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Research Article

First report of highly pathogenic *Cladosporium cladosporioides* isolates on durum wheat plant under controlled conditions in Algeria

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Abstract: Phytopathogens isolated from durum wheat grains collected across twenty-eight locations in northeastern Algeria revealed, for the first time, the presence of *Cladosporium cladosporioides* isolates in durum wheat seeds in Algeria. These isolates were identified through macroscopic and microscopic examinations, followed by molecular identification confirmation. Under controlled conditions, pathogenicity assessments were conducted on three durum wheat varieties, focusing on the effects on germination rate, coleoptile length, root length, and the basal parts of the wheat seedlings. The findings demonstrated that *C. cladosporioides* isolates induced a significant reduction in the germination inhibition rate to 100% and decreased root and shoot length by 35.44% and 45.41%, respectively. Moreover, they diminished root and shoot fresh weight by 85.56% and 47.18%, respectively.

Keywords: Algeria, *Cladosporium cladosporioides*, Durum Wheat, Coleoptile, Pathogenicity, Root

Introduction

Durum wheat ranks tenth among the most widely cultivated cereals globally, with total production of around 38 million tons (Xynias *et al*., 2020). Various biotic and abiotic factors, including fungal diseases, significantly influence wheat yield and quality (Khan *et al.*, 2023).

Among fungal pathogens, *Cladosporium* is recognized as a significant genus that causes various infections in cereals, particularly wheat (Ogórek *et al*., 2012). It encompasses species widely distributed as molds in various global environments (Heuchert *et al.*, 2005). Several species act as plant pathogens, while others serve as spoilage agents or contaminants in food or industrial goods. Additionally, they can be found as endophytic fungi (El-Morsy, 2000).

Previously, the genus *Cladosporium* was associated with over 772 identified names (Dugan *et al.* 2004). Following a comprehensive revision by Bensch *et al.* in 2012, only 170 species names were recognized within the strict sense of *Cladosporium.* Increased research interest led to the discovery of several new species, documented in studies from 2014 to 2017 by many researchers

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(Crous *et al.,* 2014; Bensch *et al.,* 2015; Braun *et al.,* 2015; Razafinarivo *et al.,* 2016; Sandoval-Denis *et al.,* 2016; Ma *et al.,* 2017; Marin-Felix *et al.*, 2017). According to the most recent updates by Iturrieta-González *et al.* (2021) and Pereira *et al.* (2024), *Cladosporium* now includes 230 recognized species. The olive-green to brown or sometimes black colonies are usually the easiest characteristics for recognizing species of this genus (Gcobisa, 2019). *C. cladosporioides* is a commonly encountered saprophyte with a wide distribution (Musa *et al*., 2018). They are regarded as heterogeneous complexes of several genetically and morphologically distinct species (Braun *et al*., 2003).

Many studies have used traditional morphological methods to identify species in this genus, but this approach is challenging given their striking morphological similarities, especially among closely related species. (Ghiaie *et al*., 2017). To surmount these challenges, molecular identification techniques, notably DNA sequencing, have become increasingly essential for accurately classifying *Cladosporium* species (Walker *et al.,* 2016).

Unfortunately, no studies or statistics have been conducted on *Cladosporium* species and their impact on durum wheat in Algeria. Thus, this study aimed to isolate and identify some endophytic *Cladosporium* species from durum wheat seeds and evaluate their pathogenicity on wheat plants.

Materials and Methods

Plant materials

Twenty-eight samples of durum wheat grains from three varieties, Waha (Wah), Boussellam (Bous), and Oued El Bared (OEB), were obtained from the National Center for Seeds and Plants Certification and Control (NCSPCC) in Setif, Algeria, during the 2019-2020 crop season. The samples were collected from various provinces in Algeria's northeastern region.

Endophytes isolation

Cladosporium isolates were isolated from seeds following the method established by the National Laboratory of Plant Protection, France (NPPL, 2008). Initially, the seeds were superficially disinfected by immersion in a 1.5% sodium hypochlorite solution for 5 minutes. Subsequently, the grains were rinsed three times with sterile distilled water, thoroughly drained, and air-dried on sterilized absorbent paper.

Finally, 100 seeds were placed in Potato Sucrose Agar (PSA) petri dishes, with 10 seeds per dish, then incubated at 28 °C for 5-7 days.

Cladosporium species are distinguished by producing small, velvety colonies that range from olive green to brown and even black (Gcobisa, 2019).

Macroscopic and microscopic characterization

Following isolation and purification using the single-spore technique, the *Cladosporium* fungal genera were identified utilizing determination keys developed by Nasraoui (2006), Botton (1990), and Remi (1997). Various features were examined for identification, including colony size and appearance, and the color (pigmentation) on both the surface and the back of the colonies.

