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DETECTION AND CHARACTERIZATION OF *BOTRYTIS CINEREA* ISOLATES FROM VEGETABLE CROPS IN EGYPT

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A B S T R A C T

Botrytis cinerea is a necrotrophic plant pathogen that causes plenty of crop losses in Egypt and worldwide. Fifteen isolates of *B. cinerea* were collected from cabbage, pepper and lettuce grown in different locations in Egypt and subjected to investigation. Diversity in phenotypic, pathological and molecular characteristics was detected among isolates, leading to categorizing them into different groups. Molecular variation was demonstrated in all isolates by transposable elements (TEs) analyses. Four TE types, based on the presence or absence of two transposable elements, *boty* and *flipper*, were recognized among *B. cinerea* isolates in which *transposa* type (having both TEs, *boty* + *flipper*) was predominant (40%), while only *boty* and only *flipper* types appeared with distribution values of 26.7 and 20%, respectively and *vacuma* type (Lacking both TEs) showed the lowest distribution value (13.3%). Furthermore, *vacuma* population demonstrated the lowest pathogenic potential comparing to others. A correlation was found between TE type and virulence level of isolate, but no impact of TE type was observed on phenotypic characteristics of *B. cinerea*.

Keywords: Grey mold, Molecular divergence, Transposable elements, Pathogenesis, Vegetable crops.

INTRODUCTION

Botrytis cinerea Pers.: Fr. (teleomorph, Botryotinia fuckeliana (de Bary) Whetzel) is the most common pathogen causing more destruction on mature and senescent tissues (Williamson *et al.*, 2007). This fungal pathogen causes blossom blight, leaf spots/blight, fruit rot, bud rot, damping-off, stem rot/canker, tuber/root rot and bulb rot. When no application of chemical control is adopted (Villa-Rojas *et al.*, 2012), it could cause post-harvest losses, up to more than 40%, in both field- and greenhouse-grown horticultural crops (Pedras *et al.*, 2011).

Worldwide, *B. cinerea* may cause more than $10 billion worth losses annually (Weiberg *et al.*, 2013). *B. cinerea* attacks wounded, weakened or senescent tissues of leafy/fruity vegetables, and also healthy tissues of plants in maturity. Infections may be invisible at harvest, but they may develop rapidly at low temperatures and high relative humidity during storage. On pepper, although grey mold starts in the field, infection appears during storage. Additionally, grey mold of white cabbage (*Brassica oleracea*) causes losses under bad storage conditions and considers as the main disease rapidly colonized by *B. cinerea* responsible for losses of cabbage (Leifert *et al.*, 1993). Moreover, old leaves and damaged stems of lettuce could be good target tissues to be rapidly colonized by *B. cinerea* (Davis *et al.*, 1997). Controlling this pathogen is difficult due to its different infection modes, the ability to produce sexual and asexual spores to survive under favourable and unfavourable conditions and its wide host range. Furthermore, it may elicit noticeable disease symptoms either during pre-harvest period or remain latent to induce later severe symptoms during the post-harvest period (Fillinger and Elad, 2016). The conidia of *B. cinerea* quickly spread by water or wind, but the sclerotia are indispensable for survival under adverse environmental conditions (Brandhoff *et al.*, 2017).

Severe grey-mold occurs in the field after prolonged duration of rain, fog and more dew leading to major crop losses. Furthermore, *Botrytis* blight is probably the most common disease of greenhouse-grown crops where the cool moist weather and lower ventilation are among the most favourable factors for disease development. Likewise, vegetables and fruits could be infected by grey mold during cold storage and shipment if the conditions in the storage house are not suitable for a particular commodity. Genomic variability in terms of fungal morphology (Chardonnet *et al.*, 2000), genomic instability (Dufresne *et al.*, 2006; LÓPez-Berges *et al.*, 2009), pathogenicity (Aboelghar *et al.*, 2019), and ploidy (Buttner *et al.*, 1994) was previously demonstrated. Additionally, different genomic typing of *B. cinerea* was conducted based on restriction fragment length polymorphism (Giraud *et al.*, 1997), microsatellite (Fournier *et al.*, 2002), amplified fragment length polymorphism (Moyano *et al.*, 2003), DNA fingerprinting repetitive sequences (Ma and Michailides, 2005), and transposable elements (Dufresne *et al.*, 2006; LÓPez-Berges *et al.*, 2009). The purpose of the current study was to look for a possible relationship between some distinct characteristics of *B. cinerea* isolates infected different vegetable crops with respect to geographical locations in Egypt.

