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Identification and characterization of fungal pathogens associated with black root rot on saffron from Southern Khorasan, Iran

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AbstractSaffron, a spice derived from the dried stigmas of Crocus sativus flowers, is renowned for its high cost and culinary value. As a perennial herbaceous plant belonging to the Iridaceae family, saffron is susceptible to diseases such as corm rot, which has previously been attributed to various fungal pathogens including Fusarium, Penicillium, and Botrytis. It has been observed that soil-borne fungi are consumed by the bulb mite. Nonetheless, a significant amount of saprophytic fungi can be found in the soil of saffron fields. This may lead one to wonder if the saffron corm mite is a main or secondary pest. In this study, we aimed to identify the underlying causes of the early yellowing of saffron leaves and the reduction in the quantity and quality of this valuable product. A total of 100 plant samples displaying symptoms of infection, along with their corms, were collected and analyzed from various saffron cultivation areas in Qaen City, Southern Khorasan Province. Our findings revealed that the fungal genera Rhizoctonia and Curvularia were the primary causes of saffron root rot. This is the first report of Curvularia associated with saffron in Iran and other countries. The benefits of the disease-related information presented here extend to a diverse group of stakeholders, including farmers, learners, researchers, plant protection organizations, development departments, extension workers, policymakers, government agencies, and public organizations.

Keywords: Curvularia, Qaen city, Rhizoctonia, Rhizoglyphus robini Claparede, Saffron

1.Introduction

The most precious and valuable plant in the world, and indeed in Iran, is undoubtedly saffron (Cardone et al., 2020; Kalha et al., 2008). For approximately 3500 years, saffron has been highly valued as a precious spice. Its extensive medicinal benefits and versatile pharmacological applications have earned it the moniker "golden condiment," resulting in increased demand and usage over time. While cold temperatures can stimulate vegetative growth, saffron thrives best in dry and moderate regions. With a significant contribution of 88.80%, Iran leads the world market for saffron, with India coming in second at 5.80%. Other countries that produce saffron include Greece, Afghanistan, Morocco, Italy, Spain, China, and

Azerbaijan, with respective contributions of 1.90, 1.58, 0.68, 0.60, 0.26, 0.26, and 0.06% (Gupta et al., 2021). Saffron, a spice of great esteem, has numerous culinary and medicinal applications (Vo et al., 2021), and its supply has been gradually increasing in some countries. In sustainable agriculture, saffron can provide an additional source of income, enabling farms to be more multipurpose, diversify their output, and encourage gastronomic tourism. A decrease in saffron production has resulted from several factors. Over the last ten years, Iran has produced more saffron, with an average yield of 3.53 kg/ha; production went from 59,000 ha and 230 t in 2007 to 108,000 ha and 376 t in 2017. Iran (48%), Spain (27%), Greece (2.9%), France (2.7%), Afghanistan (7.7%), Hong Kong (2.5%), and Portugal (2%) are the major exporters of saffron. The biggest importers are Saudi Arabia (4.2%), Sweden (3.8%), Argentina, and the United Arab Emirates (2.5%). Spain (23%), Hong Kong (8.7%), the United States (7.6%), China (6.2%), France (5.2%), and India (7.0%) are the next highest importers (Cardone et al., 2020). The main obstacle to its successful growth is the failure to generate sufficient viable corms to be used as seeds. This crop also needs a lot of work to be cultivated (Agayev et al., 2009), and the global decline in saffron production has been attributed to several factors, including persistent genetic erosion, lack of mechanization, continued use of outdated farming practices, and corm rot disease (FernÃ¡ ndez et al., 2006). Owing to these limitations, saffron-producing countries have found it more difficult to produce, which has limited the crop's industrial output and kept it out of reach for consumers at an affordable price. Due to these and other considerations, saffron has become a highly researched crop in terms of growing techniques, possible therapeutic uses, and biotic stress management, all of which harm saffron output (Chen et al., 2020).

