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The impact of plastic mulches on *Aspergillus flavus*-clade populations in pistachio orchard soils

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Abstract

Plastic mulches on the soil surface can impact the population and activity of soil microorganisms, such as Aspergillus species, by altering the temperature and humidity of the soil. In the present study, the effects of white and black mulches were examined on the population of *Aspergillus flavus*-clade in the soils of two pistachio orchards (35-year-old Fandoghi cultivar) with a surface drip irrigation system in Rafsanjan (Kerman Province, Iran). The experiment was performed as a randomized complete block design with three replications. The population of *A. flavus*-clade in the soil was measured monthly using the serial dilution method and SPDA culture medium. Identification and toxigenic isolates were screened using a coconut agar medium. The results indicated that white and black plastic mulches decreased the population density of *A. flavus*-clade in all sampling months except for December in the two pistachio orchards. In garden No. 1, the population of *A. flavus*-clade decreased 64 and 46 percent in August compared to the control in black and white mulches, respectively. However, in garden No. 2, this reduction was 48 and 35% in black and white mulches, respectively. Additionally, in plastic mulches, mainly black mulch, the population of toxigenic isolates exhibited a significant decrease in the soil compared to the control, especially during hot months. It is concluded that using plastic mulches, mainly black mulches, can reduce the population of *A. flavus*-clade in pistachio orchard soils and minimize contamination of pistachios with *Aspergillus* and aflatoxin.

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Introduction

The use of mulches in agriculture has been interesting since ancient times because mulches improve the growth and productivity of crops and optimize water consumption (Yu et al., 2018). Agricultural mulches refer to materials that create a protective cover on the ground or around the roots of plants. Plastic mulches have been used since around 1960 and their use is still increasing all over the world (Bhardwaj, 2013). Mulches play an important role in soil fertility, accumulation of soil particles, and moisture retention in dry periods as well as improving the physical, biological, and chemical properties of soil (Kader et al., 2017; Lalruatsangi et al., 2019). These effects also cause quantitative and qualitative changes in the population of soil microorganisms (Li et al., 2004; Wang et al., 2020). The use of mulch can affect plant growth, the activity of soil microorganisms, the inoculum of plant pathogens, as well as their aggressiveness and survival via changes in the solubility of minerals in the soil (DeVay, 1995; Wang et al., 2021). The use of mulches also leads to a slight increase in the diversity of arbuscular mycorrhizal fungi (AMF) and bacteria (Liu, et al., 2012; Chen et al., 2014). Additionally, transparent plastic mulches controlled for the white root rot of apple trees caused by *Rosellinia necatrix* before and after planting trees over a long period (Freeman et al., 1990). Increasing the soil temperature is one of the mechanisms of the effect of mulches on soil-borne pathogens, influenced by factors such as soil moisture, day length and intensity of sunlight, air temperature, thickness and transparency of mulches, soil radiation, and soil color (DeVay, 1995;

Mahrer et al., 1984). During soil solarization, the temperature of the soil varies depending on the depth of the soil. DeVay (1991) showed that the maximum temperature at depths of 5 and 45 cm in the soil of fields varies between 42 to 55 °C and 32 to 36 °C. At depth 5 cm of the soil, the pathogen population reduced from 94 to 100 percent (DeVay, 1991). The use of transparent plastic mulch in the seedbed of a forest nursery eliminated entirely the population of *Fusarium solani*, *F. oxysparum, Penicillium* spp. and *Rhizopus* spp. after one month at depth of 0 to 5 and 5 to 10 cm in the soil (Verma et al., 2010). The population of Aspergillus species was completely eliminated at a depth of 0 to 5 cm and reduced by 53 times compared to before mulch treatment at a depth of 5 to 10 cm (Verma et al., 2010).

