

Molecular Characterization of *Botrytis cinerea* Isolates from Different Host Plants

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Abstract

In Iraq, a study was conducted by researchers to investigate the different species of *Botrytis* found in faba bean fields throughout the country, including 26 isolates of *Botrytis fabae* and *B. cinerea*. Variations in characteristics were noted among the *fabae* isolates, with a sporulation rate of 18.1×10^6 /plate, while the *cinerea* isolates had a sporulation rate of 19.5×10^6 /plate. The *fabae* isolates produced multiple sclerotia of varying sizes, whereas the *cinerea* isolates either did not produce any sclerotia or had fewer, larger ones. Two particular *fabae* isolates, numbered 28 and 29, were identified as the most harmful, resulting in the most severe cases of disease with scores of 5.1 and 5.7, respectively. These isolates were collected from a research farm in Kufa. On the other hand, the *cinerea* isolates were associated with less harmful strains, which were found on plants in two locations in the Al-Abbasyah district and one location in the Kufa district. The virulence of a variety of additional isolates exhibited intermediate levels within the range. For all *Botrytis fabae* isolates, except for isolate 4 from Kufa which exhibited poor response, Primers Bc 6 (r & f) amplified a single band at 90 bp. The PCR products generated by *Botrytis cinerea* fungus were determined to have a size of 120 base pairs. Primers Bc 9 (r & f) were utilized to amplify a single band of 90 bp for *Botrytis fabae* isolates, excluding those from the Kufa region (isolates no. 4 and 7). In the case of isolates from *Botrytis cinerea* and *Botrytis fabae*, PCR products of 90 base pairs may be produced. Primers BC6 and BC7 demonstrated the highest level of specificity in differentiating between *Botrytis fabae* and *Botrytis cinerea* samples, respectively.

Key words: Molecular Characterization, *Botrytis cinerea*, Isolates, Plants

INTRODUCTION

The faba bean is considered one of the most important legumes that can be grown in Iraq. In the northern regions of the delta area, where the combination of low temperatures and high relative humidity create ideal conditions for its growth, the chocolate leaf spot disease is a well-known and highly destructive threat to faba bean crops, causing significant damage and loss. The presence of low temperatures and high relative humidity in this region promotes the rapid spread and severity of the disease, leading to widespread outbreaks [1,2]. In most cases, chocolate leaf spot is caused by *Botrytis fabae*, although *Botrytis cinerea* may also play a role. A variety of *Botrytis* species have been recognized, capable of infecting plants at various growth stages. Due to the potential for the disease to lie dormant in host plants, identifying it early is challenging, adding complexity to epidemiological research [3,4]. Thus far, researchers have identified 22 distinct species of *Botrytis* and a single hybrid within the genus. The classification primarily relies on physical characteristics, although physiological parameters and host range are also taken into consideration. The inclusion of numerical data further supports this classification (2005). The primary pathogen *Botrytis cinerea* Pers. is known to infect around 200 different hosts across various environments, such as fields, greenhouses, and storage facilities, showing no clear specialization towards a specific host. While certain species exhibit a broad host range, others have more specific requirements. Typically, only one or two closely related species within the same plant genus are susceptible to infection, with the exception of *B. fabae*, which has the ability to infect a variety of species belonging to the Fabaceae family, such as *Vicia*, *Lens*, *Pisum*, and *Phaseolus* [5]. Chemical treatment becomes challenging when dealing with field isolates of *Botrytis* spp. grown on artificial media, as they exhibit a diverse range of pathogenicity, morphological traits, and resistance to fungicides. This variability complicates the ability to specifically target particular strains of *Botrytis*. The substantial genetic diversity observed is likely a result of mutations and heterokaryosis. However, the emergence of resistant strains has facilitated the identification of genetic markers indicating distinct genotypic populations of *Botrytis* spp. The polymerase chain reaction (PCR) is a valuable technique developed for the detection and differentiation of genetic variations in a wide array of harmful fungi. In situations where

identification of plant diseases in untreated materials is required, PCR amplification of genomic DNA using specific primers can be beneficial. The objective of this study was to conduct a comparative analysis of 29 isolates of *Botrytis* spp. obtained from various regions of Iraq, focusing on their morphological, physiological, pathological, and molecular characteristics [6-8].