In Southern Khorasan Province, saffron is vital to both social and economic ties. Many harmful factors affect saffron, as they do in other plants, and these factors affect the amount and quality of the plant (Gupta et al., 2021). Corm rot is one such limiting factor that reduces the area under cultivation and production of saffron. The disease adversely affects the formation of daughter corms and drastically reduces the yield. Because of the existing knowledge regarding the epidemiology and etiology of the disease, a thorough understanding of the condition is necessary. As resistant genotypes are not widely available and there are no superior cultural practices, there are currently no effective and long-lasting management approaches in place (Vo et al., 2021). According to reports from France and Spain, *R. crocorum* is a fungus that damages corms by piercing their outer sheaths (García et al., 2006). Numerous fungi, such as *Rhizoctonia crocorum* and *Fusarium oxysporum* f. sp. gladioli, *Fusarium solani*, *Fusarium moniliforme*, *Macrophomina phaseolina*, and *Pythium ultimum*, are responsible for saffron root and corm rot. Since these fungi live so long in the soil, it will take some time to eradicate them from the affected fields. Corm rot can also be brought on in fields and warehouses by *Aspergillus niger*, *Penicillium digitatum*, and *Rhizopus stolonifer* (Mirghasempour et al., 2022; Ren et al., 2023). The degradation of Kashmir's saffron farms has been linked to the fungi *Rhizoctonia crocorum* and *Fusarium solani* (Kalha et al., 2008). Research has also revealed that these fungi are present on Iranian saffron farms (Kafi et al., 2002).

The fungal genus *Curvularia*, belonging to the Pleosporaceae family, is ubiquitous in various environmental habitats such as water, soil, and plants and can cause infection in both human and animal hosts. *Curvularia* species are predominantly saprobic organisms that are commonly found in tropical regions in association with both host and non-host living animals. Some of these species also can act as opportunistic pathogens globally. Moreover, certain Curvularia organisms are known to reside within different plant species as endophytes. The genus *Curvularia* was first described in 1933, with *C. lunata* being designated as the type species (Heidari et al., 2018). The fungus in question consists of approximately 59 identified species. Its spores are noteworthy for their unique and visually appealing characteristics, and the name of the fungus derives from its distinctive bent shape. When examined under a microscope, the spores exhibit smooth exterior walls that are non-parallel and transverse, and they adopt a curving, boomerang- to roughly spindle-shaped form. At the macroscopic level, the fungus produces fluffy mycelia on agar culture substrates. Members of the genus *Curvularia* are divided into three groups: geniculata, lunata, and maculans, based on the number of transverse walls in their spores and the hosts to which they are assigned (Hosokawa et al., 2003). Because the environment in which spores are formed has a significant influence on their morphology, differentiating between the species within each group can be difficult. As a result, the demarcation between several species within a group can sometimes be arbitrary and subjective. Some species of this particular fungus are hazardous to humans, animals, and plants. Due to their exceptionally large size, spores from the species *C. lunata* have been linked to human infection. After inhalation, spores remain in the respiratory system and cause hay fever and recurrent allergies that cause the nasal mucosa to grow. Diseases caused by this fungus are especially dangerous for monocotyledonous plants, including sorghum, corn, and wheat. In 1948, Majie was the first to report that the *Curvularia* pandemic on the iris was the source of the sickness. *Curvularia trifolii* f. sp. Gladioli is one of the significant soil fungi that Maji identified in 1975 as causing iris rhizome rot, vascular tissue damage, and root damage in tropical climates (Parmelee et al., 1956). Certain *Curvularia* species have been identified as the etiological agents responsible for leaf spots on grasses. Additionally, multiple *Curvularia* species have been characterized as pathogens that lead to seed rot and seedling blight, root rot, leaf spot and blight, and grain discoloration and deformation (Karimzadeh et al., 2021). The following Iranian species were identified through the application of molecular phylogeny and morphology: *C. americana*, *C. muehlenbeckiae*, and *C. verruculosa*, which were recovered from cowpea, maize, and bottlebrush plants in the Khuzestan province (Janbozorgi et al., 2019); *C. spicifera*, *C. inaequalis*, and *C. nicotiae* causing spot symptoms on *Salvia officinalis* from Chaharmahal and Bakhtiari province (Karimzadeh et al., 2021); and *C. inaequalis* on *Pyrus communis* causing leaf blight and black root rot symptoms from Kermanshah province (Jamali et al., 2020);. It has just come to light that *Curvularia shahidchamranensis*, a unique species in the genus *Curvularia*, has been discovered and examined on soil affected by crude oil in Ahvaz, which is located in the Khuzestan province. This type of fungus was shown to tolerate crude oil and only displayed minor growth inhibition when exposed to conditions modified with up to 50% (v/v) of crude oil (Dehdari et al., 2018). To the best of our knowledge, there is no evidence linking any species of this fungus to saffron.

