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Research paper

Some physiological changes of fresh pistachio upon foliar application of sulfur

Fatemeh Nazoori[1]*, Elaheh Zamani Bahramabadi[1], Seyed Hossein Mirdehghan[1], and Maryam Afrousheh[2]

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[1]Department of Horticultural Sciences, College of Agriculture, Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran
[2]Pistachio Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rafsanjan, Iran
*Corresponding author; Email: f.nazoori@vru.ac.ir; ORCID: 0000-0003-4026-7220

Abstract
Application of sulfur as pesticide is a strategy in plant protection but in addition to their many benefits for pest control, pesticides may affect crop characteristics. In this study, Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), and refinery micronized (R) and mineral (M) sulfur (30 and 60 kg/1000 L in water) were sprayed on pistachio trees of the cultivar 'Ahmadaghaei' twice (in middle May and after 50 days) for controlling the psylla. Pistachios were harvested in late September and stored at 4 °C for 25 and 50 days. The effect of pesticides on hulls and kernels was then evaluated. Weight of pistachios, phenolic content of kernel, and chlorophyll and carotenoid contents of hull and kernel declined over time, while phenolic content of hull displayed an increasing trend. DPPH scavenging capacity of hull and kernel remained stable over time. Refinery micronized sulfur at both concentrations caused a higher weight loss on day 25 but it was similar to control after 50 days. It caused higher chlorophyll and lower carotenoid of hull and kernel. Mineral sulfur at both concentrations caused higher chlorophyll content of hulls. All sulfur treatments reduced antioxidant activity of hull and kernel, while elevating the hull phenolic content. Movento and Confidor pesticides stimulated loss of weight. Although the chemical pesticides and sulfur cannot easily penetrate the kernel, the physiological status of the hull and the whole plant may influence the kernels and the yield.

Keywords: Confidor; Fruit physiology; Movento; Pesticide; Sulfur

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Abbreviations: R30: Refinery micronized sulfur (30 kg/1000 L); R60: Refinery micronized sulfur (60 kg/1000 L); M30: Mineral sulfur (30 kg/1000 L); M60: Mineral sulfur (60 kg/1000 L)

Introduction
Pistachio (*Pistacia vera* L.) tree produces edible nuts rich in nutritional components (Tsantili *et al.* 2011). Sulfur application as a pesticide has been widely used in pistachio cultivation in recent decades (Bakhtiari *et al.* 2016). Therefore, different forms of sulfur as a pesticide have been produced and used in pistachio farms, mainly against psylla. Sulfur in the commercial form is produced in various ways such as mining process and petroleum refinement. Its effect is a non-systemic contact (Norton *et al.* 2013). Sulfur has some advantages over alternatives including low cost, high efficacy, low probability of resistance development, and its acceptability in different production systems (Kwasniewski *et al.* 2014). For sulfur application, the type, concentration, and time of exposure should be determined for each plant to prevent the toxic and deleterious effects. For example, when blueberries were fumigated with a high concentration (250-350 µL L^{-1} h) of SO_2, higher weight loss than the control was observed after 45 days of cold storage, but lower concentrations (50-200 µL L^{-1} h) had no adverse

effects (Rivera *et al.* 2013).

The efficacy of sulfur on controlling pistachio psylla as an important pest has been observed (Bakhtiari *et al.* 2016). However, a pesticide mustn't affect the product quality. The kernel which is the edible part of the pistachio is covered by the shell and hull that protect it from sulfur penetration. But it is clear that the kernels receive signals from their surrounding tissues and are influenced by the physiological conditions of the hulls and the whole plant. Therefore, herein, mineral and refinery micronized sulfur was applied on pistachio fruits of the cultivar 'Ahmadaghaei' twice with a 50-day interval for controlling psylla. Then, the effect of sulfurs on the physiology of fresh pistachios was investigated at harvest time and also at two periods of cold storage (25 and 50 days). The hulls and kernels were investigated separately. The effects were also compared with two chemical pesticides, Movento and Confidor, which are also applied for psylla control.

Materials and Methods

Plant material and treatments

This study started in mid-May 2019 on the farms of Anar city, Iran. Pistachio trees of the cultivar 'Ahmadaghaei' were sprayed with Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), and two types of sulfur, including refinery micronized (30 and 60 kg/1000 L in water) and mineral sulfur (30 and 60 kg/1000 L in water). The concentrations were selected based on the suggestions of gardeners of the area. Control trees were not sprayed. The spraying was repeated after 50 days, in July. Each treatment included three rows of trees. The samples for each treatment were taken from the middle row. Six nearby trees were considered as one replication and the sampling was performed from four sides of each tree. The spraying operation was done in the morning and ended before the weather warmed up. At the time of pistachio harvest in late September and early October, the fruits were sampled and transferred to the laboratory. A portion of fruits was used for obtaining the data at harvest time and the rest were put in disposable tableware with closed lid and stored in 4 °C. After 25 and 50 days, the analyses were performed again.