Molecular identification

The molecular identification was performed by Gene Life Sciences Corporation (Sidi-Bel Abbes, Algeria), and the method was detailed by Bencheikh *et al.* (2020).

Pathogenicity tests

Seven *Cladosporium* isolates (CladGa2, CladGa3, CladGa4, CladGa5, CladGa6, CladGa9, and CladGa19) were judged for their potential pathogenicity. For this reason, two methods were employed to assess their aggressiveness on three durum wheat varieties—Bous, Wah, and OEB—commonly cultivated in northeastern Algeria.

Pathogenicity on coleoptile and seminal roots length

Using a modified version of Mesterhazy's (1983) technique. To obtain the homogenized mycelium, six disks of 6 mm diameter from 7-day-old *Cladosporium* isolate cultures were used

to inoculate bottles containing 50 ml of Potato Sucrose Broth (PSB). The flasks were incubated at ambient temperature (25 ± 3 °C) in an orbital shaker (200 rpm) for 7 days. Centrifugation at 5000 g for 10 min was used to harvest the mycelium, which was then homogenized, diluted using sterile distilled water to 13.3 mg/ml, and mixed with 0.2% Tween 20 surfactant.

Regarding the artificial infection, fresh PSA plates were covered with sterilized Whatman No. 1 filter paper impregnated with 8ml of the homogenized mycelium. Then, 15 surface-sterilized seeds of each wheat variety were placed at a rate of 5 seeds per petri dish and covered by a second sterilized filter paper. The dishes were then incubated at 25° C for 4 days.

On the fourth day, the pathogenicity assessment involved determining its impact on germination rate (%) and the lengths of coleoptiles and seminal roots (mm). Results were compared to control seeds, which were inoculated only with 8 ml of sterile distilled water (instead of homogenized mycelium).

Pathogenicity on the wheat plant basal part

According to the method used by Summerell *et al.* (2006), the seven-day-old cultures of the *Cladosporium* isolates were scraped using a spatula with 5 ml of sterile distilled water. The spore suspension obtained was adjusted to 10^6 spores/ml using a hemocytometer (Malassez cell). The inoculation was performed by soaking superficially disinfected wheat seeds in spore suspensions of each fungal strain for 2 hours (Mnasri *et al.*, 2017). Three seeds were then transplanted into a plastic pot containing sterilized soil. Each combination was repeated three times.

Regarding the control, the pots contain only disinfected seeds (3 seeds/pot). Irrigation was carried out as needed, knowing that the experiment was conducted in November 2022. The statistical design was a Completely Randomized Design (CRD) with three replications for both coleoptile pathogenicity and seminal root length, and for pathogenicity on the wheat plant basal part. All pots were placed under natural conditions, with temperatures between 20 and 23 °C and 12 hours of daylight and 12 hours of darkness.

Symptoms were examined 30 days post-planting (Woo *et al.*, 1996). Measurements included seedling germination rate, root and shoot length, as well as root and vegetative system fresh weight.

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics, version 25. The One-way ANOVA test was employed for "*in vitro"* tests. However, the Two-way ANOVA test was used in the growth chamber experiment. Duncan's post hoc test was used to compare means at a 5% level (P = 0.05) with a 95% confidence interval.

Results

Endophytes isolation

About 28 fungal endophytes were isolated; only seven appeared olive-grey to dull green, velvety, and tufted on the PSA medium, providing strong evidence that they belonged to the *Cladosporium* genus during initial observation.

Macroscopic characteristics

Colonies on PSA medium exhibit features such as olivaceous (olive-green) pigmentation, defined margins, and various textures ranging from floccose-felty to velvety. The reverse side of the colonies displays colors such as olive-black, iron-grey, leaden-grey, or olivaceous-black (Fig. 1). Aerial mycelia are sparse, diffuse, or sometimes abundant, forming mats with growth ranging from flat to low convex (Llorente *et al.*, 2012; Bensch, 2012; Torres *et al.*, 2017; El-Dawy, 2021).

Microscopic characteristics

The obtained isolates exhibited typical morphological features consistent with the *Cladosporium* complex species. These included straight, solitary, unbranched, terminal or lateral, and nodule-free conidiophores, ranging in color from olivaceous-brown to olivaceous, emerging terminally from ascending hyphae. Ramoconidia were observed in different shapes, usually in groups of three or four at the tips of conidiophores,

straight, cylindrical-oblong. Conidiogenous cells, typically terminal, were integrated, measuring 16-38 µm in length and 1-2 µm in diameter. Abundant conidia were observed, measuring 3–6 µm in length and 2–2.5 µm in width, arranged in chains of up to nine conidia. They are limoniform, ovoid, obovoid to subglobose, aseptate, light brown, hila conspicuous, with 0–2 septa and displaying an olivaceous-green hue (Fig. 2). In addition, chlamydospores were not observed in this species.

