Project Title:	The Causes & Prevention of Lesions (Oedema) in Herbs
Project Number:	FV 247
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Project Commenced:	30 September 2002
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The results and conclusions in this report are based on a series of experiments conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name]
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CONTENTS

GROWER SUMMARY

1.1 PROJECT BACKGROUND

A number of salad and herb crops, including coriander, suffer from a condition that manifests itself as water-soaked lesions on the leaves. This disease is known in the herb trade as 'oedema' (US spelling edema). It appears very quickly, often emerging throughout an apparently healthy crop within twenty-four hours. Retailers will not accept any cosmetic damage to leaf crops, so this problem can make the crop unsaleable.

In herb and salad crops, it is not currently known whether oedema is:

- A physiological disorder (the favoured theory being weakness of cells under turgid conditions, related to weather conditions).
- Caused by insect damage.
- Caused by pathogen damage.

According to growers, the condition occurs when the air is humid and the ground is wet. However, work during this project has disproved this theory.

A further theory involves the disturbance in calcium transportation, similar to the condition 'blossom-end rot' of tomato, although on-going work appears to challenge this theory also.

In the literature, oedema is generally described as the over-development of cells, both in number and size, resulting in the formation of swellings or galls, often corky in texture.

Anecdotally, there appears to be two conditions termed 'oedema' in coriander. One is seen as a greying of the leaf, which can then develop into yellow-brown necrosed spots. The other is a slate-blue, water-soaked, bruised-looking patch. Both sets of symptoms, if kept in a humid environment (e.g. having been packaged for sale), cause the coriander leaves disintegrate into a black, liquid mush.

AIMS OF THE PROJECT

The aim of this project is to understand the physiology and causes of oedema, in order to allow preventative measures to be investigated; e.g. selection of less susceptible varieties, use of plant protectant products (insecticides, fungicides etc.), more appropriate management of irrigation schedules. Previously, almost no work has been done on the condition, particularly in herbs.

Two strategies have been proposed: working with growers in the field and setting up controlled experiments in the laboratory.

A network of growers has been set up who will log incidences of oedema in their crop and provide samples of diseased plants for investigation in the laboratory. Logging their anecdotal evidence is also likely to provide significant clues as to the causes of the condition. As it is thought that oedema occurs in humid conditions, weather data (primarily soil and air temperatures, wind speed and direction and relative humidity) will be collected. Many of the growers collect this data for their own use, so will also be able to provide it for the project.

In the laboratory, the symptoms of the condition will be investigated and recorded. Certain experiments, based on those mentioned in the literature and from anecdotal evidence will also be set up. Conditions can be monitored closely and factors changed with relative ease. Any significant results can then be transferred to field experiments.

SUMMARY OF CURRENT PROGRESS

1.1.1 GROWER NETWORK, RECORD SHEETS & WEATHER DATA

The network of growers has continued to expand. Some grower and meteorological data was gathered for 2003, but this was more difficult to get than anticipated. 2004 data is currently being requested, although this again is proving very difficult.

1.1.2 INVESTIGATION INTO THE ROLE OF ELEVATED HUMIDITY

Three sets of experiments have been used to investigate the effects of elevated humidity on oedema and ‘blue spot’: growing coriander plants in conditions of elevated humidity (average 83%); mimicking the root pressure, as caused by high humidity, with the use of a pressure bomb to push water along the transpiration

stream and into leaf tissue; and taking sections to be studied under the light microscope.

These experiment have concluded that conditions of elevated humidity do not automatically lead to either oedema or 'blue spot'. When sections of oedematous tissue are studied under the light microscope, it can be seen that the cells have not burst, as originally hypothesised. However, there is a definite change in the intercellular structure (see figure 1.1) when compared to healthy tissue (figure 1.2).