Different species of the genus Aspergillus are native to hot-dry, semi-arid, and tropical regions. They are widely present in the soil, and climate changes cause significant fluctuations in their population (Zhang et al., 2021). Primary population of Aspergillus inoculum and management practices in the soil, such as irrigation, can highly affect the density of Aspergillus spores on the fruit of pistachio trees (Moradi et al., 2004). Contamination of pistachio nuts with different species of Aspergillus and aflatoxin in orchards and harvesting stages is one of the most important problems in pistachio production, consumption, and export (Doster and Michailides, 1999). Among the different species of Aspergillus, two species *A. flavus* and *A. parasiticus* are more important in pistachio orchards (Doster & Michailides, 1994; Heidarian et al., 2005; Rahimi et al., 2007; Mohammadi et al., 2009). Based on the polyphasic taxonomy, these two species are placed in the *Aspergillus flavus*-clade (Frisvad et al., 2019). Considering the role of plastic mulches in reducing evaporation, maintaining of soil moisture for a long time and their effect on the population and activity of soil microorganisms, especially Aspergillus species, this research evaluated the effect of white and black plastic mulches on the population density of *Aspergillus flavus*-clade in the soil of two pistachio orchards with a surface drip irrigation system.

Materials and Methods

Selection of orchards and treatments

This research was carried out in two orchards, western of Rafsanjan, with the characteristics listed in Table 1. The treatments consisted of control without plastic mulch (T1), white plastic mulch (T2), and dark plastic mulch (T3). Plastic mulches resistant to ultraviolet radiation (UV=1.5) with a thickness of 45 µm and a width of 110 centimeters were purchased from Yazd Plastic Chemical Company. These mulches were placed in the shade of the pistachio trees on the surface irrigation pipes, at the beginning of the growing season (Fig. 1). The experiment was carried out as randomized complete block design with three replications and the rows of trees on their sides were considered as guards. Sampling from soil to determine of Aspergillus population was done every month from May to December. In each replication, five subsamples (500 grams) were collected from different areas and a depth of 10 to 15 cm of soil, were thoroughly homogenized and finally 2 kg sample was prepared. Soil surface temperature changes in all treatments were measured by soil thermometers in July between two consecutive irrigations. Air temperature data during the day were also obtained from the meteorological station of Rafsanjan city, which was located 10 km away from the experimental garden.

Table 1. The characteristics of pistachio orchards

	Orchard No. 2	Orchard No.1
Region	Kosarriz Rafsanjan	Rostam Abad Harandi Rafnasjan
Soil texture	Sandy loam to sandy loam without gravels	Sandy loam to sandy loam with 15% gravels
Cultivar and age of trees	35 years Fandoghi	35 years Fandoghi
Planting dimensions (between a inside the row)	7×1	10×2
Tree density per hectare	1428	500
length of planting trees row	55	37
Irrigation system and interval	Two rows surface drip irrigation-8 days	Two rows surface drip irrigation-12 days

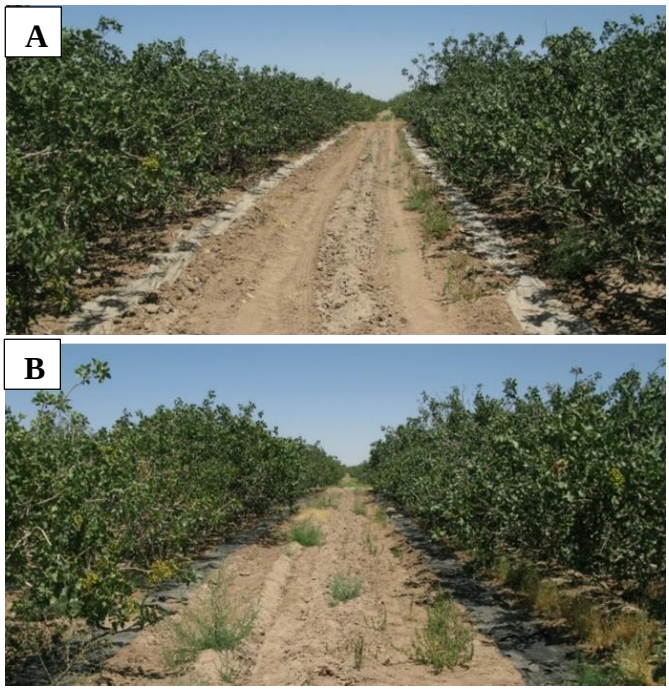

Fig. 1. White plastic mulch in T2 treatment (A) and black plastic mulch in T3 treatment (B).

