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Parasitic Bacteria and Fungi on Common Mistletoe (*Viscum album* L.) and Their Potential Application in Biocontrol

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Abstract

This study was carried out to identify pathogenic bacteria and fungi on mistletoe (*Viscum album* L.) and investigate their potential use in biological control of this parasitic plant. For this purpose, a total of 48 fungal isolate and 193 bacterial strains were isolated from contaminated *V. album* during the summers 2005–2006. The isolated bacterial strains and fungal isolates were identified by using the Sherlock Microbial Identification System (MIS; Microbial ID, Newark) and microscopic methods, respectively. The bacterial strains that induced hypersensitive reaction (HR) on tobacco (*Nicotiana tabacum* L.) and fungal isolates were tested for pathogenicity on young shoots of mistletoe by using injection methods. The pathogenic bacterial strains and fungal isolates were also tested for their activity against mistletoe using spray methods. Five bacterial strains (two *Burkholderia cepacia*, one each of *Bacillus megaterium*, *Bacillus pumilus* and *Pandoraea pulminicola*) were HR and pathogenicity positive when injected but none of them when sprayed on mistletoe. When fungi were injected, 32 isolates were pathogenic but only thirteen when sprayed on mistletoe. *Alternaria alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *Acremonium kiliense* VA-11 fungal isolates were the most effective ones and caused strong disease symptoms on mistletoe. The present study is the first report on the efficiency of potential biocontrol agents against mistletoe in Turkey.

Introduction

The European mistletoe (*Viscum album* L.) is a parasitic flowering plant with functional chlorophyll, and it is considered as a semiparasite that lives on a wide range of woody plant species (Zuber 2004; Catal and Carus 2011). Mistletoe is dispersed by birds that eat the berries and then excrete the seeds or smear them on branches by wiping the sticky pulp off their beaks. Under proper conditions, the seeds germinate and the roots penetrate the branch of the host plant. It causes a great deal of damage in forests, orchards, plantations and ornamentals worldwide. Barney et al. (1998) have listed 452 plant species as hosts of *V. album*.

Crimean pine (*Pinus nigra* Arnold) has wide natural distribution in Balkans, Syria, the Crimea, Cyprus and Turkey (Yücel 1998) and covers an area of approximately 4.2 million ha in Turkey (Catal and Carus 2011). Crimean pine is the most important timber species in Turkey as it represents approximately 25% of the standing volume (approximately 297 million m3) of the Turkish forests (Konukcu 1998). Its growth is seriously affected by parasitic/hemiparasitic plants such as mistletoe (Ergun and Deliorman 1995, 1997).

Compared to weeds, parasites are difficult to control by conventional means because of their life style. They are intimately involved and have so much metabolic overlap with the host that differential treatments are very difficult to develop (Aly 2007). Control of mistletoe is an important problem for the forest service in Turkey (Yüksel et al. 2005). Many fungi and insects are pathogens or herbivores, respectively, of mistletoes (Shamoun 1998). None, however, are sufficiently studied and developed for operational use as biological control agents.

Parasitic plants cannot be controlled effectively by traditional methods or herbicides. Several different approaches such as mechanical, chemical and use of resistant varieties to control parasitic plants are currently in use, but are only partially successful (Abbasher and Sauerborn 1992; Zuber 2004). Direct methods such as pruning infected branches or removing

infected trees are still the only practical methods. However, they are applicable only in small areas, such as parks, orchards or cities (Weber 1993).

In the recent decades, some chemicals have become available for parasitic plants control (Garcia-Torres 1998), although few herbicides are able to selectively control only parasitic plants (Goldwasser and Kleifeld 2004; Gressel et al. 2004). The systemic herbicides 2.4-D, 2.4-5 T, 2.4-MCPB and di-chloro ethane were found to kill *V. album* shoots on *Abies*, with little host damage, and tests with herbicides on *V. album* growing on various deciduous trees have given promising results (Hawksworth 1983). Baillon et al. (1988) reported detailed experiments with 2.4-DB and glyphosate. They observed that no herbicide is found in the host, but that maximum effects occur only 4–6 months after treatment. Other possible herbicides are listed by Weber (1993). The application of ethephon ((2-chloroethyl)-phosphonic acid, Florel®) has been discussed by Adams et al. (1993). However, the chemical approach poses some difficulties are as follows: lack of application technology, chemical damage to the host, continuous parasite seed germination throughout the season, marginal crop selectivity, environmental pollution, low persistence and availability. For this reason, there is some need for alternative control methods such as biological control.

