

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.,
2024, 15(2), 361–366
doi: 10.15421/022451

Rapid detection of *Alternaria* spp. by PCR in the newly created forest plantations on the drained bottom of the Aral Sea

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Article info

Received 19.03.2024

Received in revised form 02.05.2024

Accepted 17.05.2024

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Sherimbetov, A., Sherimbetov, S., Adilov, B., Ruzmetov, D., & Shavkiev, J. (2024). Rapid detection of Alternaria spp. by PCR in the newly created forest plantations on the drained bottom of the Aral Sea. Regulatory Mechanisms in Biosystems, 15(2), 361–366. doi:10.15421/022451

Salinization and drought are the most important abiotic stress factors causing significant impact to the agriculture of Uzbekistan. To eliminate the negative consequences of the drying of the Aral Sea, large-scale works are currently being conducted on this territory to create protective forest plantations of halophytic trees and shrubs. An important issue in the protection of forest plantations is the isolation, identification and monitoring of phytopathogenic fungi associated with these forest plantations. The study's objective was to isolate *Alternaria* fungi from *Haloxylon aphyllum* (Minkw.) Iljin, *Tamarix hispida* Willd., *T. ramossissima* Ledeb. and to also apply a diagnostic marker using PCR assays to detect and identify *Alternaria* pathogens in these plants. As a result of the study, 10 strains of *Alternaria* fungi were isolated from plant samples of black saxaul – *Haloxylon aphyllum*, and two tamarisk species – *Tamarix hispida*, *Tamarix ramossissima*. The dominant species was: *Alternaria tenuissima* (Kunze) Wiltshire. β -tubulin gene was used as a molecular marker to distinguish and identify *Alternaria* spp in 34 leaf samples of *Haloxylon aphyllum*, *Tamarix hispida* and *Tamarix ramossissima*. The primer set used in the PCR assay was shown to be capable of detecting the presence of *Alternaria* in the leaves. Therefore, a β -tubulin-based diagnostic marker can be widely applied for monitoring of *Alternaria* infecting a wide-range of halophytic trees and shrubs in the protective forest plantations.

Keywords: salinity; halophytic trees; halophytic shrubs; forest plantations; phytopathogenic fungi.

Introduction

Salinity and drought are considered to be the most important environmental abiotic stresses (Chen et al., 2009). It is estimated that more than 900 million hectares (over 6%) of agricultural land and 30 percent of irrigation water worldwide are exposed to the influence of salinity (www.fao.org). Desiccation of the Aral Sea is considered to be the most dramatic ecosystem collapse in Central Asia. The Aral Sea which formerly covered 68,000 km² and was the fourth-biggest lake in the world, has been gradually decreasing since the 1960s, after long-term water withdrawal for irrigation (Breckle et al., 2010). Today, this lake has lost 90% of its area.

Due to disappearance of the Aral Sea, a complex set of problems have arisen with far-reaching global consequences. On the dried-up part of the sea, vast saline areas have appeared, which established the new so-called "Aralkum" desert.

The World Meteorological Organization calls the drying out of the Aral Sea one of the largest man-made environmental crises of the 20th century. The disappearance of the sea had a systemic negative effect on the flora, fauna, landscape and climate of the Aral Sea region. Within a radius of 100 kilometers, summers became hotter and drier, and winters became colder and longer. The amount of precipitation has decreased several times. Of the 173 species of animals, only 38 have survived, the populations of which are extremely vulnerable. Enormous damage was caused to the ichthyofauna – a reduction in the sea area and an increase in salinity led to the disappearance of almost all endemic species, the number of fish in the adjacent lake systems decreased by 20 times, and there are no fish left in the Great Aral Sea.

