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Black Point of Wheat: Fungal associations, cultivar susceptibility, and biological control: case of *Alternaria* spp.

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To my mother, the dearest of all.

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For your endless love, strength, and

faith that has guided me in every step.

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الملخص

جنس *Alternaria*، من أبرز التحديات التي تواجه إنتاج القمح على مستوى العالم. فهذا المرض لا يؤدي فقط إلى تدهور جودة الحبوب، بل يثير أيضًا مخاوف تتعلق بسلامة الغذاء والأعلاف نتيجة تراكم السموم الفطرية. هدفت هذه الأطروحة إلى دراسة التنوع والقدرة السُمِّيّة لأنواع *Alternaria* المرتبطة بالقمح الجزائري، بالإضافة إلى تقييم استراتيجيات مبتكرة ومستدامة للمكافحة. في الجزء الأول من الدراسة، جرى تحليل 48 عينة من حبوب القمح جُمعت من مناطق الإنتاج الرئيسية. وقد أظهرت تحليلات التسلسل الجيني متعدد المواقع لـ 72 عزلة ممثلة أن *Alternaria alternata* هي النوع الأكثر شيوعًا، تليها أنواع من القسم Infectoriae. كما تم التعرف على ثلاثة أنواع لم تُسجَّل سابقًا في القمح الجزائري (*A. eureka*، *A. consortialis*، و *A. tellustris*). أظهرت التحاليل الكيميائية الحيوية مستويات مرتفعة من التلوث بالسموم الفطرية، حيث تم الكشف عن المونو ميثيل إثير للألترناريول، الألترناريول، وحمض التينو أزونيك في 75%، 69%، و35% من العينات على التوالي. وأكد تواجد عدة سموم فطرية في معظم العينات وجود خطر سُمِّي كبير. في الجزء الثاني من البحث، تم تقييم الفعالية المضادة للفطريات لبلازما التفريغ بالحاجز العازل (DBD) كطريقة مكافحة مستدامة. وأظهرت أربع أنواع من *Alternaria* معزولة من القمح الصلب استجابة تعتمد على الوقت والنوع. فقد انخفض النمو الميسليومي بشكل ملحوظ بعد 5 دقائق من التعريض، بينما تم تثبيط إنبات الأبواغ بشكل شبه كامل بعد 15 دقيقة لدى أكثر العزلات حساسية. وأكدت الملاحظات المجهرية حدوث تغييرات بنيوية خطيرة، شملت انهيار جدران الأبواغ وتسرب المحتويات السيتوبلازمية. وبصورة شاملة، توفر هذه النتائج أول تقييم متكامل للتنوع الحيوي لأنواع *Alternaria* والتلوث بالسموم الفطرية المرتبط بمرض النقطة السوداء في القمح الجزائري، وتُبرز إمكانية اعتماد بلازما DBD كبديل صديق للبيئة عن استراتيجيات المكافحة الكيميائية

الكلمات المفتاحية :السمية، القمح، جنس ألتيرناريا، معالجة البلازما الباردة.

Abstract

Black point disease, primarily caused by fungal species of the genus *Alternaria*, represents a major constraint to wheat production worldwide. This disease not only reduces grain quality but also raises concerns for food and feed safety due to the accumulation of mycotoxins. The present study investigated both the diversity and toxigenic potential of *Alternaria* species associated with Algerian wheat, as well as the evaluation of innovative, sustainable control strategies. In the first part of the study, 48 wheat grain samples collected across major production regions were analyzed. Multi-locus sequence analysis of 72 representative isolates revealed *Alternaria alternata* as the predominant species, followed by taxa of the I*nfectoriae* section. In addition, three species not previously reported in Algerian wheat (*A. eureka*, *A. consortialis*, and *A. tellustris*) were identified. Mycotoxin analyses showed high levels of contamination, with alternariol monomethyl ether, alternariol, and tenuazonic acid detected in 75%, 69%, and 35% of samples, respectively. The co-occurrence of multiple mycotoxins in most samples confirmed a considerable mycotoxigenic risk. In the second part of the work, the antifungal activity of Dielectric Barrier Discharge (DBD) plasma was evaluated as a sustainable control method. Four *Alternaria* species isolated from durum wheat exhibited time- and species-dependent sensitivity to plasma treatment. Mycelial growth was significantly inhibited after 5 minutes of exposure, while spore germination was almost completely suppressed after 15 minutes in the most sensitive strains. Microscopic observations confirmed severe structural alterations, including spore wall collapse and cytoplasmic leakage. Together, these results provide the first comprehensive assessment of *Alternaria* species diversity and mycotoxin contamination associated with black point disease in Algerian wheat and highlight the potential of DBD plasma as an eco-friendly alternative to chemical control strategies.

Keywords: Black point, durum wheat, Alternaria spp., mycotoxins, dielectric barrier discharge plasma.

Résumé

La maladie du point noir, causée principalement par des espèces fongiques du genre *Alternaria*, constitue une contrainte majeure pour la production du blé dans le monde. Cette maladie réduit non seulement la qualité des grains, mais soulève également des préoccupations pour la sécurité alimentaire et animale en raison de l'accumulation de mycotoxines. La présente étude a porté sur la diversité et le potentiel toxinogène des espèces d'*Alternaria* associées au blé algérien, ainsi que sur l'évaluation de stratégies de lutte innovantes et durables. Dans la première partie de l'étude, 48 échantillons de grains de blé collectés dans les principales régions de production ont été analysés. L'analyse multilocus de 72 isolats représentatifs a révélé *Alternaria alternata* comme espèce prédominante, suivie des taxons de la section Infectoriae. Trois espèces non encore signalées dans le blé algérien (*A. eureka*, *A. consortialis* et *A. tellustris*) ont également été identifiées. Les analyses mycotoxiniques ont montré des niveaux élevés de contamination, avec la détection de l'alternariol monométhyl éther, de l'alternariol et de l'acide ténuazonique dans 75 %, 69 % et 35 % des échantillons, respectivement. La cooccurrence de plusieurs mycotoxines dans la majorité des échantillons a confirmé un risque toxinogène considérable. Dans la deuxième partie du travail, l'activité antifongique du plasma à décharge à barrière diélectrique (DBD) a été évaluée comme méthode de lutte durable. Quatre espèces d'*Alternaria* isolées du blé dur ont montré une sensibilité dépendante du temps et de l'espèce. La croissance mycélienne a été significativement inhibée après 5 minutes d'exposition, tandis que la germination des spores a été presque totalement supprimée après 15 minutes pour les souches les plus sensibles. Les observations microscopiques ont confirmé des altérations structurales sévères, incluant l'effondrement des parois sporales et la fuite du contenu cytoplasmique. Dans l'ensemble, ces résultats fournissent la première évaluation complète de la diversité des espèces d'*Alternaria* et de la contamination mycotoxinique associée à la maladie du point noir dans le blé algérien, et mettent en évidence le potentiel du plasma DBD comme alternative écologique aux stratégies chimiques de lutte.

Mots-clés : Toxicité, Blé, *Alternaria spp.*, traitement par plasma froid.

List of Abbreviations

AAL	AAL toxins
ACT	Actin gene
AhR	Aryl hydrocarbon receptor
ALT	Altenuene
Alt a1	Allergen Alt a1 gene
AME	Alternariol monomethyl ether
ANOVA	Analysis of variance
AOH	Alternariol
ATX-I, II, III	Altertoxin I, II, III
BLAST	Basic Local Alignment Search Tool
BLS	Bacterial Leaf Streak
BP	Black Point
CAL	Calmodulin gene
CNNC	National Center of Control and Certification
CP	Cold Plasma
DBD	Dielectric Barrier Discharge
DHN	Dihydronaphthalene
EFSA	European Food Safety Authority
EndoPG	Endopolygalacturonase gene
FAO	Food and Agriculture Organization

GAPs	Good Agricultural Practices
GAPDH/gpd	Glyceraldehyde-3-phosphate dehydrogenase gene
HPLC	High-Performance Liquid Chromatography
HSD	Honest Significant Difference
HSTs	Host-Specific Toxins
ITS	Internal Transcribed Spacer
LC	Liquid Chromatography
LSU	Large Subunit ribosomal RNA
MBB	Mohamed Ben Bachir
MEA	Malt Extract Agar
MEB	Malt Extract Broth
mtSSU	Mitochondrial Small Subunit
NCBI	National Center for Biotechnology Information
nHSTs	Non-Host-Specific Toxins
OPA10-2	OPA10-2 gene
PCA	Principal Component Analysis / Potato Carrot Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PKS	Polyketide Synthase
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPB2	RNA polymerase II gene

SCAR	Sequence Characterized Amplified Region
SD	Standard Deviation
SMs	Secondary Metabolites
SNA	Spezieller Nährstoffarmer Agar
SPE	Solid Phase Extraction
SSU	Small Subunit ribosomal RNA
STTX-I, III	Stemphyltoxin I, III
TAE	Tris-Acetate-EDTA
TeA	Tenuazonic Acid
TEF1-α	Translation Elongation Factor 1-alpha gene
TEN	Tentoxin
THN	1,3,6,8-tetrahydroxynaphthalene gene
Tsr1	Tsr1 gene
TTC	Toxicological Threshold Concern
USDA	United States Department of Agriculture

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Introduction

Wheat stands as a primary staple food crop globally, fundamentally underpinning international food security. According to FAO (2025), the anticipated global wheat harvest for 2025/26 has been revised downwards to 804.9 million tons. In contrast, the Food and Agriculture Organization of the United Nations has elevated its projection for the 2025/26 agricultural year's global grain harvest to an unprecedented 2 billion 961 million tons (FAO, 2025).

In Algeria, wheat and barley are the principal cereal crops. Durum wheat occupies approximately 45% of the cultivation area, followed by barley at just over 33%, and bread wheat at around 15% over the last ten years. Projections indicate that the 2025-26 wheat harvest will be maintained at 3 MMT, with barley production anticipated to reach 1.2 MMT. These production estimates are consistent with the current land use, where wheat is cultivated on slightly more than 2 million hectares and barley on 1 million hectares. Wheat constitutes 60% of the national food ration, signifying its importance in the Algerian diet (USDA, 2025).

However, wheat yields are under constant threat from biotic stresses. Fungal, bacterial, and viral pathogens cause an average global yield loss of 21.5% (Nemati et *al.*,2022). While Figueroa et *al*. (2018) emphasized yield reduction, Abdullah et *al*. (2019) highlighted seed quality deterioration. Together, these findings suggest that Black Point threatens both production and post-harvest viability, indicating the need for integrated management strategies beyond yield protection. Pathogenic fungi commonly linked to wheat black point disease include species from the *Fusarium, Alternaria, Bipolaris, and Drechslera* genera (El-Gremi et *al*., 2017).

Black Point disease in wheat is mostly caused by *Alternaria* species, which can harm wheat grains both during and after harvest. Alternaria infections typically result in lower cereal grain quality rather than a decline in yield (Kosiak et *al*., 2004; Vučković et *al*., 2012). The embryo section of wheat kernels infected with Alternaria species usually exhibits black coloring, which detracts from the grain's nutritional value and flour quality. (Maisello et *al*., 2022). Environmental factors, particularly meteorological conditions, significantly influence the incidence of *Alternaria* species in grains. Weather and climatic factors are identified as the primary drivers of *Alternaria* infection rates. Specific conditions during the flowering-ripening period that contribute to *Alternaria* occurrence include heavy precipitation, long-term dew, abundant watering, and high air humidity (Gannibal, 2018).

Along with the pre-harvest challenges that negatively impact primary crop production, *Alternaria* species can also cause significant post-harvest losses through food spoilage and the biosynthesis of hazardous mycotoxins such as alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and tentoxin (TEN), which can contaminate food products and consumer goods (Mujahid et *al.*, 2020; Aichinger et *al*., 2021). Although limited information is currently available regarding the perylene quinones altertoxin I, II, and III (ATX-I, -II, and -III), stemphyltoxin I and III (STTX-I and STTX-III), and alterperylenol/alteichin (ALP), most existing data on *Alternaria* mycotoxins in food concern AOH, AME, ALT, tenuazonic acid (TeA), and TEN. Multiple *Alternaria* mycotoxins frequently co-occur in contaminated food products; in some instances, up to six distinct toxins have been simultaneously detected, as reported in tomato sauce, wheat flour, and sunflower seed oil (Crudo et *al.*, 2019; Puntscher et *al*., 2018). Moreover, climate change and the adoption of reduced tillage practices have been associated with an increased prevalence of *Alternaria* mycotoxins in cereal crops (Müller & Korn, 2013).

International regulatory bodies, such as the European Food Safety Authority (EFSA), have emphasized the public health risks posed by *Alternaria* mycotoxins, particularly in grains and grain-derived products, and have concluded that further targeted research on their toxicity and occurrence is essential (EFSA, 2011; EFSA, 2016). Unlike most synthetic chemicals, the primary data used to evaluate the hazards of naturally occurring toxins are derived from peer-reviewed scientific studies. EFSA's Panel on Contaminants in the Food Chain (CONTAM Panel) has issued two scientific opinions on *Alternaria* toxins: the first assessed the potential health risks to humans and animals from *Alternaria* toxins in food and feed (EFSA, 2011), while the second evaluated dietary exposure to these toxins within the European population (EFSA et *al*., 2016).

The management of wheat Black Point disease necessitates a multifaceted approach, often involving the application of fungicides or biological control agents. However, fungicides can result in undesirable environmental and health impacts due to residues, while biological control methods may exhibit limited efficacy in practical field settings (Lamichhan et *al*., 2017) modern agricultural disease management strategies are increasingly prioritizing integrative approaches and innovative technologies (Yodpitak et *al*., 2019). Consequently, attention has turned to innovative approaches, including nonthermal (cold) plasma technology. This method has emerged as a promising tool, with applications spanning biology, medicine, agriculture, and the food industry (Fridman et *al*., 2012; Attri et *al*.,2020) Its utilization in agricultural research

and industry is expanding, focusing on areas such as modifying seed surface characteristics, eliminating seed-borne pathogens, neutralizing mycotoxins, and promoting seed germination and plant growth (Mildaziene et *al.*,2022).

Despite the agronomic importance of durum wheat in Algeria and its central role in the Mediterranean diet, there remains a notable lack of comprehensive research addressing the fungal disease known as Black Point, particularly when caused by *Alternaria* spp. While *Alternaria* is globally recognized as a major contaminant of cereal grains, its prevalence, species diversity, and toxicological impact on Algerian wheat have only recently begun to receive attention. Until the 2025 study by Daichi et al., there was no detailed molecular characterization of *Alternaria* species nor systematic mycotoxin profiling in Algerian durum wheat. Moreover, the relationship between black point symptoms, fungal colonization, and grain quality, especially in terms of nutritional degradation and food safety, remains poorly understood in the Algerian context.

Therefore, promptly resolving the problem of this emerging disease would support attempts to overcome Algeria's food security challenges. That is why this prospective study was designed to obtain data that will help to address these research gaps by meeting the following objectives:

- Characterizing the phylogenetic diversity and phenotypic variation of *Alternaria* species isolates responsible for black point disease in durum wheat from Algeria.

- Evaluating mycotoxin levels in toxigenic *Alternaria spp.* cultures and durum wheat grain samples from different Algerian regions using HPLC.

- Evaluating the effect of cold plasma treatment in inactivating various *Alternaria* species

Part 1
Literature review

1.Significance of durum wheat

Durum wheat (*Triticum turgidum* ssp. *durum*) is a tetraploid species (2n = 28) cultivated annually over approximately 18 million hectares, representing about 8–10% of total global wheat acreage. Its annual production averages between 35 and 40 million metric tons (De Vita et *al.*, 2019). By 2050, the global population is projected to reach 9.7 billion, and to meet this demand, wheat production will need to increase by an estimated 132 million metric tons, assuming a stable per capita consumption rate. As a key staple of the Mediterranean diet, durum wheat not only holds nutritional significance but also contributes substantially to agricultural economies, particularly in regions susceptible to drought and environmental stressors that threaten both yield and grain quality (Zaïm et *al.*, 2020).

Durum Wheat is adapted to a range of soil conditions, but it thrives best in fertile well-drained loam clay soils at elevations between 1,500 and 2,800 meters above sea level (Hailu et *al.*,2005). Additionally, to the Mediterranean basin countries such as Turkey, Italy, Morocco, Syria, Tunisia, France, Spain, and Greece; other major producers of durum wheat include Canada, Mexico, the United States, Russia, Kazakhstan, Azerbaijan, and India, with Canada, Mexico, and the United States being the leading exporters (Martínez-Moreno et *al*., 2020)

As with other cereal crops, Algeria's durum wheat production was severely affected by the prolonged drought in 2023. The country, which typically cultivates around 1.5 million hectares of durum wheat annually, experienced a marked decline in output. The total wheat production, including both durum and common wheat, was estimated at 3.3 million metric tons, with durum wheat representing a substantial share of this total. However, this figure remains below the five-year average due to widespread drought conditions that have hindered crop growth across the nation. Durum wheat semolina is a dietary staple in rural regions of Algeria, where it is traditionally used to prepare couscous, leavened flatbread, *frik*, homemade pasta, and various types of cakes (Kezih et *al.*, 2014).

2.The constraints of durum wheat production

2.1Abiotic constraints

Drought stress is a significant issue for agricultural production in many parts of the world (Ergen et *al.*,2009), particularly in regions where durum wheat is predominantly cultivated. Climate change is expected to substantially alter temperature and rainfall patterns, leading to more frequent and severe droughts. Additionally, the expansion of durum wheat cultivation into

regions with cold winters is constrained by the cold-sensitivity of current landraces. Low temperatures commonly impair plant growth and crop yields, resulting in substantial losses (Zhang Li et *al*.,2000). Additionally, plants exhibit varying tolerances to chilling (0–15 °C) and freezing (< 0 °C) temperatures at different phenological stages, with the seedling and heading stages being the most sensitive.

Other abiotic stresses, such as salinity, nutrient deficiencies, and heavy metals, can also negatively impact durum wheat production (Kabbaj et *al*., 2017). Salinity is a growing issue in many areas where durum wheat is grown, resulting in reduced grain yield and quality. Additionally, nutrient deficiencies, particularly in nitrogen, phosphorus, and zinc, are common in durum wheat-growing areas, which can limit yield potential. Furthermore, the high levels of heavy metals in the soil, which can arise from both natural and anthropogenic sources, poses a serious threat to durum wheat production, as these metals can adversely affect plant physiology and lower the yield and quality of the grain harvested (Zaïm et *al*., 2020; Ayed et *al*., 2021; Arif et *al*., 2020).

2.2. Biotic constraints

Durum wheat is susceptible to various biotic stressors that reduce crop productivity. These include the widespread use of local cultivars that have not been improved, declining soil fertility, inadequate traditional cultural practices, insect pests, and diseases that may be bacterial, fungal, or viral (Lemann et *al*.,2017). In addition, weeds are one of the main factors contributing to crop losses of up to 50% (Azad, 2003).

The presence of weeds is generally inversely proportional to wheat crop yield, so controlling weeds directly contributes to higher yields (Kristensene et *al*., 2008). Weed growth alongside heavily fertilized and irrigated crops reduces productivity and lowers produce quality through contamination with weed seeds (Yadav et *al*.,2006). They compete with the wheat crop for vital resources, such as nutrients, water, sunlight, and space, which in turn weakens the main crop and ultimately leads to reduced yields. Furthermore, the climate fosters rapid and abundant weed growth, leading to widespread infestations of weeds across all crops. the most common species of weeds in wheat fields are *Avena* sp., *Lolium* sp., *Bromus* sp., *Phalaris* sp., and *Chenopodium* sp. (Minhas et *al*.,2023).

On the other hand, a wide variety of insects have been found on wheat globally. While many of these insects cause little to no significant damage or are limited in their distribution,

some pests can inflict substantial losses in yield and forage. Certain pest issues are directly tied to the specific farming practices used in a particular region. In contrast, others are opportunistic or generalized herbivores that do not exclusively target wheat (Miller et *al*., 2002). The insect pests of durum wheat include aphids, mites, sawflies, and the Hessian fly, which can considerably harm the crop by feeding on the leaves, stems, and grains. These insect pests not only reduce yield but may also transmit diseases. It is reported that there are about 100 harmful insect species in wheat (Qayyum et *al*.,2021). Some of these insects, which are in different orders and families, have polyphagous, some oligophagous, and some monophagous feeding features. Insects, which appear in different phenological periods related to the regions, cause direct damage by feeding on the root, stem, leaf, spike, flower, and grain of wheat.

When the insects discover favorable weather conditions and food supplies, they spread epidemics. For instance, the Sunn *pest Eurygaster integriceps Puton* can result in yield losses of 90% and 100% in wheat during epidemic years if no control measures are taken (Lodos et *al*., 1961, Kıvan et *al.*, 2005). In many areas where wheat and barley are grown, the Hessian fly, Mayetiola destructor, is a serious insect pest that can infect up to 32.5% of bread wheat crops (Makni et *al*., 2011).In addition to directly harming wheat crops, wheat aphids like Rhopalosiphum padi (L.) and R. maidis (Fitch) are vectors for several viruses (Riedell et *al*., 2003, Bukvayová et *al*., 2006, Savaris et *al*., 2013, Fidan et *al.*, 2014) .Moreover, the impact of pest infestations on wheat crops, particularly durum wheat, becomes even more pronounced under the stress of climate change, which not only fosters the emergence of new pest species but also exacerbates their virulence and resistance to control measures, consequently leading to increased economic losses for farmers and affecting food security globally (Zhang et *al.*,2021).

The Sun pest can significantly impair the quality of durum wheat. Infestation causes declines in gluten quality and alterations to the protein structure, which adversely impact the milling characteristics and overall quality of semolina and pasta product (Olanca et *al*., 2009). It belongs to the genus *Eurygaster* and the order Hemiptera. A total of 16 species belonging to this genus are known worldwide, but the most significant pest species on wheat is *Eurygaster integriceps Puton.* Geographically, they are more common in Asia, Europe, North America, and North Africa. (McPherson, 1982; Critchley,1998; McPherson,2017).

Durum wheat production is threatened by a range of viral, bacterial, and fungal diseases, all of which have the potential to reduce crop yields and quality substantially. Bacterial diseases are significant biotic constraints that substantially impact wheat, a vital global source of

carbohydrates. These bacterial pathogens are estimated to reduce annual wheat production by approximately 10%, with severe infections early in the growth cycle resulting in losses of up to 40% (Tambong, 2022). The most common bacterial pathogen causing serious losses in durum wheat production is *Xanthomonas translucens*, which is responsible for Bacterial Leaf Streak (BLS**).** This disease not only reduces crop yields but also lowers grain quality and nutrient content, changing the protein content (Shane et *al*., 1987), which makes the affected crops less suitable for human consumption and animal feed. The symptoms appear on the leaves and/or spikes of wheat plants. They begin with water-soaked, necrotic streaks that eventually develop into translucent lesions. In severe cases, the entire leaf may appear blighted and wilt prematurely, with yellow streaking and browning of leaf sheaths. In addition to the bacterial diseases, there are the fungal diseases of durum wheat that pose a serious threat to productivity.

3.Main fungal diseases of durum wheat

It has long been recognized that various factors can influence the qualitative characteristics of wheat seeds, including seed germination, moisture content, seed discoloration, and prevalence of seed-borne fungi. Seed-borne fungi are prevalent in durum wheat, with the most common culprits being *Alternaria spp.*, *Fusarium spp.*, *Bipolaris sorokiniana*, and *Pyrenophora tritici-repentis*. These fungi can infect the crop at various stages of development, from seedling emergence to grain maturation, resulting in significant yield and quality losses (Table 1).

Table 1: Major fungal diseases of durum wheat, their causal agents, characteristic symptoms, and key references.

Disease	Causal Agent	Symptoms	Reference
Fusarium Head Blight (Scab)	*Fusarium graminearum*	Bleached spikelets on heads, pink or reddish fungal growth on glumes, shriveled kernels (tombstone grains), reduced yield, and mycotoxin (DON) contamination making grain unsuitable for consumption	Rakesh et al., 2016; Esse et al., 2019

Fusarium Wilt	*Fusarium oxysporum* f. sp. *Lini*	Vascular infection; wilting, stunting, and plant death	Rakesh et al., 2016; Esse et al., 2019)
Tan Spot	*Pyrenophora tritici-repentis*	Necrotic leaf lesions spreading to sheaths/glumes; reduced photosynthesis and grain filling	(Li et al., 2018; Rakesh al., 2016)
Septoria Tritici Blotch (STB)	*Zymoseptoria tritici*	Leaf blotches; yield losses up to 50%, severity dependent on temperature and moisture	(Faris et al.,2020) (Burstmayr et al.,2021)
Alternaria Leaf Blight	*Alternaria alternata*, *A. triticina*	Brown necrotic spots on leaves, shriveling of grains, seed discoloration	(Mehta,2014)
Spot Blotch	*Bipolaris sorokiniana*	Dark brown lesions on leaves and nodes; premature leaf death; shriveled grains	(Gupta et al.,2018)
Stem Rust	*Puccinia graminis* f. sp. *tritici*	Reddish-brown pustules on stems and leaves; lodging; major yield losses	(Bhattacharaya et al., 2020)
Stripe Rust (Yellow Rust)	*Puccinia striiformis* f. sp. *tritici*	Yellow pustules in stripes on leaves; reduced photosynthesis and grain weight	(Chen ,2020)

4.Black point of durum wheat

4.1. Back ground of the disease

Different countries have different names for black grain discoloration. Bolley (1913) was the first to refer to the condition as "black point" (BP) in the United States. Several scholars thereafter adopted this phrase (Coons, 1918; Evans, 1921; Drechsler, 1923; Henry, 1923; Dastur, 1932; Brentzel, 1944; Hanson & Christensen, 1953). Other names for it include "puntatura" in Italy (Curzi, 1926), "mouchetage" or "mouchetture" in Morocco and Algeria

(Miège, 1930) and France (Rosella, 1930; Ponchet, 1966), "black germ" or "black grain" in the former USSR (Ziling, 1932), and "smudge " or "kernel smudge" in Canada (Simmonds, 1930; Greaney & Wallace, 1943).

The phenomenon known as black point, manifested as kernel darkening, has been reported across all durum wheat-producing regions (Fernandez et *al*., 2011). It is typified by a brown to black discoloration localized in the embryonic tissues of the grain. This condition poses a significant concern in wheat cultivation, particularly under humid environmental conditions and periods of rainfall during grain filling and seed maturation, which have been identified as key factors promoting black point incidence and severity in wheat (Khlebova et *al*., 2019).

4.2.Symptomology

Black point is characterized by a dark brown to black discoloration that develops primarily at the embryo end of the wheat kernel. The symptoms are generally confined to the outer tissues of the grain during the early stages of infection, but under severe conditions the discoloration may extend along the crease and surrounding kernel tissues (Figure 1). The affected grains frequently exhibit grayish-brown pigmentation and reduced visual quality, making them undesirable for processing and marketing. Symptom severity varies according to environmental conditions, cultivar susceptibility, and the extent of fungal colonization (Li et *al*., 2020; Golosna et *al*., 2026**)**.



Figure 1: Black point symptoms observed on wheat kernels showing varying degrees of dark discoloration at the germ (Watkins, 2011).

Black point is a subtle yet detrimental disease that typically manifests as grains begin to lose moisture, particularly during the grain-filling or milking stages (Xu K-G et *al*., 2018; Bashyal et *al*., 2020). The symptoms generally remain inconspicuous until harvest, becoming evident only once the grain is threshed from the spike (Southwell et *al*., 1980b). Earlier studies suggested that the presence of black point does not exert a significant impact on seed germination (Dharam Vir et *al*., 1986; Rees RG et *al*., 1984). However, subsequent investigations have demonstrated that the disease can reduce germination rates, diminish the number of embryonic roots, and shorten coleoptile length (Toklu et *al*., 1999). Moreover, black point has been associated with delayed seedling emergence and decreased seedling vigor (Özer, 2005; Toklu et *al*., 2008). Importantly, several fungal pathogens implicated in black point are known producers of mycotoxins, posing potential risks to both animal and human health. Maisello et *al*.,2020).

After flowering, wheat seed infection rises (Andersen, 1952). Additionally, it may be impacted by management strategies, location (particularly in warm and humid areas), and genotype (Zhang et *al*., 1990). The ovary wall and seed coat allow penetration into the seed (Han et *al*., 2010; Ansari et *al*., 2017). The fungus survives as a resting mycelium for 5 years (Mead, 1942). The two most significant factors influencing the occurrence of black point disease are temperature and humidity. The occurrence of the disease is often increased by higher humidity (particularly above 90%), precipitation, and comparatively lower temperatures (<30c) after heading (Cromey and Mulholland, 1988; Li et *al*., 2019a; Li et *al*., 2019b).

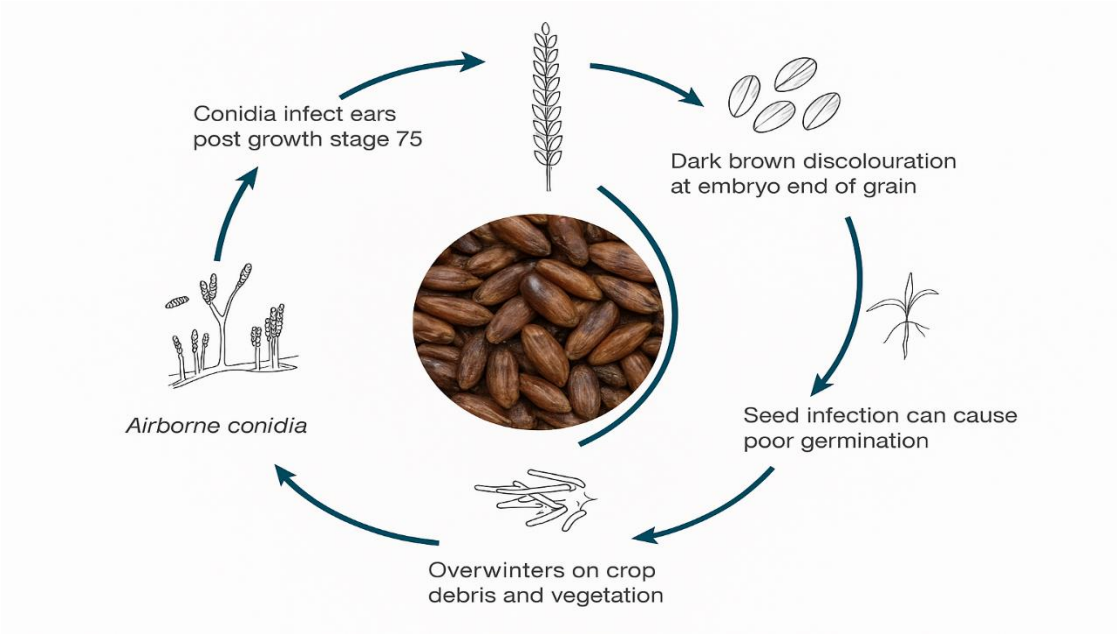

Figure 2: life cycle of Black point disease of wheat (original)

4.3.Association of fungi with black point disease

A diverse range of fungal taxa has been consistently associated with the etiology of black point disease in wheat. Predominant isolates recovered from infected kernels include *Alternaria*, *Cochliobolus*, *Fusarium*, *Cladosporium*, *Curvularia*, *Penicillium*, *Aspergillus*, and *Stemphylium* (Kailash et *al*., 1987; Fakir et al., 1989; Rossi et *al*., 1991; Ilyas et *al*., 1998; El-Khalifeh et *al*., 2002; Mihaela et *al*., 2013; Malaker and Mian, 2002; Mishra and Srivastava, 2015; Abdullah and Atroshi, 2014; Abdullah and Atroshi, 2016). These fungi have been repeatedly isolated from black point-affected kernels and demonstrated pathogenicity, either as individual agents or through synergistic interactions (Huguelet and Kiesling, 1973; Agarwal et *al*., 1993; Ahmed et *al*., 1994; Vassilev et *al*., 1997; Karwasra et *al*., 2006; Toklu et *al*., 2008; Fernandez and Conner, 2011; Pathak and Zaidi, 2013; Srivastava, 2014; El-Gremi et *al*., 2016).

4.4.*Alternaria* Genus

4.4.1. Generalities on *Alternaria*

The genus *Alternaria* is considered one of the most cosmopolitan groups of fungi, occurring on a wide range of ecological substrates including plants, soil, textiles, and stored seeds (Dang et *al*., 2016). Their ability to adapt to diverse environments is partly attributed to their production of pigmented (melanized) cell walls and stress-tolerant conidia, which enhance

survival under fluctuating environmental conditions. A key factor in their ecological success is the dispersal of airborne spores, which can travel long distances and colonize new habitats efficiently.