Biological control of parasites by using plant pathogens has gained acceptance as a practical, safe and environmentally beneficial management method applicable to agro-ecosystems (Charudattan 2001). Several fungi infect mistletoes and are shown to be potential as a biological control agent against them: *Plectophomella visci* Moesz (= *Phyllosticta visci* Sacc.), *Septoria visci* Bres. and *Sphaeropsis visci* (Fr.) Sacc. (Brandenburger 1985), *Colletotrichum gloeosporoides* (Sacc.) Penz. (Stojanovic 1989), *Botryosphaerostroma visci* (*Plectophomella visci* Moesz) (Weber 1993; Fischl 1996; Geza et al. 2009), *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not. (anamorph: *Fusicoccum aesculi* Corda = *Macrophoma* sp.), *Gibberidea visci* (Fuckel), *Sphaeropsis visci* (Alb. & Schwein.) Sacc.,) (Dragan et al. 2004) and *Phaeobotryosphaeria visci* [Syn.: *Botryosphaeria visci* (Kalchbr.) Arx & E. Müll.; anamorph: *Sphaeropsis visci* (Fr.) Sacc.] (Varga et al. 2012). Nevertheless, *V. album* is known to harbour relatively few fungal pathogens, presumably because of an effective defence system (Holtorf et al. 1998). Although there are many reports of isolation of fungal pathogens attacking *V. album*, none are sufficiently studied and developed for operational use as biological control agents (Geza et al. 2009). Nonetheless, given the potential number of agents and the advantages of the approach, development of biological control as a management option appears promising for the near future (Shamoun 1998).

The aim of this study was to determine pathogenic bacterial and fungal flora on *V. album* and investigate their potential application in biocontrol.

Material and Methods

Collection and transfer of infected plant samples

Diseased parts of common mistletoe (stems and leaves) were collected in Erzurum and Artvin Provinces of the Eastern Anatolia region of Turkey in April and September of 2005 and 2006. The samples were collected in sterilized polythene bags and brought to the laboratory for the isolation of the associated fungal and bacterial pathogens and then stored at 4°C until examined.

Bacterial and fungal isolation and storage condition

All bacterial strains and fungal isolates tested in this study were isolated from stems and leaves of diseased mistletoe plant samples (Fig. 1). The growth media used for fungi and bacteria were potato dextrose agar (PDA) and nutrient agar (NA), respectively.

Isolation was performed by cutting surface-disinfected plant materials into small pieces from the margins of spots with a sterile razor blade. The collected plant sample surfaces were washed with distilled and sterilized water to remove epiphytic bacteria and fungi. Diseased portions of plant samples (approximately 1×1 cm) were cut under aseptic condition into small bits into sterile Petri dishes. The pieces were surface sterilized with 96% ethanol for 30 s, rinsed in sterilized water for 1 min and blotted dry with sterile Whatman's filter papers.

For bacterial isolation, the cut and surface-sterilized plant tissue was inserted into and soaked in a test tube containing 2 ml of sterile NaCI solution. Cut pieces were ground, and 100 μl of homogenate was streaked on Petri dishes containing standard medium NA. Plates were incubated at 26°C for 2–3 days. Dominant colonies were subcultured on NA for pure cultures. From each infected sample, two single colonies were isolated and selected as a representative for this study. Representative colonies were purified by repeated re-streaking on NA. Isolated strains were stored in 15% glycerol and LB broth at −80°C for further studies.

For fungal isolation, cut and surface-sterilized pieces were placed on 9-cm Petri dishes containing solidified PDA. The plates were then incubated at 25°C for 5–7 days. All pieces of mycelia that developed on PDA were transferred to fresh PDA for the identification, or pure cultures were obtained using the single-spore technique. Pure culture was inoculated onto PDA slants prepared in screw-cap tubes. The cultures were incubated at 25°C for 5–7 days to allow growth to cover most of the slants. All cultures were maintained on the slants of freshly prepared PDA and stored at 4°C.

Bacterial and fungal pure cultures were deposited in the microbial collection of Department of Plant Protection, Faculty of Agriculture, Atatürk University, Erzurum, Turkey.

Identification of the bacteria

Preparation and analysis of fatty acid methyl esters (FAME) from the whole-cell fatty acids of bacterial

Fig. 1 Infected mistletoe plant samples used for isolating of bacteria and fungi

strains were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA; Miller and Berger 1985; Roy 1988). FAMEs were separated by gas chromatography (Hewlett-Packard Inc. Agilent HP 6890 series) using a fused-silica capillary column (25 m × 0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of each bacterial strain were identified by comparing the results to the commercial databases (TSBA 50) with the MIS software package. The identity of bacterial strains was revealed by computer comparison of FAME profiles of the unknown test strains with those in the library. The isolates with an index score higher than 0.5 were considered as 'identified with a high degree of certainty'.