Instead of a surface of water, the sandy-salt Aralkum desert with an area of 5.5 million hectares formed on the dried part of the Aral Sea. From

here, over 75 million tons of sand with admixtures of pesticides and chemicals that were used to irrigate cotton fields and then enter the former sea with river or underground water rise into the atmosphere every year. The dust plume reaches a length of 600 km, and the height of the rising particles is up to 4 km (<https://earthobservatory.nasa.gov/images/146487/a-dusty-day-over-the-aral-sea>). Poisonous salts from the Aral region have been found in the blood of penguins in Antarctica, in the forests of Norway, and on the glaciers of Greenland, the Tien Shan and the Pamirs. Pollution of glaciers is increasing the already active melting caused by global climate change. Dust storms from Aralkum are extremely negative environmental factors that directly affect human health. Among residents of the Aral Sea region, the prevalence of eye and respiratory diseases, anemia, diabetes mellitus, respiratory diseases and cancer is increasing.

According to experts, it will no longer be possible to fully restore the Aral Sea. The most important task is to reduce the destructive impact of the acute environmental crisis.

In recent years much attention has been paid to halophytes which are naturally adapted to survive and capable of completing their life cycle in saline habitats (Hasanuzzaman et al., 2014; Sherimbetov et al., 2020; Ergashev et al., 2022; Matniyazova et al., 2022).

Haloxylon aphyllum is a hyperhalophyte and hyperxerophyte shrub or small tree belonging to the Chenopodiaceae family. It is a member of the Asian desert flora and is commonly found in the desert regions of Uzbekistan. Its benefits include resistance to salt and alkali, resistance to wind erosion, tolerance to drought, and possession of good growth and high survival rates. These attributes have led to its widespread usage in the management of desertification in arid regions of Uzbekistan. *Haloxylon aphyllum* is regarded as a valuable plant resource with great ecological and economic value for protection against wind, saving water and soil resources, and improving saline-alkaline soils (Zare et al., 2021).

In addition, *Tamarix* species are known to be the secondary forest members that are widespread in the arid and desert locations. They have excellent germination capacity, rich roots, strong ecological adaptability, and high drought and salt resistance. Given that *Tamarix* species have robust adaptations for windproofing, sand fixing, soil and water retention (Zhang et al., 2002; Kuzminsky et al., 2014), they have a lot of potential applications in the conversion of Aralkum to forests and improve ecological environment. Planting protective plantations using local halophytic trees and shrubs that can grow in highly saline soils and survive in arid conditions is an effective method of combating salt-dust transfer, as well as for fixation of drifting sands. Therefore, currently, large-scale works are being conducted on the drained bottom of the Aral Sea to create protective forest plantations and specialized forest nurseries.

In order to improve the efficiency of afforestation, it is necessary to conduct mycological monitoring to determine the composition and biology of phytopathogenic and endophytic fungi. Among the numerous diseases in the forestry, the first place in terms of prevalence and inflicted damage belongs to fungal infections. According to the frequency of their damage to plants, sporadic, focal and epiphytic diseases are distinguished (Ghelardini et al., 2016). Massive outbreaks of disease in tree nurseries, which occur from year to year in certain areas, are caused by fungi that live and persist in the soil and plant debris and infect healthy plants. Pathogens with airborne spread and an iterative reproductive cycle during the growing season of plants are also known. Phytopathogenic fungi affect the leaves, trunks and root system of seedlings and adult plants.

Numerous fungal pathogens cause diseases of tree nursery plants but arguably the most prevalent and troublesome are those caused by *Alternaria* species. According to Lou et al. (2013), *Alternaria* is a cosmopolitan fungal genus which widely distributed in the organic matter in the soil. Some *Alternaria* species only infect plant tissue that has been weakened by stress, senescence, or injury, others are very destructive primary pathogens, some of which produce strong plant toxins. *Alternaria* species produce a large number of spores, that are spread by wind or splashing water.

Depending on the size of the conidia, *Alternaria* species were divided into two groups – "large-spored" (60–100 µm long) and "small-spored" (less than 60 µm long) species (Hannibal, 2004; Simmons, 2007). At the present time, 382 species are known in the *Alternaria* genus, which are divided into 29 sections (Woudenberg et al., 2013; Gou et al., 2022; Li et al., 2023). *Alternaria* is an important invasive pathogen, that can colonize a great variety of plants all over the world (Perello & Sistema, 2006; Lee et al., 2014; Lee et al., 2015; Thomma et al., 2023). *Alternaria* species are known to cause diseases in more than 4000 species of monocotyledonous and dicotyledonous plants (Armitage et al., 2015; Lawrence et al., 2015; Woudenberg et al., 2015). They also cause diseases in ornamental plants, fruit trees and shrubs (Thomidis et al., 2006; Windham, 2008; Kalkalikova et al., 2009; Wang et al., 2010).

