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Research Article

First Report and Molecular Characterization of *Colletotrichum gloeosporioides* Associated with Anthracnose of Olive Fruit in the Pothwar Plateau of Pakistan

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ABSTRACT

This study reports the first identification of *Colletotrichum gloeosporioides* as the causal agent of anthracnose disease on olive fruit in Pakistan. During disease surveillance in olive orchards of Pothwar Plateau (32 ° 33 ′ and 34 ° 3 ′ N, and 71 ° 89 ′ and 73 ° 37 ′ E), anthracnose symptoms were observed in multiple farmer fields with disease incidence up to 85% and prevalence of 93%. Morphological identification, followed by molecular confirmation through ITS sequencing, identified *Colletotrichum gloeosporioides* as the pathogen responsible for fruit rot/anthracnose. Pathogenicity tests confirmed its ability to infect healthy olive fruits, causing typical anthracnose symptoms. This is the first documented occurrence of *C. gloeosporioides* on olive in Pakistan, contributing significant insights into the fungal pathogens impacting olive cultivation in the region.

Keywords: Olive anthracnose, Fruit rot, *Colletotrichum gloeosporioides*, First report, Molecular characterization, Pothwar region, Pakistan

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INTRODUCTION

Olives (*Olea europaea*) are an economically important fruit crop grown worldwide, especially in Mediterranean climates. Olive cultivation is gaining traction due to the favorable climatic conditions and the growing consumer demand for olive oil in Pakistan (Ali *et al.*, 2024). However, various diseases including anthracnose impact the yield and quality of olives. Anthracnose, commonly referred to as "olive fruit rot," is caused by species of the *Colletotrichum* genus, with *Colletotrichum gloeosporioides* being one of the most aggressive pathogens responsible for substantial yield losses. This fungal disease manifests as dark, sunken lesions on olive fruit, often leading to severe rotting, premature fruit drop, and reduced oil quality (Talhinhas *et al.*, 2018). Under favorable environmental conditions of humidity and temperatures, the disease spreads rapidly, making it a major concern for olive growers. Infected fruits develop orange to pinkish spore masses, which facilitate secondary infections and further disease proliferation within orchards. Globally, *Colletotrichum gloeosporioides* causes anthracnose in a wide variety of fruits, including almonds, avocado, apple, arabica coffee, cashews, banana, citrus, grapes, guava, mango, strawberry, papaya, passion fruit, and crops including cereals, and

legumes (Bailey and Jeger, 1992; Elizabeth and Mordue, 1967). Additionally, species of *Colletotrichum* are frequently found on decaying wild fruits (Tang *et al.*, 2003) . Anthracnose not only affects olive fruit yield but also has a significant impact on oil quality. Infected olives undergo biochemical alterations that lead to increased acidity, reduced antioxidant content, and degradation of essential fatty acids, all of which compromise the overall quality and market value of olive oil (Talhinhas *et al.*, 2018). The disease-induced deterioration accelerates lipid oxidation, resulting in undesirable sensory attributes such as rancidity and off-flavors (Peres *et al.*, 2021). Moreover, anthracnose infection leads to a reduction in polyphenol and tocopherol levels, which are crucial for the oil's stability and health benefits. Studies from other olive-producing regions have shown that anthracnose-affected olives yield oil with lower extraction efficiency and altered chemical composition, making it unsuitable for extra virgin olive oil classification (Peres *et al.*, 2024). *Colletotrichum* species have been reported as causing anthracnose in olives in different parts of the world (Gharsallah *et al.*, 2023). However, limited information is available regarding their occurrence in Pakistan. This study presents the first report of *C. gloeosporioides* as the causative agent of anthracnose on olive fruit in the Pothwar Plateau, Pakistan, and details its morphological and molecular identification.

MATERIALS AND METHODS

Survey and Sample Collection

Disease surveillance was conducted in the olive orchards of Pothwar Plateau of Pakistan, specifically at the (32 ° 33 ′ and 34 ° 3 ′ N, and 71 ° 89 ′ and 73 ° 37 ′ E) coordinates. Anthracnose symptoms were observed in various farmer fields, and disease incidence and prevalence were calculated using the percentage formula below.

$$\text{Disease prevalence } (\%) = \frac{\text{Locations displayed disease symptoms}}{\text{Total no. of locations under surveillance}} \times 100$$

$$\text{Disease incidence } (\%) = \frac{\text{No of diseases plants}}{\text{Total no. of plants observed}} \times 100$$

Random sampling from 23 different farmer fields yielded 70 infected olive fruits, which were transported to the laboratory for pathogen isolation and identification.