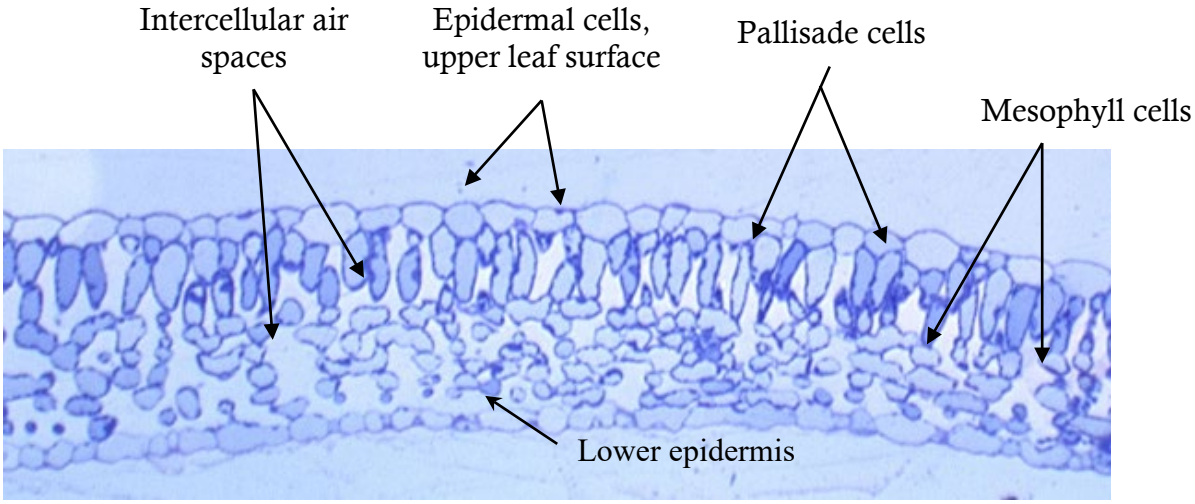

Figure 1.1: TS of a healthy coriander leaf (magnification x100) with labels of cell types.

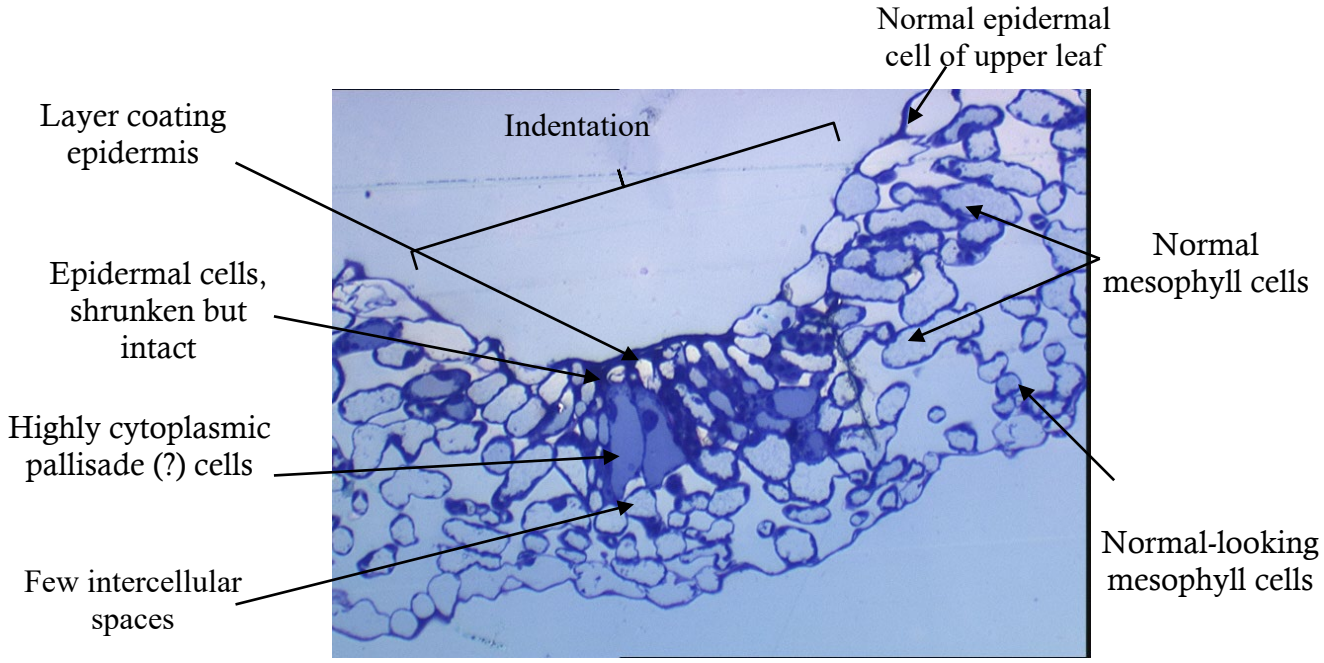

Figure 1.2: TS of indentation of oedema of coriander (magnification x100), with labels of cell types. Note: Lower epidermis came away during sectioning.