Alternaria diseases on plant leaves appear in the form of spots. In this case, the spots are large (sometimes up to 2 cm in diameter and larger), brown, dark-brown, gray, sometimes almost black, round, scaly, or irregular in shape. Toxin-producing species of *Alternaria* may have yellow borders around the spots (caused by the toxin) on the leaves (Woudenberg, 2015). The damage caused by *Alternaria* is represented by a decrease in the photosynthetic capacity of the leaves, a decrease in yield due to the mold of fruits and seeds, contamination of agricultural products with fungal metabolites, including mycotoxins, allergens and enzymes. The majority of *Alternaria* pathogens usually kills about 20% of fruit, but when the disease is extensive and severe, fruit yield loss can reach 60% (Woudenberg, 2015) or, as observed in India, 78% (Hannibal, 2011). Mycotoxins secreted by *Alternaria* species can be divided into three major structural groups: 1) derivatives of dibenzo- α -pyrones, such as alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT); 2) Perylene derivatives such as altertoxins (ATX-I, -II, and -III) and 3) Tetramic acid derivative which include tenuazonic acid (TeA).

The standard method for identifying *Alternaria* pathogens in infected plants is based on culture identification and quantification, which is laborious and time-consuming, which causes delays in the plant protection decision-making process. In addition, it requires a great deal of expertise in morphology-based taxonomy (Simmons, 2007). There is a need for a quick and accurate method to identify the pathogen at an early stage of

infection because *Alternaria* species cause various diseases in forestry. This will allow for the formulation of effective control strategies to avoid significant financial losses.

One of molecular biology's greatest discoveries is the polymerase chain reaction (PCR), which was made in the middle of the 1980s. PCR makes it possible to synthesize DNA, which allows the specific and precise replication of nucleic acid fragments in a million copies (Mullis & Faloona, 1987). Even trace amounts of DNA fragments can be found and identified using this method. For fungal pathogens, several PCR-based methods are currently available for determining their taxonomic status. Even though the morphology of some fungi could very similar, molecular approaches can distinguish between distinct species (Aoki & O'Donnell, 1999; Demeke et al., 2005). Fungal DNA can be detected in plant tissue using PCR, even prior to the manifestation of physiological symptoms (Wang et al., 2009). Thus, detection and identification of plant pathogens is possible even during a symptomless latent period of diseases (Shetty et al., 2007; Fones & Gurr, 2015).

Eukaryotic cells contain large amounts of β -tubulin, which is the main component of microtubules (Einax & Voigt, 2003). β -tubulin has been utilized to discover, identify, and analyze fungal species across the entire kingdom (Baldauf & Palmer, 1993; Zarrin et al., 2017). It is widely accepted that the β -tubulin gene is a perfect molecular marker for species identification and study of deep level phylogenies for fungal species because this gene contains 3.5 times more phylogenetic information than the small subunit rRNA gene (O'Donnell et al., 1998). The aim of this study is to isolate phytopathogenic fungi associated with halophytic plants growing on the dried bottom of the Aral Sea and also to apply a diagnostic marker using a β -tubulin gene-based PCR assay for rapid detection of *Alternaria* pathogens in these plants.

Materials and methods

Sample collection, fungal isolation and morphological identification. For the analysis, we used healthy and diseased plant samples (total number of samples – 34) of black saxaul – *Haloxylon aphyllum* (Minkw.) Iljin, and two tamarisk species – *Tamarix hispida* Willd., *Tamarix ramosissima* Ledeb., which were collected during the expedition to the dry seabed of the Aral Sea. Before sterilization, plant samples were thoroughly washed with running tap water to remove particles of sand, soil, and other debris. The roots were subjected to a three-stage surface sterilization procedure, treated with Tween 80 solution (200 µL in 100 mL of distilled water) for 10 minutes, 70% ethanol for 15 minutes, and twice with 2% sodium hypochlorite solution for 15 minutes, followed by rinsing with distilled water.

