Effect of salinity and its interactions with Verticillium albo-atrum on the disease development in tomato (Lycopersicon esculentum Mill) and lucerne (Medicago sativa L & M. media) plants.

Dikilitas, Murat

EFFECT OF SALINITY & ITS INTERACTIONS WITH VERTICILLIUM ALBO-ATRUM ON THE DISEASE DEVELOPMENT IN TOMATO (*Lycopersicon esculentum* Mill) and LUCERNE (*Medicago sativa* L & *M. media*) plants

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To my parents

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ABSTRACT

The increased levels of salinity associated with irrigation practices pose a threat to crop production, especially where water quality is marginal. Under these conditions plants are not only stressed but may also be more susceptible to various pathogens. One strategy to maintain yields in such situations, or to increase yields in marginal areas has been to develop salt tolerant strains of crops. Such strains should also be resistant to pathogens in that environment, including halophytic races of the pathogen that may have adapted to the higher salt concentrations. An understanding of the interactions between a crop plant and potential pathogens under salinity is, therefore, an important part of a project to develop strategies for disease control in crops grown in saline soils.

In this project, plant-pathogen-, plant-salt-, pathogen-salt-, plant-pathogen-salt interactions were studied. The interactions between tomato and lucerne and isolates of the vascular wilt fungus *Verticillium albo-atrum* were investigated under non-saline and saline conditions. Pathogenicity trials indicated that isolates of V1 and V2 were pathogenic to tomato, however, the degree of pathogenicity of the isolates were affected by the particular cultivar, plant age, method of inoculation and temperature. The pathogenic effects of *Verticillium* on tomato did not increase with increasing spore concentration or when isolates were co-inoculated.

NaCl both delayed and reduced the germination rate of seeds and caused severe symptoms on plants. The effect of NaCl was also detrimental on the germination and the growth rate of the fungus.

Progress of the disease was markedly accelerated by salt stress. Various growth parameters were lower in the group of plants both inoculated with *V. albo-atrum* & treated with NaCl than they were for plants inoculated only with *V. albo-atrum* or only treated with NaCl. As a defence response, an increase in PAL activity occurred when lucerne cells were treated with an elicitor from *V. albo-atrum*; a further increase was evident when cells were exposed to both elicitor and NaCl. However, the increase in PAL activity was minimized both at high concentrations of elicitor and NaCl.

Disease-resistant and salt-tolerant plants showed resistance to V1 under non-saline and saline conditions; however, disease-susceptible and salt-tolerant plants did not show resistance to V1 under those conditions. Development of salt tolerant crops should therefore, involve selection of strains that maintain or improve its resistance to pathogens.

Abbreviations

2,4-D	2,4 Dichlorophenoxy acetic acid
A & H	Arnon and Hoagland (1940) nutrient solution
ABA	Abscisic acid
ANOVA	Analysis of variance
BER	Blossom end rot
BOi2Y	Bingham *et al* (1975) regeneration medium
CAM	Crassulacean Acid Metabolism
CHL	Chlorophyll
cv	Cultivar
d.f.	Degrees of freedom
DOX	Czapek Dox Medium (modified)
Dwt	Dry weight
EC	Electrical conductivity
EDTA	Ethylenediamine tetraacetic acid
FAO	Food and Agricultural Organization
FDA	Fluorescene Diacetate
FLW	Number of Flowers
FW	Fusarium wilt
Fwt	Fresh weight
GAs	Gibberellic acids
H	Height
IAA	Indoleacetic acid
IC50	Inhibition of concentration that inhibits 50% of the population
LA	Leaf area
LAR	Leaf area ratio
M & S	Murashige and Skoog (1962) medium
M	Molar
MPa	Mega Pascal
MS	Mean Square
NAR	Net assimilation rate

ns	non significant
P	Probability
PAL	Phenylalanine lyase activity
PAR	Photosynthetically active radiation
PDA	Potato Dextrose Agar
PE	Pectin esterases
PEG	Polyethylene glycol
PG	Polygalacturonases
PL	Pectin lyase
PLP	Lipopolysaccaharide
RGR	Relative growth rate
RL	Root length
SE	Standard Error
Sig.	Significance
SS	Sum of squares
T50	Time taken for 50% total germination
UWS	University of Wales, Swansea
v/v	Ratio of volume to volume
VAM	Vesicular-arabuscular mycorrhizal
VW	Verticillium wilt
w/v	Ratio of weight to volume
WTC	Water content of the plants

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"Healthy plants, healthy planet"
(The American Phytopathological Society).

GENERAL INTRODUCTION

Humans have relied on plants for many centuries. Many civilizations are dependent on crops such as rice, wheat, barley or corn for their survival. People not only rely on plants for their own food but they also use them for animal feed. So, everything we do is affected either directly or indirectly by plants. However, plants are also affected by pathogenic or non-pathogenic stresses (Hamilton *et al.*, 1990). For example, plant diseases often seriously limit agricultural productivity and have therefore influenced the history and development of agricultural practices. On the other hand, limitation on crop productivity arising from non-pathogenic stress, such as adverse temperatures, frost damage, air pollution and accumulation of salt, are also important problems in agricultural areas. Among these stresses, osmotic or toxic stress due to salinity is the most serious problem that limits plant growth, particularly in arid regions (Fisher & Turner, 1978; Zekri & Parsons, 1990). Such regions are affected seriously by salinity (soil containing high concentrations of soluble salts) resulting in a decrease in the area of arable land (Flowers & Yeo, 1986).

Today, *ca.* 20% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity (Rhoades & Loveday, 1990). This is a substantial portion of the world's land (Chowdhury *et al.*, 1993). For example, about 25-30% of the crops grown in the United States suffer from severe salinity. On the other hand, semi-arid regions in Asia, such as those irrigated by the Indus in Pakistan, the Tigris and Euphrates flowing through Syria and Iraq, and the Ganges system in the North-West of India have the worst salinity problems (McWilliam, 1986). These figures might increase in the future and indicate the magnitude of the problem that must be tackled if future global food needs are to be met.

Since the use of arable land is decreasing, in the future, economically important plants may be bred for their resistance to disease and non-pathogenic stress agents and should be made commercially available. However, some economically important plants may tolerate abiotic stress factors but they may not show resistance to the effect of pathogens or they may show resistance to the effect of pathogens but they may not tolerate the abiotic stress factors. So, it is important to generate such crops for resistance

to diseases and environmental stresses. To do this, it is important to understand the physiological responses and the defence mechanisms such plants express under the sorts stress conditions in which the plants are required to grow.

The family Fabaceae includes many valuable forage plants, among them medics, members of the genus *Medicago*, which contains more than 50 annual and perennial species. One of the species of this genus is *Medicago sativa*, (lucerne or alfalfa), described as "The Queen of the Forages". It originated near Iran, but related forms and species are found as wild plants scattered over central Asia and into Siberia (Stuteville & Erwin, 1990). The first recorded reference to lucerne was found on brick tablets of Hittite sites in Central Anatolia around 1300 BC. From that time lucerne was spread throughout Asia and Europe and into America. High nutritional quality is one of the most important characteristics of lucerne. It is rich in protein, vitamins (A and D), and minerals and has a very high yield potential compared with that of other forage crops. Thus, it is prized by growers as a primary component in dairy cattle rations and is an important feed for horses, cattle, sheep, and milking-goats. For example, lucerne meal is made into pellets and used in mixed feeds for cattle, poultry and other animals. It may be grown as a cover crop, where it may increase the yield of succeeding crops, and improve soil structure and tilth. It also controls weeds in subsequent crops (Heichel *et al.*, 1981). The presence of effective nodules on the roots of the plants is essential to a vigorous, productive stand. These nodules, formed by bacteria (*Rhizobium meliloti*), are able to fix nitrogen from the air for use by the lucerne plants.

A mature lucerne plant may have from 5 to 25 stems, which usually reach a height of 38-63 cm, and a deep root system that may reach depths of 2 to 6 m (Heuser, 1931; Brown & Miller, 1978), thus protecting the plant from drought (Williams & Stiles, 1962; Stiles, 1966). Stems are branched and slender and bear trifoliate leaves. A wide range of soil and climatic conditions are suitable for lucerne, but for best production it requires a well-drained soil with nearly neutral pH and good fertility (Hanson *et al.*, 1988). However, lucerne is affected by a wide range of organisms. For example, more than 20 diseases are reported as serious in lucerne, these include fungal and bacterial wilts, leaf spots, crown and root rots, viruses, nematodes and insects. Wilts that are significant in lucerne include bacterial wilt caused by *Clavibacter michiganensis* subsp. *insidiosus*,

fusarium wilt caused by *Fusarium oxysporum* Schlecht. f. sp. *medicaginis* and verticillium wilt caused by *Verticillium albo-atrum* Reinke & Berth (Stuteville & Erwin, 1990). On the other hand, lucerne is also exposed to many types of environmental stress such as salinity and drought. Although, lucerne is recognized as being moderately salt tolerant (Maas & Hoffman, 1977) this response varies according to genetic and climatic factors.

Another economically important plant group is the Solanaceae family, which includes *Lycopersicon esculentum* (tomato). It is cultivated worldwide in a variety of environments. The genus *Lycopersicon* exhibits a vast reservoir of genetic variability that remains largely unexploited. Some have disease resistance and salt tolerance and are now mostly commercially available. They have been derived from related species of *Lycopersicon.* Some of these species cross readily to *L. esculentum,* but a few others have compatibility barriers, which make gene transfer by sexual means more difficult. The tomato and its close relatives are believed to have originated in the mountainous regions of Andes (Peru, Ecuador and Chile). Domestication of the tomato is believed to have occurred with the early civilizations of Mexico. European explorers brought the tomato and disseminated it throughout Europe and Asia in the sixteenth century.

The global demand for food and raw materials produced by agriculture requires the further study and optimal utilization of soil resources of the earth. Thus, improving resistance of plants to pests, disease and environmental problems has been the main objective of some plant scientists. To overcome problems with salinity in cultivated arid areas, generation of salt-tolerant plants, and new technological developments, such as drainage improvements and reclamation, have helped. However, classical plant breeding has had limited success in improving the salt tolerance of crop species (Epstein *et al.*, 1980). Consequently, for lucerne, many laboratories have selected salt-tolerant cells from cultures (for example, Croughan *et al.*, 1978; Winicov, 1991; Shah *et al.*, 1993; Chaudhary *et al.*, 1994; Al-Rawahy, 2000). In some studies, *M. media* Pers. (cv. Rambler) was initiated from leaflet explants and used to produce cell lines tolerant to high levels of NaCl (Chaudhary, 1996; Al-Rawahy, 2000). The plants regenerated from salt adapted cell lines were found to have more tolerance to NaCl than the unselected plants.

It is possible that salt-tolerant cultivars of lucerne or tomato will be of economic importance in the future. On the other hand, lucerne or tomato is affected by various diseases, particularly those in which *Verticillium* is the causal agent (Isaac, 1957; Basu, 1987; Pennypacker, 1991; Esyanti, 1993) and may still be affected under saline conditions. So, it is important to determine the pathogenicity of the fungus under saline conditions and if acquisition of salt tolerance affects disease resistance under normal and saline conditions. It is already known that salinity decreases the yield in salt-affected areas (McWilliam, 1986; Chaudhary, 1996; Dikilitas & Smith, 1999a). The pathogenicity of existing *Verticillium* strains or evolution of a new strain of the fungus that is adapted to saline conditions may cause a reduction in crop yield in salt-affected soils, in addition to the deleterious effect of salt. This will make the situation worse. Because, plants showing resistance to pathogens under saline-free conditions may be exposed to salinity in the future due to gradual accumulation of the salt in the soil. Under these conditions, reduction in the resistance of plants against pathogens may be expected or more aggressive pathogens may evolve. At that time, salt tolerant plants may not be a solution (Dikilitas, 1997).

Since little is known about the complex interactions between salinity and a plant and a pathogen (*Verticillium* sp.), this work was carried out to establish a basis for future work. To study any interaction between the fungi and plants, or fungi and salinity, or plants and salinity, or a combination of the agents in a complex environmental system, it is important to study each factor on its own, in order to gain an understanding of the problem before looking for a solution. Therefore, this study has started by giving a brief idea about the relevant subject to *Verticillium* spp., salinity and lucerne or tomato.

This thesis includes the result of studies of the interactions between isolates of the phytopathogenic fungus *V. albo-atrum* Reinke & Berth. and drought-resistant & sensitive cultivars of tomato plants (*L. esculentum* Mill.), and between the pathogen and various cultivars of lucerne (*M. sativa* L. & *M. media* Pers.) *in vitro* and *in vivo* conditions.

The results of experiments related to the pathogenicity against lucerne or tomato plants maintained in a growth chamber-, greenhouse- and laboratory conditions are included in Chapter 3, which contains the results of studies of the effect of-; temperature on fungal growth and the pathogenicity of fungus on plants; inoculation methods and

spore concentration on infection; a comparison between USA and European isolates of *Verticillium* on the pathogenicity of tomato, and the progress of disease development in plants inoculated with one of two isolates of *V. albo-atrum* (V1 and V2). The pathogenicity of *V. albo-atrum*, isolate V1 to cultivars of *M. sativa* and the salt tolerant strains of *M. media* Pers. cv. Rambler was also investigated over a 3-year experimental period to determine whether selection for salt tolerance changes the resistance of *M. media* to verticillium wilt.

Chapter 4 includes the effects of NaCl on lucerne and tomato seeds during germination and post-germination stage. *In vitro* studies on various physiological and pathological parameters of *V. albo-atrum* on a substrate enriched by Na^+ and Cl^- ions were also evaluated as well as the effect of other antifungal compounds.

Chapter 5 includes the interactions between plant, fungus and NaCl. Experiments were performed on young and mature and on various tomato cultivars including salt- and disease-susceptible ones in hydroponics. Physiological, pathological, biochemical and microscopic analyses were made on infected tissues to investigate whether a correlation exists between salt stress and pathogenicity. Pathogenicity of V1, V2, USA isolates and salt-adapted strains of *V. albo-atrum* on tomato plants was investigated under non-salt and salt conditions. The response of lucerne cultivars to *V. albo-atrum* under salt- and non-salt conditions was also investigated in a hydroponic system. Regenerated plants from the salt-adapted cell lines of lucerne (cv. Vertus) were tested for the resistance to *V. albo-atrum.* The effect of an elicitor, derived from *V. albo-atrum*, on phenylalanine ammonia-lyase (PAL) activity on lucerne cells subjected to NaCl was also investigated. Finally, the hypersensitivity of lucerne cells was tested under NaCl conditions.

CHAPTER I
INTRODUCTION

1.1. *Verticillium.*

The increased incidence of disease caused by species of *Verticillium* (a cause of vascular wilt disease) during the last century reflects the fact that the genus has become a very important plant pathogen to a wide range of economic crops including vegetables, field and ornamental crops such as cotton, lucerne, potato, cucumber, strawberry, tomato, eggplant, olive, pistachio, pea, pear, strawberry, hops, antirrhinum, dahlia, mint, red pepper, okra, chrysanthemum and Brussels sprouts (Schnathorst, 1981; Tjamos, 1989; Potti, 1998). For example, *V. albo-atrum* is a major problem in areas cultivated with lucerne, especially in Europe and North America (Heale *et al.*, 1979; Pennypacker & Leath, 1986). On the other hand, the effect of *V. albo-atrum* on tomato, similar to that of lucerne, has commonly been reported on greenhouse grown tomato (Lorenzini *et al.*, 1997). Therefore, studies to improve both the quality of lucerne & tomato and their resistance to *Verticillium* have been of great economic importance. Different cultivars in a species have different levels of resistance to pathogens (Bell, 1981). One approach for improving resistance has, therefore, been to test different cultivars of a species with a view to reducing the impact of *Verticillium*.

1.2. Taxonomy of *Verticillium.*

A description of the genus *Verticillium* was first made by Nees von Esenbeck (1816) based on the morphology of the conidiophores. The conidiophores are erect, septate and branched with the short branches being arranged in whorls to form the typical verticillate conidiophores. Conidia are arranged singly, terminally and before falling away might for a short time be associated in mucilaginous spore balls at the end of branches. They are unicellular elliptical, ovate or spherical and might be hyaline or slightly pigmented. The mycelium is septate. Because there is no sexual stage the fungus was first classified in the Deuteromycotina, Fungi Imperfecti (Barnett, 1955). However, a few sexual stages in this genus have been recorded in the group *Nectria inventa* (Hastie & Heale, 1984).

There was some confusion between *Verticillium* and the genus *Acrostalagmus* that was identified by Corda (1838) in the early literature. *Acrostalagmus* also produced verticillate conidiophores. However, Hoffman (1854), presented evidence to show that

there was no real difference between them and subsequent workers (Klebahn, 1913; Carpenter 1918) strengthened the idea that the two genera should be classified under the name of the genus *Verticillium*. Under the *Verticillium* genus, about 40 species have been described (Mace *et al.*, 1981).

The description of *V. albo-atrum* was first made by Reinke & Berthold (1879) who isolated the pathogen from a diseased potato plant in Germany. They observed that the fungus had swollen conidiophores, darkened at the base, and branched in a whorl in brown infected tissues. Conidia were hyaline and aseptate. With increasing growth, septation in the hyaline mycelium increased and they became shorter, darker, and torulose resulting in the formation of short black cells. They called these black cells "Dauermycelien or Sklerotein" and they regarded them as the resting mycelium by which the fungus could survive in the soil and in plant debris in harsh conditions of climate.

In 1913 Klebahn isolated from diseased dahlia plants a species of *Verticillium* that was different from *V. albo-atrum* because it produced microsclerotia and no dark pigmented resting mycelium. He named it *V. dahliae*. *V. dahliae* is more effective than *V. albo-atrum* in the warmer climatic regions of the world. *V. dahliae* and *V. albo-atrum* are the most important species of the genus and both species cause highly destructive diseases. *V. dahliae* has also been isolated from olive (Saydam & Copcu, 1972, 1973); sesame and okra (Esentepe *et al.*, 1972); avocado, mango, some vegetables and cotton (Karaca *et al.*, 1971).

In 1919 Pethybridge isolated two further *Verticillium* species associated with potato disease. These species produced neither resting mycelium nor microsclerotia. They produced dark pigmented chlamydospores either singly or in a group, terminally or intercalary in rows of three or four. He called these *V. nigrescens* (small chlamydospores, 7-10 μm in diameter) and *V. nubilum* (large chlamydospores, 8.5-15.5 μm in diameter). At first, they were described as saprophytes on potato, but Isaac (1949) showed that they were mild pathogens. *V. nigrescens* is a common soil fungus and a typical soil inhabitant, (mainly in Europe), that infects potato, tomato, hop, eggplant, and chrysanthemum. In contrast, *V. nubilum* has been recognized as a true soil inhabitant, only weakly pathogen to potato, tomato and antirrhinum (Isaac, 1956).

In 1951, Mason & Hughes renamed *Stachylidum theobromae*, which causes disease of banana, *V. theobromae*, based mostly on its phialide shape and size and the structure of the conidiophores.

In 1953, *V. tricorpus* was discovered and described by Isaac. He said it was pathogenic to tomato and formed, in addition to young, yellow prostrate mycelium in culture, all three types of resting structures viz. resting mycelium, microsclerotia and chlamydospores. In 1965, Smith supported the view that this isolate should be classified as another species of *Verticillium*.

In 1954 and 1958 *V. intertextum* and *V. lateritium* were discovered by Davies and Abraham, respectively.

Although speciation in *Verticillium* is based both on the type and the size of resting bodies, and the characters in culture, the taxonomic status of *V. albo-atrum* and *V. dahliae* in particular had been controversial since 1913. Some, such as Bewley (1922), Rudolph (1931), Caroselli (1957), Basu (1961) and Platt (1986) stated that the microsclerotial and the resting mycelium forms should be regarded as the characteristics of one large species called *V. albo-atrum*. They concluded that these two species were variants of *V. albo-atrum*. Others, Klebahn (1913), Van der Meer (1925), Van Beyma Thoe Kingma (1940), Robinson *et al.* (1957), Talboys (1960), Smith (1965), Isaac (1967), Clarkson & Heale (1985), Grybauskas & Dutky (1987); Thomson & Buhler (1988), remain convinced that both should be kept as separate species because of their different morphological and physiological features.

In 1967, Isaac investigated differences between *V. albo-atrum* and *V. dahliae* from many geographical areas in the UK, USA, Canada, Italy and Germany and he found that resting bodies of the two species remained constant and different. *V. dahliae* frequently formed very short strand of dark septate hyphae in direct association with microsclerotia. On the other hand, *V. albo-atrum* after three weeks growth in culture developed knots. Such knots were not formed from budding and were never observed in *V. dahliae*.

Heale & Isaac (1965) stated that continuous light could inhibit the formation of *V. albo-atrum* resting mycelia but not *V. dahliae* microsclerotia. Puhalla (1973) used UV irradiation and he stated that *V. albo-atrum* was more sensitive to light than *V. dahliae*.

Talboys (1960) suggested that selective media, prune-lactose-yeast extract, could discriminate between *V. albo-atrum* and *V. dahliae*. In *V. albo-atrum* the colony surface appeared grey and fibrous while *V. dahliae* produced fluffy mycelia.

Based on a comparative study of the morphology, physiology and pathogenicity of several isolates of *Verticillium*, Isaac (1953 and 1967) concluded that there were five distinct species of *Verticillium* viz:-

V. albo-atrum (R. & B.)	- forming dark pigmented torulose resting mycelium,
V. dahliae (Kleb.)	- forming dark pigmented microsclerotia,
V. nigrescens (Pethybr.)	- forming small chlamydospores,
V. nubilum (Pethybr.)	- forming large chlamydospores,
V. tricorpus (Isaac)	- forming resting mycelium, microsclerotia and chlamydospores.

Work by Milton *et al.* (1971) using polyacrylamide gel electrophoresis on the buffer-soluble proteins from various *Verticillium* species indicated that the patterns of proteins observed were different between these species and no single protein band was common to all five isolates.

In 1973, Levy provided further evidence to support separation of *V. albo-atrum*, *V. dahliae*, *V. nigrescens* and *V. tricorpus* into separate species.

Carder & Barbara (1991) used molecular variation and restriction fragment length polymorphism's (RFLPs) to support Isaac's (1967) conclusion. Nazar *et al.* (1991); Hu *et al.* (1993); Moukhamedov *et al.* (1994); Mahuku *et al.* (1999) used polymerase chain reaction (PCR) to detect differences between *Verticillium* species. The results of their studies accurately revealed substantial differences between *V. albo-atrum* and *V. dahliae*.

It is generally accepted that one of the most important parameters used to differentiate between *V. albo-atrum* and *V. dahliae* is the temperature reaction. Isaac (1949) studied the relationship between temperature and growth in several isolates of *V. dahliae* and *V. albo-atrum* and one isolate of *V. nigrescens*. He stated that the optimum temperature for *V. albo-atrum* and *V. dahliae* did not differ (22.5 °C) whereas *V. nigrescens* grew well between 22.5 °C and 25 °C. He also observed that the growth of *V.*

albo-atrum declined above 25 °C while *V. dahliae* and *V. nigrescens* grew relatively well at 30 °C. He also stated that none of these fungi grew at 35 °C nor below 4.5 °C. Similar reports were also made by Martinson & Englander (1967); Puhalla & Mayfield (1974); Soesanto & Termorshuizen (2001). In addition, Mamluk & Skaria (1979) compared the growth rate and the formation of resting bodies for 15 days at 28 °C and found that *V. dahliae* grew normally and formed true microsclerotia while *V. albo-atrum* grew and formed 'Dauermycelia poorly'. They concluded that the rate of growth in culture and the formation of resting bodies were the most important and reliable characteristics in identifying *Verticillium* spp.

Raynal & Guy (1977) mapped the distribution of the disease of *V. albo-atrum* in Europe. From their results, Heale *et al.* (1979) reported that the pathogenicity of *V. albo-atrum* is restricted to latitudes between 43°N and 46°N. The distribution of the disease in the northern states of the western USA and British Colombia corresponds approximately in latitude with the European distribution. The disease of *V. albo-atrum* has also been reported in the southern hemisphere in New Zealand (Smith, 1965) at a similar distance from the equator. This distribution clearly reflects a temperature effect, explained by the fact that *V. albo-atrum* is avirulent when temperatures exceed 25 °C (Isaac, 1949; Ingle & Hastie, 1974).

1.3. Vascular wilt disease.

Vascular wilt diseases are widespread- and very destructive- plant diseases; appearing as wilting, browning and dying of the leaves followed by death. Wilts occur as a result of the presence and activities of the pathogen in the xylem vascular tissues of the plant. The diseases may be caused by such species of soil-borne fungi as *Fusarium*, *Verticillium* and *Ceratocystis*, and bacteria such as *Ralstonia*, *Erwinia* and *Clavibacter* (Dey & Van Alfen, 1979; Schnathorst, 1981; Agrios, 1988). Each of them causes widespread and severe diseases on several important crop, forest, and ornamental plants. However, *Verticillium* and *Fusarium* are the two most important groups of soil fungi that are responsible for the wilting of many crops of economic importance. The genus *Fusarium* survives in warmer climates, while *Verticillium* develops well in cooler temperatures (Buxton, 1957; Nadakavukaren & Horner, 1961; Hwang *et al.*, 2000). The

pathogens frequently disturb the flow of water (transpiration) and nutrients (translocation) in the plant and consequently cause wilting. The earliest reports of vascular wilt disease occurred in the early 1900s (Mace *et al.* 1981).

Ludwig (1952) stated that blockage and wilting in vascular wilt disease is associated with the accumulation of a hyaline substance in the vessels. In later studies by Beckman (1964 & 2000); and Van der Molen *et al.* (1987), the formation of gels in vessels was observed as a general response to infection by both host-specific and non-host specific pathogens. Duniway (1971) studied the water relations of fusarium wilt (FW) of tomato, and showed that leaves of diseased and water-stressed plants wilted at similar values for water potential, and that the wilting which occurred during disease development was caused by water stress. He also showed that the transpiration rate of *Fusarium*-infected plants was less than that of healthy plants. He concluded that the transpiration was not the main reason for the wilting of diseased plants.

1.4. Verticillium wilt (VW).

The most important species of *Verticillium* are; *V. albo-atrum* and *V. dahliae* (Isaac (1967). Especially, *V. albo-atrum* is a more virulent pathogen than *V. dahliae* in the cool-temperate areas of the world (Heale *et al.*, 1979; Platt & Arsenault, 2001).

- life cycles:

The life cycle of pathogenic *Verticillium* spp. can be divided into dormant-, parasitic- and saprophytic stages. Additional phases within some of these three stages can also be identified: germination of resting structures in soil; penetration of roots; colonisation of the root cortex and endodermis, and movement to the xylem; colonisation of the xylem of stems and leaves; symptom expression; death of host tissue; and formation of resting structures (Schnathorst, 1981).

- survival structures:

Heale & Isaac (1963); Heale (1988) reported that the resting mycelium of *V. albo-atrum* could remain viable for 9 months in pieces of decaying lucerne root and in shoot tissue buried in 31 cm soil, for 7 months at 15 cm and for 5 months at the surface. They also concluded that constantly changing conditions of moisture and temperature on the soil surface decreased the viability of the resting mycelium. On the other hand, Basu

(1987) demonstrated that *V. albo-atrum* was viable between –5 °C and 5 °C in sterile or non-sterile soil for 3-years. However, the longevity of the fungus declined with increasing temperature. The fungus was capable of surviving for 18 months in sterilized, and 8 months in non-sterilized soil at 15 °C. But the longevity was reduced down to 8, 7 and 6 months at 25-, 30- and 35 °C, respectively. One of the possible reasons that the fungi may survive for some period is that they may exhibit dormancy. For example, Isaac & MacGarvie (1962, 1966) reported that resting structures of *Verticillium* spp., except *V. nigrescens*, exhibited dormancy. Vitamins, amino-acids, enzymes, detergents, soil extracts, root exudates, heat shock or freezing and thawing failed to induce germination. However, soaking resting structures in distilled water for 12 hours and plating them on nutrient agar resulted in a high percentage of germination. They suggested that dormancy resulted from inhibitors which needed to be removed by one or more agencies prior to germination. Similar findings were also made by Schnathorst, 1981.

The parasitic stage of the fungus starts when infecting hyphae gain entrance to the plant. In order for the pathogen to initiate the infection, the roots have to be in close proximity to the infection hyphae, since *Verticillium* spp. have been shown to be poor saprophytes in field soil (Schnathorst, 1981; Agrios, 1988). For example, Sewell (1959) observed that hyphae of *V. albo-atrum* in soil were not found more than 2 mm away from germinating propagules. *V. albo-atrum* is capable of entering unwounded tomato and lucerne plants (Flood, 1980; Dikilitas, 1997) and is capable of directly penetrating cells which give rise to root hairs (Smith *et al.*, 1988) and penetrates the epidermal cells in the region of elongation. Entry of the fungus sometimes is accomplished through wounds on the root or stem caused by mechanical injury, or insect damage (Pegg, 1985; Garas *et al.*, 1986; Dikilitas & Smith, 1999b). Huang *et al.* (1986) reported that the fungus might also penetrate leaves through wounds.

The pathogen produces both cellulolytic and pectolytic enzymes along with other enzymes that may attack the cell walls of host tissues, the enzymatic breakdown products may be used as a food base, and enzyme action may facilitate penetration of cell walls (Green, 1981; Pegg & Brady, 2000). Once entry to the root is gained, hyphae grows both inter- and intracellularly across the root cortex to the endodermis and ultimately reaches

the xylem. From here, the fungus distributes within the plant mainly by means of the transpiration stream, especially in the vessel lumen through pits (Schnathorst, 1981; Garas *et al.*, 1986; Smith *et al.*, 1988).

Infection and colonization of the host tissue is favoured by relatively cool temperatures; this is why most symptom expression is observed in the spring. The fungus becomes less active during hot weather. In fact, it is very difficult to isolate *Verticillium* from diseased plants during the summer months. If the plant is vigorous, it may be able to restrict the fungus within certain portions of the vascular system due to increased enzymatic activity and it may also produce new fungus-free vascular tissues for water movement. However, continued invasion by the pathogen will result in death of plants (Tisserat, 1998).

When the host dies the fungus passes the saprophytic stage in the dead tissue or in the soil (Stuteville & Erwin, 1990).

1.4.1. VW of lucerne (M. sativa L.).

The disease became more important and spread in Europe after the 1950s (Heale, 1985). The disease was first recorded in Sweden in 1918 (Hedlund, 1923) and then in Germany in 1938 (Richter & Klinowski, 1938). After World War II, there were several reports of the disease, viz. from Denmark in 1945 and Holland in 1947 (Hansen & Weber, 1948), and from France in 1950 (Kreitlow, 1962). The disease spread rapidly throughout Europe and the first report of the disease in Britain was made by Noble *et al.*, in 1953. In Britain, the disease affected 12,800 ha in 1942, 44,000 ha in 1944 and 111,000 ha in 1954. However, the area affected declined to 15,000 ha in 1970 (Rogers, 1976). Outside the European Continent, the disease was reported in Canada by Aubé & Sackston in 1964 and it was reported in Washington State, USA in 1976 by Graham *et al.*, 1977. The disease was also reported in other parts of the United States in the same year. Delwiche *et al.* (1981); Thomson & Buhler (1988) reported the disease in Wisconsin (1980) and in Utah (1985) respectively. Erwin & Khan (1988) reported that the disease had now spread through the entire northern lucerne-growing region of the United Sates, as far south as southern California.

Isaac (1957), Isaac & Lloyd (1959); Dixon (1981) made a detailed description of the disease. They established that in the disease, a high degree of specialisation exists in the pathogens. Isaac (1957); Isaac & Lloyd (1959) reported that only *V. albo-atrum* and *V. dahliae* from lucerne were capable of causing infection in lucerne; other isolates of both species obtained from other host plants were incapable of causing infection. Those results were also supported by Khan (1977); and Flood (1980). Barasubiye *et al.* (1994) reported host-specialized isolates of *Verticillium*; the strains from lucerne were more virulent on lucerne than the potato strains, though only the potato strains were virulent on potato. Tsror *et al.* (1998) reported that a highly virulent isolate of *V. dahliae* from paprika caused severe reduction in height in potato, watermelon and tomato. However, isolates from those plants neither produced symptoms nor a reduction in height in paprika plants. Similarly, Paternotte & Vankesteren (1993) reported that resistant tomato cultivars were seriously affected by new aggressive isolates but not by a control isolate.

The response of different cultivars of lucerne to infection by *V. albo-atrum* may be different. This is true even within cultivars. For example, various levels of resistance to *V. albo-atrum* was found to vary from one plant to another within the same cultivar (personal communication with Assoc. Prof. Barbara W. Pennypacker, USA, 2000). This was attributed to the genetic heterogeneity of the crop (Pennypacker, 2000). Latunde-Dada *et al.* (1987) observed that callus cultures derived from individual seedling of lucerne exhibit differential response to infection by the wilt fungus *V. albo-atrum.* Newcombe & Robb (1988) separated cultivars of lucerne into three lines according to their responses to *V. albo-atrum;*

-highly resistant symptomless plants from which the pathogen could not be reisolated 3 months after inoculation,
-moderately resistant, symptomless plants from which the pathogen could be reisolated up to 7 months after inoculation,
-susceptible plants which died within 6 weeks following inoculation.

Pennypacker *et al.* (1988 & 1990); Pennypacker & Leath (1993) studied the growth and physiological response of resistant lucerne clones inoculated with *V. albo-*

atrum and found that *V. albo-atrum* caused significant reduction in height, percentage of flowering, stem, leaf and aerial biomass in disease-free lucerne plants.

Histological examination has shown that *V. albo-atrum* sporulated freely in the xylem vessels facilitating rapid colonization of the host (Pennypacker & Leath, 1983).

Papadopoulous *et al.* (1991) used histochemical analysis to study the reaction of susceptible and resistant cultivars of lucerne to *Verticillium* species and found that the level of stem colonization in resistant cultivars was slightly lower than in susceptible ones during the first 2 weeks following inoculation. Susceptible seedlings died during the 8 weeks-period post-inoculation. Pennypacker & Leath (1986 & 1993) observed that infected plants had discontinuous alterations in xylem vessels. They observed that protoxylem and some metaxylem vessel elements were obliterated by hypertrophied xylem parenchyma.

1.4.2. VW of tomato (L. esculentum Mill.).

Tomato is a widely distributed annual vegetable crop, which is consumed fresh, cooked or after processing: by canning, making into juice, pulp, and paste or as a variety of sauces. The tomato crop is adapted to a wide variety of climates ranging from the tropics to within a few degrees of the Artic Circle. In this broad environment, tomato cultivated areas are affected by environmental and biotic factors.

The first report for VW of tomato was made by Jagger & Steward in 1918. Since then the disease has been reported in many parts of the world. *V. albo-atrum* enters tomato through root epidermal cells or wounds in stems or roots as in the case of lucerne. Since there are similarities between VW of tomato and lucerne, the detailed symptoms of the disease and its occurrence are dealt with in the Symptoms section.

1.5. Symptoms.

There is a great diversity of symptoms involved in vascular wilt disease resulting from either the presence of the pathogen or its propagules in the host vascular tissue (Mace *et al.*, 1981). Isaac (1992) reported that the vascular wilt diseases caused by members of the Deuteromycotina are particularly destructive and are very rapid in their effects. If the plant is resistant to disease, no-symptoms or a few symptoms might be observed and recovery may generally start in one or two days. On the other hand, if the

plant is moderately resistant, recovery may be observed 3-4 weeks after infection, depending on the environmental conditions and plant age (Dikilitas, 1997; personal communication with Dr. J.M. Milton, 1998). If the plant is susceptible, wilting progresses upwards until the whole plant appears desiccated; generally no recovery is observed and finally death occurs (Dikilitas, 1997; Korolev *et al.*, 2000).

An early description of the disease was made by Isaac (1957), who reported that irrespective of the identity of the pathogen, the external symptoms of wilt in all plants were identical.

- *external symptoms*;

Symptoms of VW can develop throughout the growing season, but are more common in late spring or early summer or in the autumn. The disease can be readily recognised in the field, though in the later autumn months, because of similarities between disease symptoms and the natural die-back that occurs in healthy plants, recognition of the disease can become difficult (Isaac, 1957).

Externally, leaves on diseased shoots appear 'off colour' (pale green to yellow) loose turgor and eventually desiccate. The symptoms may appear on individual shoots in a section of the plant stem, or throughout the entire plant. In some cases, the disease progresses slowly over a period of months resulting in gradual defoliation, shoot dieback and eventually the plant may die. Leaves generally loose turgor and the leaf margins roll upwards in the warmer periods of summer days, though, with the drop of temperature towards evening, some recovery may be observed (Noble *et al.*, 1953; Isaac, 1957; Graham *et al.*, 1977; Esyanti, 1993). Unlike FW, VW causes uniform yellowing and wilting of the lower leaves (Isaac & Griffiths, 1962).

Stunting is another symptom of plants infected with *Verticillium* (Pennypacker *et al.*, 1990; Esyanti, 1993). For example, growth reduction was observed by Hawthorne (1987); and Pennypacker *et al.* (1988) in some otherwise symptom-free lucerne plants inoculated with *V. albo-atrum*, though this may be due to a hormonal imbalance rather than water stress (Mepsted, 1991).

V-shaped chlorosis of leaflets, or formation of a yellow-to-red-brown lesion near the leaf tip, followed by desiccation and abscission, are other characteristics of the

disease (Pennypacker & Leath, 1986). Eventually such leaves die and turn a light reddish-brown colour (Stuteville & Erwin, 1990). Latunde-Dada & Lucas (1982) reported that VW of lucerne started as a yellowing of the cotyledons and lower leaves, then progressed upwards causing chlorosis in older leaves. In extreme cases, the affected foliage wilts, turns dry and brittle, and drops from the plant in a matter of days or weeks (Lorenzini *et al.*, 1997). At times, the initial re-growth of a diseased plant will appear healthy until the plant reaches six to eight inches in height. At this stage, the plants often begin to exhibit the symptoms described above.

Usually there are no external symptoms observed in roots.

- *internal symptoms*;

Another diagnostic symptom of VW is vascular discolouration; discolouration can be viewed by slicing vertically through the stem and root near the soil line and looking for a narrow column of browning between the central pith region and the outer portion of the stem (Graham *et al.*, 1977; Pennypacker & Leath, 1986). In severe cases, internal tissues of the petioles and pedicels of the flower can also become brown (Graham *et al.*, 1977). This brown discolouration mainly results from oxidation of polyphenols, giving rise to the dark melanin pigments (Bishop & Cooper, 1983).

1.6. The mechanism of wilting.

The conidia are carried throughout the plant in the transpiration stream (Newcombe *et al.*, 1990; Heinz *et al.*, 1998). They are usually moved up to a vessel end wall where they are trapped. They then germinate to produce new infection sites. So, the fungus moves rapidly up the plant by germinating and growing through the pores of the vessel and sporulating on the other side (Beckman *et al.*, 1976). The hyphae grow through the xylem, ramify throughout the plant, and pass from cell to cell via pit pairs. At this stage, the xylem becomes increasingly blocked with mycelium.

Movement of conidia is much more rapid than the mycelial growth in xylem tissues. For example, the Panama wilt disease pathogen, *F. oxysporum* f.sp. *cubense* can migrate from the bottom to the top of an eight-metre tall banana tree in less than two weeks (Ploetz, 1994). As the plants begin to die, sporulation is stimulated. At this point,

many spores are formed and remain in the tissue, ready to be returned to the soil as the plant decomposes (Beckman *et al.*, 1976).

There are various reports concerning wilting of the plants; some of which suggest that occlusion of xylem vessels is the primary reason for wilting (Cooper & Wood, 1980; Douglas & Machardy, 1981). In fact, the pathogen itself or tyloses (growth of adjacent xylem parenchyma into the vessel) and vascular gels associated with pathogen, are thought to be responsible for partial or complete occlusion of large vessels in the stem. Since the wilt fungus *V. albo-atrum* enters through the cortex in the apical region of the root, the endodermis is an effective barrier to the pathogen but as it is not fully developed in this region it is easily breached and the fungus then enters the xylem elements. It was reported that vascular flow was reduced in wilted tomato plants (Street & Cooper, 1984). Duniway (1976); Dey & Van Alfen (1979) made similar comments for FW of tomato and clavibacter wilt of lucerne plants respectively. Reduction in flow rate may be caused by fungal mycelium and conidia, tylosis xylem hyperplasia, gum and gel deposition, blocking of vessel pits and the permanent opening of the stomata (Pegg, 1981; Schnathorst, 1981). It is generally believed that *Verticillium* moves up a plant through spore trapping sites in the vascular tissue in a stepwise fashion. In a resistant plant, rapid expression of defence responses around the spore-trapping sites slows growth of the pathogen and often restricts the fungus to the base of the stem. However, in a susceptible plant, the fungus escapes and eventually proliferates at a logarithmic rate, in the upper stem and leaves. Heinz *et al.* (1998) reported that even in resistant plants fungal colonization was observed in the upper stems shortly after root infection. They reported that *V. albo-atrum* was not restricted to the base of resistant tomato plants. In fact, high amounts of *Verticillium* were present in the upper stems of both susceptible and resistant plants, although symptom expression in the resistant plant was restricted to the stem base. They suggested that wilt diseases are more systemic than previously thought and that basic assumptions about the nature of the wilt diseases should be re-evaluated.

Many of the symptoms associated with vascular wilt disease point to changes in the accumulation of hormones in the plant (Hillocks, 1992; Resende *et al.* 1995). For example, indoleacetic acid (IAA) or ethylene induce the formation of tylose and gels, which accumulate at perforation plates and contribute to vascular blockage. These

hormones are produced by both pathogen and susceptible host plants (Beckman, 1987; Hasan, 2002). Both of these factors stimulate the production of tyloses and cause plasticity of cell walls. As a result, tyloses may expand to such a degree and such a rate, that the xylem vessels may become completely blocked (Whitney *et al.*, 1972). However, new conducting elements may also be formed in infected plants and may partly compensate for the reduced efficiency of the blocked vessels, so that the extent to which tyloses cause wilting is uncertain. After the fungus is established in the xylem elements, it releases secondary metabolites that act as toxins to the host plant cells, disturbing cell permeability and hastening plant death. Factors that add to the disease symptoms are high molecular weight polysaccharides, produced by the pathogen or cleaved from vessel walls by hydrolytic enzymes. For example, cellulose, which is broken down by cellulase secreted by *V. albo-atrum*, may cause blockages in the host plant vascular system, thus restricting lateral transport and plugging the small vessels in petioles and leaves (Beckman, 1987 & 2000).

As a defence response, several changes in the metabolism of the diseased plant accompany the increase in respiration that occurs following infection. For example, the increase in respiration in diseased plants is accompanied by an increase in activity of the oxidative pentose phosphate pathway, which is the main source of phenolic compounds. In a similar way, hemicellulosic gels and gums may be produced, in cells adjacent to infected tissues. Not only could these block pathogen growth and spread through vascular tissues but they may also restrict solute movement through plants (Beckman, 1987).

Kiessig & Haller-Kiessig (1957) reported that two components were involved in VW of lucerne- a thermolabile wilt toxin and thermostable necrosis toxin. Nachmias *et al.* (1985 & 1987) claimed that the active toxin of *V. dahliae*, which was produced by the fungus on culture medium, was a peptide of variable composition, occurring as a component of a protein lipopolysaccaharide (PLP) that induced symptoms in susceptible but not in resistant cultivars.

Vascular wilt fungi also produce a range of extracellular enzymes in culture and some of these have been implicated in pathogenesis. In VW, pectin esterases (PE), polygalacturonases (PG) and pectin lyase (PL) are most significant. Such enzymes

provide the pathogen with essential or supplementary nutrients by degrading and removing carbon fragments from the pit membrane (Van der Molen *et al.*, 1987), resulting in colonization of the susceptible plant. For example, Cooper & Wood (1980) found higher endo-PL and endo-PG activities in tomato plants susceptible to *Verticillium* than in resistant plants. Several workers have also presented evidence for their role in vascular occlusion (Beckman, 1987; Cooper & Wood, 1980).

Another mechanism that has been proposed to lead to wilting involves ethylene (Van der Molen *et al.*, 1983). Mussell *et al.* (1982) reported that cell wall from tomato contained an enzyme that generates ethylene. The enzyme was released from the cell walls by a purified PG from *V. dahliae*. So, the attack of a pathogen could elicit ethylene release though this mechanism and, in turn, this would trigger responses in host tissues leading to the formation of tyloses and gel. IAA has also been found in *V. albo-atrum* culture filtrates and in diseased tomato plants (Pegg & Selman, 1959; Beckman, 2000) and may play a part in the development of symptoms. The apices of infected stems showed a 200% increase in IAA compared to healthy stems, and IAA treatment of cut tomato stems caused epinasty and hypertrophy in young cuttings. Since all classes of plant growth substances, IAA, ethylene, gibberellins, cytokinins and abscisic acid (ABA) are known to interact and in some cases act as substitutes in a particular effect, it is difficult to attribute an exclusive role to an individual compound (Pegg & Brady, 2000).

1.7. Spread of the disease.

Isaac (1957) reported that the fungus grows out of xylem elements to form conidiophores at the basal portions of the infected stems. These conidiophores bearing conidia are responsible for the spread of infection in the crop during harvesting. He also stated that towards the end of the season these areas become blackened with the production of resting mycelia. Similarly, others (Sheppard & Needham, 1980; Christen, 1982 &1983; Martin *et al.*, 1991) have reported that *V. albo-atrum* was introduced into a new area, in the form of resting mycelium, from contaminated plant materials, including seeds. For example, *V. albo-atrum* was isolated from *Helianthus annuus* L. and *M. sativa* L. by Sackston & Martens (1959) and Isaac & Heale (1961) respectively.

Heale & Isaac (1963) and Heale *et al.* (1979) reported that contaminated cutter blades of harvesting machinery played an important part in the spread of the disease both within and between fields. Other ways in which the wilt fungus may be disseminated are through cars, people (Nicholls *et al.*, 1987), and insects (Harper & Huang, 1984; Kalb & Millar, 1986; Huang *et al.*, 1986). Some authors such as, Lindemann *et al.* (1982), have reported that the fungal spores may be carried by wind and cause epidemics in areas of lucerne cultivation although the fungus is known to be a soil-borne-pathogen, however, this idea was not supported by Jimenez Diaz & Millar (1988).

1.8. Resistance to vascular wilt diseases.

In spite of the considerable literature on plant-pathogen interactions, there is still controversy over the basis of susceptibility and resistance to wilt pathogens and the mechanisms involved in recognition are not fully understood.

For example, in cotton plants the level of susceptibility to wilt disease caused by *V. dahliae* is determined by the rate of colonization of the vascular tissues (Beckman, 1987). The degree of resistance depends on the speed and intensity of the host defence response (Mace, 1978). In the resistant interaction, gel plugs, which restrict the movement of microconidia to the vessel endings, form within 24-48 hr following infection. The response is triggered by host-derived ethylene and IAA (Mussell *et al.*, 1982) and the gels both cut off the water flow and immobilize the parasite (Beckman *et al.*, 1962). At the same time, phenolic substances that are highly resistant to degradation start to accumulate. In subsequent stages, parenchyma cells ensheath the vessels with suberin or lignin-like coatings (Vance *et al.*, 1980; Street *et al.*, 1986; Beckman, 2000). Through pits in the lateral vessel walls, these living cells also produce tyloses that completely block the vessel above the trapping site, and then secrete stress metabolites, including phytoalexins, (Talboys, 1972). This sequence of events seals off the pathogen, preventing further colonization of the vascular system. In susceptible plants, however, the vascular gels are not produced quickly enough to stop the pathogen (Harrison, 1981). If they do form, they are either too weak or there are not enough of them.

When plants are damaged, whether mechanically or by pathogens, a series of responses is initiated, resulting in the formation of wound barriers (Barckhausen, 1978).

Damaged cells die and in the adjacent layers of cells, substances such as suberin, lignin, gum and tannin accumulate (Eldon, 1995). A few days later, a cork layer is formed which may restrict further invasion by pathogens.

Phytoalexins, which are low-molecular weight compounds that are both synthesised by and accumulated in plant cells after exposure to microorganisms (Paxton, 1981; Tang & Smith, 2001), may also be produced and inhibit the growth of microorganisms (Flood, 1980; Bianchini *et al.*, 1999).

1.9. Cross-protection or induced resistance.

Cross protection or induced resistance can be defined as a type of biological control in which inoculation of the plants with an avirulent pathogen (inducer) causes a reduction in severity of the symptoms or delays disease development caused by a virulent pathogen (challenger) (Hillocks, 1986). This resistance occurs by reducing, restricting or blocking the pathogen in the host plant (Fravel, 1989).

There are many workers in the plant pathology field who have attempted to define or give a clear picture for cross protection. It has been shown that cultivars that are highly susceptible to a race or a pathovar can be induced to a higher level of resistance (cross-protected) to that particular pathogen by pre-inoculation with a non-pathogenic or avirulent microorganism (Matta, 1989). Such disease resistance generally requires one to a few days to become optimally functional (Biles & Martyn, 1989) and persists for sometime thereafter. Pre-inoculation often results in enhancement of some defence responses such as callose deposition, gel- and tylose formation and deposition of other secondary metabolisms (Matta, 1989).

Millar *et al.* (1984) reported that the wilt disease caused by *V. albo-atrum* on lucerne could be prevented by inoculation with *Gliocladium roseum*. The cut-stems were inoculated with *V. albo-atrum* ($1x10^4$ conidia/ml), then treated immediately with *G. roseum*. They reported that the incidence of wilt was reduced by up to 4- to 5-fold by treatment with *G. roseum* (8×10^6 conidia/ml), and that the effect persisted through two harvests. Control of the disease in this way was more effective than with benomyl, chlorothalonil or maneb. Control of *V. dahliae* by *G. roseum* was also demonstrated *in vitro* by Keinath *et al.* (1991). Hall *et al.* (1984) reported that suspensions of *Bacillus*

subtilis were introduced into silver maples through stem wounds. After 3 days, stems were wound-inoculated with a conidial suspension of *V. dahliae*. After 16 weeks, there was a highly significant reduction in the frequency of isolation of *V. dahliae*. Similar cases were reported on tomato plants by Jorge (1990) in which simultaneous root dip inoculation with conidial suspensions of *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) Sn. et Hans. (inducer) and *V. dahliae* Kleb. (challenger) resulted in the expression of cross-protection in Fusarium-resistant tomato cultivars. On the other hand, Fravel (1995) used the biocontrol agents *G. roseum* and *Talaromyces flavus* with sublethal rates of metham sodium for control of *V. dahliae*. He found that the combined effect of the sublethal rate of the fumigant with either biocontrol fungus was additive for reduction of disease incidence.

Some workers used pathogens within the same genus. For example, Schnathorst & Mathre (1966) used non-pathogenic *V. albo-atrum* to control wilt disease in cotton caused by pathogenic strains of *V. albo-atrum*. Melouk & Horner (1975) used *V. nigrescens* against *V. dahliae* to control the wilt disease in mints. Similar experiments were also carried out by Matta & Garibaldi (1977) who protected greenhouse tomato against the vascular wilt caused by *V. albo-atrum*, by dipping roots of the seedlings in a spore suspension of an avirulent isolate of *V. albo-atrum*. Sato (1994) observed that pre-inoculation of lucerne with the potato strain of *V. albo-atrum* decreased the severity of wilt disease caused by the strain of *V. albo-atrum* isolated from lucerne. Hillocks (1986) used non-pathogenic strains of *F. oxysporum* f. sp. *vasinfectum* against pathogenic strains of the same species in cotton plants. Price & Sackston (1983 & 1989) also used non-pathogenic strains of *V. dahliae* to control wilt disease in sunflower caused by pathogenic strains of *V. dahliae*.

Development of cross protection may be influenced by many factors, such as, the method of inoculation (Davis, 1967 & 1968), the concentration of the inducer inoculum in relation to the challenger inoculum and the timing between inoculation with the inducing organisms and inoculation with the challenging organisms (Jorge, 1990; Jorge *et al.*, 1992). For example, Jorge (1990) reported that cross-protection was gradually lost as the time between the inoculation with the inducer (*F. oxysporum* f.sp. *lycopersici*) and challenger (*V. dahliae*) was increased. Millar *et al.* (1984) reported that control of the

disease caused by *V. albo-atrum* was not achieved if treatment with *G. roseum* was delayed 2 min or longer, or if the inoculation with *V. albo-atrum* was at a concentration of 10^5 conidia/ml or higher. However, in a recent study, Huertas-González *et al.* (1999) proposed that extensive protection of tomato cultivars by *F. oxysporum* f.sp. *lycopersici* race 1 against race 2 only occurred in cultivars carrying resistance gene *I* but not in a cv. lacking the gene. They supported that the view that the main mechanism of cross protection is not competition between the two different races during infection, which would be expected to occur in the cultivar lacking resistance gene *I* but rather the induction of plant defense through the interaction between a specific avirulence factor of race 1 with the corresponding resistance gene. They explained that cross-protection is induced by simultaneous co-inoculation with the avirulent and the virulent race without the need for a time interval between induction and challenge, suggesting that recognition and activation of the plant's defenses are rapid events that occur shortly after the pathogen has entered the vascular tissue. However, the nature and biochemical properties of cross protection still need some elucidation.

1.10. Control of the disease.

Vascular wilt disease of lucerne, especially *Verticillium* species, remains one of the most destructive diseases of lucerne, tomato and other crops. The problem of controlling VW caused by *V. albo-atrum* and *V. dahliae* may be divided into three categories (Tjamos,1989), there are problems arising from the nature of the pathogen, such as its wide distribution and virulence, there are problems related to the epidemiology of the disease, such as dissemination of the pathogen, there are problems associated with cultural practises.

The best strategy for control involves "disease-free plants in disease-free soil" (Isaac, 1957). For this reason, elimination of pieces of plant debris carrying the fungus, or soil that is harvested with the seed are important cultural practises (Isaac, 1957; Huisman & Gerik, 1989).

Seed treatment with chemicals and soil fumigation can be helpful where protection of economically highly valuable crops is concerned (Isaac & Lloyd, 1959; Christias, 1989). Fumigation of seed and seed dressing for seed treatment are other

important methods for controlling the disease (Heale *et al.*, 1979). It has been reported that storing seeds at 30 °C for one year (Huang *et al.*, 1994) and dry heat at 75 °C for 5 hours (Stuteville & Erwin, 1990) could eliminate the fungus in seeds. Although it is an expensive method, Ligoxigakis & Vakalounakis (1992) reported that in Greece they prevented the vascular wilt caused by *V. albo-atrum* and *V. dahliae*, by fumigating the soil with methyl bromide.

After the seedling stage, using fertilizers rich in nitrogen, enables the rapid formation of a thick layer of sapwood that seals the infected parts beneath. It also stimulates leaf growth (Stuteville & Erwin, 1990). An adequate supply of nitrogen is associated with vigorous vegetative growth and a dark-green colour of the plant.

Isaac (1957); Heale *et al.* (1979) reported that cleaning machinery with a suitable disinfectant and cutting healthy crops first could help prevent the disease.

Crop rotation can still reduce although not eliminate the pathogen populations in the soil. In such cases, appreciable yields from the susceptible crop can be obtained every third or fourth year of the rotation (Agrios, 1988).

Soil solarization and ploughing up the soil were also found to be effective in controlling the VW of cotton and lucerne (Nicholls *et al.*, 1987; Melerovara *et al.*, 1995). For example, when polyethylene is placed over moist soil, during sunny summer days, the temperature at the top 5 cm of soil may reach as high as 52 °C compared to a maximum of 37 °C in unmulched soil (Agrios, 1988). If the sunny weather continues for several days or weeks, the increased soil temperature from solar heat inactivates many soil-borne pathogens and thereby reduces the inoculum and the potential for disease. Tjamos & Paplomatas (1988) reported that soil solarization, either singly or in combination with a reduced dosage (34 g/m^2) of methyl bromide, was effective in controlling VW of globe artichokes for three successive cropping seasons. Furthermore, propagules of *Talaromyces flavus*, a heat-resistant *V. dahliae* antagonist, increased and survived better in solarized than untreated control soils.

Although the use of systemic chemicals for wilt control is practically impossible, chemicals that act through the host metabolism and can cause the plants to be more resistant have been intensively studied. Davis & Dimond (1952) proposed three possible actions that could make systemic chemicals effective; they could, counteract toxins;

enhance host resistance, or kill the pathogen directly within the host. So far, no systemic chemicals have been found to successfully counteract toxins, however, some chemicals have been found to enhance resistance of the host and suppress the pathogen within the host (Beckman, 1987). For example, Dimond & Davis (1953) reduced expression of symptoms in tomato caused by *Fusarium* through chemical methods.

Plant breeding is another method of controlling the disease. For example, Niemann *et al.* (1991) and Miller & Christie (1991) reported that breeding resistant lucerne cultivars is an important step in controlling wilt disease, although the mechanism of resistance is not fully understood. Finding a conventional method for breeding lucerne cultivars resistant to *Verticillium* species may be complex because lucerne is an autotetraploid crop and suffers severe inbreeding depression, which is expressed as a reduction in crop productivity after breeding (Christie *et al.*, 1985; Pennypacker, 1991). Furthermore, more virulent strains of the fungus may evolve (Flood *et al.*, 1978).

Because, *Verticillium* is generally a problem where irrigation is involved (Eldon, 1995), drought resistant plants, as well as disease resistant ones might be also solution for VW. Arbogast *et al.* (1999); Bletsos *et al.* (1999); Xiao *et al.* (2000); Jefferson & Gossen (2002) reported that an increase in VW was correlated with increasing irrigation. This has been attributed to the effect of irrigation water lowering the soil temperature and providing better conditions for fungal growth (Talboys, 1972). So, in plants that are drought resistant, water stress may be beneficial. Pennypacker (1991), for example, found that drought stress decreased the effect of the pathogen on the stem dry weights of lucerne infected with *V. albo-atrum.*

Legislation procedures could also prevent spread of VW to disease-free areas (Heale *et al.*, 1979).

1.11. Effects of hydrogen peroxide, peroxidase, NaCl and purified phytoalexins on spore germination and germ tube elongation of *V. albo-atrum* isolates.

Hydrogen peroxide (H_2O_2) is a stable, partially reduced form of oxygen produced in plant cells by dismutation of superoxide (Halliwell & Gutteridge, 1989). Generally, physiological concentrations of H_2O_2 in plant cells lie within 1 μM to 100 μM (Joseph *et al.*, 1998). Plant leaves or cell cultures generate O^{-}_2 when exposed to fungi or fungal cell

wall components (Doke, 1983; Apostol *et al.*, 1989). The H_2O_2 is involved in lignin production to form physical barriers in the host cells that inhibit fungal proliferation. For example, Tang & Smith (2001) reported the production of H_2O_2 in lucerne cells which were incubated with an elicitor extracted from *V. albo-atrum.* It is also known that H_2O_2 diffuses rapidly across cell membranes to elicit phytoalexin production in soybean cells in response to fungal infection (Apostol *et al.*, 1989). It was recorded that spore germination and mycelial growth of *Pseudocercospora* species was inhibited by H_2O_2 *in vitro* (Joseph *et al.*, 1998). It was established that peroxidase also inhibited spore germination and mycelial growth of *Pseudocercospora* species (Joseph *et al.*, 1998).

Flood (1980); Bianchini *et al.* (1999); He *et al.* (2002), on the other hand, showed phytolaexins from lucerne inhibited germination and radial extension of *Verticillium* species. Some other abiotic factors also inhibited the germination and the radial extension of fungi. It was reported that low osmotic potentials decreased the germination and the radial extension of *Verticillium* species (Chandler *et al.*, 1994). Similar findings were also made by McQuilken *et al.* (1992), they showed that decreases in osmotic potential caused a reduction in mycelial growth and oospore germination of *Pythium oligandrum.* Salinity also seems to inhibit the germination and radial extension or growth of fungi. For example, geographical distribution of *Fusarium* species were affected by salinity (Abbas & Mandeel, 1995). The decrease in growth of *Phytophthora* sp. caused by salinity was also reported by Wilkens & Field (1993).

1.12. Environmental stresses and resistance in plants.

Plants are subjected to a number of environmental stresses during their life. So, it is important to understand the terminology and mechanism(s) that concern plant stress. Stress is an abnormal change in physiological processes caused by one or a combination of environmental and biological factors (Table 1.12.1). Austin (1989) described stress as having the potential to produce injury as a result of abnormal metabolism; it may be expressed as a reduction- in growth, yield, or quality of the plant or of plant parts, or death of the plant or plant parts. On the other hand, Boyer (1982) defined a plant as 'stressed' when it was prevented from expressing its full genetic potential for reproduction. Jones (1983) subdivided stress tolerance into categories such as escape

(where the plant avoids being subjected to the stress), avoidance (where the plant avoids its tissues being subjected to the stress even though the stress is present in the environment) and true tolerance at a biochemical or physiological level.

Table 1.12.1. Sources of Environmental Stress

Physical	Chemical	Biotic
Drought	Salts	Diseases
Temperature	Allelochemicals (organic)	Allelopathy
Radiation	Nutrients (inorganic)	Lack of symbiosis
Flooding	*Pesticides (i.e. excess use)	Human activities
*Mechanical	Toxins	Competition
*Electrical	*Air Pollution (SO_2, H_2S)	Insects
Magnetic	pH of soil solution	
Wind		

* can also be defined as human activities.

Through the processes of evolution, a plant species can become fit or adapted to an environment in which it grows. So, the stress may change metabolism, which leads to a change in morphology by a process called acclimation, and through such changes plants become resistant or tolerant to that stress. In this way, survivors have a tolerance to injury from environmental factors that enables them to overcome partially or completely any adverse effects (Kramer, 1980).

1.13. Soil salinity.

A soil is considered to be a three dimensional piece of landscape having shape (form), area, and depth (Soil Survey, 1951). The concept of a soil as a profile having depth but necessarily shape or area is also a common use of the term. Scofield (1942) and Campbell & Richards (1950) considered a soil to be saline if the electrical conductivity of a solution, extracted from a saturated soil paste, had a value of 4 mmhos/cm (≅ 2.56 g/l dissolved salt, Maas & Hoffman, 1977; Abrol *et al.*, 1988, or more at 25 °C), and the exchangeable-sodium-percentage is less than 15. Generally, the pH would be less than 8.5.

Saline soils are recognizable by the presence of white crusts of salts on their surface. The kinds and amount of salts present mainly determine the chemical characteristics of saline soils. The soluble salt consists of various proportions of the cations; Na^+, Ca^{2+}, and Mg^{2+} and the anions; Cl^-, and SO^2_4. The cation K^+, and the anions HCO^-_3 and CO^-_3 and NO^-_3 occur in minor amounts. Despite the essentiality of Cl^- as a micronutrient for all higher plants and Na^+ as a mineral nutrient for many halophytes, an increase in their concentration will result in toxicity to non-salt tolerant plants. So, Na^+, especially as NaCl, is the most significant of the salts causing salt stress in plants (Levitt, 1972). Other cations such as Ca^{2+} and Mg^{2+} are usually present in sufficient quantities to meet the nutritional needs of crops; they sometimes contribute to the salinity especially at the later stages of soil development (Flowers & Yeo, 1986; Taiz & Zeiger, 1991).

There is no critical point of salinity where plants fail to grow. As the salinity increases growth decreases until plants become chlorotic and die. Plants differ widely in their ability to tolerate salts in the soil. Salt tolerance ratings of plants are based on yield reduction on salt-affected soils when compared with yields on similar non-saline soils. Soil salinity classes were given in Table 1.13.1.

Table 1.13.1. General guidelines for plant response to salinity (Adapted from FAO, 1988).

Soil salinity class	Conductivity of the saturation extract (EC, dS/m)	Effect on crops
Non saline	0 - 2	Salinity effects negligible
Slightly saline	2 – 4	Growth of sensitive plants may be restricted.
Moderately saline	4 – 8	Growth of many plants are restricted.
Strongly saline	8 – 16	Only tolerant plants grow satisfactorily.
Very strongly saline	>16	Only a few very tolerant plants grow satisfactorily.

Salts are a common and necessary component of soil, and many salts (nitrates, and potassium) are essential plant nutrients. The salts that contribute to the problem of soil salinity are derived from various sources. Firstly, water that evaporates from the sea includes salt, which then falls as rain over inland areas and may deposit these 'cyclic salts' (Teakle, 1937) in coastal regions. This source is considered to be the major cause of salt accumulation in the soil and groundwater of inland areas. Secondly, soils derived from inland seas that retreated about ten million years ago naturally contain large quantities of salts. Thirdly, the continued weathering of rocks, which involves hydrolysis, hydration, solution, oxidation, carbonation and other processes, release salts that become soluble (Abrol *et al.*, 1988). These salts move from the more-humid- to the less-humid- and relatively arid areas, by means of ground-and stream water. In arid areas, over millions of years, they gradually concentrate due to lack of leaching and so produce salt affected areas. This may results in a salt desert. However, under humid conditions these soluble salts are transported to the oceans (Abrol *et al.*, 1988). Tidal inundation of seawater also causes salinity in the low-lying areas of the world (Rowell, 1994). Soil salinity in some areas result from the restricted drainage caused by the construction of roads and rail lines, or other developmental activities. Such activities may cause a high-ground water table or low permeability of soil (Abrol *et al.*, 1988). In addition to that, important source of salts may come from ice-melters used on roads and sidewalks. Marine salts may also be brought by an underground infiltration of sea-water (infiltrating salts) (Waisel, 1972).

Accumulation of excess salts in the root zone causes partial or complete loss of soil productivity and this is the oldest and most serious environmental problem (McWilliam, 1986; Zhu, 2001). For example, the collapse of the Babylonian Empire is considered to be partly the result of failure of irrigated crops resulting from accumulation of salts (Hillel, 1992). Although irrigation practises have increased agricultural productivity it is now widely recognised that it has also contributed to the increasing salinization of agricultural lands (Sinha & Singh, 1976; Boyer, 1982; Shannon, 1997). For example, irrigation of crops with water of marginal quality due to competition between agriculture and demand by cities and industries for high quality of water also caused soil salinity (Wainwright, 1984). The presence of even small concentration of salts in good quality irrigation water leads to salt accumulation in soils unless leached

away by rain or irrigation water. On the other hand, intensive irrigation without adequate drainage results in a rise in the ground water level and capillary action draws up salts through the soil profile (Bridges, 1997). It has been reported that World wide there is more land going out of irrigation because of salinity then there is new land coming into irrigation (Vose, 1983).

Salinity may also occur in soils or compost in glasshouses in the form of potassium, nitrate and chloride, resulting from the application of water that contains fertilizers, or from the accumulation of residues of fertilizers and liquid feeds in excess of crop needs (Epstein *et al.*, 1980).

Salinity, whether natural or induced by agriculture, is a widespread environmental stress that can limit growth and development of salt-sensitive plants (Adams *et al.*, 1992; Yurtsever & Sonmez, 1996). As salinity levels increase, plants extract water less easily from soil, thus aggravating water stress conditions and resulting in accumulation of elements that are toxic to plants. An increase in salinity causes nutrient imbalances and reduction water infiltration.

The salinity problem is primarily associated with the arid and semi-arid regions of the world, where there is insufficient rain to leach away soluble salts (Fisher & Turner, 1978). Most of the salts are left behind after the extraction of water by the root, which leads to an increase in concentrations of salts that contribute to salinity in the soil. In addition to that, evaporation from the soil surface will remove water and leaves the salt behind in the soil, which eventually reaches toxic levels in the root zone.

In humid areas, the soil solution is concentrated very little; consequently root zone salinity in humid regions is rarely a problem (Abrol *et al.*, 1988).

1.14. Effects of soil salinity on plant growth.

There are many symptoms caused by salinity, some of these symptoms include; increased succulence of leaves or stems, leaf chlorosis and necrosis, leaf drop, root death, nutrient deficiency symptoms, and wilting (Johnson, 2000). Most of these symptoms may be mixed with the symptoms caused by the wilt fungus *V. albo-atrum* (Isaac, 1957; Esyanti, 1993; Pennypacker *et al.*, 1988 & 1990).

The investigations of the effects of salinity on plant species, particularly those of commercial importance, have increased rapidly during the past few years (Chaudhary, 1996; Sohan *et al.*, 1999). Salinity limits both plant growth and yield to different extents, depending on the plant species involved, salinity levels and the ionic composition of the salts.

Plants exposed to saline environments are subjected to several adverse conditions, which can be categorised as follows (Levitt, 1980; Fitter & Hay, 1987; Romero & Maranon, 1994; Romero *et al.*, 1994):

Direct toxicities of ions (excessive ion accumulation) e.g. Na^+ and, Cl^-, Boron.
Ion-specific effects (ion imbalance in the plant).
Osmotic effects (a reduction in the availability of water resulting from salt).

An increase in the external salinity decreases water flow into the plant and limits water uptake to cells. It also causes a reduction in turgor potential and reduces cell volume (Tal, 1984). This has been termed physiological drought, because plants are affected by a lack of water even though the water content of the soil is apparently adequate for crop needs (Greenway & Munns, 1980). There is a close correlation between salt concentration and growth. For optimal growth, plants must receive all the required elements, in a form that is easily available and must absorb them in the right proportions. When the concentration of the salt in the surrounding medium is increased, water absorption is reduced, and as a consequence, growth tends to diminish. Consequently, plants have to acclimatise to the lowering of water potential in order to survive in a saline environment. For example, *Avicennia germinans*, a maritime halophyte, grows in a soil where the salinity can vary from less than half the concentration of sea water, during the rainy season, to more than double that in the dry season (Smith *et al.*, 1989).

In the past there has been considerable argument as to whether the primary injury caused by salt stress was mediated through ion toxicity or osmotic effects. While Bernstein & Hayward (1958) emphasised osmotic stress as the primary cause of growth reduction, later workers considered toxicity of Na^+ and Cl^- ions to be more important (Chaudhary, 1996; Al-Rawahy 2000). Santa-Cruzz *et al.* (1997) compared the effect of

salinity and non-ionic osmotic stress induced by mannitol on the growth of several tomato species. They concluded that the primary stress induced by salinity was osmotic stress; hence both stresses had similar effects in the short term. Continual exposure to high salt concentrations in the root zone has been shown to cause a build-up of potentially toxic ions within the plant cells, and to disrupt the uptake of other essential micronutrients, so limiting plant growth and in severe cases resulting in necrosis (Passioura, 1986). However, in many herbaceous crop species growth inhibition and injury occurs even at low levels of NaCl salinization (Maas, 1993). Under this condition water deficit is not a constraint (Greenway & Munns, 1980). Certainly there is good evidence for ion toxicity having a major effect on plant growth in some species. In a number of species, such as avocado (Downton, 1978), growth is reduced by concentrations of NaCl (20 mmol l^{-1}) that are so low osmotic stress can be ruled out. In these species at least ion toxicity must be major stress. For example, Strogonov (1964) found that NaCl depressed the germination of lucerne (*M. sativa*) much more than isoosmotic solution of mannitol. The growth of beans, maize and barley was much better in Polyethylene glycol (PEG) solutions than in isoosmotic salt solutions (Greenway & Munns, 1980). According to Levitt (1980), different salts supplied at isoosmotic concentrations often inhibit growth of plants at different threshold osmotic concentrations. This again indicates that ion toxicity plays a part in overall stress. Especially, high concentrations of Na^+ and Cl^- may cause disruption in membrane function, protein synthesis, enzyme activity, and assimilation and photosynthesis (Flowers *et al.*, 1977).

One of the negative effects of salt stress, which might be responsible for the reduction in growth, is induction of deficiencies in other essential nutrients, or imbalances in ionic content. For example, high external sodium reduces the activity of Ca^{2+} ions in the root medium and so decreases the quantity of Ca^{2+}, which is available for uptake by the plant (Cramer & Lauchli, 1986). As a result, root growth and function may be inhibited and the translocation of Ca^{2+} from root to shoot may be impaired (Grieve & Maas, 1988). In addition to that ionic imbalance, particularly Na^+:Ca^{2+} and Na^+:K^+ ratios may affect cell metabolism and function and impairs the membrane integrity causing cell death (Cuartero *et al.*, 1992; Perez-Alfocea *et al.*, 1996; El-Iklil *et al.*, 2002). It has often

been observed that salt stress causes a decline in the potassium concentrations of various plants (for example, *A. stolonifera*, tomato, cucumber; Ahmad *et al.*, 1981; Del Amor *et al.*, 2001; Alpaslan & Gunes, 2001). It is possible that tissue potassium concentration declines to the extent that potassium deficiency causes growth reduction in some cases. It has also been reported that salinity increased the Cl^- content of the leaves (Inal *et al.*, 1997; Del Amor *et al.*, 2001; Essa, 2001; Inal, 2002). Thus, it caused a reduction in uptake of NO^-_3 by replacing it.

Salinity can cause changes in photosynthetic pigment composition. High concentrations of NaCl were responsible for the inhibition of photochemical reactions of isolated chloroplast (Reddy *et al.*, 1992). In halophytes and salt tolerant species, the chlorophyll content increased (Reddy *et al.*, 1992) while in salt sensitive species it decreased (Salma *et al.*, 1994; El-Iklil *et al.*, 2002; Kaya & Higgs, 2002; Kaya *et al.*, 2002). The reduction in chlorophyll in salt sensitive species was correlated with Cl^- accumulation (Velagaleti *et al.*, 1990). It has been reported that in salt sensitive cultivars of *M. sativa* at 170 mM NaCl treatment, photosynthesis was reduced by the accumulation of Cl^- in the chloroplast (Seemann & Chritchley, 1985) and as a result of that productivity and quality of the crops decreased (Satti & Yahyai, 1995; Stoop *et al.*, 1996; Jumberi *et al.*, 2002).

Salinity occurring during the day, or in the spring or summer cultivation causes higher reductions in yield than if it occurs during the night or in autumn cultivation (Van Ieperen, 1996). This results, because the higher temperatures and illumination and lower relative humidity in summer time lower water potential in the plant by inducing faster transpiration. As well as high transpiration affecting water potential, high salinity also lowers it, which will reduce the water flow into the fruit and therefore the rate of fruit expansion (Johnson, 2000; Johnson *et al.*, 1992; Del Amor *et al.*, 2001).

Nitrogen uptake by tomato plants is not affected at relatively low salt concentrations (70 mM NaCl) but at 140 and 200 mM NaCl, nitrogen uptake drops to a third of that observed in non-saline conditions (Pessarakli & Tucker, 1988). It has also been reported that uptake of NO^-_3 from the root solution is strongly inhibited by salinization; consequently NO^-_3 concentration in leaf and stems as well as nitrate

reductase activity within the leaves are lower in salinized than in control plants (Cramer *et al.*, 1995; Flores *et al.*, 2002).

Salinization has been observed to alter the hormone balance in plants. An increase in salinity caused a decreased transport of kinetin from roots to leaves, and an increase in leaf content of abscisic (ABA) acid. Both changes decrease stomatal aperture (Aspinall, 1980). ABA appears to modulate the response of plants to a variety of stresses (Zeevaart, 1988). Drought, NaCl, and 'cold tolerance' induce a two-to four fold increase in the ABA content of tomato leaves (Plant *et al.*, 1991; Yurekli *et al.*, 2001). This similarity in the response suggests that ABA may be a common signal for mediating the response to all three environmental stresses in tomato. The increase in ABA can be due either to higher ABA production in the roots or by a decrease in ABA metabolism in leaves (Jackson, 1997).

The (IAA) content, either rises slightly or remains unchanged under saline conditions (Dunalp & Binzel, 1996). The hormone causes reduction of the movement of water in the roots and therefore, it may play a role in protecting tomato plants from water deficit and decreasing plant turgor (Tal & Amber, 1971). Plants might response to salinity-mediated water stress by reducing water losses through ABA-regulated stomatal closure while IAA may perform independently (Dunlap & Binzel, 1996). Besides stomatal closure, the increased ABA concentration in leaves causes a reduction in leaf expansion while a lower root IAA content promotes root growth. These two effects would partially explain the increased root/shoot ratio in tomato plants grown in saline conditions. On the other hand, ethylene was also detected in tomato plants that were exposed to salinity (Jones & El-Beltagy, 1989). As a result of that epinasty was observed in leaves at the bottom of the stems.

Salinity, reduce the water potential of the external environment which can lead to the depletion of cellular water content and the loss of cell turgor (Hare *et al.*, 1998). A decrease in cell turgor can result in stomatal closure so inhibiting gas exchange and photosynthetic efficiency (Shannon, 1997). Decreases in cell turgor may also negatively affect cell division and elongation (Shannon, 1997).

Salinity causes blossom end rot (BER) in tomato plants, which makes fruits unacceptable for both the fresh market and the processing industry. BER symptoms

begin with slight browning at the distal placental tissue, which progressively invades the pericarp; the fruit stops growing and starts ripening too early. The main cause of symptoms is a local Ca^{2+} deficiency due to the result of excessive salinity in the irrigation solution or growing media (Adams & Ho, 1992). This is made worse in high temperatures because, under saline conditions, the increased transpiration causes more Ca^{2+} to move the leaves and less to the fruit (Adams & Ho, 1993). However, Saure (2001) reported that 2 important factors are responsible for the symptoms of BER.