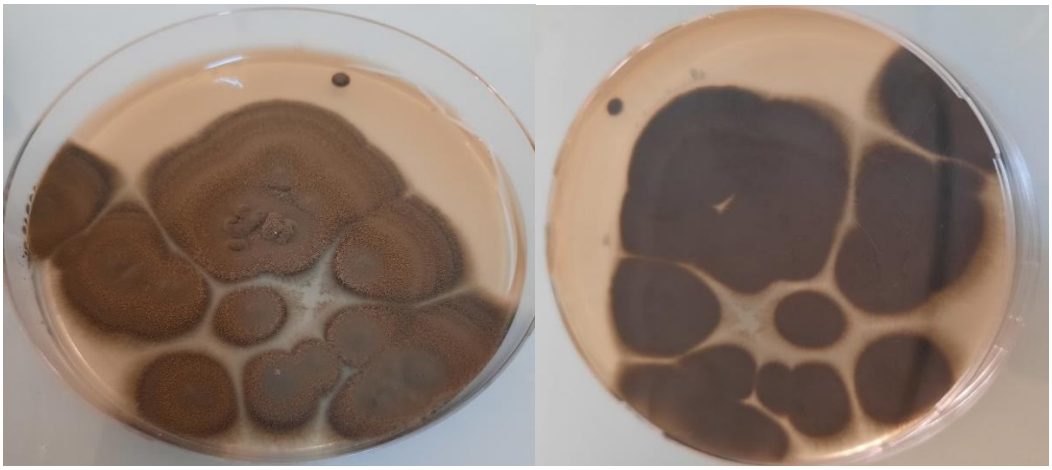

Figure 1 Macroscopic aspect of *Cladosporium cladosporioides* isolates colony cultures on PSA.

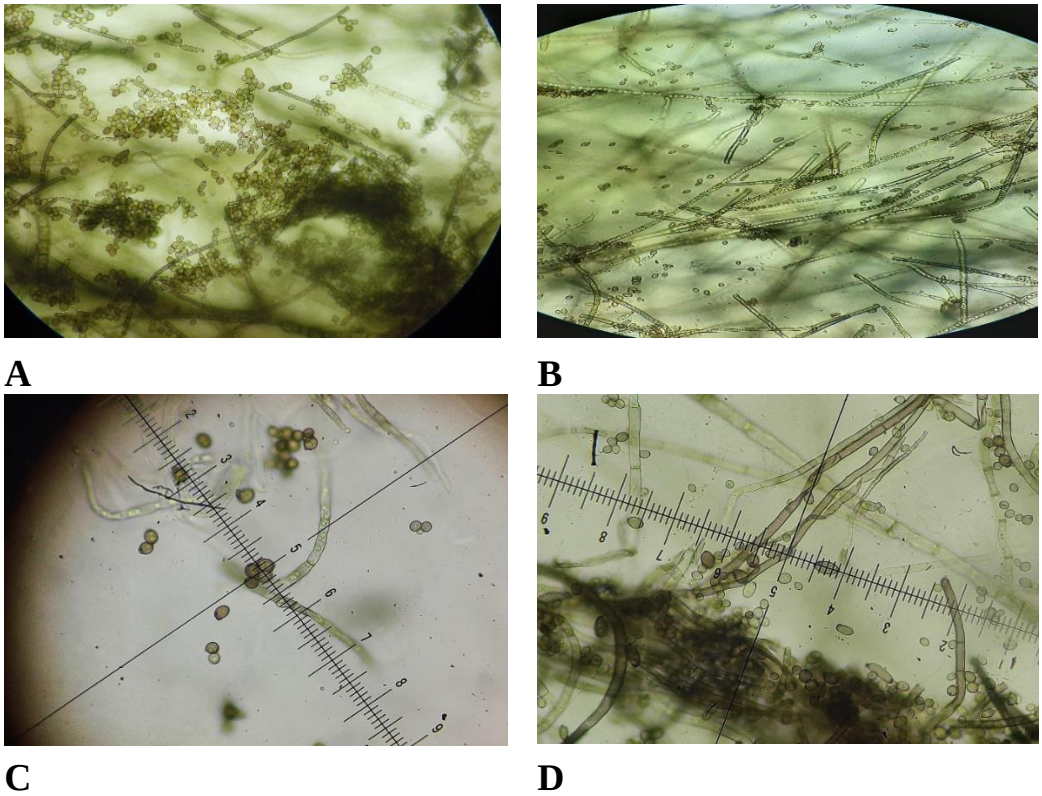

Figure 2 Spores of *Cladosporium cladosporioides* isolates. A: CladGa2; B: CladGa5; C: CladGa4; D: CladGa19 under an optical microscope fitted with a micrometric eyepiece at magnification (x40).