Moisture content

One kilogram of whole fruits of each replication was weighted at harvest and reweighted after 25 and 50 days. The weight loss was expressed as a percentage of the first weight (Cantin *et al.* 2012).

Extraction for phenolic content and antioxidant activity

Each sample consisted of small parts of at least 10 hulls or kernels. One g of each sample was ground in a small volume of 80% methanol by a mortar and pestle. The mixture was centrifuged at 10000 rpm for 10 min. Extraction was repeated three times. The supernatants were collected, brought to 10 ml volume by 80% methanol and kept at -20 °C until usage (Orthofer and Lamuela-Raventos 1999).

Phenolic content

Total phenolic concentration was determined according to the Folin–Ciocalteu procedure, using gallic acid for preparing the standard curve. Gallic acid was diluted as 20, 40, 60, 80, 100, 120, and

140 mg ml^{-1}. An amount of 5 ml of Folin agent (1:10 in water) and 4 ml sodium carbonate (7.5%) were added to 0.5 ml of plant extracts or standard solutions. Absorbance was read at 760 nm by a UV-visible spectrophotometer (Lambda-Elmer Perkin, American). The results were expressed as mg of gallic acid in 100 g of fresh weight (Orthofer and Lamuela-Raventos 1999).

Antioxidant activity

Antioxidant activity was determined by the 2,2-diphenyl-1- picryl-hidrazil (DPPH) radical-scavenging method. An amount of 900 µl of DPPH solution (500 µM) was added to 100 µl of the plant extract. For the correction factor, 900 ml of distilled water was added to 100 µl of the plant extract. The absorption of these was read at 517 nm. A mixture of 100 µl of distilled water and 900 µl of DPPH solution was used as blank. The absorbance was read at 517 nm, using a UV-visible spectrophotometer. The results were concluded by the fallowing formula and expressed as the inhibition percentage of the DPPH radical (Serrano *et al.* 2005).

$$\text{Antioxidant activity (\%)} = 1- \frac{\text{Sample absorbance} - \text{Correction factor}}{\text{Blank}} \times 100$$

Photosynthetic pigments

Each sample consisted of small parts of at least 10 hulls or kernels. One g of each sample was ground in a small volume of 80% acetone (in water) by a mortar and pestle. The mixture was centrifuged at 10000 rpm for 10 min. Extraction was repeated three times. The supernatants were collected and brought to 10 ml volume by 80% acetone. The absorbance was read at 470, 646.8, and 663.2 nm using a UV-visible spectrophotometer. The chlorophyll and carotenoid concentrations were calculated using the following formula and expressed as mg g^{-1} of fresh weight (Olsson *et al.* 2004).

$$\text{Chl a} = (12.25\ A663.2 - 2.79\ A646.8)$$

$$\text{Chl b} = (21.21\ A646.8 - 5.1\ A663.2)$$

$$\text{Chl T} = \text{Chl a} + \text{Chl b}$$

$$\text{Car} = [(1000A470 - 1.8\ \text{Chl a} - 85.02\ \text{Chl b})/198]$$

Statistical analysis

The study was a factorial experiment based on a completely randomized design with four replications. Factors were storage time (0, 25, and 50 days) and sulfur treatments (control, Movento, Confidor, R30, R60, M30, and M60). Data were analyzed by the SAS 9.1 statistical software (SAS Institute Inc. 2013), and Duncan's multiple range test at $p \leq 0.01$ probability was used to compare the means. The graphs were plotted by the MS-Excel software package.

Results and Discussion

Weight loss

The interaction of treatments and storage times had a significant effect ($p \leq 0.01$) on weight loss (Table 1). This trait increased over time. Confidor and Movento caused higher weight loss at both storage times compared with the control. Both concentrations of refinery micronized sulfur (R30, R60) caused higher wight loss than the control after 25 days but were similar to it on the 50th day (Figure 1). Some research showed the adverse effects of sulfur on fruit weight, which was dependent on the concentration. For example, in cultivars 'Brigitta' and 'ONeal' of blueberry, fumigation with high concentration (250-350 µL L^{-1} h) of SO_2 resulted in higher weight loss than the control after 45 days of cold storage, but fumigation with lower concentrations (50-200 µL L^{-1} h) was similar to the control (Rivera *et al.* 2013). In this study, 30 and 60 kg/1000 L of refinery micronized sulfur revealed adverse effects on weight but mineral sulfur was not harmful. Mineral sulfur with its lower concentration (M30) even caused less weight loss than the control on the 50th day. This result may be due to the longer persistence of refinery micronized sulfur residues on the fruit surface. In grapes, residue concentrations were generally lower for a wettable powder of elemental sulfur in comparison to a micronized formulation that was applied with the same rate and at the same time for the last application before harvest (Kwasniewski *et al.* 2014). Contrastingly, our results showed that the effects of sulfur on water loss were omitted sooner than that of Movento and Confidor. It is known that chemical pesticides have various degrees of phytotoxicity along with their benefits. They can lead to the destruction of the plant cell walls and membranes causing elevated transpiration and water loss (Parween *et al.* 2014).

