

EFFECT OF SOIL WATER SATURATION ON THE INCIDENCE OF INFECTION BY SYSTEMIC *Botrytis cinerea* IN LETTUCE PLANT

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ABSTRACT

*Stress has enormous implications on plant survival by lowering defensive mechanisms which predisposed the plant to attack by pathogenic micro-organisms. The effects of stress caused by soil water saturation on the spread of systemic Botrytis cinerea Pers. (Teleomorph: Botryotinia fuckeliana) was investigated at Reading, UK, using model plant lettuce (Lactuca sativa L.) Tom Thumb variety. Soil water saturation at 25%, 50%, 75% and control 100% was imposed for seven weeks starting from three weeks after germination. Chlorophyll fluorescence (Fv /Fm) which is the measure of plant stress decreases significantly with increased water stress ($F_{3,79} = 55.93, P < 0.001$). There was also a significant decrease in the rate of photosynthesis between stressed and control plants ($F_{1,39} = 797.52, P < 0.001$). The decrease in the rate of photosynthesis with raised water stress was significant ($F_{3,79} = 204.01, P < 0.001$) and between stressed plants and control ($F_{1,39} = 10.79, P < 0.001$). Root weight determined after harvest showed a significant decrease in root weight with raised water stress ($F_{3,79} = 204.01, P < 0.001$), also a significant decrease in the root weight was recorded between stressed and control plants ($F_{1,39} = 10.79, P < 0.001$). High amount of systemic *B. cinerea* was detected in stressed plants which increase with raised water stress however, the difference was not significant between the treatments ($F_{1,59} = 1.23, P = 0.676$). However, a significant difference ($F_{1,39} = 225.27, P < 0.001$) was found between stressed plants and the control plants. Highest proportion of *B. cinerea* was recorded in roots, followed by leaves and stems. Statistically, the differences between the plant parts on the expression of systemic *B. cinerea* was not significant ($F_{2,59} = 0.77, P = 0.470$). The results indicate that water is not an important factor in the spread of systemic infection caused by *B. cinerea*.*

KEYWORDS: *Botrytis cinerea*; chlorophyll fluorescence; photosynthesis; stress; systemic infection.

INTRODUCTION

Lettuce (*Lactuca sativa*, (Asteraceae (Compositae)) is one of the most important salad crops and horticulturists regard it as one of the most profitable to grow (O'Neil *et al.* 1997). The four major types of lettuce are crisphead, butter head, cos or romaine, and leaf or bunching (Zepp *et al.* 1994). There is also limited production of a fifth type called stem lettuce (Zepp *et al.*, 1994). However, the most frequently produced lettuce is crisphead, which is characterized by a firm head and a brittle leaf texture (Zepp *et al.*, 1994). The crisphead is referred to commercially as "iceberg" or "head lettuce" (Zepp *et al.*, 1994). Lettuce diseases are a serious problem to growers; for many diseases there are no effective methods for their control once the crop has been infected (Zepp *et al.* 1994). Lettuce diseases may result from attack by viruses, fungi, bacteria, nematodes or a non-pathogenic source. Some disease are seed-borne (Sowley *et al.* 2010), others are air-borne (Cho, 2008), while others are transmitted by insects or microorganisms (Cho, 2008). Soft rot, caused by fungal *Botrytis cinerea*, is one of the most serious diseases of lettuce and often starts on bruised leaves (Cho, 2008). O'Neil *et al.* (1997) reported that *B. cinerea* is the most common disease affecting lettuce and other protected crops, Elias *et al.* (2010) isolated systemic *B. cinerea* from different sections of healthy lettuce plants and as the plants grew infections spread to newly produced

tissues from previously infected areas, concluding that *B. cinerea* exhibits an asymptomatic type of growth in lettuce.

