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ORIGINAL ARTICLE

Effect of Sulfur on Toxigenic *Aspergillus flavus In Vitro*

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A R T I C L E I N F O

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A B S T R A C T

Application of sulfur to control the common pistachio psylla has been nowadays popular. The psylla is one of the main pistachios' pests. *Aspergillus flavus*, the most important aflatoxin producer, has a strong impact in the mycoflora of pistachio orchards. The present study has been conducted to determine the effects of sulfur on *A. flavus*. Therefore, a toxigenic *A. flavus* strain was cultured on potato dextrose agar (PDA) medium, and the effects of mineral sulfur, refinery sulfur and sodium metabisulfite were closely monitored on fungal mycelial dry weight, germination of spores, germ tube and mycelium germination. The Fungal parameters measurement for sulfur vapor exposure and sulfur addition to the culture medium declared no significant effect on the growth of *A. flavus* mycelium in the direct addition. But sulfur in liquid medium as well as in high concentrations caused mycelial dry weight loss. Importantly, the sublimation of 0, 0.5, 1, 2, 5, 10, 25 and 50 gm^{-3} from the refinery sulfur, prevented completely the growth of germinated spores and mycelial. Significantly, the complete inhibition of spore germination was observed at a concentration level of 2 g m^{-3} and also sodium metabisulfite prevented the growth of *A. flavus* at a concentration of 15 g l^{-1} culture medium. The sulfur usage in culture medium declared no inhibition of *A. flavus* growth, but at high concentrations in liquid medium, it reduced mycelial dry weight. Therefore, it is found that the sulfur application in any kind to control of psylla would not probably effect significantly on the aflatoxigenic *A. flavus*.

Introduction

Pistachio is one of the most important, widely grown commercial and economical nut for Iranian agriculture that is mainly traded as dry nut (Shamshiri and Hasani, 2015; FAO, 2020; Sharifkhah *et al.*, 2020; Hosseini *et al.*, 2022; Nazoor *et al.*, 2022a, b). It has been found that aflatoxin contamination can be a fatal challenge for its safety and trade (Moradi and Fani, 2018; Mahbobinejhad *et al.*, 2019). In recent years, the sulfur has been widely used to control of common pistachio psylla (*Agonoscena pistaciae* Burckhardt & Lauterer) known as a key pest. The usage of SO_2 as a food preservative is well-known for a long time. For instance, sulfur is the most widely used to protect crops around the United States (Gammon *et al*., 2010; Nazoor *et al.*, 2022a, b). Meanwhile, it is

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used in different forms to control pre- and post-harvest diseases. These forms may be in the formulation of dry sulfur used as a powder on crops and also in SO_2 gas on post-harvest crops in storage spaces. According to the CODEX standard for food additives, the maximum sulfur dioxide allowed for dried apricots and raisins are 2000 and 1500 mg kg^{-1}, respectively. In addition, according to the European guidelines on food additives, (except for colors and sweeteners) the maximum allowed amount of sulfur dioxide for dried fruits (apricots, peaches, grapes and figs) is 2000 mg kg^{-1}. It is also reported that the Recommended Daily Intake (RDI) for sulfur from dietary sources is 800 mg kg^{-1} (Gunduz *et al*., 2014). It is noted that sulfur gas is applied to preserve color and to protect against mold in the processing of figs, raisins, apricots and other fruits during drying seasons (Aktas, 2015). The Food and Drug Administration has professionally recommended sulfur dioxide as a food additive in fruits and vegetables, juices, syrups, meats and fish (Gammon *et al*., 2010). It should be noted that the sulfur dioxide has a greater effect on the growth of gram-negative bacteria such as *Echerichia coli* and *Pseudomonas* in respect to gram-positive bacteria such as *Lactobacillus*. It is also used as a fungicide against a number of molds which may infect fruits during storage and transportation to the market processes. These fruits are included *Botrytis*, *Cladosporium*, *Alternaria*, *Penicillium*, *Aspergillus* and *Rhizopus* (Melanitou, 1995). The excessive sulfur consumption may also have adverse effects on animals and humans for instance; the thiamine deficiency for animals feeding cats and dogs sulfur-contaminated food (Studdert and Labuc, 1991) and cerebral necrosis (PEM) for ruminants (Olkowski, 1997). For human, its negative effects on the eyes, skin and respiratory system can be emphasized and meanwhile the threshold for exposure to sulfur is 2 ppm (Gammon *et al*., 2010). Sodium metabisulfite in water produces SO_2 which is highly toxic to microorganisms as its mutagenic effects, inactivates mRNA reacts with disulfide bonds in proteins, enzymatic co-factors, aldehydes, and ketone structures of five and six carbon sugars. On the other hand, it converts cytosine derivatives to uracil by detrimental effects on membranes (Jiang *et al*., 2015).