Isolation and enumeration of *Aspergillus flavus*-clade population in soil

The population of *Aspergillus flavus*-clade in the soil was measured by serial dilution method and using a sucrose-peptone-dichloran agar (SPDA) medium (Dhingra & Sinclair, 1995). For each replication three soil samples (10 grams) were collected and blended separately with 0.1 % sterile peptone water (90 ml). Soil suspensions were shaked (90 rpm) for one hour and a serial dilution of 10−2 and 10−3 in 0.1 ml aliquots were spread on six petri dishes containing SPDA medium. After incubation of the plates for three to four days at 25 °C in darkness, the population of *Aspergillus flavus*-clade fungi (per gram of dry soil) was calculated by counting green fungal colonies. The isolates were cultured on CYA (Czapek yeast autolysate agar), CZA (Czapek Dox agar), CYA20S (CYA+20% sucrose), and MEA 2% (Malt extract agar) media at 25°C and (CYA) medium at 37 °C for 7 days in the darkness (Samson &Frisvad, 2004; Arzanlou et al., 2016) and morphological identification was done according to Kilch (2002). To confirm the identity of the fungal isolates, genomic DNA was extracted using a CTAB extraction procedure (Doyle & Doyle, 1987). A 580 bp portion of calmodulin gene was amplified from Aspergillus isolates using the primers cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) (Glass and Donaldson 1995). Twenty-five µL PCR reactions contained 1X reaction buffer, 0.4 mM of each primer (Metabion, Germany), 200 mM dNTPs, 2.5 mM MgCl2, 20 ng of DNA and 1 unit of *Taq* polymerase. PCR amplifications were performed in a Biometra TAdvanced Thermal Cycler (Biometra, Göttingen, Germany) with the cycling conditions consisting of 95°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and then 5 min at 72°C.

Identification of toxigenic isolates

Coconut agar medium (CAM) was used for identification and screening of toxigenic isolates. Two hundred grams of coconut powder (for one liter medium) was poured into boiling water for 10 minutes and the extract was filtered using three layers' cheese cloth. After adding 18 g/L of agar, medium was sterilized for 20 minutes at 121 °C and 15 pounds per square inch (Davis et al., 1987). Totally 150 A. flavus-clade isolates in each treatment were randomly selected and cultured on petri dishes containing CAM medium and was kept at 30 °C in the dark. After three days, Petri dishes were placed upside down and a drop of 25 % ammonia solution was placed into the lid of each petri dish to release ammonium vapor (Saito & Machida, 1999). Color development (pink pigmentation) was indicative of toxigenic isolates (Fani et al., 2014).

Results

Based on morphological and molecular characteristics, the collected isolates were identified as Aspergillus

flavus, deposited in the GeneBank (MT882333 and MT882334). The results showed that the population of A. flavus in the control treatment (without mulch) in orchard No. 1 had a decreasing trend with the increasing the temperature and this trend continued until August. The fungi population in August with 24% reduction showed a significant difference ($p \leq 0.01$) comparison with May, the beginning of the experiment. (Fig. 2). In September and October, the fungi population showed an increasing trend compared to August, which was only in October with a 13% increase and had a significant difference from August. The fungi population showed a decreasing trend in November and December compared to October, with a significant difference (Fig.2). The population of A. flavus in the white and black mulch treatments also had a decreasing trend from May to September, a significant difference compared to May, but from October to December, the fungal population increased and showed a significant difference compared to August and September (Fig. 2). In both white and black mulch treatments, the fungal population was significantly lower compared to the control during June to October, so that in August white and black mulches showed 46 and 64% reduction of fungal population respectively in comparison with the control. Also, in July to December, the lowest fungal population was observed in the black mulch treatment, which had a significant difference ($p \leq 0.01$) to white mulch and the control treatment. The lowest fungal population was observed in black mulch in August compared to white mulch and it showed a 33% reduction (Fig. 2). In garden No. 2, the population of A. flavus in the control treatment (without mulch) had a decreasing trend during June to August, so that fungal population decreased by 33.7% to May (the beginning of the experiment). It also indicated a significant difference (Fig. 3).