Isolation and Identification of Pathogen

Infected olive fruits were surface sterilized with 70% ethanol and then placed on potato dextrose agar (PDA) plates and incubated at 25C° with 12h alternate light and dark cycle to isolate the pathogen. The pathogen was identified based on its morphological characteristics. (Weir *et al.*, 2012)

Molecular Identification

For molecular confirmation, DNA was extracted from fungal colonies by Zymo Research Fungal /Bacterial Miniprep Kit following the steps of standard protocol (Ekpa *et al.*, 2016) and amplified using the internal transcribed spacer (ITS) primers ITS1 and ITS4 (Mills *et al.*, 1992). Agarose gel (1%) was prepared with SYBR Green dye (5µl), and after solidifying, DNA samples (15µl) were loaded into the gel for electrophoresis at 100 Volt, 60 mAmp, 6 Watt. After 30 minutes, DNA bands were visualized using a UV transilluminator (Lee *et al.*, 2012). Positive samples were sequenced at Biodesign Institute, Arizona State University, USA, and sequences were analyzed using MEGA 11 and BLAST, with phylogenetic relationships inferred through the Maximum Likelihood method (Garrido-Cardenas *et al.*, 2017; Tamura *et al.*, 2011).

Pathogenicity Test

To confirm the pathogenicity of the isolated fungus, mycelial plugs (5 mm in diameter) were placed on disinfected, healthy olive fruits of the Gemlik variety (Chliyeh *et al.*, 2014). These fruits were placed on three layers of moist blotter paper inside food-grade, sterilized plastic boxes (5L × 3W × 2H inches) and incubated at 25°C under a controlled photoperiod of 12 hours of light and 12 hours of darkness, using fluorescent tube lights. Fruits inoculated with PDA plugs without the pathogen serve as control. After 7 days, typical anthracnose symptoms, such as dark, sunken lesions with concentric rings, were observed on the inoculated fruits. Fungal re-isolation from these fruits confirmed that the pathogen was *C. gloeosporioides*, fulfilling Koch's postulates (Walker *et al.*, 2006).

RESULTS

Symptomatology Incidence and Prevalence

The surveyed orchards demonstrated a disease incidence of up to 85% and prevalence of 93% (Figure 1). The initial symptoms of anthracnose on olive fruits included small, dark, sunken spots or lesions. As the fruit matured, these lesions expanded and developed concentric rings (Figure 2). A distinctive orange or pink spore mass was observed on the surface of the lesions, typical of anthracnose infection as shown in Figure 2 (Rhouma *et al.*, 2010).

The fungal isolates from the infected fruits were identified based on colony morphology and conidial characteristics. The colonies on the PDA were white from the front and turned pink and orange from the inverted view (Figure 3 a&b). Conidia were aseptate, hyaline, oval, and elongated, measuring 12.38 ± 2.48 µm to 14.85±2.48µm in length and 4.95 ± 0.00 µm in width, (Figure 3c) consistent with the genus *Colletotrichum*. The conidial morphological features and size align with the description provided by Ritchie and Cannon (2003). According to their key, the conidia of *C. gloeosporioides* measure between 9–24 µm in length and 3–4.5 µm in width.