Molecular identification

After amplifying the rDNA region using the ITS4 primers, the phylogenetic analysis was done by the use of seven sequences of 525b, 520b, 525b, 527b, 524b, 523b, and 553b length for the CladGa2, CladGa3, CaldGa4, CladGa5, CladGa6, CladGa9 and CladGa19 isolates, respectively (Fig. 3). Comparing the obtained sequences to GenBank reference sequences revealed 99 to 100% similarity values. The isolate most similar to ours was *C. cladosporioides* Sp-XI-1.1.

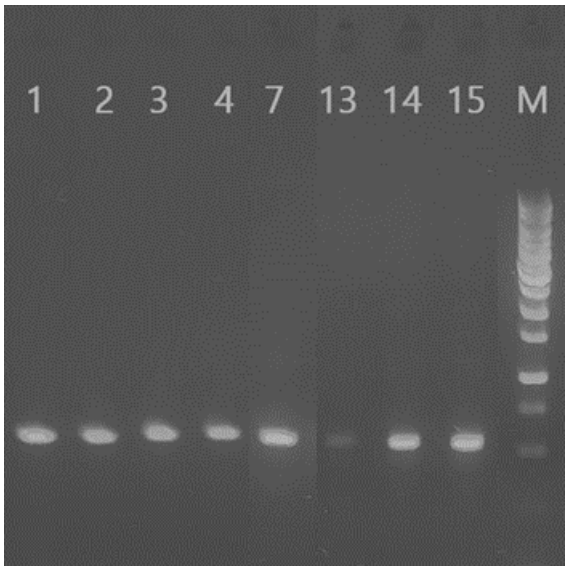

Figure 3 Agarose gel figure of PCR-amplified ITS4 gene region of the (1) CladGa2, (2) CladGa3, (3) CaldGa4, (4) CladGa5, (7) CladGa6, (13) CladGa9 and (15) CladGa19 isolates.

Based on the previous results and the phylogenetic tree, the isolates were grouped with many reference isolates of *C. cladosporioides* (Fig. 4). Thus, our isolates were confirmed as *C. cladosporioides*. Finally, the isolates were named *Cladosporium cladosporioides* CladGa2, *Cladosporium cladosporioides* CladGa3, *Cladosporium cladosporioides* CaldGa4, *Cladosporium cladosporioides* CladGa5, *Cladosporium cladosporioides* CladGa6, *Cladosporium cladosporioides* CladGa9, and *Cladosporium cladosporioides* CladGa19. The sequences were submitted to GenBank under the accession numbers OQ780867, OR510891, OR510955, OR510957, OR510958, OR511430, and OR512395, in the order listed.

Pathogenicity tests

Effect on germination rate, coleoptiles, and root length

After 4 days, the results showed that the 7 *Cladosporium* isolates negatively affected germination rates, with the CladGa5 isolate exhibiting the greatest aggressiveness, inhibiting germination in Wah, OEB, and Bouss varieties at 100%, 92.59%, and 85.51%, respectively. On the other hand, the less aggressive isolate was CladGa3 by inhibition of germination rates of 48.15%, 28.32%, and 20.29% with OEB, Wah, and Bouss, respectively (Fig. 5). In addition, the isolate CladGa5 was significantly more aggressive ($P < 0.05$) by a reduction in root number of 100, 94.67, and 86.67% with Wah, OEB, and Bouss varieties, respectively. In contrast, the CladGa3 isolate was significantly less aggressive, with 60, 28.47, and 14.67% OEB, Wah, and Bouss, respectively (Fig. 6a).

Regarding root length, the isolate CladGa4 appears to be the more aggressive, without a statistical difference ($P > 0.05$); it gave inhibition rates of 97.65, 97.62, and 91.53% with the OEB, Bouss, and Wah varieties, respectively. The CladGa2 isolate showed low root length inhibition rates of 60.48, 49.72, and 43.19% in the Bous, Wah, and OEB varieties, respectively (Fig. 6-b).

When we consider coleoptile length reduction rates, the CladGa5 isolate shows the highest pathogenicity, with inhibition percentages of 100, 98.17, and 97.95% for Wah, OEB, and Bouss, respectively. Conversely, the CladGa2 isolate showed the lowest pathogen effect, with coleoptile length reduction rates of 57.54, 52.01, and 35.38% in Wah, OEB, and Bouss varieties, respectively (Fig. 7).

