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Research Article

DOES INFECTED SEED SERVE AS INOCULUM SOURCE FOR *BOTRYTIS CINEREA* INFECTION?

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ABSTRACT
Isolations were made from lettuce seed and seedlings to determine effect of seed in the transmission of *Botrytis cinerea* infection in lettuce plant. Commercially purchased lettuce seed Tom Thumb variety was used. Isolation of the pathogen after surface-sterilisation of the seed indicated that the *Botrytis cinerea* was present within the seed rather than on the surface. It was found that 87% of the seed pathogen detected was *B. cinerea* and it was able to pass from seed to the resultant seedling, initially appearing in the cotyledon than roots and subsequently in the stems, true leaves or leaf bases and in to the seed. A relationship between level of seed infection by *B. cinerea* and seedling infection was established ($F_{1,38}$ = 51.22, $P < 0.001$), with higher seed infections resulting in greater seedling infection and leaf rot. This shows that seed is an important source of inoculum for *B. cinerea* infection.

Keywords: *Botrytis Cinerea, Dry Spores, Lettuce Seed, Seedling*

INTRODUCTION
Many fungal diseases are seed transmitted where the seed borne infection can spread upward in to the seedling and infect the whole plant body (Stewart and Franicevic, 1994; Barnes and Shaw, 2003; Elias *et al.,* 2010). Although, seed infection can be transmitted in to the seedlings however, it appears seed infection does not always result in seedling infection. This is supported by the work of Elad *et al.,* (2004) who isolated *Botrytis fabae*, the cause of chocolate spot of *Vicia faba* from bean seeds. Elad *et al.,* (2004) found that the levels of *B. fabae* conidia on most infected seeds, which were tested, were too low to cause an aggressive lesion at 15^0C and the fungus dies without seriously damaging the plant. He concluded that for an aggressive infection to occur seeds would have to carry a high amount of inoculum. Burgess *et al.,* (1997) reported that not all seed infections by endophytic *B. cinerea* results in seedling infection. The evidence was based on their inability to isolate *B. cinerea* from sections of surface sterilized asymptomatic epicotyl from seedlings with visible root lesions and healthy seedlings were grown from infected seeds. Stewart and Franicevic (1994) reported that *Aspergillus flavus* causes seedling infection in young maize plant grown from contaminated seeds. However, it was found that the rate of germination was considerably lower when *Aspergillus flavus* entered and contaminated the seed. The distribution of the fungus suggests that initially the organism may have followed the meristem of the plant. Ochoa and Ellis (2002) found that *Fusarium oxysporum* was seed-borne in common naranjilla (*Solanum quitoense*) and can be transmitted from seed to seedling. *Fusarium oxysporum* was isolated from seeds and plant parts, which were surface sterilised before plating. In another study Koycu and Ozer (1997) isolated several fungi from onion seeds and seedlings, which were seed-borne, however, only *Aspergillus niger* and *Fusarium oxysporum* were transmitted from seed to onion sets.
In 1992 Singh *et al.,* found that *Botryodiplodia theobromae* can be transmitted from seed to seedling in maize. However, they found that heavily infected seeds failed to germinate and were covered with profused growth of pycnidia on their surfaces. While seed with weak to moderate inoculum of *Botryodplodia theobromae* germinated and their histopathology showed that the fungus readily invades growing seedling from adjoining pericarp, scutellum or closing tissue. They concluded that the fungus grows internally in the seedlings.
The fungus was shown to infect the cotyledon immediately after germination and remain as a symptomless infection in the tissue. There have been unpublished reports of high levels of infection of

lettuce seed by *Botrytis cinerea*. However, the relative importance of the seed borne infection in causing the lettuce seedling rot disease has not been clearly established. In addition, the ability of *Botrytis* to move from infected seed into the seedling tissue was assessed and an attempt was made to correlate levels of seed infection with extent of rot disease at pre and post harvest. Barnes and Shaw (2003) similarly, reported .seed-borne infection of *B. cinerea* in Primula plant. According to them seed infection lead to seedling infection even when the external inoculum was excluded. Since *B. cinerea* was isolated from the surface sterilised seeds of lettuce that means the infection was internal.

In agreement with the present study the work Sowley (2006) shows that *B. cinerea* can be transmitted from seed to seedling in lettuce plant. His experiments showed that *B. cinerea* could be recovered from plants grown from infected seeds under sterile air flow. His outdoor experiments in 2005 showed that *B. cinerea* could be transmitted from infected plants to seeds, and the same was true for uninoculated plants. This was confirmed by the recovery of *B. cinerea* from seeds of plants, which were initially grown from apparently uninoculated seeds and later inoculated with the dry *B. cinerea* spores. However, uncertainties remain with Sowley (2006) experiments due to lack of continuity between his 2005 and 2006 experiments. In 2005 experiments, he used lettuce Little Gem variety and harvested the plants at 1.5 month, but in 2006; he changed the lettuce variety to Tom Thumb and harvested the plants at two months. Furthermore, the environmental condition under which the 2005 experiment was conducted differs from that of 2006 experiments. These differences have put doubt on the results and necessitate the need for the repeat of the experiment using similar lettuce variety under the same environmental condition. The present study hopes to achieve that. Therefore, the study tested two hypotheses. First Infected seed is inoculum source for *Botrytis cinerea* infection in lettuce. Second that seed infection which grows into the seedling can be transmitted in to the seed.