Looking at the meteorological and grower data gathered from the 2003 season, appear to support these findings, as incidences of oedema and 'blue spot' occurred at many levels of humidities. Comparison of the three seasons covered by the project will provide a more reliable picture.

1.1.3 INVESTIGATIONS INTO ION BALANCE

The hypothesis that a lack of calcium within the plant causes oedema is still under investigation. Analysis of cation composition of oedematous, 'blue spotted' and healthy tissue from the 2003 season showed that there was a significant increase in calcium concentration in the oedematous tissue when compared to the healthy samples, which obviously does not support the calcium deficiency theory.

This is supported by the findings from the hydroponics experiment, in which coriander plants were grown in a calcium-free medium. As expected, growth and development was seriously affected, with the plants being stunted and chlorotic, but no oedema or 'blue spot' developed.

1.1.4 INVESTIGATION INTO THE ROLE OF PATHOGENS

There is no literature to indicate that oedema or 'blue spot' is caused by a particular pathogen, but it is a potential cause that needs to be investigated.

The bacterium *Pseudomonas syringae* pv. *coriandricola* is known to cause various blights of coriander although most studies of *P. syringae* diseases refer to seed, rather than the leaf varieties. The symptoms reported include: necrotic lesions with some water soaking on the leaves in the early stages, petals becoming brown and falling and lesions developing on unripened fruits, before they become blackened and shrivelled.

Isolation of bacteria from inside oedematous leaf samples gave interesting results. Copious quantities of a pink-pigmented bacterium grew on the agar isolation plates. This bacterium was tentatively identified as a *Methylobacterium*, a non-pathogenic species which is present on the leaf surface of many species. Tests were carried out which, have not, as yet, conclusively proved the identity of this bacterium. More recent experiments to identify the proportion of this bacterium as a percentage of the whole bacterial load showed that it was not very common, but grew fastest on the agar plates and eclipsed other bacteria present.

None of the bacteria isolated were *Pseudomonas syringae*, thus this bacterium can be ruled out as the causal agent of oedema.

PRELIMINARY CONCLUSIONS & FUTURE WORK

These preliminary results would suggest that neither hypotheses linked to transpiration (i.e. 1) that humidity causes oedema and 2) during the condition, cells burst) - is correct.

It would also appear that a calcium deficiency is not the trigger, although further analysis of ion content will be carried out on samples from the 2004 and 2005 seasons to confirm this.

The investigations into bacterial causes are still at an early stage, but indicate that *Pseudomonas syringae* is not the cause. Re-inoculation of healthy plants with isolated bacteria is the next step in identifying any causal agent.

SCIENCE SECTION

INTRODUCTION

A major part of the study of the causes of oedema in coriander involves investigating the role of pathogens. No anecdotal evidence links a particular type of pathogen to the condition and, of course, there is no literature on the subject.

The bacterium *Pseudomonas syringae* pv. *coriandricola* has been documentsed to cause various blights of coriander (Taylor and Dudley 1980; Diederichsen 1996; Dennis and Wilson 1997; Refshauge and Nayudu 2001) Most studies of *P. syringae* diseases refer to seed coriander, rather than the leaf varieties. The symptoms reported include: necrotic lesions with some water soaking on the leaves in the early stages (Taylor and Dudley 1980), petals becoming brown and falling and lesions developing on unripened fruits, before they become blackened and shrivelled.

It has also been known for a number of years that leaf surfaces are colonised by many species of bacteria, yeast, filamentous fungi, mollicutes, actinomycetes algae and protozoa (Jacques & Morris 1995; Yang *et al*., 2001; Lindow & Brandl, 2003). These organisms grow commensally with the plant and are not pathogenic.

Strains of bacteria from the genus *Methylobacterium* are some of these leaf surface organisms. *Methylobacterium* are pink-pigmented facultative methylotrophic (PPFM) bacteria, so called as they are capable of utilising single carbon atom-containing compounds (e.g. methane, methanol and formaldehyde) as their sole energy source (Lidstrom 2004; Omer, Tombolini et al. 2004). They are often isolated from plant material, particularly leaf surfaces (Corpe and Rheem 1989) and have been associated with more than 70 plant species (Corpe 1985; Holland and Polacco 1994), although never as a plant pathogen.