Infection of lettuce plants by *B. cinerea* is favoured by cool moist weather conditions (Kern *et al.* 2006; Halasi *et al.* 2008). Infected lettuce plants show abundant white, cottony mycelial growth and hard, black sclerotic bodies on the underside of the lower leaves and the basal part of the stem (Halasi *et al.* 2008). The head and stem of infected plants often disintegrates into a soft watery mass covered with sclerotia (Cho, 2008). Grey mould produces a firm, watery, brown decay on the underside of the head. It can be identified by the presence of dense grey or light brown airborne spores of (ascospores or conidia), which require high humidity and free water for infection to occur (Cho, 2008).

Stress resulting from over/under watering (Sinclair, 1991; Zhang *et al.* 2004) can damage tissues, especially their membranestructures, leading to an increased leakage of solutes and increased availability of nutrients which favours infection by necrotrophic pathogens such as *Botrytis cinerea* (Shafia, 2009). Water is essential for lettuce and is therefore likely to affect plant defence allocation; water stress is one of the major causes of crop losses worldwide (Wang *et al.* 2003; Nazarli *et al.* 2010). Under water stress (too little water) a water deficit develops in plant tissues leading to a significant inhibition of photosynthesis (Zhang *et al.* 2004).

To avoid further water loss via transpiration plants react with rapid closure of stomata (Wang *et al.*, 2003). As consequences, diffusion of CO₂ into the leaf is restricted resulting in a lower internal CO₂ concentration, which causes a decrease in net photosynthesis (Chaves, 1991; Cornic *et al.*, 1992; Nogues and Baker, 2000; Shafia, 2009).

Measures of chlorophyll fluorescence are used to study the rate of plant photosynthesis and identify injury to the leaves where there are visible symptoms (Santos *et al.* 2000; Sayed, 2003; Wagner *et al.*, 2006). Therefore the measurement of chlorophyll fluorescence serves as a good indicator of environmental stress and also serves as a screening method for resistant plants (Sayed, 2003; De-ping *et al.* 2004). Root weight can also be adversely affected by water stress (Tayeb, 2011). Root length increases under mild water stress, but then decreases with increasing intensity of water stress (Tayeb, 2011). There are several studies of the effects of water stress on plant physiological traits (e.g. Sayed 2003; Zlatev and Yordanov 2004; Tayeb 2011) however, it is not clear how this can influence the systemic *B. cinerea* infected plant. Therefore we tested three hypotheses. First soil water saturation stresses the plants decreasing plant defensive mechanisms which encourage pathogenic infection of the plants. Second spread of systemic *B. cinerea* increases with increased water stress. Third expression of *B. cinerea* in plant increases with raised water stressed.

MATERIALS AND METHODS

Plant Material

The study was conducted at Reading United Kingdom, using the lettuce plant (*Lactuca sativa*, Tom Thumb variety) grown in a controlled environmental chamber from seed infected with *Botrytis cinerea* at 18-20°C and 12-14h respectively. A completely randomized factorial design was used with 4 levels of soil water saturation, (100%, 75%, 50% and 25%). The plants (80) were individually grown for ten weeks in 20cm diameter pots containing 300g of vermiculite soil (Tayeb, 2011). At the end of the experiment, the effect of stress on the incidence of systemic infection of *B. cinerea* was determined by measurement of the rate of photosynthesis and chlorophyll fluorescence before harvest, while root weight and the biomass of *Botrytis cinerea* were determined after harvest.

Measurement of rate of Photosynthesis

To obtain a measure of the degree of stress induced by the watering regime photosynthesis rates were measured for each plant before harvest. The photosynthesis rate was determined as the amount of CO₂ assimilated per m² leaf surface area by intact leaves, using a Red Gas Analyser (ADC Bioscientific LCI Analyser No. 31109) equipped with a standard broadleaf

chamber measuring an area of 6.5cm². The level of CO₂ in the analyser varied between 400-655 µl CO₂/mol air, determined by position of the inlet of the analyser outside the controlled environment. Leaves were given 2-4 min in the chamber to reach equilibrium before the readings were taken.

