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ENDOPHYTIC *Botrytis cinerea* ESTABLISHES INTERNAL INFECTION IN LETTUCE PLANT (*Lactuca sativa* L.)

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ABSTRACT

Understanding the epidemiology of the wide spread of necrotrophic pathogen *Botrytis cinerea* is of considerable horticultural interest, as the design of control strategies is of great economic importance. Here it was showed that seed is the source of systemic *Botrytis cinerea* in lettuce plants. This was determined by the detection of seed infection from plants which were grown in controlled conditions from infected seed collected from flower inoculated plants. Fragment analysis found that 62.5% of seed isolates detected from plants which were grown from non fungicide treated seeds was identical to the inoculated isolate. In contrast, all seed isolates detected from plants which were grown from fungicide treated seed have different genotypes to the inoculating isolate. The results show that with time as the plant grew infection from the seed spread to stems and leaves systemically without exhibiting symptoms. This confirmed the systemic spread of the seed isolate, but showed that other *B. cinerea* isolates were present and that single plants can host multiple isolates. The results of these experiments show that the age of the plant affects the spread of seed infection to other parts of the plant. This shows that the endophytic phase is an important component of the population dynamics of *B. cinerea*.

Keywords: *Botrytis cinerea*, Endophyte, *Lactuca sativa*, Systemic infection.

INTRODUCTION

Botrytis cinerea is a member of the phylum Ascomycetes, family Sclerotiniaceae. The fungus is designated by its anamorphic (asexual form) name, because the perfect sexual stage (teleomorph) known as *Botryotinia fuckeliana* is rarely observed (Beever and Weeds, 2004). *Botrytis cinerea* has a

fairly simple life cycle; it produces copious clear or grey conidia on long branched conidiophores (Agrios, 2005). Symptoms of the diseases vary depending on the host and plant part infected. However, general symptoms include soft rots, accompanied by water soaking and browning of tissues, followed by the appearance of grey masses of conidia on rotted tissues (Williamson *et al*., 2007). Elad and Stewart (2004) reported that *B. cinerea* can enter into the host body and remain dormant or quiescent for a varying periods resulting in a symptomless endophytic infection. During quiescence, further fungal growth and colonization are halted, and signs of the pathogen are not visible (Cadle and Davidson, 2008; Elias *et al.,* 2010).

Endophytic fungi colonize healthy plant tissues inter- and or intra- cellularly, persisting for the whole or part of their life cycle without causing visible disease symptoms in the host plant (Arnold *et al.* 2000; Bacon and White, 2000; Samir and Amnon, 2007; Tejesvi *et al*., 2007; Oses *et al*., 2008). The endophytic colonisation of plants by *B. cinerea* lead to, systemic infection of the host and has become a great concerned for the food production industry as it results in serious and unexpected post-harvest problems due to the decay of the produce without any prior warning.

Fully endophytic *B. cinerea,* like other endophytic fungi, are only disseminated by seed; being highly and efficiently dispersed by their host seed they need not have spores as a means of dissemination (Saikkonen, *et al*., 2002). With the start of seed production, the endophyte grows upward in the plant and infects the outer layers of the seed; these transfer endophytes from the plant batch to the seed, (Sowley, 2006; Shafia, 2009). However, seed infection is only important in circumstances where the pathogen can spread into the plant (Barnes and Shaw, 2002; Barnes and Shaw, 2003). Elias *et al.,* (2010) reported that highest rate of infection of *B. cinerea* occurred in the roots of plants grown from infected seeds, but as the plants grew the infection moved upwards into newly produced tissues. The general trend is that infections progress from the roots, through the stems to the leaves and finally into the seed without causing symptoms (Elias *et al*., 2010). Generally, internal infection may depend on which part of the flower is infected thus affecting the likelihood of the pathogen transferring into the seed.