From an agricultural perspective, *Alternaria* species are notorious as plant pathogens, particularly in cereals, vegetables, and fruits. They are responsible for economically important diseases such as leaf blight, leaf spot, fruit rot, and seed discoloration. In wheat, species like *A. alternata* and *A. triticina* are associated with seed-borne infections that compromise germination, grain quality, and storage stability (Meena et *al*., 2017). These fungi are also prolific producers of secondary metabolites, including host-specific toxins (HSTs) and mycotoxins, which not only play a role in pathogenesis but also raise concerns regarding food safety (Dang et *al.*, 2016).

In addition to their agricultural significance, *Alternaria* spp. are now recognized as important human health hazards. Their airborne spores are well-documented allergens and are strongly associated with the development and exacerbation of asthma, rhinitis, and hypersensitivity reactions, particularly in children and sensitized individuals (D'Amato et *al*., 2016; Crameri et *al.*, 2014). Clinical studies have identified *Alternaria* as one of the most potent outdoor fungal allergens, with high spore counts correlating with peaks in asthma-related hospital admissions (Bush, 2020). Moreover, spores of *Alternaria* can occasionally act as infectious agents. Though rare, cases of cutaneous, subcutaneous, and even invasive infections have been reported, especially in immunocompromised patients such as those with poorly controlled diabetes or under long-term corticosteroid therapy (Pastor & Guarro, 2008; Mercier et *al*., 2020). Taken together, *Alternaria* represents a genus of dual importance: as a widespread plant pathogen causing substantial agricultural losses, and as a clinically relevant allergen and opportunistic pathogen in humans.

4.4.2. Taxonomy

The taxonomy of the genus *Alternaria* has evolved significantly since its original classification, reflecting advances in morphological understanding, as well as more recent molecular and genomic techniques. Initially, *Alternaria* was classified based primarily on morphological features of its conidia, including shape, size, septation, and pigmentation. The early taxonomic framework, initiated by Nees and subsequently adopted by Fries, primarily utilized microscopic observation and host association. This approach led to inconsistencies in

species delineation due to significant variability in morphological traits under diverse cultural and environmental conditions (Müller, 1991**).** As a result, a significant number of species were described, though many were later recognized as morphological or ecological variants rather than separate species.

During the mid-20th century, significant efforts were directed toward systematizing the genus *Alternaria*. E.G. Simmons was instrumental in redefining species concepts within *Alternaria* through extensive examination of conidial morphology and sporulation patterns under controlled growth conditions. He provided a monograph of *Alternaria*, recognizing 275 species in the genus and establishing species groups such as *A. alternata, A. tenuissima,* and *A. infectoria*, which have since become fundamental in mycological studies. Nevertheless, the reliance on morphological data proved inadequate for resolving species boundaries, especially among closely related or cryptic taxa, owing to phenotypic plasticity and the impact of environmental conditions (Lawrence et *al*., 2015).

Lawrence et *al*. (2014, 2016) clarified the modern taxonomy of *Alternaria*, characterizing the asexual morph as alternarioid dematiaceous hyphomycetes that produce effuse, pigmented colonies with hyaline hyphae. These fungi are distinguished by mononematous to caespitose, macronematous conidiophores, which may be simple or branched, pale brown to brown in coloration, and bear monotretic or polytretic, sympodial conidiogenous cells. The conidia are typically darkly pigmented, multicellular, predominantly dictyosporous, though occasionally phragmosporous, and occur singly or in catenate chains. The sexual morph, documented in sections such as *Alternaria*, *Crivellia*, *Embellisioides* Woudenb. & Crous, *Eureka* Woudenb. & Crous, *Infectoriae* Woudenb. & Crous, *Nimbya*, and *Panax* D.P. Lawr. et *al*., is defined by small, dark brown, erumpent to superficial, globose to ovoid, glabrous, uni-loculate ascomata with papillate ostioles. These structures consist of thin-walled peridia enclosing fissitunicate, cylindrical to cylindric-clavate asci, which are embedded within broad cellular pseudoparaphyses, and contain muriform, ellipsoidal to fusoid, pigmented ascospores (Woudenberg et *al*., 2013; Lawrence et *al*., 2015; Ariyawansa et *al.*, 2015)

4.4.3. Different taxonomic approaches to Alternaria

4.4.3.1.Morphological and phenotypic approaches

The genus *Alternaria* was originally defined in 1816, with *A. tenuis* identified as the type and only representative species at that time (Nees, 1816). The asexual form of *Alternaria*

species generally exhibits spreading colonies that display a broad spectrum of colors, including gray, greenish, dark olive, brownish-black, or jet black. The mycelium is typically embedded within the substrate, consisting of hyphae that may appear hyaline, olive-brown, or dark brown. Stromatic formations are uncommon, and specialized structures such as setae and hyphopodia are absent.

The conidiophores are macronematous and mononematous, either unbranched or branched, light to dark brown in hue, and may occur singly or in compact clusters. Conidiogenous cells are continuous with the conidiophores, positioned terminally or intercalarily, and are polytretic, sympodial, or at times monotretic with visible scars. Conidia are produced individually or in chains, are dry, and show a wide morphological diversity ranging from ovoid and obovoid to cylindrical, narrowly ellipsoidal, or obclavate, often tapering into a beak. Their coloration spans from pale to medium olive-brown or brown, and their surfaces may be smooth or roughened (verrucose). They typically possess transverse septa, occasionally accompanied by oblique or longitudinal divisions. These septa can be thick, dark, and firm, sometimes developing an internal, cell-like structure, and certain species display meristematic growth.

The sexual stage is represented by small, dark brown ascomata that occur singly or in groups, becoming erumpent to superficial upon maturity, and are globose to ovoid in form. These ascomata may be smooth or setose, apically papillate, and ostiolate, featuring a short, blunt papilla and a thin outer wall (peridium). The hamathecium consists of cellular pseudoparaphyses. Each ascoma contains few to numerous asci, which are generally 8-spored, basal, bitunicate, fissitunicate, and cylindrical to cylindro-clavate in shape, either straight or slightly curved, and supported by a short, forked pedicel. The ascospores are muriform, ellipsoidal to fusiform, slightly constricted at the septa, and yellowish-brown, smooth-surfaced, and devoid of guttules. They exhibit 3–7 transverse septa and 1–2 longitudinal septal series within the two central cells. The terminal cells are either non-septate or bear one longitudinal, oblique, or Y-shaped septum (Lawrence et *al.*, 2016) (Figure 3).

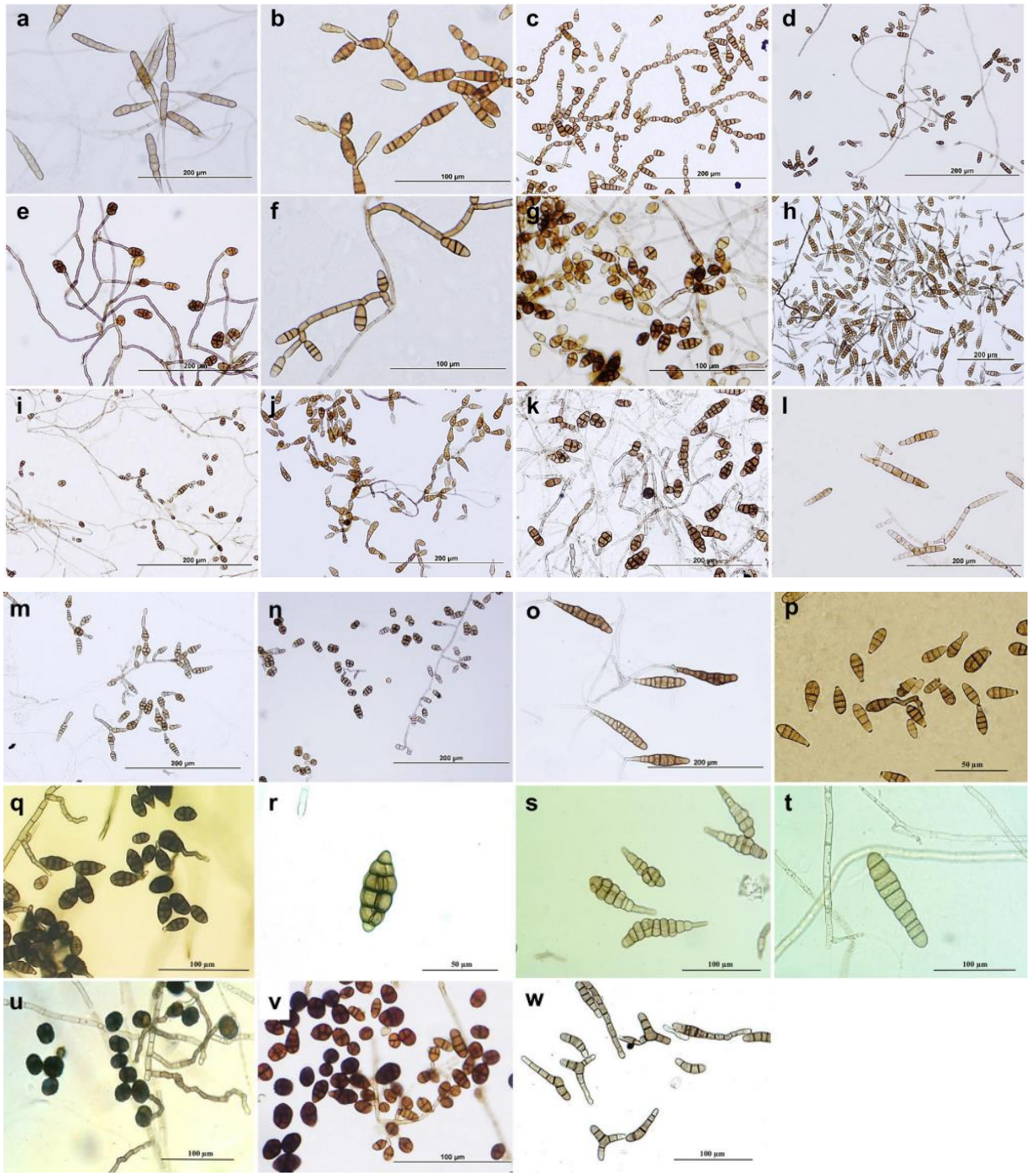

Figure 3: Conidia of a Alternaria alternantherae EGS 52–039 (sect. Alternantherae), b A. arborescens EGS 39–128 (sect. Alternaria), c A. brassicicola BMP 0325 (sect. Brassicicola), d A. abundans BMP 1928 (sect. Chalastospora), e A. cheiranthi EGS 41–188 (sect. Cheiranthus), f A. embellisia EGS 38–073 (sect. Embellisia), g A. proteae EGS 39–031 (sect. Embellisioides), h A. limicola BMP 2239 (sect. Euphorbiicola), i A. eureka EGS 36–103 (sect. Eureka), j A. infectoria EGS 27–193 (sect. Infectoriae), k A. japonica BMP 0332 (sect. Japonicae), l A. scirpicola EGS 19–016 (sect. Nimbya), m A. calycipyricola EGS 52–072 (sect. Panax), n A. mouchaccae EGS 31–061 (sect. Phragmosporae), o A. linariae CBS 109156 (sect. Porri), p A.

arrhenatheri BMP 1942 (sect. Pseudoalternaria), q A. chartarum BMP 0359 (sect. Pseudoulocladium), r A. selini EGS 35–178 (sect. Radicina), s A. cinerariae EGS 33–169 (sect. Sonchi), t A. leucanthemi EGS 17–063 (sect. Teretispora), u A. atra BMP 0353 (sect. Ulocladioides), v A. alternariae BMP 0352 (sect. Ulocladium), w Alternaria sp. BMP 0951 (sect. Undifilum). (Lawrence et *al*.,2016).

4.4.3.2.Molecular Approaches

The introduction of molecular phylogenetics in the early 21st century marked a turning point in *Alternaria* taxonomy. Current DNA-based classification of *Alternaria* relies on multiple gene loci, including nuclear ribosomal DNA, intervening ITS regions, mtSSU, protein-coding genes like ACT, Alt-a1, CAL, GAPDH, RPB2, TEF1-α, THN, Tsr1, and the plasma membrane ATPase gene (Ghafri et *al*.,2019; Jayawardena et *al*.,2019). Various molecular methods, such as random amplified polymorphic DNA (Roberts et *al*.,2000), amplified fragment length polymorphism (Somma et *al*.,2011), selective subtractive hybridization (Robert et *al*.,2012), and sequence-characterized amplified genomic regions (Stewart et *al*.,2013), have been investigated or proposed for distinguishing *Alternaria* species. However, the standard gene regions and other protein-coding loci are not able to delineate species within all the sections, such as small spore species-groups like sect. *Alternaria* and sect. *Infectoriae* (Woudenberg et *al*.,2015; Peever et *al*., 2004; Patriarca et *al*.,2019). Hong et *al*. (2005) illustrated that the *Alt-a1* locus is reliable for *Alternaria* species identification. Lawrence et al. (2013) used five protein-coding loci for clarifying the phylogenetic hypothesis among *Alternaria* and revealed that the plasma membrane ATPase and CAM genes were the most suitable phylogenetic markers for molecular identification. In a landmark study, Woudenberg et *al*. (2013) came up with 16 new Alternaria sections. They outlined phylogenetic lineage within *Alternaria* and related genera based on multi-locus phylogeny of SSU, LSU, ITS, GAPDH, RPB2, and TEF1-α gene areas. Since then, whole genome sequencing has emerged as a crucial technique for distinguishing equivocal species in *Alternaria* and other complex species. So, to distinguish between the species in the *Alternaria* sect. Woudenberg et al. (2013) used the whole genome and transcriptome comparisons combined with multi-locus phylogeny based on ITS, GAPDH, RPB2, TEF1-α, ALT-a1, endoPG, and OPA10-2 gene loci. In their thorough taxonomic treatment of *Alternaria* with multi-locus phylogeny, Lawrence et *al*. (2016) accepted 27 sections in Alternaria (Figure 4), but later revised it to 28 accepted sections (Ghafri et *al*.,2019; Lawrence et *al*., 2015). Gannibal et *al*. (2022) and Ghafri et *al*. (2019) recently introduced two new sections (i.e., sects. Helianthiinficiens and Omanenses) of

Alternaria, resulting in the acceptance of 29 sections (Gannibal et *al*.,2019; Ghafri et *al*.,2019; lawrence et *al*., 2015).

The use of phylogenomic has helped to clarify relationships within the genus in recent years. Whole-genome sequencing was employed in studies by Dettman & Eggertson and Li et al.,2022 to evaluate evolutionary divergence and lineage sorting across sections. These studies found areas of gene tree discordance that reflect complex evolutionary histories, while also validating many of the previously established categories. In addition to supporting the section-based categorization, these high-resolution studies offered potential loci for stronger species delimitation (Dettman & Eggertson, 2021; Li et *al*., 2022).

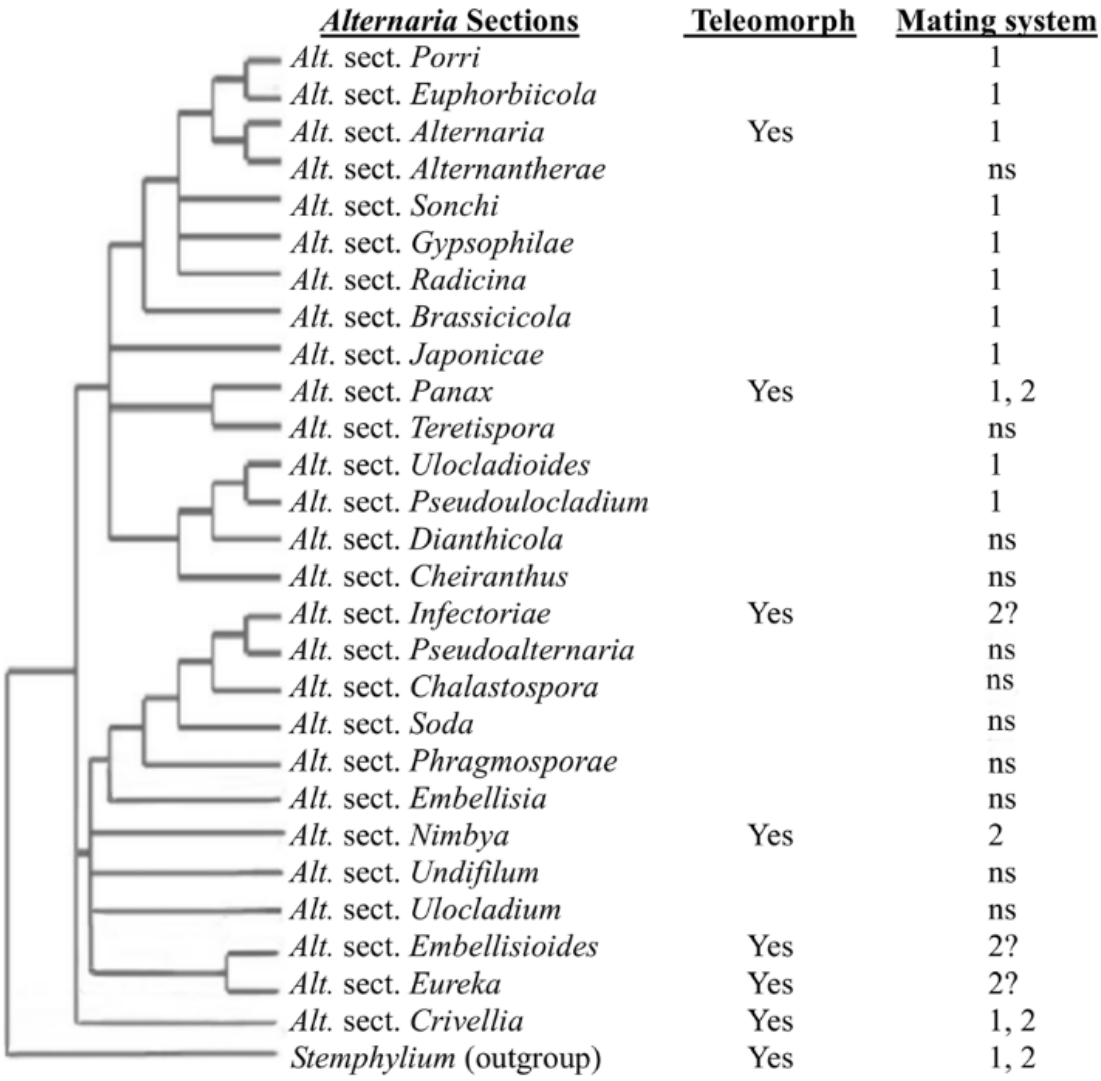

Figure 4: Phylogeny, taxonomy, and mating system of *alternarioid hyphomycetes*.

Cladification derived from manual combination of four phylogenetic trees based on analysis ofsequences ofseven nuclear genes (Lawrence et *al.*, 2013, 2014; Woudenberg et *al.*,2013; Grum-Grzhimaylo et *al.*,2015). Type of mating system: 1 heterothallism, only one of two idiomorph of MAT1 locus present in a genome; 2 homothallism, both idiomorphs of MAT1 locus present in a genome; ns not studied (data for sect. *Infectoriae* obtained from Andersen et al. 2009;datafor sect. Nimbya obtained from Johnson et al. 2002; data for sect. C*rivellia* obtained from Inderbitzin et *al.*, 2006; data for *Stemphylium* obtained from Inderbitzin et *al.*, 2005; other data obtained from Gannibal and Kazartsev 2013 and Gannibal .

Some taxonomic problems still need to be overcome despite this progress. The species *Alternaria alternata*, which is frequently isolated from a wide range of hosts and settings, nonetheless poses challenges because of its high intra-species genetic diversity and great ecological adaptability. As a result, there is continual debate on whether it represents a single species or a group of cryptic taxa. DeMers (2022) emphasized the taxonomic instability of *A. alternata*, pointing out that species delimitation is made more complicated by its varied host associations and lifestyle as an endophyte and pathogen, especially when depending on ecological or nutritional criteria. Today, the taxonomy of *Alternaria* is characterized by a consensus-based, polyphasic approach that incorporates morphological, molecular, ecological, and metabolomic data. The current classification into phylogenetic sections provides a stable framework for identification and research. However, ongoing genome-based studies and better understanding of species boundaries are still needed, especially for taxa of agricultural, ecological, and clinical significance.

4.4.4. Ecology and epidemiology of Alternaria causal agent of Black point disease

The *Alternaria* spp. are cosmopolitan fungi found in soil, plant debris, and air, and they act as saprophytes, endophytes, or pathogens. In cereals, especially wheat and barley, *Alternaria* species are the primary cause of black point disease. Alternaria contamination of wheat kernels has been documented in many nations, including Tunisia, Slovakia, Argentina, China, Russia, and Italy (Vučković et *al.*, 2012; Li et *al.*,2000; Mašková et *al.*,2012). Although it does not affect harvest production, this disease can reduce grain quality and its market value, especially when kernels are exposed to the milling and processing pathways.

Environmental factors, host development, and pathogen biology interact intricately in the epidemiology of black point disease in wheat, which is mainly brought on by *Alternaria* species. The quality of grains and market value are significantly decreased by the disease, which is typified by a dark brown to black discoloration on the germ end of wheat kernels. A variety of fungi can cause Black Point symptoms, although the most often isolated species from infected kernels are *Alternaria alternata*, *Alternaria tenuissima*, and *Alternaria infectoria* (Somma et *al*., 2019).

The late phases of plant development, particularly from anthesis to the late milk stage, are when the infection process is mainly initiated. The timing of inoculation has a significant impact on disease incidence, with plants inoculated closer to grain maturity being more vulnerable, according to a traditional glasshouse study. Additionally, the study showed that the length of the leaf wetness or dew period and inoculum density were positively correlated with the occurrence of black point. According to Southwell et *al*. (1980), infection required a minimum dew time of three to six hours, with peak symptoms appearing 48 hours following leaf wetness.

Conidia spread by wind or splashing rain are the main source of inoculum for Alternaria species. These spores are frequently detected on the phyllosphere of healthy wheat plants and can survive in soil and crop debris, indicating that Alternaria functions as an opportunistic pathogen or latent endophyte under the right circumstances (Turzhanova et *al*., 2020). The fungus typically begins the infection at the rachilla and spreads to the embryo and surrounding tissue via exterior or vascular pathways (Ma De-ying, 2006).

Additionally, epidemiological research demonstrates that Alternaria species are regularly found in areas of the world that grow wheat. 97% of the fields studied in Lebanon had contamination, according to surveys, with the Alternaria and *Infectoriae* sections accounting for the majority of the fungal community. However, there was no discernible correlation between the geographic origin and the fungal population structure, indicating genetic homogeneity and broad diffusion across regions (Masiello et *al.*, 2022). According to Perelló et *al*. (2008), *A. infectoria* was also shown to be the primary cause of black point infections in Argentina, where its occurrence has increased in contrast to previous records that primarily identified *A. alternata* as the causal agent.

The high genetic variability among Alternaria strains further complicates black point epidemiology. Molecular fingerprinting methods, such as retrotransposon-based iPBS profiling, have revealed significant intra- and inter-species diversity, which may influence pathogenicity and mycotoxin production capacity (Turzhanova et *al.*,2020). Despite this genetic variation, most isolates from black point-infected kernels cluster into two main groups: one comprising *A. alternata*, *A. tenuissima*, and *A. arborescens*, and another containing *A. infectoria* and *A. triticina* (Somma et *al*., 2019).

4.4.5. Effect of climate change on *Alternaria* spp. occurrence

Changes in humidity and rising temperatures have a direct impact on *Alternaria* spp. life cycles and spore formation. Temperature continued to be a major factor influencing daily spore loads. Still, a 26-year aerobiological study in Copenhagen found that, despite rising temperatures, airborne *Alternaria* spore concentrations had significantly decreased due to urbanization and changing agricultural practices (Olsen et *al.*, 2019). This implies that although temperature can encourage fungal sporulation, environmental and land-use conditions influence its impact.

Predictive modelling indicates that as warming trends continue, *Alternaria* spp. will probably become more common in formerly cooler places. According to a study on climate modelling, fungal growth on tomatoes was predicted to rise significantly in northern Europe (like Poland) in the future, mirroring the conditions that exist now in southern Europe (like Spain). On the other hand, in areas that are already warm, too much heat may inhibit fungal development beyond optimal temperature thresholds (Van de Perre et *al*., 2015).

In controlled environments, it has also been demonstrated that heat and elevated CO_2 levels increase the incidence and severity of *Alternaria japonica*-caused disease. According to Pugliese et *al*. (2012), plants cultivated in environments with higher CO_2 (800 ppm) and higher temperatures displayed more severe disease on both rocket salad and basil, indicating that climate conditions have a synergistic influence on pathogen aggression.

Black point is becoming more common, mainly due to climate change. Variations in temperature and precipitation patterns create microenvironments that are warm and humid for extended periods, extensively promoting *Alternaria* spp. growth and sporulation. The late phases of grain development, especially from anthesis to the late milk stage, are a critical

window for infection. Warm temperatures and dew periods longer than six hours significantly raise the risk of infection during this time (Southwell et *al*., 1980).

Additionally, recent research shows that the geographical distribution of *Alternaria* species implicated in black point is altering due to global warming. Because of altered cropping patterns and changing agroecological conditions brought on by climatic variability, studies conducted in Argentina revealed an increase in the occurrence of *A. infectoria*, a species group that was less prevalent in previous surveys (Perelló et *al.*, 2008). According to research conducted in Europe and the Mediterranean, climate-related stressors that weaken plant defenses and prolong pathogen survival on developing heads are also responsible for the increased incidence and severity of black point in wheat (Amatulli et *al*., 2013).

Furthermore, host vulnerability and mycotoxin production can be impacted by stress reactions brought on by climate change. For example, postharvest warmth and increased CO_2 made lettuce cultivars more vulnerable to *A. alternata* invasion and toxin buildup. A connection between abiotic stress, weakened plant defense, and fungal secondary metabolism was shown by the increased mycotoxin synthesis, particularly tentoxin and tenuazonic acid, in susceptible cultivars under stress (Miranda-Apodaca et *al.*, 2023). In addition, Somma et *al*. (2019) highlighted that climate change is contributing to the global rise in black point, pointing out that altered environmental conditions may also encourage *Alternaria* spp. to produce mycotoxin, which would exacerbate the problem by posing additional risks to grain safety and marketability. As a result, control efforts are made more difficult by climate-induced changes in fungal ecology, including altered phenology, competitive interactions with other diseases, and enhanced saprotrophic activity (Vučković et *al.*, 2012).

4.4.6. Diversity of *Alternaria* pathogenicity

Alternaria species exhibit diverse life strategies, functioning as saprophytes, endophytes, and pathogens (Thomma, 2003; Dang et *al.*, 2015). Their pathogenic success is evident in the vast array of economically significant plants they infect, including apple, broccoli, cauliflower, potato, tomato, citrus, pear, strawberry, and tobacco. The broad host range and global distribution of *Alternaria* result in substantial economic losses (Meena et *al*., 2016).

The pathogenicity of *Alternaria* species is highly diverse, reflecting a strong evolutionary ability to infect numerous host plants. This genus can infect over 400 plant species,

causing significant damage to crops and ornamental plants both in the field and after harvest through various biochemical and ecological processes. This diversity is evident both among species, such as *A. alternata, A. tenuissima*, and *A. infectoria*, and within species, where isolates can show considerable variations in virulence, host range, and toxin production (Keller, 2019; Meena et Samal, 2019).

Alternaria species' pathogenicity relies on both host susceptibility/resistance and the production of host-specific (HSTs) and non-host-specific (nHSTs) toxins. These fungi can invade various plant hosts by producing over 70 diverse phytotoxins, categorized by the disease symptoms they induce (Friesen et *al*., 2008). These toxins are chemically defined as low-molecular-weight secondary metabolites (SMs). Their deleterious effects primarily target multiple cellular organelles and structures, including mitochondria, chloroplasts, the plasma membrane, the Golgi apparatus, and the nucleus (Menna et *al*., 2017).

Host-selective toxins (HSTs) and non-host-selective toxins (nHSTs) synthesized by *Alternaria* species function as key virulence determinants during plant pathogenesis. Pathogenic *Alternaria* strains capable of producing these toxins frequently harbor supernumerary chromosomes, which are absent in non-pathogenic counterparts (Akamatsu et al., 1999; Akamatsu, 2004).The HTSs are particularly well studied in several *A. alternata* pathotypes, which serve as key effectors that determine host range and disease severity, and induce necrosis only in susceptible host cultivars, often at nanomolar concentrations, and are entirely ineffective in resistant or non-host plants (Meena et *al* Samal,2019). To date, over twelve distinct HSTs have been identified in *Alternaria*, each associated with a specific pathotype and host (Figure 5).

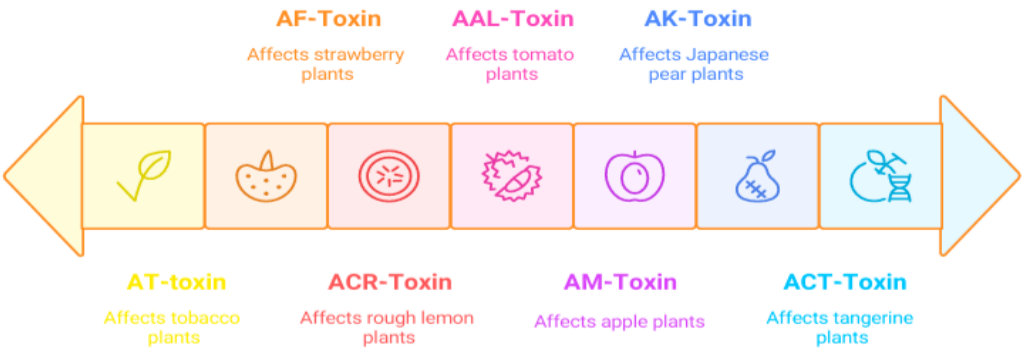

Figure 5: Schematic Diagram of Alternaria sp. HSTs toxins, and their specific host's (Elena et *al*., 2017).

These toxins operate by targeting critical cellular organelles or metabolic pathways within host tissues. For instance, AK- and ACT-toxins compromise the plasma membrane through lipid peroxidation. At the same time, AM-toxin inhibits photosynthetic electron transport within the chloroplast (Otani et *al*., 1995). The genes responsible for their biosynthesis are typically located on conditionally dispensable chromosomes, which are accessory genomic regions not essential for saprophytic survival but crucial for pathogenicity on specific hosts (Tsuge,2003). On the other hand, nHSTs have a multifaceted impact on virulence and are capable of affecting a diverse array of plants, even those that are not typically their hosts. In *vivo* and *in vitro* studies of nHSTs have also been performed using human cell lines and animal models (Hussain et *al*., 2024) .

Nonetheless, non-host-selective toxins have been found in *Alternaria* isolates causing black point in wheat. Several studies have been conducted on the pathogenicity of *A. alternata*, *A. tenuissima*, and *A. infectoria*, which are often involved in wheat black point, and no evidence of pathotype-specific HSTs in this pathosystem has been found (Somma et *al*., 2019; Masiello et *al*., 2020). In contrast, while virulence seems not to be strictly host-specific, it is associated with necrotrophy and opportunism, and by the production of non-host selective mycotoxins such as alternariol (AOH), alternariol monomethyl ether (AME), TeA, and altenuene (ALT) (Hussain et *al.*, 2024). *Alternaria* mycotoxins employ various cytotoxic mechanisms that impair host tissue integrity and immune responses. Specifically, TeA functions as a potent inhibitor of protein biosynthesis in eukaryotic cells, resulting in rapid necrosis and the suppression of active defense mechanisms within plant tissues (Kumari et *al*.,2019). Likewise, research indicates that

AOH and AME induce DNA damage and oxidative stress, further compromising plant resistance (Menna et *al*.,2017).

4.4.7. Alteranaria Mycotoxins

Alternaria species are capable of producing a diverse array of secondary metabolites with toxic properties, collectively known as *Alternaria* toxins (Dall'Asta et *al*.,2014). While only a fraction of these toxins has been chemically characterized (Barkai-Golan et al.,2008), many have been reported to exhibit myotoxic effects in animals and humans (Logrieco et *al*.,2009). Several *Alternaria* species, including *A. tenuissima, A. citri, A. brassicicola, and A. alternata,* are known to produce these toxic secondary metabolites. Notably, *A. alternata* is recognized as a key mycotoxin-producing species (EFSA, 2011).

Alternaria mycotoxins have been detected in various food and feed products, including fruits, grains, vegetables, processed foods, coffee, animal feed, and alcoholic beverages (Figure 5) Tomato-derived products exhibit particular vulnerability to *Alternaria* mycotoxin contamination, attributable to their elevated water content, suitable pH, and nutrient composition, which collectively foster *Alternaria* species proliferation and subsequent mycotoxin production (Zhang et *al*.,2025). Ongoing research into the toxicological effects of *Alternaria* mycotoxins indicates that these compounds can be categorized into two groups. The first category consists of genotoxic compounds including AOH, AME, and ATXs that operate at the DNA level to cause DNA damage in a variety of cell lines, such as Caco-2, HepRG, and HT29 (Aichinger et *al*., 2018; Fernández-Blanco et *al.*, 2016). Non-genotoxic compounds that have shown cytotoxicity in vitro, such as TeA and TEN, make up the second group. As of right now, Alternaria mycotoxins in food and feed products are not subject to globally regulated regulatory restrictions. The only organization that has released regulations outlining the highest permissible levels of AOH, AME, and TeA in specific food items is the European Union (EU,2022).

