http://dergipark.ulakbim.gov.tr/trkjnat/index
Trakya University Journal of Natural Sciences, 17(1): 65-70, 2016
ISSN 2147–0294, e-ISSN 2528–9691

**Research Article/Araştırma Makalesi**

# SEVERAL PESTA TABLET TRIALS WITH *Aspergillus alliaceus* Thom & Church FOR EFFECTIVE UNDERGROUND AND ABOVEGROUND *Orobanche* L. BIOCONTROL

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Received (Alınış): 08 March 2016, Accepted (Kabul Ediliş): 02 May 2016, Published (Basım): 15 June 2016

**Abstract:** The present study was performed in order to determine the efficiency of the fungi *Aspergillus alliaceus* in fighting broomrape by the pesta-tablet trials under below- and above-ground conditions and to determine the appropriate tablet formulations for both conditions. For this purpose, different tablets were used in different experimental steps and their efficiencies were evaluated. The results showed that the tablets T-1, T-2, T-3, T-5, T-12 and T-13 greatly reduced the amount of broomrape in below-ground conditions, particularly with previous and high amount or crushed-tablet applications before crop-sowing. In above-ground conditions, any tablet formulation or directly sclerotial contacting were very effective in broomrape control and it was determined that close presence to or direct contact with broomrape is essential for successful biocontrol. In conclusion, considering the durability of the fungal agent against harsh environmental conditions and its sclerotial structure, it was deduced that if the fungi could be implemented in a stand-alone or especially integrated with each other, it will provide long-term, high-level broomrape biocontrol, indicating the advantages of *A. alliaceus* against other alternative fungal agents.

***Key words:*** *Orobanche*, broomrape, *Aspergillus*, biocontrol, mycoherbicide, bioherbicide.

## *Aspergillus alliaceus* Thom ve Church ile Toprak Altı ve Toprak Üstü Etkili *Orobanche* L. Biyokontrolü için Çeşitli Pesta Tablet Denemeleri

**Özet:** Bu çalışmada; hem toprak altı hem de toprak üstü şartlarda pesta tabletlerin *Orobanş* kontrolünde ne derecede etkili olabileceklerinin tespit edilmesi ve her iki ortam şartında da uygun tablet formülasyonlarının belirlenmesi amaçlanmıştır. Sonuçlara göre T-1, T-2, T-3, T-5, T-12 ve T-13 tabletleri, özellikle ekim öncesi yüksek doz kırık tablet uygulaması ile toprak altı orobanş bulaşmasını büyük oranda indirgemiştir. Toprak üstü uygulamalarda ise herhangi bir tablet uygulamasının veya doğrudan sklerot temasının orobanş biyokontrolünde önemli olduğu ve başarılı bir toprak üstü uygulama için küfün orobanşa yakınlığının veya temasının şart olduğu anlaşılmıştır. Sonuç olarak; fungusun sert çevresel koşullara dayanıklılığı ve onun sklerotial yapısı dikkate alındığında, toprak altı veya toprak üstü olmak üzere tek bir ortamda veya özellikle birbiri ile eşgüdümlü olarak her iki ortamda uygulandığında uzun dönemde onun yüksek seviyede orobanş biyokontrolünü sağlayacağı tespit edilmiştir. Sklerotial özelliği ve orobanş biyokontrolündeki etkinliği, *Aspergillus alliaceus*'a diğer alternatif fungal ajanlara göre üstünlük kazandırmaktadır.

***Anahtar kelimeler:*** *Orobanche*, Canavarotu, *Aspergillus*, biyokontrol, mikoherbisid, biyoherbisid.

## Introduction

*Orobanche* (broomrape) is an obligate root parasitic plant that can cause severe yield reduction in many important crops (Rubiales & Fernández-Aparicio 2012). Fungal pathogens display their pathogenic effects only on parasitic weeds without damaging the host plant and therefore they are known as bioherbicides (Charudattan 2001). Microorganisms are an inexpensive method of control but their use is governed by regulatory constraints. We have recently detected a new fungal biocontrol agent *Aspergillus alliaceus* Thom & Church, on natural orobanche samples (Aybeke et al. 2014). *A. alliaceus* is a very interesting fungus due to its sclerotial form, being comprised of high fungal mycelium and food reserves. Sclerotia can remain dormant in the soil for many years under unsuitable environmental conditions and when the conditions turn to normal it can create new micellary forms on parasitic weeds, which makes it superior to other non-sclerotial fungal biocontrol agents. So, our efforts have been directed to preparation of suitable sclerotial *A. alliaceus* formulations and to applications of a pesta tablet pathogenic to broomrape. Some of the pesta tablet formulations tested so far showed a high toxic effect