Identification of the pathogenic fungi

The fungi observed as potential biocontrol agents against mistletoe were also identified on the basis of colony characteristics, such as diameter, colour, mycelial texture and conidial morphology under optical microscope using Kendrick (1971) and Barnett and Hunter (1975).

Hypersensitivity tests of the bacteria

All of the bacterial strains identified by MIS were tested for hypersensitivity on tobacco (*Nicotiana tabacum* L.) as described by Klement et al. (1964). The bacterial suspension (10^8 cfu/ml) prepared in sterile distilled water and infiltrated into the intercostal area of the leaves of tobacco plants by using a 3-cc syringe (Becton Dickinson, Franklin Lakes, NJ, USA). The inoculated plants were incubated in a completely randomized design on the greenhouse bench for 24–48 h at 20–28°C. The presence of rapid tissue necrosis at the inoculation site was recorded within 24–48 h after infiltration. This test was repeated at least three times for each strain. For HR tests, sterilized distilled water (sdH2O) was used as a negative control.

Pathogenicity tests of the bacteria and fungi

The bacterial strains that induced HR on tobacco plants and fungal isolates were tested for pathogenicity on the young leaves of mistletoe by using injection methods in field conditions.

For each bacterial experiment, a single colony was transferred to 500–ml flasks containing nutrient broth (NB) and grown aerobically in flasks on a rotating shaker (150 rpm) for 48 h at 26°C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of 10^8 cfu/ml. The tested fungus was grown on PDA medium for the sporulation on Petri dishes at 25°C for 5 days (light during the day and dark at night). The concentration of spores was determined by using a hemocytometer, adjusted to 1×10^6 spores/ml with an appropriate dilution and used fresh as a stock suspension. The resulting bacterial and fungal suspensions were used to treat young leaves.

The bacterial and fungal suspensions were infiltrated into young leave by using a 3-cc syringe. They were covered with polyethylene bags for 2 days. After this, they were studied for necrotic spots. If there was a necrotic spot on any of the leaves, the pathogenicity would be evaluated as positive, or not negative. For each bacterial strain and fungal isolate, three shoots were used. For pathogenicity tests, sterilized distilled water (sdH2O) was used as a negative control.

Field experiments

The pathogenic fungal isolates resulting in necrotic spots on leaves by using injection method in field conditions were also tested for their biological control activity using spraying. Pathogenicity tests were performed by using fungal suspensions as described above. The suspensions were sprayed on young shoots of mistletoe by using a bottle sprayer. Each of the fungal isolates was applied on a mistletoe shoot at the same height in four different directions on the branches of a pine tree at three different locations

(Şenkaya, Ispir and Uzundere) in Erzurum Province. The applied shoots were covered with polyethylene bags for 2 days. Then, they were observed for necrotic spots on leaves. For each fungal isolate, 12 shoots and three trees were used. Sterilized distilled water (sdH2O) was used as a negative control. Infection rate was recorded in 8 months after using a 1–5 scale in which 1: healthy plant and all leaves green, 2: 1–25% of leaves showing wilt symptoms, 3: 26–50%, 4: 51–75% and 5: >75% of plants displaying wilt symptoms or plants dead (Kelman and Person 1961). Percentages of infected mistletoe (%) and infection rates on mistletoes were given as the average of five different locations 8 months later. The experiments were repeated for three times in 3 years. The results were given as the average of 3 years.

Pathogenicity tests of the most effective fungal isolates on non-target host plants

The four most effective pathogenic fungal isolates were also tested for their pathogenicity using spray method on three non-target host plants (*Pinus sylvestris* L., *Prunus armeniaca* L. and *Prunus cerasus* L.) in field conditions. The pathogenicity tests were carried out on 3-year-old trees and performed by using fungal suspensions as described above. Each of the fungal isolates was applied on young shoots of the non-target host plant at the same height in four different directions of a non-target host plant in Erzurum Province. They were covered with polyethylene bags for 2 days. After 20 days, the pathogenicity test results were evaluated. If there was a necrotic spot on any leave, the pathogenicity would be evaluated as positive, or not negative. Sterilized distilled water (sdH2O) was used as a negative control. This test was repeated at least three times for each fungal isolate.