The principal mycotoxins of *Alternaria* were initially isolated, structurally characterized, and confirmed between 1953 and 1986. These compounds are classified into three major structural groups (Bottalico and Logrieco, 1998): dibenzopyrone derivatives (including AOH, AME, and ALT), perylene derivatives (ATX-I, ATX-II, ATX-III), and tetramic acid derivatives (TeA). Beyond these classes, *Alternaria* species are also capable of synthesizing cyclic tetrapeptides, such as tentoxin (TEN) (Figure 6).

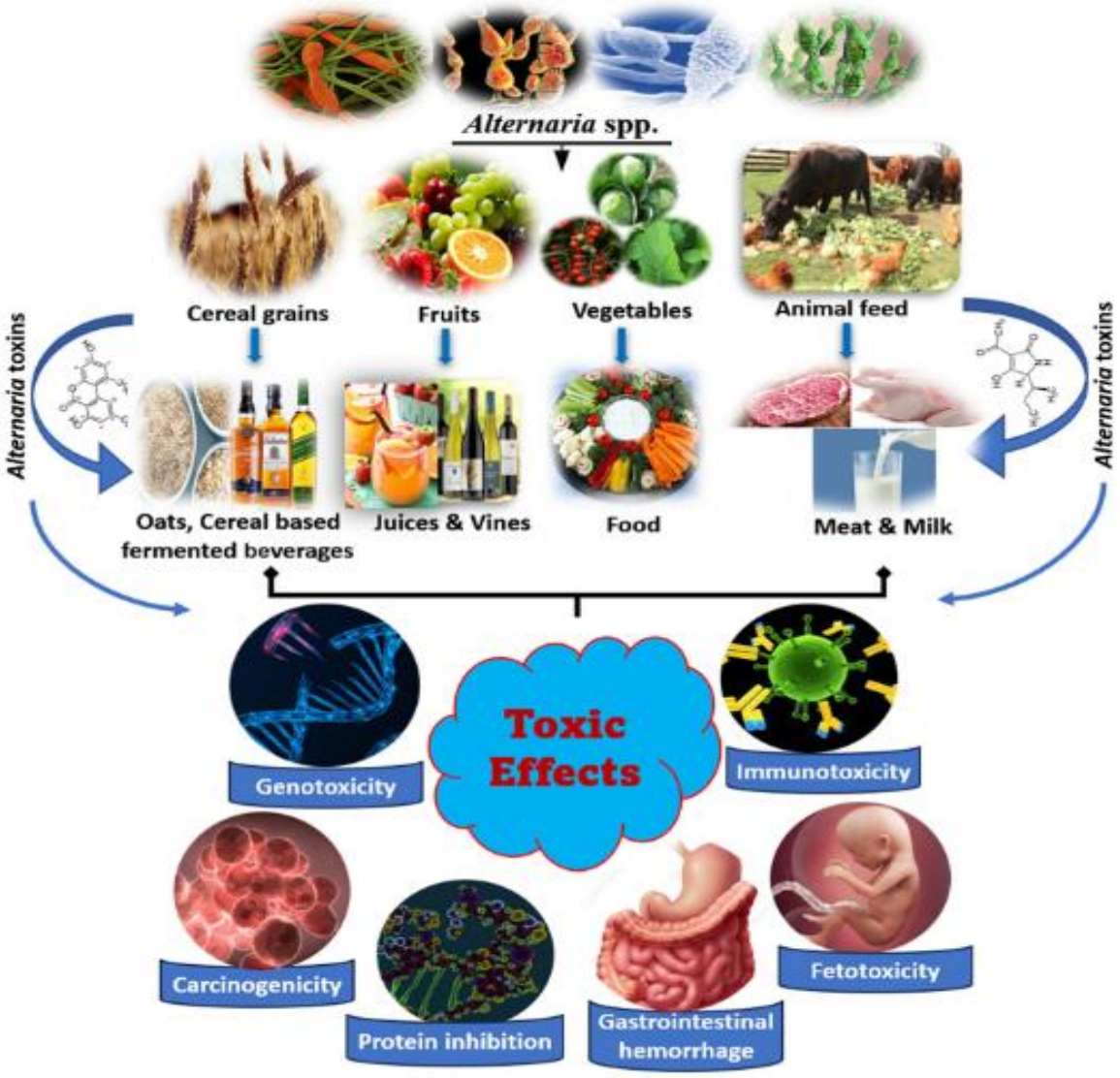

Figure 6: Flow diagram of Alternaria mycotoxin contamination and their hazardous effects on health (Hussain et *al.*,2024).

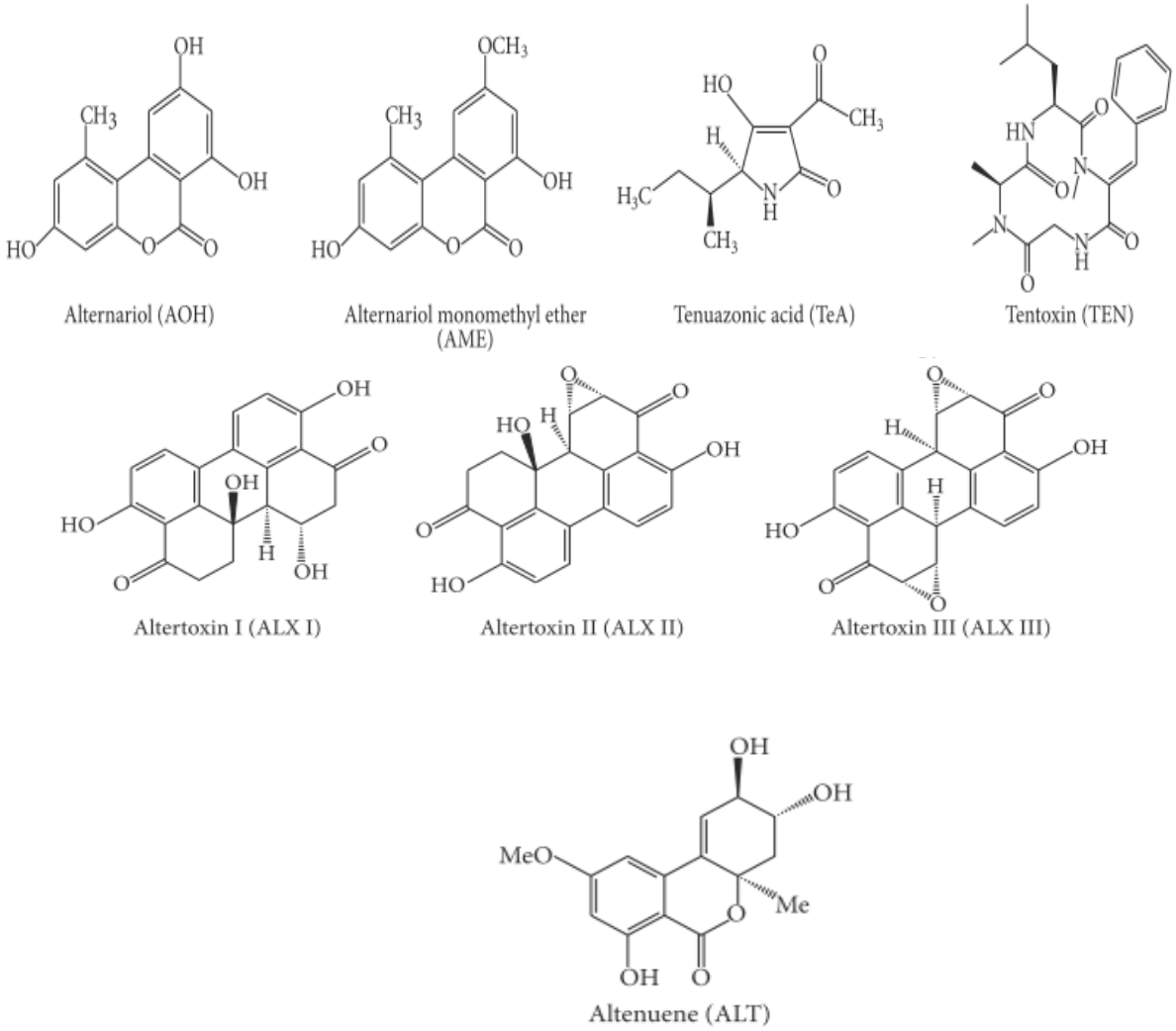

Figure 7: Chemical structure of the most common Alternaria sp. mycotoxins (Ostry, 2008).

4.4.7.1. Dibenzopyranones

Alternaria synthesizes three primary dibenzopyranones: alternariol monomethyl ether (AME), altenuene (ALT), and alternariol (AOH) (Pinto and Patriarca ,2017). These toxins are commonly detected in various food commodities (Puntscher et *al*., 2019; Zhang et *al*.,2021). AME and AOH are the most abundant, constituting approximately 20% of the total content in *Alternaria* extract from most strains. Conversely, ALT is present in considerably lower concentrations, contributing only 1–3% of the extracts (Aichinger et *al*.,2021). AOH and AME exhibit a broad spectrum of genotoxicity and cytotoxicity and can induce oxidative stress in biological systems. (Tiessen et *al*.,2017; Vila-Donat et *al*.,2015). Epidemiological studies

suggest a potential correlation between the high incidence of oesophageal cancer in Linxian, Henan Province, China, and the consumption of grains heavily contaminated with AOH and AME (Zheng et *al*., 2022). In *vitro* studies indicate that human fetal esophageal epithelial cells exhibit increased proliferation upon exposure to AOH and AME due to their capacity to induce cellular mutations and bind to DNA, thereby accelerating cellular proliferation. Research has indicated that AOH can induce squamous cell carcinoma in fetal esophageal cells and inhibit the metabolism and proliferation of porcine granulosa cells, potentially leading to reproductive cycle defects (Tiemann et *al*.,2009). Furthermore, AOH has been shown to induce histone phosphorylation in various cell lines, a phenomenon associated with DNA double-strand breaks and indicative of genotoxicity (Crudo et *al*.,2023). Androgen reporter bioassays have revealed that AOH can fully trigger the androgenic response, with higher concentrations potentiating the androgenic effects of testosterone in a dose-dependent manner, suggesting an additive effect (Stypula-Trebas et *al*.,2017). This finding represents the first report of the androgenic potential of a naturally occurring nonsteroidal chemical, which may influence future research directions. While AOH and AME are often studied in combination due to their synergistic toxic effects, some reports suggest that ALT exhibits relatively low toxicity compared to AME and AOH (Siegel et *al*.,2010). Gene silencing experiments using CRISPR/Cas9 in A. alternata have elucidated a 15-kb gene cluster (comprising PksI, omtI, moxI, sdrI, doxI, and the transcription factor gene aohR) involved in the biosynthesis of AOH and AME. Coexpression of PksI with different tailoring enzymes results in the production of diverse dibenzopyranones (Wenderoth et *al*.,2019).

4.4.7.2. Tetramic acid derivative

Three classical analogues of tetramic acid are produced by Alternara: 3-acetyl-5-isobutyltetramic acid, 3-acetyl-5-isopropyltetramic acid, and TeA (Chen et *al*., 2011). TeA, the most basic tetramic acid, was initially isolated from the culture filtrate of *A. tenuis* (now known *as A. alternata*) (Motoyama et *al*., 2021). Numerous *Alternaria* species, such as *A. alternata* (Sun et *al*., 2022), *A. longipes, and A. tenuissima* (Chen & Qiang, 2017), have been found to contain TeA. Numerous polluted crops, fruits, and vegetables have been found to contain TeA (Siegel et *al*., 2009; Van de Perre et *al*., 2014). TeA significantly boosts the pathogen's pathogenicity and encourages the spread of invasive hyphal infections (Shi et *al*., 2021). TeA causes the production of ROS from chloroplasts in host plants, which consequently causes chromatin condensation, lipid peroxidation, DNA cleavage, organelle disinfection, rupture of

the plasma membrane, chlorophyll degradation, irreversible cell death, and leaf necrosis (Chen et *al*., 2010; Shi et *al*., 2021). TeA causes necrotic spots on rice leaves and impairs the growth of radish, turnips, mung bean, soy bean, and rice seedlings (Bjørk et *al*., 2020; Chen et *al*., 2005). It also has herbicidal qualities. Thus, TeA can be used as a bioherbicide on cotton plants (Zhou et *al.*, 2019). By preventing the ribosome from producing and releasing polypeptides, TeA restricts the production of proteins (Vejdovszky et *al*., 2016). When exposed, cell lines from both humans and animals become toxic. It is associated with weight loss and causes bleeding in dogs and chickens. According to the results, TeA is more toxic than AME, ALT, and AOH and harmful to broiler chicken embryos (Broomhead et *al*., 2002). Precancerous esophageal mucosal changes seen in mice experiments suggest that it might be a mutagen (Yekeler et *al.*, 2001). Additionally, three mammalian cell lines, the root tips of Vicia faba, and Chlamydomonas reinhardtii were used to assess TeA toxicity. The results demonstrated that all samples had reduced protein production. TeA suppressed the synthesis of chlorophyll in C. reinhardtii and the generation of Chinese hamster lung cells and human hepatocytes (Zhou and Qiang, 2008). The EFSA assessed the toxicity of TeA and established a toxicological concern limit of 1500 ngkg−1 body weight per day (Asam and Rychlik, 2013). It was recently discovered that M. oryzae carries the TeA biosynthesis gene, TAS1, which produces a hybrid protein of NPRS and PKS (Mo & Gulder, 2021; Yun et *al*., 2015).

4.4.7.3. Perylene derivatives

Cytotoxicity, photochemical characteristics, and fungal pathogenicity are important bioactivities of aromatic polyketides (Daub et *al*., 2013). ATXs are perylene derivatives that can be secreted by a number of species, including *A. eichorniae, A. mali, and A. alternata* (Zwickel, Kahl, et *al*., 2016). It is believed that ATX-II is the most lethal of the toxins in this category (Fleck et *al*., 2012). In addition to causing DNA damage and cytotoxic and oxidative effects, ATX-I activates the NRF2 transcription factor and antioxidant-responsive element pathway. A number of ATXs, including ATX-II and stemphyltoxin III, have been linked to DNA damage through reactive epoxy moieties that form DNA adducts (Kiefer et *al*., 2023).

ATXs have been demonstrated to be acutely fatal in mice and have a broad spectrum of activities, in addition to being more potent mutagens than AME and AOH. According to Fleck et al. (2012), ATX-II was 50 times more potent as a mutagen than AME and AOH and showed mutagenicity in Chinese hamster V79 cells. One study found that intact tomatoes treated with ATX-II changed into the less toxic ATX-I, indicating that the plant's enzymes aided in this

transformation (Puntscher, Marko, et *al*., 2019). AME had no impact, but in MCF-7 breast cancer cells, ATX-I and ATX-II together increased Cytochrome P450 activity or activated the aryl hydrocarbon receptor (AhR) pathway (Hohenbichler et *al.*, 2020). Caco-2 cells serve as the foundation for one of the most popular models of intestinal metabolism and absorption because they resemble human epithelial cells. When tested in Caco-2 cells, ATX-I exhibited a higher absorption rate than ATX-II. The results showed that ATX-II primarily functions in the gastrointestinal system, whereas ATX-I is engaged in blood circulation (Fleck et al., 2014). Male Sprague-Dawley SD rats were administered oral ATX-I at low doses (1.10 μg/kg bw/d) and high doses (5.51 μg/kg bw/d) for 28 days in a row. The kidney, liver, and spleen all showed histopathological damage, although the blood profile and serum biochemical features did not significantly alter. Testing for genotoxicity indicates that ATX-I may damage DNA. Although ATX-I may not have obvious genotoxic effects in vivo due to its narrow dose range, it may harm the liver, spleen, and kidneys (Zhu et *al*., 2022a). There is no comprehensive research on ATX-I, and its genotoxic consequences are unknown.

4.4.7.4. Cycle peptides

The cyclic peptide TEN is synthesized by the following species: *A. alternata, A. longipes, A. citri, A. porri, and A. mali* (Ntasiou et *al*., 2015; Rodríguez-Carrasco et *al*., 2016). TENs are phytotoxins that can cause dicotyledonous plant seedlings to develop chlorosis. Chloroplast structural changes, reduced levels of lipids and proteins specific to chloroplasts, and disruption of chloroplast function could result from the location of the TEN receptors being unique to susceptible species (Noelting et *al*., 2022; Wang et *al*., 2022). The European Food Safety Authority (EFSA) used the toxicological threshold concern (TTC) approach, setting the daily threshold for TEN at 1500 ng kg−1 body weight. (Puntscher, Cobankovic, and others, 2019) As stated by Peng and colleagues (2021), Particular effects of TENs on plant metabolism include changes in internal ion concentrations, stomata opening and closing, and ion absorption and transport. According to some researchers, the development of TEN in C. miyabeanus is caused by the NRPS gene CmNps3, whereas *Alternaria* species may produce TEN due to the AaNps3 gene. DeBruyne and associates (2016). The cytochrome P450 gene (TES1) and the NPRS gene (TES) are two genes that have been shown to be involved in the production of TEN in A. alternata (Li et *al*., 2016).

4.4.7.5. Perylenequinone derivatives/ATXs

Aromatic polyketides are recognized for their diverse biological activities, including cytotoxic effects, photochemical properties, and contributions to fungal virulence (Daub et *al*., 2013). Among these compounds, altertoxins (ATXs) constitute a group of perylene-derived metabolites produced by several *Alternaria* species, including *A. eichhorniae, A. mali,* and *A. alternata* (Zwickel, Kahl, et *al*., 2016). Of these, ATX-II has been identified as the most toxic representative (Fleck et *al*., 2012).

ATX-I has been shown to exert cytotoxic and oxidative effects, induce DNA damage, and activate the NRF2 transcription factor along with the antioxidant response element signaling pathway. The genotoxic potential of certain altertoxins, particularly ATX-II and stemphyltoxin III, has been associated with the presence of reactive epoxide groups capable of forming DNA adducts (Kiefer et *al*., 2023). Furthermore, altertoxins have demonstrated a broad spectrum of biological activities and acute toxicity in murine models, with mutagenic potency exceeding that of alternariol (AOH) and alternariol monomethyl ether (AME). Notably, ATX-II was reported to exhibit mutagenic activity in Chinese hamster V79 cells and was approximately 50-fold more potent as a mutagen than both AOH and AME (Fleck et *al*., 2012).

Studies investigating toxin metabolism in plant tissues have shown that ATX-II can be converted into the less toxic compound ATX-I in intact tomato fruits, suggesting that endogenous plant enzymatic systems may contribute to detoxification processes (Puntscher, Marko, et *al*., 2019). In addition, synergistic interactions between ATX-I and ATX-II have been observed in MCF-7 breast cancer cells, where co-exposure enhanced activation of the aryl hydrocarbon receptor (AhR) signaling pathway and increased cytochrome P450 activity, whereas AME did not produce similar effects (Hohenbichler et *al*., 2020).

The intestinal absorption and metabolic behavior of altertoxins have also been investigated using Caco-2 cell models, which are commonly employed as an in vitro approximation of human intestinal epithelial barriers. These studies demonstrated that ATX-I exhibits greater absorption efficiency than ATX-II, suggesting differential toxicokinetic behavior, with ATX-II exerting more localized gastrointestinal effects while ATX-I may enter systemic circulation more readily (Fleck et *al*., 2014).

In vivo toxicological assessment further supports the harmful potential of ATX-I. Repeated oral administration of ATX-I to male Sprague–Dawley rats over 28 days, at both low (1.10 μg/kg

bw/day) and high (5.51 μg/kg bw/day) doses, resulted in histopathological alterations in the liver, kidneys, and spleen, despite the absence of significant changes in serum biochemical parameters or hematological profiles. Genotoxicity analyses suggested DNA-damaging potential, although the relatively low exposure doses may have limited the detection of overt in vivo genotoxic effects (Zhu et *al.*, 2022a). Nevertheless, current knowledge regarding ATX-I remains limited, particularly concerning its long-term genotoxic and systemic toxicological consequences.

4.5. Control strategies for *Alternari* spp. causing Black point

Black point disease of wheat, attributed mainly to *A. alternata*, *A. tenuissima*, and *A. infectoria*, presents a complex challenge due to its seedborne nature, necrotrophic behavior, and association with mycotoxins. Successful disease management requires an integrated approach that includes cultural practices, chemical and biological interventions, biophysical methods, and the use of resistant cultivars (Figure 8).

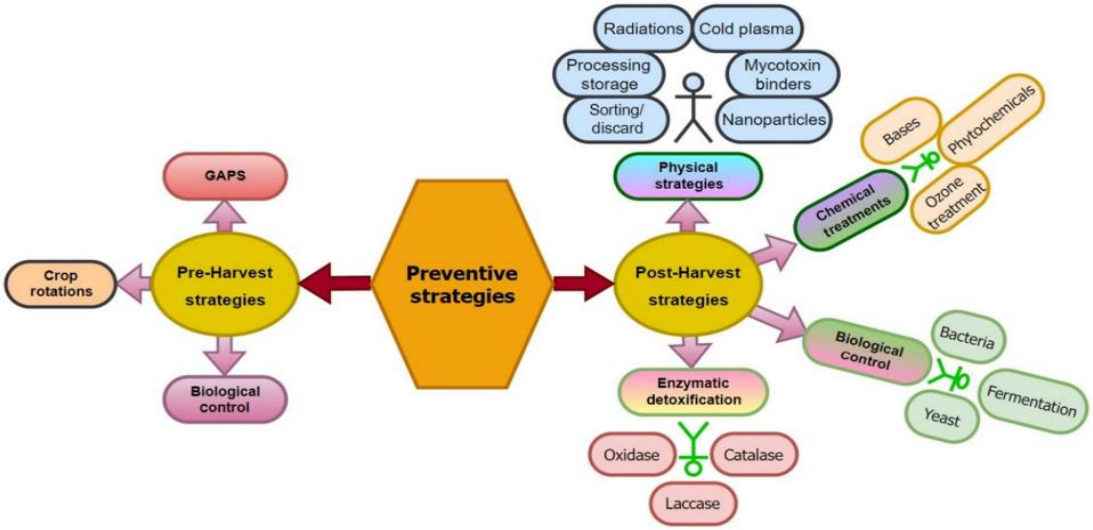

Figure 8: A schematic presentation of preventive strategies and their types (original).

4.5.1. Cultural practices

Cultural control forms the foundation of sustainable black point management in wheat. This disease is highly influenced by environmental factors during the grain-filling and maturation stages, especially humidity, rainfall, and delayed harvesting, which favor both *Alternaria* colonization and mycotoxin biosynthesis. It has been shown that prolonged dew periods, even as short as 6–48 hours during the milk to dough stage, significantly increase black

point incidence (Southwell et *al*., 1980). Thus, early sowing and timely harvesting are critical to escaping peak pathogen pressure and limiting the moisture window conducive to fungal development. Avoiding late-season irrigation, especially after heading, also helps reduce humidity in the canopy and spikelets, thereby lowering infection risk (Sisterna & Sarandón, 2010). Crop rotation and residue management further reduce inoculum levels. Because *Alternaria* spp. survive as saprophytes on crop debris, practices like deep plowing or residue removal can significantly reduce primary inoculum in the following season (Masiello et *al*., 2022). Proper field sanitation and avoidance of excessive nitrogen fertilization can also enhance plant resilience and limit overly lush canopies that trap moisture, which favors infection. Furthermore, early maturing varieties that reach grain maturity under drier conditions may inherently escape the critical infection window. These practices not only lower black point severity but are also linked to reduced levels of Alternaria mycotoxins such as *alternariol* (AOH) and *tenuazonic acid* (TeA), which accumulate primarily during late grain development under wet conditions (Amatulli et *al*., 2013).

4.5.2. Chemical Control

The application of fungicides in agricultural settings is a common global practice, crucial for inhibiting fungal proliferation and disease progression (Achilonu et al., 2023). Propiconazole, a systemic fungicide, exhibits efficacy against black point, powdery mildew, and leaf spot (Kassaw et *al*., 2021). Given that *A. alternata* possesses an esterase enzyme implicated in its pathogenicity, understanding the interaction between this esterase domain and fungicides is of importance (Hou et *al*., 2022). Moreover, feruloyl esterases contribute to *A. alternata's* pathogenicity by degrading plant cell walls, particularly the lignocellulose component (Crepin et *al*., 2004). Nevertheless, there is a paucity of information regarding the effects of fungicides on *A. alternata'*s esterase enzymes. In addition, Zhen-yue (2005) demonstrated that Tilt® (propiconazole) had the best inhibition of *Alternaria* hyphal growth with an EC_{50} of 3.38 mg/kg, while mancozeb showed superior suppression of spore germination at an EC_{50} of 0.25 mg/kg (Zhen-yue, 2005). A greenhouse and field trial by Zhen-sheng (2006) confirmed that propiconazole and mancozeb significantly reduced disease incidence and were more effective when applied 4 days after pathogen inoculation than when used preventively (Zheng-sheng,2006). The effectiveness of pesticides in controlling black point disease can be discerned through the examination of pairwise interactions between humidity, temperature, and precipitation. Propiconazole, Metalaxyl-M + Mancozeb, and Cymoxanil + Mancozeb

demonstrate the most excellent efficacy under conditions of high humidity and precipitation, rendering them suitable for application in tropical or subtropical regions (Nil et *al.*, 2025).

Food security is at risk from crop diseases. Still, it is challenging to identify and diagnose them in their early stages, which is crucial for disease control, because of a lack of infrastructure (Mohanty et *al.*,2016; Long et *al*., 2023) To swiftly and affordably detect disease occurrences on every agricultural plot, AI-based computer vision techniques need to be researched (Abade et *al*., 2021). AI-based methods minimize unintentional chemical use, perform routine plant health checks, and provide information on the bare minimum of pesticides needed to treat plant diseases (Ferentinos, 2018). According to recent research, deep machine models identify diseases in the dataset with 98% accuracy (Naik et *al*., 2022).

4.5.3. Biological control

Biological control strategies offer an environmentally conscious alternative to synthetic chemical interventions, primarily by mitigating the risks associated with chemical residues (Liccardo et *al.*, 2020). This approach aims to reduce reliance on conventional fungicides, thereby minimizing their ecological impact and the development of pathogen resistance (Deshi et *al.*, 2014; Becker et *al.*, 2021). The escalating problem of fungicide resistance, particularly with single-site fungicides, underscores the urgent need for robust biological alternatives. This concern is further amplified by the detrimental effects of chemical fungicides on human and animal health, alongside their substantial environmental toxicity (McLaughlin et *al.*, 2023).

Several microbial antagonists, notably species of *Pseudomonas*, have shown potential in reducing mycotoxin production by *Alternaria* under both in *vitro* and field conditions. For instance, *Pseudomonas simiae* was reported to significantly suppress the production of alternariol and tenuazonic acid in wheat grains, although it had a limited impact on fungal colonization in the field (Müller et *al.*, 2019). Similarly, fluorescent *Pseudomonas* isolates obtained from wheat heads exhibited strain-specific antagonism against *Alternaria* and significantly reduced toxin levels in treated wheat kernels (Müller et *al.*, 2018). Natural antifungal proteins, such as PgAFP, have also shown potential by not only limiting fungal growth but also suppressing the biosynthesis of major mycotoxins like alternariol and alternariol monomethyl ether, outperforming some synthetic fungicides in mycotoxin suppression (Cabral et *al.*, 2019). These results highlight the dual benefit of certain biological

control agents in both managing disease symptoms and reducing health risks from food contamination.

Biological control agents such as *Trichoderma* species have demonstrated efficacy in impeding the progression of infections through specialized mechanisms, including parasitism, antibiosis, and resource competition within the rhizosphere (Gajera et *al*.,2023). *Trichoderma* species have been employed to mitigate the proliferation of phytopathogenic fungi and promote plant development in various field and vegetable crops (Heflish et *al*.,2021). The effectiveness of *Trichoderma* is attributed to its rapid growth and resilience to unfavorable environmental conditions (Tapwal et *al*.,2016). The antagonistic and mycoparasitic activities of *Trichoderma* on plant pathogens, particularly through hyperparasitism, are considered crucial in reducing disease severity (Halifu et *al*.,2020). During the hyperparasitic phase, *Trichoderma* produces cell-wall-degrading enzymes such as chitinases, glucanases, and proteinases, which compromise the structural integrity of the plant pathogen's cell wall (Berini et *al*., 2016).

4.5.4. Physical control: Plasma treatment

4.5.4.1. Plasma overview

Sir William Crookes first described “radiant matter” in 1879, a phenomenon later termed plasma by Irving Langmuir in 1932, which is now recognized as the fourth state of matter. Plasma is defined as a quasi-neutral ionized gas, with two principal categories distinguished: thermal plasma, operating at temperatures several hundred to thousands of degrees above ambient, and non-thermal plasma, functioning within a range of 25–450 °C (Niemira, 2020). In the literature, non-thermal plasma is variably referred to as atmospheric cold plasma, low-pressure cold plasma, atmospheric pressure plasma, or non-thermal atmospheric plasma, with the term cold plasma (CP) being most commonly employed.

Cold plasma is generated by subjecting a gas or gas mixture commonly argon, helium, nitrogen, oxygen, or air to an electric current (Laroussi, 2005; Herceg et *al*., 2015). This process leads to the formation of ions, excited molecules, charged particles, and reactive oxygen and nitrogen species (RONS), including superoxide, ozone, hydroxyl radicals, hydroperoxyl, nitric oxide, nitrogen dioxide, dinitrogen pentoxide, as well as UV photons (Yu et *al*., 2006; Sakiyama et *al*., 2012; Machala et *al*., 2013; Mai-Prochnow et *al*., 2014). These reactive species are highly transient, with lifespans ranging from milliseconds to nanoseconds (Filaire & Toumi, 2012). The resulting plasma composition establishes a hostile microenvironment for microorganisms

such as bacteria and fungi, while leaving no residues or harmful by-products that could compromise either the environment or the treated commodity (Mai-Prochnow et *al.*, 2014).

All cold plasma (CP) generation systems share three fundamental components: a feed gas, the operating pressure during plasma formation, and a power supply. Regardless of the specific configuration employed, CP is generally applied at room temperature (Klampfl et *al*., 2012).

4.5.4.2. Feed gas

The choice of feed gas significantly influences the efficacy of cold plasma. Studies have shown that gases like sulfur hexafluoride and argon are more effective for fungal control than air (Basaran et *al.*, 2008). Although air is commonly used, either alone or in mixtures, for research on postharvest fungi, its effectiveness can be surpassed by other gases.

4.5.4.3. Pressure

Cold plasma can be generated either under atmospheric pressure or at reduced pressure within vacuum chambers prior to surface application (Niemira, 2012a). While low-pressure systems offer advantages in terms of purity, precision, and high energy yield, their reliance on specialized vacuum chambers results in increased operational costs, extended processing times, and greater spatial requirements. Consequently, such systems are often cumbersome and stationary, limiting their suitability for commercial deployment (Klampfl, 2014). In contrast, atmospheric-pressure systems (1 bar, 100 kPa) facilitate the treatment of vacuum-sensitive materials, including living tissues, which can be conveyed through a treatment zone (Niemira, 2012a). Furthermore, the feasibility of designing portable devices enhances the practicality and versatility of atmospheric cold plasma applications (Klampfl, 2014).

4.5.4.4. Power supply

Stryczewska et al. (2013) reported that cold plasma (CP) power supply systems can be based on transformers, converters (e.g., AC to DC), inverters, or alternative configurations such as radio frequency systems. Furthermore, CP generation is feasible through renewable energy sources, including solar power and batteries (Li & Tanielian, 1999; Pei et *al*., 2012). Although variations in applied power or voltage may modulate antibacterial efficacy, the intrinsic properties of CP remain largely consistent regardless of the supply or generation system employed (Dasan et *al*., 2017; Won et *al*., 2017).

4.5.4.5. Cold plasma application

Niemira (2012a) states that CP systems designed for food processing can be divided into three different systems based on the way the plasma is administered to the food product: remote, direct, and electrode contact. In conclusion, Mai-Prochnow et *al*. (2014) distinguished between direct and indirect CP systems. Here, the CP generating systems will be referred to as direct, indirect, or hybrid in order to treat postharvest fungal infections. Direct plasma systems work by generating CP between a single electrode on a dielectric surface and the sample, which serves as a second electrode and allows for direct contact between the sample and the plasma (Joshi et *al*., 2011; Mai-Prochnow et *al*., 2014). High quantities of RONS are delivered to the treated surface via the direct system, which quickly inactivates or kills microorganisms (Fridman et *al*., 2007; Niemira, 2012a). Another benefit of this plasma system is that it emits weak UV light and has a relatively low temperature (room temperature) at the production site (Heinlin et *al*., 2010). However, as the current passes through the sample, direct plasma might damage the product while it is being treated (Hojnik et *al.*, 2017).

