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Morphological, cultural, growth characterization and molecular studies of *Alternaria brassicae* isolate I-1B20 cause leaf spot of mustard

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Abstract
Leaf spot disease caused by the fungus *Alternaria brassicae* is a major threat to mustard production worldwide. This disease affects all parts of the mustard plant, including leaves, stems, and pods, leading to reduced yield and quality of the crop. Conventional methods of identifying leaf spot disease in mustard by *Alternaria brassicae* have their advantages and limitations. Preceding methods of detecting leaf spot disease in mustard caused by *Alternaria brassicae* have several limitations including visual inspections, time-consuming processes and need for trained personnel. To overcome these issues, Molecular techniques, remote sensing, image processing, and biosensors have all proven to be effective in accurately detecting and quantifying leaf spot disease in mustard. In order to gain a better understanding of this pathogen, the present study aimed to investigate the morphological, cultural, growth characteristics, and molecular aspects of the *Alternaria brassicae* isolate I-1B20. As the result of this research, the radial growth and sporulation of the leaf spot of mustard is present in large numbers. In culture medium of OMA, its growth is high with best sporulation results. The pH and temperature in the plant is 8.0 and 24°C of best sporulation and radial growth results. The humidity rate of the growth of the plant is 100. This information will aid in the development of effective management strategies for controlling leaf spot disease in mustard plants. The sequences, BLAST, genetic expression and phylogenetic tree are performed through the isolate of the Alternaria brassicae to develop the disease resistant strategies.
Keywords: Molecular techniques, culture medium, different parameters, leaf spot of mustard, *Alternaria brassicae*

1. Introduction

Mustard, also known as Brassica nigra, is a popular vegetable crop that is widely cultivated for its leaves and seeds. However, like any other crop, mustard is susceptible to various diseases caused by different pathogens. One of the most common diseases that affect mustard plants is leaf spot, which is caused by a fungus known as *Alternaria brassicae* [1]. *Alternaria brassicae* is a fungal pathogen that belongs to the genus Alternaria, which is a common cause of plant diseases worldwide [2]. This fungus is known to cause leaf spots on a variety of plants, including mustard, broccoli, cabbage, and cauliflower [3]. The disease which is caused by *Alternaria brassicae* is a major concern for mustard farmers as it can result in significant yield losses if not managed properly. Among the various strains of *Alternaria brassicae*, isolate I-1B20 is known to be a highly virulent strain that causes severe leaf spot symptoms on mustard plants. This particular strain has been isolated and identified in various regions of the world, including Europe, Asia, and North America, where mustard is extensively grown [4].

I-1B20 isolate of *Alternaria brassicae* was first identified in mustard fields of Allahabad, Uttar Pradesh. It is a necrotrophic fungus that thrives in warm and humid conditions [5]. It is commonly found in the soil and plant debris, making it a major threat to crops such as mustard, broccoli, and cabbage [6]. The fungus can survive in the soil for several years, making it challenging to control. However, it is the airborne spores that are responsible for the spread of the disease, making it a significant concern for farmers. It is a highly virulent strain, causing severe leaf spot symptoms in mustard plants [7]. The fungus attacks leaves, stems, and pods of the plant, leading to development of dark brown or black lesions. These lesions can coalesce, causing extensive damage to the foliage and reducing the photosynthetic area of the plant. As a result, the plant's growth is stunted, and the yield is significantly reduced.

Conventional methods for identifying *Alternaria brassicae* involve visual inspection, microscopic examination, isolation and culture, and serological and molecular techniques [8]. These methods not only confirm the presence of *A. brassicae* but also aid in understanding its diversity and virulence, which can contribute to the development of effective management strategies [9]. Cultural methods involve the isolation and cultivation of the fungus on specific growth media. *Alternaria brassicae* is known to grow well on potato dextrose agar (PDA) and malt extract agar [10] at room temperature. The colony characteristics, such as colour, texture, and growth rate, can be used to differentiate it from other fungi. Biochemical tests, such as the utilization of different carbon sources and production of enzymes, can also aid in the identification of *Alternaria brassicae* [2]. One example is this microorganism is able to create cellulose enzyme, which can be identified through a reagent that changes colour when this enzyme is present. In recent years, advances in technology have led to the development of molecular techniques for identifying *Alternaria brassicae* [11]. These methods involve the amplification of specific DNA sequences using polymerase chain reaction (PCR) and comparing them to a database of known sequences. This allows for a more rapid and accurate identification of the pathogen. In addition to DNA-based and immunological techniques, there are also several other modern methods that have been developed for the identification of *Alternaria brassicae* [12]. These include polymerase chain reaction (PCR) based techniques, loop-mediated isothermal amplification (LAMP), and enzyme-linked immunosorbent assay [13]. Each of these methods has its own advantages and limitations, but collectively they have greatly improved the efficiency and accuracy of identifying *Alternaria brassicae*.

The identification of *Alternaria brassicae* has been carried out using various methods, including morphological, cultural, and biochemical techniques. These methods involve the observation of morphological characteristics, growth patterns, and biochemical reactions of the pathogen to distinguish it from other similar fungi. Various pathogens in the leaf are predicted and foremost pathogen which causes the disease are identified. Using various culture medium, radial growth and sporulation are estimated. With the help of different pH, radial growth and sporulation are identified. By variation in the temperature range, the radial growth and sporulation of the plant is identified. The sporulation and radial growth are detected with the aid of different humidity. A pathogenicity test is conducted to assess the pathogen's disease severity. DNA extraction, purification and sequencing process is evaluated for determining the sequences of the detected pathogen.

The study on *Alternaria brassicae* isolate I-1B20, the causal agent of leaf spot disease in mustard, targets to discuss about several research gaps. The morphological characterization of this isolate is limited, and its cultural characteristics, such as growth rate and sporulation patterns, are not well stated. Additionally, there is a lack of molecular-level studies on the genetic and genomic features of this isolate. The objectives of this study are to conduct comprehensive morphological and cultural characterization of the I-1B20 isolate, measure its growth parameters, and achieve molecular studies to identify key pathogenicity-related genes and molecular markers. This comprehensive understanding will enlighten the development of effective disease management strategies for Alternaria blight in mustard crops.

2. Literature review

Literature review provides an overview from the preceding studies of morphological, cultural, growth, and molecular studies on *Alternaria brassicae*, highlighting its morphological features, cultural characteristics, growth patterns, and molecular properties.

The goal of this study is to assess how bio-agents and elicitors impact *Alternaria brassicae* in mustard plants using the dual culture technique and poison food technique in a completely randomized design (CRD). The research was carried out in the Plant Pathology Laboratory of Sam Higginbottom University of Agriculture, Technology and Sciences, located in Prayagraj. The findings showed that Trichoderma viride + Pseudomonas fluorescens had the highest mycelia growth inhibition of *Alternaria brassicae* at 87.70mm among bio-agents, with Trichoderma viride following closely at 82.60mm. Salicylic acid at 150 ppm showed the highest percentage of pathogen inhibition (75.33%) among all elicitors, with salicylic acid at 100 ppm following closely behind (58.69%) [14].

