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THE CAUSES AND PREVENTION OF OEDEMA (LESIONS) IN HERBS

FV 247

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1.2 THE CAUSES AND PREVENTION OF OEDEMA (LESIONS) IN HERBS

Coriander: two sets of symptoms called oedema – each characterised and described. Role of meteorological conditions, ion imbalance and micro-organisms investigated.

Project Background

Coriander suffers from a condition that manifests as foliar lesions, which 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. As retailers will not accept any cosmetic damage to leaf crops, this disease can make the crop unsaleable and can cause losses in excess of £200,000 per annum (HDC, 2002).

At the onset of this project, it was not known whether oedema was caused by insects, by microbes, or was a physiological disorder. In scientific literature, oedema is generally described as the over-development of cells, both in number and size, resulting in the formation of swellings or galls, which are often corky in texture.

Aims and Strategies

The aims of this project were to characterise the physiology and to determine the causes of oedema, in order to allow preventative measures to be investigated; e.g. selection of less susceptible varieties, use of plant protectant products and more appropriate management of irrigation schedules. Previously, almost no work has been done on the condition, particularly in herbs.

Three strategies were employed: 1) gathering data and coriander samples from growers; 2) gathering meteorological data; 3) setting up controlled experiments in the laboratory.

A network of growers was set up to log incidences of oedema in their crops and to provide samples of diseased plants for investigation in the laboratory. It was hoped that logging anecdotal evidence would provide significant clues as to the causes of the condition. Of the 14 growers who contributed, 11 provided crop data, 8 gave meteorological data and 9 sent samples.

As it was thought that oedema occurred in humid conditions, weather data was collected. Many of the growers collect this data for their own use, so were able to provide it for the project.

In the laboratory, the symptoms of the condition were investigated and recorded. To look for the causes, three subject areas were studied: the effect of humidity and other environmental conditions; ion imbalance focusing on calcium; and the role of micro-organisms. These were based on scientific literature and anecdotal evidence gathered from growers: Many growers felt that oedema occurred around periods of high humidity, which resulted in leaf cells bursting. It is known that other conditions exist which are exacerbated by environmental conditions and nutrient imbalances. There is no literature of oedema being caused by micro-organisms, although some crops are known to be infected with *Pseudomonas syringae*. A link between this bacterium and the symptoms produced was examined

Summary of Project Findings

Identification of Symptoms

At the start of the project, oedema was identified as grey foliar lesions. However, during the first season, it became clear that a second set of symptoms was also being termed oedema. Neither set matched with those recorded as oedema in other plant species. For clarity, the original, grey symptoms are termed 'oedema' and the secondary blue ones, 'blue spot'.

Oedema appeared as angular grey patches of the leaf lamina, between veins, on the upper side of the leaf (figure 1a). A tan, pinprick indentation (approximately 0.8mm diameter) generally developed within this area, usually one per grey patch. Neither the patch nor the indentation appeared to increase in size once developed.

a) _____ 3cm.

b) _____ 1cm

Figure 1a: Coriander exhibiting symptoms of oedema. The indentations are faintly visible within each grey lesion (indicated by arrows).

Figure 1b: Coriander leaves exhibiting slate-blue blue spot symptoms (indicated with arrows).

Blue spot lesions were irregular, slate-blue patches on the upper leaf lamina, spanning the veins (figure 1b). Once developed, the blemishes did not expand and the leaves were not usually covered completely.

Under the light and scanning electron microscopes, further differences became obvious, particularly between oedematous and healthy tissues. In oedema, the cells did not burst, as originally hypothesised, although the cellular structure is seriously disrupted. Instead of the regular layout of cell types, the cells appear to slump on top of each other, loosing their ordered positioning, resulting in the indentation. Under the light microscope, a layer coating the bottom of the indentation was also seen, which was thought to be leakage of cell contents. No differences are apparent in the structure of the grey area when compared to healthy tissue.

Cellular differences between tissue with blue spot and healthy samples was minor. The predominant distinction was the increased visibility of chloroplasts under the light microscope. In addition, the cuticle of tissue with blue spot was also approximately double the thickness of that in healthy leaves. The reasons for this were not determined.

Investigation into the Effect of Humidity

Four sets of experiments were used to investigate the effects of humidity on oedema and blue spot: 1) growing coriander plants in conditions of elevated humidity (average 83%); 2) subjecting coriander plants to a step change in humidity (63% and 83%); 3) subjecting coriander plants to a step change in temperature (10ºC and 20ºC) under two levels of humidity (67% and 86%); 4) increasing the internal water pressure of the leaf, as caused by high humidity, by mimicking the root pressure with the use of a pressure chamber to push water along the transpiration stream and into leaf tissue.

Only one plant of the hundreds used in the *in vivo* experiments developed oedema and none blue spot. Increasing the internal pressure directly with the pressure chamber did not result in symptoms of either kind. It can therefore be concluded that changing

environmental conditions to result in elevated humidity does not automatically lead to either oedema or blue spot.

Analysis of Meteorological Data

Correlating the occurrence of symptoms in the field with local meteorological conditions was a vital part of this project, presenting a complementary approach to the laboratory experiments into the relationship of humidity to oedema and blue spot. Crop data was supplied by a number of growers and included such information as sowing and harvesting dates, soil type and pH and the amount, if any, of the crop affected by either condition. Meteorological data (soil and air temperatures, wind speed and relative humidity (RH)) was also collected, either by the growers themselves or other organisations situated close by.

Over the three seasons (March-October 2003, 2004, 2005), 15 outbreaks of oedema were reported over 4 sites, 17 of blue spot from 5 sites, and 235 healthy all 11. At no sites did both conditions occur at the same time. Oedema outbreaks decreased over the study period (2003: 13; 2004: 5; 2005: 0), although this cannot be concluded of blue spot, as outbreaks were only logged from the middle of the first season (2003: 3; 2004: 9; 2005: 5).

Half of all outbreaks occurred in June, with 16% of these being oedema and 34% blue spot. Both conditions declined over the summer months, although oedema does re-appear in December (6%) on experimental (rather than commercial) crops. The trend in severity of symptoms paralleled that of frequency for both conditions; i.e. when there are most outbreaks, they are most severe.

Oedema occurred at most stages of growth, from seedlings (fewer than 2 true leaves) to harvest-stage plants and those approaching flowering. Blue spot, in contrast, was were not seen in plants smaller than 8cm and over half of all outbreaks developed at harvest stage (10-12cm). The time of year the plants reached these stages had no effect on the conditions.

Of the records of soil pH received, all ranged from 5.8 to 7.2, regardless of whether any condition was seen. Weak patterns emerged when soil type was linked to outbreak frequency. 92% instances of blue spot occurred in sandy-clay-loam, with the rest being on

medium-heavy loam. Oedema occurred on a range of soils, although 57% were on heavier soils. The other 43% emerged on the lighter sandy loam.

When the meteorological variables (average minimum air temperature, average daily average RH etc.) were plotted against whether disease was seen or not, the results were very interesting. There was **no** range of any variable at which **all** crops suffered from either oedema or blue spot. Instead, we can say that at certain values (figure 3), it is *more likely* that the conditions will occur, although the actual probability could not be determined and neither could the interaction of the meteorological variables.

Meteorological Variable	Range seen in Oedema	Range Seen in Blue Spot
Air temperature	**2.1ºC to 21.9ºC**	**7.0ºC to 20.8ºC**
Soil temperature	**9.4ºC to 18.2 ºC**	**14.3ºC to 17.7ºC**
Relative Humidity	**72.7% to 80.4%**	**72% to 80%**

Figure 2: Range of each meteorological variable at which oedema and blue spot are *more likely* to occur.

This would indicate that another variable, termed an 'x-factor' is the trigger. It is this x-factor which is under closer influence of the meteorological conditions and will trigger the symptoms, subject to the weather conditions being within the critical range. The x-factor may be a micro-organism, as most bacteria and fungi prefer damp conditions for infection and most have a specific temperature range in which they are viable.

Investigations into Ion Balance

The hypothesis that a calcium imbalance within the plant causes oedema and/or blue spot would tie in with the proposal of an x-factor, closely related to weather conditions, as ion absorption and transport is dependant on water uptake and flow. Coriander plants were grown in hydroponic solutions containing a range of calcium concentrations Ion chromatography analysis of cation composition of oedematous, blue spotted and healthy tissue was also carried out.

Neither eliminating calcium from the growing medium, nor varying its concentration triggered oedema or blue spot, although symptoms of deficiency were induced.

Analysis of ion content did not show any significantly different levels of calcium in either blue spot or oedematous tissue when compared to healthy. No conclusions can be drawn

on the relationship between the concentration of other ions and oedema as the results were not clear, however, an increase in potassium levels in tissue with blue spot was revealed, although it is not known whether this increase caused the blue spot, or the blue spot caused the increase.

The Role of Micro-Organisms

There is no literature to indicate that oedema or blue spot is caused by a specific pathogen, but it is a potential cause that needs to be investigated, especially as a potential candidate for the x-factor revealed earlier. The microscopic analysis did not expose the presence of a fungus, therefore these experiments concentrated on bacteria, in particular the bacterium *Pseudomonas syringae*. *P. syringae* pv. *coriandricola* is known to cause various blights of coriander and some growers involved in the project have crops infected with this micro-organism.

Bacterial colonies were isolated from inside leaf tissue, were quantified and then identified using DNA sequencing. The colonies were then used to infect healthy coriander plants, in an attempt to trigger either oedema or blue spot.

The quantification experiments showed that, as expected, there were significantly more bacteria in tissue with oedema and blue spot than in healthy samples, but it could not be determined if this was primary infection (i.e. that which caused the initial disease) or secondary infection (i.e. other bacteria taking advantage of wounded tissue).

DNA sequencing revealed four different genera of bacteria in oedematous tissue, including *Pseudomonas*, *Pantoea*, *Delftia* and *Erwinia*. These genera were also found in tissue with blue spot, along with *Bacillus* and *Acidovorax*. All of these bacterial genera comprise pathogenic species, however most bacteria have specific plant host ranges. Whether coriander is included in this host range was tested by infecting healthy coriander plants with purified cultures. Although not all colonies were tested due to time constraints, those that were, did not result in either oedema or blue spot. In some cases, the plant's resistance mechanism, known as hypersensitive response, was induced, blocking the growth of the pathogen by killing its own infected cells.

Conclusions

Overall, it can be concluded that oedema and blue spot are separate conditions and should be classified as such in the scientific press. It does not appear that a calcium imbalance is responsible for either oedema or blue spot and, although significantly higher ion concentrations were found in blue spotted tissue, it is not clear whether this is responsible for the condition, or *vive versa*. In addition, neither condition is due solely to a particular meteorological condition. Instead, it seems more likely that the presence of an additional variable (the x-factor), under close influence of its environment, is the cause of the symptoms. This x-factor has many characteristics of a micro-organism and, although none were identified, the growers' theory that the symptoms my have been produced by the bacteria *Pseudomonas syringae* was not upheld.

Action Points

This project has not revealed any findings that indicate a change of practice would be beneficial.

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References

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INTRODUCTION

PROJECT BACKGROUND

Over the last decade, the increased popularity of Asian cuisine and the encouragement of many celebrity chefs has lead to the steady rise in demand for fresh-cut herbs. In 2004, the total UK herb market was approximated at £160 million, producing 50,000 tonnes fresh leaves in an area of 7800 hectares (Marriott, 2005).

Of this, UK-produced coriander (*Coriandrum sativum*) now exceeds 2000 tonnes per year. Three quarters of this is grown in the UK as a field crop, in poly-tunnels or under glass and has a farmgate value in excess of £1.8 million (HDC, 2002).

A number of salad and herb crops, including coriander, suffer from a condition that manifests itself as foliar lesions. 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 disease can make the crop unsaleable and can cause losses in excess of £200,000 per annum (HDC, 2002).

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

- A physiological disorder.
- Caused by insect damage.
- Caused by pathogen damage.

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, which are often corky in texture. These are also known as intumescences.

In talking to growers over the duration of the project, it has emerged that, in coriander, there are two sets of symptoms being termed oedema. One appears as an angular greying of the leaf in which a tan-coloured, pin-prick indentation forms, the grey area then turns yellow-brown and dies off. The other develops as irregular slate-blue patches on the leaves, with no indentation appearing. In both cases, if tissue is kept in a humid

environment (e.g. having been packaged for sale), the coriander leaves disintegrate into a black, liquid mush.

There appears to be little or no literature on the condition 'oedema' in herbs. There have been some studies carried out on oedema of other plant species, but their descriptions do not seem to match that of the condition in coriander, as described by growers. These studies are described later in the chapter.

Having spoken to growers, a number of theories as to the cause of oedema have been put forward. They feel that the condition occurs when the air is humid and the ground is wet. This is consistent with the theory that lack of transpiration, but continuing root water pressure causes cells to burst. However, as will be discussed later, this is not supported by the scientific evidence of mechanism of water transport in plants.

A further theory involves the disturbance in calcium transportation, similar to the condition 'blossom-end rot' of tomato.

Although a pathological origin to oedema has been investigated by horticultural consultants, no responsible micro-organism has been found. There is some anecdotal evidence that the bacterium *Pseudomonas syringae* is present in commercial coriander crops and is controlled through the use of seed dressings. It is therefore possible that oedema is, in fact, previously unrecorded symptoms of this infection.

TAXONOMY & PHYSIOLOGY OF CORIANDER

Taxonomy

Coriander is an umbelliferous plant, of the subfamily *Apioideae* (Diederichsen, 1996) and tribe *Coriandreae*. Within this tribe only 8 genera (21 species) are found, including that of *Coriandrum*, into which the cultivated species *Coriandrum sativum* L..

Physiology

The base temperature for emergence of coriander is between 4°C and 6°C (Luk'janov and Reznikov, 1976, cited in Diederichsen, 1996), although this is slow. At temperatures of 15°-17°C, emergence will occur approximately 2 weeks after sowing, which is optimal for crop production.

During the vegetative stage in particular, coriander requires a continuous supply of water (Diederichsen, 1996). Once the flower stem has been produced, the plant becomes far more drought tolerant, although high humidity and rainfall during the period of seed maturity can also reduce seed yield.

Although some authors state that coriander is a long day plant (Nawata *et al.*, 1995), this is disputed (Diederichsen, 1996). Nawata and co-authors found that on much of the Indian subcontinent, the warm and humid winter (rainy season) is shorter than the hot, dry summer (dry season). This does not allow for enough vegetative growth to have developed and accumulated adequate resources for the provision of a good seed crop in general coriander varieties. To produce seed, local Indian cultivars have developed short critical day length requirements for flowering.

Although its climate is similar to that of India and Bangladesh, Thailand is situated at a lower latitude than India, thus the day length, even in the wet season, is longer. Even the Indian cultivars did not flourish, as flowering occurred before the formation of adequate vegetative growth and seed yield was poor. Nawata's group felt that there was a subtle difference between critical day length requirement of both the Indian and Thai local varieties.

In contrast, trials at Gatersleben in Germany (Diederichsen, 1996) appear to show that the plant is not sensitive to day length. Diederichsen also states that, as coriander can be grown in some parts of India at any time of the year and in others there are two growing seasons, this shows that the species has no, or only a slight photosensitive adaptation to long days. This author states that flowering is triggered after the accumulation of 1700-1800 day degrees (d°) during the vegetative period (Luk'janov and Reznikov, 1976; cited in Diederichsen, 1996) although light appears to also exert an influence, as the vegetative period is always longer when the plants are grown under glass.

Nawata's 1995 study states that heat inhibits all stages of growth, from the leaf production to flower bud formation, fertilisation and fruiting (Nawata *et al.*, 1995). It could be that it is not heat *per se* which inhibits development, but the speed of day degree accumulation. If the environment is hot, the day degree requirement is quickly attained, not allowing a

great deal of time for the plant to amass adequate nutrients to support successful flowering and seed production.

Lifecycle Physiognomy and Physiology

Cultivated coriander is an annual plant with two main stages in its growth cycle. It grows in a rosette, directly from the base of the plant. These leaves form an alternate phyllotactic pattern.

Germination

Germination is epigaeal, with the shoot producing two cotyledons, which are long and narrow.

Vegetative Stage

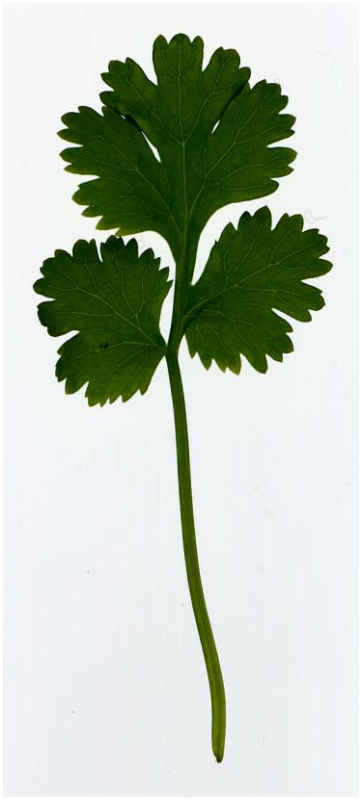
The vegetative (leaf) phase, as explained previously, grows best under conditions which allow a long period of assimilation of nutrients in preparation for seed production. The leaves are bright green, turning reddish or purple and starting to wither with the onset of flowering.

The first two true leaves are simple in structure; quite rounded and undivided but with three lobes, with a softly, but irregularly serrated edge (Figure 1a).

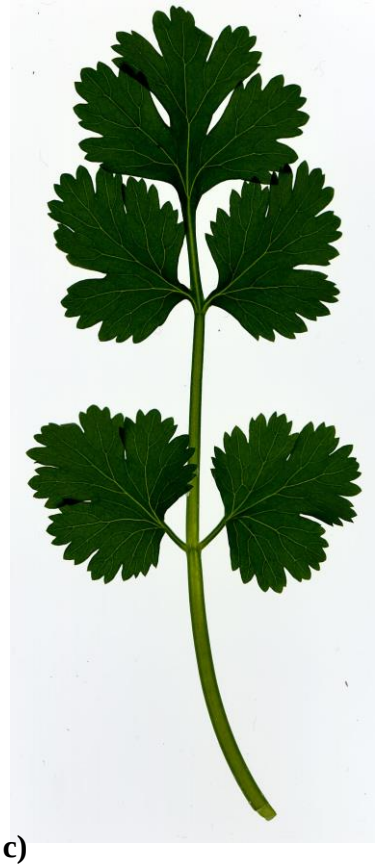
Later leaves are pinnatifid, in that they are made up of a number of leaflets emerging from different points down the length of each petiole.

The third, fourth and fifth leaves are tri-pinnatifid, i.e. they have three leaflets on each petiole (Figure 1b). The terminal leaflet is of a similar shape to the first two leaves. The two lateral leaves are positioned opposite each other, at the base of the terminal leaf. They are also rounded, with an irregularly serrated edge, but are less lobed than the terminal leaf. Sometimes three parts can be seen, other times two uneven parts and at others the leaves are one complete piece.

a)



b)



c)

d)

e)

Figure 1: Physiognomy of coriander at different stages of its lifecycle.
a) first two true leaves; b) third to fifth leaves of vegetative stage; c) further leaves in vegetative stage; d) leaves on the coriander flower stem; e) umbels

As the plant develops further, leaves become quin-pinnatifid, producing five leaflets per petiole (Figure 1c). The terminal and first pair of lateral leaves are similar to that of the third, fourth and fifth leaves, then two further leaves develop further down the petiole. These two leaflets, again situated directly opposite each other, are much like the first leaves, in that they are rounded, have irregularly serrated edges and are only partially lobed.

Inflorescence Stage

Once the 'bolting' phase of flowering is initiated a central stem is extended. Depending on the variety, the flower stem can reach heights between 0.2 and 1.4 metres (Diederichsen, 1996). The leaves, although still alternately arranged on the stem, are more deeply incised and far more feathery in nature (Figure 1d).

The umbels, the style of flower arrangement after which the family is named, are compound and produced on the tip of the stem. Each umbel has between two and eight primary rays, each of a different length, allowing each umbellet (group of flowers on a bracteol or flower petiole) to be located at the same level (Figure 1e).

The globular or oval-shaped fruit of the coriander plant is actually a capsule, which contains up to four seeds, although two are more usual in cultivated varieties.

There are schizogenic channels in all parts of the plant , which contain essential oils and give the plant its characteristic smell (Diederichsen, 1996). The oils contain a variety of compounds, which change as the plant matures. The green plant contains aldehydes, which give the 'bug' smell after which a number of cultures name the plant. During ripening, the aldehydic components disappear from the fruits and in the vittae, linalool predominates, counting for 67.7% of the total essential oils in the ripe fruits (Diederichsen, 1996).

ORIGINS & USES OF CORIANDER

Etymology

The name 'coriander' is derived from the ancient Greek 'koros' meaning 'bug', (Grieve, 1931) in reference to the smell of the crushed leaves and unripe fruits! The word was then adapted by the Latin language to '*coriandrum*', from whence the modern Western

name originates (Katzer, 1998). However, Spanish-based languages know the plant as 'cilantro', which the above source considers to derive from the Medieval Latin '*celiandrum*'.

History of Use

Coriander has been grown around the world for many centuries and both the aromatic leaves and the seeds have been used in medicine and cookery in many diverse cultures. The oldest seeds discovered date to 6000 BC and were found in the Nahal Hemar cave in Israel (Diederichsen, 1996). Ancient Egyptian notes on coriander date back to the 5th dynasty (2500 BC) and coriander fruits were found in the tomb of Tutankhamun. References are found in Sanskrit writings dating from approximately 1500 BC. It was noted that in China, coriander is mentioned in a 5th century book of vegetables (Li, 1969, cited in Diederichsen, 1996). Classical Greek and Latin authors such as Hippocrates and Pliny, wrote about coriander (Diederichsen, 1996). Hippocrates and other Greek physicians used the plant and wrote about it in their herbals and Pliny tells us that the best coriander came from Egypt, where it was cultivated (Grieve, 1931). Nicholas Culpeper's Herbal (Culpeper, 1653 (reprinted 1995)) gives coriander seed as being helpful for wind. However, Culpeper notes that it is 'hurtful to the head, sending unwholesome vapours to the brain' and also mentioned that it was dangerous for mad people!

The essential oils contained in the fruits can be steam distilled and used as a flavouring for the liqueurs Benedictine and Chartreuse (Davis, 1997) and, more recently, in the cocoa and chocolate industries. The essential oils can also be found in soaps and perfumes (Diederichsen, 1996).

The cultivated varieties have been bred for either their leaf characteristics or the essential oil component of their seeds.

Nawata's study (1995) indicates that the climate (particularly day length) have resulted in the traditional uses seen. For example, in Bangladesh and India, both the dried seed and fresh leaves are commonly used and are of equal importance, while the root is not used at all. In these regions, seeds of the local cultivar are generally sown at the end of the dry season (November to December). The young plants are thinned six to eight weeks later, and sold as the leaf herb. The remaining plants are left to flower and set

seed, which is then harvested a further four to eight weeks later (January to March). The dried fruits can be kept for use or for sowing the following year. Hence coriander seed is available most of the year, yet the leaves are only available for a short time.

In Thailand however, the leaves and roots are used most often, while the use of the seeds is rarer. The roots are also frequently used in Chinese cookery. Diederichsen (1996) agrees that the climate doesn't support seed production, concluding that this is due to humidity adversely affecting seed production and maturation.

Nawata's studies also found that that Thai local varieties cannot satisfy this domestic demand for coriander leaves, so Thailand imports large quantities of seed from abroad.

Dissemination Pathway

The exact origin of coriander is unclear and no certain information of the wild species appears to exist to aid the investigation (Diederichsen, 1996). This author states that, even though several authors, as far back as 1780 (Raspe, 1780, translated by C. von Linnaeus) reported that coriander appeared as a weed in areas of Europe and southern Russia, investigation of available 'wild' plants has revealed them to be usual cultivated forms of *C. sativum* , which had escaped captivity. Yet the behavioural characteristics which allow this to happen, e.g. the ripe fruit shattering when the umbel is completely ripe, are common to weeds and support the hypothesis that coriander is a secondary cultivated plant, originating as a weed (Diederichsen, 1996).

A number of authors have hazarded theories as to its background. Nawata and co-authors (Nawata *et al.*, 1995) consider the plant to have originated around the Mediterranean, moving outwards to Europe, Russia, China and the Indian subcontinent with domestication. Indeed these authors found ancient Chinese literature to show that coriander came to China, via the silk road, from the Mediterranean (Nawata, 1992; cited in Nawata *et al.*, 1995). A Russian study (Ivanova and Stoletova, 1990) mentions that the Persian name for coriander was used in China, indicating that the plant was introduced from this area. This supports the evidence of the silk road as the entrance route.

When the names given to the plant in other countries are compared, it can be seen that they are similar, e.g. Kishnish (Turkey), geshnes (Persian), kishnets (Russian), gishniiz

(Pahlawi) (Diederichsen, 1996; Katzer, 1998). This also implies that introduction followed a certain pathway, in this case to Russia from the Caucasus or even the areas east of the Caspian Sea (Diederichsen, 1996).

Nawata *et al.* (1995) studied the dissemination pathway of coriander in Asia by looking at the morphological and physiological characteristics of local varieties of coriander from China, India, Bangladesh and Thailand

In Bangladesh, a big and ellipsoidal morphology was found, while in India, two morphologies were found: big and ellipsoidal and small and globular. The authors felt that the former type was from local cultivars, whilst the latter was recently bred for essential oil production.

Three morphologically separate seed types were found in Thailand: small and globular (considered to be real local Thai varieties); big and globular (introduced from abroad, but used commonly for fresh leaf production in central Thailand) and big and oval (only found locally in the northern regions, near the border with Myanmar). In China, local cultivars appeared to have small and globular-shaped seeds.

The authors concluded that the Myanmar (big and oval) cultivars originate from India, whilst the Thai local varieties (small and globular) do not, despite the geographical proximity of India and Thailand. Instead, the Thai cultivars appear to be closely related to Chinese coriander (small and globular-shaped seeds), possibly brought from southern regions of China.

Most European cultivars are known to have a similar shape to the Thai and Chinese varieties. This would fit with other sources who believe that coriander was brought, by the Romans, from the East along the spice routes (Grieve, 1931; Spice Advice: Coriander, 2003).

STUDIES OF OEDEMA IN NON-HERB SPECIES

Effect of light

Certain authors (Lang and Tibbitts, 1983; in Morrow and Tibbitts, 1988) found that, in tomatoes (*Lycopersicon hirsutum*) 'water congestion did not induce neo-plastic growth, but makes tumour development more pronounced' (presumably water congestion refers to an

increase in water pressure in the plant). A further study (Morrow and Tibbitts, 1988) confirmed that red light initiated intumescences on tomato leaves, while far-red light inhibited them. Phytochrome was indicated as a primary regulator of this response, although further investigation was needed.

Investigations into oedema of ivy-leaf geranium (*Pelargonium peltatum*) (Rangarajan and Tibbits, 1994) were based on the above findings in tomato. The study wished to establish whether treating the greenhouse-grown plants with far-red radiation would protect from oedema. It was found that no treatment reduced the development of oedematous galls by more than 50%, unlike tomatoes, where gall formation was inhibited. This led the authors to conclude that oedema injury of geraniums has different causal factors to that of tomatoes and treating commercially grown geranium plants with far red light would not be beneficial.

Pest Damage

The Horticultural Development Council project into oedema of cabbages (Ellis and Kazantzidou, 1993) concludes that the corky lesions or calluses seen on cabbages are a reaction by the plant to thrip damage. This is in contradiction to the findings of Sherf and Macnab that oedema in cabbages is again correlated with low transpiration in waterlogged conditions (Sherf and Macnab, 1986).

ALTERNATIVE THEORIES

Humidity

Background Physiology of Water Transport

For water to be absorbed effectively, the root surface must be in close contact with the soil. The apical region of the root absorbs water most readily for a number of reasons: it has the greatest surface area as root hairs are found in this region; the exo- or hypodermis, thought to be impermeable to water, have not yet been laid down in this region.

There are three mechanisms by which water (and nutrients) move across plant tissue (Toole and Toole, 1991; Taiz and Zeiger, 1998) (see Figure 2a):

- The apoplastic pathway,
- The symplastic pathway,
- The transmembrane (Taiz and Zeiger, 1998) or vacuolar (Toole and Toole, 1991) pathway.

Water moving along the apoplastic pathway travels through the cell walls (the continuum of cell walls and intercellular air spaces being known as the apoplast (Taiz and Zeiger, 1998)). It is not generally necessary for the water to cross membranes. However, in the root, this pathway is blocked by a layer of cells in the endodermis, whose cell walls are suberised. This barrier, known as the Casparian strip, (see Figure 2b) forces water to enter the protoplast of the endodermal cell, to continue its route along the symplastic pathway.

The symplastic pathway involves water moving through the cell, but unlike the transmembrane or vacuolar pathway, it remains in the cytoplasm. This is effected by the cytoplasm of one cell being connected to its neighbour by strands of cytoplasm, known as plasmodesmata, eliminating the need for water to pass through membranes. This network is known as the symplast, hence the name of the pathway. A water potential gradient exists between the xylem and the sub-stomatal air spaces, drawing water along it.

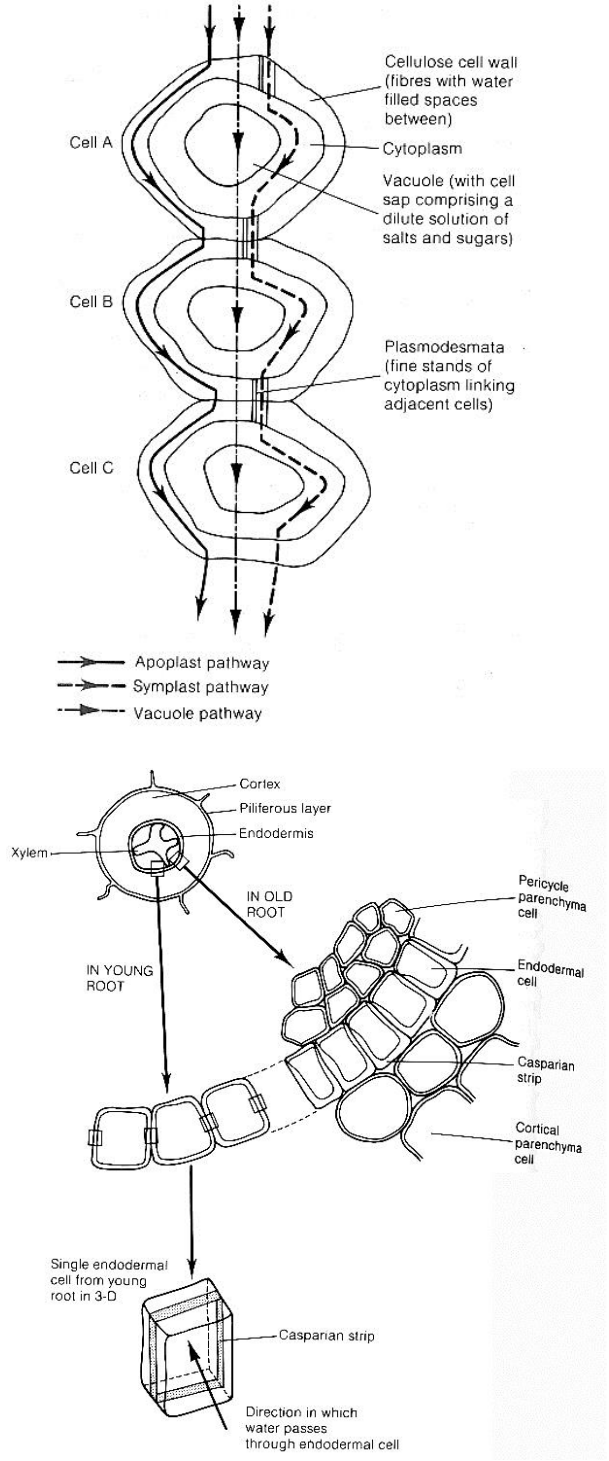


Figure 2: Routes for water transport across cells.

a) Comparison of alternative routes (Toole and Toole, 1991). The apoplastic route is thought to be the most important, although the choice of pathway may depend on the driving force through the transpiration stream.

b) Water transport across the root, showing the Casparian strip (Toole and Toole, 1991)

The transmembrane (or vacuolar) pathway describes the route taken when water passes through each cell via the cell wall, crossing the plasma membrane, cytoplasm and tonoplast and entering the vacuole, before repeating the route in reverse to exit. Again the existence of a water potential gradient between the xylem and the sub-stomatal air spaces causes the water to move along this somewhat tortuous pathway.

The relative importance of each pathway has not yet being clearly established (Taiz and Zeiger, 1998), but, due to the volumes of water carried, it is felt that the apoplastic route is the most important, followed by the symplastic and transmembrane (vacuolar) pathways respectively (Toole and Toole, 1991). It may be that the choice of pathway depends on the driving force; the apoplastic route may be favoured when plants are transpiring and water movement is fastest, whereas the symplastic or vacuolar routes may operate under osmotic driving forces.

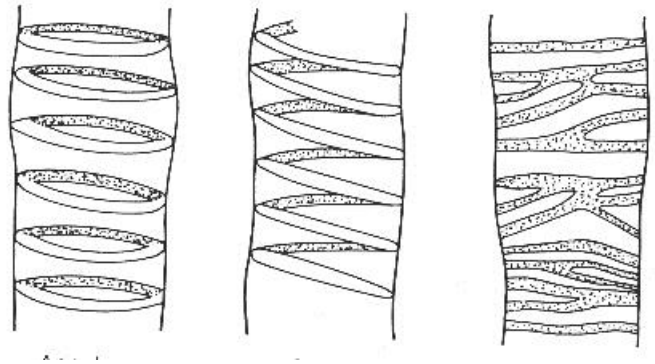
In the majority of plants, water is transported up the stem via a system of vessels know as the xylem. Compared to transport across the tissue outlined above, movement in the xylem is simple and of low resistance.

Xylem vessels are made up of two types of specialised cells or tracheary elements, called tracheids and vessel elements. Vessel elements are found only in angiosperms and a small group of gymnosperms known as Gnetales (Taiz and Zeiger, 1998), while tracheids are present in both angiosperms and gymnosperms. Both types of tracheary elements are dead and have no organelles or membranes, but have lignified secondary walls.

Tracheids are long and spindle-shaped and are arranged in overlapping, vertical files (Taiz and Zeiger, 1998). Communication and lateral water transport occurs through permeable 'pits', small areas in the lateral cell walls, which have no secondary thickening and a thin and porous primary wall. The pits of one tracheid generally line up with pits in an adjacent one, forming 'pit pairs'.

Vessel elements are shorter and broader than tracheids. There are varying degrees of lignification, which differentiates vessels. Lignin deposition in the protoxylem occurs in rings (annular) or spirals (see Figures 3a and b), to allow for further cell expansion (Toole and Toole, 1991).

In the metaxylem, lignification is more extensive and can be reticulate (see Figure 3a), sclariform (ladder-like formation of reticulate thickening) and/or pitted. The vessels are stacked end to end and their adjoining walls (cross walls) break down, resulting in perforation plates. These plates can be 'compound' if some wall remains, or 'simple' if it is fully removed (see Figure 4). These open cross walls result in a long tube throughout the length of the plant, providing a very efficient, low-resistance pathway for the flow of water, in response to a pressure gradient (either positive or negative).



Annular **Spiral** **Reticulate**

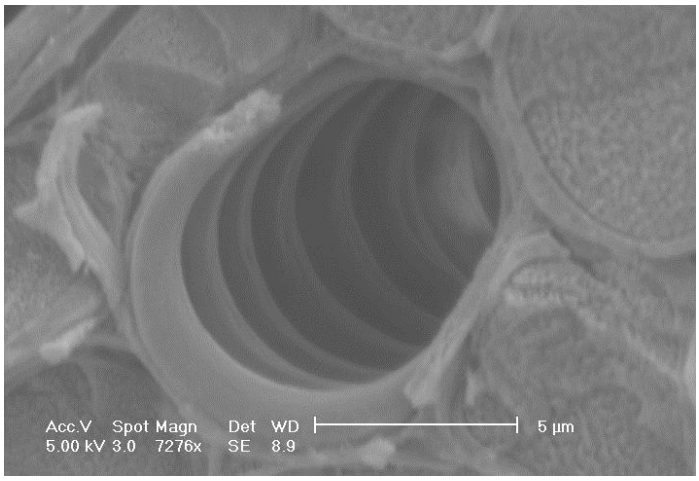


Figure 3: Forms of lignification of the xylem.

a) The 3 types of lignigfication annular: spiral and reticulate (Toole and Toole, 1991).

b) Electron micrograph of a xylem vessel of a coriander leaf, showing spiral lignification.

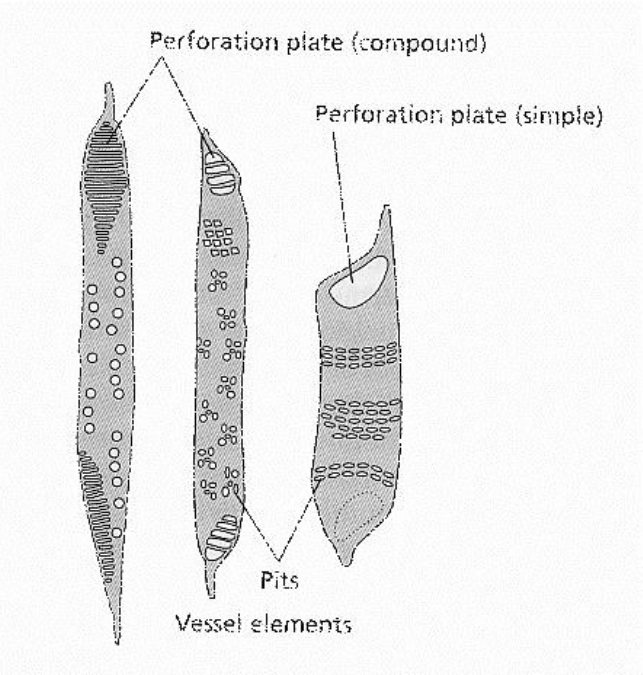


Figure 4: Vessel elements of the xylem showing various perforation plates (Taiz and Zeiger, 1998)

The primary method of moving water up the xylem is through the creation of a negative hydrostatic pressure or tension in the leaves, pulling water up the xylem vessels. This mechanism is known as 'the cohesion-tension theory of sap ascent', because it requires the cohesive properties of water to support the large tensions produced in the columns of water (Taiz and Zeiger, 1998). A number of studies have disputed the veracity of this theory (e.g. Zimmerman, 1983, in Taiz and Zeiger, 1998), questioning whether the water columns can sustain such high tensions necessary to raise water the required distance. However, in response to these questions, further studies have concluded that it is possible (Holbrook *et al.*, 1995; Pockman *et al.*, 1995; both cited in Taiz and Zeiger, 1998) and believe that the contradictions were due to technical limitations.

The negative pressure mentioned above is caused by evaporation of water from the leaf surface (transpiration), which, in turn, draws more water up the plant and across the leaf. Water, which has travelled across the leaf via the pathways mentioned previously, reaches the outer surfaces of the mesophyll cells. An extensive system of intercellular spaces surrounds these loosely packed cells, giving them direct contact with the atmosphere. Water vapour in the intercellular spaces diffuses out of the leaf through pores

known as stomata (see figure 5), through a 'boundary layer' of air then into the open, moving air. This diffusion, thus evaporation, is controlled by a concentration gradient of water vapour (vapour pressure gradient), which arises when the water vapour in the air surrounding the leaf is less than at the leaf surface (Larcher, 1995) and is affected by air and leaf temperature, air humidity and wind speed.

The higher the leaf temperature, the more water will evaporate. The higher the air humidity, the shallower the vapour pressure gradient is and less water can diffuse along it. Wind speed affects transpiration by affecting the depth of the boundary layer. This film of still air surrounds the surface of the leaf and quickly becomes vapour-saturated. Its resistance to water vapour diffusion is directly proportional to its thickness (Taiz and Zeiger, 1998). In calm air, the boundary layer resistance for large leaves such as banana, is three times that of small incised, or needle-shaped leaves (Larcher, 1995). As wind speed increases, the boundary layer is reduced and these differences are levelled out.

In addition, plants can push water up the xylem vessels by positive hydrostatic-, or root pressure. This pressure is caused by the transport of absorbed ions up the xylem, leading to a build-up of solutes, a decrease in the osmotic potential (ψ_s) and thus a decrease in xylem water potential (ψ_w). This then provides a driving force for water absorption through the roots, even without the pull from the transpiration stream. Root pressure is most prominent in well-hydrated plants under high humidity, when there is little transpiration (Taiz and Zeiger, 1998). Under drier conditions, transpiration rates are high so the turnover of water within the plant is great enough to prevent a positive pressure from developing.