**Table 1.** Pesta tablet ingredients.

| Tablet No. | Tablet formulation |
|---|---|
| T-1 | Semolina (80g) + farina (25g) + 62ml distilled water + liquid conidial solution (3 days), modified from Connick et al. (1991)'s method. |
| T-2 | fungal mycelium (5g) + 5ml distilled water + starch (2g) + farina (15g) + 2ml unrefined corn oil + silica (7g), modified from Amsellem et al. (1999). |
| T-3 | 1 M sucrose (50ml) + farina (79g) + fungal mycelium (5g) + $CaCl_2$ (5.4g) + 2ml crude corn oil, modified from Amsellem et al. (1999). |
| T-4 | 20ml liquid conidial solution (3 days) + special ground rye farina (58g) + semolina (10g) + farina (20g) + 23ml distilled water, modified from Müller-Stöver et al. (2002). |
| T-5 | semolina (32g) + farina (12.4g) + liquid conidial solution (3 days) + 23ml distilled water, modified from Connick et al. (1998). |
| T-6 | special ground *Sorghum vulgare* farina (0.49g) + semolina (11.5g) + **sclerotia** (0.6g) + 2ml potato broth (sclerotia g/*Sorghum* + semolina (0.36g), Aybeke et al. (2015). |
| T-7 | special ground *S. vulgare* farina (0.88g) + semolina (0.118g) + **sclerotia** (0.019g) + 0.75ml potato broth (sclerotia g/*Sorghum* + semolina (0.019g), Aybeke et al. (2015). |
| T-8 | special ground *S. vulgare* farina (0.075g) + 0.138g semolina (0.138g) + **sclerotia** (0.019g) + 0.2ml potato broth (sclerotia g/*Sorghum* + semolina (0.089g), Aybeke et al. (2015). |
| T-9 | special ground *S. vulgare* farina (0.05g) + semolina (0.176g) + corn starch (0.050g) + **sclerotia** (0.018g) + 0.2ml potato broth (sclerotia g/*Sorghum* + semolina (0.06g), Aybeke et al. (2015). |
| T-10 | special ground *S. vulgare* farina (0.05g) + semolina (0.065g) + special ground rye flour (0.027g) + **sclerotia** (0.10g) + 0.2ml potato broth (sclerotia g/*Sorghum* + semolina (0.06g), Aybeke et al. (2015). |
| T-11 | semolina (156g) + special ground *S. vulgare* farina (95.1g) + **sclerotia** (0.10g) + yeast extract (3.6g) + kaolin (26.7g) + 165ml potato broth, Aybeke et al. (2015). |
| T-12 | Fungus-free liquid culture + **sclerotia** (0.60g), Aybeke et al. (2014). |
| T-13 | Sclerotial solid culture with fungal mycelia, Aybeke et al. (2014). |

on broomrape and the effective areas of pesta tablets applications under soil were given in our latest study (Aybeke et al. 2015). Pesta granules were found to reduce to minimize the broomrape infection within nearly a 0.2–0.3cm diameter of granules in dense soil conditions. The histopathological effects after fungal contact to broomrape were also reported. The granules [no.1, 2, 4 and 5 granules and no. 7 and 8 granules (sclerotial)] gave the best efficient results with combination of delayed crop sowing and extra high amount tablet applications, decreasing orobanche contamination up to almost 95%. As a continuation of this recent study, in the present study, it is aimed to test *Orobanche* biocontrol by different and new fungal tablet applications untried so far in below- and above-ground conditions and to determine fungal pesta tablet formulations in these two –ground conditions. The results are expected to clarify the availability of *A. alliaceus* tablet applications in broomrape biocontrol under long-term, real environmental below- and above-ground conditions.