Results

193 bacterial and 48 fungal strains were isolated from diseased *Viscum album* during the summers 2005–2006. MIS identification results of the bacterial strains and microscopic identification results of the fungal isolates are given in Table 1 and Table 2, respectively.

According to the MIS results, identified 193 bacterial strains belong to 30 genera. Bacterial identification results are given at the genus level but fungal identification results are given at the genus or species level. The most abundant bacterial genera were *Bacillus* (19.58%), *Pseudomonas* (13.91%), *Stenotrophomonas* (13.91%) and *Acinetobacter* (13.40%). Five bacterial strains including two *Burkholderia cepacia* and one of each of *Bacillus megaterium*, *Bacillus pumilus* and *Pandoraea pulminicola* (data not shown) showed hypersensitive response (HR) and were pathogenicity positive when applied using injections. But, none of them were pathogenic when sprayed on mistletoe (Table 1).

According to pathogenicity tests of fungus injected in mistletoe, 32 isolates were pathogenic, but only 13 when applied as spray. 16 fungal isolates did not cause disease symptoms on mistletoe when applied with either method. The most pathogenic fungal species when sprayed on mistletoe was *Alternaria alternata* (53.84%). *Alternaria alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *Acremonium kiliense* VA-11 were the most effective fungal isolates detected on mistletoes showing strong disease symptoms. Percentage of infected mistletoe (%) and infection rate according to 1–5 scale varied from 35% to 45% and from 3.37 to 4.20, respectively. As shown in Table 2, the mean percentage of infected mistletoe (%) at three different locations (Ispir, Şenkaya and Uzundere) was 36.76, 22.00 and 14.38%, respectively. The mean infection rate on mistletoe (scale 1–5) in Ispir, Şenkaya and Uzundere was 4.08, 2.82 and 2.31, respectively. The pathogenicity test results of the most effective fungal isolates *A. alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *A. ciliense* VA-11 were negative in all.

Discussion

This study was carried out to investigate the possibilities of biological control of *Viscum album* using fungi or bacteria. For this purpose, a total of 48 fungal isolates and 193 bacterial strains were isolated from diseased *V. album*. A total of five bacterial strains showed hypersensitive response (HR) and proved to be pathogenically positive when tested by using injection method. But, none of them were pathogenic when sprayed on mistletoe. Our results support reports that any bacteria can parasitize and are shown to be potential as a biological control agent against mistletoes.

According to the pathogenicity test results of fungi using injection method, a total of 32 isolates were pathogenic, but thirteen of them were pathogenic when sprayed method on mistletoe. The other fungal isolates were not effective in killing the mistletoe without also injuring the host. *Alternaria alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *Acremonium kiliense* VA-11 fungal isolates were determined as the most effective isolates and were detected on mistletoe plants showing strong disease symptoms (Fig. 2). *A. alternata* appears to hold promise as a possible agent of biological control of the mistletoe also by Shabana et al. 1995;. Siddiqui et al. (2010) reported *A. alternata* leaf blight in *Chenopodium album*. In addition, *Alternaria* spp. are airborne moulds that are considered to have potential for the biological control of weeds such as *Amaranthus retroflexus* L (Ghorbani et al. 2000). There are no reports found in the literature that indicate *A. alternata* and *A. kiliense* to be pathogenic on mistletoe. Ours is the first report about *A. alternata* and *A. kiliense* on mistletoe.

Host specificity is of great importance when considering a pathogen for biological control (Sands and Pilgeram 2009). A narrow host range provides higher environmental safety of a bioherbicide, but can also limit effectiveness if more than one species has to be controlled (Elzein et al. 2006). In this study, *Alternaria alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *Acremonium ciliense* VA-11 fungal isolates were also tested for

Table 1
Number of isolated bacterial strains, their genus according to MIS, the number of hypersensitivity and pathogenicity positive strains