After these pre-treatment stages, the roots were aseptically cut into fragments with a length of 1.5–2.0 cm. Disinfected plant fragments were transferred to Petri dishes 8 cm in diameter containing agar media (PDA, PCA, WA) and incubated at 25 ± 1 °C in an artificial climate chamber for 4 weeks. All fungi that grew from inside the root samples were then transferred to media in order to isolate pure monocultures.

Transient preparations were prepared by using methylene blue, crystal violet and iodine-glycerol from the isolates and photographed under a binocular microscope Nover (NLCD-307B). The shapes and sizes of macroconidia and microconidia were measured and x40, x100, x400 images of cells and mycelium were photographed.

Morphological identification of isolated fungi was conducted based on characteristics of the macroconidia, phialides, microconidia, chlamydo-spores and the colony color and growth rate (Ellis, 1976; Simmons, 1992; Leslie & Summerell, 2006; Simmons, 2007).

DNA Extraction, PCR Amplification. DNA isolation from the mycelium of the investigated isolates was carried out using "PureLink Genomic DNA Mini Kit" (Thermo Fisher Scientific). Measurement of quantity and quality of genomic DNA was performed using a spectrophotometer NanoDrop Eight (Thermo Fisher Scientific, USA), then the DNA samples were stored at –20 °C until used for PCR.

DNA isolation from plant material. DNA isolation from the leaf tissues was carried out using "PureLink Genomic DNA Mini Kit" (Thermo Fisher Scientific).

A region of the gene for β -tubulin gene was amplified with *Alternaria* specific primers aTUB_F (5'- TCC GTC GTG CCT TCC CCC AAG

GTC TCC GAC-3') and aTUB_R (5'- GGA GCG AAT CCG ACC ATG AAG AAG TGG).

Table 1
Primers used in this study

Primer	Nucleotide sequence
aTUB_F	TCCGTCGTGCCTTCCCCAAGGTCTCCGAC
aTUB_R	GGAGCGAATCCGACCATGAAGAAGTGGAGA

For PCR, a mixture of amplification reagents "Platinum™ Taq DNA Polymerase" (Thermo Fisher Scientific, USA) was used. The PCR mixture (25 µL) contained the DNA of the investigated strain (4 µL), 14.9 µL dd H₂O, 2.5 µL 10xPCR buffer, 0.75 µL 50 mM MgCl₂, 0.5 µL 10 mM dNTP mixture, 0.5 µL 10 mM forward primer, 0.5 µL 10 mM reverse primer, 1.25 µL extender KB, 0.1 µL Platinum Taq DNA polymerase.

The following thermocycling program was used for PCR: initial denaturation (95 °C, 10 min), denaturation (94 °C, 10 s), annealing (65 °C, 30 s), elongation (72 °C, 1 min) – 30 cycles: for amplification of β-tubulin in fungal isolates; 40 cycles: for direct PCR detection in plant samples), final elongation (72 °C, 5 min).

The resulting PCR products were examined by gel electrophoresis with 1xTBE buffer (pH 8.3) in 2% agarose gel, followed by gel staining with 0.5 µg/mL ethidium bromide (EtBr) solution. Electrophoresis was performed using horizontal electrophoresis system SE-1 (Helicon, Russia)

Table 2
Cultural and morphological characteristics of *Alternaria* isolates isolated from halophytic trees and shrubs

Name of the isolate	Species	Aerial mycelium growth	Mycelium texture	Mycelium colour	Conidia L, µm	Conidia W, µm
AN-Aralkqum-1	<i>Alternaria tenuissima</i>	Moderately compact	cottony	Gray white	32.2	8.3
AN-Aralkqum-2	<i>Alternaria tenuissima</i>	Moderately compact	cottony	Gray white	41.5	11.7
AN-Aralkqum-3	<i>Alternaria tenuissima</i>	Moderately compact	cottony	Gray white	25.3	10.7
AN-Aralkqum-4	<i>Alternaria tenuissima</i>	Moderately compact	cottony	Gray white	47.0	12.6



Fig. 1. *Tamarix hispida* plants, healthy on the left, diseased on the right side



Fig. 2. *Haloxylon aphyllum* plants, healthy on the right, diseased on the left side

at 100 V for 100 minutes. PCR products were visualized by UV light and photo documented by using a gel document imaging system BK-AG100 (Biobase Kings Co., Ltd, China).

