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Effect of temperature on the morphological characteristics of *Botrytis cinerea* and its correlated with the genetic variability

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PEER REVIEW

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Comments

This is a good work, the authors take a focus on the research to evaluate the establish relationships between the morphological characteristics, pathogenesis and resistance to fungicide-iprodisone, and its correlated with the genetic variability according presence or absence of transposable elements.

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ABSTRACT

Objective: To study the effect of temperature on the morphological characteristics of *Botrytis cinerea* (*B. cinerea*) and its correlated with the genetic variability. *B. cinerea* is a plant-pathogenic fungus that produces the disease known as grey mould in a wide variety of agriculturally important hosts in many countries.

Methods: Six strains from different host collected have been isolated and characterized by several methods as mycelial growth, fungicide resistance, pathogenicity and the effects of the temperature. Also was analyzed by PCR and distinguished by the presence or absence of transposable elements.

Results: Results showed that clear morphological differences exist between strains at the temperature of 4, 12 and 28 °C. All strains analyzed molecularly were classified as Group II (transposa-type). Demonstrating a negative correlation between mycelial growth and other characteristics as the fungicide resistance and pathogenicity. Lastly, it is difficult to establish relationships phenotypic and genotypic between strains of *B. cinerea*.

Conclusions: The results indicated that the mycelial growth, resistance at fungicide and pathogenicity are independent of the characteristics molecular, however, are dependent of a factor such as temperature.

KEYWORDS

Botrytis cinerea, Transposable elements, *Boty* and *flipper*, Temperature, Mycelial growth, Pathogenicity, Resistance to fungicide-iprodisone

1. Introduction

Botrytis cinerea (*B. cinerea*) Pers. Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a phytopathogenic fungi responsible for grey mould, which is an important disease in more than 200 species of fruits, vegetables, ornamental crops and affecting a great number of organs[1]. Grey mould, cause important economic losses

before and after the harvest[2], worldwide losses from this fungus account for 20% of the harvest of the affected crops, and the cost is estimated at 10–100 billion euros per year. The market size for anti-*Botrytis* products has been 15–25 million US dollars in recent 3 years[3]. An important biological feature is that the process of infection and growth by *B. cinerea* is often associated with prior colonization of dead or dying plant debris as a nutrient-

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providing saprophytic base[4]. There have been few reports on the effects of environmental factors like water activity, temperature and the influence of solutes on *B. cinerea* growth under conditions *in vitro*[5]. Being that the environmental conditions favorable for mold growth gray is temperature and water free[6]. *B. cinerea* presents cardinal temperatures optimal between 15–25 °C[7], nevertheless, the pathogen is also active at lower temperatures since conidium germination and mycelial growth can occur at temperatures as low as 0 °C. Under favourable conditions, a complete infection cycle can occur in 3 to 4 d depending on the type of host tissue attacked. Also required a relative humidity of >95% for disease development. It has been shown in previous experiments that high humidity is required for germination of conidia and infection of the host[8]. The use of synthetic chemicals as fungicides is the primary method of the control of *B. cinerea*. Fungicides can provide disease control through both pre- and post-infection activity. Pre-infection activity is commonly known as protectant (preventive) activity and post-infection activity comprises a curative action that can involve both pre- and post-symptom expression activities[9]. Gray mould is difficult to control satisfactorily with fungicides because the fungus is genetically variable and has developed strains resistant to many of the chemicals introduced in the last 20 years. Chemical control of diseases caused by *B. cinerea* has largely depended upon the use of fungicides: benzimidazole and dicarboximide such as iprodione. However, today it is common to detect dicarboximide-resistant *B. cinerea* isolates[10]. In addition, there are also pressures from consumers to reduce pesticides in the food chain. For these reasons, there is an increased interest in alternatives to fungicides for disease control[10]. Biological control can be used as an alternative to synthetic fungicides, employs antagonistic microorganisms that protect fruits, vegetables and ornamental crops from infection of the pathogen. Nevertheless, it appears that the control of the disease with antagonistic microorganisms is not often as consistent as chemical control[11].

B. cinerea is a haploid and heteroallelic ascomycete[12]. Analysis genetic such as RFLP-PCR[13,14], ARMS-PCR[15], allele-specific PCR[16], RAPD-PCR[4], nested PCR, and detection transposable elements (TEs)[13], amongst other molecular typing techniques, have characterized to *B. cinerea*. They have molecular analysis identify two groups based on the presence or absence of two TEs in the genome of *B. cinerea*, *vacuma* (isolates without either TEs) and *transposa* (isolates with the TEs *boty* y *flipper*)[13].