Phenolic content

The interaction of treatments and storage times was significant ($p \leq 0.01$) for the content of phenolics of the hull (Table 1). In control, this parameter remained statistically stable, but the samples that were treated by sulfur showed an increasing trend over time so that R30, R60, and M60-treated samples had 57.3%, 38.4%, and 40.3% higher phenolic content of the hull, respectively, after 50 days compared to the harvest time (Figure 2a). Pistachio hull is a rich source of phenolics being ranked among the first 50 food products highest in antioxidants (Tomaino *et al.* 2010). The increasing effect of sulfur on the phenolic content of hulls can be related to the higher water loss which imposes higher drought stress. In wheat plants, the phenolic content significantly increased after fumigation by 0.06 ppm SO_2 for 8 h daily from germination time to grain maturity (Agrawal and Deepak 2003). But in blueberries, SO_2 fumigation did not influence the phenolic content (Cantin *et al.* 2012). According to Bolling *et al.* (2010), total phenolics of almond skins increased progressively or stayed constant during 15-months storage at 4 or 23 °C.

The main effect of storage time was significant ($p \leq 0.01$) on the phenolic content of kernels (Table 1). In kernels, the phenolic content was totally lower than that of hulls (Figure 2) which has also been reported in another research (Tomaino *et al.* 2010). Phenolic content of kernels decreased at both storage times compared to the harvest time, which reveals the difference in metabolic status between hulls and kernels (Figure 2a,b). In another study on pistachios, the phenolic content of kernels indicated a 12.2% decrease after high water loss (Tsantili *et al.* 2011).

Table 1. Analysis of variance of the effect of sulfur treatments and storage times for the measured physiological traits of fresh pistachios

Sources of variation	Degrees of freedom	Mean squares				
		Weight loss	Hull phenolic content	Hull antioxidant activity	Hull chlorophyll	Hull carotenoid
Treatments (T)	6	11.80**	0.09**	14.88**	177.93**	33.48**
Storage (S)	2	965.47**	0.09**	1.42^{ns}	153.28**	215.65**
T × S	12	6.04**	0.02**	1.13**	91.67**	29.03**
Error	42	2.12	0.006	0.37	3.94	2.15
Coefficient of variation (%)		12.87	19.44	0.64	12.92	11.41

Table 1 continued

Sources of variation	Degrees of freedom	Mean squares			
		Kernel phenolic content	Kernel Antioxidant activity	Kernel chlorophyll	Kernel carotenoid
Treatments (T)	6	0.001^{ns}	2.49**	75.12**	36.91**
Storage (S)	2	0.01**	0.62**	159.43**	21.20**
T×S	12	0.0003^{ns}	0.21**	105.77**	43.27**
Error	42	0.13	0.03	3.66	1.99
Coefficient of variation (%)		14.95	0.19	12.08	10.14

^{ns} and ** non-significant and significant at 0.01 probability level, respectively

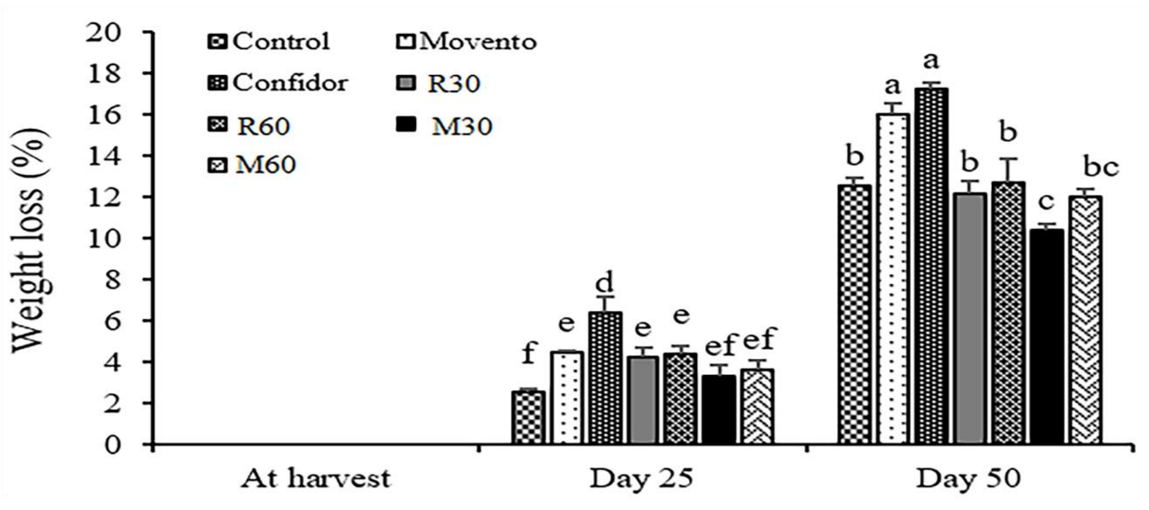

Figure 1. Weight loss of pistachio cultivar 'Ahmadaghaei' treated preharvest with Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), refinery micronized sulfur at two concentrations (30 and 60 kg/1000 L) (R30, R60), and mineral sulfur at two concentrations (30 and 60 kg/1000 L) (M30, M60) during storage