The ability of endophytic *B. cinerea* to be disseminated by seed makes it the most widely distributed disease of vegetables, ornamentals, fruits, and field

crops throughout the world (Williamson *et al*., 2007). Therefore in order to increase availability and to reduce cost of production it is important to study the systemic endophytic spread of *B. cinerea* which will provide information in designing of control strategies. The main aim of the present study was First to investigate the role of seed borne endophytic *B. cinerea* in the establishment of internal infection in lettuce plants. Second to determine whether there is a co-ordination in the spread of infection from the root to the upper parts of the plants in lettuce. Third to investigate the effect of time i.e. effect of plant age in the spread of internal infection.

MATERIAL AND METHODS

Botrytis cinerea Culture and Isolation of the Fungus from Plant Tissues

Botrytis cinerea isolate, B1.2 (isolated at Reading in 2005) was maintained on *Botrytis* selective medium (BSM) before being transferred to 3% malt extract agar (MEA CM0059, Oxoid, Basingstoke). After the culture covered approximately 1/3 of each plate, they were exposed to continuous UV-light to encourage sporulation. *Botrytis* selective media (Edward and Seddon, 2001) was used in the detection and isolation of the fungal pathogen from plant tissues.

Seed Stock

The seed used in this experiment was collected from flower inoculated lettuce plants which were initially grown from lettuce seed (Tom Thumb variety, Fothergills Seeds, Newmarket), which were harvested in 2009. The seed was sterilized by soaking in 100ml of (0.1g/l) the systemic fungicide Shirlan (active ingredient 500g/l Fluazinam, Sygenta Crop Protection UK limited) for 2hrs and drying over night before sowing.

Plant Growth and Inoculation

Infected seed from flower inoculated plants were divided into two treatments one was treated with the fungicide Fluazinam following the same method used to sterilize the initial seed stock. Fungicide and non-fungicide treated seeds were separately grown in twenty, 15cm pots, each with four seedlings per pot. Pots were arranged in two blocks, each consisting of 20 pots (10 pots each for fungicide and non- fungicide treated seeds). At the two leaf stage Block one was selected and inoculated with fungal spores by gently tapping the back of Petri plates containing a 14-day-old sporulating culture of *B. cinerea* B1.2. The seedlings were left for 10 min to allow spores to settle, then transferred into black polyethylene bags and stored in the laboratory under ambient conditions

(to prevent overheating and condensation) for 24hrs before removing the polyethylene bag (Fig 1). Seedlings were reared in a controlled environment room (22^{0}C +/-, 2 ^{0}C, RH 66).

Fig. 1: Seedlings Left for 24hrs in a Black Polythene Bag

Isolation of *Botrytis cinerea* from Lettuce Seedlings

The experiment was performed twice (April/May 2011 and June/July, 2011). In each case, one week after inoculation one seedling was collected from each of the 40 pots in the two blocks and divided into one cm-long sections of secondary root, 1cm diameter leaf discs and hand-cut 1 mm sections of stems. These were sterilized and individually plated in BSM plates. This was repeated at intervals of three, six and eight weeks after inoculation. The growth of *B. cinerea* from the seedling sections after each plating test was counted and recorded.

Genetic Characterizations

DNA was extracted from sections of roots, stems and leaves isolated 8 weeks after inoculation. Mycelium (100mg) was scratched from Petri plates and manually ground in a pestle and mortar in the presence of liquid nitrogen. The resulting fine powder was transferred into a 200ml effendorf tube and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, West Sussex, UK), stored at -20^{0}C and the concentration of the DNA as adjusted to 10 ng µl using a Nanodrop ND 1000 Spectrophotometer (Applied Biosystems UK). The *B. cinerea* DNA isolates were characterised using nine microsatellite markers designed by Fournier *et al.,* (2002) which were labelled with FAM (Blue), HEX (Green) and NED (Yellow). Each reaction mixture contained 25 µl of Biomix (Bioline, UK), 20 µl of water, 1.5 µl of each forward and reverse primer and 2 µl of DNA template. The PCR of the SSR was repeated several times for successful amplification. The products from successful amplifications were multiplexed in three combinations; (BC1, BC4, BC9), (BC3, BC6, BC10), (BC2, BC5, BC7). These groups were chosen to avoid overlapping the allele size ranges of