The TEs are mobile, repetitive DNA sequences of genomes that can alter the sequence, expression and chromosome rearrangements of genes. These changes have the potential to cause wide-ranging genetic variation, giving populations the flexibility to adapt successfully to environmental conditions, particularly in species that do not have a sexual phase[3]. TEs are grouped into two classes by their transposition intermediate: class I or retrotransposons (RNA) and class II or DNA transposons (DNA)[3].

Transposable element *boty* is a retrotransposons, contains 596 bp long terminal repeats and one internal putative *gag pol* gene that encodes a polyprotein with sequences homologous to the reverse transcriptase and rnaase H

domains of retroelement *pol* genes. Transposable element *flipper* is a transposons, contains 1842 bp long with perfect inverted terminal repeats of 48 bp and an open reading frame encoding a transposase[3]. Recent studies of DNA polymorphism of genes of isolates of *B. cinerea* have grouped them in two phylogenetic species groups. Group I strains belonged exclusively to the *vacuma* type, whereas Group II strains included both *vacuma* and *transposa* types[4,12]. The phylogenetic analyses between Group I and Group II have shown differences in morphology, phenology and host range but has not identified any differentiation between *vacuma* and *transposa* strains within Group II[17]. The aim of this work was to evaluate the possibility of establish relationships between the morphological characteristics as the mycelial growth, pathogenicity and resistance to fungicide-iprodione, at three different temperatures of incubation and its correlated with the genetic variability according to presence or absence of TEs (*flipper* and *boty*).

2. Materials and methods

2.1. Microorganisms

B. cinerea strains recovered from apple and grape fruits were used in this study. The isolates were maintained on potato dextrose agar (PDA), containing 200 g boiled potato, 20 g glucose and 20 g agar-agar in 1 L of distilled water, added with 0.012 g of cloranfenicol and 0.5 mL lactic acid. For conidial production, *B. cinerea* was grown on PDA at (28±1) °C during 7 d; isolates were maintained on PDA at 4 °C[10].

2.2. DNA extraction, PCR and Molecular analyses of the subpopulations

For the molecular determination of the subpopulations of *B. cinerea*, was extracted Genomic DNA following the procedure described by Möller *et al.* (1992)[18]. Mycelia were collected from 7-day-old *B. cinerea* cultures grown at (28±1) °C in the dark. The yield and integrity of the DNA were checked by agarose gel electrophoresis. The genomic DNA was used as a template for the PCR reactions.

PCR amplification was performed using primers for the molecular marker of the ribosomal region 18S (ribosomal intergenic spacer, IGS), transposable element *flipper* and *boty* previously described by Muñoz *et al.* (2008). All the primers that were used, their annealing temperatures, and the sizes of the amplicons are listed in Table 1. Each PCR mixture contained 6 µL of primers, 2.5 µL of dNTP, 2.5 µL of DNA, 2.5 µL of Mg²⁺ and 0.5 µL of Taq polymerase in a total volume of 50 µL. Finally, the resulting products were analyzed on agarose gel 2%, stained with ethidium bromide and then were observed under UV light. The reactions repeated three times for each PCR reactions.

Table 1
PCR specifications and conditions.

Target	Primer Sequence	First	Number of cycles	Temperature	Final elongation step	Product size
		denaturation step		Annealing		
IGS	ATG AGC CAT TCG CAG TTC	4 min at 94 °C	35	59 °C	4 min at 72 °C	2941 pb
<i>flipper</i>	GCA CAA AAC CTA CAG AAG A	4 min at 94 °C	35	60 °C	4 min at 72 °C	1230 pb
<i>boty</i>	CCC AAT TTA TTC AAT GTC AG	4 min at 94 °C	35	60 °C	4 min at 72 °C	630 pb

2.3. Mycelial growth assays in PDA

A cut 4 mm mycelial disc from a 3-day-old culture was placed in the center of each plate with PDA. The diameter of each mycelial growth was measured at 7 d at the temperatures of 4, 12 and 28 °C, in three perpendicular directions without opening the Petri dishes, and the mycelial area was calculated. The results were expressed in square centimeters. Three replicates of each isolate were analyzed.