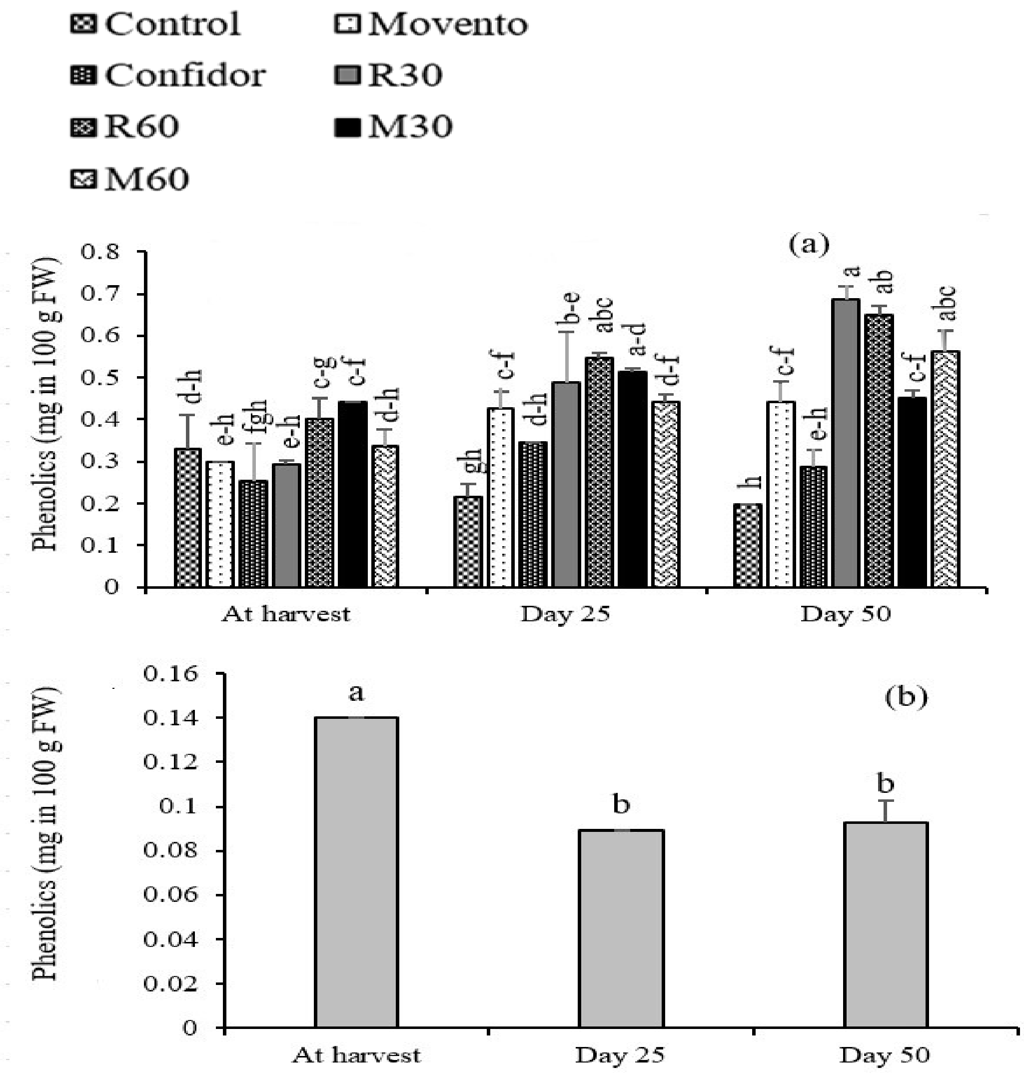

Figure 2. Phenolic content of (a) hulls and (b) kernels of pistachio cultivar 'Ahmadaghaei' treated preharvest with Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), refinery micronized sulfur at two concentrations (30 and 60 kg/1000 L) (R30, R60), and mineral sulfur at two concentrations (30 and 60 kg/1000 L) (M30, M60) during storage

Antioxidant activity

The interaction of treatments and storage times was significant ($p \leq 0.01$) for the antioxidant activity of hulls and kernels (Table 1). The hulls that were treated with chemical pesticides were similar to the control, but sulfur-treated hulls showed lower antioxidant activity than the control at all storage times (Figure 3a). In kernels, sulfur treatments had lower antioxidant activity than the control, while Movento revealed an effect more similar to the control at 25 and 50 days after harvest (Figure 3b). These results are not in agreement with the results of phenolic amount implying that in sulfur treatments, a significant decline must have occurred in the content of other compounds with antioxidant properties, likely antioxidant enzymes,