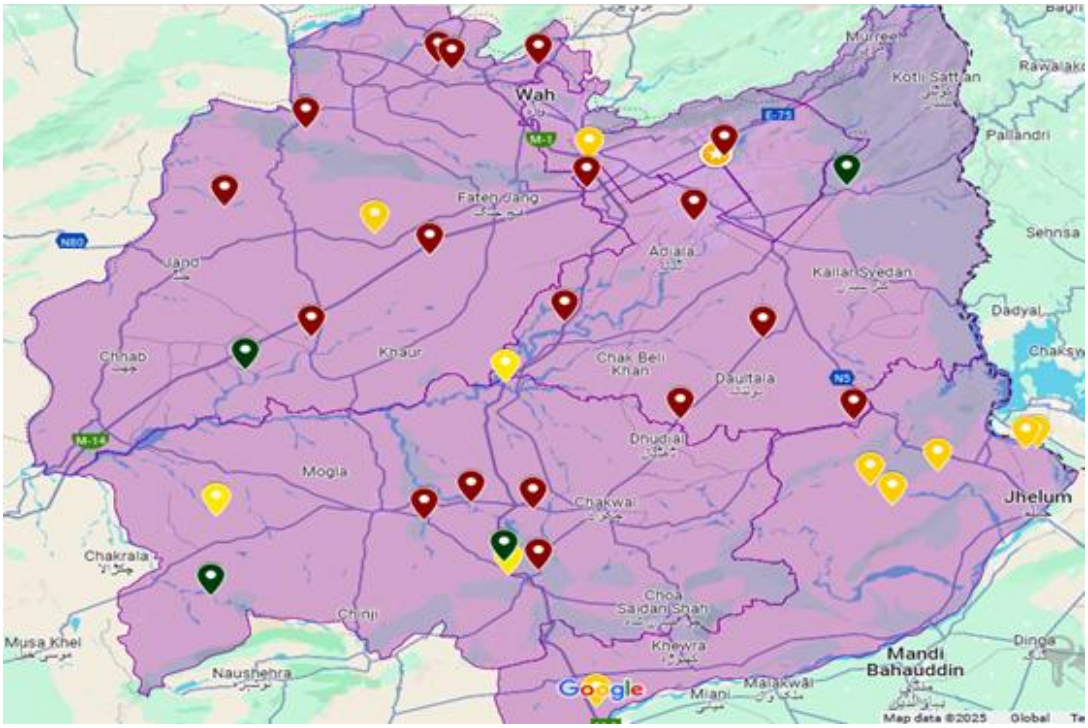

Figure 1. Geographical distribution olive anthracnose in Pothwar. Red pin (disease), yellow pin (no fruiting), and green pin (no disease).

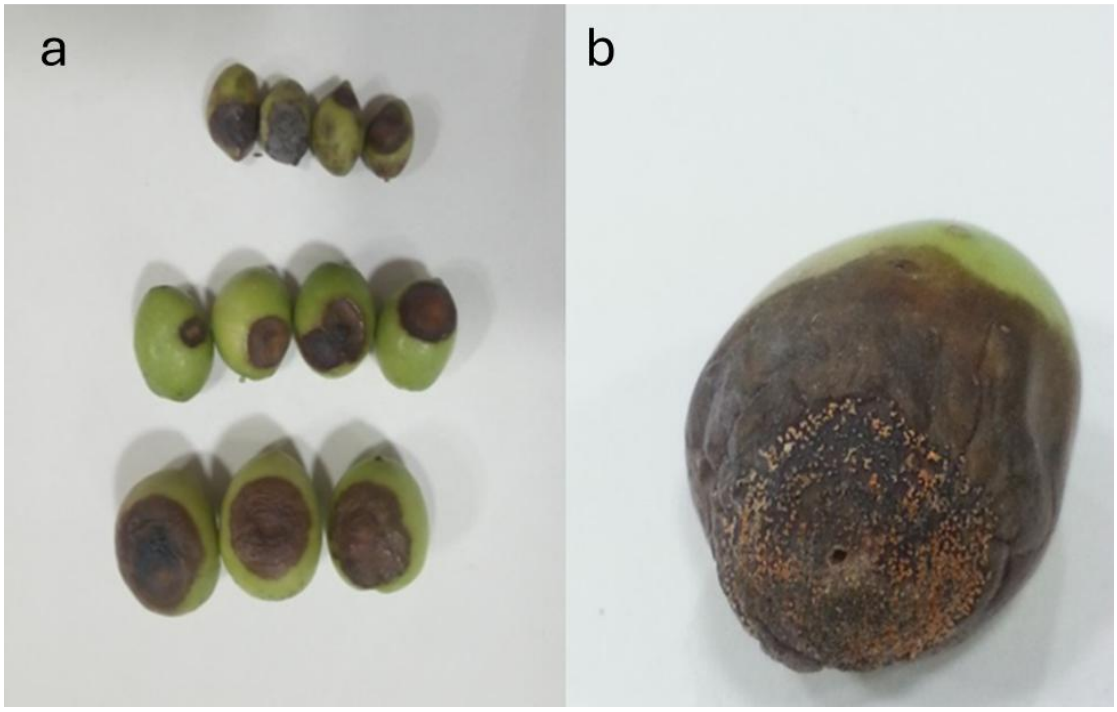

Figure 2. Morphological Identification based on symptoms (a) Anthracnose symptoms on small medium and large size varieties of olive, (b) Orange spore mass on olive fruits.