Pathogenicity on the wheat plant basal part

In contrast to the in vitro test results, the findings of this experiment varied widely. They revealed that C. cladosporioides isolates reduced root length (by 3-35%) more than shoot length (by 0-19%). The isolate CladGa5 appears to be more aggressive on root length (with no significant

difference between isolates, P > 0.05), reducing root length by 35.44% in the OEB variety. However, the CladGa6 isolate was significantly (P < 0.05) more aggressive in terms of shoot length, reducing shoot length by 45.41% in the Wah variety (Fig. 8).

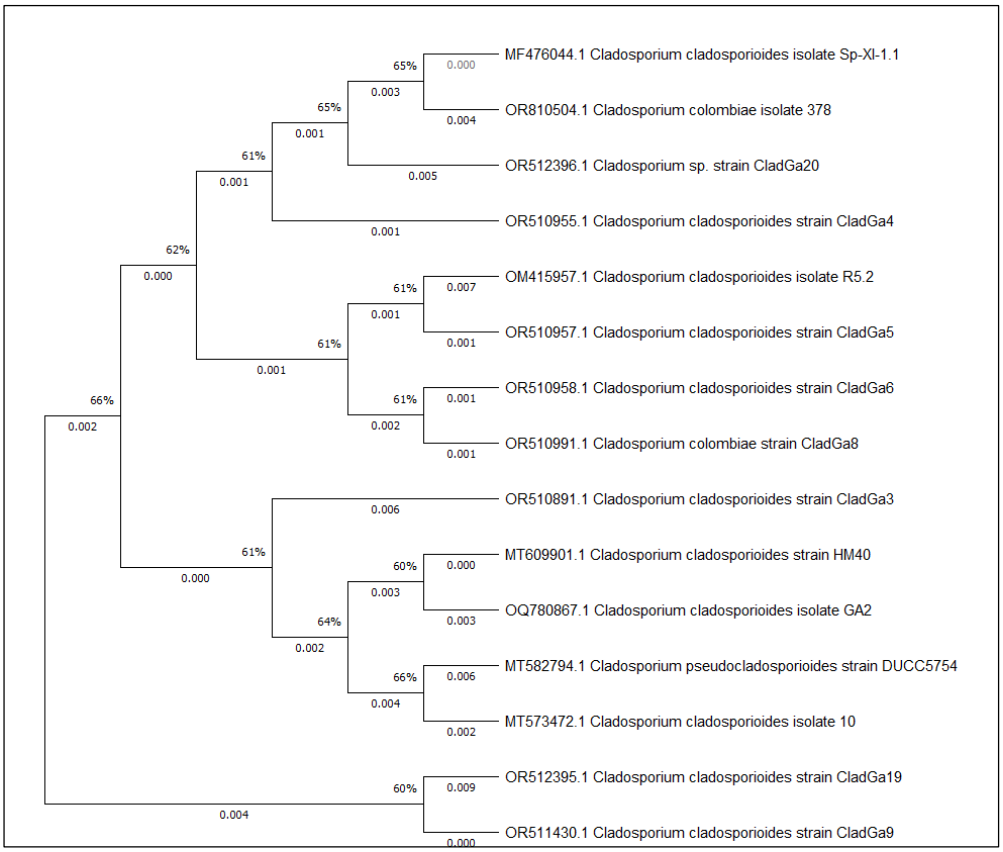

Figure 4 Phylogenetic relationships of *C. cladosporioides* isolates (CladGa2, CladGa3, CaldGa4, CladGa5, CladGa6, CladGa9 and CladGa19) inferred by Neighbour-Joining (NJ) analysis of ITS4 sequences.

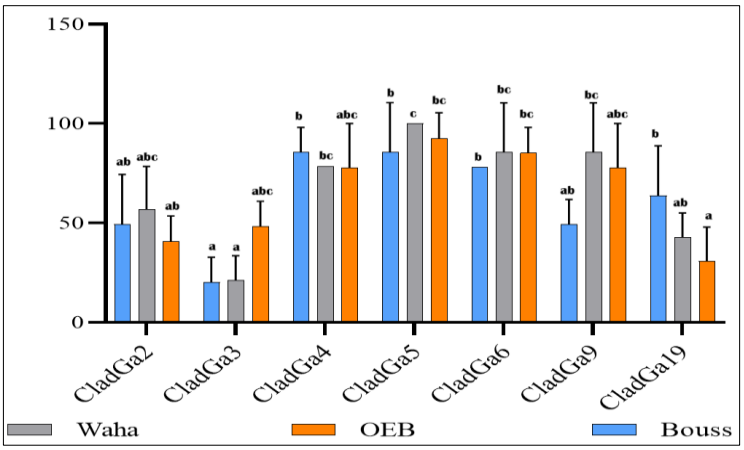

Figure 5 Germination inhibition rates* in the presence of the *Cladosporium* isolates.