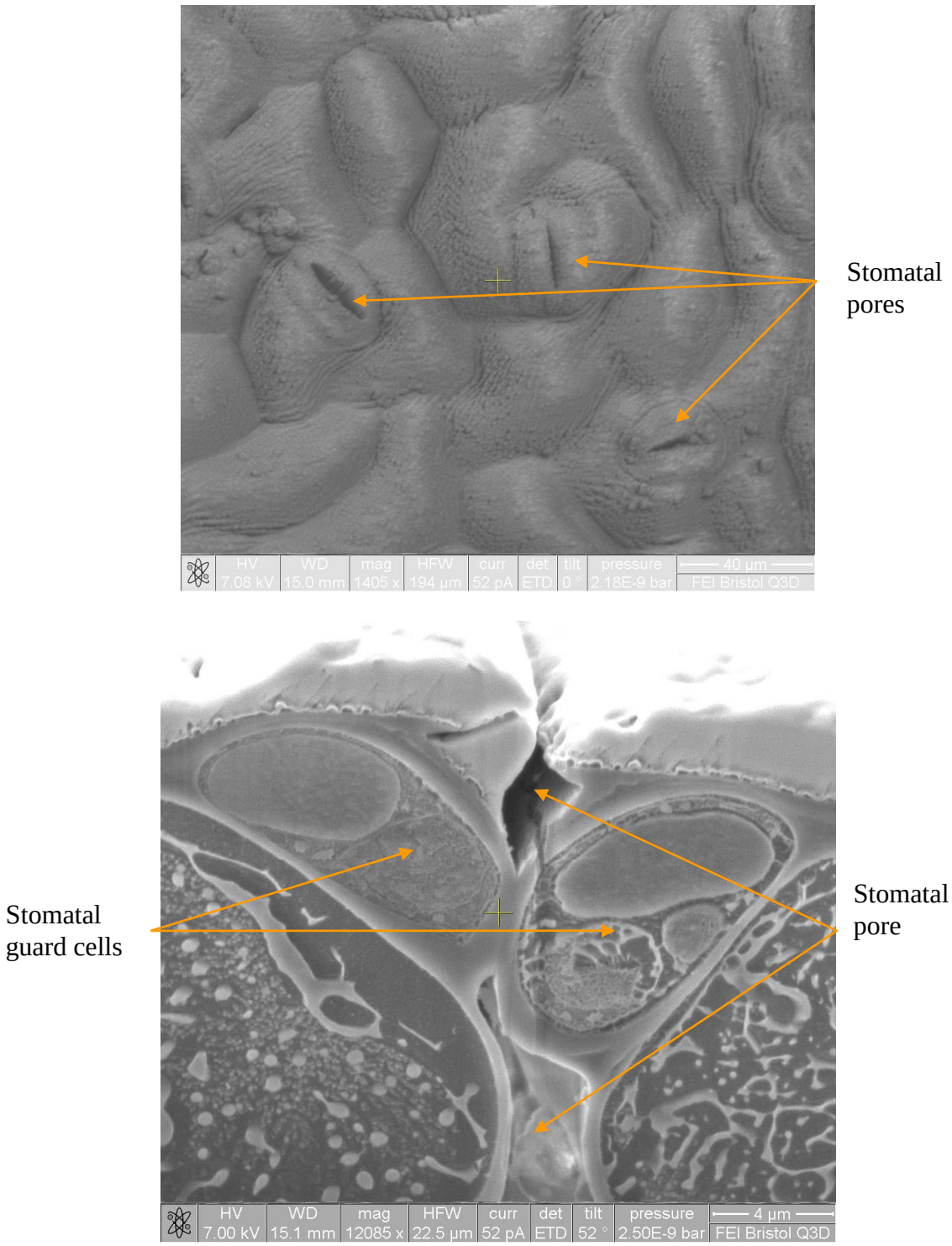


Figure 5: Electron micrographs showing stomatal pores of a coriander leaf

a) surface view

b) FIB SEM showing stomatal guard cells either side of the stomatal pore

Humidity in Relation to Oedema

Agrios describes oedema as 'numerous small bumps on the underside of leaves or on stems' (Agrios, 1997). These 'bumps' arise from the division and expansion of cells, resulting in greenish-white swellings or galls, as the affected cells break through the leaf surface. The exposed surfaces of the galls later develop a corky texture and a rusty colour. Agrios states that oedema is caused by excess watering, especially in cloudy, humid weather. Other authors (e.g. Brooks, 1953; Sherf and Macnab, 1986) agree with this finding, in that, in conditions of extreme atmospheric humidity or if root uptake of water is greater than water-loss through the shoots, cells in young leaves and fruit may proliferate, forming intumescences. However, it is unclear what the mechanistic link might be between high humidity and increased cell proliferation.

Other plants are affected by humidity. Torre and co-workers state that roses grown in high relative humidity (RH) showed a shorter post-harvest life than those grown at moderate RH (Torre *et al.*, 2001). High humidity also adversely affects the fruit quality and final yield of both tomato (*Lycopersicon esculentum*) (Lipton, 1970) and cucumber (*Cucumis sativus*) (Bakker, 1984; both cited in Choi *et al.*, 1997).

It has been suggested anecdotally, that oedema in coriander is caused by high root pressures in the xylem bursting leaf cells. However, as has been outlined above, there is no direct connection between the xylem vessels and the vacuole of the cell; water must diffuse through semi-permeable membranes to reach it. Therefore, high water pressure can develop in the cell walls and intercellular spaces and cannot cause the vacuoles to burst. Instead, the pressure is more likely to upset the macro leaf structure, breaking the cell-cell integrity.

Disturbances in Calcium Homeostasis

Background Physiology of Calcium Utilisation

Large amounts of calcium can be stored within the plant, mainly bound within the cell wall (particularly in the middle lamellae, where it is thought to aid cell-to-cell adhesion) or sequestered in organelles, such as the vacuole (Clarkson, 1984; cited in Rengel, 1992). A small (micromolar) amount remains free within the cytosol to carry out various metabolic and signalling activities e.g. carbohydrate translocation, as a cofactor by some enzymes

involved in the hydrolysis of ATP and phospholipids and in the formation of the mitotic spindle during cell division (Taiz and Zeiger, 1998).

Although these cellular processes only require small amounts of calcium, the supply must be uninterrupted (Gilroy *et al.*, 1993). However, calcium has low mobility, moving predominantly with the transpiration stream in the xylem, whilst being largely absent in the phloem (e.g. Marschner, 1995). In slowly transpiring organs (e.g. fruits) and in periods of low water uptake and movement, i.e. under conditions of soil salinity, in periods drought or high humidity, transpiration is decreased, so calcium requirement can outstrip supply and calcium starvation may occur. This starvation can seriously disrupt growth and development and is most apparent in the growing tips - leaf and root meristems and maturing fruits - where growth and development is most rapid and transpiration is low. Resulting diseases include blossom-end rot of tomato, which causes leathery, dark-coloured patches occur at the blossom end of the fruit (Hessayon, 2002).

There is also evidence that calcium is not retranslocated to the root, once it has reached the aerial parts of the plant (Marschner and Richter, 1974; cited in Jakobsen, 1993).

The interaction of nutrients, both specific and non-specific, can affect the critical deficiency levels of one or all components (Marschner, 1995). This is most important when each mineral is near deficiency range and increasing the supply of only one can exacerbate the problem, as growth is stimulated, increasing the requirement for the other. Optimal ratios are therefore just as important as the specific concentration. Most pertinent to agriculture and horticulture is the fact that calcium and magnesium absorption and availability is disrupted by the presence of high concentrations of potassium (e.g. Marschner, 1995). Growers often use a nitrogen-phosphate-potassium (NPK) fertiliser and, as potassium is not easily leached, it can build up when application outstrips demand (Hansen and Petersen, 1975; cited in Jakobsen, 1993).

Balancing the concentration of nutrients in the soil is also important from a disease perspective. It is well known that high levels of nitrogen result in 'soft' tissue, which is more susceptible to infection by biotrophic micro-organisms (although the opposite is true for hemi-biotrophs). High levels of potassium and calcium, however, increase the resistance to attack from both types of micro-organism (Marschner, 1995). Calcium in

particular, is known to inhibit the action of extracellular pectolytic enzymes (e.g. polygalacturonase) produced by the attacking pathogen, with the aim of dissolving the middle lamella (Bateman and Lumsden, 1965; cited in Marschner, 1995).

In conditions of soil acidity, aluminium ions are released into solution and can be absorbed by plants, resulting in toxicity. Amongst other things, aluminium interferes with membrane transport of divalent cations (especially calcium and magnesium) (Grimme, 1983; Rengel and Robinson, 1989a; Rengel and Robinson, 1989b; Rengel, 1992), reducing their accumulation within cells. It has also been shown that in acidic conditions, breakage of calcium cross-bridges between the uronic acids in the pectins situated in the inner layers of the cell wall of roots can occur (Taiz, 1984). This may reduce the load-bearing ability of the cell wall, weakening the call-to-cell integrity.

The Involvement of Micro-organisms

Plant-Associated Micro-Organisms

Large numbers of micro-organisms are associated with plants, from the roots (rhizosphere) to the leaves (phyllosphere). Indeed some phyllosphere bacterial communities have been found to consist of more that 88 species from 37 genera (Legard *et al.*, 1994). Most do not influence the host plant in any way, some even establishing large populations without any apparent repercussions. A few however, have obvious effects - some are pathogenic and cause disease, while others alter plant growth by inducing the production of plant hormones. It is generally recognised that endophytic (inside leaf tissue), rather than of epiphytic (leaf surface), populations are directly responsible for disease (Beattie and Lindow, 1999).

Beattie and Lindow (1999) also suggest that water is the most important factor to influence microbial growth. Larger populations of epiphytic bacteria are produced in the continuous presence of water (Haefele and Lindow, 1987; cited in Beattie and Lindow, 1999). This is also true of the endophytic environment. Young showed that continually water-soaking leaves after infiltration with bacteria resulted in larger populations than leaves that were left to dry (Young, 1974; cited in Beattie and Lindow, 1999).

A number of studies show that bacteria can modify their environment in order to enhance colonisation. For example, nutrient leakage is thought to be increased through the

production of indole-3-acetic acid (IAA) by certain types of bacteria (Brandl and Lindow, 1998; Manulis *et al.*, 1998). In these studies, epiphytic populations of *Pantoea agglomerans* (formerly *Erwinia herbicola*) increased to approximately twice that of an isogenic IAA-deficient mutant after co-inoculation onto greenhouse-grown bean plants and pear flowers in the field. Additionally, under stressful conditions, the production of IAA was also associated with increased survival (Manulis *et al.*, 1998).

The micro-environment may also be adapted through production of a layer of extracellular polysaccharide (EPS) on the leaf surface, which many studies have illustrated through scanning electron micrographs (e.g. Morris *et al.*, 1997). It is thought that EPS may act to anchor bacterial cells to leaves, whilst also protecting them from desiccation (Wilson *et al.*, 1965), EPS being highly hygroscopic.

Within the leaf, modifications also occur, most often caused by phytopathogenic strains. A common occurrence is for the pathogen to increase host cell membrane permeability (Burkowicz and Goodman, 1969; Addy, 1976) and raising the pH (Atkinson and Baker, 1987b), which in turn is hypothesised to increase the amount of water and nutrients available to the bacteria living in the intercellular spaces (Atkinson and Baker, 1987a).

EPS are also produced within the intercellular spaces, resulting in water soaking of leaf tissues. Like on the leaf surface, this increases the water available to the bacteria cells, as well as reducing contact with toxic molecules. (Denny, 1995)

In addition to these environment-modifying activities, bacteria can co-operate amongst both homogeneous and heterogeneous populations (quorum sensing), through production of and response to particular chemicals (Greenberg, 2003) e.g. acyl-homoserine lactones (Bainton, 1992; cited in Greenberg, 2003). Population density itself can cause behaviour to vary. Wilson and Lindow's study into the survival of a strain of *Pseudomonas syringae* when exposed to desiccation stress (Wilson and Lindow, 1994; cited in Beattie and Lindow, 1999) found that inocula with high cell concentrations showed up to 100-fold better survival than cells from the low-concentration inoculum. Interestingly, when two *P. syringae* strains were applied to different plant species in a field situation, survival rates of the bacteria varied greatly (Kinkel *et al.*, 1996). A strong correlation was also apparent between the population size of indigenous species and that of the *P. syringae* applied.

This suggests that colonised leaves present a different habitat to uncolonised ones, with the bacterially-induced modifications facilitating both their own colonisation and that of immigrant cells landing on the same leaf.

That the internal bacterial population is directly responsible for the induction of disease has been recognised for many years. In 1949, Allington and Chamberlain recorded that there was a close correlation between bacterial leaf infection and bacterial multiplication in intercellular spaces (Allington and Chamberlain, 1949). However, it is widely presumed that endo- and epiphytic populations for a continuum (Beattie and Lindow, 1999) and studies of a number of different bacteria have shown that large surface populations can be linked with the onset and severity of foliar diseases, including *Pseudomonas syringae* pathovars (pvs.) *coronafaciens* (Hirano *et al.*, 1981), *glycinea* (Mew and Kennedy, 1982), *syringae* (Hirano and Upper, 1990) and *tomato* (Smiteley and McCarter, 1982), *Erwinia amylovora* (Thomson *et al.*, 1976; all cited in Beattie and Lindow, 1999) and *Xanthomonas campestris* pv. *undulosa* (Duveiller and Maraite, 1995).

Hypersensitive Response to Pathogen Attack

The hypersensitive response (HR) occurs in incompatible plant-pathogen interactions, i.e. those where an avirulent pathogen attacks a resistant host (Alvarez, 2000). In compatible reactions (susceptible host and virulent pathogen), the response is either absent or delayed and disease develops. The term 'hypersensitive response' is credited to Stakman in 1915 (Stakman, 1915; in Heath, 2000a) and originally described the rapid and localised death of plant cells of rust-resistant cereals caused by attack from a *Puccinia* rust fungus. This cell death was preceded by chloroplast degeneration and subsequent total loss of cytoplasmic structure (Goodman and Novacky, 1994).

Goodman and Novacky also state that the most important aspects of HR are the rapid rate of reaction, the limited number of necrotic cells and the fact that the progress of the disease is halted (Goodman and Novacky, 1994). These authors believe that neither extensive necrosis (i.e. the collection of dead cells present at the infection site, becoming large and discoloured enough to be visible to the naked eye) nor death of all the pathogenic structures are necessary. In addition, the HR may or may not be restricted to those cells that have actually been invaded by or have had contact with the pathogen (Heath, 2000a).

Studies have determined that this resistance occurs on a 'gene-for-gene' basis (de Wit, 1997), where race-specific elicitors, coded by pathogen avirulence (*avr*) genes, are recognised by plant receptors, coded by resistance (*R*) genes.

However, since the first identification of HR, it has been demonstrated that the programmed cell death (PCD) response varies, depending on the host-pathogen combination. There are separate HR models proposed for fungi, bacteria and viruses (Goodman and Novacky, 1994). Various morphological, physiological and molecular changes have been shown to appear at the same time as the PCD, e.g. the production of phytoalexins (Dixon, 1986), hydrolytic enzymes (Boller, 1987), pathogenesis-related (PR) proteins (Stircher *et al.*, 1997), protease inhibitors (Ryan, 1990) and the deposition of lignin (Friend, 1976) and callose (Kauss, 1987) into the plant cell wall (all cited in Richael and Gilchrist, 1999). Yet Richael and Gilchrist point out that it is difficult to decide how each contributes

specifically to disease resistance, or even if it is involved at all, as many are induced concurrently during pathogen attack.

There are a large number of elicitors involved in HR, some of which are specific and others are which are not (Heath, 2000a). Few specific elicitors have been isolated, even though products from pathogen avirulence (*avr*) genes may only be expected to trigger HR in plants that carry the equivalent resistance (R) gene. In viruses, specific elicitors have been identified as coat proteins, the helices domain of a replicase gene or a movement protein (Dawson, 1999). In fungi, these elictors are specially produced peptides that are either known or presumed to be *avr* gene products (Laugé and de Wit, 1998). Although many more bacterial *avr* genes have been cloned, their products are difficult to identify. They appear to be secreted directly into the plant cell via a type III secretion system, for which the *hrp* (hypersensitive reaction and pathogenicity) genes encode some parts (He, 1998).

Non-specific elicitors include salicylic acid (SA), an oxidative burst, ion influx/eflux, nitric oxide (NO) and jasmonic acid (JA). Their binding sites appear to be connected with the plant plasma membrane (Ebel and Scheel, 1997; cited in Heath, 2000a).

Diseases of Coriander Caused Directly by Pathogens

Coriander is attacked by both bacterial and fungal diseases (Taylor and Dudley, 1980; Mahor *et al.*, 1982), but there is little literature of significant virus infection. This is corroborated by growers involved in the project. One in particular feels that he only suffers from attacks from *P. syringae* and not oedema. It is therefore wise to investigate the possibility that the symptoms of oedema and/or blue spot are actually as yet unreported symptoms of a bacterial or fungal infection.

There does not appear to be any literature studying pathogenic causes of oedema. However, other diseases do result in watery lesions, including blights caused by strains of the bacteria *Xanthomonas* and *Pseudomonas* and the fungus *Alternaria*.

Alternaria is thought to be a persistent disease of coriander (Dennis and Wilson, 1997; Sutton, 2003), infecting leaves, stems, flowers and fruits and roots (Agrios, 1997). The water-soaked lesions have a bluish tinge, which enlarge, turn black and often develop

concentric rings (Agrios, 1997). The affected leaves will senesce and dry up, or fall off. Dark, sunken spots developing on the stem of seedlings may degenerate into cankers, eventually girdling the stem and killing the plant.

It is known that Coriander suffers from various diseases (bacterial blight, flowerstand blight, petal blight) caused by the *coriandricola* pathovar of *P. syringae,* (Taylor and Dudley, 1980; Toben and Rudolph, 1996; Dennis and Wilson, 1997; Refshauge and Nayudu, 2001; Cazorla *et al.*, 2005). This disease is known to be the cause of large losses of coriander seed in Australia, Europe and America.

In the early stages of *Pseudomonas* infection, leaves, petioles and young shoots show brown necrotic lesions with some water-soaking – bruised-looking areas due to the increase in water in the tissue (Taylor and Dudley, 1980), which can lead to a blackening of leaf veins and wilting (Refshauge and Nayudu, 2001). Petals become brown an drop and the unripened fruits exhibit water-soaked lesions (Taylor and Dudley, 1980). In severe cases, plants can become stunted, yellowed and may die.

Refshauge and Nayudu confirmed that *P. syringae* pv. *coriandricola* spreads through the vascular system before penetrating the surrounding tissues (Refshauge and Nayudu, 2001). Ultrastructural studies described in this paper showed large populations of the pathogen in xylem vessels in the stem, which these authors suggested could cause a blockage and thus wilting. No bacteria were seen in the phloem vessels. Bacterial cells were apparent in the interstitial spaces, verifying that the pathogen had the ability to spread between or through the xylem cells. Their presence within cells would suggest that *P. syringae* pv. *coriandricola* produces wall degrading enzymes or pectinases, as observed in other, closely related pathovars. Once in the leaf tissues, the pathogen appears to destroy large numbers of chloroplasts, which would account for the yellowing and reduction of productivity.

P. syringae pv. *coriandricola* is seed-borne (Taylor and Dudley, 1980; Toben *et al.*, 1991, cited in Dennis and Wilson, 1997). The invasion mechanisms of closely related pathovars, such as *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *pisi*, which infect bean and pea respectively, have been well studied (Legard and Hunter, 1990; Grondeau *et al.*, 1996; both in Refshauge and Nayudu, 2001). These pathovars reside epiphytically

until opportunity for invasion occurs. They then develop in the seed coat and may then contaminate the growing cotyledons.

The treatment of seed-borne disease with seed treatments has been studied by Dennis &n Wilson (1997). They looked at soaking infected seed with dilute acid and found that both tartaric acid and hydrochloric acids were successful, although HCl was preferred because of the cost, availability and ease of preparation. They recommended that infected seed should be soaked in 0.5% HCl for 24 hours, washed with water then dried. Indeed, commercially treated seed is available through UK seed merchants, such as Tozer Seeds (Surrey, UK) and CN Seeds (Cambridgeshire, UK).

Taylor and Dudley (1980) carried out a non-replicated trial of antibiotic treatment for *P. syringae* on coriander. Both streptomycin and kasugamycin reduced seed infection at harvest from 1.3% in untreated seed to less than 0.3%. However, antibiotics are not currently approved for use in food crops and are unlikely to be in the future, for fear of the development of widespread resistance.

Bacterial diseases in high-value horticultural crops are also often controlled with the use of copper-based fungicides (Dennis and Wilson, 1997).

However, with the increased concern over the use of chemicals on food crops and the rise of the demand for crops to be grown according to such protocols as the Assured Produce Scheme (Assured Food Standards, London, UK) by supermarkets and other buyers, the role of pesticides is waning. Instead, the use of cultural practices and resistant varieties is ever more necessary.

AIMS

The aim of this project was 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.) and more appropriate management of irrigation schedules.

Three strategies were employed:

1. Gathering data and samples from growers in the field;
2. Gathering meteorological data;
3. Setting up controlled experiments in the laboratory.

A network of growers was set up who logged incidences of oedema and blue spot in their crops and provided samples of diseased plants for investigation in the laboratory. It was hoped that logging their anecdotal evidence would provide significant clues as to the causes of the condition.

As it was thought that oedema occurs in humid conditions, weather data (primarily soil and air temperature, wind speed and direction and relative humidity) was also collected. Many of the growers collect this data for their own use, so have also been able to provide it for the project.

In the laboratory, the symptoms of the condition were investigated and recorded. Based on the literature discussed in this chapter and anecdotal evidence gathered from growers, three subject areas were studied: The effect of humidity and other environmental conditions; ion imbalance focusing on calcium and the role of micro-organisms.

SCIENCE SECTION - PART 1

IDENTIFICATION OF SYMPTOMS IN CORIANDER

INTRODUCTION

At the start of the project, oedema was identified as grey foliar lesions. However, as the project progressed and more growers offered their experiences of the condition and tissue samples, it emerged that other, blue-ish symptoms were also being called oedema. This condition also appears to be far more serious, in frequency, timing of occurrence and severity of lesions, than the grey oedema. Neither set of symptoms match up with those recorded as oedema on other plant species. It was therefore essential, particularly as there is so little published literature on the condition, to identify and record the symptoms in detail. The symptoms of the two conditions can then be compared and contrasted, with a view to re-naming them and publicising their differences. For the purposes of this study, the grey lesions have been termed 'oedema' and the blue-ish ones termed 'blue spot'.

Symptoms were studied with the naked eye and using various kinds of microscopy. Logging symptoms visible to the naked eye is essential for preliminary identification of the condition in the field. Once in the laboratory, various other microscopy techniques were used: looking at the intact tissue via a binocular zoom microscope, in order to look at the surface structure; fixing and sectioning tissue to study the internal structure, using a compound microscope with magnifications in the order of tens and hundreds of times; and looking at sectioned tissue under an environmental scanning electron microscope (ESEM), with magnifications of between 200 and 12,000 times.

Microscopic analysis can uncover further anomalies that invite investigation, In this case, ESEM examination of coriander with blue spot required analysis of chlorophyll content.

METHODS

Surface Symptoms

The surface of coriander leaves were studied with the naked eye and with a binocular zoom microscope (magnification of 0.7 to 4.5 times), without being treated in any way.

Symptoms at Cellular Level

Study of Lesions with a Light Microscope

Fixing Tissue

Small sections of tissue (approximately 2mm x 4mm) were cut with a scalpel blade, placed in a 1.5ml tube of buffered 3% glutaraldehyde solution. This was left for a few hours, then put under a vacuum until the sections sank, the solution having been forced into cells.

The tissue was then dehydrated by being transferred to a series of ethanol solutions of increasing concentrations (25%, 50%, 70%), being left for approximately 24 hours in each solution.

Sectioning Tissue

Fixed tissue was transferred to glass vials and the ethanol drawn off. The vials were then half-filled with LR white hard-grade resin and placed on a rotator for approximately 24 hours. The resin was replaced a further two times, leaving the vials on the rotator for 48 and 24 hours respectively.

Each section was transferred to a separate gelatine capsule filled with out-gassed resin (to aid polymerisation) and the capsule lids replaced. The full capsules were then placed under a UV lamp at room temperature for a minimum of 48 hours, being turned two or three times during this period, until the resin hardened.

The tissue was located within the resin under a light microscope and excess resin was sawn off the capsule. The capsule was positioned as required and then glued to a resin block using Quick-Setting Araldite®. The gelatine capsule was picked off and the resin surrounding the tissue was trimmed until trapezoidal in shape. The sample was then placed in the Reichert-Jung Ultracut microtome (Leica Microsystems GmbH, Wetzlar, Germany) and the tissue orientated to a suitable position.

A glass knife was cut and placed in the microtome and positioned as necessary. Sections of between 1 & 2μm were cut using the manual action and placed on a glass slide. The sections were stained using methylene blue and photographed under a compound light microscope.

Study of Lesions with an Environmental Scanning Electron Microscope

A sample of coriander leaf tissue was introduced onto the ESEM stub, using carbon cement. The stub was then positioned in the cryo shuttle. A vacuum was pulled on a small cup of liquid nitrogen to create nitrogen slush, into which the stub was plunged. The stub holding the sample was then quickly transferred into the preparation chamber (Polaron Polar Prep 2000, Quorum, Ontario, Canada) set to −140°C. The sample was fractured using the appropriate tool, then the stub tapped to remove loose fragments. The sample was then introduced into the chamber of the Philips XL-30 SEM (Philips /FEI, OR, USA) where the temperature was etched by raising the temperature from -140° C to -90° C for 15 minutes. Clarity of viewing was further improved by transferring the samples to the preparation chamber and sputter coating with gold particles. The samples were relocated to the ESEM chamber and viewed at an acceleration voltage of between 2 and 5 kV.

Analysis of Chlorophyll Content

Method adapted from (Knudson *et al.*, 1977)

Both dried and fresh tissue was used in this experiment. Dried tissue was re-hydrated before chlorophyll extraction by immersion in de-ionised water for approximately 3 h and application of a vacuum for 4 minutes. Fresh tissue was used immediately after harvest.

All leaflets were stripped from each coriander stem and placed into a universal bottle, to which 25ml of absolute ethanol was added. The bottle was then stored in the refrigerator (4°C, in the dark) for 24 hours. The chlorophyll-ethanol (chl-ethanol) solution was then poured off into a 100ml Durant bottle and the leaf tissue rinsed with approximately 10ml absolute ethanol, which was added to the chl-ethanol solution. The leaf tissue in the universal bottle was re-submerged with 25ml absolute ethanol and again stored in the refrigerator for 24 hours, before being rinsed for a final time. The leaf tissue was dried for 3 days in a drying oven set at 70°C and then weighed.

The final solution was made up to 70ml with absolute ethanol and a 10ml aliquot of this was drawn off. This aliquot was measured with a spectrophotometer at 665nm and 649nm.

The absorbance is converted to total chlorophyll content for each type of chlorophyll with the following equations:

$$\frac{\mu g \text{ chl a}}{\text{ml solution}} = (13.70)(A_{665nm}) - (5.76)(A_{649nm})$$

$$\frac{\mu g \text{ chl b}}{\text{ml solution}} = (25.80)(A_{649nm}) - (7.60)(A_{6665m})$$

Finally, the amount of chlorophyll (µg) per mg dry weight was calculated and the means were subjected to a two-sample t-test assuming unequal variances.

RESULTS

Surface Symptoms

Visible Symptoms

Oedema appeared as a slight greying of the leaf lamina, between veins on the upper side of the leaf (figure 1.1a). Once formed, the patches did not seem to increase in size. A tan, pinprick indentation generally developed within this area, usually one per grey patch, which also remained a constant size.

If the leaves were cut and left in the open air, the patch dried out, turning papery and necrotic, before the whole leaf withered and died. The symptoms usually occurred outside, although occasionally, coriander over-wintering in polytunnels was also afflicted.

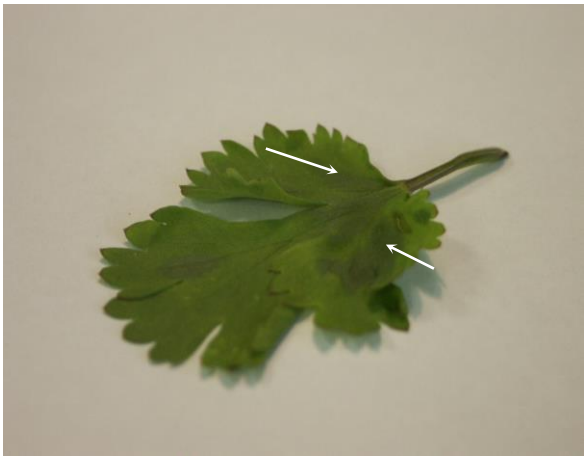
Blue spot lesions appeared as irregular, slate-blue patches on the upper leaf lamina, spanning the veins (figures 1.1 b and c). Once developed, the blemishes did not seem to expand and the leaves were not usually covered completely.

They appeared as soon as the weather warmed and continued all through the season, both outside and under cover. Once cut, affected leaves soon blackened and the tissue broke down and started to rot.

a) 3cm

1cm

b)



1cm

c)

Figure 1.1a: Coriander exhibiting symptoms of oedema – angular grey patches, located between the veins. The tan, pinprick indentations are faintly visible within each lesion (indicated by arrows).

Figures 1.1b & c: Coriander leaves exhibiting blue spot symptoms of slate-blue, irregular lesions (indicated with arrows).

Symptoms as Seen Under the Binocular Zoom Microscope

Under x90 magnification, the greyness of oedema was not particularly distinguishable. The indentation was revealed as a pale brown-beige sunken lesion, with a slimy, water-soaked appearance. The indentation measured approximately 0.8mm in diameter.

The blueness of blue spot samples was again not particularly visible under x90 magnification and neither were there any distinguishable characteristic at this magnification.

Symptoms at Cellular Level

Study Of Lesions With A Light Microscope

Figure 1.2 shows a transverse section through a healthy coriander leaf. The different cell types are clearly visible, as are the intercellular spaces. Epidermal cells tend to be round or oval, while palisade cells are rectangular and mesophyll cells vary greatly in shape and size. Their predominant common characteristic is their roundness. They are distributed loosely and randomly, leaving large air spaces.

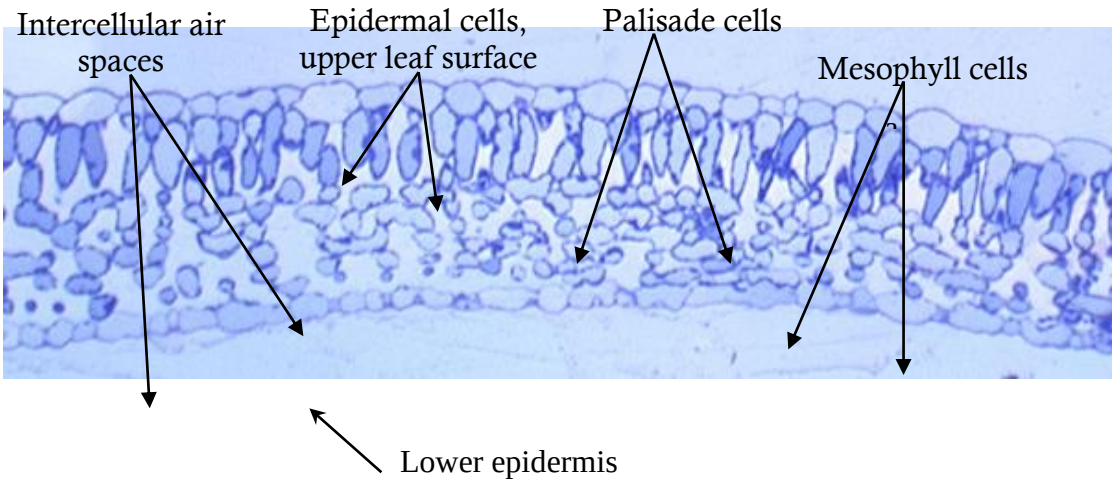


Figure 1.2: TS of a healthy coriander leaf (magnification x100). Tissue was fixed with a 3% glutaraldehyde solution and embedded in LR White hard-grade resin. Sections cut 1-2µm thick and stained with methylene blue.

Figure 1.3 shows transverse sections through the indentations seen in coriander leaf with oedema. Epidermal cells are still present and appear intact, yet they are much smaller than unaffected cells. The cells below the affected epidermis are highly cytoplasmic. The identity of these cells is not immediately obvious, as they have no regular shape, size or arrangement, but they appear to be a mixture of palisade and mesophyll cells that have

lost their intercellular integrity, collapsing into one another. In addition, a layer develops on the surface of the epidermis. This could be material produced as the epidermal cells degrade. To either side of the indentation, the cells appear to have maintained their usual characteristics and structure.

The three slides in figure 1.4 show transverse sections of oedematous tissue, before the indentation is visible with a hand lense. Again, a change in the cells can be seen. The epidermal cells elongate considerably, whilst the palisade cells become shorter and more closely packed, gradually disappearing. The mesophyll cells also become elongated and enlarged, crowding out the intercellular air spaces.

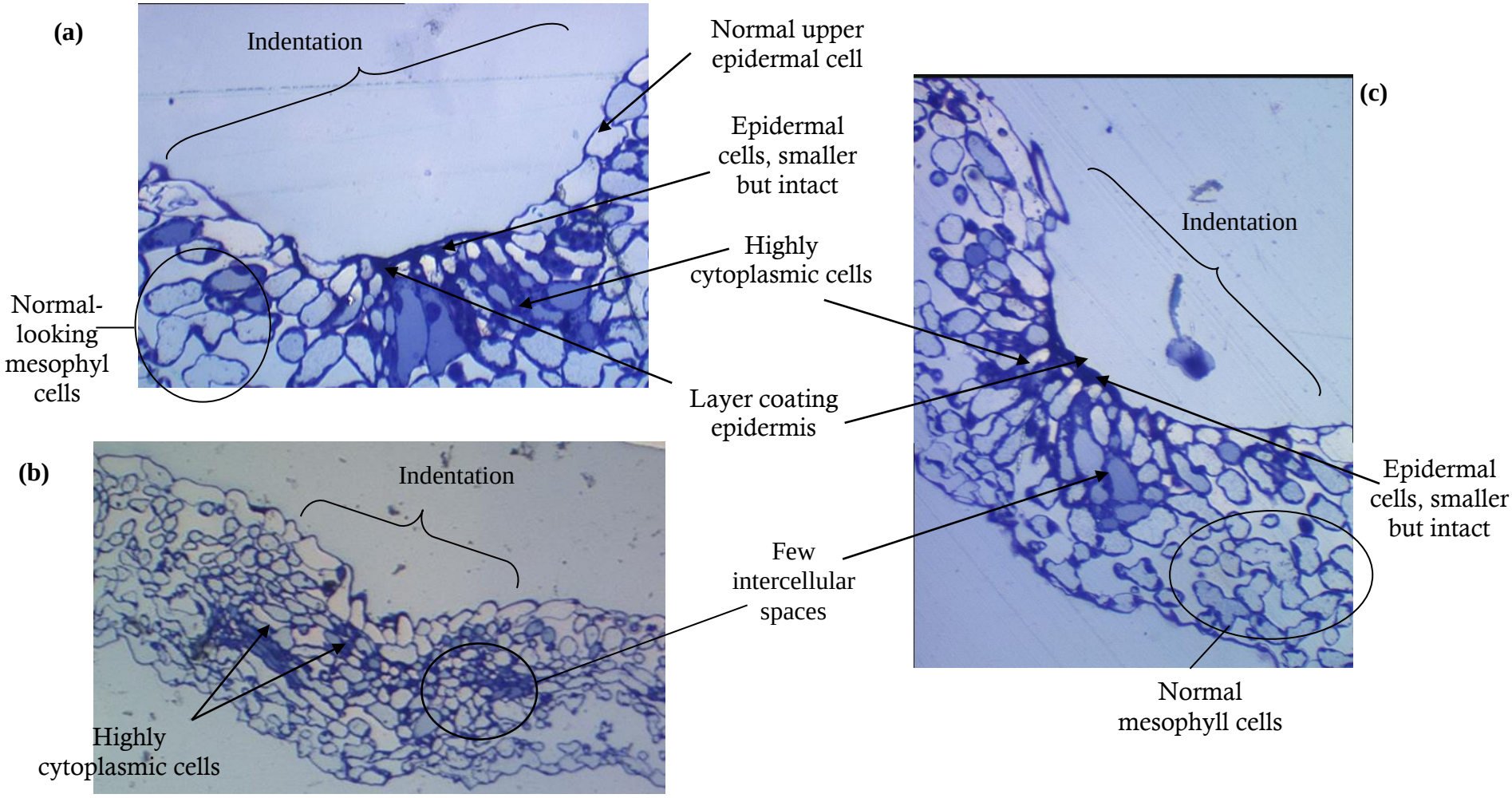


Figure 1.3 a–c: TS of indentations seen in oedema of coriander (a: mag. X200; b & c: mag. x100). Tissue fixed with a 3% glutaraldehyde solution & embedded in LR White hard-grade resin. Sections cut 1–2µm thick & stained with methylene blue.

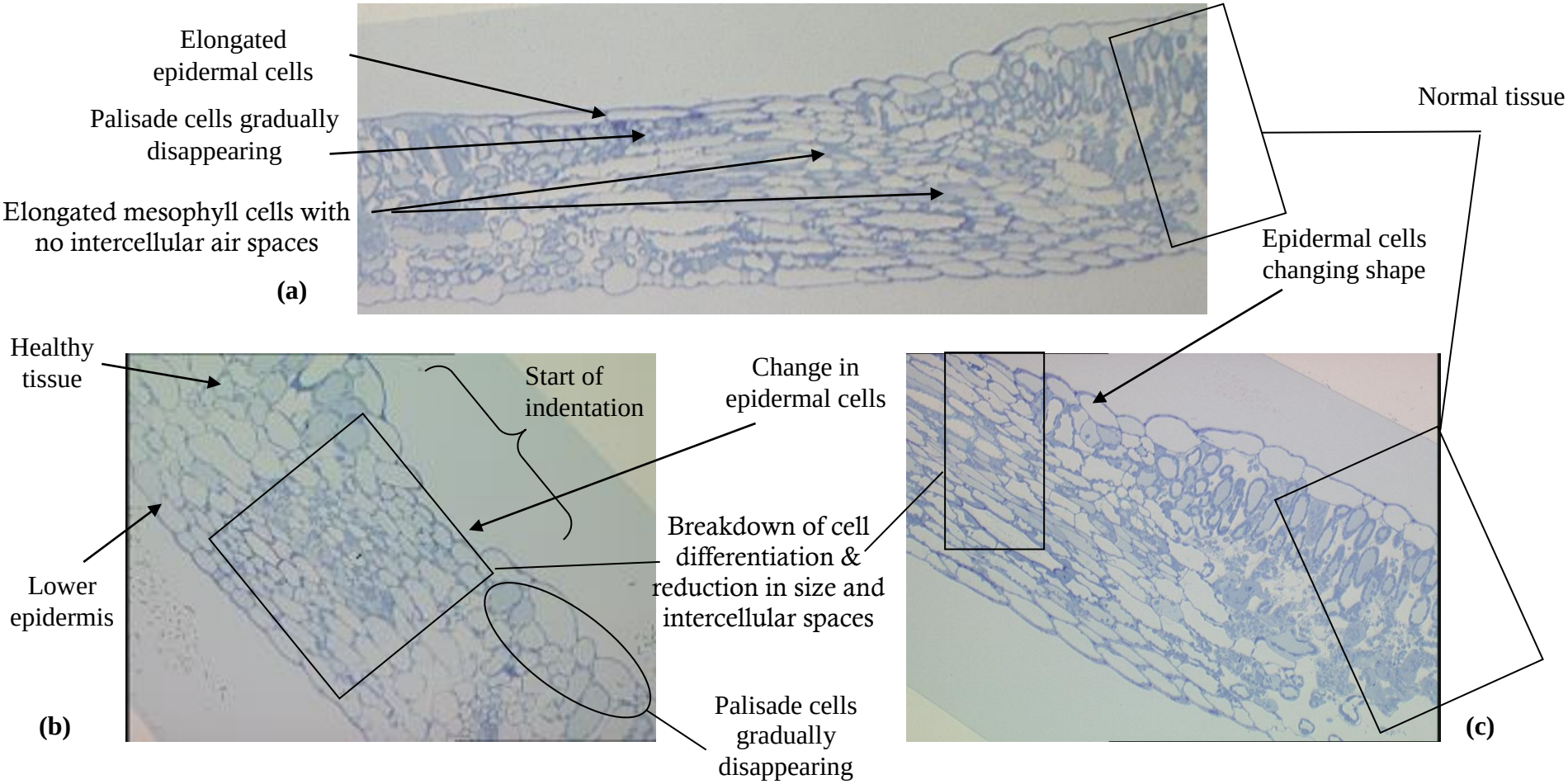


Figure 1.4 a–c: TS of oedematous tissue prior to visible development of indentation (mag. x100) Tissue was fixed with a 3% glutaraldehyde solution and embedded in LR White hard-grade resin. Sections cut 1–2µm thick and stained with methylene blue.

The sections in figures 1.5 and 1.6 were taken through areas of blue spot on coriander. The cells appear normal and there is no apparent breakdown of intercellular integrity like in the oedematous tissue. However, the cuticle is thicker on these leaves than on the healthy tissue. In addition, the chloroplasts in both palisade and mesophyll cells are more visible than in healthy tissue. Whether this is due to an increase in size, number or pigmentation cannot be deduced from these pictures, although the colour change seen in affected tissue could possibly be due to a breakdown of one of the chlorophyll pigments. This could account for the blue colour typical of the condition.

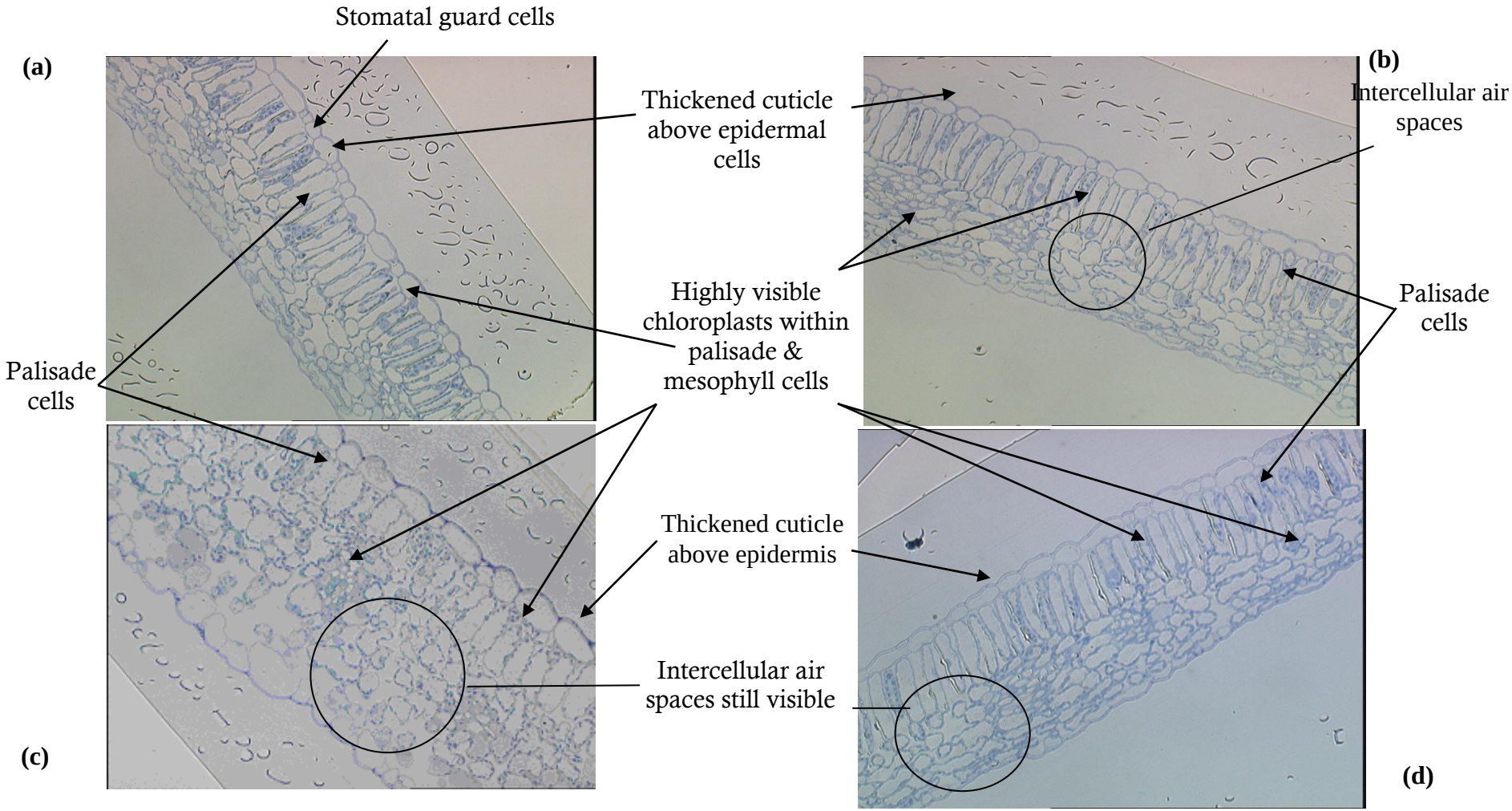


Figure 1.5 a–d: Slides showing TS through blue spot lesions of coriander (magnification x100). Tissue fixed with a 3% glutaraldehyde solution & embedded in LR White hard-grade resin. Sections cut 1–2µm thick & stained with methylene blue.