MATERIALS AND METHODS

Plant samples, fungal isolation & identification: Symptomatic and asymptomatic plant samples were collected from different parts of cabbage (*Brassica oleracea)*, lettuce (*Lactuca sativa*) and pepper (*Capsicum annuum*) which were grown in different locations in Egypt during the season of 2015-2016 (Table 1). All samples were cultured on the selective medium, m1KERS, previously developed by Abdel Wahab and Helal (2013) for *Botrytis* isolation and then identified microscopically.

Table 1. Collection of *Botrytis cinerea* isolates from different vegetable crops.

Host Plant	Plant Part	Location	Isolate code
Cabbage	Leaf	Kafr El-Sheikh	BCC1 - BCC5
Lettuce	Leaf	Qalyubia	BCL100 - BCL103
Pepper	Fruit	EL-Beheira	BCP1- BCP7

Phenotypic characteristics of *B. cinerea* isolates: Cultural and morphological characteristics of *B. cinerea* isolates were determined using Czapek's Dox medium. Mycelial agar plug (6 mm in diameter) was cut out from each colony margin of 4 days old culture using a sterilized cork-borer and transfered to the surface of the medium at the center of a 9 cm- diametere Petri plate. Three replicates of each isolate were prepared, and then incubated at 23 °C for seven days. Conidial dimension of each isolate was measured according to the methodology describe by Shirane *et al.* (1989) at 40X objective of the light microscope (Olympus microscope, BH2) and calculated using the following formula: Conidial volume (μm^3) = $L.W^2.\pi/6$, where W = conidial width (µm), L = conidial length (µm) and π= 3.14159. Isolated sclerotia were also described for their shape, size, and number per plate. Mycelial growth rate (MGR) of each isolate culture was determined by cutting out mycelial plug (6 mm diameter) from the colony margin and placing it onto the surface of potato dextrose agar (PDA medium) at the center of the Petri plate. Three replicates were used for each isolate. Plates were then incubated at 23 °C for three days and the MGR was measured on a daily basis.

Molecular identification of *B. cinerea* isolates: Genomic DNA of each isolate was extracted according to Möller *et al.* (1992). Molecular identification was done by PCR using the specific primers, C729F and C729R (Rigotti, 2002) and the ITS primers, ITS1 and ITS4 (White *et al.*, 1990) (Table 2). PCR was performed in 25 µl of total reaction mixture made up of 2 µl genomic DNA (50 ng/µl), 0.5 µl of each primer "Bio-search Technologies" (10 µM), Red PCR master mix (Bio-line) (12.5 µl) and 9.5 µl H_2O, using the thermocycler, Techne-Progene. The amplification condition was as follows: Initial denaturation for 4 min at 94 °C followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, extension for 1 min at 72 °C and terminated with a final extension for 10 min at 72 °C.

Molecular typing of *B. cinerea* isolates using Transposable Elements (TEs): The two transposable elements, *Boty* and *Flipper*, were detected using specific primers for each according to Diolez *et al.* (1995) and Levis *et al.* (1997), respectively (Table 2). PCR was

carried out in a total volume of 25 µl reaction mixture consisting of 2 µl genomic DNA (50 ng/ µl), 0.5 µl of each primer "Bio-search Technologies" (10 µM), 12.5 µl of Red PCR master mix (Bioline) and 9.5 µl of H_2O. Amplification was done in a thermocycler (Techne-Progene) using the following programme: an initial denaturation at 95 ˚C for 5 min, followed by 40 cycles of denaturation for 1 min at 94 ˚C, annealing during 1 min at 68 ˚C, extension for 1 min (for *boty* primers) or 3 min (for *flipper* primers) at 72 ˚C, and terminated with a final extension for ten min at 72 ˚C.