1- an increase in the concentration of physiologically active gibberellins (GAs) and a resulting decrease in Ca^{2+} cause enhanced permeability of cell membranes. Increased level of GAs that caused BER has also been reported in many studies (Bangerth, 1973; Vettakkorumakanav *et al.*, 1999).

2- soil water deficit, high salinity or high NH^{+}_{4} activity also causes the deterioration of cell membranes with loss of turgor and leakage of cell liquids.

Salinity also has a detrimental effect on germination. It may affect germination in two ways; by creating a low osmotic potential which reduces or prevents water uptake; or by providing conditions for the entry of ions which may be toxic to the embryo or developing seedling (Bewley & Black, 1982; Bliss *et al.*, 1986a). Hilhorst & Toorop (1997) defined germination as the sum total of the processes preceding and including the protrusion of the radicle through the surrounding seed structures. Water uptake by the seed has been regarded as the starting point of germination; with the end point being the emergence and elongation of the radicle through the testa (Bewley & Black, 1994). In many studies, it has been reported that a low osmotic potential or the toxicity of the ions involved had a detrimental effect on the germination of seeds (Emmerich & Hardegree, 1990; Johnson, 2000; Essa, 2001; Esechie *et al.*, 2002). Bliss *et al.* (1986b) showed that inhibitory effect of NaCl and betaine (a non-toxic solute) were similar before germination began, but they were different subsequently. They proposed that the difference between isotonic betaine and NaCl might be the toxic effect of NaCl, which is obvious after the hydration threshold had been surpassed. It has also been reported that salinity not only causes a reduction in germination but also delays the germination (Kent & Lauchli, 1985).

It appears, then, that all three main components (osmotic effects, ion toxic effects and nutritional effects) are responsible for reduction of growth of plants in saline conditions (Mahmoud, 1992).

The effects of salinity are not always negative; salt treatment has also been shown to improve tomato fruit quality (Mirzahi *et al.*, 1988; Del Amor *et al.*, 2001). The improvement of quality through irrigation with saline waters has also been reported in grape (Watzman, 1999) and in celery (Pardossi *et al.*, 1999). The application of brackish water (2 dS m^{-1}) to vines was reported to result in an increase in wine quality whilst maintaining the crop yield (Watzman, 1999). It was also reported that the application of moderate salinity during the development of fruit, such as melon and tomato, caused an increase in soluble solids. Shannon & Grieve (1999) concluded that a small decrease in crop yield resulting from salinity might be partially offset by the increased marketable quality of the fruit.

1.15. The effects of salt stress on cell membranes.

It has been reported that many adverse effects of salinity are related to the structural and functional integrity of membranes (Laszlo *et al.*, 1980; Balsamo & Thomson, 1995; Alpaslan & Gunes, 2001). Binzel *et al.* (1988) reported that growth inhibition resulting from salinity is due to changes in the physical and chemical properties of cell membrane. Mansour *et al.* (1993) reported that alteration in membrane permeability resulted in cell death. For example, Na^+ increased the permeability of cell membrane and caused K^+ leakage from barley and bean roots (Nassery, 1975 & 1979). Leopold & Willing (1984) reported that the leakage organic solutes from salt-stressed soybean leaves increased with the increase of NaCl concentration, while almost no leakage was observed resulting from osmotic effects caused by sorbitol. Similar findings were made by Alpaslan & Gunes (2001), who reported that the membrane permeability of cucumber and tomato plants was increased by increasing salinity.

1.16. Mechanism of salinity tolerance.

Although plant responses to salinity are one of the most widely researched subjects in plant physiology the mechanisms that impart salt tolerance are still unresolved (Cheeseman, 1988; Munns, 1993).

Plants, which were able to obtain more water than others from a soil under low water potential, would grow better in saline conditions (Cruz & Cuartero, 1990). So, plants have developed various mechanisms for survival under high salinity stress. Some tolerate the high concentrations of toxic ions present in their root environment by exclusion or compartmentation of ions into the vacuole, and the production of high concentrations of organic solutes in the cytoplasm that lower the osmotic potential (Greenway & Munns, 1980). These organic solutes such as proline (Perez-Alfocea *et al.*, 1993a) and *myo*-inositol (Sacher & Staples, 1985) are generally non-toxic to enzymes.

It has been reported that Na^+ and Cl^- ions were accumulated in the vacuolar sap of halophytes (Austin, 1989). As a result of this, plants become succulent. Succulence is usually defined as the thickening of the leaves of the plants exposed to salinity, although this condition is also applicable to the stem and the root. It is expressed as an increase of water content per unit dry weight, fresh weight or water content per unit area (Jennings, 1976). It has been proposed that increases in succulence in response to salinity could be a characteristic indicative of an increased degree of salt tolerance (Tal & Shannon, 1983).

An increase in salt uptake generally depends on transpiration loss, because the water loss will increase the flux of saline water into the root system. Consequently, most plants, especially halophytes, show morphological features that prevent water loss, such as increased succulence, a thick cuticle on leaves, a reduced number of stomata, or sunken stomata, altered stomatal distribution and rolled leaves (Begg, 1980; Flowers *et al.*, 1986; Cruz & Cuartero, 1990), which would thereby reduce the uptake of ions and would improve salinity tolerance. Preventing water loss, by this way, might also reduce the toxic effect of excessive ion concentration (Flowers *et al.*, 1991).

The metabolism of CO_2 in succulents is unusual, and because it was first investigated in members of the family Crassulaceae, it is called crassulacean acid metabolism (CAM). CAM plants usually grow where water is scarce or is difficult to get, including deserts and semi-arid regions, salt marshes and epiphytic sites (as when certain orchids grow attached to other plants). In these habitats CAM plants (like all plants) must obtain water and CO_2, but if they fully open their stomata during daylight and thereby obtain CO_2, they transpire too much water. Therefore, they open their stomata and fix CO_2 into malic acid primarily at night, when temperatures are cooler and relative

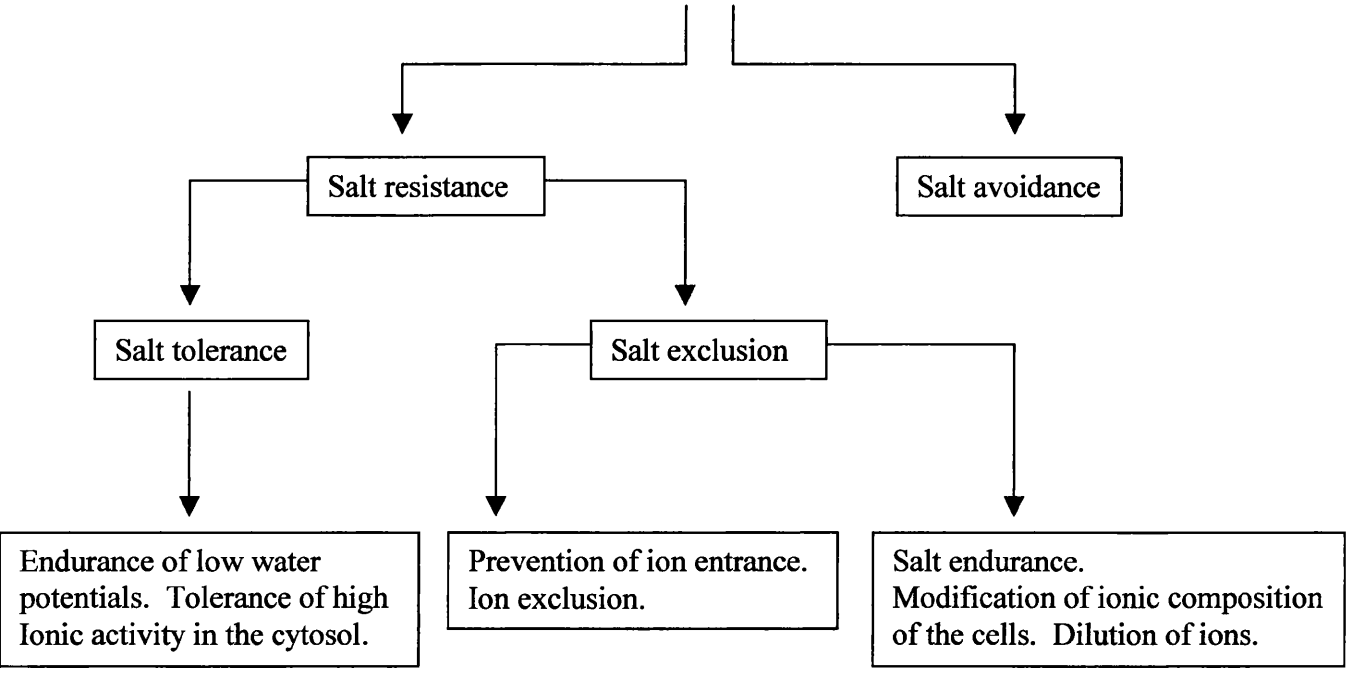
humidities higher. The formation of acid at night is detectable as a sour taste and is accompanied by a net loss of sugars and starch. As a result of accumulation of malic acid in CAM plants, sometimes even at concentrations of 0.3 M or more, until sunrise, the osmotic potential of the cells become quite negative, so they can absorb and store the water when the plant exists in dry or salty soils (Ting, 1985).

Some species possess and use the C_4 pathway of photosynthesis as a mechanism for decreasing transpiration and limit salt intake (Long & Mason, 1983).

Apart from these physiological adaptations, some developmental traits (e.g. the time of flowering) may also counteract the effects of salinity stress. Therefore, selection and breeding for salt tolerant cultivars requires an understanding of such mechanisms of salt tolerance.

Climate and irrigation also influence salinity tolerance. As the soil dries, salts become concentrated in the soil solution, increasing salt stress. Therefore, salt problems are more severe under hot, dry conditions than under cool, humid conditions. Detailed description of adaptation to salinity was given in Figure 1.16.1 by Waisel (1991).

Fig. 1.16.1. Modes of plant adaptation to salinity (Waisel, 1991).



Further research on metabolism of salt tolerance needs to be carried out in order to find out the traits governed at both plant and the cellular level.

1.17. Glycophytes and Halophytes.

On the basis of their tolerance or sensitivity, plants commonly are distinguished as halophytes and glycophytes. Glycophytes ("sweet" plants) tolerate only low concentrations of salt, while halophytes (halas=salt, salt plants) tolerate relatively high concentrations of salt (Levitt, 1972; Greenway & Munns, 1980; Flowers & Yeo, 1986; Flowers & Yeo, 1988). It was estimated by Flowers *et al.* (1986) that there were at least 800 species of halophytic angiosperms in more than 250 genera. This illustrates the point that there are many species of plants that possess the necessary features to enable them to grow and survive in a saline environment (Austin, 1989).

Some halophytes possess glands and bladders, which actively excrete excess salts. Examples of these are *Spartina, Armeria, Limonium* and *Glaux* (Long & Mason, 1983). Each gland may excrete up to 0.5 μl of salt solution in an hour. Obligate halophytes, for example *Halogeton glomeratus*, only grow in saline soil, and *Salicornia europaea* grows well in the presence of NaCl (Wainwright, 1984). For example, saltbush, indigenous to Australia, has developed a mechanism to control the Na^+ and Cl^- ion concentration of its tissues. The epidermal bladders on the surface of the aerial parts of the plant are specialised cells that accumulate salt. As the leaf ages the salt concentration in the cell increases and eventually the cell bursts or falls off the leaf, releasing the salt outside the leaf (Troughton & Donaldson, 1972).

In non-halophytes, resistance to salinity is commonly correlated with the ability to restricted entry of ions into the shoot. Their growth will be retarded when the salt content of the soil exceeds a rather low value (Levitt, 1972). Glycophytes lack specialized anatomical features as well as tolerance to ions accumulated in the tissues. Typical of glycophytic dicotyledons is the uptake of ions from the external medium but the upward movement of these ions through the shoots is restricted by mechanisms of varying effectiveness (Greenway & Munns, 1980).

In most halophytes osmotic adjustment results from the increase in concentrations of Na^+ and Cl^- in the tissue. In glycophytes tolerance to salinity is related to the

exclusion of these ions from tissues. This became clearer by comparing ionic concentrations in the tissues of salt-tolerant and non-salt tolerant cultivars of the same species. Many salt tolerant non-halophytes tend to restrict Na^+ uptake and take up more K^+ than do the less tolerant ones (Greenway & Munns, 1980). For example, salt tolerant clones of *Agrostis stolonifera* contained lower Na^+ in the shoots than a salt-sensitive inland clone (Ahmad *et al.*, 1981). This showed that restricted Na^+ uptake and maintenance of high Na/K ratios were features of salt tolerance in *A. stolinifera*, a result later confirmed by Hodson *et al.* (1981).

However, Na^+ "exclusion and accumulation" have often been implicated, as mechanisms of salt-tolerance in non-halophytes but this conclusion cannot be generalized. The wild maritime tomato species *Lycopersicon chesmanii* was a salt accumulator but the commercial species *L. esculentum* exhibited salt exclusion (Rush & Epstein, 1976).

The high concentrations of the ions in the tissues of halophytes suggest that their metabolic process may be tolerant to salt stress when compared to glycophyte metabolism. However, comparison shows the enzymes of halophytes and glycophytes have a similar degree of sensitivity to salt (Weber *et al.*, 1980; Gibson *et al.*, 1984). The sensitivity of enzymes from halophytes to salt, despite the presence of high ionic concentrations, suggests that plant cells have the capability to compartmentalize the toxic ions away from sensitive metabolic sites (Flowers *et al.*, 1977).

1.18. The role of proline accumulation under salt stress.

Proline accumulation has occupied a special position in plant physiological research, particularly in response to different stresses. Its accumulation as whole plant level under salt stress in halophytes has been reported by many workers such as Smirnoff & Stewart (1985) in coastal plants; Stewart & Lee (1974) in *Triglochin maritime* and *Armeria maritima*. In glycophytes proline accumulation has been reported under salt stress in *Hordeum vulgare* (Buhl & Stewart, 1983) in *M. media* (Chaudhary, 1996), in *A. stolonifera* (Ahmad *et al.*, 1981), in tomato (El-Iklil *et al.*, 2002).

Several hypotheses have been put forward to explain the role of proline accumulation in stress metabolism. Proline acts as a compatible solute regulating and

reducing water loss from the cell during episodes of water deficit. Proline may have also a role as a sink for the nitrogen from nitrogenous compounds derived from the net loss of protein, and lastly it may represent merely a manifestation of the damaging effects of stress (Aspinal & Paleg, 1981) and may act as a substrate for respiration that might provide energy needed for recovery from stress (Hare & Cress, 1997). Proline accumulation may be a general response to stress, especially under salinity-, water- and temperature stress (Stewart, 1981; Heuer 1994; Aziz *et al.*, 1999). For example, salinized tomato plants are able to produce osmotically active organic substances that help to alleviate the salinity-mediated osmotic stress. Storey & Wyn Jones (1975) detected no increase in choline or betaine in the shoots and roots of tomato plants grown with- or without salt. In contrast, the proline concentration was 10-fold higher in shoots and 18-fold higher in the roots of plants grown at 100 mM NaCl than in plants grown in the absence of salinity. Similar findings were also made by Gunes *et al.* (1995), who reported that the proline contents of potato was almost 10 times higher than the control plants. Proline accumulation in salt-stressed plants could be due to the low activity of the oxidising enzymes (Sudhakar *et al.*, 1993) and its accumulation in leaves and mainly in roots is considered as a salt sensitive trait in tomato that may be used to select plants with different degrees of tolerance (Bolarin *et al.*, 1995).

However, the physiological significance of proline accumulation is rather poorly understood. It has been assigned the role of cytosolute or a protective agent for cytoplasmic enzymes and cellular structures (Stewart & Lee, 1974; Aziz & Larher, 1995). It has been considered to play a central role as an osmoregulatory solute in plants subjected to hyperosmotic stresses, primarily drought and salinity (Delauney & Verma, 1993). Some workers suggested that proline accumulation is neither a sensitive indicator of salinity nor of protective value but merely a symptom of injury (Hadson & Hitz, 1982). However, most investigations have indicated a positive correlation between proline accumulation and adaptation to salt or drought stress (Weimberg *et al.*, 1982; Watad *et al.*, 1983; and Rhodes *et al.*, 1986). Under salt stress conditions, a salt marsh ecotype of *A. stolonifera* accumulated more proline in roots and shoots than an inland ecotype (Ahmad, 1978). In the apices of maize seedlings growing at -1.6 MPa, proline accumulation reached 120 mmolal, accounting for almost 50% of the total osmotic

adjustment (Voetberg & Sharp, 1991). Such observations clearly suggest that in some plants, proline accumulation may play a direct, adaptive role in countering the effects of osmotic stress.

Proline may also prevent damage caused by cellular dehydration by increasing the water binding capacity of proteins (Vanlerberghe & Brown, 1987).

Addition of proline to salt supplemented medium has also been shown to enhance the growth and survival of unselected cells in a number of species (Pandey & Ganapathy, 1985; Handa *et al.*, 1986; Van Swaaij *et al.*, 1986). For example, exogenous proline showed beneficial effects during recovery of barley plants from water stress (Itai & Paleg, 1982), and in cultured tomato cells during water stress (Handa *et al.*, 1986). Similarly, proline (10 mM) in the external medium of NaCl-selected and unselected cell lines of *Cicer arietinum* under 100 mM NaCl stress, increased fresh and dry weights (Pandey & Ganapathy, 1985). A similar treatment increased the growth of salt-unadapted callus of rice (Kishor, 1988).

Synthesis and accumulation of proline also occur in cell suspension cultures of both glycophytes and halophytes. For example, salt tolerance correlates well with proline accumulation in NaCl-tolerant callus lines in *C. arietinum* (Pandey & Ganapathy, 1985), salt-tolerant cultured cells of eggplant (Jain *et al.*, 1987) and NaCl-tolerant calli in *M. sativa* (Shah *et al.*, 1990). Increased proline accumulation in response to NaCl stress, also occurs in suspension cultures of *Mesembryanthemum crystallinum* (Thomas *et al.*, 1992), while a positive correlation was found between proline accumulation and the capacity of cell cultures from chili pepper (a mesophyte) and creosote bush (a xerophyte) to grow under water stress (Santos-Diaz & Ochoa-Alejo, 1994).

Osmotic adjustment has been defined as the decrease in plant osmotic potential through the production and accumulation of solutes in the plant cells in response to the decrease in external water potential (Shannon, 1997). These solutes are often termed 'osmoprotectants' or 'osmoregulatants' and have been described as soluble organic osmolytes that are non-toxic even at high concentrations (McNeil *et al.*, 1999).

The organic solutes have been produced by plants have been studied extensively and has focused mainly on the production and accumulation of specific sugars, polyamines and glycine-betaine. Levels of glycine betaine (also as referred as betaine)

(Holmstrom *et al.*, 2000), mannitol (Stoop *et al.*, 1996), inositol (Karakas *et al.*, 1997), and proline (Perez-Alfocea *et al.*, 1993b; Cano *et al.*, 1996) have all been reported to increase in response to salinity. The primary role of these compounds is considered to be osmotic adjustment. However, Yeo (1998) have reported that these compounds have a role to protect to tissue against oxidative damage.

1.19. *In vitro* selection for salt tolerance.

The generation of salt tolerant plants has potential application to semi-arid and arid soils. Plant tissue cultures techniques have been used successfully to develop variant lines from somatic cell cultures (Ben-Hayyim & Kochba, 1983; Ben-Hayyim *et al.*, 1985; Rumbaugh & Pendery, 1990). Many salt tolerant somatic cell lines have been isolated in a number of plant species, including *Nicotiana sylvestris* and *Capsicum anuum* (Dix & Street, 1975), Citrus (Ben-Hayyim & Kochba, 1983), Cicer (Pandey & Ganapathy, 1984), *Lycopersicon peruvianum*, (Hassan & Wilkins, 1988). It is generally accepted that a mechanism regulating Na^+/K^+ selectivity exists in plant cells, which show salt tolerance (Chaudhary, 1996).

Many countries depend heavily on irrigation for food production, however, much of the food productivity is affected by soil salinity (Brown, 1981). If the problem of soil salinity decreases food production, whilst the population growth increases, then the rate of food production cannot keep the pace with the growth of the population for the world as a whole. Therefore, improvement of salt tolerance in crop plants is an important challenge to biotechnology.

Salt tolerant cell lines of lucerne have been selected in several laboratories (Croughan *et al.*, 1978; Smith & McComb, 1983; Bingham & McCoy, 1986; Shah *et al.*, 1990, Al-Rawahy, 2000). Studies with the first salt-tolerant cell line of lucerne showed a halophytic type of salt tolerance which was selected in the cell line that required salt for optimal growth (Croughan *et al.*, 1978). In some cases, the selected lucerne cell lines were maintained "*in vitro*" for several years and the plants were finally regenerated, the somaclones were so stunted that whole-plant tolerance was not determined (Stavarek & Rains, 1984). Similarly, one disappointing example has been with *Pennisetum purpureum* Schum. where plants regenerated from NaCl tolerant callus were even more

NaCl sensitive than plants regenerated from unselected callus (Chandler & Vasil, 1984). Smith & McComb (1981) screened four lucerne cultivars at the whole-plant and cellular level. One cultivar W75RS (Regen S), which showed "*in vitro*" tolerance also had a higher level of whole-plant tolerance. However, following selection of a NaCl cell line capable of plant regeneration, it was found that the regenerated plants were as salt sensitive as the initial plants (Smith & McComb, 1983). This may have resulted from loss or interchange of chromosomal segments during the cellular selection process, a process that was observed "*in vitro*" (McCoy *et al.*, 1982). However, in one study, the salt-tolerant lucerne plants that were regenerated from salt-adapted cell lines apparently showed dominant salt tolerance and it was transmissible through seed (Winicov, 1991).

1.20. Biotic stresses and plant diseases in salt-affected conditions.

Salinity can also cause a combination of complex interactions that affect plant metabolism and susceptibility to injury of wounds caused by insects or mechanical (Grattan & Grieve, 1999).

Salinity induces several metabolic changes in plants, such as accumulation of proline and glycine-betaine (Bray *et al.*, 1991). Wheat and barley seedlings irrigated with saline Hoagland solution (0-700 mM NaCl) accumulated Na^+, Cl^- and metabolites such as glycine betaine, etc. in the leaves, which correlated with a decreased the population and growth rate of the aphid *Schizaphis graminum* and *Rhopalosiphum padi* feeding on the plants (Araya *et al.*, 1991). However, it was noted that glycine-betaine was not harmful to aphids, as it increased the survival and reproduction of the aphids on both salt treated and untreated plants. It appears that is the accumulation of NaCl that is the real cause for the reduction in the survival and reproduction of aphids (Araya *et al.*, 1991). Similar findings were made by Kostandi & Soliman (1998). They showed that the effect of saline irrigation water containing NaCl or Na_2SO_4 reduced susceptibility to smut by 22.7 and 10.8% respectively. In contrast, however, high soil salinity or saline irrigation has been reported to increase the severity of stem rot caused by *Phytophthora citrophthora* in citrus roots (Sulistyowati & Keane, 1992). Although high salinity did not stimulate growth of the pathogen *in vitro*, the increase in disease under saline conditions was attributed to a direct effect in reducing resistance in the host. The similar results of the

effect of salinity stress on the development of plant diseases have also been reported in phytophthora root rot of chrysanthemum and citrus (Blaker & MacDonald, 1986; MacDonald, 1982 & 1984) and in pythium blight of penncross creeping bentgrass (*Agrostis palustris* Huds. 'Penncross') (Rasmussen & Stanghellini, 1988). The increase in disease severity under saline conditions has sometimes been attributed to an increase in virulence of the pathogen (Ragazzi *et al.*, 1994). However, reports related to modification in the tolerance potentials of plants to biotic stresses in the presence of salinity are rare. Consequently, it is difficult to draw any conclusion at this stage and when developing strategies for crop improvement, it must be kept in mind that there are two factors contributing, resistance of the plant and virulence of the pathogen. Furthermore, studies at cellular and molecular level are needed to understand the overall response of the plant.

1.21. Possible solutions to salinity.

The cost of salinity control is considerable but unavoidable if agricultural production is to be sustained at current or higher levels. In some parts of the world land that was once agriculturally productive has been abandoned due to induced salinity, which can occur through mismanagement and incorrect irrigation practices. Thus, in order to utilize or re-utilize such land, it is necessary first to correct the negative effect of salinity or sodicity (soils that contain sufficient exchangeable sodium to degrade soil physical properties) and, secondly, to introduce management practices that will prevent their recurrence (Qadir *et al.*, 2000).

If soil is saline and not sodic; good quality water can be used to leach salts out of the profile (Ayers & Westcot, 1985). The sodium is then leached down below the root zone, and the soil structure in the upper layers is restored. The management of salt-affected soils would be a reasonably a simple matter if the only consideration was the need to leach away salts. However, Na^+ has a reduced mobility in comparison to the divalent cations such as Ca^{2+} and Mg^{2+} and as salt is washed down through the soil, some sodium is left behind stuck to clay particles. It displaces more useful substances such as Ca^{2+}, and as a result Ca^{2+} uptake by plants is reduced (Cramer & Lauchli, 1986; Adams & Ho, 1989). Furthermore, Na^+ builds up in the soil and reduces the soil stability, causing

the soil to set hard when it dries (Soil Survey, 1951; Ellis & Mellor, 1995; Bridges, 1997). Application of gypsum or macro elements (N, P, Ca and K) to the soil surface will increase their concentrations in the root zone and supply extra calcium ions to displace sodium in the soil solution (Soil Survey, 1951). For example, a beneficial effect of a Ca^{2+} supplement on the growth of crops in saline media has been reported in many studies (barley, Lynch & Lauchli, 1985; cotton, Cramer *et al.*, 1986; cucumber, Kaya & Higgs, 2002; strawberry, Kaya *et al.*, 2002).

In many areas of the world drainage problems arise due to accumulation of rainfall or excess irrigation water on the soil surface (FAO, 1972). In such areas drainage ditches may take off the excess water before it enters the soil. Alternatively, subsurface drainage may be used. Another solution is to lower the water table, by planting more deep-rooted plants such as trees or perennial plants like lucerne.

Other effective measurements to prevent salinity in agricultural areas are; the use of drippers or micro-irrigation, to reduce the loss of water through wind drift, irrigation at night; or using pipes instead of channels to reduce loss of water by evaporation. Romero *et al.* (2002) reported that the detrimental effect of salinity could be alleviated by increasing humidity by misting the greenhouse atmosphere.

Mixing fresh and saline waters will help to obtain high crop yield under high salinity conditions throughout the cropping season. Alternatively, good quality water could be used for irrigation at the more critical stages of growth, e.g. germination, and the saline water at the stages where the crop has relatively more tolerance. Del Amor *et al.* (2001) reported that salt tolerance of tomato plants increased when the application of saline irrigation water was delayed.

Scraping or flushing the salts are important methods to keep the salt under control. However, the amount of salts removed from the soil is rather small (Qadir *et al.*, 2000). Leaching is one of the most effective methods for removing salts from the root zone of soils. It should be done when the soil moisture content is low and the groundwater table is deep. Summer months are less effective for leaching because large quantities of water are lost by evaporation. Biological reduction of salts by harvest of high salt accumulating aerial plant parts are also helpful to solve the salinity problem (Qadir *et al.*, 2000).

Cultural practices and crop selection to prevent salinity problems are just as important are corrective actions after the development of problems. There are several management alternatives available to prevent or reduce salinity problems. One of the first alternatives relates to the choice of crop (Zonneveld, 1976).

Cuartero & Fernandez-Munoz (1999) showed that, for a given EC of the irrigation water, the decreases in crop yield were lower in soil cultivation than the decreases reported for plants grown in hydroponic culture. This was proposed to result from the delay in build-up of salt in the soil. However, Shannon *et al.* (1987) demonstrated that the EC threshold tolerance of *L. esculentum* cv. Heinz was almost two-fold greater when in solution culture than when in sand culture, being 8.1 and 3 dS m^{-1}, respectively.

Harrington & Alm (1988) noticed that tobacco cell culture increases its resistance to high NaCl level after a preliminary high temperature treatment. In *Gossypium hirsutum* and *G. barbadense* a tolerance to the salt stress was noticed due to heat shock treatment at 47 °C (for 3 h), which induced a salt resistance that was related to the rapid and intensive release of ethylene, and accumulation of proline and osmotin (Kuznetsov *et al.*, 1993).

Strogonov (1964) pointed out that plant salt tolerance could be increased by treatment of seeds with NaCl solution prior to sowing. Similar findings were also made by Alvarado *et al.* (1987) for tomato and by Ashraf & Rauf (2001) for maize seeds. They stated that soaking of seeds in NaCl- or KCl-containing solutions alleviated the adverse effects of salt stress, accelerated germination, seedling emergence, and seedling growth. Cano *et al.* (1991) reported greater fruit yield in some cultivars of tomato grown with salty water when seed were primed with 1 M NaCl for 36 h. However, any benefits of seed priming for later stages of development and in yield is still unclear.

Nitrogen fixing plants may also be used to enrich soil nitrogen (Cordovilla *et al.*, 1999). Recently, many microorganisms, especially nitrogen fixers, have been shown to change the properties of salt-affected soils, resulting in bioremediation of the salinity (Zahran, 1991). The major groups of salt tolerant microorganisms reported from many salt-affected soils include free nitrogen fixing bacteria e.g. *Azotobacter*, *Alcaligens*, *Azospirillum*; cyanobacteria e.g. *Anabaena*, *Nodularia*, *Nostoc* etc. and symbiotic *Rhizobia* species (Zahran, 1991). Vesicular-arbuscular mycorizhal (VAM) fungi seem to

increase salt tolerance in some crops such as onions and bell peppers (Hirrel & Gardemann, 1980). In tomato, some soil samples with VAM originating from saline soils significantly improved the growth of tomato cultivar 'H-1350' irrigated with 10 dS/m water but other samples failed to show an effect or even produced slower growth than the non-mycorrhizal control (Prud *et al.*, 1984). However, to date the use of mycorrhizae is still controversial and cannot be definitely recommended.

Soliman & Doss (1992) showed that tomato fruit yield increased by approximately 14% despite the inhibitory effect of salinity, when grown with frequent application of liquid fertiliser compared to solid fertilisers.

Moreover, cultivars with enhanced growth of the root system would also have a higher capability to replace roots killed by deleterious saline conditions.

High or moderate salinity reduces the fruit size and yield (Johnson, 2000; Del Amor *et al.*, 2001). Hence, producing small size tomatoes or even cherry tomatoes could be an alternative way in these conditions.

CHAPTER II
GENERAL MATERIALS and METHODS

2.1. Growth of fungi.

2.1.1. Preparation of culture media.

Table 2.1.1. Potato Dextrose Agar (PDA).

Compound	Concentration (g/l)
Peeled and chopped potatoes	200
Glucose	20
Oxoid Agar No:3	25

200 g of peeled potatoes were chopped into small pieces and boiled for 30 minutes with 1000 ml of distilled water. The resulting mash was filtered through Mira Cloth (Calbiochem) and 20 g of glucose was added to the filtrate (Table 2.1.1). The volume was made up to 1 litre with distilled water and 250 ml of this medium was transferred to each of four 500 ml conical flasks containing 6.25 g of agar (2.5% w/v). The medium was autoclaved at 121 °C for 20 minutes (2.68 kg cm^{-2} pressure) and poured into 12 Petri dishes.

Table 2.1.2. Czapek Dox Medium (modified).

Compound	Concentration (g/l)
Sucrose	30.0
$NaNO_3$	2.0
KCI	0.5
Magnesium glycerophosphate	0.5
$FeSO_4$ $7H_2O$	0.01
K_2SO_4	0.35
Difco Agar	25.0

The ingredients were dissolved in a small volume of distilled water and the volume adjusted to 1 litre (Table 2.1.2). 250 ml medium was placed in each of four 500 ml conical flasks containing 6.25 g of Difco agar and the medium was then sterilized by

autoclaving at 121 °C for 20 minutes (2.68 kg cm^{-2} pressure). The medium from each flask was poured into 12 Petri dishes.

- *Chemicals*

Unless otherwise stated, chemicals were obtained from Sigma Ltd., Dorset, England and were of analytical reagent grade quality. Commercially available Czapek Dox agar (modified), manufactured by Oxoid Ltd. was also used in some experiments.

2.1.2. Fungal isolates.

Isolates of *V. albo-atrum* used in this study were obtained from lucerne and tomato plants. They were the isolates V1 and V2 obtained from *M. sativa* and *L. esculentum*, respectively. Isolate V1 can cause disease in lucerne and tomato whilst V2 can cause disease only in tomato (Isaac, 1957; Heale & Isaac, 1963; Dikilitas, 1997). In some studies of this dissertation, isolates of *V. albo-atrum* (Sevcik, Freitag, Arl 86B & Loken) from *M. sativa* that were obtained from the culture collection of Professor Craig Grau (University of Wisconsin, Madison-USA) were also used in some pathogenicity and salinity experiments.

Periodically, fresh isolates of V1 and V2 were obtained in the following way; for V1, plants of lucerne, cv. Europe were inoculated with *V. albo-atrum*, isolate V1 by the root-dip method. The inoculated plants were planted in the field and maintained in the gardens at UWS, Plate 2.1. Inoculated plants were incubated for 8 weeks in field conditions and the reisolations were made at end of this period. For V2, plants of tomato, cv. Ailsa Craig were inoculated with *V. albo-atrum*, isolate V2 by the root-dip method. The inoculated plants were incubated in the greenhouse at UWS and resisolations were made at the end of 6 to 8 weeks (section 2.2.5).

2.1.3. Morphology of fungus.

To identify the fungus, its morphology was examined under a light microscopy. Mycelia of the fungus were observed while growing on Dox or PDA agar by removal of a square of fungus (*ca.* $1cm^2$) from the plate with a sterilized scalpel. The morphology of conidiophores that grew into this empty area from the edge of the agar was observed under a light microscope, Plate 2.2a. Fungal hyphae were also observed by removing mycelium and mounting in water on a glass slide, Plate 2.2b.

Plate 2.1.
Lucerne plants inoculated with *V. albo-atrum*, isolate V1 and maintained as a source of stock in the garden (UWS).

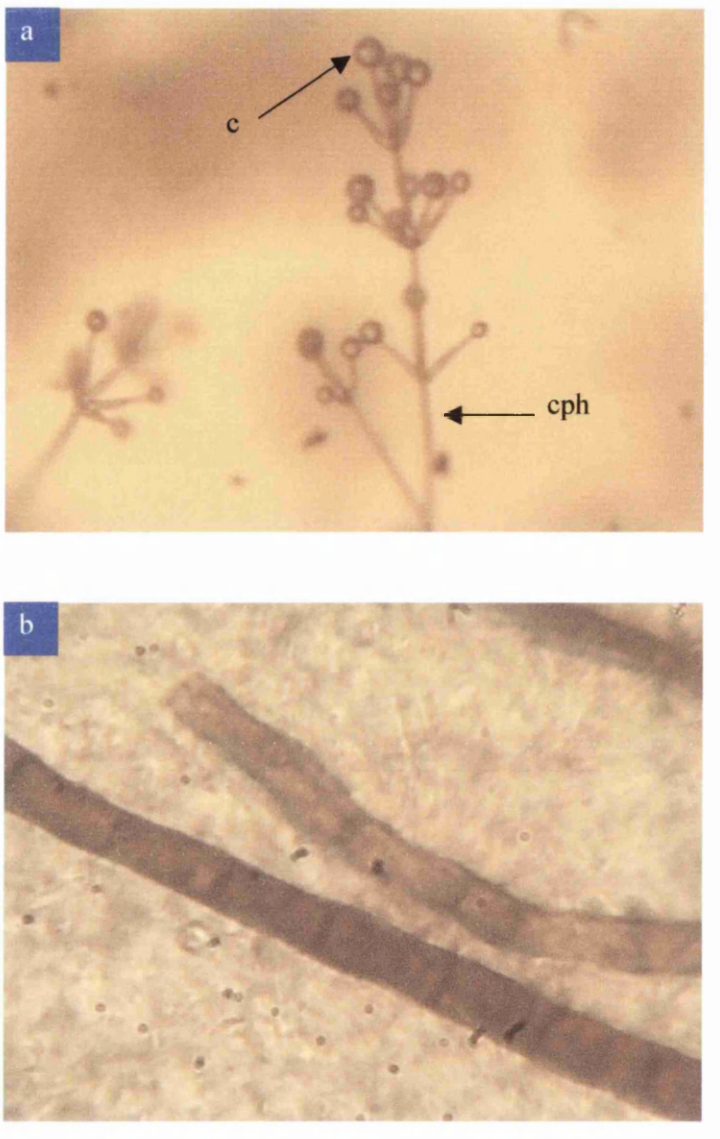


Plate 2.2.
(a); An erect verticiliate conidiophore of *Verticillium* (x 80) -c: conidia in 'spore ball'; -cph: conidiophore; (b);. Resting mycelium of *V. albo-atrum* (x 100).

2.1.4. Slope agar.

To maintain fungi as stock cultures they were kept on agar slopes in glass tubes. For this purpose, 15 ml slopes of Dox or PDA sterile media were stored at 4 °C ± 1 °C. These cultures were renewed every 6 months by transfer of an inoculum to Petri dishes of the same medium. The Petri dishes were maintained at 23 °C ± 1 °C in the dark. Cultures were checked visually for contamination and new slope cultures of the fungi were inoculated from the resulting pure colonies.

2.1.5. Preparation of spore suspension.

Cultures of *V. albo-atrum*, maintained on either Dox or PDA medium and subcultured every 23 days, were used for inoculation. Spores were removed from the surface of the 23-day old cultures by addition of 10 ml of sterile distilled water followed by gentle stroking of the mycelial surface with a sterile glass rod. The resulting suspension was transferred to a beaker and the concentration of spores was determined under a microscope using a neubauer haemacytometer slide and counter. The concentration of spores in the suspension was adjusted with sterile distilled water to give approximately 10^7 conidia ml^{-1}

2.2. Growth of plants.

The various cultivars of *M. sativa*, strains of *M. media* and cultivars of *L. esculentum* and *L. lycopersicon* were used in this study and were germinated in trays of John Innes No. 1 unsterilised compost in a greenhouse (in the Botany Garden at UWS) at 23 °C ± 2 °C, under supplementary daylight fluorescent tubes (150 $\mu E\ m^{-2}\ s^{-1}$), with a 16 hour per day photoperiod (when necessary). The trays were covered with transparent plastic domes to protect the seedlings from desiccation. When the plants showed significant growth, the plastic domes were removed. After 2 weeks, the seedlings were transferred to individual 15-cm plastic pots containing John Innes No. 2 unsterilised compost. Growth was continued for a further four weeks before seedlings were used for experiments.

Seedlings of salt tolerant *M. media* were grown from the cuttings derived from the parent plants maintained in the greenhouse. The original salt tolerant strains of *M. media* Pers. cv. Rambler were generated from cells, adapted to different levels of NaCl in liquid

culture (Chaudhary, 1996) in this department. A new salt line was also prepared by using the leaf of original salt tolerant plants (250 mM NaCl-tolerant plant) by Al-Rawahy (2000).

The new cell line was adapted to different levels of salt in liquid culture as well. Plants which were generated from the first salt line was called "the original salt tolerant plants" while the plants which were generated from the second salt line was called "the new salt tolerant plants". In text the plants are designated by the letter "R"- for 'Rambler', followed by a number indicating the molarity (in millimoles) of the salt concentration to which the line is tolerant and either 'O' to indicate 'original', or 'N' to indicate the new generation of salt tolerant plants.

Salt tolerant plants were propagated by rooting the 12-cm cuttings with the help of rooting powder either in a test tube containing 1/5 dilution of Arnon and Hoagland (A&H) (1940), solution with a ferric-EDTA iron source, or in trays of perlite in a heated mist propagator (Table 2.2.1). In both cases shoots were maintained for 16 days in the greenhouse. Cuttings in the trays of perlite were also watered at three-days intervals with A&H solution with a ferric-EDTA. Non-salt tolerant plants were also propagated by cuttings to avoid from genetic differences in the population although the seeds were available, because the genetic differences in plants, grown form seed would vary (personal communication with Assoc. Prof. Barbara W. Pennypacker, USA, 2000). After 2 weeks the rooted-cuttings were transferred to pots of John Innes No. 2 compost. These plants were then used for pathological experiments after four weeks of growth.

M. media Pers. cv. Rambler is a synthetic cultivated variety resulting from the combination of seven separate lines. Some of these lines resulted from the F1 of Ladak (*M. media*) X Siberian (*M. falcata*) being back crossed to Ladak because of its drought tolerance, a feature that could be of value in salinity problems. The seeds and cultivar information were provided by the Seeds Division of the Plant Health and Plant Products Directorate, Canada (Chaudhary *et al.*, 1994). Chaudhary *et al.*, 1994 and Al-Rawahy (2000) stated that the cultivar Rambler exhibited a high degree of creep, some resistance to bacterial wilt disease and drought. However, Dikilitas (1997) showed that cultivar Rambler was quite susceptible to VW.

During the course of these studies, some salt tolerant lucerne plants were also generated from the cells of cv. Vertus by using the method of Chaudhary (1996) with minor

modifications. The resistance of cv. Vertus, a *Verticillium*-resistant cultivar, was assessed against *V. albo-atrum* when the cultivar became salt tolerant.

Table 2.2.1. Arnon and Hoagland Stock Solution (A&H) (1:5).

Compound	Concentration (g/l)
1. Macronutrients: -	
Potassium Nitrate (KNO_3)	10.100
Calcium Nitrate ($Ca(NO_3)_2.4H_2O$)	4.9230
Ammonium Dihydrogen Orthophosphate ($NH_4H_2PO_4$)	2.3008
Magnesium Sulphate ($MgSO_4.7H_2O$)	4.9300
2. Micronutrients: -	
Manganous Chloride ($MnCl_2.4H_2O$)	0.3870
Boric Acid(H_3BO_3)	0.6310
Zinc Chloride ($ZnCl_2$)	0.0240
Ammonium molybdate ($(NH_4)_2Mo.4H_2O$)	0.0154
Cupric Sulphate ($CuSo_4.5H_2O$)	0.0154
3. Iron Solution: -	
Ethylenediaminetetraacetic acid (FeNa EDTA)	6.5652

From the above stock solutions 1ml of micronutrients, 20 ml of macronutrients and 1 ml of iron solution were taken and made up to 1 litre with distilled water. Lucerne cuttings were sprayed with the A&H solution every three days to promote root development. The stock solutions were stored at 4 °C.

2.2.1. Growth conditions of stock salt tolerant plants.

During the course of experiments, the stock plants were grown in a greenhouse with John Innes Compost No. 2. The temperature of the heated, ventilated greenhouse was regulated to about 23 °C ± 2 °C. The humidity fluctuated from about 50 to 70%.

Shoots of salt tolerant plants were cut 15-20 cm from the soil level and kept as stock in the greenhouse.

2.3. Inoculation of plants.

Tomato and lucerne plants were inoculated with spores of *V. albo-atrum* with the isolates V1 and V2 using one of the following methods.

(i) Root dipping;

Plants were removed from pots and the soil was removed by shaking the plants gently, followed by washing in tap water. The roots of the plants were placed in the spore suspension for 10 minutes. The inoculated plants were re-planted using the same soil. Sterile distilled water was used in place of the spore suspension to treat the roots of control plants. At 1-week interval the heights and disease symptoms of the plants were recorded over a six- or seven-week period following inoculation.

(ii) Wound inoculation;

The stem of each plant just beneath the soil level was wounded with a razor blade. The wounded area was inoculated by fixing a block of agar medium, which had been cut from PDA or Dox cultures of the fungus onto the wound (culture face towards to the wound). The heights and disease symptoms of the plants were recorded at weekly interval over a six-week period following inoculation. Control plants were treated with blocks of agar that had not been inoculated with the fungus.

(iii) Soil drenching;

Unless otherwise stated, each plant was inoculated with 100 ml of 1×10^7 spores/ml, applied around the base of tomato seedlings. Control seedlings received 100 ml of distilled water in place of the spore suspension. Following inoculation tomato plants were watered on demand like other treated plants.

(iv) Stem puncture method;

Tomato plants were inoculated with a stem puncture method following the procedure described by Resende *et al.* (1995), with minor modifications. The conidial suspension (1×10^7 spores/ml) was injected from a sterile syringe to the base of the stem.

Approximately 0.5 ml of the inoculum suspension was absorbed in 5 punctures. The drop of inoculum that formed between the stem and the needle disappeared rapidly into the cortex. Control seedlings were similarly injected, with distilled water in place of the spore suspension.

(v) Root dipping with shaved roots;

Plants were removed as for the root dipping method, their roots were shaved to facilitate the entry of conidia to plant roots prior to the inoculation and the roots were placed in spore suspension for 10 minutes. Sterile distilled water was used in place of the spore suspension to treat the roots of control plants.

2.3.1. Disease symptom assessment.

Plants were scored for symptoms of a disease using a system adapted from Dixon & Doodson (1971) and Moller-Nielson & Andreasen (1971) using the following scale:

0- no wilt symptoms.

1- trace of infection: chlorotic yellowing visible on the cotyledons and first unifoliate leaf only.

2- slight infection: chlorosis and epinasty affecting less than 50% of leaves.

3- moderate infection: widespread symptoms including chlorosis, epinasty and necrosis, new largely symptom free branches emerging.

4- severe infection: plant weak and stunted; both main stem and branches show advanced symptoms.

5- extremely severe infection: branches and stem necrotic but some greenness still visible at the shoot apex.

6- plant completely dead.

Intermediate scores i.e. 1.5 and 2.5 were used when symptoms did not fall into the main categories.

Plants which scored between 0 and 2 were categorised as resistant, between 2.5 and 3.5 as moderately susceptible, and between 4 and 6 as susceptible (Latunde-Dada & Lucas, 1982).

From these scores a symptom index, which records the time of onset of symptoms and their rates of progression in the plants, was calculated as a percentage for each group of plants in a single treatment. The number of plants showing a particular value (from 0 to 6) were multiplied by that value and the figure obtained for all plants summed and the total multiplied by 100. This value was divided by a maximum value of symptoms i.e. 6, times the total number of plants for that treatment

Therefore,

$$\%\,SI = \frac{\sum SI}{6 \times \sum n} x100$$

SI: Symptom index

n: number of plants

2.3.2. Root symptom index.

To assess the effect of the pathogen or salt or both in plant roots, a root symptom index was recorded according to the method described by Graham *et al.* (1977).

1- clean root,

2- trace of discoloration,

3- moderate discoloration,

4- severe discoloration,

5- dead.

2.3.3. Reisolation of fungi from inoculated plants.

Reisolation of the fungus was made from the plants that had been inoculated, either to establish whether infection had taken place, or as part of a program to maintain the pathogenicity of the fungi. Inoculated and control plants were removed from the soil and their roots were washed carefully under tap water to remove soil particles. Small portions of root and stem segments were removed, and the sections were chopped into pieces 2 to 3 cm in length then surface sterilized by immersion for 1 minute in $HgCl_2$ solution (0.25% w/v). The sections were transferred to fresh $HgCI_2$ solution (0.25% w/v) for another 1-minute period. After this treatment, the sections were washed twice by immersing in a large

amount of sterile distilled water for 1 minute using flame-sterilized forceps to handle the material. Following this treatment the sections were cut into small pieces and transferred to a Petri dish containing Dox agar medium. The cultures were maintained at 23 °C ± 0.5 °C in the dark for 3 to 4 days during which the plates were examined daily for the appearance of characteristic verticillate conidiophores. When the presence of *Verticillium* was indicated, a dilution series were prepared to produce a pure single-spore colony. These spore colonies were removed as appropriate.

2.3.4. Streaking technique (a method to obtain a pure culture).

A small portion of the fungus identified as *V. albo-atrum* was removed from plant tissue using a sterile loop. The inoculum was then streaked over Dox medium in a Petri dish. The plates were incubated for 4 days at 23 °C ± 0.5 °C in the dark and then examined for the presence of the fungus. Then a dilution series were prepared to obtain a pure-single colony.

2.3.5. Serial dilution.

Spores of colonies identified as *V. albo-atrum* on the basis of their conidiophore morphology were removed from the surface of a culture grown on Dox agar medium to a tube containing sterilized water, by means of a loop that was pre-wetted with sterile distilled water. Spores removed from the surface of mycelia were transferred from the loop by immersion in 5 ml sterile water. The spores were dispersed by gentle shaking and 1 ml of suspension was transferred with a sterile pipette to a second 5 ml aliquot of sterile water. From this aliquot 1 ml suspension was transferred to a third aliquot and the process was repeated until the 7th dilution. Each spore suspension was poured into a Petri dish containing 10 ml Dox agar medium, which was held at just above setting point. The medium was mixed and the dish was transferred to an incubator at 23 °C ± 0.5 °C in the dark. The cultures were observed daily for colonies of *V. albo-atrum*. Colonies that were identified as originating from a single spore were transferred to Dox and PDA for subsequent culture.

2.4. Histological analysis.

To examine and observe the changes in the internal structures or spread of mycelium in xylem tissues, 1-cm plant stems from tomato were cut by a razor and fixed overnight in 2.5% (v/v) gluteraldahyde (Agar Scientific, Stansted, UK) in 0.1 M phosphate buffer, pH 7.0, at 4 °C. Fixed tissues were washed with 0.1 M phosphate buffer at 4 °C, four changes were made over a period of an hour then rinsed with distilled water and dehydrated through a graded ethanol series in room temperature following by embedding in LR white medium grade resin series (London Resin Co., Reading, UK). Then samples were placed under a vacuum chamber for several hours at maximum vacuum and fixed in absolute resin over a night at 60 °C. Semi-thin (2 μm) sections were cut on glass knives, collected on glass slides and stained in 0.5% (w/v) cotton blue in lactophenol (2 min, 60 °C) prior to light microscopy.

2.5. Preparation of elicitor from *Verticillium*.

V. albo-atrum isolates from lucerne (V1) and from tomato (V2) were reisolated and grown on Dox medium as described previously. Elicitor from culture filtrate was prepared as follows: 1 cm^2 discs were cut from 4 week-old fungal cultures of isolates grown on Dox agar plate. 1 disc was transferred aseptically to 100 ml Czapex Dox liquid medium in a 250 ml conical flask and the flasks (25 for each isolate) were placed on an orbital shaker, (100 rpm), in the dark at 23 °C ± 2 °C for 6 weeks. After this time the contents of a number of flasks were combined to make a total of *ca.* 2.5 litres of culture. This was filtered through 2 layers of cheesecloth and the mycelium discarded. The filtrate was centrifuged at 10,000g for 20 min at 4 °C and kept overnight at –20 °C, then it was freeze-dried. The resulting residue was re-suspended in 200 ml distilled water and the suspension was dialysed exhaustively against distilled water at 4 °C. After the second freeze-drying the crude elicitor was re-dissolved in a known amount of distilled water and then centrifuged (20,000g, 20 min, 4 °C). The preparation was stored at –20 °C following assay of carbohydrate and protein content from 5 ml aliquots.

2.6. Determination of protein and carbohydrate.

2.6.1. Protein.

Two methods were used to determine the protein content of the samples, the Lowry and the Bradford methods.

(i) Lowry method (Lowry *et al.*, 1951);

The protein content of various samples was determined using the method of Lowry (Lowry *et al.*, 1951).

Preparation:

Solution A, 100 ml
0.5 g $CuSO_4.5H_2O$
1 g $Na_3C_6H_5O_7.2H_2O$ (Na-citrate).
Solution may be stored indefinitely at room temperature.

Solution B, 1 litre
2% Na_2CO_3 in 0.1 M NaOH
Solution may be stored indefinitely at room temperature.

Solution C, 51 ml
1 ml Solution A
50 ml Solution B

Solution D, 20 ml
20 ml Folin-Ciocalteu phenol reagent

Solution E, 50 ml
Bovine Serum Albumine (0.5 mg/ml)

5 ml of fresh mixed solution C was mixed with 1 ml of protein sample (containing between 0-500 μg protein). The mixture was incubated for 10 min at room temperature, and 0.25 ml of Folin-Ciocalteau reagent was added to the solution, which was mixed immediately. After a further 30 min, the absorbance at 500 nm was recorded. A calibration curve was constructed using bovine serum albumin (BSA, Sigma type V) as standard protein. The response was linear over the range 0 to 500 μg.

All protein concentrations from elicitor preparation were determined by this method.

(ii) Bradford method (Bradford, 1976);

Dye Reagent: 100 mg of Coomassie Brilliant Blue G-250 (Sigma) was dissolved by agitation in 50 ml of 95% ethanol then the solution was mixed with 100 ml of 85% w/v phosphoric acid (H_3PO_4) than it was diluted with distilled water to 1 litre and filtered. The reagent is stable at room temperature for at least 2 weeks.

Sample (100 µl containing 10-100 µg of protein) was mixed with 5 ml of Coomassie blue reagent. The absorbance (595 nm) was measured after 10 min and before 1h in 3 ml cuvette against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent.

A standard curve was prepared using Bovine Serum Albumin fraction V (Sigma) and measuring absorbance at 595 nm. The response was linear over the range 10 to 100 µg protein. All protein concentrations from lucerne suspension cells were determined by this method.

2.6.2. Carbohydrate.

The carbohydrate content was determined using the Phenol Sulphuric Acid method described by Hodge & Hofreiter (1962). A standard curve was prepared using D-Glucose over the range of 10-100 µg and distilled water was used as a control.

1 ml of 5% (w/v) phenol solution (Sigma) was added to 1 ml carbohydrate solution (containing 10-100 µg carbohydrate), the solution was mixed and 5 ml concentrated sulphuric acid (analytic reagent) was carefully added using a fast flowing pipette. After 10 minutes, the tubes were mixed thoroughly and placed in a water bath at 30 °C for 20 minutes after which time the absorbance at 490 nm was recorded. Glucose was used to prepare a standard curve. The amount of carbohydrate in a sample is expressed in glucose equivalents.

2.7. Effect of NaCl on lucerne and tomato.

2.7.1. Seed viability.

According to Moore, (1973), the reaction between tetrazolium and hydrogen atoms released by the dehydrogenase enzymes involved in the respiration of living tissues, results in the production of the water-insoluble, oil-soluble red pigment, formazan. The testing solution is prepared by dissolving 2% tetrazolium salt in distilled water, at a pH of approximately 6.5-7.0. Seeds were placed between two sheets of Whatman[1] No.2 filter paper to which 0.5 ml of distilled water was added. These were incubated at room temperature in the dark for 24 h. Each seed was then cut longitudinally and the halves were placed in a micro centrifuge tube to which 0.3 ml of 2% tetrazolium solution was added. The tubes were incubated at 25 °C ± 1 °C in the dark for 24 h. After this period the viability of each seed was scored. Ten seeds from lucerne and five from tomato were used for the viability of seeds.

*2.7.2. Evaluation of different lucerne (*M. sativa *L. &* M. media*) and tomato (*L. esculentum*) cultivars under different concentrations of NaCl during germination.*

The development of cultivars with the ability to germinate under high salt stress would be useful in the reclamation of saline soils. One of the major problems has been the development of uniform, repeatable methods for selecting for the ability to germinate under salt stress conditions. However, the most common selection technique was used involving, Whatman filter paper in closed containers and moistening with salt solution (Al-Niemi *et al.*, 1992), since the preliminary experiments with the other methods were either inconvenient for the screening of large number of seeds or carrying high risks for contamination during the experimental period. As Carlson *et al.* (1983) stated that blotter or filter paper are useful however, localised evaporation and seed positioning might change the microenvironment of the seeds but he did not comment about any other disadvantages using filter paper. If localised evaporation is reduced by a cling film over the Petri dishes, and seeds were positioned carefully, the disadvantages of using filter paper would be minimised.

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[1] The use of trade names in this document does not imply endorsement by the University of Wales, Swansea, nor criticism of similar ones not mentioned.

So, rapid screening techniques would be useful if the data would be used for further stages. Whatman filter paper was successfully used as saline medium to rapidly screen large numbers of lucerne or tomato seeds for ability to germinate under salt stress.

2.7.3. Effect of salinity on germination and seedling growth in Petri dishes.

(i) Lucerne;

Seeds of *M. sativa* from 18 cultivars and one cultivar from *M. media* (Table 2.7.1) were scarified with sandpaper and exposed to 0, 25, 50, 100, 150, 200, 250, 300 and 350 mM NaCl during germination using standard test procedures (Rumbaugh *et al.* 1993). A sample of seed from each cultivar was obtained from the originating company or from research institutes in different countries on the basis of contract. It is not known whether the environment during seed development affects the ability of the seed to germinate in saline solutions. Two replicates of 25 scarified seeds of each cultivar were placed in 100-mm glass Petri plates (surface sterilised with 95% ethanol) containing a single Whatman No. 2 filter paper. Then 4.5 ml of appropriate NaCl solution was added, and the plate was sprayed with 0.75 ml of 0.8% aqueous solution of a fungicide (0.006 g phenyl mercuric ammonium acetate per plate). Petri plates were sealed by cling film to prevent evaporation and they were stacked in twos in a dark growth chamber used as a germinator at 25 °C.

Germinated seeds were counted after 7 days. Germination in each plate was compared to the control group (0 mM NaCl), which was set as 100, and expressed as a percentage.

The experiment was conducted in a randomized complete block design with two replications. Data were analysed by analysis of variance procedures. The adjusted germination data also were analysed by regression (Bliss *et al.*, 1986a) and probit procedures, as suggested by Carlson *et al.* (1983). The IC_{25} and IC_{50} values were determined for each cultivar by probit analysis of the germination percentages, i.e., the IC_{50} value is the NaCl concentration in mM that inhibits germination of 50% of the viable seeds for a particular cultivar. The corresponding standard error was computed to provide a measure of precision.

The estimated inhibitory concentration of NaCl that prevents 50% of the seeds from germinating (IC_{50}) was estimated from the quadratic curves for each cultivar. Formulae used to calculate the IC_{25} and IC_{50} and approximation of its associated standard error are:

$Y = ax + b$ A typical linear regression equation

$Y = ax^2 + bx + c$ A typical quadratic equation

$$IC_{25} = \frac{-b - \sqrt{b^2 - 4a(c - 75)}}{2a}$$

$$IC_{50} = \frac{-b - \sqrt{b^2 - 4a(c - 50)}}{2a}$$

$$SE(IC_{value}) = \frac{SE(\frac{y}{IC_{value}})}{(b + 2aIC_{value})}$$

$IC_{value} = IC_{25}$ or IC_{50}

(ii) Tomato;

Same sterilisation and germination technique were used as in lucerne. However, amount of germination solution and germination time was twice as much of lucerne (Bradbeer, 1988). 20 seeds were plated per treatment and arranged as in lucerne germination experiment (Table 2.7.2).

Table 2.7.1. Source of lucerne seeds.

Country of origin	Name	Cultivar	Specifications
England	*M. sativa*	Euver[1]	moderately resistant to VW.
England	*M. sativa*	Europe[1]	moderately resistant to VW.
England	*M. sativa*	Vela[1]	moderately resistant to VW.
England	*M. sativa*	Kabul[1]	resistant to VW.
Canada	*M. media*	Rambler[1]	susceptible to VW, moderately tolerant to NaCl
France	*M. sativa*	Vertus[1]	response to salinity unknown, resistant to VW.
Turkey	*M. sativa*	Elci[2]	cold & drought tolerant and produces high yield, bred in Urfa-Turkey by Prof. Dr. Sehabettin Elci (1976).
Turkey	*M. sativa*	Peru[2]	grows well in the greenhouse, response to salinity and VW unknown.
Turkey	*M. sativa*	Bilensoy-80[2]	drought tolerant, response to salinity but resistant to the wilt fungus, bred in Turkey by N. Celalettin Bilensoy (1984).
Turkey	*M. sativa*	Mesa Sirsa[2]	naturally resistant to VW.
Turkey	*M. sativa*	Kayseri[2]	cold tolerant, response to salinity and VW unknown, bred in University of Ankara (16.5.1964).
Turkey	*M. sativa*	Bitlis[2]	response to salinity and VW unknown.
USA	*M. sativa*	13R Supreme[3]	salt tolerant and resistant to VW, anthracnose and aphids. Specially selected for lucerne growers in desert areas of USA.
USA	*M. sativa*	Redgreen[3]	response to salinity unknown but resistant to VW, *Phytophthora* and nematodes. It was specially selected to meet the demands of lucerne growers in the areas where semi-dormant types are most suited, especially Southern Europe.
USA	*M. sativa*	Protea[3]	response to salinity unknown but resistant to VW, FW and Bacterial wilt. It is a synthetic cultivar specially selected for lucerne growers in Southern Europe.
USA	*M. sativa*	Lobo[3]	response to salinity unknown but resistant to VW, FW and Bacterial wilt. It is a synthetic cultivar specially selected for lucerne growers in Southern Europe.
South Africa	*M. sativa*	SA Standard[4]	response to salinity and VW unknown.
Canada	*M. sativa*	Barrier[5]	response to salinity and VW unknown but resistant to VW.
Canada	*M. sativa*	AC Blue J[5]	response to salinity and VW unknown.

Table 2.7.1. (Continue).

Lucerne seeds were derived from;

[1]Murat Dikilitas, University of Wales, Swansea-UK, 1997.
[2]Assoc.Prof. Aziz Karakaya, University of Ankara, Ankara-Turkey, 1999.
[3]David Walker, ABI Alfalfa Inc., Cambridge-UK, 1999.
[4]Roger Bills, JLB Smith Institute of Ichthyology, Grahams Town-South Africa, 1999.
[5]Dr. Surya Acharya, Lethbridge Research Center, Alberta-Canada, 1999.

Sources; University of Ankara, Faculty of Agriculture/Turkey- 1990 (Prof. Dr. Sehabettin Elci). ABI Alfalfa Inc. 27.1.1999. National Alfalfa Variety Review Board. ABI Alfalfa Inc. 6700 Antioch, PO Box 2962. Shawnee Mission Kansas 66201-1362.

Table 2.7.2. Source of tomato cultivars used for salinity and the pathogenicity tests.

Country of origin	Name	Cultivar	Specifications
England	*L. esculentum*	Ailsa Craig[1]	Produces heavy crops, with medium sized fruit. Response to salinity unknown. Susceptible to VW.
England	*L. esculentum*	Cyclon Hybrid F1[1]	Naturally resistant to virus and VW and FW.
England	*L. esculentum*	Hybrid Sweet 100 F1[1]	Response to salinity and VW unknown.
Israel	*L. esculentum*	Margarita (Fa-558)[2]	Response to salinity and VW unknown.
Turkey	*L. esculentum*	Fantastic F1[2]	Response to salinity and VW unknown.
Turkey	*L. esculentum*	Falkon[2]	Response to salinity and VW unknown.
Turkey	*L. lycopersicon*	Tomato[2]	Response to salinity and VW unknown.
Turkey	*L. esculentum*	Simge F1[3]	It grows well in the greenhouse. Response to salinity and VW unknown.
Egypt	*L. esculentum*	Edcawy[4]	Salt tolerant, response to VW unknown.

Tomato seeds were derived from;
[1]Murat Dikilitas, University of Wales, Swansea-UK, 1997.
[2]Murat Dikilitas, Ankara/Adana-Turkey, 1998.
[3]Prof. Yuksel Tuzel, University of Ege, Izmir-Turkey, 1999.
[4]Dr. Helen Johnson, Univeristy of Aberyswyth, Aberyswyth-UK, 1999.

2.7.4. Effect of salinity on germination in soil conditions.

The effect of salinity on seedling emergence was conducted according to the method of Johnson (2000).

(i) Lucerne;

From the results of previous experiments (the effect of salinity on seed germination and seedling growth in Petri dishes) the effect of NaCl and various osmotic potentials on seed germination were assessed in five lucerne cultivars (13R Supreme, Vertus, Mesa Sirsa, Vela and Rambler) in seed trays, under greenhouse conditions.

Seed trays were filled with John Innes No. 1 compost to a weight of 500 g. A volume of 100 ml of either distilled H_2O or various concentrations of NaCl or Mannitol

was applied to the compost until the seed tray weight reached 600 g. A spray bottle was used, to ensure an even application of the solution (Table 2.7.3). NaCl concentrations chosen in this experiment were selected from the conclusion of germination experiments carried out in Petri dishes. All the chemicals used in this section were purchased from BDH (Merck Ltd, Merck House, Poole, Dorset-UK) and Fisher Chemicals (Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, Leics.-UK), unless otherwise stated.

A grid 10 x 10 mm was marked on the compost using a metal template and a single seed was sown into each square of the grid. Prior to sowing, the seeds were soaked in running distilled water in a filtration flask for overnight. Each seed was sown to a depth of 5 mm and was covered with the surrounding compost (25 seeds for each treatment was used). The seed trays were covered with black polyethylene bags and incubated in the greenhouse at an average temperature of 23 °C ± 1 °C. Each day the seed trays were weighed and the treatments were applied until the tray weight reached 600 g. The black polyethylene bags were removed when hypocotyl emergence was first observed. Emergence, the point at which 2 mm of the hypocotyl was visible above the soil surface, was scored daily. Percentage germination seeds was calculated over a 9-day period.

(ii) Tomato;

The same method was also used for germination of tomato seeds (20 seeds were used for each treatment). However, concentrations of NaCl or mannitol solutions were different from the solutions used for lucerne seeds (Table 2.7.3). Five tomato cultivars were assessed: Margarita (Fa-558), Fantastic F1, Ailsa Craig, Simge F1, and *L. lycopersicon.*

Table 2.7.3. The concentrations of salt or mannitol treatments and their osmotic potentials on lucerne or tomato seed germination.

NaCl		Mannitol*		Osmotic potential
(% wt/v)	(mM)	(% wt/wt)	(mM)	(MPa)
0.438	75	2.727	150	-0.36
0.585	100	3.644	200	-0.48
0.880	150	5.466	300	-0.72
1.168	200	7.26	400	-0.96

*non-ionic-penetrating substance.

The osmotic values of the germination media were measured as mmol kg^{-1} with a ChemLab Scientific Product Ltd. 5500 vapor pressure osmometer.

2.8. Root formation of the lucerne cultivars in saline medium.

In this experiment cultivars that showed tolerance or susceptibility to salt [Protea, 13R Supreme (USA), Bilensoy-80, Mesa Sirsa, Peru (Turkey) and Vertus (Europe)], were used in an experiment to test their ability to form roots in saline conditions. The aim of this experiment is also to confirm the results of previous experiments and observe the responses of the cultivars during rooting stage.

Cuttings were derived from parent (stock) plants that had been grown from seed. Shoots were cut 10 to 12 cm from the top of those plants and were individually transferred to 10-cm pots of perlite standing on Petri dishes (8 replicates per treatment). Rooting ability of the cultivars was tested under the stress of 0, 50, 100 and 150 mM NaCl containing A&H (Table 2.2.1) nutrient solution. Plants were flushed through with 100 ml of A&H solution every other day for 2 weeks. NaCl content of the nutrient solution was increased by increments of 50 mM daily until the required salinity of medium was reached. Watering was carried out by holding the plant pots above a bucket, slowly pouring over excess solution and allowing it to drain. By this method salt accumulation in the perlite was prevented.

The plants were grown in a growth room throughout the experiment with a photo flux density of photosynthetically active radiation (PAR) of 250 $\mu E\ m^{-2}\ s^{-1}$, relative humidity of 60%, temperature of 22 °C and a 16 photoperiod per day.

Beside the growth parameters, proline concentration in leaves was also measured.

2.9. Effect of temperature, NaCl, and various antifungal compounds on germination and growth of *V. albo-atrum.*

2.9.1. Effect of temperature on fungal growth of V. albo-atrum.

The flasks were inoculated with mycelial disk (0.5 cm) of the isolates V1 or V2 and incubated at 18, 22, 25 and 30 °C for 6 weeks in a rotary shaker (100 rpm/min) after that mycelial mats were harvested and mycelia weight in mg was recorded. 10 replicates were used for each treatment per isolate and the mean value was calculated.

2.9.2. Effect of NaCl on fungal growth of V. albo-atrum.

V. albo-atrum isolates from lucerne (V1) and from tomato (V2) were reisolated and grown on Dox medium as described previously in Materials and Methods.

1-cm diameter discs were cut from 4 week-old fungal cultures of isolates grown on Dox agar plate. 1 disc was transferred aseptically to 100 ml Czapex Dox liquid medium containing, 0, 25, 50, 100, 150 and 200 mM NaCl in a 250 ml conical flasks and the flasks (10 flasks for each treatment of each isolate) were placed on an orbital shaker, (100 rpm), in the dark at 23 °C ± 1 °C for 6 weeks.

After this time the contents of a number of flasks were combined to make a total of *ca.* 1 litre of culture. This was filtered through 2 layers of cheesecloth. Filtrate was used for elicitor assay (see section, 2.5) and the residue was washed with distilled water to remove residual solutes, and then dried in an oven at 60 °C for 24 h. The mycelial mats were removed from the oven and cooled before weighed. Fungal growth is expressed as mg dry weight/culture. 10 replicates were taken and the mean value was recorded.

2.9.3. Effect of NaCl on the linear growth of fungi.

Various concentrations of NaCl were tested for their effects on the radial growth and sporulation of the *V. albo-atrum*, isolates V1 and V2. Various concentrations of NaCl (0, 50, 100, 150, 200, 250, 300 and 350 mM) were incorporated with Dox medium content and autoclaved at 121 °C for 20 min (2.68 kg cm^{-2} pressure). The plates containing Dox medium at various NaCl concentrations were inoculated with mycelial

disks (3 mm) cut from the outer margin of 3 week-old cultures on Dox and the plates were then incubated at 23 °C ± 1 °C for 3 weeks. One disc was transferred aseptically to each plate and five replicates were used for each concentration and isolate. The linear growth of fungi was measured in mm diameter.

(i) Sporulation;

Sporulation was determined by pouring 10 ml of sterile water on to the surface of a culture, gently stroking the surface with a flame sterilized glass rod, and collecting the liquid containing spores and hyphal fragments in a sterile flask. Suspension was filtered through a Whatman No. 1 filter paper and counting was made with a neubauer haemacytometer. Five replicates were used for each concentration of each isolate.

2.9.4. *Viability of* V. albo-atrum, *isolates V1 or V2, in various concentrations of liquid Dox medium.*

1 x 10^5 conidia/ml of *V. albo-atrum*, isolates V1 or V2, were incubated at 23 °C ± 1 °C for 24 h in sterile boiling tubes containing 10 ml liquid Dox medium at different concentration of NaCl (0, 50, 100, 150, 200 and 250 mM). After the period of incubation, 100 µl of treated spores were smeared on sterile microscope slides and counted with a neubauer haemacytometer under a microscope. Spores germinated counted as alive.

2.9.5. *Selection procedures for salt-adapted fungal isolates.*

Initially, isolates V1 and V2 were grown on Dox medium containing 50 mM NaCl. Every 3 weeks, the cultures were transferred to medium in which the concentration of NaCl was increased by an increment of 50 mM. Each isolate was grown in this way until the NaCl concentration reached 150 mM. At this concentration, both isolates V1 and V2 were maintained for 5 months prior to the start of the experiment. Those isolates, which had been maintained on medium containing 150 mM NaCl are designated V1-150 and V2-150. A sample of each V1-150 and V2-150 was transferred to medium in which the NaCl concentration was increased to 200 mM. Isolates were then maintained on 200 mM NaCl for 2 months and are designated V1-200 and V2-200.

2.9.6. *Effects of hydrogen peroxide and purified phytoalexins on the germination of conidia and germ tube elongation of* V. albo-atrum *isolates.*

Conidial suspensions (10^5 spores ml^{-1}) of *V. albo-atrum* were prepared as described by Dikilitas (1997). The method of Lu & Higgins (1999) was adopted with slight modifications. A known quantity of H_2O_2 or phytoalexins (medicarpin and sativan) was added to 1 ml samples of the spore suspension in plastic centrifuge tubes and 100 µl of these treated spores were smeared on sterile micro cavity microscope slides (76 mm x 26 mm) which were incubated at 23 °C ± 1 °C for 24 h in Petri dish moisture chambers. Three replicates were used for each concentration per treatment per isolate. Spores were considered germinated when the length of the germ tube exceeded the diameter of spore. The percentage of spore germination was determined by counting the number of germinated spores in every 100 spores under a microscope. Three counts were made for each replication and the average was calculated.

In the case of phytoalexins; the residue of purified phytoalexins, medicarpin (0.09 mg/ml) and sativan (0.12 mg/ml), were re-dissolved in 1 ml of 5% v/v ethanol. An equivalent of sterile-distilled water and 5% v/v ethanol were used for H_2O_2- and phytoalexins-treated groups, respectively.

2.9.7. *Effects of hydrogen peroxide and purified phytoalexins on hyphal growth of germ tubes of* V. albo-atrum.

The effect of various concentrations of H_2O_2 and phytoalexins (medicarpin and sativan) on germ tube growth of *V. albo-atrum* was tested on slides with untreated spores previously germinated for 24 h as described above. A known quantity of H_2O_2 or phytoalexins (medicarpin and sativan) or 50 µl H_2O was added to the 50 µl of spore suspension on sterile micro cavity microscope slides and was well mixed with the germinating spores by gently rotating the slides. The spores were incubated for a further 18 h and the lengths of the germ tubes were measured microscopically. The average length of germ tubes measured just before the treatment was used to determine the germ tube length at the time of treatment (Lu & Higgins, 1999). After the period of incubation the average length of germ tubes (100 germ tubes per replicate) were determined using an ocular micrometer and from these values a mean germ tube length was calculated.

2.10. Effect of various NaCl concentrations on disease severity of tomato plants inoculated with *V. albo-atrum.*

The resistance of 4- (young) and 8-week (mature) old tomato plants that were inoculated with *V. albo-atrum*, isolate V2 was assessed under saline conditions. The seeds of cv. Ailsa Craig was germinated as described before and transferred individually to 15-cm pots of perlite and Levington's Universal Compost mixture (4:1). The surface of the pots was covered with vermiculate to keep the moist in pots thus preventing from drought.

The plants which were inoculated with V2 or used as controls were watered with 1/5 A&H solution for 3 days before the salinity treatment started. After this period both inoculated and non-inoculated tomato plants were treated with various concentrations of NaCl. The plants were watered every other day (250 ml) with 1/5 A&H solution, containing different concentrations of NaCl (0, 25, 50, 100, 150 and 200 mM). The concentration of NaCl in the culture solution was increased by increments of 50 mM NaCl until the required salinity of the medium was reached. Watering was carried out as described before. Two control groups consisting of NaCl- and non NaCl-treated groups (only tap water used) were set up for the inoculated plants. The plants were maintained in the greenhouse and were harvested after a total of 5 or 8 weeks depending on the experimental procedure.

For each NaCl concentration 10 l of stock solutions were prepared.

2.10.1. The drip irrigation system.

Human beings must increase and extend the growing period of agricultural crops to compensate for the increase in world population. However, many factors, environmental and biological, limit the crop productivity. In many countries regenerated or modified plants for the resistance to stress are now available to increase the crop production even in marginal lands. Although these developments are promising only one factor is considered to be important when plants are developed against stress. However, many stress factors can deteriorate the development of plants by acting synergistically. For example, irrigated lands may be exposed to salinity by many reasons in the near future therefore salt-tolerant plants, which tolerate the high levels of salt, may be of economic importance. However, the stress factors, other than the salinity, may still have

the potential damage on those plants that tolerate the effect of salt. If one of those factors is considered to be a plant pathogen, which has always limitation on economic crops, this means that the problem is not solved yet. Since a living organism is involved, adaptation or in a long term, evolution of the pathogen may create bigger problems for the agricultural lands or in a short term the toleration of the level of salt by regenerated plants might be less than that of the plant pathogen.

So, to evaluate the problem in detail and assess the each stress factor for commercial crops, a drip irrigation system was used. In this system, both water and nutrients can be delivered to the crop as required.

For this project, valuable information was obtained from a research group, Prof. Mike Hall, Dr. A.R. Smith and a Research Assistant Helen Johnson, in the Biological Sciences Department in Aberystwyth University. As a result of this research, preliminary plans were prepared for the development of an open drip irrigation system. The system was designed for the purpose of assessing plant, pathogen and salt interactions. Unless otherwise stated all equipment required for the system was obtained from Aquaplast Irrigation Co. (26 Penketh Place, Skelmersdale, Lancashire and B & Q, Swansea).

The pre-diluted nutrient solution containing NaCl or not was pumped from two central tanks (Aqua Butt, Sankey Home & Garden Products, Nottingham) via control valves and filtered to the main pipes. Each pipe has 112 drippers arranged at set, intervals in groups of four. It is recommended by the Irrigation Company that two drippers should be assigned to one plant; one of them went into the pot and the other went into between pots and troughs (each dripper has the capacity to deliver 2 l h^{-1}). After calibration, each dripper delivered 12 ml nutrient solution/min in four different time scale (morning, mid morning, noon and evening, total 22 min irrigation per day), therefore, 264 ml were delivered to the troughs from each dripper. The scheduling of drip irrigation was programmed by a digital timer (Smith Industries Plc). After each irrigation period, water run-through was observed under the troughs. The same timer controlled the two hydroponic systems, so ensuring simultaneous irrigation.

