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Possibilities of Using Myconanotechnology in Combating *Verticillium dahliae* Kleb. Fungal Pathogen

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Abstract: *Verticillium dahliae* is a soil-borne plant pathogen and its control is limited with chemical fungicides. The disease causing fungus, which causes significant economic agricultural losses all over the world, has a very wide host range. The scarcity of successful methods in the control of the disease further increases the importance of the disease. In addition, the effort to develop alternative control methods is based on the inadequacy of environmentally friendly fungicides, the emergence of fungicide resistance in pathogens and the breaking of host resistance of pathogen populations. Today, the popularity of nanoparticle applications, which provide better efficiency in the control of diseases, is increasing day by day. The current research aims to synthesize a bionanomaterial (RDEK) with the wild mushroom *Russula delica* Fr. methanol extract (RDE) without using any hazardous substances, with a new, simple, cost-effective and environmentally friendly method, and to point out the use of this synthesized material in agricultural applications as an antifungal agent for the first time. aims.

In the study, it was determined that RDE and RDEK applied in different concentrations showed antifungal activity against *Verticillium dahlia*. RDEK bionanomaterial, which shows the best activity at 0.02 mg/ml concentration, can be evaluated as a fungicide in agricultural applications.

Key words: *Verticillium dahliae, Russula delica*, Bionanomaterial, Fungicide

Verticillium dahliae Fungal Patojeni İle Mücadelede Mikonanoteknolojinin Kullanım Olanakları

Öz: *Verticillium dahliae* toprak kökenli bir bitki patojenidir ve mücadelesi kimyasal fungusitler ile sınırlıdır. Tüm dünyada önemli ekonomik tarımsal kayıplara neden olan hastalık etmeni fungusun çok geniş bir konukçu dizisine sahiptir. Hastalığın kontrolünde başarılı yöntemlerin azlığı, hastalığın önemini daha da arttırmaktadır. Ayrıca alternatif mücadele yöntemleri geliştirilme çabası çevre dostu fungusitlerin yetersizliği, patojenlerde fungisit dayanıklılığının ortaya çıkışı ve patojen popülasyonlarının konukçu dayanıklılığını kırması nedenlerine dayanmaktadır. Günümüzde ise hastalıklarının kontrolünde daha iyi etkinlik sağlayan nanoparçacık uygulamalarının popülerliği her geçen gün artmaktadır. Mevcut araştırma, yabani mantar olan *Russula delica* metanol ekstresi (RDE) ile herhangi bir tehlikeli madde kullanmadan yeni, basit, uygun maliyetli ve çevre dostu bir yöntem ile biyonanomateryal (RDEK) sentezlemek ve ilk defa sentezlenen bu materyalinantifungal madde olarak tarımsal uygulamlarda kullanımına işaret etmeyi amaçlamaktadır.

Çalışmada, farklı konsnatrasyonlarda uygulanan RDE ve RDEK'nın *Verticillium dahlia*'ye karşı antifungal aktivite gösterdiği tespit edilmiştir. 0.02 mg/ml konsanrtasyonda en iyi aktivite gösteren RDEK biyonanomateryali tarımsal uygulamlarda fungusit olarak değerlendirilebilir.

Anahtar kelimeler: *Verticillium dahliae, Russula delica*, Biyonanomateryal, Fungusit

Introduction

Plant pathogenic fungi in agriculture are known to cause many important diseases in various plants and limit the superior quality and high production worldwide. Plants infected by pathogens accumulate harmful toxins and metabolites that threaten the safety of agriculture and food industries. *Verticillium dahliae* (Avcı şamdanküfü), is a plant pathogen that affects more than 300 woody and herbaceous plant species and causes wilting. Plant protection methods, including the control of V. dahliae, unfortunately, continue with the use of chemical fertilizers, fungicides, herbicides, and pesticides. Incorrect and unnecessary use of these harms the ecological environment and causes serious economic losses. It also causes the emergence of drug-resistant strains (Sun et al., 2012; Sesli et al 2020). It is necessary to develop environmentally friendly products and techniques to reduce the use of chemical agents such as herbicides, insecticides, and fungicides and to ensure healthy sustainable agriculture. To overcome the above-mentioned challenges, the discovery and evaluation of new sources of bioactivity and the development of new types of formulations are necessary. Based on the latest developments in nanotechnology, nanoparticles seem to have great potential for the development of new generation products called nano plant protection products (Kah, 2015; Balaure et al., 2017). Although numerous applications of nanotechnology in medicine have been reported (Boisseau and Loubaton, 2011; Morigi et al., 2012), their application in research and development (R&D) is still in its infancy (Kah and Hofmann, 2014; Walker. et al., 2017). Clay minerals have received wide attention due to their unique biological properties that include broad-spectrum antimicrobial activity (Choy et al. 1999; Choi et al. 2018; Choy et al. 2000;). biodegradability biocompatibility non-toxicity (Baek et al. 2012; Gaharwar et al., 2013; Xavier et al., 2015).