Table 2. The primers used for molecular identification and characterization of *Botrytis cinerea* isolates.

Primer	Primer sequence, 5' to 3'	Amplicon size (bp)	Reference
ITS1	TCC GTA GGT GAA CCT GCG G	509	White *et al.* (1990)
ITS4	TCC TCC GCT TAT TGA TAT GC		
C729F	CTGCAATGTTCTGCGTGGAA	700	Rigotti (2002)
C729R	AGCTCGAGAGAGATCTCTGA		
BotyF4	CAG CTG CAG TAT ACT GGG GGA	510	Diolez *et al.* (1995)
BotyR4	GGT GCT CAA AGT GTT ACG GGA G		
Flipper F300	GCA CAA AAC CTA CAG AAG A	1250	Levis *et al.* (1997)
Flipper F1550	ATT CGT TTC TTG GAC TGT A		

Artificial infection test of *B. cinerea* isolates: Pathogenicity test was conducted using detached lettuce leaves (*Lactuca sativa* L. cv. 'Baladi'). Three replicates were used of leaves taken from the central part of lettuce heads and placed in a moist sterilized plastic box. Discs from the colony margin of each isolate culture aged seven days were cut out using a sterilized cork-borer (6 mm diameter) and placed with the mycelial side facing the lettuce leaf surface. The covered plastic boxes were incubated under humid conditions at 23 °C for three days. The degree of virulence was recorded daily by measuring lesion diameter around each plug. This test was repeated twice.

Statistical analysis: Results were analyzed statistically by analysis of variance (ANOVA) to determine the significance in differences within phenotypic and pathogenicity assays. Data means were analyzed at *P* .05 level using the least significant difference test.

RESULTS

Phenotypic variability of *B. cinerea* isolates collected from vegetable crops: Morphological characteristics of *B. cinerea* showed diversity among isolates (Table 3). The growth texture of isolates was classified into three classes: light warty, fluffy, and cottony (Figure 1). Whereas all conidia were described as ovate, their measurements were significantly varied in dimensions, ranging from 10.7 to 12.6 µm in length, 7.3 to 8.8 µm in width and 297.6 to 498.3 μm^3 in volume. (Table 3). Furthermore, morphological diversity of sclerotia was found in their size, distribution pattern, and number per plate. The size and distribution pattern of the sclerotia were described as either large irregularly (Figure 2A) or numerous small and scattered (Figure 2B) in the culture plate. The shape of all sclerotia was cerebriform (Table 3, Figure 2C), while their number varied among isolates and ranged from 21 to 65 sclerotia /plate. Moreover, mycelial growth rate (MGR) showed significant variation which ranged from 1 to 2.9 cm/d (Table 4).

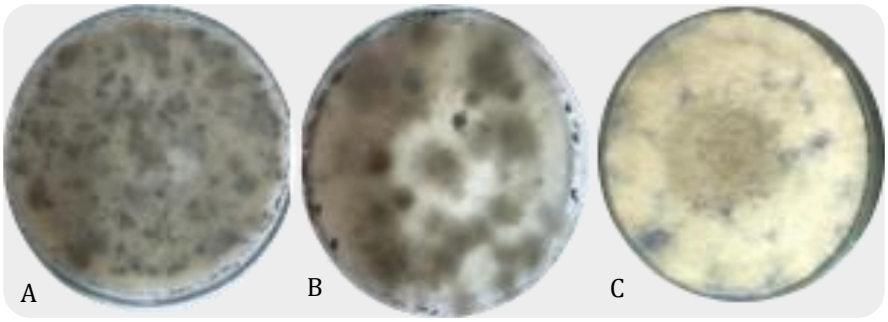


Figure 1. Morphological characteristic of mycelial growth texture, (A) Light warty, (B) Fluffy, (C) Cottony.

