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Impacts of Lavandula angustifolia Mill. and Thymbra spicata L. essential oils on postharvest gray mold of strawberries

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Abstract

Antifungal activities of two essential oils (EOs), derived from the *Lavandula angustifolia* L. and *Thymbra spicata* L. plant leaves were tested in current study against two isolates (M1-5 and M3-5) of *Botrytis cinerea* in Potato Dextrose Agar (PDA). These studies were performed *in vitro* and a further *in vivo* test with vapor contact application of the EOs was performed with strawberry fruits to confirm the antifungal activities in postharvest storage. *In vitro* studies were conducted with four different application doses (0.25, 0.50, 1.00 and 2.00 mL L-1) of both EOs with poisoned food technique. The highest dose (2.00 mL L-1) of *L. angustifolia* had a 92.50% mycelial growth inhibition on M1-5, where the same dose of same oil had 0.00% mycelial growth inhibition on M3-5. On the other hand, the highest dose (2.00 mL L-1) of *T. spicata* had 16.76% and 51.18% of mycelial growth inhibition on M1-5 and M3-5, respectively. The lower doses had less or no antifungal activity, thus only the highest doses were tested in the consecutive *in vivo* studies. Results suggested that both of the EOs had moderate impact on the prevention of disease severity at strawberry cv. Camarosa fruits, inoculated with M1-5 and M3-5 isolates. The EOs were also noted to have a significant influence on the prevention of the weight loss and loss of soluble solids concentration. Results suggested that the vapor contact application of *L. angustifolia* and *T. spicata* essential oils have potential to be alternative to synthetic fungicides for controlling gray mold in strawberry fruits caused by *B. cinerea*.

Keywords: Alternative control, *Botrytis cinerea*, Fruit storability, Poisoned food technique, Vapor contact

Introduction

Berry fruits, including mainly strawberry, blackberry, blueberry, cranberry and raspberry, are rich in a wide variety of nutrients and phytochemicals (Skrovankova et al., 2015; Usanmaz, 2019). The high and diverse contents phenolics, flavonoids and alkaloids gives significant antioxidant capacity to the berries and help to fight with various diseases including cardiovascular diseases and cancer (Mukherjee et al., 2020). Among the berries, strawberry (*Fragaria* x *ananassa* Duch.) fruits are the most popular because of their flavors and adaptability to different environmental conditions. However, strawberry fruits are very sensitive to postharvest storage and have a very short storage life (2-5) which obstruct its marketability (Parvez and Wani, 2018). The high respiration rate and sensitive fruit skin makes the strawberries susceptible to mechanical injuries and pathogen infections (Caleb et al., 2016). The main causes of the pathogenic decay on the strawberries is the gray mold caused by *Botrytis cinerea* (Parvez and Wani, 2018). *B. cinerea* causes soft rots and water-soaked paranchyma tissues on the fruits, which is followed by the growth of gray conidia, decayed fruits and rendered marketability (Williamson et al., 2007). Fungicide application is the most widely used technique for fungus control (Adaskaveg et al., 2021), but its acceptability by the consumers is decreasing due to the scientifically confirmed hazards on human health and nature (Koch

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et al., 2017). Besides to that, under excessive- and mis-use of fungicides have been reported to cause the development of resistant genotypes and reduce their efficacy (Hao et al., 2011). Therefore, safely and healthy alternatives for fungicides have become very important in the last decades (Huang et al., 2021).

Use of plant derived products, including proteins, lipids, polysaccharides and secondary metabolites, as a protective coating material or different means of treatment have been reported to provide success in improving storability of fruits and vegetables (Wan et al., 2021). The most important advantage of these materials is that they are biodegradable (Nor and Ding, 2020) while their correct use have no known negative impact on human health. Biodegradable materials lower petroleum consumption, help to reduce carbon dioxide levels in atmosphere, reduce wastes and require less energy during production. Application of plant natural products as edible coatings helps to reduce respiration and transpiration of harvested products and maintain storage quality (Wan et al., 2021). Several plant essential oils have been reported to control *B. cinerea* infections at strawberry fruits, including lemongrass oil, lemon oil and orange oils (Kahramanoğlu, 2019). The different biochemical compounds of the essential oils are known to provide antifungal activity to the oils. Some examples to these compounds are eucalyptol, linalool, carvacrol and γ-terpinene (Kordali et al., 2016; Moazeni et al., 2021). *Lavandula angustifolia* Mill. and *Thymbra spicata* L. are rich in these biochemical compounds. *L. angustifolia* contains eucalyptol (15.10%) and linalool (11.98%) (Karadağ et al., 2021), while *T. spicata* contains carvacrol (56.03%) and γ-terpinene (6.87%) (Sengun et al., 2021). Several studies reported antifungal activity for the essential oils of these plant species, i.e. *L. angustifolia* against *Aspergillus niger* (Stupar et al., 2014) and *T. spicata* against *Aspergillus parasiticus* (Gumus 2010). However, according to authors' knowledge, no information available about the postharvest efficacy of *L. angustifolia* and *T. spicata* essential oils against *B. cinerea*. Therefore, present study was conducted to determine the *in vitro* antifungal activity of *L. angustifolia* and *T. spicata* against *B. cinerea* and test their further impacts on fruit quality in *in vivo* studies on strawberry fruits cv. Camarosa.