2. Materials and Methods

Sample collection, fungal sectioning, and morphological characterization. Corms from saffron plants that showed early symptoms of yellowing in 2022 may have been affected by a disease. To study these corms, the entire plant was carefully transported to the laboratory in plastic bags at each sampling stage. The corms were then cleaned with tap water. Rotting roots, approximately one to two centimeters in length, were treated by immersing them in 70% ethanol for 30 seconds, followed by a one-minute soak in 2% sodium hypochlorite. After three rinses with sterile distilled water, the corms were allowed to air-dry at room temperature. Sample plates were prepared by combining 200 grams of potato, 20 grams of dextrose, and 15 grams of agar per liter of distilled water. These plates were then incubated in the dark at 25 °C. Fungal colonies began to form on and around the roots after three days. After ten days, the colonies were purified on fresh PDA plates using the single-spore technique. To isolate individual spores, a piece of the culture medium with sporulation was transferred to a test tube containing 9 cm^3 of sterilized distilled water. The mixture was then stirred to create a homogenous environment. One milliliter of this suspension was added to nine cm^3 of sterilized distilled water in another test tube. The spore density in the suspension was examined using a light microscope. The dilution process was repeated until the desired spore density was achieved. Pure colonies were then analyzed for morphological characteristics and the structure of conidiogenesis after being incubated on 2% water agar.

Pathogenicity tests. For healthy saffron corms, a pathogenicity test was conducted on five representative isolates selected from the surveyed origins. Pathogenicity testing was performed following Koch's postulates. After surface sterilization of the corms for 10 minutes with 5% sodium hypochlorite and 1 minute with 75% ethanol, they were washed three times with distilled water. To inoculate the intact plant material, a final suspension containing 1×107 conidial/ml was prepared. The corms were then transplanted into sterile substrates (black/white peat, perlite, and vermiculite; 2:1:1) after being immersed in a 200 ml conidial suspension for 24 hours. The substrates were kept at a controlled temperature of 25 °C with 12 hours of light and darkness for three weeks. The last observation of disease progression was performed approximately three weeks after inoculation. Sterile water-inoculated corms were used as negative controls (Chen et al., 2020; Gale et al., 2005; Hu et al., 2022).

3. Results and Discussion

Given that saffron corms are underground organs, they are more vulnerable to bacterial and fungal invasion. Apart from the microbes in the soil, the production of saffron and the quality of corms are greatly influenced by the surrounding conditions, soil chemistry, irrigation, and fertilizer use. The saffron corm rot complex has been associated with several plant diseases. The microbial population and its interactions in the rhizosphere and cormosphere of *Crocus sativus* are not only specific to individual plants but are also affected by the different stages of plant growth. The climate conditions affect the seasonal dynamics of the microbial community. Saffron suffers significant yield losses owing to several plant diseases that can cause corm rot disease, either individually or in combination. In this study, 90 of the 100 samples of saffron plants displaying disease symptoms showed evidence of fungal colonization after growth on a laboratory culture medium. Five infected samples contained *Rhizoctonia* and *Curvularia* spp.. *Curvularia* infection was observed in 85 independent samples.

Because of the previously published data on *Rhizoctonia*, the high frequency of root infection with *Curvularia*, and the lack of reports on saffron, this study focused on *Curvularia*. The majority of *Curvularia* species were detected in plant and human tissues, both living and dead (Dehdari et al., 2018). In this study, plants infected with *Curvularia* display burning at the tips of their leaves, as well as dark burned scars on their margins, and indications of ablation. Additionally, the roots of these affected plants showed symptoms of black rot. The length and diameter of the leaves of the infected plants were substantially smaller than those of healthy plants (Figure 1). Notably, leaf

Figure 1. Symptoms of saffron root rot associated with *Curvularia* spp. and *Rhizoctonia* spp.; (A) Black root rot; (B) Symptoms on leaves in the field; (C) Healthy plant

Characteristics of *Curvularia* colonies on PDA. The fungal mycelia on the PDA culture medium were puffy and had a dark olive green color. The growing rings of the mycelium were not concentric, and there were no signs of stroma on the surface (Figure 2).

Figure 2. A colony of *Curvularia* on PDA

Morphology of conidia, conidiophores, and conidiogenous cells. Conidiophores ranged in length from 15 to 67.5 (54.1) µm and width from 5 to 7.5 (5) µm. They are light brown, solitary, sometimes two-branched, zigzag, and smooth. Additionally, they can occasionally be distinct from, and similar to, vegetative mycelia. For smooth conidiophores, the length ranged from 8 to 12 (10.2) µm, whereas for zigzag conidiophores, it was 45 to 112.5 (50) µm. Conidia usually have smooth surfaces with three to five distinct black transverse walls. It has occasional-

Figure 3. A-C, Conidum with 6, 7 and 8 transverse walls, respectively.

It is challenging to distinguish between distinct species of *Curvularia* because of the darker middle cells compared to the end cells, the oval, curved oval, or egg-shaped conidia, as well as other shapes that appear to vary depending on environmental circumstances (Figure 4).