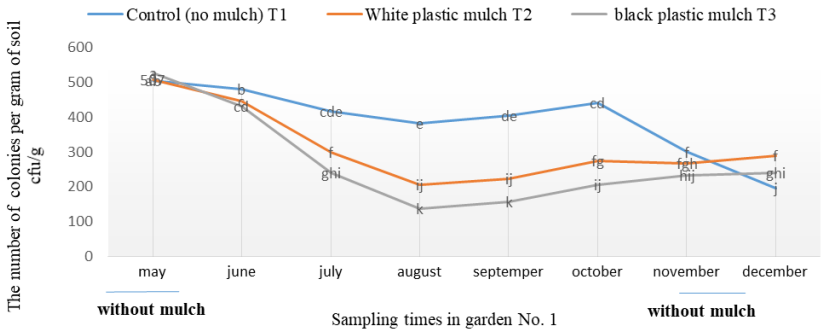

Fig. 2. The population changes of *Aspergillus flavus* at sampling times in control (without mulch), white plastic mulch and black plastic mulch in garden No 1. Means with the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

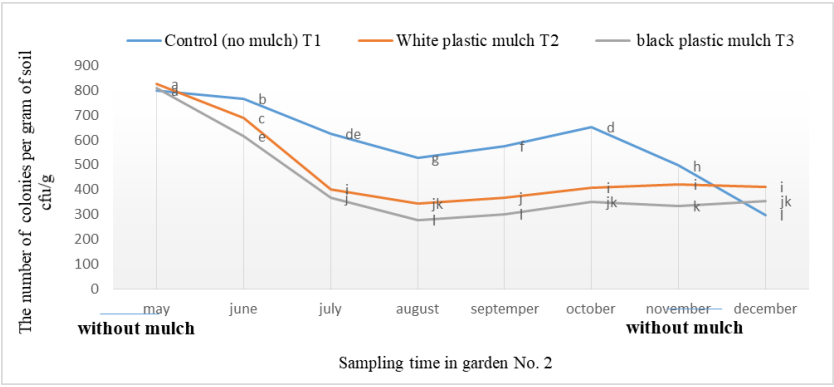

Fig. 3. The population changes of *Aspergillus flavus* at sampling times in control (without mulch), white plastic mulch and black plastic mulch in garden No. 2. Means with the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

The decreasing trend of the fungal population was observed in white and black mulch treatments from June to August, and the *A. flavus* population was significantly less compared with the control treatment. In August, the fungal population in white and black mulch showed 34.9% and 47.8% reduction, respectively, compared to the control. In the control treatment, the fungal

population showed a significant increase in September and October compared to August, and then again a significant decrease until December, while in the white and black mulch treatments, there was a significant increase from October to December compared to the control. Except May and July, the lowest fungal population density was observed in the dark mulch in all sampling months (Fig. 3). As the air temperature increased during the day, the soil surface temperature increased in all treatments, so that in the control treatment, the soil surface temperature in the middle of the day was equal to the air temperature (Fig. 4-6). However, in the treatments with plastic mulch, the increase in soil surface temperature under the mulch was much more severe, so that this temperature difference reached a maximum (33 °C) at 14:30 in the afternoon. In addition, the difference between the control treatment and the black and white plastic mulches also reached 29 and 21 °C, respectively. At the end of the irrigation cycle, with a decrease in soil moisture, the soil surface temperature at 9:30 was higher than the air temperature, even in the control treatment. This increase in soil surface temperature compared to the air on the eleventh day after irrigation was 6, 9 and 16 °C in the control, white and black mulch treatments, respectively (Fig. 4-6).

