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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Isolation and identification of *Podospora flexuosa* (syn. *Cladorrhinum flexuosum*), a potential biocontrol agent detected in sugar beet cultivation areas in Türkiye

Türkiye'de şeker pancarı ekim alanlarından tespit edilen potansiyel biyokontrol ajanı *Podospora flexuosa* (syn. *Cladorrhinum flexuosum*)'nın izolasyonu ve tanımlanması

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ABSTRACT

Sugar beet (*Beta vulgaris* L.) is widely cultivated in Konya province of Türkiye and about one third of Türkiye's sugar beet production is obtained from this province. As a result of the isolations made from plants showing severe root rot and desiccation symptoms in sugar beet fields in the region in 2015-2017, a new endophytic fungal isolate was obtained from leaves along with soil and foliar disease agents belonging to different fungal species. The fungal isolate has dull yellow, light olive to light brown, fast growing colony-like, flexible conidiophores and septate branched hyphae. The representative fungal isolate did not cause disease symptoms on host plant leaves. Molecular identification of the fungal isolate was carried out using primers specific to the ITS gene region, and it was identified as *Podospora flexuosa* (syn. *Cladorrhinum flexuosum*) based on morphological and molecular characteristics. Since *P. flexuosa* is reported to be a biological control agent living as a saprophyte in soil and plant materials, it is thought that the fungal isolate obtained in our study can be used as a potential biocontrol agent in the biological control of plant disease agents in the future. To the best of our knowledge, this fungal species is reported for the first time for Türkiye.

ÖZET

Şeker pancarı (*Beta vulgaris* L.) Türkiye'nin Konya ilinde yaygın olarak yetiştirilmekte ve Türkiye şeker pancarı üretiminin yaklaşık üçte biri bu ilden elde edilmektedir. 2015-2017 yıllarında bölgedeki şeker pancarı tarlalarında şiddetli kök çürüklüğü ve kuruma belirtileri gösteren bitkilerden yapılan izolasyonlar sonucunda, farklı fungal türlere ait toprak ve yaprak hastalık etmenleri ile birlikte yapraklardan da yeni bir endofitik fungal izolat elde edilmiştir. Fungal izolat besiyerinde donuk sarı, açık zeytinden açık kahverengiye kadar değişen renklerde, hızlı büyüyen koloni benzeri, esnek konidiyoforlara ve septat dallı hiflere sahiptir. Temsili fungal izolat konukçu bitki yapraklarında hastalık belirtilerine neden olmamıştır. Fungal izolatın moleküler tanımlaması ITS gen bölgesine özgü primerler kullanılarak gerçekleştirilirken, morfolojik ve moleküler özelliklerine göre *Podospora flexuosa* (syn. *Cladorrhinum flexuosum*) olarak tanımlanmıştır. *P. flexuosa*'nın toprakta ve bitki materyallerinde saprofit olarak yaşayan bir biyolojik kontrol ajanı olarak bildirildiğinden, çalışmamızda elde edilen fungal izolatın gelecekte bitki hastalık etmenlerinin biyolojik kontrolünde potansiyel bir biyokontrol ajanı olarak kullanılabileceği düünülmektedir. Bildiğimiz kadarıyla, bu fungal tür Türkiye için ilk kez rapor edilmektedir.

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INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is a dicotyledonous plant that is widely cultivated for sugar production in temperate regions (Trebbi & McGrath, 2004). About 29% of the total sugar beet production of 17.436.100 tonnes in Türkiye is produced in Konya province (TÜİK, 2019). There are two sources of sugar production: sugar beet and sugar cane (*Saccharum officinarum* L.), which have an important place in human nutrition (Akar & Yavuz, 2020; Üstüner, 2022). Türkiye ranks fifth in the world in terms of sugar beet production after France, Russia, the USA and Germany (FAOSTAT, 2019).

Species of the anamorphic genus *Cladorrhinum* Sacc. & Marchal form a slimy form consisting of unicellular conidia with lateral phialidic openings. Fertile hyphae may accumulate into more or less branched conidiophores. In culture microsclerotia can sometimes be seen in addition to terminal and lateral phialides (Hoog et al., 2000; Domsch et al., 2007).