DNA from pure Alternaria cultures was isolated, and ITS primers were used in PCR amplification to produce 700 bp fragments. Variations among Alternaria species were observed in morphological characteristics Pathogenicity experiments verified that all Alternaria isolates were pathogenic. Analysis at the molecular level using ITS regions revealed that the nucleotide sequences showed a 99% similarity to sequences already in NCBI database. Three types of Alternaria were detected in mustard leaves which are affected by grey blight. Furthermore, differences were noted in the quantity of conidia, longitudinal division, horizontal division, conidial size, and width across the various isolates. The research emphasized the strong genetic link between the Alternaria isolates and their connection to grey blight disease in mustard [15].

A thorough field study was carried out to document the disease occurrence and seriousness in Indian mustard due to the fungal pathogen *Alternaria brassicae*. The organism was identified both macroscopically and microscopically, and grown in the lab on PDA medium. To investigate the effectiveness of the botanical fungicide, water-based extracts from 14 diverse were examined in a laboratory setting against pathogens. Among the 14 plants, the only one that showed antifungal properties was the water-based garlic extract, which completely stopped conidial germination within 5 hours in cavity slides placed in a germination box. The garlic extract demonstrated a MIC of 0.75% against the pathogen [16].

To control Alternaria infection in mustard seeds for increasing yields, a study was carried out in the plant protection lab with four replicates. Five different methods were used to treat seeds of the local Mustard variety. Four hundred seeds were divided into treatments with 25 seeds per petri plate, each plated on triple layers of moistened blotter paper. The petri plates were then incubated for 2 days before being subjected to deep freezing for 24 hours. Information on disease occurrence and seed sprouting was documented on post incubation. Seedling vigour and seedling weight were measured at 5 DAI as well. Different treatments showed a notable variance in the presence of Alternaria species on seeds at different Days after Imbibition (DAI). The seed-borne infection of Alternaria spp. was significantly reduced by using Uthane M-45 and T. harzianum compared to the control. By day 3, M-45 thoroughly examined the pathogen, but only noticed a disease incidence of 4% and 5% by day 7 and day 10 [17].

Findings indicated that the most severe cases of Alternaria blight occurred when the mean of maximum temperature, mean minimum temperature, average temperature and average relative humidity was over 70%. Variations in nutrient management led to varying levels of disease severity on leaves and pods, with disease severity increasing significantly as the plant aged. The most significant rise in disease severity occurred at 95 days after sowing, with next highest increases seen at 80 DAS and 65 DAS, regardless of the treatments applied. The lowest level of disease severity (13.83%) was seen when the plots received Azotobacter @ 250 g kg-1 seed, Phosphobacteria @ 250 g kg-1 seed, and FYM @ 7.5 t ha-1 [18].

In the blotter method, the highest seed reduction achieved was 85.91%, fungi were found in seeds treated with neem in contrast to ata, Ata and Castor yielded quantities of 83.52%, 82.59% and 82.39%, respectively. Reduction in fungi compared to the control. Achieving a seed germination rate of 93% was the highest possible noted in seeds treated with akondo (1:1) mixture, along with neem , castor and akondo led to seed germination rates of 92%, 92%, and 90% respectively. A 24.67% level of seriousness was documented after applying neem for 28 days. Following seeding, the plants exhibited the greatest shoot length at 16.09 cm, root length of 4.69 cm, and overall vigor. Comparison of index and seed yield to control showed an increase of 1842.56% and 33.70%, respectively, in the net dwelling place. Neem extract was discovered to be a potent botanical solution for the environmentally-friendly control of seed-borne fungi and Alternaria leaf spot diseases of mustard that is black in colour [19].

3. Research methodology

Samples of infected leaves were gathered from various areas in Allahabad such as Karchhana, Koraon, Phulpur, Sahso, Bara, Sankargarh, Meja, Sadar, Soraon, Handia, covering a total area of around 5,482 square kilometres. The overall methodology of study is depicted in Figure 1.

Preparation of the inoculum for Alternaria brassicae:

The fungus *Alternaria brassicae* was isolated from mustard plants showing black leaf spots in the winter months from February to March. Infected leaves were collected and putty in 4ºC ice bath. They were then cut into 2 mm pieces, sterilized with 4% NaOCl for 1-2 minutes, washed with SDW 3-4 times, and finally placed on PDA medium in culture plates and in a BOD incubator for 4-5 days. Fungi colonies were seen in PDA plates with diseased leaf fragments after being incubated at 24º C– 25º C for 4 -5 days. During pouring, streptomycin antibiotics were included in the culture medium to prevent bacterial growth. The mycelia found at the edges of the leaf spot fragments, which seemed to have developed distinct colonies on the substrate, were subsequently transferred into individual Petri dishes containing PDA medium under aseptic conditions. In the BOD incubator, they were cultured for a week at a temperature of 23±2ºC. The pathogen was identified as *Alternaria brassicae* (Berk.) Sacc and separated using a method of isolating single spores to evaluate the conidiophore and conidial morphology. Isolated fungal pathogen cultures have been stored on PDA slants at 4°C.

In-vitro analysis by their sequences and genetic expression:

Variation in morphological characteristics was noted in strains of A. brassicae through the examination of their individual spore cultures in a laboratory setting. The study evaluated cultural variability under various conditions such as temperature, relative humidity (RH), light duration, pH, and culture media. *A. brassicae* isolates were analysed for their variety in culture at seven specific pH levels (5, 6, 7, 8, 9, and 10), four varied relative humidity conditions (25, 50, 75, and 100%), and six diverse temperatures (6, 12, 18, 24, 30, and 36 degrees Celsius). A PDA medium at pH 7.0 was contaminated with a 4 mm mycelia disc from developing A. brassicae colonies. After being injected with the inoculum, Petri dishes were incubated for 28 days at seven varying temperatures, under conditions of complete humidity and a light/dark cycle of 12 hours each day (1000 lux).Each treatment was carried out on three occasions. Different levels of humidity were controlled in order to study how relative humidity affects the growth of mycelium and the production of spores. Following the process of inoculation, Petri dishes were left to incubate for a period of 28 days under the condition of 12 hours of darkness and 12 hours of light each day (1000 lx), along with being kept at the optimal temperature range (24-25°C) based on the specific cultures being studied. Five distinct growth mediums such as Corn meal agar (CMA), Czapex dox agar, V-8 juice agar (V-8J), Oat meal agar (OMA), and Carrot agar (CA) were created, with pH levels adjusted to (5, 6, 7, 8, 9, and 10) based on the specific culture. This was done to analyse how different culture media affect mycelia growth and sporulation.