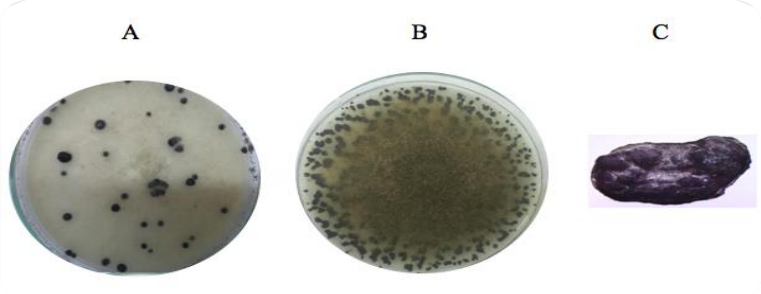


Figure 2. Sclerotial distribution of different *Botrytis cinerea* isolates in plates. Two sclerotial patterns: large irregularly shape (A), numerous small and scattered (B), sclerotial shape is cerebriform (C).

Table 3. Phenotypic characterization of *Botrytis cinerea* isolates collected from different vegetable crops.

Isolate	Growth texture	Sclerotial shape	Distribution pattern of sclerotia	No. of sclerotia / plate*	Conidial shape	Dimensions of conidia (µm)**		
						Length	Width	Conidial volume (µm3)
BCC1	Cottony	Cerebriform	Large-irregularly	24±6.8	Ovate	10.8±0.8	7.9±0.7	358.6±59.6
BCC2	Cottony	Cerebriform	Large-irregularly	21±5.1	Ovate	11.2±0.9	8.2±1.2	404.6±109.5
BCC3	Cottony	Cerebriform	Large-irregularly	27±6.7	Ovate	10.8±0.8	7.9±0.7	358.6±59.6
BCC4	Cottony	Cerebriform	Large-irregularly	23±4.2	Ovate	12.1±0.7	8.7±0.8	475.4±84.6
BCC5	Cottony	Cerebriform	Large-irregularly	23±5.3	Ovate	10.7±0.5	7.3±0.6	297.6±44.9
BCL100	Light warty	Cerebriform	Large-irregularly	56±5.9	Ovate	10.8±0.8	7.7±0.6	335.8±69.3
BCL102	Light warty	Cerebriform	Large-irregularly	59±6.4	Ovate	11.9±0.9	8.1±0.8	413.7±93.7
BCL103	Light warty	Cerebriform	Large-irregularly	64±6.1	Ovate	11.9±0.8	8.2±0.9	425.1±109.9
BCP1	Fluffy	Cerebriform	Large-irregularly	57 ±12.7	Ovate	11.7±0.6	8.2±0.6	413.5±60.2
BCP2	Fluffy	Cerebriform	Large-irregularly	57±9.5	Ovate	11.9±0.5	8.3±0.6	426.5±56.4
BCP3	Fluffy	Cerebriform	Large-irregularly	62±7.0	Ovate	11.2±0.7	8.5±0.5	424.8±58.5
BCP4	Fluffy	Cerebriform	Large-irregularly	65±7.0	Ovate	10.8±0.5	7.3±0.6	298.9±43.3
BCP5	Fluffy	Cerebriform	Large-irregularly	54±5.7	Ovate	12.3±0.8	8.8±0.8	498.3±97.9
BCP6	Fluffy	Cerebriform	Large-irregularly	62±7.2	Ovate	12.0±0.7	8.4±0.8	450.9±77.2
BCP7	Fluffy	Cerebriform	Large-irregularly	59±5.0	Ovate	12.6±0.5	8.4±0.8	469.5±106.7

*All data are means of triplicate measurements± standard deviation (SD) at L.S.D.$_{.05}$

** All data are means of 20 conidial measurements± standard deviation (SD) at L.S.D.$_{.05}$

Molecular identification of *B. cinerea* isolates using PCR: The confirmation of morphological identification was investigated by amplifying the ITS1-5.8S-ITS4 region of all isolates using the primer pair, ITS1 and ITS4 which produced an amplicon with the expected molecular length of approximately 509 bp (Figure 3).

While, the specific primers for *B. cinerea* (C729F/C729F) designed by Rigotti (2002), amplified an amplicon of 700 bp long (Figure 4).