Cladorrhinum species are found as saprophytes in fertilisers, plant material and soil (Lewis & Larkin, 1998; Madrid et al., 2011). This fungus has been associated to with humans and animals (Zapater & Scattini, 1979; Chopin et al., 1997; Gajjar et al., 2011). It represents a group of fungi of great importance because some species of the genus *Cladorrhinum* have biocontrol potential or promote plant growth and also produce phytases, enzymes useful in animal feed processing (Carmaran et al., 2015). Until 2001, the evolutionary relationships of *Cladorrhinum* species with other ascomycetes and the phylogenetic position of their anamorphic species were not fully known. *C. flexuosum* was first identified by isolation it from soil in Spain (Madrid et al., 2011). Wang et al. (2019 a,b) redefined three genera, *Cladorrhinum*, *Podospora*, and *Triangularia*, and established Podosporaceae based on phylogenetic analysis. Known related teleomorphs belong to the genus Sordariales, Lasiosphaeriaceae (Bell & Mahoney, 1997; Miller & Huhndorf, 2001). The current name of this fungus has been recorded as *Podospora flexuosa* (syn: *Cladorrhinum flexuosum*) (Madrid et al., 2011). This fungus belongs to the phylum Ascomycota, class Sordariomycetes, subclass Sordariomycetidae, order Sordariales, family Podosporaceae (Huang et al., 2021).

Cladorrhinum species are characterised by conidiophores of pale grey, dark green to brownish colour, rapidly growing colonies and often with widening collars and lateral openings. Conidia are described as unicellular, hyaline or subhyaline, smooth-walled, generally spherical and slimy masses (Mouchacca & Gams, 1993).

Cladorrhinum flexuosum was isolated from soil in Spain in 2011 and it was noted that *Cladorrhinum* species could be used as biocontrol agents (Madrid et al., 2011). Another study, it was reported that *C. flexuosum* could be used as an agent with a high potential to be developed for use in the control of the disease caused by *Fusarium graminearum* (Abaya et al., 2021). According to the studies conducted by Lewis and Larkin (1998), it was stated that *C. foecundissimum* was found to have strong antagonistic activity against *Rhizoctonia solani* Kuhn and *Pythium ultimum* Trow and could therefore can be used as a potential biocontrol agent. As many organisms have the ability to exert antagonistic effects against plant diseases, the use of these microorganisms against plant diseases is considered to be more natural, more environmentally friendly and safer. More effective biological control can only be achieved by using the right microorganism that is antagonistic to the target pathogens (Xue, 2003; Xue et al., 2009). Fungal biological control agents are considered important to protect valuable crops worldwide that can be infected by fungal diseases (Butt et al., 2001).

Therefore, although there are very few studies on these fungi, this study will shed light on future biological studies due to its potential use as a biocontrol agent. The work aims to contribute to the mycobiota of Türkiye by adding a new record.

MATERIALS and METHODS

Sampling, fungal isolation and phenotypic characterization

Disease samples were collected from approximately 30,000 hectares of sugar beet production areas in Konya province in two consecutive years between 2015 and 2017. Plants showing symptoms of root rot such as wilting and marginal necrosis of leaves at the seedling (spring) and mature plant (autumn) stages were included in the sampling, and the samples were taken to the laboratory in ice boxes for isolation. The *Podospora flexuosa* isolate CF 41-871 was obtained from a sample of sugar beet seedlings collected in Konya province, the major sugar beet growing area in Türkiye. Infected tissues (2–3 mm long) were kept in 1% sodium hypochlorite solution for 2 min to ensure surface sterilisation and then rinsed in distilled water (Uysal et al., 2022). After air drying, they were transferred to Petri dishes containing potato dextrose agar (PDA) medium (Merck, Darmstadt, Germany). The petri dishes were incubated for 7-10 days at 25-26 °C in a 12 h dark/light cycle. Phenotypic characterisation of the fungus was carried out by considering its colour, shape, colony structure, conidia and sclerotia structures in the petri dish.

DNA sequence analysis

DNA isolation of these species isolates was carried out according to the method of Lee and Taylor (1990). The ITS region of the DNA was amplified with the ITS 1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS 4 (TCC TCC GCT TAT TGA TATGC 3') primers described by White et al. (1990). They are described and their sequences and those of the 5.8S and 28S ribosomal DNA and internal transcribed spacers 1 and 2 of the ribosomal RNA gene cluster are analyzed (Gilgado et al., 2005). Sequencing was performed by Macrogen Biotechnology. ITS sequence analysis was performed using BLAST via http://www.ncbi.nlm.nih.gov (Kurt et al., 2020). Sequences of each isolate were deposited and recorded in GenBank.