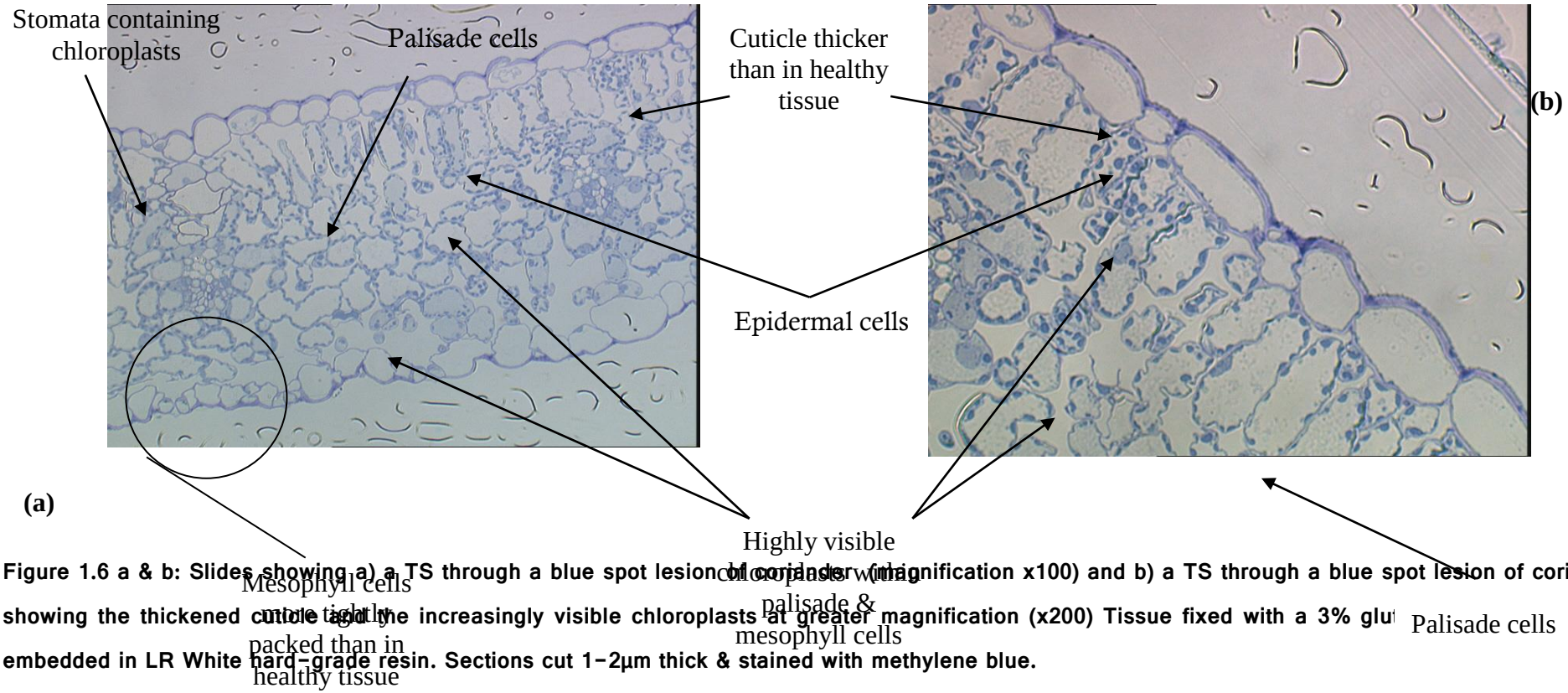
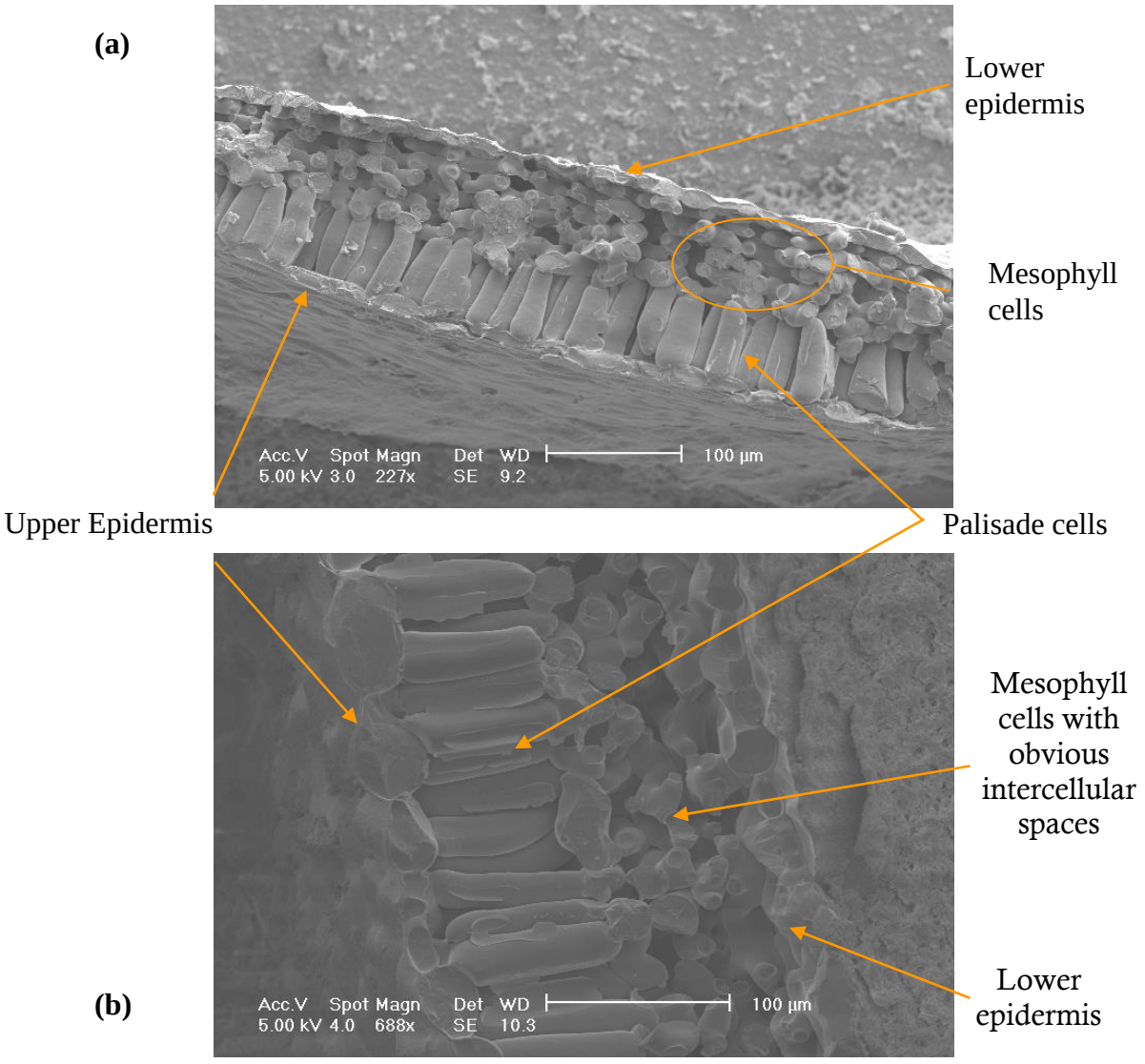


Figure 1.6 a & b: Slides showing a) a TS through a blue spot lesion of coriander (magnification x100) and b) a TS through a blue spot lesion of coriander showing the thickened cuticle and the increasingly visible chloroplasts at greater magnification (x200) Tissue fixed with a 3% glu ɔn & embedded in LR White hard-grade resin. Sections cut 1-2µm thick & stained with methylene blue.

Study of Lesions with a Scanning Electron Microscope

Figure 1.7 shows electron micrographs of sections through healthy coriander leaves at two magnifications. All different cell types are clearly visible. The palisade cells are columnar in shape and regularly arranged, while the mesophyll cells are irregularly shaped, but well spaced. The upper and lower epidermises are one cell thick, and no excessive cuticle is visible.



Figures 1.7 a & b: Electron micrographs of transverse sections across a healthy coriander leaf at two magnifications. The palisade cells are columnar in shape and regularly arranged. The mesophyll cells are irregularly shaped, but well spaced.

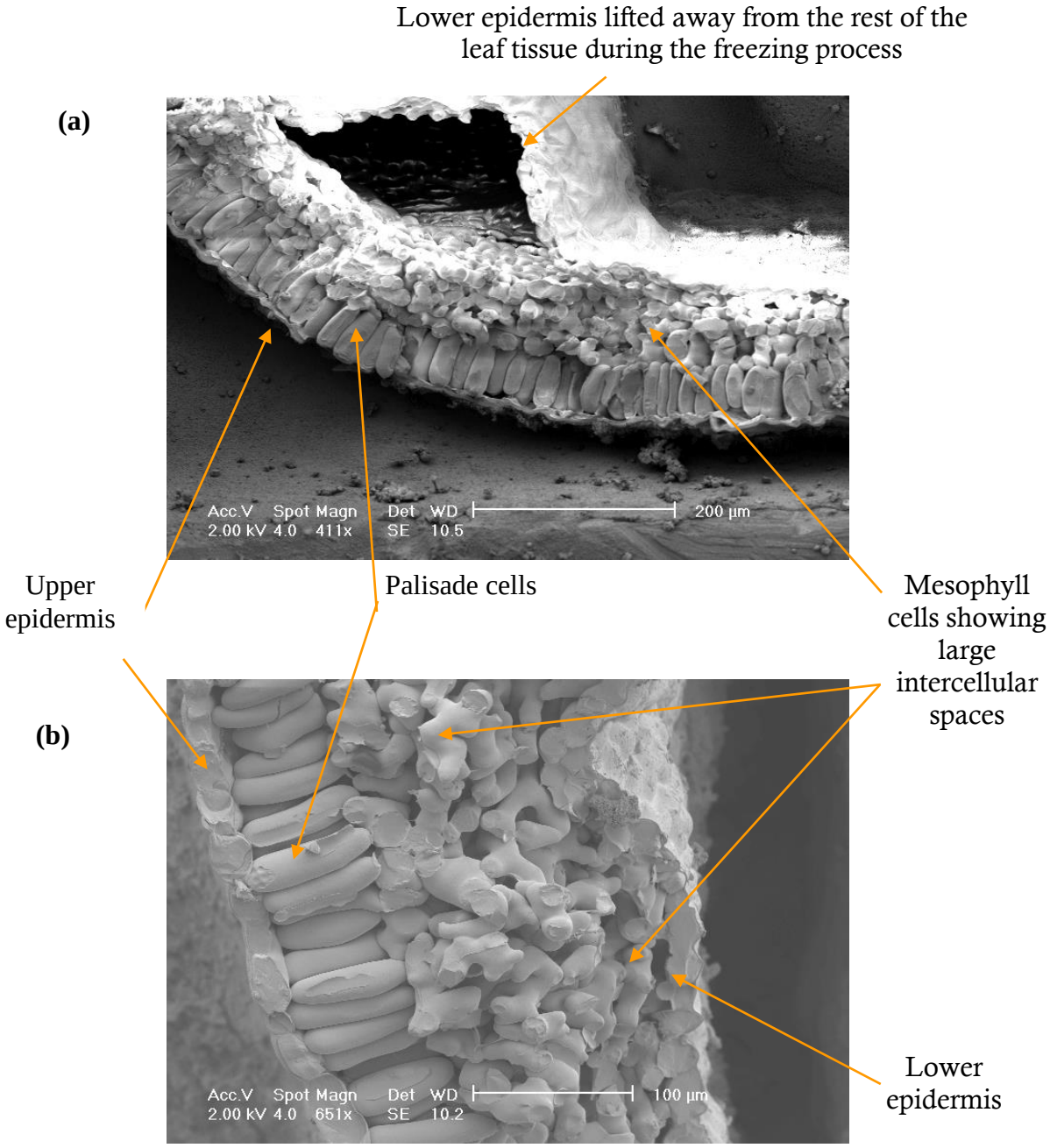
Figures 1.8 and 1.9 show electron micrographs through a coriander leaf showing symptoms of oedema. The first two figures were taken through the grey patch, whilst the latter two

show the lesion. In the grey area, there is no obvious difference in the cells, when compared to the healthy sample. This is particularly apparent at higher magnification (figure 1.8 b).

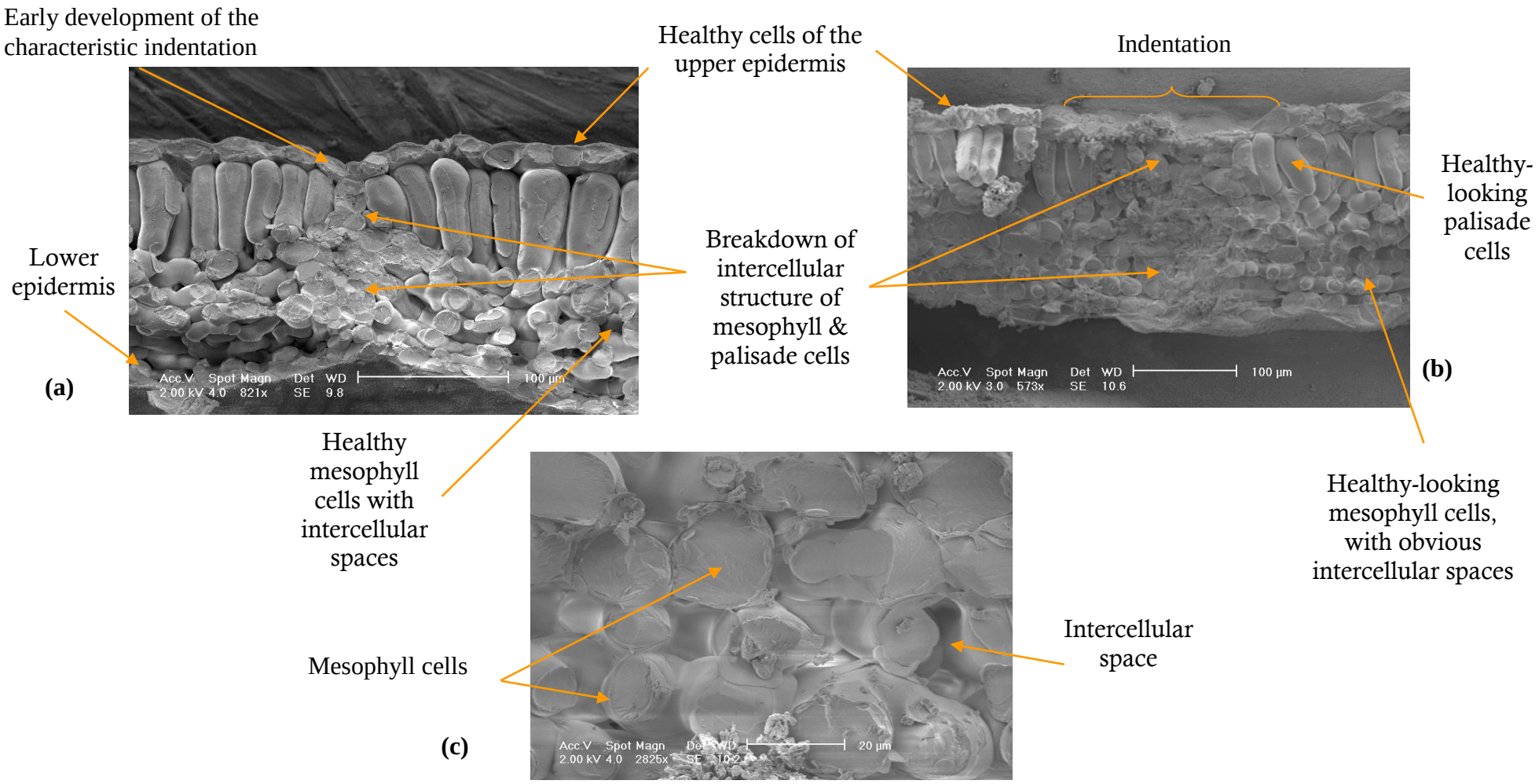
Even early in the development of the oedematous lesion, the intercellular structure had broken down (figure 1.9a). The palisade cells had lost their characteristic columnar shape and regular positioning, while the air spaces between the mesophyll cells were lost. Corroborating the findings of the light microscope sections, this disruption only occurred over a very small area, with the area either side of the lesion looking unaffected.

As the condition developed, the indentation enlarged. This is manifest in the increased cell disruption shown in figure 1.9b, which, even in these later stages, still remained restricted. The surrounding cells appeared normal, when compared to micrographs of healthy tissue.

A closer look at the mesophyll cells in the lesion confirms that the cells are intact, with little or no intercellular air spaces (figure 1.9c).

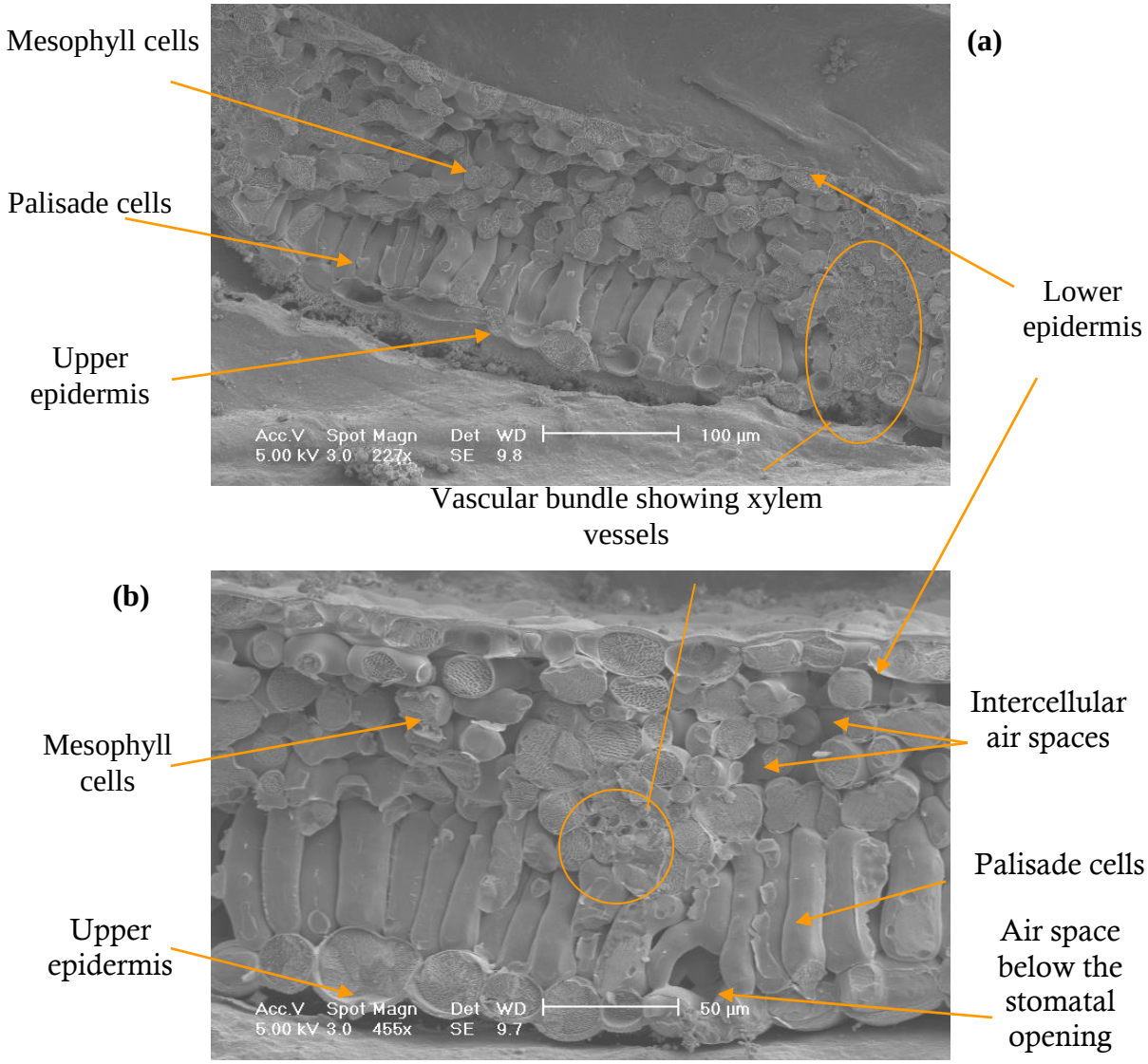


Figures 1.8 a & b: Electron Micrographs through the grey patch of oedematous coriander leaves at two magnifications. There is little difference in intracellular structure when compared to healthy tissue (note: sample 1.18 is upside down).



Figures 1.9 a, b & c: Electron micrographs through oedematous coriander leaves. a) taken through the grey patch showing the onset of indentation development; b) through the characteristic indentation and c) shows mesophyll cells in the indentation of an oedema at high magnification, with the breakdown in intercellular structure giving few intercellular spaces.

Electron micrographs of tissue suffering from blue spot are shown in figures 1.10 to 1.12. From the transverse section shown in figure 1.10a, it can be seen that there is little difference in cellular structure, when compared to the healthy leaf (figure 1.7). The cells show no collapse either in intercellular structure or within the cells themselves, further evidenced under higher magnification in figure 1.10b.



Figures 1.10 a & b: Electron micrographs of TS through a coriander leaf suffering from blue spot at two magnifications. Different cell types are evident, as are xylem vessels and the stomatal aperture There is no difference in appearance between this and healthy tissue, neither is there any cellular distortion characteristic of oedema.

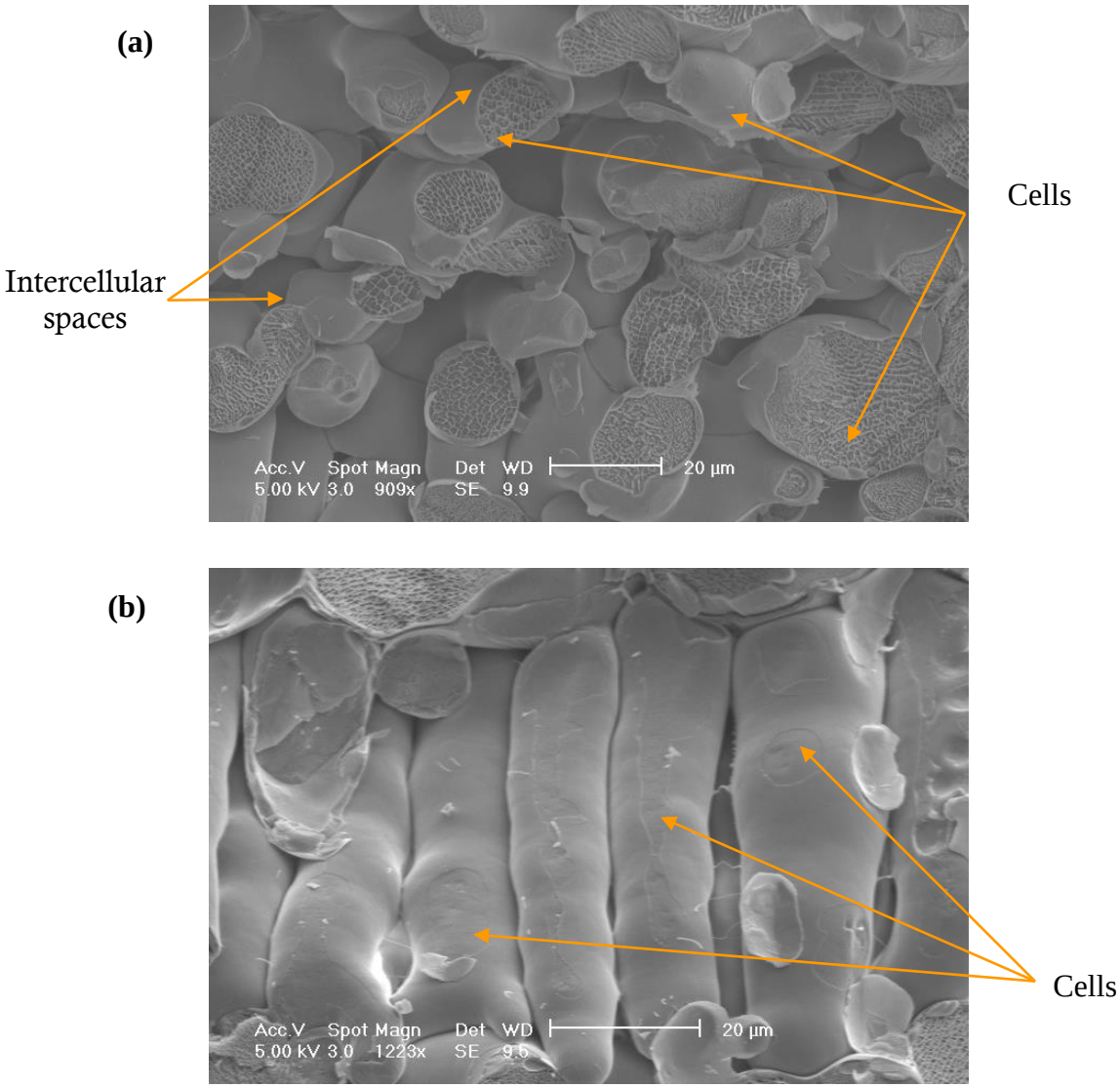


Figure 1.11 a & b: Electron micrographs through a region of coriander leaves suffering from blue spot. There is no evidence of cellular disruption in either the mesophyll cells (a) where intercellular spaces are fully visible, or palisade cells (b) which appear healthy, with a columnar shape and regular positioning.

Figure 1.11 shows the mesophyll and palisade cells in perfect condition. Again, the mesophyll cells are well spaced, with the intercellular spaces clearly visible, while the palisade cells are again regularly positioned and columnar in shape.

The thickened cuticle seen in the light microscope sections of blue spot tissue (figure 1.6) is more clearly visible when studied under the electron microscope (figure 1.12b). When compared to that of a healthy coriander leaf (figure 1.12a), it can be seen that the healthy cuticle is approximately half the thickness of the one suffering from blue spot.

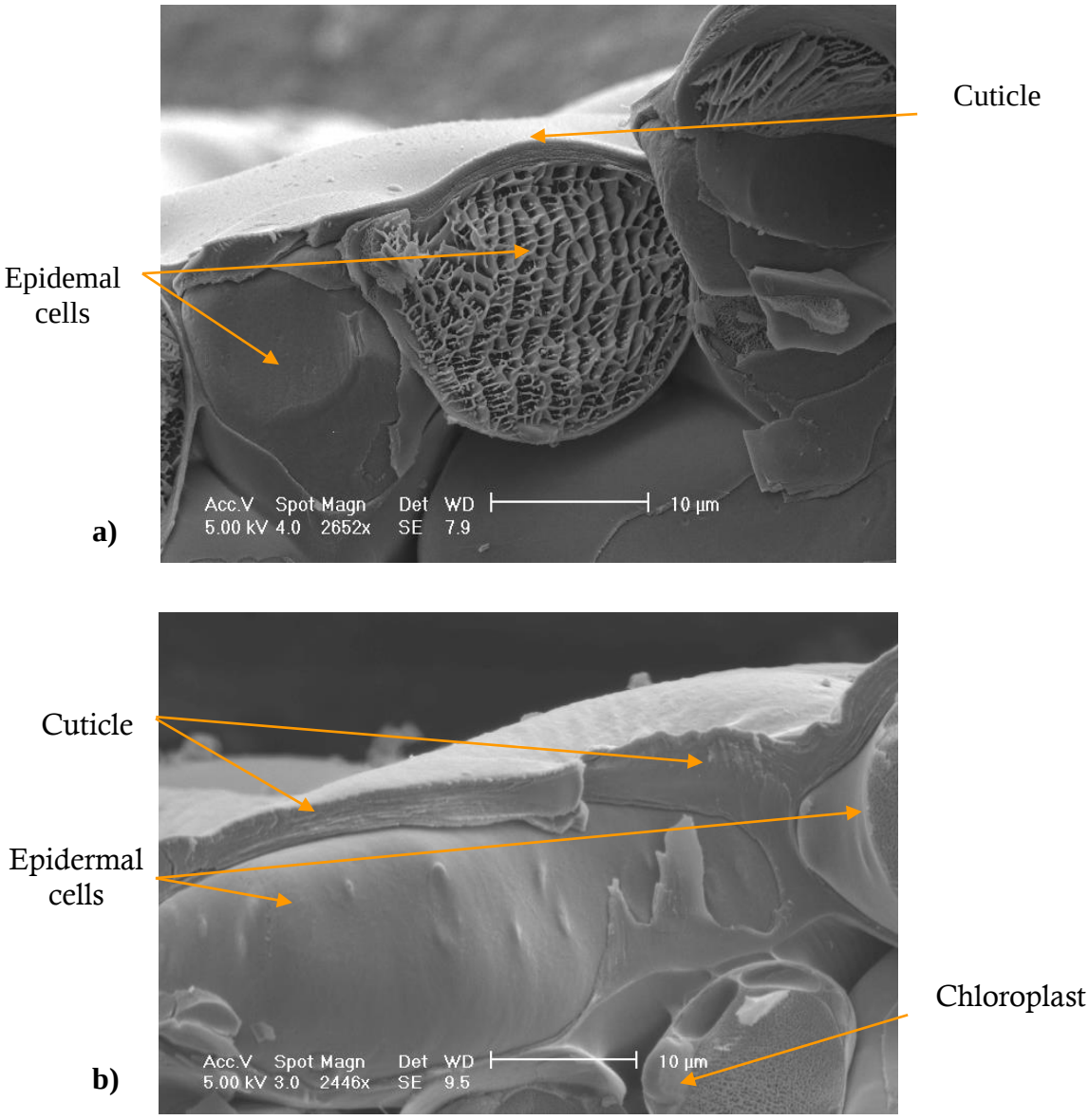


Figure 1.12a & b: Electron micrographs of the waxy cuticle of the upper surface of a) a healthy coriander leaf and b) a leaf suffering from blue spot. The cuticle on the leaf with blue spot is approximately twice as thick as that on healthy tissue. The reason for this is unknown.

Analysis of Chlorophyll Content

Following the increased visibility of chloroplasts in blue spotted tissue when studied under the light microscope (section 1.3.2.1), the amount of chlorophyll found in this tissue was compared to that found in healthy tissue.

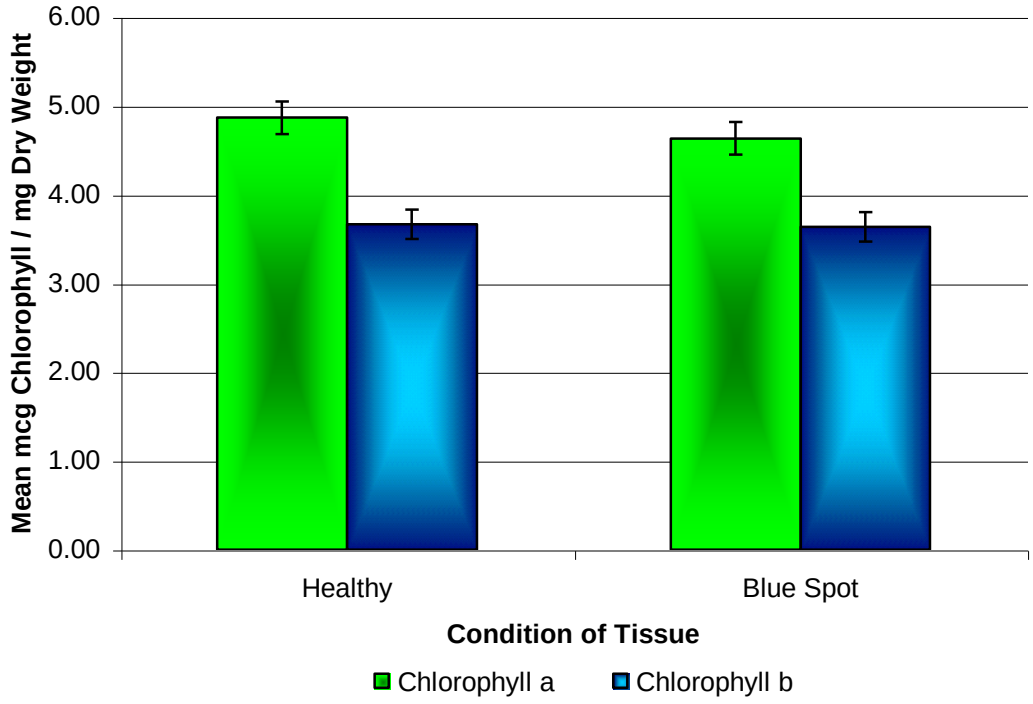


Figure 1.13: Mean differences in contents of chlorophylls a & b in healthy tissue and that with blue spot, from coriander samples from different sites and sown at different times. (Mean ± SE; $n_{healthy}$=5; $n_{blue\ spot}$=4)

The proportion of leaf area showing blue spot symptoms could not easily be recorded for this experiment, but it should be noted that all leaves chosen were showing sizeable patches of blue spot.

The mean differences in the contents of chlorophylls a and b between healthy (chl 4.87µg mg^{-1} dw; chl b=3.67µg mg^{-1} dw) and blue spotted tissue (chl a=4.63µg mg^{-1} dw; chl b=3.64µg mg^{-1} dw) were not significant ($p_{chl\ a}$=0.554; $p_{chl\ b}$=0.941). This indicated that neither the amount of chlorophyll nor the type was affected by the blue spot condition. However, it cannot be deduced from this experiment if chloroplasts were more numerous but smaller.

DISCUSSION

At all levels of investigation, there were distinct differences between oedema and blue spot, the most obvious to the naked eye being the colour. The area of leaf lamina affected also varied – oedema was constrained by the veins, whereas blue spot spanned them. Oedema also developed the tan indentation shown in the above pictures, where as these were never present in blue spot.

At the cellular level, these differences were even more apparent. The sections of oedematous tissue examined under both a light microscope and those scanned with the ESEM show that serious physiological changes had taken place. Although cells had not burst, (a theory originally put forward by growers – see Chapter 1), the deposits visible in the pit of the indentation under the light microscope, indicated that there is leakage of cell contents. Two suggestions can be made, which are not necessarily mutually exclusive: firstly, the middle lamella joining two cells broke down, allowing adjacent cells to move relative to each other, without the cells themselves collapsing. Secondly, the cell membranes were compromised in some way, again, without the cell walls collapsing allowing the contents to seep out.

In contrast, the cellular differences between tissue with blue spot and healthy samples were relatively minor. The predominant distinction shown up by microscopic examination was the visibility of chloroplasts in tissue with blue spot – they were not perceptible in the healthy samples. This was further investigated through analysis of chlorophyll content. However, the content of neither type of chlorophyll in the two tissues was significantly different. It should be pointed out that the structure and number of chloroplasts in blue spot tissue was not determined with this analysis.

It was not deemed necessary to compare the chlorophyll content of oedematous tissue with either healthy samples or those with blue spot, for two reasons: firstly, only one sample of coriander suffering from oedema was available for analysis, meaning that any result would not definitively be characteristic of the condition; secondly, the sample was very badly affected by the condition, showing severe chlorosis and necrosis, thus the amount of chlorophyll would have been affected.

Leaf tissue with blue spot also exhibited a cuticle which was approximately twice as thick as that seen on a healthy leaf.. This was most obvious when viewed with the ESEM, however the cause of the thickening is not clear. The cuticle is composed of a two-layered cuticular membrane (CM) and a superficial layer of epicuticular wax (Jeffree, 1996). The outer, continuous layer, secreted onto the epidermal cell wall (Lee and Priestly, 1924; cited in Jeffree, 1996), comprises mainly soluble and polymerised aliphatic lipids. It is free of cellulose and other substances found in the cell wall (Ristic and Jenks, 2002) The inner part of this bi-layer impregnates the cell wall and thus contains various embedded cell wall polysaccharides in significant quantities (Jeffree, 1996). The epicuticular wax can either develop amorphously or can develop a crystalline structure characteristic of the plant species. Coriander appears to have the former type of wax.

As the samples studied under the light microscope were taken from a different site to those studied with the ESEM, it can be confirmed that this is due to the condition, rather than the growing location.

In all sections, under both light and electron microscopes, there was no evidence of bacterial or fungal cells in or around the lesions either in oedematous tissue or that with blue spot.

CONCLUSIONS

From these studies of the symptoms of both conditions, it is obvious that oedema and blue spot do not exhibit similar symptoms. It can therefore be inferred that they are entirely separate conditions that could benefit from being officially described.

Quantifying the number of chloroplasts in the blue spot tissue and investigating their structure may shed more light on the trigger for the blue spot lesions, as may examination of the presence of other pigments.

The tissue was studied at a sufficiently high magnification to allow for visualisation of any bacterial of fungal cells. However, as it cannot be proved categorically from these microscopic studies that pathogens are not causing the conditions, further, appropriate analyses will be carried out.

Although these studies have provided no clue as to the causes of each condition, the growers' hypothesis that oedema is caused through cells bursting under high internal pressure was confirmed as incorrect. This thesis will continue to investigate the hypotheses presented, using targeted experiments.

SCIENCE SECTION - PART 2

INVESTIGATIONS INTO THE EFFECT OF HUMIDITY

INTRODUCTION

As both the blue and the grey symptoms were both termed oedema by growers, no particular theory was put forward for blue spot. However, it has been suggested that oedema in coriander is caused by high water pressures in the xylem, bursting leaf cells. Yet, as previously outlined (see Chapter 1), the protoplast of the cell is not in direct connection to the transpiration stream, as semi-permeable membrane barriers exist. The vacuoles therefore, cannot be burst directly by elevated xylem pressure; nevertheless, high water pressure can develop in the cell walls and intercellular spaces and could break cell-cell integrity and so upset the macro leaf structure.

Transpiration from the leaf surface causes a negative pressure within the transpiration stream, which, in turn, draws more water through the plant and across the leaf. Water vapour in the intercellular spaces within the leaf diffuses out of stomatal pores and through a 'boundary layer' of air, into the open, moving air. This diffusion, thus evaporation, is controlled by a concentration gradient of water vapour (vapour pressure gradient), which arises when the water vapour in the air surrounding the leaf is less than at the leaf surface (Larcher, 1995) and is affected by air and leaf temperatures, air humidity and wind speed. The higher the air temperature the greater the amount of water vapour that that air can hold and raising leaf temperature increases evaporation. However, raising air humidity, reduces the vapour pressure gradient, thus less water can diffuse along it. Wind speed influences transpiration by affecting the depth of the boundary layer, as its resistance to water vapour diffusion is directly proportional to its thickness (Taiz and Zeiger, 1998).

Despite the lack of a clear mechanistic link between xylem pressure and cell bursting, elevated humidity does have adverse effects on plants. Roses grown in high relative humidity (RH) showed a shorter post-harvest life than those grown at moderate RH (Torre *et al.*, 2001). The fruit quality and final yield of both tomato (*Lycopersicon*

esculentum) (Lipton, 1970) and cucumber (*Cucumis sativus*) (Bakker, 1984) is also adversely affected by high humidity (both cited in Choi *et al.*, 1997).

Brooks (1953) and Sherf and Macnab (1986) state that, in conditions of extreme atmospheric humidity or if root uptake of water is greater than water-loss through the shoots, cells in young leaves and fruit may proliferate, forming intumescences. However, it is unclear what the mechanistic link might be between high humidity and increased cell proliferation.

It is therefore possible that humidity does play a causal role in either oedema and/or blue spot, either alone, or in conjunction with other environmental factors, such as low temperature.

Temperatures below that at which the plant in genetically programmed to survive disrupt all metabolic and physiological processes (Salisbury and Ross, 1985), although it appears that the most affected area and that which causes most damage if upset, are cell membranes. The currently accepted model for membrane physiology is the fluid mosaic (Singer and Nicolson, 1972). It indicates that the membrane is generally made up of a fluid bi-layer of phospholipids and sterols, with each molecule's hydrophobic tail into the centre and hydrophilic head facing out. Dispersed across this membrane are two types of proteins. Integral proteins, containing some or all hydrophobic amino acids, are tightly bound within the membrane, some only penetrating into the lip bi-layer, while others extend all the way through. Peripheral proteins are more loosely bound to the surface of the membrane.

The fluidity of the membrane is dependant on the ratio of saturated fatty acids (SFA's) to unsaturated fatty acids (UFA's), as SFA's cannot maintain their fluidity at low temperatures (Salisbury and Ross, 1985). In chill-sensitive plants (those with a higher proportion of SFA's), membrane lipids solidify at a critical temperature dictated by the SFA:UFA ratio and the fluid membrane transforms into a solid-gel state. This results in cracks and channels forming in the lipid bi-layer and disruption in enzyme activity, leading to ion and solute leakage.

Some plants are able to develop 'frost tolerance', which involves increasing the proportion of UFA's or sterols in the membrane, allowing the membrane to remain functional. Giving

the plant a period of slowly decreasing temperature, known as 'hardening –off' in horticulture, allows these changes to take place.

This chapter aims to confirm the literary evidence that elevating humidity cannot trigger either oedema or blue spot. It will also investigate the effects of abruptly changing humidity on these conditions and coupling elevated humidity with low temperatures.

METHODS

Plant Maintenance

6cm pots were filled with planted with 2:1 vermiculite:perlite mix. Each coriander capsule (variety Santo, untreated) was split by applying gentle pressure and one half was sown in the centre of each pot.

The plants were maintained in a growth room set at 20°C and 8 hours dark / 16 hours of light (8 PAR) and the medium was kept moist by watering from the base. To provide necessary nutrients, ½ strength Long Ashton Nutrient Solution (see section 2.2.2) was provided once a week at a rate of 0.5l per 20 pots.

Long Ashton Nutrient Solution

NH_4	3mM	Mo	0.00175 mM
PO_4	2 mM	K	4.05 mM
Na	0.015 mM	NO_3	12 mM
B	0.25 mM	Mg	1 mM
Mn	0.002 mM	Ca	1 mM
Cu	0.0015 mM		

The Role of Elevated Humidity in the Occurrence of Oedema and / or Blue Spot - Experiment 1

21 pots were prepared and maintained for as outlined in section 3.2.1 above, placed in the propagator tray and subjected to the following treatments:

No propagator lid (atmospheric humidity – average 67%relative humidity (RH))

Propagator lid, vents open (elevated humidity - average 86% RH)

Propagator lid, vents closed (very elevated humidity – average 90% RH)

The propagator trays were placed in a Latin square.