Isolation is relatively easy because of the ability of the PPFM bacteria to use the single carbon compounds. The methanol mineral salts (MMS) medium (From Green 2001) is one such medium that is simple to prepare

In the absence of any evidence of fungal attack from the sections taken previously, it was decided that the presence of bacteria should be investigated, The use of particular media, gram staining, the presence of fluorescence and finally PCR can detect the presence of and identify any bacteria present. The re-inoculation of plants (following

Koch's postulates) will attempt to indicate if any of the isolated bacterial strains are the cause of either oedema or 'blue spot'.

AIMS & OBJECTIVES

- To identify the presence of bacteria, in particular *Pseudomonas syringae*, present in oedematous and 'blue spotted' coriander in comparison to healthy tissue.
- To identify any bacteria which could be causing either oedema or 'blue spot'.
- To re-inoculate healthy coriander plants with any isolated cultures in order to further investigate the link between the bacteria and the development of oedema and 'blue spot'.

METHODS

PRELIMINARY INVESTIGATION INTO THE PRESENCE OF BACTERIA IN OEDEMATOUS CORIANDER TISSUE

Oedematous and healthy coriander tissues were compared. Plates of 5% sucrose nutrient agar (SNA) and Kings B medium (KB) were used.

Surface Bacteria

Five coriander leaves were each washed in 10ml sterile distilled water for 30 seconds. The washings were then streaked out onto plates of each of the 3 media.

The plates were incubated at 28°C for 48 hours. The resulting colonies were subjected to UV analysis (254nm and 365nm).

Intercellular Bacteria

Five leaves were surface sterilised for 30 seconds in a 1% sodium hyperchlorite solution, without agitation. The leaves were then washed twice in sterile distilled water. Discs approximately 0.7cm diameter, were cut by laying the leaf over the opening of an Eppendorf tube, then closing the lid.

Each leaf disc was then macerated within the tube for 15 seconds, 500µl LB broth added, then the tissue was macerated for a further 5 seconds in order to suspend it.

1/10, 1/100 and 1/1000 dilutions were made, of which 150µl of each was spread onto plates of each agar and incubated at 28°C for 24 hours.

The colonies were subjected to UV analysis (254nm and 365nm).

QUANTIFICATION OF BACTERIA PRESENT IN OEDEMATOUS TISSUE

The method for isolating intercellular bacteria outlined in section 2.2.1.2 above, was repeated for this investigation

The colonies were counted after 24hours.

PRELIMINARY IDENTIFICATION OF THE PINK BACTERIUM ISOLATED FROM OEDEMATOUS CORIANDER

Methanol Mineral Salts (MMS) Agar

K_2HPO_4	1.20g
KH_2PO_4	0.62g
$CaCl_2.6H_20$	0.05g
$MgSO_4.7H_2O$	0.2.g
NaCl	0.10g
$FeCl_3.6H_2O$	1.0mg
$(NH_4)_2SO_4$	0.5µg
$CuSO_4.5H_20$	5.0µg
$MnSO_4.5H_2O$	10.0µg
$Na_2MoO_4.5H_2O$	10.0µg
H_3BO_3	10.0µg
$ZnSO_4.7H_2O$	70.0µg
$CoCL_2.6H_2O$	5.0µg

15g Oxoid purified agar

The mixture was adjusted to pH 7.0, autoclaved at 121°C for 20 minutes, then cooled to approximately 50°C before adding 1ml of filter-sterilised methanol.

Colonies were streaked onto the MMS plates from SNA and KB plates isolated previously and incubated at 28°C. The plates were studied after 24, 48 and 60 hours before being stored in a 4°C cold room for one week, when they were looked at once again

DIFFERENTIATION AND IDENTIFICATION OF BACTERIA ISOLATED FROM OEDEMATOUS CORIANDER TISSUE

Colonies were transferred from the SNA and KB plates isolated in the previous experiment (section 2.4) to plates of MMS agar and either SNA or KB in corresponding positions in a regular grid pattern. This enables any lack of growth on the MMS to be confirmed as being due to the medium, rather than the transfer process.

The plates were incubated at 28°C. The colonies on SNA and KB were counted after 24 hours, but those on MMS were left for 1 week before being counted.