The colony's expansion in diameter was measured daily for 10 days after being introduced to different temperatures, RH levels, pH levels, and cultural conditions. Take into account the culture that was developed in seven various media plates and determine the conidial concentration of each isolate. Add ten millilitres of sterile distilled water to the culture plate. The sterile glass slide was used to gently scrape the culture surface in order to create a conidial suspension. The haemocytometer was employed to measure the conidial concentration in every culture. The haemocytometer is made of a thick glass microscope slide with a rectangular indentation, creating a precise volume chamber. It is a counting-chamber device typically utilized for blood cell counting and observations for sporulation were conducted 28 days after inoculation.

A pathogenicity experiment was conducted on mustard plants by administering a conidial suspension of A.brassicae on the leaves to monitor any symptoms. In October 2020, mustard seeds were sown in the fields of the Department of Botany botanical garden at Allahabad University, Prayagraj Uttar Pradesh. After seven weeks, some plants will grow out from the dense vegetation, with a distance of 25-30 cm between them. The leaf surface received the perfect dose of inoculum, a concentration of $1x10^6$ conidia/ml. The data was gathered to classify group A. brassicae isolates based on the diversity seen in culture during mycelia growth on the 10th day and sporulation on the 25th day. As a result, separate dendrograms were generated using information about isolates' mycelia growth and spore production. Figure 2 illustrates about the recognising the pathogen of *Alternaria brassicae* from the mustard crop.

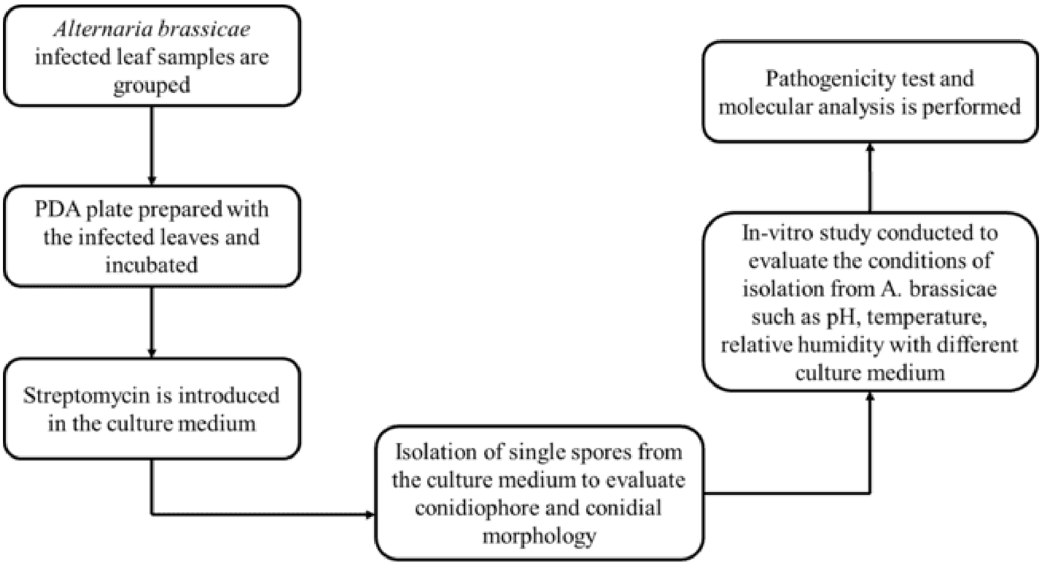

Figure 1 Overall research study of *Alternaria brassicae* identification in leaf spots of the mustard

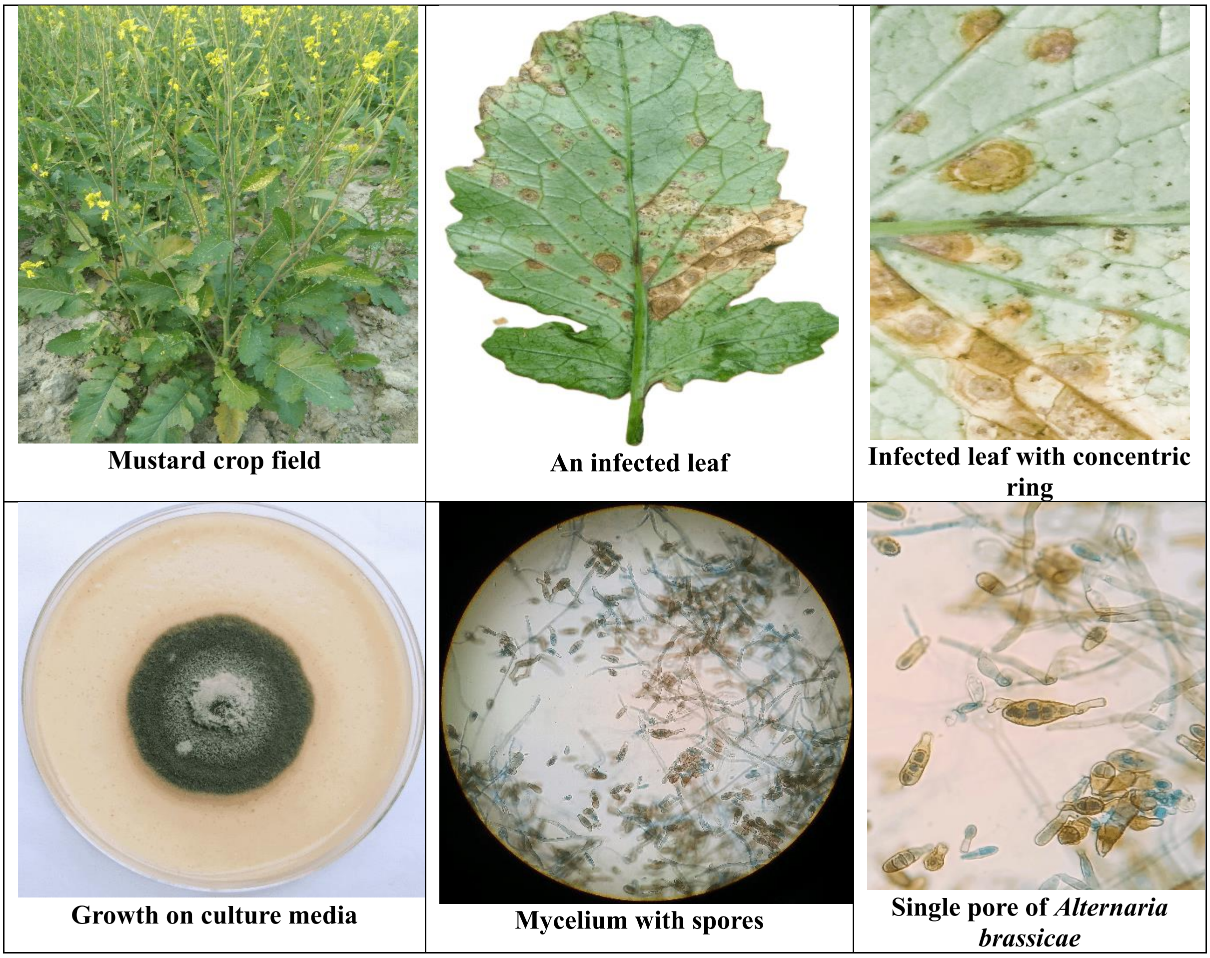

Figure 2 Identification of *Alternaria brassicae* pathogen from mustard crop field