Figure 4. Different types of conidium in *Curvularia*.

It was observed that the conidia at the tip germinated apical or axillary (Figure 5).Conidia were found to be enormous, measuring 35–57.5 (35) and 10.5–12 µm (13 µm) in length and width, respectively. Some of the conidia in this collection have a somewhat enlarged base cell of the conidiophores toward the conidiogenous cell, where it is connected to the conidiophore (hilum) (Figure 6).

Upon staining with cotton blue, the conidiophores and conidia did not exhibit any pigmentation or paleness. The conidiogenous cells continued to grow beneath the preceding conidia, and the conidiophores had a zigzag shape. There was a scar where a fall occurred when the conidia were separated (Figure 7).

Figure 5 Germination manners in *Curvularia* conidium

Figure 6. Connection points of conidiophore and conidium

Figure 7. Conidiogenous cell and Scar

Conidia were arranged in a single, terminal, and sub-terminal pattern on the conidiophores. A maximum of four conidia, ranging in color from olive green to pale brown, were visible on each conidiophore in the water-agar culture medium (Figure 8).

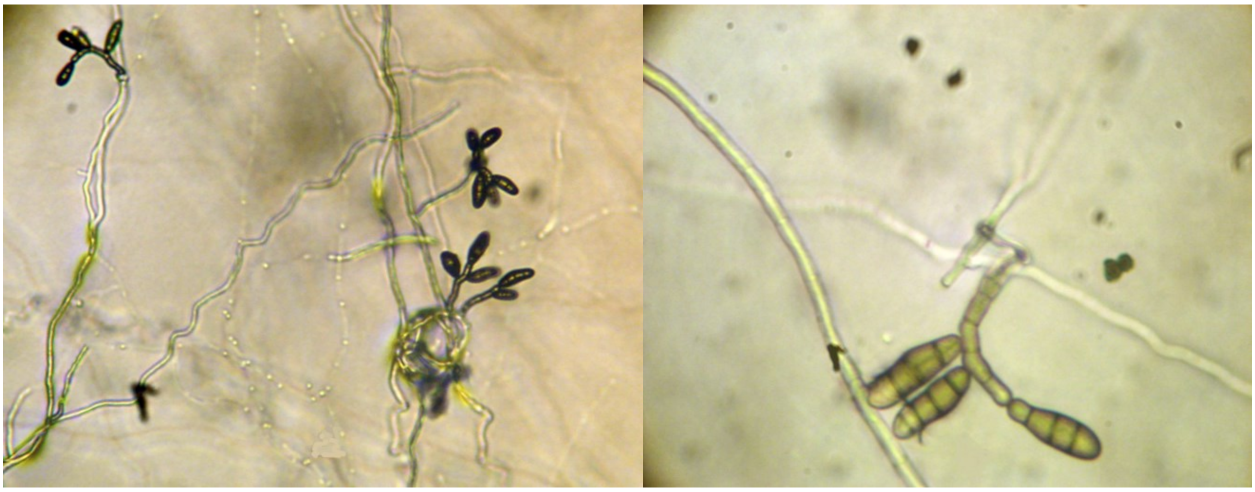

Figure 8. Arrangement of conidia on conidiophores in the water-agar culture medium

4. Conclusion

In summary, this study suggests that these species are heterothallic, as no sexual forms developed in cultures with monospored populations. Based on the characteristics of mycelium on the culture medium, conidia on conidiophores, conidiogenic cells, and conidia size and morphology, these species seem to be members of the group of *geniculata*.

It is thought that a significant contributing cause to the increase in this infection is the saffron mite (*Rhizoglyphus robini* Claparede) (Palevsky et al., 2022). The saffron bulb mite has long been regarded as a major pest, and conventional means of control have included the use of chemical miticides (for disinfection, among other purposes) in addition to certain non-chemical techniques .For the acarine pest to infiltrate and establish itself on the saffron corms, it needs fungi that are born in the soil. Otherwise, there is a direct correlation between fungal infection and *R. robini* damage to saffron bulbs. It implies that to improve management tactics for this pest, we need to take into account the involvement of saprophytic fungi as a primary factor that creates the conditions necessary for bulb mite colonization and damage to occur. It is suggested that more research be done utilizing suitable techniques to inhibit soil-born fungus and the damage that bulb mites cause to saffron (Amiri-Jami, 2023). As a result, little emphasis has been paid to the function of fungi that are native to the soil. Finding the appropriate fungicide to treat this condition and correctly identifying the species of this fungus are the main goals of the next part of this research, which should soon be made available to users.

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