Materials and methods

The research took place between December 2024 and March 2025 in areas where strawberries were ripening. To obtain accurate samples of grey mold illness, farmers in the Duhok region, Erbil, and Sulaymaniyah in Iraq were interviewed. The fields were carefully examined for signs of the illness on the leaves, flowers, and fruits.

Isolation, purification and morphological identification

Between December 2024 and March 2025, a total of fifty deteriorated strawberry samples were gathered from each farming area and transported to the Plant Disease Diagnostic Laboratory at the University of Agriculture to ascertain the presence of the *Botrytis cinerea* fungus. Infected strawberries were sliced thinly (1-2 centimeters) and placed on potato dextrose agar (PDA) for cultivation. Once the pathogenic fungi were identified, they were purified using the hyphal tips technique on PDA medium, and fungal slants were established to continue the research. Utilizing a Nikon AZ100 microscope at magnifications of 10, 40, and 100 times, the germinating spore and fruiting body of the pathogen were observed and documented. [9].

Pathogenicity test

After undergoing sterilization with 70% alcohol, the pristine fruits were then purified with distilled water. Subsequently, the fruit was immersed in a 1% hypochlorite solution for an extended duration, followed by a thorough rinsing in distilled water. A spore suspension was introduced into each fruit through the insertion of a sterile needle and subsequent sprinkling of the needle's tip. The inoculated fruit was placed in moist chambers and monitored for 24 hours at ambient temperature. Regular inspections were carried out to detect any signs of disease in the injected strawberries before the onset of the characteristic gray mold. Additionally, further isolation from affected fruits was performed to corroborate Koch's postulates. [10].

Molecular identification of grey mold of strawberry

Genomic DNA was extracted and purified from a pathogenic fungus using a modified version of the CTAB method, which proved to be effective. The isolated DNA samples from the pathogen were then subjected to electrophoresis using 1.0% agarose gels in 0.5X TBE buffer with ethidium bromide at a concentration of 100 g/ml. The electrophoresis of the DNA samples from the pathogen was conducted on 1.0% agarose gels in 0.5X TBE buffer, and the resulting DNA bands were visualized and documented using a computerized Gel DocEZ imager. Subsequent analysis involved the amplification of DNA fragments using polymerase chain reaction [11, 12].

PCR Amplification

Involves the utilization of Internal Transcribed Spacer (ITS) to manage the process. Primers ITS1 (5TCCGTAGGTGAACCTGCGG 3) and ITS4 (5) TCCTCCGCTTATTGATAGC 3) will be employed for the amplification of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) of the relevant pathogen. To achieve this goal, a total of 15 distinct isolates were examined. The final step of the Polymerase Chain Reaction (PCR) amplification was conducted using a 15 µl aliquot of green master mix (2X Dream Taq Green PCR), containing 4 mM MgCl₂ and 0.4 mM dNTPs (dATP, dCTP, dGTP, and dTTP). Following the addition of 1–10 µl of nuclease-free water to the PCR reaction, the amplification of DNA templates was carried out utilizing forward and reverses (ITS1 and ITS4) primers at concentrations ranging from 0.1–1.0 M.

Sequencing and phylogenetic

The sequence data acquired from the amplified PCR products was utilized to construct a phylogenetic tree in the software MEGA 7.0. Subsequently, the NCBI database was queried using BLAST to locate sequence matches. [13, 14].

Management of grey mold of strawberry

Benzimidazole (1,3-benzodiazole), anilide (aniline), cyprodione (cyprodinil + pyrimethanil), dicarboximide (N-octylbicycloheptene), anilinopyrimidine (cyprodinil + pyrimethanil), triazole (metconazole + propiconazole), and pyrazole are all types of fungicides that were assessed for the management of strawberry grey rot in a study involving Petri dish fungicide evaluation. A detailed analysis of these fungicides, including benzimidazole (1,3-benzodiazole), dicarboximide (N-octylbicycloheptene), anilide (aniline), triazole (metconazole + propiconazole), anilinopyrimidine

(cyprodinil + pyrimethanil), and pyrazole, was carried out using the "poisoned food technique" (4-hydroxypyr).