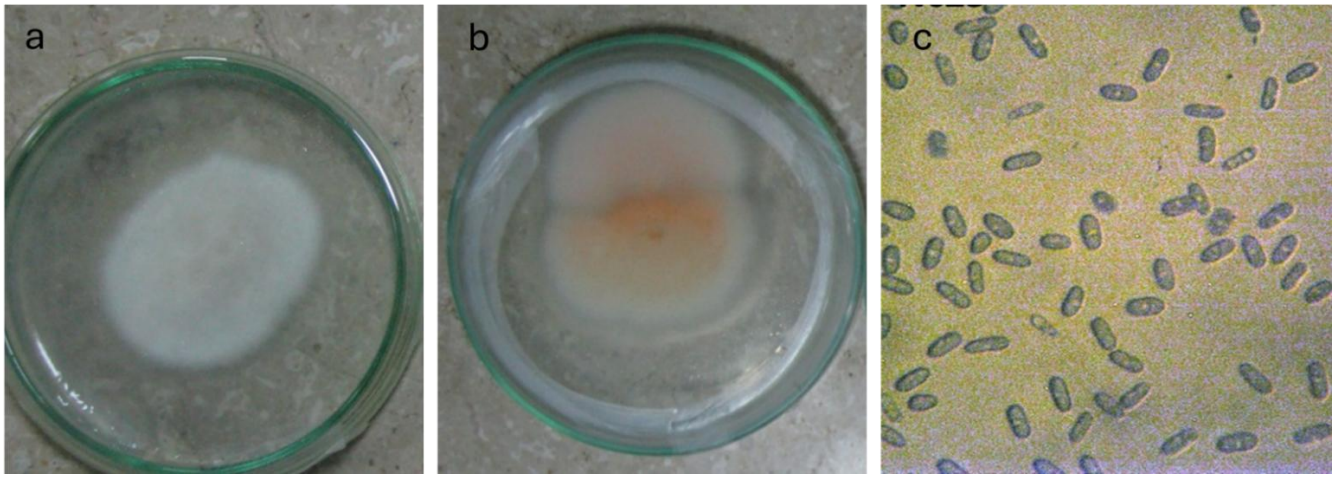

Figure 3. Morphological character of *Colletotrichum gloeosporioides* (a) Front view (b) Inverted view (c) Conidia

Molecular Identification

The PCR product was run on a gel, where a clear band around 450-500 bp indicates the amplification of the specific region from the DNA (Fig 4). This size range suggests that the amplification was successful for the ITS1-5.8S-ITS4 region, which is often used for identifying fungal species, particularly in molecular diagnostics.

Molecular identification using Sanger sequencing confirmed the pathogen as *Colletotrichum gloeosporioides*. Sequencing of the 5.8S region revealed a 100% similarity to the reference sequences of *Colletotrichum gloeosporioides* (MF380788.1, MH370162.1, MH156758.1, MF380828.1, MF380675.1, KX463018.1) available in GenBank (Figure 4). The sequence data revealed similarity to multiple reference sequences in GenBank, thereby identifying the fungus at the species level.