## Materials and Methods

*Orobanche cernua* Loefl., which is a widespread species in Thrace Region of Turkey, and the sunflower HA89-B (*Helianthus annuus* L.) were used as the broomrape and the host plant, respectively. The fungal biocontrol agent was *Aspergillus alliaceus* Thom & Church (CBS collection; code: CBS 563.65). *A. alliaceus* isolates were grown on 20% Czapek Yeast Sucrose Agar (CY20S) media. A liquid suspension was prepared according to the method given in Aybeke et al. (2014) as "in Malt Extract Broth (malt extract, 20g; peptone, 1g and distilled water, 1000 ml) with 2.5 × 106 spore $ml^{-1}$ (the spore number was determined by Thoma counting method)" (Aybeke et al., 2014). For sclerotial solid culture with fungal mycelia, the isolated microfungi were inoculated on 20% CY20S media and this sclerotial agar were used (Aybeke et al. 2014). All experiments were conducted in small plastic pots filled with soil for their two-thirds volume. The soil consisted of sand and clay (1:1 ratio). In the experiments, broomrape seeds were scattered on the soil and stirred.

The pesta-tablet (see Table 1 for ingredients) mixtures were prepared using a stainless-steel pasta maker. Very thin sheets (1-1.5mm) were dried in the room conditions and then cut into small pieces (250μm - 2mm) (Zahran et al. 2008).

*Below-ground conditions tablet trials*

The first experimental setup is shown in Table 2. In this experiment, efficacy of different dosages and application methods of 5 tablets (T-1 to T-5) (late sunflower sowing, irrigation before crop sowing, tablet crushing, etc.) were investigated in the broomrape biocontrol. In all experiments, pots including normal field soil (non-sterile) were used. The tablets and orobanche seeds were mixed with the soil where sunflower seeds were also planted in the pots.

**Table 2.** 1st below-ground experiments (pot) focused on tablet amounts and its applications efficacy. The compositions of the tablets are described in Table 1. The text written in bold style represents the difference with the other alternative options.

| Exp. No. | Application method and amounts** |
|---|---|
| Control-1 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg), 3 sunflower seeds. |
| Control-2 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg), **the pots were watered, and 3 sunflower seeds were sown one week later (alternative to Control-1) (**modified from Aybeke et al. 2015). |
| 1-1 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-1 tablet + 3 sunflower seeds. |
| 1-2 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-1 tablet + 3 sunflower seeds **(the pots were watered, late sowing + crushed tablet, as alternative to 1-1 (**modified from Aybeke et al. 2015). |
| 1-3 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-2 tablet + 3 sunflower seeds. |
| 1-4 | No: 3 pots, soil (560g) + **10g T-2 tablet** + *Orobanche* seeds (215mg), the pots **were watered, and the sunflower seeds were sown one week later + crushed tablet; as alternative to 1-3 (**modified from Aybeke et al. 2015). |
| 1-5 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-3 tablet + 3 sunflower seeds. |
| 1-6 | No: 3 pots, soil (560g) + T-3 tablet **(10g)** + *Orobanche* seeds (215mg), the pots **were watered, and the sunflower seeds were sown one week later + crushed tablet**; **as alternative to 1-5 (**modified from Aybeke et al., 2015). |
| 1-7 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-4 tablet + sunflower seeds. |
| 1-8 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-5 tablet + sunflower seeds. |
| 1-9 | No: 3 pots, soil (560g) + *Orobanche* seeds (215mg) + 4.5g T-5 tablet + sunflower seeds (the pots **were watered, and the sunflower seeds were sown one week later + crushed tablet**; **as alternative to 1-8**) **(**modified from Aybeke et al. 2015). |

**Table 3.** 3rd experiment (in small size pot, no: s-80), "T-13" only were used in all these trials.

| Appl. code | Application |
|---|---|
| Control | soil (80g) + *Orobanche* seeds (0.08g) + 3 sunflower seeds. |
| 1 | soil (80g) + *Orobanche* seeds (0.08g) + tablet (0.04g; 0.5g tablet/kg soil) + 3 sunflower seeds. |
| 2 | soil (80g) + *Orobanche* seeds (0.08g) + tablet (0.04g; 1g tablet/kg soil) + 3 sunflower seeds |
| 3 | soil (80g) + *Orobanche* seeds (0.08g) + tablet (0.29g; 3g tablet/kg soil) + 3 sunflower seeds. |
| 4 | soil (80g) + *Orobanche* seeds (0.08g) + tablet (0.48g; 6g tablet/kg soil) + 3 sunflower seeds. |
| 5 | soil (80g) + *Orobanche* seeds (0.08g) + tablet (0.72g; 9g tablet/kg soil) + 3 sunflower seeds. |
| 6 | soil (80g) + *Orobanche* seeds (0.08g) + tablet (0.96g; 12g tablet/kg soil) + 3 sunflower seeds. |