Different dilutions were used for each fungicide, ranging from 50 g/mL to 250 g/mL. The extent of inhibition of mycelial growth was calculated by employing the formula: Inhibition of mycelial growth = $100 \left(\frac{X - Y}{X} \right)$ (Equation 1), with X and Y representing the radial growth rates of the control and fungicide-treated plates, respectively. The inhibition of mycelial growth reached 100 percent.

Preparation of plant extracts

Plant components were harvested, subjected to surface sterilization, and subsequently dried in a shaded environment. Following this, the materials were finely ground into powder using liquid nitrogen and extracted in a Soxhlet apparatus for 48 hours utilizing 100% ethanol and methanol. The extraction process involved individual solvent extractions at 50 °C under reduced pressure in a rotating evaporator (Heidolph VV2000). The resulting crude extracts were stored at 20 degrees Celsius for subsequent analysis. Dimethyl sulfoxide (DMSO) stock solutions and serial dilutions were employed to prepare the extracts [15]. In these experiments, DMSO functioned as the placebo for the control group. The susceptibility of grey mold pathogens was assessed using the agar well diffusion method, which involved studying the antifungal properties of medicinal herbs such as *Allium sativum*, *Mentha* spp., *Rosmarinus officinalis*, *Achillea millefolium*, *Tagetes patula*, and *Syzygium cumini* [16]. After subjecting the petri dishes to sterilization in an autoclave for fifteen minutes at a pressure of fifteen atmospheres and a temperature of 121 degrees Celsius, twenty milliliters of PDA medium were added to each dish. The autoclave operating conditions were carefully controlled as outlined. Subsequent to the medium being set in each petri dish, a sterile cork borer was utilized to create wells measuring six millimeters in diameter in the dishes. Various concentrations of solution were prepared in DMSO and pipetted into each well: 5, 15, 25, and 50 g/mL. Each Petri dish housed a single, uncontaminated fungal colony at the center, maintaining a temperature of 25 degrees Celsius. Three separate experiments were conducted as replicates to ensure accuracy. A comparative analysis was conducted by applying a control treatment containing DMSO at the same concentration as the extracts under investigation. The extent of inhibition in radians was calculated using Eq.2. In vitro research has demonstrated the ability of certain bacteria to inhibit the growth of gray mold. The specific pathogenic microorganisms utilized in the study are detailed in

the subsequent paragraphs. The comprehensive list of adversaries is provided in Table 2 for reference.