the primers in each mix. The PCR was run with an initial denaturing step of two minutes at 94^{0}C for BC1, BC2, BC3, BC5, BC6 and BC9 followed by 35 cycles of one minute at 94^{0}C, one minute annealing at 53^{0}C, 30 seconds at 72^{0}C and 5 minutes at 72^{0}C. While BC4, BC7 and BC10 an annealing temperature of 59^{0}C was used. The products were submitted for fragment analysis by Source Bioscience, UK. The results of the fragment analysis were scored by using Genemapper software (Applied Biosystem).

Experimental Design and Statistical Analysis

Fungicide and non-fungicide treated seeds were sown in 20 pots, each with four seeds per pot. The experiment was conducted in April/May 2011 and June/July, 2012. The experimental design was a factorial block design with 3 main factors (a) seed fungicide treatment (b) seedling inoculation and (c) time. One block of seedlings was dry inoculated two weeks after sowing with fungal isolate B1.2. Although the density of the spore deposition was not measured, the technique was similar to the one used for the initial commercially purchased lettuce seeds in 2009. One week after inoculation one seedling was collected from each of the 40 pots, washed under running tap water and sterilized by spraying with 70% ethanol and then allowed to air dry in a sterilized lamina flow. Seedlings were divided into roots, stems and leaves and plated in *Botrytis* selective media (BSM) at 18^{0}C for one week. The remaining seedlings were sampled and plated at interval of three, six and eight weeks after inoculation. At eight week after inoculation, the 61 colonies isolated from roots, stems and leaves were subcultured and fragment analysis was performed following the previously described protocols.

Colony counts at the eight week after inoculation were compared between the four different treatments using generalised linear model approach (GLM) using statistical software Mini Tab (2009).

RESULTS

Establishment of Internal Infection

Botrytis cinerea was recovered from the surface–sterilized sections of healthy leaves, stems and roots for all treatments in April/May 2011 and June/July 2011, with the exception of a single sample plated one week after inoculation in April/May 2011, where all samples became contaminated (Table 1). Although growth of *B. cinerea* was recorded from the second, third and fourth sampling of April/May 2011, the difference between the treatments was very close to

significant (P= 0.06). In June/July 2011 experiments, infection was initially most common in the roots with clean stems and leaves. However, as the plants grew the infection spread into stems and then into leaves. In the last plating test eight weeks after inoculation colony count from the roots stems and leaves was relatively one. (Table 1, time on the spread of infection P <0.002).