2.4. Pathogenicity test in apple

Six batches each with 5 red delicious apples were used, harvested at commercial maturity, selected for uniformity of size and any apparent injury or infection was removed. The fruit were washed with non ionic detergent, were rinsed with tap water and allowed to air dry at environmental. To determine the uniformity of maturity of apples was determined the firmness with a penetrometer. The force (N) was defined as firmness. The firmness was measured in apples ten at random, three times on different sides in each apple. Fruits were wounded (3 mm×3 mm×3 mm) with a punch on two opposite sides of each fruit, and inoculated with 20 µL of each conidial suspension (10⁶ conidia per mL) and 20 µL of water as control[19]. After 7 d of incubated at temperatures of 4, 12 and 28 °C, by measuring the diameter of the decayed area as the mean of its width and length and computing the total area of disease. Areas of disease were analyzed by analysis of variance. Each experiment was performed in triplicate.

2.5. Test fungicide sensitivity

All isolates were screened for resistance to iprodione at different temperatures in PDA amended with iprodione. The chemical, iprodione “Rovral 50 WP” (Bayer Crop Science, AG Leverkusen, Germany).

Inhibition of mycelial development was assessed by measuring the radial growth and area is calculated, on PDA containing in a range of growing concentrations of fungicide. Concentrated stock solution was prepared progressively diluted in sterile distilled water to appropriate concentrations levels for the assay of four different fungicide concentrations at 100, 500, 1000 and 2000 mg/L. In controls sterile distilled water was used instead of the suspension of fungicide. A cut 4 mm mycelial disc from a 3-day-old PDA culture was placed in the center of each plate. The dishes were incubated for 7 d at 4, 12 and 28 °C. Colony diameters were measured in three perpendicular directions without opening the Petri dishes and the inhibition of mycelial growth was calculated on the basis of the area in compared with controls: % inhibitions= $[(\phi \text{ control} - \phi \text{ treatment}) / \phi \text{ control}] \times 100$. The minimum dose of fungicide required for inhibiting mycelial growth (MID) was determined as the concentration of fungicide at which 100% inhibition of mycelial growth. Isolates that were able to grow actively on PDA amended, with a MID superior at 0.5 g/L (500 mg/L) were considered resistant to iprodione. The experiment was repeated twice.

3. Results

3.1. Molecular analyses of the subpopulations

All were analyzed strains for evaluated in determining presence or absence of TEs, *boty* and *flipper*; the amplified PCR products showed the expected size for all the targets tested. Five of the strains contain both the *boty* and *flipper* TEs, while one strain contains only the *boty* transposable element.

In the strain B7, it only contains the *boty* element (Figure 1), while strains BNM 0527, BNM 0528, B8, B9 and B10 presented both the *boty* and *flipper* elements shown in Figure 1.



Figure 1. Lane 1–5: marker IGS, Lane 2–6: *boty* transposable element (strains B7, BNM 0527, BNM 0528, B8, B9 and B10), Lane 4: standard molecular weight marker, line 3–7: *flipper* transposable element (strains BNM 0527, BNM 0528, B8, B9 and B10).

Esterio, *et al* (2007), classified as a different variant those strains possess a single transposable element, however, in our case we have classified the strains in the transposon-type subpopulation according to Giraud, *et al* (1997), none of the strains, turned out to belong at the vacuma-type subpopulation[20,21]. According to classification between phylogenetic species groups, all strains analyzed in this study were classified as Group II as proposed by Fournier, *et al*(2005)[22]. (According to the most recent proposed classification) none of the strains, turned out to belong to the vacuma-type subpopulation.

3.2. Mycelial growth in vitro

The study about the effect of the temperature on the mycelial growth of *B. cinerea*. The growth of the isolates of *B. cinerea* on PDA and its origins are shown in Table 2. The results showed a markedly reduce of the mycelial growth area at 4 °C compared with the temperatures of 12 and 28 °C. It can be seen that the strain B7 revealed significant effect ($P < 0.05$) in mycelial growth area at a temperature of 4 °C compared with the other strains. At the temperature of 12 °C, it was shown that strains: BNM 0527, B7 and B10 presented

significant value effect ($P<0.05$) in the same way that the B9 strain at the temperature 28 °C in compared with the other strains. The B7 strain was characterized by presenting the greatest growth area at the temperatures of 4 and 12 °C (11.22 and 64.51 cm² respectively), with exception of the temperature of 28 °C, where B9 strain presented greatest growth area (66.72 cm²). The smallest mycelial growth area are presented in the strains: BNM 0528 (3.04 cm²) and BNM 0527 (3.06 cm²) at 4 °C, B8 (36.35 cm²) and BNM 0528 (41.63 cm²) at 12 °C and 28 °C, respectively. Figure 2 is observed the effect of temperature in mycelial growth areas.