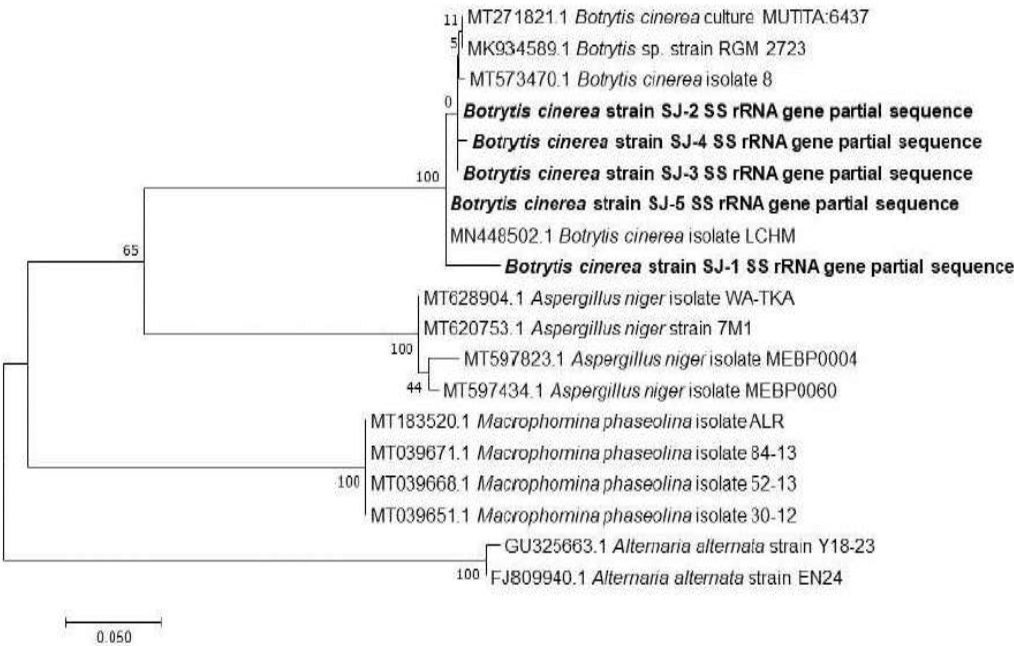
Statistical analysis

Each trial was conducted twice, employing a completely randomized design on both occasions. None of the experimental conditions yielded a statistically significant interplay between the two evaluations. Subsequently, the outcomes from both trial rounds were combined. The statistical examination was carried out utilizing Statistix® 8.1 software. A prior investigation in 1997 (Steel et al.) utilized ANOVA to determine data significance, with mean distinctions between treatments assessed through Tukey's test ($P < 0.05$). [17].

Results

There were three replicates for each of the three concentrations, seven treatments, and sterile water was utilized as a control in the experiments. The in-vitro studies were conducted using the Poisoned Food Technique. At concentrations of 100, 200, and 300 g/mL, *Alternaria alternata* was the most prevalent fungus (10.09%), followed by *Macrophomina* (4.27%), *Rhizoctonia* (3.49%), *Mucor* (3.21%), *Rhizopus* (1.05%), *Curvularia* (0.79%), and *Aspergillus* (0.98%). The findings are illustrated in Charts 1 and 2. Chart 1 provides a visual representation of the data from Tables 3a and 3b in a stacked bar chart format. Strawberry plants are susceptible to various fungal diseases, with hybrid inoculum sourced from different environments as shown in Figure 1. A compound microscope was employed to visually confirm the identity of Conidiophores following morphological validation of the culture. Even at maximum magnification, a conidiophore containing egg-shaped, ellipsoidal conidia was clearly visible, demonstrating the presence of the grey mold pathogen. The infectious agent was verified through pathogen screening and a pathogenicity test based on Koch's postulates following morphological analysis [18]. In the experiment, a greenhouse containing flourishing plants was utilized, with the population divided into two groups: one group received an injection of a concentrated pathogen solution (4 106 CFU/ml), while the other group remained uninfected. After seven days, signs of soft rot were detected in the plant tissues of the infected group; by day ten, the functional tissues began to collapse rapidly, indicating a surge in infection rate; and by day fifteen, the fruit organs disintegrated, suggesting a complete takeover by *B. cinerea*. Conversely, the control group exhibited no symptoms of the disease, demonstrating

full resistance. A substantial reduction in crop yield (ranging between 24 and 25 percent) was observed when comparing the infected group to the control group. A summary outlining the variances in pathogenicity between Narowal and NARC is detailed in Table 4. The primary objective of the pathogenicity assessments was not to assess yield loss, but rather to confirm the existence and identification of a pathogen. Identification through molecular data and the creation of a phylogenetic tree was conducted for gene analysis. Previous studies on fungal pathogen colonization frequency indicated that *Botrytis cinerea* was the sole pathogen responsible for grey mold disease in strawberries, leading to a focused research effort on this fungus for future investigations. The ITS region of the 18S rRNA gene from five representative *B. cineria* isolates was selected for genetic analysis, with amplification performed using ITS 1 and ITS 4 primers as outlined in the materials and methods section. Utilizing the Maximum Likelihood approach through Mega7 software, phylogenetic studies were conducted, and BLAST searches on the NCBI database confirmed that all five isolates shared nucleotide sequence similarity with *B. cinerea*. In Figure 3, the evolutionary trees for all variations can be observed. The sequences, identified as SUB8901212 Seq1 MW485211, SUB8901212 Seq2 MW485212, SUB8901212 Seq3 MW485213, SUB8901212 Seq4 MW485214, and SUB8901212 Seq5 MW485215, can be located in the GenBank database. Utilizing molecular information and constructing phylogenetic trees play a crucial role in the identification process.



