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Original Article

Comparison of Different Media for Isolating Endophytic Fungi from Plant Tissues of *Citrus* Species

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ABSTRACT

Endophytic fungi play an ecological role in promoting plant growth and controlling diseases. This study compared the efficacy of different media for isolating fungal endophytes. We used potato dextrose agar (PDA), corn meal agar (CMA), water agar (WA), czapek dox agar (CDA), and sabouraud agar (SB). This study resulted in the isolation of 43 endophytic fungal isolates from healthy leaf and fruit tissues of mandarin and orange, with 69.7% of these isolates coming from leaf tissue. Morphological identification revealed these isolates belonged to *Alternaria* spp., *Colletotrichum* spp., Botryosphaeriaceae, and *Trichoderma* spp. Principal component analysis (PCA) showed that PDA and SB were effective for isolating culturable endophytic fungi. The unweighted pair-group method of arithmetic (UPGMA) dendrogram showed that PDA was in the same group as the SB, while CDA and CMA were separated. The WA was distinct from all other media, forming an outgroup. The results of this investigation demonstrated the influence of different media on the isolation of endophyte fungi from various plant tissues of *Citrus* species. Future research should focus on the efficacy of plant disease of the endophyte fungi and their potential for biocontrol under field conditions.

Keywords: Botryosphaeriaceae, *Trichoderma*, media effectiveness, fungal endophytes

Turunçgil Türlerinin Bitki Dokularından Endofitik Fungus İzolasyonunda Farklı Besi Ortamlarının Karşılaştırılması

ÖZ

Endofitik funguslar, bitki gelişimi ve bitki hastalıklarının mücadelesinde ekolojik role sahiptir. Bu çalışmada fungal endofitlerin izolasyonunda farklı ortamların etkinliği karşılaştırılmıştır. Patates dekstroz agar (PDA), mısır unu agar (CMA), su agar (WA), czapek doks agar (CDA) ve sabouraud agar (SB) kullanılmıştır. Bu çalışmada mandalina ve portakal bitkisinin sağlıklı yaprak ve meyve dokularından 43 adet endofitik fungal izolat izole edilmiştir, bu izolatların %69.7'si yaprak dokusundan elde edilmiştir. Morfolojik tanımlama bu izolatların *Alternaria* spp., *Colletotrichum* spp., Botryosphaeriaceae, ve *Trichoderma* spp.'ye ait olduğunu ortaya koymuştur. Temel bileşen analizi (PCA), PDA ve SB'nin kültüre alınabilen endofitik fungusların izolasyonunda başarılı olduğunu göstermiştir. Ağırlıklandırılmamış çift grup aritmetik yöntemi (UPGMA) dendrogramı, PDA'nın SB ile, CDA'nın CMA ile ayrıldığını göstermiştir. WA ise diğer tüm besi yerlerinden farklılaşarak dış grup oluşturmuştur. Bu araştırmanın sonuçları, turunçgil türlerinin çeşitli bitki dokularından endofit fungusların izolasyonunda farklı besi ortamlarının etkisini ortaya koymuştur. Gelecekteki araştırmaların, endofit fungusların bitki hastalıklarındaki etkinliğini ve saha koşullarında biyolojik mücadele potansiyeline odaklanması gerekmektedir.

Anahtar kelimeler: Botryosphaeriaceae, *Trichoderma*, besi ortamının etkinliği, fungal endofitler

INTRODUCTION

A significant amount of commercially fresh citrus fruits (2.3 million tons) is produced in Türkiye and it ranks eighth in the world (Anonymous, 2024). Among the seventeen citrus species, *Citrus sinensis* (sweet orange), *C. lemon* (lemon), *C. reticulata* (mandarin orange, tangerine), *C. aurantium* (bitter orange), *C. maxima* (pummelo), and *C. paradisi* (grapefruit) are the most common (Albrigo et al., 2019). The Washington variety of *Citrus sinensis* L. is particularly popular, and mandarin are the second most popular preferred citrus fruit after oranges (Giménez-Sanchis et al., 2022). However, biotic factors significantly limit citrus production. Fungal plant pathogens, such as *Colletotrichum* (Ramos et al., 2016), *Penicillium* (Moraes Bazioli et al., 2019), *Alternaria* (Garganese et al., 2018), *Phytophthora* (Narayanasamy, 2011), *Botrytis* (Saito and Xiao, 2017) can infect various *Citrus* tissues both before and after harvest. Since most cirtus are consumed fruits, chemical disease control is often restricted. Consequently, strategies that do not negatively impact human health, such as biological control, are gaining importance (Arora et al., 2018; De Silva et al., 2019; Ortega et al., 2020).