Closed-circuit loops and a self-contained electrode system produce indirect plasmas, which are then delivered to the sample as a blown arc, flame, or jet. The treated surface comes into touch with the plasma discharge rather than the plasma directly. There is less exposure to the antimicrobial components in this system since many of the short-lived RONS are unable to reach the treatment surface (Fridman et *al*., 2007). Direct and indirect plasma generating are both used in hybrid plasma systems. Typically, a grounded wire-mesh electrode is used to create the plasma directly, preventing direct current passage through the sample during treatment and preventing product damage. Additionally, these devices emit weak UV radiation, generate low temperatures during emission, and are independent of the distance from the surface to be treated (within certain bounds) (Heinlin et *al*., 2010). Although the fungal species managed was not specified, Khamsen et *al*. (2016) treated rice seed using a hybrid plasma system to completely inactivate seedborne fungus in the field of postharvest pathology.

4.5.4.6. Mode of action of cold plasma

Although the precise antibacterial mechanism of cold plasma (CP) remains unclear, it is generally believed to act directly on pathogens and, in the case of fresh produce, indirectly by stimulating host defense responses (Perni et *al*., 2007; Mai-Prochnow et *al.*, 2014; Guo et *al*., 2015). Numerous studies have demonstrated that air-based cold plasma effectively

inactivates bacteria, spores, and other microorganisms on both food and non-food surfaces. Cold plasma treatment has been shown to efficiently eliminate a broad spectrum of microorganisms through the generation of reactive species that are lethal to cells (Nicol et al., 2020). During plasma formation, the oxidation of gases produces reactive oxygen species (ROS), reactive nitrogen species (RNS), UV radiation, energetic ions, and charged particles (Dobrynin et *al*., 2009; Han et *al.*, 2016). Among these, the activity of reactive species is considered the primary factor underlying the antimicrobial properties of cold plasma (Laroussi et *al*., 2004). The primary antimicrobial mechanism of radical species generated during plasma discharge is the induction of oxidative stress, which ultimately results in cell lysis and loss of cellular function. Oxidative stress intensifies cellular damage through processes such as membrane portion, lipid peroxidation, enzyme inactivation, and DNA cleavage. Cold plasma species act simultaneously on multiple bacterial and fungal targets, producing both structural and functional alterations that culminate in cell death. In addition, these reactive species can chemically degrade toxins such as aflatoxin, yielding less harmful by-products (Misra et *al*., 2015). Studies on microbial inactivation have shown that ROS markedly increase oxidative stress in both eukaryotic and prokaryotic cells, leading to rapid bacterial inactivation, whereas NO and ions contribute minimally to the overall cellular effects (Pai et *al*., 2018). The sequence of events by which free radical–induced oxidative stress causes cellular damage and lysis is depicted in Figure 8. Additional plasma byproducts contributing to microbial inactivation include UV radiation, hydrogen peroxide (H_2O_2), and electromagnetic effects. Cold plasma treatment for 5 min reduced viable *Clostridium difficile* spores by approximately three logarithmic units (Connor et *al*., 2017). Optical emission spectroscopy revealed that plasma-generated light contained atomic oxygen, atomic nitrogen, hydroxyl radicals, nitric oxide, and nitrate, all of which are implicated in cell death. Furthermore, scanning electron microscopy (SEM) confirmed structural damage to microbial cell membranes (Charoux et *al*., 2020). In *Botrytis cinerea*, SEM observations demonstrated that cold plasma induced mycelial folding and collapse depressions (Zhou et *al*., 2019).

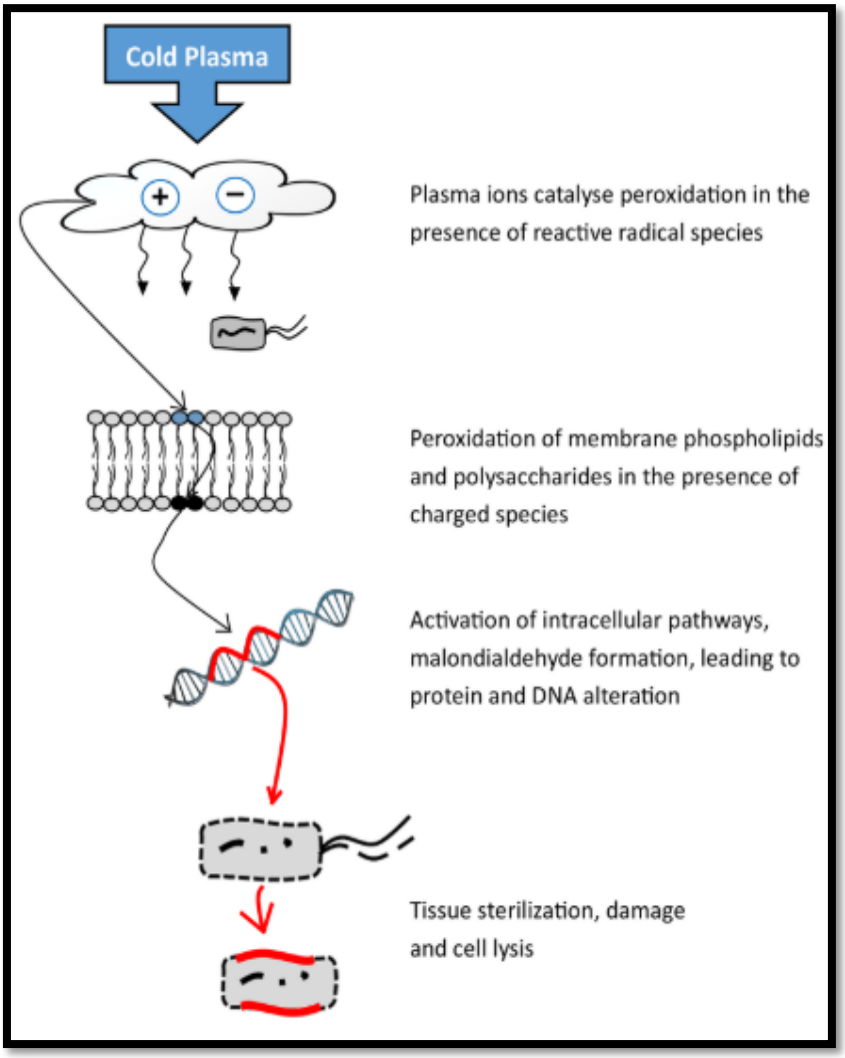

Figure 9: Mechanism of microbial inactivation by cold plasma treatment (Dobrynin et *al*., 2009).

4.6. Preventive strategies for Alternaria-derived mycotoxins

Effective prevention and reduction strategies are essential to lower the toxicity of mycotoxins in food commodities and lessen the exposure of humans and animals to such dangerous contaminants. Food quality is directly impacted by the possibility of contamination in agricultural goods both before and after harvest. Adhering to Good Agricultural Practices (GAPs) before harvesting is a crucial preventative measure (Paterson et *al*., 2018). Crop rotation strategies, the use of registered fungicides to manage fungal infection and insect damage, avoiding crops sensitive to fungus and adopting resistant cultivars to prevent fungal infestation, and appropriate seedbed analysis and treatment are all examples of GAPs (Sarrocco & Vannacci, 2018). To lower the risk, environmental variables like temperature, moisture content,

and mechanical damage to fruits or foods should be tracked (Adebiyi et *al*., 2019). Furthermore, the danger of contamination can be significantly decreased by preharvest neutralization of mycotoxins and the separation or disposal of contaminated food items, such as fruits and crops, from healthy ones (Aoun et *al*., 2020; Sundh & Goettel, 2013).

Cocultivation techniques can counteract fungal mycotoxins and modify defense metabolic pathways in plants with possible GMOs and microorganisms (Berthiller et *al*., 2013). According to Dana et *al*. (2018), vitamin C also inhibits the enzymes that produce fungal toxins, which controls the synthesis of mycotoxin. According to international rules for the permissible levels of mycotoxin in foodstuffs, postharvest methods for handling and incorporating logistic conditions, storage and harvesting requirements, sampling, and analysis have also been taken into consideration (Zain, 2011). Important considerations include storage space cleanliness, humidity management, and temperature (Pavicich et *al*., 2020). In addition to posing health risks, dirty and inappropriate storage can result in significant financial and economic losses. Even if poisons are stable above 150–200◦C, physical techniques like heating are crucial factors that can successfully reduce the concentrations of toxins (Karlovsky et *al*., 2016). High temperatures, however, have the potential to damage certain heat-sensitive nutrients in food or other essential components. Due to its extreme difficulty in implementing on a broad scale, radiation treatment such as exposure to gamma rays is used as a final step following food packaging. Another efficient method for producing free oxygen radicals, which have a chemical impact on various pollutants, is ozonation (Conte et *al*., 2020).

However, because these free radicals are unable to profoundly permeate food items, they cannot be used when there is a substantial amount of food present. A promising, affordable, and eco-friendly method for mycotoxin detoxification is cold atmospheric-pressure plasma (Hojnik et *al*., 2017). 100% degradation of AOH and AME was seen within 180 and 300 seconds of exposure, respectively, when the cold plasma method was applied (Wang et *al*., 2020). It has also been utilized to break down many mycotoxins, especially AAL and T2 toxins (Mohammadi et *al*., 2021). According to Pereyra et *al*. (2019), mycotoxin binders are absorbent substances that cling to mycotoxin and stop it from passing through the intestines and into the bloodstream. Chemical decontamination techniques are affordable and efficient. Thus, the food processing sector uses a variety of chemicals, including citric, succinic, acetic, formic, and tartaric acids (Wu & Khlangwiset, 2010). Ammonization, acidification, and ozonation are examples of chemical treatment techniques. *Calendula officinalis* and *Eucalyptus* globulus phytochemical

extracts showed fungistatic effect against *A. arborescens* and *A. alternata* when tested on tomato fruits. They also significantly reduced the generation of mycotoxin (TeA, AOH, and AME) (Pane et *al.*, 2016).

One possible strategy for reducing toxicity levels is the biological suppression of mycotoxins (Medina et *al.*, 2017). It has been demonstrated that several toxin-producing fungi and their toxins are inhibited by biocontrol agents or competitive inhibitors, such as yeast and *Lactobacillus* species (Habschied et *al.*, 2021). AOH and AME can be extracted from aqueous solutions by inactivated yeast cells (Wang et *al.*, 2020). This study offers a theoretical framework for further investigations into the processes by which *Alternaria* mycotoxins are eliminated from food systems. Similarly, toxin biosynthesis gene clusters can be silenced by bacteria that have been altered to produce vitamin C (Gerke & Braus, 2014). Using nanoparticles to combat mycotoxins is one of the novel detoxifying techniques (González-Jartín et *al.*, 2019). Furthermore, for enzymatic detoxification, oxidase, laccase, microbial manganese peroxide, and catalase have been employed (Hassan & Zhou, 2018). The scientific world is now focusing on mycotoxin contamination as a growing hazard to humanity, and the development of new analytical methods requires significant funding and attention. Campaigns for education and awareness are successful in educating farmers and major food-producing nations about mycotoxin exposure, safe food handling, and logistics.

Part 2

Material and methods

Chapter 1

Diversity and Toxicity of Alternaria, one of the causal agents of Black point disease of Durum wheat in Algeria

1.1. Sampling of wheat kernels and isolation of *Alternaria* Strains

During 2021/2022 crop season, the National Center of Control and Certification (CNCC), regional laboratory of Sétif, provided Black pointed symptomatic durum wheat kernels of six varieties, namely Bousselem, Mohamed Ben Bachir (MBB), GTA dur, Oued el Bared, Viteron, and Sersou (Simeto). They were randomly collected from fields located in six different geographic locations in sixteen regions of the north-east to the south east of Algeria, including: Msila, Sétif, Biskra, Batna, Bejaia, and Khanchela (Appendix 1) According to the Köppen–Geiger climate classification, these regions are characterized by distinct climatic conditions (Figure 10). Wheat sampling sites were georeferenced and visualized using QGIS (version 3.44.5), with Köppen–Geiger climate classification data obtained from Beck et al. (2018). A total of 48 durum wheat grain samples were collected from different agro-climatic regions of Algeria. One sample originated from Bejaia, characterized by a temperate warm climate with dry, hot summers. Five samples were obtained from the Batna region, which is classified as arid steppe with cold conditions. Additionally, five and two samples were collected from M'sila and Khenchela, respectively, both regions characterized by an arid desert climate with cold winters. Finally, seven samples were collected from Biskra, an area known for its arid desert climate with hot conditions. All samples were stored in hermetic paper bags at room temperature until further processing. These 48 samples were directly used for fungal isolation (Table 2).

Table 2: Geographical origin of Algerian durum wheat samples, including the sampling regions, associated climate conditions, number of collected samples, and wheat varieties analyzed .

Region	**Climate conditions**	**N. of total samples**	**Wheat variety**	**N. of samples**	**Wheat sample name**
Bejaia	Temperate, dry and warm summer	1	Boussalem	1	V30
Sètif	Temperate, dry and hot summer	28	Boussalem	11	V8, V10, V12, V14,

					V21, V23, V25, V26, V27, V36, V42
			GTA Dur	1	V9
			Mohamed Ben Bachir	1	V32
			Oued El Bared	15	V4, V15, V16, V17, V18, V19, V20, V22, V28, V31, V39, V43, V46, V47, V48
Batna	Arid, steppe, cold	5	Oued El Bared	2	V29, V35
			Simeto	3	V24, V33, V34
M'Sila	Arid, desert, cold	5	Vitron	5	V1, V2, V3, V7, V11
Khenchela	Arid, desert, cold	2	Vitron	2	V41, V44
Biskra	Arid, desert, hot	7	Oued El Bared	2	V38, V40
			Vitron	5	V5, V6, V13, V37, V45

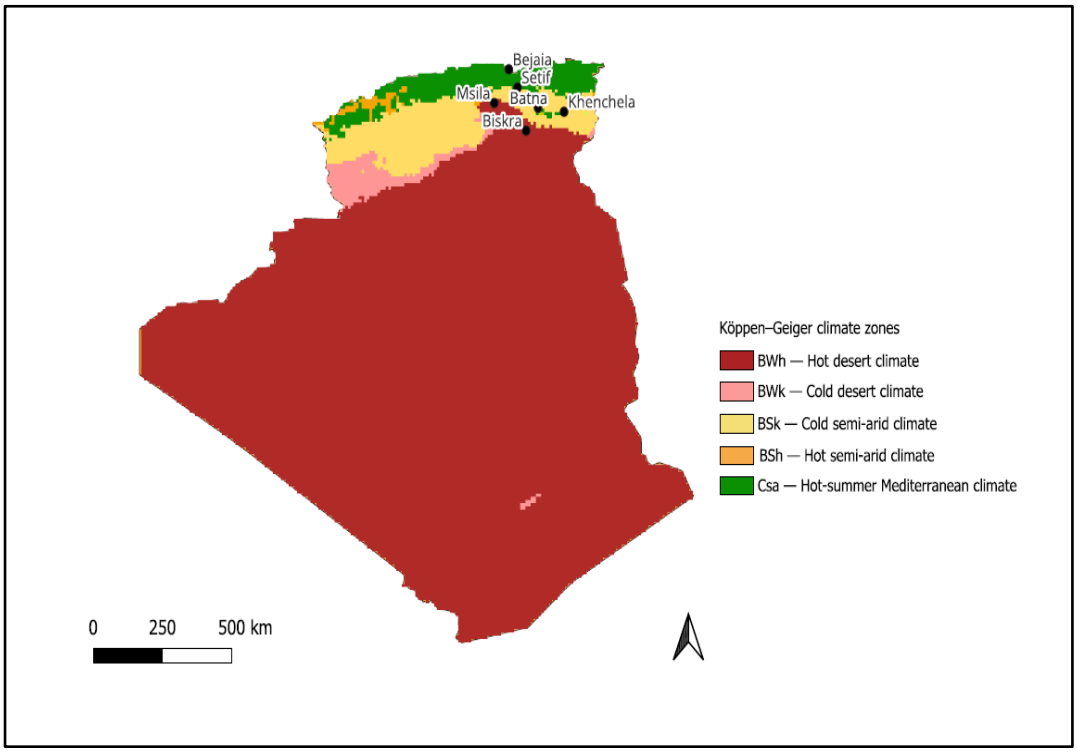

Figure 10: Map of Algerian regions where wheat sampling fields were located, illustrating climate zones according to the Köppen–Geiger classification (Beck et *al*., 2018).

Fungal isolation was conducted using randomly selected kernels. They were sterilized by dipping them in a 2% sodium hypochlorite solution for 2 minutes, followed by two rinses in sterile distilled water, then dried on sterile filter paper (Masiello et *al*.,2020). Six kernels were deposited onto Petri dishes containing (PDA) medium and incubated at 25ºC for 5-7 days. Although various fungal colonies grew on each seed, only Alternaria isolates were kept for further research.

1.2.Purification and conservation of isolates

Pure cultures were obtained from each isolate by successive subculturing on PDA medium. The pure isolates were cultured on (PDA) medium and incubated at 25ºC for 7 days to perform a single spore culture as described by Ke ZHANG and Yuan-Ying SU (2013) with some modifications. From spore suspension, single drops were placed and spread evenly over the PDA petri dishes. After 6-12h incubation at 25º, plates were observed under a binocular stereomicroscope, and one germinated spore per isolate was picked and sub-cultured in a new PDA Petri dish. The plates were incubated at 25ºC for 7 days in order to be conserved.

The conservation technique of isolates was done by subculturing pure isolates in SNA cryotubes, incubated at 25ºC for 7 days, then stored at 4ºC.

1.3.Morphological characterization

Morphological characteristics of the fungal pathogens found in black-pointed durum wheat kernels were based on criteria that include cultural characteristics (such as mycelium texture, colony color, or the reverse side of the colony).

After 5-7 days on PDA, observations were conducted. The macroscopic exam, including the morphological characteristics of the fungal colony such as the mycelium nature (color and thallus aspect), was carried out to extract certain specific characters. Thus, depending on the species and morphotypes, the mycelium may be shallow, underdeveloped, velvety, slightly fluffy, or airy, or it may be cottony and brown, olive to light grey, or dark. Even though the macroscopic characters of colonies appear different, it is challenging to distinguish and identify this fungal pathogen considering these criteria alone. Distinguishing between closely related *Alternaria* species, such as *A. alternata*, *A. tenuissima*, and *A. arborescens* from the *Alternata* section, as well as species like *A. solani* and *A. tomatophila* from the *Porri* section, is particularly challenging, also due to their similar spore morphologies. To address this, isolates are examined microscopically by analyzing the morphology and organization of the spores produced. This is done using an optical microscope and referencing established literature (Simmons, 2007). The three-dimensional structures of conidial chains are evaluated after a 10-day incubation period on PCA medium at 25ºC. The conidial chains are then observed under the microscope at magnifications of ×40 or ×100 for detailed analysis. The typical sporulation for each species is photographed using a camera.

1.4.Molecular characterization

1.4.1. DNA extraction

For each monosporic strain, five mycelial plugs were excised from the margins of actively growing colonies on PDA and transferred onto sterilized cellophane disks overlaid on PDA plates. Following incubation at 25 °C for 2–3 days, the resulting mycelia were scraped into 2 mL tubes, frozen at –20 °C, and subsequently lyophilized. Approximately 10–15 mg of powdered lyophilized mycelium was used for total DNA extraction with the *Wizard Magnetic DNA Purification System for Food* kit (Promega Corporation, Madison, WI, USA).

Briefly, mycelium was disrupted using a 5 mm iron bead in a TissueLyser (Retsch, Germany) at 15 osc/s for 30 s in the presence of 250 µL Lysis Buffer A and 2.5 µL RNase A. Samples were incubated at 37 °C for 15 min, followed by the addition of 125 µL Lysis

Buffer B. After mixing in the TissueLyser (10 osc/s, 30 s), tubes were incubated again at 37 °C for 10 min, then supplemented with 375 µL Precipitation Solution, vortexed, and centrifuged at 13,000 rpm for 15 min at room temperature (18–22 °C). The supernatant was transferred to a new tube, combined with 25 µL MagneSil® PMPs and 0.8 vol isopropanol, inverted 10–15 times, and incubated for 3 min at room temperature.

Magnetic separation was performed using the MagneSphere® Technology Magnetic Separation Stand, discarding the liquid phase. The pellet was resuspended in 125 µL Lysis Buffer B, mixed gently, and subjected again to magnetic separation. The pellet was then washed twice with 750 µL of 70% ethanol, dried at 65 °C for 6 min, and resuspended in 100 µL nuclease-free water. After a final incubation at 65 °C for 5 min, DNA was recovered by magnetic separation and transferred to a fresh tube.

Genomic DNA quality was assessed by electrophoresis on 0.8% agarose gel in 1X TAE buffer, using a 1 kb DNA Ladder (ThermoFisher Scientific, Carlsbad, CA, USA) as reference. Prior to loading, each sample was mixed with 2 µL of 2X loading dye and visualized under UV light.

1.4.2. PCR with species-specific primers

For the molecular identification of the *Alternaria* genus, two informative gene fragments were selected: the allergen-coding gene (*alt-a1*) and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene. Amplification was performed using specific primers: Alt-for (5'-ATGCAGTTCACCACCATCGC-3') and Alt-rev (5'-ACGAGGGTGAYGTAGGCGTC-3') (Hong et al., 2005) for *alt-a1*; and gpd1 (5'-CAACGGCTTCGGTCGCATTG-3') and gpd2 (5'-GCCAAGCAGTTGGTTGTGC-3') (Berbee et al., 1999) for *gpd*.

PCR reactions were prepared in a mixture containing 0.125 µL of Start Taq DNA Polymerase (1 U/µL; Fisher Molecular Biology, Italy), 0.45 µL of each primer (10 µM), 1.2 µL of 10 mM dNTPs, 1.5 µL of fungal genomic DNA, and 1.5 µL of 10X buffer. Amplification of the *alt-a1* gene was carried out in a Mastercycler EP Gradient thermal cycler (Eppendorf, Hamburg, Germany) with the following program: initial denaturation at 95 °C for 2 min; 33 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 50 s; followed by a final extension at 72 °C for 7 min. Amplification of the *gpd* gene was performed under the following conditions: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 59 °C for 50 s, and

extension at 72 °C for 60 s; with a final extension at 72 °C for 7 min (Masiello et al., 2020). Water was included as a negative control in all reactions.

PCR products were separated by electrophoresis on 1.5% agarose gel in 1X TAE buffer, visualized under UV light, and compared against a 100 bp DNA Ladder (ThermoFisher Scientific, Carlsbad, CA, USA).

1.4.3. Sequencing

PCR products were enzymatically purified using a mixture of Exonuclease I and FastAP thermosensitive alkaline phosphatase (ThermoFisher Scientific), following the manufacturer's instructions. Specifically, 0.5 µL of Exo and 1 µL of FastAP were added to 5 µL of each PCR product, incubated in a thermal cycler for 15 min at 37 °C (optimal enzyme activity), and subsequently for 15 min at 85 °C to inactivate the enzymes.

Purified PCR products were then labeled using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Each reaction mixture contained 3 µL of PCR product, 0.16 µL of primer, 3.84 µL of nuclease-free water, 2 µL of buffer, and 1 µL of master mix with ddNTPs labeled by four distinct fluorophores.

Following labelling, samples were purified by filtration through Sephadex G-50 (5%; Sigma-Aldrich, Milan, Italy) columns, and sequencing was performed using an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

1.4.5. Phylogenetic analysis

DNA sequences were analyzed using Sequencing Analysis 5.2 software (Applied Biosystems, Waltham, MA, USA). Consensus sequences were generated by aligning forward and reverse strands with Bionumerics software (Applied Maths, Kortrijk, Belgium). Preliminary comparisons of FASTA sequences were performed against available entries in the National Center for Biotechnology Information (NCBI) database using the BLASTN tool. Phylogenetic analyses were subsequently conducted in MEGA7 software employing the Maximum Likelihood method, with bootstrap support based on heuristic search using 1000 replicates and gap removal (Kumar et *al*., 2016). To resolve phylogenetic relationships among and within the detected species, reference sequences identified through BLAST searches were retrieved from the NCBI database and incorporated into the analyses.

1.5. Production of mycotoxins by *Alternaria* isolates

Seventy-two *Alternaria* isolates molecularly identified and characterized, isolated from discolored black point durum wheat kernels, were tested for mycotoxin production. For mycotoxin production evaluation as described by Li and Toyozaki (2001), flasks containing 30g of rice brought up to 45% moisture with distilled water were incubated at ambient temperature overnight, and the next day, they were autoclaved at 121 °C for 30 min. Five mycelium plugs from each fresh Alternaria PDA culture were inoculated in each rice flask and incubated in darkness at 25°C for three weeks (21 days) with daily shaking. Then, the inoculated rice kernels were dried in the oven at 60° or 65° for 48h and finely milled with an Oster Classic grinder (220- 240V,50/60 Hz, 600W; Madrid, Spain).

1.5.1. Extraction of mycotoxins from rice cultures

Following the natural occurrence of toxigenic secondary metabolites produced by *Alternaria* species, as reported by Ostry (2008), cultures were analyzed for the presence of alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxin I (ATX-1), tenuazonic acid (TeA), and tentoxin (TEN). For extraction, 2 g of homogenized samples were placed in 15 mL plastic tubes and mixed with 10 mL of an extraction solvent composed of acetonitrile/methanol/water (45:10:45, v/v/v), adjusted to pH 3 with hydrochloric acid. The mixture was shaken for 60 min on an orbital shaker, centrifuged at 4500 rpm for 10 min, diluted with distilled water (D10), and centrifuged again at 15,000 rpm for 15 min at 4 °C. The resulting extract was subsequently injected into the high-performance liquid chromatography system for analysis.

1.5.2. Extraction of the mycotoxins in wheat samples

For accurate detection of mycotoxins in diseased durum wheat kernels, forty-eight samples representing different varieties and geographic origins were finely ground. From each sample, 5 g were weighed and extracted with 30 mL of solvent mixture (acetonitrile:methanol:water, 45:10:45, v/v/v), adjusted to pH 3 with hydrochloric acid. The mixtures were shaken for 60 min, after which the supernatants were filtered through Whatman No. 4 paper (Whatman, Maidstone, UK) and collected for subsequent analyses.

1.5.2.1.Solid phase Extraction (SPE) clean-up for TeA

For the clean-up of TeA, 5 mL of filtrate was transferred into a 25 mL flask and mixed with 10 mL of sodium dihydrogen phosphate solution. An Oasis HLB column (Waters Corporation, Milford, CT, USA) was connected to a vacuum manifold and conditioned with 2 mL of acetonitrile until air passed through the column. Subsequently, 7.5 mL of the diluted filtrate was loaded into the reservoir and allowed to pass through the column at a controlled rate of 1–2 drops per second. The eluate was discarded, and a 4 mL vial was positioned beneath the column. TeA was then eluted with 1 mL of solvent mixture (acetonitrile: methanol:0.05 M sodium dihydrogen phosphate solution, 35:35:30, v/v/v). The eluate was collected, capped, and stored at approximately 4 °C until LC analysis.

1.5.2.2. Solid Phase Extraction (SPE) clean-up for AME, AOH, ATX-1, TEN, and ALT

For the clean-up of AME, AOH, ATX-1, TEN, and ALT, a C18 column was connected to a vacuum manifold and conditioned with 6 mL of acetonitrile until air passed through the column. Subsequently, 2.5 mL of filtrate was applied to the column and allowed to flow at a controlled rate of 1–2 drops per second. The eluate was discarded, and a 4 mL vial was positioned beneath the column. Mycotoxins were eluted with 2 mL of solvent mixture (acetonitrile: glacial acetic acid, 99:1, v/v). The eluate was evaporated to dryness under a nitrogen stream at approximately 60 °C, and the purified residue was resuspended in 500 µL of methanol by vertexing for 1 min. Finally, the solution was diluted with 500 µL of LC mobile phase Solvent A for subsequent analysis.

1.5.2.3. Standard solutions

A mixed stock standard solution containing 10ng/µL AME, AOH, TEN, and ALT was prepared by diluting single standard solutions of mycotoxins in methanol. The stock solution was then appropriately diluted to obtain calibrant solutions in the concentration range of 0.1-1.0 ng/µL of AME, AOH, TeA, ATX-1, TEN, and ALT in mobile phase solvent A.

1.5.2.4. Spiking protocol

Four (5 g aliquots) of wheat were weighed, consisting of 1 blank and 3 replicates. Three samples were spiked with 500 µL each of the mixed standard solution (10ng/µL). The spiking solvent was allowed to evaporate for 30 min before proceeding with the extraction step. For TeA and ATX-1, 5mL of filtrate (corresponding to 0.5g matrix[22]) was spiked with

50 ul TeA (9.5 µg/mL) and 50 µL ATX-1(10 µg/ml). The final spiking level was 1 µg /g for all toxins.

1.5.3. HPLC Analysis

HPLC analysis was carried out using a UV diode array detector set at 279 nm for TeA and TEN, and at 256 nm for the remaining toxins. The mobile phase consisted of Solvent A (acetonitrile:0.027 M sodium dihydrogen phosphate, 50:50, v/v). The 0.027 M sodium dihydrogen phosphate solution was prepared by dissolving 3.75 g of $NaH_2PO_4 \cdot 2H_2O$ in 990 mL of LC-grade water, adjusting the pH to 3.0 with concentrated o-phosphoric acid, and completing the volume to 1 L with LC-grade water. The solution was then filtered through a 0.22 µm membrane filter for aqueous solutions.

The gradient program consisted of pumping Solvent A for 5 min at a flow rate of 1 mL/min, followed by Solvent B for 20 min at the same flow rate. Chromatographic separation was performed on a Waters Symmetry Shield C18 analytical column (150 × 4.6 mm, 5 µm; Waters Corporation, Milford, MA, USA), preceded by a 0.45 µm pore-size filter. Retention times for the analyzed toxins are reported in Table 3.

Table 3: Retention times for Alternaria mycotoxins

Mycotoxins	Retention Times
ALT	7.8min
AME	18min
AOH	12.5min
TeA	8.35min
ATX-1	9.2min
TEN	8.6min

Chapter 2

Effect of barrier discharge plasma treatment on four Alternaria species

Chapter 2: Effect of barrier discharge plasma treatment on four *Alternaria* species

2.1. Source of the fungal pathogen material

Four Alternaria species, namely: *A. alternata, A. arborescens, A. angustiovoidea, and A. mali /A. rhadina/Aternaria citriarbusti* complex were isolated from diseased Algerian durum wheat kernels using a single-spore isolation technique at the Applied Microbiology Research Laboratory, Setif University (Algeria). Species identification was performed based on morphological characteristics and confirmed by molecular analysis (Daichi et *al*., 2025). Fungal cultures were maintained on Potato Dextrose Agar (PDA) and incubated at 25–28 °C for 4 days prior to experimental use.

2.2.Discharge system

Fungal samples were treated using a custom-built dielectric barrier discharge (DBD) plasma device operating at atmospheric pressure. The system consisted of planar electrodes mounted on a layered acrylic structure, with one electrode covered by a dielectric barrier to prevent arc formation. Electrical excitation was provided by an alternating current (AC) power source with a frequency of 20 kHz, a duty cycle of 15%, and a current limit of 9.0 A. Plasma was generated in ambient air, producing reactive oxygen and nitrogen species (RONS), UV photons, and charged particles. Samples were positioned directly beneath the discharge zone to ensure uniform exposure. Treatment durations ranged from 1, 2,5 to 5 min for mycelial growth and from 5,10, to15 minutes for spore germination (Figure 11).