Table 1: Incidence of *B. cinerea* in Different Plant Parts

N = 10 per Treatment

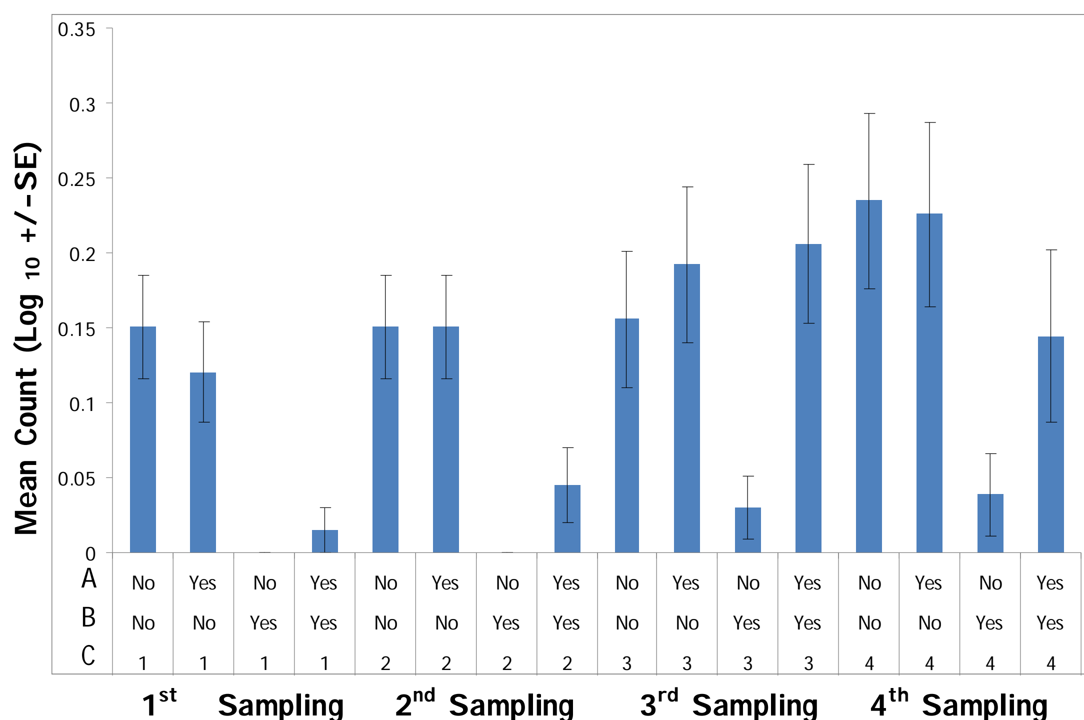
Plant	Weeks After Inoculations																							
	1 Week			3 Week			6 Week			8 Week			1 Week			3 Week			6 Week			8 Week			
	April / May												June / July												
	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L	Total
-N/-F		-		7	3	0	7	4	3	9	4	5	10	0	0	10	0	0	8	5	0	10	6	6	97
-N/+F		-		4	0	0	5	3	0	8	4	2	0	0	0	0	0	0	2	0	0	2	1	0	31
+N/+F		-		5	1	1	6	4	4	6	1	5	1	0	0	3	0	0	5	4	1	5	5	4	61
+N/-F		-		4	1	2	5	4	3	6	4	6	8	0	0	10	0	0	10	4	2	10	6	6	91
Total				**20**	**5**	**3**	**23**	**15**	**10**	**29**	**13**	**20**	**19**	**0**	**0**	**23**	**0**	**0**	**25**	**13**	**3**	**27**	**18**	**16**	**280**

-N/-F = Non Fungicide Treated Seed/Un-inoculated Seedling

-N/+F = Fungicide Treated Seed/Un-inoculated Seedling

+N/+F = Fungicide Treated Seed/Inoculated Seedling

+N/-F = Non- fungicide Treated Seed/ Inoculated Seedling



A - Fungicide Seed Treatment

B - Seedling Inoculation

C - Time

Fig. 2: Effect of Seed Fungicide Treatment, Seedling Inoculation and Time on the Spread of Internal Infection in Lettuce Plant

Seed fungicide treatment

Fungicide treatment of the seed before sowing cleared most of the seed infections and greatly reduced the incidence of infections in all the plant tissues plated at first and second plating test. (Table 1 and Fig. 2). At three weeks after inoculation plants grown from fungicide treated seed in June/July had no stem and leaf infection but moderate levels in their roots; the stems and leaves were significantly different from the ones grown from the untreated seed (Table 2, $F_{1, 319}$ = 32.65, P = 0.000,).

Table 2: Effect of Seed Fungicide Treatment on the Spread of Endophytic *B. cinerea* in Different Plant Part

Fungicide Treatment	Plant Part			
	Root	Stem	Leaves	Total
Treated	52	23	17	92
Untreated	120	41	33	194

At the fourth plating test, eight weeks after inoculation leaves had less infection but the roots and stems were not affected by the seed fungicide treatment. The effect of seed fungicide treatment interacted significantly with the effect of seedling inoculation (Table 3, $F_{1, 319,}$ 4.76, P= 0.030,).