Pathogenicity test

The pathogenicity of the fungus was tested under in vitro conditions using sugar beet seeds of the universally susceptible Aranka variety and was assesed in the test using the inoculum layer technique in the test (Schmitthenner & Hilty, 1962; Herr & Roberts, 1980). The soil mixture consisted of sterile soil, sand and peat moss (1:1:1), and this mixture was filled two-thirds of the way into 10 cm diameter sterile plastic containers. Culture discs of fungal isolates growing in PDA were placed on the soil mixtures, and 10 sterile seeds were arranged in a circle in each pot, with the soil mixture was placed on top. For the control group, seeds were planted in sterile soil mix. The experiment was performed in four replicates. The pots were incubated at 24±2°C under 12 hours of light and 12 hours of darkness, and watered regularly. Plants that died before and after emergence were recorded. In the final assessment, at the end of the 6th week, the remaining plants were removed and scored according to the Hanson scale of 0-4, where: 0=no lesions; 1=mild growth retardation and wilting of leaves; 2=there are chlorotic leaves and necrotic appearance at the leaf margins; 3=drying of roots, browning, death of leaves; 4=dead plant (Hanson et al., 2006). Four pots were used for each isolate in all pathogenicity tests. Pots containing seeds were placed in a growth chamber in four replicates according to a completely randomised experimental design. Disease index values were calculated using the following formula;

$$\text{Disease severity index} = \frac{\sum (\text{no of the seedlings at each scale} \times \text{scale value})}{\text{The highest scale value} \times \text{total seedling evaluated}}$$ (Towsend & Heuberger, 1943)

In this way, the percentage of disease in the plant caused by the isolate was determined. Disease severity is calculated using the Townsend-Heuberger formula. The percentage of disease severity was calculated by

multiplying the Disease Severity Index value by 100. The pathogenicity of these isolates is expressed as a percentage of disease severity.

RESULTS and DISCUSSIONS

Cladorrhinum-like colonies that developed from the tissues of the samples were subcultured on PDA, purified and microscopically examined microscopically for morphological characteristics. *Cladorrhinum* colonies were dull yellow, light olive to light brown, fast-growing and flexible conidiophores and globose to dacryoid conidia on the media (Figure 1). Hyphae were divided and branched. Microsclerotia were absent.