4. Results

4.1 Detection of pathogens in the leaf

Mustard is susceptible to various diseases caused by pathogens. One such pathogen is *Alternaria brassicae*, a fungus that causes leaf spots in mustard plants. These leaf spots not only reduce the yield and quality of the mustard plant, but they can also serve as a source of infection for other plants in the same field. Therefore, early detection of *Alternaria brassicae* in mustard leaf spots is crucial for effective disease management.

1. Detecting *Alternaria brassicae* in mustard leaf spots is to identify the symptoms. The early symptoms of Alternaria leaf spot include small, circular and dark brown spots on the leaves. As the disease progresses, the spots become larger and develop a greyish-white centre with a dark brown border. The affected leaves may also become distorted, turn yellow, and eventually die. These symptoms are similar to other leaf spot diseases and making it important to confirm the presence of *Alternaria brassicae* through laboratory testing.
2. The most common method for detecting *Alternaria brassicae* in mustard leaf spots is through visual inspection of the diseased plant tissue. This involves collecting a sample of the affected leaves and examining them under microscope. Enzyme-Linked Immuno Sorbent Assay and PCR are used to detect the pathogen precisely, detect the specific proteins or antigens in the sample and extraction of DNA from leaf spot sample and analysed with gel electrophoresis to confirm the presence of pathogen with the aid of the appearance of the specific band in the gel. PCR, on the other hand, is a molecular technique that amplifies specific DNA sequences of the pathogen. This method requires the extraction of DNA from the leaf spot sample and its amplification using specific primers designed for *Alternaria brassicae*. The amplified DNA is then analysed using gel electrophoresis, and the presence of the pathogen can be confirmed by the appearance of a specific band on the gel.

4.2 Radial growth and sporulation in different media

Table 1 Measurement of Radial growth and sporulation with aid of different media

S.N.	Different media	Radial growth (mm)	Sporulation
1.	PDA	17.50	20.50 x10^5/ml
2.	CMA	16.50	20.0x10^5/ml
3.	OMA	19.50	22.25x10^5/ml
4.	CZA	18.75	20.75 x10^5/ml
5.	CA	14.50	17.75 x10^5/ml
6.	V-8J	13.50	17.25 x10^5/ml

Measurement of radial growth and sporulation with different medium are presented in Table 1. Totally, six different culture are utilized for the research. When comparing the six different culture medium, OMA culture medium presents high radial growth and sporulation rate. V-8J culture medium shows lowest radial growth and sporulation rate.

4.3 Radial growth and sporulation in different pH

Table 2 Amount of Radial growth and sporulation with aid of different pH

S.N.	pH	Radial growth (mm)	Sporulation
1.	5.0	14.40	17.75x10^5/ml
2.	6.0	15.00	19.25x10^5/ml
3.	7.0	15.50	21.25x10^5/ml
4.	8.0	17.25	24.25x10^5/ml
5.	9.0	13.24	15.75x10^5/ml
6.	10.0	12.25	14.50 x10^5/ml

Extent of Radial growth and sporulation with aid of different pH is illustrated in Table 2. In the pH value of 8, radial growth is determined in 17.25 and in pH value of 10, radial growth is 12.25. From the results, in the pH range of 8 it has the highest growth when compared with others.

4.4 Radial growth and sporulation in different temperature

Table 3 Representation of varied temperature and its growth and sporulation

S.N.	Temperature (0ºC)	Radial growth (mm)	Sporulation
1.	6	10.96	12.75x $x10^5$/ml
2.	12	11.56	13.25 $x10^5$/ml
3.	18	10.50	11.25$x10^5$/ml
4.	24	19.50	22.25$x10^5$/ml
5.	30	18.50	21.25$x10^5$/ml
6.	36	12.50	16.75$x10^5$/ml

Table 3 depicts about the radial growth and sporulation in the diverse temperature. In the temperature 18, it shows lowest growth and in 24 temperature shows highest temperature. When comparing with others, temperature 24 shows highest results in growth and sporulation.

4.5 Radial growth and sporulation in different humidity

Table 4 Diverse humidity range in sporulation and radial growth

S.N.	Relative humidity	Radial growth (mm)	Sporulation
1.	20	9.96	10.75$x10^5$/ml
2.	40	10.56	12.25$x10^5$/ml
3.	60	11.96	13.75$x10^5$/ml
4.	80	15.86	18.75$x10^5$/ml
5.	100	18.96	22.40$x10^5$/ml

Table 4 illustrates about the varied humidity range in radial growth and sporulation. In the range of 20 humidity, it shows lowest growth rate and 100 range of humidity, it shows highest sporulation rate and growth.

4.6 Pathogenicity test

Pathogenicity test is an essential tool in the study of plant diseases caused by fungal pathogens. In the case of the Alternaria species, which includes over 300 plant pathogenic fungi, pathogenicity test plays a crucial role in understanding the disease mechanisms and developing efficient management strategies.

The pathogenicity test for Alternaria species involves several steps. The first step is to obtain a pure culture of the pathogen. This can be done by isolating the fungus from infected plant tissues or by obtaining a culture from a culture collection. The pure culture is then grown on a suitable medium, such as potato dextrose agar, and incubated at optimal temperature and humidity conditions.

The next step is to prepare the inoculum. This can be done by either growing the fungus on a suitable host plant or by harvesting spores from the culture. The inoculum is then applied to the plant tissues in various ways, depending on the type of disease being studied. For foliar diseases, the inoculum can be sprayed onto the leaves, while for soil-borne diseases, the inoculum can be applied to the roots.

After inoculation, the plants are monitored for disease symptoms and development. The symptoms can vary depending on the host plant and the specific Alternaria species. To confirm the pathogenicity of the fungus, the disease symptoms are compared to those observed in the control plants, which were not inoculated with the fungus.

In some cases, the pathogenicity test may also involve re-isolating the fungus from the infected plant tissues and re-inoculating it onto healthy plants to confirm its role in causing the disease. This step is crucial in ruling out the possibility of other pathogens or environmental factors causing the disease.