The synergistic reaction of antibiotics and their resistance-inducing effects make. clay derivatives materials promising antimicrobial materials to prevent and combat plant diseases caused by pathogenic microorganisms (Mansilla et al., 2013). Recent studies show that such nanoparticles are more favorable than the corresponding solution regarding antimicrobial activities (Saharan et al., 2015; Hosseini, et al.,2016). This study, it was aimed to synthesize environmentally friendly, economical, simple clay nanoparticles using the mushroom *Russula delica* (Akçıntar), extract, which has strong bioactive properties, and to investigate the antifungal activity of the synthesized nanomaterial against *Verticillium dahliae*, which has a wide host range (Sesli et al 2020).

Material and Method

Materials

Sodium tripolyphosphate (TTP) was obtained from Acros Organics (New Jersey, USA). Dimethyl sulfoxide (DMSO) was provided by Sigma-Aldrich. In addition, HCl (hydrochloric acid) and CH3COOH (acetic acid) acids, and NaOH (sodium hydroxide) used in the experiment were graded analytically.

The *Russula delica* (RD) mushroom was collected from Mardin province between April and May 2020. Macroscopic examinations, ecological structural features, and key artifacts were used to make the diagnosis (Christensen, 1981). The phytopathogenic fungus (isolate names in parenthesis) used in the study was *Verticillium dahlia* (OVd82). This isolate that was found pathogenic to their original hosts was deposited in the culture collection of Mardin Artuklu University's "The Microbiology-Biochemistry Research Laboratory" (Mardin, Turkey). The antifungal activity tests were carried out in the Microbiology-Biochemistry Research Laboratory at Mardin Artuklu University.

Extraction of Mushroom

First, the RD was dried at room temperature for 10–15 days before being pulverized into small particles. The mixture of 20 g RD and 200 ml methanol was then placed in a 500 ml Erlenmeyer flask and stirred overnight. The mixture was then filtered, and the filtrate was extracted with methanol again by stirring overnight. The final mixture was filtered, and all of the methanol mixtures were collected in an evaporation balloon to dry up the methanol at 40°C. The obtained *R. delica* extract (RDE) was stored at 4°C for further use (Acay, 2021).

Synthesis of RDEK

2 g of RDE was dissolved in 20 ml of methanol with stirring for 1h. Then, the same amount of bentonite clay (C) was dissolved in DMSO and both mixtures were mixed. After the pH value of the mixture, which was stirred for 2-3 hours, was adjusted to approximately 5.5, 0.4 g of TTP was dissolved in 10 ml of pure water and slowly added to this mixture. 20 ml more methanol was added to the mixture, which was mixed homogeneously for 3 hours, and it was precipitated in the centrifuge. And finally, the obtained filtrate was dried in an oven at 60 °C and bionanomaterial (RDEK) was obtained.

Determination of the antifungal effect of RDE and RDEK

The disk diffusion method was performed using Malt extract agar (MEA) (Merck) medium. The fungal isolate was inoculated into 20 mL of Sabouraud Dextrose Broth medium and incubated in a water bath at 28°C and 120 rpm. To solidify properly, 25 mL of MEA was sterilized and cooled to 45–50°C before being poured into 9 cm diameter sterile petri dishes. It was stored overnight in a 37°C oven. After culturing the mushrooms (107 conidia/mL) in the water bath, 150 µL was taken and homogeneously distributed in the medium using sterile cotton swabs and sterile petri dishes. 30 µL (210 µg/disc) of different concentrations (0.01, 0.02, 0.04 mg/ml) of RDE, RDEK and clay solutions were impregnated on 9 mm sterile blank paper discs. Discs impregnated with solutions were gently placed on solidified agar. The petri dishes were incubated in an oven at 28°C for 48/72 hours, and the inhibition zone diameters on the medium were measured in mm using a ruler. The experiment was repeated three times, and petri dishes without RDE and RDEK served as controls.