Pathological and TE typing of *B. cinerea* isolates: Virulence variation was revealed by the pathogenicity assay which was conducted on fifteen B. cinerea isolates collected from cabbage, lettuce and pepper using the detached lettuce leaf technique (Table 4). Isolates showed statistically significant variation in terms of lesion diameter which ranged from 0.4 to 4.4 cm and the degree of virulence was thus divided into three categories (Figure 5), namely, highly virulent with a lesion diameter ranging from >2.5 to 4.4 cm (53.3%), moderately virulent with a lesion diameter from >1 to 2.5 cm (33.3%) and low virulent with a lesion diameter ranging from 0.4 to 1 cm (13.3%). The detection of TEs of all *B. cinerea* isolates was done by the specific primers: Flipper F300 & Flipper F1550 to amplify *flipper,* or BotyF4 & BotyR4 to amplify *boty* element and demonstrated four types of TEs (Table 4). PCR revealed 510 bp for *boty* element in 10 out of 15 isolates tested (Figure 6A). Whereas, for *flipper* detection, PCR generated 1250 bp in 9 out of 15 isolates tested (Figure 6B). Six isolates demonstrated the two TEs (*transposa* type: *boty* and *flipper*), confirmed that *transposa* isolates were predominant (40%) among *B. cinerea* populations which were collected from various plant organs of different vegetable crops. Whereas, four isolates have only *boty* element (*boty* type, 26.7%), three have only *flipper* element (*flipper* type, 20%) and two have neither of them (*vacuma* type, 13.3%).

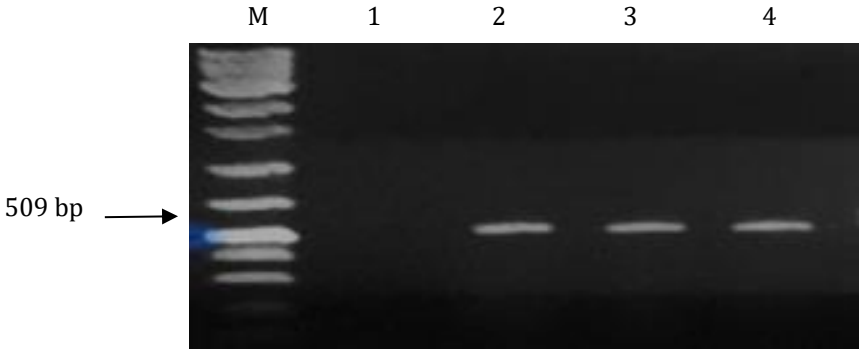


Figure 3. PCR amplification product of 509 bp using the primers ITS1 and ITS4. Left lane (M), represents 1Kbp DNA ladder marker. 1, negative control, a reaction mixture lacking DNA template. The 2, 3 and 4 lanes correspond to *Botrytis cinerea* isolates (BCL102, two positive controls).

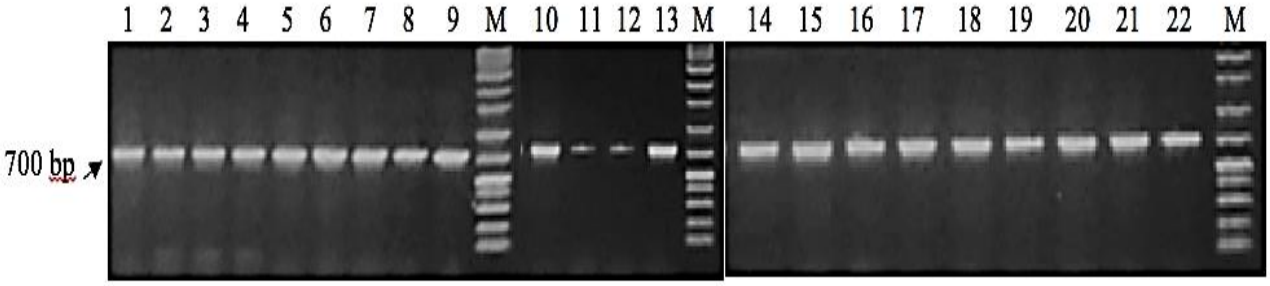


Figure 4. PCR amplification product of 700 bp using *Botrytis cinerea* specific primers. Right lane (M): 1Kbp DNA ladder marker; lanes 1 to 5 are loaded with samples corresponding to cabbage isolates, BCC1 to BCC5; lanes 6 to 9 correspond to positive controls; lanes10 to 12 represent lettuce isolates, BCL101 to BCL103; lanes13 correspond to the positive control; lanes 14 to 20 stand for pepper isolates, BCP1 to BCP7; lanes 21 and 22 correspond to positive controls.