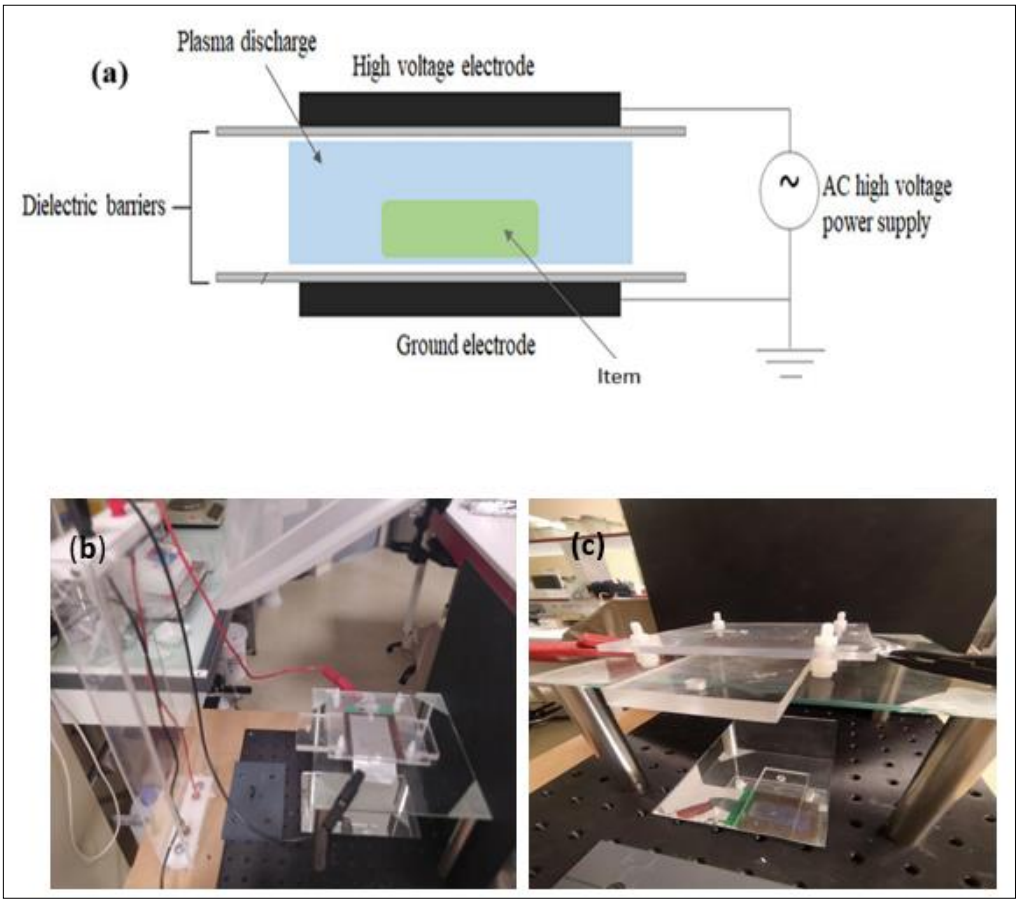

Figure 11: Schematic and experimental configuration of the dielectric barrier discharge (DBD) plasma system for *Alternaria sp.*

Treatment**. (a)** Schematic diagram of the DBD plasma setup. **(b-c)** Photographs of the experimental DBD plasma system from top and side views, respectively. These images show the transparent dielectric barrier, electrode wiring, sample placement on the treatment platform, and structural components including spacers used to maintain the discharge gap.

2.3.Fungal isolates and Impact on mycelium growth test

The inhibitory impact of DBD plasma on *Alternaria* species, including *Alternaria alternata, Alternaria arborescens, Alternaria angustiovoidea, and Alternaria mali /A. rhadi/A. cirtiabusti*, was assessed through mycelium growth assays conducted at three different exposure times. MD 9: *A. arboresens,* MD14: *A. mali/A. rhadi/A. cirtiabusti,* MD 40: *A. alternata,* MD6: *A. angustiovoidea.*

The stored cultures were revitalized on Malt Extract Agar (MEA). Plugs (5 mm) from the actively growing mycelium of the cultures (24 h) were cut and placed on a sterile

microscopic coverslip, and then submitted to plasma treatment. The overall treatment times used were 1 min, 2,5 min, and 5 min. The mycelial plugs were seeded in the center of MEA plates and incubated in the dark at 28 °C for 5 days. The mycelial plug, unexposed to plasma treatment, was used as an additional untreated control (0 min) and placed in the center of MEA plates.

The determination of mycelium growth inhibition was performed by measuring the colony diameter zones using scanned colonies. Images were captured and analyzed using ImageJ software. The diameter was evaluated each day by calculating the average of the two perpendicular diameters of each fungal colony. Three replicates were performed for each treatment/control. The Inhibition of mycelial growth was compared with the control, following the calculation method proposed by Philippe et *al.* (2012).

$$\text{Inhibition of mycelial growth (\%)} = \left(\frac{dc-dt}{dc}\right) \times 100$$

The radial growth of non-thermal plasma (NTP) treated mycelia was compared with the untreated control by calculating the mean diameter **(dc)** of the colony in the control sample and the mean diameter **(dt)** of the colony in the treated sample.

2.4.Conidial germination tests

The dielectric barrier discharge plasma (DBD) treatment effect against the four species of *Alternaria* was evaluated through conidia germination assays. Conidia were harvested from 7-day-old MEA cultures after gently rubbing the colony surface with a policeman. Dislodged conidia were removed, flooding the plates with 0.01% Tween 80, which prevented the conidia from sticking to the agar surface or the petri dish walls during collection.

The resulting spore suspensions were filtered through two layers of sterile cheesecloth and centrifuged at 4500 rpm for 10 min. The settled pellets were resuspended in 1ml of Tween. The spore concentrations of each species were then determined using a hemacytometer.

Aliquots (10 µL) of each fungal conidial suspension were spotted on Whatman-graded paper and placed on a sterile microscopic coverslip, then submitted to plasma treatment. The overall treatment times used were 1 min, 5 min, 10 min, and 15 min. The papers inoculated with conidia that were not exposed to DBD were used as an additional untreated control (0min). The treated papers with spore suspension were placed in 2 mL Eppendorf tubes containing malt extract broth (MEB) Media autoclaved at 110 °C for 10 min, and incubated at 28 °C in the dark.

The germination of spores was observed under a light microscope after 6 and 18 h. The germination percentage was evaluated by counting at least 200 spores in random fields of view for each treatment; a spore was considered germinated when the germ tube length was larger than half of the diameter of the spores. The percentage of conidial germination inhibition was calculated using Equation (10) from (Adhikari et *al*., 2020).

$$\text{Inhibition of sporangiospore germination (\%)} = \frac{(gc - gt)}{gc} \times 100,$$

where **gc** is the number of germinated conidia in the untreated control, and **gt** is the number of germinated conidia for each plasma treatment.

2.5. Statistical analysis

Data on mycelial growth and spore germination were analyzed using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). A two-way analysis of variance (ANOVA) was performed to assess the effects of plasma exposure time and incubation period, and post hoc comparisons among treatment means were conducted using Tukey's HSD test at a significance level of $p < 0.05$. For data visualization, including bar graphs with error bars, as well as multivariate analysis, OriginPro 2024 (OriginLab Corp., Northampton, MA, USA) was used. Principal component analysis (PCA) was carried out in Origin to explore interspecific variation in plasma sensitivity across the four *Alternaria* strains .

Part 3

Results

Chapter 1

Diversity and Toxicity of Alternaria, one of the causal agents of Black point disease of Durum wheat in Algeria

1.1. *Alternaria* contamination in durum wheat samples

Significant fungal contamination was found in all wheat kernel samples, ranging from 80% to 100%. The genera Alternaria, Fusarium, and Cladosporium were found in all six areas of Algeria. The M'sila region has low levels of Fusarium and Cladosporium, with mean contamination rates of up to 7% and 13%, respectively (Figure 12).

Alternaria was the most common fungus found, with contamination levels ranging from 7% in sample V18 from the Setif region to 97% in samples V13 and V24 from Biskra and Batna, respectively. Figures 2 and 3 show the regional mean contamination levels, which ranged from 32% in Khenchela, 35% in Setif, 53% in Batna and Biskra, and up to 63% in M'sila. The quantity of wheat samples collected varied greatly despite these regional variations in Alternaria contamination; just one or two samples were acquired from Bejaia and Khenchela, respectively (Table 1). Additionally, there was a lot of variation in the contamination levels within each region. (Figure 13).

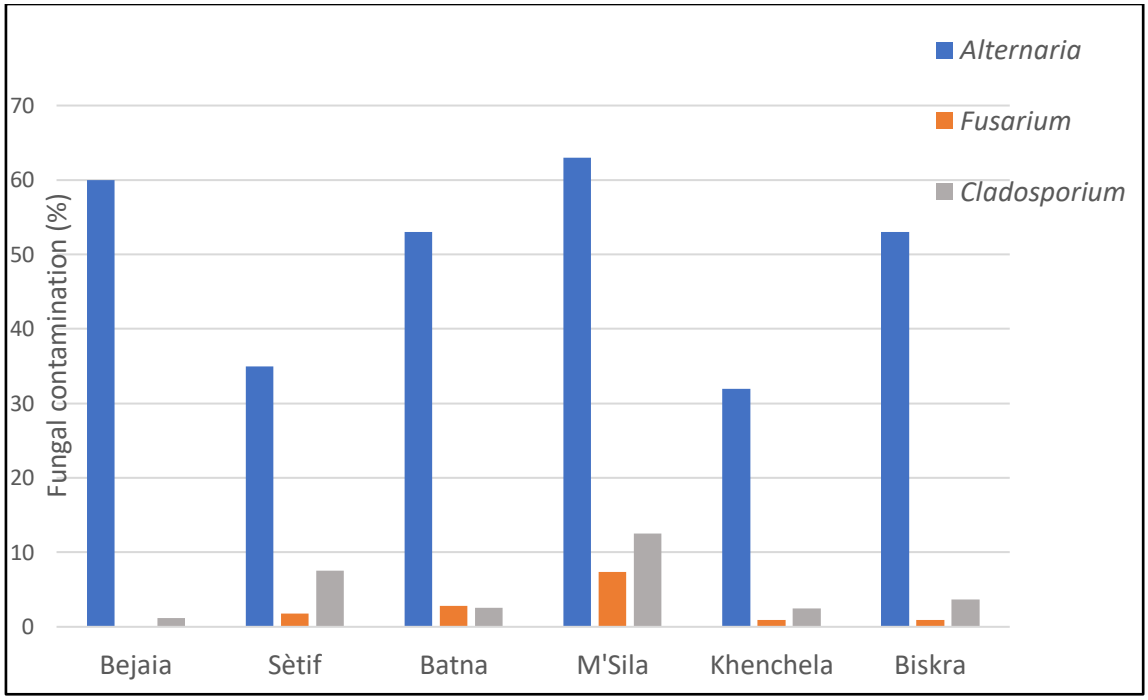

Figure 12: Percentage of fungal contamination by *Alternaria*, *Fusarium*, and *Cladosporium* genera detected in wheat kernels collected from six southeastern regions of Algeria.

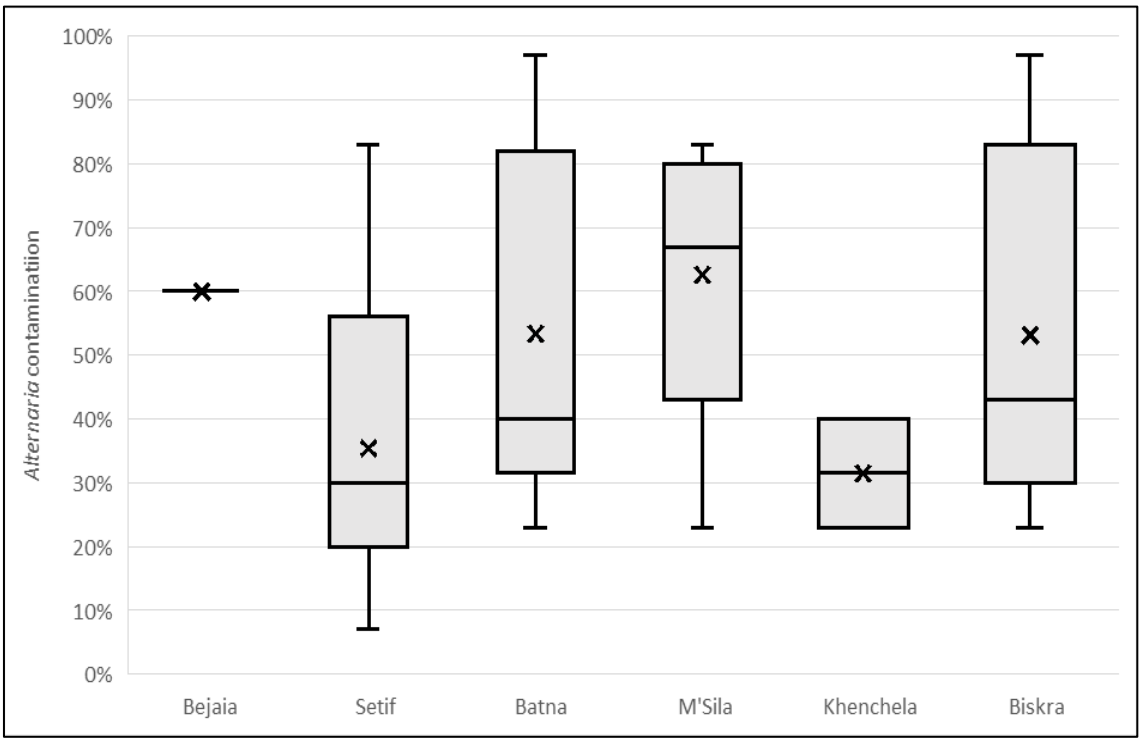

Figure 13: The percentage of wheat kernels taken in six Southeastern districts of Algeria that were contaminated by Alternaria species. The minimum and maximum values in each plot, which represents a single region, are shown by the limits of whiskers; the mean value is represented by X, and the median value is represented by the line inside the boxes.

Regarding the influence of wheat varieties on *Alternaria* contamination, Figure 14 indicates that the highest incidence was observed in two varieties: Simeto, cultivated in Batna region, and Vitron, grown in Msila, Khenchela, and Biskra. The mean contamination levels for these varieties were 59% and 58%, respectively.

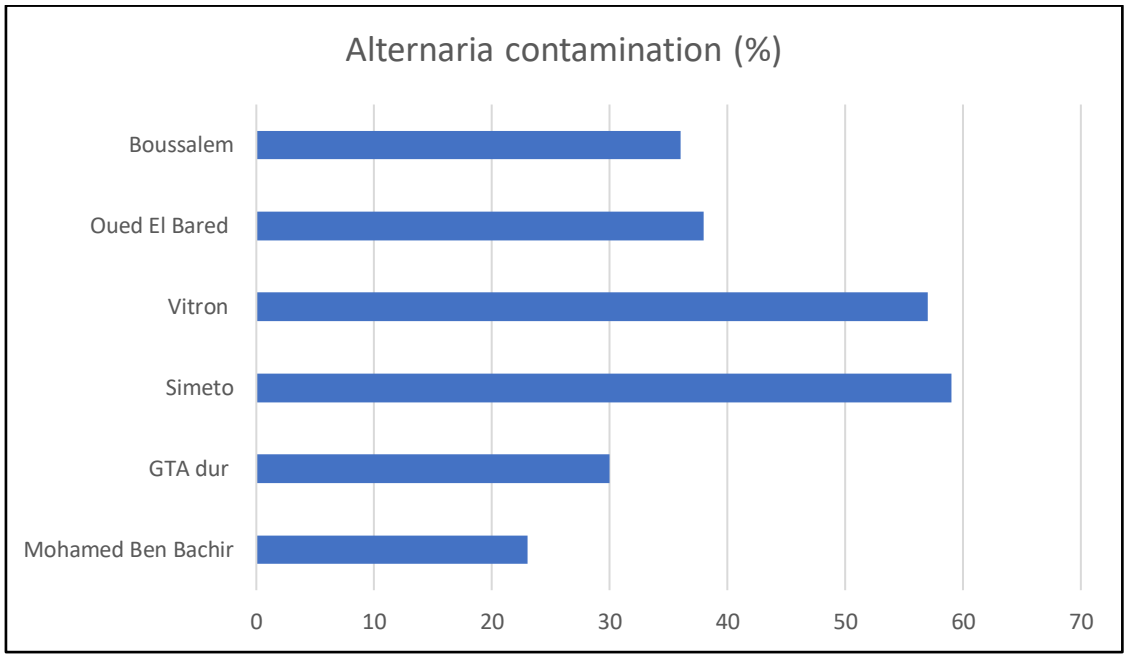

Figure 14: Percentage of *Alternaria* contamination in six different wheat varieties grown in Algeria.

1.2. Morphological characterization of *Alternaria* isolated species

Based on macroscopic characteristics, 72 of the 608 Alternaria isolates from wheat kernels were chosen as representatives of the total population. 72 isolates were chosen for PDA media colony morphological characterization. The findings showed that the isolates' macroscopic characteristics varied significantly. Single conidiophores and catenulate muriform, brown to golden brown conidia were generated by every Alternaria isolate. Nonetheless, eight isolate groups (A, B, C, D, E, F, and G) were distinguished by their colony morphology, conidiophores, and conidia traits.

Isolates in Groupe A were identified as *Alternaria alternata* in sec*tion Alternaria* commonly referred to small spores as was introduced by Lawrence et al. (2013), producing cottony, light gray to green colonies. These observations aligned with those of Zhao et al. (2024), who reported that cultures of *A. alternata* grown on (PDA) exhibited a dark olive color with loose, cottony mycelium on the surface. Similarly, Xu et al. (2024) observed that colonies on PDA initially appear white but later develop an olive-green coloration with a rim. These descriptions align with previous morphological characterizations of *A. alternata*, which typically exhibit dark green to black pigmentation as the colony matures (Figure 15a). The conidia of 3 to 6 cells of the isolates appear as dense clusters in branched chains. The shape of the conidia was obpyriform (club-shaped) to oval or obclavate, with colors ranging from

yellowish-brown to dark brown and featuring 3-7 transverse septa and 0-3 longitudinal (or oblique) septa (Figure 15a).

Isolates in Groupe B identified as *A. arborescens,* exhibited a fluffy or woolly texture with a dense mycelial growth, the surface of the colonies were often velvety or powdery due to the production of conidia, olive-green to dark brown in color as confirmed Pavon et al. (2020), its dark olive-green to black colony color and velvety texture. They also highlighted its role in causing plant diseases and its adaptability to various environmental conditions. Also, Zhang et al. (2021) described the colony morphology of *A. arborescens* as dark green to black with a woolly texture, emphasizing its distinct conidial chains and septation patterns. Isolates produced conidial chains of 2-7 conidia, with oval to ellipsoid shape, 1-4 transepta and no longi-septa, or, rarely, 1 (Figure 15b).

Isolates in Groupe C identified species in the Alternaria section, produced olive green-dark brownish, fluffy texture with slightly granular surface colonies that grew radially with irregular edges. These colonies produced larger conidia with a more pronounced beak, comparable to *A. alternata*, which is characterized by small conidia. According to Woudenberg et al. (2013), species in section Alternaria, such as *A. alternata* and *A. anguitovoidea*, can be differentiated by subtle variations in conidial size, shape, and septation patterns. *A. anguitovoidea* exhibits larger conidia with more pronounced beaks and a higher degree of branching in conidial chains. The isolates of this group produced conidial chains that were branched and tree-like, conidia with 3-6 transverse septa and 1-3 longitudinal septa (Figure 15C).

Isolates in Groupe D were difficult to identify morphologically. isolates produced olive-brown to dark, woolly texture, velvety surface, and brownish- black reverse colonies. Conidia are produced in short chains or singly, unlike the long-branched chains in the Alternaria section, like *A. alternata* and *A. arborescens*. Conidia were wide ovoid, ellipsoid in shape, and short beak in the apical end with 3-6 transverse septa, 1-3 longitudinal septa.

Isolates in Group E were identified as species in the section Ulocladioides. Isolates produced dark olive-green to dark colonies, fluffy surface due to the aerial mycelium and black brownish revers. Conidia were produced in abundance, giving the colony a granular appearance under low magnification. They were arranged in short, non-branched conidiophores and without a beak, which is a key characteristic of *Ulcoladioides.* These observations aligned with Simmons (2007), who reported species with intermediate characteristics between *Alternaria* and *Ulocladium,* which were later reclassified into the

genus *Ulocladioides* based on molecular phylogenetic studies. Also, as Lawrence (2016) described, the section *Ulocladioides* 3-dimensional sporulation pattern is usually characterized by simple or branched conidiophores. Conidia were with 0-3 transverse septa and 0-3 longitudinal septa.

Isolate in Groupe F, were difficult to identify which section it belongs, because conidia were not produced very abundantly. Conidia were solitary or in short chains with cylindrical and ovoid shape. Isolate produced green olive to dark and fluffy gray surface colonies. Conidia had several septa; transverse no or many longitudinal septa.

Isolates in Groupe G, were identified species belonging to section *Infectoriae*, produced gray to yellow green, white colonies. Conidiophores were short with ovoid to ellipsoid shape and 2-5 tran-septa and 0 to 1 longisepta in 0 to 3 transverse segments.

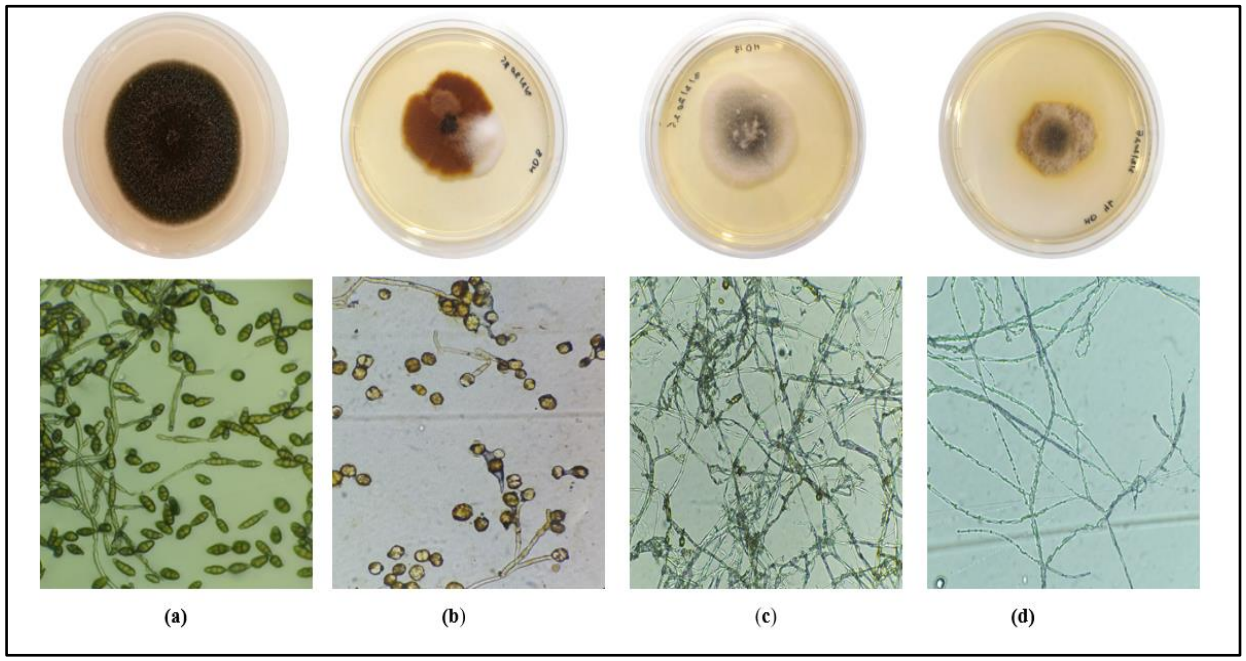

Figure 15: Morphological characteristics of Alternaria isolated from Durum Wheat kernels; (a) A. *alternata,* (b) *A. consortialis*, (c) *A. eureka* (d) *A. arborescen.*

1.3. Molecular and phylogenetic analyses of *Alternaria* strains

When amplified using the primers *gpd1/gpd2*, all 72 Alternaria strains tested produced the anticipated PCR products of roughly 600 bp. Conversely, 66 strains produced the anticipated 500 bp product when amplified using the *Alt-for/Alt-rev* primer pair, but 6 out of 72 strains

were unable to amplify the fragment. Thus, 66 Alternaria strains were subjected to phylogenetic analysis using a multi-locus sequence technique. The *gpd* and *alt-a1* genes' matched sequences produced shared segments that were roughly 470 and 400 bases long, respectively. Reference sequences were carefully chosen based on BLAST search results from the NCBI database and then incorporated into the studies to determine the phylogenetic relationships between and within the isolated species. The final sequence alignment of the combined dataset comprised 23 sequences of Alternaria reference strains that were obtained from NCBI-BLAST and 66 sequences of Alternaria strains that were collected in this investigation. The concatenated sequences of the two segments were also subjected to a phylogenetic analysis in order to further determine the strains' identities. The *gpd* and *alt-a1* sequences were aligned at a total of 871 places in the combined dataset.

Based on reference strains, the generated phylogenetic tree revealed five different clades, designated A through E (Figure 16). High bootstrap values (>85%) supported these clades. Additionally, each clade was distinct from the others and matched taxonomy categories such as *Alternaria*, *Ulocladioides*, *Brassicicola*, *Eureka*, *Embellisia*, *Pseudoalternaria*, and *Infectoriae*. (Figure 16).

Four well-supported sub-clades comprise 44 of the 66 Alternaria strains (67%) that clustered in section *Alternaria*. In the sub-clade A1, twenty-eight strains were grouped with reference strain *A. alternata* ATCC 66891. Only the strains MD7, MD10, MD16, MD28, MD29, MD65, MD30, MD51, and MD64 were found to have low variability in this sub-clade. These strains exhibited notable similarities with one another but somewhat differed from the reference strain of *A. alternata*.

In subclade A2, three field strains were grouped with reference strain *A. arborescens* BMP 0308, while in subclade A3, two field strains were grouped with reference strain *A. angustiovoidea* strain EGS 36-172. The reference strains *A. gaisen* BMP 0243, *A. grisea* CBS 107.36, and A. betae-kenyensis CBS 118810 were all grouped together. Eleven field strains and the reference strains *A. citriarbusti* BMP 2343, *A. mali* BMP 3064, and *A. rhadina* CBS 595.93 were included in Sub-clade A4; nevertheless, it was not possible to distinguish between the various morpho-species. As a result, the isolates included in this cluster could not be recognized at the species level.

On the other hand, every strain that clustered inside the sections of *Ulocladioides*, *Eureka*, and *Embellisia* (clades B, C, and D, respectively) was well discriminated and supported by

bootstrap values. Specifically, the reference strain *A. consortialis* JCM 1940 was connected with two field strains, MD19 and MD8; *A. eureka* EGS 36-103 with field strain MD13; and *A. tellustris* BMP 0396 with two field strains, MD46 and MD34. There was no clustering between reference strains from the *Brassicicola* (*A. brassicicola* ATCC 96836) and *Pseudoalternaria* (*A. rosae* EGS 41-130) sections that were included in the analysis.

Out of 66 *Alternaria* field strains analyzed, 17 (approximately 26%) clustered within the *Infectoriae* section (clade E, Figure 16). Most isolates, however, exhibited considerable variability both among themselves and in comparison, with reference strains, forming two distinct sub-clades. Sub-clade E1 comprised eight field strains, four of which (MD39, MD50, MD38, and MD47) showed close affinity to the reference strain *A. ventricosa* EGS 52-075 (Figure 16). Sub-clade E2 included nine field strains together with six reference strains: *A. infectoria* EGS 27.193, *A. intercepta* EGS 49-137, *A. conjuncta* EGS 37-139, *A. ethzedia* EGS 37-143, *A. californica* EGS 52-082, and *A. metachromatica* bmp 0045. Within this group, strains MD42 and MD60 displayed high homology with *A. infectoria*, while strain MD70 was clearly identified as *A. metachromatica* (bootstrap value 87%). Overall, clade E was marked by pronounced variability and weak internal branch support, complicating the resolution of phylogenetic relationships between Algerian isolates and reference species.

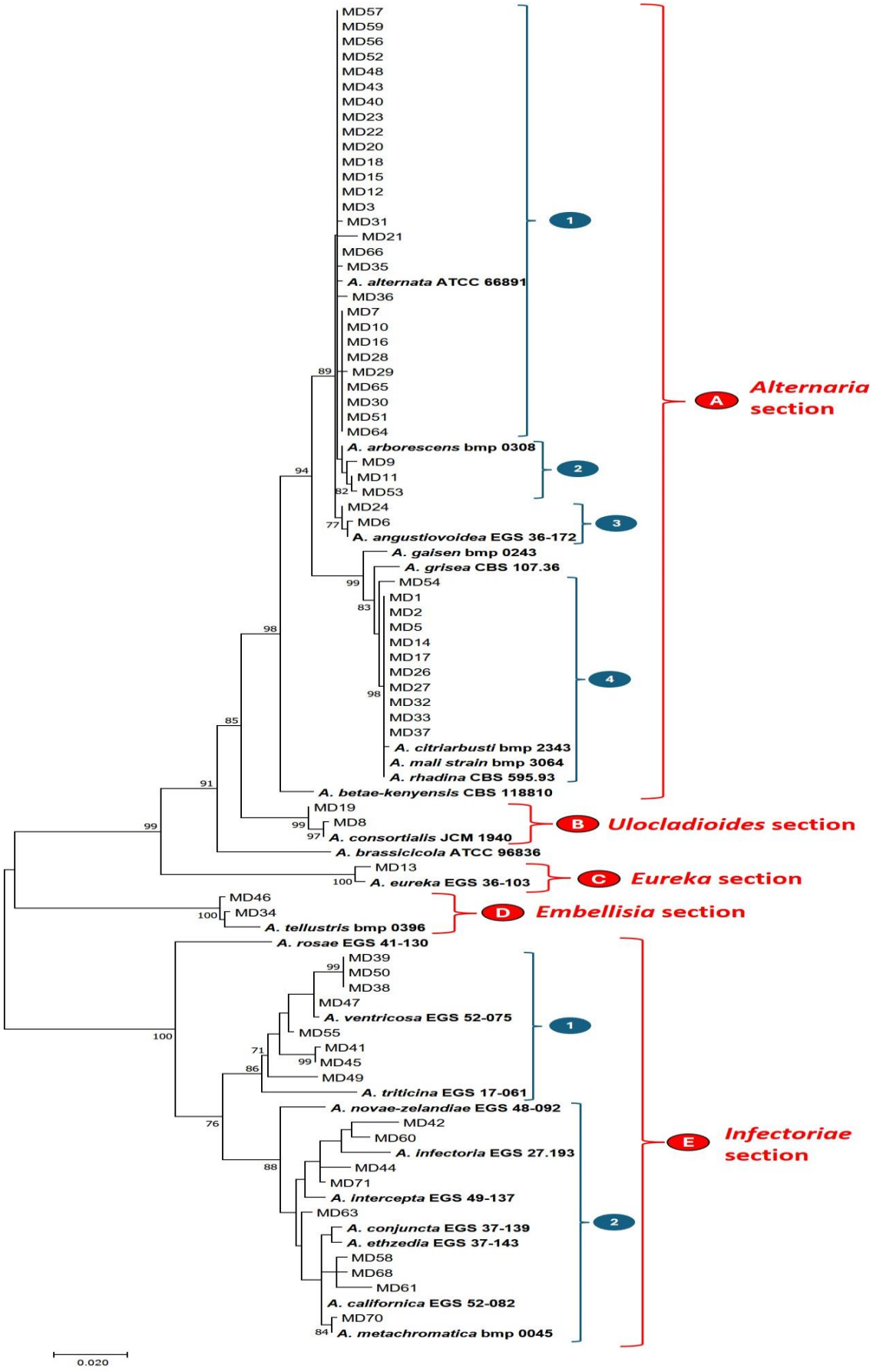
MD57
MD59
MD56
MD52
MD48
MD43
MD40
MD23
MD22
MD20
MD18
MD15
MD12
MD3
MD31
MD21
MD66
MD35
A. alternata ATCC 66891
MD36
MD7
MD10
MD16
MD28
MD29
MD65
MD30
MD51
MD64
A. arborescens bmp 0308
MD9
MD11
MD53
MD24
MD6
A. angustiovoidea EGS 36-172
A. gaisen bmp 0243
A. grisea CBS 107.36
MD54
MD1
MD2
MD5
MD14
MD17
MD26
MD27
MD32
MD33
MD37
A. citriarbusti bmp 2343
A. mali strain bmp 3064
A. rhadina CBS 595.93
A. betae-kenyensis CBS 118810
MD19
MD8
A. consortialis JCM 1940
A. brassicicola ATCC 96836
MD13
A. eureka EGS 36-103
MD46
MD34
A. tellustris bmp 0396
A. rosae EGS 41-130
MD39
MD50
MD38
MD47
A. ventricosa EGS 52-075
MD55
MD41
MD45
MD49
A. triticina EGS 17-061
A. novae-zelandiae EGS 48-092
MD42
MD60
A. infectoria EGS 27.193
MD44
MD71
A. intercepta EGS 49-137
MD63
A. conjuncta EGS 37-139
A. ethzedia EGS 37-143
MD58
MD68
MD61
A. californica EGS 52-082
MD70
A. metachromatica bmp 0045
0.020
A
Alternaria section
B
Ulocladioides section
C
Eureka section
D
Embellisia section
E
Infectoriae section
1
2
3
4

Figure 16: 66 Alternaria strains isolated from Algerian wheat were used to create a phylogenetic tree using the maximum likelihood approach (bootstrap 1000 repetitions) of the combined allergen *Alt a1* and *glyceraldeyde-3-phosphate dehydrogenase* genes. The branches are accompanied by bootstrap values greater than 70. The research comprised twenty-three Alternaria reference sequences. Additionally displayed are sub-clades and clades that indicate sections.