Measurement of Chlorophyll Fluorescence

To investigate if the rate of chlorophyll fluorescence was affected by the water stress treatment, chlorophyll fluorescence was determined at the end of the experiment before harvest. The rate of chlorophyll fluorescence was determined as the amount of re-emitted light from the leaf, measured using a Handy Pea Data Chlorophyll Fluorometer (Hansatech Instrument Ltd. Pea plus version: 1.02). Leaves were placed in the chamber for 20 min before taking the measurement. The rate of chlorophyll fluorescence was determined as S11 photochemical efficiency Fv/Fm where Fv the maximal variable chlorophyll fluorescence, Fm is the maximal chlorophyll fluorescence).

Measurement of Root weight

A measurement of root weight was taken from all the plants after harvest. Roots were cut and washed under running tap water and dried for 1h in an oven before taking the measurement using a balance (Kern scale Technic, 440-21N).

Determination of biomass of systemic *B. cinerea*

A total of twenty plants, four from each block were randomly selected and roots, stems and leaves were separately disrupted in liquid nitrogen, 100mg of the resulting fine powder was then transferred into 200ml eppendorf tubes. The DNA was extracted by using Qiagen DNeasy plant mini kit following the manufacturer's instructions. The isolate was quantified using a Nanodrop ND 100 spectrophotometer (Applied Biosystems). A quantitative polymerase chain reaction (qPCR) was performed on genomic DNA with scar primers of *Botrytis cinerea* as designed by Suarez *et al.* (2005) following established protocol (eg Sowley, 2006; Rajaguru 2008; Shafia, 2009) for *Botrytis cinerea*. A master mix containing 12.5 µl qPCR master mix (Qiagen UK), 1µl of each forward and reverse scar primers (Invitrogen), and 5µl of water was prepared and 20 µl was aliquoted into each well. To each well 5µl of DNA from individual sections of the lettuce plant were added. A standard curve (10ng/µl, 1ng/µl, 0.1ng/µl, 0.01ng/µl, 0.001ng/µl, and two water controls) was prepared with the previously quantified DNA extracted from a clean lettuce plant grown from tissue culture propagation. The plates were covered and pulsed in a centrifuge before carrying out the run at 95°C for 10 minutes and 40 cycles of 60°C for 1 minute, 95°C for 15 seconds Suarez *et al.* (2005) using qPCR rotor gene machine (Applied Biosystems).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and tested for normality using Genstat version 13(Tayeb, 2011).

RESULTS

Effect of soil water saturation on the rate of photosynthesis

DCO₂ was used as a measure of net photosynthesis and there was a significant difference in the rate of photosynthesis between water stressed plants ($F_{3,79} = 204.01, P < 0.001$). Stressed plants showed a reduction in photosynthesis rate (Figure 1). There was also a significant difference between infected and control plants ($F_{1,39} = 10.79 P < 0.001$).

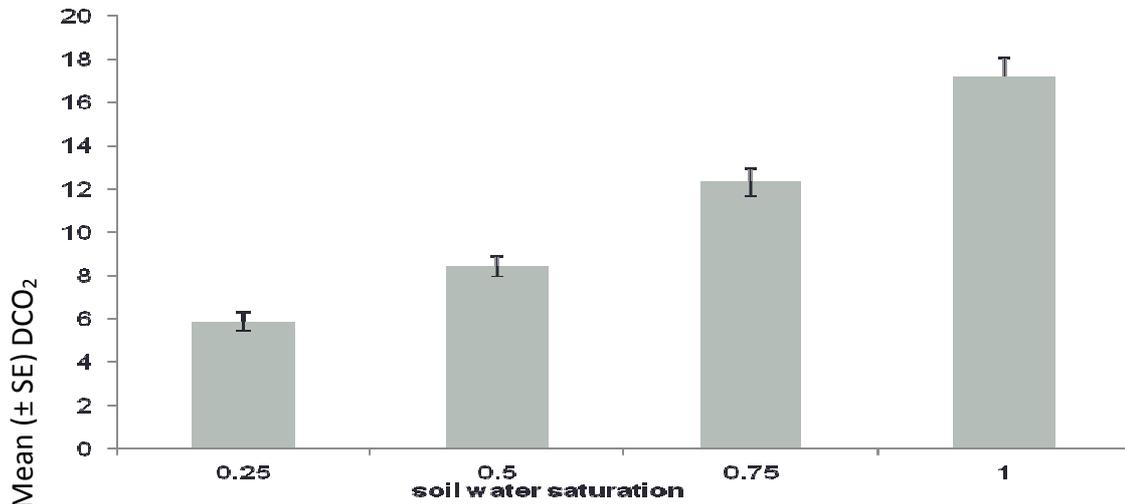


Figure 1: Effect of soil water saturation on the rate of photosynthesis. 0.25-stand