Figure 5. Three virulence categories of *Botrytis cinerea* on detached lettuce leaves. A, highly virulent; B, moderately virulent; C, low virulent.

Table 4. Pathological, mycelial growth rate and molecular characteristics of *B. cinerea* isolates from vegetable crops.

Isolate	MGR (cm/d)*	Lesion diameter (cm)**	TE type***
BCC1	2.9±0.3a	3.4±0.3a	T
BCC2	2.9±0.3a	2.5±0.3b	F
BCC3	1.9±0.7b	0.4±0.0d	V
BCC4	1.9±0.6b	2.6±0.1b	B
BCC5	1.0±0.1c	2.2±0.3c	T
BCL101	1.5±0.2b	3.3±0.3b	F
BCL102	1.5±0.2b	1.5±0.4c	B
BCL103	1.6±0.4b	1.5±0.2c	B
BCP1	2.2±0.6b	3.2±0.1b	F
BCP2	1.9±0.5b	3.7±0.3a	T
BCP3	1.5±0.1b	4.4±0.2a	T
BCP4	1.9±0.9b	3.6±0.1a	B
BCP5	1.9±0.9b	3.8±0.1a	T
BCP6	2.1±0.3b	1.8±0.2c	T
BCP7	1.9±0.7b	0.5±0.1d	V

*MGR, mycelial growth rate. Data followed by the same letter are not statistically different. **all data are means of triplicate measurements ± standard deviation (SD) at L.S.D.$_{.05.}$ ***transposable elements type: T, *transposa*; F, *flipper*; B, *boty*; V, *vacuma*.

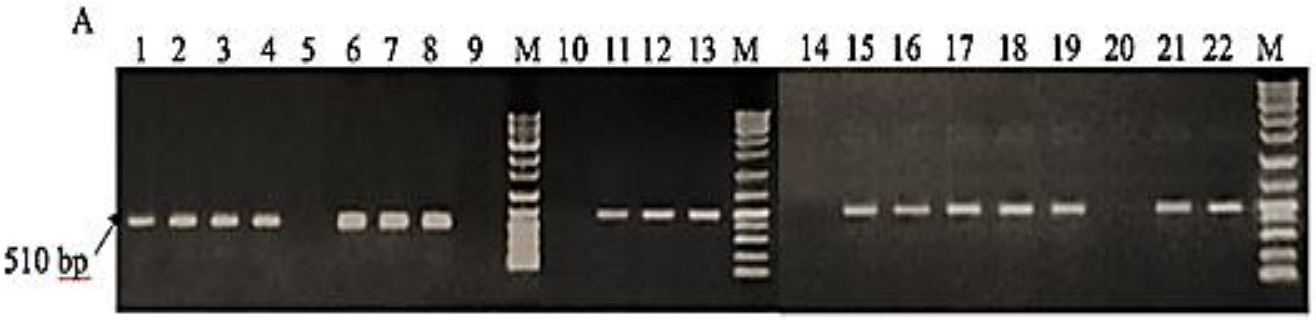
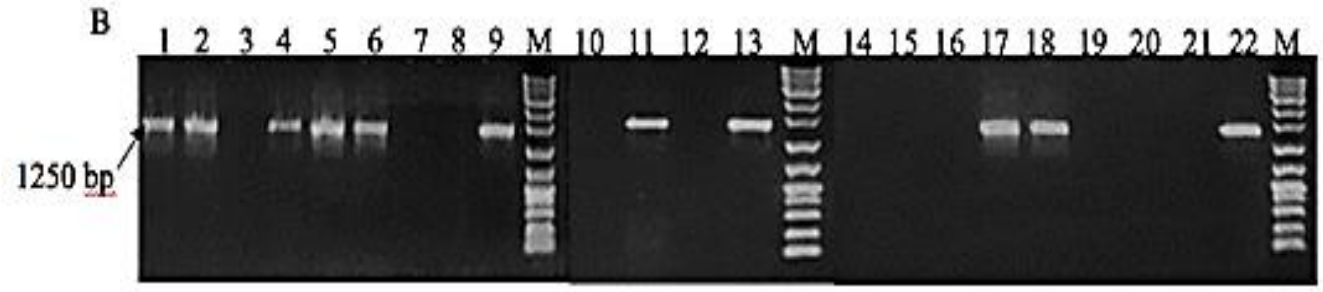