An in vitro study was conducted to assess the effectiveness of various fungicides, antagonists, and plant extracts on the mycelial growth of *Botrytis cinerea* NARC and Narowal isolates. The statistical analysis revealed that numerous fungicides, antagonists, and plant extracts had a notable impact on the development of *Botrytis cinerea*'s mycelia at both NARC and Narowal sites. In comparison to the control group, all fungicide treatments exhibited significant inhibition of fungal growth, with Dicarboximide proving to be the most potent at both locations. Following Dicarboximide, Benzimidazole, Anilinoptrimidine, Trizole, Pyrazole, and anilide also displayed inhibitory effects. The substantial disparity in growth rates between control and treated samples indicates a considerable suppressive effect of the treatments on the pathogen's growth. Certain chemicals exhibited greater efficacy against Narowal isolates than NARC isolates, while others actually facilitated the pathogen's growth, potentially due to the development of resistance by the fungus. In laboratory conditions, the fungus was subjected to treatment with seven different compounds, consisting of six antagonists and one control. Employing botanical extracts to hinder the progression of certain ailments is perceived as an environmentally conscious approach. An investigation was conducted to determine the efficacy of botanical extracts in preventing the growth of two strains of *Botrytis cinerea*. The inhibitory properties of six distinct botanical extracts and a placebo were assessed against microorganism growth. It has been determined that botanical extracts sourced from *Achillea millefolia*, *Allium sativum*, *Mentha spicata*, *Tagetes patula*, *Syzygium cumini*, and *Rosmarinus officinalis* display the highest effectiveness against NARC and Narowal strains. The notable disparity in growth rates between the treatment groups and the control group serves as compelling evidence that the treatments are successful in halting the spread of the disease.

Discussion

The economically detrimental disease known as grey mold has a significant impact on strawberry crops and is attributed to the pathogenic fungus *Botrytis cinerea* [19]. During the initial stages of the investigation, our focus was primarily on identifying disease symptoms and conducting a survey with a strong visual analysis component. We gathered samples of infected plants that exhibited

symptoms described by Barnes and Shaw, such as water-soaked surfaces, collapsed plant organs and cells, and a grayish mold-like appearance on fruit surfaces. The fungus development in the purified sample was meticulously examined under a microscope, producing reliable results. Our research took us to various locations in Narowal and NARC to collect samples of the common grey mold disease, with NARC showing a higher prevalence due to its consistently higher humidity levels compared to Narowal throughout the study period [20]. Card [21] The findings were corroborated by other reports. Throughout the research, various morphological and cultural traits were observed among *B. cinerea* isolates. The global origin of the isolates examined in the study was found to contribute to these differences, as determined by the researchers. Numerous studies utilizing cultural and morphological characteristics have provisionally identified *B. cinerea* [22]. In an attempt to reduce production losses, a series of in vitro studies were conducted to investigate the effectiveness of various fungicidal medications, antagonists, and botanical extracts as potential treatments for this disease. The response to each of the six fungicides tested for their ability to inhibit fungal growth was significant. Dicarboximide emerged as the most effective fungicide, closely followed by benzimidazole. Several studies have indicated that the bacterium *B. cinerea* has developed resistance to the dicarboximide antibiotic. Numerous other fungicide varieties, including benzimidazole, are available for consideration. Anilide, and triazole, among others [23-25] Plant scientists are currently revisiting historical biological and agricultural methods as a result of the growing resistance to multiple fungicides and the escalating environmental concerns. One proposed solution is the utilization of pathogenic bacteria as agents of biological control to combat a variety of disorders that may impact the health of strawberry plants. Throughout our study, we tested six different antagonists to evaluate their effectiveness in inhibiting the pathogen in laboratory conditions. Among the antagonists examined, *Trichoderma* spp. demonstrated the highest efficacy against the pathogen, while *Bacillus subtilis*, *Penicillium* spp., *Burkholderia* spp., and *Colletotrichum* spp. also exhibited some ability to influence the disease. The conclusions drawn from our research align with previous studies conducted by other experts in the field [26-28]. In an experimental setting, a total of six distinct extracts were prepared and evaluated; each of these extracts exhibited significant