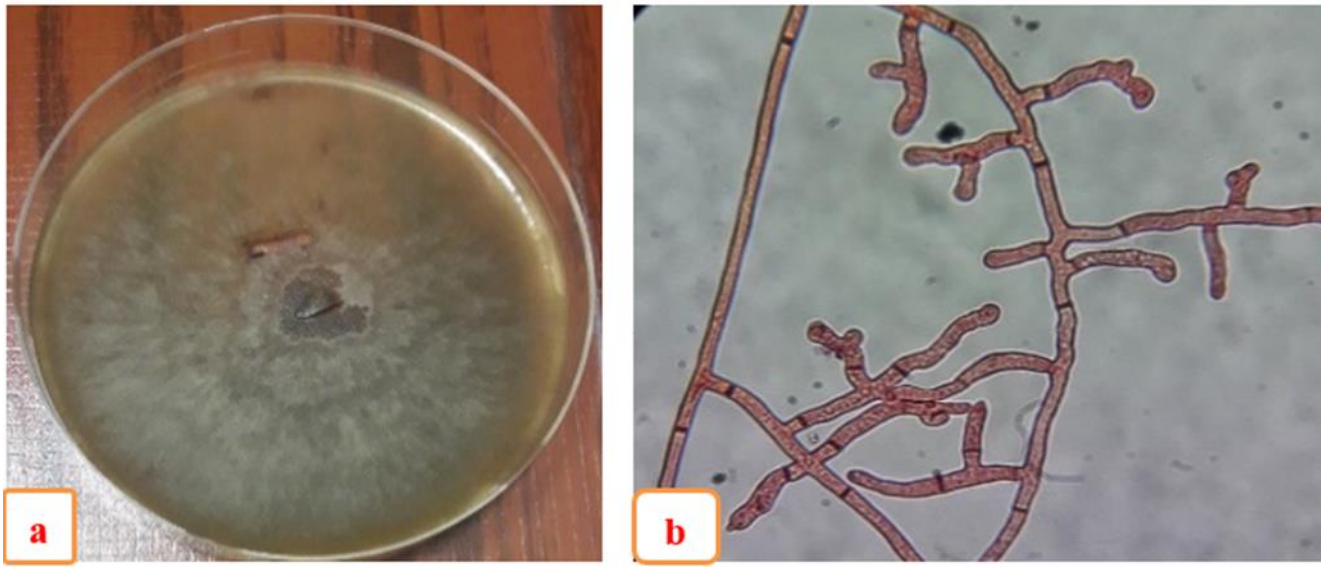

Figure 1. Microscopic features of *Podospora flexuosa*. a) *Podospora flexuosa* colony growth on PDA medium, b) conidiophore *of Podospora flexuosa*

Şekil 1. Podospora flexuosa'nın mikroskobik özellikleri. a) PDA besi yeri üzerinde Podospora flexuosa'nın koloni gelişimi, b) Podospora flexuosa'nın konidioforu

The resulting *P. flexuosa* isolate sequences (MN900535) were approximately 99% similar to other isolates on GenBank (MH864075). As a result of the pathogenicity test performed under *in vitro* conditions, it was found that the *P. flexuosa* isolate did not develop any disease symptoms in sugar beet leaves and roots after inoculation (Figure 2).

a) Inoculated pot with *Podospora flexuosa* isolate for pathogenicity test

b) Appearance of seedlings as a result of pathogenicity test

c) Control group

Figure 2. Pathogenicity test steps of *Podospora flexuosa* isolate and pathogenicity results. a) Sugar beet seeds placement on the *P. flexuosa* fungal disk; b) Growth of sugar beet seeds in pots with the fungus *P. flexuosa*; c) Growth of sugar beet seeds in pots where *P. flexuosa* fungus was not applied (control group)

Şekil 2. Podospora flexuosa izolatının patojenite test adımları ve patojenite sonuçları. a) Şeker pancarı tohumlarının P. flexuosa fungal diskine yerleştirilmesi; b) Saksılarda şeker pancarı tohumlarının P. flexuosa fungusu ile saksıda yetiştirilmesi; c) P. flexuosa fungusunun uygulanmadığı saksılarda şeker pancarı tohumlarının yetiştirilmesi (kontrol grubu)

The genus *Cladorrhinum* has become a group of fungi of primary importance for agriculture and animal husbandry, because some species produce phytases, enzymes useful in animal feed processing, as well as promoting plant growth and having the potential as biocontrol agents (Carmarán et al., 2015).

As a result of our studies, the isolate, which was determined to be non-pathogenic, is a first record for Türkiye for both the genus *Podospora* and the species *P. flexuosa*. In previously published studies, fungal species was identified as a biocontrol agent with strong antagonistic activity against fungi such as *C. foecundissimum* Sacc & Marchal, *Rhizoctonia solani* Kühn and *Pythium ultimum* Trow, which are among the *Cladorrhinum* species that are new to our country and have only limited studies in the world (Lewis & Larkin, 1998). Carmarán et al. (2015) also reported *Cladorrhinum* species as biocontrol agents. Abaya et al. (2021) found that *C. flexuosum*, one of the endophytic fungal isolates, could inhibit the pathogenicity of *Fusarium graminearum* and *Waitea circinata* in wheat seedling leaves by 31-86%.

There are very few studies on this fungal pathogen worldwide. Therefore, it is important to evaluate the biological control potential of this fungal species against potential pathogens. This study demonstrated the presence of *P. flexuosa* from sugar beet growing areas. Result is believed to guide researchers using this fungus as a biological control agent in future studies.

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STATEMENT OF CONFLICT OF INTEREST

The authors of the article declare that they do not have no conflicts of interest.

AUTHOR'S CONTRIBUTIONS

The authors declare that they have contributed equally to the work.

STATEMENT OF ETHICS CONSENT

Ethical approval is not required as this article does not contain any studies with human or animal subjects.

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