Figure 6. PCR amplification using TE specific primers in 15 isolates of *Botrytis cinerea.*
A, PCR product of 510 bp corresponding to *Boty* element. Right lane (M), 1Kbp DNA ladder marker in each gel; lanes1 to 5 correspond to cabbage isolates (BCC1 to BCC5); lanes 6 to 8, the positive controls; lane 9: the negative control; Lanes 10 to 12 represent lettuce isolates (BCL101 to BCL103); lane 13 corresponds to the positive control; Lanes 14 to 20 correspond to seven pepper isolates (BCP1 to BCP7); lanes 21 and 22 represent the positive controls.



B, PCR amplification product of 1250 bp representing the *Flipper* element. Right lane (M) in each gel, represents 1Kbp DNA ladder marker; lanes1 to 5 correspond to cabbage isolates (BCC1 to BCC5); lanes 6 and 9, the positive controls; lanes 7 and 8, the negative controls; lanes 10 to 12 represent lettuce isolates (BCL101 to BCL103); lane 13 corresponds to the positive control; lanes 14 to 20 correspond to seven pepper isolates (BCP1 to BCP7); lanes 21 and 22 represent the negative and positive control, respectively.

DISCUSSION

Symptomatic and asymptomatic samples collected from the tested vegetable plants, cabbage, lettuce and pepper revealed their infection with *B. cinerea,* which showed a phenotypic diversity among isolates regardless the location and host plant. Such divergence was observed in cultural characteristics like mycelial texture, mycelial growth rate, conidial dimensions and sclerotial characteristics such as size, pattern and number per plate. All conidia observed were oval as previously recorded with *B. cinerea* isolates collected from grape and strawberry (Wagih *et al.*, 2019). Although the same sclerotial shape (cerebriform) was observed in all *B. cinerea* isolates collected from the vegetable crops under study, the divergence in sclerotial shape was previously found among strawberry isolates (Wagih *et al.*, 2019). Similarly, variability in virulence was detected when isolate aggressiveness was tested, but no relationship was found between isolate virulence and location/host plant. Interestingly, the isolates collected from lettuce like those collected from cabbage and pepper demonstrated different virulence levels and indicated that there is no host preference for grey mold infection by *B. cinerea* isolates even if the re-inoculation was done on the same original host plant. Molecular analysis showed various distribution values of TEs depending on TE type in agreement with those previously reported (Abdel Wahab, 2015; Wagih *et al.*, 2019). However, this investigation contradicts other documented studies (Samuel *et al.*, 2012; Kumari *et al.*, 2014). *Transposa* type demonstrated predominance (40%) and this was in agreement with what was reported before (Esterio *et al.*, 2011), followed by *boty* type (26.7), *flipper* type (20%) while *vacuma* type being the lowest distribution value (13.3%) in this respect. A clear correlation was found between virulence and *vacuma* isolates which possess low pathogenic potential as previously reported (Martinez *et al.*, 2005; Muñoz *et al.*, 2010; Schilling *et al.*, 2013; Kumari *et al.*, 2014; Wagih *et al.*, 2019). Future efforts should, therefore, be focused on investigating the effect of host plant on the severity level of grey mold using the same isolate type. The TE/virulence relationship should also be studied through the host-pathogen interaction.

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