Results

Morphological and cultural characterization of Alternaria pathogens. As a result of the current study, 4 strains *Alternaria* fungi were morphologically characterized. Based on morphological and cultural traits, they were assigned to the species *Alternaria tenuissima* (Kunze) Wiltshire.

Alternaria tenuissima – on PDA agar forms initially grayish-white aerial mycelium and then turned olive green. The surface of the colony is even, the edges are uneven, fast-growing. Under the microscope: Mycelium hyphae have transverse septa, which is characteristic of higher fungi of the Dothideomycetes class. Primary conidiophores have size 60–135 × 4.4–6.7 µm and grow from aerial mycelial hyphae or directly from the agar surface, up to 70(115) µm long, 4–6 µm wide, bent 1–3 times. Conidia are simple (unbranched), moderately long or in long chains, more than 9 conidia are found in each chain, later conidia chains are branched, but not strongly branched. The color of conidia is light brown and golden brown, covered with smooth or very fine scales, inverted tuft or ellipsoid, 9.8–60.2x8.6–15.5 µm, 3–5 (9) transverse and (0)1–2(4) longitudinally or obliquely septate in broad part of conidia.



Fig. 3. Condition after 72 hours of incubation

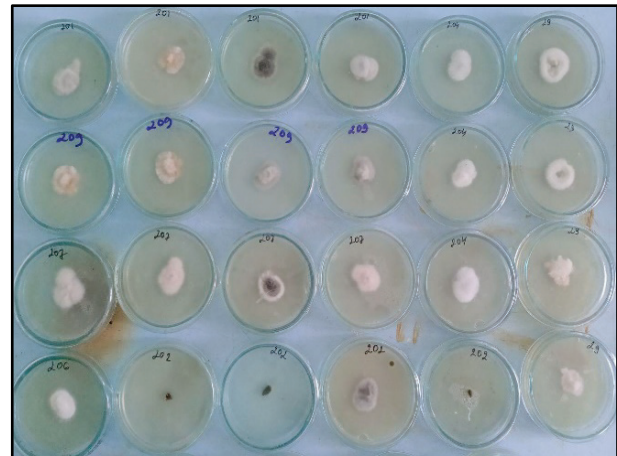


Fig. 4. Monospore fungal specimens

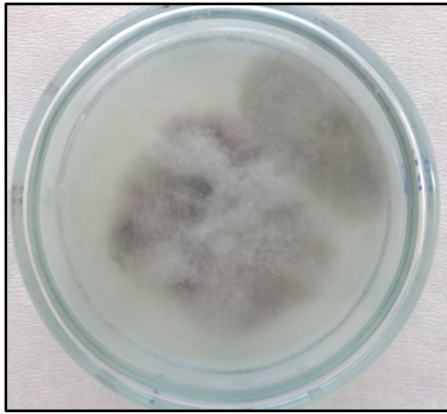


Fig. 5. *Alternaria tenuissima* on the PCA media

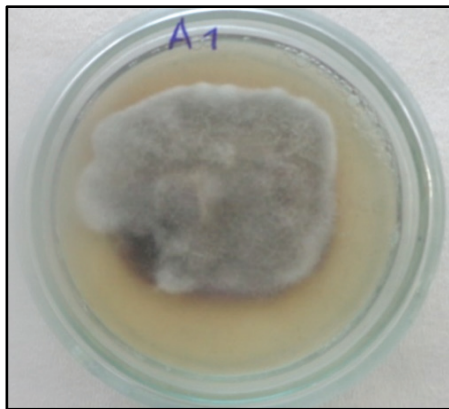


Fig. 6. *Alternaria tenuissima* on the PDA media



Fig. 7. Conidia of *Alternaria tenuissima* on PCA medium (magnification x400)



Fig. 8. Mycelium of *Alternaria tenuissima* on PCA medium (x100 magnification)

PCR analysis. PCR was performed to amplify the region of B-tubulin gene on 4 investigated isolates of *Alternaria tenuissima*. PCR products were obtained, as predicted ~300 bp long (Fig. 3).