Table 3: Effect of Seed Fungicide Treatment and Seedling Inoculation on the Incidence of Internal *B. cinerea in* Lettuce

Inoculation	Fungicide Treatment							
	Treated				Untreated			
	R	S	L	Total	R	S	L	Total
Inoculated	31	15	15	61	59	19	19	97
Uninoculated	21	8	2	31	61	22	14	97

However, there was no significant interaction between seed fungicide treatment, seedling inoculation and time on the spread of infection ($F_{3, 319}$ = 0.38, P = 0.771).

Seedling Inoculation

Seedling inoculation in both the April/May and June/July experiments did not affect infection levels at the 1st and 3rd weeks after inoculation, but alter infection frequency at 6th and 8th weeks after inoculation. However, there was

no significant interaction between seedling inoculation and time on the spread of internal infection (Table 4, $F_{3,319} = 1.50$, $P = 0.215$).

Table 4: Effect of Inoculation on the Spread of *B. cinerea* in Different Plant Part

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

Influence of Time on the Spread of Infection

The initial seed source was infected seed. All the plants which were grown without seed fungicide treatment initially had a high rate of root infection, but stems and leaves were free of infection in tests conducted after inoculation (Table 1). However, as the plant grew the infection moved into stems and then leaves. There was a significant interaction between time and location of infection (Fig. 2, $F_{3,319} = 4.92$, $P= 0.002$).

Genotyping of Recovered Isolates

Isolates recovered from roots, stems, and leaves of experimental plating in the June/July experiment eight weeks after inoculation were genotyped at nine microsatellite loci. The isolate detected from the initial seed used to grow the plants (haplotype coded A), was recovered from 62.5% of the plants which were not inoculated at seedling stage and in some cases the isolate was recovered throughout each plant. All isolates recovered from inoculated plants had a different genotypic identity from the isolate recovered from the source seed.

DISCUSSION

The present research establishes that seed is the source of systemic *B. cinerea* infection in lettuce. Infection is initially confined to the root in plants grown from infected seed, and as the plant grows infection moves into developing tissues. In Aril/May, 2011, when the plants were grown from fungicide and non-fungicide treated seed, although no colony count was recorded at week one after inoculation due to the contamination of the samples, subsequent plating tests show that roots carried more infection of *B. cinerea.* When the experiment was repeated in June/July 2011, the distribution of *B. cinerea* in the parts of lettuce plant was somewhat different (Table 1). From the plating test at one and three weeks after inoculation the infection was confined to the roots while the stems, and leaves were infection free in plant grown from non-

fungicide treated seeds. However, the plating test at one and three weeks after inoculation shows that roots, stems and leaves were clean in plants grown from fungicide treated seed. At six weeks after inoculation both treatments showed that roots have the highest infection rate, but there was also a movement of infection from the roots to the upper parts of the plant. The plating test at eight weeks after inoculation showed a balance of infection between the plant tissues. In a similar experiment, Sowley, (2006), Shafia, (2009) and Elias *et al*. (2010) found that healthy lettuce plants without any visible disease symptoms may harbour live *B. cinerea* in their roots, stems and leaves, and as the plant grows infection from the seed or seedling moves to the upper part of the plant. In a related experiment Barnes and Shaw (2003) found that *Primula* x *Polyantha* plants grown from infected seeds had a high level of root infection. Since the plants were grown in isolation, they concluded that the infection in the roots was likely to have spread to other part of the plant.

In this study it is established that as plants grow, *B. cinerea* infection moves to newly produced tissues. It is possible that *B. cinerea* goes into a quiescent phase before spreading to the stems and leaves Barnes and Shaw (2003). Seed fungicide treatment was effective in removing surface contamination. The presence of the pathogen in plants grown from fungicide treated seeds suggests that the pathogen is systemic. Infection arises from both internal and external seed infection (Table 3). Sowley (2006) found that seed fungicide treatments significantly lower seedling infection rate therefore most of the infection comes from the seed coat. Surface sterilization or seed washing confirmed that infection was largely due to surface contamination (Burgess et al 1997). Kabeere *et al* (1997) reported that infection of maize seeds by *Fusarium subglutinans* was reduced from 96% to 8% after surface sterilization. They showed that the majority of seed inoculum was on the seed surface because there was a substantial reduction in recovery of *F. subglutinans* when seeds were surface sterilized.