MATERIALS AND METHODS

Experimental Plant

Seed from a commercially developed lettuce (Tom Thumb variety, Fothergills Seed, Newmarket, UK) was purchased and used for the experiment using the following protocols. Half of the seed was treated with fungicide by soaking the seed in 100ml of the systemic fungicide Shirlan (active ingredient 500g/l Fluazinam, Sygenta Crop Protection UK limited; 0.1g dissolved per litre of water) for 2h, followed by overnight drying (following Shafia, 2009).

Prior to sowing in 200 15cm pots, seed sterilisation was carried out to determine if the *Botrytis cinerea* detected were present on the surface or within the seed. Seeds were blotted dry on sterile filter paper and placed onto 50 plates of Botrytis selective media (25 seeds per plate). Plates were incubated in the dark at 18^{O}C on *Botrytis* selective media in a 9cm Petri plate and examined daily for 7 days. Seeds from both fungicide and non-fungicide treated plants were tested. The Petri plates were observed micro- and macroscopically for the presence of *B. cinerea*.

Plant Growth and Seedling Inoculation

Seed was sown in 16 trays filled with autoclaved, sterile compost potting mix in the glasshouse facilities of the University of Reading. At the two leaf stage, four trays of seedlings grown from both fungicide and non-fungicide treated seeds were selected and covered with black polyethylene bags. Dried *Botrytis cinerea* spores collected from a two–week-old sporulating culture of *B. cinerea* in 9cm Petri plates were used for the inoculation. Dry spores were harvested from the Petri plates by tapping gently on an autoclave-sterile piece of aluminium foil. The spores were transferred into 10ml autoclave-sterile syringes (BD Plastipak, UK). The syringes were fitted with 25mm 63/100 23GX1 needles. The needles were inserted into the bag before forcibly delivering the spores into the enclosed area and then left for 24h to facilitate the germination of spores through the build-up of high humidity. An acetate paper on which a graph sheet was photocopied and attached to a glass slides was placed in each tray and examined under a microscope to estimate the number of spores deposited per unit area (an average of 12 spores/mm^2 was found). A week after inoculation, 200 seedlings were transplanted into 15cm pots and maintained under controlled conditions.

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Isolation of Botrytis Cinerea from Lettuce Seedling

To confirm the spread of *B. cinerea* within the plant body, at intervals of 2, 4, 8 and 12 weeks after sowing 10 seedlings were removed. The roots stems and leaves were surface sterilised for 1 min with 1% NaOCl, followed by three rinses in distilled water.

One cm-long sections of secondary root, 1cm diameter leaf discs and hand-cut 1 mm sections of stems were blotted dry on sterile filter paper and segments plated onto Botrytis selective media plates. Plates were incubated in the light at 18°C and examined over a 7-day period. Fungal colonies growing from the section of seedlings were subcultured onto plates of malt extract agar and *Botrytis cinerea* recorded and identified.

Isolation of Botrytis Cinerea from Seed

To confirm that *B. cinerea* had been successfully transferred into the seeds of lettuce plants, ten seeds from the 50 infected and uninfected plants were collected and sterilized by soaking in 20ml of 70% ethanol in a Duran bottle for 30 minutes, and allowed to dry for one hour in a laminar flow cabinet. The seeds from each plant were separately crushed and plated in *Botrytis* selective medium and incubated (25 seed per plate) at 18^{o}C for one week.

Growth of *Botrytis cinerea* from each seed was monitored and recorded. Confirmation of the presence of *B. cinerea* was based on the sporulation of the pathogen and morphological observation of the colonies under the microscope from where BSM stained brown. The colonies isolated were subcultured into MEA in 9cm Petri plates and incubated at 18^{o}C for one week with alternating UV-A light (12h/day) and dark (12h/day) to allow for the growth of mycelia. The growth of *B. cinerea* from the seeds to seed was recorded and the data were analysed using ANOVA (Sowley, 2006).

Isolation of Botrytis Cinerea from Plant Grown from Infected Seed

The seed collected from the inoculated plants which were confirmed to have infection of *Botrytis cinerea* was grown in controlled environmental room up to the flower stage. The seed was collected from all the 50 plants and tested for the presence of *Botrytis cinerea* by plating on Botrytis selective media and incubated at 18 ^{o}C for one week colony identified micro and macroscopically.