Table 2

Comparison of the mycelial growth and rot isolates of *B. cinerea* at different temperatures of incubation.

Isolates	Host	Mycelial growth areas (cm ²) ^a			Areas of rot (cm ²) ^b		
		4 °C	12 °C	28 °C	4 °C	12 °C	28 °C
BNM 0527	apples	3.06 ^a	62.11 ^c	48.62 ^b	0.65 ^{ab}	5.38 ^d	18.74 ^f
BNM 0528	apples	3.04 ^a	49.07 ^c	41.63 ^a	0.70 ^{bc}	4.70 ^b	15.98 ^e
B7	grape	11.22 ^c	64.51 ^e	47.83 ^b	1.14 ^c	8.68 ^e	14.84 ^d
B8	grape	3.37 ^a	36.35 ^a	56.71 ^c	0.98 ^d	3.89 ^c	11.18 ^d
B9	unknown data	3.33 ^a	43.92 ^b	66.72 ^d	0.59 ^a	1.79 ^a	4.62 ^a
B10	grape	4.00 ^b	56.04 ^d	48.23 ^b	0.72 ^c	8.48 ^e	7.05 ^b

^aAverages mycelial growth areas of *B. cinerea* on potato dextrose agar.

^bAverages areas of rot in five fruit per treatment were used (15 inoculation sites).

Values in the same column followed by the same letter are not significantly different ($P=0.05$). Three replicates of each isolate were analyzed.

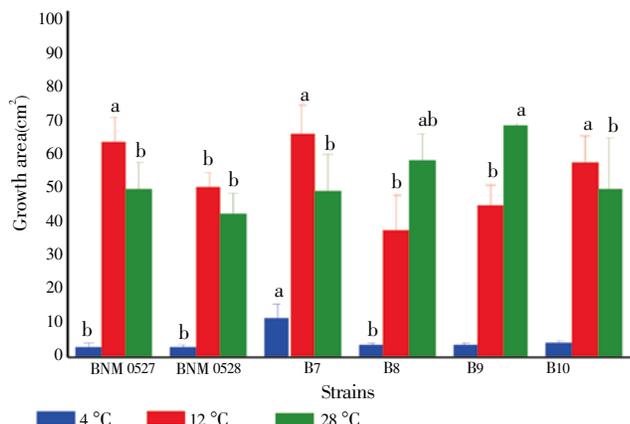


Figure 2. Effect of temperature in mycelial growth areas *in vitro*, showed a significant effect ($P<0.05$) at different temperature.

Favourable temperatures for *B. cinerea* was between 12–28 °C and limiting temperatures a 4 °C. Values in the same column followed by the same letter are not significantly different.

3.3. Pathogenicity

The pathogenicity of each isolate, expressed as the area of rot, was tested on apple at three temperatures of incubat (4, 12 and 28 °C). Firmness values of fruit were between 3.3 and

3.8 N. The results of rot were expressed in square centimeters of disease, 7 d after inoculation. There were also significant differences ($P<0.05$) in virulence between the *B. cinerea* strains. All strains at the temperature of 28 °C were more virulent, except the B10 strains are more virulent at 12 °C. Strains B7 was more virulent at 4 and 12 °C with the values of 1.14 and 8.68 cm², respectively, while at the temperature 28 °C, the strain BNM 0527 present the largest disease area with 18.74 cm². Strains B9 was less virulent at the three temperatures with values of 0.59 (4 °C), 1.79 (12 °C) and 4.62 cm² (28 °C). The areas of diseases are shown in Table 2.

The most pathogenic strains colonized more than 70% of the apples surface at 28 °C, whereas the weaker one colonized less than 2.5% of the surface at 4 °C (Figure 3).