Apart from determining the pathogenicity of a particular Alternaria species, the pathogenicity test also helps in understanding the disease cycle and the factors that influence its development. This information is essential in developing effective disease management strategies, such as cultural practices, chemical control, and genetic resistance.

<u>Grade</u> <u>Extent of infection</u>

0	=	Healthy leaves
1	=	1 – 10 per cent of the leaf area infected
2	=	11 – 25 per cent of the leaf area infected
3	=	26 – 50 per cent of the leaf area infected
4	=	51 – 75 per cent of the leaf area infected
5	=	more than 75 per cent of the leaf area infected

The pathogenicity test results for *Alternaria brassicae*, the causal agent of leaf spot disease in mustard, are as follows:

- Grade 3: 26 – 50 per cent of the leaf area infected
- Grade 4: 51 – 75 per cent of the leaf area infected
- Grade 5: More than 75 per cent of the leaf area infected

These results specifies that *Alternaria brassicae* cause substantial infection and harm to mustard plants, leading to yield losses. The pathogen is known to affect most Brassica species and can spread by means of spores on infected plant debris under warm and wet conditions.

4.7 Molecular analysis

Molecular analysis is a powerful tool that allows scientists to study the structure and function of molecules at a microscopic level. It has become an indispensable tool in modern science, providing valuable insights into the structure and function of molecules. It has a wide range of applications and continues to drive advancements in various fields, ultimately leading to a better understanding of the world and improved quality of life.

DNA purification

DNA purification is a crucial step in many scientific experiments and processes, as it allows for the isolation and extraction of pure DNA from a sample. This purified DNA can then be used for various downstream applications, such as sequencing, cloning, and gene editing. The process of DNA purification involves several steps, each of which is designed to remove impurities and contaminants from the DNA sample. These impurities can include proteins, RNA, enzymes, and other molecules that may interfere with subsequent experiments or analyses.

- The first step in DNA purification is to obtain a sample containing the desired DNA. This can be a tissue sample, blood sample, or a cell culture. The sample is then lysed, or broken down, to release DNA. Various methods can be used for lysis including morphological disruption, enzymatic digestion, or chemical lysis.
- Once the DNA is released from the cells, it is important to remove any proteins, RNA, and other impurities that may be present. This is typically done through a process called precipitation, where the DNA is mixed with a salt solution and a high concentration of alcohol. The DNA molecules will then clump together and can be separated from the rest of the solution through centrifugation.
- After precipitation, DNA is often treated with an enzyme called RNase, which specifically degrades RNA molecules. This step ensures that any remaining RNA is removed from the sample.
- The next step in DNA purification is called purification or extraction. This is where DNA is separated from the other components of the sample. There are several methods for DNA extraction, including column-based purification, silica membrane purification, and magnetic bead purification. These methods all involve binding the DNA to a solid support and then washing away the impurities.
- Once the DNA is purified, it is important to quantitate and assess the quality of the sample. This can be done through various methods, such as spectrophotometry, agarose gel electrophoresis, or real-time PCR. These techniques can determine the concentration and purity of the DNA, as well as any potential contaminants that may still be present.

Table 5 Primer details of the PCR and sequencing

Primer Details (For PCR):

No.	Oligo Name	Sequence (5`→ 3`	Tm (°C)	GC- Content
1	ITS 1	5' – GGAAGTAAAAGTCGTAACAAGG – 3'	56.3	47.5%
2	ITS 4	5' – TCCTCCGCTTATTGATATGC – 3'	55.3	45%

Primer Details (For Sequencing):

No.	Oligo Name	Sequence (5`→ 3`)	Tm (°C)	GC- Content
1	ITS1	GGATTAGATACCCTGGTA	56.3	47.5%

Table 5 represents the primer details of the PCR and sequencing of the species *Alternaria brassica*. PCR and sequencing have revolutionized the field of genetics and have had a profound impact on our understanding of the genetic makeup of living organisms. These techniques have played a crucial role in the development of personalized medicine and have led to numerous breakthroughs in genetic research.

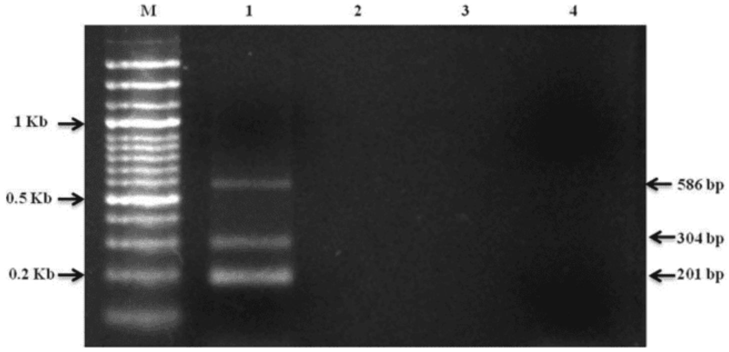

Figure 3 Depiction of Gel electrophoresis of *Alternaria brassica*

Alternaria brassica gel electrophoresis result is represented in the Figure 3. The gel electrophoresis result of A. brassica isolates provides valuable insights into the genetic diversity and population structure of this important plant pathogen. M band represents a control group of DNA fragments that is present in all A. brassica isolates. It could be a conserved region of the genome that is essential for the pathogen's survival.1, 2, 3 and 4 bands represent DNA fragments that are present in some isolates but not others. They could be associated with specific virulence factors or genetic markers that differentiate between different strains or populations.

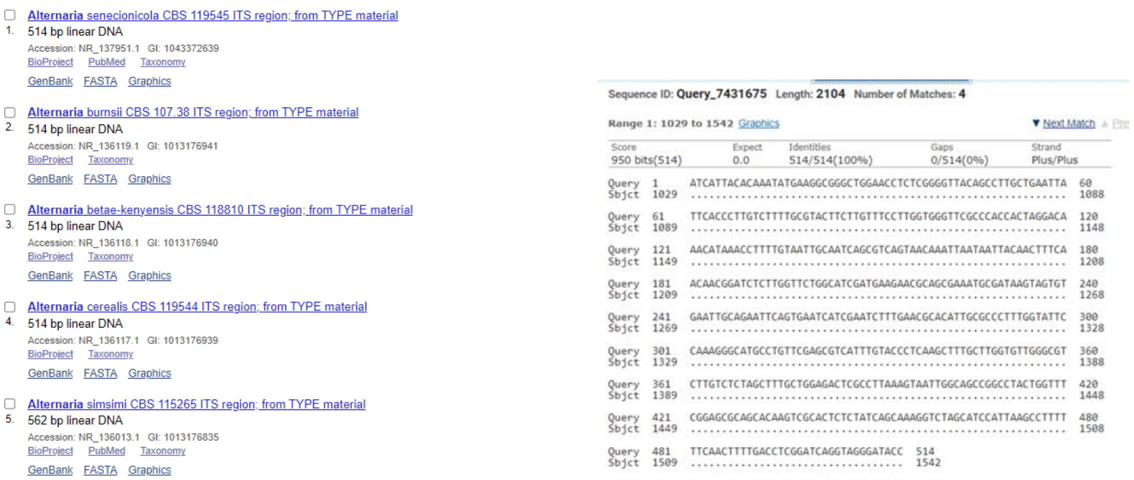

Figure 4 Description of the sequences and CDS feature of the sequences with pairwise identities in dots