For 25% water saturation, 0.5-stand for 50% soil water saturation. 0.75 stand for 75% soil water saturation and 1-stand for 100% soil water saturation. There was no significant difference in sub-stomatal CO₂ and transpiration rates between plants reared under the differing watering regimes.

Effects of soil water saturation on the rate of chlorophyll fluorescence

There was a significant difference in the rate of chlorophyll fluorescence (Fv /Fm) between plants reared under differing watering regimes ($F_{3,79} = 55.93, P < 0.001$). In plants stressed by a reduction in soil water saturation, an increase in Fo (minimal chlorophyll fluorescence) was accompanied by a decrease in Fm (maximal chlorophyll fluorescence).

An increase in Fo is one of the characteristics indicating inactivation of PSII the system (Figure 2), while a decline in Fv indicates a non-photochemical quenching process at or close to the reaction center (Zlatko and Yordanov, 2004). Also a significant difference was observed in the rate of chlorophyll fluorescence between infected plants and control plants ($F_{1,39} = 797.52 P < 0.001$).

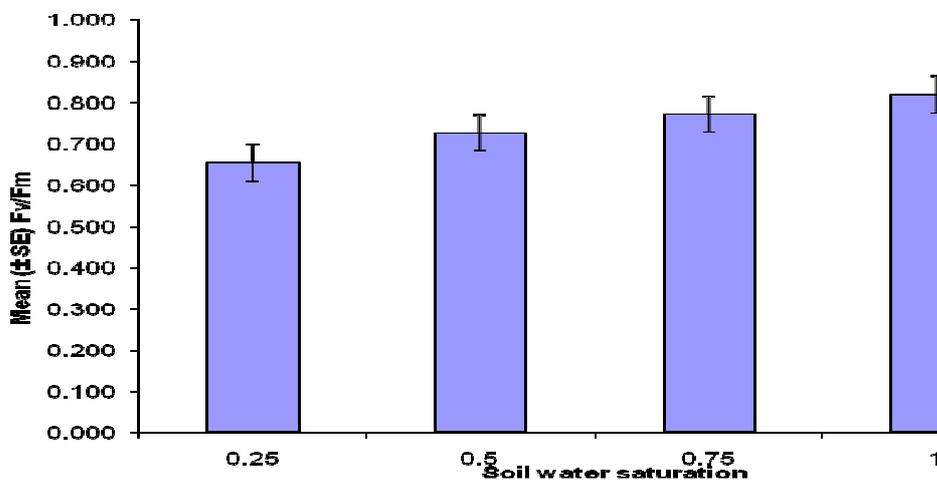


Figure 2: Effect of soil water saturation on the rate of chlorophyll fluorescence

Effect of soil water saturation on the root weight

There was a significant difference in root weight between the water stressed plants ($F_{3,79} = 110.60$, $P < 0.001$), indicating that water stress can halve

the root weight of stressed plants. There was also a significant difference between weight root of infected and control plants (Fig 3, $F_{1,39} = 79.78$, $P < 0.001$).