The different species of *Aspergillus* have naturally high saprophytic potential and are able to grow in most substrates and may produce different mycotoxins. These include aflatoxins, ochratoxin and gliotoxin, which may cause severe disease in humans and animals. It is noted that aflatoxins are more toxic than other mycotoxins and can be more frequently seen in pistachios (Moradi and Hokmabadi, 2011). Aflatoxins can be produced by more than 15 different species of *Aspergillus*, especially *A. flavus* and *A. parasiticus*. Exposure to consumption of aflatoxins may lead to liver cancer, genetic mutations, immunosuppression, Reye's syndrome, chronic hepatitis, and tongue tie in children (Wang *et al*., 2019). Based on the climatic conditions, different species of *Aspergillus* can be observed in air and soil in pistachio orchards and may cause contamination of pistachio nuts leading to aflatoxin production. Aflatoxin production rate is affected by various biotic and abiotic stresses. Aflatoxins are produced before harvest and under orchard conditions. Delay in harvesting, lack of proper product processing and poor storage conditions can lead to increase fungal growth and aflatoxin production (Moradi and Hokmabadi, 2011). Use of sulfur to control of common pistachio psylla (*Agonoscena pistaciae* Burckhardt & Lauterer), one of the key pests of pistachios, has nowadays become very popular. The aim of this study was to investigate the effects of sulfur element on different aspects of vegetative and reproductive parameters of *A. flavus* including radial growth, mycelium dry weight and spore germination.

Materials and Methods

Sulfures and fungal strain

Two micronized sulfurs with particle diameters of 225µm (based on the manufacturer's statement) from Barish (refinery sulfur) and Zarkouh (mineral sulfur) companies were used in the assays. Toxigenic *A. flavus* (P1646 strain, Pistachio Research Center) was also utilized. The strain was sub-cultured on the potato dextrose agar (PDA) medium in the Petri dishes.

For daily use, the spore suspension was prepared from a concentration of 10^5 containing 1 gram of peptone, 1 gram of yeast extract and 0.1 gram of agar in 100 ml of distilled water stored at 4°C.

Determination of the radial growth of A. flavus

Concentrations of 0, 2.5, 5, 7.5, 10, 15 and 20 g of refinery and mineral sulfur were prepared in 100 ml of GPYAP culture medium (*i.e.*, glucose 20 g, potato extract 100 ml, agar 20 g, peptone 2.5 g, yeast extract 2.5 g and water 1000 ml) and was poured into petri dishes after sterilization in autoclave at 1 atmosphere and 121°C. 5 µl of *A. flavus* spore suspension contained 500 spores was inoculated as a spot in the center of the Petri dish containing the GPYAP medium. Petri dishes were incubated in dark witn 29°C for one week and radial growth was measured using a ruler and the rate of inhibition compared to the control was calculated.

Determination of mycelia dry weight of A. flavus

Concentrations of 0, 100, 250, 500 and 1000 mg of mineral and refinery sulfur were poured into 250 ml flasks containing 100 ml of PDB culture medium (glucose 20 g, potato extract 100 mg, water 900 ml). The flasks were sterilized in autoclave with 1 atmosphere at 121°C and inoculated with 200µl of *A. flavus* spore suspension (containing $2x10^4$ or 20,000 spores) at 10^5µl concentration and shaken at 250 rpm for two weeks. The resulting suspension was then washed by sterilized distilled water and was passed through filter paper (Whatman No. 1, UK). The Mycelium was dried at room temperature and the weight was measured using an analytical balance (Kern ADB/ADJ, Germany) and was compared in comparison with the control.