Endophyte microorganisms play an important role in the ecology of the host plant, colonizing various tissues without causing harm. It is estimated that each of the Earth's approximately 270,000 plant species with one or more endophytes (Clay et al., 2016; Soltani, 2017; Wani et al., 2015). Fungal endophytes are particularly effective, not only in managing plant pathogens (Du et al., 2022; Priyashantha et al., 2023; Pazarlar and Şimşek, 2024), but also in promoting plant growth and enhancing resilience to abiotic stresses like drought and salinity (Korkom, 2023; Moghaddam et al., 2021; Mattoo and Nonzom, 2021). Fungal endophytes can be isolated in various plant tissues, including roots, shoots, leaves, flowers, bark, and fruits (Yan et al., 2019). Their diversity in plants varies according to species, different physiological conditions, tissue type, climatic characteristics, pH value, and geographical regions (Chand et al., 2020; Lugtenberg et al., 2016; Vega et al., 2010). Molecular techniques are also used in identification of endophytic fungi. However, problems such as sporulation, slow growth and contamination continue in the cultivation process of endophytic fungi. Therefore, investigating the effect of media in obtaining endophytic fungi will contribute to the solution of these problems. While potato dextrose agar is frequently used for isolating culturable fungal endophytes (Sharma et al., 2016; Dhayanithy et al., 2019; Sadeghi et al., 2019; Fu et al., 2024), other media like agar (WA) (Talukdar and Tayung, 2021), corn meal agar (CMA) (Potshangbam et al., 2017), czapek dox agar (CDA) (Das and Mahapatra, 2019) and Sabouraud agar (SB) (Moharram et al., 2017) media have also been successfully employed in different studies.

So far, fungal endophytes have been isolated from various plants and plant tissues in many studies, but the success of different media in isolating endophytes has not been compared. Therefore, the aim of this study was to demonstrate how different media affect the isolation of *Citrus* fungal endophytes.

MATERIALS AND METHODS

Sample collection

Sampling was done in December 2022 from 10-15 year old orange (*Citrus sinensis*) (8 da) and mandarin (*C. reticulata*) orchards (5 da) located at Aydın Adnan Menderes University, Agriculture Faculty, Aydın province, Türkiye. Healthy, orange fruits of uniform ripeness were selected, and healthy leaf samples were collected from the same trees (ten trees). Mandarin sampling followed an identical procedure. All fresh leaf and fruit samples were transported to the laboratory in an icebox and processed for isolation on the same day.

Isolation of fungal endophytes on different media

Surface disinfection was carried out following the protocol in Muñoz-Guerrero et al. (2021). Briefly, the samples were washed thoroughly with the running tap water for 10 minutes to remove surface particles and dried in an aseptic condition. The fruits and leaves were cut into 0.7-1 cm parts. Surface sterilization was done using 2.5% sodium hypochlorite for 2 min., rinsing with sterile distilled water, and then implemented with 70% ethanol for 1 min., followed by three sequential washes with sterile distilled water. Samples were dried in a sterile cabinet.

Five medium were used in the isolation of fungal endophyte. The ingredients in the media are given in Table 1. The disinfected tissues of samples were placed on this media. The Petri dishes were incubated in an incubator set at 28 ± 1°C for 15 days. This study was repeated twice.