NIS (%)	Genus	HR+	PI+	PS+	NIS (%)	Genus	HR+	PI+	PS+
38 (19.58)	*Bacillus*	2	2	0	3 (1.54)	*Myroides*	0	0	0
27 (13.91)	*Pseudomonas*	0	0	0	3 (1.54)	*Paenibacillus*	0	0	0
27 (13.91)	*Stenotrophomonas*	0	0	0	2 (1.03)	*Aerococcus*	0	0	0
26 (13.40)	*Acinetobacter*	0	0	0	2 (1.03)	*Alcaligenes*	0	0	0
8 (4.12)	*Microbacterium*	0	0	0	2 (1.03)	*Kocuria*	0	0	0
5 (2.57)	*Clavibacter*	0	0	0	2 (1.03)	*Ochrobactrum*	0	0	0
5 (2.57)	*Corynebacterium*	0	0	0	2 (1.03)	*Roseomonas*	0	0	0
5 (2.57)	*Erwinia*	0	0	0	2 (1.03)	*Serratia*	0	0	0
4 (2.06)	*Enterobacter*	0	0	0	2 (1.03)	*Sphingobacterium*	0	0	0
4 (2.06)	*Micrococcus*	0	0	0	2 (1.03)	*Vibrio*	0	0	0
4 (2.06)	*Streptococcus*	0	0	0	2 (1.03)	*Yersinia*	0	0	0
3 (1.54)	*Rhodococcus*	0	0	0	1 (0.51)	*Agrobacterium*	0	0	0
3 (1.54)	*Burkholderia*	2	2	0	1 (0.51)	*Arthrobacter*	0	0	0
3 (1.54)	*Cellulomonas*	0	0	0	1 (0.51)	*Brevibacillus*	0	0	0
3 (1.54)	*Curtobacterium*	0	0	0	1 (0.51)	*Pandoraea*	1	1	0

NIS (%), Number of isolated strains and their percentage; HR+, Number of hypersensitivity positive strains on tobacco plants; PI+, Number of pathogenicity positive strains on young leave of mistletoe by using injection methods; PS+, Number of pathogenicity positive strains on young leave of mistletoe by using sprayed methods.

Table 2
Microscopic identification results of fungal isolates and their biological control activity against mistletoe

			Biological control activity of tested fungal isolates on mistletoe at three different locations							
			Ispir		Şenkaya		Uzundere		Average	
Isolates	PAT	Microscopic results	PIM%	DS	PIM%	DS	PIM%	DS	PIM%	DS
VAŞ-202	+	*Alternaria alternata*	63	4.3	46	4.3	26	4.0	45	4.20
VAŞ-205	+	*Alternaria alternata*	61	4.8	35	2.9	30	2.4	42	3.37
VAŞ-217	+	*Alternaria alternata*	66	4.9	32	2.2	22	1.9	40	4.00
VA-11	+	*Acremonium kiliense*	54	5.0	31	3.2	20	3.0	35	3.73
VAŞ-226	+	*Alternaria alternata*	43	4.1	27	3.0	11	1.9	27	3.00
VAŞ-214	+	*Alternaria alternata*	36	4.1	22	2.5	17	2.4	25	3.00
VAŞ-95	+	*Alternaria alternata*	34	3.0	21	3.9	14	2.1	23	3.00
VA-16	+	*Acremonium* sp.	28	4.1	18	2.6	14	2.3	20	3.00
VAŞ-9	+	*Geotrichum* sp.	30	4.5	19	2.5	11	2.0	20	3.00
VAŞ-200	+	*Aspergillus flavus*	17	2.9	8	1.5	5	1.6	10	2.00
VAŞ-208	+	*Ulocladium chartarum*	13	4.4	12	4.1	5	2.5	10	3.66
VAŞ-213	+	*Alternaria alternata*	16	2.9	8	1.7	6	1.4	10	2.00
VAŞ-215	+	*Nigrospora oryzae*	17	4.1	7	2.3	6	2.6	10	3.00
Mean PIM and DS			36.76	4.08	22.00	2.82	14.38	2.31	24.38	3.15
19 isolates	+	NI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16 isolates	-	NI	NT	NT	NT	NT	NT	NT	0.0	0.0

PAT, Pathogenicity test results; PIM, Percentage of infected mistletoe (%); DS, Disease severity on mistletoe according to 1–5 scale; NI, Not identified; NT, Not tested.

pathogenicity in different non-target host plants such as pine, apricot and cherry. None of them were pathogenic on these plants. *A. alternata* is one of the most cosmopolitan fungal species and is generally saprophytic (Rotem 1994), and these host-specific forms have been designated as pathotypes of *A. alternata* (Nishimura and Kohmoto 1983; Kohmoto et al. 1995). Seven varieties of the imperfect fungus have been described and these produce host-specific toxins and cause necrotic diseases on different plants (Kohmoto et al. 1995). It may be associated with crops such as *Foeniculum vulgare* Mill., *Lactuca sativa* L., *Cichorium intybus* L. and *Apium graveolens* L. (D'Amico et al. 2008).