RESULTS

PRELIMINARY INVESTIGATION INTO THE PRESENCE OF BACTERIA IN OEDEMATOUS CORIANDER TISSUE

Bacteria did develop from the oedematous tissue on both media at all dilutions, although there was little from healthy tissue. Of the three dilutions, the 1/1000 provided the clearest results, enabling single colonies to develop and to be isolated with ease.

Most notably, a pink-pigmented bacterium had grown in copious quantities and had covered many of the other bacterial colonies (see figure 1). None of the bacteria fluoresced when placed under UV light.

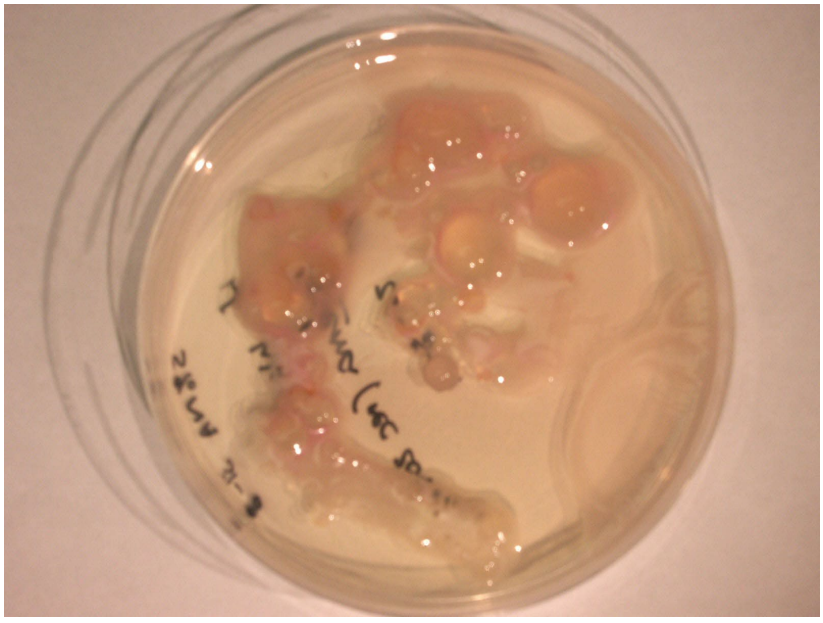

Figure 1: Copious growth of pink pigmented bacteria growing on a 90mm plate of

QUANTIFICATION OF BACTERIA PRESENT IN OEDEMATOUS TISSUE

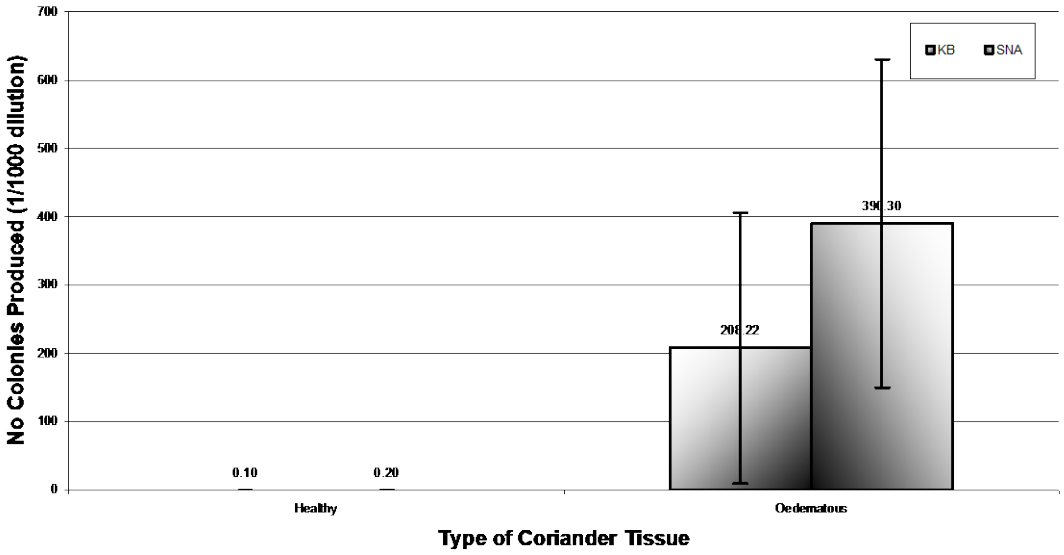

Figure 2: Number of colonies produced from oedematous & healthy tissue plated onto Kings B medium & 5% sucrose nutrient agar

($9 \leq n \leq 10$; SE $0.10 \leq p \leq 240.17$).

