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Symptomless Transmission of Seed Borne Botrytis *Cinerea* into the Seed of Next Generation Lettuce Plant (*Lactuca Sativa*)

Research article

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Abstract

Study was carried out to determine the behaviour of endophytic and systemic pathogen *Botrytis cinerea* the causative agents of grey mould disease in ornamental and vegetables. The necrotic pathogen was isolated from *Botrytis* selective media from sterilised portion of lettuce seeds collected from the plants which were dry inoculated at seedlings and flower stage of development. Seed collected from first-experiment plants, which was found to have carry the *B. cinerea* inoculum used during dry inoculation, was used to grow plants for the second generation experiment at controlled environment room after surface sterilization. Isolation of the inoculating isolate from the seed of healthy second generation lettuce plants confirmed symptomless transmission of the inoculating isolates.

Keywords: *Botrytis cinerea;* Endophytic infection; Systemic infection; Sterilization; *Lactuca sativa*

Introduction

Botrytiscinerea (teleomorph *Botryotinia fuckeliana*) have been recognized as the most important pathogen affecting nursery plants, vegetables, ornamental, field orchard and forestry plants as well as stored and transported agricultural products throughout the world [1]. At the early times of its recognition, it was referred as a temperate area pathogen, nevertheless currently the pathogen's impact has been a concern in all parts of the world ranging from tropical, sub-tropical to world cold areas. The pathogen produces clear grey conidia on Long branch conidiophores [2], the conidia infect the plant directly to cause spreading necrotic lesions. In some plant *B. cinerea* can enter and remain dormant or quiescent for varying period of time [3]. During quiescence further fungal growth and colonization are halted, and sign of the pathogen is not visible but became activated by the onset of senescence or stress of the host tissue [4,5].

In a previous research [6,7] showed that fungal diseases are mainly transmitted via seed, and seed borne infection can lead to symptomless infections after they have been transmitted to seedlings. They reported that *B. cinerea* can grow into the plant without showing symptoms and single isolates may grow throughout the healthy plant without showing symptoms. They reported that the infections appear to be seed-borne, but may also arise from air-borne spores. Study by [8] who isolated *Botrytis fabae*, the cause of chocolate spot of *Vicia faba* from bean seeds found that seed infection does not always transmit infection into seedling, [8] reported that the levels of *B. fabae* conidia on most infected seeds, which were tested, were too low to cause

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an aggressive lesion at 15^{0}C 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. This was supported by [9] who reported that not all infections by endophytic *B. cinerea* results in systemic infection. The evidence was (a) failure to detect *B. cinerea* from sections of surface sterilized asymptomatic epicotyl from seedlings with visible root lesions and (b) infected seeds gave rise to apparently looking healthy seedlings.

In this experiment, *B. cinerea* was isolated from seeds as well as leaves of symptomless lettuce plants. Similar, report by [6,10]. They found that seed infection lead to systemic endophytic infection even when the external inoculum was excluded. They concluded that the infection was internal since *B. cinerea* was isolated from the surface sterilised seeds of Primular and lettuce plants. Therefore, the present study tested two hypotheses. First, the *B. cinerea* grows systemically in lettuce plant and second, the infection from infected host seedling could be transferred to the seed in lettuce that subsequently infect seeds of next-generation plant without showing symptom.

Material and Methods

Seed Sterilization

Lettuce seed (*var*. Tom Thumb), purchased from Fothergills Seeds, New market, and was used for the experiments. Portion of the seed was sterilized in fungicide by soaking in100ml of (0.1g/l) systemic fungicide Shirlan (active ingredient 500g/l Fluazinam. Sygenta Crop Protection UK limited) for 2h and drying over night before sowing.

Inoculation and Seedling growth

Seeds were sown in16 trays filled with vermiculite growing medium. These trays were kept in a glass house at University of Reading. Eight trays were used each for sterilized and non-sterilized seeds. One week after germination, four trays of seedlings growing from sterilized and non- sterilized seeds (Figure 1) were selected and covered with black polyethylene bags. *Botrytis cinerea* spores 2.20 incubated for two weeks were used for the inoculation. Dry spores were harvested from the petri plates by tapping gently on a sterile piece of aluminium foil. The spores were transferred into 10ml 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 left for 24h, to facilitate germination of spores through building up of high relative humidity. To estimate the number of spores deposited per unit area an acetate paper on which graph sheet was photocopied was placed in each tray and examined by a microscope. There was average of 12 spores/ mm.2.A week after seedling inoculation seedlings were transplanted in to15cm pot and placed in 20 blocks of 4 treatments (Figure 2).