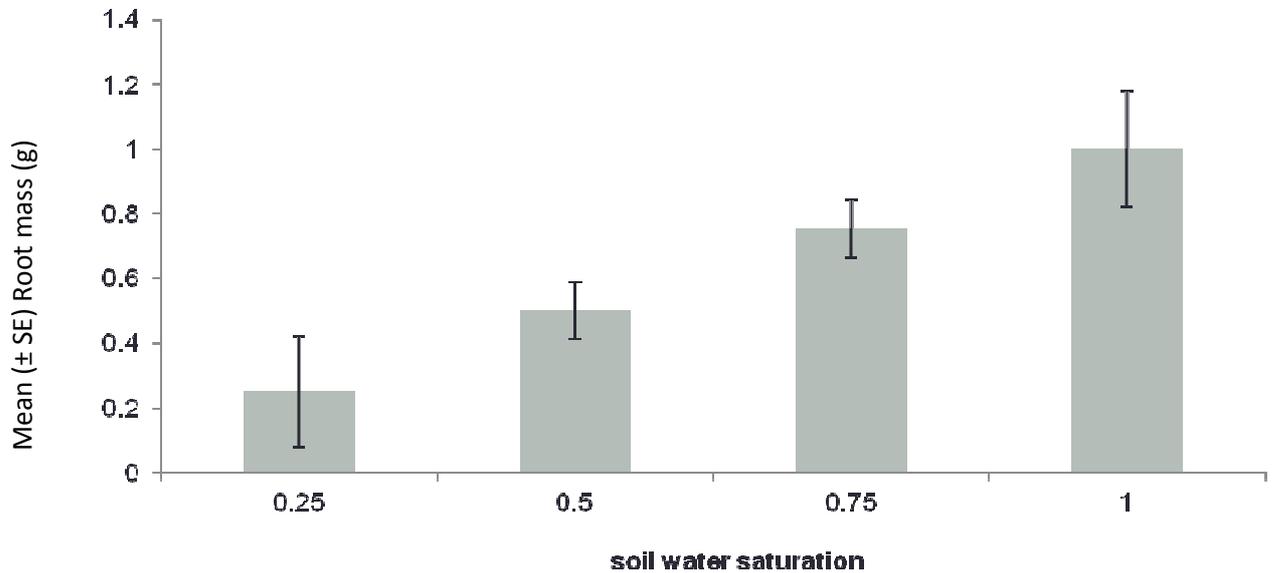


Figure 3: Influence of soil water saturation on root mass

Effects of water stress on biomass of systemic *Botrytis cinerea*

Systemic *Botrytis cinerea* was detected in all plant sections examined. The proportion of *B. cinerea* was found to increase with a reduction of water availability, with the 25% water treatment recording the highest proportion of *B. cinerea* (Figure 4.4). However, the effects of soil water saturation was not statistically significant, ($F_{1,59} = 1.23$, $P = 0.676$) on the spread of systemic *B.*

cinerea. However, a significant difference ($F_{1,39} = 225.27$, $P < 0.001$) was found between stressed plants and the control unstressed plants. The highest proportion of *B. cinerea* was recorded in roots, followed by leaves and stems. Statistically, the differences between the plant parts on the expression of systemic *B. cinerea* is not significant ($F_{2,59} = 0.77$, $P = 0.470$). The interaction term was also not significant ($F_{6,59} = 0.41$, $P = 0.865$).