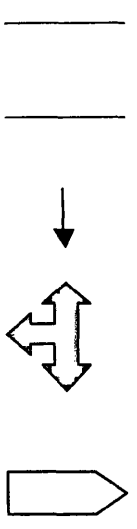
This system has the capacity to grow 56 plants per pipe holding 20 plants that were inoculated with V1, 16 with V2 and 20 as control. Plants were grown in raised troughs filled with perlite (B & Q, Swansea) as this allowed easy drainage by means of a

gutter and prevented cross-contamination between control and salt solutions. Four plants were placed in each trough (dimensions 40x76x26 cm) with holding approximately 80 l of medium perlite. To prevent direct light to the root zone and to minimise algal growth and evaporation, each trough was covered with black polyethylene. The entire system described above was illustrated in Plate 2.3.

Prior to transplanting of the plants into the hydroponic system, the perlite in the troughs was irrigated with water until run-through was observed.

6-week old tomato plants (cv. Ailsa Craig) were inoculated with *V. albo-atrum*, isolates V1 or V2. Before inoculation the roots of the plants were washed under tap water to remove excess soil. Inoculated plants were transferred individually to the pots (15 cm) of perlite that was placed in the troughs. At the time of transplantation a sample of 5 plants was harvested and the growth parameters including leaflet surface area, stem and root length and their dry weights were recorded. The initial harvest provided a standard degree of variation within the sample population. Plants were left to settle to establish the disease development for couple of days prior to the application of salt treatment. After this period, groups of plants that were inoculated with V1 or V2 were irrigated either with the nutrient solution containing 50 mM NaCl or with the nutrient solution alone. The plants were initially supported using wooden canes. As they grew, each plant was supported by a piece of string attached to long & big wooden canes.

Symbols used in drip-irrigation system



Drip irrigation feed pipes

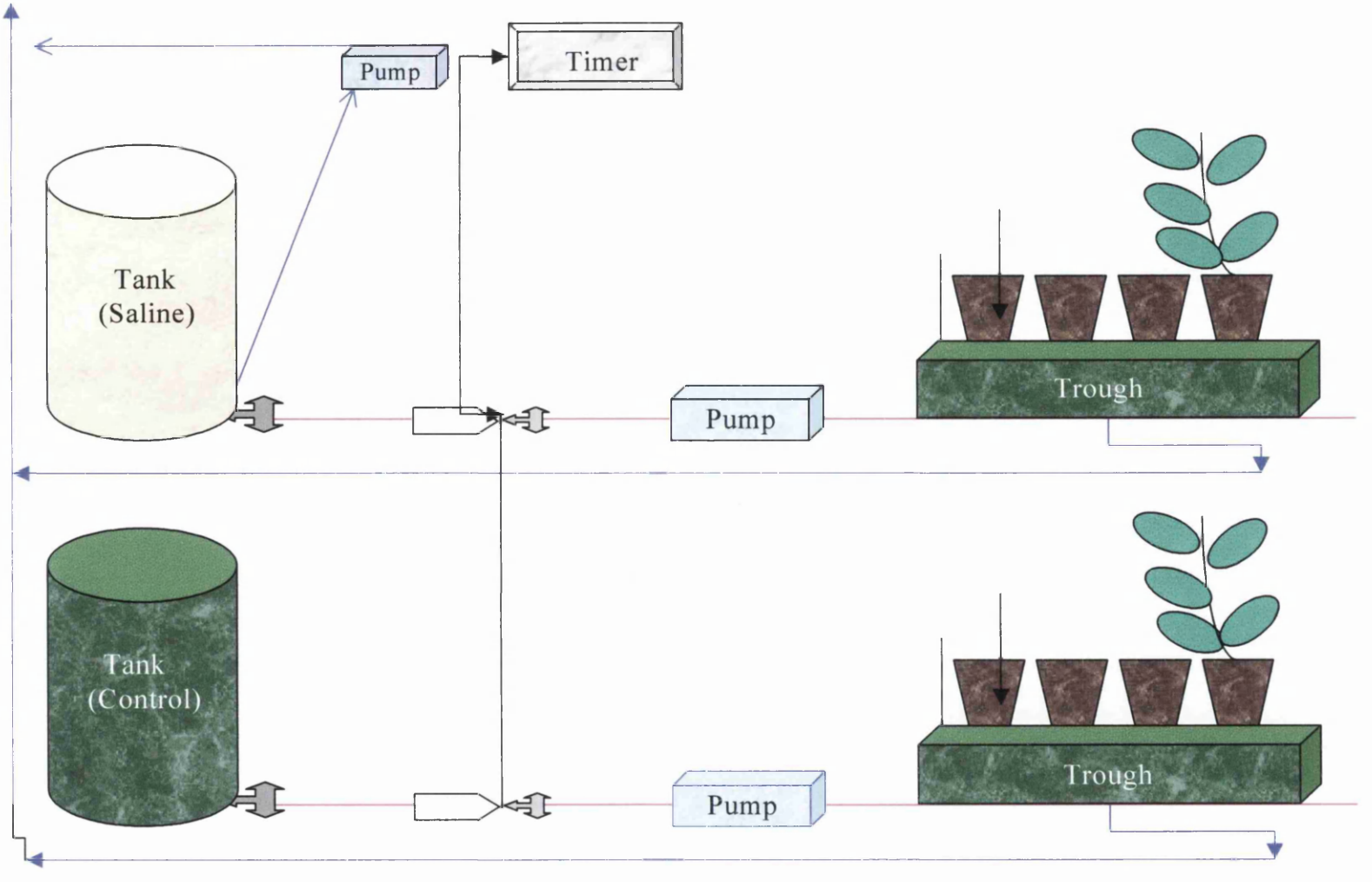
Drip irrigation waste pipes

Arrow head dripper

Valve

Filter

Plate 2.3. Schematic diagram of drip irrigation system.



2.10.2. Medium culture of drip irrigation hydroponic system.

The 'Solufeed F' culture medium was used in a hydroponic culture nutrient system. The Solufeed F (Table 2.10.1) is considered to provide a balanced and easy to manage nutrient system for commercial crop production in perlite etc. and Nutrient Film Technique (personal communication with Mr. Dick Holden, Solufeed Ltd., Saffron Walden, Norfolk House-Essex).

Table 2.10.1. Contents of Solufeed F (Analysis %).

Compound	Concentration
Total (N)	8.7
Nitric nitrogen	8.7
Phosphorus Pentoxide	6.8 (3 % P)
Potassium oxide	37.3 (30.8% K)
Magnesium oxide	4.6 (2.8% Mg)
Boron	0.024
Copper chelated by EDTA	0.016
Iron chelated by EDTA	0.088
Manganese chelated by EDTA	0.052
Molybdenum	0.004
Zinc chelated by EDTA	0.012

The stock solution of Solufeed F;
The method of use is to prepare 10% Solufeed F stock solution to be diluted according to crop requirements, normally 1:100 (final concentration 0.1%).

All chemicals with the exception of the nutrient feed were purchased from Fisher Scientific UK (Bishop Meadow Road, Loughborough, Leicestershire, UK). Stock solutions of Solufeed F and calcium nitrate [$Ca(NO_3)_2.4H_20$] were prepared. It was advised that Solufeed F and calcium nitrate mixture caused pellets in the nutrient solution (personal communication with Dr. Helen Johnson), therefore, the stock-solutions were added to the tanks separately. The concentration of calcium nitrate to be added to the feed tanks was calculated as described in the Solufeed manual, taking into account the calcium concentration in the mains water supply (20 ppm Ca, consultation with Welsh

Water Co.). This resulted in a stock solution to be added extra 80 ppm Ca. In addition to those chemicals, 50 ppm potassium nitrate was also added to the nutrient solution.

The appropriate weight of NaCl was pre-diluted in a beaker then it was completely dissolved in the tank with tap water to give the concentration of 50 mM. The nutrient stocks and the NaCl were added to the tanks when they were re-filled.

2.10.3. Monitoring of the nutrient solution.

The pH (Hanna Instruments, Woonsocket, RI, USA 02895) and EC (electrical conductivity) meter (Milwaukee, Australia) were used regularly throughout the experimental period. It was aimed to maintain pH accordance with the criteria described in Solufeed manual. With the help of the EC meter, it was possible to check whether excess salt was building-up.

A hydroponic system is a suitable testing system to compare individual and combined effect of the fungus and salt. So, when the plants were watered by salt solution no salt accumulation was detected at the bottom of the plant root area. So any toxic and physiological effects of salt concentrations applied on plants were observed accurately.

As a result of communications and preliminary experiments four tomato cultivars were selected for plant, salt & pathogen interactions study. Fantastic F1 and Margarita (Fa-558) were from Israel, Simge F1 and Ailsa Craig were supplied from Turkey and England, respectively. Although Edcawy was known to have salt tolerance (Mahmoud *et al.*, 1986a. & 1986b), because of limited seed stock, this cultivar could not be tested for plant & pathogen & salt interactions in the hydroponic system.

At 7 d intervals, plant height and symptom index were taken and in the 4^{th} week, four representative plants were selected randomly from each treatment to screen the salt and pathogen interaction in the early stage. Total harvest was made in the final week (7^{th} week). Leaf area, fresh and dry weights of stem and roots, chlorophyll contents of the leaves were recorded both in the middle and at the end of the experimental trial.

2.11. Harvesting plants.

At the start of the experiment five or ten representative plants of each cultivar were harvested, dried for a period of 3 days in an oven at 60 °C and the initial fresh and dry weights were calculated as follows:

$$Fwt_{initial}\ \text{plant} = Fwt_{initial}\ \text{shoot} + Fwt_{initial}\ \text{root}$$

$$Dwt_{initial}\ \text{plant} = Dwt_{initial}\ \text{shoot} + Dwt_{initial}\ \text{root}$$

$$Fwt_{final}\ \text{plant} = Fwt_{final}\ \text{shoot} + Fwt_{final}\ \text{root}$$

$$Dwt_{final}\ \text{plant} = Dwt_{final}\ \text{shoot} + Dwt_{final}\ \text{root}$$

During or after the experimental trial, some leaf samples were collected for chlorophyll and other laboratory determinations, the remaining fresh weight from each cultivar was recorded and the plants were then dried for a period of 3 days in an oven at 60 °C. These were then re-weighed to find the dry weight.

(i) The relative growth rate per week, RGR ($week^{-1}$);

$$RGR\,(week^{-1}) = \frac{[\ln(Dwt_{final}) - \ln(Mean\ Dwt_{initial})]}{\text{Weeks}}$$

$$Index\ of\ RGR = \frac{RGR_{Treatment}}{Mean\ RGR_{Control}}$$

(ii) Relative rate of height increase (index of height);

$$True\ height = Final\ height\ of\ shoot - Mean\ of\ initial\ height\ of\ shoot$$

$$Index\ of\ height = \frac{True\ \ height_{Treatment}}{Mean\ of\ True\ height_{Control}}$$

(iii) Relative rate of root length increase (Index of root length);

$$True\ root\ length = Final\ \ root\ length - Mean\ \ of\ initial\ root\ length$$

$$Index\ of\ root = \frac{True\ root\ lenght_{Treatment}}{Mean\ of\ True\ root\ length_{Control}}$$

(iv) Measuring leaf area;

Tomato leaf area was determined from its weight using a calibration curve (Fig. 2.11.1) constructed in the following way; about seventy five leaves were harvested from the appropriate tomato plants and each was placed and spread onto the plate of a photocopier. A copy of each leaf was made and the image of each leaf was cut out and weighed. In addition, the Fwt of each leaf was recorded.

The weight of the image of each leaf was converted to area using the information that for the paper used in the copying process, each sheet (21- x 29.5 cm) weighed 5.36 g, giving a ratio of 115.587 cm^2: 1 g.

So,

If the image of an individual leaf weighed 0.120 g. then, the total leaf area for one surface would be 1382 mm^2, for both leaf surfaces it would be 2764 mm^2.

Using this data, a calibration curve was constructed in which the area of each leaf, calibrated from its photocopied image, was plotted against its Fwt. This process was followed for leaves from 9-10 week-old- and 13-15 week old plants. In both cases, the plot was a straight line (r=0.96 and 0.99 respectively), indicating a linear relationship between area and Fwt area across a wide range of leaf size.

Consequently, Fwt was regarded as a good indicator of leaf area and the conversion was achieved using x and c determined from the two plots for young (9-10 weeks old) and mature (13-15 weeks old) tomato plants (Fig. 2.11.1) in the following formula;

Calculation of Leaf Area (LA);

- for young tomato plants;

$$LA = 81.967 \text{ x } \frac{LW}{Number\ of\ leaf} + 4.2879$$

- for mature tomato plants;

$$LA = 51.711 \text{ x } \frac{LW}{Number\ of\ leaf} + 6.3029$$

$$Index\ of\ LA = \frac{LA_{Treatment}}{Mean\ of\ LA_{Control}}$$

(v) Net Assimilation Rate (NAR): $g.cm^{-2}.week^{-1}$.

$$NAR = \frac{{}_2W - {}_1W}{Weeks} \text{ x } \frac{[\ln({}_2LA) - \ln({}_1LA)]}{{}_2LA - {}_1LA}$$

$$Index\ of\ NAR = \frac{NAR_{Treatment}}{Mean\ of\ NAR_{Control}}$$

${}_1W$; initial dry of a plant, ${}_2W$; final dry weight of a plant, ${}_1LA$; initial leaf area of a plant, ${}_2LA$; final leaf area of a plant, LW; leaf weight.

(vi) The percentage of water content (WTC) in a plant;

$$\%WTC = 100 - 100 \text{ x } \frac{Dwt}{Fwt}$$

Computation of an index of RGR, height, root length, chlorophyll content, Net Assimilation Rate etc. is a useful criteria to compare the different cultivars under the

stress of NaCl or a pathogen or both. The index for height, root length, RGR, NAR etc. was calculated for each cultivar as a proportion of the mean of the same cultivar grown in unstressed conditions and the comparison was made between treatments and control groups within each cultivar. The index figures were used in graphs and statistical analysis to minimize the calculation error when statistical analysis was made.

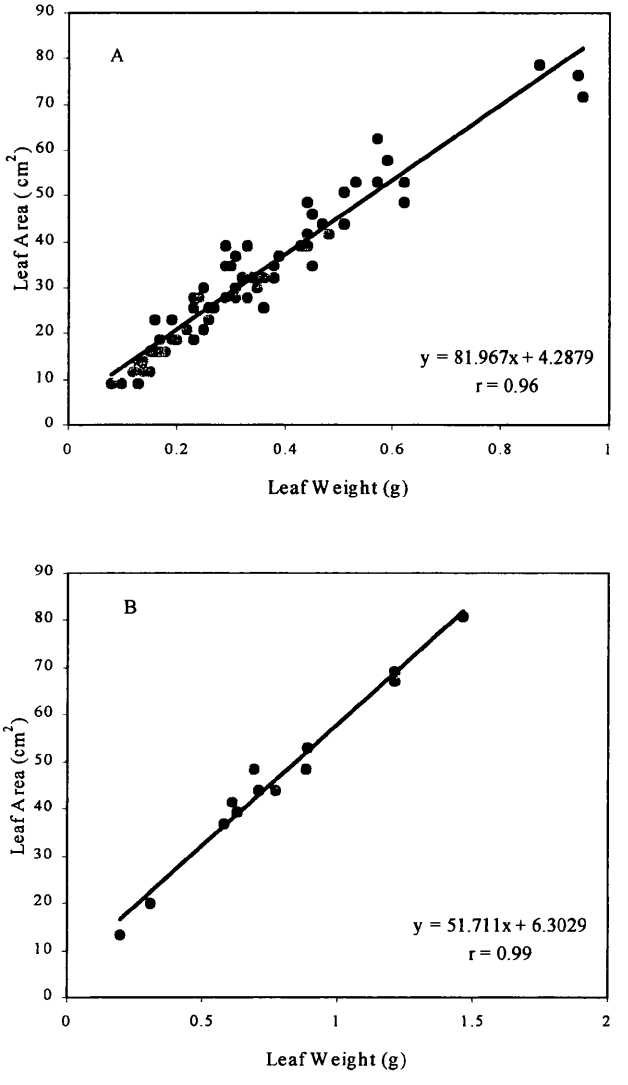


Fig. 2.11.1. Calibration between leaf weight and area. (A) young- (n=62); (B) mature-tomato plants (n=13).

2.11.1. Spectrophotometric quantitation of chlorophyll.

The procedure for determination of chlorophyll was conducted according to Arnon (1949). At the time of harvest, a sample (2 g) of leaf sample was placed into small glass tubes with 5 ml acetone:water (80% (v/v)) and covered with aluminium foil to prevent access light and placed in the fridge until use. Subsequently, a further 10 ml aliquot of aqueous acetone (80%, v/v) was added to each leaf sample, which was homogenized for 5 min with a Polytron Homogeniser, by which time all the chlorophyll had been released. The extract was filtered on a sintered glass funnel, washing with 5 ml acetone-water mixture. The extracts and washings were made up to 15 ml volume and stored in a refrigerator. Chlorophyll determination, after appropriate further dilution, if necessary, was made in a UV-visible spectrophotometer at 663.5 nm for Chlorophyll *a* and 645 nm for Chlorophyll *b* against an acetone (80%) blank. The results were expressed mg/l on a fresh or dry weight basis.

Calculation of chlorophyll concentration in acetone extracts;

$$\textit{Total}\ \text{chlorophyll (mg/l)} = 20.2A_{645} + 8.02A_{663.5}$$

$$\textit{C}\text{hlorophyll}\ \textit{a}\ \text{(mg/l)} = 12.7A_{663.5} - 2.69A_{645}$$

$$\textit{C}\text{hlorophyll}\ \textit{b}\ \text{(mg/l)} = 22.9A_{645} - 4.68A_{663.5}$$

2.11.2. Determination of proline.

Proline extraction and estimation were conducted according to Bates *et al.* (1973). Acid-ninhydrin was used as a reagent. The reagent was made by dissolving (warming and agitating) 1.25g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid (the reagent remains stable for 24 hours at 4 °C). After weighing, the leaves were frozen in liquid nitrogen and crushed immediately in a mortar and homogenized in 10ml of 3 %(w/v) aqueous sulphosalicylic acid. The homogenate was filtered through Whatman No. 2 filter paper. Two ml of filtrate was mixed with 2 ml acid ninhydrin in a test tube and boiled at 100 °C for 1 hour, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 5 ml toluene, the tubes were shaken thoroughly for 15-20 seconds and left for 20 min for separation of the two layers. The chromophore containing toluene was removed and allowed to warm to room temperature.

The absorbance was measured in a spectrophotometer (UV-1601-Shimadzu) at 515 nm against a toluene blank. The assay was calibrated with standard solution of L-Proline. The proline concentration was determined from a standard curve and expressed in μmol/g on a fresh weight basis.

2.12. *In vitro* studies with lucerne.

2.12.1. Development and maintenance of callus cultures.

Callus was initiated from leaves of lucerne plants, grown in a glasshouse or growth room under fluorescent tubes supplemented by mercury vapour lamps with 16 hours photoperiod and flux density of 110 μE m^{-2} s^{-1} at 23 °C ± 2 °C and 60-70% relative humidity, in the following way. Young terminal leaflets were excised from the plants and transferred into 100 ml conical flasks containing 50 ml of 5% bleach solution (1% hypochlorite solution) with 2-3 drops of washing up liquids. The flasks were plugged with a foam bung and were shaken gently for 5 min. Leaves were then rinsed with sterilised water (4 x 20 ml) to remove any traces of sterilising solution and the edge of the leaves were cut with scalpel to initiate the growth of callus before aseptic transfer onto M&S (Murashige & Skoog, 1962) solid medium in Petri dishes, Table 2.12.1. Cultures were then developed at 23 °C ± 2 °C in the dark. Callus cultures were subcultured every 6 weeks to maintain fresh callus stock.

2.12.2. Development and maintenance of cell suspension culture.

Liquid suspension cultures were derived from 4 week-old callus culture. Approximately 1 cm^3 of callus was transferred aseptically into 75 ml M&S liquid medium in a 250 ml conical flask and the flask incubated on an orbital shaker (100 rpm) at 23 °C ± 2 °C in the dark. Once established, this initial culture was subcultured after 4 weeks by transferring 25 ml of cell suspension and microcalli into 50 ml fresh M&S liquid medium. Once the culture was fully established it was routinely subcultured every 2 or 3 weeks.

2.12.3. The culture media.

- Preparation of cell suspension media;

A modified M&S medium was used for the induction and maintenance of callus and cell suspension cultures. The detailed composition of the medium is presented in Table 2.12.1.

Table 2.12.1. The composition of M&S medium for cell cultures of *M. sativa.*

1.	**Macronutrients**	mg/l	**3.**	**Iron Source**	mg/l
	NH_4NO_3	1650		Na_2EDTA*	74.6
	$CaCl_2.2H_2O$	440		$FeSO_4.7H_2O$	27.8
	$MgSO_4.7H_2O$	370			
	KH_2PO_4	170	**4.**	**Vitamins & Glycine**	mg/l
	KNO_3	1900		Thiamine HCl	10
				Nicotinic acid	5
2.	**Micronutrients**	mg/l		Pyridoxine HCl	10
	H_3BO_3	6.20		Glycine	2
	$MnSO_4.4H_2O$	22.30			
	$ZnSO_4.7H_2O$	10.60	**5.**	**Growth Regulators**	mg/l
	KI	0.83		Kinetin	0.25
	$Na_2MoO_42H_2O$	0.25		2,4-D**	2.00
	$CuSO_4.5H_2O^+$	0.025			
	$CoCl_2.6H_2O^+$	0.025			

$^+$Cu+Co can be made up as stock solution then added to micronurtients stock.

$^+CuSO_45H_2O$ 0.25 g/100 ml

$^+CoCl_26H_2O$ 0.25 g/100 ml

*Disodium ethylene diamine tetraacetic acid

** 2,4-D Dichloro phenoxyacetic acid

Media stock solutions were made up as follows:-

Stock Solutions;

Macronutrients and iron source; stock solutions were made at a concentration 10 times that of the final medium. Chemicals were dissolved in a 1000 ml volumetric flask using distilled water and stored at 4 °C.

Micronutrients and vitamins; stock solutions were prepared at 100 times of the final medium strength. Micronutrient stock solutions were stored at 4 °C while vitamins were dispensed in 10 ml aliquots and stored at –20 °C.

Growth regulators;

Auxin: 50 mg 2,4-D was dissolved in 2.5 ml ethanol in a warm water bath, gradually diluted to 100 ml with distilled water and stored at room temperature.

Kinetin: Kinetin stock solution was prepared by dissolving 25 mg Kinetin in 2 ml of 0.2 N HCl, warmed in a water bath, slowly diluted to 100 ml with distilled water and stored in a refrigerator at 4 °C in the dark.

Ingredients for preparation of 1 litre medium;

Macronutrients	100 ml
Iron source	100 ml
Micronutrients	10 ml
2,4-D	4 ml
Kinetin	1 ml
Vitamins & glycine	10 ml
Myo-inositol	0.1g
Sucrose	30.0 g
Ceasin hydrolysate	2.0 g
Agar (Difco Bacto)	7.5g

The components were added to 400 ml distilled water, mixed and the solution made up to 990 ml with distilled water. The pH was adjusted to 5.8 and 5.9 with 0.2 N KOH and the volume made up to 1 litre. For liquid medium, 75 ml of medium was dispensed into a 250 ml conical flask. In the case of salt containing medium, desired

concentrations of salt were added and the medium was dispensed in to the flasks as stated above. The flasks were plugged with foam bungs and covered with tin aluminium caps. For agar supported medium the nutrient solution was transferred into a 2 litre conical flask and agar was added accordingly. To dissolve the agar completely and uniformly, the flask was heated and stirred on a magnetic stirrer for 15 minutes. Then the medium was autoclaved at 121 °C for 20 min (2.68 kg cm^{-2} pressure). The cooled flasks were either inoculated or stored at 4 °C. In the case of agar containing flasks, the medium was dispensed into Petri dishes (M&S solid medium) and stored at 4 °C for further use.

2.12.4. Selection Procedure for salt tolerant cell lines.

After the pathogenicity experiments with *V. albo-atrum* on lucerne plants, it was decided that cv. Vertus or Kabul should be used for the selection of salt tolerance ability. A multi-step procedure was used for selecting salt-tolerant cell lines, by transferring the suspension cells to successively higher concentrations of NaCl as used by Chaudhary (1996) with minor modifications (not only the cells but the medium from the previous culture were also transferred to a higher NaCl concentration containing medium) to produce salt-tolerant cultures of alfalfa. Sterilization of the leaves was carried out as in section 2.12.1. The way of maintenance and subculture of cell suspension culture was prepared as described previously with the exception that cultures were illuminated under 50-60 $\mu E\ m^{-2}s^{-1}$ for 16 h photoperiod by cool fluorescent lights.

Approximately 1-2 g cells were inoculated into 75 ml of medium containing 25 mM NaCl to produce a culture containing 25 mM NaCl. After one month, the suspension cultures were subcultured into a medium of 50 mM and higher salinity (75 and 100 mM). Because of the limited time, only 50 mM NaCl-adapted cell lines (6 months) were used to produce regenerated salt tolerant strains.

2.12.5. Preparation of regeneration medium.

The cell cultures were adapted to 50 mM NaCl (approx. 6 months) then they were transferred to hormone-free BOi2Y regeneration medium, Table 2.12.2, (Bingham *et al.*, 1975). Microcalli and sedimented cells from suspension cultures were transferred to the regeneration medium by means of a fine stainless-steel mesh scoop with very little carry-

over liquid medium. During the first month on the regeneration medium, green embryoids started to develop on the surface of the starting callus. These were then subcultured onto a fresh BOi2Y medium to initiate the growth of the embryoids into small plantlets. When the plantlets were 3 cm high and had well-developed roots, they were transferred to John Innes No. 1 potting compost in small pots. The pots were covered with transparent plastic domes to protect the plantlets from desiccation and were placed in trays filled with water to a dept of 3 cm. When the plants showed significant growth, the plastic domes were removed and the plants were placed in a mist propagator for several days before being transferred to the greenhouse bench. The plants, which were generated from salt adapted cells, were watered with salt solution to which they were adapted. The regenerated and control plants were propagated by cuttings as described in section 2.2. The temperature of the heated greenhouse was regulated to about 23 °C. These clones were referred to as NaCl-selected lines.

Stock solutions were prepared as in M&S liquid culture medium.

Ingredients for preparation of 1 litre medium;

Macronutrients	100 ml
Iron source	100 ml
Micronutrients	10 ml
Vitamins & glycine	10 ml
Myo-inositol	0.1 g
Sucrose	30.0 g
Yeast extract	2.0 g
Agar (Difco Bacto)	10.0 g

The ingredients were made up to 1 litre with distilled water. The pH of the medium was adjusted to 5.9. The medium was dispensed into honey jars or small bottles and sterilised in an autoclave at 121 °C for 20 min (2.68 kg cm^{-2} pressure).

Table 2.12.2. The composition of BOi2Y regeneration medium.

1. **Macronutrients**	mg/l	3. **Iron Source**	mg/l
NH_4NO_3	1000	Ferric EDTA*	32
$Ca(NO_3)_2.4H_2O$	347		
$MgSO_4.7H_2O$	35	4. **Vitamins & Glycine**	mg/l
KH_2PO_4	350	Thiamine HCl	0.1
KNO_3	1000	Nicotinic acid	0.5
KCl	65	Pyridoxine HCl	0.1
		Glycine	2
2. **Micronutrients**	mg/l		
H_3BO_3	1.6		
$MnSO_4.4H_2O$	4.4		
$ZnSO_4.7H_2O$	1.5		
KI	0.8		

* Ferric Ethylene diamine tetraacetic acid

2.13. Histochemical staining.

2.13.1. Evans Blue procedure for quantitation of dead cells.

Cell death was determined using Evans Blue as described by (Levine *et al.*, 1994). The selective staining of dead cells with Evans Blue depends on the exclusion of this pigment from living cells by the intact plasmalemma, whereas it passes through the damaged plasmalemma of dead cells and accumulates as a blue protoplasmic stain (Turner and Novacky, 1974).

Treated and untreated cells, described above, were incubated for 15 min with 0.05% Evans Blue and then washed extensively to remove excess and unbound dye by centrifuging (1000g, 5 min). Dye bound to dead cells (0.5 g) were solubilized in 5 ml 50% methanol with 1% SDS for 30 min at 50 °C. The resulting solution was then cooled

and then the absorbance of supernatant was recorded at 600 nm. All data are either duplicates or more.

2.13.2. FDA stains of living cells;

Fluorescene diacetate (FDA) staining method was used following the methods of Larkin (1976) with some modifications. Live cells absorb FDA and consequently fluoresce when exposed to 300-500 nm. FDA was stored in an acetone stock solution (10 mg/ml in acetone) at 0 °C. It was added to the cell sample to give a final concentration of 0.05%. After 15 min incubation at room temperature, the cells were washed with distilled water to remove surplus FDA, they were then transferred to a slide and covered with slip. A fluorescence microscope with an epifluorescence condenser (Zeiss photomicroscope, Germany) was used to count the living cells. Viable cells were recognised by their fluorescence however, non-fluorescing ones were judged to be nonviable.

2.14. Assay of PAL activity from cell cultures.

-Cell Suspension;

To assess the early defense response of lucerne cells exposed to NaCl, V2 elicitor or both, PAL activity assay was performed. Cell suspension culture was used for the assay, 6 days after subculture, at a time when endogenous PAL activity was minimal (Little, 1989). The required quantity of test compound, never exceeded 2 ml, was added to the culture from a sterile syringe via a sterile membrane filter. An equivalent volume of water was used as a control. Each culture was then incubated on an orbital shaker (100 rpm) for 4 hours at 25 °C in the dark, for analysis of PAL activity. After the incubation, cells of lucerne were recovered from suspension cultures by filtration and stored in liquid nitrogen prior to extraction. The frozen cells were weighed before being transferred to a pre-chilled mortar, and homogenized (1ml buffer per 2 g fresh weight of cells) in 50 mM Tris-HCl, pH 8.4, containing 4 mM Na_2EDTA, 10 mM mercaptoethanol, 2 mM ascorbic acid and 1 mM PMSF, with a pestle with the aid of some acid-washed sand. The homogenate was filtered through two layers of wet Mira Cloth (Calbiochem), and the resulting filtrate was centrifuged (20,000 g, for 20 min at 4 °C). The supernatant was

dialyzed overnight against 2 litres of dialysis buffer (50 mM Tris-HCl, pH 8.4 containing 10 mM mercaptoethanol, 4 mM Na_2EDTA and 0.5 mM PMSF). The protein concentration was determined in a Coomassie-blue dye-binding assay using the Bradford method (1976).

PAL activity was determined (Bolwell *et al.*, 1985) by measuring, at 30 min intervals, the increase of absorbance at 290 nm of reaction mixtures. Incubation mixtures (3 ml) contained 1.5 ml of 50 mM Tris-HCl buffer (pH 8.4) containing, 4 mM Na_2EDTA, 10mM mercaptoethanol, 5 mM ascorbic acid and 1μM PMSF and 1 ml of 10mM L-phenylalanine (final concentration). The reaction was started by addition of enzyme extract (0.5 ml) containing 0.4 to 1.0 mg protein to the mixture, which was incubated at 40 °C for 2 hours. Assays were performed in duplicate and the control incubation was prepared using D-phenylalanine (10 mM final concentration) in place of the L-isomer.

Activities have been calculated from the molar absorption coefficient of cinnamic acid at 290 nm, which was determined to be 10, 900 litre.mol^{-1} cm^{-1} under the conditions of assay. PAL activity was expressed as: nmol cinnamic acid mg^{-1} protein h^{-1}.

The change in absorbance was converted to nmol of cinnamic acid using the following equation;

$$E = \frac{A}{C \times l}$$

A : Absorbance at 290 nm.

C : Concentration of cinnamic acid in Moles/L.

l : Path length of light (1 cm).

E : The molar absorption coefficient of cinnamic acid at 290 nm, which was determined to be 10,900 litres $mol^{-1}cm^{-1}$

Chemicals; All chemicals were from Sigma, and Fisher Co. except where specified.

2.15. Data Analysis.

Data were analysed using a One or Two-Way factorial design Analysis of Variance, ANOVA, (including Duncan Multiple Range Test) to determine the significance of differences between the plant cultivars and treatments using a significance level of $P < 0.050$ (*) and $P < 0.001$(**)[1]. Data were also presented as means ± Standard error (SE). Statistical analysis of the data was performed by using the SPSS for Windows statistical data analysis package (SPSS is a registered trademark of SPSS inc.).

[1] *: Significant
**: Highly significant

RESULTS

CHAPTER III

Plant Pathogen Interactions

3.1. Effect of method of inoculation, spore concentrations, age of culture and temperature on the pathogenicity of *V. albo-atrum* on tomato plants.

Environmental conditions such as temperature or conditions specific to the pathogen, such as the concentration of conidia, age of culture, or method of inoculation, may have effects on the virulence of the pathogen. These effects may increase or decrease the virulence of the pathogen. To assess those conditions on the pathogenicity of *V. albo-atrum*, the following experiments were performed.

3.1.1. Effect of the method of inoculation on disease development in tomato plants.

This experiment was performed to assess the effect of the method of inoculation and establish the pathogenicity of *V. albo-atrum*, isolate V2, towards the tomato cultivar Ailsa Craig under greenhouse conditions.

Six-week-old tomato seedlings were inoculated with spores of V2 by the root dip-, wound-, root-dip- (shaved roots prior to inoculation), stem puncture- and Soil drenching methods (see Materials and Methods). Seedlings, 8 plants per treatment, were transferred to 15-cm diameter pots and were placed in a greenhouse at 22 °C ± 1 °C. As controls, 8 seedlings were treated with distilled water. Observations on plant growth and pathogenicity of the fungus (reflected by the symptom index) were recorded weekly for 6 weeks following inoculation. The average height of the inoculated plants was compared with the average height of the control plants. The disease symptom index was also compared with the symptom index of the control plants. At the end of the experimental period, plants were harvested and procedures to reisolate the fungus from individuals of the inoculated plants were carried out. So, three different features were noted as indicators of disease progression:

Growth, indicated by the mean height of the plants, and the disease symptom index over time, is presented in Figs. 3.1.1a & b. The results of statistical analysis and attempts to reisolate the fungus from inoculated plants are presented in Tables 3.1.1 & 3.1.2.

(i) Analysis of height and symptom index.

The effectiveness of the inoculation methods was compared statistically and on the basis of the following results subsequent experiments were carried out. Briefly, the

height of all inoculated plants, whatever the method of inoculation was used, was lower compared to controls (Figs. 3.1.1a & 3.1.2). The difference in height was statistically significant compared to the control group ($P<0.001$, Table 3.1.1). The stem injection-, and soil drenching methods were not as effective as the other methods, although their height was significantly shorter than the controls ($P<0.001$, Table 3.1.1). In other words, the increase in height per week following inoculation was much higher in stem injection- and soil drenching methods compared to other inoculated plants.

The inoculated plants showed symptoms after the first week following inoculation (Fig. 3.1.1b). In the case of the plants inoculated with the root dip- and wound methods, the severity of the symptoms increased towards the end of the experimental period. However, this was not the case for those inoculated with the stem injection- and soil drenching methods. Root-dip-, wound- and root-dip (shaved roots) inoculation methods resulted in mild level (35-40 %) after the 3rd week of inoculation, and there were no significant differences between them in terms of disease progression, and the symptoms of the disease did not increase further until the end of the experiment. On the other hand, those plants inoculated with the stem injection- and soil drenching methods had slight symptoms and recovered quickly from the effect of the fungus (Fig. 3.1.1b). This was confirmed by the failure to reisolate the fungus from those plants.

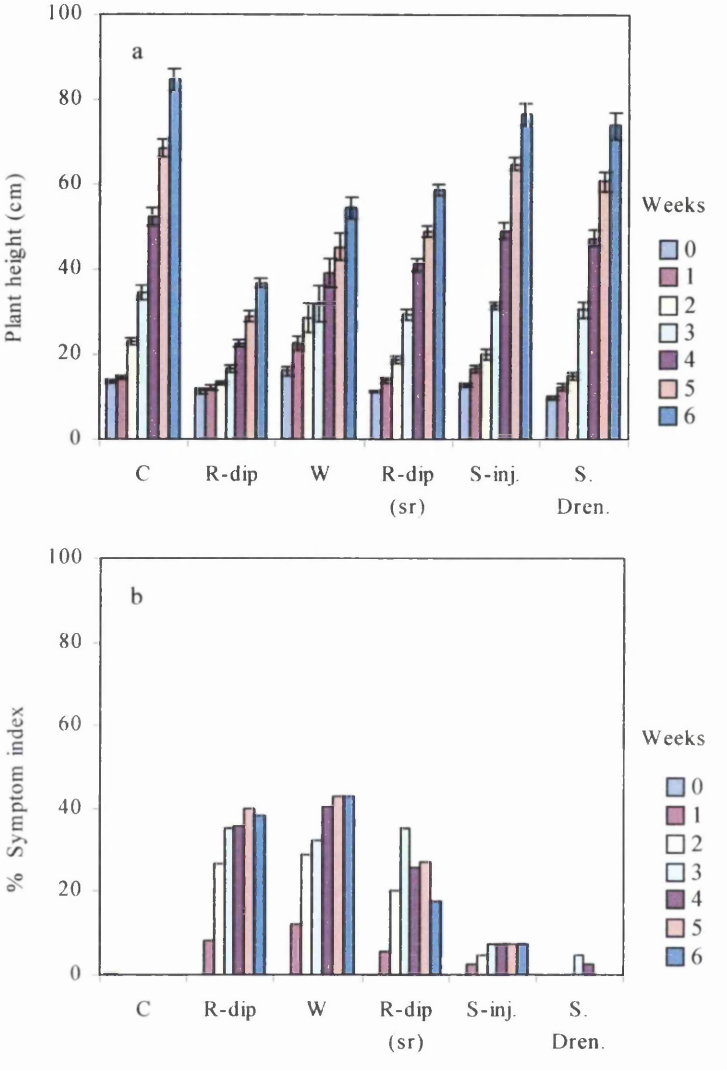


Fig. 3.1.1. Effect of method of inoculation with *V. albo-atrum*, isolate V2, on (a) the height, and (b) the disease symptom index of tomato plants (cv. Ailsa Craig).
C - Control; R-dip - Root-dip-; W - Wound; R-dip (sr) - Roots were shaved prior to inoculation by the root-dip method; S-inj - Stem puncture injection; S. Dren - Soil Drenching method. Vertical bars show ± SE of mean.

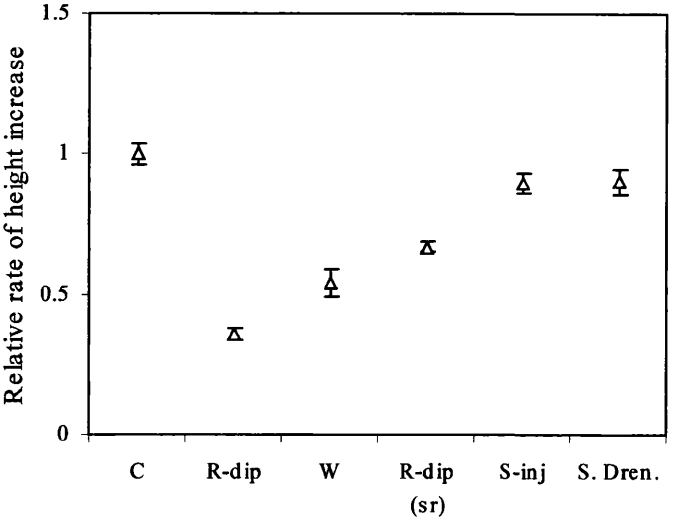


Fig. 3.1.2. Effect of method of inoculation with *V. albo-atrum*, isolate V2, on the relative rate of height increase of tomato plants (cv. Ailsa Craig) over a 6-week period. C - Control; R-dip - Root-dip-; W - Wound; R-dip (sr) - Roots were shaved prior to inoculation by the root-dip method; S-inj - Stem puncture injection; S. Dren - Soil Drenching method. Values plotted are means ± SE.

Table 3.1.1. Analysis of variance of the effect of the method of inoculation with *V. albo-atrum*, isolate V2, on the relative rate of height increase of tomato plants (cv. Ailsa Craig).

Parameters	Treatments*						ANALYSIS OF VARIANCE				
Height	C	S.Dren.	S. inj.	R. dip. (sr)	W	R. dip	SS	df	MS	*F*	*Sig.*
	a	b	b	c	d	e	2.65	5	0.53	61.3	0.000

*Between treatments, values with the same letters are *not significantly different* from each other at 0.05 level. C - Control; R-dip - Root-dip-; W - Wound; R-dip (sr) - Roots were shaved prior to inoculation by the root-dip method; S-inj - Stem puncture injection; S. Dren - Soil Drenching method.

(ii) Reisolation of the fungus.

To confirm the effect of inoculation methods, attempts were made to reisolate the pathogen from root and basal stem of the inoculated plants at the end of the experiment. For convenience, 4 plants were selected out of 8. Whatever the method of inoculation was used, plants were uprooted six weeks after inoculation and the roots were washed carefully under tap water to remove soil particles. A 3-cm section from the basal stem and root of each plant were cut and reisolation was carried out as described in Materials and Methods. The fungus was reisolated from all root-dip- and wound inoculated plants as tested. However, only 2 isolations from the basal stem and 1 isolation from the root were achieved from the plants inoculated by the root-dip (shaved roots) method. No isolation was achieved from either root or basal stems of the plants inoculated with stem injection- and soil drenching methods, although those plants showed slight symptoms during the experimental period (Table 3.1.2).

In this section, the root-dip inoculation method was found more effective than the other methods. As a result of that, this inoculation method was used in the subsequent experiments.

Table 3.1.2. Reisolation of *V. albo-atrum*, isolate V2, from inoculated tomato plants. (4 plants were chosen randomly from 8 plants of each treatment).

Inoculation Methods	Number of plants from which reisolation was successful		
	Root	Basal stem	Symptom index (%)
Root-dipping	4	4	35
Wound	4	4	40
Root-dipping (s.r.)[1]	2	1	30
Stem injection	0	0	0
Soil Drenching	0	0	0

[1]s.r.-shaved roots

3.1.2. The effect of spore concentration on disease development in five-week old tomato plants.

In order to find out the effect of various spore concentrations of *V. albo-atrum* on disease development in tomato plants, five-week old seedlings were inoculated by the root-dip method (10 plants for each treatment). Plants were placed in the greenhouse in 15-cm pots. Four different spore concentrations were used, 1 x 10^4-, 1 x 10^6-, 1 x 10^7- and 1 x 10^8 per ml. The height of the plants and the disease symptom index were recorded weekly over a five-week period, Fig. 3.1.3a & b. After harvest, the relative rate for increase in height and RGR were calculated, Table 3.1.3.

(i) Analysis of height and symptom index.

Plants inoculated with V1 or V2 by the root-dip method showed significant stunting compared to the control group, regardless of the spore concentrations used. Increasing the concentration of spores used in the V1 inoculations did not cause any further reduction in height or disease severity. However, increasing the concentration of spores in the V2 inoculations resulted in a slightly greater reduction in height but not severity of the disease, Fig. 3.1.3a & b.

At the end of the treatment, the relative rates for the increase in height and RGR were calculated. In the case of V1, all the spore concentrations that were used caused a significant reduction in height and RGR compared to the control, Tables 3.1.3 & 3.1.4, $P<0.05$. However, in the case of V2, while an increase in the spore concentration caused a slightly greater reduction in height, it did not cause a further reduction in RGR.

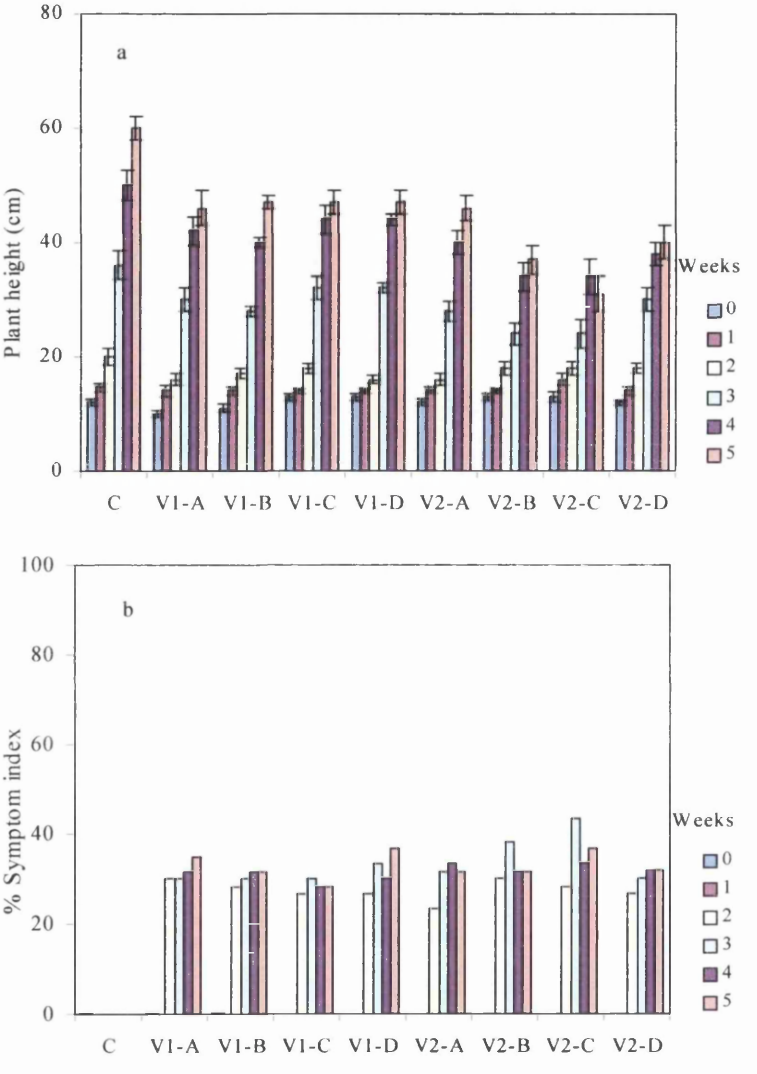


Fig. 3.1.3. The effect of various spore concentrations of *V. albo-atrum*, isolates V1 & V2, on (a) the height; (b) disease symptom index of tomato plants (cv. Ailsa Craig). Vertical bars show ± SE of mean. Spore concentrations were; V1-A or V2-A, 1 x 10^4; V1-B or V2-B, 1 x 10^6; V1-C or V2-C, 1 x 10^7; V1-D or V2-D, 1 x 10^8 spores/ml.

Table 3.1.3. The effect of various spore concentrations of *V. albo-atrum*, isolates V1 or V2, on the relative rate of height increase (H) and RGR of tomato plants cv. Ailsa Craig.

Treatments									
Parameters	Control	V1				V2			
		[1]A	B	C	D	A	B	C	D
H	1.00	0.75	0.75	0.71	0.71	0.71	0.50	0.38	0.58
RGR $Week^{-1}$	1.00	0.82	0.87	0.87	0.88	0.84	0.83	0.78	0.89

[1]A: 1 x 10^4; B: 1 x 10^6; C: 1 x 10^7; D: 1 x 10^8 spores/ml.

Table 3.1.4. Analysis of variance of the effect of various spore concentrations of *V. albo-atrum*, isolates V1 & V2, on relative rate of height increase (H) and RGR of tomato plants.

Parameters	Treatments*								ANALYSIS OF VARIANCE				
	C	V1				V2							
		A	B	C	D	A	B	C	D	SS	df	MS	*F*	*Sig.*
Height	a	b	b	b	b	b	c	c	b	1.45	8	0.18	10.7	0.000
RGR	a	b	b	b	b	b	b	b	b	0.25	8	3E-02	2.80	0.009

*Between treatments, H, RGR with the same letters are *not significantly different* from each other at 0.05 level. A: 1 x 10^4; B: 1 x 10^6; C: 1 x 10^7; D: 1 x 10^8 spores/ml.

3.1.3. The effect of continued sub-culturing on the pathogenicity of V. albo-atrum.

There are many ways to maintain fungus cultures for future use. For example, they may be stored at –80 °C under mineral oil or glycol, or they may be transferred to slope agars and stored at 4 °C or they may even be freeze-dried (CABI Bioscience-UK, 1997). One of the standard methods is to regularly transfer the fungus to fresh agar medium. Through this type of subculture, the fungus is readily available and normally will be maintained in a viable state for a long period. This was important in a project of this type where comparisons of pathogenicity of the fungus, under different cultivation conditions, were to be made over a prolonged time. However, one of the disadvantages of this method is that regular sub-culturing may cause the pathogen to loose its pathogenicity over a long period of time (personal communication with Dr. JM Milton, 1997; Thomson *et al.*, 1993; CABI Bioscience-UK, 1997; Krokene & Solheim, 2001).

Isolates of *V. albo-atrum* that had been subcultured regularly over a three-year period were available at the beginning of this project and in order to establish whether such regular sub-culturing had affected the pathogenicity of the fungus to tomato, the pathogenicity of one of these isolates was compared with the pathogenicity of freshly isolated fungus (see Materials and Methods). In this section, freshly isolated *V. albo-atrum* is referred to "New" while the isolate that had been subcultured over a three year period is referred to as "Old".

Six-week old tomato seedlings were inoculated by the root-dip method with spores (1×10^7 spores/ml) of *V. albo-atrum*, isolates V1 (New or Old) or V2 (New or Old). Plants were placed in the greenhouse in 15-cm pots (10 plants for control and 10 plants for each treatment). At the beginning of the experiment, separate batches of 10 seedlings were weighed and their fresh and dry weights were determined. Observations on the growth and development of the disease in the remaining plants were recorded weekly for a period of 6 weeks following inoculation, Fig. 3.1.4a & b. Four features were taken as indicators of disease progression: the average height of inoculated plants compared with the average height the control plants; RGR of the inoculated plants compared with the average RGR of the control group, the disease symptom index and the

ability to reisolate the fungus from individual plants from each of the inoculated groups. Mean temperature during the growth period was < 22 °C.

The relative rates of increase in height and RGR are presented in Fig. 3.1.5. All the plants inoculated with *V. albo-atrum*, isolates V1 or V2, whether the inoculum came from the freshly isolated fungus or the isolate maintained in culture for three years, caused stunting in height when compared to the control group. Inoculated plants showed symptoms from the 2nd week of the experiment. During the period of 6 weeks, inoculated plants, regardless of the source of the isolate, showed similar growth patterns although the plants inoculated with freshly isolated fungus had slightly higher growth rates, Fig. 3.1.4a. Plants inoculated with V2 had more symptoms of disease than those inoculated with V1, Fig. 3.1.4b. However, no big differences were observed between the plants inoculated either V1 (old or new isolates) or V2 (old-or new isolates) in terms of disease symptom index. After the completion of the experiment, plants were harvested and their relative rate of height increase and RGR were calculated; it was found that all the inoculated plants showed significant differences from the control plants in height or RGR, Table 3.1.5. Plants inoculated with V1 showed clear differences from the ones inoculated with V2 irrespective of the age of isolates when RGR was calculated, $P<0.05$, Table 3.1.5.

At the end of the experiment, attempts were made to reisolate the pathogen. For convenience, four plants were selected from each treatment; root sections (2-3 cm) were used, as described in the previous sections. Reisolation was positive from the inoculated plants (Table 3.1.6), though in the case of those plants inoculated with V2 the fungus was isolated from 3 plants (whether from V1- or V2 inoculated plants), whereas only one isolation was possible from the plants inoculated with V1.

It was clear that, at the temperature prevailing during the course of this experiment, the inoculated plants were affected by the fungus, whether the V1 or V2 isolate was used. Apart from confirming the results of the previous experiments, which were carried out at 22 °C, it is apparent that isolates of *V. albo-atrum* were still pathogenic to tomato plants although they had been subcultured over a three-year period.

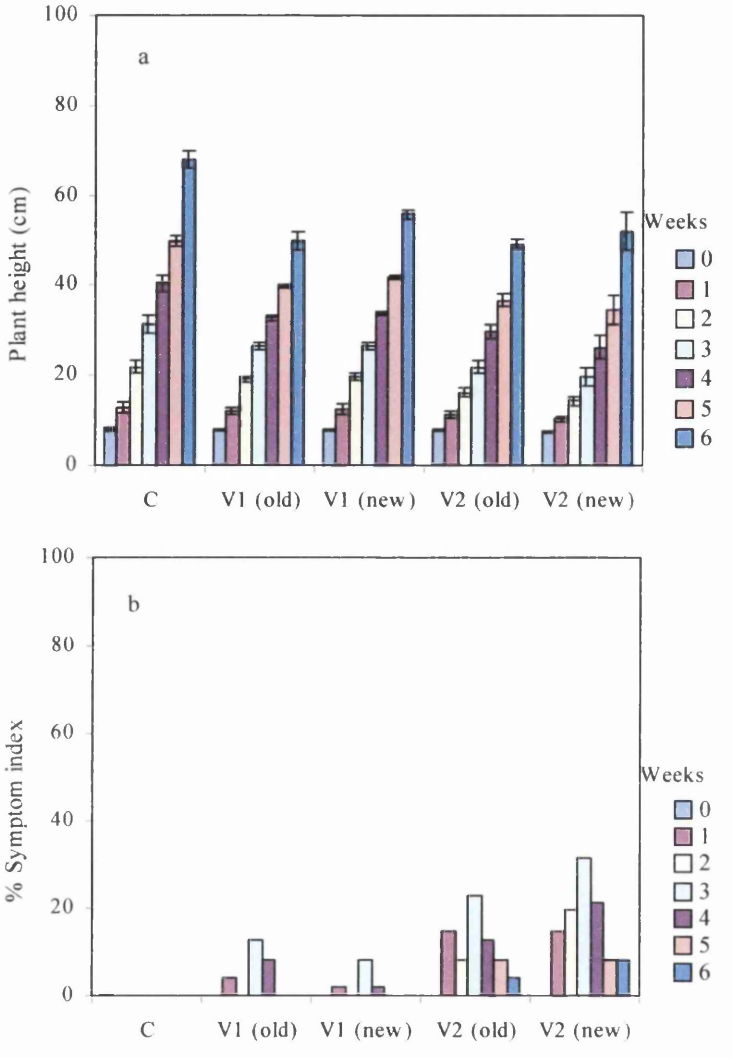


Fig. 3.1.4. Effect of old and new isolates of *V. albo-atrum* on (a) the height, and (b) the symptom index of tomato plants cv. Ailsa Craig. Vertical bars show ± SE of mean.

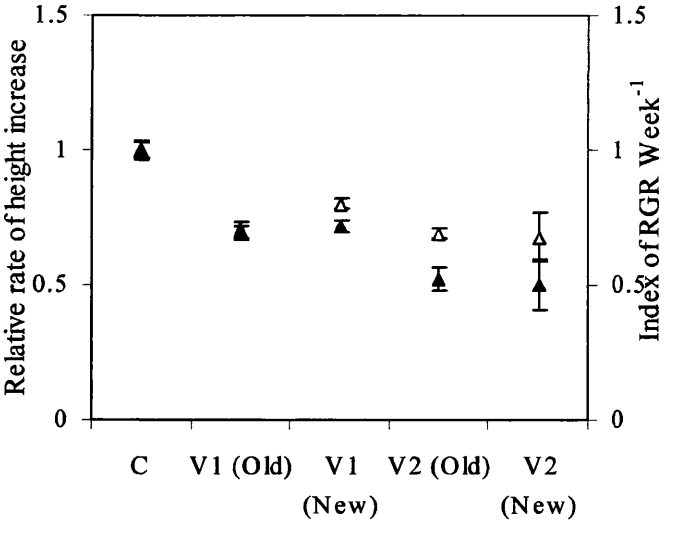


Fig. 3.1.5. Effect of old and new isolates of *V. albo-atrum* on the relative rate of height increase (Δ), and RGR (▲ of tomato plants cv. Ailsa Craig. Values plotted are means ± SE.

Table 3.1.5. Analysis of variance of the effect of old and new isolates of *V. albo-atrum* on the relative rate of height increase (H) and RGR of tomato plants.

Parameters	Treatments*					ANALYSIS OF VARIANCE				
	C	V1 (old)	V1 (new)	V2 (old)	V2 (new)	SS	df	MS	*F*	*Sig.*
H	a	b	b	b	b	0.58	4	0.14	8.38	0.000
RGR	a	b	b	c	c	1.28	4	0.32	16.2	0.000

*Between treatments, H and RGR with the same letters are *not significantly different* from each other at the 0.05 level.

Table 3.1.6. Reisolation of *V. albo-atrum*, isolate V1 and V2, from inoculated tomato plants (4 plants per treatment) with the method of root-dip.

Isolates	Number of plants from which reisolation was successful	
	Root	Symptom index (%)
V1 (Old)	1	5
V1 (New)	1	5
V2 (Old)	3	10
V2 (New)	3	10

3.1.4. The effect of temperature on disease development of tomato plants inoculated with V. albo-atrum.

Having established the most effective method of inoculation, the effect of temperature on the pathogenicity of isolates V1 & V2 of *V. albo-atrum* towards tomato, cultivar Ailsa Craig was examined. There temperatures were selected, 22-, 25- and 30 °C. In each case, plants were maintained in the greenhouse for the duration of the experiment. In the case of the experiment carried out at 22 °C ± 1 °C six-week old plants were used. For the other two temperatures, plants were five weeks old. Developmentally, however, there appeared to be no difference between plants of the two age groups and previous data (not shown) had indicated that the week difference in age had little effect on development of the disease in tomato.

In each experiment, tomato seedlings (10 plants for each treatment) of the appropriate age were inoculated with spores of the fungus (either the V1- or the V2 isolate, 1 x 10^7spores/ml) by the root dip method (see Materials and Methods) and planted in pots (15 cm). The pots were placed in the greenhouse under the conditions described. At the same time, 10 seedlings were treated with distilled water as a control group. At this point also, a separate group of 10 seedlings were taken from the same

batch of plants from which the treated plants were taken and were weighed and their fresh and dry weights recorded. Observations on both plant growth and on development of the disease were recorded weekly for a period of 6 weeks following inoculation. Five features were recorded as indicators of disease progression: the average height or root length of inoculated plants compared with the average height or root length of the control plants; the RGR of inoculated plants compared with the average RGR of the control group, the disease symptom index and the ability to reisolate the fungus from individuals of the inoculated plants.

Growth, indicated by the mean height of the plants, and the disease symptom index (severity of the wilt disease) over a 6-week period is presented in Figs. 3.1.6 (a, b, c, d, e, & f). Relative rate of height & root length increase and relative growth rate (RGR) of the inoculated and control plants are presented in Table 3.1.7. The results of statistical analysis and attempts to reisolate the fungus from inoculated plants are presented in Tables 3.1.8 & 3.1.9.

(i) Symptom development.

The symptoms of wilt disease were initially observed as wilting and yellowing of the lower leaves that occurred during the second week after inoculation, Plate 3.1a & b. This was followed by chlorosis and flaccidity of the leaves. Epinasty was clearly visible up to 2/3 heights of the infected plants, Plate 3.1c. In the following weeks those leaves became desiccated and abscised from the stem. Abscission was more common at the bottom of the stem; however, symptoms progressed to some higher leaves but did not spread through the whole plant. During the day, the upper leaves of the infected plants showed signs of wilting but recovered towards evening. From the third week onwards, inoculated plants with V1 showed recovery, Plate 3.1d, however, those inoculated with V2 expressed moderate symptoms of the disease in the following weeks. As the disease progressed, a characteristic V-shaped area at the tip of the young leaflets became visible Plate 3.2a. These areas later curled inward and shriveled, Plate 3.2b. Adventitious roots were also observed more commonly in the lower part of the stems of plants inoculated with V1 than those inoculated with V2, Plate 3.3. Towards the end of the experiment,

inoculated plants, especially V1, produced more branches compared to the beginning of the experiment.

In general terms, there were some significant differences between the symptom index of those plants inoculated with V1 and those inoculated with V2, Fig. 3.1.6b. Plants that were inoculated with V1 showed almost no symptoms, while those that were inoculated with V2 showed moderate symptoms of the disease, although the symptoms appeared to be lessening towards the end of the experiment, Plate 3.1d.

In addition to external symptoms, the fungus also caused internal disease symptoms. These symptoms and penetration of the fungus into the vascular system were observed by histological examination in which thin sections of the stems were prepared and stained with cotton blue in lactophenol and examined under the microscope (see Materials and Methods). Germination of spores and accumulation of mass hyphae were observed inside xylem vessels, Plate 3.4a & b. Spread of mycelium from one xylem vessel to another was also visible. In subsequent weeks, as the disease progressed, in response to infection a number of tyloses were observed, together with lignification of cortical cell walls, Plate 3.5a & b. Towards the end of the experiment, vascular discoloration was observed in the xylem vessels while ascent of spores and production of mycelium possibly caused blockage in the xylem vessels, resulting in wilting in stems, Plate 3.6a & b.

(ii) The effect of temperature on development of symptoms.

Development of symptoms was affected by temperature. At 25 °C (Fig. 3.1.6 d), for example, the severity of the symptoms as measured by the disease symptom index, was much lower than those of the plants inoculated with V2 at 22 °C (Fig. 3.1.6b), while the disease symptom index of plants maintained at 30 °C was again lower (Fig. 3.1.6f).

Apart from observations on the expression of such symptoms by infected plants, the heights of experimental plants were recorded and some plants were harvested. This allowed comparison between various parameters for the inoculated and control groups.

a

b

a

b

Plate 3.1.
Tomato plants inoculated with *V. albo-atrum*, isolate V2, showing typical symptoms, such as (a) wilting and (b) yellowing of the lower part of the stem leaves; (c) epinasty (clearly visible up to 2/3 heights of the infected plants); and (d) recovery of the plants towards the end of the experiment.

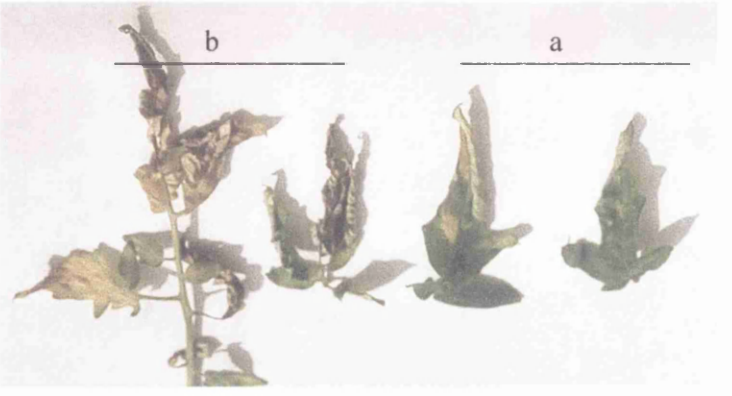


Plate 3.2.
Characteristic symptoms shown by *Verticillium*-infected plants (isolate V2). (a) characteristic yellow V-shaped area at the tip of young leaflets; (b) leaves, curled inward and shrivelled.

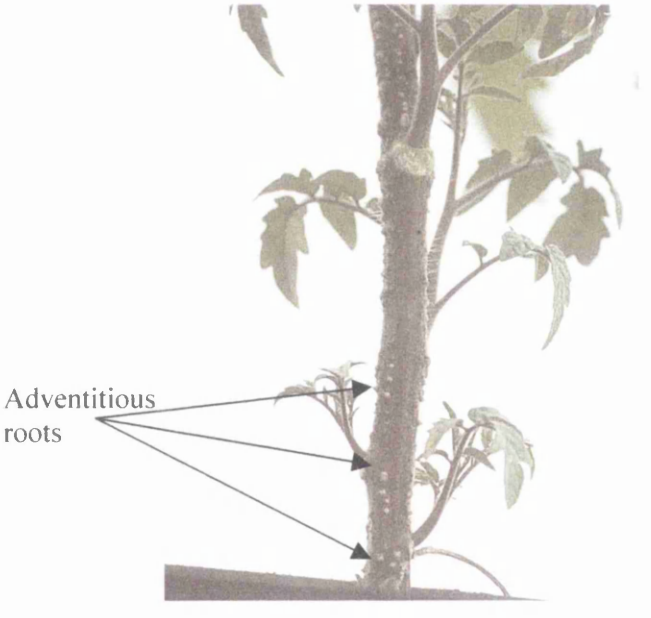


Plate 3.3.
Appearance of adventitious roots, as external defence response, on the lower stem part of tomato plants following inoculation with *V. albo-atrum* (isolate V2).

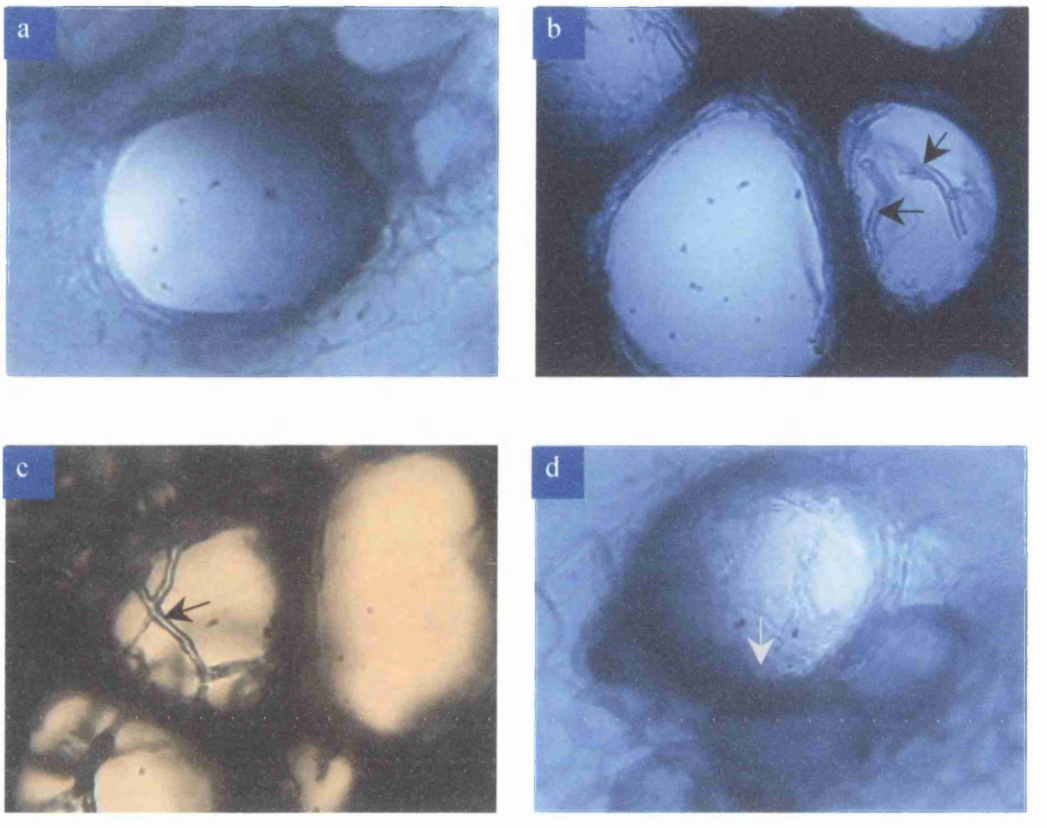


Plate 3.4.
Stages in the germination of spores, development and spread of mycelium of *V. albo-atrum* (isolate V2) in xylem vessels of tomato (cv. Ailsa Craig) (x100). (a) clear xylem free from spores; (b) germination of mycelium from spores & colonization of the xylem by mycelium; (c) spread of mycelium from one xylem vessel to another; and (d) lignifications of cell walls and blockage of xylem by mycelium ball.

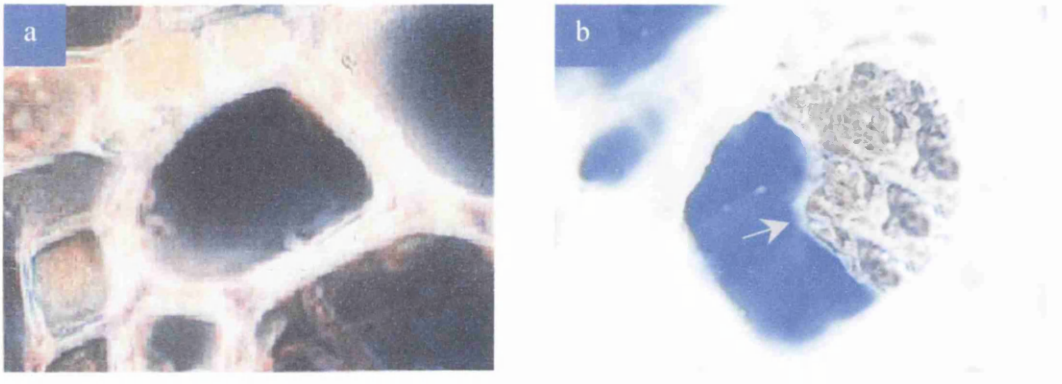


Plate 3.5.
Formation of tyloses and mucilage in the xylem vessels of tomato (cv. Ailsa Craig) inoculated with *V. albo-atrum*, isolate V2, (x 100). (a) clear xylem; (b) production of defence response structures such as tyloses and mucilage or gummosis.

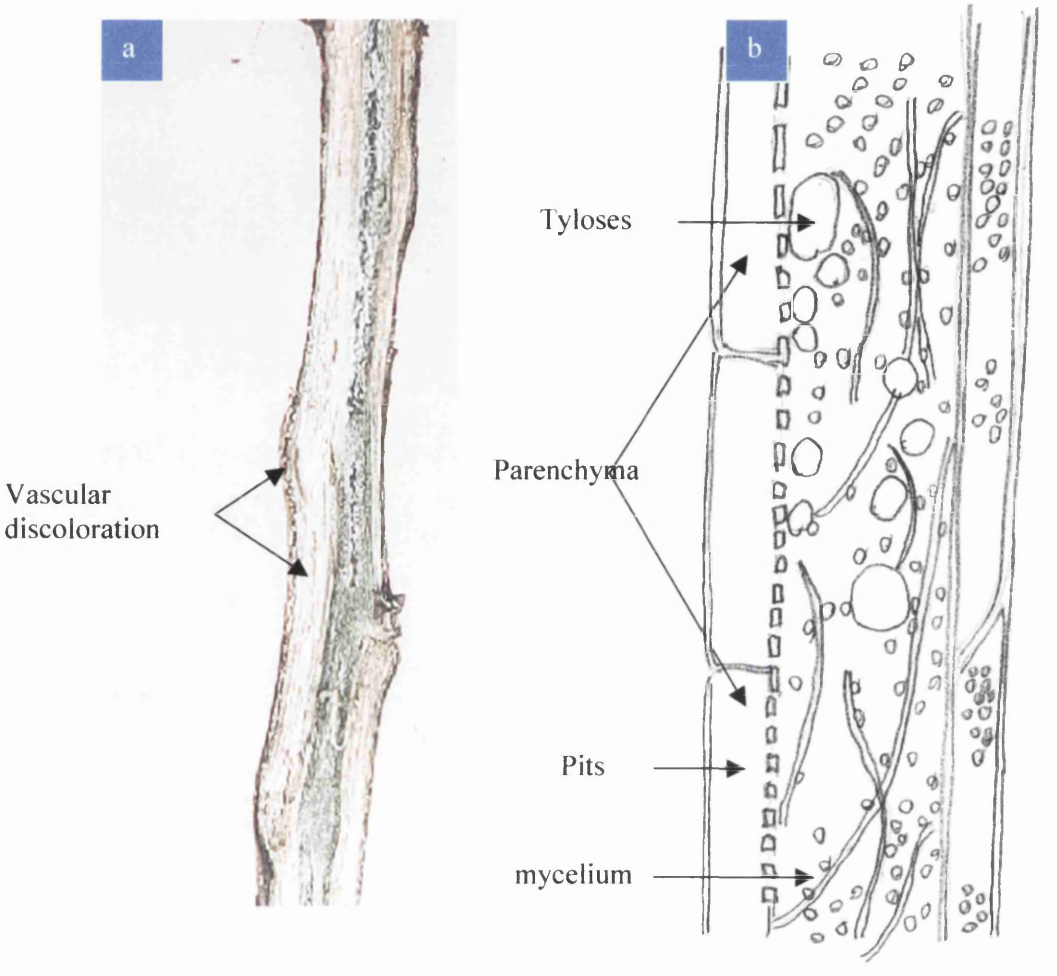


Plate 3.6.
(a) Longitudinal stem sections of tomato inoculated with *V. albo-atrum* (isolate V2) showing symptoms such as vascular discoloration (b) drawing of an internal stem of a tomato showing how spores and mycelium propagates in the xylem tissues.

(iii) The effect of temperature on height, root length and dry weight.

In the case of the plants maintained at 22 °C there was a clear difference between those plants inoculated with V2 and the control plants (Table 3.1.7). This difference was evident throughout the experiment and the difference was found to be significant ($P<0.001$) when analysis of variance was carried out (Table 3.1.8) on the relative rate for increase in height. Similarly, inoculation with V1 resulted in a decrease in height compared to control plants, though this was not obvious until week 4 and was not as severe as with V2, Table 3.1.7. Again, this difference was reflected in the relative rate for increase in height (Table 3.1.7). In the case of root length, however, there was no difference in the effect of V2 and V1, and neither V2 nor V1 significantly affected the rate of root growth (Table 3.1.7 & 3.1.8). V1 and V2 also significantly affected Dwt ($P<0.001$), expressed as RGR (Tables 3.1.7 & 3.1.8).

Temperature significantly altered the effects of V2 and V1 on the disease symptom index (Figs. 3.1.6b, d & f), height (Figs. 3.1.6a, c & e), relative rates for increase in height & root length and the RGR (Table 3.1.7) and analysis table (Tables 3.1.8), at both 25- and 30 °C. In those temperatures, V1 was not statistically different from the control groups on height and RGR (Table 3.1.8), however, V2 was still effective at 25 °C and not effective at 30 °C on height but effective on RGR (Tables 3.1.8).

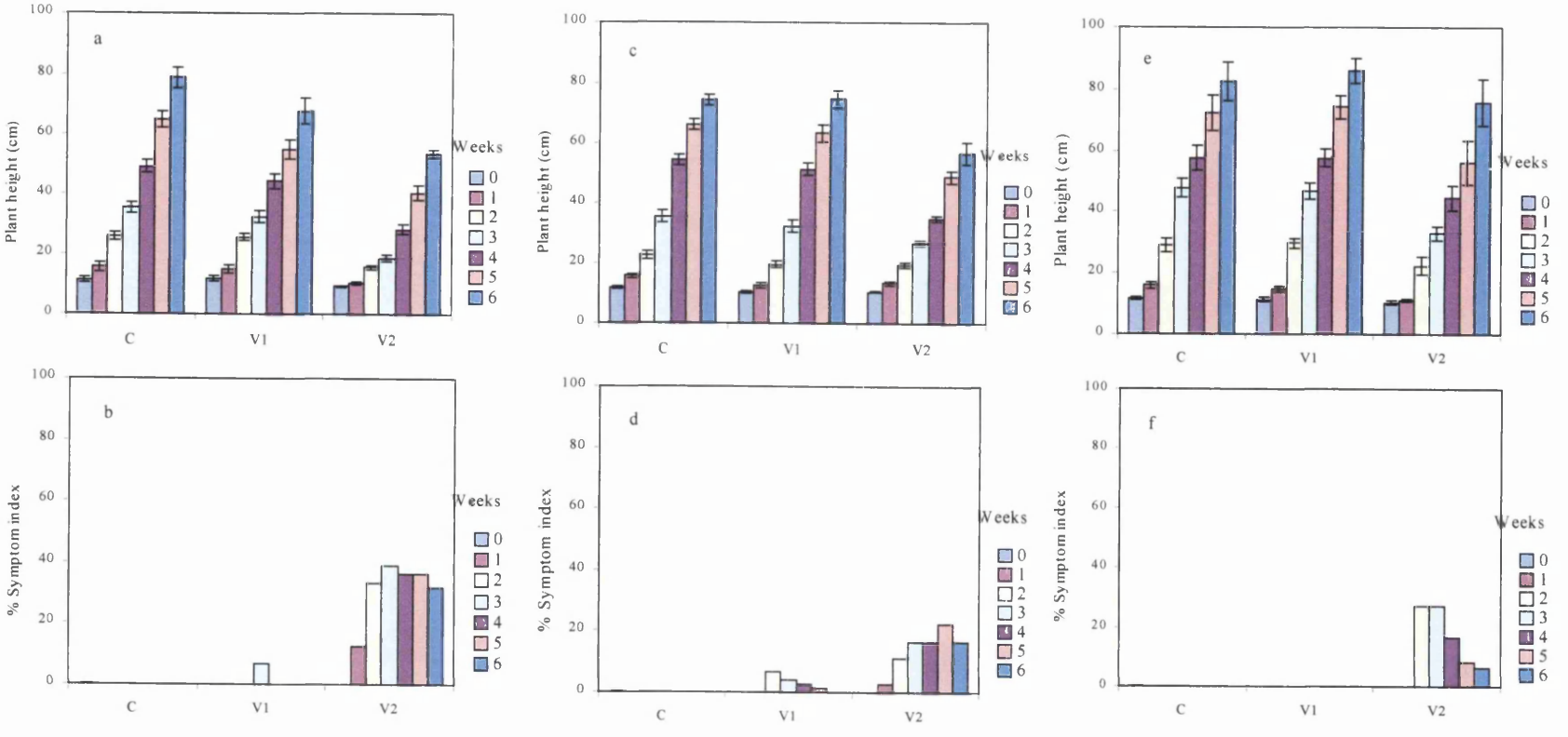


Fig. 3.1.6. The effect of temperature on the pathogenicity of *V. albo-atrum*, isolates V1 or V2; towards tomato plants cv. Ailsa Craig. a, c & e height; b, d & f disease severity – measured as symptom index; a & b (22 °C); c & d (25 °C); e & f (30 °C). Vertical bars show ± SE of mean.