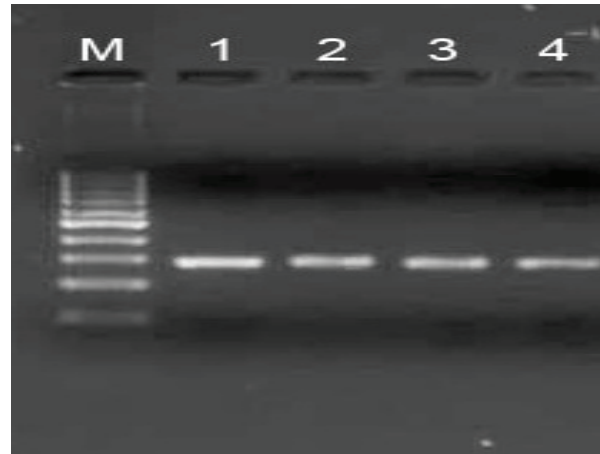


Fig. 9. Electropherogram of the results of *Alternaria* specific β -tubulin amplification of 4 investigated isolates of *Alternaria tenuissima*: M – DNA molecular weight marker 100–1000 bp, 1–4 – PCR products corresponding to 4 isolates *Alternaria tenuissima*

Then we conducted direct PCR detection of *Alternaria* pathogens in the infected leaves. In doing so, we increased the number of PCR cycles to 40 cycles in order to increase detection efficiency. When the aTUB_F and aTUB_R primer set was tested on samples of *Haloxylon aphyllum*, *Tamarix hispida* and *Tamarix ramosissima* plants, it could clearly distinguish healthy and *Alternaria* – infected plants by specific amplification of 300 bp amplicon in infected samples (Fig. 3). From 34 samples of leaves, *Alternaria* was detected in 26 diseased samples.

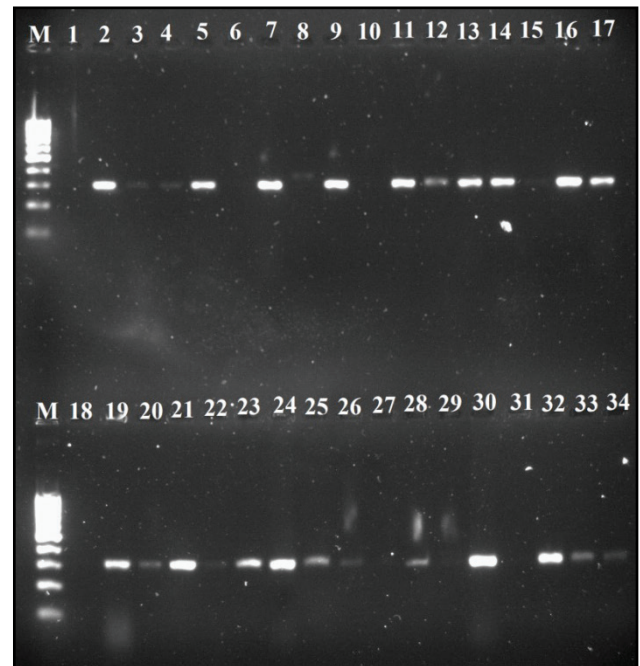


Fig. 10. Electropherogram of the results of *Alternaria* specific B-tubulin amplification in 34 leaf samples of halophytic plants: M – DNA molecular weight marker 100–1000 bp, 1–34 – results of PCR amplification B-tubulin from leaf samples

Discussion

The early artificial plantations of *Haloxylon* and *Tamarix* plants could shrink or even die due to the unfavorable abiotic and biotic factors. The main factors responsible for the degradation of these trees and shrubs

in these afforestation areas are generally believed to be a lower underground water level, soil water scarcity and diseases, which leads to their limited ability to grow and inadequate population regeneration.