The healthy tissue produced many fewer colonies than the oedematous tissue (see figure 2), however, the variability between the number of colonies produced was very large, so overrode any significant difference.

PRELIMINARY IDENTIFICATION OF THE PINK BACTERIUM ISOLATED FROM OEDEMATOUS CORIANDER

Many colonies initially form on the MMS agar, but growth is arrested within 24 hours. *Methylobacterium* will continue to grow very slowly, even at 4°C, to produce dense, levoid, pink-orange colonies (see figure 3).

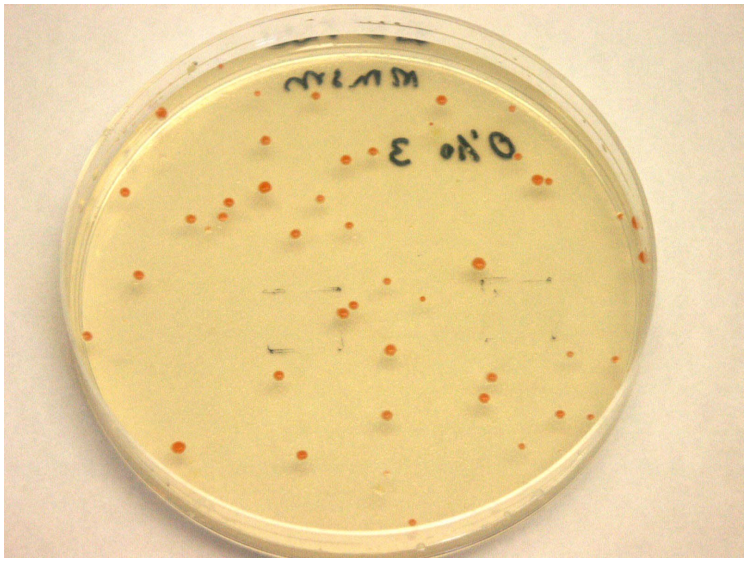

Figure 3: Pink colonies identified as *Methylobacterium* growing on methanol mineral salts medium.

DIFFERENTIATION AND IDENTIFICATION OF BACTERIA ISOLATED FROM OEDEMATOUS CORIANDER TISSUE

Five different kinds of bacteria were isolated on SNA and KB agar, from both healthy and oedematous coriander tissue:

1. Cream coloured with a slimey appearance and a denser, mounded centre with a less dense, irregular 'skirt'.
2. Cream coloured, levoid colony with a regular outline.
3. Yellow-orange, irregular colony with a slimey appearance.
4. Pink pigmented, very liquid colony, starting round before oozing over the agar surface, especially if the plate is not level.
5. Creamy-yellow colour, slightly liquid, irregular outline, but with no 'skirt'.

Between 50% and 72% of the colonies are of strain 1 in all 4 tissue/media combinations (see figure 4). It should be noted, however, that the number of colonies isolated from oedematous tissue ($n_{oed;\ SNA}$=193; $n_{oed;\ KB}$=214) was far greater than that isolated from healthy tissue ($n_{healthy;SNA}$=8; $n_{healthy;\ KB}$=6).

The pink-pigmented bacterium made up only 15-16% of the total colonies on oedematous tissue only. However, when this was transferred to the MMS medium, no growth was seen.