Table 1. Ingredients of different media used for isolation of fungal endophyte

Media	Components	Manufacturer
Potato dextrose agar (PDA)	15 g/L bacteriological agar, 20 g/L dextrose, 4 g/L potatoes,infusion from	Condalab 1022
Corn meal agar (CMA)	15 g/L bacteriological agar, 2 g/L corn meal, infusion from	Millipore C1176
Water agar (WA)	15 g/L bacteriological agar	Condalab 1802
Czapek dox agar (CDA)	15 g/L bacteriological agar, 0.01 g/L ferrous sulfate, 0.5 g/L magnesium sulfate, 0.5 g/L potassium chloride, 1 g/L potassium phosphate dibasic, 3 g/L sodium nitrate, 30 g/L sucrose	Millipore C1551
Sabouraud medium (SB)	10 g/L peptone 40 g/L dextrose 20 g/L agar	Millipore 91,249 Millipore P6685 Condalab 1022

Morphological identification of fungal colonies on growth media

The fungal colonies were transferred to PDA and obtained pure cultures. Their identification was obtinated morphological characteristics such as colony color, hyphae, and spore structure (Li et al., 2016; Liu et al., 2009) by microscope (Leica CX21, Germany). The isolation rate was calculated for each media.

Data analysis

The trials of each media were performed in five replicates and this experiment was repeated twice. The analysis of variance (one-way ANOVA) comparison was performed to the LSD test (p=0.05). The success of the media in isolating fungal endophytes was determined through Principal component analysis (PCA) (JMP 18.1.2) and clustering (PAST 4.03).

RESULTS AND DISCUSSION

Tissue isolation followed by morphological identification remain the most common approach in studies of fungal endophytes. In this study, we successfully isolated 43 culturable fungal endophytes from various *Citrus* tissues of these 39 isolates were from *C. sinensis* and four were from *C. reticulata*. The highest number fungal endophytes (30 isolates) were obtained from leaf tissue. Morphological identification revealed that the all fungal isolates belonged to four genera within Ascomycota. The *Alternaria* spp. was the most prevalent, accounting for 21 isolates both from leaf and fruit tissue (Figure 1). Thirteen fungal isolates, including *Trichoderma* sp. (found exclusively in *C. reticulata* fruit), were recovered from fruit tissue. Both Botryosphaeriaceae and *Colletotrichum* spp. were isolated from both leaf and fruit tissues (Table 2).

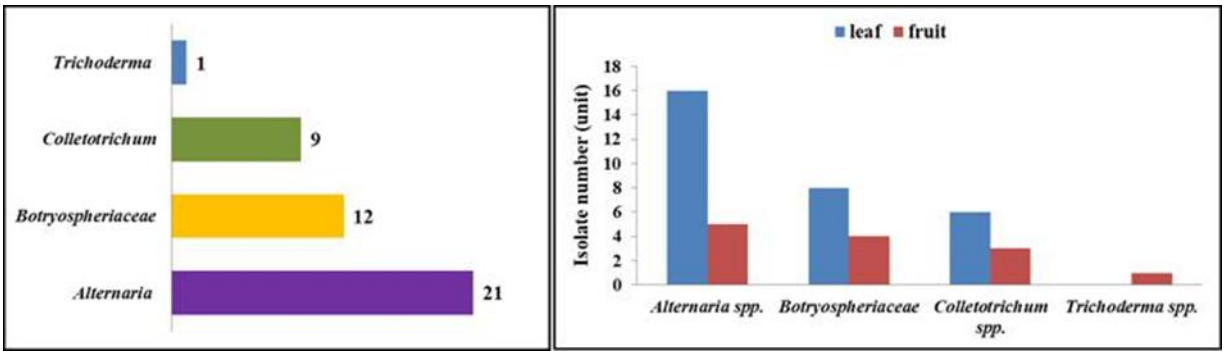

Figure 1. The effects of medium obtained from different tissue on *Citrus* and the species of fungal isolates obtained from *Citrus* species.