Determination of A. flavus spore germination and growth

Refining sulfur

As mineral sulfur was not sublimated in the laboratory, only refined sulfur was tested in this section. Petri dishes containing GPYAP medium were inoculated with 5 µl of *A. flavus* spore suspension at a concentration of 10^5 ml^{-1} and placed upside down immediately on a mesh in the center of a 0.01 m^3 desiccator. Then 0, 5, 10, 20, 50, 100, 250 and 500 mg of refining sulfur were poured on the floor of the desiccator on aluminum foil and burned using a flame and the lid was immediately closed. Petri dishes were kept in a desiccator for 30 minutes then incubated at 29°C. Four replications were considered for each treatment and the experiments were repeated twice. A graduated ruler was used to measure mycelial growth and the rate of inhibition was calculated compared to the control.

Sodium metabisulphite

Concentrations of 0, 1, 2.5, 5, 7.5, 10, 15 and 20 mg of sodium metabisulfite in 100 ml of PDA culture medium (glucose 20 g, agar 20 g, potato extract 100 ml, water 1000 ml) was prepared. Petri dishes contains sodium metabisulfite-PDA were inoculated with 5 microliters *A. flavus* spore suspension with 10^5 m l^{-1} concentrations and incubated in at 29°C in the dark for 7 days. Radial growth was measured using a ruler and the rate of inhibition compared to the control was calculated.

Results

Effect of sulfur on radial growth of A. flavus

The results of variance analysis for the effects of mineral and refinery sulfur on the mycelial growth of *A.flavus* are shown in Table 1. The results shows that sulfur had no effect on the radial growth of *A. flavus* ($P \geq 0.05$).

Table 1. Analysis of variance of the effect of mineral and refinery sulfurs on the mycelial growth of *Aspergillus flavus*.

	Source of variances	Sum of squares	DF	Mean of squares
Mineral sulfur	Concentration	2	6	0.333 ^{ns}
	Repeat	0.286	1	0.286 ^{ns}
	Error	3.714	6	0.619
	Total	28356	14	
Refinery sulfur	Concentration	3.429	6	0.571 ^{ns}
	Repeat	0.643	1	0.643 ^{ns}
	Error	2.857	6	0.476
	Total	28447	14	

ns: No significant at the 1% level

Effect of sulfur on dry mycelia weight of A. flavus in liquid medium

The same results of Table 1 are shown in Table 2 for the dry mycelia weight of *A. flavus*. Based on the results, both mineral and refining sulfur had a significant effect on the biomass of *A. flavus* in liquid medium ($P < 0.05$) (Figs. 1 and 2). The results show that by increasing the concentration of sulfur to 1 g L^{-1}, the amount of dry mycelia weight increased compared to the control and then shows a decreasing trend up to a concentration of 10 g L^{-1}. The dry mycelia weight for concentrations of 0, 1, 2.5, 5 and 10 g L^{-1} of mineral sulfur was equal to 0.375, 0.525, 0.39, 0.26 and 0.125 mg and the amount of weight of dry mycelia for concentrations of 0, 1, 2.5, 5 and 10 g L^{-1} refinery sulfur was equal to 0.35, 0.565, 0.375, 0.28 and 0.2 mg, respectively.

Table 2. Analysis of variance of the effect of mineral and refinery sulfurs on the mycelial dry weight of *Aspergillus flavus*.

	Source of variances	Sum of squares	DF	Mean of squares
Mineral sulfur	Concentration	0.181	4	0.045 **
	Repeat	0.002	1	0.002 ^{ns}
	Error	0.005	4	0.001
	Total	1.310	10	
Refinery sulfur	Concentration	0.148	4	0.037 **
	Repeat	0.003	1	0.003 ^{ns}
	Error	0.002	4	0.000
	Total	1.406	10	