Dry spore inoculation of the flower/bud

At flower stage 10 blocks consisting of 40 plants (half of the replicates,) were selected from the first four treatments (a) fungicide treated seed/uninoculated seedling (b) fungicide treated seeds/ inoculated seedlings (c) non fungicide treated seeds/ uninoculated seedlings (d) non fungicide treated seeds/ inoculated seedlings. Flower and bud of the plants were covered with black polyethylene bags and inoculated with similar spores as in seedling inoculations, and left for 24h.

Figure 1: Dry spore inoculation of seedling

Figure 2: Dry spore inoculation of seedling

Isolation of *Botrytis cinerea* from seeds of lettuce plants

To determine the systemic transmission of infection into the seed, 10 seeds from each of the plants grown in the eight treatments of lettuce plants were collected and sterilized by soaking in 20ml of 70% ethanol in a duram bottle for 30 minutes, and allowed to dry for one hour in the laminar flow. The seeds from each plant were separately crushed and plated on *Botrytis* selective media and incubated at 18^{0}C for one week. Growth of *Botrytis cinerea* from each seed was monitored, counted and recorded.

Genetic characterization of *B. cinerea* isolates

Using pestle and mortar in the presence of liquid nitrogen, 100mg mycelium of the seed isolates grown in MEA plates was scratched and ground to fine powder. DNA was extracted from the fine powder using DNeasy Plant Mini Kits (Qiagen, West Sussex, UK); the concentration of the DNA was adjusted to 10ng µl^{-1} using Nanodrop ND 1000 Spectrophotometer and stored at -20 $^{\circ}$C until needed.

The template DNA was run in polymerase chain reactions with scar primers designed by [11] which are specific for *B. cinerea.* Templates that gave DNA band were characterized by nine microsatellite markers published by [12] labelled with FAM (Blue), HEX (Green), and NED (Yellow). The volume for each reaction mixture contained 25 µl of Biomix (Bioline, UK), 20 µl of water, 1.5 µl of each forward

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Figure 3: Second generation lettuce plant at seedling stage

Figure 4: Second generation lettuce plant grown at Control environment at Flower stage.

and reverse primers 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 at initial denaturing step of two minutes at 94 °C for BC1, BC2, BC3, BC5, BC6 and BC9 followed by 35 cycles of one minute at 94 °C, one minute annealing at 53 °C, 30 seconds at 72 °C and 5 minutes at 72 °C. While BC4, BC7 and BC10 have an annealing temperature of 59^{0}C. The products were multiplex submitted for fragment analysis at Macrogen South Korea. The results from the fragment analysis were interpreted by using Genemapper V4 version software (Applied Biosystem).

Transmission of infection from seed to the next generation plant

To test whether *B. cinerea* has been successfully transferred from seed to the healthy next plant generation seed, infected seeds were collected from five flower inoculated plants D2-2, D2-3, D2-5, D2-10 and A2-6. The seed from each plant was divided into two. One-half of the seed was sterilized with fungicide as previously. Sterilized and non-sterilized seeds from all the five plants were grown in 10, 20mm pots. Five plants were grown for each of the two treatments, one plant per pot. The plants were grown up to seedling stage (four month), with seed's collection from all the plants (Figure 3 and 4).

Experimental design

First generation experiment-Glasshouse. Seedling to seed transmission of *B. cinerea*. Prior to sowing seeds, 100 seeds from both sterilized and non-sterilized were plated in BSM plate to determine the initial level of infection. Fifty seeds from each of the two treatments were crushed before plating. Plates were incubated at 18^{0}C for 1 week.