Results and Discussion

Characterization of RDEK

Fourier Transform-Infrared Spectrometer (FT-IR) (ALPHA Bruker model) was used to determine the functional groups on the RDE, C, and RDEK bionanomaterials. The FTIR spectra of RDE, RDEK, and C substances are shown in Figure 1 in the range of 400-4000 cm-1. In the FTIR spectrum of the bionanocomposite, it is similar to RDE with O-H, N-H, C-H stretches located at 3290, 2919, and 2851 cm-1 in the range of 3500-2500. It is seen that the peaks of C-H bending, Si-O-Si, and Al-O-Al groups at 1429, 998, and 871 cm-1 are similar to C'. The fact that these peaks in the C and RDE FTIR spectra are in the RDEK FTIR spectrum highlights that the bionanomaterial contains both clay and mushroom extract (Saadat, et al 2012; Laksanawati, et al 2019)

The X-ray diffraction (XRD) analysis was applied to detect the crystallinity of the prepared bionanomaterial. For this, the Rigaku Ultima-IV model XRD device was used (2θ=0°–80°). The XRD analysis showed that the basic structure of bionanocomposite is both crystalline and amorphous. It can be observed from **Figure 2** that there two diffraction peaks appeared near 2θ=11.2° characteristic crystalline structure originating from bentonite and 2θ=20.3° amorphous state indicating the presence of both bentonite clay (Abdullahi et al 2017; Babahoum and Ould Hamou, 2021) and chitin content of mushroom extract (Ssekatawa, et al 2021) In addition, the average size of the RDEK was found as 18.77 nm.

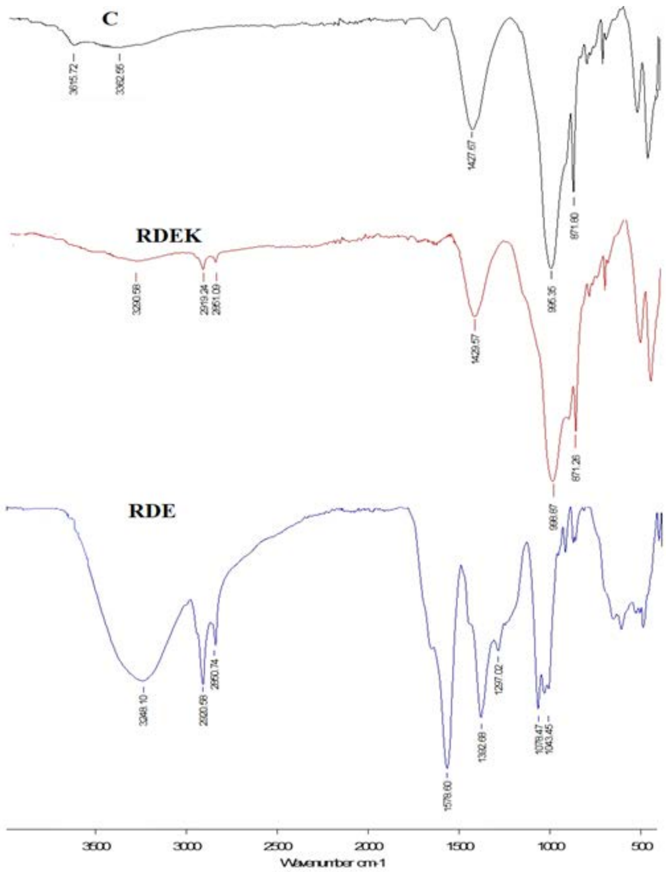

Figure 1. FTIR spectra of C, RDEK and RDE.