Table 3.1.7. The effect of temperature on the pathogenicity of *V. albo-atrum*, isolates V1 or V2; on the relative growth rate (RGR), the relative rate of height (H) & root length increase (RL) of tomato plants.

Treatments									
Parameters	C			V1			V2		
	22 °C	25 °C	30 °C	22 °C	25 °C	30 °C	22 °C	25 °C	30 °C
H	1.00 ± 0.04*	1.00 ± 0.02	1.00 ± 0.08	0.83 ± 0.07	1.02 ± 0.04	1.00 ± 0.08	0.65 ± 0.02	0.74 ± 0.05	0.92 ± 0.10
RL	1.00 ± 0.04	1.00 ± 0.02	-	1.02 ± 0.01	1.04 ± 0.04	-	0.97 ± 0.06	0.96 ± 0.04	-
RGR $Week^{-1}$	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.17	0.84 ± 0.02	0.98 ± 0.01	1.00 ± 0.12	0.84 ± 0.01	0.59 ± 0.05	0.44 ± 0.08

* represents SE (Standard Error) of the mean.

Table 3.1.8. Analysis of variance of the effect of temperature on the pathogenicity of *V. albo-atrum*, isolates V1 and V2, on the relative rate of height (H), root length (RL) and RGR of tomato plants.

Temp. (°C)	Parameters	Treatments*			ANALYSIS OF VARIANCE				
22		C	V1	V2	SS	df	MS	*F*	*Sig.*
	H	a	b	c	0.353	2	0.177	10.4	0.001
	RL	a	a	a	4E-03	2	2E-03	0.29	0.750
	RGR $Week^{-1}$	a	b	b	7E-02	2	3E-02	17.7	0.001
25									
	H	a	a	b	0.619	2	0.309	12.0	0.000
	RL	a	a	a	3E-02	2	1E-02	1.16	0.331
	RGR $Week^{-1}$	a	a	b	1.181	2	0.590	35.3	0.000
30									
	H	a	a	a	7E-02	2	3E-02	0.66	0.528
	RGR $Week^{-1}$	a	a	b	1.416	2	0.708	6.45	0.008

*Between treatments, H, RL and RGR with the same letters are *not significantly different* from each other at 0.05 level.

(iv) Reisolation of the fungus.

At the end of the experiment, attempts were made to reisolate the pathogen from the group of the plants inoculated at 22 °C. For convenience, four plants were selected from each treatment. Both basal stem and root sections of the inoculated plants were used. Plants that had been inoculated by the root-dip method were uprooted after six weeks and the roots carefully washed under water to clean the root area. A section (2-3

cm) was removed from the stem and a 3 cm section from the root. Reisolation was carried out as described in Materials and Methods.

Reisolation was positive from only one plant inoculated with V2, Table 3.1.9. This may have been because the rest of the plants were heavily contaminated with saprophyte fungi, making reisolation difficult. However, despite this failure there was evidence that these sections carried the fungus. For example, those particular stem sections had accumulated dark melanin pigments, which is often an indicator that the fungus is present. No reisolation was made from plants inoculated with V1, despite the fact that these plants showed stunted height and a reduction in RGR compared to control groups.

No attempt was made to reisolate the fungus from the group of plants inoculated at 25 °C and 30 °C because they either show little or no symptoms of the disease.

Table 3.1.9. Reisolation of *V. albo-atrum*, isolate V1 and V2, from inoculated tomato plants (4 plants per treatment) with the method of root-dip.

Isolates	Number of plants from which reisolation was successful		
	Root	Basal stem	Symptom index (%)
V1	0	0	0
V2	1	1	30

3.1.5. Effect of temperature on fungal growth.

Pathogenicity of both isolates of *V. albo-atrum* was negatively affected in increasing temperatures. To assess whether fungal biomass was effected (responsible for pathogenicity), growth of fungal mycelium was recorded in various temperatures. Having tested the pathogenicity of the fungus *V. albo-atrum* in various temperatures towards tomato plants, growth of *V. albo-atrum* was also tested in liquid Dox culture *in vitro* conditions. 10 flasks of Dox medium with 0.5 cm mycelium disk of each isolate was incubated at 18-, 22-, 25- and 30 °C for 6 weeks in an orbital shaker (100 rpm).

After the incubation the mycelial mats were harvested and the amount of dry weight of mycelia was recorded.

Growth of the fungus was affected by temperature. Both isolates, V1 and V2, had highest growth at 22-, and 25 °C, Fig. 3.1.7. However, increase in temperature resulted in decrease in fungal growth. At 30 °C, fungal growth was negatively affected and had its lowest growth rate. This was also corresponded with the pathogenicity experiment; Fig. 3.1.6 & Table 3.1.7, in that *V. albo-atrum* isolates were not pathogenic to tomato plants. At 18 °C only dry weight of mycelium of isolate V1 was recorded. At this temperature, fungal growth was lower compared to the growth rate obtained at 22-, and 25 °C, Fig. 3.1.7.

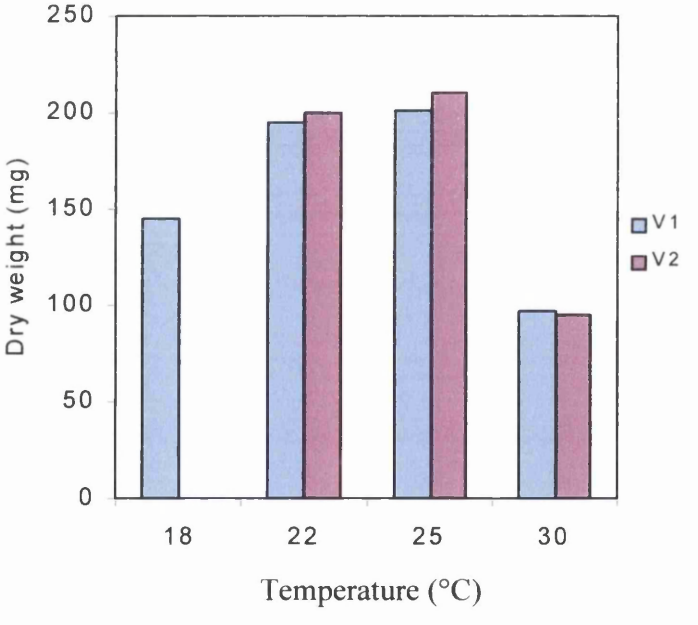


Fig. 3.1.7. Mycelium growth of *V. albo-atrum* in liquid culture (6 weeks).

3.2. Co-inoculation of *V. albo-atrum* isolates on the pathogenicity of tomato Ailsa Craig.

In the field, it is not unusual for plants to be exposed to more than one pathogen at the same time, and it is not uncommon for two or more fungi to infect a host at the same time. In such cases interactions between the fungi may occur (Jorge, 1990; Ganeshan, 1999). Such interaction may involve synergism so that the effects of the fungi on the plant are greater than the effects of infection by individuals (Dewey *et al.*, 1999). On the other hand, the fungi may have an antagonistic effect on each other so that the combined effects of the fungi are less than those of the individuals (Jorge *et al.*, 1992; Tsahouridou & Thanassoulopoulos, 1999; Schmidt *et al.*, 2001). To determine whether there was any such interaction between the two isolates of *V. albo-atrum*, V1 and V2, 6-week old tomato plants, cv. Ailsa Craig, were inoculated with either the V1- or V2 isolate, or with both isolates simultaneously. Plants were inoculated by the root dip method and were maintained in the greenhouse at 22 °C following inoculation. Observations of the growth of plants and development of the wilt disease were made weekly for a period of 6 weeks.

Growth, indicated by the mean height of the plants, and the disease symptom index are presented in Figs. 3.2.1a & b. The relative increase in height rate, RGR and the chlorophyll content of the leaves are presented Figs. 3.2.2a & b. The results of reisolation of the fungus from inoculated plants are presented in Table 3.2.1. It is known that *V. albo-atrum* causes chlorosis in leaves (DeVay *et al.*, 1997) and it is one of the early symptoms of the wilt disease (Bowden & Rouse, 1991). However, in a co-inoculation where two isolates of a fungus is involved, it is not clear whether chlorosis will be severe or mild compared to the inoculation made by one isolate.

Like the results of the previous experiment (section 3.1.4), plants inoculated with the V2 isolate showed more severe symptoms than those inoculated with the V1 isolate, Fig. 3.2.1b. A similar trend was also observed with the height increment per week, Fig. 3.2.1a. When the two isolates were used together, there was only a marginal effect on the height of the plants and no difference in the development of symptoms. The effects of the two isolates were reflected in the rate of height increase and RGR. The inoculated plants showed significant differences from the control group (Fig. 3.2.2a; $P<0.05$, Table 3.2.2. with those plants inoculated with isolate V2 or V1+V2 showing a greater reduction

in RGR and rate of height increase compared to plants inoculated with only V1 (Fig. 3.2.2a; Table 3.2.2).

The chlorophyll content of the leaves was also compared. However, there was no statistical difference between the treatments (Fig. 3.2.2b; Table 3.2.2).

Finally, reisolation was positive from the plants inoculated with V2 or V1+V2, Table 3.2.2.

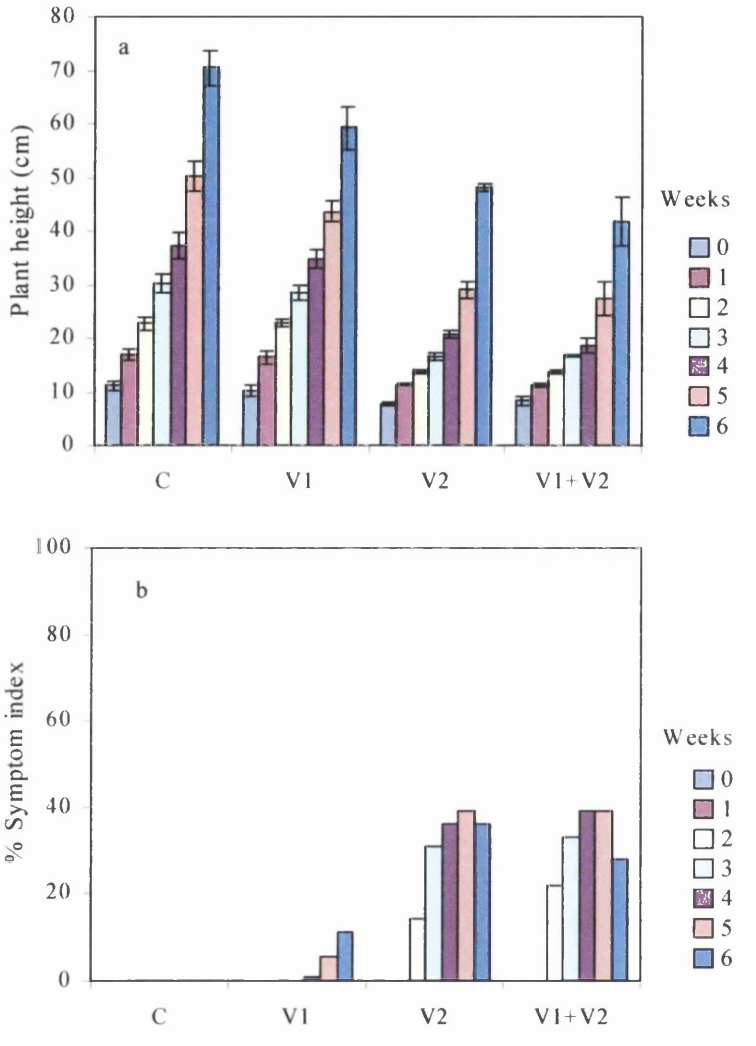


Fig. 3.2.1. The effects of *V. albo-atrum*, isolates V1 or V2, and V1+V2 on (a) the height, and (b) the symptom index for tomato (cv. Ailsa Craig). Vertical bars show ± SE of mean.

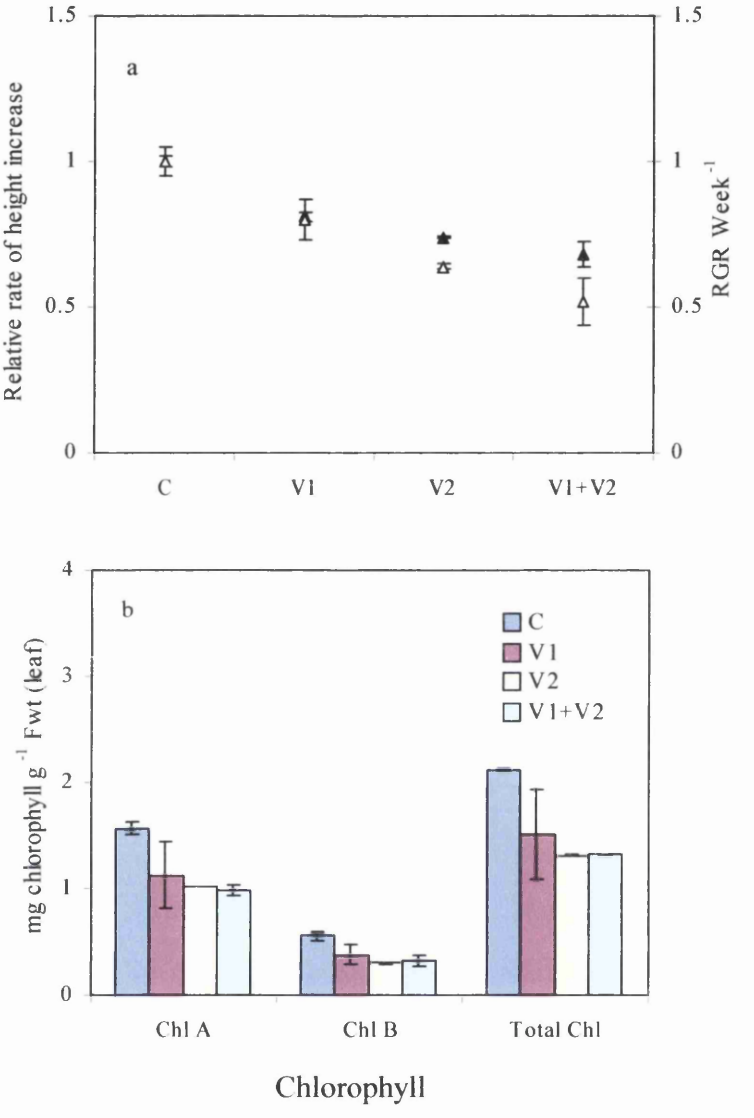


Fig. 3.2.2. The effects of *V. albo-atrum*, isolates V1 or V2, and V1+V2 on (a) the relative rate of height increase and RGR (Δ-Height; ▲-RGR), and (b) the chlorophyll content of the leaves of tomato (cv. Ailsa Craig). Vertical bars or values ± SE of mean.

Table 3.2.1. Reisolation of *V. albo-atrum*, from tomato plants (3 plants per treatment) inoculated isolate V1 or V2, or V1 and V2 in combination with the method of root-dip.

Isolates	Number of plants from which reisolation was successful	
	Basal stem	Symptom index (%)
V1	0	11
V2	3	36
V1+V2	3	30

Table 3.2.2. Analysis of variance of the effects of *V. albo-atrum*, isolates V1 or V2, and V1+V2 on the growth parameters of tomato (cv. Ailsa Craig).

Parameters	Isolates*				ANALYSIS OF VARIANCE				
	C	V1	V2	V1+V2	SS	df	MS	*F*	*Sig.*
H	a	b	bc	c	0.508	3	0.169	13.3	0.000
RGR	a	b	c	c	6E-02	3	2E-02	19.6	0.000
CHL	a	a	a	a	0.878	3	0.293	3.31	0.139

* Between isolates, growth parameters of Ailsa Craig (6-week old) with the same letters are *not significantly different* from each other at 0.05 level. H: relative rate of height increase; RGR Relative Growth Rate; CHL: Chlorophyll content of the leaves.

3.3. Cultivar response of tomato plants towards *V. albo-atrum* isolates.

The previous experiments have established that isolate V2 is more pathogenic towards tomato cv. Ailsa Craig than the V1 isolate. However, cultivars may differ in their susceptibility to pathogens (Daniel *et al.*, 1990; Dolar, 1995). Consequently, the pathogenicity of *V. albo-atrum* was tested towards various cultivars of tomato that were available. The characteristics of the various cultivars are included in Table 2.7.2. Groups of 10 plants were inoculated with spores of either isolate V1 or V2 with the root dip method and plants were maintained for 6 weeks in the greenhouse at UWS.

After 6 weeks the relative rate of height increase, RGR and water content (WTC) of the plants were recorded. The results, Tables 3.3.1 & 3.3.2, show that on the basis of height and RGR, there were no significant differences between cultivars of Cyclon Hybrid F1, Edcawy, *L. lycopersicon* and Margarita (Fa-558) and the control plants, indicating resistance to VW. Resistant cultivars produced symptom-free new branches while susceptible ones did not. On the basis of these two parameters, the three other cultivars, Hybrid Sweet F1, Falkon and Simge F1 were significantly affected by both the V1- and the V2 isolate. In general, as was the case with Ailsa Craig, the effects of the V2 isolate were greater than the V1, except in the case of Falkon (Tables 3.3.1 & 3.3.2).

After the harvest, final fresh and dry weights of plants were recorded to determine the water content of the plants. In contrast to the results for the increase in height and RGR, none of the cultivars differed in WTC from the control plants (Tables 3.3.1 & 3.3.2). It seems likely then that either blockage of the xylem tissues by either mycelium or secondary plant tissues did not take place in those cultivars, or that it was not sufficient to significantly restrict water flow. It is probable that genetic variability of the plants played a major role in this respect.

From the results of this experiment, some of the cultivars (commercially available) used in the further experiments, their response to salt or VW or both were assessed in seedling or mature plant level.

Table 3.3.1. The effect of *V. albo-atrum*, isolates V1 or V2, on selected growth parameters of various cultivars of tomato. Values for each treatment are expressed relative to the value of control group of each cultivar which was assigned a value of 1, with the exception of WTC (expressed as % water content)

	H			RGR			WTC		
Cultivars	C	V1	V2	C	V1	V2	C	V1	V2
Cyclon Hybrid F1	1.00 ± .05*	1.09 ± .02	1.11 ± .08	1.00 ± .03	0.97 ± .03	1.00 ± .04	88.4 ± .7	87.7 ± .8	87.1 ± .3
Hybrid Sweet F1	1.00 ± .07	0.86 ± .06	0.68 ± .08	1.00 ± .01	0.70 ± .11	0.56 ± .11	79.6 ± .9	80.5 ± 1	82.2 ± 1
Margarita (Fa-558)	1.00 ± .05	1.08 ± .05	0.94 ± .08	1.00 ± .02	1.07 ± .01	1.02 ± .03	78.9 ± 1	74.6 ± 1	77.5 ± 1
Falkon	1.00 ± .04	0.74 ± .06	0.80 ± .08	1.00 ± .01	0.68 ± .04	0.73 ± .06	83.7 ± .1	83.8 ± .2	83.2 ± .3
L. lycopersicon	1.00 ± .04	0.89 ± .04	0.87 ± .01	1.00 ± .01	1.01 ± .01	1.00 ± .01	78.0 ± .6	76.2 ± .8	77.7 ± 1
Simge F1	1.00 ± .06	0.93 ± .05	0.76 ± .06	1.00 ± .01	0.90 ± .02	0.67 ± .06	83.4 ± .4	83.5 ± .6	84.1 ± .4
Edcawy	1.00 ± .09	0.96 ± .05	0.99 ± .07	1.00 ± .03	0.98 ± .03	0.95 ± .01	82.8 ± .5	83.1 ± 1	82.7 ± .5

* figure represents SE of the mean.

Table 3.3.2. Analysis of variance (both one way and two-way) on the pathogenicity of *V. albo-atrum*, isolates V1 and V2, on the relative rate of height increase, RGR and WTC of tomato plants.

Parameter	Cultivars	Treatments*			ANALYSIS OF VARIANCE				
H		C	V1	V2	SS	df	MS	*F*	*Sig.*
	Cyclon Hybrid F1	a	a	a	7E-02	2	3E-02	1.19	0.318
	Hybrid Sweet F1	a	ab	b	0.230	2	0.115	5.06	0.028
	Margarita (Fa-558)	a	a	a	9E-02	2	4E-02	1.66	0.210
	Falkon	a	b	b	0.254	2	0.127	4.72	0.022
	L. lycopersicon	a	a	a	5E-02	2	2E-02	2.913	0.088
	Simge F1	a	ab	b	0.215	2	0.107	4.22	0.031
	Edcawy	a	a	a	4E-03	2	2E-03	0.07	0.929
	Sig.+	ns	s	s					
RGR Week^{-1}									
	Cyclon Hybrid F1	a	a	a	4E-03	2	2E-03	1.16	0.848
	Hybrid Sweet F1	a	ab	b	0.427	2	0.214	4.76	0.032
	Margarita (Fa-558)	a	a	a	3E-02	2	1E-02	4.42	0.026
	Falkon	a	b	b	0.429	2	0.214	16.8	0.000
	L. lycopersicon	a	a	a	6E-04	2	3E-04	0.82	0.451
	Simge F1	a	a	b	0.411	2	0.206	21.2	0.000
	Edcawy	a	a	a	1E-02	2	5E-03	2.14	0.151
	Sig.+	ns	s	s					
WTC									
	Cyclon Hybrid F1	a	a	a	7.59	2	3.79	0.90	0.419
	Hybrid Sweet F1	a	a	a	16.0	2	8.01	0.88	0.440
	Margarita (Fa-558)	a	a	a	77.2	2	38.64	2.65	0.098
	Falkon	a	a	a	1.00	2	0.504	1.18	0.329
	L. lycopersicon	a	a	a	15.1	2	7.552	1.28	0.298
	Simge F1	a	a	a	2.13	2	1.067	2.60	0.102
	Edcawy	a	a	a	0.40	2	0.203	0.06	0.940

* Between treatments, height, RGR and WTC with the same letters are *not significantly different* from each other at 0.05 level.
+ One-Way ANOVA test between cultivars within a treatment at 0.05 level.
ns: not significant; s: significant

3.4. Comparison of the effects of European- and USA isolates of *V. albo-atrum* on young (4-week old) tomato seedlings.

The pathogenicity of two isolates of *V. albo-atrum* from Europe (V1 and V2) towards young tomato seedlings was compared with that of four isolates from the USA (Sevcik, Arl 86 B, Freitag and Loken obtained from the culture collection of Prof. Craig Grau (Madison-USA).

Seedlings of tomato plants were inoculated with spores of the isolates V1, V2, VS (Sevcik), VA (Arl 86 B), VF (Freitag) and VL (Loken) by the root-dip inoculation method (10 plants for each treatment, 1 x 10^7 spores/ml) and placed in a greenhouse in 15-cm pots. While the pathogenicity of V1 and V2 on tomato plants has been reported many times since the 1980's, the pathogenicity of USA isolates has not previously been tested on tomato plants. Comparisons of V1 and V2 isolates for pathogenicity test were recorded both on tomato and lucerne in 1997 by Dikilitas.

Observations on relative rate of increase in height and root length and RGR were recorded for a period of 6 weeks after inoculation. At the start of the experiment, ten representative plants were harvested, the initial mean fresh and dry weights of shoots and roots and root lengths were recorded and the initial mean of dry weights and RGR were calculated.

The relative rate of height and root length increase and RGR are presented in Table 3.4.1 and the result of the statistical analysis is presented in Table 3.4.2.

All isolates of *V. albo-atrum*, regardless of their country or plant origin caused significant reduction in height, root length and RGR in young tomato seedlings Tables 3.4.1 & 3.4.2. However, the isolates from Europe (V1 and V2) caused greater reductions in height, root length and RGR than the isolates from the USA $P<0.001$.

Table 3.4.1. Pathogenicity of *V. albo-atrum*, isolates V1, V2, VS, VA, VF & VL, on young seedlings (4-week old) of tomato plants.

Parameters	C	V1	V2	VS	VA	VF	VL
H	1.00 ± .04*	0.29 ± .03	0.25 ± .05	0.65 ± .03	0.72 ± .03	0.66 ± .03	0.60 ± .02
RL	1.00 ± .06	0.39 ± .02	0.17 ± .01	0.59 ± .02	0.70 ± .01	0.70 ± .02	0.68 ± .02
RGR	1.00 ± .01	0.40 ± .01	0.50 ± .01	0.40 ± .01	0.60 ± .01	0.60 ± .01	0.40 ± .01

* figure represents SE of the mean.
Isolates from Europe: V1 (lucerne); V2 (tomato).
Isolates from USA: All isolates are from lucerne; VS (Sevcik); VA (Arl 86 B); VF (Freitag); and VL (Loken).

Table 3.4.2. Analysis of variance of the effect of *V. albo-atrum*, isolates V1, V2, VS, VA, VF & VL, on young seedlings (4-week old) of tomato plants on the relative rate of height & root length increase and RGR.

Parameters	Treatments*							ANALYSIS OF VARIANCE				
	C	VA	VF	VS	VL	V1	V2	SS	df	MS	*F*	*Sig.*
H	a	b	b	b	b	c	c	3.52	6	0.58	57.0	0.000
RL	a	b	b	b	b	c	d	3.50	6	0.58	45.8	0.000
RGR $Week^{-1}$	a	b	bc	d	d	d	cd	2.53	6	0.42	67.3	0.000

* Between treatments, H, RL and RGR with the same letters are *not significantly different* from each other at 0.05 level.

3.5. Pathogenicity of *V. albo-atrum*, isolate V1, towards *M. media* cv. Rambler strains.

The Pathogenicity of the V1 isolate of *V. albo-atrum* had previously been tested on strains of *M. media* cv. Rambler, which were tolerant to various concentrations of NaCl, (Dikilitas, 1997). In that study, the salt tolerant strains (150, 200, 250 and 300 mM salt tolerant plants) showed significant differences from the control groups in terms of height and RGR. Progressively, the more salt tolerance they gained the more susceptible they became to the fungus (Dikilitas, 1997).

However, after a period of time, salt tolerant plants may loose their ability to survive in the concentration of salt in which they were generated, if they have been maintained subsequently in non-saline conditions. To keep the salt lines fresh for the salinity tests a new salt line should be generated (personal communication with Dr. Salim Al-Rawahy, 1999). Therefore, in the present study, two generations of salt tolerant *M. media* lines were again tested for resistance to V1, one was generated from the original salt-adapted cell lines of *M. media* (Chaudhary, 1996). The other was regenerated by Al-Rawahy (2000) from the original salt tolerant plants of *M. media* generated by Chaudhary (1996). In the text, plants are designated by the letter "R"- for 'Rambler', followed by a number indicating the molarity (in millimoles) of salt to which the line is tolerant. The letter 'O' indicates the original line of Chaudhary, while 'N' to indicates the newly generated salt tolerant plants by Al-Rawahy.

The original salt tolerant strains of *M. media* were tested against the isolate V1 both in the first- and third year of the study in order to assess any change in resistance to infection over time. The original and the new generation of salt tolerant plants were compared in the third year of the study.

The cuttings of Rambler strains were rooted under a misting bed, inoculated with *V. albo-atrum*, isolate V1, by the root-dip method and then grown in the greenhouse for 10 weeks. Batches of ten plants from each strain were used as treatment and control groups. The symptom index of the inoculated plants was recorded weekly for a period of 10 weeks following inoculation, Fig. 3.5.1. The relative rate increase in height and RGR of the plants are presented in Fig. 3.5.2a, b, c, d, e & f.

Rambler strains, either original, newly-generated or non salt tolerant ones showed significant differences from the control plants in terms of height and RGR, Figs. 3.5.1 & 3.5.2; Tables 3.5.1 & 3.5.2. All the inoculated plants showed wilting in the first or second week after inoculation, leaves became yellow and desiccated, however, no recovery was observed during the course of the experiments in the salt tolerant plants. There was no interaction apparent between strains and treatments, in terms of height and RGR measurements either in the experiment of the first- or third year (Table 3.5.1). This means that all the strains showed a similar response to the pathogen. However, newly generated salt tolerant plants showed significant interaction between strains and treatments in terms of RGR measurement; this means that, the parental Rambler plants (although it was susceptible to the disease) showed better defence reaction than the newly generated salt tolerant plants, Table 3.5.2.

When the symptom index values were recorded in each experiment, it was obvious that Rambler plants showed great susceptibility to the disease. The original salt tolerant plants showed similar trends both in the first and third year. Again, newly generated salt tolerant plants also showed great susceptibility to the disease. Symptoms of the disease of the inoculated plants became more severe towards the end of the experiment.

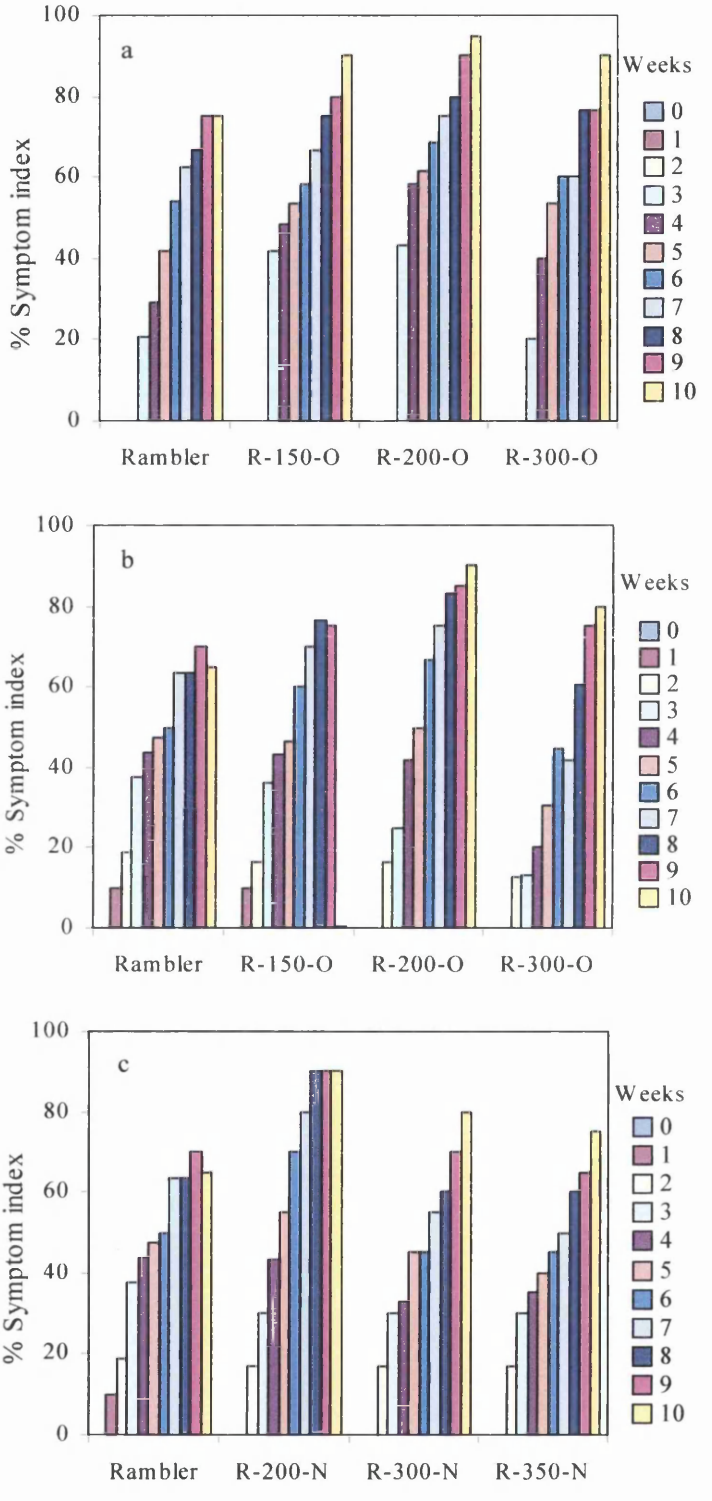


Fig. 3.5.1. Effect of pathogenicity of *V. albo-atrum*, isolate V1 on disease symptom index of lucerne cv. *M. media*. a (first year); b (third year); c (new salt line).

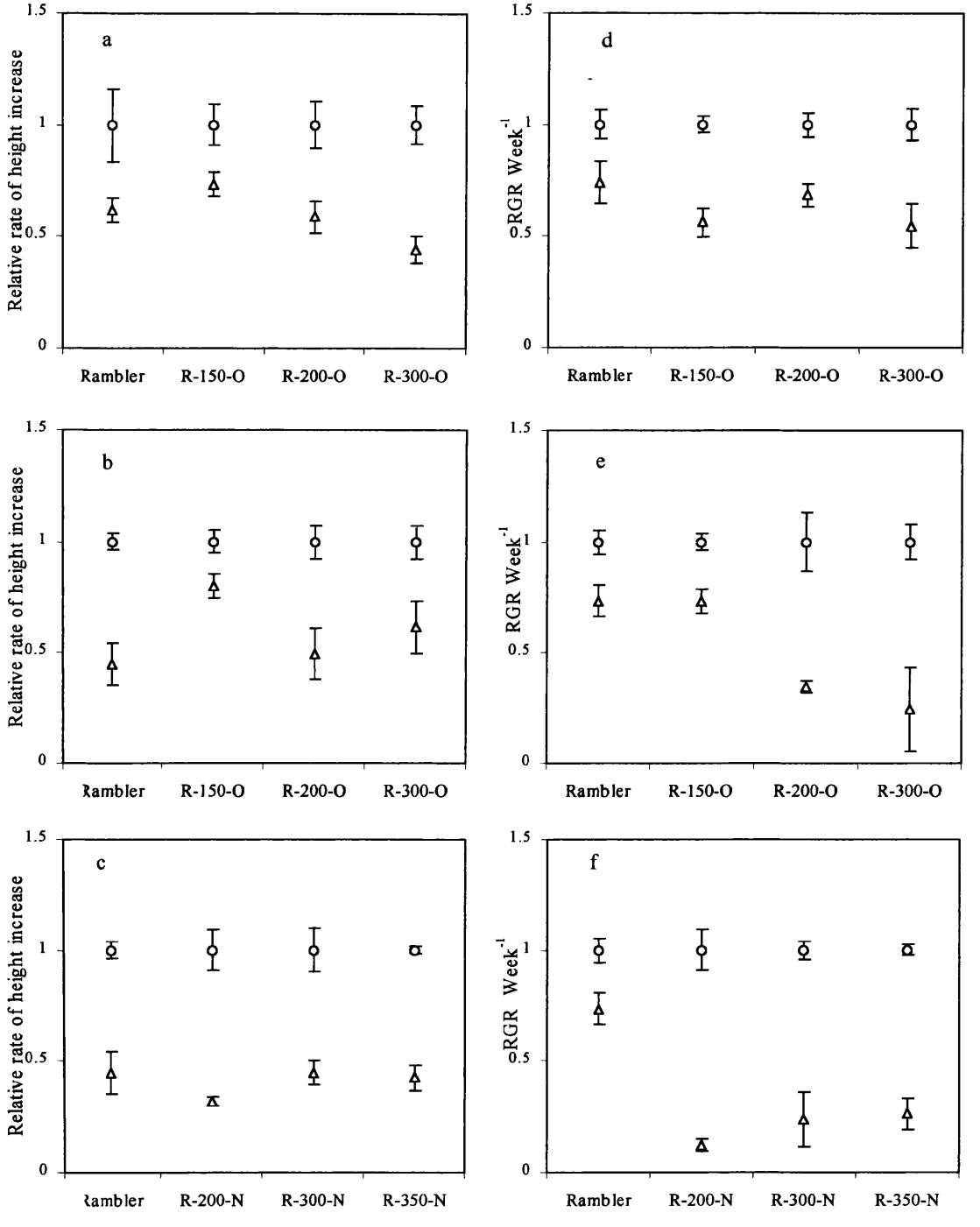


Fig. 3.5.2. Effect of pathogenicity of *V. albo-atrum*, isolate V1 towards lucerne plants cv. *M. media* strains. a, b, & c relative rate of height increase; d, e, & f index of RGR. a&d (first year); b&e (third year); c&f (new salt line) results. (o-control; Δ-inoculated plants). Values plotted are means ± SE.

Table 3.5.1. Effect of *V. albo-atrum*, isolate V1, on the relative rate of height increase of *M. media* cv. Rambler strains.

Year	Strains	Treatments*		ANALYSIS OF VARIANCE				
1st		C	V1	SS	df	MS	*F*	*Sig.*
	Rambler	a	b	0.513	1	0.513	4.92	0.047
	R-150 mM-O	a	b	0.198	1	0.198	7.15	0.025
	R-200 mM-O	a	b	0.756	1	0.756	9.33	0.008
	R-300 mM-O	a	b	1.445	1	1.445	23.3	0.000
	Sig.+	ns	ns					
						2-Way interaction		
	Strains x Treatment			0.156	3	5E-02	0.72	0.539
3rd								
	Rambler	a	b	0.670	1	0.670	46.7	0.000
	R-150 mM-O	a	b	0.102	1	0.102	7.01	0.027
	R-200 mM-O	a	b	0.381	1	0.381	11.7	0.014
	R-300 mM-O	a	b	0.329	1	0.329	8.32	0.023
	Sig.+	ns	ns					
						2-Way interaction		
	Strains x Treatment			0.169	3	5E-02	2.38	0.088
New isolates								
	Rambler	a	b	0.670	1	0.670	46.7	0.000
	R-200 mM-N	a	b	1.176	1	1.176	30.8	0.000
	R-300 mM-N	a	b	0.610	1	0.610	13.8	0.000
	R-350 mM-N	a	b	1.131	1	1.131	14.6	0.000
	Sig.+	ns	ns					
						2-Way interaction		
	Strains x Treatment			3E-02	3	9E-03	0.37	0.774

* Between treatments, RGR of lucerne with the same letters are *not significantly different* from each other at 0.05 level.
+ One-Way ANOVA test between cultivars within a treatment at 0.05 level.
ns. Not significant

Table 3.5.2. Effect of *V. albo-atrum*, isolate V1, on the RGR of *M. media* cv. Rambler strains.

Year	Strains	Treatments*		ANALYSIS OF VARIANCE				
1st		C	V1	SS	df	MS	*F*	*Sig.*
	Rambler	a	b	0.238	1	0.238	5.32	0.038
	R-150 mM-O	a	b	0.553	1	0.553	38.7	0.000
	R-200 mM-O	a	b	0.369	1	0.369	15.2	0.002
	R-300 mM-O	a	b	0.576	1	0.576	13.7	0.003
	Sig.+	ns	ns					
						2-Way interaction		
	Strains x Treatment			9E-02	3	3E-02	0.96	0.417
3rd								
	Rambler	a	b	0.152	1	0.152	7.35	0.024
	R-150 mM-O	a	b	0.114	1	0.114	12.0	0.010
	R-200 mM-O	a	b	0.901	1	0.901	9.91	0.014
	R-300 mM-O	a	b	1.080	1	1.080	18.9	0.005
	Sig.+	ns	ns					
						2-Way interaction		
	Strains x Treatment			0.379	3	0.126	2.87	0.053
New isolates								
	Rambler	a	b	0.152	1	0.152	7.35	0.024
	R-200 mM-N	a	b	1.961	1	1.961	49.4	0.000
	R-300 mM-N	a	b	1.394	1	1.394	46.9	0.000
	R-350 mM-N	a	b	1.909	1	1.909	104	0.000
	Sig.+	ns	ns					
						2-Way interaction		
	Strains x Treatment			0.505	3	0.168	6.40	0.001

* Between treatments, RGR of lucerne with the same letters are *not significantly different* from each other at 0.05 level.
+ One-Way ANOVA test between cultivars within a treatment at 0.05 level.
ns. Not significant

RESULTS

CHAPTER IV

Effect of NaCl on plant development and fungal growth.

In subsequent experiments it is the intention to determine the combined effects of NaCl and pathogen on the growth of tomato and lucerne. Prior to those experiments the effects of NaCl on germination and development of lucerne- and on tomato seeds was assessed as a means to screen the tolerance of various cultivars to salinity. The effects of NaCl on fungal growth were also assessed, to determine what concentration of NaCl inhibits and reduces fungal germination and hyphal growth. The results of these experiments were then used to select the concentration of NaCl used in experiments involving plant-pathogen-salt interactions.

4.1. Effect of NaCl on germination and development of tomato seed.

4.1.1. Germination.

Before testing the pathogenicity of *V. albo-atrum* on various tomato cultivars under saline conditions, seeds of tomato were initially screened, in a germination assay, for their response to salinity and to water stress. Germination assays are a recognized method for assessing salt tolerance at seed germination stage (Carlson *et al.*, 1983) although the salt tolerance at this stage may not always reliably predict the tolerance status at other stages (Greenway & Munns, 1980). However, it was one of the aims of this experiment to identify such cultivars in order to test them in later experiments. In order to make a wide comparison between tomato cultivars, seeds were collected from different sources, Table 2.7.2. At the time of selection, little was known concerning their level of tolerance to salinity or drought conditions, or the level of their resistance to *V. albo-atrum*. Selection was made mainly on the basis of their commercial importance, availability and cost.

The main aim of this experiment was to determine the tolerance of these seeds to salinity prior to testing the resistance of the plants to *V. albo-atrum* under both saline and non-saline conditions, so that proper conclusions could be made about their resistance to the fungus and the combined effects of salinity and the fungus. *V. albo-atrum* is a soil borne pathogen and the fungus is widespread in moderate climate conditions. Similarly, tomato and lucerne are cultivated widely in those same climatic conditions. However, as discussed earlier, external factors such as salinity and drought may also represent a threat to such crops, in addition to the fungus. It is timely, therefore, that the effects of *V. albo-*

atrum on tomato and lucerne are assessed against under conditions of salinity and drought.

Viability of the seed stocks was routinely assessed with the tetrazolium method (see Materials and Methods) and found to be more than 85% for all the cultivars.

In order to study the effect of NaCl on tomato seedlings, seeds were germinated in a series of glass Petri dishes containing one of 0-, 50-, 100-, 150- and 200 mM NaCl (see Materials and Methods).

Samples of the six cultivars of tomato seeds, (Ailsa Craig, Sweet 100 F1, Simge F1, Fantastic F1, Edcawy, Margarita (Fa-558) and one cultivar of *L. lycopersicon*, were germinated over a 2-week period on filter paper containing the NaCl solution, in glass Petri dishes. At the end of that period, germination was assessed by counting germinated seeds. Tables 4.1.1 & 4.1.2 show the total germination and the result of analysis of variance respectively, for the tomato seeds after 2 weeks of incubation.

Cultivars showed significant differences in their ability to germinate on the various salt media ($P<0.001$, Table 4.1.2). Maximum differences were observed at 50, 100 and 150 mM NaCl concentration (Table 4.1.1). The germination of most cultivars showed significant differences from the control group at 100 and 150 mM NaCl, with the exception of Sweet 100 F1, which also showed a significant difference from the control group in the presence of 50 mM NaCl.

On average, 87% of the seeds germinated in the presence of 50 mM NaCl though the figure for Sweet 100 F1 was only 50%, making it significantly different from the control group (Table 4.1.2). This salt concentration corresponded to a 5.20 dS/m (EC) at 21 °C (Table 4.1.3). With 100 and 150 mM, NaCl level, the figure for average germination was decreased (48 and 17 %, respectively). In general, concentrations of NaCl over 50 mM caused a significant reduction in germination ($P<0.001$, Table 4.1.2). The lowest germination was observed with 200 mM NaCl (corresponding to 18.10 dS/m at 21 °C -Table 4.1.3), in which only 7% of the seeds germinated.

The relationship between percentage germination and the concentration of salt for any cultivar showed almost a quadratic fit. Correlations ranged from 0.93 to 0.98, which is a highly significant correlation. This meant that an increase in concentration of NaCl in the medium resulted in a significant reduction in germination of tomato seeds.

The concentration of NaCl that prevents 50% of the seeds from germinating (IC50) was estimated from the quadratic curves for each cultivar and are presented in Table 4.1.4. It is apparent that the germination of the cultivars Edcawy, Simge F1 and *L. lycopersicon* were less affected by NaCl-, while Fantastic F1 and Ailsa Craig were moderately –affected. In contrast, Sweet 100 F1 and Margarita were most affected by NaCl.

There were also highly significant interactions ($P<0.001$, Table 4.1.2) between tomato cultivars and NaCl concentration. This means that different cultivars responded to salinity differently, some of them were resistant and some of them were not.

4.1.2. Hypocotyl elongation.

To see the effect of NaCl after post-germination, the length of hypocotyl elongation was also recorded after 2 weeks incubation. In this case, all the seedlings showed severe effects at 50 mM NaCl concentration (Table 4.1.5) unlike the effects on their germination (Table 4.1.1). Most seedlings showed significant differences at 50 mM NaCl concentration ($P<0.01$, Table 4.1.6), whereas *L. lycopersicon* showed tolerance up to 150 mM NaCl.

There were also highly significant interactions ($P<0.01$) between cultivars and NaCl concentration (Table 4.1.6).

In general, cultivars of tomato were more tolerant to NaCl at germination stage than at the post germination stage. No hypocotyl elongation was recorded at NaCl concentrations of 150 mM and above, whereas, *L. lycopersicon* showed some growth at 150 mM NaCl however, this growth was significantly different from the control group, ($P<0.05$, Table 4.1.6).

Table 4.1.1. Mean adjusted percent germination of tomato cultivars after 2 weeks incubation on saline conditions.[1]

Cultivars	NaCl (mM)				
	0	50	100	150	200
Ailsa Craig	100	93	14	0	0
Hybrid Sweet F1	100	50	25	0	0
Margarita (Fa-558)	100	92	8	0	0
Fantastic F1	100	100	42	0	0
L. lycopersicon	100	86	71	42	29
Simge F1	100	86	72	29	0
Edcawy	100	100	100	50	20
Mean	100	87	48	17	7

[1]Germination expressed as a percent of the no-salt control within each cultivar.

Table 4.1.2. Analysis of variance of the effects of [NaCl] on the germination of tomato cultivars grown at 0-, 50-, 100-, 150-, 200- mM NaCl for 2 weeks.

Cultivars	[NaCl] mM*					ANALYSIS OF VARIANCE				
	0	50	100	150	200	SS	df	MS	*F*	*Sig.*
Ailsa Craig	a	a	b	b	b	20489	4	5122	50.2	0.000
Hybrid Sweet F1	a	b	bc	c	c	14000	4	3500	14.0	0.006
Margarita Fa-558	a	a	b	b	b	20944	4	5236	94.2	0.000
Fantastic F1	a	a	b	c	c	20099	4	5024	226	0.000
L. lycopersicon	a	a	ab	bc	c	7020	4	1755	7.16	0.027
Simge F1	a	a	b	b	b	13881	4	3470	14.0	0.006
Edcawy	a	a	a	b	c	4040	4	2760	69.0	0.000
							2-Way Interaction			
					Cultivars	12554	6	2092	15	0.000
					[NaCl]	94494	4	23623	171	0.000
					Cultivars x [NaCl]	12981	24	540	4	0.000

*Between NaCl concentrations, percentage of germination of tomato cultivars with the same letters are *not significantly different* from each other at 0.05 level.

Table 4.1.3. Electrical conductivity of various salt solutions at 21 °C.

NaCl (mM)	EC (dS/m)
0	0.01
25	2.32
50	5.20
100	9.40
150	14.35
200	18.10
250	23.10
300	27.80
350	-[1]

[1]Electrical conductivity (EC) could not have been measured because of the high salinity.

Table 4.1.4. Concentration of NaCl which would reduce germination by 50% (IC50) for tomato cultivars.

Cultivars	IC50 (NaCl mM)
Ailsa Craig	70
Hybrid Sweet F1	58
Margarita (Fa-558)	65
Fantastic F1	95
L. lycopersicon	146
Simge F1	128
Edcawy	165

Table 4.1.5. Mean adjusted hypocotyl length of tomato cultivars after 2 weeks incubation on saline conditions.[1]

Cultivars	NaCl (mM)				
	0	50	100	150	200
Ailsa Craig	1.00	0.69	0.32	0	0
Hybrid Sweet F1	1.00	0.77	0.32	0	0
Margarita (Fa-558)	1.00	0.83	0.04	0	0
Fantastic F1	1.00	0.85	0.07	0	0
L. lycopersicon	1.00	0.79	0.64	0.53	0.26
Simge F1	1.00	0.57	0.15	0.04	0
Edcawy	1.00	0.70	0.10	0.07	0.03
Mean	1.00	0.74	0.23	0.10	0.04

[1]Hypocotyl expressed as 1 unit of the no-salt control within each cultivar.

Table 4.1.6. Analysis of variance of the effects of [NaCl] on the hypocotyl elongation of tomato cultivars grown at 0-, 50-, 100-, 150-, 200- mM NaCl for 2 weeks.

Cultivars	[NaCl] mM*					ANALYSIS OF VARIANCE				
	0	50	100	150	200	SS	df	MS	*F*	*Sig.*
Ailsa Craig	a	b	c	d	d	1.537	4	0.384	234	0.000
Hybrid Sweet F1	a	b	c	d	d	1.648	4	0.412	168	0.000
Margarita Fa-558	a	b	c	c	c	1.980	4	0.495	668	0.000
Fantastic F1	a	b	c	d	d	1.981	4	0.495	1610	0.000
L. lycopersicon	a	ab	abc	bc	c	0.610	4	0.153	5.29	0.048
Simge F1	a	b	c	d	d	1.465	4	0.366	399	0.000
Edcawy	a	b	c	c	c	1.571	4	0.393	331	0.000
							2-Way Interaction			
					Cultivars	0.60	6	0.10	19	0.000
					[NaCl]	10.1	4	2.54	492	0.000
					Cultivars x [NaCl]	0.629	24	2E-02	5.0	0.000

*Between NaCl concentrations, hypocotyl length of tomato cultivars with the same letters are *not significantly different* from each other at 0.05 level.

4.1.3. The effect of NaCl and mannitol on germination and development of tomato seeds in soil.

The previous experiment had shown that NaCl inhibited germination of the selected tomato seeds, to a greater or lesser extent. That experiment was carried out *in vitro* rather than the conditions normally encountered by tomato seeds. Furthermore, two factors may have been operating in that experiment; the toxicity of the NaCl and the osmotic potential of the solutions used. In order to learn more about the inhibitory effect of NaCl on germination NaCl on germination was assessed using seeds germinated in soil. In addition, the effect of mannitol, as an osmoticum, on germination was also assessed.

The percentage germination was recorded over a 13-day (see Materials and Methods). The total percentage germination for each cultivar at the end of the experiment is presented in Table 4.1.7, while the cumulative percentage germination is shown in Figs. 4.1.1-4.1.5.

Germination of the seeds from the control treatments were assigned a value of 100 and the germination observed in the other treatments of the same cultivars is expressed as a percentage of that. Whichever cultivar is considered, the percentage germination decreased with increasing- NaCl or mannitol concentration, (Figs. 4.1.1. – 4.1.5, Table 4.1.7), though some were more affected than others. Margarita (Fa-558) and Fantastic F1 showed similar responses to salinity and were more affected by 75 mM NaCl than Ailsa Craig, Simge F1 and *L. lycopersicon.* However, in the presence of 100 mM NaCl, the percentage germination of all the cultivars decreased to a minimum, with Fantastic F1 being most affected (5%, Table 4.1.7), though *L. lycopersicon* was the most resistant

Mannitol, used at the same osmotic potential as the NaCl, also caused a significant decrease in germination over the 2-week period of the experiment (Figs. 4.1.1-4.1.5 and Table 4.1.7), though with the exceptions of Ailsa Craig at –0.36 MPa and *L. lycopersicon* at both –0.36- and –0.48 MPa the effect of mannitol was not as severe as NaCl. The data suggest, therefore, that at least a part of the inhibition caused by NaCl resulted from the toxicity of the ions, rather than from an osmotic effect. The results of the determination of cumulative germination, normalized for each cultivar to a figure of 100 for the control (distilled water), are presented in Figs. 4.1.1 - 4.1.5. In the cases of

Margarita (Fa-558), Fantastic F1 and Ailsa Craig the majority of germination in the control treatments occurs over the period 4 to 8 days after sowing. In the cases of Simge F1 and *L. lycopersicon* maximum germination is not achieved until day 10. NaCl significantly reduced the percentage germination whether it was used at 75- or 100 mM. In some cases, the time taken for germination to occur was increased. The exception was Simge F1 in the presence of either 75- or 150 mM NaCl (-0.36- & -0.48 MPa respectively, Fig. 4.1.4), in which the maximum level of germination was achieved before the control plants, albeit at a lower level. In the case of Fantastic F1, there was an increase in the initial time taken for germination to occur (Fig. 4.1.2). The data is expressed in Table 4.1.8 as the time taken for 50% germination to occur and is compared with the same data for treatment with mannitol. Few generalizations can be made, though the delay caused by isoosmotic mannitol appears not to be so marked as with NaCl, except in the case of Ailsa Craig at 150 mM- and *L. lycopersicon* at 200 mM mannitol (-0.36- & -0.48 MPa respectively). Again, this suggests that the effect of NaCl results both from toxicity of the ions and an osmotic effect.

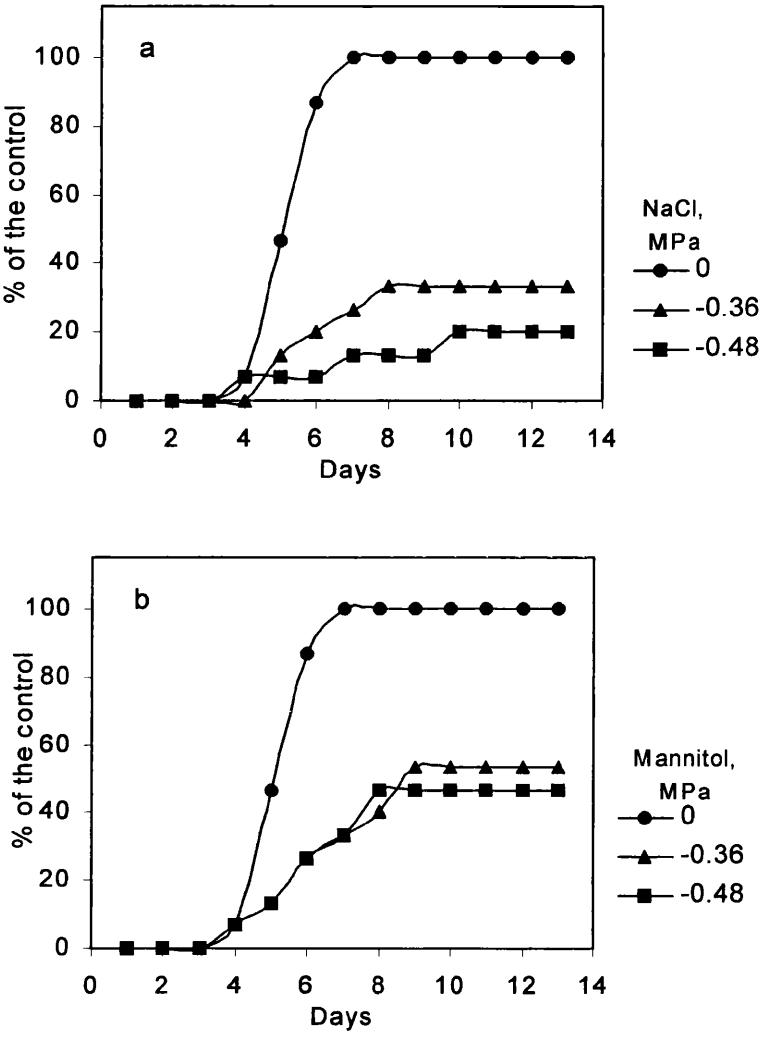


Fig. 4.1.1. Germination of seeds of Margarita (Fa-558) in response to a) NaCl and b) mannitol at different osmotic potentials (MPa).

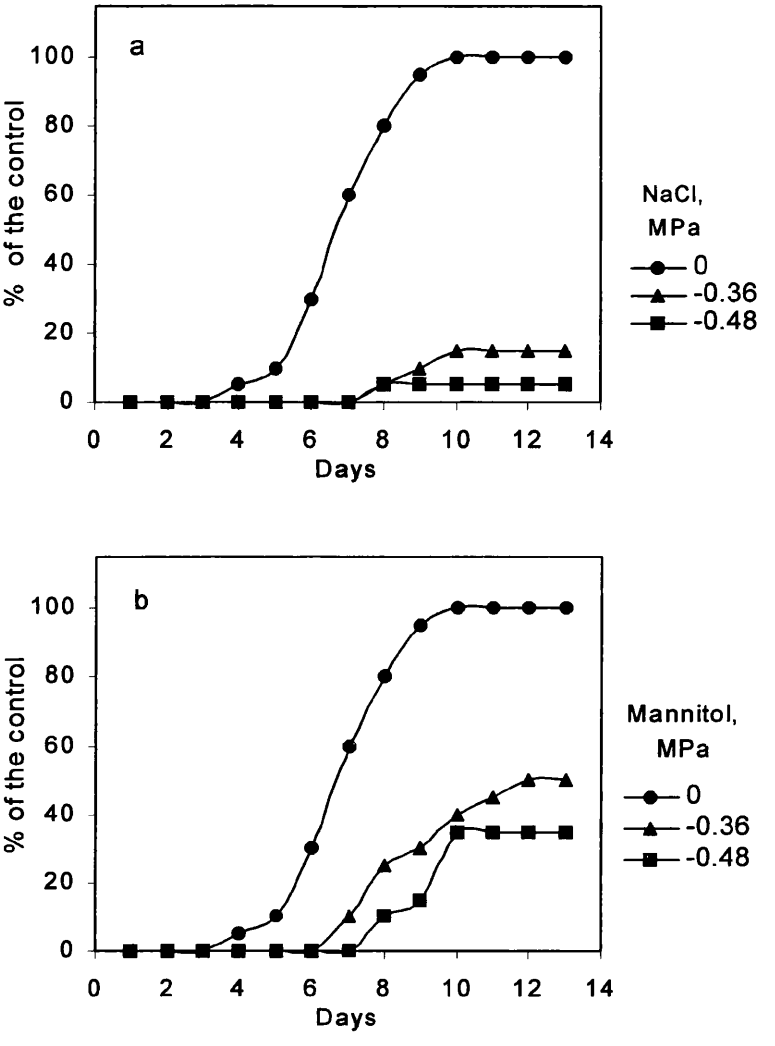


Fig. 4.1.2. Germination of seeds of Fantastic F1 in response to a) NaCl and b) mannitol at different various osmotic potentials (MPa).

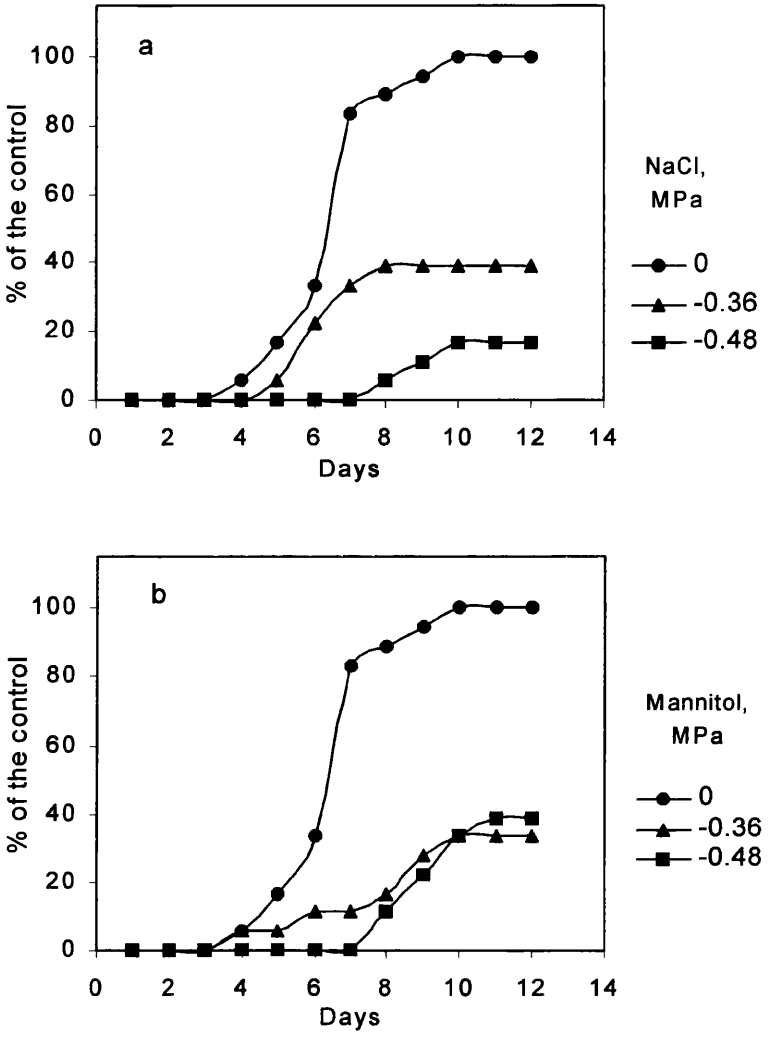


Fig. 4.1.3. Germination of seeds of Ailsa Craig in response to a) NaCl and b) mannitol at different osmotic potentials (MPa).

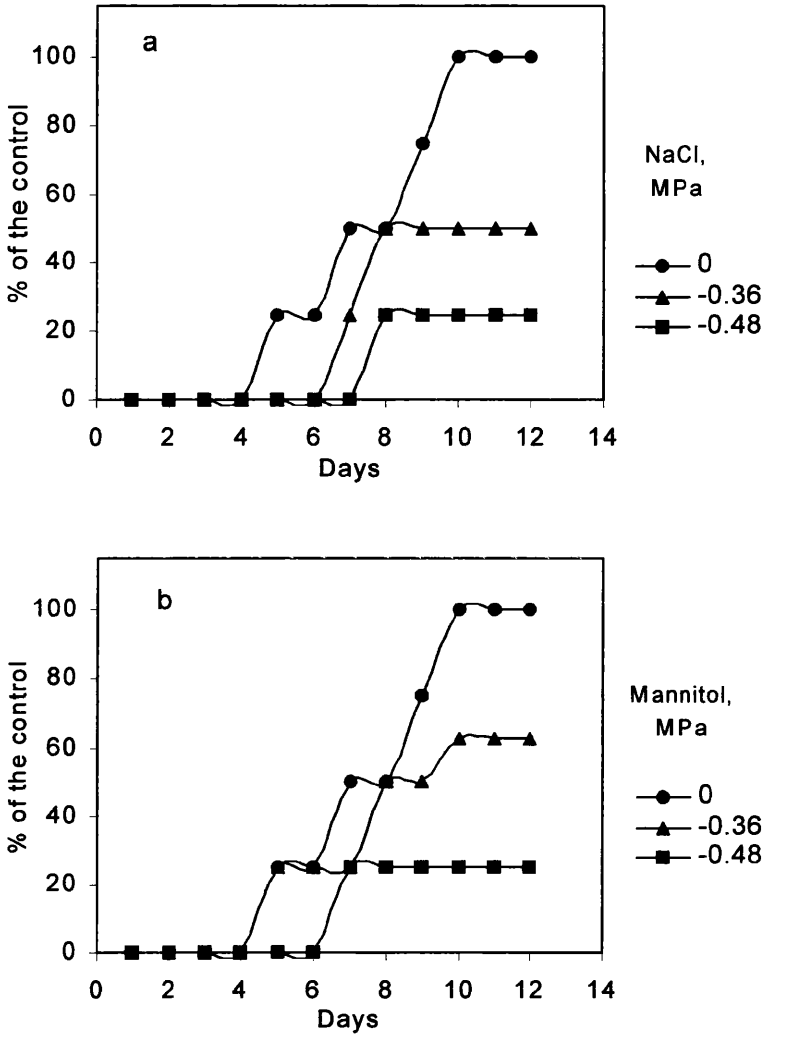


Fig. 4.1.4. Germination of seeds of Simge F1 in response to a) NaCl and b) mannitol at different osmotic potentials (MPa).

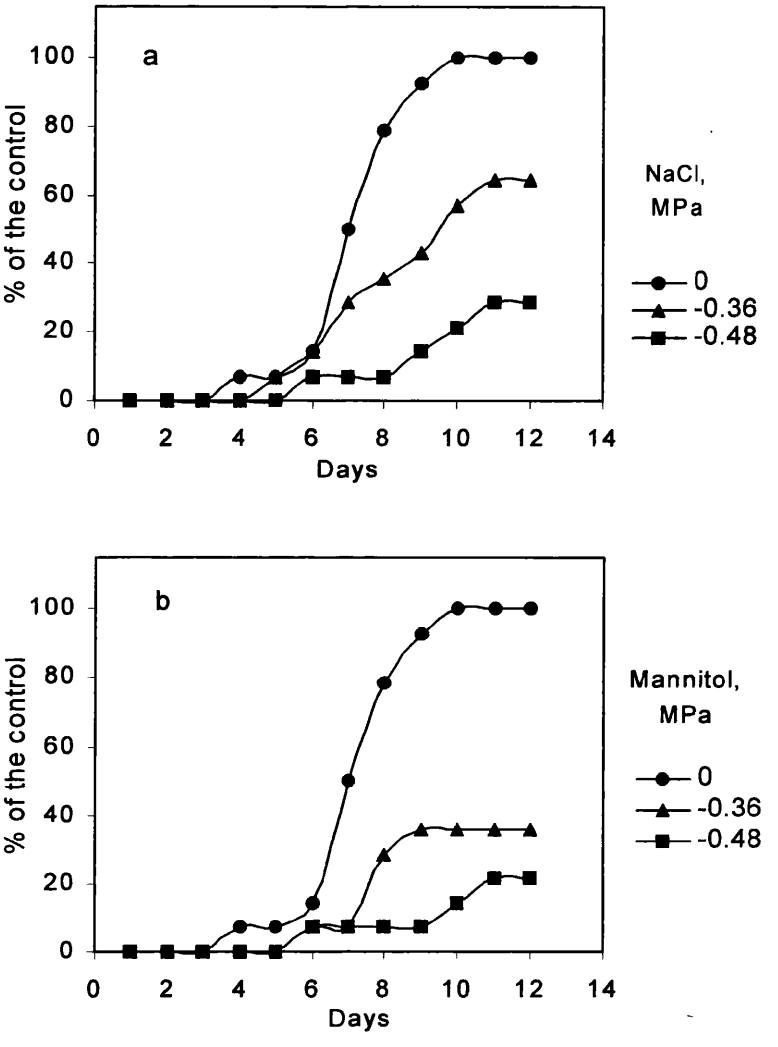


Fig. 4.1.5. Germination of seeds of *L. lycopersicon* in response to a) NaCl and b) mannitol at different osmotic potentials (MPa).

In general, percentage germination of tomato seeds under NaCl or mannitol treatment was significantly increased over time and was significantly decreased with increasing salt or mannitol concentration (Figs. 4.1.1-4.1.5).

The results of the germination experiment indicated that NaCl or mannitol were inhibitory to the tomato seeds. The effects of NaCl or mannitol were also observed after post-emergence such as hypocotyl elongation.

The effects of NaCl or mannitol on hypocotyl elongation are presented in Table 4.1.9. Hypocotyl elongation was inhibited by treatment with NaCl in all cultivars in a pattern that was closely similar to its effect on germination (Table 4.1.7); Fantastic F1 treated with 100 mM NaCl showed the greatest inhibition (final hypocotyl length was 3% of the control treatment), while *L. lycopersicon* showed the most resistance (final hypocotyl length was 19.5% of the control treatment). Similarly, mannitol at the same osmotic potentials inhibited hypocotyl elongation, though as in the case of its effect on germination, not to the same extent as NaCl. Again, this data suggests that in the case of NaCl both toxic effects from the ions and osmotic potential play a part in the inhibition. Curiously, hypocotyl elongation in *L. lycopersicon* was more affected by the higher concentration of mannitol than it was by the iso-osmotic concentration of NaCl, just as was the case with seed germination (Table 4.1.7).

Table 4.1.7. The effect of NaCl or mannitol on percentage seed germination of cultivars of *L. esculentum* and *L. lycopersicon*. Percentage germination was calculated from 20 seeds per cultivar per treatment after 13-day incubation. Control mean germination was assigned a value of 100 and the germination observed in the other treatments of the same cultivars is expressed as a percentage of that.

NaCl (mM)	Osmotic Potential (MPa)	Margarita (Fa-558)	Fantastic F1	Ailsa Craig	Simge F1	*L. lycopersicon*
0	0	100	100	100	100	100
75	-0.36	33	15	39	50	64
100	-0.48	20	5	17	25	29
Mannitol (mM)	Osmotic Potential MPa	Margarita (Fa-558)	Fantastic F1	Ailsa Craig	Simge F1	*L. lycopersicon*
0	0	100	100	100	100	100
150	-0.36	53	50	33	63	36
200	-0.48	47	35	38	25	21

Table 4.1.8. Number of days taken for 50% germination of seeds of cultivars of *L. esculentum* and *L. lycopersicon* treated with NaCl or mannitol.

NaCl (mM)	Osmotic Potential MPa	Margarita (Fa-558)	Fantastic F1	Ailsa Craig	Simge F1	*L. lycopersicon*
0	0	4	5	5	5	6
75	-0.36	5	7	5	6	6
100	-0.48	5	7	9	6	7
Mannitol (mM)	Osmotic Potential MPa	Margarita (Fa-558)	Fantastic F1	Ailsa Craig	Simge F1	*L. lycopersicon*
0	0	4	5	5	5	6
150	-0.36	5	7	6	5	6
200	-0.48	5	7	8	5	9

Table 4.1.9. The effect of NaCl or mannitol on hypocotyl length (mm) of cultivars of *L. esculentum* and *L. lycopersicon* after 2 weeks incubation.

NaCl (mM)	Osmotic Potential MPa	Margarita (Fa-558)	Fantastic F1	Ailsa Craig	Simge F1	*L. lycopersicon*
0	0	54	67	63	73	77
75	-0.36	11	6	22	18	38
100	-0.48	10	2	6	9	15
Mannitol (mM)	Osmotic Potential (MPa)	Margarita (Fa-558)	Fantastic F1	Ailsa Craig	Simge F1	*L. lycopersicon*
0	0	54	67	63	73	77
150	-0.36	28	25	17	29	19
200	-0.48	25	19	22	15	10

4.2. The effect of NaCl on germination and development of lucerne.

4.2.1. Germination.

The main aim in this study, is to see the tolerance level of seeds to NaCl and choose the resistant and susceptible ones for further experiments for a number of experimental approaches.

Little had been established concerning the tolerance to salinity or resistance to VW of the range of lucerne seeds that were available in this project Table 2.7.1. So, prior to their use in later experiments, the seeds of the various cultivars were screened to determine the effect of salinity and mannitol (which simulates water stress), on their germination. Again, the purpose of this assessment was to identify suitable plants for use in experiments designed to investigate resistance to the combined effects of a pathogen and salinity.

The seeds were germinated on filter paper in glass Petri dishes, as in the experiment with tomato seeds, described above, and incubated for 1 week with different concentrations (0, 25, 50, 100, 150, 200, 250, 300 and 350 mM) of NaCl (see Materials and Methods).

The percentage germination and the analysis of variance are presented in Tables 4.2.1 & 4.2.2. Hypocotyl- and radicle length and the respective analysis of variance are presented in Tables 4.2.4-4.2.7, respectively.

Seeds of cv. Kabul failed to germinate in concentrations of NaCl above 100 mM. Subsequently, it was established that the germination rate was less than 40% in the absence of NaCl. Consequently no data is presented for Kabul.

The remaining cultivars showed significant differences with regard to their ability to germinate on media containing NaCl (Tables 4.2.1 & 4.2.2). The biggest differences were observed in the treatments in which the concentration of NaCl was 150 mM or higher, (Tables 4.2.1 & 4.2.2). While small decreases in percent germination compared to the control occurred at 25- and 50 mM NaCl, with the exceptions of Europe, Vela and Rambler more than 90% of the seeds germinated at these concentrations. Up to 150 mM further increases in NaCl concentration caused only small decreases in the germination rate of the seeds. However, above 150 mM NaCl there was a marked, decrease in germination for most of the seeds and most of the cultivars showed significant differences

from their control groups ($P<0.001$, Table 4.2.2). In the presence of 350 mM NaCl none of the seeds germinated. Cultivars Euver, Vela, Mesa Sirsa, 13R Supreme and Lobo showed the greatest tolerance to the effects of 300 mM NaCl, 13R Supreme showing 43% of the germination of the control at that concentration. Curiously, Vela was one of three cultivars, including Rambler and Bitlis the germination of which was less than 70% in the presence of 150 mM NaCl.

At the same concentration Peru, Mesa Sirsa, Lobo, Vertus and SA Standard showed germination rates of 90% or better compared to the controls, yet at 300 mM NaCl the germination for Peru and Vertus was 8% and for SA Standard it was 0. Clearly there is not a straightforward linear relationship between the concentration of NaCl and its effect on germination across the range of cultivars tested. In some cases the change in germination caused by increasing NaCl is stepped, suggesting the presence either of a protective- or adaptive mechanism.

Response of lucerne seeds to NaCl was similar to the response of tomato seeds to NaCl. The graph showed quadratic shape between percentage germination and NaCl concentrations. Correlations of the germination of seeds under various NaCl conditions were significantly high ranging from 0.94 to 0.99.

The data from Table 4.2.1 was plotted as a series of graphs and were used to calculate the concentration of NaCl, which would inhibit germination of the seeds by 25- and 50% (IC25 and –50, respectively), (Table 4.2.3). From this table four cultivars emerge as showing particular sensitivity to the effects of low concentration of NaCl; Vela, Rambler, Bitlis and AC Blue J, with IC25s of 71.2- 84.0-, 79.1- and 106.5 mM (compared to a mean for the group as a whole of 160 mM. This position is reflected in the values for IC50 (164.8-, 164.5-, 164.5- and 174.3 mM for Vela, Rambler, Bitlis and AC Blue J respectively, compared to a value of 230 mM for the whole group). These differences in the response of the individual cultivars to treatment with NaCl is highly significant ($P<0.001$, Table 4.2.2).

4.2.2. Hypocotyl elongation.

In addition to measurement of germination, the length of the hypocotyl and radicle were also recorded, at the end of the experiment, Tables 4.2.4-4.2.7, in order to assess the effect on these two growing tips of the seedling. Again, increasing concentration of NaCl caused a decrease in hypocotyl and radicle length. In contrast to the effects of NaCl on germination (Tables 4.2.1 & 4.2.2) where many cultivars were tolerant to 150mM NaCl and a few of them were tolerant to 200 mM NaCl, Tables 4.2.5 & 4.2.7, 50% of the cultivars showed significant differences in the length of their hypocotyls from their control groups at 50 mM NaCl and all of them at 100 mM NaCl. Similar results were obtained for radicle length; 50% of the cultivars showed significant differences in the length of their hypocotyls from their control groups at 50 mM NaCl and all of them at 100 mM NaCl (Tables 4.2.6 & 4.2.7).

In general, when comparison was made, in terms of hypocotyl or radicle length, all the cultivars from the USA showed greater tolerance to NaCl than those of other cultivars. From other countries, only Vertus and Bilensoy-80, Barrier and SA Standard showed tolerance up to 100 mM NaCl ($P<0.001$, Table 4.2.5 & 4.2.7).

The interaction between cultivars and NaCl concentrations was found important in hypocotyl length, however this case was not important in radicle length measurement ($P<0.001$, Table 4.2.5 & 4.2.7).

Table 4.2.1. Mean adjusted percent germination of 18 lucerne cultivars after one-week incubation in saline conditions. †

Cultivars	NaCl (mM)								
	0	25	50	100	150	200	250	300	350
Euver	100	100	90	78	78	78	14	17	0
Europe	100	89	89	89	80	82	62	4	0
Vela	100	97	66	62	62	31	31	17	0
Rambler	100	90	85	85	50	30	20	5	0
Vertus	100	100	98	94	90	90	79	8	0
Elci	100	92	90	88	88	86	66	0	0
Peru	100	100	100	98	96	94	73	8	0
Bilensoy-80	100	100	100	87	83	80	40	7	0
Mesa Sirsa	100	100	98	92	90	88	81	13	0
Kayseri	100	98	94	89	87	87	60	6	0
Bitlis	100	94	94	88	53	31	6	3	0
13R Supreme	100	100	100	90	87	80	53	43	0
Redgreen	100	95	88	86	86	71	52	2	0
Protea	100	97	94	81	77	77	35	3	0
Lobo	100	95	95	95	92	81	65	14	0
SA Standard	100	98	95	93	93	86	20	0	0
Barrier	100	100	100	98	83	68	24	5	0
AC Blue J	100	97	94	94	75	39	0	0	0
Mean	100	97	93	88	81	71	44	9	0

† Germination expressed as a percent of the no-salt control within each cultivar.