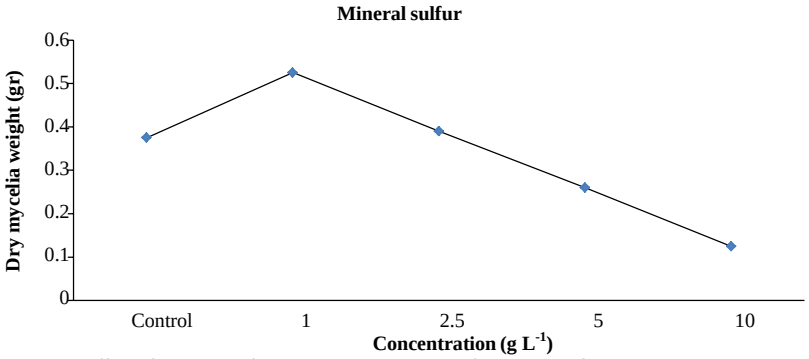

Fig. 1. Effect of mineral sulfur on mycelia dry weight of *Aspergillus flavus* in liquid medium.

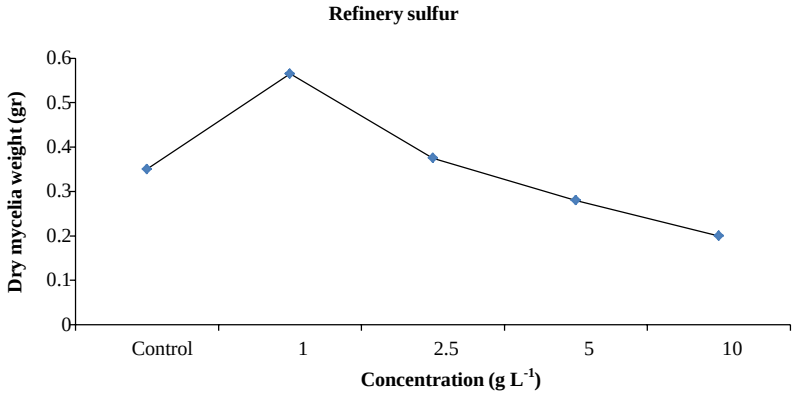

Fig. 2. Effect of refinery sulfur on mycelia dry weight of *Aspergillus flavus* in liquid medium.

Effect of sulfur sublimation on germination and growth of A. flavus spores

The variance analysis for the effects of sulfur sublimation on germination and growth of *A. flavus* spores are shown in Table 3. The results show that sulfur sublimation has a significant effect on the germination of *A.flavus* spores ($P < 0.05$) (Figs. 3 and 4). Sublimation of 2-50 g m^{-3} of refined sulfur prevented germination of *A. flavus* spores. At concentrations of 0.5 and 1 g m^{-3}, refining sulfur sublimation did not completely inhibit the germination of *A. flavus* spores.

Table 3. Analysis of variance of the effect of refining sulfur sublimation on *Aspergillus flavus* spores.

Source of variances	Sum of squares	DF	Mean of squares
Between groups	17618.667	3	8809.333**
Within groups	874.222	9	97.136
Sum	18492.889	11	

**: significant at the 1% level

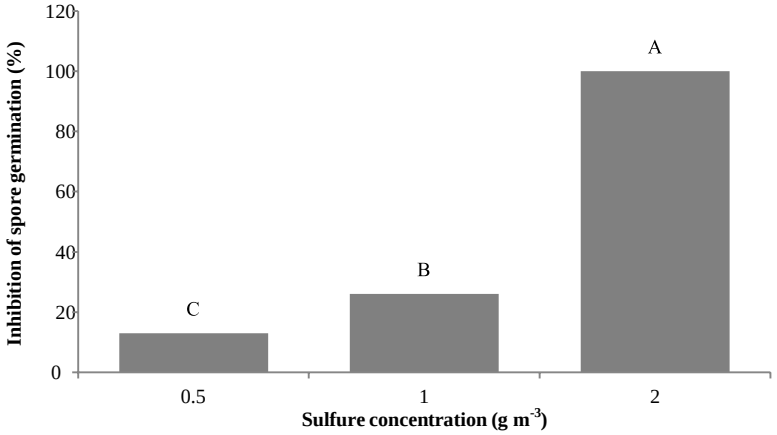

Fig. 3. Effect of refining sulfur sublimation on *Aspergillus flavus* spores.