efficacy against pathogens when compared to the control group. Among these extracts, *Allium sativum* extract demonstrated the highest effectiveness in inhibiting fungal growth, followed by extracts from *Mentha spicata*, *Tagetes patula*, *Syzygium cumini*, *Rosmarinus officinalis*, and *Achillea millefolium*. Our research findings support the conclusions of Fufa and Kutawa's study, highlighting the potential of *Allium sativum* extract as a viable alternative to conventional pesticides in combating pathogens. Effective management of fungi responsible for grey mold disease necessitates a combination of pre- and post-harvest strategies. Our study findings recommend dicarboximide and benzimidazole as highly effective fungicides. The utilization of *Allium sativum* and *Mentha spicata* extracts as alternatives to synthetic fungicides for treating grey mold disease is proposed. Additionally, the use of biological controls like *Trichoderma* spp. and *Bacillus subtilis* has shown promising results in reducing the dependence on chemical treatments [16, 27-30]

Table 1. directory of variety medical plant extract used for strawberry grey mold.

NO.	Ordinary name	Family group	Scientific Name	Part of plant used
1	Jambolan	Myrtaceae	<i>Syzygium cumini</i>	Leaves
2	Garlic	Amaryllidaceae	<i>Allium sativum</i>	Pods
3	Marygold	Asteraceae	<i>Tagetes Patula</i>	Leaves
4	Mint	Lamiaceae	<i>Mentha spp</i>	Leaves
5	Yarrow	Asteraceae	<i>Achillea millefolium</i>	Leaves
6	Rosemary	Lamiaceae	<i>Rosmarinu sofficinalis</i>	Leaves

Table 2. shows directory antagonists used against strawberry grey mold.

No.	type	Antagonists
1	Fungi	<i>Penicillium spp</i>
2	Fungi	<i>Colletotrichum gloeosporioides</i>
3	Fungi	<i>Trichoderma spp</i>
4	Fungi	<i>Aspergillus fumigatus</i>
5	Bacteria	<i>Burkholderia cepacian</i>
6	Bacteria	<i>Bacillus subtilis</i>