Sequences description and its CDS features with pairwise identities in dots are represented in the Figure 4. Pairwise identities in dots are a valuable tool for understanding sequences and their features. They provide a visual representation of the alignment between two sequences, highlighting regions of similarity and dissimilarity. Sequences with high pairwise identities in dots can also be used to identify potential functional regions within a sequence. These regions may include binding sites for proteins or regulatory elements that control gene expression.

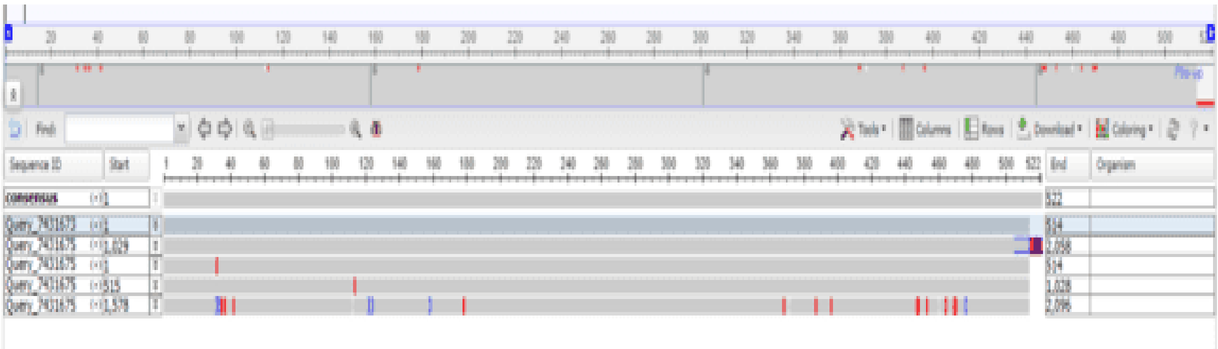

Figure 5 Sequences in NCBI MSA Viewer 1.25.0

Sequences of the NCBI MSA viewer are described in the Figure 5. The NCBI MSA Viewer 1.25.0 is a powerful and versatile tool that has revolutionized the way researchers analyse and visualize sequence alignments. Its user-friendly interface, advanced features, and seamless integration with other NCBI tools make it an essential tool for any bioinformatics research project.

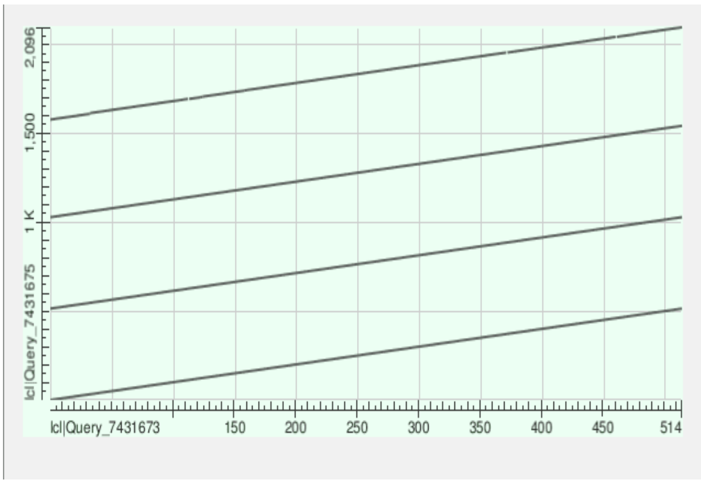

Figure 6 Dot plot of the sequences

Figure 6 illustrates the dot plot of the sequences of the research. Dot plot sequences are a valuable tool in bioinformatics and have a wide range of applications in sequence analysis. They provide a quick and intuitive way to compare sequences and can aid in the identification of important regions and features.

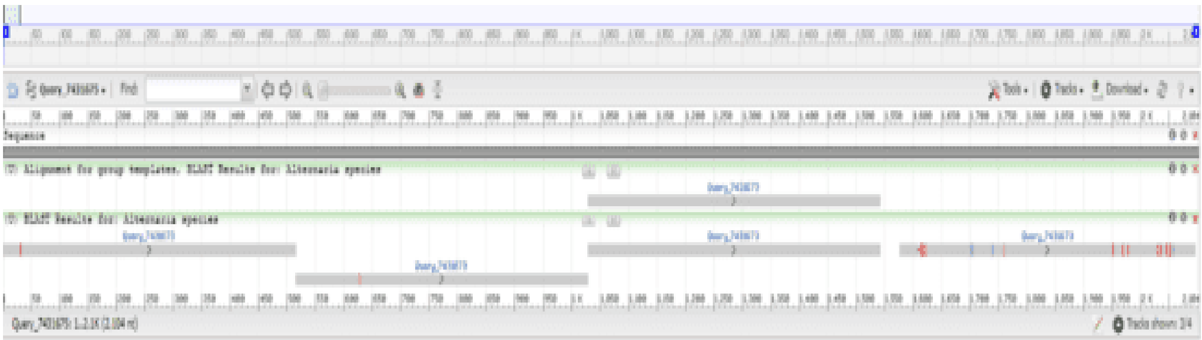

Figure 7 BLAST results OF the Alternaria Species

Figure 7 presents the results of the BLAST of the Alternaria species. BLAST results have provided valuable insights into the genetics and evolution of the Alternaria species. They have revealed the presence of repetitive elements, highly variable genomes, and conserved genes involved in secondary metabolite production. These findings not only enhance our understanding of these fungi but also have practical implications for agriculture and human health.