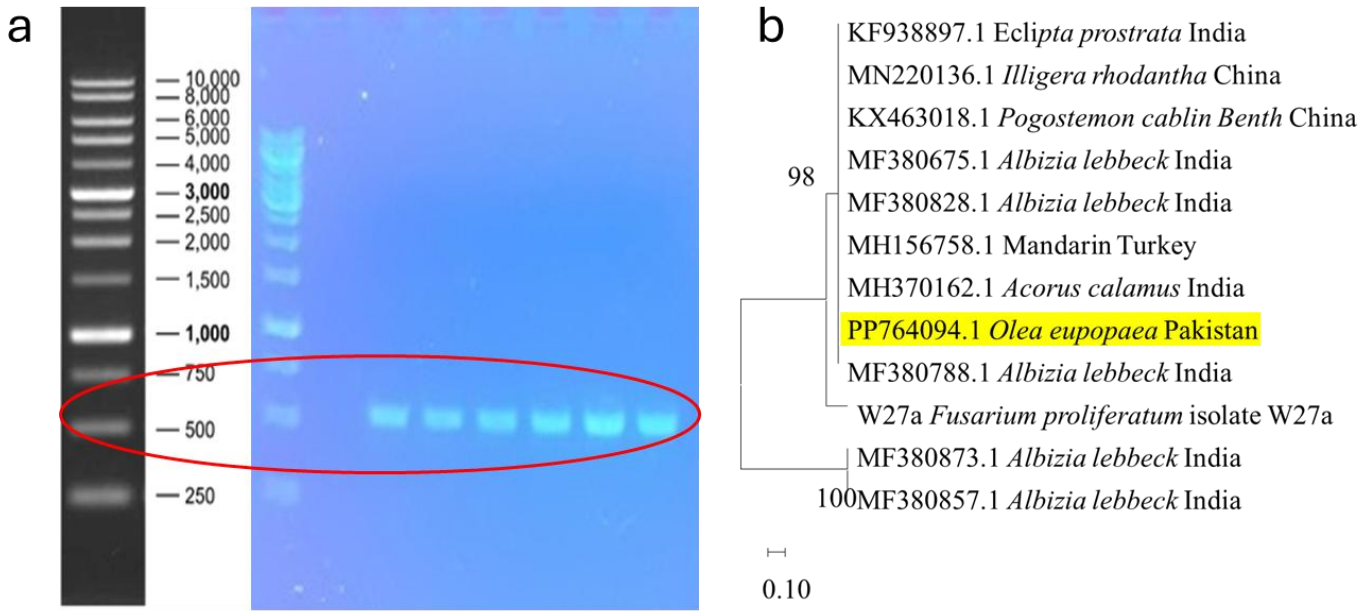

Figure 4. Molecular characterization of *Colletotrichum gloeosporioides* (a) 450-500 bp amplicon of ITS1-5.8S-ITS4 from highly virulent isolates by PCR, (b) Phylogenetic analysis of *Colletotrichum gloeosporioides* causing anthracnose of olive. The DNA ladder is 1Kb ladder.

Pathogenicity

After 7 days, typical anthracnose symptoms, such as dark, sunken lesions with concentric rings, were observed on the inoculated fruits shown in Figure 5. The pathogenicity test successfully reproduced the typical anthracnose symptoms on healthy olive fruits, confirming that *C. gloeosporioides* is capable of causing anthracnose on olive. This was further supported by the re-isolation and morphological identification of the pathogen from the symptomatic fruits.