Table 4.2.2. Analysis of variance of the effects on the germination of lucerne seeds of NaCl (at 0-, 25-, 50-, 100-, 150-, 200-, 250-, 300- and 350 mM. Seeds were grown for one-week.

Cultivars	[NaCl] mM*									ANALYSIS OF				
	0	25	50	100	150	200	250	300	350	SS	df	MS	*F*	*Sig.*
Euver	a	a	a	a	a	a	b	b	b	25396	8	4174	11.8	0.001
Europe	a	a	a	a	ab	ab	b	c	c	22691	8	2836	25.3	0.000
Vela	a	a	b	bc	bc	cd	cd	d	d	18929	8	2366	13.7	0.000
Rambler	a	a	ab	ab	bc	cd	cd	d	d	24700	8	3087	12.4	0.001
Vertus	a	a	ab	ab	ab	ab	b	c	c	25216	8	3152	453	0.000
Elci	a	ab	ab	ab	ab	ab	b	c	c	24919	8	3114	22.0	0.000
Peru	a	a	a	a	a	a	b	c	c	26510	8	3313	229	0.000
Bilensoy-80	a	a	a	a	a	a	b	c	c	25886	8	3235	35.4	0.000
Mesa Sirsa	a	a	a	ab	ab	ab	b	c	c	23937	8	2992	66.0	0.000
Kayseri	a	a	a	a	a	a	b	c	c	24501	8	3062	28.1	0.000
Bitlis	a	a	a	a	b	c	d	d	d	29335	8	3666	44.4	0.000
13R Supreme	a	a	a	ab	ab	abc	bc	c	d	18612	8	2326	7.98	0.003
Redgreen	a	a	a	ab	ab	ab	b	c	c	23751	8	2968	14.9	0.000
Protea	a	a	a	a	a	a	b	b	b	24936	8	3117	10.4	0.001
Lobo	a	a	a	a	a	ab	b	c	c	22861	8	2857	27.0	0.000
SA Standard	a	a	a	a	a	a	b	c	c	31404	8	3925	65.7	0.000
Barrier	a	a	a	a	a	a	b	b	b	29101	8	3637	19.7	0.000
AC Blue J	a	a	a	a	b	c	d	d	d	33302	8	4162	93.3	0.000
Sig. +	ns	ns	ns	ns	ns	ns	s	s	ns					
												2-Way Interaction		
										Cultivars	17310	17	1018	7.22	0.000
										[NaCl]	420110	8	52513	372	0.000
										Cultivars x [NaCl]	35887	136	264	1.87	0.000

* Between NaCl concentrations, percentage germination of lucerne seeds with the same letters are *not significantly different* from each other at 0.05 level.
+ One-Way ANOVA test between cultivars within a [NaCl] at 0.05 level.
s. Significant
ns. Not significant

Table 4.2.3. Concentration of NaCl calculated to reduce germination by 25 (IC25) and 50% (IC50) for lucerne cultivars. Values presented are ± SE of the mean.

Cultivars	NaCl (mM)	
	IC (25)	IC (50)
Euver	139.7 ± 2.9	219.6 ± 8.4
Europe	181.4 ± 1.1	246.4 ± 2.3
Vela	71.2 ± 2.9	164.8 ± 1.8
Rambler	84.0 ± 18	164.5 ± 20
Vertus	220.1 ± 0.2	273.1 ± 2.8
Elci	198.1 ± 17	257.9 ± 13
Peru	215.5 ± 5.7	264.0 ± 6.3
Bilensoy-80	175.6 ± -	244.2 ± -
Mesa Sirsa	215.9 ± 9.0	269.5 ± 1.6
Kayseri	199.9 ± 6.9	260.5 ± 5.9
Bitlis	79.1 ± 16	164.5 ± 12
13R Supreme	199.5 ± 9.0	265.3 ± 3.0
Redgreen	167.8 ± 1.6	237.7 ± 1.2
Protea	151.0 ± 33	225.7 ± 9.9
Lobo	203.8 ± 7.2	261.6 ± 3.4
SA Standard	172.8 ± 5.1	234.9 ± 1.9
Barrier	156.1 ± 3.7	202.1 ± 1.9
AC Blue J	106.5 ± 10	174.3 ± 6.2
Mean	160	230

† Germination expressed as a percent of the no-salt control within each cultivar.

Table 4.2.4. Mean hypocotyl length of 18 lucerne cultivars after one week incubation on saline conditions. †

Cultivars	NaCl (mM)								
	0	25	50	100	150	200	250	300	350
Euver	1.00	0.81	0.73	0.64	0.55	0.32	0.12	0.05	0
Europe	1.00	0.81	0.76	0.56	0.51	0.32	0.16	0.03	0
Vela	1.00	0.90	0.65	0.64	0.50	0.17	0.12	0.09	0
Rambler	1.00	0.88	0.57	0.55	0.39	0.23	0.04	0.03	0
Vertus	1.00	1.00	0.93	0.76	0.59	0.50	0.29	0.15	0
Elci	1.00	0.98	0.79	0.72	0.59	0.52	0.26	0	0
Peru	1.00	0.81	0.75	0.69	0.53	0.48	0.20	0.06	0
Bilensoy-80	1.00	1.00	1.00	0.89	0.68	0.41	0.11	0.00	0
Mesa Sirsa	1.00	1.00	0.70	0.56	0.49	0.46	0.32	0.06	0
Kayseri	1.00	0.91	0.64	0.51	0.36	0.29	0.19	0.09	0
Bitlis	1.00	0.98	0.83	0.76	0.27	0.11	0.00	0.00	0
13R Supreme	1.00	0.95	0.88	0.77	0.51	0.37	0.19	0.17	0
Redgreen	1.00	1.03	1.00	0.90	0.68	0.44	0.27	0.04	0
Protea	1.00	0.83	0.81	0.66	0.38	0.35	0.13	0.01	0
Lobo	1.00	0.97	0.92	0.56	0.37	0.31	0.16	0.06	0
SA Standard	1.00	0.94	0.94	0.79	0.54	0.30	0.09	0.00	0
Barrier	1.00	0.98	0.98	0.63	0.63	0.39	0.23	0.09	0
AC Blue J	1.00	1.00	0.61	0.57	0.41	0.31	0.00	0.00	0
Mean	1.00	0.93	0.81	0.68	0.50	0.35	0.16	0.05	0

† For each cultivar hypocotyl length expressed relative to a unit of 1 for the no-salt control.

Table 4.2.5. Analysis of variance of the effects of [NaCl] on the hypocotyl length of lucerne cultivars grown at 0-, 25-, 50-, 100-, 150-, 200-, 250-, 300- and 350 mM NaCl for a period of 1 week.

Cultivars	[NaCl] mM*									ANALYSIS OF				
	0	25	50	100	150	200	250	300	350	SS	df	MS	*F*	*Sig.*
Euver	a	ab	b	b	bc	cd	de	de	e	2.087	8	0.261	21.0	0.000
Europe	a	ab	bc	cd	de	ef	gh	h	h	1.969	8	0.246	24.4	0.000
Vela	a	a	b	b	b	c	cd	cd	d	2.211	8	0.276	64.1	0.000
Rambler	a	a	b	b	bc	cd	d	d	d	2.195	8	0.274	17.6	0.000
Vertus	a	a	ab	b	c	c	d	de	e	2.255	8	0.282	50.8	0.000
Elci	a	a	b	b	c	c	d	e	e	2.341	8	0.293	170	0.000
Peru	a	ab	bc	bcd	cd	d	e	e	e	1.944	8	0.243	26.4	0.000
Bilensoy-80	a	a	a	b	c	d	e	f	f	3.130	8	0.391	598	0.000
Mesa Sirsa	a	a	b	c	c	c	d	e	e	2.040	8	0.255	70.1	0.000
Kayseri	a	a	b	bc	cd	de	ef	gh	h	1.963	8	0.245	54.0	0.000
Bitlis	a	a	b	c	d	e	f	f	f	3.173	8	0.397	1110	0.000
13R Supreme	a	ab	ab	b	c	cd	de	de	e	2.253	8	0.282	37.2	0.000
Redgreen	a	a	a	b	c	d	e	f	f	2.823	8	0.353	239	0.000
Protea	a	ab	ab	b	c	c	d	d	d	2.255	8	0.282	40.6	0.000
Lobo	a	a	a	b	c	c	d	de	e	2.518	8	0.315	90.7	0.000
SA Standard	a	ab	ab	b	c	d	e	e	e	2.864	8	0.358	51.6	0.000
Barrier	a	a	a	b	b	c	cd	de	e	2.455	8	0.307	46.0	0.000
AC Blue J	a	a	b	b	c	c	d	d	d	2.541	8	0.318	85.7	0.000
Sig. +	ns	ns	s	s	s	s	s	s	ns					
												2-Way Interaction		
										Cultivars	0.916	17	5E-02	9.27	0.000
										[NaCl]	41.33	8	5.1660	888	0.000
										Cultivars x [NaCl]	1.686	136	1E-02	2.13	0.000

* Between NaCl concentrations, percentage germination of lucerne seeds with the same letters are *not significantly different* from each other at 0.05 level.
+ One-Way ANOVA test between cultivars within a [NaCl] at 0.05 level.
s. Significant
ns. Not significant

Table 4.2.6. Mean radicle length of 18 lucerne cultivars after one week incubation in saline conditions. †

Cultivars	NaCl (mM)								
	0	25	50	100	150	200	250	300	350
Euver	1.00	0.78	0.52	0.42	0.35	0.09	0.06	0.03	0
Europe	1.00	0.77	0.47	0.40	0.23	0.14	0.11	0.03	0
Vela	1.00	0.97	0.74	0.52	0.42	0.18	0.16	0.11	0
Rambler	1.00	0.85	0.44	0.26	0.29	0.12	0.03	0.03	0
Vertus	1.00	0.93	0.73	0.54	0.54	0.44	0.15	0.05	0
Elci	1.00	0.92	0.76	0.64	0.60	0.40	0.24	0.00	0
Peru	1.00	0.61	0.48	0.48	0.40	0.31	0.09	0.04	0
Bilensoy-80	1.00	0.98	0.95	0.75	0.60	0.35	0.09	0.00	0
Mesa Sirsa	1.00	0.77	0.65	0.35	0.33	0.30	0.14	0.05	0
Kayseri	1.00	0.94	0.86	0.69	0.61	0.50	0.25	0.08	0
Bitlis	1.00	0.94	0.88	0.75	0.25	0.08	0.00	0.00	0
13R Supreme	1.00	0.91	087	0.75	0.33	0.19	0.05	0.05	0
Redgreen	1.00	0.99	0.87	0.56	0.42	0.37	0.10	0.02	0
Protea	1.00	0.94	0.74	0.54	0.54	0.21	0.08	0.03	0
Lobo	1.00	0.96	0.70	0.62	0.58	0.36	0.19	0.07	0
SA Standard	1.00	0.95	0.91	0.73	0.66	0.14	0.09	0.00	0
Barrier	1.00	0.95	0.92	0.66	0.63	0.40	0.21	0.08	0
AC Blue J	1.00	0.97	0.70	0.6	0.50	0.33	0.00	0.00	0
Mean	1.00	0.89	0.74	0.57	0.46	0.27	0.11	0.03	0

† For each cultivar radicle length is expressed relative to a unit of 1 for the no-salt control.

Table 4.2.7. Analysis of variance of the effects of [NaCl] on the radicle length of lucerne cultivars grown at 0-, 25-, 50-, 100-, 150-, 200-, 250-, 300- and 350 mM NaCl for a period of 1 week.

Cultivars	[NaCl] mM*									ANALYSIS OF				
	0	25	50	100	150	200	250	300	350	SS	df	MS	*F*	*Sig.*
Euver	a	a	b	b	b	c	c	c	c	2.018	8	0.25	20.4	0.000
Europe	a	a	b	bc	bc	cd	d	d	d	1.922	8	0.24	17.2	0.000
Vela	a	ab	bc	cd	d	e	e	e	e	2.278	8	0.28	27.8	0.000
Rambler	a	ab	bc	bc	c	c	c	c	c	2.137	8	0.267	4.79	0.015
Vertus	a	a	ab	b	b	bc	cd	d	d	2.128	8	0.266	14.5	0.000
Elci	a	ab	bc	c	c	d	d	e	e	2.202	8	0.275	48.3	0.000
Peru	a	ab	bc	bc	bc	bc	c	c	c	1.614	8	0.202	4.81	0.015
Bilensoy-80	a	a	a	b	c	d	e	e	e	2.888	8	0.361	170	0.000
Mesa Sirsa	a	ab	b	c	cd	cd	cde	ef	f	1.857	8	0.232	15.5	0.000
Kayseri	a	ab	b	c	cd	d	e	f	f	2.185	8	0.273	99.3	0.000
Bitlis	a	ab	b	c	d	e	e	e	e	3.183	8	0.398	193	0.000
13R Supreme	a	a	ab	b	c	c	d	d	d	2.759	8	0.345	95.7	0.000
Redgreen	a	a	a	b	b	bc	cd	d	d	2.577	8	0.322	20.2	0.000
Protea	a	a	ab	b	b	c	c	c	c	2.441	8	0.305	17.6	0.000
Lobo	a	a	ab	b	b	bc	cd	cd	e	2.150	8	0.269	12.8	0.000
SA Standard	a	a	a	b	b	c	c	c	c	3.001	8	0.375	79.7	0.000
Barrier	a	a	a	b	b	c	cd	d	d	2.358	8	0.295	28.1	0.000
AC Blue J	a	a	b	b	bc	c	d	d	d	2.556	8	0.319	27.5	0.000
Sig. +	ns	ns	ns	s	s	s	s	ns	ns					
										2-Way Interaction				
										Cultivars	1.305	17	7E-02	5.22	0.000
										[NaCl]	40.07	8	5.00	340	0.000
										Cultivars x [NaCl]	2.179	136	1E-02	1.09	0.298

* Between NaCl concentrations, percentage germination of lucerne seeds with the same letters are *not significantly different* from each other at 0.05 level.
+ One-Way ANOVA test between cultivars within a [NaCl] at 0.05 level.
s. Significant
ns. Not significant

4.2.3. The effect of NaCl and mannitol on germination of lucerne in soil.

The previous experiment has established that NaCl is inhibitory to the germination of lucerne seeds and can affect the subsequent elongation of both radicle and hypocotyl *in vitro*. The following experiment was performed to establish the response to NaCl of lucerne seeds grown in soil. The effect of mannitol on germination was also assessed, to establish the osmotic effect on germination. Cultivars that showed a degree of high tolerance (Vertus and 13R Supreme), moderate tolerance (Mesa Sirsa) and susceptibility (Vela and Rambler) to NaCl, either in the germination or post-germination stage, were selected and used in this study.

The percentage germination was recorded over a 9-day period and the total percentage seed germination for each cultivar is presented in Table 4.2.8.

Although there was a decline in seed germination in response to treatment with NaCl (Figs. 4.2.1-4.2.5 & Tables 4.2.8 & 4.2.9), it was not as great as occurred in Petri dishes (above). With the exception of Vertus in the presence of 200 mM NaCl, in all cases in which the NaCl treatment was equivalent, inhibition was less when the seeds were germinated in soil. As in the experiment *in vitro*, 13R Supreme, Vertus and Mesa Sirsa showed some tolerance to salinity. However, in contrast to their germination *in vitro*, Vela and Rambler also showed a degree of tolerance. The rate at which germination occurred is not greatly affected by treatment with NaCl, as indicated by the calculations of the days taken to achieve 50% germination (Table 4.2.9).

Treatment with mannitol, at the same osmotic potentials as the NaCl used in the experiment, also inhibited germination (Figs. 4.2.1-4.2.5 & Tables 4.2.8 & 4.2.9). In all cases, the effect of mannitol was greater than that of NaCl. However, this was not the case with tomato seeds in which the effect of mannitol was lower than that of NaCl. The same pattern that occurred to lucerne seeds with NaCl was also observed with mannitol, 13R Supreme, Vertus and Mesa Sirsa showed some tolerance, Vela and Rambler less so. As was the case with NaCl treatment, there was no significant difference in delay of germination (T50) between NaCl- and mannitol-treated seeds, Table 4.2.9.

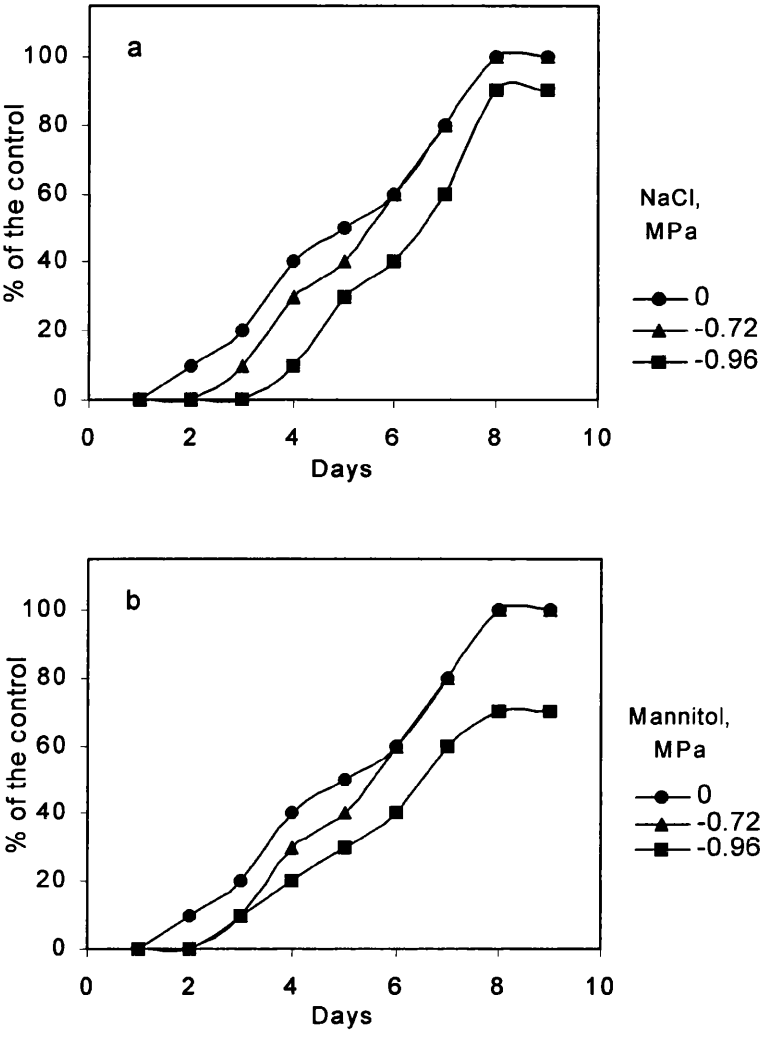


Fig. 4.2.1. Germination of *M. sativa* cv. 13R Supreme in response to a) NaCl and b) mannitol of different osmotic potentials (MPa).

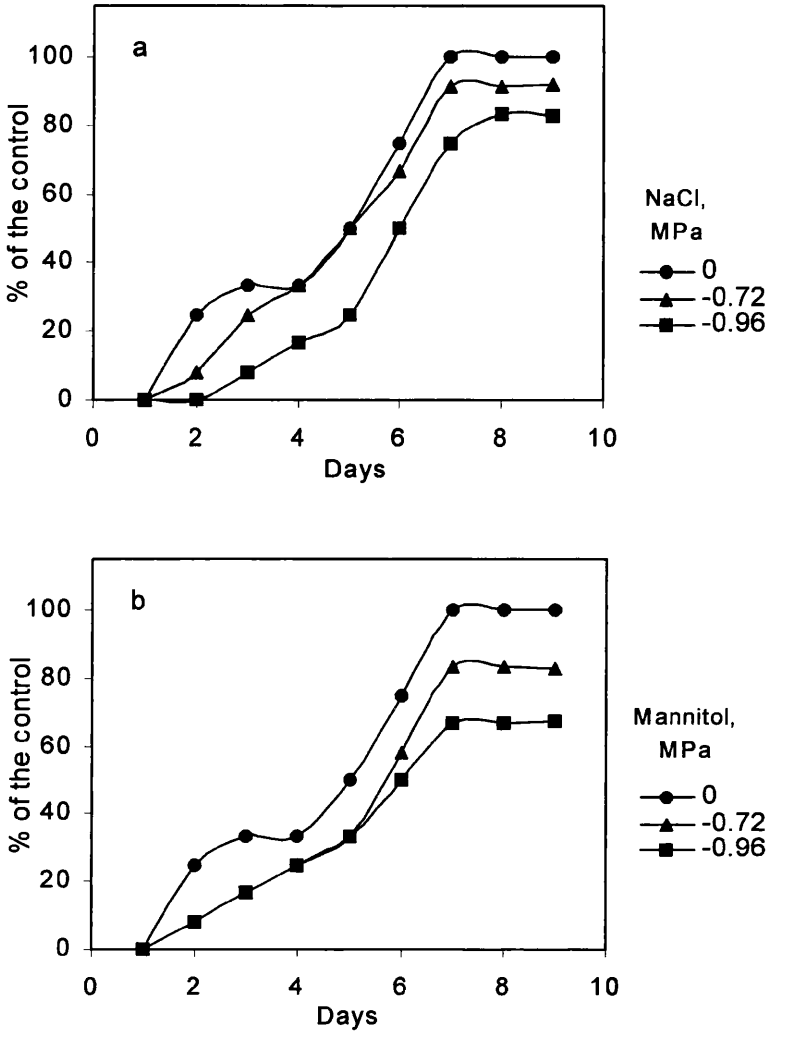


Fig. 4.2.2. Germination of *M. sativa* cv. Vertus in response to a) NaCl and b) mannitol under various osmotic potentials (MPa).

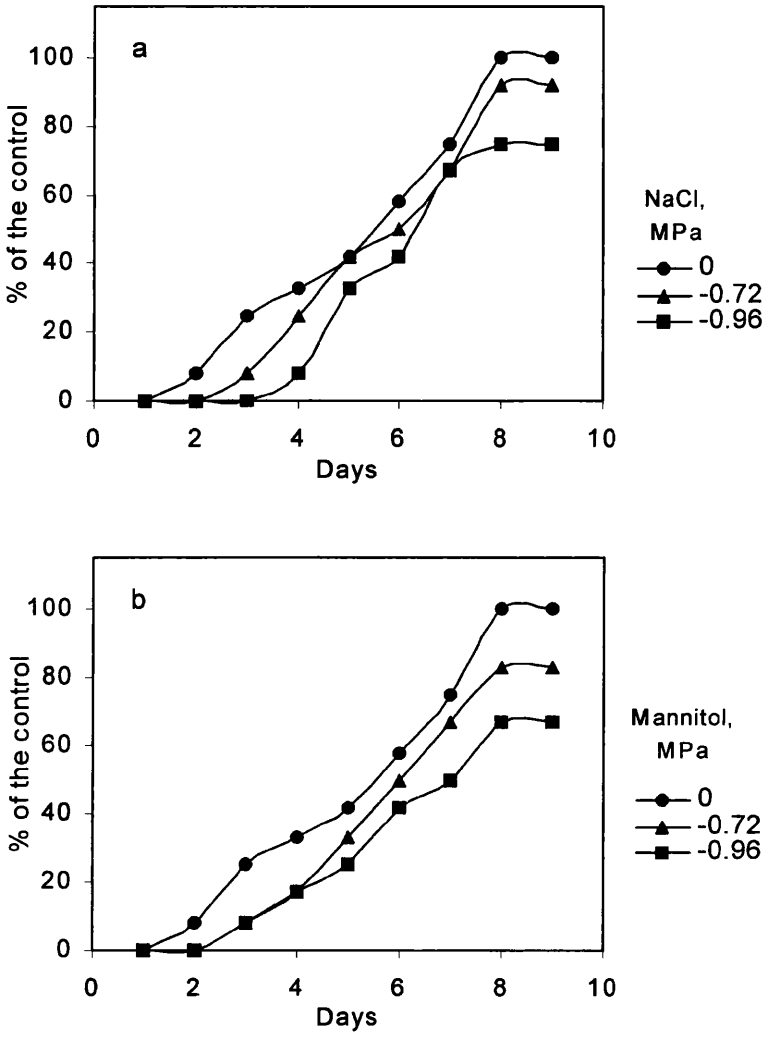


Fig. 4.2.3. Germination of *M. sativa* cv. Mesa Sirsa in response to a) NaCl and b) mannitol under various osmotic potentials (MPa).

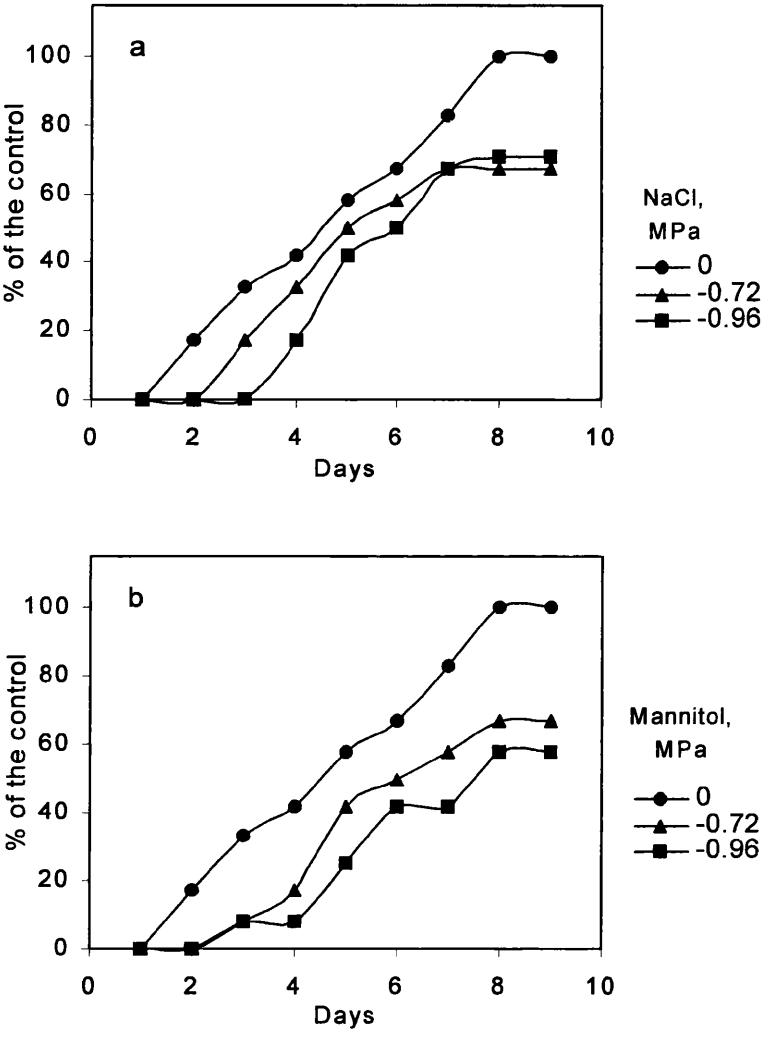


Fig. 4.2.4. Germination of *M. sativa* cv. Vela in response to a) NaCl and b) mannitol under various osmotic potentials (MPa).

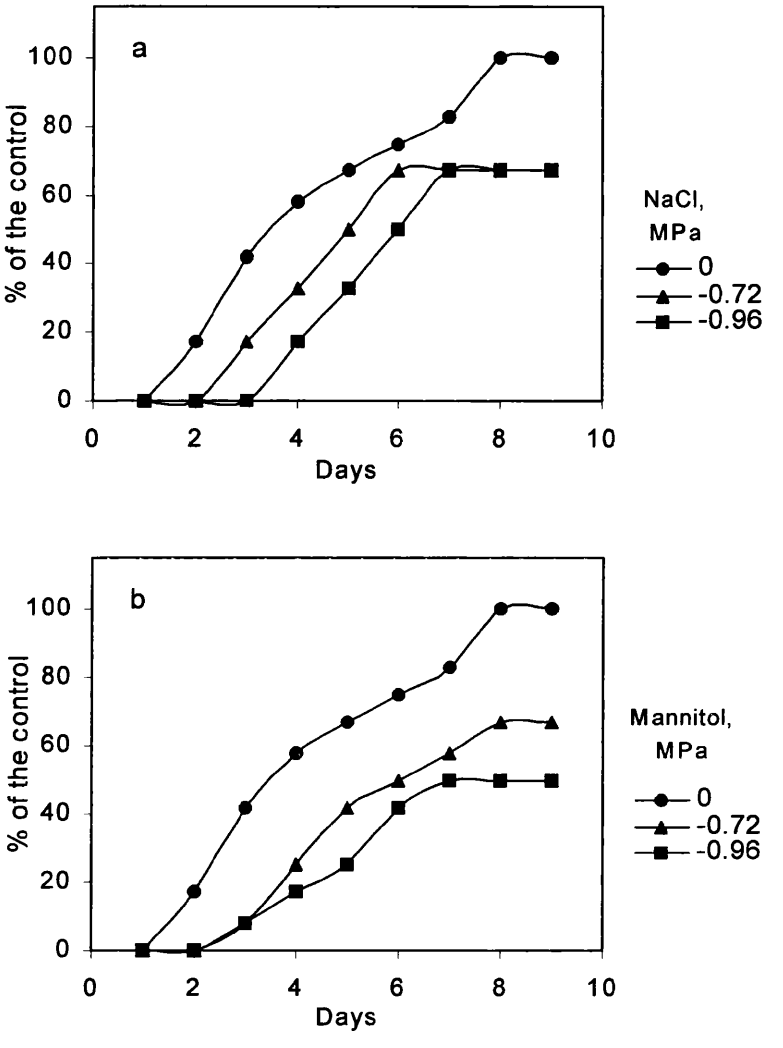


Fig. 4.2.5. Germination of *M. sativa* cv. Rambler in response to a) NaCl and b) mannitol under various osmotic potentials (MPa).

Table 4.2.8. The effect of NaCl or mannitol on percentage seed germination of cultivars of *M. sativa* and *M. media*. Percentage germination was calculated from 25 replicates per cultivar per treatment after 9 days incubation. The germination of the control is expressed as 100 and the germination observed in the other treatment of the same cultivars is expressed as a percentage of that.

NaCl (mM)	Osmotic Potential MPa	*M. sativa* cv. 13R Supreme	*M. sativa* cv. Vertus	*M. sativa* cv. Mesa Sirsa	*M. sativa* cv. Vela	*M. media* cv. Rambler
0	0	100	100	100	100	100
150	-0.72	100	92	92	67	67
200	-0.96	90	83	75	71	67
Mannitol (mM)	Osmotic Potential MPa	*M. sativa* cv. 13R Supreme	*M. sativa* cv. Vertus	*M. sativa* cv. Mesa Sirsa	*M. sativa* cv. Vela	*M. media* cv. Rambler
0	0	100	100	100	100	100
300	-0.72	100	83	83	67	67
400	-0.96	70	67	67	58	50

Table 4.2.9. Days to 50% germination of tomato seeds treated with NaCl and mannitol.

NaCl (mM)	Osmotic Potential MPa	*M. sativa* cv. 13R Supreme	*M. sativa* cv. Vertus	*M. sativa* cv. Mesa Sirsa	*M. sativa* cv. Vela	*M. media* cv. Rambler
0	0	4.0	3.5	4.2	3.6	3.3
150	-0.72	4.4	3.7	4.6	3.6	3.4
200	-0.96	5.0	4.5	4.5	4.2	4.1
Mannitol (mM)	Osmotic Potential MPa	*M. sativa* cv. 13R Supreme	*M. sativa* cv. Vertus	*M. sativa* cv. Mesa Sirsa	*M. sativa* cv. Vela	*M. media* cv. Rambler
0	0	4.0	3.5	4.2	3.6	3.3
300	-0.72	4.4	4.0	4.6	4.1	4.0
400	-0.96	4.4	3.8	4.5	4.4	3.9

4.3. Root formation of the lucerne cultivars in saline medium.

In the previous experiments, it was established that some lucerne cultivars showed tolerance to up to 100 mM NaCl in the seedling stage following germination (Tables 4.2.6 & 4.2.8). In this experiment, cultivars that had showed the greatest level of tolerance or susceptibility to NaCl were used in an experiment to test their ability to produce roots in saline conditions, which has been used as an indicator of tolerance or susceptibility previously (Taufikrahman, 1993).

Root formation, as indicated by the length of the longest root (Taufikrahman, 1993; Dessalegne, 1996; Douirani, 1998), of cultivars was assessed in the presence of a nutrient solution (A & H) containing one of 0-, 50-, 100- or 150 mM NaCl. The un-rooted cuttings of tolerant (Protea, 13R Supreme, Bilensoy-80, Mesa Sirsa, Vertus) and susceptible (Peru) cultivars (10 cm, 8 replicates per treatment) were placed individually in perlite in 10 cm pots that stood on Petri dishes. Each treatment was watered with 100 ml A & H solution for 2 weeks every other day (see Materials and Methods). The NaCl content of the nutrient solution was increased by increments of 50 mM daily until the required salinity was reached. In addition to measurement of root length and dry weight of the entire plant, the proline concentration of the leaves was measured.

Tables 4.3.1-4.3.5 summarize the results and the statistical analysis for root length, dry weight and proline content of the cultivars. Root length and dry weight of all the cultivars were greatly affected by 150 mM NaCl. However, root formation and dry weight of cultivar Peru (the root length and hypocotyl elongation were affected at 50 mM NaCl in the previous experiment, Table 4.2.6 & 4.2.8) was also affected at 50 mM (Tables 4.3.1, 4.3.2, 4.3.3 & 4.3.4).

The accumulation of proline was considered as a sign of stress by many workers (Aziz *et al.*, 1999; El-Iklil *et al.*, 2002). However, in this study proline content of the leaves slightly increased at 100 mM NaCl treatment when comparison was made to the control group. No statistical table was produced for this parameter since there were no significant differences observed between treatments (Table 4.3.5). Plants treated with 150 mM NaCl had showed severe symptoms of the salinity and died before the measurement was taken.

Table 4.3.1. The effect of NaCl (at 0-, 50-, 100- and 150 mM) on the index of root length of lucerne (cuttings). The formation was assessed over a two-week period.

Cultivars	NaCl (mM)*			
	0	50	100	150
Protea	1.00 ± 0.08	0.95 ± 0.08	0.97 ± 0.08	0.77 ± 0.05
13R Supreme	1.00 ± 0.07	0.86 ± 0.06	0.86 ± 0.05	0.75 ± 0.05
Bilensoy-80	1.00 ± 0.09	0.95 ± 0.05	0.96 ± 0.05	0.78 ± 0.04
Peru	1.00 ± 0.02	0.83 ± 0.05	0.82 ± 0.06	0.69 ± 0.03
Mesa Sirsa	1.00 ± 0.06	1.02 ± 0.06	0.84 ± 0.03	0.79 ± 0.08
Vertus	1.00 ± 0.03	1.00 ± 0.06	0.87 ± 0.09	0.71 ± 0.05

Table 4.3.2. The effect of NaCl (at 0-, 50-, 100- and 150 mM) on the index of root length of lucerne (cuttings). Their ability to form roots was assessed over a two-week period.

Cultivars	[NaCl] mM*				ANALYSIS OF VARIANCE				
	0	50	100	150	SS	df	MS	*F*	*Sig.*
Protea	a	ab	ab	b	0.202	3	6E-02	2.06	0.135
13R Supreme	a	ab	ab	b	0.144	3	4E-02	2.83	0.071
Bilensoy-80	a	ab	ab	b	0.164	3	5E-02	1.99	0.144
Peru	a	b	b	b	0.240	3	7E-02	5.40	0.009
Mesa Sirsa	a	a	ab	b	0.191	3	6E-02	3.73	0.033
Vertus	a	a	ab	b	0.338	3	0.113	5.38	0.007

* Between NaCl concentrations, index of root length of lucerne cultivars with the same letters are *not significantly different* from each other at 0.05 level.

Table 4.3.3. The effect of NaCl (at 0-, 50-, 100- and 150 mM) on the index of dry weight of the entire lucerne plants. Their ability to form roots was assessed over a two-week period.

Cultivars	NaCl (mM)*			
	0	50	100	150
Protea	1.00 ± 0.08	1.00 ± 0.12	0.81 ± 0.13	0.52 ± 0.07
13R Supreme	1.00 ± 0.11	0.83 ± 0.13	0.73 ± 0.03	0.34 ± 0.06
Bilensoy-80	1.00 ± 0.25	0.68 ± 0.10	0.60 ± 0.04	0.43 ± 0.07
Peru	1.00 ± 0.16	0.46 ± 0.12	0.46 ± 0.12	0.31 ± 0.03
Mesa Sirsa	1.00 ± 0.10	1.39 ± 0.15	0.81 ± 0.20	0.48 ± 0.07
Vertus	1.00 ± 0.12	1.06 ± 0.15	0.73 ± 0.15	0.26 ± 0.05

Table 4.3.4. The effect of NaCl (at 0-, 50-, 100- and 150 mM) on the index of dry weight of the entire lucerne plants. Their ability to form roots was assessed over a two-week period.

Cultivars	[NaCl] mM*				ANALYSIS OF VARIANCE				
	0	50	100	150	SS	df	MS	*F*	*Sig.*
Protea	a	a	ab	b	0.93	3	0.313	4.82	0.010
13R Supreme	a	a	a	b	1.02	3	0.342	8.51	0.001
Bilensoy-80	a	ab	ab	b	1.05	3	0.351	2.43	0.092
Peru	a	b	b	b	1.38	3	0.461	6.75	0.004
Mesa Sirsa	a	ab	bc	c	1.93	3	0.646	6.40	0.005
Vertus	a	a	a	b	2.36	3	0.789	8.62	0.001

*Between NaCl concentrations, dry weight of lucerne cultivars with the same letters are *not significantly different* from each other at 0.05 level.

Table 4.3.5. The effect of NaCl (at 0-, 50-, 100- and 150 mM) on proline content of lucerne (cuttings). Their ability to form roots was assessed over a two-week period.

Cultivars	NaCl (mM)*			
	0	50	100	150
Protea	2.50 ± 0.50	2.50 ± 0.50	3.50 ± 0.50	-[1]
13R Supreme	2.50 ± 0.50	2.00 ± 0.00	3.00 ± 0.00	-
Bilensoy-80	2.50 ± 0.50	2.00 ± 1.00	3.00 ± 0.00	-
Peru	2.50 ± 0.50	3.00 ± 0.00	3.00 ± 0.00	-
Mesa Sirsa	2.00 ± 0.00	2.00 ± 0.00	2.50 ± 0.50	-
Vertus	2.50 ± 0.50	3.50 ± 0.00	3.50 ± 0.00	-

[1]No data obtained.

4.4. Effect of NaCl on radial growth-, germination of conidia- and mycelial weight of *V. albo-atrum.*

Source of isolates;

In this study, fungal isolates of *V. albo-atrum* were obtained from lucerne and tomato plants. The isolates V1 and V2 were isolated from lucerne and tomato, respectively (UWS). Other isolates, which were isolated from lucerne (VS, VF and VL), were obtained from the culture collection of Prof. Craig Grau (University of Wisconsin-Madison).

4.4.1. Effect of growth media and NaCl on radial growth.

Many of the semi-arid and arid areas in the world are characterized with salinity. Salinity may interact with the fungus in soil fauna and might reduce the effectiveness of the fungi. For example, Amir *et al.* (1996) reported that the salinity induced soil suppressiveness to the vascular fusariois. It reduced sporulation and germination of *Fusarium oxysporum.* Therefore, the negative effect of salinity could prevent the pathogen propagules from reaching the root infection sites. For example, Engel & Grey (1991) stated that chloride fertilizers increased the yield of winter wheat and reduced the severity of root diseases caused by *Fusarium culmorum.* It has also been reported that salinity might negatively affect the prevalence and distribution of the pathogen (Mandeel, 1996). However, it has been reported that salinity might also prevent the development of symbiotic bacteria in the soil (Botsford, 1983).

On the other hand, salinity had positive effect on fungal growth. For example, it increased the development of motile zoosporangia of *Perkinsus* sp. and the developmental rates increased with increasing salinity (Ahn & Kim, 2001). Similar findings were made by Ragazzi *et al.* (1994), who reported *F. oxysporum* f.sp. *vasinfectum* chlamydospores and their germ tubes have been found to grow better in a saline medium and the pathogen was more virulent when it had been cultured on a saline-enriched medium.

So, the effect of NaCl is not clear on growth and sporulation of fungi, especially on *V. albo-atrum.*

In the first instance, the radial growth of the isolates of *V. albo-atrum* was compared over a 3-week period on Dox- and PDA media. The radial growth rates of isolates are presented in Fig. 4.4.1a & b and their statistical analysis is presented in Table 4.4.1. The results indicate that isolate V2 grew faster than the other isolates during the incubation period; its radial growth rate was significantly different from the other isolates (Table 4.4.1). However, isolates from lucerne, irrespective of their origin of country, showed similar growth rates and no significant differences were observed between them (Table 4.4.1), although the radial growth rates of the lucerne isolates were slightly higher in Dox medium than in PDA (Fig. 4.4.1).

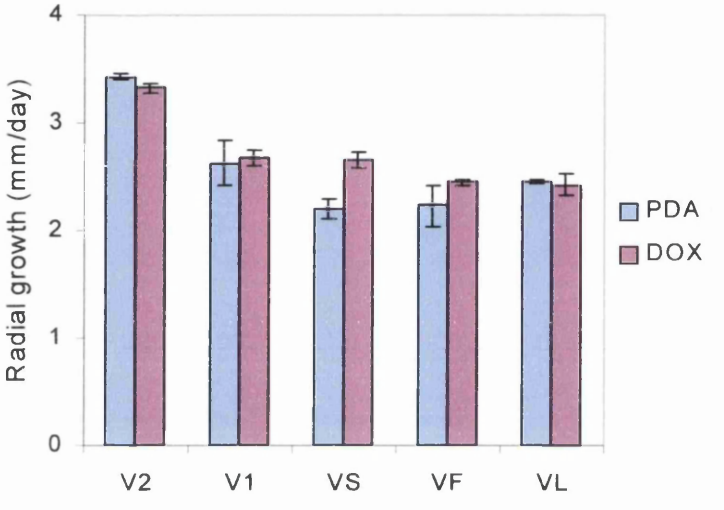


Fig. 4.4.1. Radial growth of *Verticillium* isolates grown on PDA and Dox media. V1, VS, VF and VL-isolates from lucerne. V2-isolate from tomato cv. Ailsa Craig. Vertical bars show ± SE of mean.

Table 4.4.1. Analysis of variance of the growth rates of isolates of *V. albo-atrum* on PDA and Dox media.

Media	[Isolates]*					ANALYSIS OF VARIANCE				
	V2	V1	VL	VF	VS	SS	df	MS	*F*	*Sig.*
PDA	a	b	b	b	b	1635	4	408	11.4	0.001
Dox	a	b	b	b	b	903.	4	226	18.0	0.000

*Between treatments, isolates with the same letters are *not significantly different* from each other at 0.05 level.

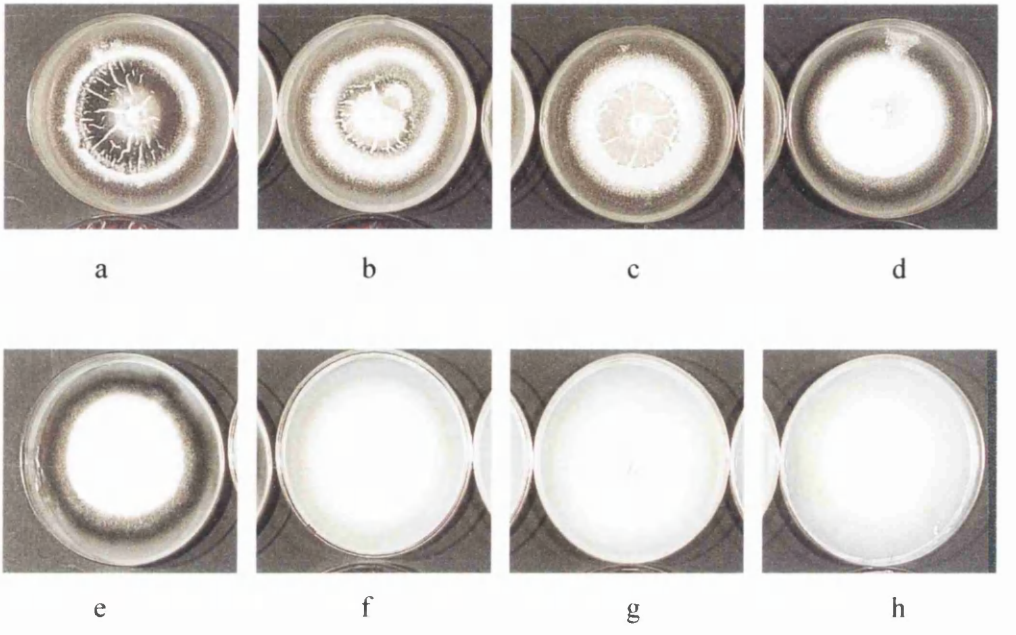


Plate 4.1.
Development of mycelium of *V. albo-atrum* (isolate V1) on Dox medium containing different concentrations of NaCl. (a) Control, 0 mM NaCl-; (b) 25 mM-; (c) 50 mM-; (d) 100 mM-; (e) 150 mM-; (f) 250 mM-; (g) 300 mM-; and (h) 350 mM NaCl.

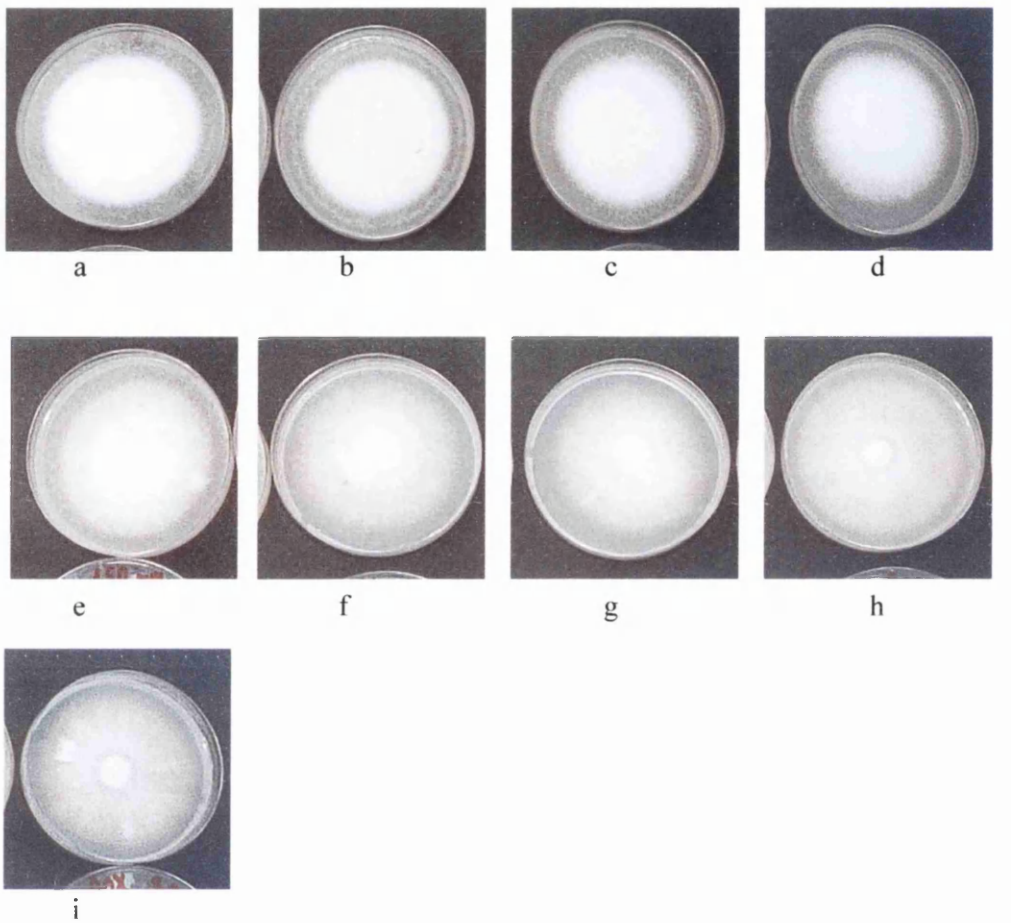


Plate 4.2.
Development of mycelium of *V. albo-atrum* (isolate V2) on Dox medium containing different concentrations of NaCl. (a) Control, 0 mM NaCl; (b) 25 mM-; (c) 50 mM-; (d) 100 mM-; (e) 150 mM-; (f) 200 mM-; (g) 250 mM-; (h) 300 mM-; and (i) 350 mM NaCl.

Having established that there were no significant differences in the growth rate of the various isolates whether they were grown on Dox- or PDA medium, the radial growth rates of the two isolates V1 and V2, which would be used in subsequent experiments were assessed under various concentrations of NaCl (0-, 25-, 50-, 100-, 150-, 200-, 250-, 300-, and 350 mM) in Petri dishes of Dox medium. NaCl was incorporated into Dox medium at the required concentration, which was then autoclaved, and plated in Petri dishes as described in Materials and Methods. The plates were inoculated with mycelial disks (3-mm in diameter) cut from the outer margin of 3-week old cultures on Dox and the plates were then incubated at 23 °C ± 1 °C.

At the end of the 3-week period, the growth of each colony was measured and the amount of spores present was determined. Sporulation was determined by rinsing each culture with 10 ml of distilled water, scraping the spores with a glass spatula, and counting them with a neubauer haemacytometer. The results and statistical analysis are presented in Fig. 4.4.2 and Table 4.4.2, respectively.

The results showed that both isolates V1 and V2 had reduced growth rate with increasing NaCl concentrations. The radial growth rate of the isolates were almost similar and significantly decreased at 200 mM and above NaCl concentrations (Fig. 4.4.2a & b). The growth rate of isolates, although it decreased with increasing salinity, did not show statistical difference from each other and the control group up to 150 mM NaCl concentration (Table 4.4.2). Although the growth rate of both isolates declined significantly after 150 mM NaCl concentration, the decline in growth rate of isolate V1 was more rapid than the isolate V2. Not surprisingly, the slowest growth rate was observed in the highest concentration of NaCl (350 mM). The appearances of the colonies are presented in Plates 4.1 & 4.2. From the plates, it can easily be seen that after 150 mM NaCl the colour of the colonies of both isolates V1 and V2 change suddenly and become feathery.

A decrease in sporulation was also observed in response to increasing concentrations of NaCl (Fig. 4.4.2a & b). Spores of both isolates were slightly affected at 25 and 50 mM NaCl. Significant decrease in number of spores was observed in both isolates when the concentration of NaCl reached 100 mM. The decrease in sporulation in response to NaCl was greater in V2 than in V1. However, it should be pointed that the

area of colonial mat where isolate V1 grew (in Petri) was less than that of isolate V2. So, the number of spores measured in colonial mat was, of course, lower in isolate V1 than that of V2. For example, isolate V1 produced 1200 x 10^5 conidia/ml while isolate V2 produced 5620 x 10^5 conidia/ml, in control conditions. In the presence of 100 mM NaCl, isolate V1 produced 826 x 10^5 conidia/ml while isolate V2 produced 2912 x 10^5 conidia/ml. The decline in amount of spores of isolate V2 was doubled at 100 mM whilst, the decline in amount of spores of isolate V1 was 1.5 times. At the highest NaCl concentration (350 mM), isolate V1 produced 70% less spores than that of control conditions while V2 produced 80% less spores than that of non-salt conditions. In general, up to 300 mM NaCl, the growth rate of V2 was almost equal to the growth rate of V1.

The production of conidia by isolate V2 was 4 or 5 times greater than that of V1 up to 50 mM NaCl concentration. Above 50 mM NaCl concentration, although V2 produced more conidia than V1, the ratio between V2/V1 was reduced down to 3.50 at 100-, 2.65 at 150-, 2.17 at 200 mM NaCl level. Although isolate V2 was capable of producing more spores per unit area than V1 and produced more colonial mat in Petri dishes, which would make larger surface area to provide more spores, it is clear that the negative effect of NaCl (above 150 mM) was more on V2. From pathological point of view, it is important that the sporulation and the colonial growth of the fungus are affected significantly at 100 and 200 mM NaCl, respectively. This study indicated that the pathogen might sporulate under moderately saline conditions and possibly play an important role as a pathogen under those conditions.

Table 4.4.2. Analysis of variance of the growth rates of isolates of *V. albo-atrum* grown at various concentrations of NaCl conditions in Dox medium.

Isolates	NaCl] mM*									ANALYSIS OF VARIANCE				
	0	25	50	100	150	200	250	300	350	SS	df	MS	*F*	*Sig.*
V1	a	a	a	a	a	b	b	c	d	4136	8	517	47.1	0.000
V2	a	a	a	a	a	b	b	b	b	2817	8	352	18.5	0.000

*Between NaCl treatments, concentrations with the same letters are *not significantly different* from each other at 0.05 level..

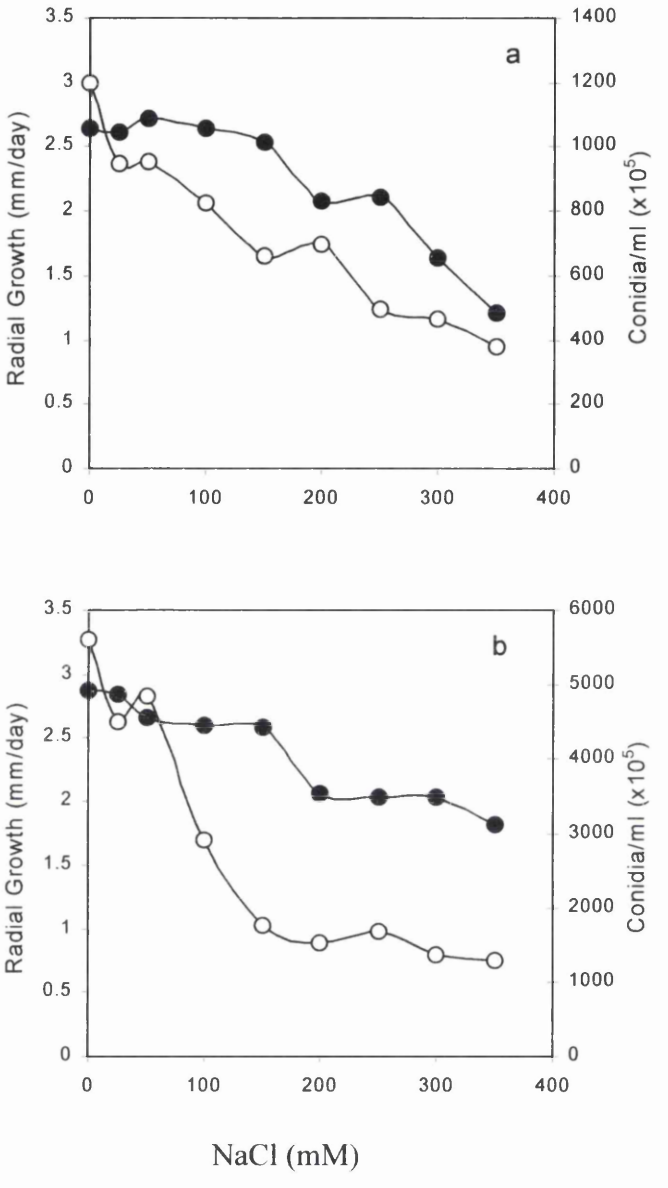


Fig. 4.4.2. Effect of NaCl (mM) on radial growth and sporulation of *V. albo-atrum.* (a) Isolate V1 and (b) Isolate V2 on Dox agar after 21 days incubation. Radial growth-(●-●); sporulation (○-○).

4.4.2. Radial growth rates of non-salt adapted and salt-adapted Verticillium isolates.

In the previous experiments, the effect of NaCl on the radial growth of isolates V1 and V2 was determined. In this section, the effect of NaCl was determined on the growth of isolates of *V. albo*-atrum that have been adapted to grow on saline medium.

Initially, isolates V1 and V2 were grown on Dox medium containing 50 mM NaCl, then every 3 weeks, the cultures were transferred to medium in which the concentration of NaCl was increased by an increment of 50 mM. Each isolate was grown in this way until the NaCl concentration reached to 150 mM. At this concentration, both isolates V1 and V2 were maintained for 5 months prior to the start of the experiment. Those isolates, which had been maintained on medium containing 150 mM NaCl are designated V1-150 and V2-150. A sample of each V1-150 and V2-150 was then transferred to medium in which the NaCl concentration was increased to 200 mM. Isolates were then maintained on 200 mM NaCl for 2 months and are designated V1-200 and V2-200.

In this experiment, the radial growth of isolates V1 and V2, and their salt-adapted strains were grown in the respective conditions under which they were normally maintained. Three mm mycelial disks of each isolate, V1 and V2, V1-150 & V2-150, V1-200 & V2-200, were transferred to the appropriate medium; in the case of V1 & V2, normal Dox medium – no salt; in the case of V1- and V2-150, Dox medium plus 150 mM NaCl; in the case of V1- and V2-200, Dox medium containing 200 mM NaCl. The cultures were grown for 3 weeks, at the end of which radial growth and spore formation were assessed, (Fig 4.4.3 & Table 4.4.3).

Isolates V1 and V2 and their salt adapted strains, V1-150 and V2-150 showed similar radial growth rates over the 3-week period, (Fig. 4.4.3), statistical analysis showing that there were no differences in growth rates between the original isolates, V1 & V2, and their salt-adapted strains V1-150 & V2-150 (Table 4.4.3). However, the growth rates of both V1-200 or V2-200 (grown on 200 mM NaCl) were significantly different from those of the original isolates (V1 or V2, which were grown under no-salt conditions) Table 4.4.3.

Adaptation to growth on medium containing NaCl cause a marked decrease in the ability of the isolates to form spores, in all the salt-adapted isolates, Fig. 4.4.3. The effect was more marked in V1- and V2-200 than in the corresponding –150 strains, and V1 was

more affected than V2. This study indicated that the fungus was not affected by NaCl up to 150 mM NaCl in a long term. Although the sporulation of both isolates declined, it should be considered that the production of spores even under stress conditions might still play an important role and cause pathogenicity. From the results, Fig. 4.4.3, both isolates produced enough conidia to spread and cause pathogenicity under saline and non-saline conditions. The pathogenicity of those salt strains on tomato plants was studied in the following chapter.

Table 4.4.3. Analysis of variance of the growth rates of isolates of *V. albo-atrum* and their salt-adapted strains.

[Isolates]*			ANALYSIS OF VARIANCE				
V1	V1-150	V1-200	SS	df	MS	*F*	*Sig.*
a	a	b	181	2	90.71	11.0	0.002
V2	V2-150	V2-200	SS	df	MS	*F*	*Sig.*
a	a	b	416	2	208	10.4	0.004

* Between treatments, isolates with the same letters *are not significantly different* from each other at 0.05 level.

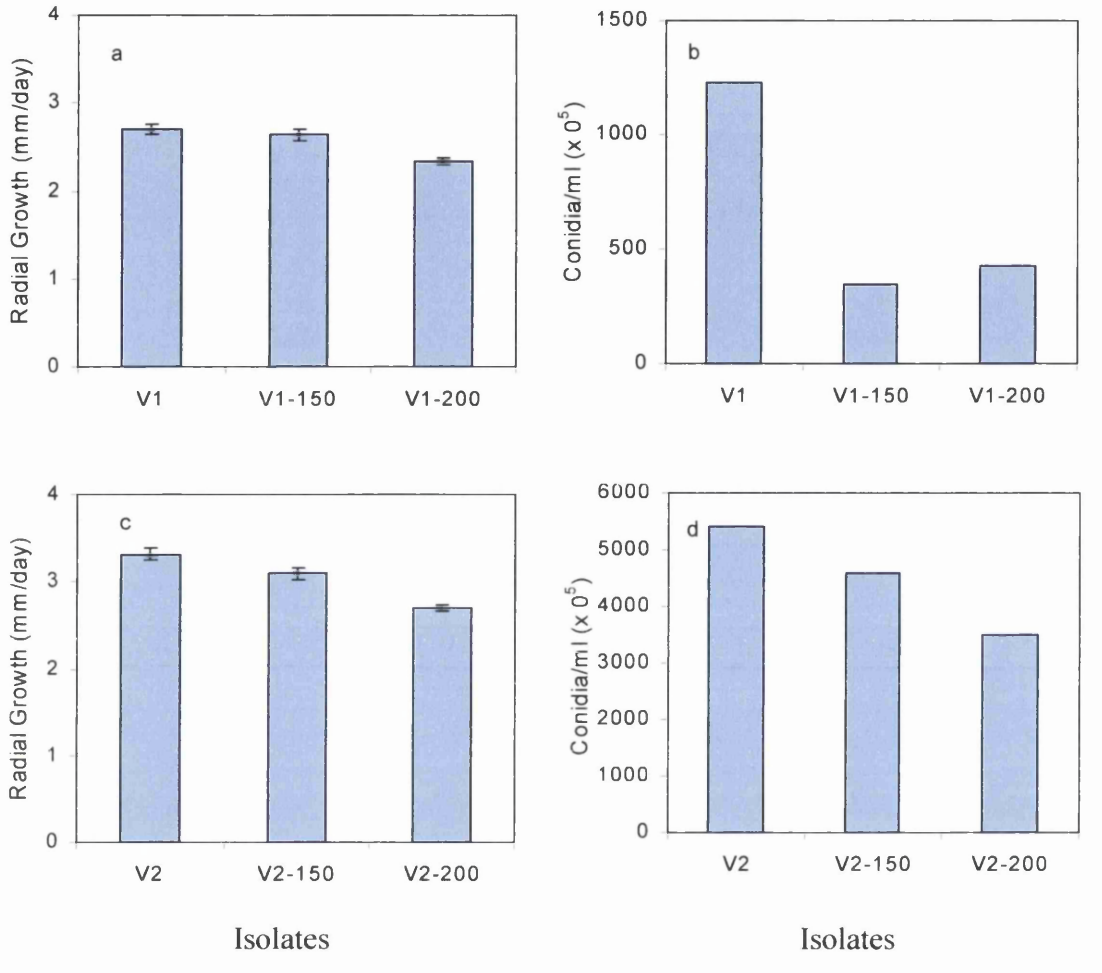


Fig. 4.4.3. Radial growth and spore formation of *Verticillium* isolates and their salt-adapted strains. Isolates were grown as described in the text and at the end of the 3-week growth period growth and spore formation were measured as described in Materials and Methods. a & c-radial growth; b & d-number of conidia produced by isolates. Vertical bars show ± SE of mean.

4.4.3. The effect of NaCl on spore germination and mycelial dry weight.

The result of the previous experiment have shown that at the higher concentration of NaCl used, there was a significant reduction in the ability of the salt-adapted strains to form spores. This may affect the ability of *V. albo*-atrum to infect plants under conditions of salinity. Another factor that may also influence infectivity is germination of the spores. Consequently, this was assessed for both the original V1 & V2 isolates. In the previous experiments also, growth was reported as radial growth. While a good indicator of growth for some purposes, it has the disadvantage that it affords no indication of the amount of mycelium in the fungal colony (Milton, 1966). Therefore, isolates V1 and V2 were also tested for their growth in various concentrations of NaCl in liquid culture (Dox) medium.

For measurement of germination, a suspension, (1 x 105^5 conidia/ml) of *V. albo-atrum*, isolates V1 or V2, was transferred to each of a series of sterile boiling tubes, each of which contained 10 ml liquid Dox medium and NaCl at 0-, 50-, 100-, 150-, 200- or 250 mM. The tubes were incubated in the dark at 23 °C ± 1 °C for 24 h and at the end of the incubation period, 100 µl of each of the cultures were smeared onto a sterile microscope slide and the germination of spores was determined microscopically, Plate 4.3. For determination of mycelial dry weight, a 1-cm diameter disk from a culture of each isolate was transferred aseptically to a 250 ml conical flask containing 100 ml Czapex Dox liquid medium with NaCl at one of the concentrations; 0, 25, 50, 100, 150 and 200 mM (10 flasks for each treatment). The flasks were placed on an orbital shaker 100 rpm, in the dark at 23 °C ± 1 °C for 6 weeks. After the period of incubation mycelium mats were harvested (see Materials and Methods) and dry weights determined. The results show that germination was largely unaffected by NaCl up to a concentration of 150 mM (Table 4.4.4). However, increasing the concentration to 200 mM caused a decrease in germination. Nevertheless, even at the highest concentration of NaCl (200 mM) significant germination occurred and at 100-150 mM NaCl, a substantial level of germination occurs, sufficient that fungus may still become a pathogen.

Mycelium dry weights are presented in Fig. 4.4.4. From the results, it was observed that both isolates showed similar growth curves. Increasing NaCl

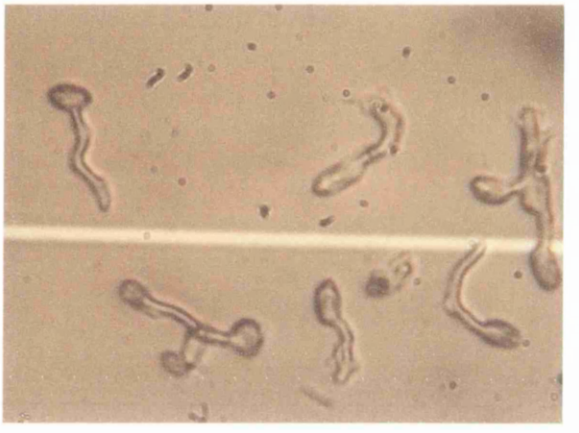


Plate 4.3.
Germination of *V. albo-atrum* (isolate V2-control group) spores, 12 h after the start of the incubation period.

concentrations in the medium caused a decrease in mycelial dry weights, though the decrease was not large up to 50 mM NaCl. At 100 mM and above, however, growth of the isolates decreased markedly.

Table 4.4.4. Spore germination at different concentrations of NaCl.

NaCl (mM)	V1 (%)	V2 (%)
0	90>	90>
100	90>	90>
150	85	85
200	70	70
250	50<	50<

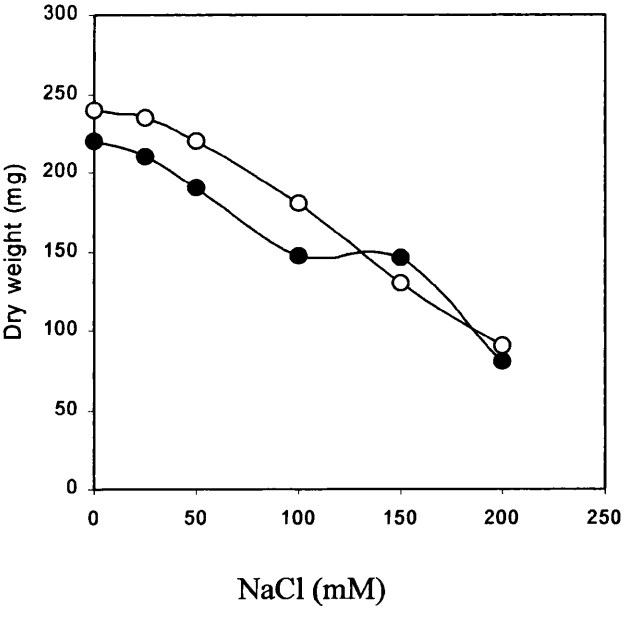


Fig. 4.4.4. Mycelium growth of *V. albo-atrum* in liquid culture (6 weeks). (● —●) V1; (○—○) V2.

4.5. Effects of hydrogen peroxide (H_2O_2) and purified phytoalexins on spore germination and germ tube elongation of *V. albo-atrum* isolates.

In the previous section, the effect of NaCl *in vitro*, was tested on the germination, viability, mycelium growth and radial growth of the *V. albo-atrum* isolates. Both isolates showed similar patterns in response to NaCl, even the salt-adapted strains, although sporulation and the radial growth per day was lower in the isolate V1 than that of V2. In this study, the effect of antifungal compounds was tested on the fungal isolates to establish if there is a difference between isolates in terms of germination and mycelium development *in vitro*.

Spore germination and germ tube elongation of *V. albo-atrum*, isolate V1 and V2, were inhibited by the effects of antifungal compounds such as H_2O_2 and the purified phytoalexins.

4.5.1. The effect of H_2O_2 on conidial germination and germ tube elongation.

Conidial germination started after 12 h incubation in the control group. The germinated conidia were counted after 24 h at which time most of the length of germ tubes exceeded the length of conidia. An increase in concentration of H_2O_2 resulted in a decrease in germination for both isolates (Fig. 4.5.1a). Significant inhibition of germination for the isolate V1 started at 50 μM H_2O_2, while the significant inhibition for the isolate V2 took place at a concentration of 5 μM H_2O_2. Germination was almost inhibited at 1000 μM H_2O_2 for both isolates. When LD50 (lethal dose which inhibits the 50 % of the population from germinating) was calculated V2 was more sensitive to H_2O_2 than V1 isolate, Table 4.5.1.

The effect of H_2O_2 on germ tube elongation of germinated conidia was tested with the same concentrations of H_2O_2 used for the previous experiment. Germ tube elongation was significantly inhibited at 5 μM H_2O_2 for the isolate V2 (Fig. 4.5.1b). However, effective concentration for V1 was 10 μM H_2O_2. Again, increase in concentration of H_2O_2 caused decrease in germ tube elongation for both isolates. No germ tube elongation was observed at 1000 μM H_2O_2 for both isolates.

4.5.2. The effect of purified phytoalexins on conidial germination.

The effect of medicarpin and sativan on the percentage germination of isolates V1 and V2 are presented in Fig 4.5.2a & b. Medicarpin significantly inhibited the germination of conidia at a concentration of 30 μg/ml for V2, and 40 μg/ml for V1, (Fig 4.5.2a). LD50 of V2 was lower than that of V1, Table 4.5.1. In the case of sativan, inhibition of germination of conidia was markedly affected as compared to the effect of medicarpin. The significant inhibition for sativan started at a concentration of 10 μg/ml. It was observed that sativan was more inhibitory to both isolates than that of medicarpin. Increase in concentration of sativan also caused severe decrease in germination of conidia for both isolates (Fig. 4.5.2b).

Table 4.5.1. The calculated LD50 of the antifungal compounds for germination of the conidia of *V. albo-atrum*, isolates V1 or V2.

Isolates	Antifungal Compounds		
	H_2O_2 (μM)	Medicarpin (μg/ml)	Sativan (μg/ml)
V1	500	35	17
V2	48	25	17

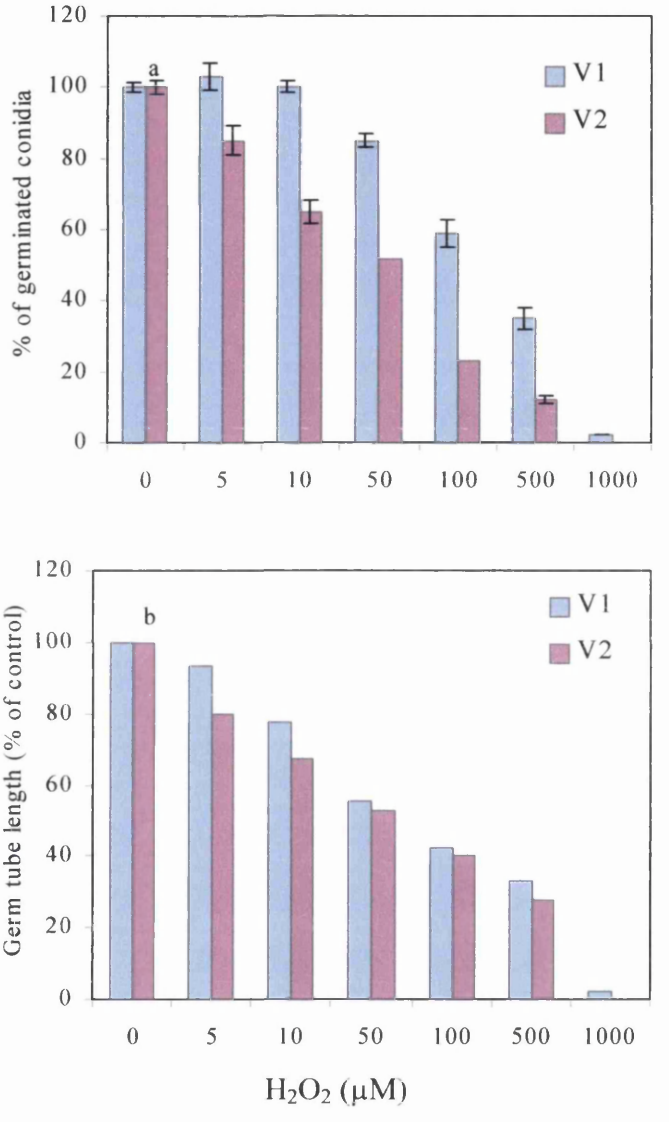


Fig. 4.5.1. The effect of H_2O_2 germination & mycelium length of *V. albo-atrum*, isolates V1 or V2. (a) % of germinated conidia; (b) % germ tube length. Germinated spores were counted microscopically. Vertical bars show ± SE of mean.

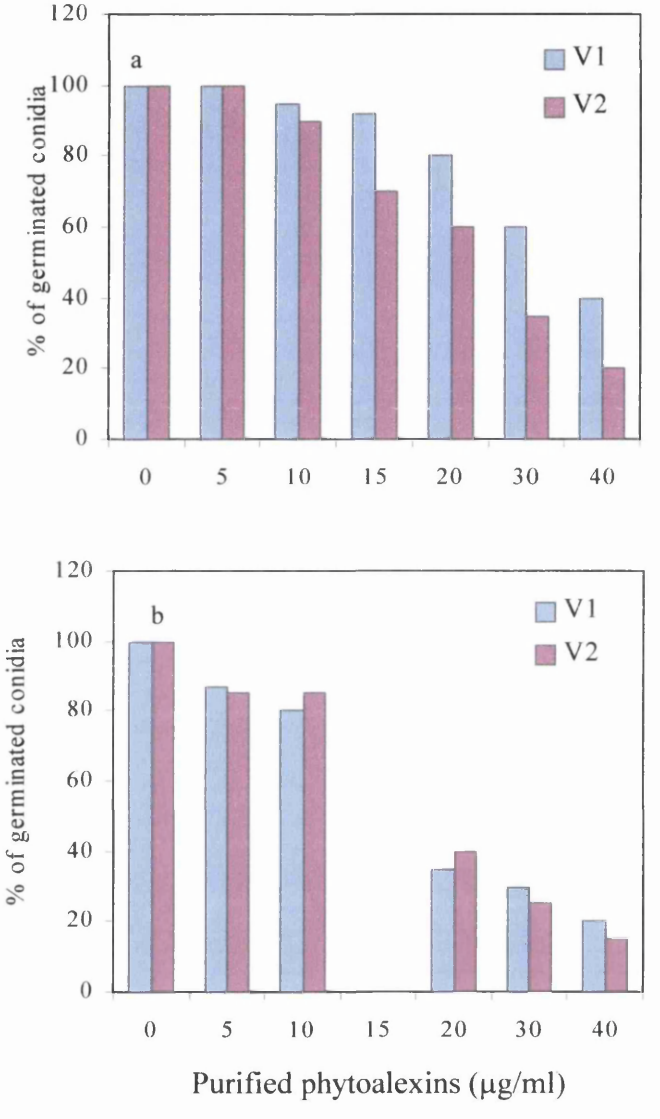


Fig 4.5.2. The effect of purified phytoalexins on conidial germination of *V. albo-atrum* isolates. (a) medicarpin; (b) sativan.

RESULTS

CHAPTER V

Interactions between the effect of salt and pathogen on plant disease development

5.1. The effect of age and NaCl on the pathogenicity of *V. albo-atrum* towards *L. esculentum.*

The separate effects of the pathogen *V. albo-atrum* and of NaCl on growth and development of various tomato cultivars were reported earlier. In this study, the combined effect of *V. albo*-atrum & NaCl are reported. The studies were conducted with both 4- and 8-week old plants of cv. Ailsa Craig, to establish whether the age of the plants has any effect on development of the disease, and in soil.

Seeds were germinated in trays of John Innes No. 1 compost and at the first opportunity, seedlings were transplanted into 15-cm pots of a mixture of perlite and Levington's Universal Compost mixture (4:1).

In the initial experiment, 4-week old plants were treated with NaCl at 0-, 25-, 50-, 100-, 150- and 200 mM. Five replicate pots, one seedling per pot, were used for each treatment. A replicate series of plants treated with the same NaCl concentrations were inoculated with $1x10^7$ conidia/ml of *V. albo-atrum*, isolate V2 by the root-dip method (see Materials and Methods). Due to the severe effects of *V. albo-atrum* and NaCl on young tomato seedlings, the plants were incubated in the greenhouse for 5 weeks. Subsequently, the response of 8-week old plants to the same treatments was assessed with the exception that, the treatments with 25- and 200 mM NaCl concentrations were omitted. In the case of the 4-week old tomato plants, treatment with 25 mM NaCl produced very few signs of symptoms while in the case of treatment with 200 mM NaCl, the effects were too severe. In the second experiment, the experimental plants were incubated in the greenhouse for a period of 8 weeks following inoculation. The conditions of the greenhouse were described in Materials and Methods.