Stewart and Franicevic (2005) investigation of the spread of internal infection by *Botrytis* species, revealed transmission of *B. allii* from infected onion seed into the seedling tissue. However, the percentage of seedling infection was less than that of seed infection which suggests that not all seed infection results in seedling infection. Also no differences were found in the amount of *B. allii* infection between plants grown from water-washed and fungicide treated seeds.

This suggest that *B. allii* is present within the seed rather than on the seed surface.

The recovery of infection in plants grown from *B. cinerea* infected seed varies with plant age (Shafia, 2009; Elias *et al.,* 2010). Sowley, (2006) found that the recovery of *Acremonium coenophiilum* from infected plants varies with seedling age, root type and medium. Similarly, Kelly and Wallin (1986) found that *Aspergillus flavus* is confined to the roots of maize at the 3-leaf stage while by the 4-leaf stage it is uniformly distributed in the root, stem and leaves. *Aspergillus flavus* became confined to the root and lower portions of the stem at the 5-leaf stage

In the present study seedlings grown from non fungicide treated seeds tended to have a higher spread of fungal infection as shown in Table 3. In both April/May 2011 and June/July2011, there was a general increase in the spread of infection from one week after inoculation to eight weeks after inoculation as shown in Table 1, in both fungicide and non fungicide treated seedlings. The general increase in infection could be due to *B. cinerea* spreading within plant tissues without causing symptoms.

There are two alternative hypotheses for the pattern of infection described. First, each part of the plant could be independently infected and secondly the pattern in the result of systemic infection resulting from the initial source infection. These data support the later hypothesis, given the timing of expression of infection and the results from the fragment analysis.

The SSR results shows that isolates recovered from roots, stems and leaves of 62.5% of uninoculated plants was similar to the genotype of *B. cinerea* from the initial seed source. Out of that 6.4% of uninoculated plants have isolates in their roots, stems and leaves similar to the *B. cinerea* isolated from the source seed. Therefore, Isolates from the inoculated plants have different genotypic identities from that of the seed source (Table 5).