* The given values are means (n = 3). Bars with the same color, marked with the same letter (s), are considered not significantly different at (P < 0.05) Duncan's test for significant differences.

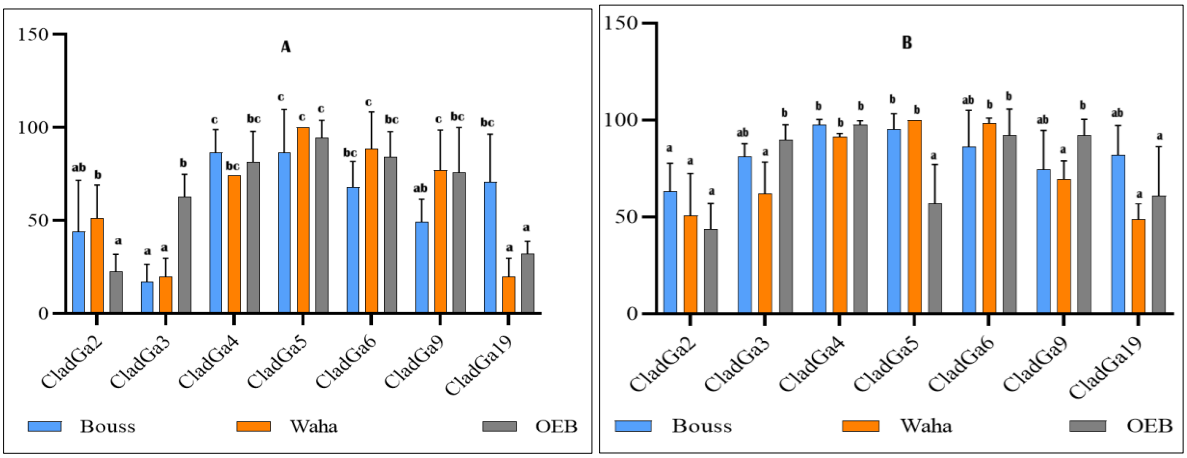

Figure 6 Inhibition rates* in the presence of the *Cladosporium* isolates: A: Reduction in root number; B: Reduction in root length. * The given values are means (n = 3). Bars with the same color, marked with the same letter (s), are considered not significantly different at (P < 0.05) Duncan's test for significant differences.

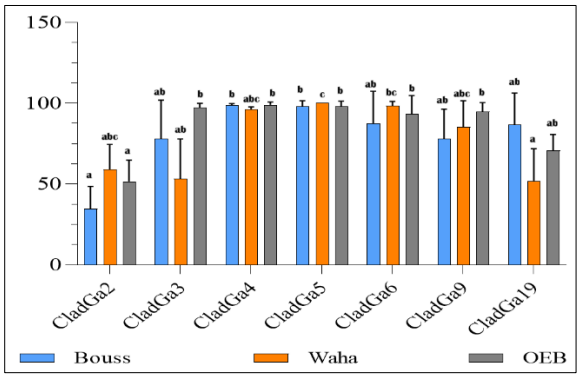

Figure 7 Coleoptile length reduction rates* in the presence of the *Cladosporium* isolates. * The given values are means (n = 3). Bars with the same color, marked with the same letter (s), are considered not significantly different at (P < 0.05) Duncan's test for significant differences.

Regarding fresh weight, the tested isolates had a detrimental effect on both the root and shoot of wheat seedlings; reductions were more than 85% and 47% for the root and shoot, respectively, with the varieties OEB and Wah. The isolate CladGa6 was significantly (P < 0.05) the most pathogen on both root and shoot fresh weight with a reduction rate of 85.56% for the OEB variety and 47.18% for the Wah variety, respectively (Fig. 8). On the other hand, the CladGa3 isolate was significantly the lowest pathogen, with average root and shoot weight reduction rates of 37.33 and 8.33%, respectively.

Statistically, the results indicated no significant difference (P > 0.05) among the three durum wheat varieties regarding root length and weight. However, a notable difference (P < 0.05) was observed among the three durum wheat varieties in shoot length and fresh weight.

Discussion

Cladosporium is a genus containing multifarious species, some of which are plant pathogens, and they have a proven capacity for causing various plant diseases, particularly in crops (Ayoubi *et al.*, 2017; El-Dawy *et al.*, 2021; Qi *et al.*, 2023). It is one of the most abundantly studied and the largest genus of hyphomycetes (Dugan *et al.*, 2004; Razak *et al.*, 2021). Pathogenic species of the genus *Cladosporium* that can negatively affect various parts of the wheat plant across different growth phases have not yet been studied in Algeria.