Table 2. Information of culturable isolated of fungal endophytes in *Citrus* tissues

Isolate	Host	Tissue	Fungal species	Isolate	Host	Tissue	Fungal species
PM2PD	orange	fruit	*Trichoderma* spp.	P1YCD	orange	leaf	*Alternaria* spp.
PM1CD	orange	fruit	*Colletotrichum* spp.	P5YPD	orange	leaf	*Alternaria* spp.
PM1WA	orange	fruit	*Colletotrichum* spp.	P3YCM	orange	leaf	*Alternaria* spp.
PM5PD	orange	fruit	*Colletotrichum* spp.	P3YSB	orange	leaf	*Alternaria* spp.
PY6PD	orange	leaf	*Colletotrichum* spp.	P3YCD	orange	leaf	*Alternaria* spp.
P4YSB	orange	leaf	*Colletotrichum* spp.	P2YSB	orange	leaf	*Alternaria* spp.
P4YSB	orange	leaf	*Colletotrichum* spp.	P3YPD	orange	leaf	*Alternaria* spp.
P2YPD	orange	leaf	*Colletotrichum* spp.	P4YPD	orange	leaf	*Alternaria* spp.
PY5SB	orange	leaf	*Colletotrichum* spp.	P1YSB	orange	leaf	*Alternaria* spp.
PY4PD	orange	leaf	*Colletotrichum* spp.	P2YSB	orange	leaf	*Alternaria* spp.
P4YPD	orange	leaf	Botryosphaeriaceae	P3YSB	orange	leaf	*Alternaria* spp.
P5YPD	orange	leaf	Botryosphaeriaceae	P4YSB	orange	leaf	*Alternaria* spp.
P2YPD	orange	leaf	Botryosphaeriaceae	P1YCD	orange	leaf	*Alternaria* spp.
P4YSB	orange	leaf	Botryosphaeriaceae	P5YPD	orange	leaf	*Alternaria* spp.
P4YCD	orange	leaf	Botryosphaeriaceae	P5YSB	orange	leaf	*Alternaria* spp.
P3YPD	orange	leaf	Botryosphaeriaceae	PM3CD	orange	fruit	*Alternaria* spp.
P2YCD	orange	leaf	Botryosphaeriaceae	M1MPD	mandarin	leaf	*Alternaria* spp.
M1YPD	mandarin	leaf	Botryosphaeriaceae	M1YPD	mandarin	fruit	*Alternaria* spp.
M3MPD	mandarin	fruit	Botryosphaeriaceae	PM4CM	orange	fruit	*Alternaria* spp.
M4YPD	orange	fruit	Botryosphaeriaceae	PM4CD	orange	fruit	*Alternaria* spp.
PM3SB	orange	fruit	Botryosphaeriaceae	PM1CD	orange	fruit	*Alternaria* spp.
PM4PD	orange	fruit	Botryosphaeriaceae				

Colletotrichum gloeosporioides, primarily known as fungal pathogens in *Citrus* species, was also isolated as endophytes in a few studies (Douanla-Meli et al., 2013; Durán et al., 2005; Juybari et al., 2019). Studies on mandarin have frequently isolated *Alternaria* species as endophytes, alongside genera such as *Penicillium*, *Phomopsis*, *Fusarium*, *Cladosporium* (Glienke-Blanco et al., 2002; Sadeghi et al., 2019). Juybari et al. (2019) similarly isolated endophytic *Alternaria* spp*., Colletotrichum*, *Fusarium*, and Botryosphaeriaceae from different *C. sinensis* tissues across different seasons, noting greater fungal diversity during winter sampling. Our study's high isolation rate of endophytic *Alternaria* aligns with these previous findings.

Fungal isolates obtained from orange and mandarin leaf and fruit tissues were evaluated across five growth media: potato dextrose agar (PDA), sabouraud agar (SB), corn meal agar (CMA), czapek dox agar (CDA), and water agar (WA) media. There was a statistically significant difference in the number of isolates obtained in across these media. The highest number of fungal endophytes was isolated using PDA ($F = 191.5$; $p < 0.05$) (Figure 2). Media/fungal isolate correlation based on plant species and tissue was assessed by principal component analysis (PCA). As a result of the PCA-biplot, the first principal component (PC1) explains 67.1% of the variance (eigenvalue = 3.352), and the second principal component (PC2) explains 32.9% (eigenvalue = 1.647) (Figure 2). Together, these two components account for 100% of the variance and there was a strong representation of the data set. PDA, SB, CDA, and CMA were positively and strongly correlated with PC1. WA is consistent with PC2 and does not correlate with other media (Figure 2). Additionally, clustering analysis (Jaccard similarity index) verified the results acquired by PCA. The endophyte fungal isolates obtained from SB, and PDA grouped into one clade, whereas CMA and CDA grouped into a single clade. The WA was set apart from all other media as an outgroup (Figure 2).