The experiment design used a factorial block design in which all the treatments were assigned to each block. The main factors (a) fungicide treatment of the seed, (b) seedling inoculation, (c) flower inoculation. At two leaf stage, seedlings were dry inoculated with spores of isolate 2.20. A week after inoculation seedling were transplanted into 80, 15 cm pots and arranged in randomized blocks. Two benches were used all in a single glasshouse (twenty blocks of four treatments). Each block has one plant from each of the initial four treatments: (a) seed sterilization/uninoculated seedling, (b) sterilized seed/inoculated seedling, (c) non fungicide treated seed/ uninoculated seedling, (d) non sterilized seed/ inoculated seedling. When the plants were two month old using 15mm cork borer leaves samples was collected from all the 80 plants and plated on BSM. At flower stage, plants were divided into two portions each consisting of 10 blocks of 40 plants. Flower and bud of the second portion was covered with black polyethylene bag and dry inoculated with spore isolates of *B. cinerea* 2.20 as in seedling inoculation. Matured seeds were collected from all the plants.

The Second generation experiment transmission of *B.cinerea* was from first generation to second generation plants without showing symptoms. To determine whether *B. cinerea* can be transmitted from seed to the plant without showing symptom, infected seed from flower inoculated plants were grown in a controlled environment which has fewer chances of external contaminations. The experimental design was randomized block design with seed sterilization as the main factor. Each block has two replicates. The two treatments were grown up to seedling stage. Matured seeds were collected from all the plants and genetically characterized. Peak height of the microsatellite data was converted into scores of one and zeros. One and zeros represented the presence or absence of bands of a given size respectively.

Statistical analysis

Significance of treatment effects on incidence of seed infection by *B. cinerea* was determined by factorial analysis of variance. A significant value in the ANOVA indicates a significant relationship in the flower inoculation $P< 0.05$.

Results

Seed infection

One hundred seeds of Lettuce var. Tom Thumb were placed on *Botrytis* selective media but no infection was detected. Infection was also not recorded on 100 sterilized seeds of this variety before plating. However, infection was detected when both fungicides treated and untreated seeds were crushed before planting on *Botrytis* selective media.

Systemic infection of *B. cinerea* in lettuce leaves

Botrytis cinerea was detected from leaves of plants grown from sterilized and non-sterilized seeds at Glass house. Infection was detected from the two treatments. More than half of the plants carried leaf infection of B. cinerea, the incidence of B. cinerea in leaf of plants grown from sterilized seeds [22% (9/40)] was similar to leaf of plants

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grown from non-sterilized seeds [30%(12/40)] but not significantly so. While detection of B. Cinerea from new lettuce leaves in seedlings grown from sterilized seed and inoculated with fungal spores at 2 leaf stage shows that the proportion of infected leaves was the same in plants grew from treated and untreated seed. But inoculated plants had a higher incidence of leaf infection. This was unaffected by seed sterilization (Table 1).

Transmission of *B. cinerea* from lettuce Seedling to the seed in glass house

Out of the seed plated on BSM plate a total of 364 seeds a cross the eight lettuce treatment shows infection of*Botrytis cinerea.* Seed infection in plants grown from sterilized seed was 32% (117/364), while in seeds collected from plants inoculated at seedlings stage was 27% (100/365), while infection in seeds collected from inoculated at flower/buds stage was 41 % (148/364). More infected seeds were recorded from plant inoculated at flower/buds stage irrespective of seed sterilization and seedling inoculation $F_{1, 79}$ =23.71, P< 0.001 (Table 2). The effects of flower/bud inoculation and seedling inoculation were independent of one another on the seed infection by *B. cinerea* $F_{1,79}$=0.60P = 0.441(Table 2).

Microsatellite of seed isolates

Isolates recovered from seed of the glass house grown lettuce plant were confirmed as *B. cinerea* isolates by polymerase chain reaction using scar primers designed by [13]. The isolates were then genotyped using nine microsatellite markers published by [13]. The isolates used to artificially inoculates seedlings, 2.20 (coded haplotype A), was recovered from some of the plants, whether inoculated or not. However, the length of the isolates detected with microsatellite loci BC1 and BC3 were found to be lower than what was obtained by the previous workers (result not shown). The microsatellite primers separated isolates into 17 different haplotypes. The frequency of haplotype's existence within the populations is depicted in Figure 1. One haplotype (47%) was recovered from seed of few flower inoculated plants and seed of some non flower inoculated plants result not shown.

Out of the isolates collected from seed of plants grown from sterilized seed 8.3% of the haplotypes matches the isolate 2.20 used in the inoculation, while 91% of the isolates were different from the inoculated isolates. While in seed collected from seedling inoculated plant, 15% of the haplotypes detected genetically matches isolate used for inoculating the plants, while 85% were different.