The studies by Bensch *et al.* (2018) and Sandoval-Denis *et al.* (2015) demonstrate that relying solely on morphological characteristics for identifying *Cladosporium* species is no longer sufficient; molecular data must be included.

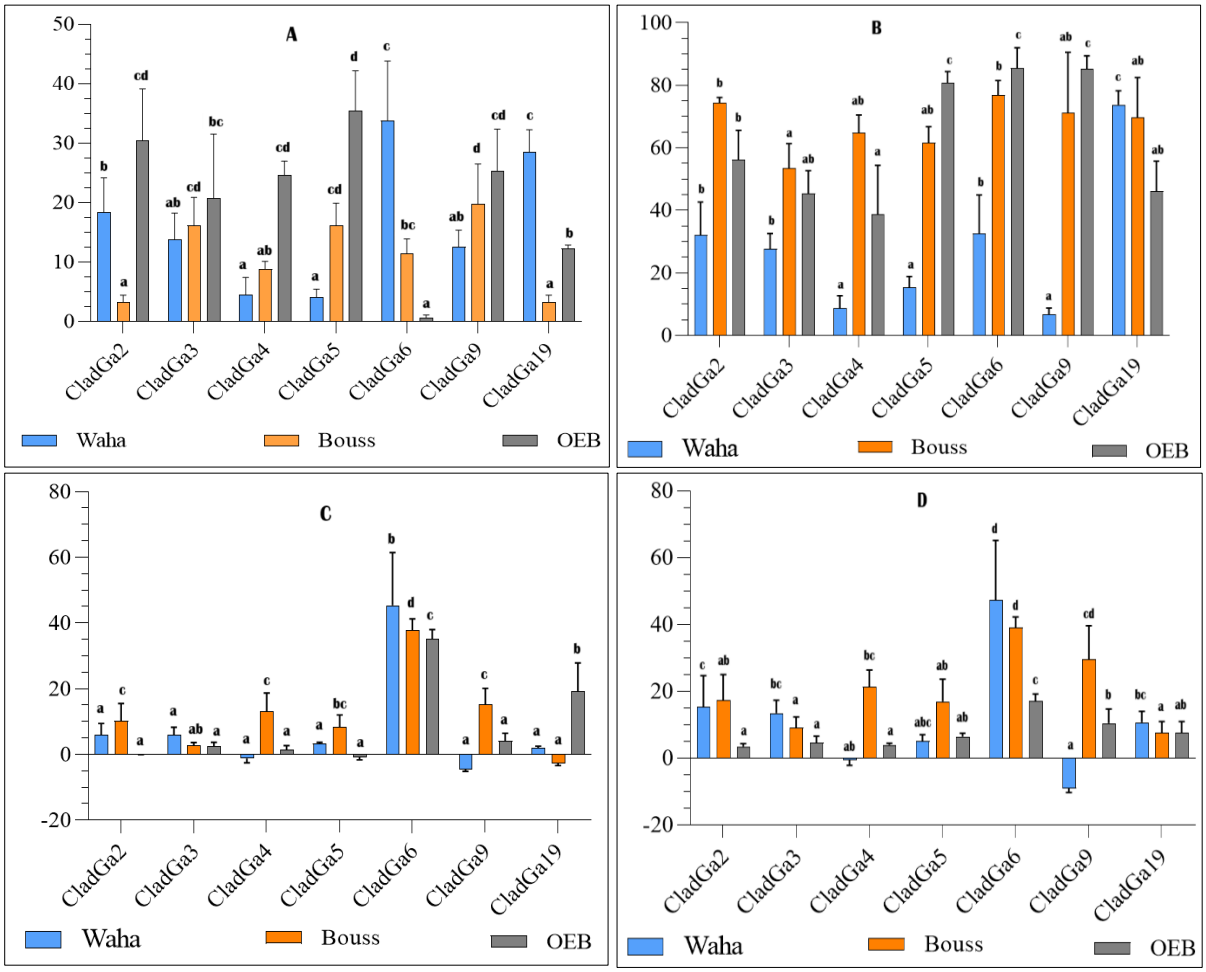

Figure 8 Effect of the *Cladosporium* isolates on wheat seedlings (reduction rates*): A: Root length reduction; B: Root weight reduction; C: Shoot length reduction; D: Shoot weight reduction. * The given values are means (n = 9). Bars with the same color, marked with the same letter (s), are considered not significantly different at ($P < 0.05$) Duncan's test for significant differences.

