batch 2

D1.6 (month 13) Supply of material to WP3 for orchard experiments (chosen mainly from batch 1)
Material supplied to WP2 for microscopy
D1.7 (month 16) First screening of batch 2 of scab control materials in greenhouse
D1.8 (month 19) Second screening of promising materials from batch 2
D1.9 (month 17-28) In co-operation with WP2, supply of leaf material to WP4 (molecular study of induced resistance)
D1.10 (month 24-28) Supply of material to WP3 for orchard experiments (chosen from batch 1 and 2)

Milestones:

M1.1 (month 2) Materials for batch 1 collected
M1.2 (month 2) Growing and inoculation procedures established
M1.3 (month 4) First screening of batch 1 completed. Material for microscopy supplied to WP2
M1.4 (month 7) Second screening of batch 1 completed
M1.5 (month 10) Materials for the second batch of screening collected
M1.6 (month 16) Supply of materials to WP3 for orchard testing completed.
M1.7 (month 16) First screening of second batch completed
M1.8 (month 19) Second screening of batch 2 completed. Material supplied to WP2 for microscopy
M1.9 (month 28) Supply of materials to WP3 for orchard trials completed
M1.10 (month 28) Materials to WP3 for orchard trials supplied
M1.11 (month 28) Supply of leaf material to WP4 completed

WP2: Histopathological investigations of host-pathogen interactions

Workpackage number:	2
Start date or starting event:	(3) June 1st 2002
Responsible person:	HJLJ
Contributing persons:	(MVB)
Person-months:	Scientist 8, (6 MVB, 2 HJLJ financed from other sources), Technician 2

Description of work:

Investigations on induced resistance aim at finding evidence that natural defence responses in plants are enhanced by application of the inducer. A natural way to start such studies is to compare the infection course of the pathogen in the host with and without previous application of the inducer. This is performed by microscopy and will clearly identify at which step in pathogen development growth is stopped. Furthermore, important defence reactions can be studied and it may be determined whether their expression is enhanced (e.g. cell wall appositions, hypersensitive reactions, callose accumulation etc.). The reference material for demonstrating induced resistance will be pathogen inoculated, non induced leaves, as described by Jørgensen *et al.*(1998).

As a first essential step, a protocol will be worked out for determining whether the inducers (control materials), found to reduce disease severity, in fact operates by induced resistance or whether antibiosis, competition and/or hyperparasitism is involved in the protection. For example, inhibition of pre-penetration growth of the pathogen indicates involvement of antibiosis/competition/hyperparasitism whereas inhibition of penetration combined with enhanced expression of defence responses indicate that induced resistance is involved. The clearing technique of Carver *et al.*, (1991) and Lyngkjær (1995) will be followed and appropriate histological staining techniques applied. In order to spread the work load it will be necessary to store some leaf samples from the two batches (WP1) for a period of time. Thus it is anticipated that much of the histological examination work will be carried out in the winter months. Leaves cleared by the method mentioned above can safely be stored for long periods of time. All promising treatments from WP1 (batches 1 &2) will be examined using the above Protocol in order to establish which type(s) of mechanism is operating in the material.

Deliverables:

- D2.1 (month 6) A protocol or a set of criteria to be used to test whether induced resistance is involved in protection offered by a control agent.
- D2.2 (month 11) Results of test of selected batch 1 material
- D2.3 (month 24) Results of test of selected batch 2 material

Milestones:

- M2.1 (month 6) A protocol for examining whether induced resistance is involved in the protection against disease is established.
- M2.2 (month 11) Test of control material in batch 1 for ability to induce resistance completed
- M2.3. (month 24) Test of material in batch 2 for ability to induce resistance is completed

WP3: Orchard testing of selected control compounds and secondary metabolite characterisation

Workpackage number:	3
Start date or starting event:	(13) April 1 st , 2003
Responsible person:	MB
Contributing persons:	EL. KP, technician
Person-months:	Scientist 5, technician 5.