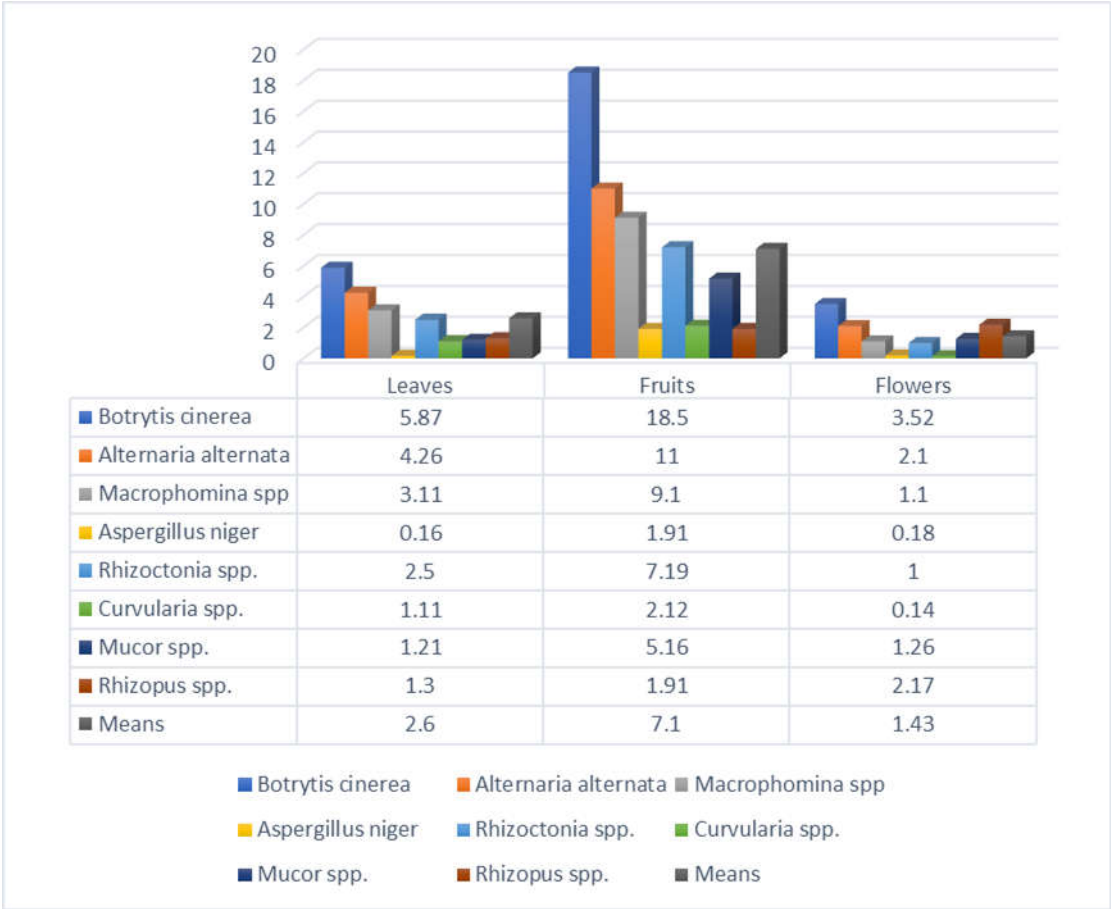


Figure 1 isolated fungi percentage from different part of strawberry in Duhok district