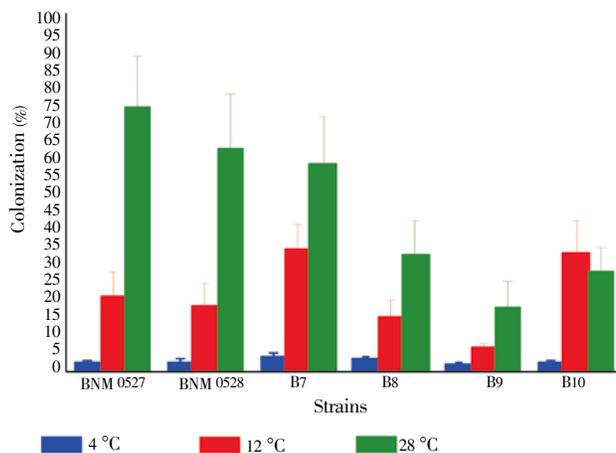


Figure 3. Greater colonization at temperature of 28 °C and after a reducing to 12 and 4 °C.

3.4. Fungicide sensitivity of isolates

The effects on mycelial growth caused by treatment with fungicide iprodione at the temperatures of 4, 12 and 28 °C showed different behavior. Resistance levels were different for the same strain at different temperatures. It was observed that the iprodione concentration increased along with the increase of the percentage of inhibition, which is observed the effect of fungicide clearly. The isolates BMN 0527 and B7 were considered resistant the fungicide at temperature 28 °C and sensitive at 4 and 12 °C. Also the isolated B10 has the same behavior but was considered resistant at 12 and 28 °C and sensitive 4 °C. Isolated BMN 0528 was considered highly resistant at iprodione because it was not presented the minimum concentrations of the fungicide required to inhibit mycelial growth (MID) at all the temperatures of incubations. The concentrations of iprodione required to cause the 100% inhibitions of mycelia growth are show in Table 3.

Table 3

Inhibition mycelial areas by iprodione at 4, 12 and 28 °C(%).

Isolates	4 °C				12 °C				28 °C			
	0.1 g/L	0.5 g/L	1.0 g/L	2.0 g/L	0.1 g/L	0.5 g/L	1.0 g/L	2.0 g/L	0.1 g/L	0.5 g/L	1.0 g/L	2.0 g/L
BMN 0527	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	97.20	99.14	99.25	100.00
BMN 0528	11.67	64.14	79.92	86.42	88.91	94.81	94.93	97.08	88.51	84.02	84.07	91.11
B7	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	95.71	97.63	100.00	100.00
B8	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	98.47	100.00	100.00	100.00
B9	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	93.99	100.00	100.00	100.00
B10	100.00	100.00	100.00	100.00	92.15	94.87	97.01	96.84	87.99	91.71	95.23	96.33

% inhibition of mycelial growth was calculated on the basis of the area in compared with controls. Data were pooled from two experiments.

4. Discussion

B. cinerea is an important phytopathogenic fungus, haploid and heteroallelic ascomycete^[12,20–22] with a large amount of genetic variability^[22]. In this study we evaluated six *B. cinerea* strains and demonstrated that isolated from the same or different origins that presented different characteristics, where it was found that all of the isolates studied belong to the Group II (transposon-type) based on the presence or absence of two TEs (*boty* and *flipper*). Modeling of *B. cinerea* growth was carried out on the basis of the growth data obtained on PDA medium, where the results from the present study showed that mycelial growth was differently affected by temperature. The range of favourable temperatures for *B. cinerea* studies was between 12–28 °C, where strains showed biggest growth areas compared with the temperature of 4 °C. The greatest mycelial growth areas was observed at 28 °C then in a decreasing order at 12 °C and the smallest mycelial growth areas was noted at 4 °C. In contrast to the proposed by Martinez *et al.* (2003), who reported limited temperatures of mycelial growth between 5 and 28 °C, where the smallest growth was noted at 28 °C^[4]. At both optimal temperatures, four strains (BMN 0527, BMN 0528, B7 and B10) showed higher growth areas at 12 °C, while the other two remaining strains (B8 and B9) showed the highest areas at 28 °C. The mycelial growth on a nutritive medium as PDA can be correlated an indirect assessment of the colonization potential of each strain. It could indicate a better ability of strains to spread on host (saprophytic colonization)^[4]. And it could also indicate the influence of temperature in a better ability of strains to spread saprophytically on nutritive moribund substrates.