BLAST analysis was used to identify the six strains MD4, MD25, MD62, MD67, MD69, and MD72, that were left out of the phylogenetic analysis. Additionally, a single gpd gene was used to create a phylogenetic tree. Each of these strains was determined to be a member of the *Infectoriae* section. (Figure 6).

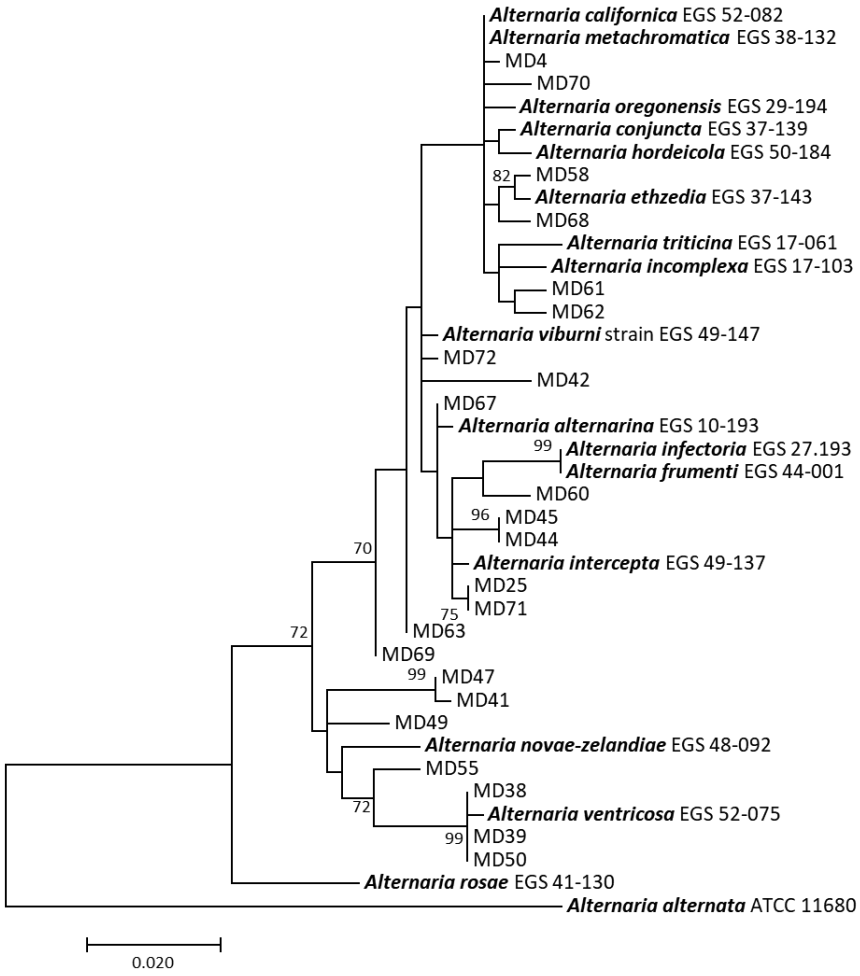

Figure 17: Glyceraldehyde-3-phosphate dehydrogenase gene sequences of 23 Alternaria Algerian isolates from the *Infectoriae* section, isolated from Algerian wheat, were used to create a phylogenetic tree using the maximum likelihood approach (bootstrap 1000 replicates). Bootstrap values greater than 70 are displayed alongside the branches. The research comprised

seventeen Alternaria reference sequences from type material. The reference strain of *Alternaria alternata* ATCC 11680 served as the outgroup.

1.4.Mycotoxin production of *Alternaria* strains

With the exception of strains from *Ulocladioides, Embellisia, and Infectoriae*, all examined strains isolated from various parts of Algeria were shown to contain at least one of the mycotoxins AME, AOH, Tea, ATX-I, TEN, and ALT (Table 3). AME and AOH production ranged from 2 to 1019 mg kg-1 for AME and 9 to 15950 mg kg-1 for AOH, depending on the strain. Furthermore, 81% of the isolates that produced AME or AOH also included these mycotoxins.

The majority of strains (70–100%) belonging to the A1 and A2 phylogenetic sub-clades produced high amounts of AME and AOH, as shown in Table 4. A1 subclade strains had mean values of 211 and 980 mg kg-1, whereas A2 subclade strains had mean values of 449 and 574 mg kg-1, respectively. Though at lesser concentrations 109 and 34 mg kg-1 for A3 and 272 and 203 mg kg-1 for A4, all strains of the A3 and A4 subclades produced AME and AOH. The strains from the other Alternaria sections did not produce any AME or AOH.

TeA production was probably observed at high levels for nearly all Alternaria section (clade A) strains, with values ranging from 16 mg kg-1 (MD10) to 13165 mg kg-1 (MD26). The strains belonging to the subclade A4 (*A. citriarbusti*/*A. mali*/*A. rhadina*) were found to produce the most TeA. With the exception of one strain (MD5), every strain in the A4 subclade showed a considerable capacity to generate TeA, with values ranging from 90 mg kg-1 to 13165 mg kg-1 (mean value of 6794 mg kg-1). Additionally, it was observed that strains of *A. alternata* (sub-clade A1) produced substantial levels of TeA, with mean values of 2915 mg kg-1 and values ranging from 16 to 9032 mg kg-1; only five of the 28 strains of *A. alternata* did not produce this toxin. TeA was produced by two of the three strains of *A. arborescens*, with values ranging from 413 to 670 mg kg-1 (mean value of 361 mg kg-1), whereas the two strains of *A. angustiovoidea* (sub-clade A3) produced between 507 and 1100 mg kg-1 (Table 4). Only one strain (MD70) of the Infectoriae division (clade E) generated TeA at a low level (291 mg kg-1).

Out of the 65 strains examined, only 25 have ATX-I, with levels ranging from 1 to 114 mg kg-1. *A. angustiovoidea* strains (sub-clade A4) had the highest mean values within the Alternaria section, however no production was seen in *A. arborescens*.

Only nine strains of Alternaria and *Infectoriae* sections were found to produce TEN, which ranged from 8 to 136 mg kg-1.

About 35% of the strains in the Alternaria section were found to produce ALT, with varying levels. On the other hand, a single strain of *A. eureka* (MD13) and a single producer strain of *Infectoriae* section (MD44) showed modest values (1 and 15 mg kg-1), respectively.

While no mycotoxin production was observed in strains from clade B, a few strains within clade E produced low levels of mycotoxins. The sole strain of *A. eureka* (clade C) produced very low amounts of ATX-I (5 mg kg-1) and ALT (1 mg kg-1). However, as previously reported, mycotoxin synthesis was only found in four strains within clade E, which represents the *Infectoriae* branch, albeit at low levels. Specifically, ATX-I (21 mg kg-1), TEN (135 mg kg-1), and ALT (15 mg kg-1) were generated by strains MD60, MD44, and MD63. Lastly, co-production of TeA, ATX-I, and TEN (291, 7, and 12 mg kg-1, respectively) was demonstrated by strain MD70, which was identified as *A. metachromatica*.

The ability to create alternariol monomethyl ether (AME), alternariol (AOH), tenuazonic acid (TeA), altertoxin I (ATX-I), tentoxin (TEN), and altenuene (ALT) mycotoxins is also described, along with the phylogenetic clade derived from *alt-a1* and *gpd* gene sequencing (Table 5).

Table 4: Alternaria strains isolated from wheat in Algeria produce alternariol (AOH), alternariol methyl ether (AME), tenuazonic acid (TeA), altertoxin I (ATX-I), tentoxin (TEN), and altenuene (ALT).

	Phylogenic clade		**AME**	**AOH**	**TeA**	**ATX-I**	**TEN**	**ALT**
***Alternaria* section**	Sub-clade A1 (27 strains)	**Producer strains (%)**	67	70	85	41	15	55
		Mean (mg kg-1)	211	980	2915	27	63	85
		Range (mg-kg-1)	18-564	9-15949	16-9032	1-87	8-136	1-395

	Sub-clade A2 (3 strains)	**Producer strains (%)**	100	67	67	0	0	100
		Mean (mg kg-1)	449	574	542	0	0	344
		Range (mg-kg-1)	192-878	423-724	413-670	0	0	212-423
	Sub-clade A3 (2 strains)	**Producer strains (%)**	100	100	100	100	50	100
		Mean (mg kg-1)	109	34	804	111	10	206
		Range (mg-kg-1)	17-200	21-48	507-1100	109-114	10	99-313
	Sub-clade A4 (11 strains)	**Producer strains (%)**	100	100	91	73	18	36
		Mean (mg kg-1)	272	203	6794	34	19	71
		Range (mg-kg-1)	2-1019	20-508	90-13165	5-76	8-30	7-192
***Eureka* section**	Clade C (1 strain)	**Producer strains (%)**	0	0	0	100	0	100
		Mean (mg kg-1)	0	0	0	5	0	1
		Range (mg-kg-1)	0	0	0	5	0	1

***Infectoriae* section**	Clade E (17 strains)	**Producer strains (%)**	0	0	6	18	12	6
		Mean (mg kg-1)	0	0	291	11	74	15
		Range (mg-kg-1)	0	0	291	4-21	12-135	15

The percentage of producer strains, along with the relative mean values and production range, are presented based on phylogenetic clades and expressed in mg kg-1.

5. Content of *Alternaria* mycotoxins in grain samples

Five Alternaria mycotoxins (AME, AOH, TeA, ATX-I, and TEN) were detected in the analyzed grain samples, whereas ALT was not detected in any of the durum wheat grain samples that were examined (Table 5).

Generally, AME and AOH were detected in 75% and 69% of grain samples, respectively. Mean mycotoxin concentrations were calculated based on all analyzed samples (n = 48), with non-detected samples implicitly considered as zero values. Their concentrations ranged from 22 to 980 µg kg^{-1} and 134 to 425 µg kg^{-1}, respectively. TeA was detected in 35% of the grain samples, with concentrations ranging from 381 to 705 µg kg^{-1}, while ATX-I was found in only four samples (8%), with concentrations up to 1758 µg kg^{-1}. TEN was detected in 21% of the grain samples, with a mean concentration of 38 µg kg^{-1} (Table 5).

All of the Algerian regions under investigation were found to contain a high level of both AME and AOH. The Khenchela region had the highest mean amount of AME (415 µg kg-1) (Table 6), whereas M'Sila had the highest mean content of AOH (203 µg kg-1). ATX-I and TEN toxins were only found in Sètif and Biskra regions, with mean values of 71 and 57 µg kg-1, respectively, in Sètif and between 8 and 31 µg kg-1, respectively, in Biskra. The presence of TeA was reported in four regions, with values of 489, 210, 261, and 120 µg kg-1 in Bejaia, Sètif, Batna, and Biskra, respectively (Table 6).

Table 5: Alternaria strains isolated from wheat in Algeria produce alternariol (AOH), alternariol methyl ether (AME), tenuazonic acid (TeA), altertoxin I (ATX-I), tentoxin (TEN), and altenuene (ALT).

Strain code	Wheat sample	Wheat variety	Region	Section	Phylogenetic clade	Produced mycotoxins
MD56	V30	Boussalem	Béjaia	*Alternaria*	A1	AME, ALT
MD26	V30	Boussalem	Béjaia	*Alternaria*	A4	AME, AOH, TeA, ATX-I
MD43	V42	Boussalem	Sétif	*Alternaria*	A1	AME, AOH, TeA
MD3	V36	Boussalem	Sétif	*Alternaria*	A1	AME, AOH, TeA, ATX-I, ALT
MD10	V27	Boussalem	Sétif	*Alternaria*	A1	AME, AOH, TeA, ATX-I, TEN, ALT
MD9	V21	Boussalem	Sétif	*Alternaria*	A2	AME, TeA, ALT
MD37	V12	Boussalem	Sétif	*Alternaria*	A4	AME, AOH, TeA
MD32	V12	Boussalem	Sétif	*Alternaria*	A4	AME, AOH, TeA, ALT
MD33	V14	Boussalem	Sétif	*Alternaria*	A4	AME, AOH, TeA, ALT
MD14	V25	Boussalem	Sétif	*Alternaria*	A4	AME, AOH, TeA, ATX-I, ALT
MD44	V26	Boussalem	Sétif	*Infectoriae*	E	ALT
MD63	V10	Boussalem	Sétif	*Infectoriae*	E	ATX-I, TEN
MD45	V12	Boussalem	Sétif	*Infectoriae*	E	n.d.
MD50	V23	Boussalem	Sétif	*Infectoriae*	E	n.d.
MD58	V12	Boussalem	Sétif	*Infectoriae*	E	n.d.
MD68	V26	Boussalem	Sétif	*Infectoriae*	E	n.d.
MD71	V14	Boussalem	Sétif	*Infectoriae*	E	n.d.
MD25	V27	Boussalem	Sétif	*Infectoriae*	-	n.d.

MD62	V12	Boussalem	Sétif	*Infectoriae*	-	-
MD70	V12	Boussalem	Sétif	*Infectoriae*	E	TeA, ATX-I, TEN
MD36	V9	Gta Dur	Sétif	*Alternaria*	A1	TeA
MD46	V9	Gta Dur	Sétif	*Embellisia*	D	n.d.
MD69	V9	Gta Dur	Sétif	*Infectoriae*	-	ALT
MD72	V9	Gta Dur	Sétif	*Infectoriae*	-	n.d.
MD12	V46	Oued El Bared	Sétif	*Alternaria*	A1	ALT
MD57	V31	Oued El Bared	Sétif	*Alternaria*	A1	AME, AOH, TeA, ALT
MD16	V17	Oued El Bared	Sétif	*Alternaria*	A1	AME, AOH, TeA, ATX-I
MD35	V47	Oued El Bared	Sétif	*Alternaria*	A1	AME, AOH, TeA, ATX-I, ALT
MD7	V39	Oued El Bared	Sétif	*Alternaria*	A1	AME, AOH, TeA, ATX-I, ALT
MD29	V48	Oued El Bared	Sétif	*Alternaria*	A1	AOH, TeA, ALT
MD22	V28	Oued El Bared	Sétif	*Alternaria*	A1	AOH, TeA, ATX-I
MD24	V22	Oued El Bared	Sétif	*Alternaria*	A3	AME, AOH, TeA, ATX-I, ALT
MD6	V17	Oued El Bared	Sétif	*Alternaria*	A3	AME, AOH, TeA, ATX-I, TEN, ALT
MD17	V46	Oued El Bared	Sétif	*Alternaria*	A4	AME, AOH, TeA, ATX-I
MD2	V46	Oued El Bared	Sétif	*Alternaria*	A4	AME, AOH, TeA, ATX-I, TEN
MD19	V20	Oued El Bared	Sétif	*Ulocladioides*	B	n.d.
MD13	V39	Oued El Bared	Sétif	*Eureka*	C	ATX-I, ALT

MD34	V43	Oued El Bared	Sétif	*Embellisia*	D	n.d.
MD42	V22	Oued El Bared	Sétif	*Infectoriae*	E	n.d.
MD49	V47	Oued El Bared	Sétif	*Infectoriae*	E	n.d.
MD4	V16	Oued El Bared	Sétif	*Infectoriae*	-	n.d.
MD67	V18	Oued El Bared	Sétif	*Infectoriae*	-	n.d.
MD31	V29	Oued El Bared	Batna	*Alternaria*	A1	AME, AOH, TeA, ALT
MD5	V24	Simeto	Batna	*Alternaria*	A4	AME, AOH, ATX-I, ALT
MD60	V34	Simeto	Batna	*Infectoriae*	E	ATX-I
MD61	V33	Simeto	Batna	*Infectoriae*	E	n.d.
MD23	V38	Oued El Bared	Biskra	*Alternaria*	A1	AME, AOH, TeA, TEN, ALT
MD21	V38	Oued El Bared	Biskra	*Alternaria*	A1	TeA, ATX-I
MD48	V3	Vitron	M'Sila	*Alternaria*	A1	AME, AOH, TeA
MD66	V7	Vitron	M'Sila	*Alternaria*	A1	AME, AOH, TeA, ALT
MD20	V1	Vitron	M'Sila	*Alternaria*	A1	AME, AOH, TeA, ATX-I
MD64	V11	Vitron	M'Sila	*Alternaria*	A1	AME, ATX-I
MD40	V7	Vitron	M'Sila	*Alternaria*	A1	AME, TeA
MD15	V7	Vitron	M'Sila	*Alternaria*	A1	TeA
MD11	V2	Vitron	M'Sila	*Alternaria*	A2	AME, AOH, ALT
MD54	V7	Vitron	M'Sila	*Alternaria*	A4	AME, AOH, TeA, ATX-I
MD1	V1	Vitron	M'Sila	*Alternaria*	A4	AME, AOH, TeA, ATX-I, TEN

MD38	V3	Vitron	M'Sila	*Infectoriae*	E	n.d.
MD39	V3	Vitron	M'Sila	*Infectoriae*	E	n.d.
MD47	V7	Vitron	M'Sila	*Infectoriae*	E	n.d.
MD18	V44	Vitron	Khenchela	*Alternaria*	A1	AME, AOH, TeA, ATX-I, ALT
MD52	V44	Vitron	Khenchela	*Alternaria*	A1	AME, AOH, TeA
MD28	V44	Vitron	Khenchela	*Alternaria*	A1	ALT
MD27	V44	Vitron	Khenchela	*Alternaria*	A4	AME, AOH, TeA, ATX-I
MD55	V41	Vitron	Khenchela	*Infectoriae*	E	n.d.
MD59	V40	Oued El Bared	Biskra	*Alternaria*	A1	AOH, TeA, TEN, ALT
MD65	V6	Vitron	Biskra	*Alternaria*	A1	-
MD30	V37	Vitron	Biskra	*Alternaria*	A1	AME, AOH, TeA, ATX-I, TEN, ALT
MD51	V5	Vitron	Biskra	*Alternaria*	A1	AOH, TeA
MD53	V6	Vitron	Biskra	*Alternaria*	A2	AME, AOH, TeA, ALT
MD8	V5	Vitron	Biskra	*Ulocladioides*	B	n.d.
MD41	V5	Vitron	Biskra	*Infectoriae*	E	n.d.

Table 4: Mycotoxin occurrence in 48 durum wheat samples from Algeria

Mycotoxin	**Contaminated samples (%)**	**Mycotoxin content (µg kg-1)**	
		Mean value	**Range**
AME	75	314	22-980
AOH	69	44	134-425
TeA	35	177	381-705
ATX-I	8	43	58-1758
TEN	21	38	52-321

Mean concentrations were calculated by dividing the total detected concentration of each mycotoxin by the total number of analyzed samples (n = 48); thus, non-detected samples were implicitly treated as zero.

Table 5: Mycotoxin content (mg kg-1) found in durum wheat samples gathered from six Algerian locations.

Each Algerian region's mean values, range of mycotoxin occurrence computed on positive samples, and percentage of contaminated samples on all samples analyzed are displayed.

Region	**N. of samples**		**AME**	**AOH**	**TeA**	**ATX-I**	**TEN**
Bejaia	1	**Contaminated samples (%)**	0	100	100	0	0
		Mean value (µg kg-1)	0	35	489	0	0
		Range (µg kg-1)	0	35	489	0	0
Sètif	28	**Contaminated samples (%)**	82	93	39	11	32
		Mean value (µg kg-1)	348	18	210	71	57
		Range (µg kg-1)	50-820	25-45	381-705	82-1758	52-321
Batna	5	**Contaminated samples (%)**	40	40	60	0	0
		Mean value (µg kg-1)	370	13	261	0	0
		Range (µg kg-1)	870-980	25-40	407-471	0	0

M'Sila	5	**Contaminated samples (%)**	60	60	0	0	0
		Mean value (µg kg-1)	224	203	0	0	0
		Range (µg kg-1)	205-365	275-425	0	0	0
Khenchela	2	**Contaminated samples (%)**	100	100	0	0	0
		Mean value (µg kg-1)	415	40	0	0	0
		Range (µg kg-1)	370-460	40	0	0	0
Biskra	7	**Contaminated samples (%)**	71	86	29	14	14
		Mean value (µg kg-1)	218	15	120	8	31
		Range (µg kg-1)	180-380	25	394-444	58	216

AME=alternariol monomethyl ether

AOH=alternariol

TeA=tenuazonic acid

ATX-I=altertoxin -I

TEN=tentoxin.

Chapter 2:

Effect of barrier discharge plasma treatment on four Alternaria species

2.1.Inhibition of mycelium growth

After five days of incubation, the inhibitory effect of DBD on Alternaria species (MD 6, MD 40, MD 09, and MD14) was assessed under different exposure durations (1, 2.5, 5, and 5 min) in comparison to untreated controls.

The findings showed that the mycelial growth of *A.angustiovoidea* (MD 6) was considerably suppressed by the plasma treatment in a time- and dose-dependent manner (two-way ANOVA, $p < 0.001$), with a significant interaction between treatment duration and incubation day ($p = 0.012$). On the other hand, inhibition was insignificant at 1-2.5 minutes; nevertheless, growth was consistently reduced by more than 40% after 5 minutes of exposure (Figure 18), creating a distinct statistical group in Tukey's post hoc analysis ($p < 0.05$; Figure 20). These results suggest that *A.angustiovoidea* is comparatively resistant to plasma, necessitating extended treatment to produce significant inhibition.

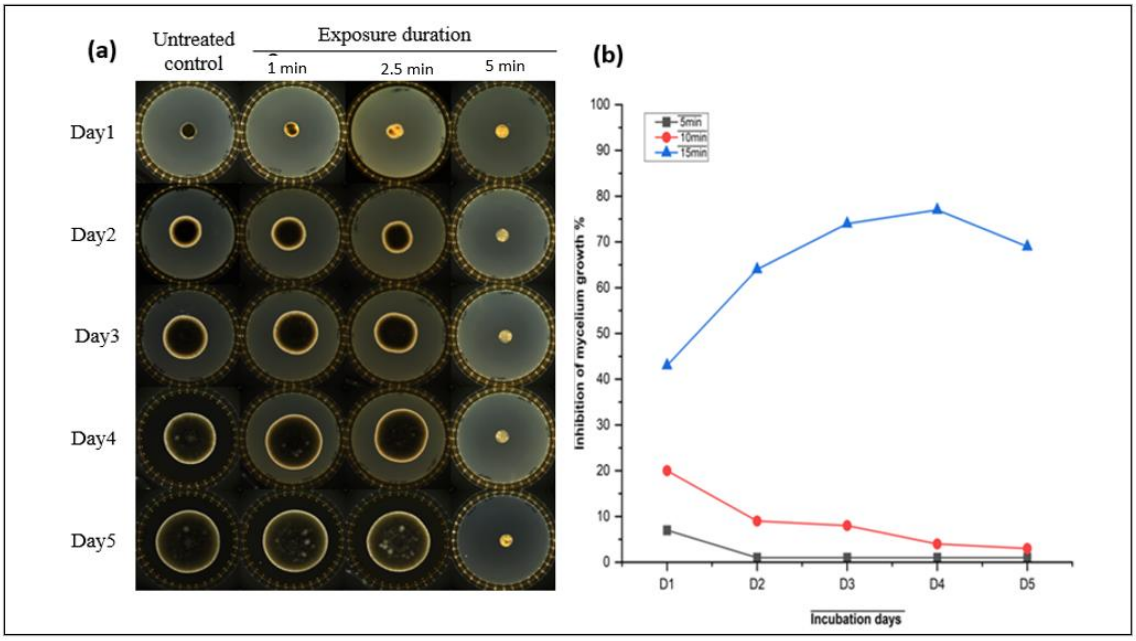

Figure 18: Mycelial growth inhibition of *A.angustiovoidea* (MD6) at 5 days incubation through DBD plasma treatment after exposure for 1, 2,5, or 5 min. (a) Mycelial growth of *A.angustiovoidea* on PDA. (b) Mycelial growth inhibition percentages, the presented values are means (n=3).

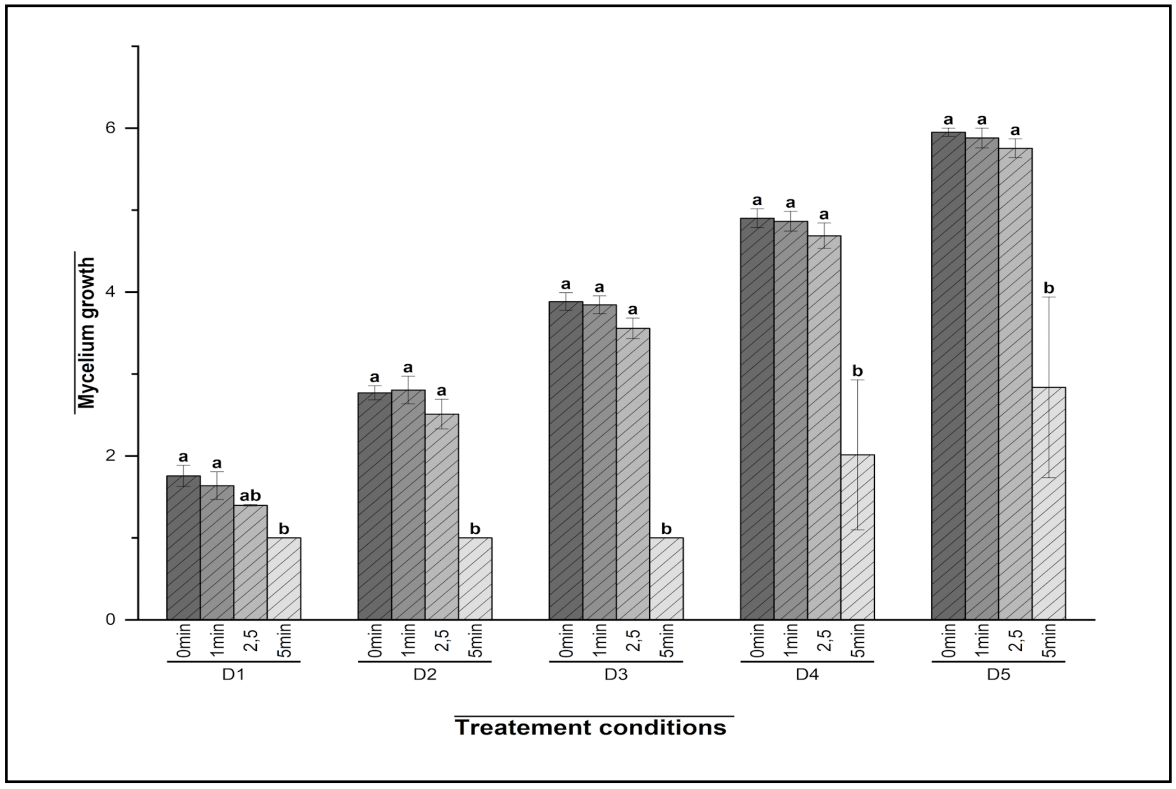

Figure 19: Mean mycelial diameter (± SD) of *A.angustiovoidea* strain (MD6) after different DBD plasma treatment durations. Bars that do not share the same letter are significantly different (Tukey's test, $p < 0.05$).

The *Alternaria* species complex MD14 (*Alternaria citriarbusti*, *A. mali*, and *A. rhadina*) exhibited strong and time-dependent sensitivity to plasma exposure (two-way ANOVA, $p < 0.001$). Both treatment duration and incubation day had significant effects, with a strong interaction ($p < 0.001$). Short exposures (1 min) had little effect, whereas 2.5 min showed intermediate inhibition. Complete suppression was achieved at 5 min (84%) (Figure 20), which clustered separately in Tukey's test ($p < 0.05$; Figure 21). These results demonstrate that MD14 is highly susceptible to plasma treatment, with even moderate exposures producing marked inhibition.

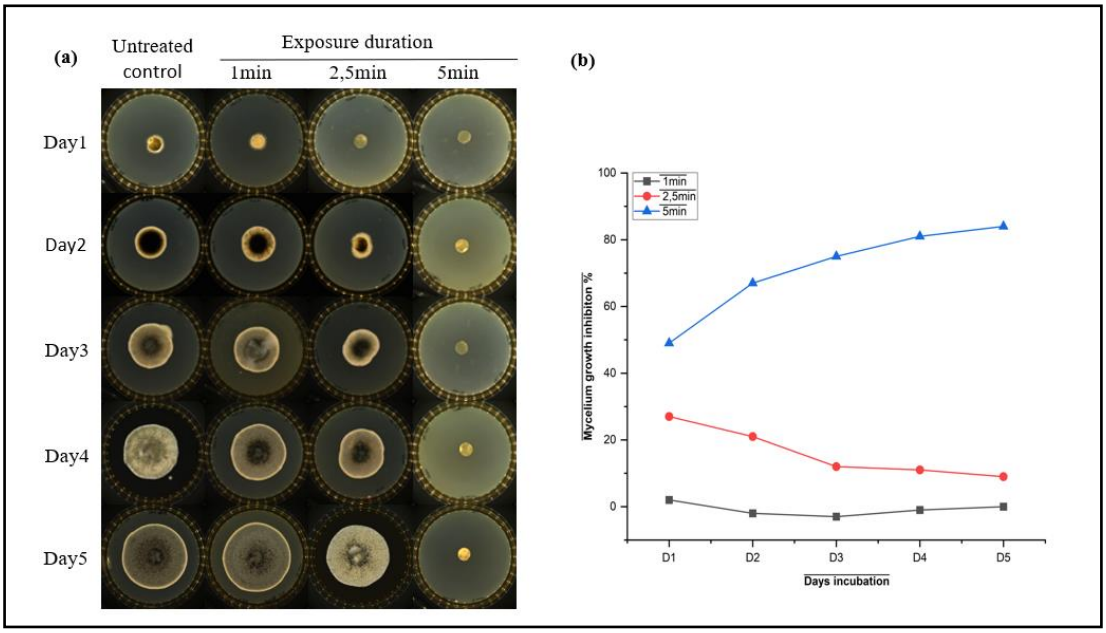

Figure 20: The inhibition of mycelial growth of the complex *Alternaria* species (MD 14) at 5 days of incubation through DBD plasma treatment after exposure for 1, 2,5 or 5 min.(a) Mycelial growth of the complex *Alternaria* species on PDA. (b) The percent inhibition of mycelial growth of the complex *Alternaria* specie.

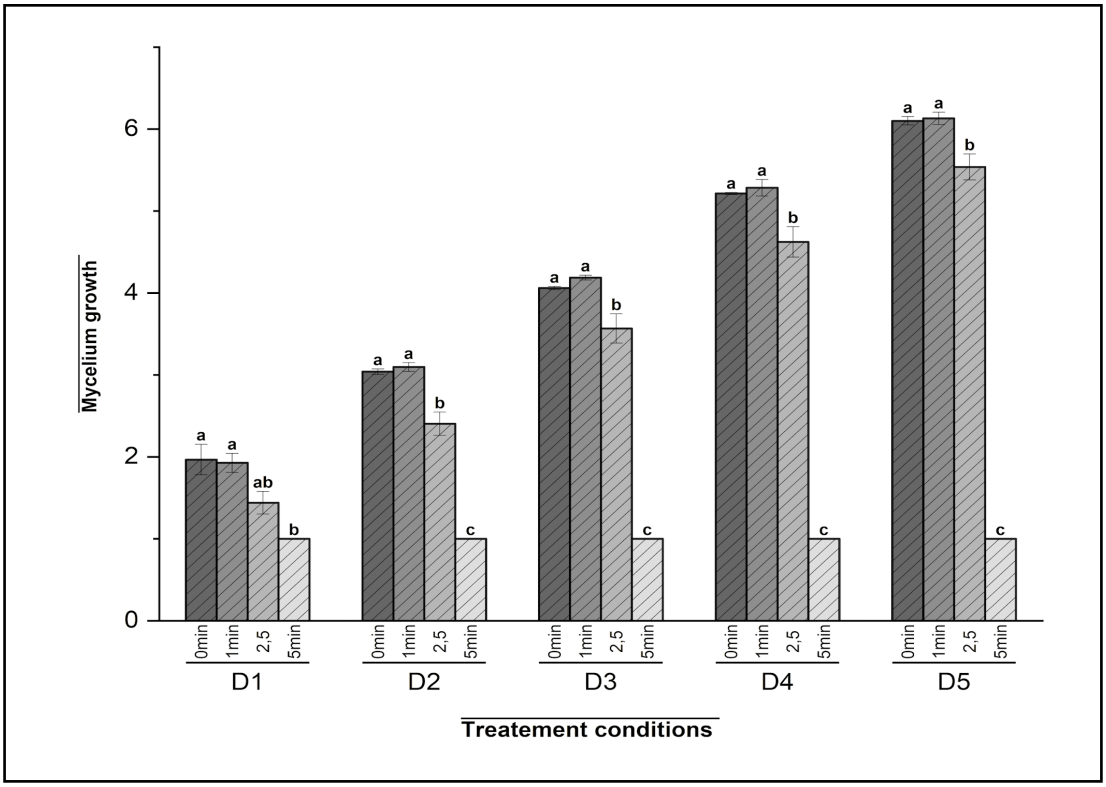

Figure 21: Mean mycelial diameter (± SD) of the complex *Alternaria* species (MD 14) after different DBD plasma treatment durations. Treatments not sharing the same letter are significantly different (Duncan's test, $p < 0.05$)

Mycelial growth of *A. alternata* (MD40) was significantly inhibited by plasma in a time-dependent manner (two-way ANOVA, $p < 0.001$), with a strong interaction between exposure duration and incubation ($p < 0.001$). Minimal inhibition was observed at 1 minute of treatment, whereas 2.5 minutes produced moderate suppression. The 5-minute treatment resulted in the strongest inhibition (81%) (Figure 22), forming a distinct statistical group ($p < 0.05$; Figure 23). This indicates that *A. alternata* is among the most sensitive species tested, showing robust suppression even at moderate exposure duration.