As a gauge of physiological progress, the number of days for each plant to emerge (hypocotle emergence through surface of growth medium) and to reach the 2 leaf and 6 leaf stages was logged. The data generated was subjected to an ANOVA to test its significance.

The Role of Elevated Humidity in the Development of Oedema and / or Blue Spot - Experiment 2

Plants were prepared as described in section 2.2.1 and subjected to the following treatments:

1. No propagator lid (control humidity - average 63% RH)
2. Propagator lid, vents open (elevated humidity - average 83% RH)

The propagator trays were placed in a Latin square.

The number of days for each plant to emerge and to reach the 2 leaf stage was recorded, as was the fresh weight of plants at 35 days after sowing and dry weight after 3 days in a drying oven (set at 80°C). The data generated was subjected to an ANOVA to test for significance between both replicates and treatments. A Tukey pairwise comparison was also carried out to provide a more detailed statistical diagnosis.

The Role of a Step Change in Humidity on the Development Of Oedema and / or Blue Spot

Plants were prepared as described in section 2.2.1 and subjected to the following treatments:

1. Propagator lid on for 35 days and removed for 10 days
2. Propagator lid off for 35 days and replaced for 10 days
3. Control - no propagator lid (average 63% RH)
4. Control propagator lid, vents open (average 83% RH)

Trays were placed in a Latin Square layout.

The propagator lids were removed/replaced 35 days after sowing. The new treatment was left for 10 days, before the fresh weight of plants was taken. Dry weights were taken after 3 days in a drying oven set at 80°C. The data generated was subjected to a General Linear Model ANOVA to test for significance between both the replicates and the treatments. A Tukey pairwise comparison was also carried out to provide a more detailed statistical diagnosis.

The Role Of a Step Change in Temperature on The Development of Oedema and / or Blue Spot Under Two Levels of Humidity

Plants were propagated as described in section 2.2.1 and subjected to the following treatments:

1. Propagator lid on, vents open. Moved to 10° growth room.
2. No propagator lid. Moved to 10° growth room.
3. Control - no propagator lid (average 63% RH). Remained in 20° growth room.
4. Control propagator lid, vents open (average 83% RH). Remained in 20° growth room.

Trays were placed in a Latin Square layout.

All plants in all treatments were germinated and maintained in the 20° growth room for the first 30 days, then the treatments were moved to an alternative growth room, where the temperature was set to 10°C, but other conditions remained constant. The new treatment was left for 10 days, before being returned to the 20°C growth room for 10 days. The fresh weight of the aerial parts of the plants was then taken, then dried in an oven set at 80°C for 3 days before dry weights were taken.

Use of a Pressure Chamber to Elevate Xylem Pressure

The pressure chamber unit was attached to a 7 litre pressurised cylinder filled with air and was utilised in the 0-20 range.

A leaf specimen over the two-leaf stage, was excised from the plant, close to the rosette and the fresh weight measured, before being pressure stressed.

The leaf was inserted, petiole-inwards, through the hole in the vessel lid. The cut petiole was then located in the split bung and held in place with Blu-Tak® (see figure 2.1).

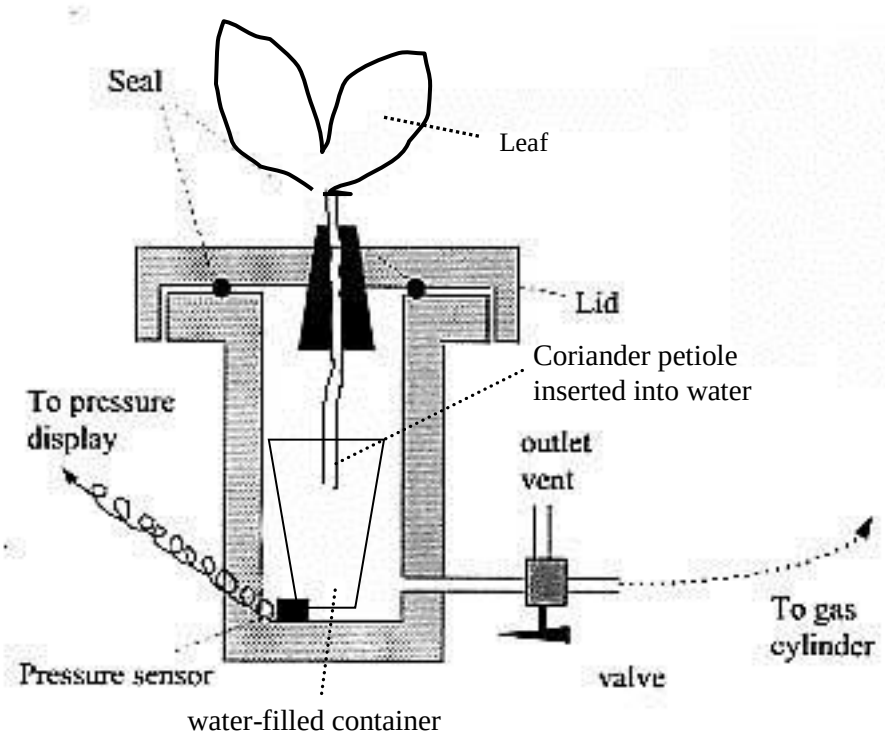


Figure 2.1: Illustration of the mechanism of the pressure chamber when used to elevate xylem pressure.

The bung was then very carefully fitted into the indentation in the lid and the lid was securely screwed onto the pressure vessel, ensuring that the petiole dips into a beaker of water placed inside the vessel. The flow-rate knob was opened just enough to allow the pressure to increase slowly. A constant pressure of between 10 and 20 bars, was maintained in the vessel for at least one hour.

After the hour, the pressure was released, the leaf removed from the vessel and immediately re-weighed to prove water had been forced into the leaf tissues. The leaf was then stood in a container of ½ strength Long Ashton Solution for up to 76 hours. Any

sign of oedema symptoms were recorded at any stage. The process was repeated 12 times.

RESULTS

The Role of Humidity in the Occurrence of Oedema and / or blue spot - Experiment 1

Measurements of plants in the high humidity environment (RH=90%) were abandoned before the 6 leaf stage as the plants had rotted. The first moderate humidity replicate (RH=83%) was also abandoned before the 6 leaf stage could be measured, as the lower leaves were dying off and the plants started to bolt; this was not repeated in the later 2 reps. The 6 leaf stage results are therefore an average of 2, rather than 3 treatments.

The graph in figure 2.2 shows that the plants in the high humidity (closed vent) environment took significantly longer to reach recorded stages than both the control plants (+1.4 days to emergence (P=0.002) and +4.55 days to reach the 2 leaf stage (P=0.000)) and the open vent treatment (+1.79 days to emergence (P=0.023) and +4.85 days to the 2 leaf stage (P=0.000)). The plants in the highest humidity environment were also very stunted, the leaf laminae were often less than 1cm across and were also chlorotic and necrotic. Some evidence was seen of both pre- and post-emergence damping off of young seedlings, usually caused by fungal pathogens, such as *Pythium* and *Rhizoctonia* (University of Illinois Extension, 1987; Agrios, 1997; Cornell University, 2006; Clothier, 2006)

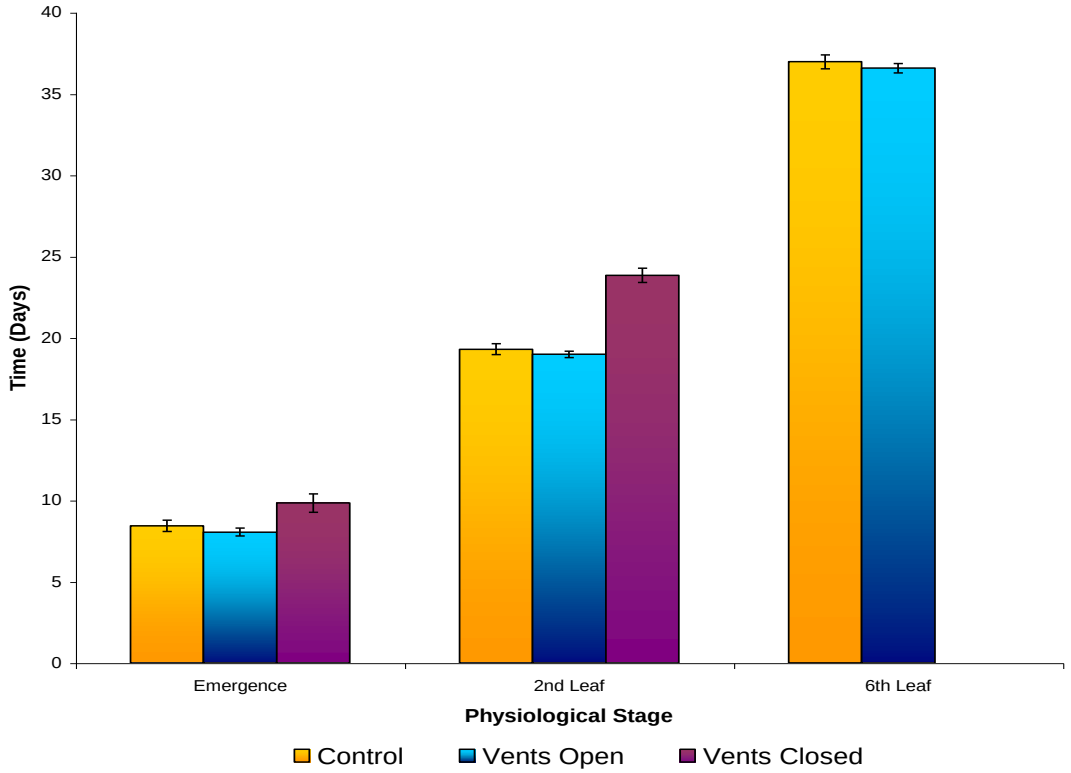


Figure 2.2: Time taken for coriander plants to emerge and reach the 2 and 6 leaf stages at atmospheric, moderate and high humidity treatments. Growth room set to 22°C, 16h day, 8h night. (Mean ± SE; 38 ≥ n ≤59)

There was no significant difference in the times taken for plants grown under moderate humidity reach the recorded growth stages in comparison to the control (emergence: $P_{emergence}=0.75$; $P_{2\ leaf}=0.78$; $P_{6\ leaf}=0.82$).

Despite the significant effects of humidity on plant development, no symptoms of oedema or blue spot were seen on any of the plants in any treatment.

The Role of Humidity in The Development of Oedema and / or Blue Spot - Experiment 2

There was a highly significant difference (P=0.002)in the time taken for the coriander to emerge when grown at atmospheric humidity (average 63%) and elevated humidity (average 83% - figure 2.3). The difference in the time taken to reach the two-leaf stage under the same conditions was also highly significant (P=0.000).

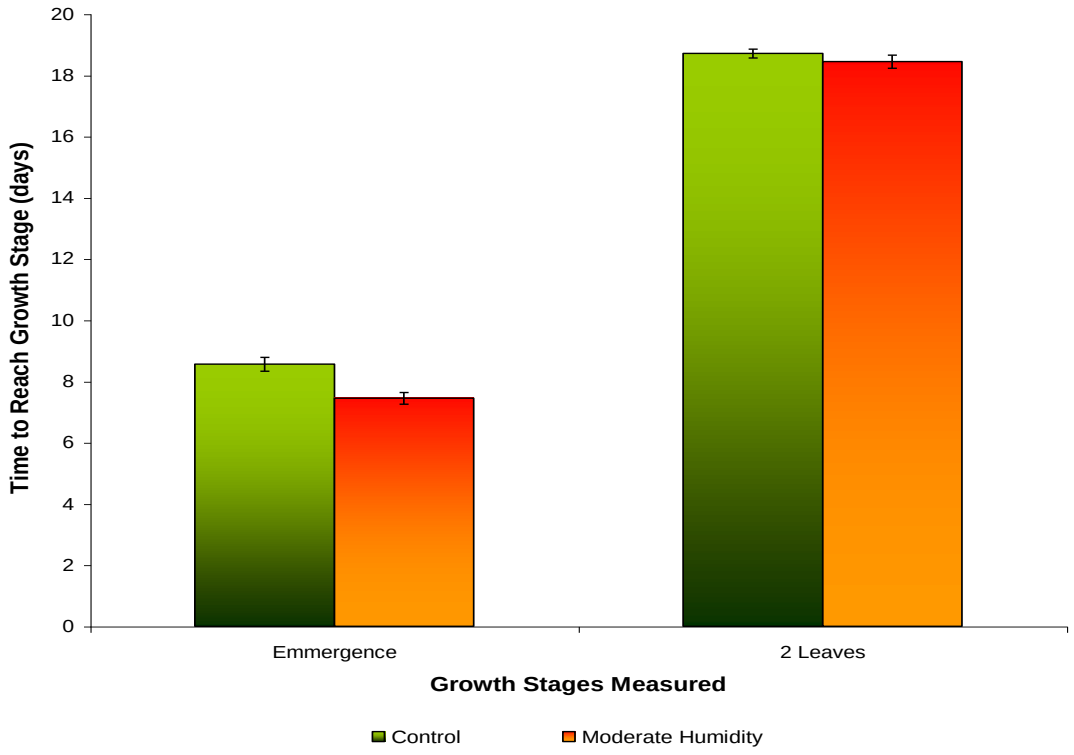


Figure 2.3: Comparison of the number of days to emergence and to the 2 leaf stage of coriander grown at atmospheric humidity (average 63 %) and elevated humidity (average 83%). Growth room set to 22°C, 16h day, 8h night. (Mean ±SE; $n_{63\%\ RH}$ =122; $n_{83\%\ RH}$ =148).

The difference in the ratio between fresh weights and dry weights of coriander grown at two levels of humidity (figure 2.4 – averages 61% and 83% RH) was highly significant (control ratio = 8.32, moderate humidity ratio = 12.73; P=0.000). As the average dry weights were not significantly different (P=0.557), any difference in the ratio was due to the water content in fresh plants.

Despite the clear differences between the treatments, neither symptoms of oedema nor blue spot were seen on any of the plants in any treatment.

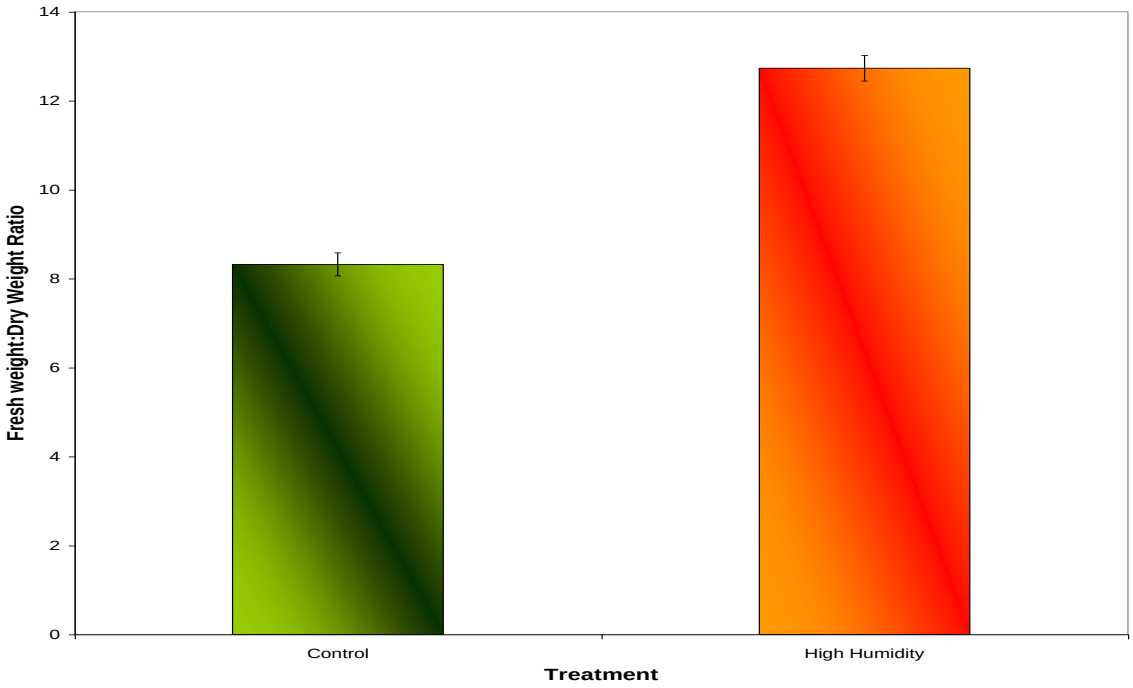


Figure 2.4: Comparison of the fresh weight:dry weight ratios of coriander grown under control level humidity (average 61%) and elevated humidity (average 83%). Growth room set to 22°C, 16h day, 8h night.(Mean ±SE; $n_{Control\ humidity}$ =122; $n_{Moderate\ humidity}$ =148).

The Role of a Step Change in Humidity on The Development of Oedema and / or Blue Spot

The ANOVA test showed that there is a highly significant difference (P= 0.000) in fresh weight:dry weight ratios of the treatments when compared to the control (T1 mean ratio = 8.33, T2 mean ratio = 11.11, C1 mean ratio = 7.11, C2 mean ratio = 12.94). The Tukey comparison showed more precisely that this difference occurs in each of the interactions between treatments and controls.

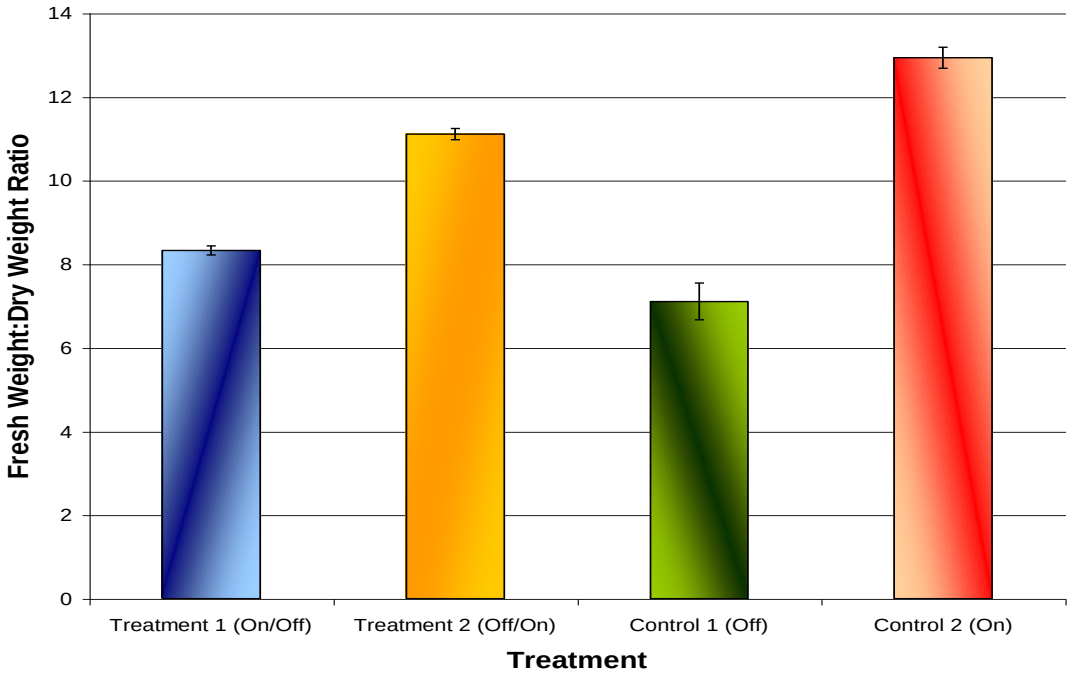


Figure 2.5: Fresh weight to dry weight ratios of coriander plants subjected to an increase or decrease in humidity, in comparison to controls of either elevated or atmospheric humidity. Growth room set to 22°C, 16h day, 8h night. (Mean ± SE; 52≥ n ≤72).

For the first time in the laboratory experiments, one plant in the third replicate showed symptoms of oedema. The affected leaves were excised and taken for fixing and sectioning. No blue spot symptoms were recorded. No other case of oedema or blue spot was seen in this or any other replicate.

The Role of a Step Change in Temperature on The Development of Oedema and / or Blue Spot Under Two Levels of Humidity.

When the data was subjected to an ANOVA test, no significant difference was found (P=0.416) between the replicates, thus data was pooled for the remainder of the analysis.

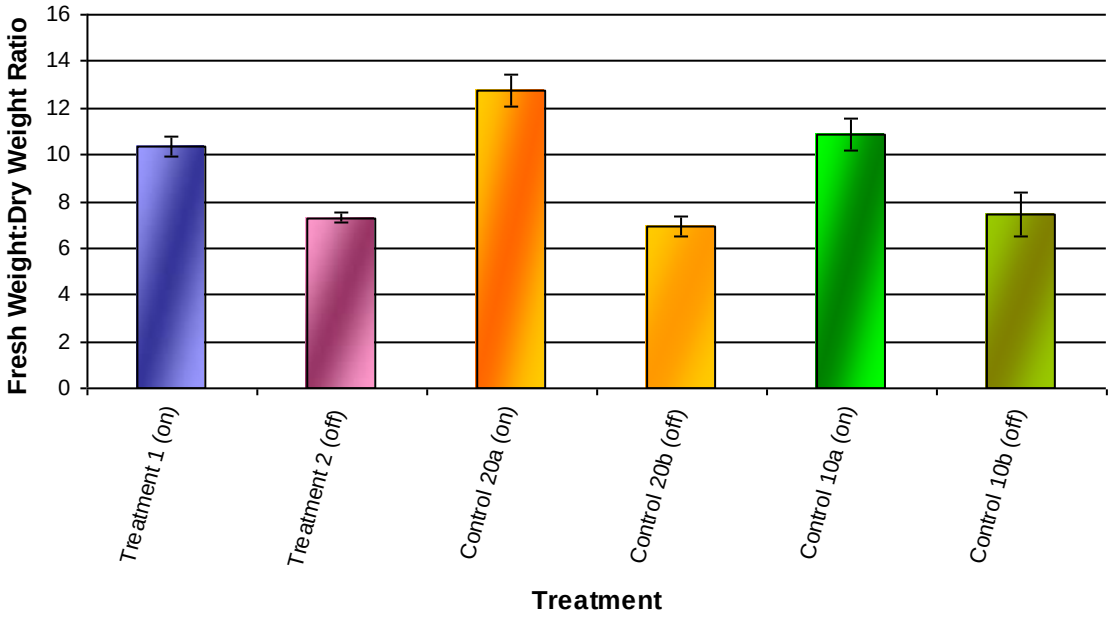


Figure 2.6: Fresh weight:dry weight ratio of coriander after subjection to a step change in temperature in comparison to controls of either 10°C or 21°C. (Mean ±SE; 57≥ $n_{treatment}$ ≤60; 27≥ $n_{control}$ ≤32)

Further analysis showed that subjecting coriander plants to changes in temperature resulted in no significant difference between treatments and controls (P=0.137).

However, under the two different humidities, the mean fresh weight:dry weight ratios (mean $ratio_{T1}$= 10.34; mean $ratio_{T2}$= 7.23; mean $ratio_{C20a}$ = 12.78; mean $ratio_{C20\ b}$ = 6.92; mean $ratio_{C10a}$ = 10.87; mean $ratio_{10b}$ = 7.45) between treatments and controls was highly significantly different (P=000). The interaction between temperature and humidity was also significant (P=0.021).

No symptoms of either oedema or blue spot were seen on any of the plants in any treatment, despite the clear differences between the treatments.

Use of a Pressure Chamber to Elevate Xylem Pressure

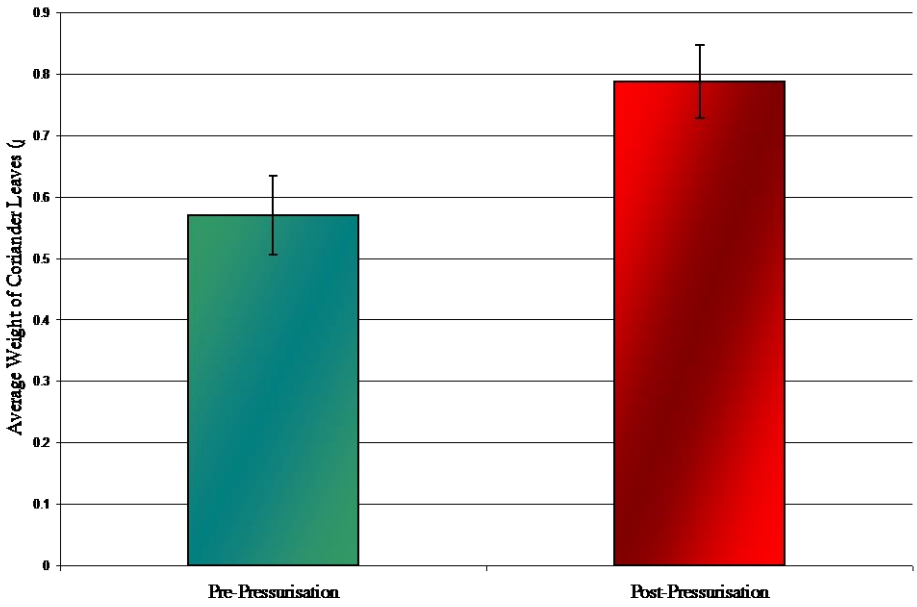
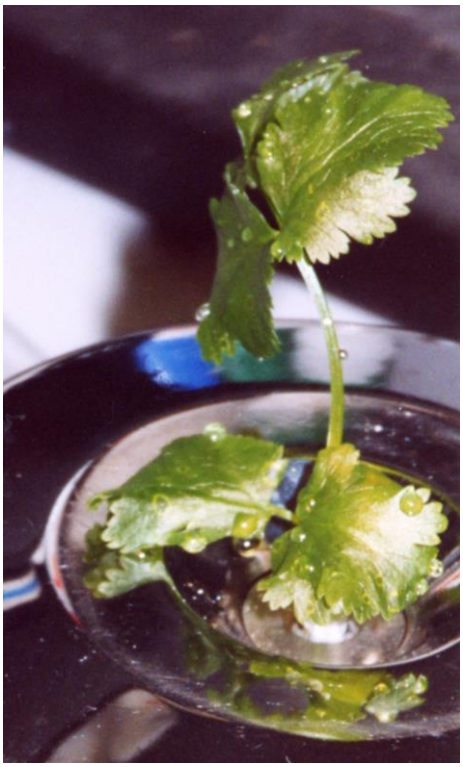


Figure 2.7: Average weights of coriander leaves before and after pressurisation, illustrating that water is being forced into leaf tissues, when being subjected to pressure in the pressure chamber (Mean ± SE; n=12).

The difference in weight of leaves before and after pressurisation ($weight_{before}$ = 0.57g; $weight_{after}$ = 0.79g; figure 2.7) provided clear evidence that water had been forced into the leaf. This is further confirmed by water guttating out of stomatal pores under elevated pressure in the pressure chamber (photograph in figure 2.8).

However, irrespective of the elevation in pressure, no oedema or blue spot symptoms were seen, either during, or in the following 72 hours after pressurisation.



2cm

Figure 2.8: Photograph of a coriander leaf in the pressure chamber. Water is being forced out of the stomata on the leaves, clearly illustrating the elevated pressure to which it is being subjected.

DISCUSSION

It could not be determined if the symptoms seen in the propagator experiments were due only to humidity or rather to water logging or to lack of nutrients. The stunting, chlorosis and necrosis are known symptoms of water logging (Royal Horticultural Society, 2004), as root function is diminished from lack of oxygen and excessive, trapped carbon dioxide. Although water alone was only provided when the trays were dry, in order to ensure that all treatments were given the same amount of nutrients, a specified amount of Long Ashton Nutrient solution was given over a weekly period. As the humidity was high, transpiration was minimal, so little of the water in the solution would have been taken up by the plant, resulting in water logging around the roots. Nutrient absorption would not necessarily have been affected as, in addition to ions are absorbed passively along the concentration gradient, absorption can also be active, against it (Toole and Toole, 1991).

The stunting, chlorosis and necrosis seen in the plants in the high humidity treatments of the first experiment provided clear evidence that coriander is not able to grow successfully in very wet environments. In addition, warm wet environments encourage the growth of certain infections such as species of *Pythium* and *Rhizoctonia* (University of Illinois Extension, 1987; Agrios, 1997; Criddle *et al.*, 1997; Cornell University, 2006; Clothier, 2006).

It is well known that a plant's growth and development will be affected by changing humidity (Hirai *et al.*, 1998; Mortensen, 2000) and this is supported by the results of these experiments. Although one case of oedema was seen amongst the plants in the humidity experiments, this was so rare a case that it cannot be said that it was caused by the environmental conditions.

Temperature also has a direct effect on growth and development (Myster and Moe, 1995; Criddle *et al.*, 1997). Although the change was not significant in this experiment, a small decrease was seen. This is to be expected as the coriander plants were only subjected to the decrease in temperature for 10 days, 18% of their total growing period. The change in temperature experiment was expressly designed to test the grower-based hypothesis that oedema or blue spot occur in periods of temperature fluctuation, which was disproved.

In the propagator experiments, an increase in atmospheric humidity caused transpiration to reduce, allowing water to accumulate in the plant, which in turn increases xylem pressure. Using the pressure chamber allowed xylem pressure to be increased artificially, at the same time allowing the amount of pressure applied to be recorded, which is a very valuable tool and important in showing scientific proof of a cause to the conditions.

The results of the pressure chamber experiment support those of the propagator, in that increasing xylem pressure does not trigger either oedema or blue spot.

CONCLUSIONS

The various parts of this study into the effects of humidity provide clear evidence that it has a direct effect on plant growth and development.

However, with only one of the many plants subjected to an increase in relative humidity developing an oedema lesion and none developing blue spot, it is clear that conditions of

elevated humidity do not trigger either oedema or blue spot, in contrast to the hypothesis presented by growers.

Following further grower suggestions that the disorders develop when the weather 'turns thundery', i.e. when relative humidity increases, an experiment was set up involving an abrupt change in humidity during the growth cycle. However, this treatment did not induce either oedema or blue spot, disproving this hypothesis.

The theory that a sharp decrease in temperature, was also disproved, with neither condition developing when coriander plants were moved into an environment 10°C cooler than the usual growth room.

Overall it can be concluded that neither elevating nor suddenly changing humidity, nor abruptly decreasing temperature induce either oedema or blue spot in coriander.

However, that is not to say that neither humidity nor temperature are involved in triggering the onset of the conditions, when coupled with other factors, such as in the field growing environment. This will be investigated in chapter 3, using meteorological and crop data over the three year period of the project.

SCIENCE SECTION - PART 3

ANALYSIS OF METEOROLOGICAL AND CROP DATA IN RELATION TO SYMPTOM OUTBREAK

INTRODUCTION

The prevalent opinion amongst growers is that oedema and blue spot are physiological disorders, linked to meteorological conditions (outlined in Chapter 1). This is mainly due to anecdotal evidence of the timing of, and weather immediately preceding, outbreaks.

Plants are directly affected by the conditions in their surrounding environment. Many studies record the effect of such parameters as air and soil temperature and relative humidity on various plant species, including cucumber (*Cucumis sativus*) (Bakker, 1984), tomato (*Lycopersicon esculentum*) (Lipton, 1970) and strawberry (*Fragaria grandiflora* Ehrh. cv. Bogyo) (Choi *et al.*, 1997).The soil type and pH are also of vital importance in gaining optimal growth. Most plants have a certain range of soil acidity which they will tolerate, some narrower than others. For example, cranberries (*Vaccinium* sp.) grow on acidic peat bogs (Salisbury and Ross, 1985), while marigolds (*Tagetes* sp.) will thrive on highly calcareous soils (Brickell, 1996). Soils at either end of the structure spectrum can also be problematic. Clays have a high water holding capacity (Moor, 1998), so, as well as becoming quickly waterlogged, they are also late to warm up in the spring. Sandy soils, in contrast, allow water to drain rapidly, leading to drought stress and leeching of nutrients if not monitored. The ideal soil for growing crops is a medium loam, which has a good balance of being well aerated and nutrient rich, without the need for constant monitoring and attention.

In the long term, pinpointing a link between growing environment, meteorological conditions and disease development can allow a forecasting system to be created to warn growers when conditions favour disease. This strategy has been used for a number of pest and diseases, the most well known in the UK being the monitoring of aphid numbers, as this insect is the primary vector of many important viruses that affect arable and other crops, such as seed potatoes and sugar beet. This programme, co-ordinated by Rothamsted Research, allows the forecasting of infestations to enable informed control decisions to be made.

In this study one approach to understanding the causes of oedema and blue spot was a series of laboratory experiments attempting to reproduce the conditions under controlled conditions. In a complementary approach, occurrence of the syndromes in the field was correlated with local meteorological conditions.

With the increasing requirement for traceability of foodstuffs and the use of modern technology during crop cultivation, growers are collecting and computerising more data than ever before and this was exploited to create databases to hold information for this project.

Data from crop-growing records was collected from each grower, along with meteorological data from either the grower themselves, or another organisation located in the immediate vicinity. The proximity is important, as climate can vary considerably, even within small distances. These pockets of clement microclimate are often exploited by growers and farmers, allowing the production of crops in locations which would usually be considered inhospitable. An example of this is Morayshire, Scotland, which is warmed by the Gulf Stream swirling round the northern tip of the UK and into the Moray Firth. Such crops as summer fruits and potatoes are produced here, at a latitude ordinarily considered too northerly.

METHOD

Data Collection

Crop Data

Following discussion with growers, a record sheet was created to include information about crop cultivation which can affect crop growth and development and therefore possibly oedema or blue spot occurrence:

- Was the crop grown in the field or in polytunnels?
- Which variety was planted?
- What was the previous crop?
- When was the crop sown?
- What was the aspect of the sowing location?
- What was the sowing density (kg/ha seed)?
- What was the soil type and pH
- Which chemicals were applied (fertiliser, foliar feed or protection products)?

- What was the irrigation regimen?
- Were any symptoms seen? If so which ones (blue or grey)?
- When were the symptoms first noticed?
- At what stage of crop development were the symptoms first noticed?
- What was the approximate percentage of crop affected?
- Symptoms appeared before which harvest?
- Was crop saleable?

The record sheets were designed to be completed both for crops that developed symptoms and also those which remained healthy through to harvest, enabling a comparison of diseased and healthy crops from the same site.

The record sheets were either posted or e-mailed to participating growers at the start of the growing season, along with a guidance sheet (Appendix 1a) explaining the reasons for the questions, the importance of including data for both diseased and healthy crops and particular points to bear in mind when completing each point. The initial version of the record sheet (Appendix 1b), sent out in spring 2003, only requested recognition of the grey oedema symptoms, because the confusion between the two sets of symptoms was not recognised until samples of symptomatic coriander were being sent to the laboratory. The record sheets were updated in consecutive seasons to reflect changes in data requirement that had arisen from laboratory experimentation and comments from growers (Appendices 1b and 1c). These changes included the differentiation between the grey and blue symptoms and the removal of chemical application data request.

Meteorological Data

Meteorological data for each site were collected either from the grower themselves, or from another organisation in the immediate locality (e.g. Scottish Agricultural Research Institute, Perth).

Data collected included:

- Daily rainfall (mm)
- Minimum and maximum daily temperatures (°C)
- Soil temperature (°C) taken at 10cm at 0900h
- Mean daily wind speed (mph)

- Mean daily relative humidity (%)

Data Processing

Two separate Microsoft Access® databases were created, one for the crop data and the other for the meteorological data. Queries were then raised which cross-referenced relevant data from both tables. This was then subjected to various statistical analyses in order to look at the relationship of certain crop growth data points, such as the date of outbreak and date sown and the association of any outbreak with each meteorological data parameter, either alone or in combination.

RESULTS

Data Analysis

Fourteen growers from throughout England and Scotland contributed to the project, crop data being gathered from eleven, meteorological data from eight and samples from nine. 276 crop records were input onto the database, over the three seasons of the study (March-October 2003, 2004, 2005). Of these, there were 15 outbreaks of oedema reported over four sites, 17 records of blue spot from five sites, 235 healthy and nine with unrecorded outcome. Interestingly, there was no record of crops suffering from both sets of symptoms. When comparing different growing environments and the meteorological conditions, only the crops with equivalent meteorological parameters could be used, often reducing the overall sample size.

The graph in figure 3.1 shows how this pooled data is spread over the three seasons. Oedema decreased over the study period, while blue spot appeared not to, although, as explained earlier, blue spot outbreaks were only recorded towards the end of the season, when the confusion over terms was determined. The author believes that blue spot was more prevalent than this data reveals. In 2003, there were 13 crops recorded as showing disease, 10 of these being recorded as oedema and three as blue spot. In 2004, 14 disease outbreaks were noted, with the number of oedematous reports decreasing to five and blue spot increasing to nine. In 2005, the total number of diseased crops was considerably lower than in previous years, with only five cases of blue spot recorded and no oedema.

Figure 3.2 demonstrates the number of each condition as a proportion of the total number of outbreaks over the growing season. Data from all three periods is combined. Half of all outbreaks were recorded in June, with 16% being oedema and 34% blue spot. Both conditions declined gradually over the following three months, with blue spot disappearing entirely by September and oedema by October, although the latter does make a small re-appearance (6%) in December. These cases were experimental rather than commercial, as growers do not usually have crops growing over the winter.

The same graph also shows the severity of symptoms in relation to this distribution and frequency. The trend in severity of both conditions paralleled that of frequency; i.e. when there are most outbreaks, the severity of the symptoms is worst. In five of the 16 blue spot cases where severity was recorded, over 80% of the crop was affected. All these cases occurred in June. In the other nine cases, under 10% of the crop exhibited symptoms. In the fifteen crops that developed oedema, five were over 50% affected, of which only two were over 80%. The remaining ten crops manifested less than 10% disease. When subjected to a general linear model ANOVA, a significant relationship was revealed between the month of outbreak and the severity in cases of oedema ($P=0.033$) but not blue spot ($P=0.076$).

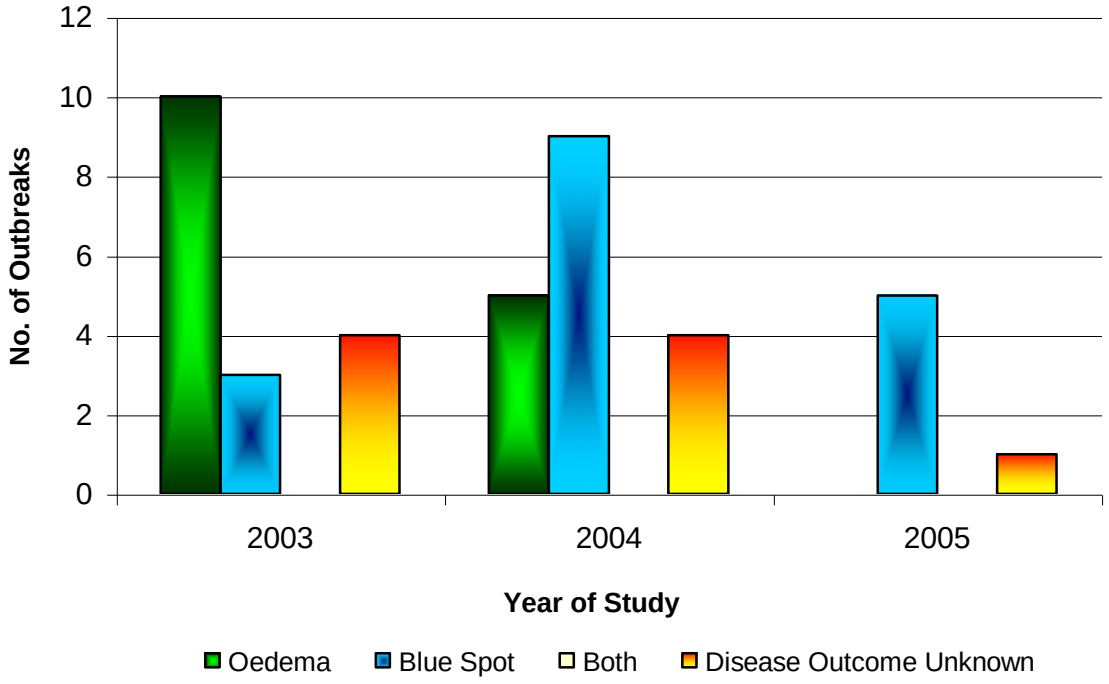


Figure 3.1: Number of reported outbreaks of oedema (n=15; n_{sites}=3), blue spot (n=17; n_{sites}=5), both conditions (n=0) and unknown state of crop (n=9; n_{sites} =3) as a proportion of the total number of crops (n=276; n_{sites}=11).

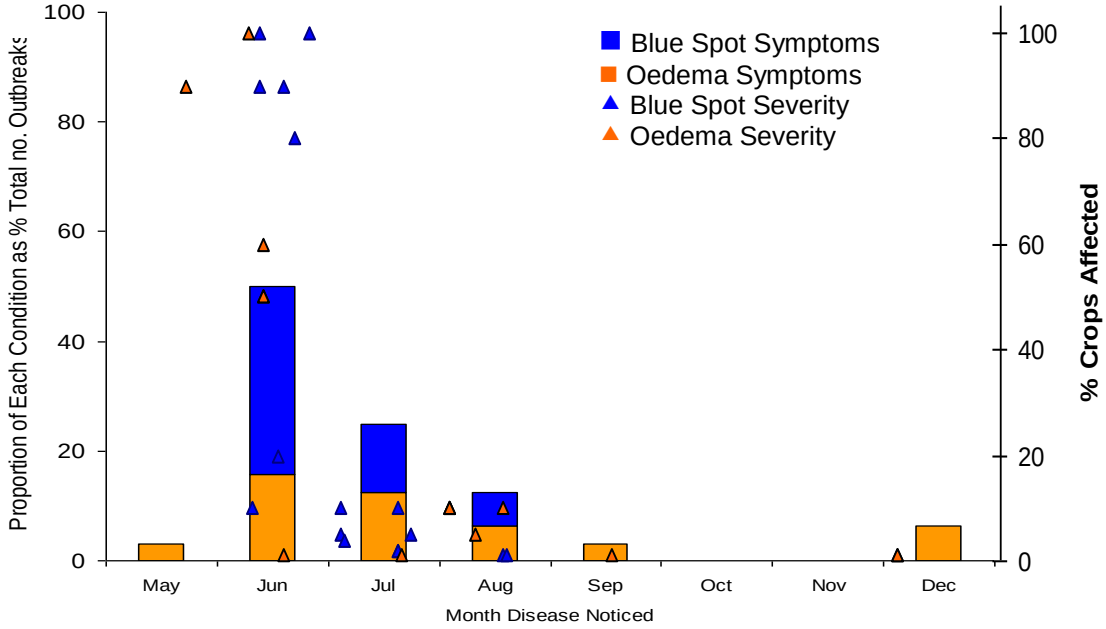


Figure 3.2: Proportion of cases of oedema (■, n=15, n_{sites}=3) & blue spot (■, n=17, n_{sites}=5) as a % of the total outbreaks occurring in each month and the severity of those outbreaks, as the % of the crop showing symptoms (▲oedema, n=15; ▲blue spot, n=16). Data pooled from all three years of the study.