Statistical Analysis

Factorial analysis of variance was used to evaluate the significance effects of seed infection on the spread of *B. cinerea* from seed to seedling and back to the next seed (Sokal and Rohlf, 1981; Sowley, 2006). A significant F pr- value in the ANOVA indicates a significant relationship between seed infection and seedling infection $P< 0.05$.

RESULTS AND DISUSSION

Results

Initial Seed Infection

One hundred seeds of lettuce Tom Thumb variety as supplied plated on *Botrytis* selective media but no infection was detected. Furthermore, no infection was recorded from one hundred seeds of Tom Thumb variety treated with fungicide before plating. However, infection was detected when both fungicides treated and untreated seeds were crushed before plating on *Botrytis* selective media. There was no significant difference (paired sample t-test, $P<0.05$)) in the level of *Botrytis cinerea* isolated from infected and uninfected seed.

Isolation of B. cinerea in Lettuce Roots, Stems, and Leaves of Lettuce

B. cinerea was detected from root, stems and leaves of plants grown from infected and uninfected seeds. The isolations of *B. cinerea* at week two and four arose from the root. At week eight and twelve, in addition to the detection of *B. cinerea* in the root, the fungus was also detected in the stems and leaves. More than half of the plants carried leaf infection of *B. cinerea*, the incidence of *B. cinerea* in leaf and root of plants grown from infected seed (75%) was significantly higher than in leaf of plants grown from uninfected seeds (33%) $F_{1,38} = 61.29$, $P < 0.001$ (Table 1).

Table 1: Isolation of *B. cinerea* from parts of seedlings grown from infected seed

Inoculation	Plant Part			
	Root	Stem	Leaves	Total
Inoculated	90	34	34	158
Uninoculated	82	30	16	128

Isolation of B. cinerea from Seed of Lettuce Plant Grown from Infected Seed

Seeds from all the lettuce plant carried the infection of *B. cinerea.* A total of 364 infections were recorded by plating methods in *Botrytis* selective media. Seed infection in plants grown from infected seed was statistically significant $F_{1,38} = 51.22$, $P < 0.001$, while in seeds collected from plant grown from uninfected seed was not significant (Table 2).

Table 2: Isolation of *Botrytis cinerea* from seed of lettuce plants grown from infected seed

	Infected	Uninfected	Total
Fungicide treated	56	58	112
Non fungicide treated	159	24	183

Discussion

Botrytis cinerea was not isolated from water-washed and surface-sterilised source seed. However, when the seeds were crushed before plating infection was detected from both fungicide and non fungicide treated seed. This shows that most of the *Botrytis cinerea* is present within the seed rather than on the surface. Maude and Presly (1 977a) reported similar results.

They found that treatment with Chloros (3% free chlorine) reduced but did not eliminate *B. cinerea* and they concluded that the majority of the *B. cinerea* was internal infection rather than surface contamination. This corresponds with the results of isolations made from *Primula Polyantha* where *B. cinerea* was the predominant *Botrytis* species isolated (Barnes and Shaw, 2003).

However, it was noted that the proportion of seedlings yielding *B. cinerea* was less than the proportion of seeds infected with *B. cinerea*, indicating that not all seed infections result in seedling infections. This observation was supported by the results of Burgess *et al.*, (1997) who observed that seed of chickpea infection did not result in seedling infection. In a related experiment Maude and Presly (1977b) reported similar observations.

They found that only 18% of seedlings were infected when seed with a reported 45% infection level was sown. The present study suggests that *B. cinerea* infects the cotyledon first and subsequently moves down to infect the leaves.

This is consistent with the findings of Maude and Presly (1977a and b); Tichelaar (1967); Barnes and Shaw (2003), Shafia (2009); Elias *et al.*, (2010), Tichelaar (1967). In this study, however, a clear relationship between the level of seed infected with *B. cinerea* and seedling infection was established, with the higher seed infections resulting in greater levels of seedling infection.

This evidence suggests that infected seed is an important source of inoculum for infection of lettuce by *B. cinerea*. However, the present study do not preclude airborne spores of *B. cinerea* from acting as an additional source of inoculum more over the fact that *B. cinerea* was not detected in the in some of the original seeds, shows that sometimes it can act as airborne contaminant but spore traps, set out in the field plots, to detect infection shows that *B. cinerea* were routinely detected. *B. cinerea* is highly sensitive to the benzimidazoles and dicarboximide group of fungicides.

Benomyl (Benlate) and iprodione (Rovral) have been used successfully in the United Kingdom as seed treatments to reduce levels of *Botrytis* and prevent carry over after harvest (Elias *et al.*, 2010). Therefore given the role of seed borne infection identified by this study, it is recommended that similar seed treatments be used on infected seed lines in an attempt to protect seedlings from *Botrytis* infection and ultimately reduce the risk of lettuce and other vegetables from rot caused by *B. cinerea* which may results in wastage, scarcity and high cost of the commodity.

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