Objectives:

1. Organic in-field testing of selected resistance inducing compounds and contact compounds to reduce apple scab infection in combination with varied growth conditions.
2. To characterise secondary metabolites in apples harvested from untreated apple trees and from trees treated with anti-fungal preparations.

Description of work:

DIAS, Research group for Pomology has at its disposal specific organic production areas. These areas were assigned to organic production in 1996 in order to accommodate organic trials. Apple trials at present include both an older planting established in 1994 and 2 new apple fields planted in 2001 under the FØJØ-II Research program (FØJØII-DJF-2). Activities described in the current research proposal will be incorporated into these existing trials. That will serve several purposes: Costs will be reduced significantly and appropriate mature trees will be available for in-field testing of selected materials(WP I).

In these existing trials, varied growth conditions in terms of water and nutrient supply will be created as part of the effort to develop sustainable production systems for apple. Testing resistance inducing compounds under such conditions will provide additional valuable information to both the original trial as well as the testing trial, since it is believed that tree growth and tree resistance interact.

In the first year of field testing (2003), the apple orchard established in 1994 will be used for screening. In the second year of field testing (2004), selected compounds will be tested in combination with trees grown under varied growth conditions.

Methods

The above-mentioned trials consist of a large number of 3-tree-plots and it is therefore possible to subdivide the plot without jeopardising the original trial design. Only one of the trees in each plot will be treated with the compounds by means of nap-sac sprayer and protection shield, the two other trees will serve as an untreated control both for the application trial and the original trial. At one level of tree growth apple scab control with sulphur will serve as a secondary control.

For resistance inducing compounds and contact compounds application will be made before apple scab infection periods occur (both primary and secondary infections). Most attention will be given the development of primary apple scab infections.

The PC-warning program RIMpro will be used to predict application time in the primary infection period. RIMpro is designed to predict the release of ascospores. Timing of preventive applications is thereby improved.

The following data are to be collected:

Assessment of scab infection on leaves after heavy primary scab infection periods will be carried out. Leaf scab infections in secondary infection periods will be carried out once and scab evaluation on fruits will be carried out at harvest. Detailed investigations of the development of scab will be carried out in connection with WP1.

Yield and fruit size per tree are to be recorded. Leaf samples will be taken in June and August to determine the nitrogen levels in leaves. If some of the compounds influence the vegetative growth of the tree or the skin quality of the fruits, appropriate data will be recorded.

In 2004 characteristic secondary metabolites are to be measured in selected fruits from each treatment:

1. The phenolic profile of the apples will be determined by HPLC and categorised as free and bound

compounds, comprises phenolic acids and flavonoids.

2. Volatile compounds will be measured in apples by dynamic headspace sampling followed by GC and GC-MS.

Deliverables:

- D.3.1. (month 19) Results of field screening of selected compounds 2003.
- D.3.2. (month 29) Results of field testing of selected compounds in combination with tree growth in 2004.
- D.3.3 (month 29-30) Analyses of volatiles and phenolic compounds of apples.
- D. 3.4 (month 30) An international publication in 2004/2005. (Together with WP1, 2 & 4).

Milestones:

- M.3.1 (month 22) Selections of promising compounds in 2003.
- M.3.2 (month 25) Testing of promising compounds in 2004
- M.3.3 (month 30) The analyses of secondary metabolites are completed 2004.
- M.3.4 (month 30) National publication 2004.
- M.3.5 (month 30) International publication 2004/2005.

WP4: Molecular analysis of induced defence responses in apple

Work package number: 4
Start date or starting event: (17) August 1st, 2003
Responsible person: DBC
Contributing persons: NN
Person-months: 15 (financed from other sources)

Objectives:

To characterise the gene expression induced in apples by treatment in relation to the known defence response.