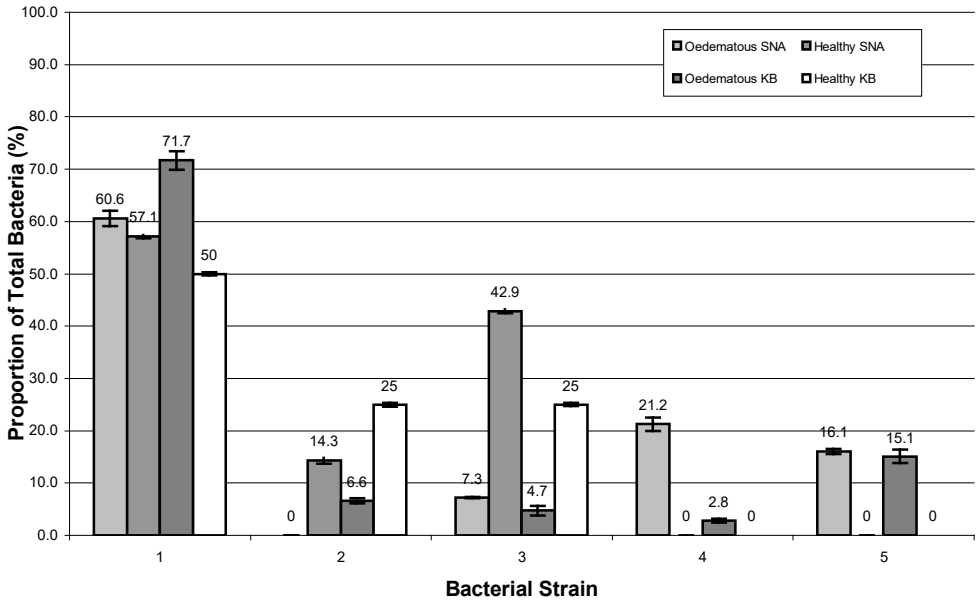

Figure 4: Proportion of each bacterial strain identified as a percentage of the total isolated ($0 \leq n \leq 152$; SE $1.77 \geq p \geq 0.12$)

DISCUSSION

The preliminary experiment indicates that bacteria are present in greater quantities in oedematous tissue than in healthy leaves. This is supported in the second experiment, although, as mentioned previously, the variability in results prevented any significance. Further repeats using more choice tissue and tissue from alternative sites will give far better results and will reduce this variability.

The use of the three dilutions in the second experiment allowed the bacterial load present within the coriander tissue to be assessed. Future experiments used a 1/1000 dilution to enable the colonies to develop separately and thus be counted and identified.

The presence of the pink bacterium was surprising and was not immediately identifiable. The quantities seen, initially suggested that this bacterium could be a causal factor in the oedema. However, further literature searches raised the possibility of it being a *Methylobacterium* (Wood, Donovan et al. 1998; Green 2001; Omer, Tombolini et al. 2004) and this bacterium is ubiquitous, but not pathogenic. The use

of the methanol mineral salts (MMS) agar appeared to be a simple means of isolating the M*ethylobacterium* and suggests that *Methylobacterium* is present in oedematous tissue. The copious growth seen on the Kings B and sucrose nutrient agar plates was not echoed on MMS agar, so can therefore be attributed to faster growth, eclipsing any other colonies.

Further quantification of the pink bacterium in relation to the general bacterial population, through 'spotting' individual colonies onto either KB or SNA and MMS agar in a corresponding grid pattern was only partially successful. On the KB and SNA plates, the pink-pigmented bacterium was clearly visible and again was not present in large quantities, but grew faster than the other four strains isolated. However, when transferred to the MMS agar, very few colonies grew and none of these were the pink-pigmented bacterium. This could of course, be due to the pink bacterium not being a M*ethylobacterium*, although this disagrees with the findings of the previous experiment, in which the pink bacterium did develop on the MMS. The small number of colonies produced reduces the likelihood of the M*ethylobacterium* being the cause of the oedema.

One of the initial objectives was to investigate the presence of *Pseudomonas syringae*. As none of the colonies were fluorescent, this bacterium was not present, therefore can be ruled out of the causes of the condition.

These experiments did not differentiate between primary and secondary infection. It is therefore possible that much of the bacteria colonising the oedematous tissue as a result of the lesions (i.e. is secondary infection).

FUTURE WORK

These experiments will be repeated using more oedematous tissue as it is sent in next season. The use of samples from alternative sites will increase the reliability of the results presented here.

To determine if any of the bacteria are responsible for the oedema, each, isolated strain will be inoculated into healthy plants, following Koch's postulates.

Tissue with 'blue spot' will also be subjected to these studies, in order to continue the comparison and contrast between the two conditions. This is particularly useful as some of this tissue comes from the same site as the oedematous tissue.

CHAPTER 4PART 3

TECHNOLOGY TRANSFER

Progress and findings of this project are regularly shared with the growers, University staff and students and other members of the herb trade who are involved with data collection.

This take the form of::

- Presentations – BHTA Annual Conference January 2004
- Written progress reports - University 21 Month Report
- Posters – University 2nd year poster presentation April 2004; BSPP Annual conference, September 2004
- Informal discussions - BHTA Technical meetings
- Newsletter articles - BHTA Newsletter, HDC Newsletter

This sharing of information will continue in the future, along similar lines.

PART 4

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4.2 PERSONAL COMMUNICATIONS

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