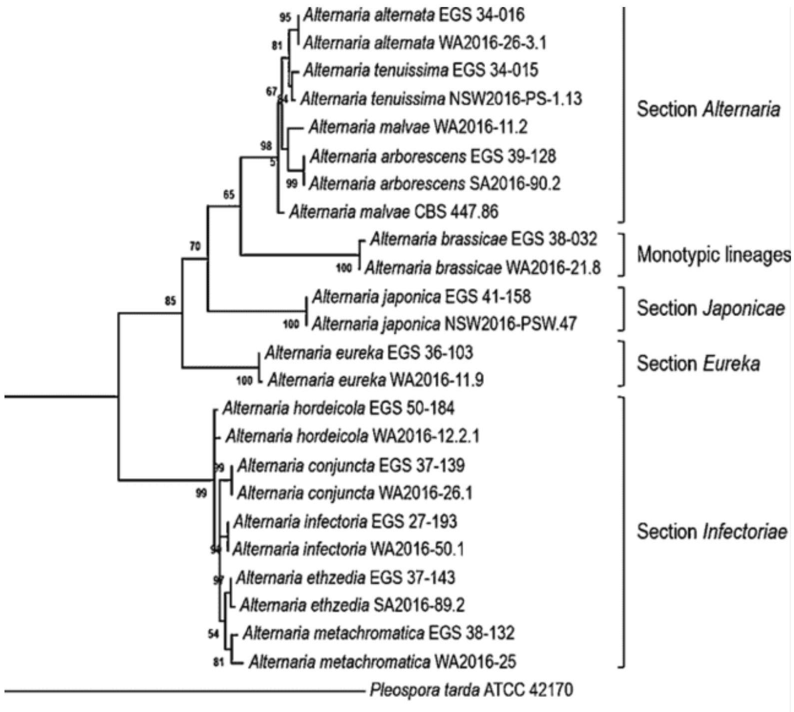

Figure 8 Phylogenetic analysis of the Alternaria species

Figure 8 illustrates about the phylogenetic analysis of Alternaria species. Phylogenetic tree based on ITS sequences of Alternaria species. The tree was constructed using the maximum likelihood method with 1000 bootstrap replicates. Bootstrap values are indicated at the nodes. The phylogenetic analysis provides valuable insights into the evolutionary relationships and diversity of Alternaria species. Additionally, the tree can aid in the identification of new Alternaria species and help to clarify the taxonomy of this complex genus.

5. Discussions

A study was conducted to investigate effective methods of managing Alternaria leaf spot in Indian mustard which is caused by *Alternaria brassicae*. The illness initially manifested in the initial week of November as tiny, yellow-brown spots surrounded by a pale green-yellow halo. The microorganism was easily cultured on a 2% Potato Dextrose Agar medium and its ability to cause disease was confirmed according to Koch's principles. Characteristics of both cultural and morphological aspects included colonies that were entirely black, grew radially, and had an effused growth pattern. Conidia ranged in colour from light brown to black. The conidia ranged in size from 110.16 to 162.0 µm, 94.08 to 120.72 µm, and 25.44 to 46.32 µm, with septal variations of 5-11, 0-8, and 2-6, respectively [20] [21].In the conventional study, Alternaria was found in the potato. But in the present study, Alternaria was studied in the leaf spot of the mustard. It was grown in the culture medium and in-silico analysis were performed to identify their gene expression.

31 samples from mustard fields of the Allahabad, Uttar Pradesh region which are linked to leaf spot disease are experimented. Analysis of ITS, GAPDH, Alt a1, and ATP sequences indicated that *A. brassicae* and *A. japonica* have one shared multilocus haplotype, whereas *A. brassicicola* and *A. alternata* have three and five haplotypes, respectively. Pathogenicity experiments demonstrated that *A. brassicicola* was the most aggressive, whereas *A. brassicae* and *A. japonica* exhibited an equivalent amount of pathogenicity. In general, the *A. alternata* population showed limited pathogenicity, with one group of isolates being genetically unique yet closely related and nonpathogenic, indicating variability in pathogenicity within this evolutionary group. Information from observing how temperature affects the development and spore production of isolates at various temperature levels showed a variety of evolutionary strategies among species. A. alternata displayed the widest temperature range and fastest growth rate, *A. brassicicola* showed the highest intensity of spore production, and *A. brassicae* had lower optimal temperatures for spore production compared to other species [22]. Preceding study was focused on variety of the Alternaria species. The present study focused specifically on *Alternaria brassicae* species and experimented their pathogenicity, gene expression, growth rates in different parameters and relationships between other species by phylogenetic tree analysis.

Previously identified primers specific to each species are not successful in differentiating between *A. macrospora* and *A. alternata* at the species level. Two SSR primers were found to effectively show variation among the isolates. Six categories were formed using ISSR primers with a 71 percent genetic variation among 15 Alternaria isolates. Analysis of diversity in ITS sequences led to the creation of five clusters. Sequencing ITS of 15 selected isolates at NCBI showed that they all belong to *A. alternata*. This happened because there were very few *A. macrospora* sequences either present or missing in the NCBI database. Further examination of genes like Alt a1, Plasma membrane ATPase, GAPDH, and TEF-1 α sequences would help confirm the identification of A. macrospora at the species level [23].Current study focused on the description of the sequences and CDS feature of the sequences with pairwise identities in dots. It also concentrated on the association with the related species of *Alternaria brassicae*.

Twenty Alternaria isolates were collected from infected tomato fruits through a baiting technique, identified morphologically to the species level, and confirmed using Internal Transcribed Spacer (ITS) gene sequencing. The HPLC technique was employed to investigate the mycotoxin production of Alternaria species. The HPLC analysis results showed mycotoxins in four out of five Alternaria species, present in the fungal extract at different retention times. *Alternaria alternata* exhibited the most activity and produced three distinct kinds of toxins. Polyketide synthase genes, which are accountable for the production of Alternaria toxin, were also found in the DNA of Alternaria species [24].The present study concentrated on the specific gene expressions which is present in the *Alternaria brassicae* and examined the dot plots and sequences of this particular species.

The pathogen is able to invade soybean leaves, leading to the development of leaf spots. We gathered sick soybean leaves and separated four pathogens, all determined to be *Alternaria alternata* through morphological and molecular examination. Koch's postulates were employed to verify the presence of disease-causing pathogens. To our understanding, this is the primary documentation of soybean leaf spot disease from *A. alternata* in northeast China. In addition, *A. alternata* exhibited a variety of hosts and resulted in leaf spot in the majority of leguminous plants. Nevertheless, it failed to infect treated lentil or tobacco. The sensitivity of *A. alternata* to fungicides was assessed through the spore germination method, revealing that *A. alternata* strains were most responsive to flusilazole, as indicated by their EC50 values [25]. Current study was considered with specific Alternaria strain such as *Alternaria brassicae* to determine the infected range and pathogenicity of the leaf spot by the in-vitro analysis.

6. Conclusions

The study on *Alternaria brassicae* isolate I-IB20 has provided valuable insights into the morphological, cultural, growth characterization, and molecular aspects of this pathogen. The findings of this study have shed light on the mechanisms and factors that contribute to the development of leaf spot disease in mustard plants. This information is crucial in understanding the biology and pathogenicity of this pathogen and can aid in the development of effective management strategies to control leaf spot disease in mustard plants. The future directions of research on *Alternaria brassicae* isolate I-1B20 should focus on developing more accurate and efficient methods for morphological identification, identifying key environmental factors for its growth and sporulation, characterizing its virulence and genetic diversity, and understanding its gene expression and pathogenicity mechanisms. This information can aid in the development of effective management strategies for leaf spot disease of mustard caused by *Alternaria brassicae* isolate I-1B20. Additionally, collaboration between researchers and farmers can help in the transfer of knowledge and implementation of these strategies in the field, leading to improved disease management and increased crop yields.