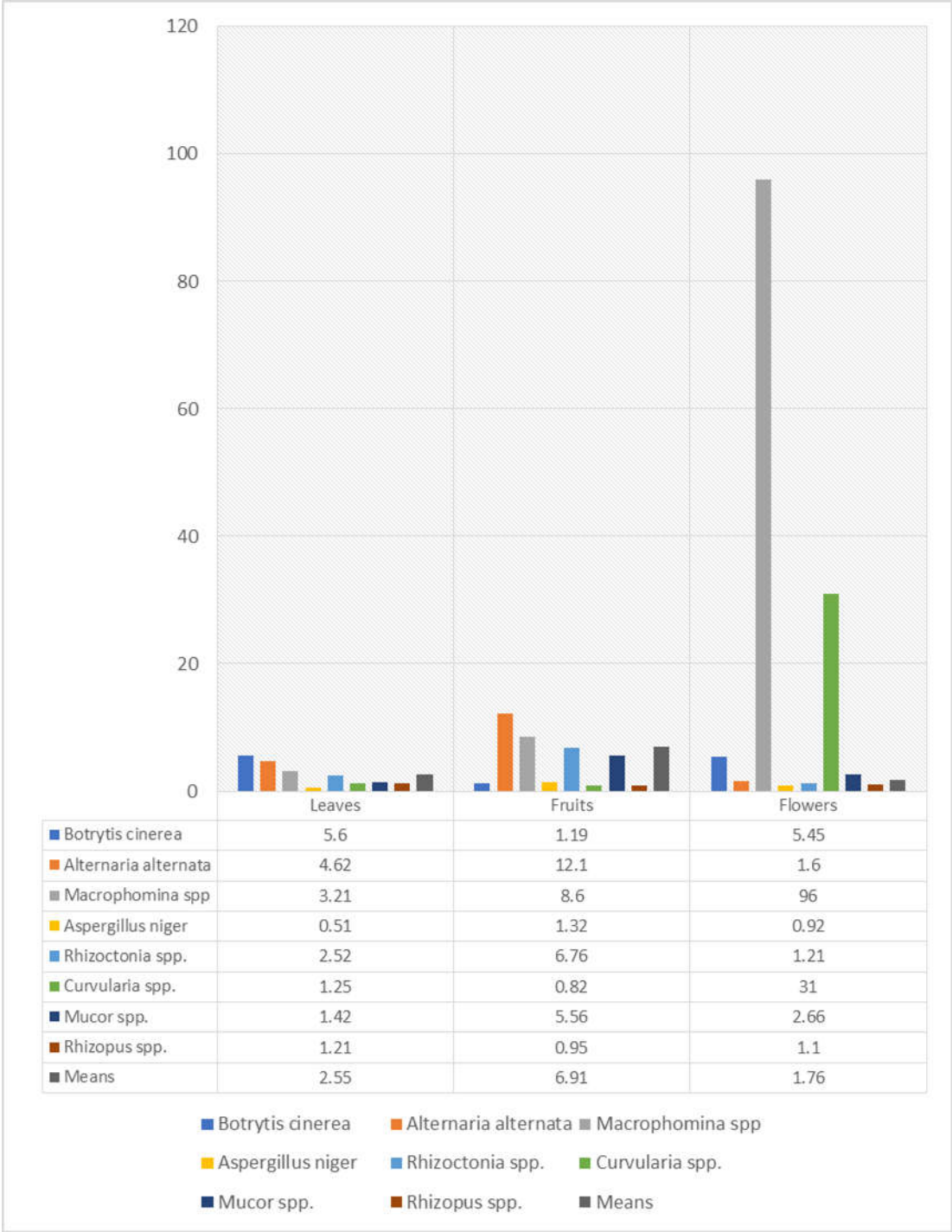


Figure 2 isolated fungi percentage from different part of strawberry in Erbil district

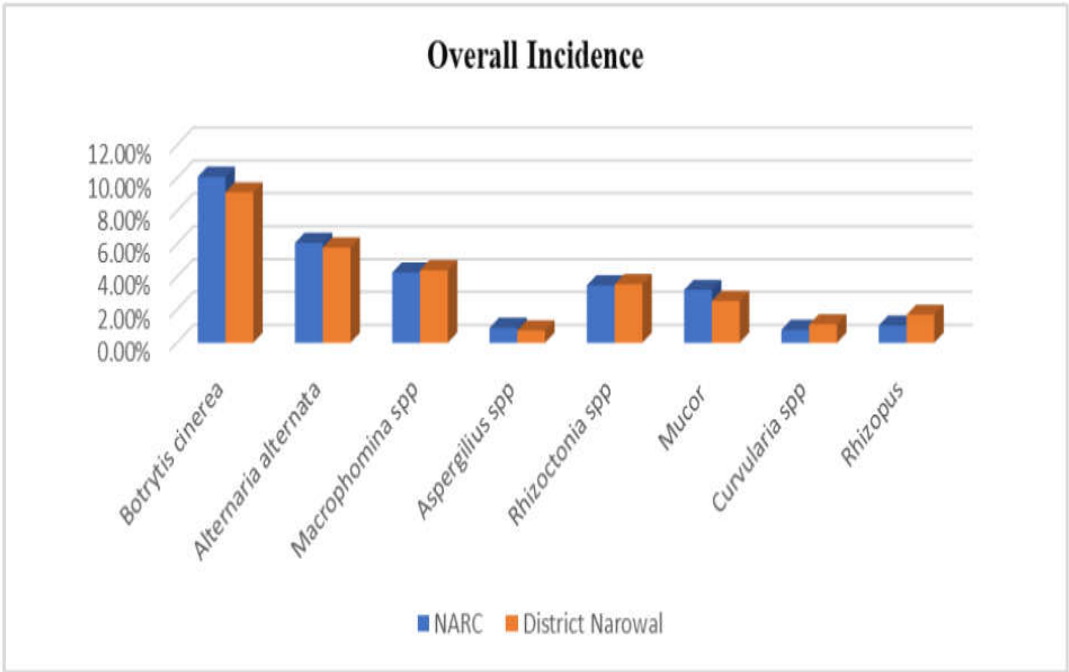


Figure 3 Overall incidence

Table 3. phylogenetic comparison in different days

Day	Infection stage	Control crop	NAROWAL ISOLATE	NARC ISOLATE
7	Visible soft rot	No	Yes	Yes
11	Collapse of parenchyma	No	Yes	Yes
17	Shriveled fruit	No	Yes	Yes
23	Greyish fungal growth	No	Yes	Yes

Table 4. In - vitro evaluation of various antagonists, plant extracts and fungicide, on the mycelia growth of *Botrytis cinerea*.

Sours	df	Fung Narc	Fung Narowal	Anti narc	Anti narowal
Trt ¹	8	550.32	953.09	588.48	486.83
Error	18	0.535	0.669	1.84	1.44
Rep	4	2.33	0.271	3.67	4.88
total	30				

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