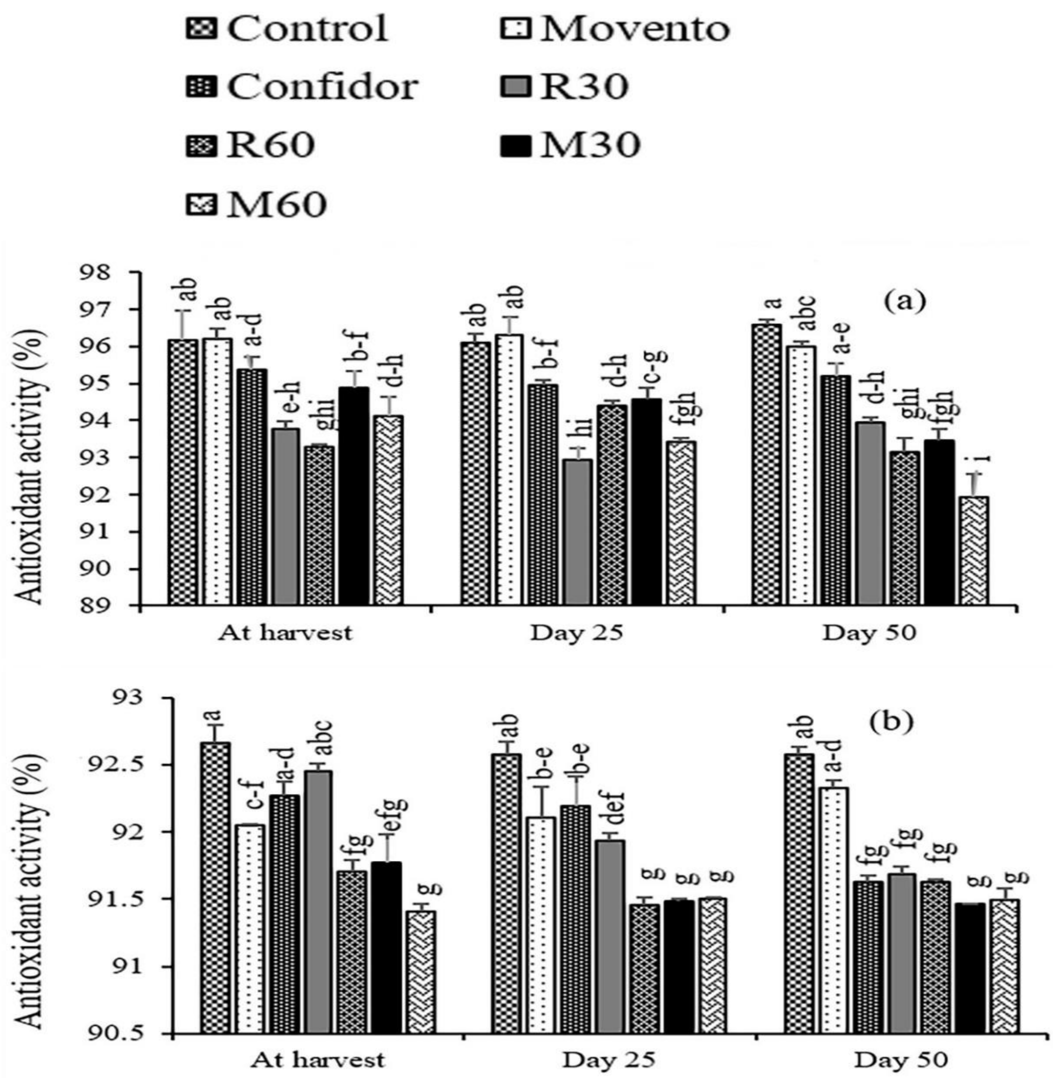

Figure 3. Antioxidant activity of (a) hulls and (b) kernels of pistachio cultivar 'Ahmadaghaei' treated preharvest with Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), refinery micronized sulfur at two concentrations (30 and 60 kg/1000 L) (R30, R60), and mineral sulfur at two concentrations (30 and 60 kg/1000 L) (M30, M60) during storage

vitamins or carotenoids (carotenoid content was lower in R30 and R60 sulfur treatments of hulls at harvest as compared to the control) (Figure 4). In a study, the antioxidant activity of 10 different fruit extracts was mainly related to the content of vitamin C and carotenoids rather than phenolic compounds (Olsson *et al.* 2004). Similar to hulls, the antioxidant activity of kernels stayed stable over time for most treatments except the samples treated with Confidor and R30, in which a decline was seen after 50 days compared to the harvest time. The stability of antioxidant activity over time is probably because of the low temperature (4 °C) of the storage, in which the antioxidant enzymes have low activity. In strawberry fruits, the antioxidant activity stayed stable during storage as a result of cold temperature (Jin *et al.* 2011).