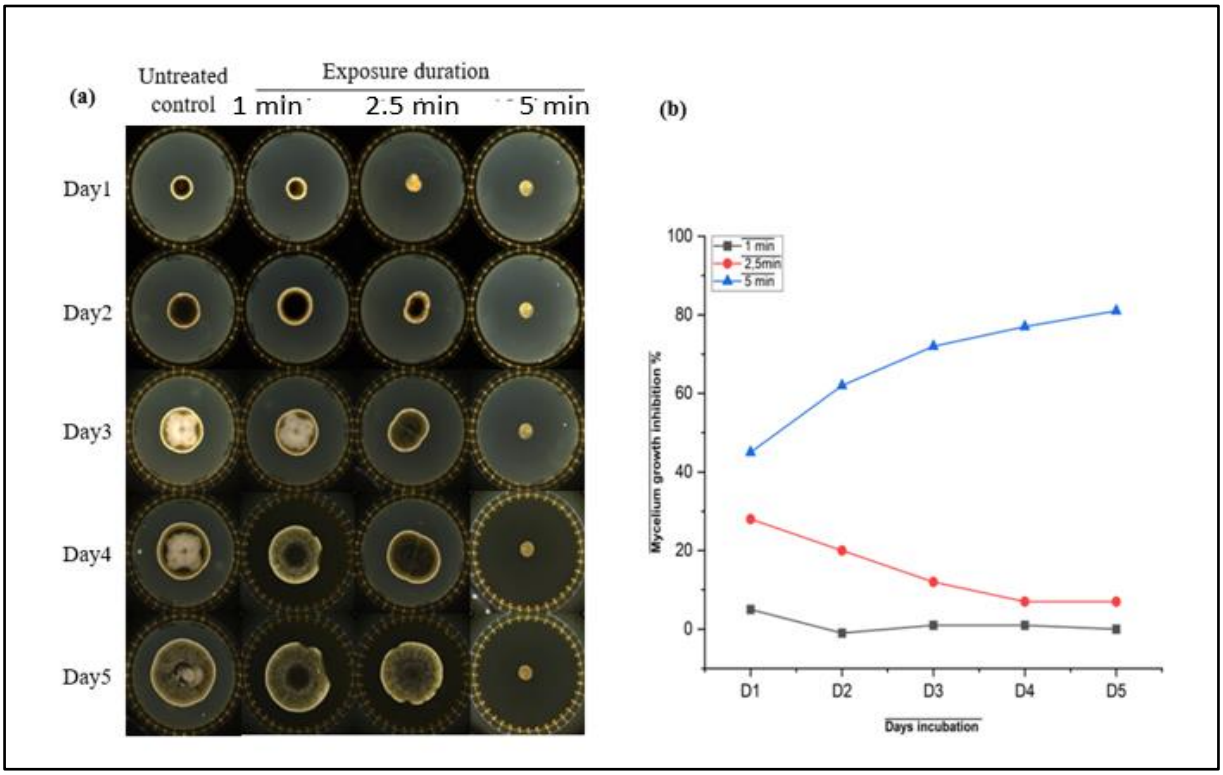

Figure 22: The inhibition of mycelial growth of *Alternaria alternata* (MD 40) at 5 days incubation through DBD plasma treatment after exposure for 1, 2,5 or 5 min (a) Mycelial growth of *A. alternata* on PDA. (b) The percent inhibition of mycelial growth of *A.alternata*.

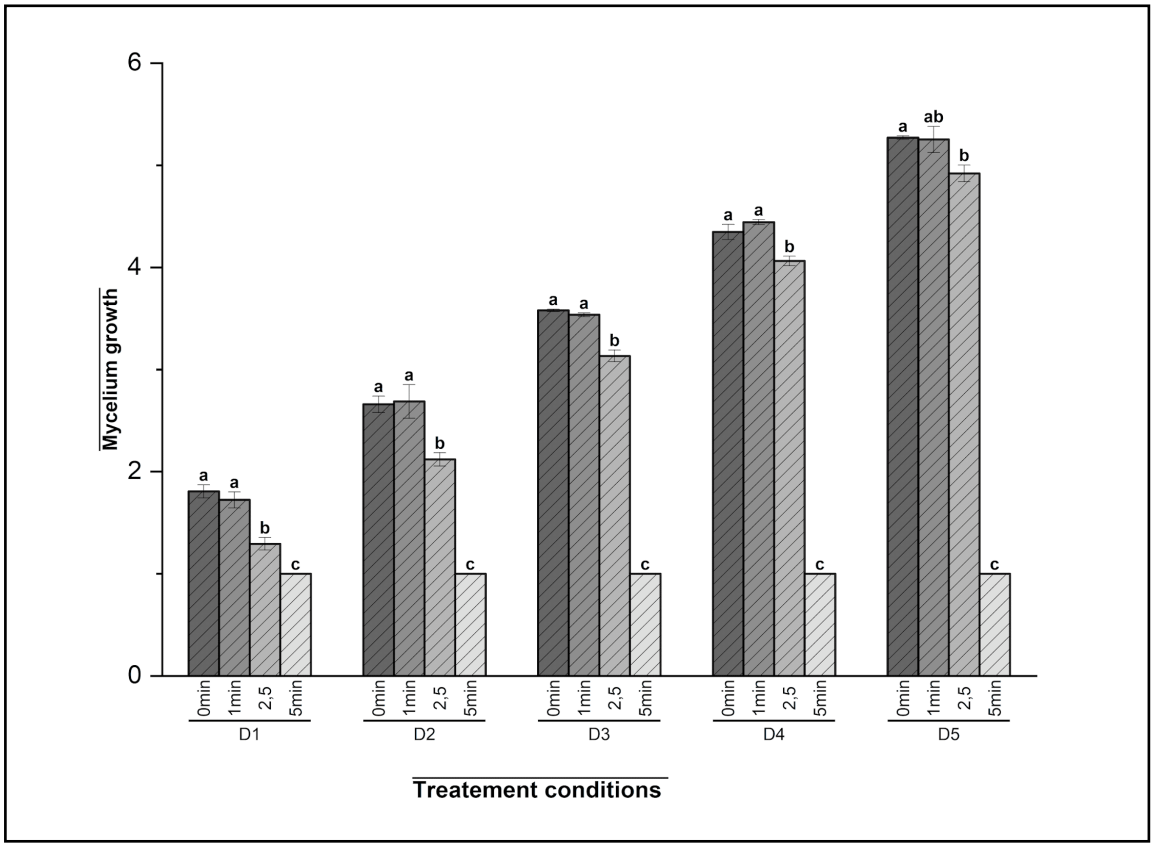

Figure 23: Mean mycelial diameter (± SD) of *Alternaria alternata* strain (MD 40) after different DBD plasma treatment durations. Treatments not sharing the same letter are significantly different (Tukey's test, $p < 0.05$)

Plasma treatment significantly inhibited the growth of *A. arborescens* (MD9) (two-way ANOVA, $p < 0.001$), with treatment duration and incubation day as main effects, but without significant interaction ($p = 0.108$). Inhibition was negligible at 1–2.5 min, whereas the 5 min treatment consistently suppressed growth by >90% across all incubation days (Figure 24). Tukey's test confirmed that 5 min formed an independent group ($p < 0.05$; Figure 25). These results indicate that *A. arborescens* is highly sensitive to plasma, with strong inhibition achieved only under the longest exposure.

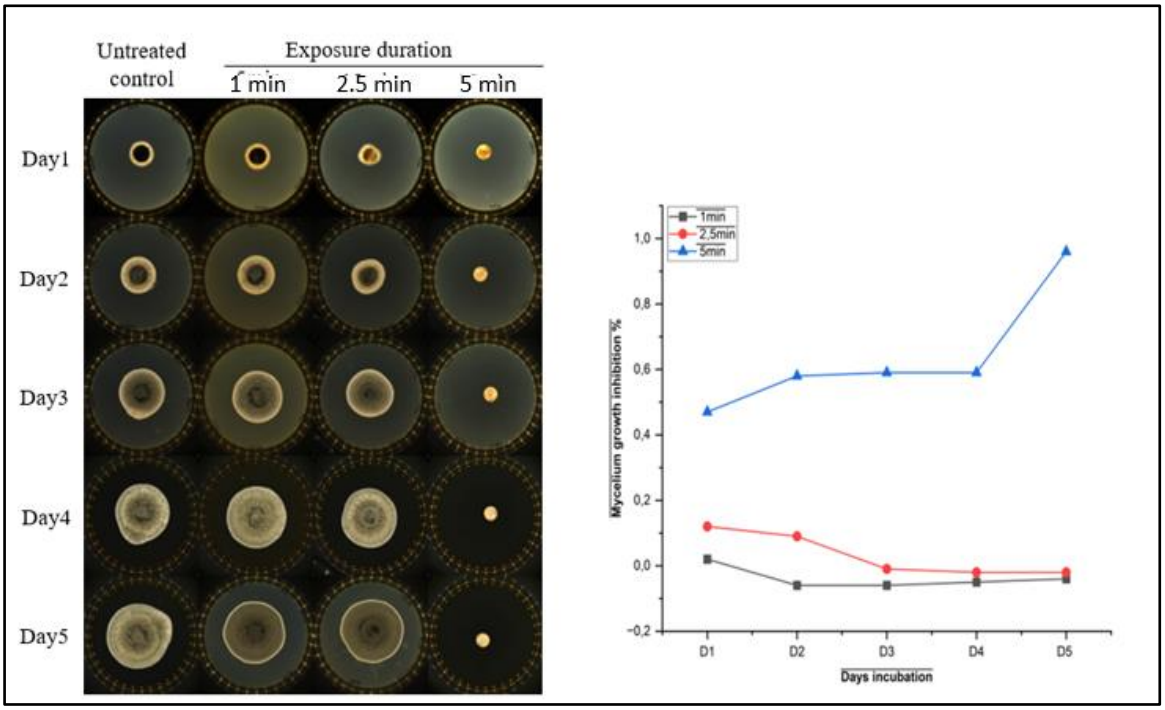

Figure 24: The inhibition of mycelial growth of *Alternaria arboresens* (MD 9) at 5 days incubation through DBD plasma treatment after exposure for 1 ,2,5 or 5 min. (a) Mycelial growth of *A. arborescens* on PDA. (b) The percent inhibition of mycelial growth of *A. arborescens.*

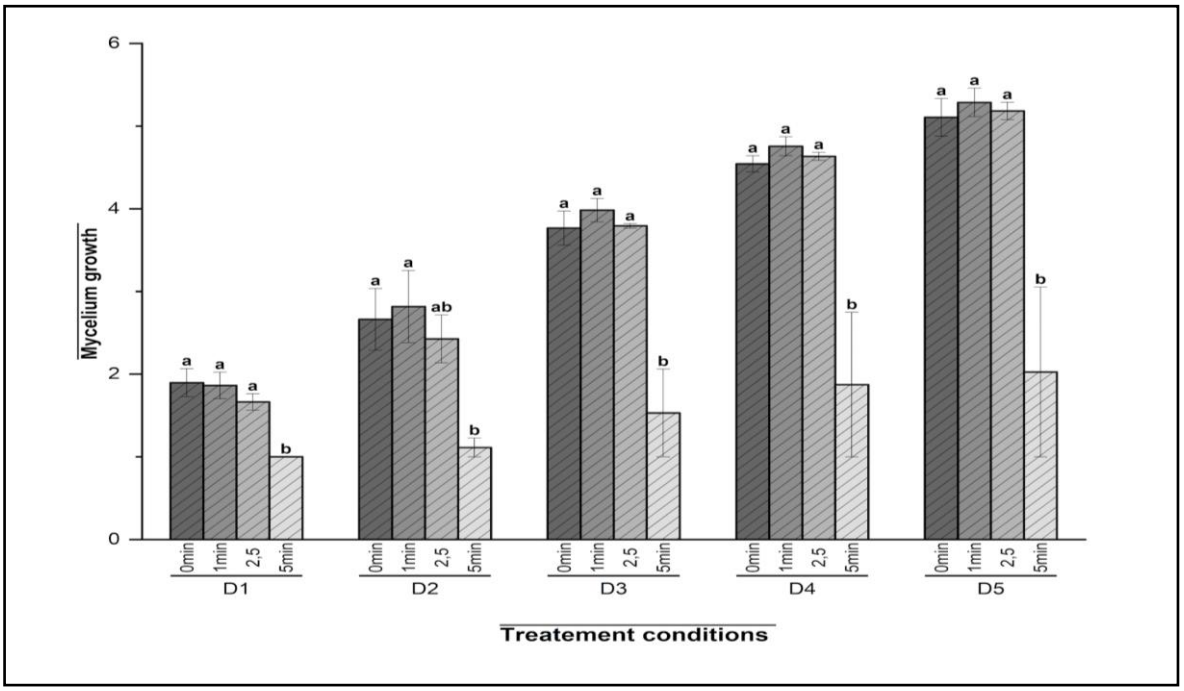

Figure 25: Mean mycelial diameter (± SD) of *Alternaria arborescens* strain (MD9) after different DBD plasma treatment durations. Treatments not sharing the same letter are significantly different (Duncan's test, $p < 0.05$).

2.2.Inhibition of spore germination

The results revealed that the treatments with plasma significantly inhibited conidia germination of the four species of *Alternaria*. Therefore, DBD exposure significantly reduced spore germination in *Alternaria alternata* (*MD 40)* in a time-dependent manner. After six hours of incubation, plasma exposure for 5 min resulted in about 77% inhibition of germination, while longer exposures of 10 and 15 min further enhanced inhibition to 80% and 90 % compared to the untreated spores (0min), respectively. A similar trend was maintained after 18 h, with inhibition remaining high (63–90%), demonstrating that the plasma treatment produced a sustained antifungal effect over time (Table 8). Statistical analysis using a two-way ANOVA indicated that the duration of plasma exposure had a highly significant impact on spore germination ($p < 0.001$), although no significant interaction was found with the incubation time. Post-hoc Tukey's test revealed that all plasma exposure durations (5, 10, and 15 minutes) formed a distinct group with substantial inhibition, clearly differentiated from the untreated control group, which maintained high germination rates (Figure 26a). These findings highlight the pronounced susceptibility of MD 40 spores to DBD plasma treatment, with increasing exposure times leading to enhanced inhibitory effects, reaching near-maximal reduction after 15 minutes.

In contrast, MD 14 displayed an even stronger sensitivity to plasma treatment. The ANOVA test confirmed a significant effect of treatment duration ($p < 0.001$), and although no significant differences were detected among the 5-, 10-, and 15-min treatments, all were grouped separately from the control (Figure 26b). Indicating a strong inhibitory effect that reached a plateau. Consistent with this, the percentage of inhibition increased from 74% after 6 h to 95% after 15 min exposure, and remained high (82–85%) even after 18 h of incubation (Table 8). These results suggest that MD 14 is highly sensitive to plasma treatment; even minimal exposure (5 min) substantially suppressed germination, and longer treatments maintained strong inhibition without further significant differences.

In the case of MD 6, plasma exposure demonstrated a statistically significant inhibition of spore germination, with the effect being dependent on the duration of exposure ($p = 0.012$) and incubation time ($p = 0.007$), although there was no significant interaction

between these factors (p = 0.531). Post-hoc analysis using Tukey's test indicated that all plasma-treated groups exhibited significantly lower germination rates compared to the untreated control, but were not significantly different from each other, suggesting that even brief DBD exposure was highly effective in reducing germination (Figure 26c). Specifically, after 6 hours of incubation, germination inhibition rates were recorded at 74%, 82%, and 95% for exposure times of 5, 10, and 15 minutes, respectively. A similar inhibitory trend was observed after 18 hours of incubation, with inhibition levels of 75%, 82%, and 85%, indicating the sustained efficacy of the plasma treatment over time (Table 8).

Concerning MD 9, two-way ANOVA indicated no significant effect of plasma exposure time on spore germination. inhibition (p = 0.112), while incubation time exerted a modest influence (p = 0.042). No interaction between the two factors was observed. Consistent with these findings, Tukey's test revealed no clear subgroup separation (Figure 26d). The inhibition percentages showed moderate activity after 6 h (54–69%), which decreased at shorter exposures after 18 h (32–52%) but increased at 15 min (82%) (Table 8).

Table 6: Percentage inhibition of spore germination (mean ± SD) of four Alternaria species following dielectric barrier discharge (DBD) plasma treatment after 6 h and 18 h of incubation

Treatment duration	**MD40 (*A. alternata*)**		**MD14 (*A. mali/A. rhadina/A. citriarbusti*)**		**MD6(*A.gustiovoidea*)**		**MD9 (*A. arborescens*)**	
	6 h	**18h**	**6 h**	**18h**	**6 h**	**18h**	**6 h**	**18h**
5 min	77± 2%	63±2%	74±2%	75±2%	50±1%	35±2%	54±1%	32±5%
10 min	80± 4%	85±3%	82 ±1%	82 ±4 %	-21±1%	-9±4%	69±1%	52±2%
15 min	90± 1%	90±0%	95±1%	85±3%	65±1%	65±1%	65±1 %	82±1%

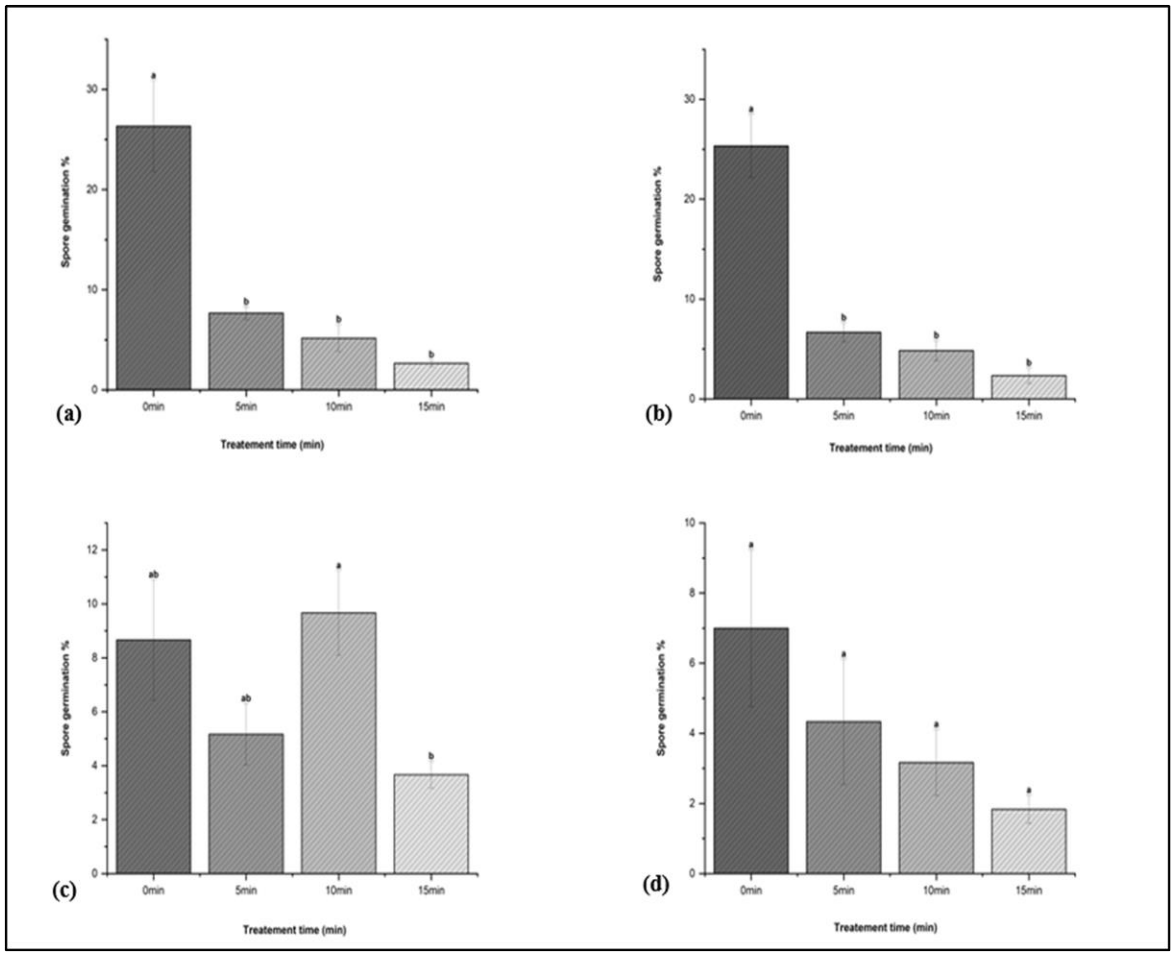

Figure 26: Effect of DBD plasma treatment on spore germination of *Alternaria* species. Spore germination (%) of the four species (a) MD40 (b) MD14 (c)MD6 (d)MD9 following different plasma exposure times (0, 5, 10, and 15 min). Bars represent mean values ± SE (n = 6). Different letters above bars indicate significant differences among treatments according to Tukey's HSD test ($p < 0.05$). Note that Y-axis scales differ among species to optimize visualization of within-species effects.

On the other hand, microscopic observations further corroborated the inhibitory effect of plasma on spore germination. Untreated spores (0 min) germinated normally with intact morphology, whereas spores exposed to plasma for 15 min showed almost complete suppression of germination after 18 h. In addition, pronounced morphological alterations were evident in treated spores, including collapsed walls and leakage of cytoplasmic contents (Figures 27 and 28).

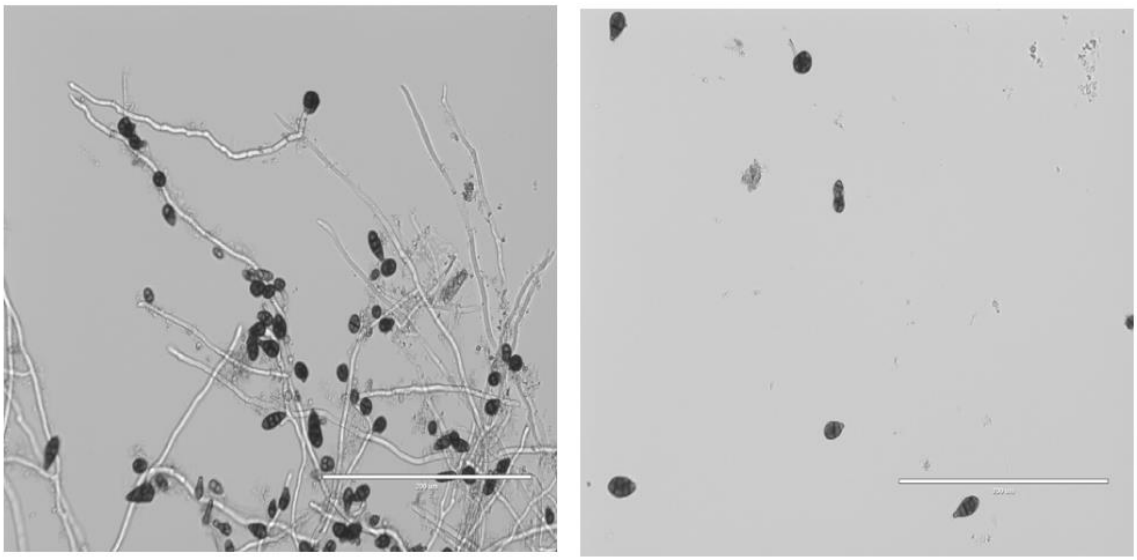

Figure 27: Side-by-side microscopic comparison of *A. alternata* spores: untreated spores (0 min) and treated spores with DBD for 15 min, after 18 h incubation.

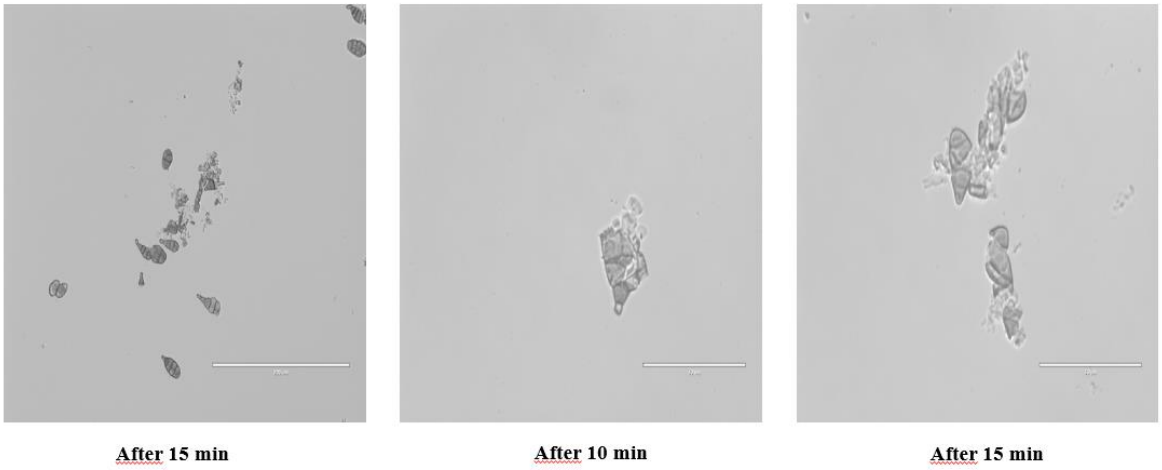

Figure 28: Pronounced morphological alterations in *A. alternata* spores following DBD plasma treatment, showing collapsed spore walls and cytoplasmic leakage

Principal component analysis (PCA) was conducted to summarize the overall response patterns of the four *Alternaria* species to DBD plasma exposure (Figure 29) . The first two principal components explained 86.8% of the total variance (PC1 = 62.5%, PC2 = 24.3%), indicating that most of the variability in spore germination inhibition can be effectively described by these two axes. Along PC1, species with higher susceptibility to plasma treatment (MD40 and MD14) were clearly separated from the less responsive species (MD6 and MD9). This separation reflects the ANOVA and Tukey's post-hoc results, where

MD40 and MD14 showed strong and statistically significant reductions in spore germination across exposure times, while MD6 and MD9 exhibited weaker or nonsignificant responses. PC2 mainly captured additional variation related to incubation time, with 18 h samples generally distributed further along this axis than 6 h samples, suggesting a time-dependent modulation of plasma effects. The clear species clustering in the PCA score plot therefore provides an integrative view of plasma sensitivity: MD40 and MD14 emerge as the most sensitive species, whereas MD6 and MD9 display relative tolerance.

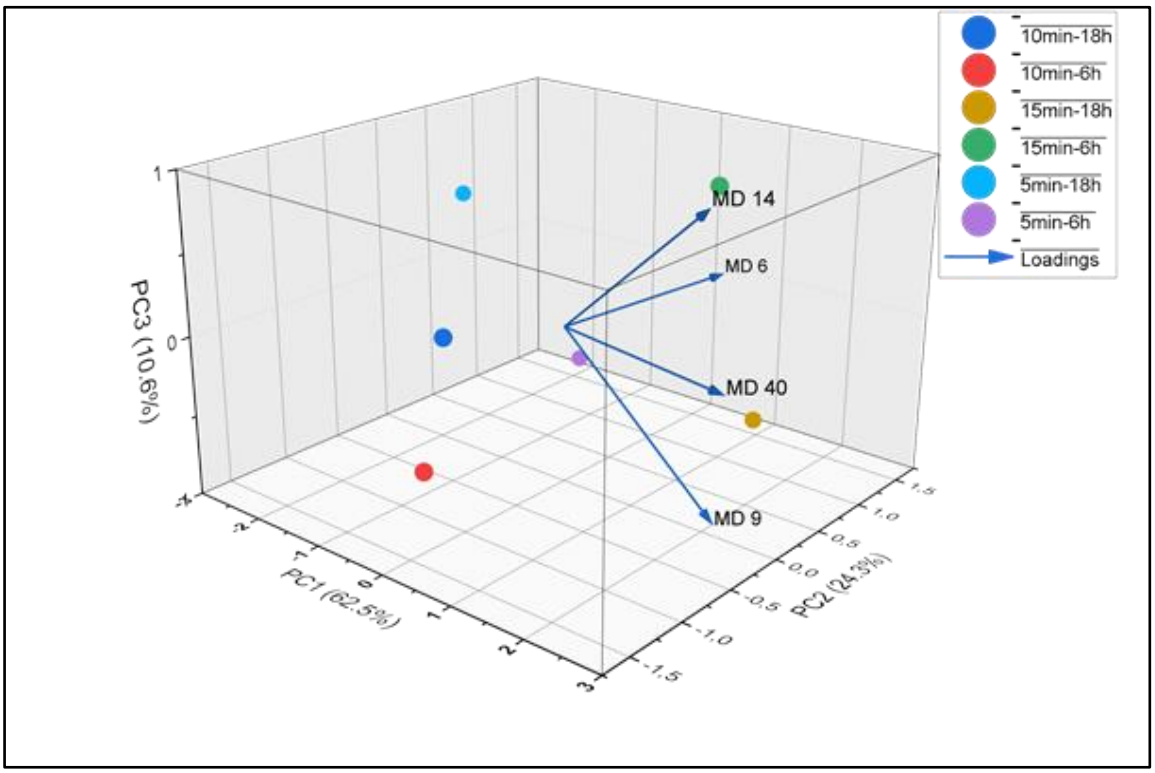

Figure 29: Principal Component Analysis (PCA) of the effects of DBD plasma exposure on spore germination of four *Alternaria* species.

Discussion

The most common fungus genus now affecting wheat is Alternaria (Gonzalez et al., 1999; Azcarate et al., 2006; Masiello et al., 2020; Tralamazza et al., 2016; Pinto et al., 2021). This conclusion is supported by the high percentage of Alternaria contamination found in the current study when compared to other fungal genera like Fusarium and Cladosporium. Additionally, as noted in earlier studies (Masiello et *al*., 2020; Muller et *al*., 2016), the opposite incidence between Alternaria and Fusarium in wheat kernels is also evaluated, indicating competitive interactions between the two fungal taxa. Bensassi et *al*. (2009) were the first to report the presence of Alternaria species linked to wheat black point in Algeria. Even yet, it has been emphasized how crucial it is to evaluate the possible risk of Alternaria toxin exposure in wheat meant for human consumption. The levels of Algerian wheat contamination found in this study indicate that this fungus genus need additional investigation. The percentage of contaminated wheat kernels in each region ranged from 30 to 60, with 40% of the samples exhibiting contamination levels exceeding 50%.

Phylogenetic analysis revealed that the Alternaria (51%) and *Infectoriae* (40%) sections contained the majority of the strains found in this investigation. Similar findings were reported by Masiello et *al*. (2020) and Ramires et al. (2018), who found that the most common species on wheat belonged to the *Alternata* and *Infectoriae* sections. *A. alternata* is the most widely spread species in the Alternaria genus. In addition to being a significant saprophyte on decomposing organic materials, this plant pathogen causes leaf spot disease in over 100 different host plant species (Woudenberg et *al*., 2015). We were able to differentiate between different species in the Alternaria section by using gpd and alt-a1 in the multi-locus analyses. 28 field strains made up the major group, *sub-clade 1: 19* were recognized *as A. alternata, 9 were clustered closely with A. alternata* but could be distinguished, 3 were identified as *A. arborescens, and 2 were identified as A. angustiovoidea. Since A. alternata, A. arborescens, and A. angustiovoidea species may be easily distinguished in the Alternaria section,* the results of this study are supported by numerous prior investigations. On the other hand, even when employing numerous genes *like alt-a1, gpd, translation elongation factor (tef), and β-tubulin (tub), it is frequently impossible to distinguish between A. alternata, A. tenuissima, A. turkisafria, and A. limoniasperae* (Somma et *al*., 2019; Masiello et *al*., 2020, 2022).