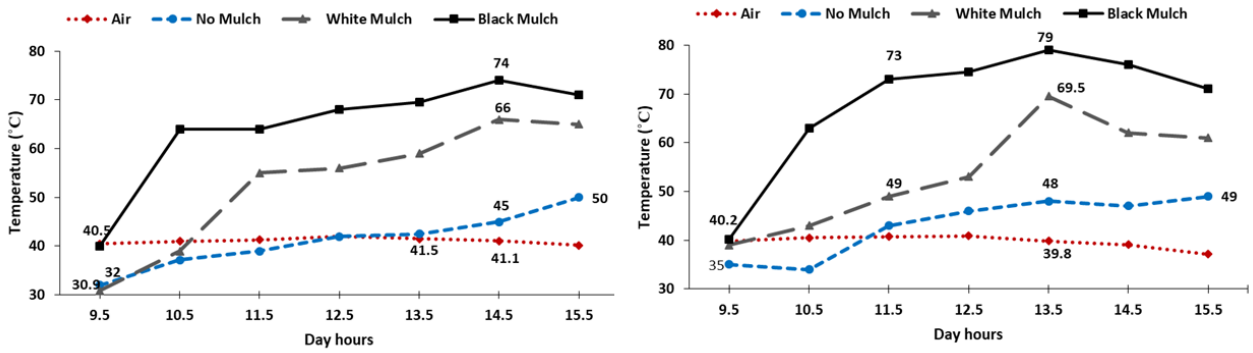

Fig. 4. Changes in soil surface temperature one day (left) and three days (right) after irrigation.

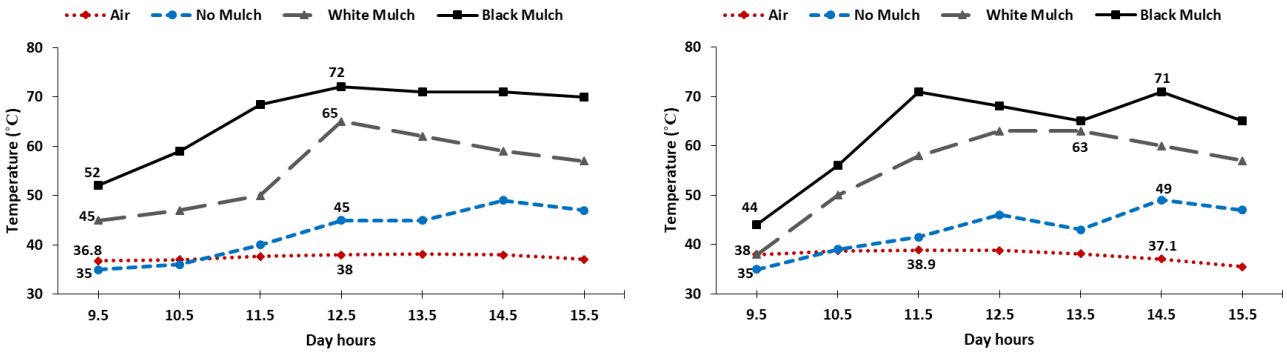

Fig. 5. Changes in soil surface temperature five days (left) and seven days (right) after irrigation.