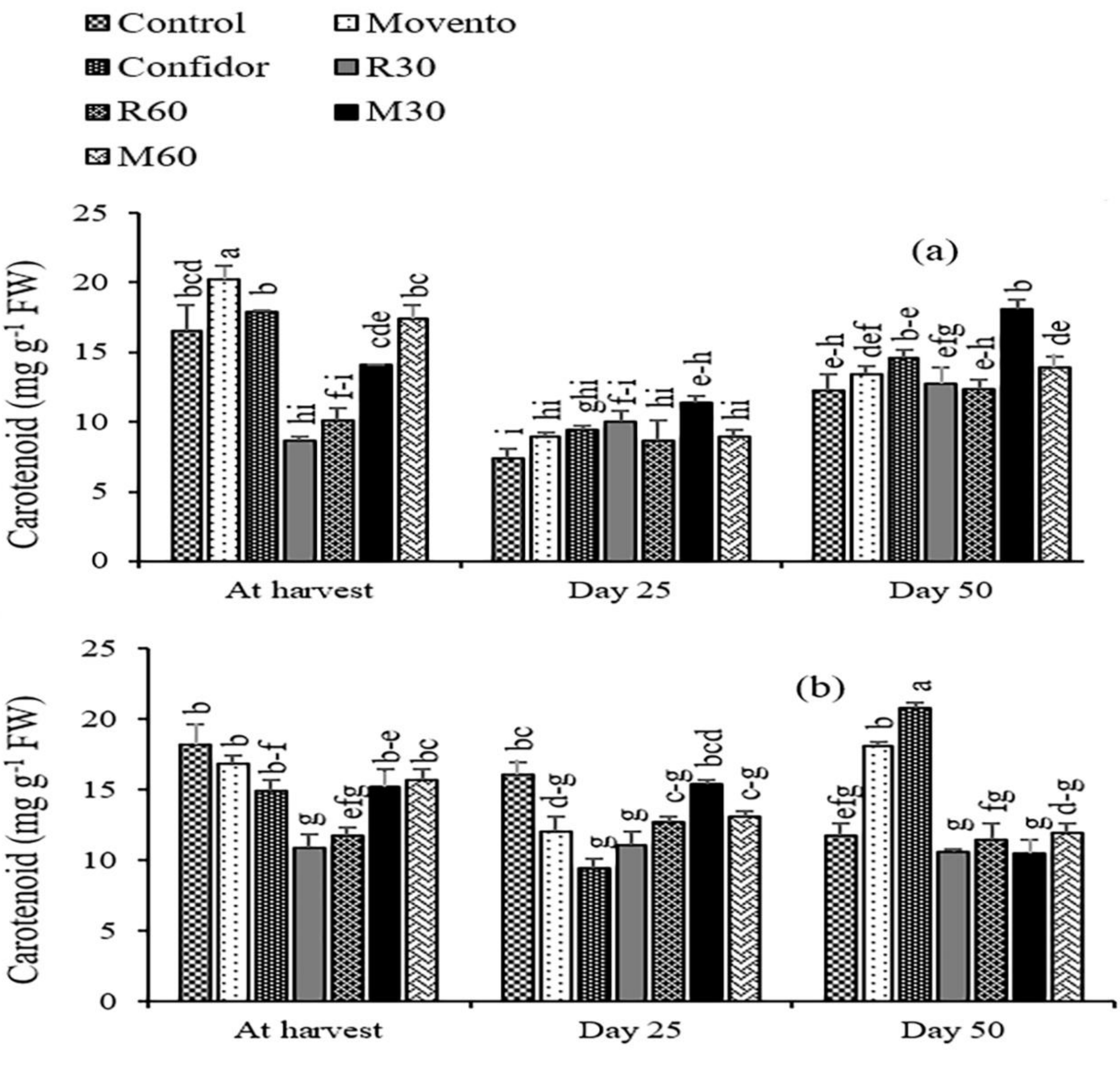

Figure 4. Carotenoid content of (a) hulls and (b) kernels of pistachio cultivar ‘Ahmadaghaei’ treated preharvest with Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), refinery micronized sulfur at two concentrations (30 and 60 kg/1000 L) (R30, R60) and mineral sulfur at two concentrations (30 and 60 kg/1000 L) (M30, M60) during storage

Chlorophyll

The interaction of treatments and storage times was significant ($p \leq 0.01$) for the chlorophyll content of hulls and kernels (Table 1). Confidor resulted in lower chlorophyl content of kernels at the harvest time and after 25and 50 days among the treatments. Both concentrations of refinery micronized sulfur (R30, R60) indicated mostly the highest chlorophyll content compared to other treatments. Chlorophyll content of some treatments showed a decline during storage (Figure 5a,b). The higher chlorophyll content in the sulfur treatments