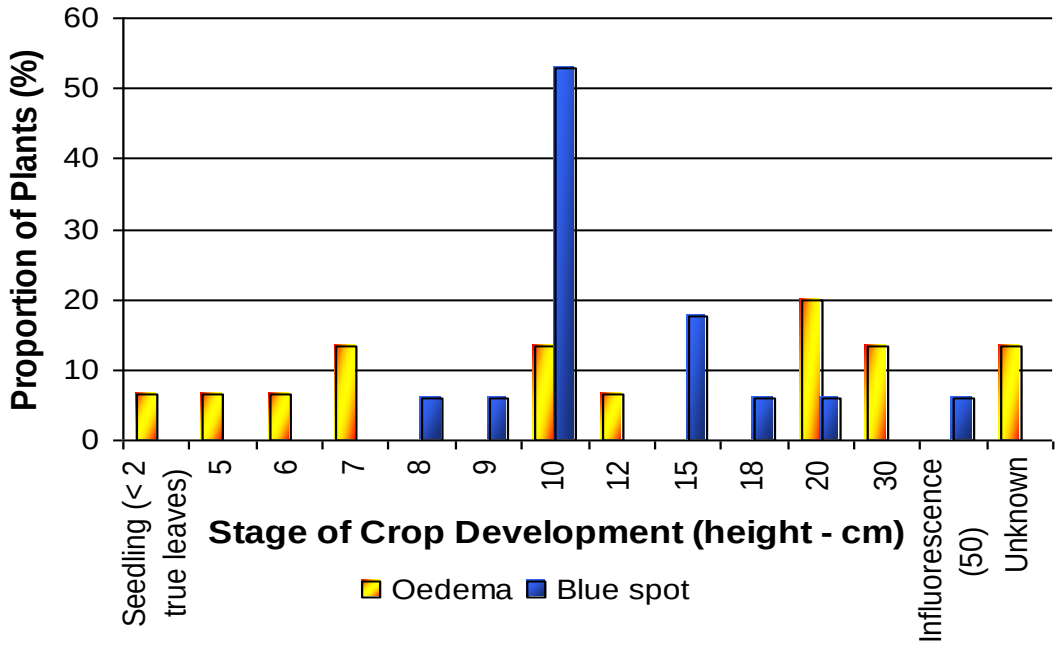


Figure 3.3: Frequency of occurrence of oedema (n=15; n_{sites}=3) and blue spot (n=17; n_{sites}=5) at various stages of plant development, as a proportion of the total number of disease outbreaks. Three year's data pooled.

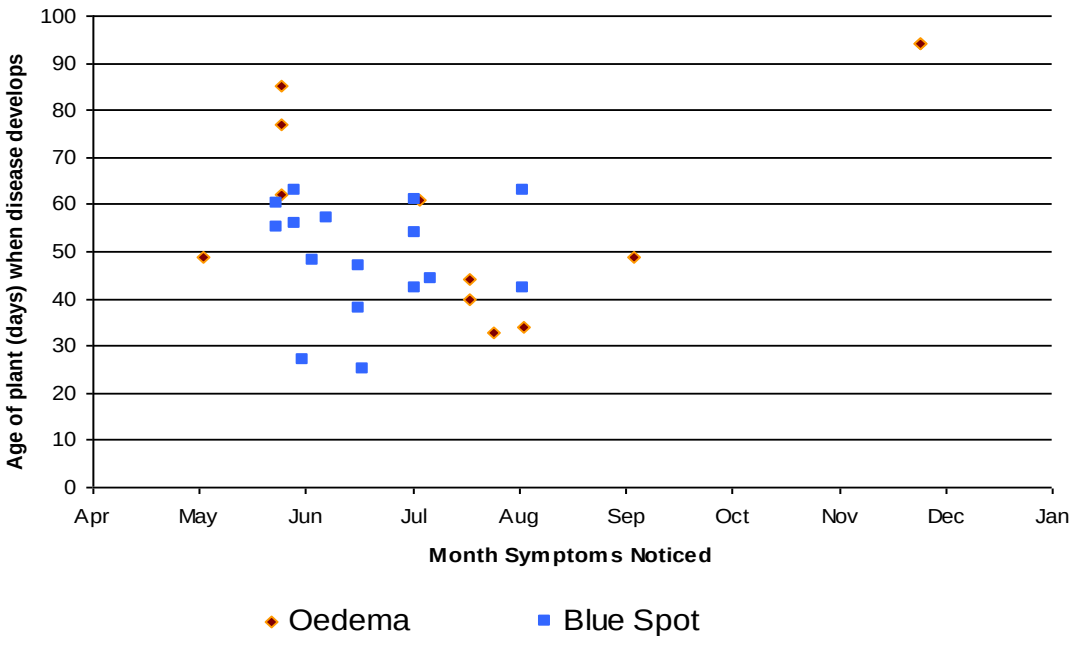


Figure 3.4: Age of plants (days) when outbreaks of oedema (♦; n=10; n_{sites}=3) and blue spot (■; n=16; n_{sites}=4) occurred during the growing season.

When the point of disease outbreak for both oedema and blue spot was analysed in relation to the plant's stage of development (figure 3.3), interesting differences were revealed between the two conditions. Recorded cases of oedema developed at most stages of vegetative growth, with symptoms seen in seedlings (fewer than two true leaves), harvest-stage plants (10-12cm) and those approaching flowering (20-30cm). The number of outbreaks increased slightly over the development period, with one third occurring after 20cm. No occurrences were noted during inflorescence. In plants with blue spot however, the distribution was more varied. Symptoms were not seen before the middle of the vegetative period (8cm), with 53% of recorded outbreaks occurring when the plants had reached 10cm. Outbreaks tailed off as plants got bigger, but nearly 6% were seen during inflorescence. These findings are not as critical as they may first appear, as commercial crops are harvested by the time they have reached approximately 15cm, before flowering.

It was then decided to explore whether the age of the plant at which disease occurs is affected by the time of year. As physiological development depends on environmental conditions, particularly the thermal time (day degrees; Dufour and Guérin, 2002; Struik *et al.*, 2006), plants will take longer to reach a particular developmental stage at the start and end of the season, when the temperatures are lower, than in the middle. If symptom development is dependant on the stage of plant development rather than age or any other variable, a sigmoid curve should be produced, with the oldest plants affected early and late in the season and the youngest in the summer months, however this was not seen (Figure 3.4). In oedema, , there was a difference of 61 days between the oldest and youngest plants that develop the condition and in blue spot data, the maximum range of ages is 38 days.

Classifying soil type is subjective, with the names attempting to reflect the proportions of each constituent present, which of course, can vary significantly over quite small areas. When the effect of soil type on the number of outbreaks was investigated for any link between the heavier, more water-holding types and cases of oedema and blue spot, weak patterns emerged (figure 3.5). 92% blue spot outbreaks occurred on sandy clay loam, with the other 8% seen on medium-heavy loam. No outbreaks were seen on lighter soils.

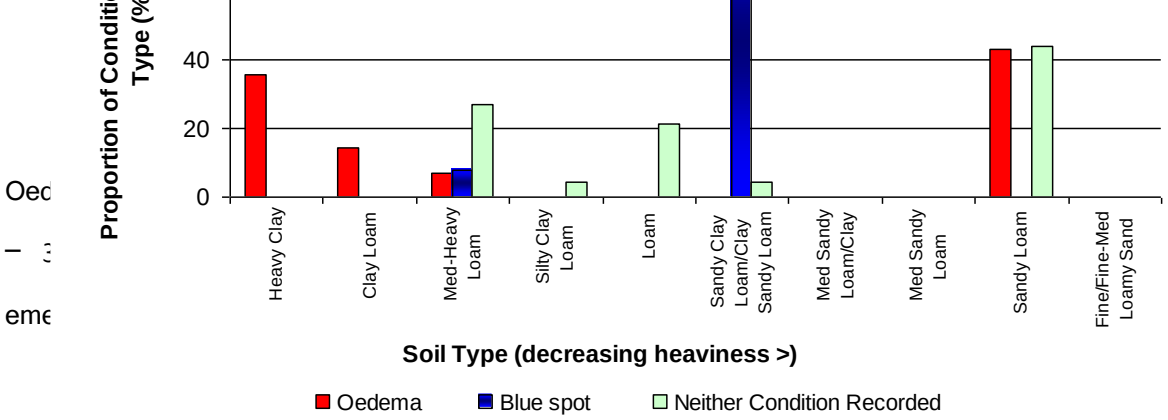


Figure 3.5: The proportion of each condition found in particular soil types (n_{oedema}=14; $n_{blue\ Spot}$=13; $n_{neither\ condition}$ =71; $n_{data\ not\ provided}$=178 - not shown)

Very few records of the soil pH were received. All those that were, ranged from pH5.8 to pH 7.2, regardless of whether any condition was seen or not (data not shown).

Analysing certain variables of meteorological data over the life of the crops in relationship to the timing of any disease outbreaks should have enabled any direct relationship to become clear.

The graphs in figures 3.6, 3.7 and 3.8 show the occurrence of oedema and blue spot plotted against meteorological parameters (mean daily mean relative humidity (RH - %), mean daily mean soil temperature (ST - ºC), mean maximum daily temperature (T_{max} - ºC), mean minimum daily temperature (T_{min} - ºC) and mean daily mean temperature ($\check{T}$ - ºC)) calculated over the life of the crop.

These figures were worked out from the *available* meteorological data provided, i.e. some sites provided daily data, while others only provided daily data once a week. In some cases, the recording equipment malfunctioned for short periods, so data could not be logged. It was felt, however, that there was enough data to give an accurate picture of the meteorological trends over the life of the crop. It should also be noted that the mean daily temperatures were tallied by averaging the daily maximum and daily minimum.

The minimum T_{min} at which oedema occurred was 2.1ºC and the maximum T_{max} was 21.9ºC and ST ranged between 9.4ºC and 18.2 ºC. The variation in RH at which oedema was seen was quite narrow, between 72.7% and 80.4%. The range of mean daily mean temperatures occurring when blue spot developed was smaller than for oedema, with T_{min} being 7.0ºC and T_{max} 20.8ºC and also for ST (14.3ºC to 17.7ºC), although RH was similar (72% to 80%).

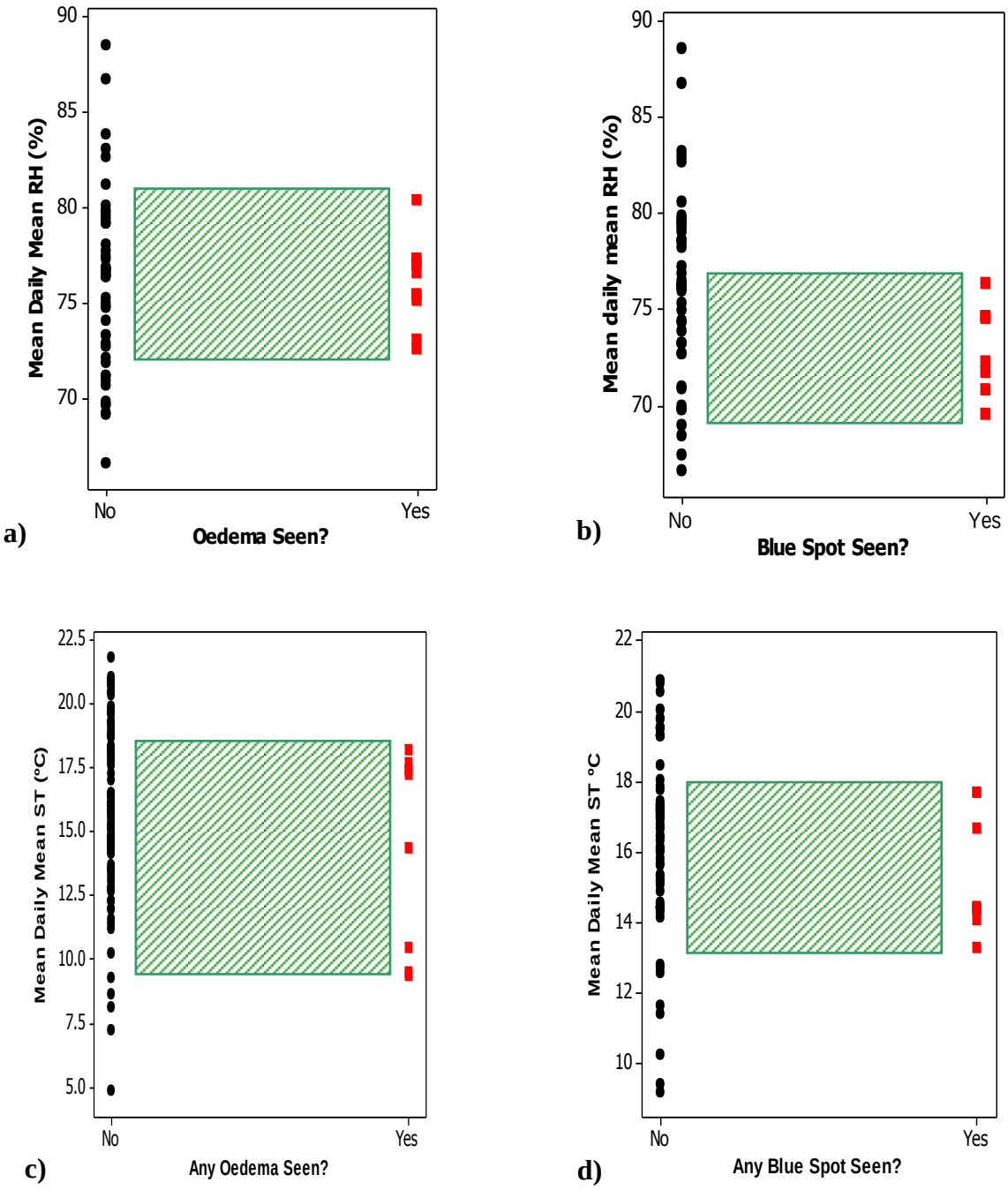


Figure 3.6 a–d: Mean meteorological variable recorded over the life of crops with (■) and without (●) symptoms.

a) the effect of mean daily mean relative humidity (RH %) on oedema and b) on blue spot.

c) the effect of mean daily mean soil temperatures (ST ºC) on oedema and d) on blue spot.

Their distributions indicate that, between certain points (indicated by the hatched area ▨), plants are *more likely* to develop the conditions, but that there is no exact point at which disease can be expected.

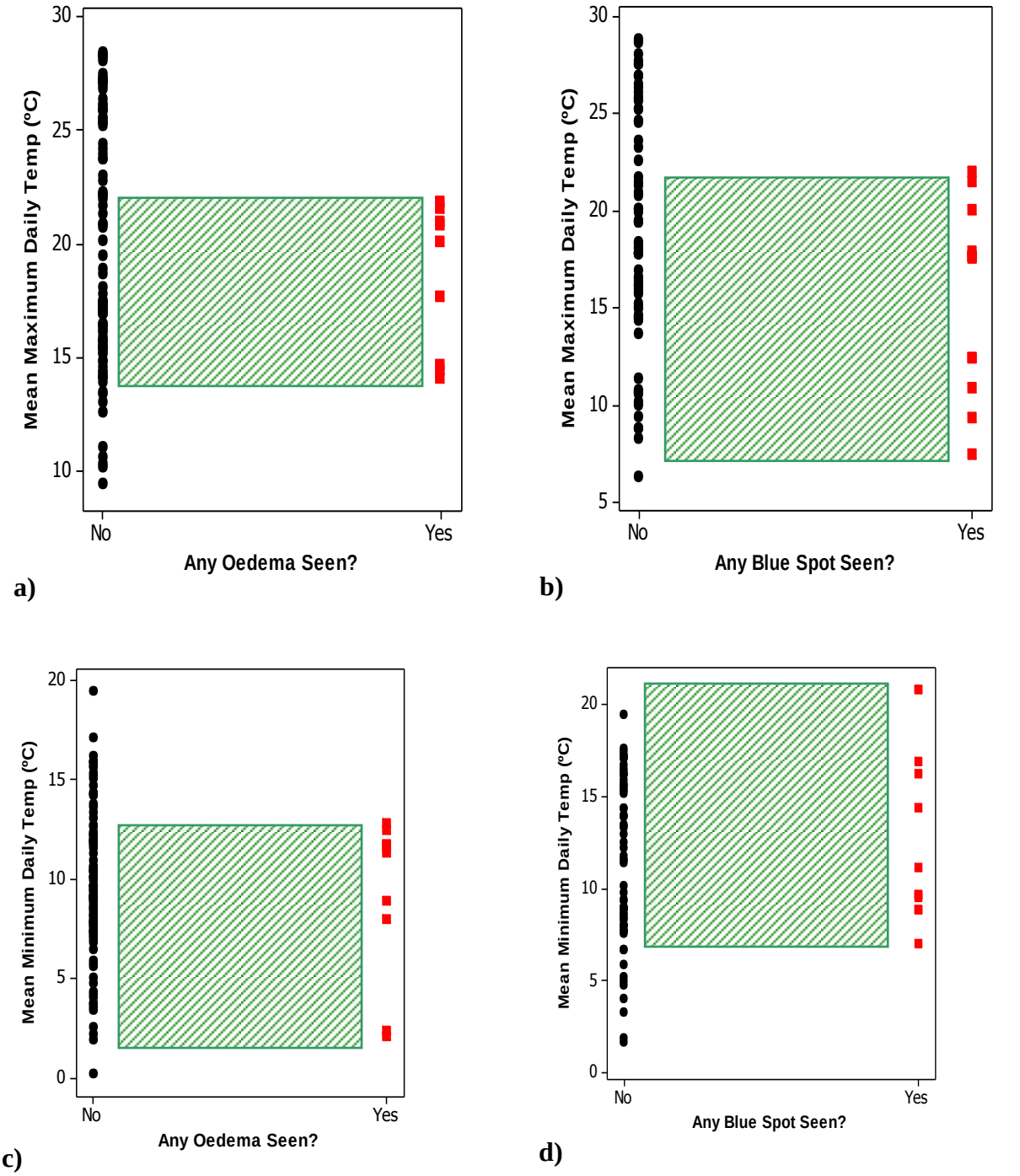
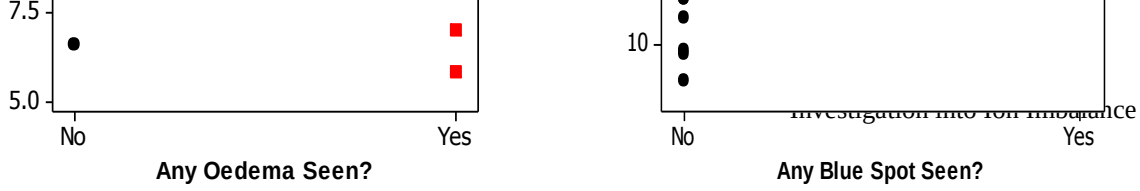


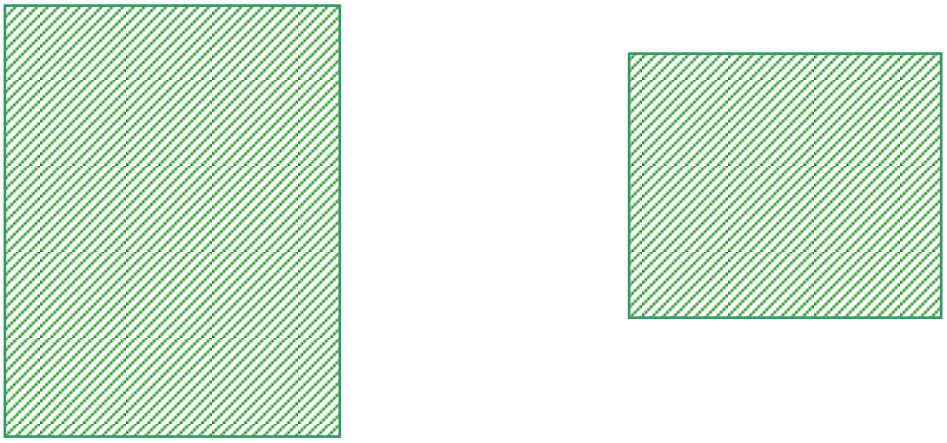
Figure 3.7 a–d: Mean meteorological variable recorded over the life of crops with (■) and without (●) symptoms.

a) the effect of mean maximum daily temperatures (T_{max} ºC)on oedema and b) blue spot.

c) the effect of mean minimum daily temperatures (T_{min} 0C)on oedema and d) blue spot.

Their distributions indicate that, between certain points (indicated by the hatched area ▨), plants are *more likely* to develop the conditions, but that there is no exact point at which disease can be expected.





a) **b)**

Figure 3.8a & b: Mean meteorological variable recorded over the life of crops with (■) and without (●) symptoms.

a) the effect of mean daily mean temperatures (T̆ ºC) on oedema and b) blue spot. Their distributions indicate that, between certain points (indicated by the hatched area), plants are *more likely* to develop the conditions, but that there is no exact point at which disease can be expected.

There is considerable heterogeneity of the variances for both the disease-free and the diseased data for both oedema and blue spot. From this, two things can be inferred; firstly, the heterogeneous variances are such that neither ANOVA nor covariance analysis are suitable. Secondly, the relationships, instead of being linear, are quadratic (both the within-group and the between-group variances are clearly different), which would require more complicated statistics, such as quadratic discriminant analysis (QDA). However, the presence of quadratic variables within the QDA, also discounts this method of analysis from being of any use.

Even without statistical analysis, further information may still be gleaned from the graphs. The overlap of the values of the meteorological variables between the two conditions, rather than any direct differences, indicates that no one variable alone is linked directly to outbreaks of either oedema or blue spot. Instead, it can be said that at certain values, blue spot and oedema are *more likely* to occur. This is illustrated by the hatched areas on the graphs, when there is both disease and no disease at the same values. However, the actual probability of seeing disease cannot be deduced from these figures.

DISCUSSION

Overall, studying the effects of the meteorological data on crop growth and development has not lead to any major breakthrough in finding a cause for either oedema or blue spot. However, any knowledge of crop physiology is advantageous for optimising crop production.

As discussed previously, the main problem with the development of blemishes on the leaves is the reduction in saleability. In the cases of oedema and blue spot recorded for this study, there did not appear to be any pattern to whether the severity of disease affected saleability. For example, for one field judged 90% diseased and another as 100%, the growers were able to sell some of the crop, while in other cases, with 50% symptomatic plants, the crops were unsaleable. This is most likely due to the target market. If leaf herbs are to be sold whole to the consumer, they must be blemish free, where as if they are to be processed and used in food products, then blemishes are less of a problem, as long as they will not cause illness.

From the grower's point of view it is important to note that the highest number and the most severe blue spot outbreaks occur in June. Combining this data with the fact that over

50% of blue spot cases occur at the 10-leaf stage of coriander development, the grower may be able to stagger sowing dates to minimise the losses from this condition. This prediction is less solid for oedema, as the frequency is more even over the season and developmental stages. It can be said however, that the condition is much more severe in May and June.

Although pH did not appear to have any bearing on conditions frequency, soil type may be a contributing factor. Over 90% of blue spot cases were seen in sandy clay loam soils, the type which is favoured by growers of most crops for being the ideal growing medium. In addition to having an optimum balance between its water-holding capacity and good drainage and aeration, it also holds has good supplies of nutrients and is of a neutral pH. The soil structure is such that that can be worked without damage in most weather conditions.

The link between soil type and oedema outbreak is less clear, as outbreaks were logged over the range of soil types. However, over half the outbreaks occurred in the clay soils, indicating that these heavier conditions may be associated with the disease.

The ranges of mean temperatures at which blue spot and oedema were seen, varied. The mean daily mean minimum temperature of oedema was nearly 7ºC lower than for blue spot, although the mean daily mean maximum temperature one degree higher. The difference between the lowest mean daily mean soil temperatures was nearly 5ºC different, oedema again occurring at the lower temperature. The mean daily mean relative humidities at which blue spot and oedema occur are very similar – 72% to 80% and 73% to 80% respectively.

Graphically representing the meteorological data allows immediate understanding of a potentially complicated situation. Firstly the graphs show clearly the high heterogeneity of values within all data sets, allowing certain statistical tests to be discounted. It is also clear that there is no area within any meteorological parameter that is distinctly different between each disease outcome (disease or no disease). If this circumstance were to have arisen, then there would have been values on the graph at which only disease would have been recorded.

The trend exhibited in these graphs, that at certain values of a parameter, blue spot and oedema are *more* (but by no means definitely) likely to occur, would indicate that another variable, to be termed an 'x-factor', is the trigger. It is this x-factor which is under closer influence of the meteorological conditions, thus will elicit the symptoms, subject to certain meteorological conditions being within a critical range. For example, certain bacterial and fungal infections occur more readily and at higher frequencies in damp conditions (Agrios, 1997) and most pathogens have a specific temperature range in which they are viable.

In the case of blue spot, this would also tie-in with the findings of the outbreak/stage of disease development data. If the x-factor is an inoculum, then not only would specific conditions be required, but a specific developmental stage of the host is preferred for the infection process, i.e. it can be supposed that the meteorological conditions are optimal in May/June, and plants of the 10-leaf stage are the ideal hosts. The same theory cannot be proposed for oedema, as disease is seen at most plant developmental stages.

Evaluation of Data Collection System

The theory behind the data collection was sound and using Access® databases provides a powerful tool for cross-referencing and extracting the required data. There are however, a number of points that need to be taken into account during their set up and use; data entry needs to be precise, as the queries will not tie in any spelling or numerical errors with their intended data group. Care is also required when creating the queries. Correct terminology must be used when building expressions, otherwise a) incorrect data is extracted or b) thousands of incorrect records are produced. These complications can usually be avoided with training and practice.

Although the record sheets were created in liaison with two of the participating growers, others who had initially agreed to participate either failed to return any data or samples, or only supplied minimal data or plant tissue. To reduce this occurrence, any form of records that the growers kept were accepted and input onto the system as best as possible and growers were also contacted as often as possible This did result in large gaps in information, as well as few samples of oedema and blue spot being sent to the laboratory. Most disappointingly, it cannot be assumed that the number of outbreaks recorded provides a true picture of the occurrences of oedema and blue spot coriander crops in the UK.

CONCLUSIONS

Although it is certain that not every outbreak of either oedema or blue spot were recorded in the study, there is some evidence that oedema has reduced in frequency over the last few years, although the reasons for this are not clear. This same conclusion cannot be made for blue spot, as the failure to record incidences in the first half of 2003 has caused the results to be skewed. If a reduction continues, then the importance of the conditions to the herb industry will also decline, meaning that finding the cause becomes less necessary.

It cannot be definitively concluded that soil type does or does not have a direct effect on the occurrence of either oedema or blue spot. However, it can be said that oedema is more likely to occur in heavier, clay and, surprisingly, some lighter sandy soils. No differences in soil pH are seen between crops which develop disease and those which do not.

Meteorological conditions are not directly responsible for either oedema or blue spot however, they appear to contribute to the likelihood of disease occurring. This invokes the hypothesis that there is another variable – an 'x-factor'- which triggers the symptoms and whose development and virulence require particular meteorological and environmental conditions. The presence of a pathogenic cause will be investigated in chapter 6, while the alternative theory, the association of ion imbalance with oedema and blue spot, will be studied in the following chapter.

SCIENCE SECTION - PART 4

INVESTIGATIONS INTO ION BALANCE

INTRODUCTION

Of the three initial hypotheses of the causes of oedema and blue spot of coriander, the first (looking at the effect of certain meteorological variables on the incidence of oedema and blue spot) did not reveal a direct link. However the presence of an additional variable was proposed, which is more closely under the control of environmental conditions. This would tie in with the second theory that a calcium imbalance results in a weakened intercellular structure, leading to the conditions described, as ion absorption and transpiration is dependent on water uptake and flow.

There are a number of conditions, such as blossom-end rot of tomato (*Lycopersicon esculentum*), in which calcium deficiency can seriously disrupt growth and development and it may be that either oedema and/or blue spot are such conditions.

Although a micromolar amount of calcium remains free within the cytosol to carry out various metabolic and signalling activities (Taiz and Zeiger, 1998), the majority is stored, mainly bound within the cell wall (particularly in the middle lamellae, where it is thought to aid cell-to-cell adhesion) or sequestered in organelles, such as the vacuole where the concentration can reach tens of millimoles (Clarkson, 1984; cited in Rengel, 1992).

Calcium is also required for cellular processes and, although only small amounts are required, the supply must be uninterrupted (Gilroy *et al.*, 1993). Calcium has low mobility and is moved predominantly with the transpiration stream in the xylem. In slowly transpiring organs (e.g. fruits) and in periods of low water uptake and movement (i.e. periods of drought or high humidity), transpiration is decreased, so calcium requirement can outstrip supply and deficiency may occur. This can seriously disrupt growth and development and is most apparent in the growing tips - leaf and root meristems and maturing fruits - where growth and development is most rapid, but transpiration is low. Calcium absorption can be disrupted by the presence of high concentrations of potassium (Srømme *et al.*, 1994; Marschner, 1995; Bar-Tal and Pressman, 1996; both in Torre *et al.*, 2001).

Environmental conditions affect xylem transport by influencing the rate of transpiration and ion concentration may therefore be different under elevated humidity. If oedema or blue spot also occur following an increase in humidity, as is believed by the growers, a causal link might be suggested. Analysis of the ion status of tissue with these conditions, in comparison to that of healthy tissue was used to test this hypothesis.

In addition, coriander was grown under hydroponics, in media containing different concentrations of calcium in order to see if these conditions induce either set of symptoms.

METHODS

Investigations into the Effect of Removing Calcium from the Growing Medium

Seeds were sown and plants maintained as outlined in section 2.2.1. At the two to three leaf stage, plants were removed from the pots, the growing medium knocked off and transferred to the hydroponic vessel. The plants were held in place by encasing the stems in sponge bungs. The vessels of the control plants were filled with half-strength Long Ashton solution (see section 2.2.2), while the treatment vessels were filled with half-strength Long Ashton solution modified by withholding $Ca(NO_3)_2$ (solution 8) and doubling the amount of KNO_3 (solution 6). The Long Ashton solutions in both sets of vessels were changed every other day and aeration was not required. 25 days after transfer, the shoots (just below the rosette) were harvested and weighed, before being placed in a drying oven (set to 80°C) for 3 days. The dry weight was then taken. The data was subjected to a one-way ANOVA in order to test for significance.

Investigations into the Effect of Varying the Calcium Concentration in the Growing Medium

Seeds were sown and plants maintained as outlined in section 2.2.1 and transferred to hydroponics vessels as described in section 4.2.1 above. One set of plants were deemed controls and the vessels filled with half-strength Long Ashton solution (see section 2.2.2). A second set, grown with low calcium, were provided with a solution of half strength Long Ashton modified by reducing the amount of $Ca(NO_3)_2$ added to 0.1mM. The final set of plants were grown with high calcium, where $Ca(NO_3)_2$ was increased to 10mM. The Long Ashton solutions in all sets of vessels were changed every other day and aeration was not required. 25 days after transfer, the shoots (just below the rosette) were harvested and weighed, before being placed in a drying oven (set to 80°C) for 3 days. The dry weight was then taken. The data was subjected to a one-way ANOVA in order to test for significance.

Ion Chromatography

A Dionex DX-120 Ion Chromatograph (Dionex Corporation, CA, USA) was used to analyse the inorganic ions. The cation detection system required an IonPac CS12a 4x250mm column and a CSRS-Ultra 4mm suppressor.

The eluent required for cation analysis was made up a 4mM solution of H_2SO_4.

Leaf samples were stored frozen in 1.5ml tubes. When required, they were defrosted and the leaf tissue crushed using a sterile homogenising stick. The sample was then centrifuged at 13,000rpm for 10 minutes.

The liquid supernatant was then removed by pipette to another 1.5ml tube and re-centrifuged at 13,000rpm for 5 minutes. 950µl eluent was then added to 50µl of the supernatant, giving a 1ml 1:20 dilution. Half of this was then injected into the machine and the sample run as per machine instructions.

The peak area readings for each set of standards and samples were averaged.

The molarity of the samples was calculated by taking a ratio of the [sample ion]:[standard ion]. The molarity was then multiplied by 20 to compensate for the 1:20 dilution carried out in preparation of the sample.

The data for each cation was subjected to a separate General Linear Model ANOVA to test for differences in each cation between each diseased sample and the control. The cation concentrations from each location were then compared with Tukey pairwise comparisons to detail where the significances lay.

RESULTS

Investigations into the Effect of Removing Calcium from the Growing Medium

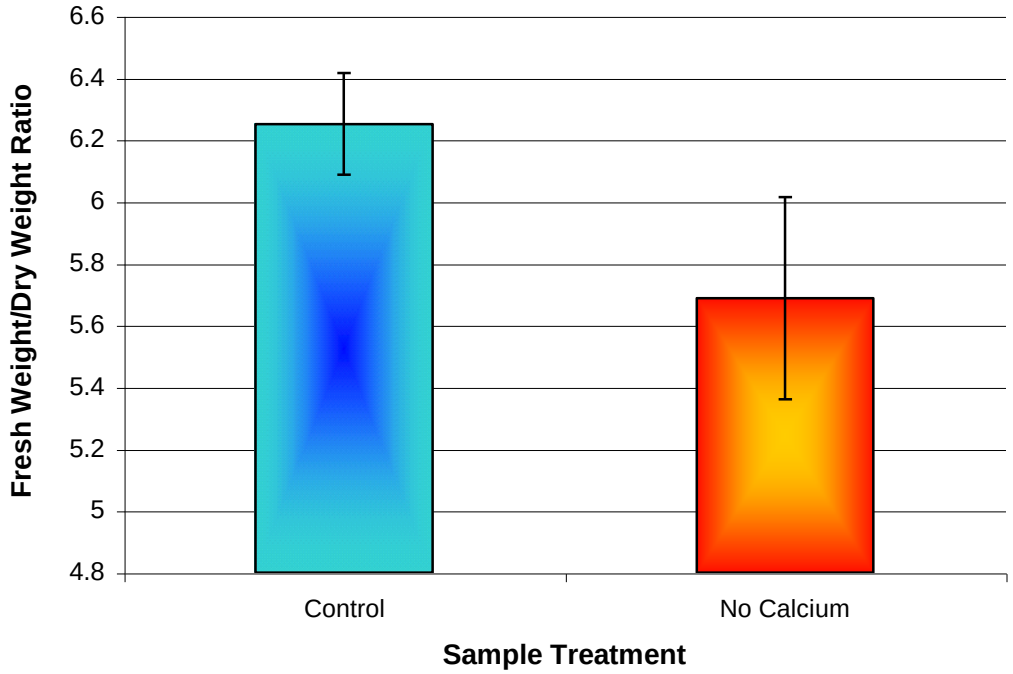
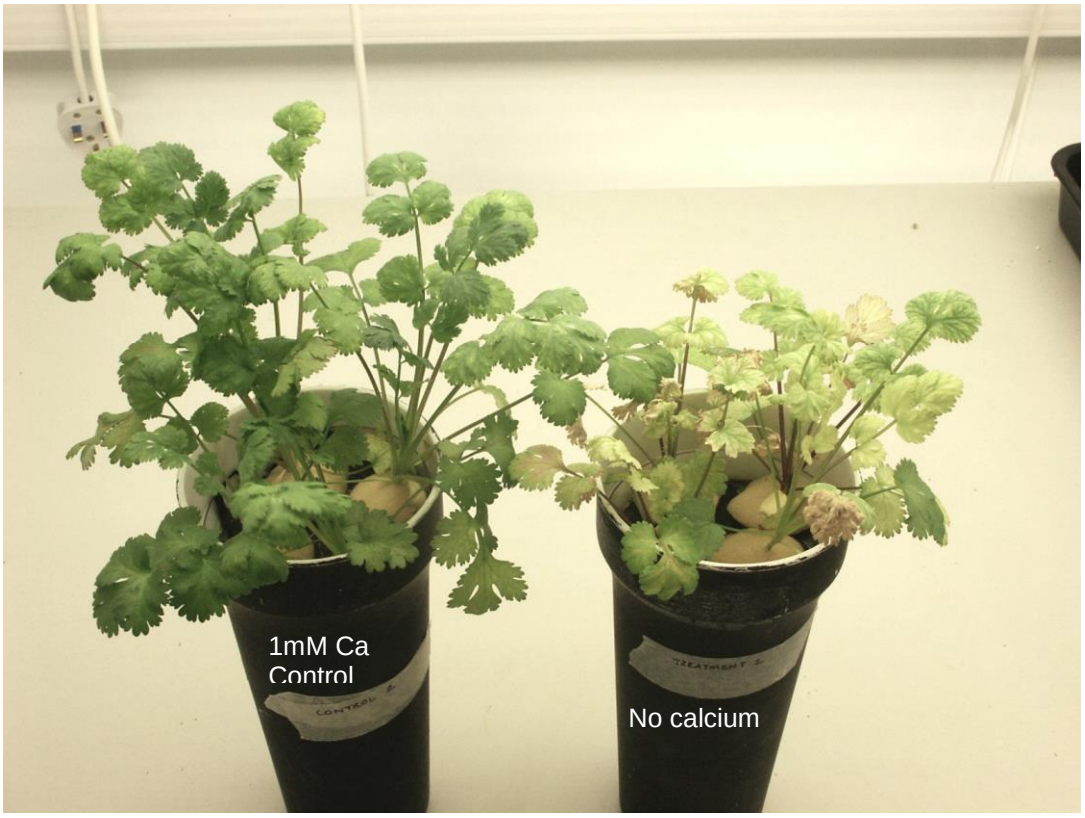


Figure 4 1: Fresh weight:dry weight ratio for coriander plants grown with and without calcium (mean ± SE; n=16).

The difference in fresh weight:dry weight (fw:dw) ratio between the control and the calcium-free treatment was highly significant (P=0.005 – see figure 4.1), showing that withholding calcium from the plant had a major effect on growth and development. In addition to being visibly smaller (figure 4.2), the new leaves produced by the calcium-deficient plants were small, highly chlorotic, with dark veins. Older leaves also started to die back more quickly. However, no oedema or blue spot developed on any plant in either the control or the treatment at any point during the experiments.



= 5cm

Figure 4.2: Coriander growing in medium containing no calcium (right) and 1mM calcium (control - left). The treatment plants are stunted and chlorotic, with older leaves showing necrotic edges and shoot dying back. The control plants are healthy, dark green and showing no necrosis or chlorosis.

Investigations into the Effect of Varying the Calcium Concentration in the Growing Medium

Following the investigation into the effect of a calcium-free medium on the growth and development of coriander (section 4.3.1), the outcome of adding different amounts of calcium to the growing medium was analysed, with a view to inducing either blue spot or oedema.

The mean fw:dw ratio of shoot tissue from plants grown at each calcium concentration was very similar ($fw:dw_{0.1mM\ Ca}$= 6.23 ± 0.39; $fw:dw_{1mM\ Ca}$= 6.94 ± 0.17; $fw:dw_{10mM\ Ca}$ =6.51 ± 0.09), even though at harvest, the appearance of the leaves did vary (figure 4.3). The coriander grown in solutions containing 10mM and 1mM calcium was a very lush green, where as that grown with 0.1mM calcium was a paler, with smaller leaves and two plants

died before harvest. Analysis of variance of the shoot data confirmed that calcium has no significant effect on fw:dw ratio (P=0.380).

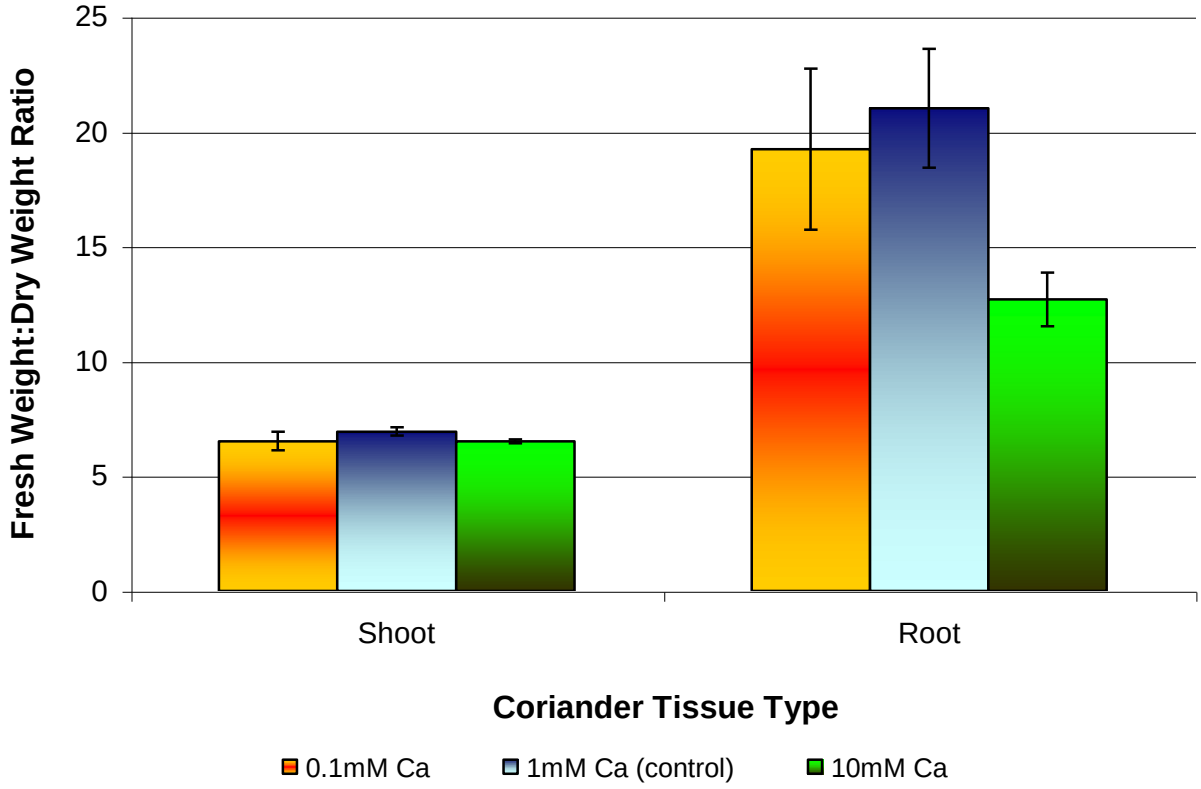


Figure 4.3: Mean fw:dw ratio of root and shoot tissue from coriander plants grown hydroponically in Long Ashton nutrient solution containing 0.1mM, 1mM & 10mM calcium. Mean ± SE; $n_{0.1mMCa}$ =10, $n_{1\ \&\ 10mMCa}$ = 12.