**Table 4.** *Orobanche* infection quantities in the 1st below-ground experiment.

| | The number of healthy broomrapes | | | | |
|---|---|---|---|---|---|
| **Exp. No** | **Mean** | **Std. Deviation** | **Mean Diff.** | **Std. Error** | **Sig.** |
| Control 1 | 32.6 | 2.6 | - | - | - |
| Control 2 | 31.5 | 1.9 | - | - | - |
| 1-1 | 17.8 | 4.1 | -15.0 | 1.9 | 0.001** |
| 1-2 | 5.2 | 3.7 | -27.6 | 1.9 | 0.001** |
| 1-3 | 25.2 | 2.5 | -7.6 | 1.9 | 0.001** |
| 1-4 | 13.8 | 1.6 | -18.8 | 1.5 | 0.004** |
| 1-5 | 28.4 | 0.8 | -4.4 | 1.9 | 0.163 |
| 1-6 | 22.8 | 3.1 | -9.8 | 1.5 | 0.006** |
| 1-7 | 34.2 | 3.9 | 1.4 | 1.9 | 0.970 |
| 1-8 | 14.6 | 3.5 | -18.2 | 1.9 | 0.002** |
| 1-9 | 9.6 | 1.8 | -23.2 | 1.9 | 0.001** |

****** The mean difference between the control and the experimental groups is significant at the level of 0.05.

**Table 5.** *Orobanche* infection quantities in the $2^{nd}$ below-ground experiment.

| | The number of healthy broomrapes | | | | |
|---|---|---|---|---|---|
| **Tablet No** | **Mean** | **Std. Deviation** | **Mean Diff.** | **Std. Error** | **Sig.** |
| Control | 33.0 | 3.4 | - | - | - |
| T-6 | 35.8 | 1.6 | -1.2 | 2.0 | 0.1 |
| T-7 | 30.3 | 1.5 | -5.6 | 2.0 | 0.5 |
| T-8 | 29.6 | 2.7 | -2.6 | 2.0 | 0.1 |
| T-9 | 29.8 | 5.6 | -3.2 | 2.0 | 0.4 |
| T-10 | 31.4 | 3.1 | -3.6 | 2.0 | 0.6 |
| T-11 | 30.3 | 2.6 | -3.0 | 2.1 | 0.6 |
| T-12 | 23.8 | 2.7 | -17.4 | 1.2 | 0.002* |
| T-13 | 14.8 | 1.1 | 0.5 | 1.4 | 0.001* |

***** The mean difference between the control and the experimental and groups is significant at the 0.05 level.

**Table 6.** *Orobanche* infection quantities in $3^{rd}$ below-ground experiment.

| | The number of healthy broomrapes | | | | |
|---|---|---|---|---|---|
| **Appl. Code*** | **Mean** | **Std. Dev.** | **Mean Diff.** | **Std. Error** | **Sig.**** |
| Control | 31.8 | 3.1 | | | |
| 1 | 28.8 | 2.3 | -3.0 | 2.2 | 0.54 |
| 2 | 30.4 | 2.3 | -1.4 | 2.2 | 0.95 |
| 3 | 26.2 | 3.7 | -5.6 | 2.2 | 0.07 |
| 4 | 24.0 | 4.7 | | | |
| 5 | 24.6 | -3.5 | 4.2 | 1.9 | 0.082 |
| 6 | 15.8 | 4.1 | -16.0 | 2.2 | 0.001** |

***** Refer to Table 3 for a detailed description of the codes, ** The mean difference between the control and the experimental and groups is significant at the 0.05 level.

In the second experiment (pot), five pots were used for each sclerotial tablet trial (T 6-13) and the tablets were placed in the pots in two parallel rows at a depth of 6 and 8cm from soil surface (see Aybeke et al. 2015). In this experiment, tablets were not distributed randomly into the soil but were put in the pots as 2 horizontal and parallel rows. to assure the contact of broomrapes at emerging status with the tablets.