Assays of fungicide showed that *B. cinerea* was difficult to control satisfactorily with iprodione (dicarboximide) pre- and post- harvest it was also seen the different effects of resistance at the three temperatures of incubation. Strains considered resistant is that at that maximum concentration of the fungicide used is four times higher than the concentration normally recommended for standard postharvest treatments (500 mg/L)^[10].

The results from the present study related to pathogenicity demonstrated the aggressive or virulent qualities of *B. cinerea* that may be changed by the temperature environment in which it is grown. Differences in virulence were consistent at three assessment temperatures. At 28 °C, it is shown the optimum temperature of pathogenicity, where showed the largest lesion areas. Therefore it could be concluded that the temperature has an effect on aggression as in ability to colonize intercellularly and to overcome the defensive barriers of tissues of apples by this fungus.

It is difficult to correlate these characteristics *in vitro* with their effects in the field, but such correlation is inevitable for designing strategies for disease control caused by *B. cinerea*.

In the paper, it is demonstrated a negative correlation between mycelial growth and other characteristics as the fungicide resistance and pathogenicity. Because not all strains showed an optimum temperature of growth, greater pathogenicity or greater resistance to fungicides at that temperature. It also observed a similar negative correlation in the characterization molecular because it could not establish a relationship between the above characteristics and the presence of TEs (Group II).

From the results presented in the work, it is observed an effect of temperature on the mycelial growth, chemical control and pathogenicity of strains *B. cinerea*. Strongly noted that not all strains showed the same behavior; and it could explain the different life cycles and epidemiology present in *B. cinerea* due to the environmental factors as temperature.

In addition, the activity of TEs may have influenced those genomic regions that are involved in vegetative growth. Integration of TEs in fungal chromosomes has been shown to have influenced chromosomal features, DNA sequence and gene expression^[22,23]. Presence and/or activity of TEs may negatively influence the fungal fitness^[24,25]. The features would also indicate the different compartments with the strains, as it was predicted by Martinez *et al.* (2003, 2005)^[4]. In addition to the genetic differences, significant biological and phenotypic differences between the two TE types within Group II, as: i) mycelial growth rate, ii) aggressiveness and iii) fungicide resistance.

In conclusion, *B. cinerea* showed a high degree of diversity which makes it a species that is difficult to establish relationships between phenotypic and genotypic unless a large number of strains are studied. Some of these differences, such as the degree of pathogenicity and chemical control effects, may have great economic importance. It also can be concluded that most of the morphological characteristics such as mycelial growth, resistance to fungicides or pathological are independent of the characteristics molecular according to these characteristics dependent of a factor such as temperature.

In view of the genetic, biological and phenotypic diversity of *B. cinerea* populations, isolate responses is often ambiguous. The information that obtained different data suggested the usefulness of a further study at molecular level to establish control strategies that reduce the biological impact caused and opened new avenues of research taking the temperature of incubation into account.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

B. cinerea is a phytopathogenic fungi which is responsible for grey mould and it is an important disease problem in agriculture sector. There is a need to study on the pathogenesis, genetic diversity and virulent pattern of the fungi to recover this problem and control the production of diseased crop.

Research frontiers

The present research work illustrated an establishment of the relationship between morphological characteristics as the mycelial growth, pathogenicity and resistance pattern at different temperatures and its genetic variability.

Related reports

McDonald (1993) and Daboussi (1997) have carried out various researchers on the transposable elements and genome evolution of fungi. Moller *et al.*, (1992), Fournier and Giraud (2008), Munoz *et al.*, (2009) and Ziogas *et al.*, (2009) have been reported on the aspects of pathogenesis and drug resistant pattern of the fungi.

Innovations and breakthroughs

The activity of transposable elements may have influenced genomic regions that are involved in vegetative growth, disease resistant and genetic variations. The present study concluded that most of the morphological characteristics, resistance to fungicides or pathological are independent of the molecular characteristics. However, it can be differentiate due to temperature.

Applications

In the present study, different data and various informations were obtained. It will be useful for further study at molecular level and establish the control strategies to reduce the biological impact caused and open new possibilities of research.

Peer review

This is a good work, the authors take a focus on the research to evaluate the establish relationships between the morphological characteristics, pathogenesis and resistance to fungicide–iprodione, and its correlated with the genetic variability according presence or absence of transposable elements.

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