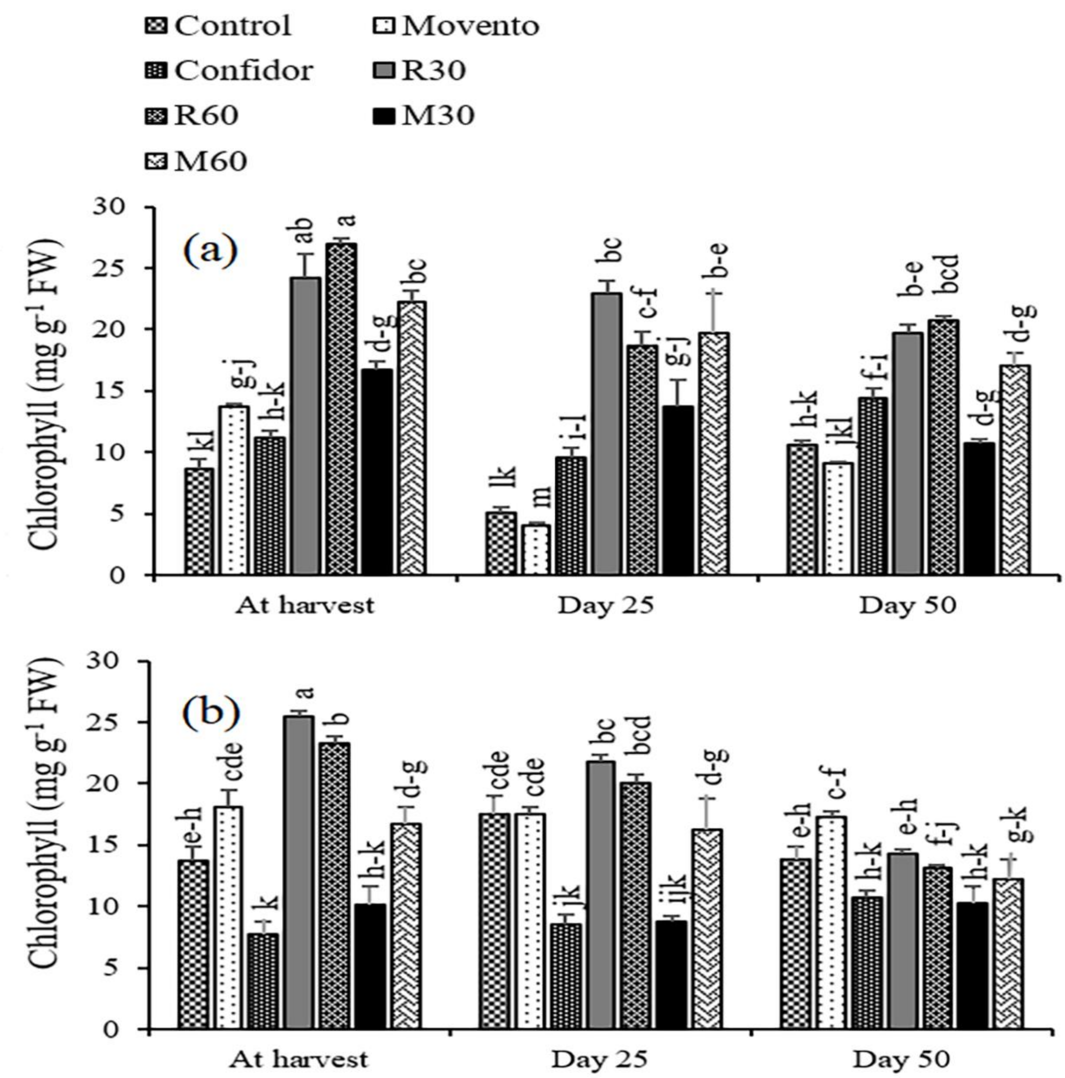

Figure 5. Chlorophyll content of (a) hulls and (b) kernels of pistachio cultivar 'Ahmadaghaei' treated preharvest with Movento (0.5 L/1000 L), Confidor (0.4 L/1000 L), refinery micronized sulfur at two concentrations (30 and 60 kg/1000 L) (R30, R60), and mineral sulfur at two concentrations (30 and 60 kg/1000 L) (M30, M60) during storage

was not due to the difference in the ripening process because all of the pistachios were completely ripe at the harvest time. Chlorophylls react to harvest light quanta and transfer the energy during photosynthesis. The way that the plants react to the SO_2 stress depends on their physiological state and their need for energy (Li and Yi 2020). Usually, researchers report the negative effect of SO_2 on chlorophyll content and photosynthesis, but some indicate the stimulation of chlorophyll synthesis under the SO_2 treatments. For instance, when Arabidopsis plants were exposed to 30 mg/m^3 SO_2 for 72 h, the total chlorophyll content was significantly increased in their leaves (Li and Yi 2020). In another study, 200 µg (active ingredient) kg^{-1} of sulfosulfuron stimulated the chlorophyll synthesis in fresh leaves of green gram (Khan *et al.* 2006).