Young forest plantations are particularly vulnerable to bacterial, viral and fungal diseases. Among the fungal diseases of forest nurseries, *Alternaria* leaf blight caused by *Alternaria* spp. is the most damaging one that causes significant reduction of growth and productivity of trees and shrubs. *Alternaria* is a soil inhabiting airborne pathogen responsible for leaf blight, collar and fruit rot of trees and shrubs disseminated by fungal spores. It should be noted that global climate change is strongly impacting plant pathogens. Aspects of pathogen population dynamics including overwintering and survival, rates of population expansion, or the number of generations in fungal species can all be greatly affected by climate change. Increased pathogen abundance throughout the growing season is the consequence of warming temperatures shortening the pathogen's incubation time. The disease severity of fungal pathogens is associated with higher temperatures. In North America, for instance, elevated mean winter temperatures have significantly increased the fungus *Phytophthora* spp.'s infection rates of American chestnut, leading to severe tree death incidents (Gustafson et al., 2022).

Fungi of the genus *Alternaria* are plant pathogens that capable of infecting plants at all stages of development – from seeds to adult plants and fruits. According to the current data, about 382 species of fungi of the genus *Alternaria* have been characterized in the world, from which 50 species cause economically significant diseases (Simmons, 1992). A growing trend of economic damage to the agriculture caused by fungi of the genus *Alternaria* is observed in all regions of the world. *Alternaria alternata* has been shown to be a potent pathogen of poplar trees (Uniyal et al., 2018).

In addition, our results are consistent with the recent study performed in Sri Lanka (Thambugala et al., 2017) in which *Alternaria tenuissima* reported for the first time on *Tamarix* spp.

It should be pointed out that recently severe disease outbreaks in the population of *Ailanthus excelsa* caused by *Alternaria alternata* were recorded in the nursery of the Forest Research Institute, Dehradun. *Ailanthus excelsa* is a very drought resistant tree and native to India (Kant et al., 2020). Previous research conducted in Iraq performed by Tawfik M. Muhsin revealed twelve species of *Alternaria* that were isolated from six salt marsh halophytes, *Atriplex patula*, *Glaux maritima*, *Hordeum jubatum*, *Puccinellia nuttalliana*, *Salicornia europaea*, *Suaeda depressa* (Muhsin et al., 2011). Precise and quick detection of pathogens is critical for effective plant disease control in the woodlands.

It should be noted that identification of mycopathogens in plants, especially in the early symptomless period of infection is very difficult, though it can facilitate timely application of corresponding fungicides to greatly reduce the losses in young forest plantations.

We have successfully applied a β -tubulin gene-based molecular marker for identification of *Alternaria* spp. in halophytic trees and shrubs. Such molecular markers can be very helpful for monitoring of phytopathogenic fungi and for determining the best plant disease control approach before they caused a significant damage.

The traditional methods depended on plant disease diagnosis based on specific symptoms in the affected plants, followed by isolation and morphological identification of phytopathogens from them. Because such a strategy relies heavily on taxonomic knowledge and takes a lot of time, it might not be helpful to develop control strategies in advance. In order to minimize financial losses, it is very important to detect pathogens at an early stage of infection by using molecular markers.

Therefore, a β -tubulin PCR assay can be of great assistance for the precise identification of *Alternaria* spp. from a variety of forest plantations of halophytic trees and shrubs, which facilitates correct selection of fungicides to manage corresponding diseases in these plantations. Their effectiveness, particularly in the early phases of disease development, will undoubtedly aid in the development of control strategies for minimizing economic losses.

The scientific research work was supported by the Ministry of Innovative Development of the Republic of Uzbekistan and was carried out on the basis of the project FZ 20191 (2275+3015) "Development of agrotechnology for the protection of newly established forest and pasture plantations from diseases in the dried-up part of the Aral Sea".

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