Overall, the most commonly occurring haplotype which dominated isolates collected from the seed of flower inoculated plants (22%) was similar to the isolate used for seed and flower inoculation at the first experiment. However, the inoculating isolate was not dominant in uninoculated plants (9%).

Transmission of infection from Seed of first generation plants to the healthy next generation plant

Seed infection was recovered from all the second generation lettuce plant grown at controlled environment room. Out of the isolates recovered from seed of plant grown from untreated seeds 59.1% of the isolated resemble the inoculating isolate, while 40.9% differ from the isolate used in the inoculation of first-generation plants. However, all the seed isolates recovered from plants grown from sterilized seeds have different genotypic identification with the isolates used in the inoculation of first-generation plants. Surprisingly, like isolates of the first generation plants, the length of the genotype obtained with microsatellite loci BC1 and BC3 were lower than the length reportedby the previous workers.

Table 1: Count of plants where *B. cinerea* was recovered after seed sterilization and dry spore inoculation at seedling stage.

No. of plants with B. cinerea				No.of plants without B. cinerea		
Treatment	sterilized	non sterilized	Total	sterilized	non sterilized	Total
Seed sterilization	7	3	10	5	4	9
Non sterilized seed	8	4	12	7	5	12
Total	15	7	22	12	9	21

P= 0.002 for interaction between inoculation and seed fungicide treatment

Table 2: Effects of flower/bud inoculations and seedling inoculation seed infection by *B. cinerea*

Flower Treatment	Seedling		Total
	Inoculated	Inoculated	
Seed sterilization	85	29	114
Non seed sterilization	59	28	87
Number of infected seed	117	100	217
Total	144	57	201

Discussion

The study found that first healthy lettuce plants without any visible symptoms may harbour *B. cinerea* in leaves, stems, root and seeds, second that as the plant grows*B. cinerea* infection initially in lettuce seeds or seedlings spread to the seed. Third seed infection transmitted into the next seed generation is a complete systemic and endophytic infection. Systemic, symptomless progression has also been demonstrated earlier in lettuce apart from commercial hybrid Primula [6,7].

In 1992 Singh [14] reported positive evidence of transmission of *Botryodiplodia theobromae* from maize seed to seedlings. Seeds which were heavily infected failed to germinate and were covered with profuse growth of pycnidia on their surfaces. However, those 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. Richard et al. showed that *Aspergillus flavus* become systemic in young maize seedlings grown from contaminated seed. It was shown that germination rates were considerably lower when *Aspergillus flavus* entered and contaminated the seed. The distribution of the fungus suggested that initially the organism may follow the meristem of the plant.

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Table 3: Size of alleles isolated from seeds of first generation lettuce plant against the nine (or ten)Microsatellite Markers.

Isolate	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC9	BC10	HHAP
Stock 2.20	162	162	156	97	156	162	117	164	165	A A
A1-5	162	162	156	97	156	162	117	164	165	BA
A2-8	162	162	156	97	156	162	117	164	165	BA
A2-6	162	162	156	97	156	162	117	164	165	BA
C2-7	162	162	156	97	156	162	117	164	165	BA
C2-4	162	162	156	97	156	162	117	164	165	BA
D2-2	162	162	156	97	156	162	117	164	165	BA
D2-3	162	162	156	97	156	162	117	164	165	BA
D1-3	162	162	156	97	156	162	117	164	165	BA
D1-2	162	162	156	97	165	162	82	164	165	BB
D1-8	162	162	156	97	165	162	82	164	165	BB
DD1-5	162	162	156	97	165	162	82	164	165	BB
- D1-4	162	162	156	97	165	162	82	164	165	BB
AA2-1	1162	1162	1115	997	1165	1162	8117	1164	1165	**D**C
AA2-5	1162	1162	1115	997	1165	1162	8117	1164	1165	**D**C
AA2-6	1162	1162	1115	997	1165	1162	8117	1164	1165	**D**C
CC2-2	1162	1162	1115	997	1165	1162	8117	1164	1165	**D**C
CC2-9	1162	1162	1115	997	1165	1162	8117	1164	1165	**D**C
AA2-4	1162	1122	1156	997	1156	1122	1117	1164	1178	**D**D
BB1-8	1162	1122	1156	997	1156	1122	1117	1164	1178	**D**D
AA2-7	1162	1122	1156	997	1156	1122	1117	1164	1178	**D**D
B1-5	1162	1122	1156	997	1156	1122	1117	1164	1178	DD
B1-9	1162	1122	1156	997	1156	1122	1117	1164	1178	DD
B1-6	1162	1162	1156	997	1156	1122	1117	1164	1178	DD
D1-6	1162	1162	1156	997	1156	1122	1117	1164	1178	DD
A2-5	1162	1162	1156	997	1156	1162	1117	1164	1165	DE
A1-8	1162	1162	1156	997	1156	1162	1117	1164	1165	DE
C1-5	1162	1162	1156	997	1165	1122	1117	1164	1165	DE
B2-1	162	162	156	97	156	122	117	164	165	BF
B2-6	162	162	156	97	156	122	117	164	165	BF
B2-10	162	162	156	104	156	162	117	164	165	BG
B2-2	162	162	156	104	156	162	117	164	165	BG
BB2-7	162	162	156	104	156	162	117	164	165	BG