Carotenoid

The interaction of treatments and storage times was significant ($p \leq 0.01$) for the carotenoid content of hulls and kernels (Table 1). In the hulls, the refinery micronized sulfur treatments (R30, R60) showed a lower carotenoid content at the harvest time. For most samples, the carotenoid content of hulls revealed a decreasing trend over time, followed by a slight increase at 50 days after harvest (Figure 4a). Similar to hulls, R30 and R60 resulted in a lower carotenoid content of kernels at harvest time. For some treatments, the carotenoid content of kernels stayed stable during storage while some others showed a slight decreasing trend (Figure 4b). Carotenoids act as natural defense compounds under stress conditions, apart from their function as antennae (Parween *et al.* 2014). The decrease in carotenoid content through the SO_2 treatment has been reported in other crop plants such as rice (Agrawal *et al.* 1982) and strawberry (Muneer *et al.* 2014). Lower carotenoid content in hulls that were treated with R30 and R60 can reveal the decoloring property of sulfur as well. In the cultivar 'Brown Turkey' of figs, 50-100 (µL/L) h SO_2 resulted in bleaching of fruits in the cold storage with a trend that was concentration-dependent (Cantin *et al.* 2011). In our study, the Movento-treated hulls showed the highest carotenoid content at harvest, which may be correlated with the higher water loss and higher phytotoxic effect of this pesticide.

Conclusion

The kernel which is the edible part of the pistachio is covered by the shell and hull, so sulfur and other pesticides cannot easily penetrate the kernel. However, the physiological status of the hull and the whole plant may influence the kernels and the yield. Our results showed that the refinery micronized sulfur at concentrations of 30 and 60 kg/1000 L caused higher wight loss, higher chlorophyll of the hull and kernel, and lower carotenoid of the hull and kernel. All sulfur treatments reduced the antioxidant activity of the hull and kernel, while elevating the hull phenolic content. Movento and Confidor also caused higher weight loss at both storage times, but the effects of sulfur on water loss were eliminated sooner than that of Movento and Confidor (on the 50th day, sulfur treatments were similar to the control). Movento-treated hulls contained the highest carotenoid content at harvest. Confidor resulted in less chlorophyl content of kernels at harvest. Therefore, the gardeners should take this into account that besides the benefits on controlling the pest, sulfur and other pesticides may have some adverse effects on the product quality.

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Conflicts of interest

All authors agree on the content of the paper and have no conflict of interest to disclose.

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برخی تغییرات فیزیولوژیکی پسته تازه بعد از محلول‌پاشی گوگرد

فاطمه ناظوری[1*]، الهه زمانی بهرام آبادی[1]، سید حسین میردهقان[1]، مریم افروشه[2]

۱- گروه علوم باغبانی، دانشکده کشاورزی، دانشگاه ولیعصر (عج) رفسنجان، رفسنجان

۲- پژوهشکده پسته، موسسه تحقیقات علوم باغبانی، سازمان تحقیقات، آموزش و ترویج کشاورزی، رفسنجان

چکیده

استفاده از گوگرد به عنوان آفت‌کش یک استراتژی در حفاظت از گیاهان است، ولی آفت‌کش‌ها علاوه بر مزایای فراوان برای کنترل آفات، می‌توانند بر ویژگی‌های محصول تأثیر بگذارند. در این مطالعه، مونتو (۰/۵ لیتر در ۱۰۰۰ لیتر)، کونفیدور (۰/۴ لیتر در هزار لیتر) و گوگرد پالایشگاهی میکرونیزه و گوگرد معدنی (۳۰ و ۶۰ کیلوگرم در ۱۰۰۰ لیتر آب) روی درختان پسته رقم احمدآقایی دوبار (در اواخر اردیبهشت و ۵۰ روز بعد از آن) برای کنترل پسیل پاشیده شدند. پسته‌ها در اوایل مهرماه برداشت شدند و در دمای ۴ درجه سانتی‌گراد به مدت ۲۵ و ۵۰ روز به منظور ارزیابی تأثیر سموم بر برخی ویژگی‌های پوست و مغز ذخیره شدند. وزن پسته‌ها، میزان ترکیبات فنولی مغز و میزان کلروفیل و کاروتنوئید پوست و مغز در طی زمان کاهش یافتند، در حالی که میزان ترکیبات فنولی پوست روند افزایشی نشان داد. ظرفیت رادیکال‌زدایی (رادیکال DPPH) پوست و مغز در طی زمان ثابت ماند. گوگرد پالایشگاهی میکرونیزه در هر دو غلظت کاهش وزن را در روز ۲۵ام بالا برد، ولی بعد از ۵۰ روز مشابه شاهد بود. این گوگرد، کلروفیل بالاتر و کاروتنوئید پایین‌تری در پوست و مغز نشان داد. گوگرد معدنی در هر دو غلظت مقدار کلروفیل بیشتری در پوست نشان داد. همه تیمارهای گوگرد ظرفیت رادیکال‌زدایی پوست و مغز را کاهش دادند، در حالی که میزان ترکیبات فنولی پوست را بالا بردند. آفت‌کش‌های مونتو و کونفیدور کاهش وزن را افزایش دادند. گرچه آفت‌کش‌های شیمیایی و گوگرد نمی‌توانند به آسانی به مغز نفوذ کنند، ولی وضعیت فیزیولوژیکی پوست و کل گیاه ممکن است روی مغز و محصول موثر باشد.

واژه‌های کلیدی: آفت‌کش؛ فیزیولوژی میوه؛ کونفیدور؛ گوگرد؛ مونتو