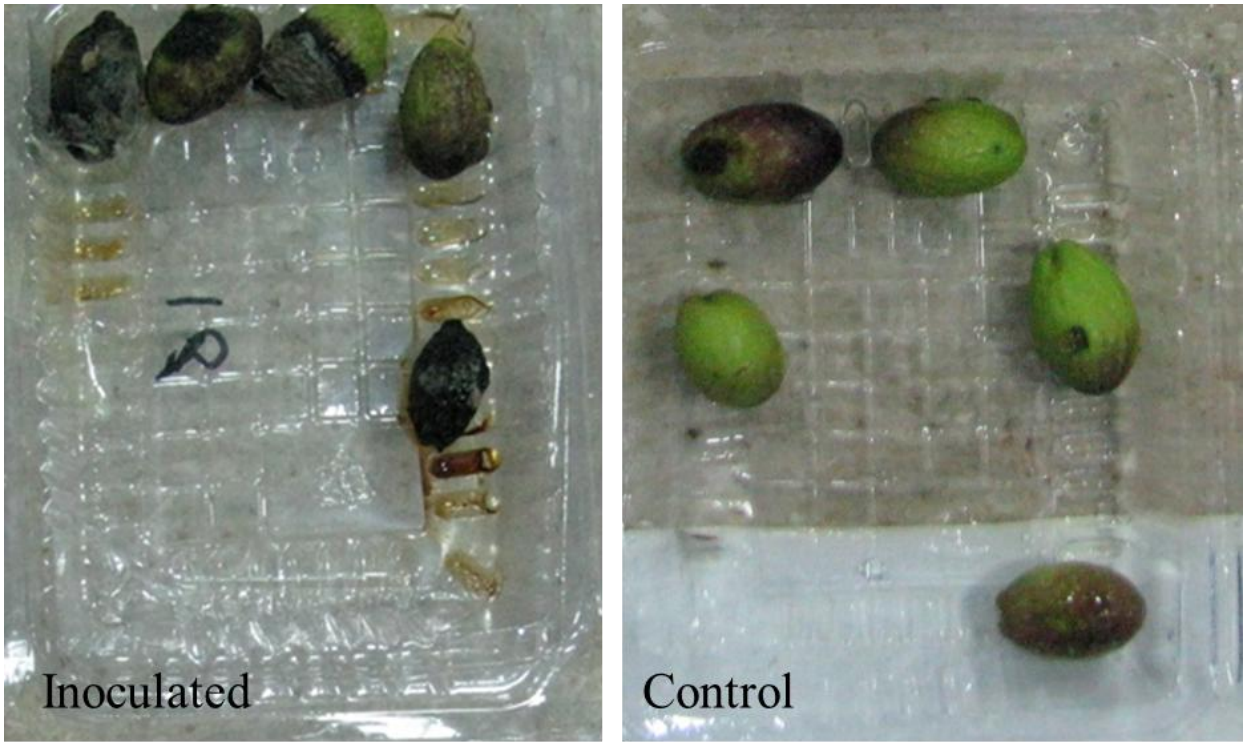

Figure 5. Pathogenicity test of *Colletotrichum gloeosporioides*, comparison between inoculated and control

DISCUSSION

Colletotrichum gloeosporioides is a widely distributed fungal pathogen that causes anthracnose disease in olives (*Olea europaea*) and other crops (Moral *et al.*, 2021). This pathogen is commonly found in Mediterranean climates, particularly in Italy, Spain, Greece, and Tunisia (Licciardello *et al.*, 2022; Markakis *et al.*, 2021; Rhouma *et al.*, 2010). Additionally, it has been reported in regions outside of the Mediterranean, including South America (notably Brazil), Australia, and other parts of the world (Duarte *et al.*, 2010; Sergeeva *et al.*, 2008). The severity and spread of the disease caused by this pathogen are mainly determined by climatic factors, particularly the humidity and fruit ripening stages, which contribute to the disease's intensification.

This study provides maiden efforts to report *Colletotrichum gloeosporioides* as a pathogen responsible for anthracnose disease in olives. The disease symptoms observed in the Pothwar Plateau are similar to those reported in other olive-growing regions, where *C. gloeosporioides* has been identified as the primary pathogen. The ability of the pathogen to infect olive fruits at various stages of maturity suggests its potential to cause significant yield losses if not managed properly. Accurate disease diagnosis and management strategies rely heavily on the molecular confirmation of *C. gloeosporioides* through ITS sequencing. The elevated incidence and prevalence of anthracnose in the orchards surveyed points to the urgent requirement for effective control approaches, including cultural practices, the use of resistant cultivars, and fungicide application.

CONCLUSION

This study represents the first documented instance of *Colletotrichum gloeosporioides* causing anthracnose on olives in Pakistan. Due to the high disease incidence and prevalence observed in the Pothwar Plateau, it is crucial to introduce integrated pest management strategies to control anthracnose in olive orchards. Further research is needed to explore the pathogen's genetic diversity, resistance mechanisms in olive cultivars, and the development of effective control measures tailored to local conditions.

AUTHOR CONTRIBUTIONS

Syed Kamil Husnain: Surveillance Writing – original draft, Writing – review & editing, Formal analysis. Farah Naz: Conceptualization, Validation, Supervision. Ramzan Anser: Resources, Validation. Azhar Mustafa: Writing – review & editing. Sajid-ur-Rehman: Writing – review & data analysis. Absar Alum: Conceptualization, Validation, Supervision, sequencing and phylogeny.

CONFLICT OF INTEREST

There is no potential conflict of interest in the research and manuscript.

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