The fw:dw ratio of the roots differed more than that of the shoots ($fw:dw_{0.1mM\ Ca}$= 19.23 ± 3.51; $fw:dw_{1mM\ Ca}$= 21.01 ± 2.59; $fw:dw_{10mM\ Ca}$ =12.68 ± 1.17). Again there was a visible difference between the coriander roots at each calcium concentration at harvest. Those in the 0.1mM treatment were smaller, yellowish and slimy, whereas those in the 10mM treatment were much bushier, appeared to be healthier and were covered in a white powder, presumably precipitated calcium. Analysis of variance (ANOVA) of the root data showed that the results from the three replicate experiments differed significantly (P=0.000), so each experiment was analysed separately. This showed that in one of the three replicates, calcium concentration had no significant effect on root fw:dw ratio ($P_{rep\ 1}$= 0.795), while in the other two, the effect was highly significant (P_{rep2}= 0.007; $P_{rep\ 3}$= 0.000). A Tukey pairwise comparison of this data showed that in replicate 2, the significant difference occurred between plants grown in 0.1 and 10 mM calcium (P =

0.005) and in replicate 3 the significant differences occurred between 0.1mM and 1mM calcium solutions (P = 0.000) and 0.1mM and 10mM calcium solutions (P = 0.000). Despite the significant changes in plant pathology, neither oedema nor blue spot was induced in any plant in this experiment.

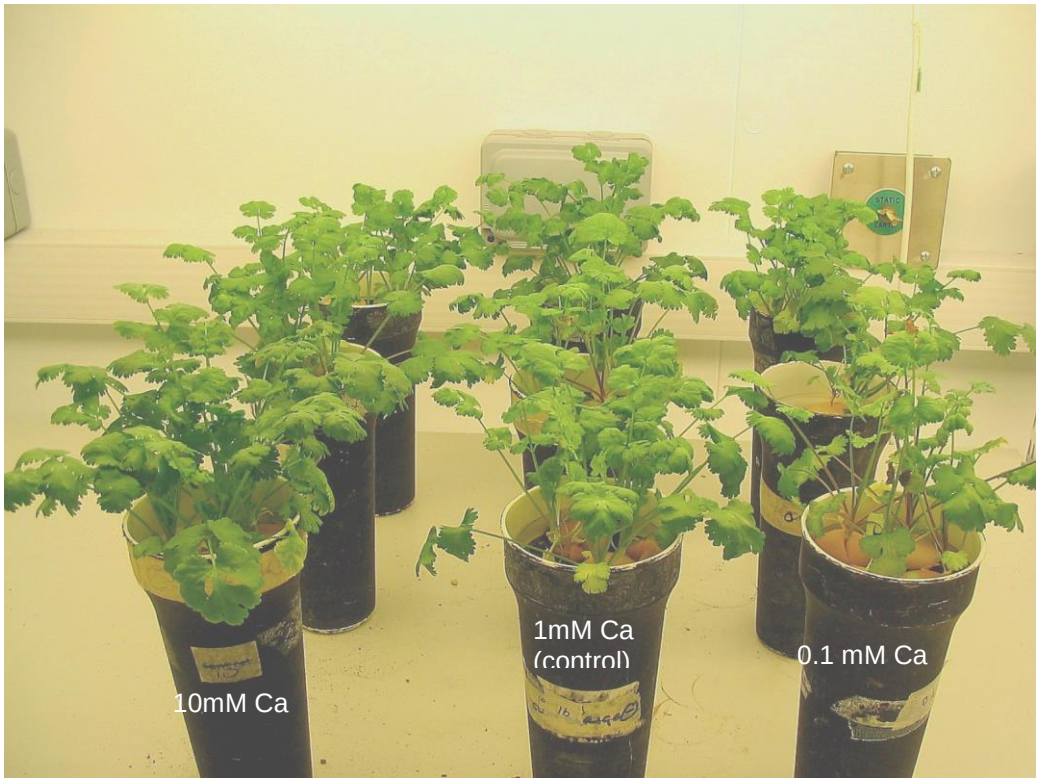


Figure 4.4: Coriander growing in medium containing 0.1mM, 1mM and 10mM calcium arranged in a Latin Square design. The physiological differences between the treatments can be seen; the 10mM plants are leafy and dark green and the 0.1 mM plants are less bushy, with smaller leaves. However, fw:dw ratio shows no significant difference between treatments. (1cm≡5cm)

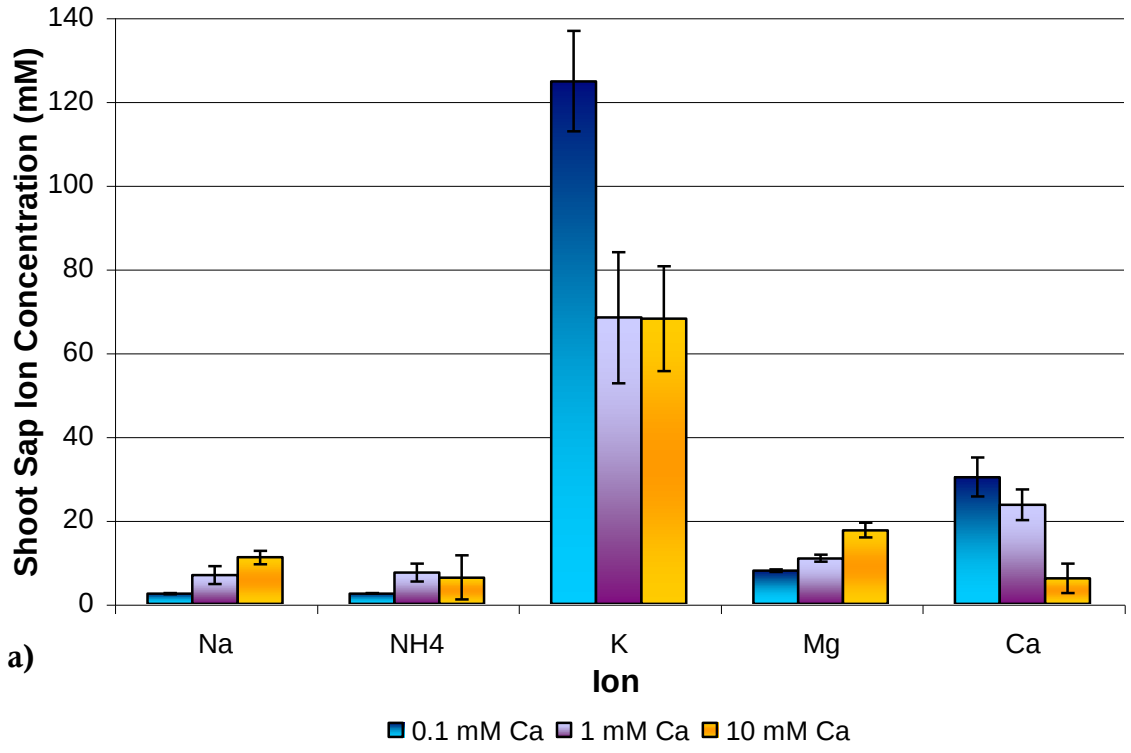
A further replicate of the experiment was harvested and the sap extracted and subjected to ion chromatography. It should be noted that this gave only the cation concentration of the *sap*, rather than the total concentration of cations present in the tissue.

The results were subjected to a nested ANOVA comparing all variables. The data from the roots and the shoots was, as expected, significantly different ($P = 0.040$), therefore was analysed separately.

There was a significant difference in ion concentration in both root and shoot tissues ($P_{root} = 0.000$; $P_{shoot} = 0.000$). Tukey pairwise comparisons demonstrated that this significance occurred between the concentrations of potassium and all other ions, in both sets of tissue. This is visualised in figure 4.5, where potassium was present at between two and 16 times that of other cations. Even though this cation was added in identical amounts to each medium (it was only calcium which was varied), the actual amount extracted from sap differed. In shoot tissue, the amount of potassium decreased as the concentration of calcium increased ($[K]_{0.1mM\ Ca} = 124.8 \pm 11.96$; $[K]_{1mM\ Ca} = 68.3 \pm 15.71$; $[K]_{10mM\ Ca} = 68.1 \pm 12.54$), while in roots, this trend was not only reversed, but more evident ($[K]_{0.1mM\ Ca} = 36.2 \pm 1.25$; $[K]_{1mM\ Ca} = 43.4 \pm 0.78$; $[K]_{10mM\ Ca} = 101.2 \pm 4.85$). Magnesium however, showed trends which increased as potassium concentration increased, even though it, like calcium, is a divalent cation,.

ANOVA demonstrated that the concentration of calcium in the growth medium significantly affected the ion concentration in root tissue ($P = 0.000$), but not in shoot tissue ($P = 0.134$). Further study showed that this significance occurred between medium concentrations of 10mM and both 0.1mM ($P = 0.000$) and 1mM ($P = 0.001$) calcium, but not between 0.1mM and 1mM ($P = 0.089$).

There was also a significant interaction between the concentration of calcium in the growth medium and the ion concentration in the coriander tissue ($P_{root} = 0.000$; $P_{shoot} = 0.016$). Again this was due to the significant differences between potassium and the other cations.



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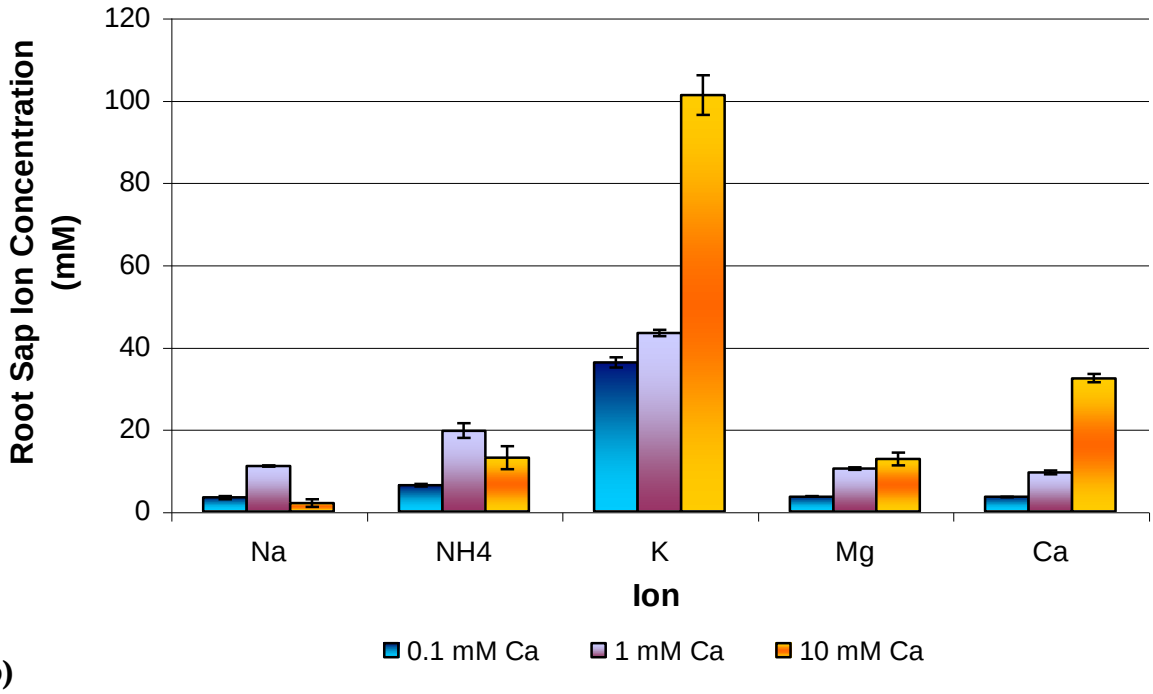


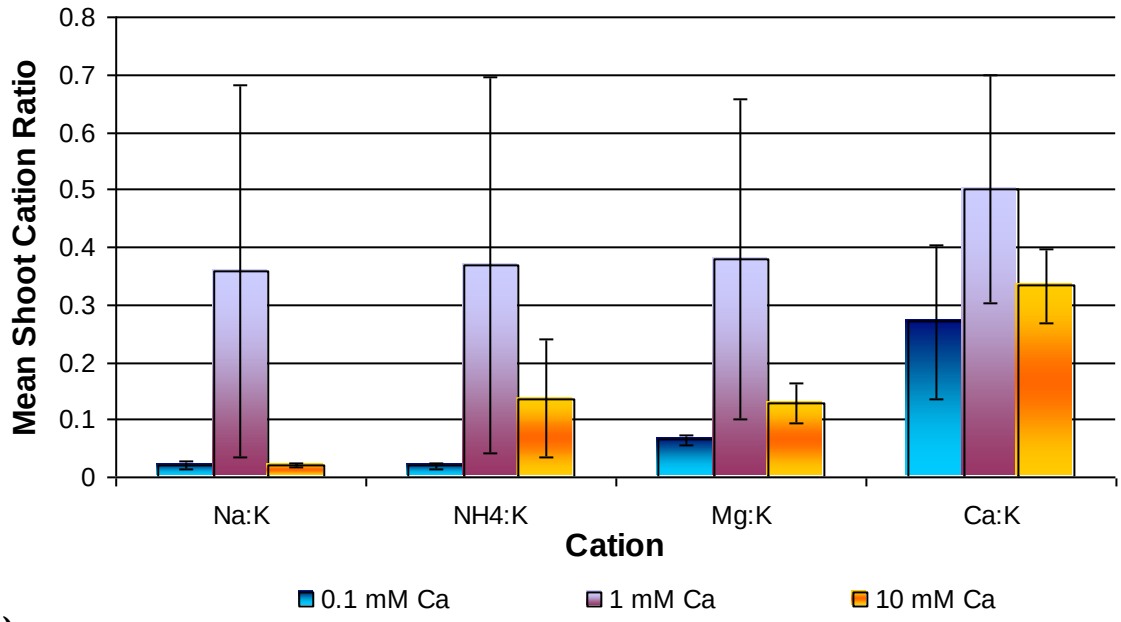
b)

Figure 4.5 a & b: Cation content of coriander leaf sap from a) shoot tissue and b) root tissue from plants grown in hydroponic solutions containing 0.1mM, 1mM & 10mM calcium ($n_{0.1mM\ Ca2}$ = 2; $n_{1mM\ Ca2}$ = 3; $n_{10mM\ Ca2}$ = 4;; mean ± SE)

Interaction between ions may be more important than the actual amount present, particularly between potassium and both calcium and magnesium (Marschner, 1995), therefore the ratios of cations present in the tissue was also analysed (figure 4.6).

The ratios showed the proportion of sodium, ammonium, magnesium and calcium to potassium. The larger the figure, the smaller the proportion of potassium in the ratio. In the shoot tissue, the Ca:K ratio was the highest of all cation:K ratios in tissue from all three hydroponic solutions, where as this was not the case in root tissue, where the figures did not show a trend.

When subjected to ANOVA, a significant difference was revealed between cation ratios from tissue grown in the different concentrations of hydroponic solution in shoot tissue ($P = 0.000$). A Tukey pairwise comparison showed that this difference occurred between the plants grown in 1mM calcium and those grown in both 0.1mM and 10mM calcium ($P_{0.1mM\ Ca} = 0.0005$; $P_{10mM\ Ca} = 0.0007$). In root tissue however, even though the overall difference was significant ($P=0.031$), the Tukey comparison did not show any significant values ($P_{1:0.1mM\ Ca} = 0.052$; $P_{1:10mM\ Ca} = 0.869$; $P_{0.1:10mM\ Ca} = 0.065$).



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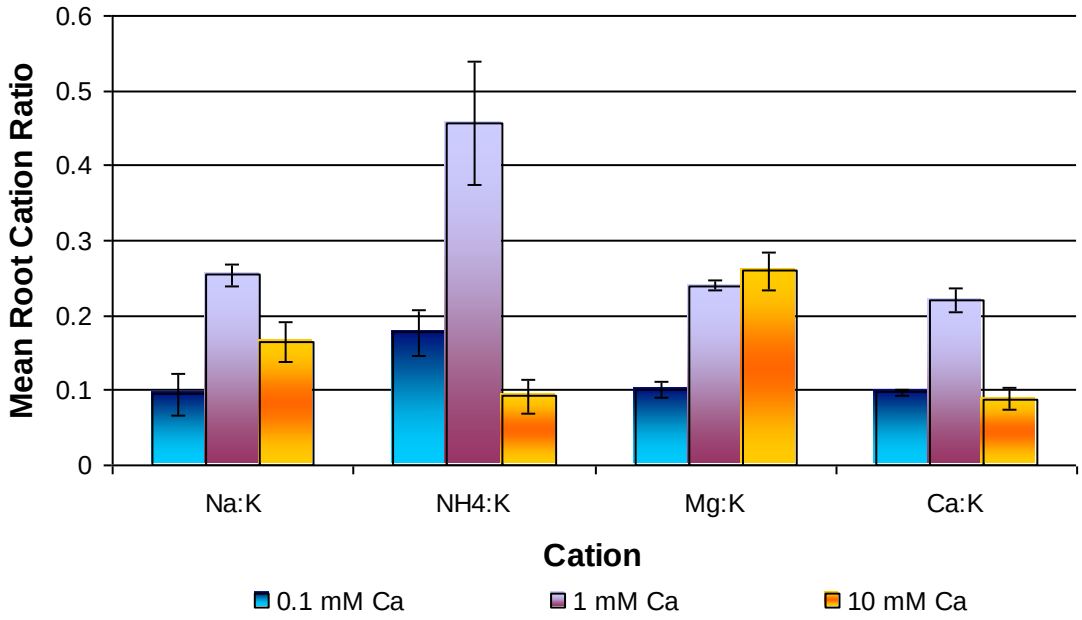


b)

Figure 4.6 a & b: Ratio of potassium ion concentration to the concentration of other cations in a) shoot tissue and b) root tissue from coriander plants grown in hydroponic solutions containing 0.1mM, 1mM & 10mM calcium ($n_{0.1mM\ Ca2}$ = 2; $n_{1mM\ Ca2}$ = 3; $n_{10mM\ Ca2}$ = 4; mean ±SE)

Ion Chromatography of Commercially-Grown Samples

Comparison of Actual Ion Concentrations

When samples from all sites were combined, there was significant variation ($P_{healthy}$ = 0.000; $P_{blue\ spot}$ = 0.000; P_{oedema} = 0.003), so each site was analysed separately. At one site (SH), samples were gathered from each of the 2003, 2004 and 2005 seasons. Analysis showed no significant variation between years (P=0.324), so the samples were combined.

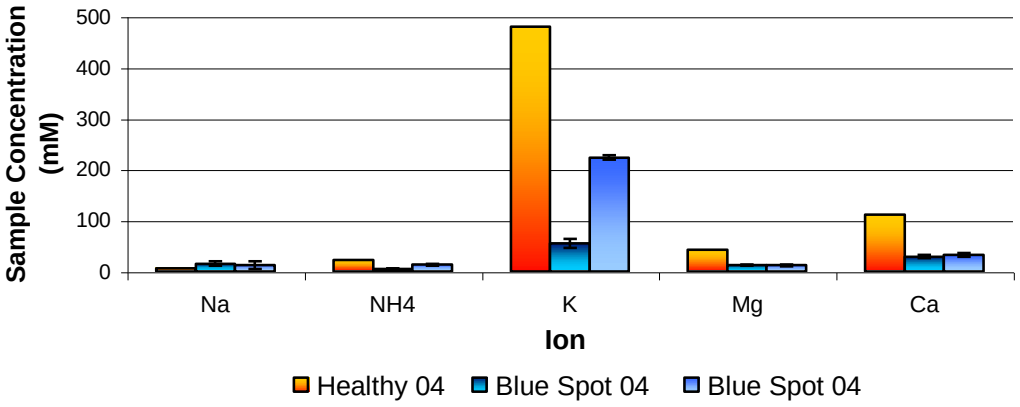
At three sites, tissue samples from both healthy and blue spotted samples were analysed (figure 4.7). At two sites samples from only tissue with blue spot were available (figure 4.8), although site LH (figure 4.8b) provided samples of differently aged coriander plants from adjacent plots, all suffering from blue spot. Two sites produced samples of tissue with both blue spot and oedema, as well as healthy samples (figures 4.9 and 4.10) and three sites did not have disease, so only healthy samples were gathered (figure 4.11).

At all eight sites, ANOVA revealed highly significant differences between ions ($P_{all\ sites}$ = 0.000). When data from tissue with blue spot and/or oedema were compared to those from healthy samples where all have been provided from one site, ANOVA exposed significant differences ($P_{all\ sites\ BS}$ = 0.000; P_{O} = 0.026; $P_{O\ \&\ BS}$ = 0.003). Tukey comparisons of data from the site with both conditions showed that the differences in ion concentration are significant between healthy and blue spotted tissues (P= 0.033), but not between healthy and oedematous tissue (P= 0.237). The difference between oedematous and blue spotted tissue is also significant (P= 0.012).

Tukey comparisons of the interaction between condition and particular ions showed that, in all cases of blue spot, this is only due to the interaction between potassium and other cations. This is also the case for healthy tissue at all sites except SH, where no interaction between potassium and the other cations was logged. In oedematous tissue, there was no significant difference between potassium and the other cations, in the two sites where this condition was found.

These differences are illustrated in the graphs in figures 5.7 to 4.11, where potassium was present at between 0.6 and 95 times other cations in healthy tissue, 0.4 and 103 times in blue spotted tissue and 2.3 and 228 times in oedematous tissue.

At site LH, samples of coriander at different stages of development were collected, all suffering from blue spot (see figure 4.9a). Analysis of the cation content showed that these varied significantly with the age of the plant (P= 0.000). Further comparisons showed that the ion concentrations from sample 1 (6 leaf stage) and sample 5 (8 leaf stage) differed significantly from the other samples, but not from each other. Tukey comparisons of the interaction between samples and cations showed that in samples 1, 2 and 4, there was a significant difference between potassium and sodium, ammonium, magnesium and calcium and also calcium and sodium, ammonium and magnesium. Sample 3, however, showed all the potassium interactions, but not the calcium-sodium, calcium-ammonium or calcium-magnesium ones and sample 5 also had the potassium interactions, but no calcium ones at all.



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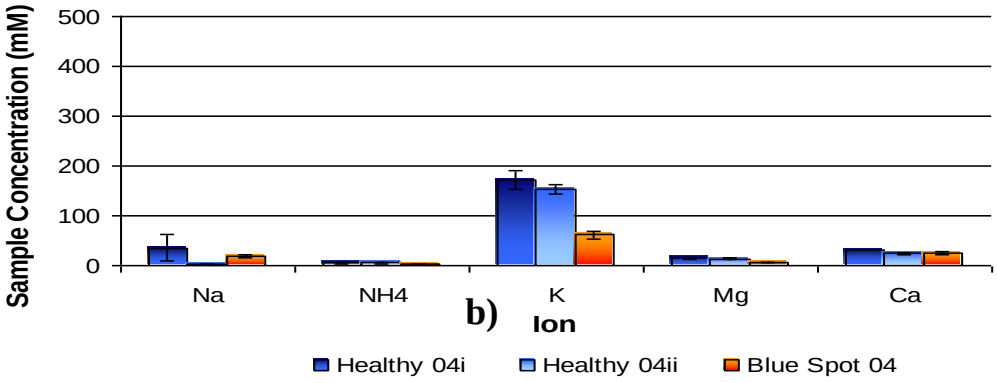

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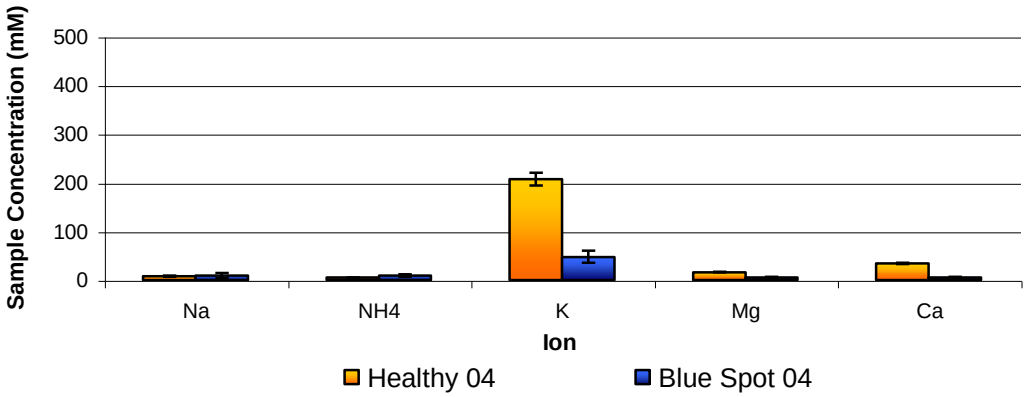
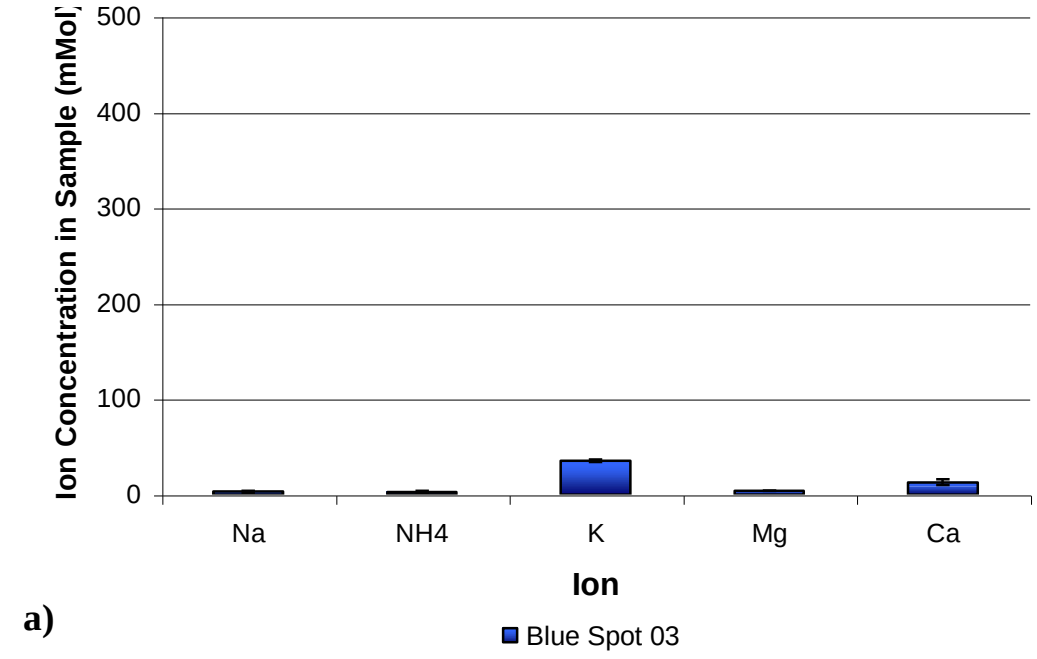


c)

Figure 4.7 a & b: Ion concentration in coriander leaf sap from healthy and blue spot plants from 3 sites:

a) AmcD, Wiltshire (field crop) n_H= 1, n_{BS1}=4, n_{BS2}=2; b) IC, Kent, (glass-house crop) n_{H1}= 3, n_{H2}= 9, n_{BS}= 2; c) VHB, W.Sussex (pot-grown, glasshouse crop) n_H= 2, n_{BS}= 4. Mean ± SE where n>1.



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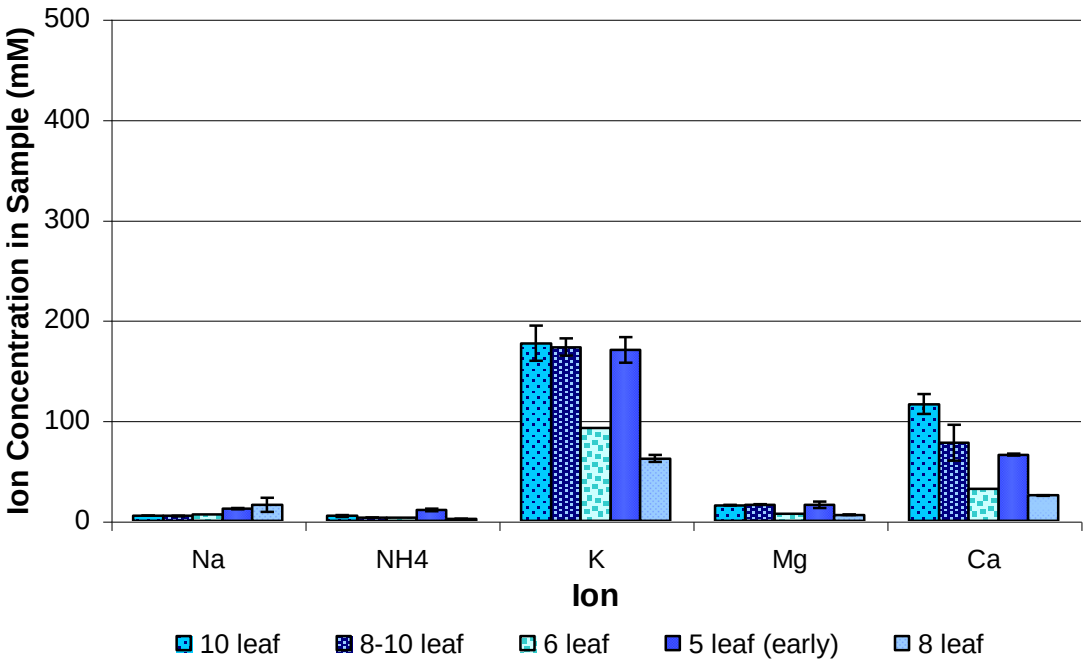


b)

Figure 4.8 a–b: Ion concentration in coriander leaf sap from blue spot plants from 2 Sites:

a) K/W, Norfolk (field crop) n_H= 2; mean ± SE where n>1; b) coriander at different ages, all suffering from blue spot, site LH, Oxfordshire ($n_{all\ samples}$=2). Mean ± SE where n>1

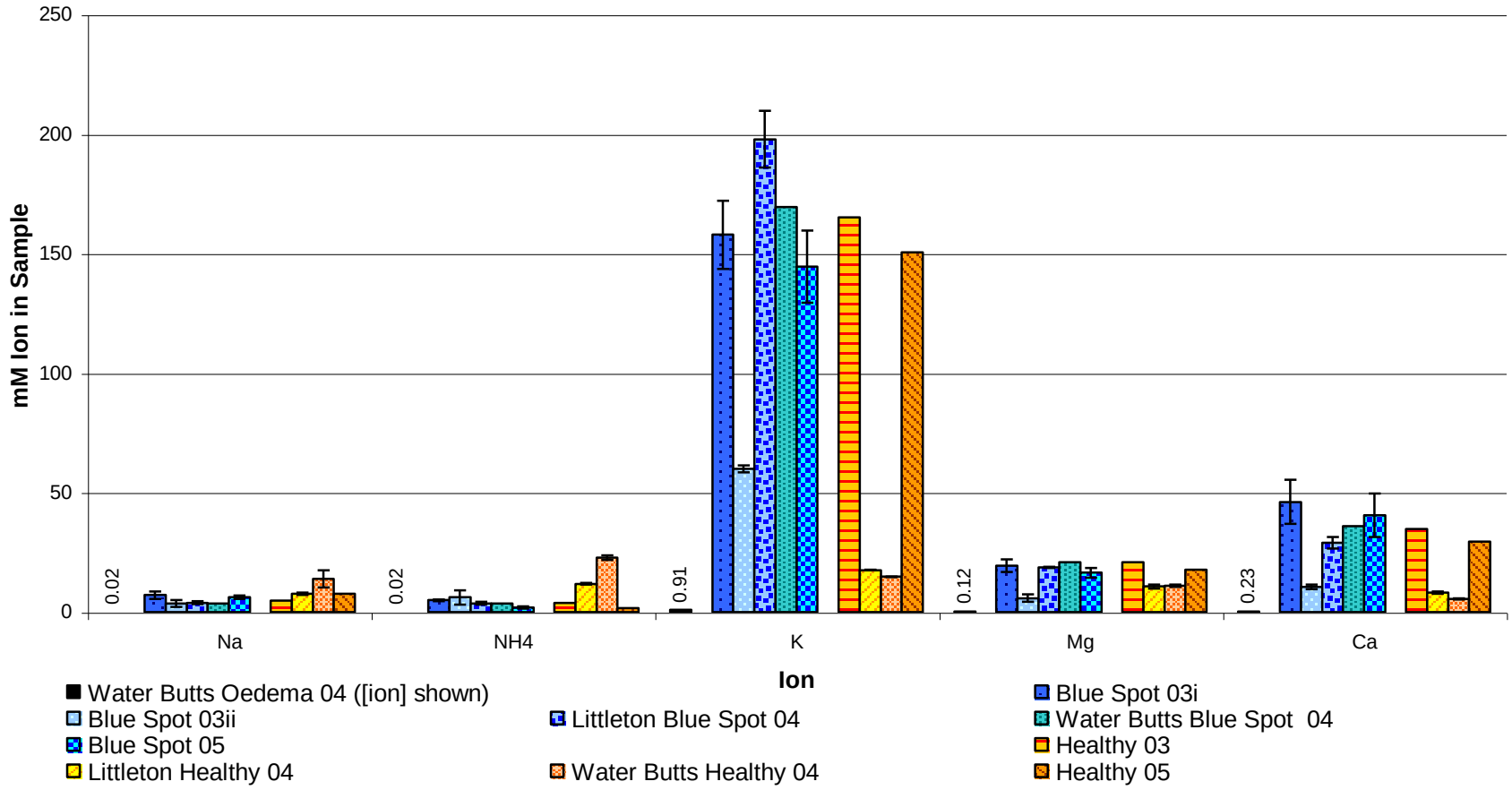


Figure 4.9 Ion concentration in sap of coriander leaf tissue from healthy, blue spotted and oedematous field-grown plants over the seasons 2003 to 2005 from site SH, Scotland; ($n_{BS\ 03i}$= 2, n_{O}= 1, $n_{BS\ 03ii}$= 2, $n_{H\ 03}$= 1, $n_{BS\ Littleton\ 04}$= 2, $n_{BS\ Water\ Butts\ 04}$= 1, $n_{BS\ 05}$= 2, $n_{H\ Littleton\ 04}$= 2, $n_{H\ Water\ Butts\ 04}$= 2, $n_{H\ 05}$= 1; mean ± SE where n>1). Note the scale is smaller than the other graphs.

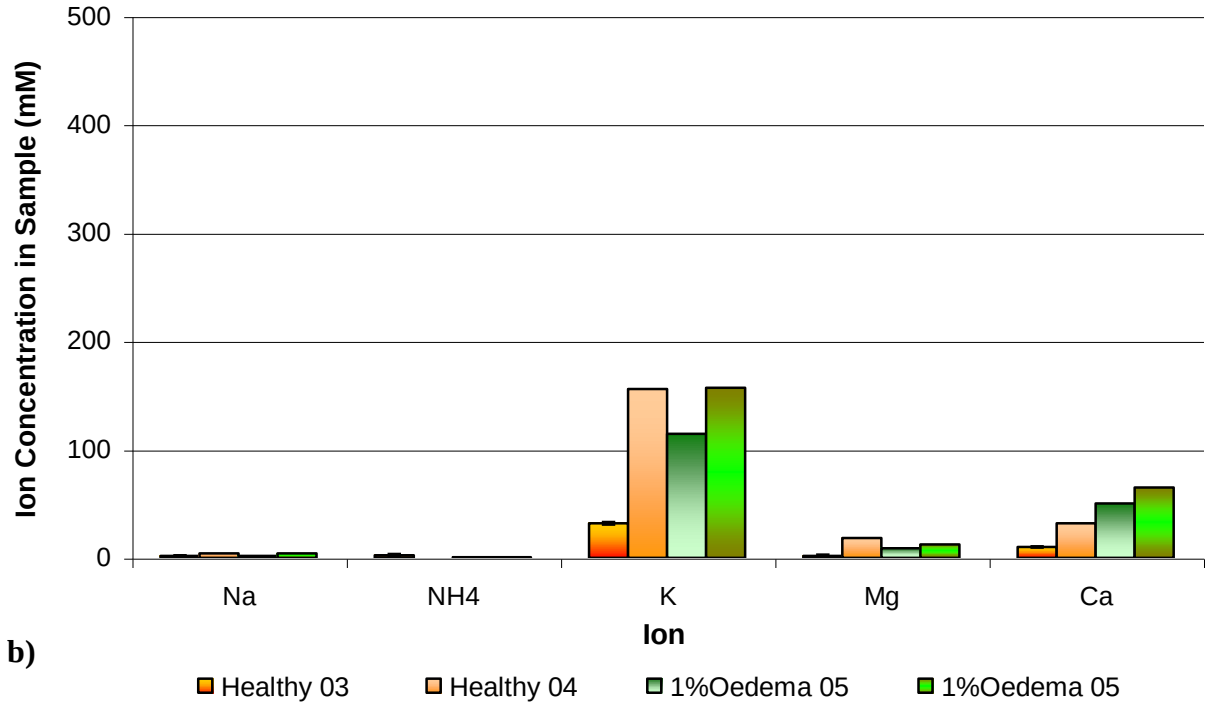
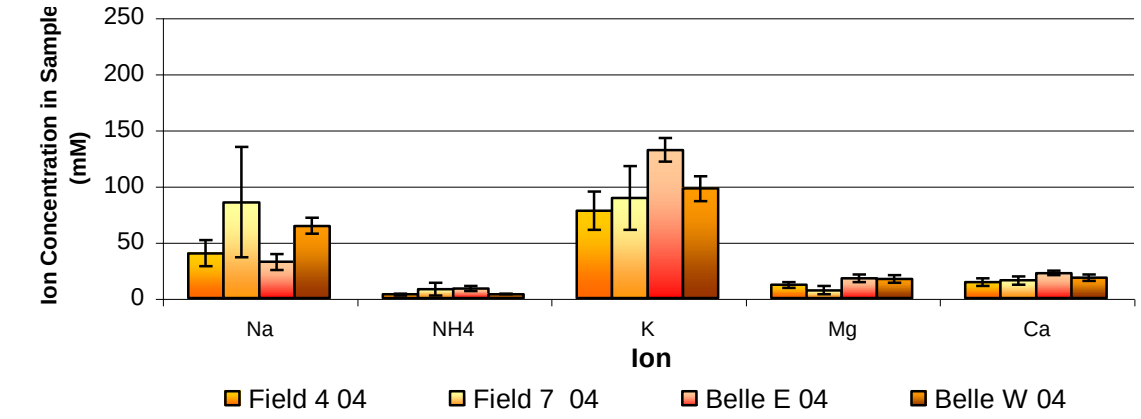


Figure 4.10: Ion concentration in sap of field-grown healthy and oedematous coriander leaf tissue from site UBf, W. Midlands ($n_{H\ 03}$= 3, $n_{H\ 04}$ = 1, $n_{O\ 05i}$= 1, $n_{O\ 05ii}$= 1). Mean ± SE where n>1.



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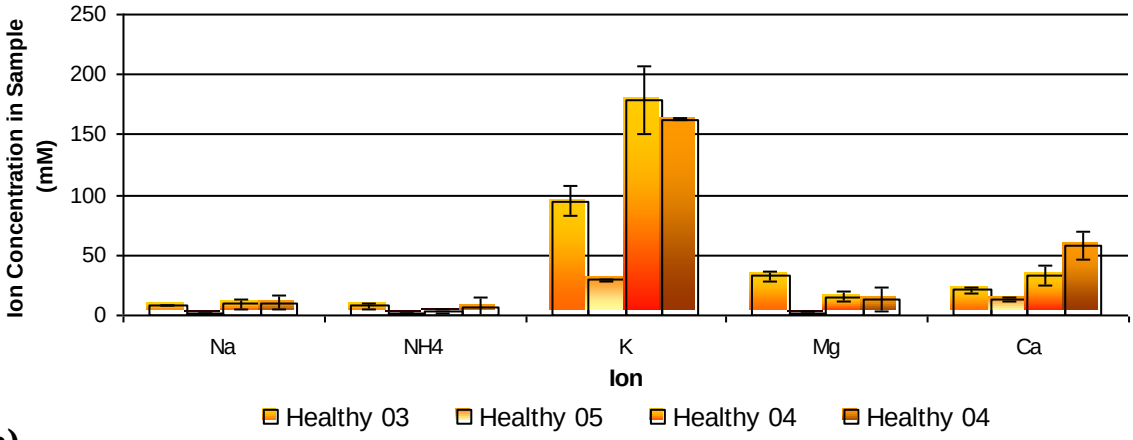

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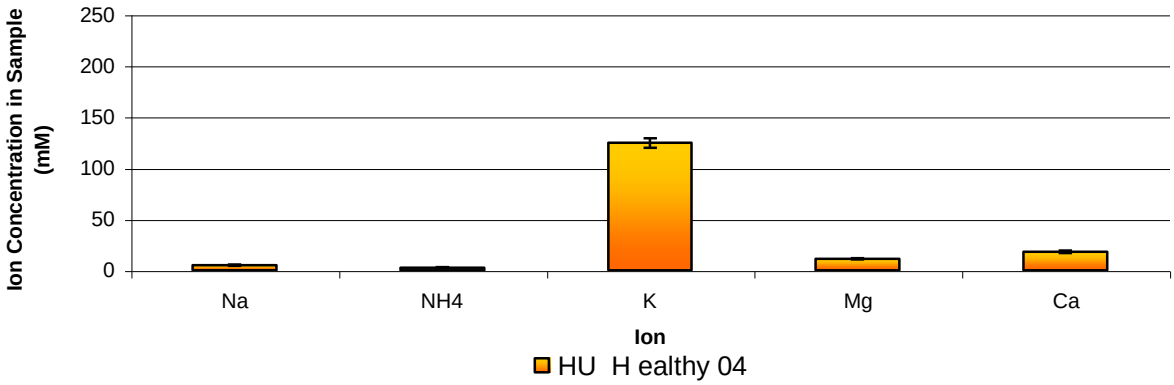


c)

Figure 4.11 a–c: Ion concentration in sap of coriander leaf tissue from healthy plants from 3 different sites. Note the sale is smaller than previous graphs.