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- C2-3	162	162	164	104	156	162	117	164	165	BG
CC2-8	1160	1160	1164	996	1164	190	885	1150	1180	**D**H
CC1-2	1160	1160	1164	996	1164	190	885	1150	1180	**D**H
CC1-6	1160	1160	1164	996	1164	190	885	1150	1180	**D**H
DD2-7	1162	1162	1156	997	1156	196	8117	1164	1165	**D**I
DD2-10	1162	1162	1156	997	1156	196	8117	1164	1165	**D**I
AA1-4	1162	1162	1156	997	1165	196	8117	1164	1165	**D**I
AA1-7	1162	1162	1156	997	1165	196	8117	1164	1165	**D**I
BB1-3	1230	195	1115	997	1165	1122	1117	1164	1165	**D**J
AA1-6	2230	**1**162	1115	997	1165	**1**162	**1**117	**1**164	**1**165	JK
CC1-9	2211	1162	1156	997	1165	995	885	1164	1165	KL
BB2-8	1162	**1**162	1109	997	1114	1122	NNA	1164	1165	**L**M
DD2-8	1162	1162	NNA	(97	1165	NNA	!117	!156	NNA	NN
DD2-3	1162	1162	1156	997	1165	NNA	*85	1164	1165	NO
BB2-4	1162	1162	1115	997	NNA	1122	1117	1164	1165	**O**P
CC1-3	1162	1162	1156	997	1156	1162	885	1164	1165	PQ
BB1-4	1162	1162	1156	997	1156	1122	1117	1164	1178	QR

Details of allele sizes of isolates obtained from seed of eight different treatments of lettuce plants. A1- fungicide treated seed uninoculated seedling uninoculated flower. A2- fungicide treated seed uninoculated seedling inoculated flower. B1- fungicide treated seed inoculated seedling uninoculated flower. B2- fungicide treated seed inoculated seedling inoculated flower. C1- non fungicide treated seed uninoculated seedling uninoculated flower. C2- non fungicide treated seed uninoculated seedlings inoculated flower. D1-non fungicide treated seed inoculated seedlings uninoculated flower. D2- non fungicide treated seed inoculated seedlings inoculated flower. B- Inoculated with B. cinerea 2.20.

Table 4: Sizes of allele isolates of second generation lettuce plant against nine (or ten) Microsatellite Markers.

Stock	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC9	BC10	HHAPL
Stock	162	162	167	97	165	121	117	150	177	A A
D2-2-1-F	162	162	167	97	165	121	117	150	177	A A
D2-2-2-F	162	162	167	97	165	121	117	150	177	A A
D2-2-3-F	162	162	167	97	165	121	117	150	177	A A
D2-2-4-F	162	162	167	97	165	121	117	150	177	AA
D2-2-5-F	162	162	167	97	165	121	117	150	177	AA
D2-3-1-F	162	162	167	97	165	121	117	150	177	AA
D2-3-2-F	162	162	156	97	165	121	117	150	177	AB
D2-3-3-F	162	162	156	97	165	121	117	150	177	AB
D2-3-4-F	162	162	156	97	165	121	117	150	177	AB
D2-3-5-F	162	162	167	97	156	121	117	150	177	C
DD2-5-1-F	1162	1162	1167	997	1156	9121	887	1150	1 177	**A**D
DD2-5-2-F	1162	1162	1167	997	1156	9121	887	1150	1 177	**A**D
DD2-5-3-F	1162	1162	1156	997	1165	1121	8117	1150	1177	**D**C
DD2-5-4-F	1162	1162	1167	997	1156	9121	8117	1150	1177	**A**A
DD2-10-1-F	1162	1162	1167	997	1165	9121	8117	1150	1177	**A**A