The macroscope study results confirmed this difficulty, especially during morphological group classification. Besides, the morphological characteristics of *Cladosporium* sp. observed in this study were consistent with several research findings (Iturrieta-González *et al.*, 2021; Razak *et al.*, 2021; Pereira *et al.*, 2024)

Although macro- and microscopic studies can provide an idea of the studied fungal genus, gaining knowledge of the species through them is very difficult. However, molecular identification is more precise and can distinguish between the different fungal species. The ITS4 gene was amplified for phylogenetic analysis. According to numerous researchers, the ITS gene could discriminate between species of *Cladosporium* (Schubert *et al.* 2007; Bensch *et al.* 2012).

The results of this study revealed the highest sequence similarity (> 99%) with GenBank *Cladosporium* sequences, and the BLAST search confirmed the precise identity as *C. cladosporioides*.

Therefore, this is the first report of this species as a pathogenic endophyte of durum wheat plants in Algeria.

On the other hand, comparing our results with other research was difficult because few studies

have examined the pathogenicity of *C. cladosporioides*. Nevertheless, other species belonging to the same genera were declared as plant pathogens of strawberries (Ayoubi *et al.*, 2017), faba beans (El-Dawy *et al.*, 2021), maize (Qi *et al.*, 2023), pine trees (Paul and Yu, 2008), tomato (Abedy *et al.*, 2022), and black point on wheat (Ogórek *et al.*, 2012; Golosna, 2022).

Although the obtained results showed that there were no disease symptoms in wheat seedlings, the re-isolation of the same fungal isolates from seedlings treated with the fungal isolates (in fulfillment of Koch's postulate) indicates that the *C. cladosporioides* isolates have an essential role in reducing both the length and fresh weight of the vegetative and root systems.

By comparing in vitro and greenhouse pathogenicity results, it can be concluded that fungal isolates (especially isolate CladGa5) were more pathogenic on wheat seeds than on seedlings, whose roots appeared very weak despite being close to the control seedlings' length.

Cladosporium is a mycotoxin-producing, potentially pathogenic fungus that can cause adverse effects on plants (Alwatban *et al.*, 2014). Mycotoxin, such as cladosporin and isocladosporin, was found by Jacyno and his collaborators (1993) to inhibit the growth of the wheat coleoptile by more than 50%. These findings are consistent with the observed coleoptile length reduction rates, which exceeded 60% in five of seven tested isolates. Therefore, there is a high likelihood that the mycotoxins produced by the tested isolates are the primary cause of the decrease in coleoptile length.

Conclusion

This study represents the first identification of seven *C. cladosporioides* isolates as endophytes within durum wheat grains under controlled conditions in Algeria. It demonstrates their adverse impact on three durum wheat varieties: Waha, Boussellam, and Oued El Bared. A significant reduction in coleoptile and root length characterizes this negative effect. Additionally, the *C. cladosporioides* isolates negatively affect various growth parameters of wheat seedlings, including the length of both the vegetative and root systems and their respective fresh weights.

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Compliance with ethical standards

Conflict of interest: All authors declare no conflict of interest.

Informed consent: Not applicable.

Additional information

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اولین گزارش از جدایه‌های بسیار بیماری‌زای *Cladosporium cladosporioides* روی گیاه گندم دوروم تحت شرایط کنترل شده در الجزایر

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چکیده: براساس پژوهش‌های انجام شده روی جمع‌آوری عوامل بیماری‌زای قارچی بذرهای گندم دوروم از ۲۸ منطقه در شمال شرقی الجزایر، برای اولین بار حضور جدایه‌های *Cladosporium cladosporioides* در بذر گندم دوروم در الجزایر گزارش شد. شناسایی این جدایه‌ها از طریق بررسی‌های ماکروسکوپی و میکروسکوپی انجام و سپس با شناسایی مولکولی تأیید شد. در شرایط کنترل شده، ارزیابی‌های بیماری‌زایی روی سه رقم گندم دوروم روی میزان جوانه‌زنی، طول کلئوپتیل، طول ریشه و بخش‌های پایینی گیاهچه‌های گندم انجام شد. یافته‌ها نشان داد که جدایه‌های *C. cladosporioides* باعث کاهش معنادار در میزان جوانه‌زنی تا ۱۰۰٪ و به‌ترتیب طول ریشه و ساقه را ۳۵,۴۴ و ۴۵,۴۱ درصد کاهش دادند. علاوه‌براین، عمل بیماری‌زا وزن تر ریشه و ساقه را به‌ترتیب ۸۵,۵۶ و ۴۷,۱۸ درصد کاهش دادند.

واژگان کلیدی: الجزایر، *Cladosporium cladosporioides*، گندم دوروم، کولئوپتیل، بیماری‌زایی، ریشه