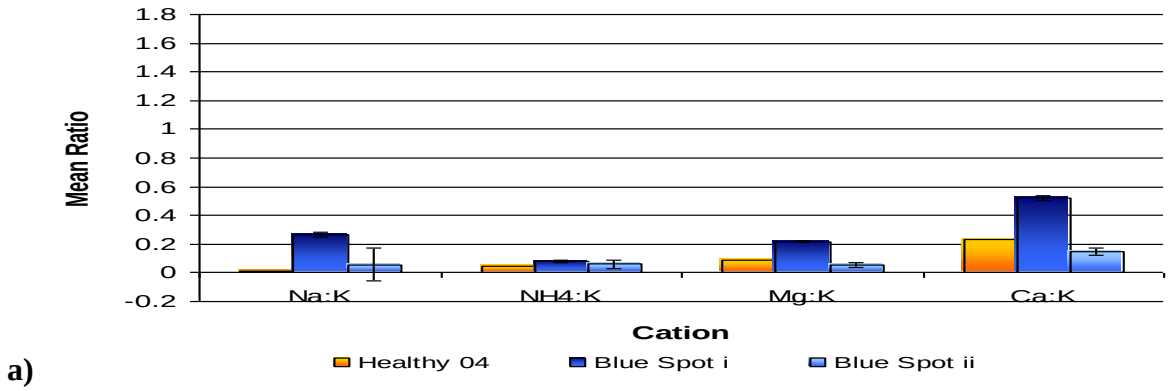
a) FGH, Guernsey (glasshouse crop) $n_{F4\ 04}$=4, $n_{F7\ 04}$=3, $n_{Belle\ E\ 04}$=4, $n_{Belle\ W\ 04}$= 3 ; b)UBp, W. Midlands (polytunnel crop) $n_{H\ 03}$= 17, $n_{H\ 04i}$= 3, $n_{H\ 04ii}$= 2, n_{H05}= 3; c) HU, N. Yorkshire (field crop) n= 12; mean ± SE where n>1.

Comparison of Ion Ratios

The differences between the ratios of cations in healthy tissue and that with oedema and blue spot were calculated from sites where more than one type of tissue. The graphs produced (figures 4.12 to 4.14) do not show any apparent trends. When healthy and blue spot tissue were compared with ANOVA, there was no statistical significance evident between cation ratios at sites AMcD (P=0.492) and site VHB (P=0.386 – figures 4.12a and c, respectively). However, at sites IC (figure 4.12 b) and SH (figure 4.14), statistical differences were found (P_{IC}= 0.000; P_{SH}= 0.000).

At site LH, the differently aged plants showing blue spot symptoms were subjected to ANOVA (figure 4.13a). A high degree of significance was present between the samples and a Tukey comparison specified that these occurred only between the 5 leaf sample suffering from early stage blue spot and both the 10 leaf and 8 leaf samples ($P_{10\ leaf}$ = 0.0196; $P_{8\ leaf}$ = 0.0409).

The two sites that suffered from oedema (SH – figure 4.14 and UBf- figure 4.13b) presented different results when subjected to ANOVA. Site SH showed a significant difference between oedematous and healthy tissue (P=0.0195), whereas site UBf did not (P=0.572).



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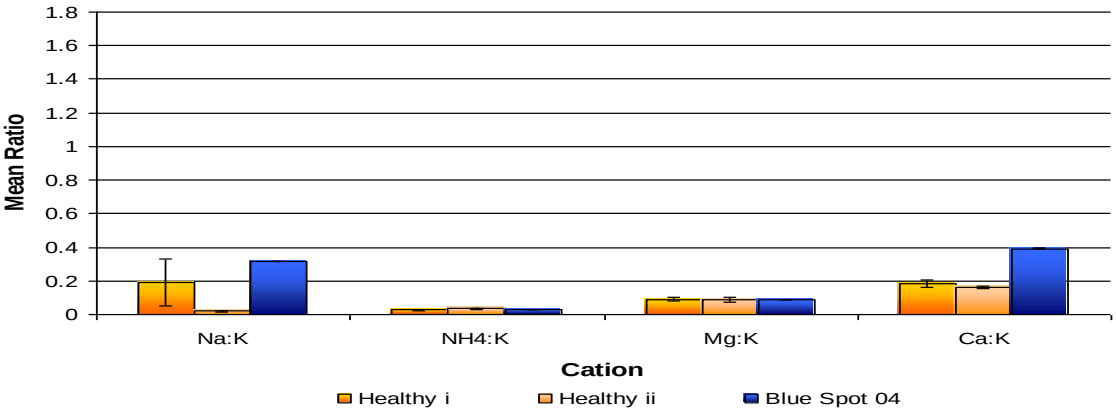

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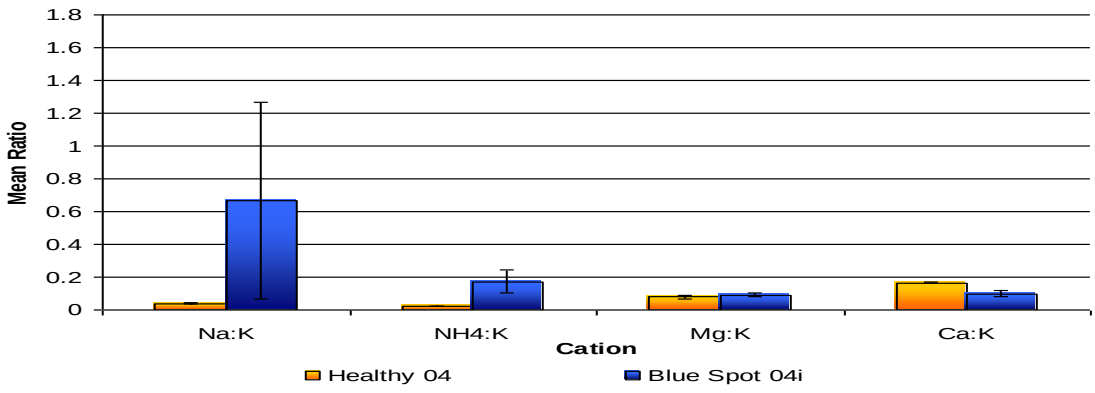


c)

Figure 4.12 a-c: Ratio of potassium to other cations from the sap of healthy and blue spot coriander leaf tissue from 3 sites:

a) AMcD, Wiltshire (field crop) n_H= 1, n_{BS1}=4, n_{BS2}=2; b) IC, Kent, (glass-house crop) n_{H1}= 3, n_{H2}= 9, n_{BS}= 2; c) VHB, W.Sussex (pot-grown, glasshouse crop) n_H= 2, n_{BS}= 4; mean ± SE where n>1.

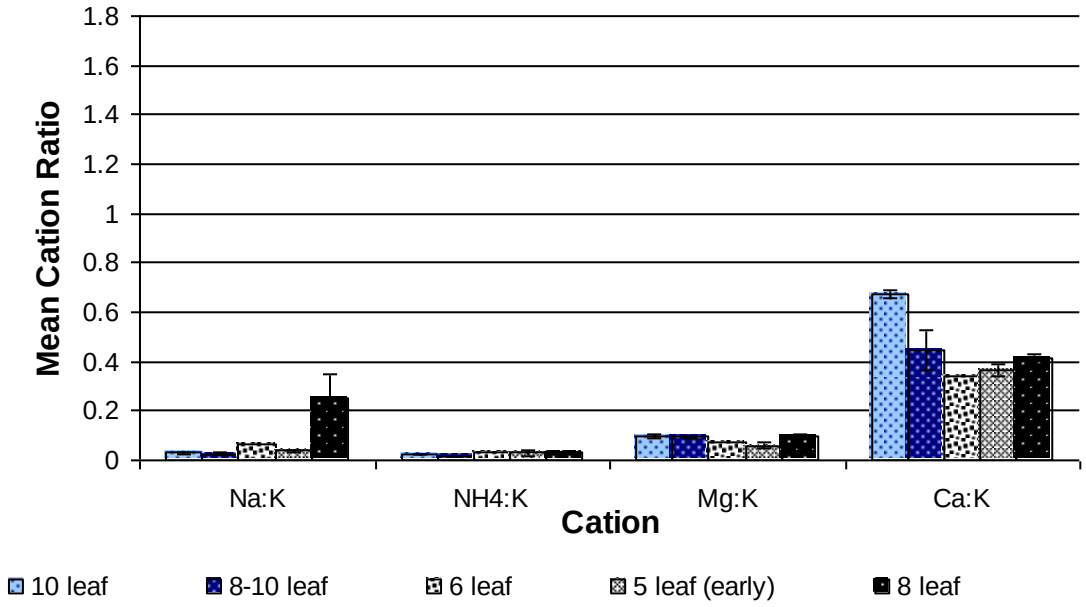


Figure 4.13: Ratio of potassium to other cations from the sap of field grown coriander leaf tissue suffering from blue spot. Samples taken from plants at different ages from site LH. $n_{all\ samples}=2$; mean ± SE.

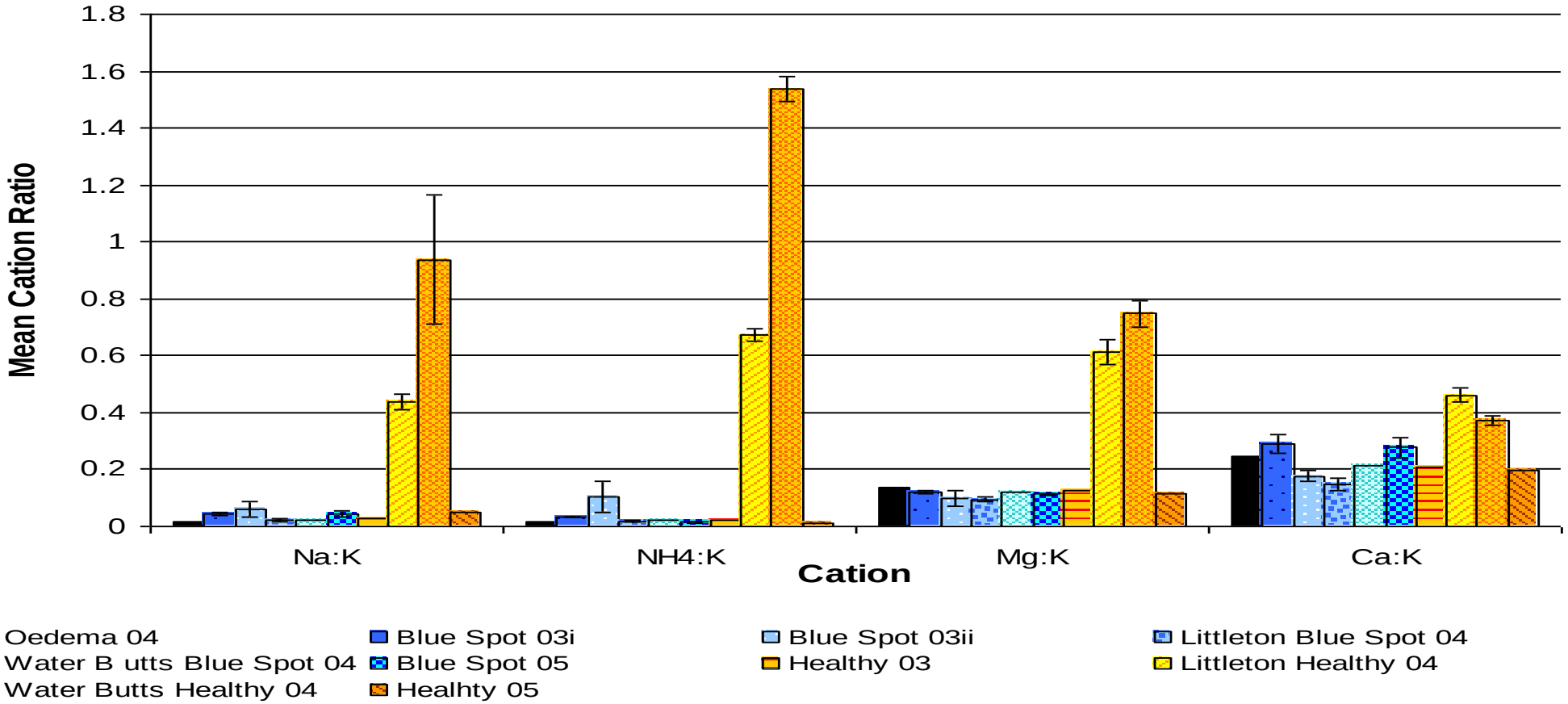


Figure 4.14: Ratio of potassium to other cations from the sap of healthy, blue spotted and oedematous field-grown coriander leaf tissue over the seasons 2003 to 2005 from site SH, Scotland ($n_{BS\ 03}$= 2, $n_{H\ 03}$= 1, n_{O}= 1, $n_{BS\ 03}$= 2, $n_{BS\ Littleton\ 04}$= 2, $n_{BS\ Water\ Butts\ 04}$= 1, $n_{BS\ 05}$= 2, $n_{H\ Littleton\ 04}$= 2, $n_{H\ Water\ Butts\ 04}$= 2, $n_{H\ 05}$= 1. Mean ± SE where n>1).

DISCUSSION

Neither eliminating calcium from the growing medium, nor varying its concentration triggers oedema or blue spot in coriander, although symptoms of nutrient deficiency were induced. As previously discussed, calcium is essential for the functioning of all tissues, but there is evidence to suggest that this ion is not retranslocated from aerial parts to the plant to the roots (Marschner and Richter, 1974; cited in Jakobsen, 1993). This is likely to be the reason for the small, decaying roots seen on the plants growing at the lowest calcium concentration, where signalling and cellular strengthening functions are not being carried out. It has been recorded that, when under attack from certain fungi and bacteria, the presence of calcium in tissues inhibits enzymes such as polygalacturonase from dissolving thee middle lamella (Bateman and Lumsden, 1965; cited in Marschner, 1995), therefore this hypothesis is possible.

The fw:dw ratio of coriander tissue under the influence of different concentrations of calcium confirmed that the recommended amount of calcium in Long Ashton nutrient solution is optimal for plant development. At the low calcium concentration, the roots appeared to have started to decay, whereas at the higher concentrations this did not happen. This is likely to be due to calcium being available to strengthen the cell walls preventing degradation.

The ion chromatography utilised in these experiments measured the ion concentration of the leaf sap, rather than of the entire tissue. In this way it does not include the calcium that is laid down in the cell walls. However, as ions are transported in the xylem sap, any current deficiencies or excesses should be noticed.

The different concentration of potassium in the tissues was interesting, given that the concentration in the growing medium was the same. It is known that when a plant has access to high levels of potassium, the uptake and physiological availability of calcium is reduced, resulting in deficiencies (Jakobsen, 1993 ; Marschner, 1995).

In these experiments, significant differences were seen in shoot tissue between cation ratios from low and control calcium plants and between control and high calcium plants. If Ca:K ratio was at problematic levels in the low calcium solution, then a significant difference would have also been seen in the ratios between the plants grown in 0.1mM calcium and those grown in 10mM calcium, but this was not so.

The agreement of the significantly high levels of potassium in coriander tissue grown hydroponically with that grown commercially in soil and compost confirms that this is normal for this species of plant.

The cation content results from the coriander at different ages (LH site) is interesting for two reasons: Firstly, samples 1 to 4 were all growing at the same time of year, in adjacent plots and suffering from the same 'batch' of blue spot, while sample 5 was a later crop afflicted with the condition. Secondly, samples 3 and 4 were sown at the same time, but sample 4 did not have blue spot as far advanced as sample 3. If the condition alone caused the differences seen in cation concentration, then sample 4 would have seemed the obvious candidate for significance to occur. Why this is so cannot be ascertained here.

The differences in cation ratios only occurred between the one of the two youngest samples and the oldest. The other young sample was suffering from blue spot at a slightly more advanced stage, which raises the question whether the condition causes certain physiological properties in the plant to age, accounting for the non-significant finding. Further study would be required to clarify this.

Comparing the findings for each site, it can be seen that cation concentration is significantly different in tissue with blue spot when compared to healthy, although whether the condition causes these differences or these differences trigger the condition cannot be ascertained from this data. However, no clear indication is given of whether the ratio of cations to potassium content is significantly affected, or indeed significantly affects the blue spot condition, as the findings from the different sites analysed were split equally.

The relationship between oedema and both cation content and cation ratio is not so clear-cut, primarily because there were only a small number of samples to be compared and then the results did not agree. More samples would need to be analysed in order to gain a full picture.

CONCLUSIONS

From the laboratory experiments it can be concluded than neither an excess nor a deficiency of calcium directly causes blue spot or oedema.

In tissue with and without blue spot, the concentration of cations is significantly different, although it cannot be ascertained whether this causes blue spot, or whether blue spot results in this difference. Further work would be required to confirm this.

No conclusions can be drawn between the concentration of cations, other than calcium and the development of oedema.

The results of the analysis of the ratio of cations in healthy tissue and that with blue spot and oedema varied, so that no clear conclusions can be drawn as to their importance in the development of either condition.

It would also be advantageous to study the effects and interactions of cations such as magnesium, sodium and potassium on the development of blue spot and oedema, as well as that of certain anions such as phosphate and sulphate.

These conclusions, along with those of the previous two chapters, looking at the effect on meteorological conditions on oedema and blue spot, do appear to indicate that the conditions are not simply physiological, but that another factor, possibly a micro-organism is also involved. This is the subject of the following chapter.

SCIENCE SECTION - PART 5

THE ROLE OF MICRO-ORGANISMS

INTRODUCTION

The initial aims of this project were based on the anecdotal evidence of growers involved in producing coriander and mainly involved investigating physiological afflictions, such as the effects of elevated humidity and/or temperature (chapters 3 and 4) and ion imbalance (chapter 5). However, when searching for the causes of particular symptoms, the role of micro-organisms should not be ruled out.

This is even more important since no physiological mechanism has been identified that elicits either oedema or blue spot. In addition, analysis of meteorological data (chapter 4) suggests that an unidentified variable, termed an 'x-factor', is responsible for outbreaks of oedema and blue spot, subject to certain meteorological conditions being within a critical range.

This would tie-in with a micro-organism being responsible, as their growth and development, like that of plants, is reliant on the environmental conditions being within a certain range (Agrios, 1997).

Large numbers of micro-organisms are associated with plants, from the roots (rhizosphere) to the leaves (phyllosphere). Most do not influence the host plant in any way, some even establishing large populations without any harmful consequences. A few, however, have obvious deleterious effects by causing disease. It is generally recognised that endophytic (inside leaf tissue), rather than epiphytic (leaf surface) populations are directly responsible for disease (Beattie and Lindow, 1999). This pathogenic group comprise fungi, bacteria, viruses, protozoa (e.g. plasmodia and oomycetes), viroids and mollicutes (e.g. mycoplasmas and spiroplasmas). These classifications can be further sub-divided by their colonisation and feeding mechanisms. Biotrophs are only able to grow and reproduce in living hosts, from which they obtain their main sources of energy and nutrition. They cannot be cultured on artificial media in the laboratory. Necrotrophs by contrast, thrive on dead organic matter, which they obtain by killing the host cells. Hemi-biotrophs have characteristics of both; they spend part of their lifecycle living biotrophically, often undetected, before becoming necrotrophic and killing the host.

Coriander is known to be attacked by both bacterial and fungal diseases (Mahor *et al.*, 1982; Diederichsen, 1996; Dennis and Wilson, 1997; Refshauge and Nayudu, 2001), although there does not appear to be any literature studying pathogenic causes of oedema. In order to ascertain whether there was a link between pathogenesis and oedema or blue spot, it was decided to concentrate only on the role of bacteria, rather than fungi or viruses. There were a number of reasons for this:

Firstly, one grower involved in the project, felt that his crops did not suffer with oedema or blue spot, but were subject to attack from the bacterium *Pseudomonas syringae*, which he controlled by the use of treated seed. It was therefore deemed sensible to investigate the possibility that the symptoms of oedema and/or blue spot are actually as yet unreported symptoms of *P. syringae*.

Secondly, the microscopy investigations described in chapter 2 did not show the presence of any fungal hypha, although could not rule out the presence of smaller bacterial cells.

Thirdly, the development of symptoms of blue spot in particular and, to some extent, oedema, are not characteristic of viral infection, where, although lesions are a common symptom, spread throughout the plant via the vascular vessels is rapid (Agrios, 1997), resulting in a systemic infection. In addition, most viruses appear to cause some degree of dwarfing or stunting of the entire plant, which was not seen in the conditions being investigated.

Identification of Plant Pathogenic Bacteria

The identification of plant pathogenic bacteria has, until recently, been a lengthy and labour-intensive process, involving a number of steps. It has tended to rely on a degree of recognition of both macro- and microscopic symptoms, before deciding on the methodology used. Furthermore, the interpretation of test results are highly subjective (Stager and Davis, 1992; cited in Drancourt *et al.*, 2000).

Methods of identifying pathogenic bacteria have improved dramatically over the past twenty years, with the development of serological techniques, such as the Enzyme-Linked Immunosorbent Assay (ELISA - Nome *et al.*, 1981), biochemical strip tests, e.g. the API® system (Biomérieux, Rhone, France) and, of course, DNA sequencing technologies. In this study, it was decided to use a number of different techniques: morphological

comparison on different selective media, microscopy and biochemical assays, as well as DNA sequencing, in order to provide a more complete understanding of the procedures available in the identification process.

Traditional Methods of Identification

With all methods of identification, the pathogen needs to be isolated, so that pure cultures can be used for identification. Sometimes, the bacteria can be identified by looking at such characteristics as:

- Morphology – colony shape, colour, texture and smell; cell size, shape, arrangement and the presence of any special structures (e.g. flagella)
- Reactions to staining – Gram staining, acid fastness
- Effects of growth conditions– differences in appearance and amount of growth on different media, including changes as a result of age (Johnston and Booth, 1983; in Fox, 1993).

However, as there are many closely related strains in any particular genus these methods alone are not usually relied upon.

Following original methodologies, a number of media were used to compare morphological and physiological characteristics of isolated bacteria. The particular types of media were chosen because of the selectivity for bacteria grown upon them. With the intention of investigating the role of *P. syringae* (a Group I, pathogenic, green-fluorescent Pseudomonad), the production of fluorescent, diffusible pigments was investigated. Most pathogenic strains of green-fluorescent Pseudomonads produce diffusible pigments on King's B medium that fluoresce green to blue under ultraviolet (UV) light (Lelliott and Stead, 1987). Sucrose nutrient agar (SNA) is also used in the isolation of green-fluorescent Pseudomonads as several of these bacteria produce large, domed, creamy-white, smooth, shiny colonies on this medium (Lelliott and Stead, 1987).

MacConkey agar is used chiefly to differentiate between Gram positive bacteria (which are inhibited) and Gram negative bacteria (which grow) and between lactose fermenters (red colonies) and non-fermenters (white colonies). However, in this study, it was used to reduce the amount of mucus produced by certain strains. Methanol Mineral Salts (MMS) medium, as its name indicates, contains methanol. The vast majority of bacteria cannot grow in the presence of methanol. However, certain types of facultatively methylotrophic

bacteria do exist. These bacteria can use single carbon compounds (e.g. methanol, methane, methylated amines) as their primary carbon source (Lidstrom, 2004).

A key method of classifying eubacteria is through the use of the Gram stain. Christian Gram devised the staining procedure in 1884 as a means of differentiating bacterial cells. At the time he did not recognise the taxonomic value (Difco, 1995). The Gram stain is now used to differentiate intact, morphologically similar cells into two groups after staining. Cell form, size and some structural details are also more visible.

Gram positive cells appear purple, as they retain the crystal violet-iodine complex which forms in the protoplast (Difco, 1995). Their cell wall is thick, composed of many layers of the polymer peptidoglycan (Deacon, 2005). This blocks the dispersal of the violet-iodine complex when washed with alcohol.

Gram negative bacteria (including all Pseudomonads) appear pink as they only have a thin layer of peptidoglycan surrounded by a lipopolysaccharide outer membrane, which does not retain the violet-iodine complex. When counterstained with safranin, the cells stain pink.

Biochemical Methods

Analyses that test for the production of certain biochemical substances have been known for many years (e.g. Lelliott and Stead, 1987), but it is only in the last few decades that these tests have been developed into rapid, standardised pre-packaged kits. These kits are generally aimed at the identification of medical bacteria, but can be useful in the recognition of plant pathogens (Fox, 1993).

The API® system is one such kit, introduced in 1970 by French company Biomérieux (Rhone, France). It combines 'a strip of biochemical tests and a database' using 'a calculation method, known as numerical identification' to make bacterial identification 'simple, rapid and reliable'. There are now a number of strips that aim to identify many different types of bacteria, predominantly of clinical origin (API Strips, Biomérieux, 2006)}. With identification of *P. syringae* as the aim of these tests, two types of strip, API 20NE® (for the identification of non-enteric Gram negative rods) and API 20E® (for enteric Gram negative rods), were used in this study and compared to other methodologies.

Nucleic Acid-Based Methods

Since the development of the polymerase chain reaction (PCR) in the 1980's (Mullis, 1987; in Schaad and Frederick, 2002), the identification process of plant pathogens has become considerably easier. There are now a number of PCR-based techniques that can be used, but for this study, the use of primers to amplify the 16S ribosomal DNA (rDNA) was chosen.

Each bacterial ribosomal RNA (rRNA) is made up of a 5S, a 16S and a 23S subunit, separated by mostly non-functional internal transcribed spacer (ITS) sequences. Primers targeted to the DNA coding for areas of the 16S region flanking the ITS sequences have been used for a number of years to identify and characterise pathogenic bacteria (Widmer *et al.*, 1998; Coenye *et al.*, 2001; Jonasson *et al.*, 2002; Spilker *et al.*, 2004). There are a number of reasons for this:

- The 16S DNA sequence is very highly conserved (Jonasson *et al.*, 2002), therefore makes a reliable target;
- There are between 1 and 15 copies of the 16SRNA gene on the bacterial chromosome, depending on the species (Jonasson *et al.*, 2002), increasing the sensitivity of the technique over that of single copy genes;
- Many thousands of sequences have been catalogued and are available in publicly accessible databases e.g. Genbank (www.psc.edu/general/software/packages/ genbank/ genbank.html), allowing easy comparison of sequences and identification of isolated colonies.

The technique also has its drawbacks.

- Evolution has resulted in very different copies of 16S rDNA being found in the same organism and conversely, organisms with identical 16S rDNA show considerable phenotypic variation (Hagström *et al.*, 2002). This technique is not able to differentiate them.
- In some genera, the species, subspecies and pathovars are so genetically similar that this method cannot differentiate further than genus level (Jonasson *et al.*, 2002).

Differentiation Between Primary and Secondary Infection

Most bacteria live on plant surfaces and do not induce disease in the host. However, once a plant is weakened, for example through nutrient or drought stress, physical injury, insect damage or disease, entry into plant tissues by pathogenic species is easier, particularly if the outer surface is broken. As this bacterial attack was not the original cause of the plant's reduced condition, it is termed a 'secondary infection'. An important part of the identification of a disease's causal organism is the differentiation between primary and secondary infection.

In 1876, Robert Koch proposed a set of four rules, known as Postulates, which apply to all types of micro-organism and must be satisfied before a particular micro-organism can be said to be the direct cause of the disease (Fox, 1993; Agrios, 1997):

- Association of the pathogen with the disease in all diseased plants examined.
- Isolation of the micro-organism and growth in pure culture or nutrient media.
- Inoculation of the micro-organism from pure culture onto healthy plants of the same species as the original, with the same disease symptoms being produced.
- Isolation of the micro-organism from the re-inoculated plant and growth in pure culture, with the characteristics being identical to those in step 2.

At the time the postulates were formed, less was known about the effect of one type of bacterium on the pathogenic capabilities of others. For example, there is literary evidence that the presence of *Pantoea agglomerans* affects the infection process of *P. syringae* pv. *atrofaciens*, leading to an intensification of symptoms in the development of basal bacteriosis of wheat (Pasichnyk *et al.*, 2005). In this case, the Postulates can be extended to include mixing bacterial strains before re-inoculation.

METHODS

1.1.1 Isolation of Bacteria From Diseased Tissue

Five leaves were surface sterilised for 30 seconds in a 1% sodium hypochlorite solution, without agitation. The leaves were then washed twice in sterile distilled water. Discs approximately 0.7cm in diameter, were cut by laying the leaf over the opening of a sterile 1.5ml tube, then closing the lid.

Each leaf disc was then macerated with a homogenising stick within the tube for 15 seconds, 500µl Luria-Bertani (LB) broth (section 5.2.2.1) was added, then the tissue was macerated for a further 5 seconds in order to suspend it.

Media

Luria-Bertani (LB) Broth

Tryptone 10.0g l^{-1}

Yeast Extract 5.0g l^{-1}

NaCl 5.0g l^{-1}

Kings B Agar (KBA)

Proteose peptone (Difco No. 3/Oxoid L46) 20.0g l^{-1}

Glycerol 10.0g l^{-1}

K_2HPO_4 1.5g l^{-1}

$MgSO_4.7H_2O$ 1.5g l^{-1}

Agar 15.0g l^{-1}

The pH was adjusted to 7.2 and autoclaved at 121°C for 15 minutes. To make broth, the agar was omitted (Lelliott and Stead, 1987).

5% Sucrose Nutrient Medium (SNA)

5% w/v sucrose was added to Oxoid nutrient agar CM3 and made up as per manufacturer's instructions.

To make broth, 5% sucrose was added to nutrient broth.

Both media were autoclaved at 121°C for 15 minutes (Lelliott and Stead, 1987).

Methanol Mineral Salts (MMS) Agar

(From Green, 2000)

K_2HPO_4 1.20g l^{-1}

KH_2PO_4 0.62g l^{-1}

$CaCl_2 . 6H_2O$ 0.05g l^{-1}

$MgSO_4 . 7H_2O$ 0.20g l^{-1}

NaCl 0.10g l^{-1}

$FeCl_3 . 6H_2O$ 1.0mg l^{-1}

$(NH_4)_2SO_4$ 0.5µg l^{-1}

Agar 15g l^{-1}

$Cu SO_4 . 5H_2O$ 5.0µg l^{-1}

$MnSO_4 . 5H_2O$ 10.0µg l^{-1}

$Na_2MoO_4 . 5H_2O$ 10.0µg l^{-1}

H_3BO_3 10.0µg l^{-1}

$ZnSO_4 . 7H_2O$ 70.0µg l^{-1}

$CoCl_2 . 6H_2O$ 5.0µg l^{-1}

The mixture was boiled, then allowed to cool before the pH was adjusted to 7.0. The medium was then autoclave at 121°C for 20 minutes, then cooled to approx 50°C and 1ml filter-sterilised methanol added.

MacConkey Agar

Peptone 200.0g l^{-1}

Lactose 100.0g l^{-1}

NaCl 5.0g l^{-1}

Bile Salts 5.0g l^{-1}

Neutral Red 0.03g l^{-1}

Agar 15g l^{-1}

Colony Numbers, Fluorescence and Morphology

150µl of the suspension prepared in section 5.2.1 was spread onto plates of KBA (section 5.2.2.2.) and 5% SNA (section 5.2.2.3) and the plates were incubated at 28°C for 24 hours. The number of colonies was counted before the plates were placed under a UV light and the presence of any fluorescent colonies or diffusible pigments was recorded. The plates were then incubated for a further 24 hours before the number of colonies was

recounted and the fluorescence checked again. The differences in morphologies of the colonies were noted.

Isolation of Colonies for Pure Culture

After 48 hours' incubation (section 5.2.3), up to 5 colonies of each non-mucoid morphology type were streaked onto separate plates of KBA and SNA.

The mucoid colonies were streaked onto plates of MacConkey's agar (section 5.2.2.5) after 24 hours' incubation. All colonies chosen were discrete, ensuring that only one type of bacterium was transferred. The plates were then incubated for 24-48 hours, before being sealed and stored at 4°C.

Further Identification of Pink Bacteria

Bacterial cells were isolated from oedematous tissue according to the protocol outlined in section 5.2.1 and the 1:1000 dilution was plated up and incubated as per section 5.2.3. After 24 hours, each discrete colony was spotted onto a plate of both MMS agar (section 5.2.2.4) and the medium from which it was taken (SNA or KBA), the spot being placed in the same position on each plate, according to a grid pattern.

The plates were then incubated at 28°C for 24 hours. It was then noted which colonies grew on the MMS agar. To confirm the results, the MMS plates were returned to the incubator for up to 7 days, being checked for further growth each day. Any colonies which exhibited continuous growth were taken to be a methylotrophic species.

Gram's Staining

Bacto® 3-Step Gram Stain Set-S (Difco Laboratories, Detroit, USA)

A thin smear of the pure bacterial culture prepared in section 5.2.4 was fixed to a glass slide by heating gently over a flame. The fixed smear was flooded with Crystal Violet (primary stain) and stained for 1 minute before being removed by gentle washing with cold water. The slide was then flooded with Stabilised Gram Iodine (mordant) and retained on the slide for 1 minute, before being washed off with Gram Safranin-T (decolouriser/counterstain). More Safranin-T was added to the slide and left for 20-50 seconds before once again being washed off with cold water. The slide was finally blotted

with a paper towel and allowed to air dry. The sample was examined with an oil immersion lens at 200x magnification.

It should be noted that some Gram positive bacteria (mainly *Bacillus* spp.) can give a Gram negative result. The presence of red cells amongst a predominance of Gram negative ones is an indication of dead, unstainable cells.

Identification of Bacteria by Sequencing 16S Ribosomal DNA (rDNA) Genes

DNA Extraction

A small amount of a pure bacterial colony (section 5.2.4) was transferred to 500µl sterile distilled water (SDW) and was re-suspended by vortexing. The mixture was then heated at 100°C for approximately 5 minutes, before being briefly centrifuged momentarily. The supernatant was then used as a DNA template for PCR.

Polymerase Chain Reaction (PCR)

Each 30µl sample was made up of 24µl 1x ReddyMix PCRMaster mix (Abgene, Epsom, UK), and 1µl each primer (10µM) and 4µl DNA template (section 5.2.8.1). Amplification was performed using a Hybaid Omn-E thermocycler (Thermo, Waltham, MA, USA) and the following programme: initial denaturation at 96°C for 5 minutes; 39 amplification cycles of 1 minute at 96°C, 1 minute at the annealing temperature and 1 minute 30seconds at 72°C; a final extension temperature of 72°C for 10 minutes; and a holding temperature of 4°C.

The general 16S rRNA primer set pA-530R (pA 5'-AGA GTT TGA TCC TGG CTC AG-3', 530R 5'-GTA TTA CCG CGG CTG CTG-3', Schue, 2005), with annealing temperatures of 58°C, was employed in the identification of isolated colonies.

DNA Purification

A QIAquick PCR Purification Kit (50; Qiagen, Hilden, Germany) was used for purifying the PCR product, following the manufacturer's micro-centrifuge protocol.

Agarose Gels

A 1.2% gel, was made (120g l^{-1} agarose powder; 1x TBE (21.6% Tris, 11% boric acid, 1.83% EDTA) 2µl ethidium bromide (10mg ml^{-1})). Wells were loaded with either 10µl of PCR product (loading buffer included with the PCRMaster mix) or 5µl ladder (O'GeneRuler 100base pair (bp) DNA ladder, 0.1µg ml^{-1}, Fermentas, Ontario, Canada).

DNA Sequencing

The amount of purified DNA was estimated by comparing product bands with ladder bands seen on the photograph of the agarose gel. According to the information supplied with the ladder, 5µl of ladder produced a 500bp band comprising 82ng DNA. The size and brightness of this band was then compared to the band produced by PCR and the quantity of DNA estimated.

Each sample was then prepared for sequencing as follows, with separate samples for each of the forward and reverse primers: 8µl de-ionised water, 1µl DNA diluted to give approx 20 ng ml^{-1} and 1µl of primer diluted to 1pM.

The samples were then sequenced using the chain termination method in the genomics laboratory of the University of Birmingham, with an ABI 3700 sequencer.

Comparison With Known Sequences

The forward and reverse-complement sequences of the 16S rDNA were aligned to confirm the success of the sequencing process, then one of the sequences, usually the forward-primed, was subjected to a nucleotide-nucleotide (BLAST-n) homology search (Nucleotide-nucleotide BLAST, NCBI, 2005) to compare it to known sequences. An Expect (E) value of less than 0.05 describes statistically significant homology. The proportion of sequence successfully aligned was noted.

API® Strip Identification

Both API 20E® and 20NE® strips were used, following the manufacturer's protocol included with the kits. The findings were recorded onto the sheets provided and the resulting code translated into an identification using the Analytical Profile Indexes (Biomérieux, 1999).

Due to the un-tested nature in the identification of plant pathogens and their cost, these strip tests were used only for samples that were not successfully sequenced by PCR.

Inoculation of Healthy Tissue

Inoculation With Single Colonies

A small quantity of a pure bacterial colony (section 5.2.4) was transferred from the agar plate into approximately 20ml liquid media of the type on which it had been growing. The tubes were incubated on a shaker (250 rpm) at 28°C for 24 hours.

The tubes were then centrifuged at 3600rpm for 10 minutes and the supernatant drawn off. The remaining pellets were re-suspended in 10mM $MgCl_2$.

The suspension was diluted 1/10 and the optical density at 600nm was ascertained. From this, the number of colony forming units (CFU) could be estimated (an absorbance of 0.2 is equivalent to $1x10^8$ CFU ml^{-1} *Pseudomonas*). The concentration of each suspension was then diluted to 10^6, 10^4 and 10^2 CFU ml^{-1} with 10mM $MgCl_2$.

Inoculation was carried out on plants grown as outlined in section 2.2.1 and watered thoroughly immediately before the process was carried out. The leaf to be inoculated was marked by ringing the petiole with a narrow strip of masking tape. Approximately 0.1ml of each suspension was forced into the underside of the leaf via a syringe.

The plants were maintained in the growth room in the same conditions in which they were grown, with watering and feeding being carried out as in section 2.2.1 and any changes on both the inoculated leaf and the rest of the plant logged for the following 2 weeks.

Inoculation With a Mixture of Colonies

A small quantity of each of two pure bacterial colonies (prepared as in section 5.2.4) were transferred from the agar plates and combined into approximately 20ml liquid media of the type on which they had been growing. The protocol outlined in section 5.2.9.1 was then continued.

RESULTS

Colony Numbers

The number of bacterial colonies isolated from oedematous tissue in comparison to that isolated from healthy tissue after 24 and 48 hours was transformed using the inverse hyperbolic sine (arcsinh), before being plotted on a graph (figure 5.1a) and subjected to two sample t-tests (unequal variances) of the combined KBA and SNA data. From this, it could be seen that there was a highly significant difference (P_{24h} = 0.00; P_{48h} = 0.00) between the number of bacterial colonies found on oedematous tissue and that found on healthy tissue at both time periods. This experiment did not distinguish between pathogenic and non-pathogenic species, nor whether infection was primary or secondary. It should also be noted that the oedema samples were taken only from one outbreak.

The same transformation and analysis was carried out on the number of bacterial colonies isolated from blue spot tissue in comparison to that isolated from healthy tissue after 24 and 48 hours (figure 5.1b). Here, a significant difference was found between the number of bacterial colonies found on oedematous tissue and that found on healthy tissue at both time periods (P_{24h} = 0.01; P_{48h} = 0.05). Again, this experiment did not distinguish between pathogenic and non-pathogenic species, nor primary or secondary infection.

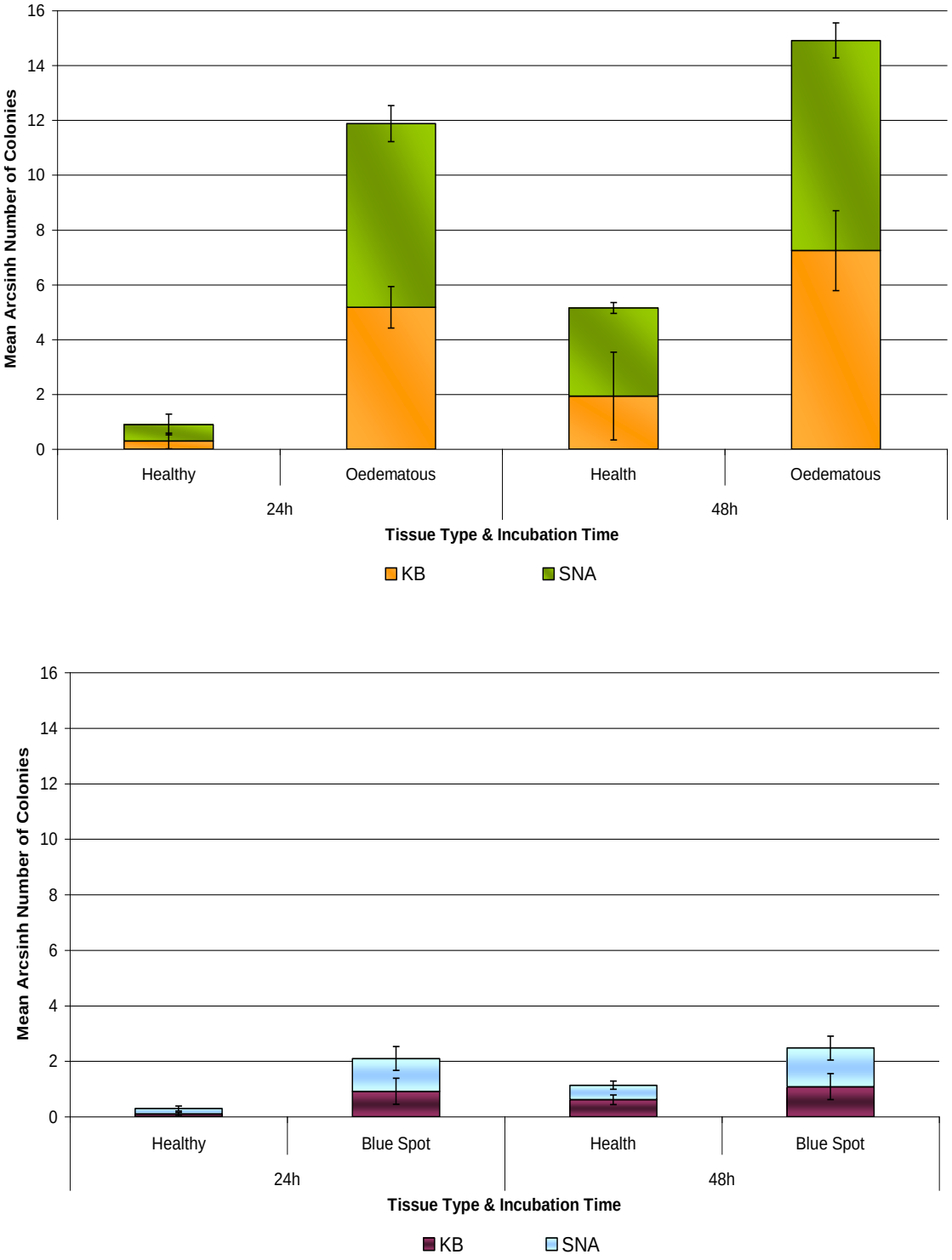


Figure 5.1 a & b: Mean number of colonies (with arcsinh transformation) isolated from healthy coriander tissue and that with a) oedema (n_{24h}=10; n_{48h}=5) and b) blue spot (n_{24h}=45; n_{48h}=30), showing the proportion of the total grown on KBA and SNA (mean ± S.E).

Colony Morphology

The table in figure 5.2 shows the characteristics recorded for bacterial colonies isolated from oedematous and blue spotted tissue after 48 hours incubation at 28°C.