In the case of both the 4- or 8-week old seedlings, non-salinity stressed & non-inoculated control plants or non-salinity stressed & inoculated control plants were watered with 250 ml of 1/5 A & H culture solution for 3 days before the salinity treatment started. After this period, plants were watered with 250 ml 1/5 dilution of A & H solution, containing the appropriate NaCl concentrations (described above), every other day. The concentration of NaCl in the culture solution was increased by increments of 50 mM NaCl until the required salinity of medium was reached.

Two control groups; NaCl- and non-NaCl- treated groups, were used to make a comparison against inoculated plants.

Observations of height and symptom index development were recorded weekly for all plants. In addition, a further 8 features were taken as indicators of disease or physiological development.

- The average height of the plants,
- the symptom index of plants above pot level and root symptom index,
- the ability to reisolate the fungus from individuals of the inoculated plants,
- water content (WTC) of the plants,
- the chlorophyll content (Total Chl) of the leaves,
- relative rate of height increase & root length increase (H & RL),
- relative growth rate (RGR),
- Leaf area (LA),
- Net assimilation rate (NAR).

Tomato plants were harvested after a total of 5 or 8 weeks depending on the experimental procedure.

5.1.1. *Effect of salinity on the severity of symptoms resulting from inoculation with* V. albo-atrum, isolate V2, *of 4-week old tomato plants.*

Observations of height of the 4-week old tomato plants inoculated with *V. albo-atrum*, or treated with NaCl, or both are presented in Fig. 5.1.1. The symptom index for shoot and root, indicating the severity of the wilt disease, or the effect of NaCl, or both are presented in Fig. 5.1.2. Leaf chlorophyll content is presented in Fig. 5.1.3. The parameters root symptom index, H, RL, RGR, LA, NAR and WTC of the plants, recorded at the end of 5-week experimental period, are presented in Tables 5.1.1 & 5.1.2. Analysis of variance tables, which compare the effect of salt and fungus, are presented in Tables 5.1.3 & 5.1.4. The results for reisolation of the fungus from plants in the various treatments are presented in Table 5.1.5.

Treatment of plants with NaCl at all the concentrations used (0-, 25-, 50-, 100-, 150- and 200 mM) inhibited the increase in height after the 3rd week of the experiment, compared to the controls (Fig. 5.1.1a). Increasing concentrations of NaCl were more inhibitory, although the effect of 25 mM was marginal over the 5-week period. At 200 mM NaCl there was very little increase in height (Fig. 5.1.1a).

By itself, *V. albo-atrum*, also inhibited the growth Fig. 5.1.1b, however, in combination, NaCl and *V. albo-atrum* were more inhibitory than either of the treatments alone (Fig. 5.1.1b). In fact, there was no significant increment in height above a NaCl concentration of 50 mM. Analysis of variance of the relative rate of increases in height and root length, Table 5.1.3, shows that NaCl concentrations of 50 mM and above caused a significant reduction in both, $P<0.05$. However, inoculated plants exposed to NaCl showed significant differences at 25 mM NaCl, $P<0.001$, Table 5.1.4.

Symptoms on the plants that were exposed to NaCl (0-, 25-, 50-, 100-, 150-, 200 mM) were visible to some degree after the first week of the treatment, Fig. 5.1.2a. While plants treated with 25- and 50 mM NaCl showed few symptoms until the last two weeks of the experiment, in general, increasing the salt concentration in the medium resulted in the appearance of more severe symptoms in the following weeks. In general, treatment with the higher concentrations of NaCl also resulted in an earlier appearance of symptoms. The effect was especially noticeable at 100 mM and above. These symptoms were observed initially as yellowing of the lower leaves that spread up to the upper leaves resulting in general chlorosis in the plants. Chlorosis was also observed in leaf petioles, which became thinner in the following weeks, causing the leaves to be abscised. Some lower leaves were rolled-inwards and abscised towards the end of the experiment. Towards the end of the experiment, plants treated with 200 mM NaCl were almost dead; only some stem parts and leaves remained green.

The symptoms of disease in response to inoculation with *V. albo-atrum*, isolate V2, were observed after 2 weeks in tomato as yellowing of the lower leaves Fig. 5.1.2c. The disease progressed (as recorded in previous experiments) as wilting, flaccidity and epinasty. Some of the upper leaves of the infected plants also showed wilting; however, no adventitious roots appeared in the lower part of the stems. No recovery was observed

in the inoculated plants, by the end of the experiment. Control plants showed no external symptoms of the disease.

Plants inoculated with V2 and treated at the same time with NaCl solutions showed more severe symptoms than either of the treatments alone. For example, plants that were inoculated with V2 and treated with 25 mM NaCl showed almost the same severity of symptoms as plants inoculated with the fungus alone (Fig. 5.1.2c); while those plants inoculated with V2 and treated with 50 mM NaCl showed more severe symptoms than either of the treatments alone, Fig. 5.1.2b. Above 50 mM, treatment with NaCl alone resulted in marked symptoms but again; these symptoms were more severe in the plants inoculated with V2 at the same time. Towards the end of the experiment, the symptom index values increased markedly in all plants inoculated with V2 and treated with NaCl. Plants inoculated with V2 and treated with 200 mM NaCl were almost dead by the end of the experiment.

Root symptom index showed a similar pattern; NaCl-treated plants showed some browning and this symptom increased with increasing concentration of NaCl, Fig. 5.1.2b. With the exception of those plants inoculated with V2 and treated with 25 mM NaCl, plants inoculated with V2 and treated with NaCl had more severe symptoms than the plants either inoculated with V2 alone or treated with NaCl alone, Fig. 5.1.2d. At higher salt concentrations, especially 200 mM, whether inoculated or not, plants did not show root development, by the end of the experiment the roots were almost dead, Fig. 5.1.2b & d.

The water content of almost all the plants was 90-95 %, whether they were inoculated, or treated with NaCl, or both, Tables 5.1.1, 5.1.2, 5.1.3 & 5.1.4, suggesting a mechanism for managing the higher external osmotic potential. In contrast, an increase in the NaCl concentration resulted in a decrease in chlorophyll content of the leaves, Fig. 5.1.3a. Again, all treatments with NaCl at 25 mM and above resulted in a significant difference in chlorophyll content from the control group, Tables 5.1.3. & 5.1.4. Plants inoculated with V2 and treated with NaCl had lower chlorophyll contents than either of the treatments alone, Fig. 5.1.3b.

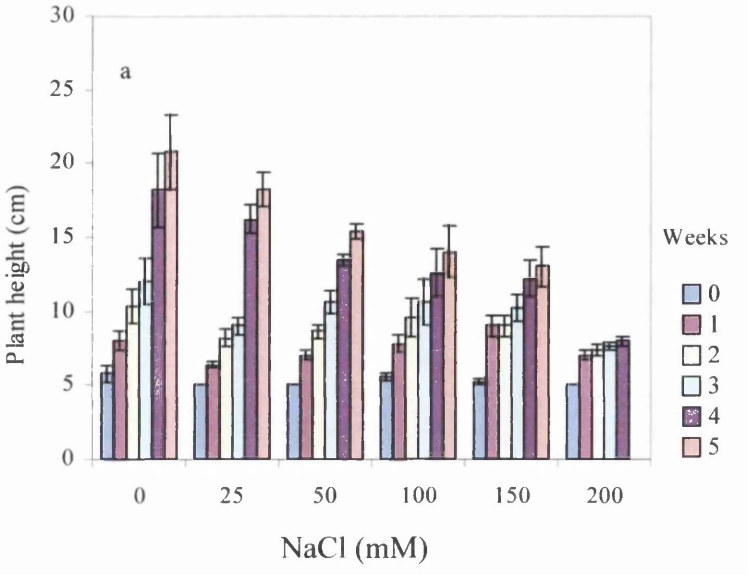
These combined effect of V2 & NaCl are reflected in the values for the relative rate of increase in height and in root. An increase in the NaCl concentration inhibited the

increase in height and root length compared to the control group, Table 5.1.1. The same trend was also observed for RGR, Table 5.1.1. Plants inoculated with V2 and treated with NaCl suffered greater reductions in height and root length compared to the control group, Table 5.1.2. Plants treated with NaCl at 50 mM showed significant differences for both height and root length parameters, Table 5.1.3. Plants inoculated with V2 whether exposed to salt or not showed significant differences in height from the control group $P<0.050$, Table 5.1.1, 5.1.2, 5.1.3 & 5.1.4. However, the root length of plants inoculated with V2 did not show significant differences from the control plants. However, under salinity (50 mM and above), they showed significant differences from the control plants, Tables 5.1.3 & 5.1.4.

RGR was also decreased with increasing NaCl concentration, Table 5.1.1. Again, the decrease was more severe when plants were treated with NaCl & inoculated with V2, in all salt concentrations, Table 5.1.2. At 50 mM and above, the RGR of plants that were treated with NaCl was significantly different from control plants, $P<0.050$, Table 5.1.3, while plants inoculated with V2 showed significant differences from the control plants, $P<0.050$, Table 5.1.4. Inoculated plants under saline conditions were more affected than either of the treatment alone, Table 5.1.4.

Significant differences were also observed in leaf area (LA) and Net assimilation rate (NAR) at NaCl concentrations of 50 mM and higher, Tables 5.1.1, 5.1.2, 5.1.3 & 5.1.4. The LA and NAR of plants inoculated with V2, whether treated with salt or not showed significant differences from the control plants, $P<0.050$, Tables, 5.1.1, 5.1.2, 5.1.3 & 5.1.4.

At the end of the experiment, the reisolation procedure was followed to establish whether the fungus was present in the inoculated or inoculated- & salt-treated plants. Reisolation was possible from the inoculated plants, Table 5.1.5. However, inoculation was not made from the inoculated plants that were treated with 150 mM or 200 mM NaCl. Their basal stems and roots were almost dead before the harvest and were contaminated with saprophytic fungi.



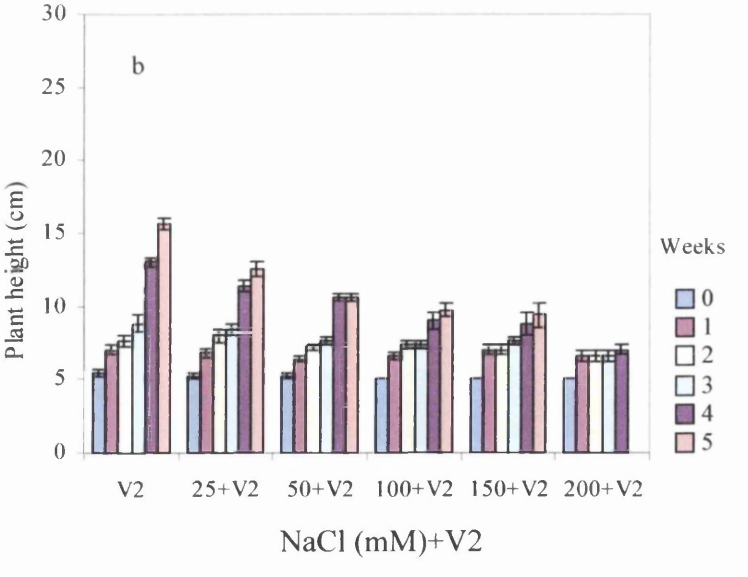


Fig. 5.1.1. The effect on the height of tomato cv. Ailsa Craig (4-week old) of (a) NaCl and (b) *V. albo-atrum,* isolate V2. Vertical bars show ± SE of mean.

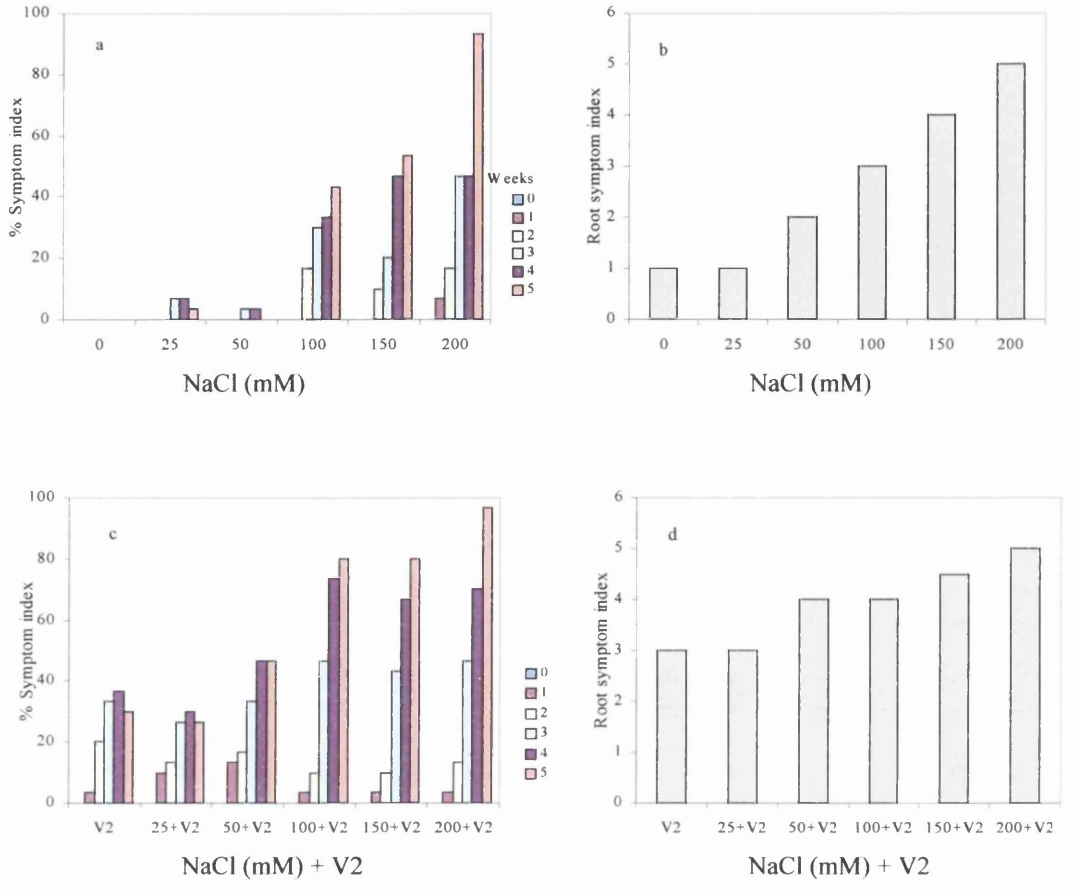


Fig. 5.1.2. The effect on disease development of 4-week old tomato cv. Ailsa Craig of *V. albo-atrum*, NaCl or both. a & c shoot symptom index; b & d root symptom index.

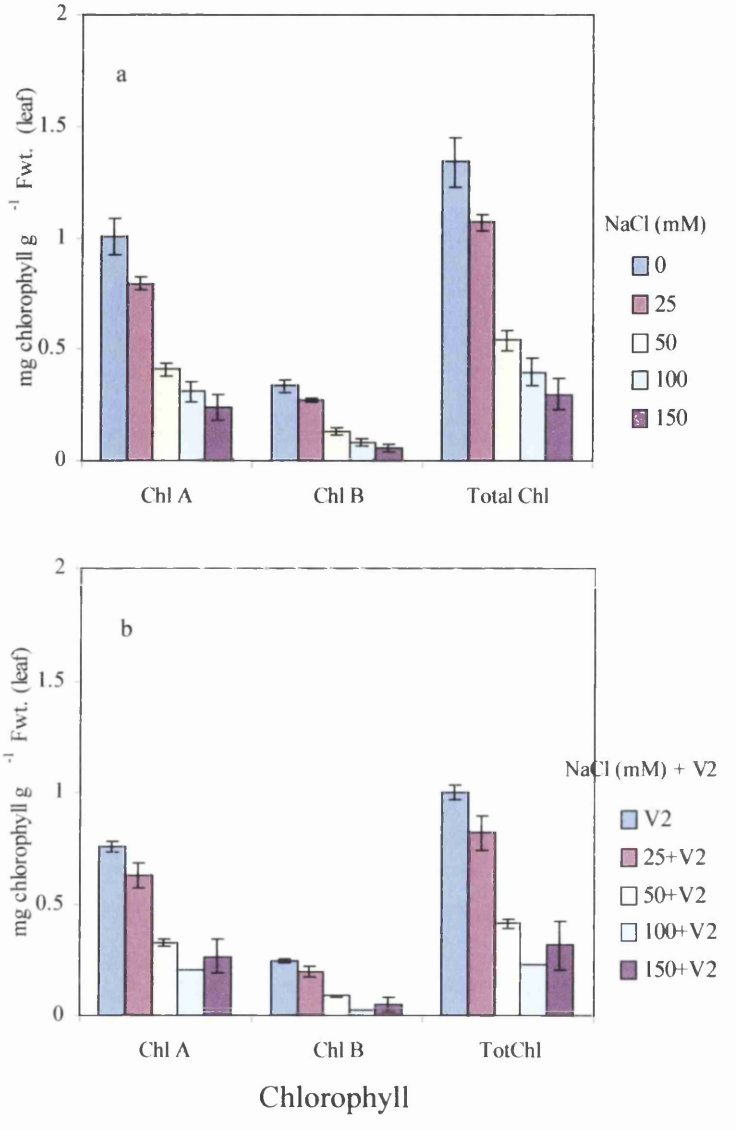


Fig. 5.1.3. The effect on the chlorophyll content (Chl) of tomato cv. Ailsa Craig (4-week old) of (a) NaCl and (b) V2 & NaCl. Vertical bars show ± SE of the mean. Measurement was taken at the end of 5-week experimental period.

Table 5.1.1. The effect of NaCl on selected growth parameters of tomato cv. Ailsa Craig (4-week old). Data presented in black is derived from uninoculated plants; data in blue is derived from the plants inoculated with *V. albo-atrum*. Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC (expressed as % water content). This colour template was used throughout this chapter where necessary.

Parameters	NaCl (mM)					
	0	25	50	100	150	200
H	1.00 ± 0.15*	0.88 ± 0.08	0.69 ± 0.03	0.56 ± 0.11	0.52 ± 0.09	-
RL	1.00 ± 0.11	0.90 ± 0.30	0.89 ± 0.14	0.57 ± 0.07	0.57 ± 0.07	-
RGR	1.00 ± 0.14	0.96 ± 0.14	0.57 ± 0.03	0.38 ± 0.19	0.43 ± 0.15	-
LA	1.00 ± 0.12	1.07 ± 0.07	0.82 ± 0.03	0.81 ± 0.03	0.70 ± 0.03	-
NAR	1.00 ± 0.24	0.93 ± 0.24	0.35 ± 0.03	0.35 ± 0.15	0.31 ± 0.11	-
WTC	90.5 ± 0.39	92.4 ± 1.28	92.3 ± 0.44	92.2 ± 1.38	89.8 ± 0.60	-

Table 5.1.2. The effect of *V. albo-atrum*, isolate V2, NaCl or both V2 & NaCl on selected growth parameters of tomato cv. Ailsa Craig (4-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group.

Parameters	NaCl (mM)					
	0	25	50	100	150	200
H	0.68 ± 0.02*	0.49 ± 0.04	0.36 ± 0.02	0.32 ± 0.03	0.29 ± 0.05	-
RL	0.95 ± 0.12	0.93 ± 0.11	0.58 ± 0.04	0.46 ± 0.03	0.49 ± 0.05	-
RGR	0.54 ± 0.07	0.47 ± 0.09	0.09 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	-
LA	0.68 ± 0.00	0.68 ± 0.01	0.46 ± 0.00	0.43 ± 0.02	0.47 ± 0.03	-
NAR	0.38 ± 0.07	0.31 ± 0.08	0.06 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	-
WTC	90.9 ± 0.53	92.3 ± 0.52	90.6 ± 2.32	92.1 ± 2.40	92.8 ± 1.99	-

- measurement was not taken due to stage of the plants.
* figure represents SE of the mean.

Table 5.1.3. Analysis of variance of the effects of NaCl on selected growth parameters of tomato cv. Ailsa Craig (4-week old), grown at 0-, 25-, 50-, 100-, 150- and 200 mM NaCl for 5 weeks. The results of analysis presented in black are derived from uninoculated plants, the ones in blue are derived from the plants inoculated with *V. albo-atrum*

Parameters	[NaCl] mM*						ANALYSIS OF VARIANCE				
	0	25	50	100	150	200	SS	df	MS	*F*	*Sig.*
H	a	ab	bc	c	c	-	0.849	4	0.212	4.34	0.011
RL	ab	a	b	b	b	-	3.209	4	0.802	3.61	0.025
RGR	a	a	b	b	b	-	1.276	4	0.319	4.04	0.016
LA	ab	a	bc	bc	c	-	0.416	4	0.104	4.25	0.014
NAR	a	a	b	b	b	-	2.168	4	0.542	4.02	0.017
WTC	a	a	a	a	a	-	28.513	4	7.128	1.61	0.213
CHL	a	b	c	c	c	-	1.635	4	0.409	40.5	0.001

- Data is not available

Table 5.1.4. Analysis of variance of the effects of NaCl, *V. albo-atrum* or both on growth parameters of tomato cv. Ailsa Craig (4-week old), grown at 0-, 25-, 50-, 100-, 150- and 200 mM NaCl for 5 weeks.

Parameters	[NaCl] mM*						ANALYSIS OF VARIANCE				
	0	0^+	25^+	50^+	100^+	150^+	SS	df	MS	*F*	*Sig.*
H	a	b	bc	c	c	c	1.830	5	0.366	15.1	0.000
RL	a	ab	ab	bc	c	c	0.955	5	0.191	3.91	0.016
RGR	a	b	b	c	c	c	2.632	5	0.526	15.1	0.000
LA	a	b	b	bc	c	bc	0.907	5	0.181	9.68	0.000
NAR	a	b	bc	bc	bc	c	2.753	5	0.551	9.10	0.000
WTC	a	a	a	a	a	a	16.894	5	3.379	0.67	0.651
CHL	a	b	b	c	c	c	1.935	5	0.387	36.4	0.000

* Between treatments, growth parameters of Ailsa Craig (4-week old) with the same letters are *not significantly different* from each other at 0.05 level.

$^+$ Ailsa Craig was exposed to [NaCl] and inoculated with *V. albo-atrum*, isolate V2.

- Data is not available

CHL: Leaf Chlorophyll Content (Chlorophyll A, B and Total).

Table 5.1.5. Reisolation of *V. albo-atrum*, isolate V2, from inoculated tomato (4-week-old) plants, grown at 0-, 25-, 50-, 100-, 150- and 200 mM NaCl. Reisolation was made at the end of the experimental period.

Isolates	Number of plants from which reisolation was successful	
	Fungal reisolation	Symptom index (%)
Control	0	0
V2	3	30
25 + V2	3	27
50 + V2	2	47
100 + V2	1	80
150 + V2	0	80
200 + V2	0	97

3 plants were examined for reisolation.

5.1.2. Effect of NaCl on the severity of symptoms resulting from inoculation with V. albo-atrum, *isolate V2 of 8-week old tomato plants.*

The measurements of height and symptom index of the 8-week old tomato plants inoculated with *V. albo-atrum*, isolate V2 or treated with NaCl or both are presented in Fig. 5.1.4. The chlorophyll content of the leaves is presented in Fig. 5.1.5. The results of the relative rate of height & root length increase (H&RL), RGR, LA, NAR are presented in Tables 5.1.6 & 5.1.7. Analysis of variance tables, which compare the effect of salt and fungus, are presented in Tables 5.1.8 & 5.1.9. No reisolation was made from the inoculated plants at the end of the experimental period.

The 8-week old plants were treated with three concentrations of NaCl, (50-, 100- and 150). Since 25 mM NaCl had little effect on 4-week old plants and the effects of 200 mM NaCl were rather severe, these concentrations were omitted in this experiment. Six replicates were used for each treatment. In another set, tomato plants treated with the same series of salt concentrations were also inoculated with $1x10^7$ conidia/ml of *V. albo-atrum*, isolate V2 by the root-dip method.

Increasing the concentration of NaCl had a marked effect on height and on the visual symptoms of the plants. An increase in the concentration of NaCl inhibited the increase in height but caused an increase in the symptom index, Fig. 5.1.4a & b. This effect was more severe in plants that were also inoculated with V2 at the same time that they were treated with NaCl, Fig. 5.1.4c & d.

In general, the symptoms resulting from the fungus or from treatment with NaCl were the same as those observed on 4-week old tomato plants. However, the appearance of some lower leaves was succulent, especially in the group of plants treated with 100 mM NaCl and above. However, this had no effect on the calibration curve constructed for determination of the leaf area from the leaf weight (see Materials and Methods). Towards the end of the experiment, those inoculated plants that were treated with 100 mM- and 150 mM NaCl were almost dead and their symptom index values were higher than the other group of plants, Fig. 5.1.4d.

An increase in the NaCl concentration resulted in a decrease in chlorophyll content of the leaves Fig. 5.1.5a. Plants inoculated with V2 and treated with NaCl at all concentrations (0-, 50- and 100 mM) showed a reduced chlorophyll content Fig. 5.1.4b.

Relative rate of increase in height and root length were recorded at the end of the experiment. An increase in the NaCl concentration inhibited the increase in height and root length compared to the control group, Tables 5.1.6. Concentrations at or above 50 mM NaCl caused significant differences in height of plants from the control group, Table 5.1.8. However, no significant difference was observed between salt-treated and control plants in root length measurement up to 150 mM NaCl concentration, on the other hand, plants inoculated with V2 and treated with NaCl showed significant differences from the control group at 100 or above (150 mM) NaCl concentrations. It is clear that the root length of the plants was less affected than the aerial parts of the plants, Tables 5.1.8 & 5.1.9. The significant effect of both V2 & 100 mM NaCl on the development of the roots clearly indicated that the fungus was still pathogenic under 100 mM NaCl concentration in a long-term.

The RGR was significantly affected at all the concentrations, Tables 5.1.6 & 5.1.8. However, inoculated plants with V2 alone did not show significant differences from the controls. It is probable that the plants recovered from the effect of the fungus

after some time. However, inoculated and NaCl-treated plants showed significant differences from the control plants at as low as 50 mM NaCl concentrations, Table 5.1.7 & 5.1.9.

Significant differences were also observed in LA and NAR at 100 mM NaCl concentration (comparisons were made between control and 100 mM NaCl-treated or control and inoculated & 100 mM NaCl-treated plants) Table 5.1.6. Significant differences were observed between control and treatment groups, Tables 5.1.7 & 5.1.9.

The result showed that the fungus had severe effect on young seedlings, however, the effect was found to be more severe with the additive effect of NaCl. On the other hand, NaCl itself also affected the plant growth and caused severe symptoms, especially at and above 100 mM NaCl concentration. No recovery was observed in plants inoculated with V2 and treated with NaCl at all the concentrations used, although some recovery was recorded from the effect of fungus in 8-week old plants.

The results indicated that the fungus is still pathogenic under salt conditions and delays the recovery of plants. Increased NaCl concentration causes more severe symptoms in both young and mature tomato plants and reduces the resistance to the effect of the pathogen thus making them more susceptible to the fungus.

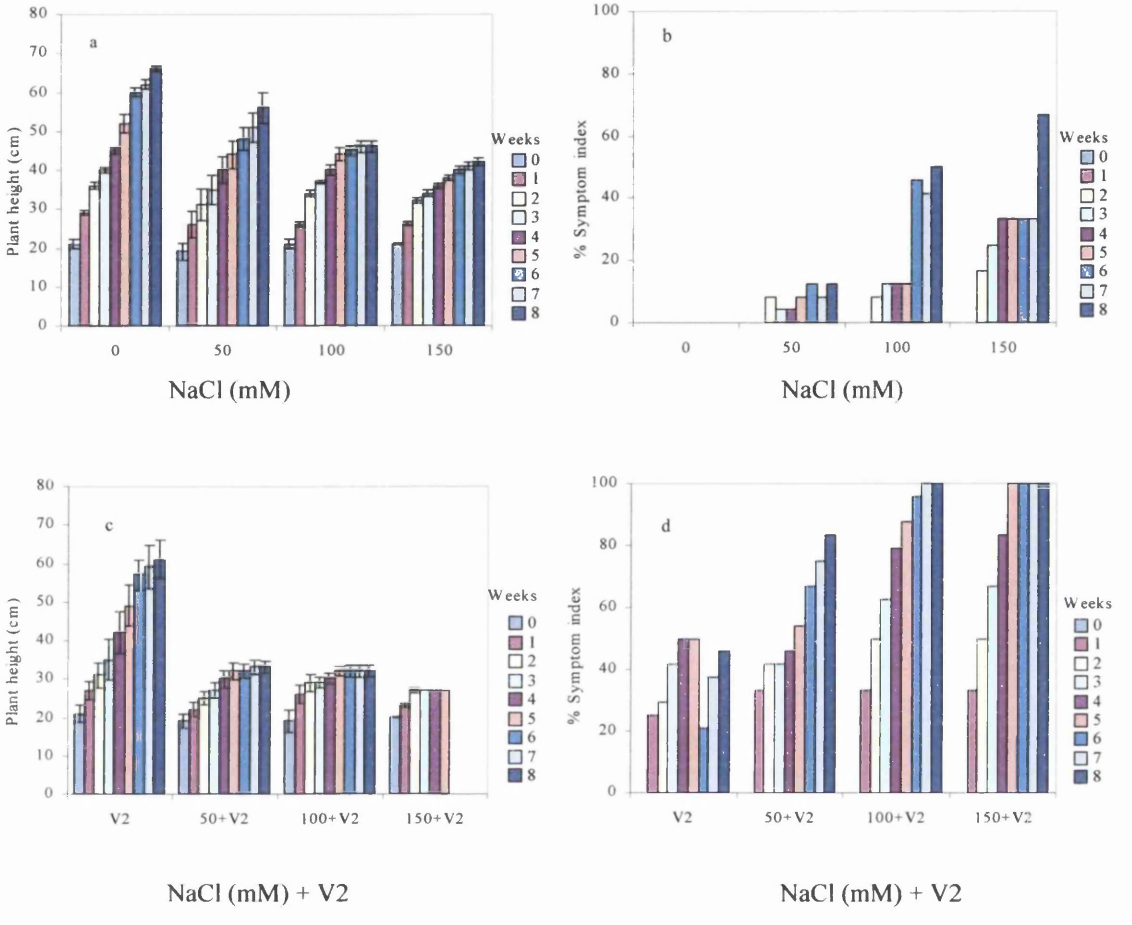


Fig. 5.1.4. The effect on the height and disease development of tomato cv. Ailsa Craig (8-week old) of (a&b) NaCl and (c&d) *V. albo-atrum,* isolate V2. Vertical bars show ± SE of mean.

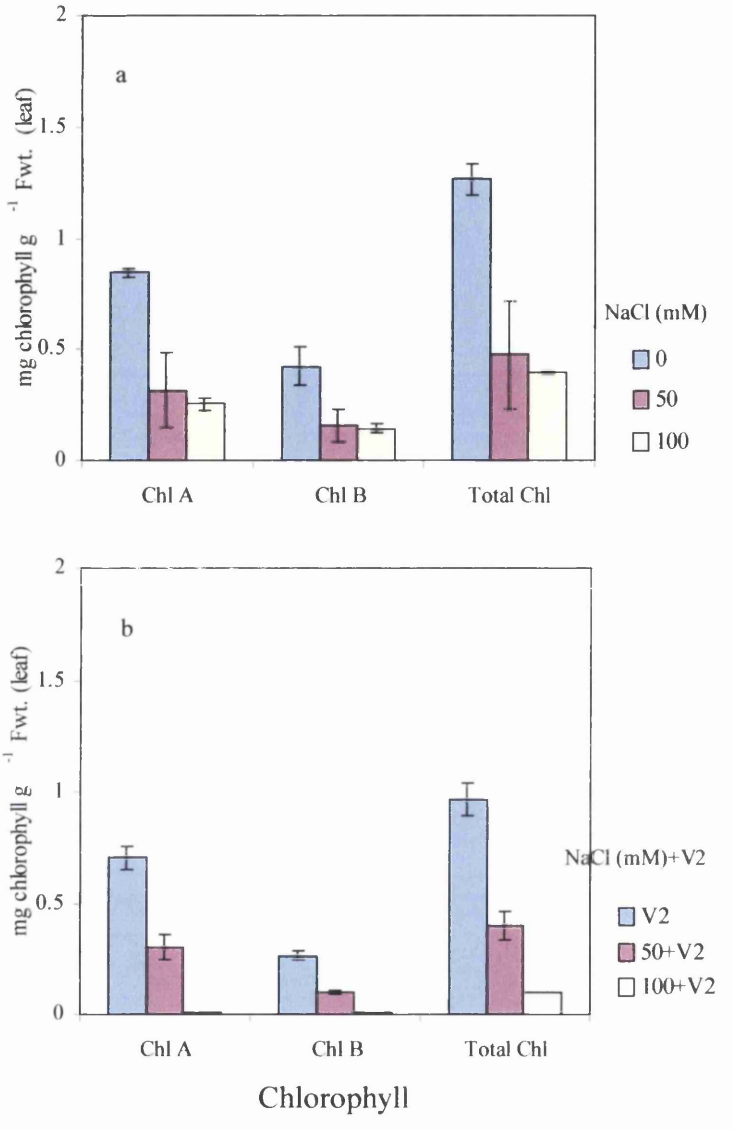


Fig. 5.1.5. The effect on the chlorophyll content (Chl) of tomato cv. Ailsa Craig (8-week old) of (a) NaCl and (b) V2 & NaCl. Vertical bars show ± SE of the mean. Measurement was made at the end of 8-week experimental period.

Table 5.1.6. The effect of NaCl on selected growth parameters of tomato cv. Ailsa Craig (8-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1.

Parameters	NaCl (mM)			
	0	50	100	150
H	1.00 ± 0.04*	0.81 ± 0.05	0.54 ± 0.03	0.46 ± 0.03
RL	1.00 ± 0.13	1.02 ± 0.13	0.71 ± 0.09	0.57 ± 0.03
RGR	1.00 ± 0.03	0.83 ± 0.05	0.70 ± 0.04	0.63 ± 0.06
LA	1.00 ± 0.00	-	0.87 ± 0.00	-
NAR	1.00 ± 0.00	-	0.64 ± 0.00	-

- measurement was not taken due to stage of the plants.

Table 5.1.7. The effect of V2, NaCl or both V2 & NaCl on selected growth parameters of tomato cv. Ailsa Craig (8-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1.

Parameters	NaCl (mM)			
	0	50	100	150
H	0.89 ± 0.09*	0.31 ± 0.06	0.29 ± 0.04	0.24 ± 0.03
RL	1.05 ± 0.12	0.86 ± 0.01	0.24 ± 0.05	0.16 ± 0.06
RGR	1.02 ± 0.10	0.55 ± 0.06	0.48 ± 0.05	0.47 ± 0.02
LA	1.00 ± 0.00	-	0.45 ± 0.00	-
NAR	1.00 ± 0.00	-	0.24 ± 0.00	-

- measurement was not taken due to stage of the plants.
* figure represents SE of the mean.

Table 5.1.8. Analysis of variance of the effects of [NaCl] on selected growth parameters of tomato cv. Ailsa Craig (8-week old), grown at 0-, 50-, 100- and 150 mM NaCl for 8 weeks.

Parameters	[NaCl] mM*				ANALYSIS OF VARIANCE				
	0	50	100	150	SS	df	MS	*F*	*Sig.*
H	a	b	c	c	0.741	3	0.247	45.43	0.000
RL	a	a	ab	b	0.577	3	0.192	4.235	0.029
RGR	a	b	bc	c	0.323	3	0.108	12.67	0.000
LA					0.303	1	0.303	5E+6	0.000
NAR					0.579	1	0.579	5E+5	0.000
CHL	a	b	b	-	0.929	2	0.464	10.9	0.042

Table 5.1.9. Analysis of variance of the effects of [NaCl] or *V. albo-atrum* or their combined effect on selected growth parameters of tomato cv. Ailsa Craig (8-week old), grown at 0, 50, 100 and 150 mM NaCl for 8 weeks.

Parameters	[NaCl] mM					ANALYSIS OF VARIANCE				
	0	0^+	50^+	100^+	150^+	SS	df	MS	*F*	*Sig.*
H	a	a	b	b	b	2.168	4	0.542	43.60	0.000
RL	a	a	a	b	b	2.936	4	0.734	19.44	0.000
RGR	a	a	b	b	b	1.266	4	0.316	23.37	0.000
LA						1E-02	1	1E-02	1E+5	0.000
NAR						0.129	1	0.129	1E+5	0.000
CHL	a	b	c	d	-	1.688	3	0.563	77.0	0.001

* Between treatments, growth parameters of Ailsa Craig (4-week old) with the same letters are *not significantly different* from each other at 0.05 level.

$^+$ Ailsa Craig exposed to [NaCl] was also inoculated with *V. albo-atrum*, isolate V2.

- Data is not available

Chl: Leaf Chlorophyll Content (Chlorophyll A, B and Total)

5.2. Interactive effects of NaCl (50 mM) and *V. albo-atrum*, isolates V1 & V2, on VW disease severity, growth and development of various tomato cultivars in a hydroponic system.

The results of the previous section, show that at some concentrations NaCl caused severe symptoms on tomato, whether on young or mature plants. The effect of NaCl, at all concentrations used, was more severe on inoculated plants. This increase in disease severity under saline conditions is unlikely to result from a direct effect of NaCl on the fungus but rather from a reduction in the resistance of host plants. It is clear that NaCl does not stimulate growth of the pathogen *in vitro* (Chapter 4; section 4.4) and does not increase the virulence of the pathogen *in vivo* (Chapter 5, section 5.4). Nevertheless, it is still not possible to conclude that the pathogen would not be more aggressive under saline conditions.

Since *Verticillium* stays viable in non-saline soil for long periods (Basu, 1987), there is always a threat to plants from the pathogen in the soil environment. If the fungus were to stay viable for a long-term under saline conditions, it is probable that it would adapt to salinity and remain pathogenic. Although there is no indication that adaptation to salt increases virulence of *V. albo-atrum*, there is a previous report of an exogenous stress, in this case an increase in temperature, causing *V. albo-atrum* to become more virulent to lucerne cultivars in southern California (Howell & Erwin, 1995).

So far, there have been no reports describing the interactive effects of salinity and *V. albo-atrum* on tomato; and only two works have been dedicated to the interactive effects of salinity and *V. albo-atrum* on lucerne (Nachmias *et al.*, 1993 and Howell *et al.*, 1994). Since it has been established that *V. albo-atrum* can survive and produce spores under saline conditions (above), and that NaCl has an adverse effect on plant health, making them more susceptible to wilt disease (above), it is important to determine the combined effect of fungus & NaCl. A variety of cultivars were selected for assessment, including resistant ones. For this work, 50 mM was used as the concentration of NaCl. This particular concentration was selected because 50 mM is the concentration of NaCl that has been reported to be the point at which problems begin in agricultural areas (Abrol *et al.*, 1988). Both V1 & V2 isolates of *V. albo-atrum* were used and the experiments were performed on 6-week old tomato cultivars, disease-resistant and susceptible

varieties. From the results of the previous experiment (section 5.1), it was concluded that 4-week old tomato seedlings were quite susceptible to both NaCl and *V. albo-atrum*, whilst 8-week old ones were only susceptible to NaCl. Individual effects of NaCl or *V. albo-atrum* or the combined effect of NaCl & *V. albo-atrum* were more severe on 4-week old seedlings than on 8-week old ones. Therefore, it was considered that the selection of 6-week old tomato seedlings might show better resistance to both NaCl and *V. albo-atrum* than 4-week old seedlings and they might as well be affected by the fungus *V. albo-atrum* unlike 8-week old seedlings. As a result of that, the combined effect of NaCl & *V. albo-atrum* might clearly be examined under the hydroponic conditions. Initially, the cultivars were selected on the basis of the results from the pathogenicity experiments (see Chapter 3); they were Ailsa Craig and Simge F1 (susceptible-) and Margarita Fa-558 (resistant to *V. albo-atrum*). Fantastic F1 was classified neither resistant nor susceptible to *V. albo-atrum*. The results of the pathology and salinity tests had indicated that cvs. Edcawy and *L. lycopersicon* were highly resistant and tolerant to both *V. albo-atrum* and NaCl, and normally both would have been selected for assessment. However, it proved impossible to obtain further stocks of the seed from the original suppliers, despite repeated attempts. A hydroponic system was used, to minimize other influences on disease development. The system not only enabled regular and even irrigation for plants, but also allowed greater replication for each treatment. The distribution of root system in pots of perlite was also monitored periodically.

Each cultivar was screened separately for the effects of pathogen and salt in the hydroponic culture system. Seeds were germinated in trays of John Innes No. 1 compost and at the first opportunity, were transplanted into 15-cm pots of soil. At 6 weeks plants were inoculated with the conidia (1×10^7 conidia/ml) of *V. albo-atrum* (isolates V1 or V2) by the root-dip method. At the same time, a group of plants were treated with distilled water as controls. Following inoculation, the plants were transferred to the 15-cm pots of perlite in the troughs of the hydroponic culture system, Plate 5.1. Plants were then irrigated with Solufeed F culture solution (Materials & Methods) for three days for an adaptation period. After this period, the pre-diluted nutrient solution, with or without NaCl, was pumped for a period of 7 weeks from two central tanks via control valves and filtered to the main pipes. Each pipe has a capacity to deliver fertigation solution to 56

pots of plants arranged at 14 troughs. A digital timer controlled the scheduling of drip irrigation system, (the entire system is described in detail in Materials and Methods. For each cultivar, a total 112 plants were used for the pathogenicity and salinity tests. The arrangement was made as follows;

(C): non-salinity stressed & non-inoculated control group (20 plants),

(V1): inoculated group with *V. albo-atrum*, isolate V1 (20 plants),

(V2): inoculated group with *V. albo-atrum*, isolate V2 (16 plants),

(S): salinity-stressed & non-inoculated group (20 plants),

(V1+S): salinity stressed & inoculated group with *V. albo-atrum*, isolate V1 (20 plants),

(V2+S): salinity stressed & inoculated group with *V. albo-atrum*, isolate V2 (16 plants).

At the start of the experiment, a sample of five plants was harvested and the growth parameters including initial stem height and root length, fresh and dry weight of the plants, and leaflet surface area were recorded.

Four plants from each treatment group were randomly selected and harvested after 4 weeks of the start of the inoculation to observe the progress of any effects of NaCl and *Verticillium* on the growth and development of the plants. No judgment was made concerning the plant selection during the experiment. At the end of the 7-week trial, plants from each treatment group were harvested. At 4^{th} and 7^{th} week of the experiment, the following selected growth parameters were assessed, including;

The relative rate of increase in height and root length (H & RL),

Relative growth rate (RGR) and water content (WTC) through stem and root fresh and dry weights,

Leaf area (LA),

Net assimilation rate (NAR),

Chlorophyll content (CHL) of the leaves,

The flower numbers (FLW) of the plants were either recorded or calculated (see Materials and Methods).

Height and symptom index values were recorded weekly for a period of 7 weeks.

5.2.1. Ailsa Craig - Growth Analysis.

The effects of *V. albo-atrum* (V1 and V2), or NaCl or both on growth and development of 6-week old tomato cv. Ailsa Craig were compared under a hydroponic system, Plate 5.1. Observations on height and the symptom index, indicating severity of the disease, or the effect of NaCl or both are presented in Fig. 5.2.1 (a & b). Leaf chlorophyll content is presented in Fig. 5.2.2. The parameters, H, RL, RGR, FLW, LA, NAR and WTC recorded at the end of 4- and 7-week experimental period, are presented in Tables 5.2.1 and 5.2.2. Analysis of variance tables, which compare the effect of salt and fungus or both, are presented in Tables 5.2.3 and 5.2.4.

In general, plants that were treated with 50 mM NaCl (S) or inoculated with *V. albo-atrum*, isolate V2 or both (V1+S or V2+S) appeared stunted in height, Fig. 5.2.1a; Plate 5.1. However, plants that were inoculated with, isolate V1 did not differ from the control plants during the experimental period, Fig. 5.2.1a.

By itself, *V. albo-atrum*, (especially isolate V2) or NaCl inhibited the growth of plants, Fig. 5.2.1a, however, the inhibition was more significant in V2 inoculated and NaCl (V2+S) treated group, Fig. 5.2.1a.

The symptoms resulting from treatment with the pathogen or NaCl or both were described in the previous sections. Here, a visual comparison of the symptoms resulting from the different treatments is presented in Plates 5.2 and 5.3. Up to the 4^{th} week, there were no significant difference in the development of symptoms resulting from the treatment with NaCl or pathogen Fig. 5.2.1, and after this period, plants started to recover from the effects of salt or the pathogen. However, plants treated with V2+S showed more severe symptoms than the other groups and no recovery was observed, although the symptoms of the disease were not as severe in the later part of the experiment as they were in the initial period, Fig. 5.2.1b.

The treatment groups, V1, V2, V1+S or V2+S, showed some chlorosis and their chlorophyll content decreased compared to the control group (C). However, the decrease in chlorophyll content was not statistically significant up to the 4th week, Fig. 5.2.2 & Table 5.2.3, and at the end of the 7-week experiment, only V2- and V2+S-treated groups showed statistical differences from the control group Fig. 5.2.2b; $P<0.05$, Table 5.2.4.

The treatment groups, V2, S, V1+S, V2+S, also showed significant differences from the control group when the relative rate of increase in height was recorded at 4th week, Tables 5.2.1 & 5.2.3. Although some reduction in the other growth parameters such as RL, RGR, LA, NAR, WTC, CHL and FLW were recorded, particularly for the V2- and V2+S-treated plants, these were not significantly different from the control group, Table 5.2.1 & 5.2.3, due to the high standard errors recorded either in control or the treated groups.

At week 7, the final harvest was made and the same parameters were recorded as in the 4th week harvest. The plants that were inoculated with V1 did not show any significant differences from the control group for any of the parameters, Tables 5.2.2 & 5.2.4. However, groups such as V2, S, V1+S and V2+S showed significant differences from the control groups in terms of H, RL, RGR (Tables 5.2.2 & 5.2.4). When FLW or CHL was compared only V2 or V2+S treatment groups showed significant differences from the control group, Tables 5.2.2 & 5.2.4.

In summary, plants inoculated with fungi and treated with salinity showed more severe symptoms and lower values in their growth parameters than either of the treatments alone suggesting that mild-salinity stress predisposes plants to infection in a long-period if not in a short period.

Plate a.

Plate b.

Plate 5.1.
Tomato plants grown in a hydroponic culture system at (a) 4-; and (b) 7 weeks following the start of the treatments. 1-control, consisting of control and plants inoculated with *V. albo-atrum*, isolate V1 or V2, (received nutrient via tubing system); 2-salt-treated, consisting of control and plants inoculated with *V. albo-atrum*, isolate V1 or V2, (received nutrient and 50 mM NaCl via tubing system).

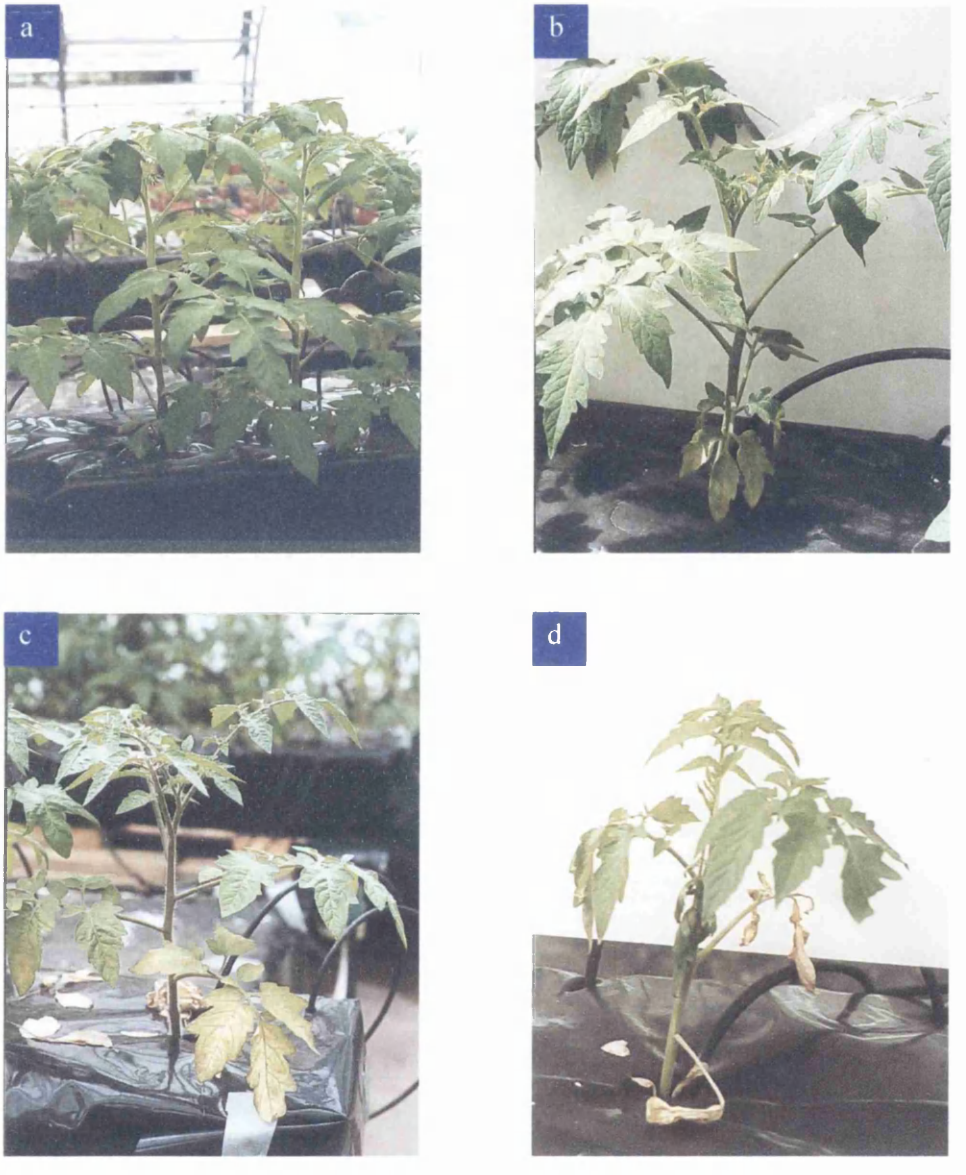


Plate 5.2.
Tomato plants (a) control; (b) inoculated with *V. albo-atrum* (isolate V2), growing under hydroponic conditions showing symptoms such as wilting and flaccidity on the lower leaves; (c) treated with NaCl showing wilting and chlorosis; and (d) inoculated with *V. albo-atrum* (isolate V2) and treated with 50 mM NaCl.

Plate 5.3.
The typical effect of *V. albo-atrum* isolates, V1 or V2, and the combined effect of isolate V2 and 50 mM NaCl on tomato plants.

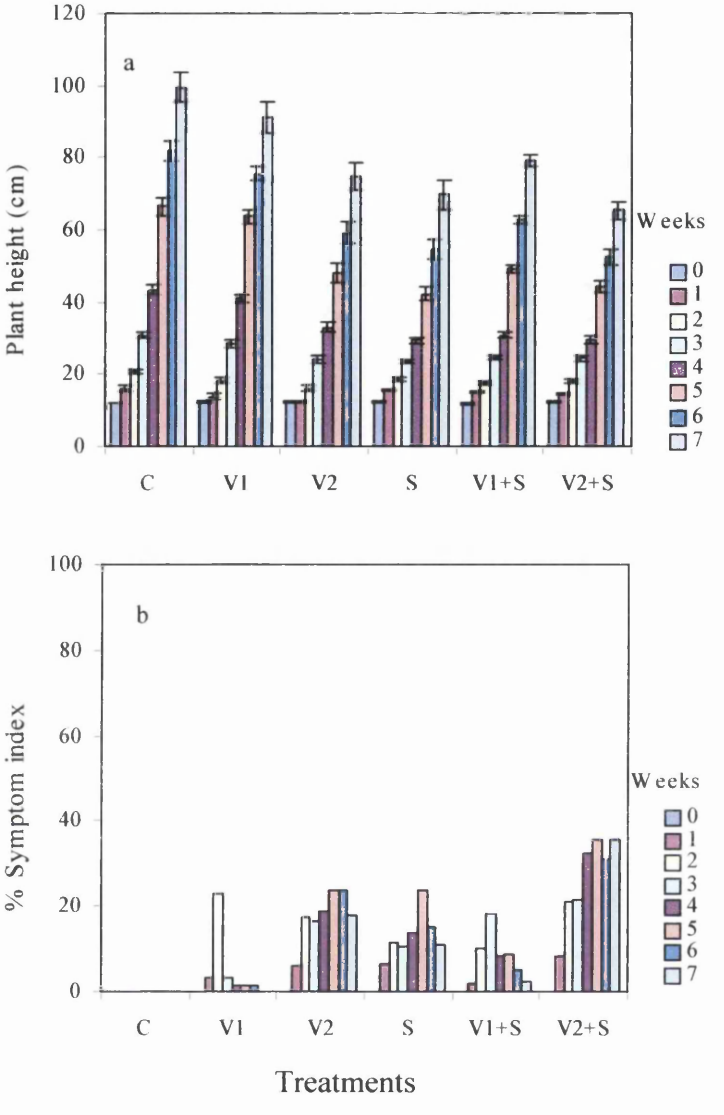


Fig. 5.2.1. The effect of *V. albo-atrum*, NaCl or both; (a) on height, (b) on symptom index of tomato cv. Ailsa Craig (6-week old). Vertical bars show ± SE of mean.

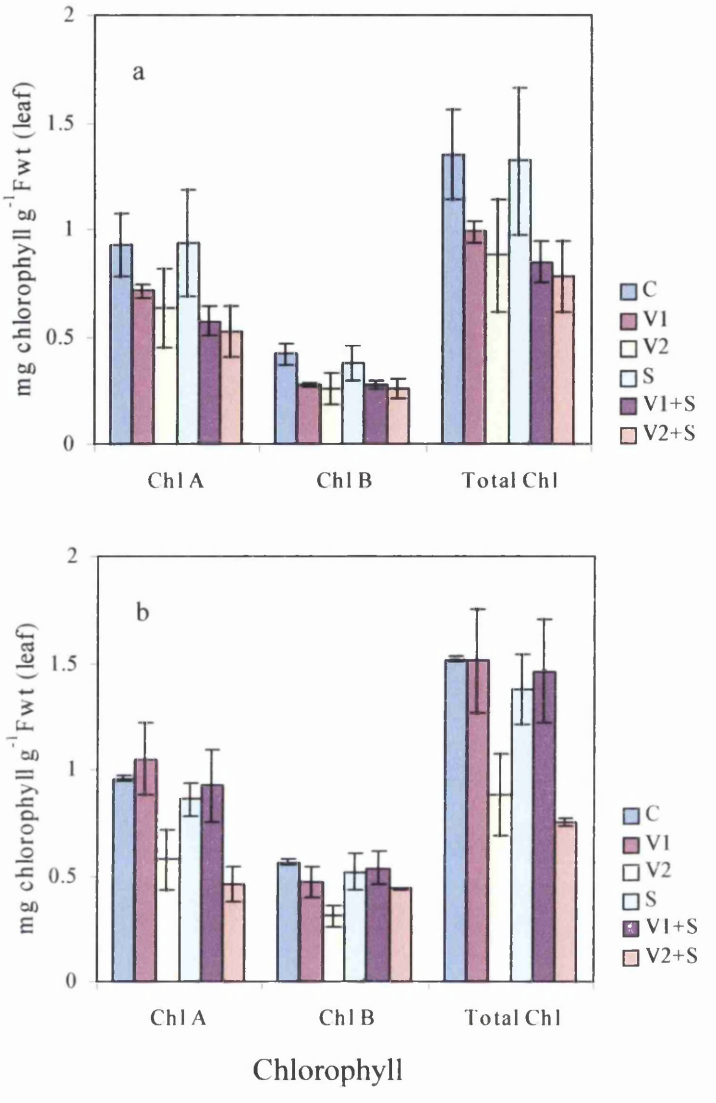


Fig. 5.2.2. The effect of *V. albo-atrum*, NaCl, or both on chlorophyll content of 6-week old leaves of tomato cv. Ailsa Craig, at; (a) 4 weeks after the start of the experiment; (b) 7 weeks after the start of the experiment. Vertical bars show ± SE of mean.

old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 4 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.08*	1.00 ± 0.19	0.67 ± 0.10	0.64 ± 0.10	0.68 ± 0.03	0.63 ± 0.01
RL	1.00 ± 0.05	0.98 ± 0.05	0.92 ± 0.05	0.92 ± 0.03	0.93 ± 0.05	0.87 ± 0.05
RGR	1.00 ± 0.15	1.01 ± 0.24	0.59 ± 0.16	0.95 ± 0.12	0.83 ± 0.11	0.82 ± 0.08
FLW	1.00 ± 0.09	1.11 ± 0.10	0.97 ± 0.05	1.00 ± 0.07	1.26 ± 0.19	0.97 ± 0.07
LA	1.00 ± 0.12	1.13 ± 0.15	0.86 ± 0.14	0.94 ± 0.10	0.80 ± 0.12	0.85 ± 0.06
NAR	1.00 ± 0.31	1.00 ± 0.32	0.42 ± 0.13	0.91 ± 0.15	0.77 ± 0.10	0.70 ± 0.08
WTC	92.0 ± 0.45	92.7 ± 0.20	92.4 ± 0.42	91.6 ± 0.23	92.4 ± 0.46	91.8 ± 0.71

Table 5.2.2. The effect of *V. albo-atrum,* NaCl, or both; on selected growth parameters of tomato cv. Ailsa Craig (6-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 7 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.05*	0.90 ± 0.05	0.72 ± 0.04	0.66 ± 0.05	0.77 ± 0.02	0.61 ± 0.03
RL	1.00 ± 0.04	0.98 ± 0.05	0.72 ± 0.04	0.56 ± 0.03	0.67 ± 0.03	0.59 ± 0.04
RGR	1.00 ± 0.03	0.99 ± 0.02	0.82 ± 0.03	0.85 ± 0.01	0.81 ± 0.02	0.72 ± 0.03
FLW	1.00 ± 0.10	1.07 ± 0.10	0.57 ± 0.09	0.87 ± 0.14	0.87 ± 0.06	0.56 ± 0.07
WTC	87.7 ± 0.86	89.0 ± 0.49	80.3 ± 9.70	90.4 ± 0.76	90.4 ± 0.41	89.3 ± 0.59

* figure represents SE of the mean.

Table 5.2.3. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Ailsa Craig (6-week old). Analysis was made 4 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	b	b	b	b	0.651	5	0.130	3.24	0.029
RGR	a	a	a	a	a	a	0.521	5	0.104	1.11	0.387
FLW	a	a	a	a	a	a	0.260	5	5E-02	1.20	0.349
CHL	a	a	a	a	a	a	0.724	5	0.145	0.71	0.506
LA	a	a	a	a	a	a	0.281	5	5E-02	1.00	0.446
NAR	a	a	a	a	a	a	0.934	5	0.187	1.29	0.312
WTC	a	a	a	a	a	a	3.545	5	0.709	0.88	0.509

* Between treatments, parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level.

Table 5.2.4. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Ailsa Craig (6-week old). Analysis was made 7 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	bc	bc	b	c	1.679	5	0.336	14.5	0.000
RL	a	a	b	c	bc	c	2.543	5	0.509	22.2	0.000
RGR	a	a	b	b	b	c	0.889	5	0.178	21.5	0.000
FLW	a	a	b	a	a	b	3.422	5	0.684	5.53	0.000
CHL	a	a	bc	ab	a	c	1.600	5	0.320	3.73	0.032
WTC	a	a	a	a	a	a	994.9	5	198.9	0.78	0.561

* Between treatments, parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level.

5.2.2. Simge F1 - Growth Analysis.

The effects of pathogenicity of *V. albo-atrum* (V1 and V2) or NaCl or both, on growth and development of 6-week old cv. Simge F1 are presented in Figs. 5.2.3 & 5.2.4; Tables 5.2.5 & 5.2.6.

All treatments, V1, V2, S, V1+S and V2+S, at week 4, resulted in stunting compared to the control group, Fig. 5.2.3a. The inhibition of growth was more significant in the group of plants treated with both V2 and NaCl (V2+S).

Symptoms of the disease or salinity on tomato plants were not observed until the 2nd week of the experiment, Fig. 5.2.3b. Symptom index values were higher in V2-, S-, and V2+S-treated groups although recovery was observed towards the end of the experiment.

In terms of the relative rate of increase in height, V1-, V2-, S-, V1+S- and V2+S-treated groups showed significant differences from the control group, Tables 5.2.5 & 5.2.7, $P<0.050$. For RL, RGR, and LA, only the V2-, S-, V1+S- and V2+S-treated groups were statistically different from the control group, $P<0.050$, Tables 5.2.5 & 5.2.7. and only the V2+S-treated group was statistically different from the controls for NAR, FLW and CHL , Fig. 5.2.4a; Tables 5.2.5 & 5.2.7. WTC was not different for any of the treated groups, Tables 5.2.5 & Table 5.2.7.

At the end of the 7-week trial, the V2-, S-, V1+S- and V2+S-treated groups of plants were significantly different from the control groups in regard to all parameters except WTC and CHL, Fig. 5.2.4b; Tables 5.2.6 & 5.2.8. Although the height of plants inoculated with V1 was affected at 4th week of the experiment, they showed recovery from the effect of V1 and they were not significantly different from the control group at the end of the experiment.

At the 4th week, there were some differences between the chlorophyll content of leaves of treated groups, however, these differences disappeared towards the end of the experiment and all chlorophyll content of the leaves were almost same, Fig. 5.2.4b.

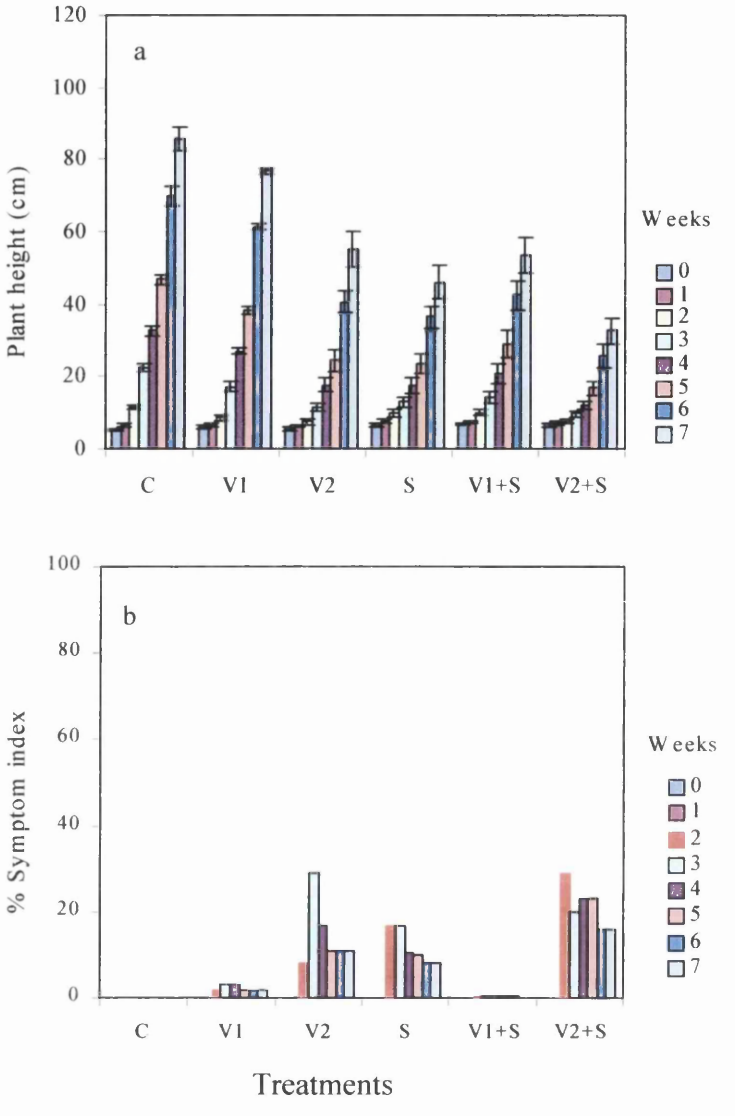


Fig. 5.2.3. The effect of *V. albo-atrum*, NaCl or both; (a) on height, (b) on symptom index of tomato cv. Simge F1 (6-week old). Vertical bars show ± SE of mean.

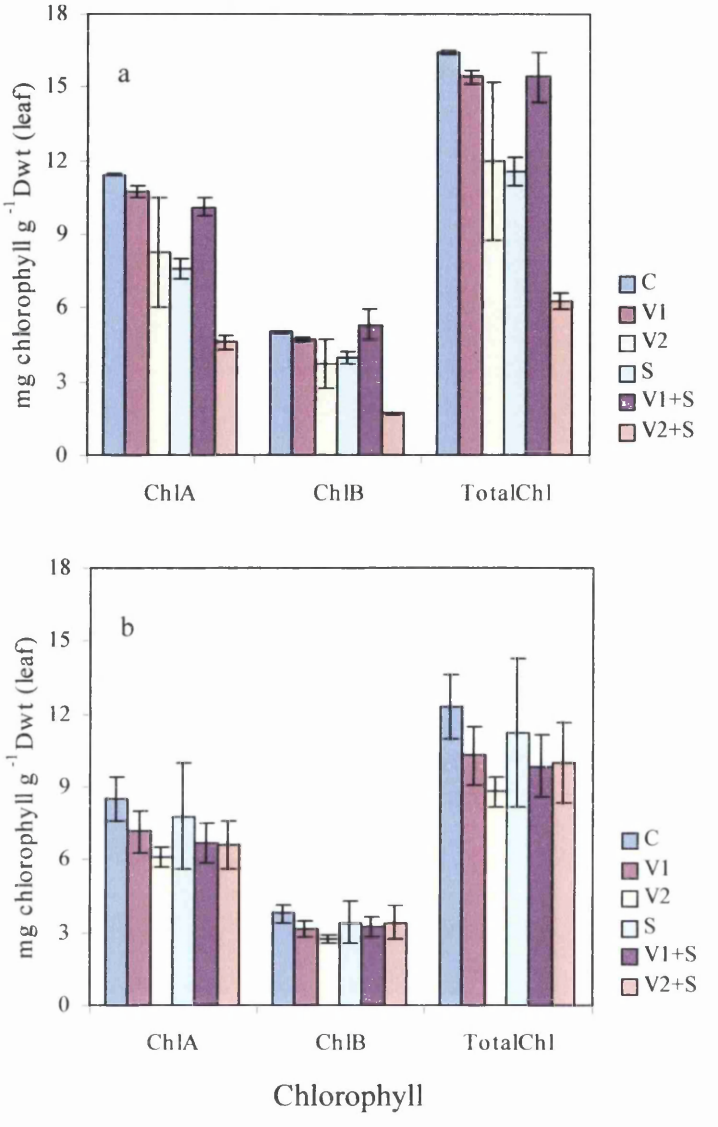


Fig. 5.2.4. The effect on chlorophyll content of *V. albo-atrum*, NaCl, or both; applied to 6-week old leaves of tomato cv. Simge F1. (a) 4 weeks after the start of the experiment; (b) 7 weeks after the start of the experiment. Vertical bars show ± SE of mean.

Table 5.2.5. The effect of *V. albo-atrum*, NaCl, or both; on the selected growth parameters of tomato cv. Simge F1 (6-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 4 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.03*	0.55 ± 0.21	0.50 ± 0.13	0.37 ± 0.10	0.52 ± 0.05	0.27 ± 0.10
RL	1.00 ± 0.04	0.91 ± 0.04	0.91 ± 0.04	0.91 ± 0.04	0.91 ± 0.04	0.87 ± 0.01
RGR	1.00 ± 0.05	0.97 ± 0.09	0.61 ± 0.08	0.63 ± 0.05	0.66 ± 0.02	0.09 ± 0.09
FLW	1.00 ± 0.03	0.81 ± 0.10	0.52 ± 0.13	0.61 ± 0.10	0.67 ± 0.03	0.61 ± 0.03
LA	1.00 ± 0.05	0.71 ± 0.15	0.60 ± 0.03	0.73 ± 0.03	0.73 ± 0.08	0.50 ± 0.04
WTC	93.0 ± 0.22	92.9 ± 0.72	91.1 ± 0.83	89.8 ± 2.70	92.4 ± 0.92	89.6 ± 0.35

* figure represents SE of the mean.

Table 5.2.6. The effect of *V. albo-atrum,* NaCl, or both; on selected growth parameters of tomato cv. Simge F1 (6-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 7 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.03*	0.89 ± 0.01	0.62 ± 0.06	0.49 ± 0.05	0.58 ± 0.06	0.32 ± 0.04
RL	1.00 ± 0.09	0.87 ± 0.09	0.76 ± 0.06	0.70 ± 0.09	0.66 ± 0.04	0.52 ± 0.04
RGR	1.00 ± 0.05	0.84 ± 0.08	0.65 ± 0.08	0.67 ± 0.09	0.73 ± 0.09	0.50 ± 0.08
FLW	1.00 ± 0.04	0.93 ± 0.05	0.75 ± 0.08	0.73 ± 0.04	0.75 ± 0.07	0.56 ± 0.04
LA	1.00 ± 0.05	0.85 ± 0.07	0.70 ± 0.09	0.73 ± 0.08	0.73 ± 0.08	0.51 ± 0.05
NAR	1.00 ± 0.13	0.72 ± 0.10	0.43 ± 0.07	0.48 ± 0.12	0.39 ± 0.11	0.33 ± 0.09
WTC	90.8 ± 0.33	91.8 ± 0.38	92.0 ± 0.38	90.7 ± 0.53	91.7 ± 0.63	90.5 ± 0.69

* figure represents SE of the mean.

Table 5.2.7. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Simge F1 (6-week old). Analysis was made 4 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	b	b	b	b	b	0.637	5	0.127	4.32	0.052
RL	a	ab	b	b	b	b	0.301	5	6E-02	4.70	0.043
RGR	a	a	b	b	b	c	1.09	5	0.218	25.5	0.001
FLW	a	ab	ab	ab	ab	b	0.351	5	7E-02	2.78	0.122
CHL	a	a	a	a	a	b	207.93	5	41.58	6.39	0.005
LA	a	a	b	b	b	b	0.422	5	8E-02	8.06	0.012
NAR	a	ab	ab	ab	ab	b	1.089	5	0.218	2.62	0.136
WTC	a	a	a	a	a	a	23.05	5	4.610	1.44	0.329

*Between treatments, growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level

Table 5.2.8. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Simge F1 (6-week old). Analysis was made 7 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	b	b	b	c	1.965	5	0.393	31.7	0.000
RL	a	ab	b	bc	bc	c	0.891	5	0.178	4.97	0.002
RGR	a	ab	bc	bc	bc	d	0.926	5	0.185	4.33	0.004
FLW	a	a	b	b	b	c	0.759	5	0.152	7.37	0.000
CHL	a	a	a	a	a	a	5.411	5	1.082	0.50	0.768
LA	a	ab	bc	bc	bc	c	0.801	5	0.160	4.64	0.003
NAR	a	ab	bc	bc	bc	c	1.912	5	0.382	5.50	0.001
WTC	a	a	a	a	a	a	13.33	5	2.666	1.68	0.167

*Between treatments, growth parameters of tomato with the same letter are *not significantly different* from each other at 0.05 level

5.2.3. Margarita (Fa-558) - Growth Analysis.

In general, treatment with V2-, S-, V1+S-, V2+S resulted in inhibition of growth compared to the control groups; inhibition in height was more severe in the S- and V2+S-treated groups, Fig. 5.2.5a, and isolate V1, as in the previous experiments, had either little or no effect on the height of the plants, Fig. 5.2.5a.

V. albo-atrum, isolate V2-, S-, and V2+S-treated groups caused symptoms. And again, the V2+S-treated group was markedly affected, 5.2.5b. The chlorophyll content (CHL) of the plants did not differ from treatment to treatment either at the 4^{th} or 7^{th} week of the experiment, although V2-, S- and V2+S-treated groups had low chlorophyll contents, Fig. 5.2.6a & b. However, they were not statistically different from the control groups.

There were significant differences in regard to H, RGR, LA and FLW at the 4^{th} week, for the V2-, S-, V1+S- and V2+S-treated, Tables 5.2.9 & 5.2.11. However, there were no statistical differences between the treatment groups in regard to RL and WTC, Tables 5.2.9 & 5.2.11. Furthermore, plants inoculated with isolate V1 did not show any differences from the non-stressed & non-inoculated control group, Tables 5.2.9 & 5.2.11.

At the end of the experiment (7 weeks), S-, V1+S- and V2+S-treated groups were all significantly different from the control group in regard to H, RL, RGR and FLW . However, the plants inoculated with isolate V2 did not show statistical differences from the control group with the exception of the relative rate of increase in height (H). Again, the effect of V1 was not significant in regard to any of the measured parameters, Tables 5.2.10 & 5.2.12., while WTC was not significantly affected in any of the treated groups, Tables, 5.2.10 & 5.2.12.

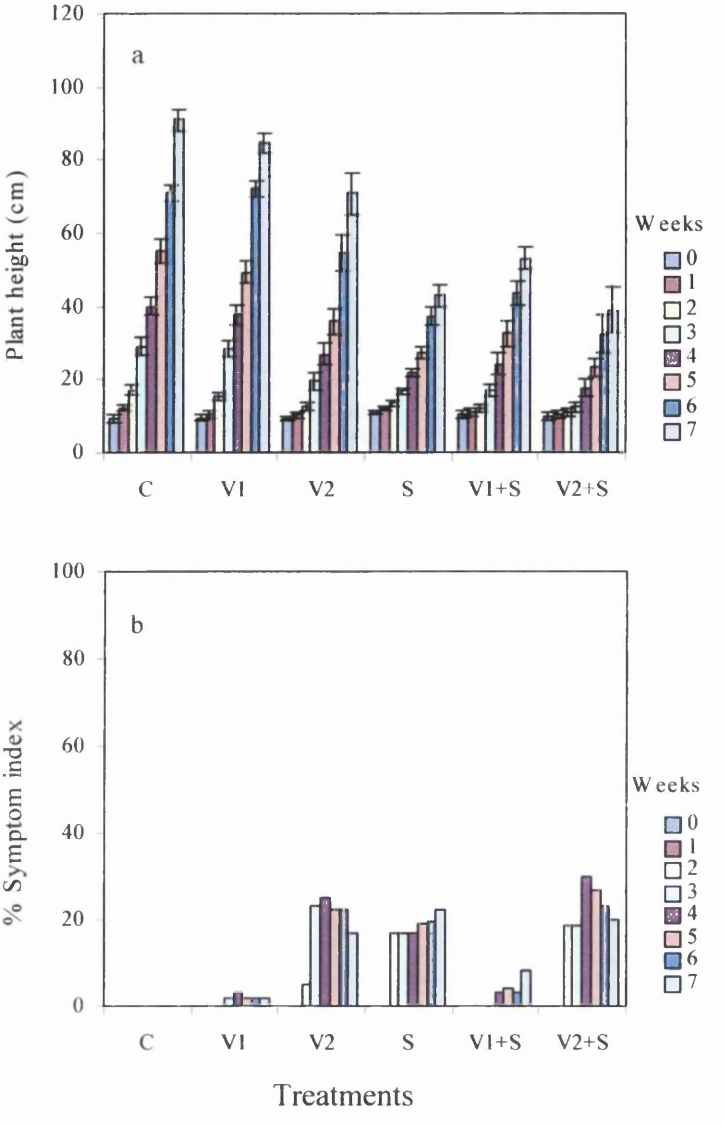


Fig. 5.2.5. The effect of *V. albo-atrum*, NaCl or both; (a) on height, (b) on symptom index of tomato cv. Margarita (Fa-558) (6-week old). Vertical bars show ± SE of mean.