Declaration

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Ethical statement for human participant: Not applicable for this research

References:

[1] R. Kumar and P. Shete, "Symptomology, biology and management of Alternaria leaf spot of mustard (Brassica spp.)," *J. Pharm. Innov,* vol. 10, pp. 264-268, 2021.

[2] P. Sharma, S. Javeria, S. Deep, M. Sharma, and R. Manokaran, "Diagnosis and Diversity Analysis of Alternaria brassicae and A. brassicicola in Vegetable Cruciferous Crops," *Innovative Approaches in Diagnosis and Management of Crop Diseases,* pp. 325-363, 2021.

[3] B. Jagadeesh, S. Maurya, P. Hemalatha, and A. Lingam, "Diseases and disorders of cole crops (Stem Brassicas) and their management," in *Diseases of Horticultural Crops: Diagnosis and Management*: Apple Academic Press, 2022, pp. 79-129.

[4] U. Riaz *et al.*, "Genomics of Mustard Crops," *Oil Crop Genomics,* pp. 271-290, 2021.

[5] J. Singh, "Mastering Mustard Health: A Guide to Diseases and Effective Management."

[6] N. Mpumi, R. S. Machunda, K. M. Mtei, and P. A. Ndakidemi, "Selected insect pests of economic importance to Brassica oleracea, their control strategies and the potential threat to environmental pollution in Africa," *Sustainability,* vol. 12, no. 9, p. 3824, 2020.

[7] D. Dev, A. Tewari, P. Upadhyay, and G. Daniel, "Identification and nomenclature of Albugo candida pathotypes of Indian origin causing white rust disease of rapeseed-mustard," *European Journal of Plant Pathology,* vol. 158, pp. 987-1004, 2020.

[8] S. Shukla, P. Singh, S. Shukla, S. Ali, and N. Didwania, "Scope of Onsite, Portable Prevention Diagnostic Strategies for Alternaria Infections in Medicinal Plants," *Biosensors,* vol. 13, no. 7, p. 701, 2023.

[9] T. X. Neik, J. Amas, M. Barbetti, D. Edwards, and J. Batley, "Understanding host–pathogen interactions in Brassica napus in the omics era," *Plants,* vol. 9, no. 10, p. 1336, 2020.

[10] A. Arteaga-Noriega *et al.*, "Sociodemographic and clinical factors related to the progression of disability in patients with multiple sclerosis," *Archivos Venezolanos de Farmacología y Terapéutica,* vol. 40, no. 9, pp. 907-912, 2021.

[11] J. Blagojević, J. Vukojević, B. Ivanović, and Ž. Ivanović, "Characterization of Alternaria species associated with leaf spot disease of Armoracia rusticana in Serbia," *Plant Disease,* vol. 104, no. 5, pp. 1378-1389, 2020.

[12] S. Das Laha, A. Kundu, and S. Podder, "Impact of biotic stresses on the Brassicaceae family and opportunities for crop improvement by exploiting genotyping traits," *Planta,* vol. 259, no. 5, pp. 1-17, 2024.

[13] J. Sundblom, E. Sandberg, and E. Ronne-Engström, "Trauma mechanisms and surgical outcomes in the elderly patient with chronic subdural hematoma," *Canadian Geriatrics Journal,* vol. 25, no. 1, p. 40, 2022.

[14] B. Barath, A. Rose, and S. Zacharia, "Effect of Bio-Agents and Elicitors on Alternaria brassicae of Mustard (Brassica juncea L.)," *International Journal of Environment and Climate Change,* vol. 13, no. 10, pp. 3724-3731, 2023.

[15] M. H. Kabir, O. A. Fakir, N. Millat, I. Ahmed, and M. B. Hossain, "Isolation and Morpho-Molecular Characterization of Alternaria spp. Causing Grey Leaf Blight of Mustard in Different Regions of Bangladesh," 2023.

[16] M. Anand, O. Akhtar, M. Shahbaan, I. Zoomi, and D. Pandey, "Evaluation of antifungal activities of some plant extracts against Alternaria brassicae causing spot disease of mustard."

[17] P. Rijal, S. Subedi, C. Adhikari, S. Shrestha, and J. Shrestha, "Seed-borne infestation and management of alternaria species in mustard seed at chitwan district, Nepal," 2020.

[18] S. Mahapatra, S. Chakraborty, M. Samanta, and S. Das, "Impacts of integrated nutrient management on epidemiology, seed yield and severity of Alternaria blight disease in Indian mustard (Brassica juncea L.)," *International Journal of Bio-resource and Stress Management,* vol. 13, no. 3, pp. 299-308, 2022.

[19] S. Tudu, M. Habib, M. Islam, M. Chowdhury, and M. Hasan, "Management of seed associated fungi and Alternaria leaf spot disease of black mustard (Brassica nigra L.) using botanicals," 2023.

[20] S. N. Patel *et al.*, "Morphological Characterization and Pathogenicity Test of Alternaria Leaf Spot of Chinese Cabbage (Brassica chinensis L.)," *International Journal of Plant & Soil Science,* vol. 35, no. 19, pp. 1616-1625, 2023.

[21] B. Mickiewicz, E. Volkova, and R. Jurczak, "The global market for potato and potato products in the current and forecast period," 2022.

[22] J. D. Blagojević, J. B. Vukojević, and Ž. S. Ivanović, "Occurrence and characterization of Alternaria species associated with leaf spot disease in rapeseed in Serbia," *Plant pathology,* vol. 69, no. 5, pp. 883-900, 2020.

[23] A. Sampathkumar and K. Raghavendra, "Molecular Identification and Genetic Diversity of Alternaria Isolates Causing Leaf Spot Disease in Cotton from Major Cotton Growing Areas of South Zone of India," *Indian Journal of Agricultural Research,* vol. 1, p. 7, 2023.

[24] A. Saleem and A. A. El-Shahir, "Morphological and molecular characterization of some Alternaria species isolated from tomato fruits concerning mycotoxin production and polyketide synthase genes," *Plants,* vol. 11, no. 9, p. 1168, 2022.

[25] W. Li, W. Huang, J. Zhou, J. Wang, J. Liu, and Y. Li, "Evaluation and control of Alternaria alternata causing leaf spot in soybean in Northeast China," *Journal of Applied Microbiology,* vol. 134, no. 2, p. lxad004, 2023.