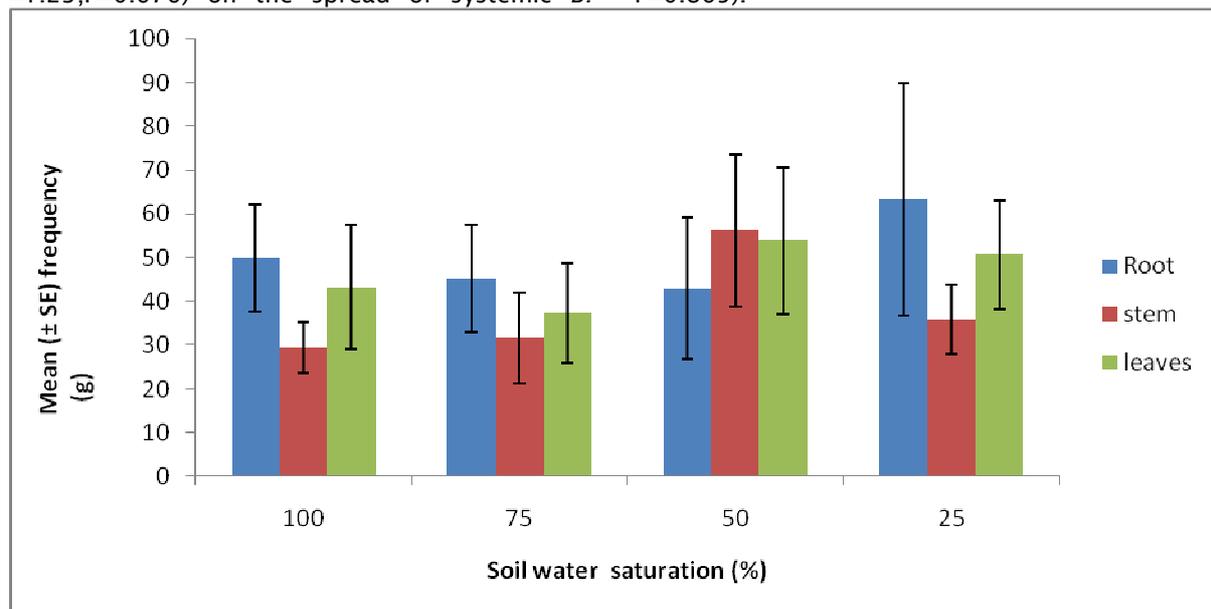


Figure 4.: Detection of systemic *B. cinerea* from different plant parts under different soil water saturation regimes.

Detection of *B. cinerea* from different parts of stressed and unstressed Lettuce plants by qPCR in (ng/μl)

Soil water	Block 1	Block 2	Block 3	Block 4	Block 5	Mean
100% R	43	10.5	75	76.5	44.5	
100% S	16.1	21.10	26.3	49.6	34	29.4
100% L	15.32	91.72	51	14.30	44.0	44.2
75% R	54	89.17	29.7	23.10	30.0	45.1
75% S	30.6	65	10.1	10.24	42.5	31.6
75% L	75.5	14.1	13.65	43	37.2	
50% R	37.5	105.95	15.1	22.95	332.2	42.9
50% S	43.72	98.1	96.62	22.13	20.05	56.1
50% L	40.11	23.84	119	43.25	43.0	53.8
25% R	46.30	24,300	167	26.0	52.3	109
25% S	42.27	405.52	13.03	24.50	58.	35.8
25% L	44	46.5	28.1	36.13	98.5	50.6

R=root, S= stem, L= leaf,

DISCUSSION

Plant stress resulting from variation in soil water saturation down regulates plant defence allocation resulting in the suppression of the rate of photosynthesis, chlorophyll fluorescence and root weight. Similar results have been found in beans *Phaseolus vulgaris* (Zlatko and Yordanov, 2004) and in Corn *Zea maize*(Tayeb 2011). The results of this study showed that the biomass of endophytic *B. cinerea* increases as the plant stress caused by change in soil water saturation increases. A significant difference in the biomass of systemic *B. cinerea* was found between infected and control plants ($F_{1,39}=225.27, P < 0.001$). Continued water stress results in the yellowing of leaves and the visible appearance of mycelium, especially on the bottom, senescing leaves. Water stress also resulted in rapid leaf death and symptomatic grey mould disease on dead leaves.

Water stress can occur in a crop when soil moisture levels are excessive and when oxygen concentration levels in the soil are lowered due to displacement by water for an extended period of time, resulting in severe damage of the root system (Marie-Laure *et al.* 2006; Sutton *et al.* 2006; Shafia, 2009; Kransensky and Jonak 2012). Lettuce plants are susceptible to water stress due to their shallow root systems, resulting in low yields and poor quality plants (Shafia, 2009). This also down regulates the defence allocation of the plant, making it more susceptible to infection by micro-organisms (Shafia, 2009).

Although the effect of soil water saturation it is not statistically significant on the spread of systemic *B. cinerea* in the present research ($F_{3,59}=0.52, P = 0.676$), the pathogen may have been aggravated by water stress through one or more mechanisms. Evidence from the physical appearance of plants shows that the fitness of plants grown from *B. cinerea* infected seed under water stress is lower than control plants and a decrease of photosynthetic rate of the stressed plants is often associated with the spread of infection from the lower to the upper part of the plants (Rodriguez *et al.* 2008).