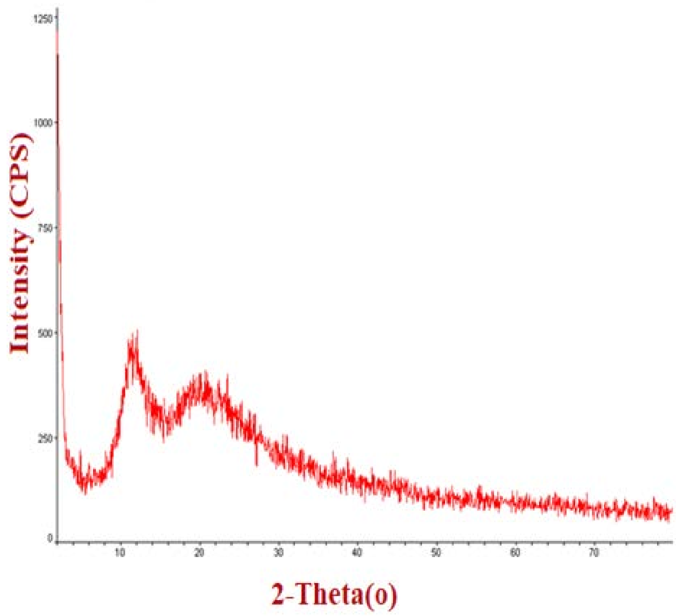

Figure 2. XRD pattern of RDEK.

Antifungal Activity of RDE and RDEK

V. dahliae is the cause of vascular wilt disease in many plant species worldwide. Considering the broad-spectrum antimicrobial activities of biosynthesized NPs, and the lack of an effective method to combat this disease, it is thought that it may have potential application against *V. dahliae*. However, few studies have so far investigated the effect of NPs on the growth of *V. dahliae.* (Fang et al 2017; Tang et al 2017; Huo, et al. 2018) found that AgNPs biosynthesized using Citrus maxima bark extract with an environmentally friendly approach effectively inhibited the growth of V. dahliae. The growth of *V. dahliae* was in a dose-dependent manner. The higher concentration of AgNPs enhanced the higher inhibition efficiency of *V. dahliae*. Thus, it was concluded that the synthesized AgNPs could potentially be used in other antifungal products.

In another study, Costa et al. (2011) investigated the antimicrobial activity of NPs formed with a type of clay (MMT) and Ag to extend the shelf life of fresh fruit salad. The results show that Ag/MMT NPs inhibit the growth of spoilage-causing microorganisms (mesophilic and psychrotrophic bacteria, coliforms, lactic acid bacteria, yeasts, and molds).

The antifungal effects of RDE and RDEK were investigated using the disk diffusion method, as shown in Table 1. The inhibition zone of RDE and RDEK against *V. dahlia* was 14±1.52, 21±2.30, mm, respectively, at a concentration of 0.02 mg/ml. Simultaneously, in the study, it was determined that clay at the same concentration (0.02 mg/ml) alone showed no antifungal activity. However, in this study, unlike Huo et al. (2019), it was observed that the inhibition was not concentration-dependent.

In the literature, mostly metal-based nanoparticles are used in the fight against plant pathogens, but the difficulties in the use of these nanomaterials push the researchers to investigate more biobased, degradable, non-toxic biocomposites (Kutawa et al 2021) In this context, the inhibition zone of 21±2.30 mm of RDEK nanoparticle against *V. dahlia* is remarkable.

Table 1 Antifungal activity of RDE and RDEK

Tested Microorganism	**Concentration (mg/ml)**	**RDE (mm±SD)**	**RDEK (mm±SD)**	**CLAY (mm±SD)***
Verticillium dahliae	0.01	NA	NA	NA
	0.02	14±1.52,	21±2.30,	NA
	0.04	NA	NA	NA

*Inhibition zone in millimeters and each treatment was carried out in triplicate. NA: No Activity.

Conclusion

The shape of the FTIR peaks of the bionanocomposite showed the interaction between its constituent mushroom extract and clay and their agreement with their peaks. XRD patterns also revealed that it had both clay crystalline and fungal amorphous features. RDEK-NPs showed satisfactory antifungal activity; In particular, this is the first time that biosynthesized RDEK-NPs show positive inhibitory ability against *V. dahlia*. The proposed method may be promising for commercial scale-up due to its low cost and efficient production. Moreover, these bionanocomposites could potentially be applied in pharmaceutical and industrial applications.

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