Table 5: Allele Sizes of Isolates Screen Against Nine Microsatellite Markers

Seed	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC9	BC10	HAPL
Seed	230	160	164	96	164	90	85	150	180	A
-N/-F1R	230	160	164	96	164	90	85	150	180	B
-N/-F2R	230	160	164	96	164	90	85	150	180	B
-N/-F3R	230	160	164	96	164	90	85	150	180	A
-N/-F4R	230	160	164	96	164	90	85	150	180	A
-N/-F5R	230	160	164	96	164	90	85	150	180	A
-N/-F6R	230	160	164	96	164	90	85	150	180	A
-N/-F7R	230	160	164	96	164	90	85	150	180	A
-N/-F8R	230	160	164	96	164	90	85	150	180	A
-N/-F9R	230	160	164	96	164	90	85	150	180	A
-N/-F10R	230	160	164	96	164	90	85	150	180	A
-N/-F1S	230	160	164	96	164	90	85	150	180	A
-N/-F2S	230	160	164	96	164	90	85	150	180	A
-N/-F3S	230	162	164	96	158	121	85	150	180	D
-N/-F4S	230	162	164	96	164	90	85	150	180	A
-N/-F5S	230	162	164	96	164	90	85	150	180	A
-N/-F6S	230	160	NA	96	164	90	85	150	180	E
-N/-F1L	230	160	164	96	164	90	85	150	180	A
-N/-F2L	230	162	164	96	163	121	117	150	180	D
-N/-F3L	230	162	164	96	164	121	117	150	180	D
-N/-F4L	230	160	164	96	164	90	85	150	180	D
-N/-F5L	230	160	164	96	164	90	85	150	180	A
-N/-F6L	230	160	164	96	164	90	85	150	180	A
-N/+F1S	230	160	164	96	164	90	85	150	180	A
-N/+F1R	230	160	164	96	164	90	100	150	180	F
-N/+F2R	230	160	164	96	164	90	85	150	180	A
+N/-F1R	230	160	156	93	164	94	85	150	180	H
+N/-F2R	230	160	164	96	164	160	85	150	180	I
+N/-F3R	230	160	164	103	164	90	85	150	179	J
+N/-F4R	230	160	164	96	164	96	85	150	163	G
+N/-F5R	230	160	164	96	164	96	85	150	163	G
+N/-F6R	230	160	164	96	156	96	85	150	163	L
+N/-F7R	230	160	164	96	164	96	85	150	163	G
+N/-F8R	230	160	164	96	164	96	85	150	163	G
+N/-F9R	230	160	164	96	164	96	85	150	163	G
+N/-F10R	230	160	164	96	164	96	85	150	163	G
+N/-F1S	230	160	164	96	164	96	85	150	163	G
+N/-F2S	230	160	164	96	164	96	85	150	163	G
+N/-F3S	230	160	158	96	164	96	85	150	164	M
+N/-F4S	230	160	164	96	164	96	85	150	163	G
+N/-F5S	230	160	164	96	164	96	85	150	163	G
+N/-F6S	230	160	164	96	164	96	85	150	163	G
+N/-F1L	230	160	164	96	164	96	85	150	163	G
+N/-F2L	230	160	164	96	164	96	85	150	163	G
+N/-F3L	230	160	164	96	164	96	85	150	163	G
+N/-F4L	230	160	164	96	164	96	85	150	163	G
+N/-F5L	230	160	164	96	156	96	85	150	163	N
+N/-F6L	230	160	164	96	164	96	85	150	163	G
+N+F1R	230	160	164	97	164	96	85	150	164	O
+N+F2R	230	160	164	96	164	96	85	150	163	G
+N+F3R	230	160	NA	96	164	96	85	150	160	P
+N+F4R	230	160	164	96	164	96	85	150	163	G
+N+F5R	230	160	164	96	164	96	85	150	163	G
+N+F1S	230	160	164	96	164	96	85	150	163	G
+N+F2S	230	160	164	96	164	96	85	150	163	G
+N+F3S	230	160	164	96	164	96	85	150	163	G
+N+F4S	230	160	164	96	164	96	85	150	163	G
+N+F5S	230	160	164	96	164	96	85	150	163	G
+N+F1L	230	160	164	96	164	96	85	150	163	G
+N+F2L	230	160	164	96	164	96	85	150	163	G
+N+F3L	230	160	164	96	164	96	85	150	163	G
+N+F4L	230	160	164	96	164	96	85	150	163	G

Alphabet A-G represents the code for the haplotypes

–N = Uninoculated Plant

-F = Non- Fungicide Treated Seed

+N = Inoculated Plant
+F = Fungicide Treated Seed
R = Root,
S = Stem,
L = Leaves.
Same number in plant column indicates isolates are collected from the same plant.

To summarise, this study has established that seed is the source of infection by *B. cinerea* in lettuce seedlings and as the plant grows infection spreads to newly produced tissues leading to a long-lived systemic endophytic infection in all parts. The finding here agreed with the results of Sowley (2006) and; Shafia (2009) that susceptibility of lettuce plant to infection increases with aging, the incidence of infection on matured leaves was higher than of younger and healthy leaves. Previous works have suggested that *B. cinerea* is root specific (Barnes and Shaw, 2002) but this study suggested that while *B. cinerea* may be initially confined to the roots it may eventually spread to other parts of the plant.

It is therefore recommended that a good quarantine system should be put in place to check all seed stocks earmarked for export and import in order to reduce the spread of systemic *B. cinerea* from one locality to another.

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