In the third experiment, the threshold limit doses of T-13 tablets, which gave the best results in a previous experiment, for effective broomrape biocontrol were tested.

The smallest pots were used in the experiments. The third experimental setup is shown in Table 3.

*Above-ground conditions tablet trials*

In the first above-ground experiment the tablets which were determined to be the lowest effective against broomrape (T-10, T-11, as indicated in Table 5) were sprinkled (not sown) on top of the soil in 10 pots after the post-emergent stage of the broomrape.

In the second above-ground experiment, dry sclerotia and fungal mycelial liquid solution were sprayed directly on the intact broomrape tubercles by means of pipette and the tubercles were kept moist by wrapping with aluminum foil, after which the pathogenic effects were observed.

After each experiment, broomrape infection rates were determined by counting the host plant roots and noting the number of healthy broomrapes. The biocontrol effect of the tablet formulations was found by comparing the healthy broomrape rates of each experiment with that of the control (Bedi 1994). A one-way ANOVA test was used for the comparison of *Orobanche* infection values among the groups (P value is statistically significant at <0.05).

## Results

The results of the experiments are shown in the Tables 4-6.

*Below-ground tablet applications*

Table 4 presents the results of the $1^{st}$ experiment. Based on the experiments 1-1, 1-2, 1-3, 1-4, 1-6, 1-8 and 1-9 “T-1, T-2, T-3 and T-5” gave relatively better results,

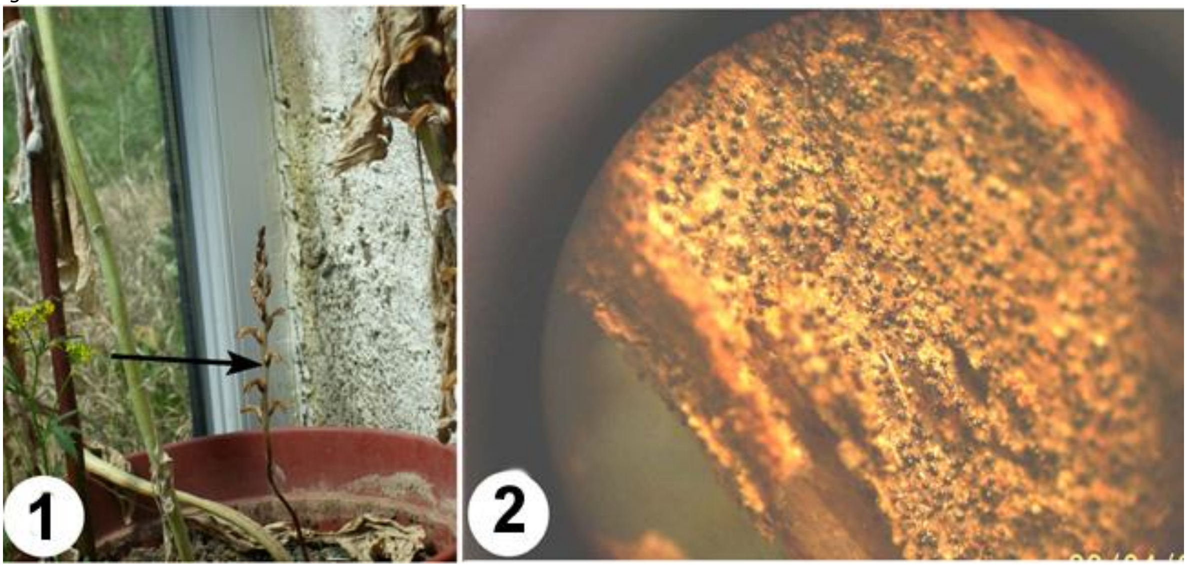


**Figure 1. (1)** Twisted and darkened *Orobanche* stem (arrow) in contact with dry sclerotia; **(2)** dense sclerotia of the *A. alliaceus* in the same broomrape stem inside.

because the mortality rate of *Orobanche* was highest or in other words, healthy *Orobanche* values were the lowest in the assays with those tablet formulations.

Table 5 presents the data on the 2nd below-ground experiment. As one can see in Table 5, healthy/intact normal *Orobanche* values were the lower in T-12 and T-13 than the other tablets meaning that these two tablets significantly reduced *Orobanche* infection compared to the others.