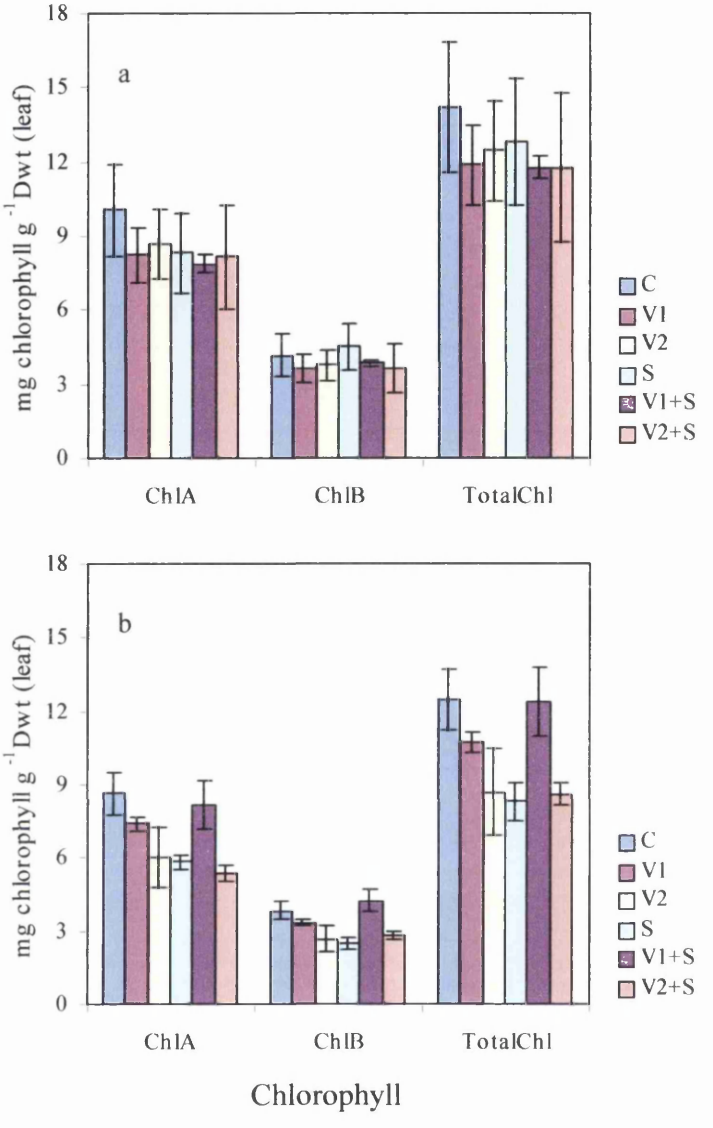


Fig. 5.2.6. The effect on chlorophyll content of *V. albo-atrum*, NaCl, or both; applied to 6-week old leaves of tomato cv. Margarita (Fa-558). (a) 4 weeks after the start of the experiment; (b) 7 weeks after the start of the experiment. Vertical bars show ± SE of mean.

Table 5.2.9. The effect of *V. albo-atrum*, NaCl, or both; on selected growth parameters of tomato cv. Margarita (Fa-558) (6-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 4 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.07*	0.90 ± 0.05	0.59 ± 0.09	0.39 ± 0.05	0.46 ± 0.06	0.24 ± 0.04
RL	1.00 ± 0.04	0.91 ± 0.04	0.91 ± 0.04	1.00 ± 0.04	0.83 ± 0.13	0.78 ± 0.01
RGR	1.00 ± 0.20	0.83 ± 0.03	0.62 ± 0.09	0.48 ± 0.05	0.59 ± 0.06	0.45 ± 0.03
FLW	1.00 ± 0.33	0.81 ± 0.03	0.61 ± 0.07	0.53 ± 0.05	0.54 ± 0.06	0.38 ± 0.05
LA	1.00 ± 0.20	0.83 ± 0.03	0.65 ± 0.07	0.49 ± 0.04	0.56 ± 0.06	0.46 ± 0.03
WTC	92.4 ± 0.29	92.1 ± 0.32	90.7 ± 0.17	89.8 ± 2.30	92.3 ± 0.21	88.9 ± 0.30

* figure represents SE of the mean.

Table 5.2.10. The effect of *V. albo-atrum,* NaCl, or both; on selected growth parameters of tomato cv. Margarita (Fa-558), (6-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 7 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.04	0.93 ± 0.03	0.75 ± 0.07	0.39 ± 0.04	0.53 ± 0.04	0.35 ± 0.07
RL	1.00 ± 0.09	0.89 ± 0.07	0.88 ± 0.09	0.56 ± 0.02	0.60 ± 0.03	0.54 ± 0.08
RGR	1.00 ± 0.03	1.00 ± 0.03	0.85 ± 0.08	0.59 ± 0.07	0.74 ± 0.07	0.48 ± 0.12
FLW	1.00 ± 0.03	0.98 ± 0.03	0.82 ± 0.06	0.60 ± 0.05	0.82 ± 0.05	0.61 ± 0.07
LA	1.00 ± 0.04	1.15 ± 0.27	0.86 ± 0.10	0.71 ± 0.09	0.85 ± 0.06	0.61 ± 0.11
NAR	1.00 ± 0.06	0.88 ± 0.14	0.74 ± 0.12	0.36 ± 0.06	0.53 ± 0.10	0.29 ± 0.09
WTC	90.3 ± 0.16	89.8 ± 0.38	90.4 ± 0.30	90.1 ± 0.42	90.0 ± 1.69	89.7 ± 1.10

* figure represents SE of the mean.

Table 5.2.11. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Margarita (Fa-558) (6-week old). Analysis was made 4 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	b	cd	bc	d	3.251	5	0.650	22.1	0.000
RL	a	a	a	a	a	a	7E-02	5	1E-02	1.92	0.224
RGR	a	ab	bc	c	c	c	0.998	5	0.200	6.87	0.000
FLW	a	ab	bc	c	c	c	1.073	5	0.215	7.34	0.000
CHL	a	a	a	a	a	a	13.72	5	2.744	0.18	0.963
LA	a	a	b	b	b	b	0.993	5	0.199	9.92	0.000
WTC	a	a	a	a	a	a	21.94	5	4.38	2.32	0.167

* Between treatments. growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level

Table 5.2.12. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Margarita (Fa-558) (6-week old). Analysis was made 7 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	b	cd	c	d	2.076	5	0.415	31.7	0.000
RL	a	a	a	b	b	b	1.103	5	0.221	8.69	0.000
RGR	a	a	ab	cd	bc	d	1.260	5	0.252	8.54	0.000
FLW	a	a	ab	b	c	c	0.882	5	0.176	12.1	0.000
CHL	a	a	a	a	a	a	53.02	5	10.61	2.55	0.091
WTC	a	a	a	a	a	a	2.139	5	0.428	0.097	0.992

* Between treatments, growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level

5.2.4. Fantastic F1 - Growth Analysis.

Before the start of the experiment, Fantastic F1 was classified neither resistant nor susceptible to VW (see section, 5.2). In this study, in terms of resistance to disease, Fantastic F1 showed some differences from the other cultivars used in the study. For example, only those plants that had been treated with salt, whether inoculated or not, or whether the inoculum was V1 or V2, showed stunting. Plants that only been treated with fungus showed no stunting throughout the experimental period, Fig. 5.2.7a. A similar pattern was observed in the symptom index values, 5.2.7b. Fantastic F1 showed complete resistance to both V1 and V2 isolates throughout the experimental period. In contrast, it showed marked susceptibility to NaCl. Again, only S-, V1+S- and V2+S-treated groups showed significant differences from the control group in regard to most of the other parameters, Fig. 5.2.7a and Tables 5.2.13 & 5.2.15. The exceptions were RL and CHL, which were not significantly different from the control groups in any of the treated groups.

CHL content of the leaves were not affected by any treatments, after 4^{th} and 7^{th} week of the experimental period, Fig. 5.2.8a & b.

At the end of the experiment, same trend was observed as in the 4^{th} week. However, WTC and CHL were still not different from the control group although RL was affected at the end of the experiment, Fig. 5.2.8b; Tables 5.2.14 & 5.2.16.

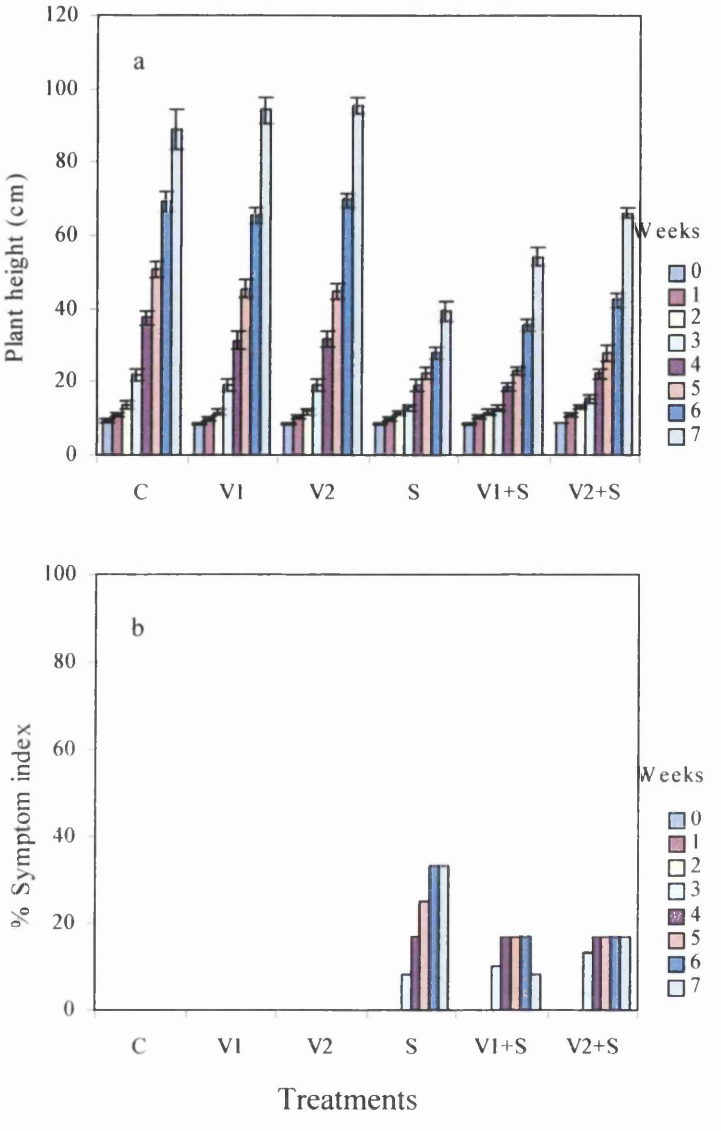


Fig. 5.2.7. The effect of *V. albo-atrum*, NaCl or both; (a) on height, (b) on symptom index of tomato cv. Fantastic F1 (6-week old). Vertical bars show ± SE of mean.

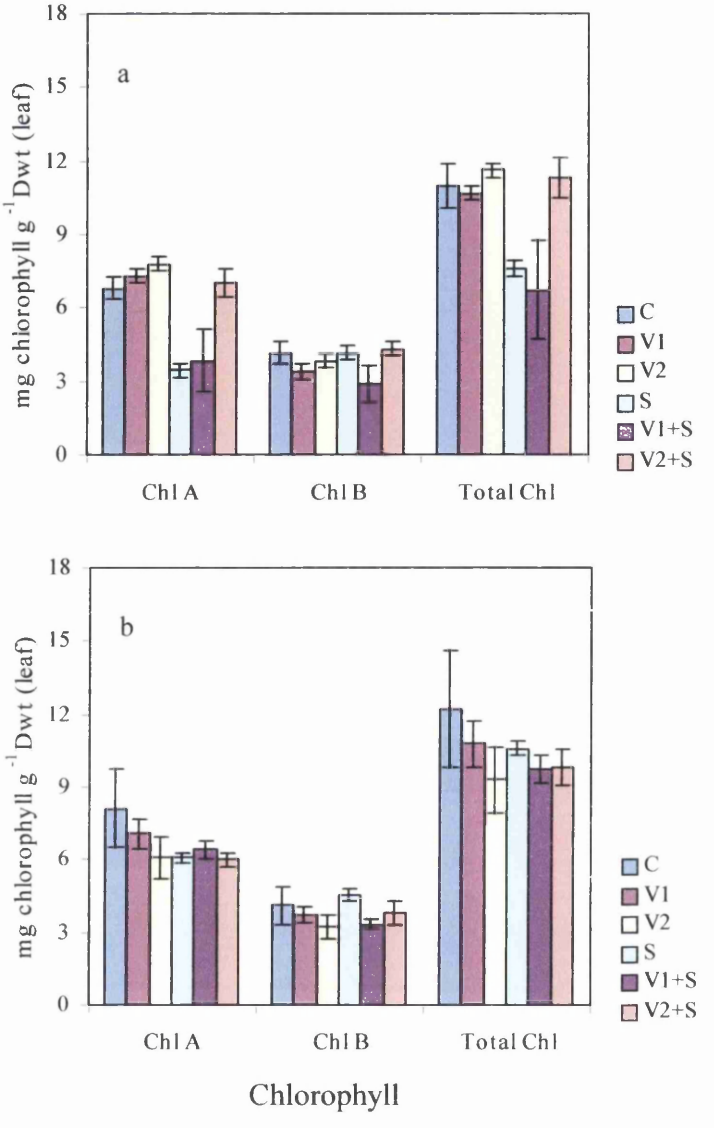


Fig. 5.2.8. The effect on chlorophyll content of *V. albo-atrum*, NaCl, or both; applied to 6-week old leaves of tomato cv. Fantastic F1. (a) 4 weeks after the start of the experiment; (b) 7 weeks after the start of the experiment. Vertical bars show ± SE of mean.

Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1. Measurement was taken 4 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.06*	1.04 ± 0.06	1.02 ± 0.05	0.46 ± 0.03	0.55 ± 0.05	0.51 ± 0.06
RL	1.00 ± 0.06	0.77 ± 0.13	1.00 ± 0.07	0.76 ± 0.11	0.82 ± 0.03	0.72 ± 0.06
RGR	1.00 ± 0.02	1.01 ± 0.08	0.96 ± 0.07	0.55 ± 0.08	0.61 ± 0.07	0.55 ± 0.13
FLW	1.00 ± 0.07	1.04 ± 0.09	1.04 ± 0.10	0.63 ± 0.08	0.67 ± 0.09	0.71 ± 0.08
LA	1.00 ± 0.07	1.00 ± 0.04	1.00 ± 0.06	0.60 ± 0.07	0.57 ± 0.06	0.55 ± 0.09

Table 5.2.14. The effect of *V. albo-atrum,* NaCl, or both; on selected growth parameters of tomato cv. Fantastic F1 (6-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 7 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.07*	1.08 ± 0.04	1.09 ± 0.03	0.39 ± 0.03	0.58 ± 0.03	0.72 ± 0.02
RL	1.00 ± 0.05	1.04 ± 0.05	1.07 ± 0.04	0.64 ± 0.04	0.64 ± 0.03	0.66 ± 0.03
RGR	1.00 ± 0.05	1.07 ± 0.01	1.05 ± 0.02	0.70 ± 0.05	0.87 ± 0.03	0.86 ± 0.03
FLW	1.00 ± 0.26	1.08 ± 0.20	0.96 ± 0.25	0.44 ± 0.09	0.27 ± 0.12	0.11 ± 0.10
LA	1.00 ± 0.02	0.93 ± 0.04	0.95 ± 0.02	0.83 ± 0.01	0.78 ± 0.01	0.70 ± 0.02
WTC	95.0 ± 0.04	95.0 ± 0.01	95.2 ± 0.11	95.1 ± 0.08	95.0 ± 0.02	95.0 ± 0.03

* figure represents SE of the mean.

Table 5.2.15. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Fantastic F1 (6-week old). Analysis was made 4 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	a	b	b	b	1.604	5	0.321	29.2	0.000
RL	a	a	a	a	a	a	0.313	5	6E-02	2.18	0.101
RGR	a	a	a	b	b	b	1.094	5	0.219	7.66	0.001
LA	a	a	a	b	b	b	1.071	5	0.214	9.92	0.000
FLW	a	a	a	b	b	b	0.694	5	0.139	5.29	0.004
CHL	a	a	a	a	a	a	5.904	5	1.181	2.68	0.115

* Between treatments, growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level

Table 5.2.16. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Fantastic F1 (6-week old). Analysis was made 7 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	a	d	c	b	4.834	5	0.967	58.2	0.000
RL	a	a	a	b	b	b	2.454	5	0.491	25.8	0.000
RGR	a	a	a	c	b	b	1.136	5	0.227	17.0	0.000
LA	a	a	a	b	b	c	0.278	5	5E-02	22.4	0.000
FLW	a	a	a	b	b	b	12.47	5	2.495	5.18	0.000
CHL	a	a	a	a	a	a	16.31	5	3.26	0.68	0.643
WTC	a	a	a	a	a	a	0.309	5	6E-02	1.27	0.290

* Between treatments, growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level

5.2.5. Fantastic F1 (8-week old) - Growth analysis.

In the previous experiment, Fantastic F1 showed great susceptibility to salinity. For example, plants that were treated with NaCl showed greater severity of symptoms, and their weekly height increments were lower than the plants that were inoculated with fungal isolates only, Fig. 5.2.7. Furthermore, the combined effect of NaCl and the fungal isolates did not exceed the effect of NaCl alone. In this experiment, 8-week old Fantastic F1 was tested for the response of salinity and disease interaction. The aim of this experiment is to determine whether susceptibility to salinity arises from the genetic feature or the age of the plant. However, the same pattern was observed as in the previous experiment when the harvest was made at 4^{th} and 7^{th} week, Figs. 5.2.9, 5.2.10; Tables 5.2.17, 5.2.18, 5.2.19 & 5.2.20. At the end of the experiment, S-, V1+S-, V2+S-treated groups showed significant differences from the control group when all parameters were recorded, Tables 5.2.18 & 5.2.20. When height increment was recorded weekly for a period of 7 weeks, the same pattern was observed as in the previous experiment, Fig. 5.2.9a. However, symptom index values were lower than that of the previous experiment Fig. 5.2.9b.

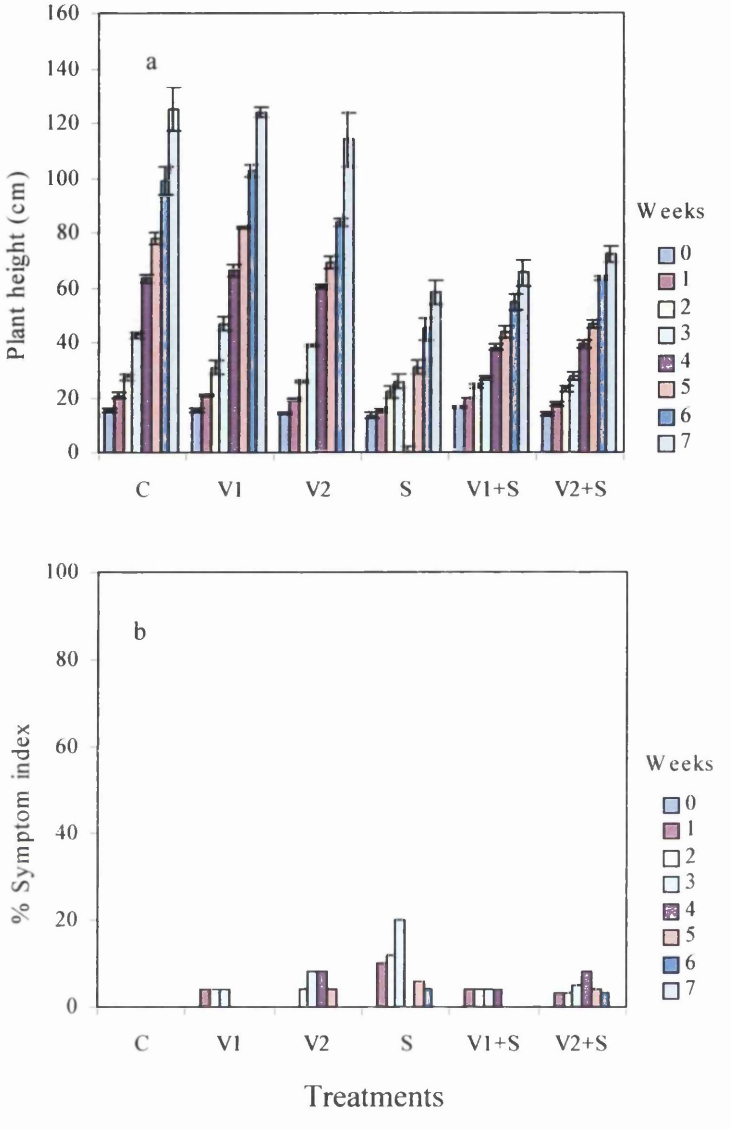


Fig. 5.2.9. The effect of *V. albo-atrum*, NaCl or both; (a) on height, (b) on symptom index of tomato cv. Fantastic F1 (8-week old). Vertical bars show ± SE of mean.

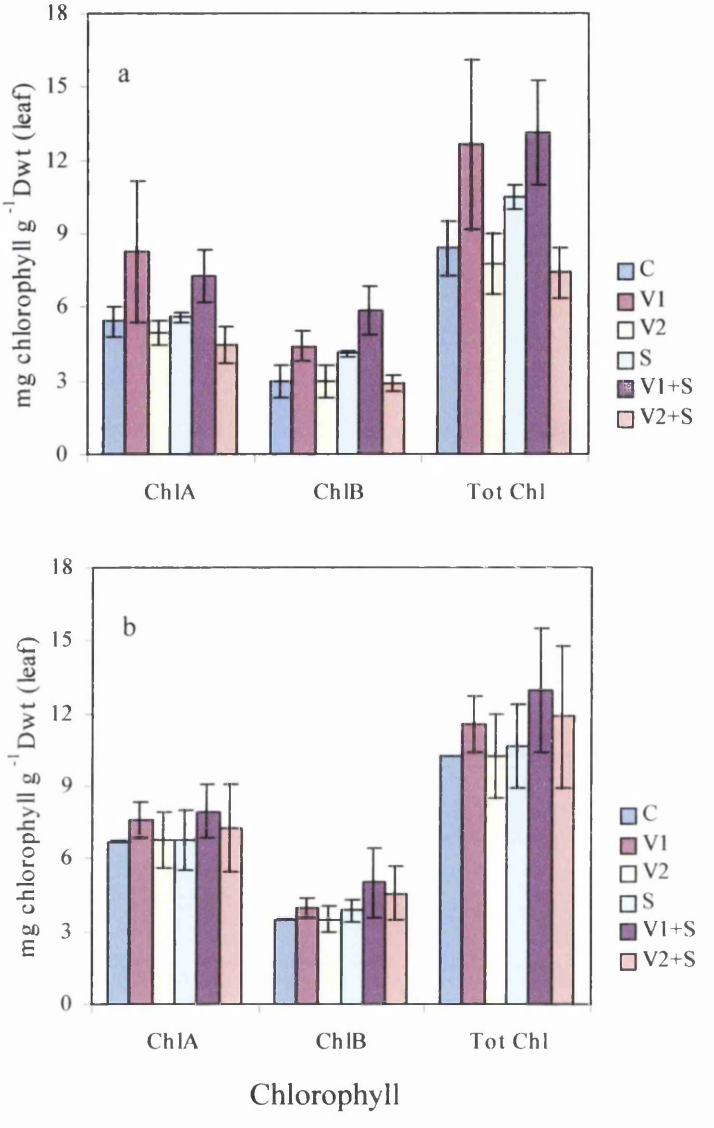


Fig. 5.2.10. The effect on chlorophyll content of *V. albo-atrum*, NaCl, or both; applied to 8-week old leaves of tomato cv. Fantastic F1. (a) 4 weeks after the start of the experiment; (b) 7 weeks after the start of the experiment. Vertical bars show ± SE of mean.

Table 5.2.17. The effect of *V. albo-atrum*, NaCl, or both; on selected growth parameters of tomato cv. Fantastic F1 (8-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1. Measurement was taken 4 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.03*	1.00 ± 0.02	0.90 ± 0.01	0.37 ± 0.02	0.41 ± 0.05	0.54 ± 0.04
RL	1.00 ± 0.10	1.12 ± 0.02	1.09 ± 0.05	0.92 ± 0.02	1.03 ± 0.10	0.96 ± 0.01
RGR	1.00 ± 0.05	1.00 ± 0.01	0.88 ± 0.11	0.44 ± 0.04	0.72 ± 0.05	0.77 ± 0.01
FLW	1.00 ± 0.01	1.00 ± 0.01	1.08 ± 0.24	0.25 ± 0.25	0.83 ± 0.50	0.92 ± 0.08
LA	1.00 ± 0.02	0.90 ± 0.03	0.91 ± 0.10	0.80 ± 0.19	0.72 ± 0.15	0.70 ± 0.03

Table 5.2.18. The effect of *V. albo-atrum*, NaCl, or both; on selected growth parameters of tomato cv. Fantastic F1 (8-week old). Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1. Measurement was taken 7 weeks after the start of the experiment.

Parameters	Treatments					
	C	V1	V2	S	V1+S	V2+S
H	1.00 ± 0.07*	1.01 ± 0.01	0.91 ± 0.09	0.28 ± 0.03	0.45 ± 0.04	0.54 ± 0.03
RL	1.00 ± 0.04	0.98 ± 0.06	0.89 ± 0.16	0.49 ± 0.05	0.51 ± 0.06	0.55 ± 0.05
RGR	1.00 ± 0.06	0.97 ± 0.03	0.96 ± 0.04	0.54 ± 0.06	0.70 ± 0.05	0.75 ± 0.02
FLW	1.00 ± 0.13	0.86 ± 0.04	0.75 ± 0.15	0.24 ± 0.04	0.00 ± 0.00	0.38 ± 0.01
LA	1.00 ± 0.01	0.99 ± 0.03	0.97 ± 0.02	0.80 ± 0.01	0.78 ± 0.01	0.83 ± 0.01

* figure represents SE of the mean.

Table 5.2.19. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Fantastic F1 (8-week old). Analysis was made 4 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	a	c	c	b	0.876	5	0.175	100	0.000
RL	a	a	a	a	a	a	5E-02	5	1E-02	1.42	0.335
RGR	a	a	ab	c	b	b	0.450	5	8E-02	13.5	0.003
LA	a	a	a	a	a	a	0.137	5	2E-02	1.61	0.287
FLW	a	a	a	a	a	a	0.927	5	0.184	1.45	0.327
CHL	a	a	a	a	a	a	69.32	5	13.86	2.12	0.165

* Between treatments, growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level.

Table 5.2.20. Analysis of variance of the effects of *V. albo-atrum*, NaCl, or both on selected growth parameters of tomato cv. Fantastic F1 (8-week old). Analysis was made 7 weeks after the start of the experiment.

Parameters	[Treatments]*						ANALYSIS OF VARIANCE				
	C	V1	V2	S	V1+S	V2+S	SS	df	MS	*F*	*Sig.*
H	a	a	a	c	b	b	1.471	5	0.294	48.9	0.000
RL	a	a	a	b	b	b	0.860	5	0.172	18.0	0.000
RGR	a	a	a	c	bc	b	0.523	5	0.105	11.8	0.000
LA	a	a	a	b	b	b	0.128	5	2E-02	25.1	0.000
FLW	a	a	a	bc	c	b	1.919	5	0.384	19.2	0.000
CHL	a	a	a	a	a	a	12.09	5	2.41	0.21	0.947

* Between treatments, growth parameters of tomato with the same letters are *not significantly different* from each other at 0.05 level.

5.3. Interactive effect of NaCl (50 mM) and *V. albo-atrum*, isolates VS, VL and VF (from USA) on VW disease severity, growth and development of cv. Ailsa Craig in a hydroponic system.

This experiment was performed to establish the pathogenicity of *V. albo-atrum*, isolates VS, VL and VF (all isolated from lucerne), towards the 6-week old tomato cultivar Ailsa Craig under non-saline and saline conditions. The plants were inoculated with isolates VS, VL or VF (1 x 10^7spores/ml) by the root-dip method. Following inoculation, the plants were transferred to the hydroponic system and irrigated with or without NaCl (50 mM) for six weeks. The set up of the plants was same as in the previous experiments as described in the beginning of this chapter. Their pathogenicity was assessed previously towards 4-week old tomato cultivar Ailsa Craig in chapter 3. It was found that they were pathogenic to young tomato seedlings. However, they were not tested towards the 6-week old tomato plants. The isolates are known to be pathogenic to lucerne (personal communication with Prof. Craig Grau, 1999), however, it is not confirmed that they are pathogenic to tomato.

Observations on height and symptom index were recorded, weekly for a period of 6 weeks, and are presented in Fig. 5.3.1. The chlorophyll contents of the plants are presented in Fig. 5.3.2. The growth parameters and their analysis are presented in Tables 5.3.1, 5.3.2 and 5.3.3.

Tomato plants inoculated with the isolates of *V. albo-atrum* from USA, did not show significant differences from the control plants, in fact, their weekly height increments were bigger than the control plants over the 6 weeks of experimental period and they showed only slight symptoms of the disease, Fig. 5.3.1a & b. Their symptom index values were all the same and low until the end of the experiment. Analysis of variance on the selected growth parameters, H, RL, RGR, LA, NAR, CHL and WTC, showed that plants inoculated with USA isolates were not significantly affected, Tables 5.3.1 & 5.3.2. However, the combined effect of NaCl & the fungus were more inhibitory than the either of the treatments alone, Tables 5.3.1 & 5.3.3. The plants inoculated and grown under 50 mM NaCl grew slowly and showed severe symptoms. No recovery was observed as compared to the other treatment groups, Fig. 5.3.1b. Their growth

parameters were significantly affected, except RL, CHL and WTC, Tables, 5.3.1, 5.3.2 & 5.3.3.

This experiment showed that isolates from USA were not pathogenic to tomato. However, the combined effect of the isolates and NaCl decreased the growth parameters causing significant differences from the control group.

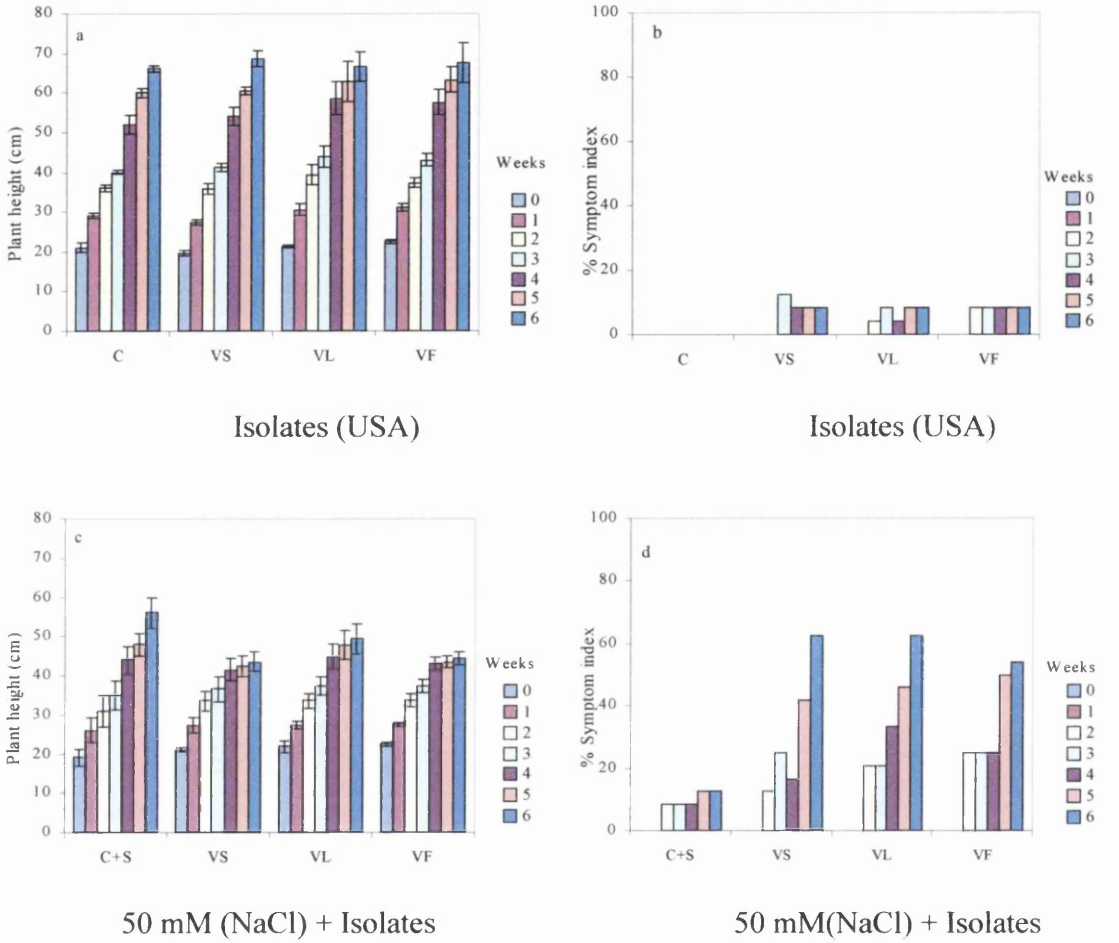


Fig. 5.3.1. The effect of *V. albo-atrum*, VS, VL, VF, (a & b) under non-salt control and (c & d) salt conditions on height and symptom index of tomato cv. Ailsa Craig. Vertical bars show ± SE of mean.

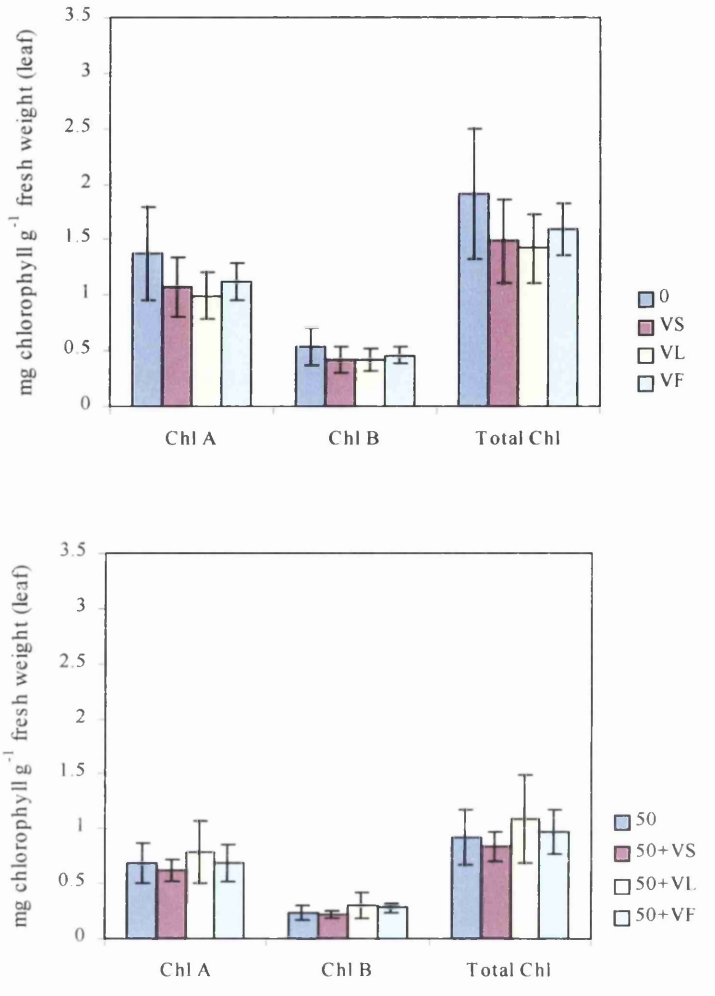


Fig 5.3.2. The effect of *V. albo-atrum*, VS, VL, VF, (a) under non-salt and (b) salt conditions on chlorophyll content of leaves of tomato cv. Ailsa Craig. Vertical bars show ± SE of mean.

Table 5.3.1. The effect of *V. albo-atrum*, isolates VS, VL, VF, under non-salt and salt conditions on selected growth parameters of tomato cv. Ailsa Craig. Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1, with the exception of WTC. Measurement was taken 6 week after the start of the experiment.

Parameters	Treatments							
	Control		VS		VL		VF	
	0 mM NaCl	50 mM NaCl	0 mM NaCl	50 mM NaCl	0 mM NaCl	50 mM NaCl	0 mM NaCl	50 mM NaCl
H	1.00 ± 0.04*	0.80 ± 0.05	1.08 ± 0.05	0.50 ± 0.07	1.00 ± 0.08	0.61 ± 0.07	1.00 ± 0.11	0.47 ± 0.05
RL	1.00 ± 0.13	1.01 ± 0.13	1.01 ± 0.13	0.75 ± 0.08	1.08 ± 0.17	0.79 ± 0.08	0.98 ± 0.12	0.83 ± 0.11
RGR	1.00 ± 0.03	0.83 ± 0.04	1.04 ± 0.03	0.58 ± 0.06	1.10 ± 0.05	0.61 ± 0.08	1.15 ± 0.02	0.62 ± 0.03
LA	1.00 ± 0.03	1.17 ± 0.12	1.05 ± 0.03	0.71 ± 0.01	1.05 ± 0.05	0.75 ± 0.01	1.10 ± 0.02	-
NAR	1.00 ± 0.09	0.51 ± 0.08	0.99 ± 0.08	0.34 ± 0.06	1.25 ± 0.15	0.38 ± 0.09	1.32 ± 0.10	-
WTC	86.8 ± 0.77	87.4 ± 0.87	82.8 ± 2.34	80.6 ± 4.48	85.2 ± 0.38	84.8 ± 1.51	82.3 ± 0.6	80.2 ± 4.17

* figure represents SE of the mean.

Table 5.3.2. Analysis of variance of the effects of *V. albo-atrum* isolates, VS, VL, VF on selected growth parameters of tomato cv. Ailsa Craig. Analysis was made 6 weeks after the start of the experiment.

Parameters	[NaCl] mM*				ANALYSIS OF VARIANCE				
	C	VS	VL	VF	SS	df	MS	*F*	*Sig.*
H	a	a	a	a	2E-02	3	7E-03	0.29	0.829
RL	a	a	a	a	2E-02	3	7E-03	0.11	0.950
RGR	ab	ab	ab	a	5E-02	3	1E-02	3.67	0.044
LA	a	a	a	a	1E-02	3	6E-02	0.99	0.427
NAR	a	a	a	a	0.350	3	0.117	2.54	0.105
CHL	a	a	a	a	0.285	3	9E-02	0.29	0.827
WTC	a	a	a	a	45.741	3	15.24	72.31	0.128

Table 5.3.3. Analysis of variance of the effects of NaCl or *V. albo-atrum* or both on selected growth parameters of tomato cv. Ailsa Craig. Analysis was made 6 weeks after the start of the experiment.

Parameters	[NaCl] mM*					ANALYSIS OF VARIANCE				
	C	C^+	VS^+	VL^+	VF^+	SS	df	MS	*F*	*Sig.*
H	a	b	c	c	c	0.785	4	0.196	14.9	0.000
RL	a	a	a	a	a	0.245	4	6E-02	1.24	0.333
RGR	a	b	c	c	c	0.527	4	0.132	11.3	0.000
NAR	a	b	b	b	-	1.103	3	0.368	13.0	0.000
CHL	a	a	a	a	a	1.527	4	0.382	1.50	0.328
WTC	a	a	a	a	a	181.6	4	45.40	1.37	0.289

* Between treatments, growth parameters of Ailsa Craig with the same letters are *not significantly different* from each other at 0.05 level.

+ Ailsa Craig was inoculated with *V. albo-atrum*, isolates USA and subsequently grown at 50 mM [NaCl] solution.

5.4. Interactive effect of NaCl (50 mM) and *V. albo-atrum*, isolates V1 or V2 and their salt-adapted strains (V1- V2-150; V1-, V2-200), on growth and development of cv. Ailsa Craig in a hydroponic system.

This experiment was conducted in the greenhouse to confirm the pathogenicity of the isolates of *V. albo-atrum* and their salt adapted strains under saline and non-saline conditions towards the 6-week old cultivar Ailsa Craig. The plants were inoculated with *V. albo-atrum* (1 x 10^7spores/ml), isolates V1 or V2 or with their salt-adapted strains. Isolate V1 or V2 were cultured on NaCl-free Dox medium. Salt-adapted isolates of the fungus were grown in NaCl enriched Dox medium (see Materials and Methods).

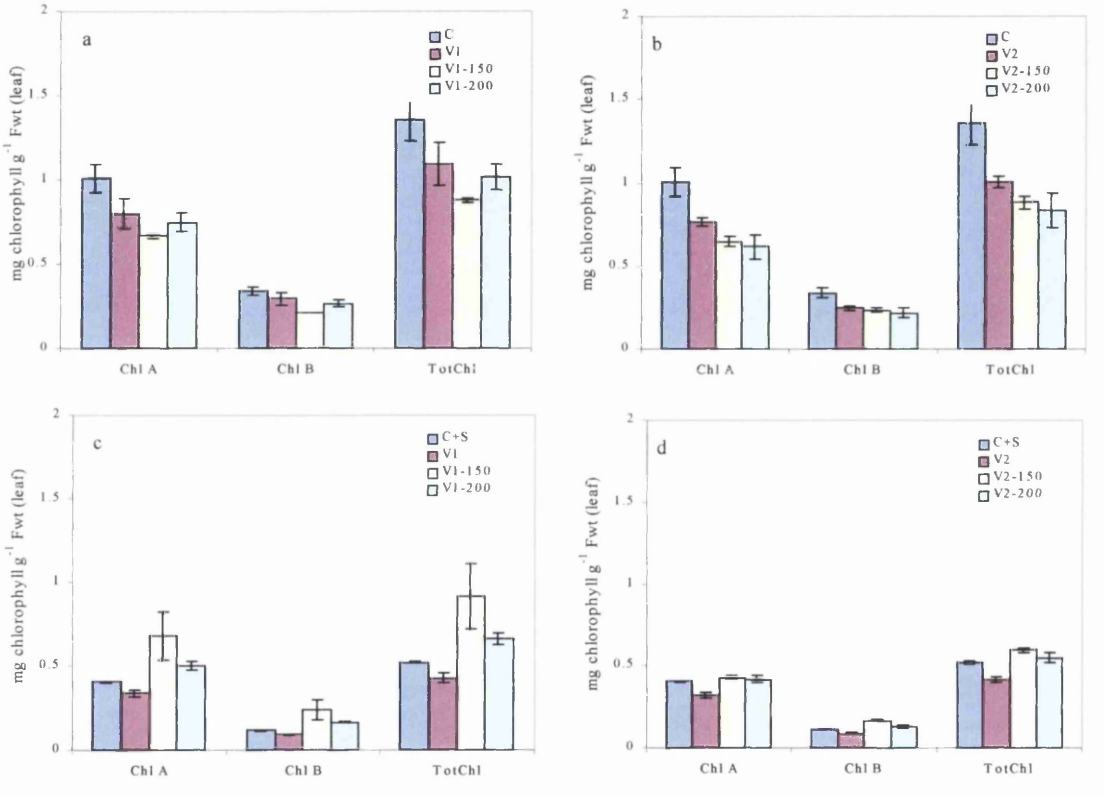
Unlike the previous experiments, no weekly observations were made on the growth of experimental plants. However, at the end of the experiment (7 weeks), height & root length and RGR, CHL contents were recorded and analysed for both isolates including the salt-adapted strains.

Previously, it was established that the fungus sporulates and grows under saline conditions. In this study, it was found that isolate V1 and its salt-adapted strains had no significant effect on the height, root length and RGR of tomato plants, Tables 5.4.1, 5.4.2, 5.4.3. However, V2 isolate and its salt adapted-strains were pathogenic to tomato, Tables 5.4.1, 5.4.2, 5.4.3.

Under salinity, the selected parameters were significantly affected. The plants inoculated with V1 and treated with NaCl at the same time, showed the same growth pattern as plants treated with NaCl alone. However, they were significantly different from the control plants, Table 5.4.4. Under salinity, the effect of V2 and its salt-adapted strains showed more inhibitory effect on plants than either of the treatments alone.

Apart from the parameters described above, at the end of the experiment, leaf samples were taken to measure the chlorophyll content of the experimental plants. The chlorophyll contents of the plants inoculated with V1 and its salt-adapted strains were not markedly reduced when compared to the control group, with the exception that the plants inoculated with strain V1-150 caused a significant reduction in chlorophyll content, Fig. 5.4.1 and Table 5.4.3. However, under 50 mM NaCl condition, the chlorophyll contents of the plants inoculated with V1 and its salt-adapted strains were markedly affected, Fig. 5.4.1 and Table 5.4.4.

The measurement of chlorophyll was also achieved for the plants inoculated with V2 and its salt-adapted strains either in non-salt or salt conditions. As it was in the height, root length and RGR parameters, all the plants here contained significantly lower amounts of chlorophyll when compared to the control group, Fig. 5.4.1 and Table 5.4.3. Again, the inoculated plants contained significantly lower amounts of chlorophyll under salinity, Fig. 5.4.1 and Table 5.4.4.



Chlorophyll Chlorophyll

Fig. 5.4.1. Effect of *V. albo-atrum*, isolates V1 and V2 and their salt adapted strains under non salt and 50 mM NaCl conditions on the leaf chlorophyll content tomato plants. a & b isolates V1 and V2 and their salt adapted strains under non-salt conditions; c & d isolates V1 and V2 and their salt adapted strains under 50 mM NaCl conditions. Vertical bars show ± SE of mean.

Table 5.4.1. The effect of NaCl on selected growth parameters of tomato cv. Ailsa Craig. Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1. Measurement was taken at the end of the 7-week experimental trial.

Parameters	Treatments						
	C	V1	V1-150	V1-200	V2	V2-150	V2-200
H	1.00 ± 0.05*	0.88 ± 0.04	0.86 ± 0.06	0.88 ± 0.07	0.74 ± 0.02	0.57 ± 0.02	0.66 ± 0.07
RL	1.00 ± 0.05	1.00 ± 0.23	0.90 ± 0.14	0.95 ± 0.25	1.12 ± 0.20	0.96 ± 0.12	1.12 ± 0.09
RGR	1.00 ± 0.06	0.89 ± 0.09	0.90 ± 0.07	0.88 ± 0.10	0.80 ± 0.04	0.82 ± 0.07	0.79 ± 0.04

Table 5.4.2. The effect of *V. albo-atrum*, isolates V1 and V2and their salt-adapted strains under 50 mM NaCl conditions on selected growth parameters of tomato cv. Ailsa Craig. Values for each treatment are expressed relative to the value of non-stressed & non-inoculated control group, which was assigned a value of 1. Measurement was taken at the end of the 7-week experimental trial.

Parameters	Treatments						
	C	V1	V1-150	V1-200	V2	V2-150	V2-200
H	0.61 ± 0.01*	0.62 ± 0.01	0.64 ± 0.01	0.64 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.54 ± 0.03
RL	0.57 ± 0.07	-	-	-	0.63 ± 0.06	0.60 ± 0.06	0.54 ± 0.04
RGR	0.78 ± 0.04	0.76 ± 0.07	0.70 ± 0.02	0.75 ± 0.08	0.64 ± 0.07	0.60 ± 0.02	0.56 ± 0.03

* figure represents SE of the mean.
+ plants were exposed to 50 mM NaCl following inoculation.

Table 5.4.3. Analysis of variance of the effect of *V. albo-atrum*, isolates V1 & V2 and their salt adapted strains on the relative rate of height & root length increase, RGR and CHL content of the leaves of tomato plants. Analysis was made 7 weeks after the start of the experiment.

Parameters	Treatments*				ANALYSIS OF VARIANCE				
	C	V1	V1-150	V1-200	SS	df	MS	*F*	*Sig.*
H	a	a	a	a	7E-02	3	2E-02	1.32	0.297
RL		a	a	a	3E-02	3	1E-02	0.06	0.978
RGR	a	a	a	a	4E-02	3	1E-02	0.38	0.767
CHL	a	ab	b	ab	0.237	3	7E-02	4.34	0.095
	C	V2	V2-150	V2-200	SS	df	MS	*F*	*Sig.*
H	a	b	d	c	0.481	3	0.160	87.2	0.000
RL	a	a	a	a	0.108	3	3E-02	0.31	0.815
RGR	a	b	b	b	0.119	3	3E-02	2.70	0.088
CHL	a	b	b	b	0.331	3	0.110	7.75	0.038

* Between treatments, height with the same letters are *not significantly different* from each other at 0.05 level.

Table 5.4.4. Analysis of variance of the effect of *V. albo-atrum*, isolates V1 & V2 and their salt adapted strains under 50 mM NaCl on the relative rate of height and RGR of tomato plants. Analysis was made 7 weeks after the start of the experiment.

Parameters	Treatments*					ANALYSIS OF VARIANCE				
	C	C+S	V1	V1-150	V1-200	SS	df	MS	*F*	*Sig.*
H	a	b	b	b	b	0.583	4	0.146	54.6	0.000
RGR	a	b	b	b	b	0.198	4	4E-02	2.94	0.058
CHL	a	bc	c	b	bc	1.106	4	0.276	12.4	0.008
	C	C+S	V2	V2-150	V2-200	SS	df	MS	*F*	*Sig.*
H	a	b	c	c	c	0.651	4	0.163	97.7	0.000
RL	a	b	b	b	b	0.687	4	0.172	6.59	0.002
RGR	a	b	c	c	c	0.586	4	0.147	13.4	0.000
CHL	a	b	b	b	b	1.147	4	0.287	44.1	0.000

*Between treatments, height with the same letters are *not significantly different* from each other at 0.05 level.

5.5. Pathogenicity of *V. albo-atrum*, isolate V1 and the effect of 50 mM NaCl in lucerne plants.

From the previous results of germination, pathogenicity and rooting ability experiments, cultivars that showed susceptibility to both NaCl and the pathogen or tolerance to both NaCl and the pathogen were selected and tested for their response to pathogen *V. albo-atrum* under 50 mM NaCl conditions.

The cultivars *M. sativa* cv. 13R Supreme, Bilensoy-80, Vertus (disease-resistant & salt-tolerant) and *M. media* cv. Rambler (disease- & salt-susceptible) and its salt tolerant strain of Rambler R-350-N (disease–susceptible & salt-tolerant) were employed.

Lucerne cuttings were rooted under a misting bed and inoculated with *V. albo-atrum*, isolate V1 (1×10^7 conidia/ml) by the root-dip method and placed individually in 10-cm plant pots containing perlite (8 replicates per treatment). They were watered with 100 ml A & H solution every 2 days for 8 weeks (see Materials and Methods).

In another set of plants, three days after the inoculation, 100 ml A & H solution containing 50 mM NaCl was applied to the pots for 8 weeks.

Upon completion of the experiment, final height and dry weights of the plants were recorded. Relative rate of increase in height and RGR are presented in Fig. 5.5.1, their results of analysis are presented in Tables 5.5.1 & 5.5.2.

Cultivars of *M. sativa*, 13R Supreme, Bilensoy-80 and Vertus showed resistance to *V. albo-atrum*, isolate V1, when height and RGR values were compared with their own control groups, Fig. 5.5.1, Table 5.5.1 & 5.5.2. However, cultivars of *M. media*, Rambler and R-350-N, showed susceptibility to the disease Fig. 5.5.1, Tables 5.5.1 & 5.5.2.

Under salinity, cultivars of *M. sativa* inoculated with V1 did not show significant differences from their control groups in terms of height and RGR measurements. However, *M. media* cv. Rambler inoculated with V1 showed great susceptibility to the fungus. The salt tolerant strain of *M. media*, R-350-N, also showed great susceptibility to the effect of fungus, Fig. 5.5.1, Tables 5.5.1 & 5.5.2.

The cultivars of *M. media* inoculated with *V. albo-atrum*, isolate V1 showed severe symptoms of the wilt disease such as wilting and chlorosis on the lower leaves, 3 weeks after in the inoculation. However, the cultivars of *M. sativa*, especially Vertus, showed symptoms of the disease but recovered quickly from the effect of the pathogen.

Cultivars 13R Supreme and Bilensoy-80 were free from those symptoms, although height and RGR were slightly lower than their controls. However, this was not statistically significant. Probably, the fungus was transported in the conducting elements but did not cause symptoms of the disease.

There were also highly significant interactions between cultivars and treatments when height measurement was taken $P<0.001$, Table 5.5.2. This means that different cultivars responded differently to the effect of salinity.

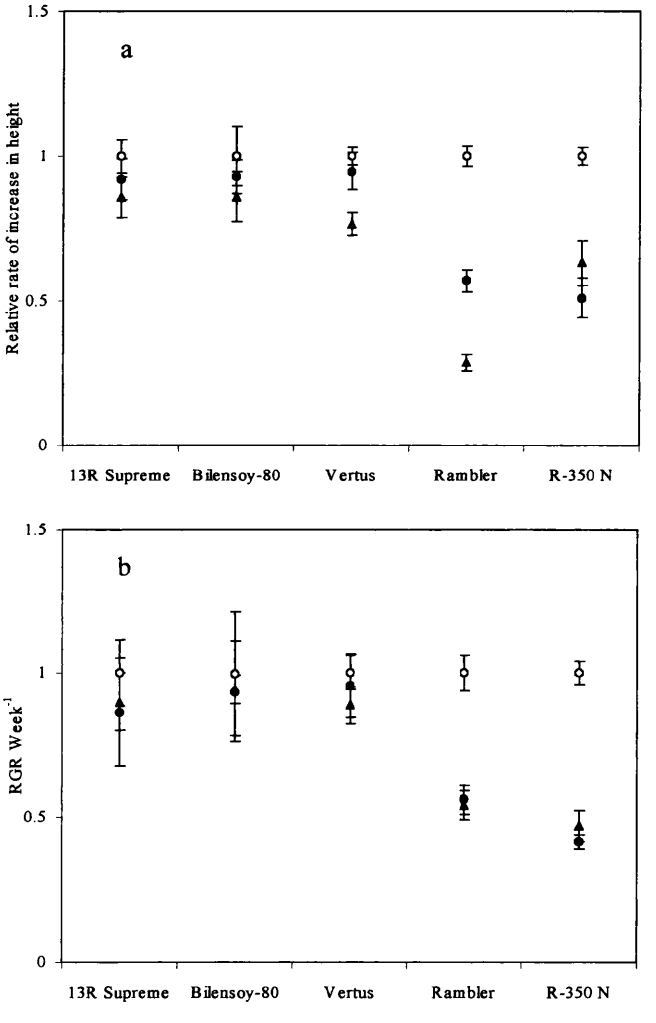


Fig. 5.5.1. Effect of *V. albo-atrum*, isolate V1 or the combined effect of 50 mM NaCl & isolate V1; (a) on the relative rate of height increase; (b) RGR of lucerne plants. ○ -control; ● -V1; ▲-V1 + 50 mM NaCl. Values plotted ± SE of mean.

Table 5.5.1. Analysis of variance of the individual and combined effect of V1 & [NaCl] on the index of height of lucerne cultivars, *M. sativa* and *M. media*.

Cultivars	Treatments*			ANALYSIS OF VARIANCE				
	C	V1	V1[1]	SS	df	MS	*F*	*Sig.*
13R Supreme	a	a	a	7E-02	2	3E-02	1.44	0.272
Bilensoy-80	a	a	a	3E-02	2	1E-02	0.31	0.737
Vertus	a	a	a	5E-02	2	2E-02	2.14	0.157
Rambler	a	b	c	1.292	2	0.646	11.4	0.000
R-350-N	a	b	b	0.651	2	0.325	16.7	0.000
Sig. +	ns	s	s					
				2-Way Interaction				
	Cultivars			1.261	4	0.315	14.1	0.000
	Treatments			1.309	2	0.655	29.4	0.000
	Cultivars x [NaCl]			0.890	8	0.111	4.99	0.000

* Between treatments, index of height of lucerne with the same letters are *not significantly different* from each other at 0.05 level.
[1] lucerne plants were treated with NaCl following the inoculation with V1.
+ One-Way ANOVA test between cultivars within a treatment at 0.05 level.
s. Significant
ns. Not significant

Table 5.5.2. Analysis of variance of the individual and combined effect of V1 & [NaCl] on the index of RGR of lucerne cultivars, *M. sativa* and *M. media*.

Cultivars	Treatments*			ANALYSIS OF VARIANCE				
	C	V1	V1[1]	SS	df	MS	*F*	*Sig.*
13R Supreme	a	a	a	5E-02	2	3E-02	0.26	0.774
Bilensoy-80	a	a	a	1E-02	2	6E-03	0.03	0.967
Vertus	a	a	a	3E-02	2	1E-02	0.39	0.681
Rambler	a	b	b	0.676	2	0.338	22.0	0.000
R-350-N	a	b	b	1.046	2	0.523	62.0	0.000
Sig. +	ns	s	s					
				2-Way Interaction				
	Cultivars			1.385	4	0.346	4.75	0.002
	Treatments			1.142	2	0.571	7.82	0.001
	Cultivars x [NaCl]			0.791	8	9E-02	1.35	0.235

* Between treatments, RGR with the same letters are *not significantly different* from each other at 0.05 level.
[1] lucerne plants were treated with NaCl following the inoculation with V1.
+ One-Way ANOVA test between cultivars within a treatment at 0.05 level.
s. Significant
ns. Not significant

5.6. The regeneration of salt tolerant lucerne from 50 mM NaCl-adapted cell cultures of *M. sativa* cv. Vertus and its resistance against VW.

M. sativa cv. Vertus has been found to be one of the most resistant cultivars to *V. albo-atrum* among the cultivars used in this study. In this section, an attempt has been made to regenerate cv. Vertus on 50 mM NaCl- containing medium (see Materials and Methods). Callus cultures used in this experiment was obtained from leaf explants of cv. Vertus. The calli were maintained on salt-free M & S medium and then transferred to liquid M & S medium containing 50 mM NaCl. After successful subculturing (6 months), the cells (0.5 g) of lucerne were transferred onto agar supported salt-and hormone-free BOi2Y regeneration medium (Bingham *et al.*, 1975), Plate 5.4. Ten vials were used for the regeneration treatment. A number of green somatic embryos was transferred onto another salt-free BOi2Y medium. Later, plantlets were transferred into small pots containing perlite and kept under a mist bed unit for 15 days. Plantlets were propagated by rooting cuttings in perlite trays for 2 weeks then they were transferred into individual pots and watered with 1/5 A & H culture solution (see Materials and Methods).

Pathogenicity test were performed with non salt-adapted and 50 mM NaCl-adapted plants. Because of the limited amount of cuttings, only height and RGR parameters were measured in this experiment.

Six-week old cuttings were inoculated with the spores of *V. albo-atrum*, isolate V1 by the root-dip method. Inoculated plants were transferred into individual 10-cm pots. After 6-week, the relative rate of height increase and RGR were taken as disease indices, Table 5.6.1.

From the results, it was concluded that there was no significant difference between non salt-adapted and 50 mM NaCl-adapted plants in response to *V. albo-atrum*, isolate V1. Both lines, non-salt and salt-adapted, were resistant to V1 under salt free conditions. However, this experiment could have been repeated with higher NaCl concentrations, and the regeneration of salt tolerant plants could have been made from the cell lines in which the adaptation of NaCl is higher than 50 mM. However, because lack of time and plant material this was not possible at that time.

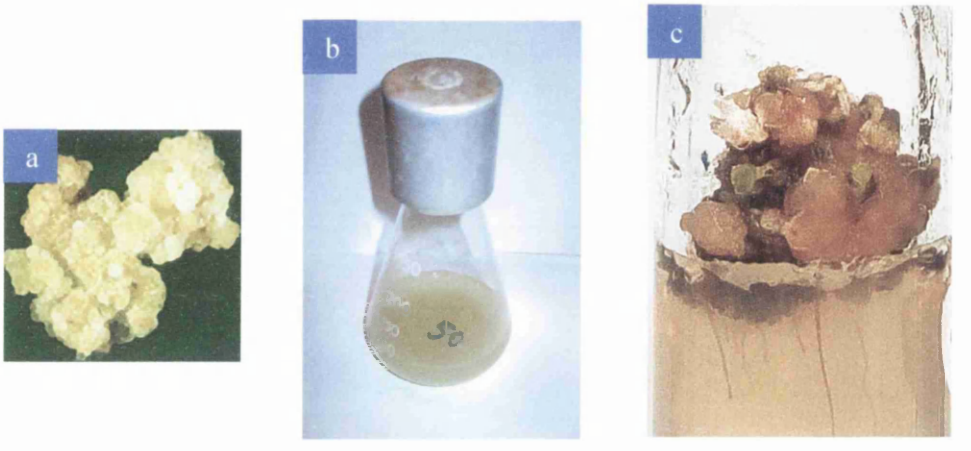


Plate 5.4.
Regeneration of lucerne plants from adapted cell lines. (a) callus cultures grown under photoperiod (16 h at 50 μmoles m^{-2} s^{-1} PAR), (b) liquid culture of lucerne cell adapted to 50 mM NaCl, (c) regeneration of plantlets from a cell line adapted to NaCl.

Table 5.6.1. Effect of inoculation with *V. albo-atrum*, isolate V1, on the relative rate of height increase and RGR of M. sativa cv. Vertus (non-salt adapted and salt adapted plants).

Parameters	Cultivars			
	Vertus		Vertus (50 mM NaCl-adapted)	
	C	V1	C	V1
H	1 ± 0.25	0.95 ± 0.15	1 ± 0.19	0.83 ± 0.10
RGR	1 ± 0.20	0.90 ± 0.15	1 ± 0.15	0.80 ± 0.12

5.7. Cell death of lucerne cells.

In general, a pathogen or its derivatives, such as, elicitors causes a hypersensitivity reaction in a host that results in cell death. In this section, lucerne cells, cv. Kabul, were treated either with an elicitor derived from *V. albo-atrum*, or NaCl or both, to assess the viability of lucerne cells.

Lucerne cells were incubated with the V1 or V2 elicitor (0.05 mg/ml) or NaCl (50 mM) or both for 24 hours, then each culture was stained with 0.05% Evans Blue for 15 min. After washing with distilled water, the percentage of dead cells was quantified from the absorbance at 600 nm.

Elicitors of *V. albo-atrum* (V1 or V2) caused an increase in percentage cell death occurring compared to the control group, Table 5.7.1. However, no difference was observed between the isolates in terms of the cell death they caused. The increase in cell death was also observed in NaCl-treated cells, the percentage cell death was even higher than the elicitors caused. Cell death was further increased (22%) with the combined effect of elicitor (V1) & NaCl, however, no difference was observed between the NaCl- and NaCl & elicitor-treated cells in terms of percentage cell death.

Fluorescene diacetate (FDA) was tested to assess the number of living cells, unlike the previous experiment in which cell death was assessed with Evans Blue. Lucerne cells were incubated with V1 or V2 elicitor, or NaCl, or both NaCl and elicitor for 24 hours.

Living cells were counted under a UV fluorescent microscope. However, from this experiment no useful data was obtained, Table 5.7.1.

Table 5.7.1. Effect of V1 or V2 elicitor or NaCl (50 mM) or both NaCl and the elicitors on viability of lucerne cells. The results are expressed as %.

Chemicals	Treatments					
	C	Elicitor		NaCl (mM)	Elicitor + NaCl (50 mM)	
	H_2O	V1	V2	50	V1+50	V2+50
Evans Blue (dead cells)	0	14	13	23	22	-
FDA (living cells)	-	-		-	-	-

- no data was obtained.

5.8. Effect of V2 elicitor or NaCl or both NaCl & V2 elicitor on PAL activity of lucerne cell suspension.

PAL was assessed in cell suspension cultures of lucerne cv. Kabul. This experiment was performed to determine if an elicitor from *V. albo-atrum* cause an increase in PAL activity in NaCl-stressed cells.

Cell suspension cultures of lucerne cv. Kabul were treated with elicitor (0.05 or 0.1 mg/ml) from *V. albo-atrum*, isolate V2 by aseptic addition of elicitor solution to the cultures. At the end of the incubation period (4 hours), PAL activity was determined in cell-free homogenates as described in Materials and Methods.

The results are shown in Fig. 5.8.1 for cultures treated with V2 elicitor (0.05 and 0.1 mg carbohydrate ml^{-1}), NaCl (50 mM), and NaCl & V2 elicitor, and NaCl (200 mM) & elicitor (0.1 mg carbohydrate ml^{-1}).

Elicitor (0.05 mg carbohydrate ml^{-1}) caused an increase in PAL activity compared to the control group, however, a further increase in elicitor concentration caused a decrease in PAL activity, Fig. 5.8.1a. It was observed that salinity also caused an increase in PAL activity and a further increase in PAL activity was evident in cells

treated with the elicitor & NaCl, Fig. 5.8.1b. However, increasing the concentration of NaCl to 200 mM (highly toxic to non-salt tolerant lucerne cells, Chaudhary, 1996; Al-Rawahy, 2000), and the addition of V2 elicitor (0.1 mg carbohydrate ml^{-1}) caused a decrease in PAL activity, Fig. 5.8.1c.

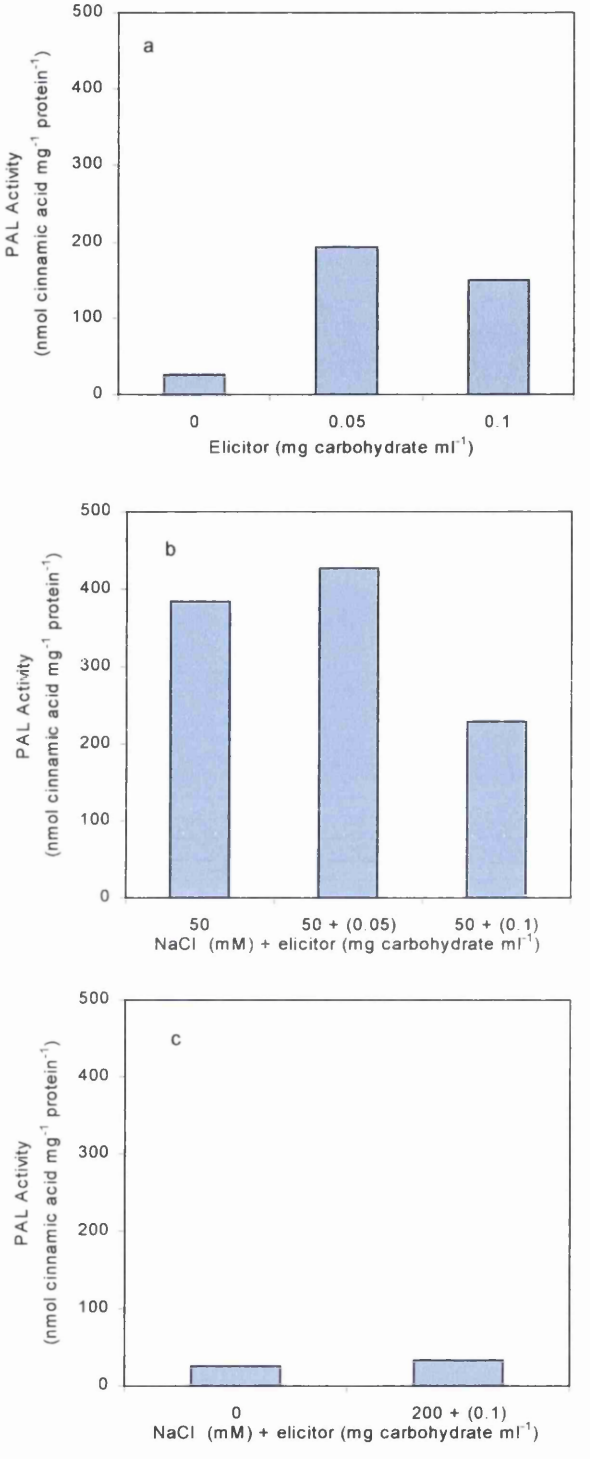


Fig. 5.8.1. (a) Effect of V2 elicitor; (b) effect of V2 elicitor and 50 mM NaCl; (c) the effect of NaCl (200 mM) and elicitor on PAL activity in cell suspension of lucerne cv. Maris Kabul. (The activity of PAL was determined after 4 hours of incubation with either elicitor or NaCl or both).

CHAPTER VI
DISCUSSION&CONCLUSIONS

6.1. Pathological response of tomato and lucerne plants to *V. albo-atrum.*

The growth and yield of plants depend upon the availability of nutrients and water in the soil where they grow and on maintenance, within certain ranges, of such environmental factors as temperature, moisture, and light. Anything that affects the health of plants is likely to affect their growth and yield and may consequently seriously reduce their usefulness to humans (Agrios, 1988). A plant is considered healthy or normal when it can carry out its physiological functions to the best of its genetic potential. Whenever plants are disturbed for example, by pathogens or by certain environmental conditions, and one or more of these functions is disturbed beyond a certain deviation from the normal, then plants become diseased. The primary causes of disease may be either pathogenic or environmental factors, or a combination of both. However, if the environmental factors are not in favour of the pathogen, it is certain that no disease will occur (Talboys, 1972).

The purpose of the programme of work reported here was to determine, under 'normal' and conditions of raised salt, the pathogenicity of the fungal pathogen *V. albo-atrum* to *L. esculentum*, *M. sativa* and to various salt tolerant strains of *M. media.* To this end, a series of experiments was carried out in which selected cultivars of *L. esculentum*, *M. sativa* and *M. media* were inoculated with *V. albo-atrum*, isolate V1 (isolated originally from lucerne), or V2 (isolated originally from tomato) under conditions of normal- and increased salinity. In addition, the effects of increased salt on growth and development of the fungus and selected cultivars of tomato and lucerne were also assessed. In preliminary experiments, the conditions for such experiments were established through a series of experiments in which the effects of age of the plants, of growth temperature, of the method of inoculation and of spore concentration on the pathogenicity of *V. albo-atrum* towards tomato were assessed. At the same time, the pathogenicity of *V. albo-atrum* towards selected salt-tolerant strains of *M. media* was also assessed.

Initially, an experiment was carried out to compare the effects on disease development in tomato cv. Ailsa Craig of different methods of inoculation (section 3.1). While root-dip, wound-, shaved root-dip- and stem injection methods led to development of the disease, however, the soil inoculation method did not cause symptoms of the wilt

disease although a reduction in height was evident. Among the inoculation methods, the root dip inoculation method was found to be the most successful as indicated by the index of height, symptom index and the number of successful reisolations (Fig. 3.1.1 & Table 3.1.2). Similar results were reported for cauliflower stems inoculated with *Verticillium*, in which a root-dipping inoculation method was more successful than such methods as stem injection or a method in which seedlings were planted alongside an agar block colonised with microsclerotia Koike *et al.* (1994). Similarly, Atibalentja & Eastburn (1997) reported that a root-dip inoculation method was most effective differentiating between susceptible and resistant horseradish cultivars. On the other hand, Malik (1978) reported that reisolation of *V. dahliae* from tulip was more successful from plants inoculated by a root-dip method than those inoculated through a wound. It is likely that more conidia enter the plant, through the broken parts of the roots and root hairs with the root-dip method, than do with a wound method. (In wound-inoculated plants, the area exposed to the fungal spores is rather small compared to the area of root exposed to spores in the root-dip method). Furthermore, the wounded area may regain mitotic activity and so form wound-cambium that replaces the damaged tissue, or produces a layer of boundary tissue, such as a periderm and so seal the wound (Barckhausen, 1978) preventing further invasion by the fungus. Despite such considerations, the wound method of inoculation used in this study was the next most effective method of inoculation as judged by the symptom index, measurement of height and reisolation (Fig. 3.1.1 & Table 3.1.2). The method in which roots were shaved prior to dipping in a spore suspension was not very effective, most likely because the root hairs, which occupy the biggest surface area of the root mass, were damaged and so the area available to the spores for penetration was greatly reduced. Whatever the reason for the differences observed, the root dip method was adopted as the method of inoculation for subsequent experiments.

The concentration of spores in an inoculum is also a factor that can determine the progress of disease in an infected plant (in this respect, the term inoculum is applied to potentially infective units of conidia of the fungus capable of starting a new infection) (Horsfall, 1932); while Garrett (1960) defined inoculum potential as 'the energy of growth of the pathogen available for infection of the host.). So, for example resistance to

wilt disease of cocoa (*Theobrama cacao* L.) depended in part on the spore concentration in the inoculum, as well as the virulence of the pathogen (Resende *et al.*, 1995). In that study, a resistant cocoa cultivar (Pound-7) was overcome by concentrations of *V. dahliae* spores greater than 10^6 conidia/ml. Similar results were obtained from maple and apricot trees inoculated with *V. albo-atrum* (Caroselli, 1957; Taylor & Flentje, 1968) and Isaac *et al.* (1971) reported that there was a positive relationship between disease severity in potato plants and fungal population of *V. dahliae* in the soil. On the other hand, with lower inoculum concentrations plants in this study showed only mild symptoms. Although susceptible cultivars may show symptoms even when very low levels of inoculum are present, with an increased spore concentration, a decrease in incubation period and an increase in incidence and severity of symptoms, including mortality, can be expected (Bell & Mace, 1981; Xiao & Subbarao, 1998). As the concentration of spores in the inoculum concentration exceeds certain critical levels, even resistant cultivars may become progressively more susceptible (Alon *et al.*, 1974). For example, Baayen & Schrama (1990), showed that higher inoculum concentrations of *F. oxysporum* f. sp. *dianthi* overcame the resistance of carnation plants and caused higher wilting rates, while Schnathorst & Mathre (1966) reported that increase in inoculum concentrations of *Verticillium* might overcome the resistance of resistant cotton cultivars. It was also reported that high populations of the pathogen could break the highest level of wilt resistant of some plants (Frank *et al.*, 1975). On the other hand, Xiao & Subbarao (1998) reported that although wilt incidence and severity of the disease caused by *V. dahliae* on cauliflower increased with increasing inoculum density, additional inoculum did not result in significantly higher disease incidence and severity.

However, in this study, even the lowest concentration of spores tested (1×10^4 conidia/ml) caused significant symptoms of disease on 5-week old tomato plants, and increasing the concentration of spores did not result in a further increase in symptom development, Fig. 3.1.3. Undoubtedly, increasing the numbers of spores in the inoculum would have presented the root with a higher number of germinating spores. The fact that in this investigation no linear relationship was established between the increase in spore concentration and symptom index values, height and RGR, must mean that even the lowest concentration of spores (10^4 conidia/ml) was sufficient to establish an effective

infection. Similarly, Atibalentja & Eastburn (1997) and Khan *et al.* (2000), working with VW of horseradish, reported that there was no correlation between inoculum density of *V. dahliae* and severity and incidence of root discoloration of horseradish. They stated that some fields infested with low inoculum densities of *V. dahliae* had high ratings of severity and incidence of root discoloration, while other fields with high inoculum densities had low ratings of severity and incidence of discoloured roots. As a result, they suggested that inoculum densities might not be a good predictor of severity of the disease. Bejarano-Alcazar *et al.* (1995) also reported that there was no linear significant correlation between the inoculum density of *V. dahliae* and the incidence of plants with vascular discoloration on cotton at the end of the crop season. These results also agreed with those of DeVay *et al.* (1974), who concluded that the inoculum density of the pathogen in soil was not the only determinant of the final incidence of foliar symptoms of VW of cotton.

Both isolates of *V. albo-atrum*, V1, isolated originally from lucerne, and V2 isolated originally isolated from tomato, caused symptoms of wilt disease when inoculated onto tomato plants. Where comparisons were made, in general terms, both V1 and V2 were effective at causing symptoms of wilt disease in tomato plants, though the degree of severity of the symptoms of each isolate depended on the temperature at which the plants were subsequently grown (Fig 3.1.6) and seedling age at inoculation (Fig 3.1.5; Tables 3.4.1 & 5.2.1).

Isolates of *V. albo-atrum* that had been subcultured regularly over a 3-year period were tested to establish whether regular sub-culturing had affected the pathogenicity of the fungus. The pathogenicity of regularly sub-cultured isolates (designated as 'old') was compared to the pathogenicity of freshly isolated fungus (designated as 'new'). The results showed that all the isolates, whether 'new' or 'old' caused stunting in height and reduction in RGR of tomato cv. Ailsa Craig (Table 3.1.5). Reisolation of the fungus was also positive from all the inoculated plants. The results, therefore, suggest that *V. albo-atrum* remains pathogenic to tomato although sub-cultured over a 3–year period. Similarly, Simpfendorfer *et al.* (1996) reported that the isolates of *Phytophthora clandestina* were still pathogenic to millet plants after 10 months storage in sterile deionised water on millet seeds at 4 °C. However, Krokene & Solheim (2001) reported

that a subculture of blue-stain fungus *Ceractocystis polonica* lost its pathogenicity to Norway spruce (*Picea abies*) after serial transfers on artificial substrates.

The effect of temperature on the nature of resistance and susceptibility is always a complex one. It does not only affect the germination and disease development of *Verticillium* spp. but also affects the geographic distribution. Here the results of experiments to determine the effects of temperatures on disease development in tomato showed that for both isolates of *Verticillium*, the pathogenicity of the fungus was more severe at 22 °C than at the two other temperature regimes (Fig. 3.1.6). The effect was especially evident with isolate V1, which failed to cause symptoms of the disease at 25 °C, though at 22 °C there was some symptom development (symptom development at 22 °C was described in section 3.1 and in this case was measured in terms of reduction in height, symptom index, reisolation of the fungus and the relative growth rate (RGR)). In contrast, the symptoms resulting from inoculation with isolate V2 (reduced height compared to controls, symptom index and reisolation of the fungus and the RGR) were more marked than from V1, and isolate V2 remained pathogenic at 25 °C, though the symptoms of the disease were reduced (Fig. 3.1.6).