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DD2-10-2-F	1162	1162	1167	997	1165	9121	8117	1150	1177	**A**A
DD2-10-3-F	1162	1162	1167	997	1165	9121	8117	1150	1177	**A**A
DD2-10-4-F	1262	1162	1167	997	1156	1121	1117	1150	1177	A**C**
AA2-6-1-F	1162	1162	1167	997	1165	1121	1117	1150	1177	**D**A
AA2-6-2-F	1162	1162	1167	997	1165	9121	8117	1150	1177	**D**A
A2-6-3-F	1162	1162	1167	997	1165	9121	8117	1150	1177	**D**A
A2-6-4-F	262	162	167	97	156	121	117	150	177	A**C**
D2-2-1+F	162	162	167	97	165	121	117	150	177	A A
D2-2-2+F	162	162	167	97	165	121	117	150	177	A A
D2-2-3+F	162	162	167	97	165	121	117	150	177	A A
D2-2-4+F	262	162	104	98	156	121	117	150	177	AE
D2-2-5+F	262	162	104	98	156	121	117	150	177	AE
D2-3-1+F	162	162	167	97	165	121	117	150	177	A A
D2-3-2+F	262	162	115	97	156	121	117	150	177	AF
D2-3-3+F	162	162	115	97	156	121	117	150	177	AF
D2-3-4+F	262	162	115	97	156	121	117	150	160	AI
D2-3-5+F	262	162	115	97	165	121	117	150	177	AF
DD2-5-1+F	1162	1162	196	997	1165	9159	885	1150	1 180	**A**G
DD2-5-2+F	1162	1162	196	997	1165	9159	885	1150	1 180	**A**G
DD2-5-3+F	1161	1161	196	997	1165	1121	896	1150	1180	**D**H
DD2-5-4+F	1161	1161	196	997	1165	1121	896	1150	1180	**D**H
DD2-10-1+F	1162	1162	1165	997	1165	9161	896	1150	1180	**A**J
DD2-10-2+F	1162	1161	1157	996	1165	9121	885	1150	1178	**A**K
DD2-10-3+F	1160	1161	1164	997	1165	990	885	1150	1164	**A**L
DD2-10-4+F	1161	1161	1164	996	1165	1121	185	1150	1177	A**M**
AA2-6-1+F	1161	1161	1164	997	1165	1159	185	1150	1164	**D**N
AA2-6-2+F	1161	1162	1164	997	1165	990	885	1150	1164	**D**O
AA2-6-3+F	1262	1162	1164	997	1165	1121	1117	1150	1177	**D**P
AA2-6-4+F	1161	1161	1164	997	1165	9121	885	1150	1177	**D**Q

The present study established that artificial inoculation of plants with *B. cinerea*allowed moderate re-isolation of the same isolate from the seed collected from the plants despite the fact that seeds from all the plants were sampled for re-isolation. However, *B. cinerea* 2.20 used for inoculation was recovered from the seeds of 22% of first-generation plants. This indicates that inoculation and natural infestation from air borne spores and /or seed infection results in a successful endophytic establishment and systemic spread of *B. cinerea* in the glasshouse grown lettuce plant. *B. cinerea* 2.20, used in the seedling and flower/bud inoculation was recovered from the seed of some flower, and few seedlings inoculated plants. These findings agree with the results of [12] who reported moderate genetic variation within *B. cinerea* isolates collected from fig plants in the same location in California but no differences between different hosts. Similarly [15] found that *B. cinerea* populations from different host's species (Strawberry, dandelion, black current, primula) sampled from different locations of the UK were genetically variable and most importantly highly variable within hosts. Nevertheless, in the study conducted [15] all the isolates recovered from inoculated and uninoculated lettuce plants were identical to the isolate used for inoculation. This indicates that a successful artificial inoculation at high concentrations can eliminate natural infestations and spread within plant populations. Mixed genotype infections are common in the plant pathogen system; however, their impact on the course of the infection and especially on the pathogen virulence and host response to infection are poorly understood.