The results of the 3rd below-ground experiment were different based on the tablet dosage, so that as long as sclerotia increased the infections in the broomrape were reduced more, as indicated in (Table 6). Also in all below-ground experiments, there were no adverse effects on sunflower plants.

<u>*Above-ground tablet applications*</u>

In the 1st above-ground experiment, 9 out of 10 broomrapes died. However, particularly in the assays with tablets close to the broomrape stems, more successful results were observed. In the 2nd above-ground experiment, *Orobanche* stems and tubercles which were in contact with sclerotia were darkened and dead (Figure 1).

**Discussion**

Based on three below-ground experiments in the present study, important results about how effective sclerotial and non-sclerotial tablets (fungal mycelial/liquid solution) as combination with different doses and application methods against *Orobanche* infection were obtained.

In the below-ground trials, T-1, T-2, T-3 and T-5 tablets, prepared with fungal mycelium/liquid conidial solution were more successful than the others. Particularly in the 1st below-ground experiment, late sunflower sowing and crushed-tablet applications as well as increasing tablet dosage on *Orobanche* biocontrol were remarkable. Similar results were obtained in other researches (Connick et al. 1998, Aybeke et al. 2015); regarding present results, “no. 1, 2, 4 and 5 granules” (according theirs tablet numbers; with fungal liquid solution) and “no. 7 and 8 granules” (sclerotial tablets) were more effective tablets reducing at nearly 95% ratio *Orobanche* contamination to host when they used in combination with delayed crop sowing and high dose tablet inoculation to soil. In the 2nd and 3rd below-ground experiments, efficacy of sclerotial tablets were tested and T-12 and T-13 tablets were found to be more effective than the others. Especially, high sclerot amounts in T-12, and sclerot as combination with fungal mycelial solution in T-13 were demonstrated to improve the success of broomrape biocontrol (Tables 2 and 3). Considering under solid soil conditions, it will be understood that the activity of tablets was certainly much lower than those of the above-ground experiments. However, late sunflower seed sowing, high dose applications of tablets including dense fungal content were very effective in overcoming broomrape infection. So, it is evident that the content of the tablet, dose of the fungus and integrated application methods with tablets increase the success *Orobanche* biocontrol. Similar results were seen in our recent studies on the subject (Aybeke et al. 2014, 2015).

In the 1st and 2nd above-ground experiments, broomrape biocontrol was successfully achieved. It was clearly understood that the proximity/contact of tablets or sclerot with broomrape is a requirement for an effective biocontrol. Considering the resistance of sclerotia to harsh climatic conditions and heat, *A. alliaceus* is ahead of other alternative fungi. *Fusarium oxysporum* is the other alternative fungi and its pesta granules were found to reduce *Orobanche* contamination to host at around 90% (Müller-Stöver et al. 2002). *Fusarium* and *Aspergillus* were alternative fungal agents for each other and no other fungal agents were reported so far (Müller-Stöver et al.

2002, Aybeke et al. 2014, 2015). Since *Fusarium* is a fungus not forming sclerotia, high amounts of tablets including *Fusarium* will be required to use in soil every year for a long time and successful *Orobanche* biocontrol. However, there is no such requirement for *A. alliaceus* because of its sclerotic structure since sclerotia ensure the viability of the fungi even in the most difficult conditions (Haarmann et al. 2009). In addition, the root rot disease caused by *Fusarium* in many crops is the handicap of using this fungus in biocontrol applications. *A. alliaceus* provides an effective host plant protection, without any fungal damage to the crop, against all development stages (from seed to flowering period) of *Orobanche* (Aybeke et al. 2014, 2015). So; it may decrease *Orobanche* seed bank in soil for a long time by high dose application (Aybeke et al. 2014, 2015).

Based on the present results, it can be concluded that an ideal biological control needs the use both below-ground tablets and aerial tablets. Future studies will be conducted as specific biochemical experiments to reveal the active chemicals secreted during the fungal infection.

**Acknowledgement**

The author thanks Prof. Dr. Necdet SÜT for his help with the statistical analysis, and to Assoc. Prof. Dr. Burhan ŞEN and Assist. Prof. Dr. Suzan ÖKTEN for growing the fungal material.

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