At 22 °C both control and plants inoculated with *V. albo-atrum* (either isolate) showed similar growth curves but when symptoms (chlorosis, wilting, formation of adventitious roots and epinasty) became apparent the growth curve of the infected plants was significantly lower than that of the control plants (Figs. 3.1.2, 3.1.5, 3.1.6), and the fungus colonised intercellularly although the host showed a reaction such as tyloses and adventitious roots on the lower stem parts (Fig 3.1.6; Plate 3.5). Similar findings were made in diseased tomato, lucerne and even in elm trees (*Ulmus* spp) (Karagiannidis *et al.*, 2002; Pennypacker *et al.* 1990; and Rauscher *et al.*, 1974). At 25 °C the effect of V2 on the rate of growth was not as marked as at 22 °C while V1 had no effect (Table 3.1.8). At 30 °C plants inoculated with isolate V1 showed no symptoms at all, while the symptoms of those plants inoculated with V2 were much less severe (Fig. 3.1.6). Although some wilting was evident on the lower part of the stems in the early weeks of inoculation and more adventitious roots were present, towards the end of the experiment a great recovery (as judged by symptom index and the weekly increments in height) was observed in those plants (Fig. 3.1.6). In fact, in subsequent weeks the inoculated plants at the higher

temperatures grew faster and produced more branches than those at the lower temperatures. The recovery of plants inoculated at 22 °C was evident four or five weeks following inoculation, while at higher temperatures recovery began *ca.* two or three weeks following inoculation. Although V2 was not effective on the relative rate of height increase at 30 °C, there was still an effect on the RGR (Table 3.1.8). Root lengths of inoculated plants seemed not to be affected by the fungus at any temperatures (Table 3.1.7).

A decrease in disease development in the host with increase in temperature, such has been observed here, may be explained by a decrease in aggressiveness of the pathogen, or by an increase in resistance of the host (Griffiths & Isaac, 1966; Brinkerhoff, 1978; Nerpin *et al.*, 1978). It is known, for example, that *V. albo-atrum* is limited in its distribution because it fails to grow at temperatures much above 30 °C (Raynal & Guy, 1977, described in the introduction). In this study it is likely that the primary effect of temperature on disease development is on the fungus. For example, the average height of the tomato plants in the control groups at 22-, 25- and 30 °C was around 80 cm, indicating that above 22 °C temperature had little effect on the growth and development of tomato. So, it is likely that the decreased effect of the pathogen on the height of plants at the higher temperatures can be accounted for by a decrease in the aggressiveness of the fungus, rather than an increase in vigour of the plant. Furthermore, reisolation of the V2 isolate was only possible from plants maintained at 22 °C; at 25 °C and 30 °C, no reisolation was possible (Table 3.1.9), suggesting that the fungus had failed to colonise the plant. Such a result is in accord with the studies of others. For example, Griffiths & Isaac (1966) failed to reisolate *V. albo-atrum* from tomato plants maintained at 30 °C and similar observations have been made for tomato and cotton plants (Virgin & Maloit 1947; Garber & Huston 1967).

It is possible that the fungus affected water transport particularly in the early weeks following inoculation causing a decrease in water uptake that resulted in a decreased rate of photosynthesis. This is consistent with the findings of Duniway & Slatyer (1971) who studied transpiration and photosynthesis in diseased tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici*. They reported that photosynthesis and

transpiration in the inoculated plants were reduced after 15 days inoculation. Similar reports were made by Mathre (1968) on cotton plants infected with *V. albo-atrum*.

A study of fungal growth also showed that the maximum production of dry weight of mycelium occurred between 22 °C and 25 °C (Fig. 3.1.7). At 30 °C mycelium growth significantly declined corresponding with the decrease in pathogenicity of the fungus on tomato plants at that temperature. The effect of high temperature was similar on the growth of both isolates. Similarly, Busch (1967) also reported that the colony growth of *V. albo-atrum* in Petri dishes was inhibited at 30 °C. Sharma (1966) reported that an increase in temperature influenced the growth of two strains of *V. dahliae* after 15 days on liquid Dox medium. He also concluded that optimum mycelium growth was obtained between 22.5 – 25 °C. Similar findings were also made by Bremner (1981). Furthermore, Isaac (1949) showed that the development of symptoms on tomato plants inoculated with *V. albo-atrum* was inhibited around 29 °C. Similar reports were made in potatoes inoculated with *V. albo-atrum* (Busch, 1967) and in cauliflowers inoculated with *V. dahliae* (Koike *et al.*, 1994). Graham *et al.*, (1977) and Wilderspin & Heale (1983) also reported that *V. albo-atrum* did not cause severe symptoms of the disease in hop plants above 30 °C.

Mepsted (1991) suggested that the reduction in height might result from a hormonal imbalance rather than water stress. However, other workers have suggested that a gradual increase in water scarcity in the plant, resulting from xylem blockage and the fungus, by causing symptoms such as wilting and chlorosis, also reduces height in infected plants (Street & Cooper 1984; Pennypacker & Leath, 1986). Occlusion of vascular elements has been reported by the many workers, for example, Bishop & Cooper (1983); Street & Cooper (1984), Hutson & Smith (1980) and Douglas & MacHardy (1981) suggested that in *Verticillium*-infected cotton, tomato or chrysanthemum plants, tyloses and gels in vascular elements might have a role in restricting the upward spread of the pathogens but also may lead to restriction in water flow thereby causing wilting. For example, Street & Cooper (1984) showed that vascular flow was reduced in wilted tomato plants and they suggested that vascular occlusion was the primary cause of water stress in Verticillium wilt. Furthermore, Hutson & Smith (1980) found that resistance to water flow in tomato, infected with *V. albo-atrum*, was greater than in uninfected plants

and this resistance was correlated with vessel blockage. Clearly, wilting is caused by water shortage; water shortage is caused by occlusion of vascular elements especially where alternative routes of flow are not present (Beckman, 1987), Beckman (1987) also suggested that a severely reduced water supply could also be responsible for some of the other symptoms. However, the cause of wilting has also been attributed to the result of toxins released by the fungus, enzymes or hormones released by the plant or the fungus (DeVay, 1989; Madhosingh, 1995; Hasan, 2002). As a result of such factors, desiccation of leaves and reduction in various growth parameters are inevitable.

Plants affected by wilt-inducing fungi and bacteria usually display a number of growth responses in addition to the typical symptoms of water deficiency. These responses include hyperplasia of the parenchyma adjoining infected vessels, epinasty, adventitious root formation etc. It has been suggested that auxins secreted by the pathogen increase the plasticity of the cell walls thus allowing pectic enzymes to damage the pits and enabling the protoplasts of adjoining parenchyma cells to bulge into the lumen of the vessel (Beckman, 1987). There is as yet no direct evidence that tyloses are major factors contributing to the wilting phenomenon, since in other wilt diseases tyloses may not be formed at all or may be associated only with mild symptoms and sparse development of the pathogen (Beckman, 1987). Ethylene, on the other hand, plays a major role in development of the symptom of vascular wilt diseases by causing epinasty (DeVay, 1989). Pegg (1981) reported that three commonly recognised symptoms of wilt diseases, epinasty, foliar abscission and adventitious roots can also be attributed directly or indirectly to the action of ethylene. On the other hand, Dimond & Davis (1953) suggested that ethylene was the cause of epinastic symptoms in FW of tomato. Gentile & Matta (1975) and Pegg & Cronshaw (1976) showed in *Fusarium*- and *Verticillium*-infected tomato plants that ethylene is produced as a pulse about a week after inoculation. They suggested that the progressive increase in ethylene correlated with senescence.

When histological examination was made in infected plants (tomato), mycelia of the fungus were observed in the xylem tissue with spores dispersed (Plate 3.4). Generally, following entry into the plant, either by hyphal growth or by uptake of conidia through broken roots or stem puncture, colonization proceeds gradually, radially and basipetally (Pegg & Brady, 2000). Similar findings were made by Pennypacker & Leath

(1983); Delwiche *et al.* (1981) and Eldon (1995) both in lucerne and cotton plants. They proposed that internal sporulation was probably the mechanism facilitating rapid colonisation of the host by *V. albo-atrum.* However, Madhosingh (1995) suggested that physical blockage with mycelia is not the main reason of onset of wilt on tomato, instead he proposed physiological and biochemical factors may cause main vessel blockage. So far, it has been shown that most of the vessel-occluding factors (gels, tyloses and phenolic products) are of host origin. Apparently the plant itself causes a water shortage, in essence, causing its own demise for the sake of a defence system (Beckman, 1987). Apart from those symptoms described, generally *Verticillium*-infected plants also showed typical dark-brown discoloration in the xylem cell walls as a response to pathogen attack (Plate 3.6). This symptom was clearly explained by Isaac (1957) and (Pennypacker & Leath, 1986), as oxidation and polymerisation of phenolic compounds leading to deposition of melanin. These symptoms are also characteristic plant responses to invasion by fungi or bacteria. In this study, epinasty of petioles, and adventitious root formation were also observed, Plate 3.1.

There were differences between cultivars of tomato in response to *V. albo-atrum.* Cultivars Ailsa Craig, Simge F1, Falkon and Hybrid Sweet 100 F1 were among the susceptible cultivars. While other cultivars were resistant to *V. albo-atrum*, often their height and RGR were lower than those of the controls. Furthermore, resistant cultivars produced new branches while susceptible ones did not (section 3.3). A similar kind of report was made by Resende *et al.* (1995), who reported that resistant cocoa plants inoculated with *V. dahliae* recovered from the disease by producing new disease-free shoots and leaves while the susceptible ones did not. On the other hand, Pegg & Dixon (1969) used the tomato (T) strain of *V. albo-atrum* to evaluate tomato cultivars for VW resistance. They found that the cultivars 'Loran Blood' and 'Moscow' showed resistance to disease expression and had little vascular colonization at all levels of inoculum concentrations with T strain. Cultivars 'Bony Best' and 'Potentate' were susceptible to the T strain. Genetic heterogeneity of the cultivars may be one of the main factors that make plants susceptible or resistant to the disease (Pennypacker, 2000). For example, the terms qualitative resistance involves a specific host resistance gene and pathogen avirulence gene. Quantitative resistance describes host resistance that lacks a recognized

gene-for-gene relationship (Heath, 1995). Because of the genetic differences, (symptom-free) resistant plants could produce more lateral and main roots that enable plants to avoid the effect of the pathogen.

Resistance to VW also varied according to the age of the plants inoculated. The present study showed that inoculation of younger seedlings (four-week old) of tomato plants with USA (VS, VA, VF & VL) or European (V1 & V2) isolates (1 x 10^7 conidia/ml) at 23 °C caused severe symptoms (section 3.5). The height of plants inoculated with European or USA isolates were extremely shortened to a height of *ca.* 25-30- and 60-70 % of the control plants, respectively, and their RGR values were again 40-50- and 40-60 % of the control group, respectively, Table 3.4.1. However, older tomato plants (6-week old) showed more resistance to *V. albo-atrum* than the younger seedlings. For example, the height of plants inoculated with European isolates, V1 or V2, was 65-85 % of the control group, and their RGR values were also 85 % of the control group, Table 3.1.7. Similarly, Evans *et al.* (1966) showed that younger cotton plants were more readily invaded by *Verticillium* than older plants. In older plants the formation of disease symptoms are often delayed. Bell (1992) also reported that cotton plants infected with *Verticillium* when young might be killed quickly or remain stunted. It is probable that the young seedlings do not possess such established root systems and strong suberin layers at the time of inoculation. In contrast, Resende *et al.* (1995) reported that older seedlings of cocoa were more susceptible to *V. dahliae* than younger ones.

When comparison was made, isolates of *V. albo-atrum* from USA (VS, VL, VF-all isolated from lucerne) or Europe (V1 or V2) had severe effect on young tomato plants under pot conditions (Table 3.4.1) though isolates from USA was less pathogenic. Furthermore, experiments in chapter 5 showed that isolates V1, VS, VL and VF were not pathogenic to 6-week old tomato under hydroponic conditions (Figs. 5.2.1 & 5.3.1; Tables 5.2.3 & 5.3.2). None of the growth parameters measured on tomato, inoculated with lucerne isolates, showed significant differences from the control plants (Tables 5.2.4 & 5.3.2). Whereas isolate V2 was pathogenic to tomato. All the growth parameters recorded for plants inoculated with V2 showed significant differences from the control plants. This may be caused by the degree of host specialisation of the pathogens. For example, Barasubiye *et al.* (1994) reported that strains *V. albo-atrum* from lucerne were

more virulent on lucerne than the potato strains, though only the potato strains were virulent on potato. Furthermore, while Tsror *et al.* (1998) reported that a virulent isolate of *V. dahliae* from paprika caused severe reduction in height in potato, watermelon and tomato, isolates from those plants neither caused reduction in height nor produced symptoms in paprika plants. Similarly, Isaac & Lloyd (1959) reported that only *V. albo-atrum* and *V. dahliae* from lucerne were capable of causing infection in lucerne; other isolates of both species obtained from other host plants were incapable of causing infection. These results were also supported by Flood (1980).

The co-inoculation of isolates V1 and V2 were also investigated on tomato plants. It is not unusual for plants to be exposed to more than one pathogen at the same time; for example, two or more fungi can infect a host at the same time (Tsor & Hazanovsky, 2001). In such cases, synergism or antagonism between the organisms can be expected (Dewey *et al.*, 1999; Schmidt *et al.*, 2001). To determine whether there was any interactions between two isolates, 6-week old tomato cv. Ailsa Craig was inoculated with the V1- or V2 isolate or both simultaneously. The results showed that the isolates V1 and V2 caused significant reductions in height and RGR, however only isolate V2 led to development of symptoms of wilt disease. No disease symptoms were observed in the group of plants inoculated with V1. On the other hand, when two isolates were inoculated simultaneously (V1+V2) on tomato, there was only a marginal effect on those parameters; the effect of simultaneous inoculation of both isolates (V1+V2) on these growth parameters was not significantly different from the effect of V2 alone. Symptom index values were almost the same in V2- or V1+V2-inoculated plants. This indicated that simultaneous inoculation of both isolates did not result in synergism or antagonism between the isolates. Simply, isolate V2 had more a marked effect than isolate V1, and the additive effect of both isolates on disease development and growth parameters was not significantly different from the effect of V2 alone. These results suggest that there were no interactions between the isolates on disease development of tomato. However, Johnson & Santo (2001) reported that two isolates of *V. dahliae* (VCG 2B-more aggressive and VCG 4A-less aggressive) were tested for aggressiveness on peppermint, Scotch spearmint and native spearmint in combination with the lesion nematode, *Pratylenchus penetrans*. They stated that in native spearmint isolate VCG 2B interacted

synergistically with *P. penetrans* while isolate VCG 4A did not. However, But they did not comment on interactions between the isolates of the fungus on development of the disease. On the other hand, Tsror & Hazanovsky (2001) reported that co-inoculation of different groups of fungi such as *V. dahliae* and *Colletotrichum coccodes*, major causal agent of potato early dying syndrome, caused more foliar disease symptoms, and crown rot and a greater colonization with *C. coccodes*, than inoculation with the pathogens separately.

The pathogenicity of the V1 isolate of *V. albo-atrum* had been previously tested on salt tolerant strains of *M. media* cv. Rambler under greenhouse conditions by Dikilitas (1997). In that study, the results showed that the susceptibility of salt tolerant strains (150-, 200-, 250- and 300- mM) to *V. albo-atrum* increased with increased tolerance to salt. Similarly, Al-Rawahy (2000) stated that plants of *M. media* regenerated from NaCl-adapted cultures were found to be less resistant to pathogens than the parent type. He observed that 29 out of 35 regenerants from NaCl-adapted cultures became contaminated with fungi in some stages of their lives, and 86 % of these eventually died within three months of their regeneration. On the other hand, this phenomenon was less marked with regenerants from non-NaCl selected (salt-sensitive) cultures, only 9 out of 37 of regenerants from non-NaCl selected cultures died as a result of contamination. Araya *et al.*, (1991) also showed that salt tolerant cultivars of barley accumulating high gylcine-betaine were more prone to aphids. These workers stressed that genetic manipulation of the cultivars for improved insect resistance needed to be considered at the same time as improved salt tolerance.

It has been reported that salt tolerance is stable in many cell lines, even after the stress agent has been removed from the medium over number of passages (Jain *et al.*, 1987), for example, in regenerated plants of *Nicotiana* (Nabors *et al.*, 1975), rice (Vajrabhaya *et al.*, 1989), sugar beet (Freytag *et al.*, 1990), wheat (Karadimova & Djambova, 1993), *M. sativa* (Winicov, 1991) and *M. media* (Al-Rawahy, 2000). However, Watad *et al.* (1991) reported that tolerance to salinity was lost when cells of tobacco were re-cultured in the absence of salt. However, there is no report for regenerated salt tolerant lucerne plants about loss of tolerance to salinity under non-saline conditions that is, if they were maintained for a number of years under normal conditions.

It has been suggested that salt tolerant *M. media* cv. Rambler plants may lose their salt tolerance under non-saline conditions (personal communication with Dr. Salim Al-Rawahy). It has also been reported that Hawaiian *Hibiscus tiliaceus*, which usually grows in conditions of high salinity, lost its salinity tolerance when grown in non-saline upland habitats in Hawaii (Santiago *et al.*, 2000). So, it is not clear whether the level of susceptibility of salt tolerant plants to *V. albo-atrum* will change after a number of years.

Because of such considerations, the experiment in which the pathogenicity of *V. albo-atrum* towards salt tolerant plants of lucerne was assessed, was performed with old- (R-Tolerance level (mM)-O) and newly- (R-Tolerance level (mM)-N) generated salt tolerant plants, under greenhouse conditions. The old-generated salt tolerant plants, in general, showed susceptibility to *V. albo-atrum.* Their height and RGR were significantly reduced (Fig. 3.5.2). Reduction in height and RGR were also reported by Pennypacker *et al.* (1990) on lucerne cultivars. For old-generated salt tolerant plants, the progress of the disease was similar both in not-salt tolerant and salt-tolerant strains of *M. media* in the first and third year of the experiments. However, the effect of *V. albo-atrum* was more marked on the newly-generated salt–tolerant lines (Fig. 3.5.2). Nevertheless, the progress of disease development more or less followed the same pattern in old and newly generated salt tolerant plants. It was once more established that when *M. media* cv. Rambler plants gained more tolerance to salt they became more susceptible to the effect of *V. albo-atrum* (see Chapter 3).

6.2. Effect of salinity on tomato and lucerne.

Soil salinity is a major and the most persistent threat to irrigated agriculture. In saline soils, crop growth is hampered by salt accumulation in the crop root zone. If the upward salt movement caused by evaporation exceeds the downward gravitational movement of water, salt will accumulate in the root zone (Mondala *et al.*, 2001). Salt in the soil interferes with crop growth when its concentration exceeds the tolerance limits of the crop (Mondala *et al.*, 2001).

In semi-arid areas, irrigation can result in large increases in agricultural productivity (Prendergast *et al.*, 1994). However, the method of irrigation in many irrigated fields has resulted in higher water tables and salinity problems (Prendergast *et*

al., 1994). Much research has been carried out to optimise the irrigation systems in terms of maximizing crop yield and minimizing water wastage. However, excessive use of irrigation without adequate drainage results in a rise in the ground water level and capillary action draws salts up through the soil profile (Bridges, 1997).

In this study the effects of salinity on the growth of tomato and lucerne were assessed, prior to evaluating their effects in combination with *V. albo-atrum*. However, the effect of salinity on plant growth is related to the stage of plant development at which salinity is imposed. For example, salt tolerance in germination is not consistently related to tolerance during emergence, vegetative growth, flowering or fruiting (Shannon, 1985).

The effect of salinity on seed germination has been carried out in both lucerne and tomato cultivars to determine for their level of tolerance to salinity, in order to make a comparison between cultivars. Screening for salt tolerance during germination is a vital criterion required for any crop variety that is to be grown in saline stressed environments (Johnson, 2000).

In the screening stage, 25 replicate for lucerne and 20 replicate for tomato seeds of each variety were used for a single treatment. Unfortunately, replicate number could not have been increased to minimise the error in the population because limited seed stock was available.

The effect of NaCl on germination of both tomato and lucerne was assessed in a series of glass Petri dishes containing 0-, 50-, 100-, 150-, and 200 mM NaCl (for tomato cultivars) and 0-, 25-, 50-, 100-, 150-, 200-, 250-, 300- and 350 mM NaCl (for lucerne cultivars). In both lucerne and tomato, whichever cultivar is considered, the percentage germination decreased with increasing NaCl concentration, though some were more tolerant than others. In the case of tomato; the germination of most cultivars showed significant differences from the control group at 100 and 150 mM NaCl, with the exception of Hybrid Sweet F1, which showed a significant difference from the control group at 50 mM NaCl concentration. The differences between cultivars in response to salinity were calculated through their IC50 values (Table 4.1.5), leading to classification of Margarita (Fa-558) and Hybrid Sweet F1 as susceptible-; Fantastic F1 and Ailsa Craig as moderate-; and Simge F1, Edcawy and *L. lycopersicon* as tolerant cultivars. However, in the case of lucerne, an increase in NaCl up to 150 mM caused only small decreases in

the germination rate of the seeds. Nevertheless, above 150 mM NaCl, there was a marked decrease in germination and most of the cultivars showed significant differences from their control groups (Table 4.2.3). According to IC50 values, Vela, Rambler, Bitlis and AC Blue J were classified as the most susceptible cultivars. Similarly, Chartzoulakis & Loupassaki (1997) reported that external salinity up to 50 mM NaCl did not reduce the final percentage germination after 7 days of eggplant seeds. However, the rate of germination of lucerne was significantly reduced at 100 and 150 mM NaCl concentrations.

Bliss *et al.* (1986a) reported that the inhibitory effect of NaCl, mannitol or betaine (non-toxic solute) on germination of barley seeds was primarily due to an osmotic effect. High concentrations of salt or other solutes such as mannitol or betaine slow down water uptake by seeds, thereby inhibiting their germination (Werner & Finkelstein, 1995). On the other hand, Bliss *et al.* (1986b) suggested that the difference between isotonic salt and betaine might be due to a toxic effect of NaCl, which is revealed only after a threshold concentration of NaCl has been surpassed. In general, seeds must reach a threshold level of hydration before the processes leading to visible germination can begin (Bliss *et al.*, 1986b). For example, Bliss *et al.* (1986b) reported that germination of barley seeds increased in both salt- and betaine treatments with increasing time of pre-imbibition in water. They have suggested that the inhibitory effect of osmotica declines as seeds begin germination. However, if it is insufficient hydration that blocks germination during the imbibition phase, then any mechanism that could boost hydration over a critical threshold value should allow germination to proceed. During seed germination cell elongation is the primary growth event, facilitating radicle extension and leading to emergence (Obroucheva, 1999). Cell elongation needs an increase in the water content of the cells. However, under stress conditions, the water availability retards cell elongation and as a result, radicle elongation and germination is delayed (Cuartero & Fernandez-Munoz, 1999).

Seed germination is also affected by the degree of sensitivity of the germination enzymes and/or hormones to the level of toxic ions, i.e. Na^+ and Cl^- in the medium (Smith & Comb, 1991; Al-Niemi *et al.*, 1992). It is probable that the high concentration of salt keeps cell water potential low and reduces protein hydration and enzyme activity

(Kramer, 1983). These physico-chemical effects upon the seed seem to result in a slower and/or lower rate of germination. For example, Flowers (1972), in the genera *Beta solicarnia* and *Suaeda*; and Cavalieri & Huang (1977), in the genera *Borrichia*, *Distichlis*, *Juncus*, *Salicornia*, *Spartina*, reported that malate and glucose-6-phosphotae dehydrogenase were among the enzymes that play critical roles in seed germination.

When comparison was made between tomato and lucerne a decrease in germination of cultivars of both species decreased with increasing salinity, however, lucerne seemed to be less affected by NaCl than tomato, although cultivars responded to salinity differently (Tables 4.1.7 & 4.2.3). In fact, the percent of lucerne seed germination in saline conditions is not higher than that of some other vegetable crops, e.g. tomato, (Miyamoto & Petticrew, 1985) but lucerne germinates faster. This is a favourable feature for establishment because the fast rate of germination reduces the time for salt accumulation (Waissman & Miyamoto, 1987). However, emergence can be reduced through post-germination salt injuries such as hypocotyl mortality, because hypocotyl and cotyledon must pass through the surface of the soil where salt is accumulated (Assidan & Miyamoto, 1987).

So far the discussion has focused on the effect of salinity on seed germination. However, hypocotyl emergence and subsequent seedling growth are also important factors influencing crop productivity, particularly when seeds are sown in conditions adverse to germination. In this study, hypocotyl elongation of tomato or lucerne cultivars was found to be less tolerant to NaCl than the germination stage (Table 4.1.7). Similar findings were made by Douirani (1998) on rice cultivars. For example, hypocotyl elongation of all tomato cultivars, except *L. lycopersicon*, showed susceptibility at 50 mM NaCl concentration though only 50 % of lucerne cultivars showed susceptibility to 50 mM NaCl and the rest are tolerant up to 100 mM NaCl concentration (Tables 4.1.7 & 4.2.6). Similarly, Chartzoulakis & Loupassaki (1997) stated that hypocotyl elongation of eggplants was more sensitive to salinity than seed germination and they observed that seedling growth was severely affected at 10 mM NaCl concentration. This reduction could be a combined effect of osmotic stress (Greenway & Munns, 1980), which is more harmful to plants during the seedling stage, and higher ion uptake (Dumbroff & Cooper, 1974). Similarly, Rhoades (1990) reported that some plants are generally relatively

tolerant during germination, but become more sensitive during emergence and early seedling stage. Any failure at this stage will reduce the plant stand and the potential yields far more than predicted. Ungar (1996) also reported that seeds of *Atriplex patula* (Chenopodiaceae) were less affected than growing plants.

In general, cultivars from USA showed greater tolerance in terms of final hypocotyl or radicle length to NaCl than those of other cultivars. From other countries; only Vertus (Europe-Switzerland), Bilensoy-80 (Turkey), Barrier (Canada) and SA Standard (South Africa) were among the tolerant cultivars (Table 4.2.6 & 4.2.8).

The effect of NaCl and mannitol (a non-ionic substance) which reduces the external water potential without the toxic effect associated with Na^+ and Cl^-, were also investigated on germinating lucerne and tomato seeds. When the seeds of lucerne and tomato cultivars were germinated in soil conditions; the percentage germination decreased with increasing NaCl or mannitol concentrations (Figs 4.1.1-4.1.5; 4.2.1-4.2.5). From tomato cultivars, Fantastic F1 was the most affected cultivar though *L. lycopersicon* was the most resistant. Mannitol, used at the same osmotic potential as the NaCl, also caused a significant decrease in germination although the effect of mannitol was not as severe as NaCl, with the exceptions of Ailsa Craig and *L. lycopersicon*. The data, therefore, suggested that at least a part of the inhibition caused by NaCl resulted from the toxicity of the ions.

When hypocotyl elongation was measured, it was inhibited in a pattern that was closely similar to its effect on germination. Fantastic F1 at 100 mM NaCl, showed the greatest susceptibility while *L. lycopersicon* showed the most resistance. Similarly, mannitol at the same osmotic potentials inhibited hypocotyl elongation but not to the same extent as NaCl. For example, the average hypocotyl length of cultivars Margarita (Fa-558), Fantastic F1, Ailsa Craig and Simge F1 dropped by 39 % and 32 % in the presence of mannitol; but by 22 % and 11 % in the presence of NaCl at –0.36 MPa and –0.48 MPa, respectively, indicating that NaCl has both toxic and osmotic effects. However, *L. lycopersicon* responded differently; the effect of NaCl was less inhibitory than that of mannitol. Strogonov (1974) reported that root growth of alfalfa dropped by 42.9 % in the presence of mannitol and by 89.3 % in the presence of NaCl. Zhao & Harris (1992) found that the seedling stage of *Prosopis chilensis* was more sensitive to

NaCl than to PEG under iso-osmotic treatments. They suggested that this might be associated with the differential permeability of roots to NaCl and PEG. Similarly, Katembe *et al.*, (1998) reported that higher concentrations of NaCl were found to be more inhibitory to germination and seedling root elongation than iso-osmotic PEG solutions on *Atriplex patula* (Chenopodiaceae). One possible mechanism is that, unlike mannitol, NaCl may readily cross the cell membrane into the cytoplasm of the cells, unless an active metabolic pump prevents accumulation of the ions. In these cases, NaCl in the cytoplasm can result in toxic accumulation of particular ions (Na^+ and Cl^-) or decreased availability of some essential nutrients (Waisel, 1972; Werner & Finkelstein, 1995) thus causing reduced growth in hypocotyl or root elongation.

Under optimal germination conditions, most tomato seeds germinate within 36-72 h of seed imbibition (Foolad *et al.*, 1999). However, in these studies, germination of the various cultivars in control conditions occurred 4 or 5 days after imbibition of the seeds, Figs. 4.1.1-4.1.5. Under saline stress, germination has been reported to be decreased or not to occur on tomato (Jones, 1986). The results presented in Figs. 4.1.1-4.1.5 clearly illustrate the inhibitory effect of salinity on germination. Not only did the total percentage germination decrease with increasing NaCl concentration, but there was an increase in the lag before germination occurred, which increased with increasing NaCl concentration and resulted in an increase in the time taken for 50 % germination to occur, Table 4.1.9. This result is supported by those of Cuartero & Fernandez-Munoz (1999), who has showed that tomato seeds need 50 % additional days to germinate at 80 mM NaCl than in a medium without salt and almost 100 % more days at 190 mM. Lengthening of the germination period can be very dangerous for a direct-sown crop because the probability of crust formation on the soil surface, which would make difficult, or even prevent emergence, and they become more susceptible to the attack of several fungi and pests.

The time taken for germination of tomato seeds was also increased through iso-osmotic mannitol solution although the effect was so marked as with NaCl, except in the case of *L. lycopersicon* at 200 mM mannitol. Similar reports were also made by Johnson (2000) on tomato cultivars. In the case of lucerne, the delay in germination caused by NaCl or mannitol was not significantly different (Table 4.2.10). The reduction in

germination and the increase in time taken for 50 % germination that resulted from salt-stress could be due to an increase in the concentration of potentially toxic ions (Torres-Schumann *et al.*, 1989) or osmotic effects of the saline medium (Alvarado *et al.*, 1987) or both. Reduction or delay in germination due to exposure to salinity stress has also been reported in a variety of crop species including *Cucumis melo* (Botia *et al.*, 1998), sunflower (Delgado & Sanchez-Raya, 1999) and soybean (Wang & Shannon, 1999).

There were also differences between cultivars in response to salinity. The most susceptible and the resistant ones to salinity were Fantastic F1 and *L. lycopersicon*, respectively. For example, Johnson (2000) reported that increasing salt concentration resulted in a decrease in the rate of germination in Fantastic F1, Simge F1 and Edcawy, however, the effect was more marked in Simge F1 and Fantastic F1 compared to Edcawy. Although Johnson (2000) reported that Fantastic F1 was not a highly salt tolerant cultivar, she recommended that this cultivar had the potential to be a viable crop to be grown in mildly salinised areas as a result of consultation with Prof. Yuksel Tuzel (Turkey).

6.3. Root formation of salt-susceptible and tolerant lucerne cultivars in saline medium

The cultivars that had showed the greatest level of tolerance or susceptibility to NaCl during germination and post-germination stage were used to test their ability to produce roots under saline conditions, which has been used as an indicator of tolerance or susceptibility (Taufikrahman, 1993). The root formation of tolerant or susceptible cultivars under saline conditions was evaluated with increasing NaCl concentrations. However, the tolerant cultivars (Protea, 13R Supreme, Bilensoy-80, Mesa Sirsa), which were established in the germination experiment, did not show significant differences from the control plants in terms of root length and dry weight up to 150 mM NaCl treatment (Table 4.3.1-4.3.4). Those cultivars initially showed susceptibility to 100 mM NaCl in terms of radicle length during post-germination stage. However, in this experiment, a concentration of 100 mM NaCl was not found really effective on tolerant cultivars. Similar results were reported by Taufikrahman (1993) on *Trifolium repens* clones in which the formation of roots was greatly reduced by 150 mM NaCl. It is probable that

the short-term effect (2 weeks) of NaCl has no significant inhibitory effect in tolerant cultivars. However, a susceptible cultivar (Peru) was significantly affected at 50 mM NaCl concentration in terms of root length and dry weight. As a result of salinity, the reduction in root growth results in a decrease in uptake of nutrients, in turn this would reduce the growth of all other organs of the plant, thus causing reduction in dry weight.

Proline content of the leaves at 50 mM NaCl concentration did not show significant difference from the control group and it slightly increased at 100 mM NaCl concentration when compared to the control group. However, this was also not significant when compared to the control group (Table 4.3.5). Similar findings were also reported by Chaudhary (1996). Delauney & Verma (1993) reported that the proline might increase as a result of low water potential to participate in osmotic adjustment. Proline can also act as a compatible solute that can exist in high concentrations without disturbing the activity of macro molecules (Ahmad *et al.*, 1982). It could also serve as a nitrogen source in the cells under stress (Kohl *et al.*, 1994).

6.4. Effect of NaCl on the growth and development of *V. albo-atrum.*

Interactions of salinity and fungus have been widely studied for some species, though there are few reports concerning interactions between *V. albo-atrum* and NaCl. Salinity may interact with the fungus in soil fauna and might reduce, or sometimes increase the sporulation and pathogenicity of the fungi. For example, Amir *et al.* (1996) reported that salinity induced soil suppressiveness to vascular Fusarium by reducing the sporulation and germination of *F. oxysporum.* Similarly, Engel & Grey (1991) reported that fertilization with chloride increased the yield of winter wheat and reduced the severity of root diseases caused by *F. culmorum.* It has also been reported that the combined effect of high temperature and high salinity suppressed the growth of *Fusarium* species and limited the distribution in an arid environment of Bahrain (Abbas & Mandeel, 1995; Mandeel, 1996). Salinity may affect fungal metabolism as reported by Omar & Abd-Alla (2000). They stated that salinization of the growth media with NaCl (85-250 mM) strongly inhibited enzymatic activity (cellulase, pectin lyase, polygalacturonase) in growth-promoting fungi such as *Cladosporium cladosporioides, F. moniliforme, F. oxysporum, F. solani, Rhizoctonia solani and Macrophominia phaseolina* on the nodules

of *Vicia faba* L. On the other hand, salinity may have positive effects on fungal growth. For example, it increased the development of motile zoosporangia of *Perkinsus* sp. (Ahn & Kim, 2001) and it created more virulent pathogens in the case of *F. oxysporum* f. sp. *vasinfectum* on cotton (Ragazzi *et al.*, 1994).

In this study, initially, the radial growth of isolates of *V. albo-atrum in vitro* was compared. The results show that under non-saline conditions the growth rate of isolate V2 (isolated from tomato) was greater than that of the isolate from lucerne, Table 4.4.1. When assessed under saline conditions it was found that with an increase in concentration of NaCl the mycelial and radial growth of both isolates of *V. albo-atrum* decreased, in particular, with 100- and 200 mM NaCl (Figs. 4.4.2, 4.4.3, 4.4.4). Sporulation and spore germination were also inhibited by 100- and 150 mM NaCl. It is interesting that the fastest growing isolate, V2, under non-saline conditions was the most affected isolate under saline conditions especially where sporulation was accounted.

The effect of NaCl has also been reported by other workers. For example, Hasan (2002) stated that the mycelial growth of *F. oxysporum* significantly increased with 1-10 % (0.17-1.70 M) concentrations of NaCl, although the production of GA and IAA by the fungus decreased above 1 % NaCl. However, neither he did report has made no attempt how he measured mycelial weight, nor discuss why his result was different from the other workers. For example, Amir *et al.* (1996) reported that concentrations of NaCl over 1 % (170 mM) negatively affected the radial growth of the *F. oxysporum* f. sp. *albedinis* and *F. oxysporum* f. sp. *lini*. They reported that sporulation and spore germination were inhibited at concentrations of NaCl above 85- and 170 mM respectively. They also noted that the inhibitory effect of NaCl increased with increasing time.

Sporulation of both isolates was inhibited at lower concentrations of NaCl than was required to inhibit radial growth (see Chapter 4, section 4.4). It has been suggested that for fungi, generally a narrower range of environmental conditions are required for sporulation than for mycelial growth and the results presented here are consistent with that of Sung & Cook (1981). Similarly, Gao & Shain (1995) reported that the conidial germination of *Cryphonectria parasitica* (Murr.), chestnut blight fungus, was more sensitive than mycelial growth to NaCl at –2.0 MPa osmotic potential. The appearance of the colonies of both isolates (V1 and V2) also became more feathery with increasing

NaCl concentrations. The morphological switch to feathery patterns in some mycelial fungi at low osmotic potentials has been attributed to their ability to adapt to drought conditions (Griffith & Boddy, 1991).

The effect of NaCl on the growth of isolates of *V. albo*-atrum that had previously been adapted to grow on saline medium was also determined. Non salt-adapted (V1 and V2) and salt-adapted strains (V1-, V2-150; V1-, V2-200) were grown in the respective conditions under which they were normally maintained. Isolates V1 and V2 and their salt-adapted strains, V1-150 and V2-150, showed similar radial growth rates over the 3-week period under the adapted conditions (Fig. 4.4.3). Statistical analysis showed that there was no significant difference between the original isolates, V1 & V2 and their salt-adapted strains V1-150 & V2-150 (Table 4.4.3). However, at 200 mM NaCl the growth rates of both V1-200 and V2-200 were significantly different from those of the original isolates with the growth of the salt-adapted strains being higher. However, although salt-adaptation resulted in this ability to maintain better growth rates under elevated concentration of NaCl than strains that had not been adapted, adaptation did coincide with a decrease in ability to form spores, in all the salt-adapted isolates, Fig. 4.4.3, though the effect was more marked in V1- and V2-200 than in the corresponding –150 strains. This may affect the ability of *V. albo*-atrum to infect plants under saline conditions. Though the lower rate of sporulation under saline conditions might still be efficient to cause pathogenicity. Furthermore, the pathogen may eventually develop an adaptation mechanism. For example, many fungal species, such as *Aspergillus*, *Penicillium*, *Rhizopus* and *Fusarium*, produce hormones, such as GA or IAA or both, as in the case of *F. oxysporum* (Hasan 2002) and at 0.5 and 1 % NaCl the content of GA and IAA was significantly increased after 5 days. The author suggested that an increase in the level of hormones might act as an adaptive response that maintains the stability of the fungus. However, at higher concentrations of NaCl, the content of GA and IAA decreased, an effect that was more marked over time. It should also be remembered that salinity does not only exert stress on fungal metabolism but also reduces the resistance of the cultivars. In such circumstances, lower germination rate for the conidia might still result in pathogenicity.

Not only might the lower sporulation rate resulting from the higher concentrations of NaCl affect pathogenicity of the fungus but another factor that may also influence infectivity is the germination rate of those spores which are produced. Consequently, this was assessed for both the original V1 & V2 isolates. The results showed that germination was mostly unaffected by NaCl up to a concentration of 150 mM (Table 4.4.4), though increasing the concentration to 200 mM caused a decrease in germination. Nevertheless, even at the highest concentration of NaCl (200 mM) 70 % germination occurred. The reduction in germination under saline conditions may be attributed to both toxic and osmotic effects of NaCl as was concluded in the seed-germination experiment. For example, Chandler *et al.* (1994) reported that reduced osmotic potentials decreased germination and the radial extension in *Verticillium* species. Similar findings were made by McQuilken *et al.* (1992) who showed that a decrease in osmotic potential caused a reduction in mycelial growth and germination of oospores of *Pythium oligandrum.* Similarly, salinity by itself also inhibits development and growth fungi. For example, a decrease in growth of *Phytophthora* sp. caused by salinity was also reported by Wilkens & Field (1993).

The amount of mycelium in the colony of both isolates was also assessed (Fig 4.4.4). The results of mycelium dry weights indicated that NaCl up to 50 mM had no effect on the growth of the pathogen although at that concentration the growth of plants was markedly affected (Fig 5.1.1 & Tables 4.1.7; 4.2.6; 5.1.3). The data, therefore, suggest that *V. albo-atrum* and NaCl may have an additive effect, if not synergistic, at least up to 50 mM NaCl and be responsible for the symptoms and reduction in growth parameters on plants. Similar findings were made by Danti & Broggio (1997) who reported that the growth of *V. dahliae* declined on aqueous and solid media containing 0.05-0.35 M NaCl, however, sporulation, in that range, was not affected.

6.5. The effect of hydrogen peroxide and purified phytoalexins on spore germination and germ tube elongation of *V. albo-atrum* isolates.

The effect of antifungal compounds such as H_2O_2, sativan and medicarpin were also tested on the fungal isolates, to establish whether there is a difference between isolates in terms of germination and mycelium development *in vitro*.

An increase in concentration of H_2O_2 resulted in a decrease in germination for both isolates (Fig. 4.5.1a). When the LD50 was calculated, V2 was found to be more sensitive to H_2O_2 than V1 isolate, Table 4.5.1. The effect of H_2O_2 on germ tube elongation of germinated conidia was also tested, with the same concentrations of H_2O_2 that was used for the germination experiment. Again, the effect was more marked in V2 than V1 (Fig. 4.5.1b). In general, the inhibitory effect of H_2O_2 was more marked on conidia than the germ tube elongation of *V. albo-atrum*. Inhibitory effect of H_2O_2 was also reported by Peng & Kuc (1992) on *Peronospora*, *Cladosporium* and *Colletotrichum* spores. Similarly, Lu & Higgins (1999) reported that higher concentrations of H_2O_2 were required to inhibit germ tubes than for inhibition of the conidia of *Cladosporium fulvum*. H_2O_2 may inhibit pathogens directly, or may generate other reactive free radicals that are themselves antimicrobial (Peng & Kuc, 1992). In this study, the extent of the effects of H_2O_2 depended on the concentration used.

Medicarpin was more inhibitory to germination of V2 than V1 (Fig 4.5.2a & Table 4.5.1) while sativan inhibited germination of the conidia of both isolates equally (Fig. 4.4.2b). Sativan was more inhibitory than medicarpin. Similarly, Flood (1980) reported that ED50's of sativan was much lower than that of medicarpin and the effect of phytoalexins was more pronounced on V2. Such inhibitory effect of phytoalexins on germination and growth of fungus are not uncommon. For example, Bianchini *et al.* (1999) reported that the phytoalexins, hemigossypol and desohemigossypol, (from cotton) inhibited conidial germination of *V. dahliae* and He *et al.* (2002) presented that phytoalexins from lucerne inhibited germination and radial extension of *Verticillium* species. Similarly, Turkusay & Onogur (1998) inhibited the germination and colony growth of *Alternaria solani* and *Botrytis cinera* with extracts from *Hedera helix*.

6.6. Interactive effect of NaCl and *V. albo-atrum* on the severity of VW disease (and yield) of tomato and lucerne cultivars.

In the past, researchers have directed their attention to either the relation between plants and abiotic stresses or that between plants and pathogens. The present study, for the first time, examined the disease incidence and the physiological developments in tomato and various salt & disease resistant or salt & disease susceptible lucerne cultivars growing under salinity stress in the presence of vascular wilt pathogen *V. albo-atrum*. For crop plants, different stresses may interact, both in their occurrence and in their effects. So, real progress in crop improvement is likely to depend on a better understanding of these interactions and their consequences for plants and ecosystems (Austin, 1989).

In this section, tomato plants were exposed either to progressively increased or continuous low levels of NaCl (50 mM-the start of salinity problems for most of the crops, Abrol *et al.*, 1988). An increase in NaCl levels in nutrient medium can be used to simulate field conditions where salt concentrations fluctuate widely over time (because, concentration of NaCl in an irrigated field gradually increases as water is lost through evapo-transpiration and quickly drops with the application of irrigation water (Rhoades, 1972)).

Although the main aim of these experiments was to study the effect of salinity and *V. albo-atrum* on the development of tomato and lucerne cultivars, classical growth analysis was also used to monitor the effect of salt on vegetative growth of plants in hydroponics.

6.6.1. Effect of NaCl on young (4-week old) and old (8-week old) tomato plants in the presence or absence of V. albo-atrum.

Exposure of plants to salt stress usually begins with the exposure of the roots to that stress. Salt stress leads to changes in growth, morphology and physiology of the roots that will in turn change water and ion uptake and the production of signals (hormones) that can communicate information to the shoot. The whole plant is then affected when roots are growing in a salty medium (personal communication with Professor T.J. Flowers; Cuartero & Fernandez-Munoz, 1999). This was the case in this

experiment. Four-week old young tomato plants (cv. Ailsa Craig) were exposed to various concentrations of NaCl (0-, 25-, 50-, 100-, 150- and 200 mM) for 5 weeks. The results showed that after 3 weeks of the start of the experiment the height of seedlings was inhibited, Table 5.1.1, and at the end of the experiment the growth parameters (H, RL, RGR, LA, NAR and chlorophyll content of the leaves, Table 5.1.1) were significantly reduced compared to the control group. At 50 mM NaCl, reduced height was the only visible response. However, at 100- and 150 mM NaCl concentration, the reduction was more pronounced and accompanied by toxicity-related symptoms of NaCl (chlorosis, rolled- and abscinded-leaves). Furthermore, at high salinity levels (100 and 150 mM NaCl) abscission of lower leaves also contributed to the reduced RGR and NAR. Inhibition of vegetative growth in tomato at high salinity levels is associated with marked inhibition of photosynthesis (Tables 5.1.1. & 5.1.6). For example, in a more detailed study, Nieman *et al.* (1988) reported that salt stress reduced the growth of pepper and suggested that it was due to reduced assimilation of photosynthate. Reduction in chlorophyll content also correlated with an increase in NaCl concentration (Tables 5.1.3 & 5.1.5). Similarly, Velagaleti *et al.* (1990) reported that the reduction in chlorophyll content of soybean correlated with increased chloride ion accumulation. Reduced chlorophyll content was also noted by Gunes *et al.* (1995), in potato. By itself *V. albo-atrum* also inhibited growth (Table 5.1.2). However, the combined effect of NaCl & *V. albo-atrum* were more inhibitory than either of the treatments (Table 5.1.2). Similarly, MacDonald (1984) reported that root systems of hydroponically grown chrysanthemums exposed to salinity after inoculation with *Phytophthora cryptogea* developed more severe symptoms than the plants that were not exposed to salinity. Swiecki & MacDonald (1988), on the other hand, showed that rooted cuttings of *Chrysanthemum morifolium* grown in nutrient solution were given a 24 h pulse exposure to salinity by amending the solution with 200 mM NaCl. They found that penetration of non-stressed roots by *Phytophthora cryptogea* was frequently limited and the plant exhibited a high degree of resistance. In contrast, hyphae of *P. cryptogea* rapidly colonized salinity-stressed roots, causing extensive necrosis after 12 h inoculation, indicating a salt-induced change from a resistant to a highly susceptible condition. Similarly, Turco *et al.* (2002) reported that a nutrient solution with an EC of 20 mS cm^{-1} increased the severity of Fusarium wilt about

34% in the cotton cultivars 'Coker 304' and 'Acala SJ2', compared with the non-saline test group.

The reduction of the growth of young tomato plants in saline conditions can be attributed largely to reduced water absorption caused by reduced water potential in the root environment. Under saline conditions, the Na^+ and Cl^- ions are taken up at high rates, which may lead to excessive accumulation in tissues. These ions may inhibit the uptake of other ions into the root and their transportation to the shoot (Mer *et al.*, 2000).

For some growth parameters (H, RL, RGR and chlorophyll content of the leaves) the effect of NaCl (50-, 100- and 150 mM) was also severe on 8-week old tomato. However, the effect of fungus, by itself, was not so marked on the growth parameters of 8-week old tomato plants as it was on the younger plants. Again, the combination of salt and the fungus was more deleterious to the development of tomato than either of the treatments alone (Tables 5.1.5, 5.1.8 & 5.1.9). Similarly, Rasmussen & Stanghellini (1988) reported that increased salinity levels predisposed Penncross creeping bentgrass to cotton blight caused by *Pythium aphanidermatum* and accelerated the onset and development of disease. Similar results were reported by Nachmias *et al.* (1993) who simulated field conditions in potato cultivars (Cara, Desiree and Nicola) by adding NaCl and $CaCl_2$ (4:1) in irrigation water (to 5 mmhos cm^{-1}). They showed that the height of potato plants were further reduced and the severity of the disease increased by *V. dahliae* in the presence of salt compared with the non-inoculated but stressed control group.

Comparison of the data for the younger and older tomato plants showed that; 8-week old plants were less affected than the 4-week old ones, in terms of growth parameters. For example, in 4-week old tomato plants RL was affected equally with the shoot, but in the case of 8-week old tomato, RL was less inhibited than the shoot. A similar case was made by Papadopoulos & Rendig (1983) and Cuartero & Fernandez-Munoz (1999) with tomato plants. The lower leaves of 8-week old tomato plants had showed signs of succulence, especially those treated with 100 mM NaCl and above, unlike 4-week old tomato plants, in which the lower leaves died or did not develop as a result of NaCl. Changes in plant tissue succulence have been shown to be a feature of salt tolerance (Flowers *et al.*, 1977) and has been attributed to accumulation of Na^+ and

Cl^- ions in the leaves (Brugnoli & Bjorkman, 1992). It has been proposed that increased succulence in the leaves aid the dilution of potentially toxic ions (Cuartero *et al.*, 1992).

The age of the plants during salinity stress affected the resistance of the plants to disease. For example, younger tomato plants under salinity showed more susceptibility to disease than the older plants. Similarly, Cruz & Cuartero (1990) reported that adaptation to salinity was higher in older plants than in younger ones. In terms of symptoms, young tomato (4-week old) plants showed more severe symptoms than did 8 week-old tomato plants.

Recovery of the plants from the effects of the either fungus or salt was quicker and earlier than the combined effect of salt & the fungus. The results, therefore, suggested that the *V. albo-atrum* was still pathogenic under salt conditions and delayed the recovery of the plants in whatever ages they are. This is in good agreement with results of Swiecki & MacDonald (1991) who reported that exposure of tomato plants (*L. esculentum* Mill.) to salinity stress either before or after inoculation with *Phytophthora parasitica* increased root and crown rot severity relative to non-stressed control. The synergy between salinity and *P. parasitica* was most pronounced on young (pre-bloom) plants and least pronounced on older (post-bloom) plants. For example, the interaction between salinity and *P. parasitica* had significantly increased root necrosis and reduced top weight, and caused a higher incidence of crown infection than corresponding non-stressed plants.

It is not clear how salinity stress has such a pronounced effect on disease severity in plants, while having relatively less effect on non-inoculated plants. This mechanism, so far, is not fully understood, but may result from an impairment of normal host defense mechanisms. For example, plants respond to pathogenic invasion in numerous ways that function to block, slow or prevent the pathogen from its successful establishment or spread in host tissue (Bell, 1981). Many of the defense responses involve biosynthesis of compounds that are toxic to the invading pathogens (Bell, 1981). However, salinity stress has many adverse effects on plants including changes in membrane permeability (Campbell & Pitman, 1971), the ultra structure of organelles, synthesis of DNA, RNA and proteins (Campbell & Pitman, 1971). It is also possible that the host is predisposed to the disease by the decreased availability of water and the accumulation of toxic ions in

tissues. As a result of that, the plant may have a lower photosynthetic activity and a lower daily growth (Brugnoli & Bjorkman, 1992). Inhibitions of these processes in dehydrated tissues have been reported to reduce plant resistance through inhibition of protein synthesis that might contribute increased host susceptibility by preventing the synthesis of important enzymes for disease resistance in the host. (Bell, 1981). However, there is still little work on enzyme synthesis for disease resistance under salinity or dehydrating condition (Boyer, 1995). Under salinity, fungus may also be more aggressive, for example, El-Abyad *et al.* (1992) reported that the activities of cell wall enzymes (xylanase and galactanase) of *Scrlerotium rolfsii* increased with increased salt concentrations when compered to non-saline conditions.

6.6.2. *Effects of NaCl (50 mM) and its interactions with* V. albo-atrum *on various tomato and lucerne cultivars in a hydroponic system.*

A hydroponic drip irrigation system was employed to assess the response of plants to *V. albo-atrum* under saline conditions. Perlite is one of the inorganic media used in soilless systems and was the substrate used in this work. In this section, the plants were exposed continuously to a low NaCl (50 mM) concentration over a period of 7 weeks. Salinity at this level is an important limiting factor for crop plants. For example, Jennings (1976) reported that NaCl at a concentration around 50 mM and above would generally suppress leaf expansion, although the level of NaCl that would affect a plant depends on the plant concerned. In the long-term, the plants also absorb and accumulate appreciable amounts of salts in their tissues that may adversely affect the growth and yield potential (Kostandi & Soliman, 1998).

Salinity tolerance of tomatoes has been studied for decades and the characteristic symptoms of salinity stress include mainly a reduction in plant height and retardation of the plant growth, increased abaxial leaf curl, and increased chlorosis and necrosis of the older leaves, as shown by Johnson (2000) in studies screening tomato varieties for salt tolerance. The findings reported here are in good agreement with her results.

Screening experiments, in this study and in much of the literature, have been performed in a greenhouse on a small-scale monitoring for a specific period of time. Experiments of this kind are suitable for preliminary identification of potentially salt

tolerant varieties, though further screening of tomato varieties via large-scale field trials focusing on fruit yield and quality is also necessary to enable the recommendation of suitable potentially salt tolerant varieties for commercial application.

Although plants may not show stress symptoms and may metabolise normally under low to moderate salinity levels, additional energy may be required to cope with the disease with an additional effect of stress caused by microorganisms such as fungi. For example, VW disease generally depends on the conditions in which the plants are grown. It has been reported that the irrigation regime may increase the incidence of wilt (Jefferson & Gossen, 2002). If the irrigation water were associated with salinity, the detrimental effects on plants would be inevitable. In fact, VW is now becoming more prevalent in many areas where an increase in the salinity of the irrigation water poses its greatest threat (Nachmias *et al.*, 1993). Since it is known that the reaction to infection by disease can be modified by environmental factors, the potential for an interaction between salinity and disease is a realistic possibility that must be considered. Indeed, it has been reported that abiotic stresses such as drought, pollution, heat or salinity may increase symptoms by directly affecting the pathogen or its host (Ayres, 1984).

In the present work, the effect of *V. albo-atrum*, isolates V1 or V2 on 6-week old tomato cvs. Ailsa Craig, Simge F1, Margarita (Fa-558) and Fantastic F1 were investigated in the presence or absence of 50 mM NaCl. In general, isolate V1 did not cause significant reduction in the growth parameters (H, RL, RGR, FLW, WTC etc.) on any of the cultivars tested (Tables 5.2.4, 5.2.8, 5.2.12 & 5.2.16). However, isolate V2 caused symptoms and reduced growth parameters and proved to be a more pathogenic isolate than V1 on cvs. Ailsa Craig and Simge F1 (Tables, 5.2.4 & 5.2.8). Cvs. Margarita (Fa-558) and Fantastic F1 showed complete resistance to V2 and none of the growth parameters for these two cultivars were significantly reduced when compared to their corresponding control groups (Tables 5.2.12 & 5.2.16). NaCl, on the other hand, affected growth parameters on all cultivars tested (Ailsa Craig, Simge F1, Margarita (Fa-558) and Fantastic F1). However, the combined effect of both NaCl & *V. albo-atrum*, especially isolate V2, were more pronounced on the growth parameters above. The combined effect of both NaCl & isolate V1 was as marked as NaCl alone, suggesting that V1 isolate did not play an important role even in the presence of NaCl (Tables 5.2.4, 5.2.8, 5.2.12 &

5.2.16). Similar results were reported by Snapp & Shennan (1994) who reported that a tomato cv. UC82B became vulnerable to infection by *P. parasitica* when subjected to salt stress and produced thinner roots and higher root-senescence rates when compared to the non-stress control group. There is also a report that soil salinity markedly increased the incidence of *Phytophthora* root rot of tomato in field conditions Snapp *et al.* (1991). The combination of salinity and enhanced disease severity led to significant reductions in fresh fruit yields, fruit size and total above-ground biomass. In some cases salinity not only increased susceptibility of plants to pathogens but also decreased their resistance against soil-borne saprophytes such as *Pythium ultimum* (Martin & Hancock, 1981).

Salt stresses have also been observed in heavy metal contaminated soils. Plants near an industrial vicinity especially in developing countries, suffer from both stresses. For example, an additive effect of NaCl (6 EC) and lead acetate (1.0 mM) on biomass accumulation was observed in *Vigna radiata* (L.) Wilczek cv. Pusa baisakhi (Singh, 1995). It is well known that plants exposed to salinity show marginal Ca^{2+} deficiency (Kostandi & Soliman, 1998; El-Iklil *et al.*, 2002). Under such conditions, the membrane becomes fragile, permitting continuous out flow of assimilates (sugars and amino acids) that facilitate fungal growth (Hancock & Huisman, 1981). In contrast, since disease severity is determined by the ability of a pathogen to produce extracellular pectolytic enzymes (e.g. polygalacturanase) that dissolve the middle lamella, it seems likely that increasing leaf Ca^{2+}content would prevent pathogen invasion and improve host resistance (Bateman & Lumsden, 1965).

Inoculation with *V. albo-atrum*, isolates from USA (VS, VL & VF-all isolated from lucerne) did not cause any symptoms or reduction in growth parameters (H, RL, RGR, NAR) on tomato cv. Ailsa Craig, as was the case for the V1 isolate (Table 5.3.2). However, in the presence of 50 mM NaCl, the growth parameters (H, RGR, NAR) again showed significant differences from the control group, while isolates of *V. albo-atrum* (VS, VL & VF) caused further reductions in the growth parameters in the presence of NaCl (Table 5.3.3). This may be explained by a reduced resistance of the host. Under saline conditions, plants may become susceptible or even non-pathogens may act as pathogens. For example, Howell *et al.* (1994) reported that *V. albo-atrum* inoculation alone had little effect on the foliage yield of a lucerne cultivar, NK-89786. Only a

salinity (simulated by irrigation with NaCl or $CaCl_2$) of 5.0 or 7.5 dS m^{-1} significantly reduced yield of the cultivar in the absence of *V. albo-atrum*. However, imposition of salinity stress (3.0 dS m^{-1}) to inoculated NK-89786 plants enhanced the progression of VW disease and resulted in a significant decrease in yield relative to yield loss resulting from inoculation or salinity alone. Their results suggest that salinity caused NK-89786 plants to become more severely compromised by *Verticillium* infection resulting in greater yield loss.

The pathogenicity of isolates of *V. albo-atrum* that had been adapted to saline conditions (V1-, V2-150; V1-, V2-200) towards tomato was also evaluated, under saline and non-saline conditions. The salt adapted strains of isolate V1 (V1-150 and V1-200) did not cause any significant reductions, in the absence of NaCl, in H, RL and RGR, just as was the case for the parent isolate V1. However, stains of V2-150 and V2-200 affected the growth parameters in a similar way to the parent isolate V2 (Table 5.4.3). Growth parameters were also significantly decreased in the presence of NaCl (50 mM) and the fungus, especially the salt adapted strains of V2 (V2-150 & V2-200) were more effective in the presence of NaCl (50 mM), as was the case for the parent isolate V2 (Table 5.4.4). These salt-adapted strains of V2 (V2-150 or V2-200) were still pathogenic to tomato even under non-saline conditions, suggesting that the fungus did not lose its pathogenicity over a period of time even when subcultured under saline conditions. It is possible, therefore, that cultivars of tomato or lucerne in areas where soil salinity is a problem could undergo an increase in susceptibility to VW and suffer a greater yield loss as a result of the combined stresses or increased pathogenicity. It has been reported that a tomato isolate of *P. parasitica* recovered from saline soil was more tolerant to salinity than one recovered from non-saline soils (Bouchibi *et al.*, 1990). Similarly, Ragazzi *et al.* (1994) reported that mycelia of *F. oxysporum* f. sp. *vasinfectum* (FOV) from non-saline medium and from saline-enriched medium both produced wilt symptoms, however, the symptoms on a medium-resistant cotton cv. GSC 20 appeared earlier and advanced more rapidly with the mycelium from the saline-enriched culture. Furthermore, Ragazzi & Vecchio (1992) reported that chlamydspore viability and pathogen virulence of FOV were enhanced when it was subcultured on NaCl-enriched media. Turco *et al.* (1999) also reported that FOV had greater enzymatic activity in saline environments, especially

with regard to pectate lyase (PL) and polygalacturonase (PG) enzymes. One conclusion that can be drawn from the present experiment is that when saline water is use to irrigate tomato or lucerne plants, where *V. albo-atrum* may be present, soil salinity should be maintained and controlled to prevent the fungus from reaching a degree of virulence that would put even medium resistant cultivars at risk.

The pathogenicity of *V. albo-atrum*, isolate V1, was also tested on various disease-resistant & salt tolerant- (13R Supreme, Vertus & Bilensoy-80; see Chapter 5); disease susceptible & salt susceptible- (Rambler, see Chapter 3) or disease susceptible & salt tolerant (R-350-N, see Chapter 3) cultivars lucerne. Cvs. 13R Supreme, Vertus and Bilensoy-80 showed resistance to V1 under non-saline and saline conditions (50 mM NaCl), whereas Rambler and R-350-N both showed susceptibility under saline or non-saline conditions, as indicated by H and RGR measurements compared to the corresponding controls (Tables 5.5.1 & 5.5.2). This result indicates that a low level of salinity was not enough to alter the resistance of cvs. 13R Supreme, Vertus and Bilensoy-80 to the pathogenicity of *V. albo-atrum*. Because of the limited time available, those cultivars were not tested under high salinity against *V. albo-atrum*. However, Besri (1990) reported that high salt levels in irrigation water in Morocco caused a total breakdown of resistance of tomato cultivars that were normally resistant to race 1 of *V. dahliae*. He also reported that cultivars that are resistant to race 2 became susceptible with increasing soil salinity. The disease-susceptible cultivars, whether they were salt tolerant or not, showed susceptibility to *V. albo-atrum* under low saline conditions. Therefore, higher concentrations of NaCl were not tested on those strains.

Plants that had been generated for tolerance to NaCl, from disease-resistant plants, were also tested for their resistance to *V. albo-atrum*, under saline and non-saline conditions (Table 5.6.1). The plants were regenerated from 50 mM NaCl-adapted cell lines of *M. sativa* of cv. Vertus. At this level of tolerance neither, *V. albo-atrum* or 50 mM NaCl caused significant reductions in H and RGR. In this regard 50 mM NaCl-tolerant cv. Vertus plants differed from the salt tolerant cvs of Rambler strains, which showed more susceptibility when the levels of tolerance to NaCl were increased (Dikilitas, 1997). This result suggests that, either level of tolerance for NaCl was not high enough for cv. Vertus to become susceptible to *V. albo-atrum* or regenerated salt

tolerant (and disease-resistant) plants might still show resistance to *V. albo-atrum* unlike susceptible cv. Rambler.

Reports concerning salt stress effects on plant diseases are still limited and incomplete. For example, Soliman & Kostandi (1998) showed that smut severity, induced by *Ustilago maydis* (DC.) Corda, was markedly reduced under salt stressed conditions on highly susceptible or resistant corn cultivars. Therefore, the results documented on chloride-disease interactions are incomplete and inconsistent, varying from a positive response (Heckman, 1995) to a negative response (Engel & Grey, 1991).

Our suggestion is that, although much work has been done on breeding alfalfa cultivars with tolerance to salinity (Carlson *et al.*, 1983), salt tolerant & disease resistant cultivars should also be included in breeding programs for tolerance to salinity and VW resistance to minimize further yield decline in areas where the two factors occur together.

6.6.3 Effect of the elicitor or NaCl or both on PAL activity of lucerne cells.

Studies with cell cultures have some advantages compared with studies at the whole plant level. Cultured cells can offer uniform conditions, thereby eliminating complications arising from morphological variability and the highly differentiated state of whole plant tissues. Suspension cells are advantageous because the osmotic and nutritional environment can be controlled and cell developmental age is relatively uniform (Binzel *et al.*, 1985). Consequently, cell cultures can provide a very useful tool in attempting to elucidate mechanisms of salt tolerance that operate at the cellular level. In this study, Kabul, a resistant cultivar reported by Flood (1980); Esyanti (1993) and Dikilitas (1997), was selected for cell culture studies.

Compounds that elicit plant defence responses are known as elicitors. Elicitors may be divided elicitors into 2 groups, abiotic and biotic elicitors. Abiotic elicitors are molecules that are not derived from the pathogen or host. Examples are fungicides, heavy metal ions and detergents. Biotic elicitors are molecules that are derived from pathogens and host plants.

L-phenylalanine ammonia-lyase is an enzyme that catalyses the first step in the phenylpropanoid pathway and is a key enzyme in control of the biosynthesis of isoflavonoids and other phenylpropanoids derived from phenylalanine. The enzyme can

be induced by a number of stimuli including, chemical elicitation, microbial, wounding or stress, light, plant growth regulators, heavy metal salts and UV radiation (Hadwiger *et al.*, 1973; Jones, 1984; Dixon *et al.*, 1983).

Frequently, pathogen attacks on plants induce alterations in enzyme synthesis in the plant, which can lead to development of various degrees of resistance around infection sites. An enzyme that generally exhibits increased activity or greater new synthesis in diseased tissues is PAL (Agrios, 1988). PAL is a key enzyme in the production of the basic molecule used for the biosynthesis of most phenolics, including phytoalexins and lignin (Agrios, 1988; Tang, 2001). The resistance of plants to the pathogen may depend on the speed and the extent of synthesis of the enzymes induced in the host by the pathogen.

Accumulation of PAL in response to *V. albo-atrum*, isolate V2 or/and NaCl was studied in cell suspension cultures of lucerne cv. Kabul (Fig. 5.8.1). Isolate V2, or NaCl, or both were effective in inducing PAL accumulation in cell cultures *in vitro* (22 °C). The combined effect of NaCl (50 mM) and the elicitor, derived from the isolate V2 of *V. albo-atrum*, (0.05 or 0.1 mg/ml) resulted in further increase in PAL activity. However, PAL activity was inversely correlated with a further increase in concentration of NaCl (200 mM) and the elicitor (0.1 mg/ml). The results presented here also indicated that the size of increase in PAL activity did not correlate with the amount of elicitor used. Similarly, Jbir *et al.* (2001) reported that salt stress increased PAL activity in wheat plants. On the other hand, Dunn *et al.* (1998) reported that after 30 days of high salinity (0.1 M NaCl), citrus plant grew more slowly and produced lower PAL activity and as a result became more susceptible to nematode attack (*Tylenchulus semipenetrans*).

6.7. Summary.

The increased levels of salinity frequently associated with irrigation practices of agricultural land pose a threat to crop production, especially where water quality is marginal. Under such conditions, plants are not only stressed but may also be more susceptible to various pathogens. One strategy to maintain yields in such situations, or to increase yields in marginal areas, has been to develop salt tolerant strains of crops. Such strains should also be resistant to pathogens in that environment, including halophytic

races of the pathogen that may have adapted to living in higher salt concentrations. An understanding of the interactions between a crop plant and potential pathogens under salinity is, therefore, an important part of any project to develop strategies for disease control for crops grown in saline soils, which may have a minimal impact on an already stressed environment.

In this project, plant-pathogen, plant-salt, pathogen-salt, plant-pathogen-salt interactions were studied. The interactions between tomato (*L. esculentum* Mill.) and lucerne (*M. sativa* L and *M. media*) and isolates of the vascular wilt fungus *V. albo-atrum* (European and USA isolates) were investigated under non-saline and saline conditions.

Pathogenicity trials indicated that isolates of V1 and V2 were pathogenic to tomato, however, the degree of pathogenicity of the isolates were affected by the cultivar inoculated, the age of the plants and the temperature at which the inoculation occurred. Plants inoculated with *V. albo-atrum* showed symptoms of wilt disease such as epinasty, wilting, chlorosis and formation of adventitious roots on the lower part of the stem. Internally, characteristic dark-brown discoloration of the stems was observed. As internal disease responses; tyloses, gum and melanin-accumulated dark xylem walls were evident.

Pathogenicity experiments showed that a root-dipping inoculation method was the most successful method for inoculation of lucerne and tomato plants. Plants inoculated with isolate V2 using the root dip method had the lowest increment in height and reisolation of the fungus was more successful from root-dip inoculated plants than from plants inoculated with one of the other methods used.

Temperature also significantly affected the growth and pathogenicity of the fungus, and altered disease development. At 22 °C, both isolates of *V. albo-atrum* were pathogenic to tomato. However, at 25 °C V1 lost its pathogenicity whilst V2 was still pathogenic to tomato. At 30 °C no disease development was observed on tomato with either isolate. Symptoms of the disease were also reduced with increase in outside temperature. Increased temperature also reduced growth of the fungus; optimum mycelial growth occurred at 22 °C and 25 °C, however, increasing the temperature to 30 °C reduced growth drastically.

No pathogenic differences were determined between isolates of *V. albo-atrum* that had been maintained in culture for many months and fresh isolates of *V. albo-atrum*. The

pathogenic effects of virulence of *Verticillium* on tomato did not increase with increasing spore concentrations or when isolates were co-inoculated. In both cases symptoms of the disease did not show significant differences from each other.

Inoculation of young seedlings with isolates of *V. albo-atrum* derived either from tomato or lucerne (including USA isolates) caused significant reductions in height, and root length and in RGR of tomato plants. All young tomato seedlings were susceptible to *V. albo-atrum.*

Salt tolerant plants of *M. media* cv. Rambler showed the highest degree of susceptibility to *V. albo-atrum* both under normal and saline conditions. The susceptibility of salt tolerant plants increased with increasing level of tolerance to salinity; newly-generated salt tolerant lines of *M. media* in particular showed great susceptibility to the pathogen.

NaCl both delayed and reduced germination in tomato and lucerne at 100 and 250 mM NaCl respectively. Differences were observed between the cultivars in response to NaCl. The cultivars of tomato which were most susceptible to NaCl were Sweet 100 F1, Margarita (Fa-558), Fantastic F1 and Ailsa Craig; the most tolerant ones were *L. lycopersicon* and Edcawy. The seedling stage was susceptible even to lower concentrations of NaCl. All the varieties of tomato except *L. lycopersicon*, showed susceptibility to NaCl at around 50 mM. NaCl both reduced the water potential of the solution resulting in delay in germination and caused toxic effects on germinating seeds. Lucerne seeds, in general, showed more tolerance to NaCl than tomato. Cultivars Bitlis, Vela, Rambler and AC Blue J showed susceptibility to NaCl while other cultivars of lucerne were tolerant to NaCl up to 250 mM. Lucerne seedlings, as was the case with tomato seedlings, showed more susceptibility to NaCl in the seedling stage. At this stage, cultivars from USA and Canada showed greater salt tolerance than those cultivars from Turkey or Europe.

Cultivars which produced radicle and hypocotyl under 100 mM NaCl were also capable of producing roots from the cuttings under the same NaCl concentration.

In this study, proline, an amino acid that generally increases in concentration in plant cells in response to water- and salt stress, did not increase under low levels of salinity (50 mM NaCl).

In vitro experiments with *V. albo-atrum* showed that isolate V1 had more resistance to antifungal compounds than that of V2. However, in radial growth experiment, isolate V2 grew better than other isolates of *V. albo-atrum* obtained from lucerne.

In terms of radial growth under saline conditions both V1 and V2 showed tolerance to NaCl up to 150 mM while production of spores was not affected up to 100 mM NaCl. Their salt-adapted strains, V1-, V2-150 did not show significant differences from the control group in terms of radial growth. However, they produced fewer spores than the control group. In contrast, V1-, V2-200 strains showed severe reduction in radial growth, in their respective medium, and produced fewer spores than the control group.

Long-term effect of NaCl on the growth of isolates of V1 and V2 in liquid culture reduced the mycelial growth (dry weight) significantly after 50 mM NaCl.

By itself, NaCl caused symptoms and reduced height, and RGR and many other growth parameters. The effect of NaCl was especially marked on young plants.

Progress of the disease caused by *V. albo-atrum* was markedly accelerated by salt stress and there was a significant differences in the degree of enhancement of the progress of the disease by salinity, were between young and mature tomato plants. Overall various growth parameters were lower in the group of plants that were inoculated with *V. albo-atrum* & treated with NaCl than in the groups that received one or other treatment. However, some differences were observed between the various cultivars in their response to the diseases in the presence of salt. For example, if a cultivar (Ailsa Craig or Simge F1) showed susceptibility separately to *V. albo-atrum* and to NaCl, the combined effect of both *V. albo-atrum* & NaCl on those cultivars was more detrimental than the either of the treatments alone. However, if a cultivar (Margarita Fa-558 or Fantastic F1) showed resistance to *V. albo-atrum* but susceptibility to NaCl, the combined effect of both *V. albo-atrum* & NaCl was not significantly different from the effect of NaCl.

The salt-adapted strains of isolate V1 did not cause significant reductions, under normal conditions when compared to control group, in height, root length, RGR or chlorophyll content of leaves of tomato cv. Ailsa Craig with the exception that strain V1-150, caused significant reduction in chlorophyll content. In contrast, the salt-adapted

strains of isolate V2 caused significant reductions in height, RGR or chlorophyll content of leaves. However, all salt-adapted strains of the fungus caused significant reductions in growth parameters under salinity.

Disease-resistant & salt-tolerant lucerne plants (Vertus, Bilensoy-80, 13R Supreme) showed resistance to V1 under non-saline and saline conditions (50 mM NaCl); however, disease-susceptible & salt-tolerant lucerne plants (R-350-N) or disease- and salt-susceptible cultivar (Rambler) did not show resistance to V1 under those conditions. Disease resistant and *in vitro* regenerated salt tolerant plants (regenerated salt tolerant cv. Vertus) did not loose its resistance to *V. albo-atrum* under non-saline and saline conditions. Development of salt tolerant crops should therefore, involve selection of strains that maintain or improve its resistance to pathogens.

PAL activity was observed to increase when lucerne cells were treated with an elicitor from *V. albo-atrum*. The increase was lower when the cells were treated with both elicitor and 50 mM NaCl. However, the increase in PAL activity was minimized both at high concentration of elicitor and NaCl (200 mM).

6.8. Limitations.

It was quite difficult to achieve everything intended. The limited time available, the nature of the experiments and problems with the seed stock prevented many questions from being answered fully. Therefore, there is a great deal of possible future work arising from work performed and data generated by this study.

Since the salt tolerant plants have already showed susceptibility to *V. albo-atrum*, under non-saline or saline (50 mM NaCl) conditions, salt tolerant *M. media* cv. Rambler (0-, 200-, 300- and 350- mM salt tolerant plants) was not tested under increasing NaCl concentrations with V1 isolate.

Salinity experiments with the plants regenerated from NaCl-selected cell lines and unselected original plants were performed under controlled conditions. It is very important to know whether this salt tolerant trait in regenerated plants can be passed through the seeds. Such information will be important for a more complete understanding of the mechanism of salt tolerance in regenerated plants.

6.9. Suggestions for future work.

Salinity is one of the most devastating problems in the irrigated regions of the world. The usual strategy for land reclamation has been either to install drainage systems to remove excess salts or to leach the salt from the soil with high quality water. Alternatively, the land could be made productive by using salt tolerant plants. However, as shown in this work, salt tolerant plants are not always a solution when they face a pathogen under saline conditions. Development of crop varieties with increased salt tolerance is crucial to provide a long-term solution to the problem of salinization, however, the problem of improving salt tolerance in plants becomes more complex when plants are also exposed to other environmental and biotic factors. Although interactions between heat and drought, and heavy metal toxicity and biotic stresses have been studied in certain cases, the interactions between salinity and fungus should also be considered carefully. For example, disease-resistant plants should be generated from suspension cells that have been adapted to higher salt concentrations. In the short term, it has been possible to regenerate 50 mM NaCl-tolerant plants from a disease resistant cultivar of lucerne. However, it is not known if further increase in the level of salt tolerance of the resistant cultivar will decrease the resistance of the plants towards the fungus under non-saline and saline conditions. Meanwhile, commercially available drought or salt tolerant plants should be considered to maximize the crop production and marketable fruit yield.

The research described here focused on the study of the effect of salinity on tomato and lucerne. The results have led to the recommendation of cultivars from USA, which are salt tolerant and disease resistant. Those cultivars should be selected for salt tolerance and disease resistance work under salinity. Fantastic F1, a tomato variety, looked promising for disease resistance work. This cultivar should be considered for the selection of salt tolerance work. However, further screening of this variety is required to assess the effect of salinity on growth, development and most importantly fruit yield and quality in a semi-arid environment.

Changes in gene expression have been reported in various crops and microorganisms when they were subjected to the various environmental stresses such as salt, drought and heat stress (Katsuhara & Kawasaki, 1996; personal communication with Dr. Tinley Basset). Therefore, salt-adapted strains of the fungus should be examined at a

molecular level to see if any changes have been made in gene-sequence by the accumulated of NaCl. If they have been made, these genes should be characterized.

The potential of the V2 strain of *V. albo-atrum* and other species of *Verticillium* that are 'weak' pathogens, should be assessed as biocontrol agents to protect the salt tolerant lucerne strains against *V. albo-atrum.*

A further objective for future research would be to assess the long-term effects of accumulation of salts within the soil profile and ground water. In order to achieve the proposed future objectives for the long-term improvement crop production in saline environments the research must be carried out with close association between scientists, commercial growers, retailers and consumers. Plant scientists including plant physiologists, molecular biologists, geneticists and plant pathologists should work together to improve agricultural productivity in many areas of the world.

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