Description of work:

Gene macroarrays (dot blots) will be prepared using known cDNA sequences which are available in the databases and probed using cDNA pool-probes prepared from mRNA extracted from tissues isolated from candidate treated material.

It is hoped that it will be possible to use microarray analysis in collaboration with Japanese researchers, though the possibility for doing this is unknown at present.

The available defence-related genes currently characterised from apple include PR-5 (thaumatin-like proteins), PR-8 (Class III chitinase), PR-10, and there are several members of each of these families of genes.

Several genes involved in secondary metabolism (phytoalexin biosynthesis and lignification).

A pathogen-induced polygalacturonase inhibitor protein.

Several protein kinases including a receptor-like kinase which is known to be induced by Ventura infection.

Blast analysis will be performed with the non-annotated EST sequences in the data base in order to identify further defence-related gene sequences. The Korean group will make clones available for these studies (G. An, *pers. comm.*).

Material of potential interest can be harvested at any stage during the project and stored at -80°C until use. On these basis of the results of induced resistance studies, mRNA will be prepared from selected material and used to prepare labelled probes to hybridise with the macroarrays.

We will prefer to do these analyses using microarrays, but are uncertain as to the status of these in apple at present. The situation can change rapidly in the period before this work package commences.

Deliverables:

D4.1 (month 27) Macroarray of apple genes

D.4.2. (month 27) Macroarray data for induced resistance in apple

D.4.3 (month 30) One scientific paper in collaboration with other participants and potentially Japanese and Korean collaborators.

Milestones:

M4.1 (month 30): The analyses are completed.

M4.2 (month 30): The analyses are completed.

M.4.3 (month 30) Publication completed

7. Implementation and time schedule

Table 3: Deliverables list

Deliverable, No	Deliverable title	Delivery date	Meeting	Nature
D1.1	Collection of materials for batch 1	month 2		
D1.2	Growing of strains of <i>V. inaequalis</i> and establishment of inoculation procedures	month 2		
D1.3	First screening of batch 1 of scab control materials in growth chambers. Material supplied to WP2 for microscopy	month 4		
D1.4	Second screening of promising materials from batch 1.	month 7		
D1.5	Collection of materials for batch 2	month 10		
D1.6	Supply of material to WP3 for orchard experiments (chosen mainly from batch 1)	month 13		
D1.7	First screening of batch 2 of scab control materials in greenhouse	month 16		
D1.8	Second screening of promising materials from batch 2	month 19		
D1.9	In co-operation with WP2, supply of leaf material to WP4 (molecular study of induced resistance)	month 17-28		
D1.10	Supply of material to WP3 for orchard experiments (chosen from batch 1 and 2)	month 24-28		
D2.1	A protocol or a set of criteria to be used to test whether induced resistance is involved in protection offered by a control agent.	month 6		
D2.2	Results of test of selected batch 1 material	month 11		
D2.3	Results of test of selected batch 2 material	month 24		
D.3.1.	Results of field screening of selected compounds 2003.	month 19		
D.3.2.	Results of field testing of selected compounds in combination with tree growth in 2004.	month 29		
D.3.3	Analyses of volatiles and phenolic compounds of apples.	month 29-30		
D.3.4	An international publication in 2004/2005. (Together with WP1, 2 & 4).	month 30		
D4.1	Macroarray of apple genes.	month 27		
D.4.2	Macroarray data for induced resistance in apple	month 27		
D.4.3	One scientific paper in collaboration with other participants and potentially Japanese and Korean collaborators	month 30		

Planning meetings are held in March and October in 2002, 2003 and the final meeting in September 2004