Thus, the two taxa *A. alternata* and *A. tenuissima* can be combined into a single species, *A. alternata*, as suggested by Woudenberg et al. (2015) following whole-genome and transcriptome analysis; Somma et *al*. (2019) later corroborated this conclusion. The sub-clade A4, which comprised 10 field strains and three reference strains of *A. mali, A.*

citriarbusti, and *A. rhadina,* is a well-supported cluster within the Alternaria section; nevertheless, no species differentiation was noted within this clade. Similarly, using the alt-a1, gpd, and tef genes, no differences between the several species of this clade were found in earlier research (Ramires et *al.*, 2018; Masiello et *al*., 2020).

However, using the combined genes alt-a1, gpd, tef, calmodulin, and tub makes it very impossible to distinguish between various species within the clade of *A. mali* (Somma et *al*., 2019; Habib et *al*., 2021). In any case, although Woudenberg et *al.* (2015) have suggested the synonymization of these two species, *A. mali* is clearly distinguishable from *A. alternata* in both this study and the numerous other examinations described above. As a result, there are various disputes over the Alternaria genus' classification based on molecular phylogeny. Evolutionary inconsistencies, such as lineage sorting and recombination, hamper the process of choosing appropriate molecular markers for phylogeny and systematic analysis, leading to conflicting results for each locus examined (Siciliano et *al*., 2018). These issues necessitate the development of more phylogenetically informative molecular markers and the exploration of alternative and more precise methods for categorizing the numerous species contained in the Alternaria divisions. For instance, Dettman et al. (2023) recently reported the discovery of particular markers (*ASA-10* and *ASA-19*) that can reliably categorize the strains in the Alternaria division according to species or lineage.

According to Lawrence et *al*. (2014) and Somma et *al.* (2019), strains within the clades of the *Infectoriae* section exhibit considerable variability and lower levels of similarity among themselves in comparison to those of other sections, particularly the Alternaria section. The *Infectoriae* division classified 17 field strains into two highly variable but poorly supported sub-clades. With the exception of strain MD70, which was identified as *A. metachromatica,* none of the strains were identified at the species level. Additionally, six more field strains that fall under this category were excluded from phylogenetic analysis due to their failure to amplify the alt-a1 gene.

Similar findings from a prior study (Masiello et *al*., 2022) support the theory that primer annealing site polymorphisms may be the reason for failure. For better results, more genes should be analyzed in order to precisely identify the species of the Algerian strains that belong to this section. However, the identification of the species included in the *Infectoriae* section has not been deemed significant in this study because the mycotoxigenic risk is nearly nonexistent due to their incapacity to create mycotoxins. However, around 40% of the contamination found in Algerian wheat kernels is caused by these species.

Only six of the field strains from Algeria belonged to sections other than Alternaria and *Infectoriae*. In the *Ulocladioides* part, two strains were recognized as *A. consortialis*; in the Eureka section, one was identified as *A. eureka*; and in the *Embellisia* section, two were identified as *A. tellustris.* Species from the *Ulocladioides, Eureka*, and *Embellisia* sections are infrequently isolated, according to earlier studies. For example, isolates from the *Ulocladioides* section were discovered in Lebanese durum wheat grains (Masiello et al., 2022) and date palms in southern Tunisia (Rabaaoui et *al*., 2022). Additionally, certain Eureka section strains were isolated from Algerian Solanaceae plants (Bessadat et *al*., 2021). But in durum wheat, the species *A. consortialis, A. eureka*, and *A. tellustris*, were initially isolated.

However, as the majority of *A. alternata* strains produce high amounts of AME, AOH, and TeA, the ability of the Algerian strains to create mycotoxins represents the common understanding based on species identification (Scott et *al.*, 2001). Additionally, under in vitro circumstances, the majority of them (55%) generated moderate levels of ATX-I, TEN, and ALT. In contrast, Rabaaoui et *al*. (2021) previously reported that no mycotoxin was produced by any strain of *A. consortialis*. Similarly, these mycotoxins could not be produced by *A. tellustris* or *A. eureka*. The presence of these Alternaria species on wheat indicates that they may be useful in controlling mycotoxigenic fungi and encouraging the growth of cereal plants. In fact, Di Martino et al. (2022) recently shown that *A. tellustris* is active against *Fusarium oxysporum* and *Rhyzoctonia solani*, two fungal diseases of wheat root. Furthermore, it has been shown that A. embellisia, which is phylogenetically closely linked to *A. tellustris*, and *A. consortialis*, which belongs to the *Ulocladioides* Section, can enhance maize seed germination and root length on maize seedlings (Hatamzadeh et al., 2023). Specifically, large concentrations of indole-3-acetic acid-like chemicals that have been linked to plant growth promotion were produced by *A. consortialis* (Wu et al., 2021).

Algerian wheat's chemical analysis made it possible to assess the presence of Alternaria mycotoxin and the ensuing actual harm to Algerian consumers. The geographic origin of the wheat samples showed a small association. Particularly, the regions of Batna, M'Sila, and Khenchela, which have arid and frigid climates, lack ATX-I and TEN. Although only one sample was taken from the Bejaia region, it is erroneous to draw conclusions about the climate because these mycotoxins are actually lacking there as well.

However, Algeria's climate is similar to that of other wheat-growing nations including Tunisia, Morocco, Egypt, Libya, Sudan, and Saudi Arabia; these nations have not been thoroughly studied for Alternaria species and their mycotoxin contamination.

Therefore, data gathered in other semi-arid and Mediterranean regions may be helpful in forecasting Alternaria distribution and recurrence as well as in creating disease control plans (Shabana et *al.*, 2021). Additionally, according to wheat variety, Simeto and Vitron had higher levels of contamination than Oued El Bared and Boussalem. However, if the impact of varietal choice needs to be precisely evaluated, specific studies including numerous samples should be conducted. It should be noted, though, that Vitron was the most common type grown in M'Sila, Khenchela, and Biskra, all areas with arid climates, while Simeto was only grown in the Batna region. Therefore, the geographic origin of the samples may also influence the differences found between kinds.

However, at least one Alternaria mycotoxin was found in 85% of the wheat samples, with AME and TeA showing the highest amounts. Furthermore, according to other research conducted in Argentina (Azcarate et *al.*, 2008), China (Li and Yoshizawa, 2000), and Egypt (Shabana et *al*. 2021), AME is the most common mycotoxin, found in 75% of the wheat samples. The mean amount of AME found in Algerian samples ranged from above 300 µg kg-1 to 980 µg kg-1. AME levels in earlier wheat research ranged from 2 in Israel to 39 in Serbia (Tralamazza et *al*., 2018), which is up to ten times lower than our findings. However, TeA has been found in Algerian wheat at extremely high concentrations (up to 705 µg kg-1), with a mean value of 177 µg kg-1, which is comparable to earlier findings in China (Xu et *al*., 2016) and Germany (Muller and Korn, 2013).

ALT and TeA show considerable acute toxicity in vitro and in animal tests, despite the fact that AME and AOH are the most commonly found Alternaria mycotoxins in wheat. The acute toxicity of AME and AOH is lower. Nonetheless, they have been reported to have cytotoxic, genotoxic, carcinogenic, and mutagenic effects. In contrast, the Algerian wheat samples showed low amounts of TEN and ATX-I with low frequency and no ALT. These mycotoxins were found at quantities below 50 µg kg-1, and only a few numbers of research monitored the toxigenic risk on grain commodities (Mašková et al., 2019; Woo et *al.*, 2022; Escrivà et al., 2017). Furthermore, earlier research has proven that wheat kernels are free of ALT contamination (Woo et al., 2022; Escrivà et *al*., 2017). The data presented in the current study on Algerian wheat highlight the urgent need for regulation, including limits for Alternaria toxins in food and feed intended for human and animal consumption, when we take into account the EU recommendation of 500 µg kg-1 of TeA and 2 µg kg-1 of AME and AOH for cereal-based food for infants.

Additionally, several poisons have been found to co-occur, which is consistent with multiple investigations (Zhang et *al*., 2025). Co-exposure to Alternaria toxins has been

shown in numerous studies to have additive or even synergistic effects, with the combination of AOH and AME causing more severe oxidative damage than either toxin alone (Marin et al., 2024). 73% of the Algerian samples had both AME and AOH, 27% had both AME and TeA, and just 6% had both TEN and TeA. Unlikely, TEN+TeA, AME+TeA, and AME+AOH together amounted for 97%, 91%, and 6% of the wheat flour samples in China, according to Jang et *al*. (2021). Only a small number of wheat samples in a ten-year survey conducted in Germany had numerous Alternaria mycotoxins co-occurring; TeA and AOH were the most common combination, but AOH and AME did not co-occur (Muller and Korn, 2013). The variations in the incidence and distribution of Alternaria mycotoxins in wheat found in samples from various parts of the world underscore the significant influence of agronomic methods and climate. Furthermore, two of the 48 samples had simultaneous contamination with five mycotoxins (AME, AOH, TeA, ATX-I, and TEN), while two more samples had four mycotoxins. On the other hand, three Alternaria mycotoxins have been found to coexist in 25% of the wheat samples. If we take into account the potential for synergistic effects resulting from co-contamination of various mycotoxins in the food and feed material, these results are extremely concerning. Common fungal pathogens called Alternaria spp. are linked to black point disease in wheat, a condition that negatively impacts grain quality and presents serious health hazards to humans because it produces mycotoxins. Alternative approaches are crucial since conventional chemical fungicides are subject to growing limitations and the emergence of resistance. Recently, low-temperature plasma has become a quick, affordable, and ecologically friendly method of microbial inactivation. Without leaving behind residues or producing hazardous byproducts, it fosters a hostile environment for pathogenic bacteria and fungi (Mai-prochnow et *al*., 2014; Ambrico et *al*., 2017; Laroussi, 2005). DBD plasma was assessed in this study as a unique method of inhibiting both mycelial growth and conidial germination to control Alternaria spp.

The results indicated that the antifungal activity of DBD plasma is strongly influenced by both treatment duration and fungal species. Short exposures of 1–2.5 min produced negligible or inconsistent reductions, whereas longer treatments of 5 min consistently resulted in significant inhibition of mycelial growth. Moreover, complete suppression of spore germination was achieved after 15 min of exposure. These observations are consistent with previous reports highlighting the dose-dependent effects of non-thermal plasma on fungi. For instance, a 3 min exposure reduced mycelial growth of *Fusarium graminearum* and *F. oxysporum* f. sp. *lycopersici* (Na et *al*., 2013), while treatments of 5–

10 min completely inhibited spore germination in *Penicillium digitatum, Fusarium fujikuroi, Colletotrichum gloeosporioides,* and *Cercospora lactucae-sativae* (Supakitthanakorn et al., 2025; Kang et *al*., 2025; Liu et *al*., 2016). Collectively, these findings emphasize the critical role of optimizing exposure time to achieve effective fungal inactivation.

Interspecific variation in susceptibility was evident. *A. alternata* (MD40) and the MD14 strain complex (*A. mali*, *A. rhadina*, and *A. citriarbusti*) were highly sensitive, exhibiting marked inhibition of both mycelial growth and spore germination after 5 min of treatment. *A. arborescens* (MD9) also showed strong suppression, with inhibition exceeding 90% under the longest exposure. By contrast, *A. angustiovoidea* (MD6) Notably, the 10-minute DBD exposure produced negative inhibition values (−21% at 6 h and −9% at 18 h), indicating a relative stimulation of spore germination compared to untreated controls. This biphasic response is consistent with the concept of plasma hormesis, in which sub-lethal doses of reactive species can promote rather than suppress biological activity, and aligns with the dose-dependent response reported by Kono et *al*. (2019), who demonstrated that DBD plasma enhanced *Aspergillus oryzae* germination at short exposure times by loosening the spore surface, while longer exposures ultimately suppressed it. Similar stimulatory effects have also been reported in plant seeds and fungal spores exposed to low-intensity plasma treatments (Mildaziene et *al*., 2018; Adhikari et *al*., 2020). In the case of MD6, the relatively high melanin content in the spore wall known to confer tolerance to oxidative stress in *Alternaria* species (Li et *al*., 2024; Zhao et *al*., 2022) may buffer lethal oxidative damage at intermediate plasma doses while simultaneously allowing mild structural modifications of the spore surface that facilitate water uptake and germination. This non-linear dose-response underscores the importance of optimizing treatment parameters on a species-specific basis.

In contrast to bacteria, fungi generally require longer plasma treatments for effective inactivation due to fundamental structural differences between prokaryotic and eukaryotic cells. The fungal cell wall, composed of chitin, α- and β-linked glucans, glycoproteins, and pigments, provides enhanced resistance to environmental stressors (Nimrichter et *al*., 2005; Latgé et *al*., 2014). During plasma exposure, reactive oxygen species (ROS) and reactive nitrogen species (RNS) attack these protective components, initiating oxidative damage. Plasma etching disrupts hydrocarbon bonds, while atomic oxygen and ozone react with exposed molecular sites, accelerating structural degradation (Ermolaeva et *al*., 2011; Fricke et al., 2012). Such processes result in morphological alterations, including reduced spore size, channel formation, and eventual cell destruction (Reis et *al*., 2012; Davies et *al*., 2016).

Once the integrity of the cell wall is compromised, plasma-generated UV photons and radicals can penetrate and directly damage nucleic acids, proteins, and membrane lipids, ultimately impairing membrane stability, permeability, and function (Montie et al., 2000). The findings are in line with earlier studies that found that DBD plasma totally prevented the hyphal growth and spore germination of fungal phytopathogens, including Alternaria alternata, Aspergillus flavus, A. oryzae, *Fusarium culmorum*, and *Fusarium oxysporum f. sp. lycopersici* (Ambrico et *al*., 2020; Perinban et *al.*, 2019; Julak et *al.*, 2018). The high correlation between mycelial growth suppression and germination inhibition seen here implies that plasma targets several stages of development, from early germination to hyphal extension, interfering with both colonization potential and infection start.

Multivariate analysis confirmed species-specific responses. Principal component analysis (PCA) revealed that the first two components explained 86.8% of the total variance, clearly separating strains according to plasma sensitivity. MD40 and MD14 clustered as highly susceptible taxa, while MD6 was positioned as relatively tolerant. This dimensional reduction not only confirmed ANOVA findings but also provided an integrated visualization of differential responses, reinforcing the conclusion that *Alternaria* spp. vary markedly in their sensitivity to plasma treatment.

Taken together, these findings highlight the strong antifungal potential of DBD plasma against *Alternaria* spp., a genus of major concern in wheat and other crops due to both economic losses and health risks associated with mycotoxins. Nevertheless, several considerations remain. The 5–15 min exposures effective *in vitro* may not directly translate to large-scale applications, where throughput, cost, and effects on product quality must be considered. Furthermore, *in vivo* evaluations under storage or field conditions are necessary to validate practical utility. Combining plasma treatment with other preservation methods (e.g., cold storage, biocontrol) may enhance efficacy while reducing treatment times. Finally, future research should investigate whether relatively tolerant species, such as *A. angustiovoidea* (MD 6), can adapt to repeated plasma exposure and identify the molecular mechanisms underlying interspecific variability in sensitivity.

General conclusion and perspectives

General conclusion and perspectives

Wheat remains a cornerstone of global food security and is particularly critical in Algeria, where durum wheat constitutes a major component of the national diet. However, its productivity and quality are severely constrained by black point disease, predominantly caused by *Alternaria* spp. Despite its agronomic importance, little was previously known about the diversity, pathogenicity, and toxicological potential of *Alternaria* isolates in Algeria. Therefore, this work was undertaken with the aim of studying the different Alternaria species involved in the black point disease complex in Algeria and, mainly, studying them at different scales.

The phylogenetic analysis of 66 field strains based on multi-locus sequencing (gpd and alt-a1 genes) revealed that the Algerian *Alternaria* population is highly diverse, clustering into five major clades corresponding to the sections *Alternaria*, *Infectoriae*, *Ulocladioides*, *Eureka*, and *Embellisia*. The majority of isolates (67%) grouped within the *Alternaria* section, predominantly as *A. alternata*, but also including *A. arborescens*, *A. angustiovoidea*, and the unresolved *A. citriarbusti/A. mali/A. rhadina* complex. The *Infectoriae* section accounted for approximately one quarter of isolates, although with high internal variability and weak phylogenetic support, suggesting the need for more robust taxonomic markers in future studies. This diversity highlights the complexity of the *Alternaria* community infecting wheat in Algeria and suggests multiple ecological niches and adaptive strategies.

Mycotoxin analyses further emphasized the pathogenic and toxicological relevance of these isolates. A high proportion of strains within the *Alternaria* section were capable of producing significant amounts of alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA), often in combination. The most toxigenic profiles were associated with the *A. alternata* and *A. citriarbusti/A. mali/A. rhadina* clusters, with some strains producing TeA at levels exceeding 13 g kg^{-1} in culture. By contrast, species belonging to *Ulocladioides*, *Embellisia*, and most of the *Infectoriae* section showed limited or no mycotoxin production. Importantly, durum wheat grain samples collected from different regions of Algeria were frequently contaminated with AOH and AME (69–75%), and to a lesser extent with TeA, ATX-I, and TEN, underlining the relevance of *Alternaria* mycotoxins for food safety and consumer health. Regional variability was also observed, with higher AME levels in Khenchela and AOH in M'Sila, suggesting an influence of local agro-climatic conditions on toxin occurrence.

The second component of this work explored cold plasma as an innovative, residue-free method to inactivate *Alternaria* spp. In vitro experiments demonstrated that dielectric barrier discharge (DBD) plasma effectively inhibited both mycelial growth and conidial germination in a time- and species-dependent manner. *A. alternata* (MD40) and the *A. citriarbusti/A. mali/A. rhadina* complex (MD14) were highly sensitive, with strong suppression (>80%) achieved after short exposures. *A. arborescens* (MD9) was also markedly inhibited at longer exposure times, whereas *A. angustiovoidea* (MD6) displayed relative tolerance, requiring extended treatments for meaningful inhibition. PCA analysis confirmed clear interspecific differences in plasma susceptibility, separating sensitive from tolerant strains. Microscopic observations revealed profound structural damage to conidia following plasma exposure, further supporting its antifungal efficacy.

Taken together, these findings provide the first integrated view of the *Alternaria* threat to durum wheat in Algeria. The high prevalence of toxigenic species, coupled with frequent contamination of harvested grains, underscores the urgent need for monitoring programs and risk assessment frameworks at national and regional levels. The demonstrated antifungal activity of cold plasma provides a promising basis for the development of innovative postharvest decontamination strategies that could complement or replace chemical fungicides, thereby contributing to sustainable wheat production and food safety.

Building upon these findings, several targeted research directions can be proposed:

-Conduct detailed pathogenicity assays to determine the extent of host–pathogen interactions, disease severity, and grain quality deterioration caused by different isolates.

- Clarify its taxonomic position through multilocus or genomic analyses.

- Use advanced methods such as whole-genome sequencing, transcriptomics, and metabolomics to identify the genes and regulatory pathways responsible for mycotoxin biosynthesis in *Alternaria* spp., with a particular focus on highly toxigenic isolates.

-Assess the potential of cold plasma not only for inactivating fungal propagules but also for degrading or neutralizing key *Alternaria* mycotoxins (TeA, AOH, AME, TEN) in vitro and in naturally contaminated durum wheat kernel

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Appendices

Appendix 1

VARIETES	COMMUNE	WILAYA	CODE LAB	CODE ANALYSE	N° Echan.
VITRON	MAARIF	MSILA	260HC/3	210260HC	V1
VITRON	MAARIF	MSILA	250HC/4	210250HC	V2
VITRON	BOUSSAADA	MSILA	251HC/6	210251HC	V3
OUED EL BARED	BENI FOUDA	SETIF	297/5	210297HC	V4
VITRON	DOUCEN	BISKRA	57HC	210057HC	V5
VITRON	ELOUTAYA	BISKRA	56HC	210056HC	V6
VITRON	MAARIF	MSILA	259HC	210259HC	V7
BOUSSAL LAM	EL OURISSIA	SETIF	284	210284HC	V8
GTA Dur	BENI FOUDA	SETIF	289	210289HC	V9
BOUSSAL LAM	EL OURISSIA	SETIF	283	210283HC	V10
VITRON	MAARIF	MSILA	249HC	210249HC	V11
BOUSSAL LAM	AIN OULMANE	SETIF	27HC	210027HC	V12
VITRON	DOUCEN	BISKRA	63HC	210063HC	V13
BOUSSAL LAM	AIN ABESSA	SETIF	35HC	210035HC	V14
OUED EL BARED	AMOUCHA	SETIF	22HC	210022HC	V15
OUED EL BARED	AMOUCHA	SETIF	21HC	210021HC	V16
OUED EL BARED	AIN OULMANE	SETIF	28HC	210028HC	V17
OUED EL BARED	BENI FOUDA	SETIF	298	210298HC	V18
OUED EL BARED	SETIF	SETIF	64	210064HC	V19
OUED EL BARED	SETIF	SETIF	71	210071HC	V20
BOUSSAL LAM	BENI FOUDA	SETIF	105	210105HC	V21

OUED EL BARED	**BENI FOUDA**	**SETIF**	**138HC**	**210138HC**	**V22**
BOUSSAL LAM	**EL OURISSIA**	**SETIF**	**34**	**210034HC**	**V23**
SERSOU (SIMETO)	**ZANA EL BEIDA**	**BATNA**	**427**	**210427HC**	**V24**
BOUSSAL LAM	**AIN ABESSA**	**SETIF**	**36HC**	**210036HC**	**V25**
BOUSSAL LAM	**BENI FOUDA**	**SETIF**	**106**	**210106HC**	**V26**
BOUSSAL LAM	**BENI FOUDA**	**SETIF**	**32HC**	**210032HC**	**V27**
OUED EL BARED	**BENI FOUDA**	**SETIF**	**139HC**	**210139HC**	**V28**
OUED EL BARED	**TAZOULT**	**BATNA**	**426**	**210426HC**	**V29**
BOUSSAL LAM	**AMIZOUR**	**BEJAIA**	**646**	**210646HC**	**V30**
OUED EL BARED	**SETIF**	**SETIF**	**674**	**210674HC**	**V31**
MOHAMED BEN BACHIR	**SETIF**	**SETIF**	**675**	**210675HC**	**V32**
SERSOU (SIMETO)	**TAZOULT**	**BATNA**	**429**	**210429HC**	**V33**
SERSOU (SIMETO)	**TAZOULT**	**BATNA**	**428**	**210428HC**	**V34**
OUED EL BARED	**TAZOULT**	**BATNA**	**426**	**210426HC**	**V35**
BOUSSAL LAM	**AMOUCHA**	**SETIF**	**487**	**210487HC**	**V36**
VITRON	**SAADA**	**BISKRA**	**362**	**210362HC**	**V37**
OUED EL BARED	**SAADA**	**BISKRA**	**582**	**210582HC**	**V38**
OUED EL BARED	**AIN ABESSA**	**SETIF**	**466**	**210466HC**	**V39**
OUED EL BARED	**SIDI OKBA**	**BISKRA**	**583**	**210583HC**	**V40**
VITRON	**BABAR**	**KHENCHELA**	**616**	**210616HC**	**V41**
BOUSSAL LAM	**AMOUCHA**	**SETIF**	**486**	**210486HC**	**V42**

OUED EL BARED	**BAZER SAKHRA**	**SETIF**	**550**	**210550HC**	**V43**
VITRON	**BABAR**	**KHENCHELA**	**617**	**210617HC**	**V44**
VITRON	**SAADA**	**BISKRA**	**361**	**210361HC**	**V45**
OUED EL BARED	**AIN ARNAT**	**SETIF**	**459**	**210459HC**	**V46**
OUED EL BARED	**BAZER SAKHRA**	**SETIF**	**551**	**210551HC**	**V47**
OUED EL BARED	**AIN ARNAT**	**SETIF**	**549**	**210549HC**	**V48**

Appendix 1

1-1 Effect of DBD plasma on mycelial growth of MD 6

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	164,391[a]	19	8,652	25,223	0,000
Constante	615,168	1	615,168	1793,363	0,000
Days	101,029	4	25,257	73,631	0,000
Treatement_Duration	53,900	3	17,967	52,377	0,000
Days * Treatement_Duration	9,463	12	0,789	2,299	0,024
Erreur	13,721	40	0,343		
Total	793,280	60			
Total corrigé	178,112	59			

a. R-deux = ,923 (R-deux ajusté = ,886)

Différence significative de Tukeya,b

Treatement_Duration	N	Sous-ensemble	
		1	2
5min	15	1,5700	
2.5	15		3,5807
1min	15		3,8053
0min	15		3,8520
Sig.		1,000	0,588

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.
Le terme d'erreur est le carré moyen (Erreur) = ,343.
a. Utilise la taille d'échantillon de la moyenne harmonique = 15,000.
b. Alpha = ,05.

2-**Effect of DBD plasma on mycelial growth of MD 14**

Tests des effets inter sujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	197,472[a]	19	10,393	341,416	0,000
Constante	606,235	1	606,235	19914,656	0,000
Days	74,274	4	18,569	609,974	0,000
Treatement_Duration	98,387	3	32,796	1077,333	0,000
Days * Treatement_Duration	24,810	12	2,068	67,918	0,000
Erreur	1,218	40	0,030		
Total	804,925	60			
Total corrigé	198,690	59			

a. R-deux = ,994 (R-deux ajusté = ,991)

Fungal_Growth

Différence significative de Tukey[a,b]

Treatement_Duration	N	Sous-ensemble		
		1	2	3
5min	15	1,0000		
2.5	15		3,5140	
0min	15			4,0760
1min	15			4,1247
Sig.		1,000	1,000	0,870

Les moyennes des groupes des sous-ensembles homogènes sont affichées.
Calcul basé sur les moyennes observées.
Le terme d'erreur est le carré moyen (Erreur) = ,030.
a. Utilise la taille d'échantillon de la moyenne harmonique = 15,000.
b. Alpha = ,05.

3-2-Effect of DBD plasma on mycelial growth of MD 40

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	137,024[a]	19	7,212	555,107	0,000
Constante	467,660	1	467,660	35996,921	0,000
Days	53,182	4	13,296	1023,390	0,000
Treatement_Duration	66,017	3	22,006	1693,826	0,000
Days * Treatement_Duration	17,825	12	1,485	114,334	0,000
Erreur	0,520	40	0,013		
Total	605,203	60			
Total corrigé	137,543	59			
a. R-deux = ,996 (R-deux ajusté = ,994)					

Fungal_Growth

Différence significative de Tukey[a,b]

Treatement_Duration	N	Sous-ensemble		
		1	2	3
5min	15	1,0000		
2.5	15		3,1060	
1min	15			3,5287
0min	15			3,5327
Sig.		1,000	1,000	1,000

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.

Le terme d'erreur est le carré moyen (Erreur) = ,013.

a. Utilise la taille d'échantillon de la moyenne harmonique = 15,000.

b. Alpha = ,05.

4- Effect of DBD plasma on mycelial growth of MD 9

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	123,830[a]	19	6,517	15,691	0,000
Constante	575,237	1	575,237	1384,911	0,000
Days	64,708	4	16,177	38,947	0,000
Treatement_Duration	50,738	3	16,913	40,718	0,000
Days * Treatement_Duration	8,384	12	0,699	1,682	0,108
Erreur	16,614	40	0,415		
Total	715,681	60			
Total corrigé	140,445	59			

a. R-deux = ,882 (R-deux ajusté = ,826)

Fungal_Growth

Différence significative de Tukey[a,b]

Treatement_Duration	N	Sous-ensemble	
		1	2
5min	15	1,5087	
2.5	15		3,5400
0min	15		3,5953
1min	15		3,7413
Sig.		1,000	0,827

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.

Le terme d'erreur est le carré moyen (Erreur) = ,415.

a. Utilise la taille d'échantillon de la moyenne harmonique = 15,000.

c. Alpha = ,05.

1-2 Effect of DBD plasma on MD 6 spores' germination

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	259,292[a]	7	37,042	3,832	0,012
Constante	1107,042	1	1107,042	114,522	0,000
T.T	145,125	3	48,375	5,004	0,012
I.T	92,042	1	92,042	9,522	0,007
T.T * I.T	22,125	3	7,375	0,763	0,531
Erreur	154,667	16	9,667		
Total	1521,000	24			
Total corrigé	413,958	23			

a. R-deux = ,626 (R-deux ajusté = ,463)

Spores Germination

Différence significative de Tukey[a,b]

T.T	N	Sous-ensemble	
		1	2
15min	6	3,67	
5min	6	5,17	5,17
0min	6	8,67	8,67
10min	6		9,67
Sig.		0,058	0,097

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.

Le terme d'erreur est le carré moyen (Erreur) = 9,667.

a. Utilise la taille d'échantillon de la moyenne harmonique = 6,000.

d. Alpha = ,05.

1-2 Effect of DBD plasma on MD 14 spores' germination

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	2137,292[a]	7	305,327	21,179	0,000
Constante	2301,042	1	2301,042	159,610	0,000
T_T	1989,125	3	663,042	45,991	0,000
I.T	57,042	1	57,042	3,957	0,064
T_T * I.T	91,125	3	30,375	2,107	0,140
Erreur	230,667	16	14,417		
Total	4669,000	24			
Total corrigé	2367,958	23			

a. R-deux = ,903 (R-deux ajusté = ,860)

Spore germination

Différence significative de Tukey[a,b]

T_T	N	Sous-ensemble	
		1	2
15min	6	2,33	
10min	6	4,83	
5min	6	6,67	
0min	6		25,33
Sig.		0,237	1,000

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.

Le terme d'erreur est le carré moyen (Erreur) = 14,417.

a. Utilise la taille d'échantillon de la moyenne harmonique = 6,000.

2.3.Alpha = ,05.

2-2 Effect of DBD plasma on MD 40 spores' germination

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	2091,125[a]	3	697,042	20,238	0,000
Constante	2625,042	1	2625,042	76,217	0,000
I_T	0,000	0			
T_T	2091,125	3	697,042	20,238	0,000
I_T * T_T	0,000	0			
Erreur	688,833	20	34,442		
Total	5405,000	24			
Total corrigé	2779,958	23			
a. R-deux = ,752 (R-deux ajusté = ,715)					

Spore germination

Différence significative de Tukey[a,b]

T_T	N	Sous-ensemble	
		1	2
15min	6	2,67	
10min	6	5,17	
5min	6	7,67	
0min	6		26,33
Sig.		0,470	1,000

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.

Le terme d'erreur est le carré moyen (Erreur) = 34,442.

a. Utilise la taille d'échantillon de la moyenne harmonique = 6,000.

2.4.Alpha = ,05.

2-2 Effect of DBD plasma on MD 09 spores' germination

Tests des effets intersujets

Variable dépendante:

Source	Somme des carrés de Type III	df	Carré moyen	F	Sig.
Modèle corrigé	165,833[a]	7	23,690	1,914	0,134
Constante	400,167	1	400,167	32,337	0,000
T_T	86,833	3	28,944	2,339	0,112
I.T	60,167	1	60,167	4,862	0,042
T_T * I.T	18,833	3	6,278	0,507	0,683
Erreur	198,000	16	12,375		
Total	764,000	24			
Total corrigé	363,833	23			

a. R-deux = ,456 (R-deux ajusté = ,218)

Spore germination

Différence significative de Tukey[a,b]

T_T	N	Sous-ensemble 1
15min	6	1,83
10min	6	3,17
5min	6	4,33
0min	6	7,00
Sig.		0,091

Les moyennes des groupes des sous-ensembles homogènes sont affichées.

Calcul basé sur les moyennes observées.

Le terme d'erreur est le carré moyen (Erreur) = 12,375.

a. Utilise la taille d'échantillon de la moyenne harmonique = 6,000.

e. Alpha = ,05.

Appendix 1

PDA medium (potato dextrose agar)

Potato	200g
Dextrose	20g
Agar	20g
Distilled water	1000mL

This medium was autoclaved at 121°C for 20 min. pH: 7 ± 0.2

SNA medium (spezieller nährstoffarmer agar)

K2HPO4	1g
KNO3	1g
MgSO4.7H2O	0.5g
KCl	0.5g
Glucose Sucrose	0.2g
Agar	20g
Distilled water	1000 mL

This medium was autoclaved at 121°C for 20 min. pH: 7 ± 0.2.

PCA (Potato carrot agar)

Potato

Carrot

Agar

Distilled water

This medium was autoclaved at 121°C for 20 min. pH: 7 ± 0.2.

MEA (Malt extract agar)

Malt extract	17g
Peptone	3g`
Distilled water	1000 ml

This medium was autoclaved at 110 °C for 10 min.

PCA (Potato Carrot Agar)

Potato	20g
Carrot	20g
Agar	15g
Distilled water	1000ml

This medium was autoclaved at 121°C for 20 min. pH: 7 ± 0.2.