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The study also establishes that inoculation of the flower/ bud made a significant difference to the infection levels with seed collected from flower/bud inoculated plant having more seed infection (P=0.001) than seed collected from non flower/bud inoculated plant, so flower/ bud inoculation has been successful (SE=0.452). The higher infection of seed collected from flower/bud inoculated plant could be due to the growth stage at which the plants were inoculated. The evidence here are many studies have shown that flower is the major point of entry of infection into the plant. Infection has also been detected in part of crop inflorescence i.e., stylar infection in grapes [16-18]. The fact that *B. cinerea* has been frequently isolated from various parts of crop inflorescence, it may probably be the most common site of entry into the seed and other part of the plants. There is a need to investigate further the growth stage and infection in flower inoculated plant. However, [14] found both seedling and flower inoculated plant to have more seed infection than uninoculated ones. Ochoa and Ellis [18] found that *Fusarium oxysporum* was seed-borne in common naranjilla (*Solanum quitoense*) and can be transmitted from seed to seedling systemically. *Fusarium oxysporum* was isolated from seeds and plant parts, which were surface sterilised before plating. Also [19,20] isolated several fungi from onion seeds and seedlings, which were seed-borne, but only *Aspergillus niger* and *Fusarium oxysporum* were transmitted from seed to onion sets.

Plant grown from sterilized seeds/uninoculated seedling/ flower/bud inoculations, had the highest seed infection, followed by seed collected from fungicide treated plants/inoculated seedlings/ inoculated flower. This indicates that seed fungicide treatment has no effect on seedling or flower/bud inoculations. Least seed infection was recorded in plants grown from non fungicide treated seed/uninoculated seedling and uninoculated flower/bud. The fact that seeds collected from plant grown from non sterilized seed with neither seedling, nor flower/bud inoculations gave rise to infected seeds means that the inoculum is either from the seed or enter from somewhere in the glasshouse. These results suggest that *B. cinerea* could move from one part of a plant to another implying that it is not localised.

A moderate level of genetic diversity was found among isolate recovered from seed collected from plant grown from fungicide treated seeds. It is possible the fungicide treatment eliminated seed borne infection, but the plant can be more frequently attached by airborne inoculum. More importantly, seed collected from plant grown from non fungicide treated plants carried seed borne infection, which systemically spread into the plants and the frequency of these plants being attached by the air borne inoculum was less. This partly supports the result of [7]. They found that 56 *B. cinerea* isolate from different glasshouses in South Carolina (USA) were all genetically different but found some clustering in relation to fungicide sensitivity. They suggested that *B. cinerea* populations were genetically very diverse even in relation to fungicide resistance. However, no fungicide resistance test was carried out in the present research. As reported [13] evidence of systemic infection spreading into plants from seed borne inoculum in lettuce plant. He discovered that isolates from leaves, stem and roots of plants were genetically similar or independent of the various parts. Results of the present study show that re-isolation of the inoculated isolated was successful, which provides evidence of systemic infection.

It was interesting to observe that one of the haplotypes occurred in high frequency among the isolate collected from seed of plants grown from non fungicide treated seed at second generation experiment. Although few air borne conidia may exist in the controlled environment room, clearly the isolates originated from the seed infection.

However, the result of the fragment analysis was somewhat different; the results of microsatellite loci BC1and BC3 at first generation were found to be lower than the length obtained by previous workers [14,20]. Furthermore, the abnormality was again noticed from the results of second generation experiments for the same primers despite the fact that a new set of microsatellite primers were used. The differences might be due to mutation during propagation which might have resulted in the changes of the microsatellite profile.

In conclusion, this study has established that the fungal inoculum used for seedling and flower inoculations has resulted in systemic endophytic infection similar to the endophytic infection of *B. cinerea* previously reported in *Primular polyantha* [2]. As reported from the previous work [21] which confirmed seed as the source of systemic *B. cinerea* infection. Therefore, resultsof the present study showed that infection in seedling grown from infected seed can be transferred into the seed and subsequently to the seed of the next-generation plant without showing symptom a complete endophytic infection. This was shown by re-isolation of the inoculum added to the plant from few seeds isolates of first and second generation plant grown from non sterilized seed at controlled environment room [22-24].

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