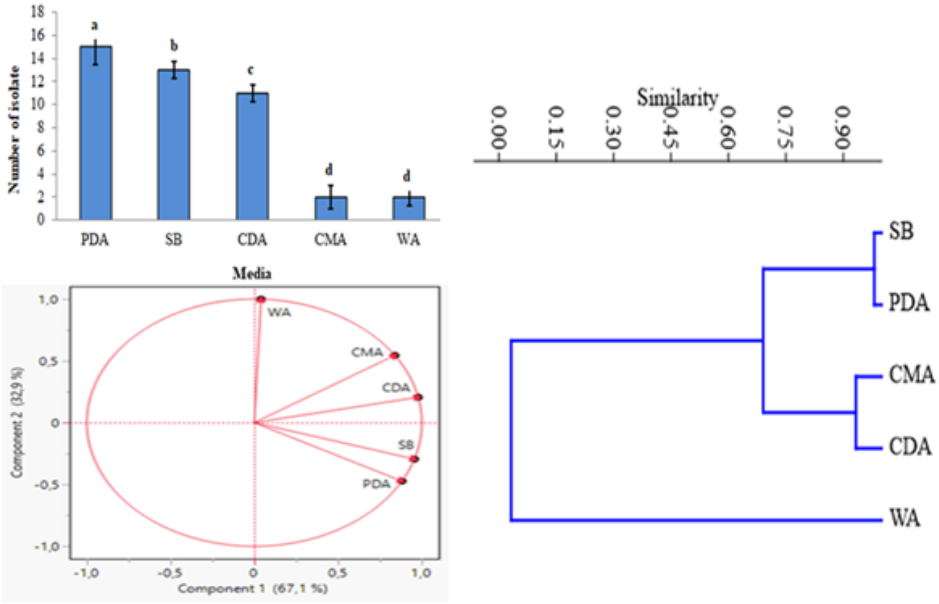

Figure 2. PCA analysis of the isolated fungal endophytes of *Citrus* species from different tissues on the five media.

Qi et al. (2012) determined successful isolation of endophytic fungi in PDA. Wang et al. (2016) acquired that among Martin agar medium, czapek's, SB and PDA media the number and variety of endophytic fungi found in PDA (178 strains of 23 genera) and SB (184 strains of 27 genera) were comparable while the strains *Phanerochaete* spp. could be only isolated from PDA. On the other hand, Syamsia et al. (2021) reported that mung bean and rice medium, showed mycelia growth of endophytic fungi the same as growth on PDA. Chen et al. (2019) found that the number of isolates obtained in CDA (90 isolates of thirteen families) was higher than in PDA (70 isolates of of two families). In this study, PDA (15 isolates) and SB media (13 isolates) were found to be successful in isolation, which follows the results of previous studies. The use of different synthetic medium cannot guarantee the isolation of all endophytic fungi, therefore plant and tissue-specific isolation procedures are important.

CONCLUSION

Main significant results of the study should be presented in a clear and concise way and should be numbered. Phytopathogen fungi are developing resistance to fungicides. As such it is necessary to investigate the potential of endophyte fungi present in the plant as alternative biocontrol agents. This study determined the impact of media type in isolating endophytic fungi from distinct plant tissues in two important *Citrus* species, orange and mandarin. In this study, PDA and SB media were found to be successful in the isolation of fungal endophytes from *Citrus* species. Future research should explore the efficacy of this endophyte fungi against plant disease and their potential of biocontrol under field conditions.

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Declaration of interests

The authors declare no competing interests.

Author Contributions

***Yunus KORKOM*:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; software; writing— original draft; writing—review and editing.

***Gizem ÖZGÜN*:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration.

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