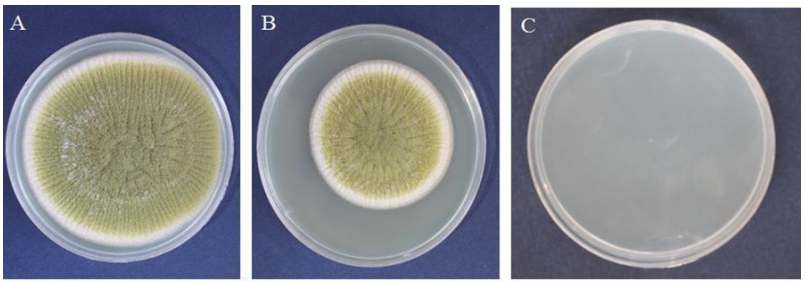

Fig. 4. Growth of *Aspergillus flavus* under sublimation of 0.5 (A) and 1 g m^{-3} (B) and 2 g m^{-3} (C) of refined sulfur

Effect of sulfur sublimation on germinated spores of

A. flavus

Sulfur sublimation at all concentrations of 0.5, 1, 2, 5, 10, 25 and 50 g m^{-3} of refined sulfur prevented the growth of germinated spores of *A. flavus* (Figs. 5 and 6). Fig. 5 shows the germinated spores that could no longer grow and their growth was terminated after sublimation of sulfur, and Fig. 7 shows the petri dishes containing these germinated spores. As it can be seen, compared to the control, in all the sulfur concentrations levels, the spores did not grow anymore.

Effect of sulfur sublimation on mycelium growth of A. flavus

Sulfur sublimation in all concentrations of 0.5, 1, 2, 5, 10, 25 and 50 g m^{-3} of refinery sulfur prevented themycelium growth of *A.flavus* (Fig. 7).

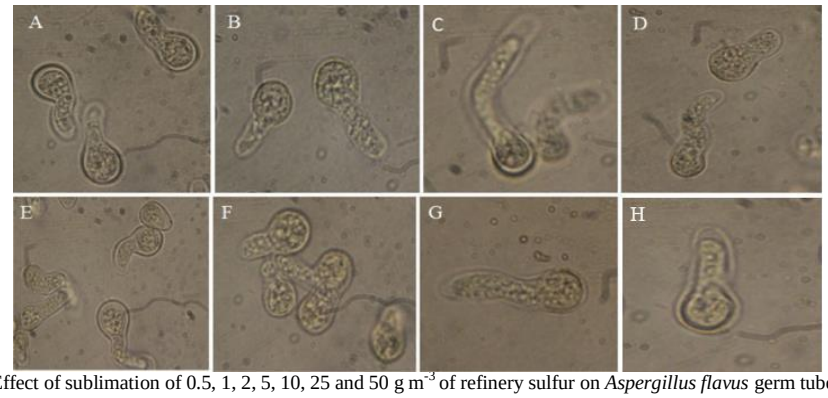

Fig. 5. A-G: Effect of sublimation of 0.5, 1, 2, 5, 10, 25 and 50 g m^{-3} of refinery sulfur on *Aspergillus flavus* germ tube, (H): Control.

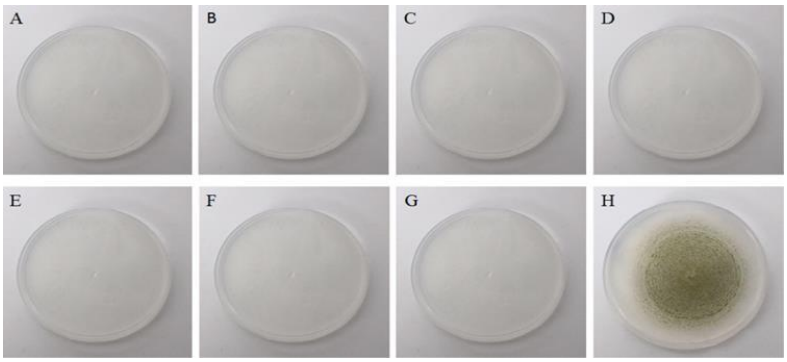

Fig. 6. A-G: Effect of sublimation of 0.5, 1, 2, 5, 10, 25 and 50 g m^{-3} of refinery sulfur on *Aspergillus flavus* colony, (H): Control.