Water stress may have resulted in changes of the physiology of the plant making it more susceptible, or reduced the ability of the plant to

synthesise defensive compounds to control the disease. Nutrient leakage due to senescence, the weakening of plant tissues, as well as decreased capacity to produced defensive chemicals may have resulted in increases susceptibility and the spread of systemic endophytic infection within the plant tissues (Luo and Zhang, 1999). Literature providing experimental evidence confirming that changes in plant physiology and other contributing factors in changes from quiescence to aggressive symptoms of diseases are scanty. Santoz *et al.* (2000) discovered that *S. sapinea* can act as a latent pathogen and physiological alteration through water stress can release the fungus from the quiescent condition resulting in rapid disease progression.

In the present study, at the end of stress treatment plants which were grown from infected seed showed a decrease in the rate of photosynthesis and chlorophyll fluorescence. However, the decrease in these traits was highest in the stressed plants. The first response of lettuce plants to water stress is the closure of stomata; this is followed by the slowing down of photosynthesis (Dodd, 2001). With continued stress more physical damage occurs such as interveinal or whole leaf yellowing.

Root weight decreased with increasing water stress. Tayeb's (2011) work on corn found a decrease in root weight due to water stress which decreases further with an increasing intensity of stress. However, it was found that root length increases under mild water stress. Mahsa *et al.* (2011) found a decrease in root dry weight and shoot growth with increasing water stress, but enhanced growth of the lateral roots in *Lolium perenne* and *Lolium barrage* grown from seed treated with paclobutrazol. Drought stress was found to have significant effect on root length, length of the root was found to be highest in well-watered plants but lower in stressed plants (Mahsa *et al.* 2011).

In this study the effect of water stress on the recovery of endophytic *B. cinerea* was slightly higher in the roots than in leaves and stems. Sowley (2006) found that plants grown from infected seed have high levels of root infection which later spreads to newly produced plant tissues.

Cosgrove *et al.* (2000) and Achua *et al.* (2006) reported that water stress inhibits the root development in woody plants and subsequently predisposed roots to infection by common pathogens; this effect also seems to occur in controlled environment-grown lettuce plants. Pszczojowska *et al.* (2003) measured fungal infection of pea and yellow lupin (*Lupinus luteus*) seed from plants grown under water and mineral stress, showing that water stress increased the rate of fungal infection. Fungal species infecting the plants included *B. cinerea*, *Alternaria alternata* and yeast. Under stress conditions a water deficit develops in plant tissues resulting in the inhibition of photosynthesis (Pszczojowska *et al.* 2003).

In the present study systemic endophytic *B. cinerea* was recovered from both stressed and unstressed symptomless plant tissues from plants reared in a controlled environment room. The detection of systemic *B. cinerea* biomass by qPCR was found to be very effective as more infection was detected from water stressed plants more than from non-stressed control plants. Elias *et al.* (2010) reported that qPCR can detect minute quantities of DNA in plant material, but

the level of detection may be limited if there is a sparse distribution of the mycelia. However, such sparsely distributed mycelia can be detected using a plating test because the pathogen is induced to grow by the medium (Sowley, 2006).

This study confirms that susceptibility to systemic *Botrytis cinerea* infection increases with raised water stress as more infection was detected in infected plants than in control plants. In general the incidence of infection was higher in stressed plants than in unstressed control plants. Also the results of the present study confirmed that with raised water stress more systemic *B. cinerea* build up in different plant part as against the control. Although the differences in the infection of plants part was not statistically significant ($F_{2, 59} = 0.77$ $P = 0.473$), roots carried higher levels of infection, followed by leaves, then stems. In their experiments Sowley (2006) and Shafia (2009) showed that commercial lettuce seeds carry high levels of systemic *Botrytis cinerea* infection; aseptic propagation can sterilize the seed stock and raise seedlings free of *B. cinerea* inoculum for experimental purposes.

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