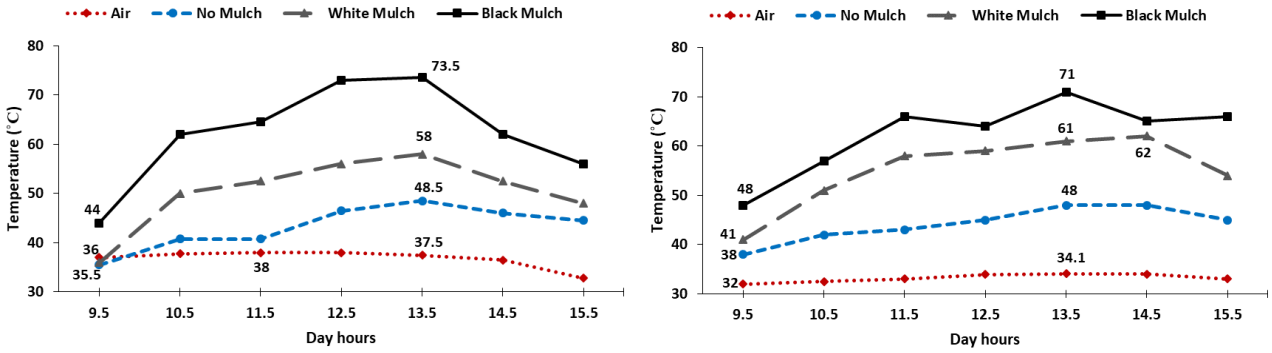

Fig. 6. Changes in soil surface temperature nine days (left) and 11 days (right) after irrigation.

On the other hand, between two consecutive irrigations, the maximum soil surface temperature in the time interval 13:30 to 15:30 for the control, white and black plastic mulch treatments was 50, 69.5 and 79 °C, respectively. A significant difference (up to 23 °C) was observed between the black and white mulch treatments in the increase in soil surface temperature (Fig. 4-6). In both gardens, the frequency of toxigenic isolates of *A. flavus* was higher than atoxigenic isolates. In orchard No. 1, frequency of toxigenic isolates in control had no significance difference between spring, summer, and autumn. The lowest frequency of toxigenic isolates in white and black mulch was observed in summer. The population of the toxigenic isolates in spring and autumn showed no significant difference in white mulch treatment. However in black mulch treatment the frequency of toxigenic isolates was significantly variable between seasons and the lowest frequency of toxigenic isolates (78.1%) was observed in summer (Table 2). The trend of changes in the frequency of toxigenic isolates in orchard No. 2 was similar to garden No. 1 (Table 3).

Table 2. Frequency of atoxigenic and toxigenic isolates of *Aspergillus flavus* in soil of pistachio orchard No. 1

	control		**white mulch**		**black mulch**	
	atoxigenic	toxigenic	atoxigenic	toxigenic	atoxigenic	toxigenic
Spring	4.67 ^{j*}	95.33 ^{a}	11.4 ^{h}	88.6 ^{c}	17 ^{g}	83.03 ^{d}
Summer	6.73 ^{ij}	93.3 ^{ab}	17.7 ^{g}	82.3 ^{d}	21.93 ^{f}	78.1 ^{e}
Fall	6.03 ^{ij}	94 ^{ab}	9.33 ^{hi}	90.7 ^{bc}	12.03 ^{h}	87.97 ^{c}

* Means with the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

Table 3. Frequency of atoxigenic and toxigenic isolates of *Aspergillus flavus* in soil of pistachio orchard No. 2

	control		**white mulch**		**black mulch**	
	atoxigenic	toxigenic	atoxigenic	toxigenic	atoxigenic	toxigenic
Spring	7.67 ^{h*}	92.33 ^{a}	12.93 ^{g}	87.07 ^{b}	17.97 ^{f}	82 ^{c}
Summer	8.73 ^{h}	91.3 ^{a}	18.33 ^{f}	81.67 ^{c}	22.93 ^{e}	77.03 ^{d}
Fall	6.33 ^{h}	93.7 ^{a}	10.03 ^{gh}	89.97 ^{ab}	12.73 ^{g}	87.27 ^{b}