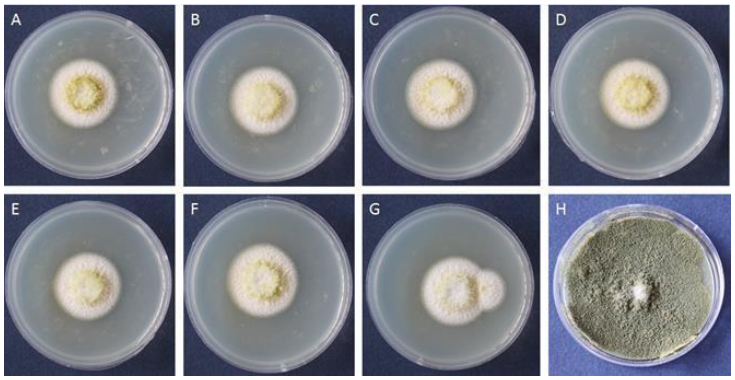

Fig. 7. (A-G): The effect of sublimation of 0.5, 1, 2, 5, 10, 25 and 50 g m^{-3} of refinery sulfur on the mycelium growth of *A. flavus*, (H): Control.

Effect of sodium metabisulphite on A. flavus

The results of the effect of sodium metabisulfite on the mycelial growth of *A. flavus* are presented in Table 4 and Fig. 8. Based on the results, sodium metabisulfite significantly reduced mycelial growth, with an increasing rate respect to sulfur concentration, where the highest amount of inhibition has been observed at a concentration of 15 g L^{-1} with 100% inhibition (Fig. 9).

Table 4. Analysis of variance of the effect of sodium metabisulfite on *Aspergillus flavus*.

Source of variances	Sum of squares	DF	Mean of squares
Between groups	14195.026	4	3548.757 **
Within groups	877.350	15	58.490
Sum	15072.377	19	

**: significant at the 1% level

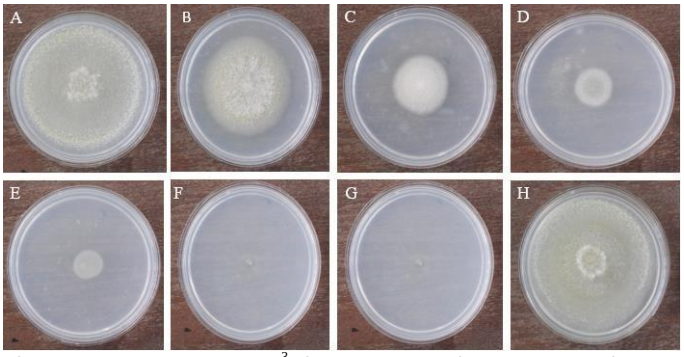

Fig. 8. A-G: Effect of sublimation of 1, 2.5, 5, 7.5, 10, 15 and 20 g m^{-3} of sodium metabisulfite on *Aspergillus flavus* mycelial growth, (H): control.

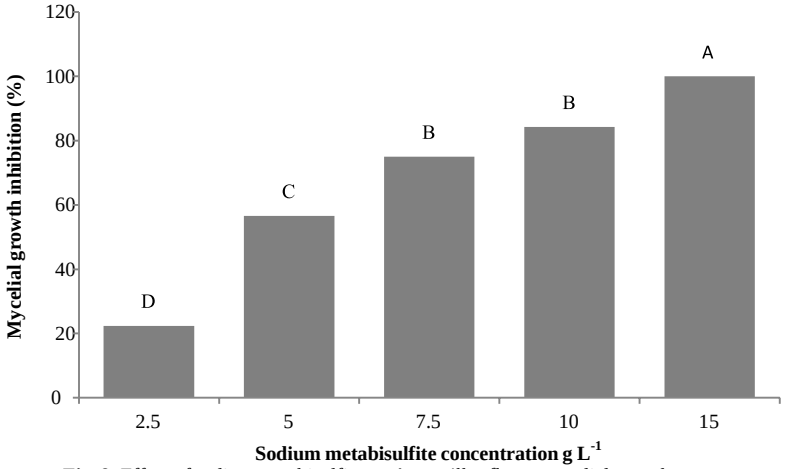

Fig. 9. Effect of sodium metabisulfite on *Aspergillus flavus* mycelial growth.