ID number		Colony Morphology
Oedema	**Blue Spot**	
O1	**B1**	Cream, mucoid surface, dense 'pimple' centre, less dense skirt
O2	**B2**	Cream, levoid colony, regular shape, shiny, uniformly dense, no skirt
O3	**B3**	Yellow/orange, mucoid surface, no regular shape
O4	**B4**	Pink, very highly mucoid
O5		Cream/yellow, slightly mucoid
	B5	Cream/yellow, uniformly dense, shiny, not mucoid, no skirt
	B6	Cream, uniformly dense, slight textured surface, dry appearance, very irregular edges

Figure 5.2: Morphologies recorded for bacterial colonies isolated from 1 sample of oedematous and 3 samples blue spotted coriander tissue, after 48 hours incubation at 28°C.

Morphology was similar between colonies grown on KBA and SNA, although those on SNA were softer and slightly more mucoid. An exception to this was seen in colony O2a grown on KBA. After 1 week's incubation a bright yellow diffusible pigment had been produced which was not seen in any other colony, nor on O2a grown on SNA.

From their morphologies, some bacterial strains isolated from oedematous tissue and from that with blue spot, appear to be similar (colonies 1, 2, 3 and 4). Colonies O5 and B5, although similar in colour, were different in texture even though grown on the same medium, indicating that they could be different species. A further bacterial strain with a distinctly different morphology was isolated only from tissue with blue spot (B6).

After the discreet colonies had been streaked onto separate plates and pure cultures isolated, their morphologies could be seen more clearly. In some colonies, additional

characteristics could be identified, while others varied from the original descriptions listed above (figure 5.2). Most importantly, colonies initially identified as the same, showed different morphologies.

Although, some colonies isolated in these experiments are comparable with descriptions of characteristics of certain bacteria (see Lelliott and Stead, 1987, pp48-49), this experiment cannot determine the identification of the colonies. To do this, the same, discreet colony would need to be grown on plates of SNA, KBA and nutrient dextrose agar (NDA), in a similar manner to the protocol outlined in section 5.2.6 and the resulting colonies compared to known characteristics. This was not carried out in this study.

Fluorescence

None of the colonies isolated from any of the tissues were either fluorescent under UV light, or produced fluorescent diffusible pigments, including that of colony O2a. This would indicate that none of the strains are green fluorescent Pseudomonads, discounting the presence of any pathogenic strains, including *P. syringae.*

Further Identification of Pink Bacteria

Only 6 colonies developed on MMS agar (figure 5.3 - highlighted in red). These colonies were salmon pink in colour, levoid and perfectly round. However, after 24h of growth, reaching between 1 and 2mm in diameter, all further growth ceased. Although these colonies were obviously able to survive in the presence of methanol, they were not able to utilise methanol as a source of carbon, therefore they were not true methylotrophic species. This finding was further studied through the use of DNA sequencing.

Condition of Coriander	Plate	No. Colonies Transferred	No. Colonies Growing on MMS After 24h	No. Colonies Growing on MMS After 7d
Oedema	KBA1	28	0	0
	KBA2	46	4	0
	KBA3	75	0	0
	KBA4	22	0	0
	KBA5	44	0	0
	SNA1	41	2	0
	SNA2	53	0	0
	SNA3	24	0	0
	SNA4	28	0	0
	SNA5	62	0	0
Healthy	KBA1	1	0	0
	KBA2	1	0	0
	KBA5	2	0	0
	SNA1	1	0	0
	SNA2	1	0	0
	SNA3	1	0	0
	SNA4	1	0	0
	SNA5	4	0	0
Blue Spot	KBA1	63	0	0
	KBA2	7	0	0
	KBA3	1	0	0
	KBA4	6	0	0
	SNA1	69	0	0
	SNA2	5	0	0

	SNA3	2	0	0
	SNA4	8	0	0
Healthy	SNA1	1	0	0

Figure 5.3: Number of bacterial colonies that developed on MMS agar, in order to identify methylotrophic species isolated from coriander tissue suffering from oedema and blue spot.

Gram's Staining

Condition	Sample	Gram Status	Cell Characteristics
Oedema	O1a	Gram -	1-2µm rods
Oedema	O1b	Gram -	2-3µm rods
Oedema	O2a	Gram -	1-2µm rods
Oedema	O2b	Gram -	2-3µm rods
Oedema	O3a	Gram -	1-3µm rods
Oedema	O3b	Gram -	1-2µm rods
Oedema	O4a	Gram -	1-2µm rods
Oedema	O4b	Gram -	1-3µm rods
Oedema	O5a	Gram -	1-3µm rods
Oedema	O5b	Gram -	2-3µm rods
Blue Spot	B1a	Gram -	1-2µm rods
Blue Spot	B1b	Gram -	1-2µm rods
Blue Spot	B1c	Gram -	1-2µm rods
Blue Spot	B1d	Gram +	3-4µm rods
Blue Spot	B2b	Gram +	2µm rods
Blue Spot	B3a	Gram -	1-2µm rods
Blue Spot	B3b	Gram -	1µm rods
Blue Spot	B4	Gram -	1-2µm rods

Blue Spot	B4b	Gram –	2-3µm rods
Blue Spot	B5a	Gram +	
Blue Spot	B5b	Gram –	2-3µm rods
Blue Spot	B5c	Gram -	1-2µm rods
Blue Spot	B6a	Gram +	3-4µm rods
Blue Spot	B6b	Gram +	2-3µm rods

Figure 5.4: Results of Gram staining of bacterial cells isolated from coriander tissue suffering from oedema and blue spot.

The results of Gram staining isolates of the unknown bacteria isolated from tissue affected by both blue spot and oedema are shown in figure 5.4. All cells isolated from oedematous tissue and 64% isolated from tissue with blue spot were Gram negative, the remaining 36% from blue spot tissue were Gram positive. The difference between Gram positive and Gram negative cells can be seen in the photographs in figure 5.5.

There were no other structures (e.g. flagella) visible on the bacterial cells.

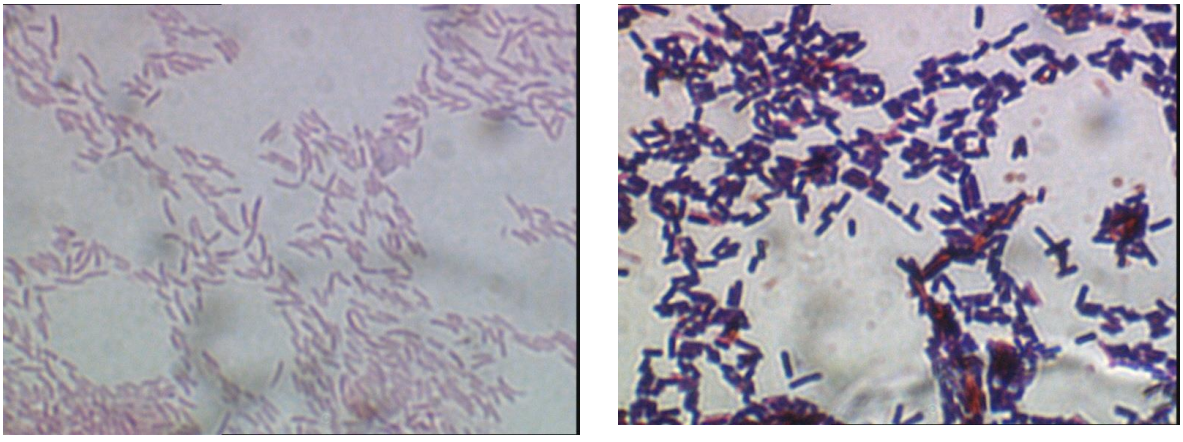


Figure 5.5: Bacterial cells stained with the Gram Stain process, showing the pink Gram negative cells (left) and the purple Gram positive cells (right).

DNA Sequencing

A number of bacteria were identified by sequencing the bacterial 16S ribosomal DNA (figures 5.6 and 5.7). As expected, identification further than the species level was rare, as the 16S rDNA is so highly conserved between genera.

There is considerable overlap between the species isolated from oedematous tissue and that with blue spot. Genera found on both types of affected tissue include Pseudomonas, *Pantoea* (some strains previously classified as *Enterobacter* and *Erwinia*), *Klebsiella* and *Delftia*. A number of colonies isolated from blue spot tissue but not oedema, were identified as species of *Bacillus*.

All sequences matched produced a very highly significant Expect (E) value ($E \leq 0.003$), confirming a high degree of homology. However, in two cases (B5c and B6a), the length of rDNA matched was so small (34/37 and 70/85 base pairs respectively). Bearing in mind that the target sequence was approximately 500bp in length, we really cannot be confident in these identities.

At least 86% of base pairs were matched in the rest of the colony sequences, with the minimum length of similar sequence being 208 base pairs. These identifications can therefore be taken with confidence.

Sample	BLAST Results Identification (Accession Number)	Best E value	No. bp's matched/ library sequence length
B1a	Various *Pseudomonas* spp. (AY882021.1)	All 0.0	420/425 (98%)
B1b	Various *Pseudomonas* spp. (e.g. AY337597.2)	All 0.0	469/471 (99%)
B2a	Various *Bacillus* spp. (e.g. AY 167861.1)	All 0.0	468/469 (99%)
B2b	*Bacillus pumilus* (AY437616.1)	0.0	464/468 (99%)
B2c	*Bacillus pumilus* (AY 4377616.1)	0.0	465/467 (99%)
B3a	*Pantoea agglomerans* (AY266430.1) (syn. *Enterobacter agglomerans* – AF130907.1) *Pantoea ananatis* (AY173023.1)	1e-140 1e-137	336/375 (89%) 333/375 (88%)
B3b	Various *Pantoea* spp. (AY579155.1)	7e-41	148/162 (91%)
B4a	*Erwinia persicinus* (Z96086.1)	1e-129	336/362 (92%)
B4b	Various *Erwinia* spp (e.g. EPJ001190)	All 0.0	415/434 (95%)
B5a	Various *Pseudomonas* spp (e.g. AY574912.1)	All 0.0	444/444 (100%)
B5b	Various *Pseudomonas* spp. (e.g. AF393463.1)	6e-165	305/308 (99%)
B5c	*P. syringae* pv. *Phaseolicola* (CP000058.1)	0.003	34/37 (91%)
B6a	Various *Bacillus* spp. (e.g. AY461745.1)	3e-62	70/85 (82%)
B6b	Various *Acidovorax* spp. (syn. *P. avenae* - e.g. AY788961.1)	All 0.0	441/460 (95%)

	Delftia acidovorans (AY133088.1) (syn. *Comamonas aciovorans / P. acidovorans*)	0.0	441/460 (95%)
B6c	Various *Bacillus* (e.g. AY167860.1)	All 0.0	455/468 (97%)

Figure 5.6: Identification results of 16S rDNA sequencing of bacteria isolated from coriander tissue suffering from blue spot.

Sample	BLAST Results Identification (Accession Number)	Best E value	No. bp's matched/ library sequence length
O1a	Various *Pseudomonas* spp. (e.g. AY 882021.1)	All 0.0	446/450 (99%)
O1b	Various *Delftia* spp. (e.g. AY965248.1) (syn. *Comamonas aciovorans* / *Pseudomonas acidovorans*)	1e-114	283/305 (92%)
O2a	Various *Pseudomonas* spp. (e.g. AY456703.1)	1e-112	208/208 (100%)
O2b	Various *Pseudomonas* spp. (e.g. AF364097.1)	All 0.0	442/443 (99%)
O3a	Various *Pantoea* spp. (e.g. DQ122350.1)	7e-174	323/326 (99%)
O3b	Various *Pantoea* spp. (e.g. DQ229105.1)	1e-41	159/184 (86%)
O4a	No hits found		
O4b	Various *Erwinia* spp. (e.g. AJ001190.1) Various Pantoea spp. (e.g. AY616179.1)	All 0.0 All 0.0	447/448 (99%) 439/443 (99%)
O5	Various *Pantoea* spp. (e.g. DQ365572.1)	All 0.0	396/408 (97%)
O5b	*Pantoea* sp. (DQ122350.1)	0.0	356/370 (96%)

Figure 5.7: Identification results of 16S rDNA sequencing of bacteria isolated from coriander tissue suffering from oedema.

It should be noted that colonies classified as being of the same morphology by traditional, morphological methods were identified by rDNA sequencing as NOT being of the same genera, e.g. B5 ≠ O5; O1a ≠ O1b. This will be discussed further later.

In addition, sample O2b was identified by API 20E® test as *Flavimonas oryzihabitans*, where as the DNA sequencing, which is more accurate, identifies it as a *Pseudomonas*.

API® Strip Identification

The colonies chosen to be identified using Bionerieux's API® system were those which were proving more difficult to sequence using the 16S rDNA protocol outlined in section 5.2.7.

The API® tests are generally simple and quick to carry out. They are not infallible however; the non-enteric (NE) tests, presumed to be the most applicable in this situation, gave no identifications.

The enteric tests, however, did generally provide an identification (figure 5.8), although in a number of cases this was not definitive (e.g. sample O3a - 61.9% probability of *Pantoea* spp. 3 and 27.9% *Pantoea* spp. 4; sample O4a - 42.2% *Pantoea* spp. 2, but may also be *Klebsiella* or *Erwinia*). The species classifications of *Pantoea* refer to the differences in 'strains' revealed by the tests and are only used by Biomérieux to allow greater accuracy in their computing algorithms; they are not true distinct strains in microbiological terms (Jones, 2006).

Sample	API® results
O2b	Flavimonas oryzihabitans
O3a	*Pantoea* spp 3* (61.9%) *Pantoea* spp 4* (27.9%)
O3b	*Pantoea* spp 3* (99.3%)
O4a	*Pantoea* spp 2* (42.2%) or Klebsiella or Erwinia
O5a	*Pantoea* spp 3* (99.3%)
O5b	*Pantoea* spp 3* (95.2%)

Sample	API® results
B3a	*Pantoea* spp 3* (99.3%)
B3b	*Pantoea* spp 3* (99.3%)
B5a	No id

Figure 5.8: Results of API 20E® tests of bacteria isolated from coriander tissue suffering from oedema (O) and blue spot (B).

***The species classifications of *Pantoea* refer to the differences in 'strains' revealed by the tests and are only used by Biomérieux to allow greater accuracy in their computing algorithms; they are not true distinct strains in microbiological terms (Jones, 2006).**

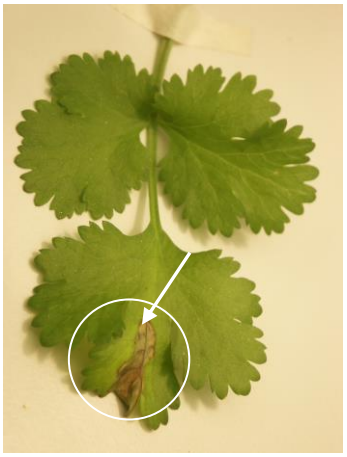
Inoculation of Healthy Tissue

Inoculation With Single Colonies

In order to differentiate between primary and secondary infection being responsible for symptoms seen, inoculation of healthy tissue with solutions of isolated bacterial colonies according to Koch's Postulates, was carried out.

Strain	No. plants inoculated	% plants showing a response	HR induced?	Symptoms after 1 weeks
O1a (*Pseudomonas*)	35	68.6	✓	Discreet areas of PCD at point of inoculation
O1b (*Delftia)*	35	65.7	✓	Discreet areas of PCD at point of inoculation
O2a (*Pseudomonas*)	19	0	×	None
O2b (*Pseudomonas*)	19	15.8	×	Some necrosis of leaf margins
O3a (*Pantoea*)	38	2.6	×	Some necrosis of leaf margins
O3b (*Pantoea*)	38	2.6	×	Some necrosis of leaf margins
O4a (unidentified)	38	0	×	None
O4b (*Pantoea* / *Erwinia*)	38	2.6	✓	Slight necrosis at point of inoculation
O5a (*Pantoea*)	38	2.6	✓	Slight necrosis at point of inoculation
O5b (*Pantoea*)	38	0	×	None

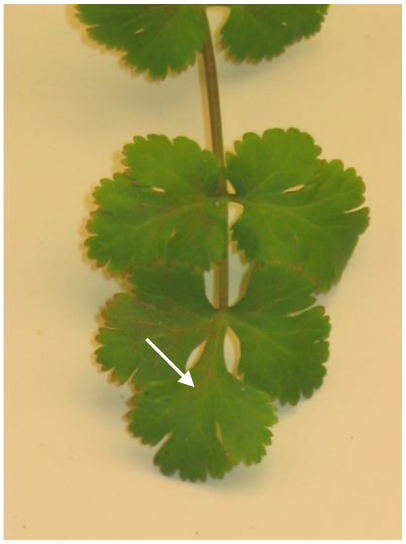
Figure 5.9: Results of inoculating coriander with single bacterial strains isolated from oedematous tissue diluted to 10^6 CFU ml^{-1} in $MgCl_2$.



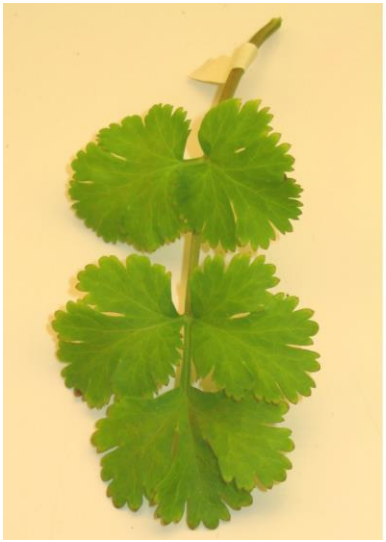
O1a

O1b

O4a



$MgCl_2$ control



-ve control

Figure 5.10: Coriander leaves inoculated with bacterial strains isolated from oedematous coriander and two controls. The point of inoculation is arrowed and the areas of hypersensitive response (HR) are circled. HR symptoms of necrosis and chlorosis are visible on the treated leaves, but not on the controls (1cm=2cm).

None of the single strains of bacteria inoculated into healthy coriander leaves caused disease of any kind, including oedema and blue spot (figure 5.9). However, strains O1a, O1b, O4b and O5a induced programmed cell death (PCD) as part of the plant's hypersensitive response (HR – see photographs in figure 5.10, visible as areas of necrosis with associated chlorois, in the area where the cell suspension entered the leaf tissue. These indicated that these strains were pathogenic to coriander, but are recognised by the plant's defence system, eliciting PCD and the HR.

As strains O1a and O1b elicited such strong responses, the inoculum of was diluted further to 10^4 and 10^2 CFU/ml to allow clearer visualisation of the development of HR lesions. These smaller, localised necrotic areas are shown in figure 5.11.

Figure 5.11: Healthy coriander leaf inoculated with bacterial strain 1a diluted to 10^4 CFU/ml in $MgCl_2$. The point of inoculation is arrowed and the area of PCD is circled (1cm=0.75cm) .

Inoculation With a Mixture of Colonies

There is much literary evidence showing that some species of bacteria, notably *Pantoea agglomerans*, can affect the survival, growth and development of others (e.g. Braun-Kiewnick *et al.*, 2000; Monier and Lindow, 2005). Therefore, it was considered necessary

to mix the strains isolated from diseased tissue and re-infect healthy coriander tissue to analyse the outcome. Unfortunately, due to lack of time, not all strains could be combined.

Of the strains chosen, O3a and B3b were identified by rDNA sequencing as *Pantoea*, while O1a, O1b, O2b, B1a and B5b were *Pseudomonas*. Colonies B2b and B6a were identified as *Bacillus* and O4a remained unclassified.

In addition, of the *Pseudomonas* strains, those which caused a hypersensitive response (HR) when inoculated alone (section 5.3.8.1) were selected to investigate if their pathogenicity would be affected in such a way as to overcome the plant's HR and induce disease.

As the table in figure 5.12 shows, pathogenicity was not influenced and HR was still stimulated. The presence of *P. agglomerans* did not appear to curb the ability of the *Pseudomonas* strains to induce HR, although no exact comparison was carried out to quantify this.

Inoculation with a mixture of *Pantoea* with *Bacillus* or the unidentified strain did not induce either disease or HR, showing that *Pantoea* does not change the pathogenicity of non-pathogenic strains.

Strains	No. plants inoculated	% plants showing a response	HR induced?	Symptoms after 1 week
O3a + O1a	4	100	✓	Spots of necrosis at point of inoculation
O3a + O1b	4	100	✓	Spots of necrosis at point of inoculation
O3a + O2b	4	0	×	None
O3a + O4a	8	0	×	None
O1a control	4	100	✓	Large area of necrosis and chlorosis
O1b control	4	1000	✓	Large area of necrosis and chlorosis
O3a control	4	0	×	None
O2b control	4	0	×	None
O4a control	4	0	×	None
B3b + B1a	4	100	✓	Spots of necrosis at point of inoculation
B3b + B2b	4	0	×	None
B3b + B5b	4	0	×	None
B3b + B6a	4	0	×	None
B1a control	4	100	×	Spots of necrosis at point of inoculation
B2b control	4	0	×	None
B3b control	8	0	×	None
B5b control	4	0	×	None

Figure 5.12: Results of inoculating coriander with mixtures of bacterial strains isolated from both oedematous and blue spotted tissue diluted to 10^6 CFU ml^{-1} in $MgCl_2$. *Pseudomonas*; *Pantoea*; *Bacillus*; unidentified

DISCUSSION

This study utilised a number of identification techniques, some of which were developed many years ago and others which are much more recent innovations. The traditional technique of plating unidentified bacteria onto particular media and comparing colony morphology to that of known strains is quite time consuming and laborious, but also dependant on the working environment (lighting etc.) and the pathologist's subjectivity and experience. Morphologies also change with colony age and the temperature at which they have been incubated (Fox, 1993).

The difficulties of identification using the traditional plating method can be seen here. When colonies of bacteria classified as being morphologically similar were analysed by 16S rDNA sequencing, they were found to be of different genera entirely and, conversely, differently morphologically-classified bacteria were found to be of the same genus.

As the results from DNA sequencing are much more reliable, even though the technique can be difficult to perfect, these results were accepted. Four genera of bacteria were identified from oedematous tissue and six from tissue with blue spot. None of these were methylotrophic, agreeing with the MMS plating experiment results outlined in section 5.3.4.

In this particular set of experiments, identification down to the pathovar level was never envisaged. In fact, identification to the species level was thought unlikely (although it was achieved in some cases). This is due to the 16S rDNA being highly conserved, thus, as variation between genera is minimal, variation within species is even more so. In addition, the primers used in this case were non-specific (i.e. they were not targeted to any particular species of bacteria), so were not expected to allow a high degree of differentiation.

Strains of *Pseudomonas*, *Pantoea*, *Erwinia* and *Delftia* were identified in both types of tissue, with *Bacillus* and *Acidovorax* only being found in blue spotted tissue. All of these bacteria are known to comprise pathogenic species and pathovars, however most bacteria have a specific host range (Agrios, 1997). It should be noted that many of these bacterial species are very closely related and have recently been re-classified following genetic analysis using the modern DNA sequencing techniques.

The non-fluorescent *Bacillus* is one of the minority of phytopathogenic bacteria which are Gram positive, confirmed by the Gram staining carried out in section 5.3.5.

Some species of *Bacillus* cause soft sots, particularly of potatoes and tobacco leaves in storage, of tomato seedlings and soya beans (Agrios, 1997), while others are known bio-control agents e.g. *B. cereus* protects legumes against damping off diseases (Agrios, 1997). The only *Bacillus* identified to species level was *B. pumilus* and that is known not to be pathogenic, but instead is a plant growth-promoting rhizobacterium (PGPR), bacteria which produce plant hormones, such as auxins, gibberellins and cytokinins (Joo *et al.*, 2004). In this case the predominant hormones produced were gibberellins. This bacterium is likely to be present on most leaf surfaces.

There are a number of species of *Acidovorax* which are pathogenic to plants. *A. valerianella* causes bacterial spot of the salad crop 'lambs lettuce' (*Valerianella locusta*)(L.) Laterr) (Gardan *et al.*, 2003) and *A. avenae* ssp.*avenae* gives rise to a number of important diseases of grain crops including rice (Shakaya *et al.*, 1985), corn (Summer and Schaad, 1977), millet (Song *et al.*, 2004) and sugarcane (Martin and Wismer, 1989; all cited in Song *et al.*, 2004). These non-fluorescent Pseudomonads, all previously classed as species of *Pseudomonas*, have recently been placed in the '*acidovorans*' DNA-rRNA homology group after genetic anlysis (Willems *et al.*, 1992).

Delftia acidovorans is the recent reclassification of *Pseudomonas acidovorans* (Wen *et al.*, 1999). Other *Comamonadaceae* include the genus *Acidovorax*. There is very little literature relating to *Delftia*, although it is apparently not pathogenic. Wen states that strains have been isolated from soil, sediment, activated sludge, crude oil, oil brine, water and various clinical samples, so it is not surprising to find it associated with leaves.

There are many fluorescent and non-fluorescent pathogenic species which remain classified as *Pseudomonas*, causing leaf spots, blights, vascular wilts, soft rots cankers and galls on most plant species (Lelliott and Stead, 1987; Agrios, 1997), some of which are of economic importance to worldwide agriculture e.g. *P. syringae* pv. *tabacci* causes wildfire disease of soybeans (University of Illinois Extension, 2006); *P. viridflava*, the source of stem and pith necrosis of tomato, causes up to 30% damage to commercial winter production in Crete (Goumas *et al.*, 1999). There is a specific pathovar which affects

coriander (*P. syringae* pv. *coriandricola*), as has been discussed previously, although testing the fluorescence indicated that it was not present (most *P. syringae* pathovars being classified as green fluorescent Pseudomonads), a finding confirmed by the lack of symptoms seen in the inoculation experiments (section 5.3.8).

Erwinia agglomerans and *Enterobacter herbicola* have recently been identified as the same bacterium through DNA-DNA hybridisation (International Society of Plant Pathology, 2005) and added to the *Pantoea* genus (Gavini *et al.*, 1989). *Pantoea agglomerans* is a minor pathogen, for example causing leaf die-back on garlic (Koch *et al.*, 1996). More importantly, it has an antagonistic effect on a number of other pathogenic bacteria. In one study, when *P. agglomerans* was applied to barley seeds as a biocontrol agent against *P. syringae* pv. *syringae*, basal kernel blight was suppressed by between 45% and 74% in field trials (Braun-Kiewnick *et al.*, 2000). In others, *Erwinia amylovora* was inhibited by strains of *P. agglomerans* (Wright *et al.*, 2001; Özaktan and Bora, 2004). Wright *et al.* state that the strain EH318 produced two antibiotics which inhibit the *Erwinia*.

The precise identification of the bacteria could have been verified by repeating the sequencing experiments using alternative, more specific primers, targeted to species and /or pathovars of the genera revealed here. This was not carried out for reasons that will be described later.

Biomérieux's API® strip tests are aimed at the clinical environment, with associated human pathogens. Using them in this study investigated the use of the technique as much as to identify the bacteria. They are generally easy to use, providing the required re-agents are kept fresh and refrigerated. The results may also be somewhat unreliable due to the different bacterial targets. The obvious examples are in the API® identification of colony O2b as *Flavimonas oryzihabitans*, when 15S rDNA sequencing identified the colony more reliably as a strain of *Pseudomonas* and the failure of the API® system to identify colony B5a. It is unlikely that these kits would be of use in the field environment as pure cultures are required, although they are adequately portable.

The significant increase in the colony numbers isolated from oedematous and blue spotted tissue over healthy coriander is interesting and high-lights the increased susceptibility of

weakened and damaged plants to increased infection. As indicated previously, the experiments on oedematous tissue were carried out on samples taken from a single disease outbreak, therefore further repeats of this experiment would be required to confirm if these results are a true representation of the general condition, or whether it is specific to this site.

There is also no indication as to whether any of the bacteria seen were pathogenic and caused the conditions, or if they were exploiting the state of the plants as secondary infection. This is carried out by inoculation of healthy tissue with pure bacterial colonies isolated from diseased tissue according to Koch's Postulates.

This technique is evidently still of use, even with modern nucleic acid methodologies. This is especially true when attempting to identify the cause of a condition as inoculation confirms that the isolated bacterial strains are truly the cause of the disorder.

The inoculations of oedema-isolated strains carried out in this investigation did not result in the onset of any disease symptoms, demonstrating that these strains were not the cause of this condition. It should be noted that due to restrictions of time, no isolates from blue spotted tissue were re-inoculated into healthy coriander.

That is not to say, however, that none of the strains were pathogenic. In certain cases, injection of the inoculum resulted in patches of necrosis and chlorosis, which are signs of HR.

As previously described, HR occurs in incompatible plant-pathogen interactions, i.e. those where an avirulent pathogen attacks a resistant host (Alvarez, 2000). In compatible reactions (susceptible host and virulent pathogen), the response is either absent or delayed and disease develops.

HR triggers a cascade of morphological, physiological and molecular changes, which contribute to PCD, with the aim of severing the source of nutrients and preventing the further spread of the pathogen.

CONCLUSIONS

From these investigations it can be concluded that oedema is not caused by the bacterium *P. syringae* pv. *coriandricola*, as indicated from anecdotal evidence. Firstly, none of the colonies isolated from diseased tissue exhibited the morphological characteristics (such as colour and fluorescence) of this bacterium and inoculation of healthy plants with these strains did not induce disease.

The inoculation experiments also show that the condition is not due to other strains of bacteria. Although the isolated colonies were identified as being part of genera comprising pathogenic strains, no symptoms were induced. Indeed some of the strains inoculated were pathogenic, as they were recognised by the plants and triggered HR.

The origins of blue spot are not so clear-cut. A lack of time prevented all bacterial colonies isolated from tissue with this condition from being re-inoculated into healthy coriander tissue, thus it cannot be determined conclusively that any of the bacteria were responsible for the condition.

Inoculation with a mixture of *Pantoea* and *Pseudomonas* did not induce disease where none was stimulated by each bacterium alone. This suggests that the interaction does not occur in all species of *Pantoea* and *Pseudomonas*.

Knowledge of traditional plating techniques remains useful even though more recent identification techniques (e.g. sequencing of 16S rDNA; fatty acid analysis) are more reliable and more accurate. The use of biochemical identification kits, such as the API® system used here, are of some use, but, as they are targeted to medical bacterial pathogens, their results can be somewhat unreliable. They are also expensive, often above the budget of plant pathology analytical laboratories.

GENERAL DISCUSSION AND CONCLUSIONS

The primary aim of this project was to understand the physiology and the causes of oedema, to then allow preventative measures to be investigated, both in coriander and other herb and salad crops.

There has been little research carried out on oedema and none of this appears to be on herbs. This gave a wide remit in which to pursue the causes of oedema in coriander. The subjects studied were the result of considerable discussions with growers, taking account of their theories and also from studying literature of other diseases and conditions with similar symptoms.

The three main hypotheses investigated were:

Elevated atmospheric humidity caused water pressure within the plant to build and cells to burst.

A calcium imbalance in the plant weakened cell walls, destroying the intercellular structure and resulting in the lesions.

The symptoms were of microbiological origin.

Before any analysis of the causes could take place, a fuller understanding of the physiology of the condition was required. During this analysis, it became apparent that growers were calling two conditions by the same name. Therefore detailing, comparing and contrasting each set of symptoms would be advantageous. For the clarity of this study, the original grey symptoms were termed 'oedema' and the secondary symptoms termed 'blue spot'.

Visual differences were obvious at all levels of study. Oedema manifested as interveinal greying of the upper leaf laminar, within which a small, brownish, sunken lesion developed. In contrast, blue spot, as its name suggests, formed irregular, slate-blue patches on the leaf laminar, which were not constrained by veins. In neither condition did the blemishes expand and spread once developed.

Under the light microscope, the sunken lesion from oedematous tissue was clearly visible. The cells appeared intact, although with altered shape and having lost intercellular structure. This was clarified through the use of environmental scanning election microscopy (ESEM).

Blue spot tissue did not exhibit any of the structural degradation seen in oedema. In fact there were few differences apparent between that and healthy tissue at a cellular level. The only disparities seen were in the leaf cuticle, which appeared thicker in blue spotted tissue than healthy, a difference clearly visible under the ESEM and the increased visibility of chloroplasts in tissue with blue spot. However, when this latter point was investigated further, neither the amount of chlorophyll nor the amount of chlorophyll-a or chlorophyll-b differed between the two tissue types.

Studying the effect of humidity and temperature on oedema and blue spot was carried out using two approaches. Firstly, environmental conditions were simulated in the laboratory and the effect on healthy plants was recorded. Additionally, meteorological data was collected over the three years of the project from as many sites as could provide it and linked to any outbreaks.

It was unfortunate that the crop and meteorological data provided by growers was incomplete, although trends were still apparent. When certain meteorological variables (mean daily mean relative humidity, mean daily mean soil temperature, mean maximum daily air temperature, mean minimum daily air temperature, and mean daily mean air temperature) were plotted against the appearance of symptoms, it was clear that no particular variable alone caused the appearance of either oedema or blue spot, however the pattern of occurrence of symptoms within the general spread of the meteorological parameters was such, that the presence of an additional variable (termed x-factor), was possible. It is this 'x-factor' which is under closer influence of the meteorological conditions and could be the cause of the disease. The most likely candidate is of microbiological origin.

Investigating a pathogenic cause to oedema and blue spot was important after the findings from the meteorological data analysis proved inconclusive. Electron microscopy did not reveal the presence of fungal hypha, so analysis was confined to looking at bacterial pathogens. The use of more traditional techniques, such as the recording of morphological

characteristics of bacteria produced by different growing media, along with nucleic acid technology was very effective and proves that old techniques are not necessarily useless. As expected, there were many more bacteria found on diseased tissue than on healthy, but it could not be determined if these were from primary infection (i.e. caused the initial symptoms) or secondary infection (the micro-organisms taking advantage of easy access provided by open wounds and the increased availability of nutrients released by the primary pathogen). Isolation and DNA sequencing of bacteria found in oedematous and blue spotted tissue did not identify any unusual strains. Some genera were known not to contain members which attack plants (e.g. *Delfia*), while others did (e.g. *Bacillus* and *Pseudomonas*). As the inoculation of healthy tissue with suspensions of the isolated bacteria did not result in the production of oedema, re-identification of the bacterial strains to species and pathovar level was not deemed necessary. The Koch's Postulate inoculation did however, result in certain strains eliciting programmed cell death, as part of the coriander plant's hypersensitive response to recognised pathogens. Some studies have indicated that the presence of one kind of bacteria can enhance the pathogenicity of others. However, mixing together the bacterial strains did not result in oedema. Due to time restrictions inoculation using bacteria isolated from tissue with blue spot were not carried out.

The laboratory experiments attempted to trigger the conditions by varying the environmental conditions (continuously elevated humidity, sudden changes in humidity and sudden changes in temperature). In addition, a pressure chamber was used to simulate increased xylem pressure within coriander leaflets. None of these experiments resulted in either oedema or blue spot, confirming the conclusion that changes in environmental conditions alone are not responsible for either set of symptoms.

Of the other growing conditions analysed, soil acidity was found to have no effect on the likelihood of outbreaks of oedema or blue spot, as pH was similar at all sites, regardless of whether symptoms developed. No clear conclusion could be drawn from the soil type data. Over 90% of blue spot cases were seen in sandy clay loam soils, the soil type usually preferred by growers for providing optimal growing characteristics and its ease of working. Oedema however, appeared in most soil types, although just over 50% were

found on heavier soils. These findings cannot be classed as conclusive without further analyses being carried out, as sample number was small.

From the studies of leaf ion balance, it was inferred that neither excess nor deficiency of calcium correlated with oedema or blue spot in coriander. Ion chromatography of sap samples from healthy, oedematous and blue spotted coriander showed a significant difference between ion concentrations in healthy and blue spotted tissue, but not between healthy and that suffering from oedema. In particular, potassium concentrations were significantly increased in blue spot tissue, although whether the difference was the cause of the blue spot, or the blue spot cause of the difference could not be determined.

Analysis of the ratios of cations:potassium did not show any obvious trend, with some sites presenting significant differences between blue spot and healthy tissue and others being non-significant. The same occurred with sites suffering from oedema.

Overall, it can be concluded that oedema and blue spot are separate conditions and should be classified as such. It does not appear that a calcium imbalance is responsible for either oedema or blue spot and, although significantly higher ion concentrations were found in blue spotted tissue, it is not clear whether this is responsible for the condition, or *vive versa*. In addition, neither condition is due solely to a particular meteorological condition. Instead, it seems more likely that the presence of an additional variable (the x-factor), under close influence of its environment, is the cause of the symptoms. This x-factor has many characteristics of a micro-organism and, although none were identified, the growers' theory that the symptoms my have been produced by the bacteria *Pseudomonas syringae* was not upheld.

The main drawback to this project was the small number of samples on which to carry out experiments. To clarify the findings, larger samples would be required. As then importance of the conditions appears to be diminishing, future work should concentrate on studying the physiology of coriander and other herbs, an area not usually studied. Recording more detailed effects of environmental conditions on coriander growth and development, e.g. the effects of certain soil types, nutrient status through the lifecycle and nutrient preferences, growers will benefit by being able to optimise of cultural practices and agrochemical use.

APPENDIX 1A

Guidance For Completing Record Sheets

- Thank you for agreeing to fill in these record sheets. They will allow me to study the interactions of environmental conditions, applications of chemicals, etc, with any occurrences of oedema.
- The record sheets are (for the time being) for crops of coriander.
- Please fill in as much detail as possible, as the more information I have, the better my analysis will be.
- Please complete a record sheet for as many different coriander crops as possible throughout the season (including those which do not develop the oedema).
- Please note that the record sheet is two-sided.

General

- The **Location ID**, refers to the reference which you have for the crop you are logging on the record sheet. Please ensure this is filled in so that I can link different record sheets to the same location.
- The **Aspect** refers to the direction the field/greenhouse/poly-tunnel is facing.
- If you do not calculate **Sowing Density** in **kg/ha**, please note it in the form you use. However, please indicate what measurement it is.

Application of Chemicals

- **Chemicals** refers to any form of fertiliser, herbicide, fungicide or insecticide which you apply. Please continue any further applications at the end of the record sheet.

Irrigation

- If you measure irrigation in a way other than **Irrigation Period** or **Amount Applied**, please indicate it on the form.
- **Type Of Irrigation System** would indicate drip feed system, overhead sprinkler etc.

Occurrence of Oedema

- As the **State of Crop Development** is quite subjective, please indicate the approximate height of the plants affected.
- Again, both **Percentage of Crop Affected** questions are subjective and need only be roughly calculated.

Any Further Comments

- Please note any other details which you feel to be important and may help in the analysis of data.

Please return the record sheets to me at the following address:

Nathalie King (Post Grad), University of Birmingham, School of Biosciences, Edgbaston, Birmingham B15 2TT

If you have any queries or problems, please do not hesitate to contact me:

0121 414 5481 NGK240@bham.ac.uk

APPENDIX 1B

The Causes & Prevention of Lesions (Oedema) in Herbs

Coriander

Company: _____ ID:_____ Date: _____

General

Grown in/under: ○ Field ○ Poly-tunnel ○ Glass house ○ Fleece

Growing medium: ○ Soil/compost ○ Gel culture ○ Other _____

Location ID: _____ Date sown: _____

Variety planted: _____ Aspect: _____

Previous crop: _____ Sowing density (kg/ha seed): _____

Soil

Soil Type: _____

Soil pH (if known): _____

Application of Chemicals (Fertiliser, Foliar Feed or Protection Products)

Any chemicals applied? ○ Yes ○ No

If Yes:

Name of chemical: _____ Name of chemical: _____

Purpose: _____ Purpose: _____

Dose: _____ Dose: _____

Date applied: _____ Date applied: _____

Name of chemical: _____ Name of chemical: _____

Purpose: _____ Purpose: _____

Dose: _____ Dose: _____

Date applied: _____ Date applied: _____

Irrigation

Was crop irrigated? ○ yes ○ no

Dates irrigated: _____

Irrigation period / amount applied: _____

Type of irrigation system: _____

Occurrence of Oedema

Date symptoms first noticed: _____

Stage of crop development when symptoms first noticed:

○ Seedling (≤ 2 true leaves) ○ Later

If later, height of crop (cm): _____

Approximate percentage of crop affected: _____

Before which harvest? ○ First ○ Second

If before second harvest:

Date of first harvest: _____

Was condition noted before first harvest? ○ Yes ○ No

Is crop saleable? ○ Yes ○ No

Any Further Comments

APPENDIX 1C

The Causes & Prevention of Lesions (Oedema) in Herbs

Coriander

Company: ID: Date: _____

General

Grown in/under: ○ Field ○ Poly-tunnel ○ Glass house ○ Fleece

Growing medium: ○ Soil/compost ○ Gel culture ○ Other _____

Location ID: _____ Date sown: _____

Variety planted: _____ Aspect: _____

Previous crop: _____ Sowing density (kg/ha seed): _____

Soil

Soil Type: _____

Soil pH (if known): _____

Application of Chemicals (Fertiliser, Foliar Feed or Protection Products)

Any chemicals applied? ○ Yes ○ No

If Yes:

Name of chemical: _____ Name of chemical: _____

Purpose: _____ Purpose: _____

Dose: _____ Dose: _____

Date applied: _____ Date applied: _____

Name of chemical: _____ Name of chemical: _____

Purpose: _____ Purpose: _____

Dose: _____ Dose: _____

Date applied: _____ Date applied: _____

Irrigation

Was crop irrigated? ○ yes ○ no

Dates irrigated: _____

Irrigation period / amount applied: _____

Type of irrigation system: _____

Occurrence of Oedema

Type of Oedema Symptom: ○ Blue Spot ○ Grey Patches

Date symptoms first noticed: _____

Stage of crop development when symptoms first noticed:

○ Seedling (≤ 2 true leaves) ○ Later

If later, height of crop (cm): _____

Did condition spread? ○ Yes ○ No

Approximate percentage of crop FIRST affected: _____

Approximate percentage of crop FINALLY affected: _____

Before which harvest? ○ First ○ Second

If before second harvest:

Date of first harvest: _____

Is crop saleable? ○ Yes ○ No

Any Further Comments

APPENDIX 1D

The Causes & Prevention of Lesions (Oedema) in Coriander

Company: ID: Date: _____

General

Grown in/under: ○ Field ○ Poly-tunnel ○ Glass house ○ Fleece

Growing medium: ○ Soil/compost ○ Gel culture ○ Other _____

Location ID: _____ Date sown: _____

Variety planted: _____ Aspect: _____

Previous crop: _____ Sowing density (kg/ha seed): _____

Soil

Soil Type: _____ Soil pH (if known): _____

Irrigation

Was crop irrigated? ○ yes ○ no

Dates irrigated: _____

Irrigation period / amount applied: _____

Type of irrigation system: _____

Occurrence of Oedema

Type of Oedema Symptom: ○ Blue Spot ○ Grey Patches

Date symptoms first noticed: _____

Stage of crop development when symptoms first noticed:

○ Seedling (≤ 2 true leaves) ○ Later - crop height (cm): _____

Did condition spread? ○ Yes ○ No

Approximate % of crop FIRST affected: _____ FINALLY affected: _____

Is crop saleable? ○ Yes ○ No

Please Add Any Further Comments Overleaf

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