Table 4: Timetable

TITL E	Co-ordination	Quarter	2002*				2003*				2004*		
			1	2	3	4	1	2	3	4	1	2	3
1	WP1:												
M.1.1	Materials for batch 1 collected		4										
M1.2	Growing and inoculation procedures established		4										
M.1.3	First screening of batch 1 completed. Material for microscopy supplied to WP2				7								
M.1.4	Second screening of batch 1 completed					10							
M.1.5	Materials for the second batch of screening collected						1						
M.1.6	Supply of materials to WP3 for orchard testing completed								7				
M.1.7	First screening of second batch completed								7				
M.1.8	Second screening of batch 2 completed. Material supplied to WP2 for microscopy									10			
M.1.9	Supply of materials to WP3 for orchard trials completed												7
M.1.10	Materials to WP3 for orchard trials supplied												7
M.1.11	Supply of leaf material to WP4 completed												7
	WP2:												
M2.1	A protocol for examining whether induced resistance is involved in the protection against disease is established.				9								
M2.2	Test of control material in batch 1 for ability to induce resistance completed						2						
M2.3	Test of material in batch 2 for ability to induce resistance is completed										3		
	WP3												
M.3.1	Selections of promising compounds in 2003..										1		
M.3.2	Testing of promising compounds in 2004											4	
M.3.3	The analyses are of secondary metabolites are completed 2004.												9
M.3.4	National publication 2004												9
M.3.5	International publication 2004/2005												9
	WP4												
M4.1	The analyses are completed.												9
M4.2	The analyses are completed.												9
M.4.3	Publication completed												9

* If convenient, indicate the actual month (can be done by numbers: January is 1 etc.)

8. Collaborative partners

Internally the research consortium involved in this project is a strong team as it links researchers with different specialisations, coming from different departments and groups at two different institutions.

Thus, at the Danish Institute of Agricultural Science (DIAS), three groups are involved, The Research Group for Food Quality and Natural Products Chemistry, (EL), The Pomology Research Group (MB) and The Research Group for Effects of Pesticides (KP).

Participants from the agricultural university (KVL) also belong to different research groups, each with its own focus on plant-pathogen-interactions. Thus JH, who is project leader, and the post doc, MVB, both are connected to the Biological Control Group at the Department of Plant Biology, while (HJLJ) works with induced resistance and histopathology and DBC's research interests are host -pathogen interactions at the molecular and physiological levels.

All the groups in the consortium have extensive research networks both in Denmark and internationally and these will benefit the project.

There is already excellent cooperation between several of the the groups and this will be further strengthened, as well as new research constellations forged, through the project. Recent research cooperation (1997-2001) on the control of the overwintering stages of the apple scab pathogen and a disease with a similar pathosystem, cherry leaf spot (*Blumeriella jaapii*) between the Pomology Group (led by Hanne Lindhard) and the Biological Control Group (led by JH) gave many interesting results (and the PhD degree to MVB) and helped to forge the excellent personal and professional relations, on which the present research proposal is based.

Our research activities will link to other FØJOII projects especially FØJOII-Djf-2: Development of sustainable production for apples.

Concerning contact to organic fruit growers we have long-standing and excellent relations with the organic fruit consultant, Maren Korsgaard.

We anticipate that the project will give research topics for several MSc and PhD students and, concerning the future, it is under consideration to extend and supplement the present application with a proposal for funding through EU.

9. Budget

KVL	2002	2003	2004	TOTAL	
Months (scientific)*	9	12	9		
Months (technical)	4	4	3		
Salary (scientific)	286 200	400 000	314 000		
Salary (technical)	97 000	102 000	80 000		
Operation – equipment					
Operation - other	35 000	70 000	55 000		
Overhead	83 600	114 400	89 800		
Total	501 800	686 400	538 800	1 727 000	

*: Scientific months based on 32 hours/ week

The institute will cover one month salary for one scientist in year 2002, 2003 and 2004 for WP2 and for one scientist and one technician in year 2003 and 2004 for WP4.

DIAS	2002	2003	2004	TOTAL	
Months (scientific)		1	4		
Months (technical)		1	4		
Salary (scientific)		38 000	161 000		
Salary (technical)		25 400	108 000		
Operation – equipment					
Operation - other		10 000	30 000		
Overhead		14 700	59 200		