Discussion

Sulfur dioxide (SO_2) is one of the oldest food additives with a long history to prevent the growth of mold, yeast and bacteria. After the inorganic chemistry development, SO_2 and its salts have been commonly used as preservatives, especially in foods and beverages (Pateraki *et al*., 2007). Also It can be used pre and postharvest in form of SO_2 generating pads inside the packages for foods, crops, ornamental plants and turfs (Nazoor *et al.*, 2022b).Heated sulfur dioxide solutions were as more effective treatments for green mold control on lemon in comparison to ethanol, sodium carbonate, and fungicide Imazalil. It has been also observed that the treatments with sulfur dioxide solutions (pH=5), eliminates satisfactorily disease caused by *Penicillium digitatum* infections without producing dangerous gases. A brief tap water washing of the treated fruits was sufficient to prevent damage to the lemon peel and to reduce residual sulphite below the threshold set (10 µg g^{-1}) for fresh produce such as grapes (Martínez-Blay *et al.*, 2020). To find the alternatives for synthetic fungicides for postharvest disease control, sulfur-containing salts were evaluated for their effects on the mycelium growth of various fungal or fungus-like pathogens and their abilities to control carrot cavity spot disease (*Pythium sulcatum*) and potato dry rot (*Fusarium sambucinum*) were monitored. The results show that salts containing metabisulfite provide strong inhibition of tested pathogens including *P. sulcatum* and *F. sambucinum*. Metabisulfite salts in concentrations of 50 and 200 mM inhibit 100% of carrot cavity spot and dry rot, respectively. Calcium sulfate and sodium sulfate also reduced carrot cavity spot at 50 mM, and ammonium sulfate, magnesium sulfate, potassium sulfate, and sodium sulfate reduced potato dry rot at 200 mM. These results show that different sulfate and metasulfite salts can be used to control these microorganisms after harvest (Kolaei *et al*., 2012). Sodium metabisulfite and potassium metabisulfite salts showed a wide range of antimicrobial activity with biocompatibility and low toxicity for humans. Sodium metabisulfite is reported to be one of the most efficient salts for microbial growth control (Kim *et al*., 2020). SO_2 deaminates cytosine derivatives to form uracil and has harmful effects on membranes. Conversely, low amounts of SO_2 may stimulate growth because sulfur is an essential element for growth (Jiang *et al.*, 2015). Sulfur used in GPYAP solid culture medium did not show any effect on *A. flavus* vegetative growth due to the lack of H_2SO_3 formation. SO_2 is converted to H_2SO_3 in water, which is the active form of sulfite in terms of antimicrobial function (Melanitou, 1995). In liquid medium, the addition of sulfur by one gram per liter increased the amount of mycelial dry weight compared to the control and then showed a decreasing trend up to a concentration of 10 grams per liter. Sulfur in low amounts causes the growth of fungi as sulfur is an essential element for the growth of microorganisms. However, with increased sulfur content, it has toxic properties for microorganisms, including mutagenic effects, inactivates mRNA, and has detrimental effects on membranes (Jiang *et al*., 2015). Sulfur is traditionally used to prevent dried figs from *A. flavus* contamination. It is also applied to kill 800 grams of sulfur per cubic meter for 2 hours and to eliminate *A. flavus* spores and other molds. But this operation is performed indoors and in rooms called sulfation chambers (Melanitou, 1995). Sublimation of 0, 0.5, 1, 2, 5, 10, 25 and 50 g m^{-3} of refined sulfur completely stopped the growth of newly germinated spores (tube mass) and mycelial, but a full effect of 2 g m^{-3} and above was observed on spore germination. Importantly SO_2 did not inhibit the growth of *A. flavus* spores at 0.5 and 1 g m^{-3}, but with the same amount of sulfur used in the experiment, it prevented the growth of newly germinated spores and mycelia. Non-germinated

spores are more resistant than newly germinated spores as well as mycelium, so SO_2 at 0.5 and 1 g m^{-3} did not inhibit the growth of *A. flavus* spores, but at the same amounts of sulfur it stopped the growth of newly sprouted spores and mycelium. The efficiency of sodium metabisulphite for inhibiting the growth of *A. flavus* isconsistent with the results of other studies (Jiang *et al*., 2015).

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