There were differences in responses of the plants against different fungal isolates in different locations. In some applications of *A. alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *A. ciliense* VA-11 isolates, plants died completely (Fig. 2). Rainfall, humidity and temperature are considered to be the most important components of the environment that limit the utilization and effectiveness of biological control agents as mycoherbicides (Siddiqui et al. 2009).

In conclusion, our results suggest that *A. alternata* VAŞ-202, VAŞ-205, VAŞ-217 and *A. ciliense* VA-11 fungal isolates have a good potential to be used as biocontrol agents of the mistletoe. This study is the first report on the efficiency of the potential biocontrol

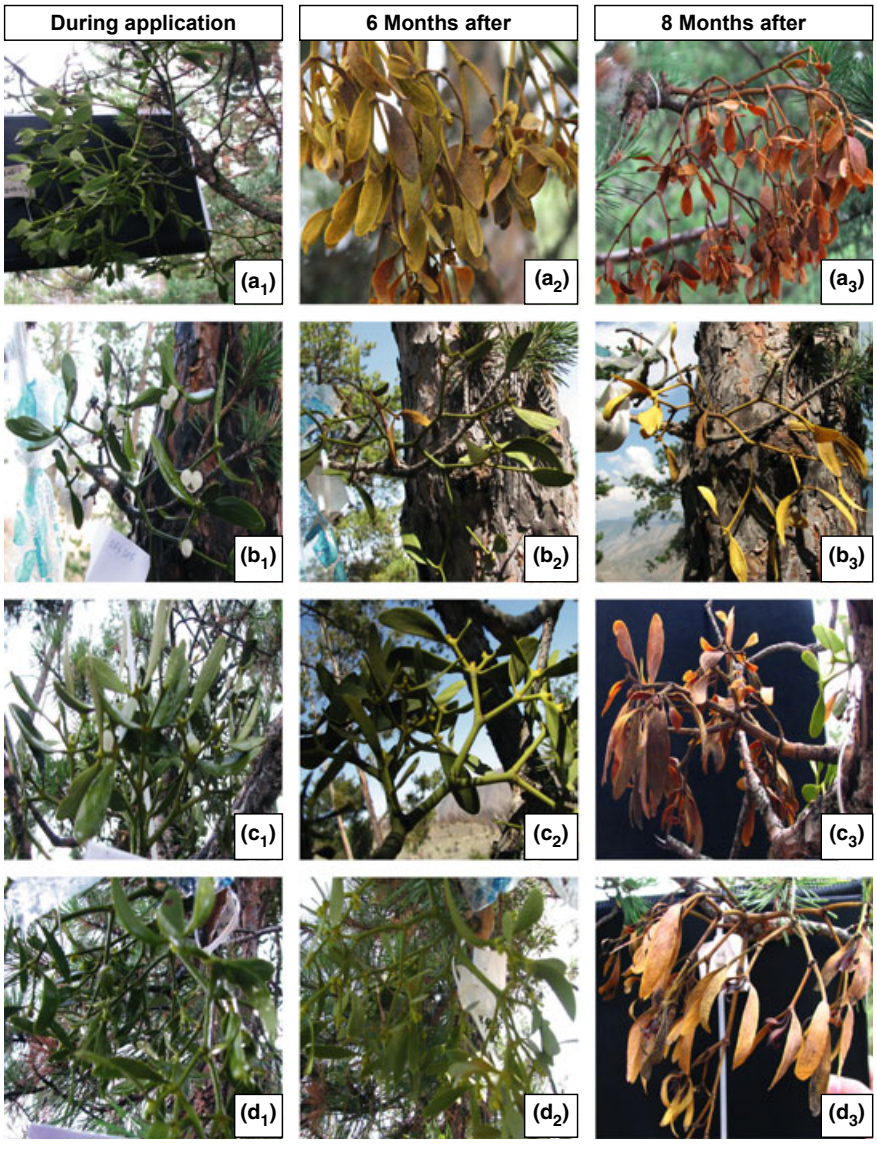

Fig. 2 Disease severity on mistletoe infected the most affective four fungal isolates during the application, the first 6 and 8 months after (a1,2,3: Alternaria alternata VAŞ-202; b1,2,3: Alternaria alternata VAŞ-205; c1,2,3: Alternaria alternata VAŞ-217; d1,2,3: Acremonium ciliense VA-11)

agents against mistletoe in Turkey. Further field studies must be conducted to analyse the real potential of these fungal agents in large areas. Studies are also needed to determine the modes of action of those fungal isolates, the population density of the applied fungi and the best form of introduction into the host.

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