* Means with the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

Discussion

The results of the current research indicated that using of white and black plastic mulches in pistachio orchard significantly reduced the population of *A. flavus*-clade compared to the control treatment. Changes in the fungal population confirm that the population density of *A. flavus* in the soil is related to agricultural practices and weather conditions (Mohammadi et al., 2009; Houshyarfard et al., 2014). The reduction of the fungal population in the hot months indicated the effect of temperature and warming of the air and soil surface. The optimal temperature for Aspergillus growth is 25 to 40°C (Klich, 2002). In the present study, the maximum temperature of the soil surface in control (without mulch treatments) was recorded as 50 to 51 °C in August. The increase in temperature and its effect on the soil surface temperature under the mulch can be considered as one of the reasons for the decreasing trend of the fungal population and its significant difference from the control, in two treatments of white and black plastic mulch, from the beginning of sampling to August. Wang et al. (2021) showed that using mulch in potato fields with drip irrigation can reduce the population of the dominant fungal population in soil (ascomycete fungi). The enhancement in the fungal population in September and October can also be related to the agricultural practices in the harvest season, because *A. flavus*-clade fungi are soil-borne and any agricultural and soil practices can increase the fungus population in the soil (Moradi et al., 2010). Of course, the more suitable temperature in these two months compared to

August should not be ignored, which can have a positive effect on the rapid growth of fungal spores. Generally, the population density of *A. flavus* fungi in the soil can increase several times after harvesting (Wicklow et al., 1984).

The higher soil temperature under black mulch can be one of the main reasons for the low population of fungi and the reduction of the frequency of toxigenic isolates compared to the white mulch treatment. Absorption of the short wavelengths and turning them into suitable heat energy makes the surface of the plastic warmer (Gill & McSorley, 2011). In black mulch treatment, the maximum temperature of the soil surface in garden No. 1 and 2 was 69 and 79°C while in white mulch treatment was 64 and 69.5°C respectively. According to the research of Katan (1981), increasing the temperature of 45 to 55 °C at a depth of 5 cm of the soil surface under transparent plastic mulch causes the death many of plant pathogens. Verma et al. (2010) also showed that using transparent plastic mulch caused the destruction of *Fusarium solani*, *F. oxysparum*, *Penicillium*, and *Rhizopus* population in the depths of 0 to 5 and 5 to 10 cm of soil. They also showed that the population of Aspergillus at a depth of 0 to 5 cm of the soil covered by transparent mulch was destroyed, but at a depth of 5 to 10 cm, the population of Aspergillus showed a 53-fold decrease compared to before the covering. The reduction of the fungal population in the white and black mulches and the incomplete disappearance of the *Aspergillus flavus* in this research, is consistent with the results of Verma et al. (2010).

The significant reduction in the frequency of toxigenic isolates in black mulch compared to the control in both gardens No. 1 and 2 also showed that soil temperature is one of the most important factors affecting aflatoxin production. The optimal temperature for the production of aflatoxin is 28 to 30 °C, therefore, increase of soil temperature can affect directly or indirectly activity of toxigenic isolates, transcription and expression of genes related to aflatoxin production (Liu & Chu, 1998; O'Brian et al., 2007). Temperature and humidity are important factors that strongly affect the biodiversity of microorganisms. Therefore, changes in soil microbiology under plastic mulches can be expected (Zhang et al., 2021).

Conclusions

The present research indicated that using white and black mulches in the pistachio orchards with a surface drip irrigation system can significantly reduce the population of *A. flavus*, especially in the hot months of the year compared to treatments without mulch. In addition to reducing water evaporation from the soil surface, the pistachio tree can absorb more water. It should also be noted that black mulch is more effective than white mulch in reducing *A. flavus* population, frequency of toxigenic isolates and contamination of pistachio fruits with aflatoxin in the orchards.

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Conflict of interest

The authors declare that there are no conflicts of interest present

CRediT author statement

M. Haghdel: Laboratory works & writing original draft. **A. H. Mohammadi:** Supervision, methodology, writing, reviewing & editing. **N. Sedaghati:** Garden works, writing, reviewing & editing. **M. Moradi:** Identification of toxigenic isolates of *Aspergillus*, writing, reviewing & editing.

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