Materials and Methods

Plant material and essential oils (EOs) production

The leaves of the *Lavandula angustifolia* Mill. and *Thymbra spicata* L. plants, both belonging to the Lamiaceae family, were gathered during their flowering stages (between 6th and 8th months in 2020) in Turkey. Leaves were air dried and ground. Then, a total of 500 g dried sample was subjected to hydro distillation with Clevenger-type apparatus to obtain oils. The *L. angustifolia* and *T. spicata* leaves resulted 2.00% and 1.47% (w w-1, dry weight basis) oil, respectively. The EOs of both plants were kept under 4 °C temperature until being used in the studies.

In vitro antifungal studies

Two different isolates of Botrytis cinerea, namely M1-5 and M3-5, which were taken from the collection of Iğdır University, Department of Plant Protection and Phytopathology Laboratory, were used in current study. The poisoned food technique (Euloge et al., 2012) was used in current research to investigate the antifungal activity of the EOs of *L. angustifolia* (LA) and *T. spicata* (TS). The EOs were tested in four different doses, which are: 0.25 mL L^{-1}, 0.50 mL L^{-1}, 1.00 mL L^{-1} and 2.00 mL L^{-1}. The EOs were dissolved in 70% ethanol in a ratio of 1:2 (v v^{-1}) and mixed into molten and cooled PDA at 45 °C. In each petri dish (9 cm in diameter), 20 mL solution was added and solidified at room temperature for 1 hour. Next, an agar disc of mycelia (with 5-mm diameter) which were cut from actively growing 7-days-old isolates (M1-5 and M3-5) of *B. cinerea* were placed in the mid-point of each petri dish. Besides to these 4 doses of EOs, three different controls were included into the current study. First control was prepared by following the same method without oil and the second control was prepared with the 70% ethanol. The third and final control was prepared with a fungicide (the treatment dose was: 100 mL 100mL-1 of 500 g L^{-1} Fenhexamid (Teldor® SC 500)). Four plates (replications) were repeated for each of the seven treatments and they were incubated at 25 °C for 7 days. During this 7 days of incubation, the colony diameter in petri plates were measured regularly on day 3, day 5 and day 7. Next, the mycelial growth inhibition (%) ability of the treatments was calculated by following the formula of Thomidis and Filotheou (2016): mycelial growth inhibition (%) = {[(dc − dt)/dc] × 100}. In this formula, the dc represents the mean radial diameter of the *B. cinerea* in control sample, and dt is the mean radial diameter of the *B. cinerea* in treated sample.

In vivo antifungal and postharvest studies

Strawberry (*Fragaria x ananassa* Duch. cv. Camarosa) fruits were used in the second part of this study to reveal the antifungal activity of EOs and to determine their impacts on fruit quality. Fruits were collected from a commercial business located in Iğdır, Turkey. These studies had been carried after evaluating the *in vitro* results, which suggested that only the highest doses can be successful. Thus, the 2.00 mL L^{-1} dose was used for both EOs. First of all, strawberry fruits were selected to eliminate any damaged fruits and then the remaining healthy fruits were disinfected in 2.0% sodium hypochlorite for 5 minutes. After that, fruits were washed 3 times under pure water and dried on sterile papers for 30 minutes. Then, 1 mm wide and 1 mm deep wound was opened on each fruit with a sterile scalpel (10 µL). Next, 10 µL conidial suspension of *B. cinerea* (1.0 × 106 conidia mL^{-1}) was infected into the wounds.

This experiment was composed of five different treatments. First two treatments of present study are

the EOs. The other three treatments are the three separate controls. One control was designed with no any treatment after artificial infection of the fruits (Control-1) and another control was designed with no artificial infection and left natural (Control-2). Final control composed of artificial infection and with the same fungicide as described in *in vitro* studies. Fungicide treatment was done by direct spraying onto the fruits. In this study, three replication (each with four fruits) was used for each treatment. The studies were designed to continue for 21 days and the measurements were done with 3 days interval. Therefore, 21 different replications were prepared on the first day and 3 replications were used in each (totally 7) measurement point. After inoculation of the *B. cinerea* isolates, the four fruits of each replication were placed in PVC boxes. The EOs application in these studies were done by contact vapor application. The application doses (2.00 mL L-1 of air) of EOs were prepared according to the air space (~500 mL) of the PVC boxes and the determined oil concentrations were soaked onto a sterile paper plate (20 mm2). Then, these oil containing plates were put onto the inner part of the box cover. Hereafter, the plastic PVC boxes were sealed with parafilm (9 mic) to prevent the loss of volatile compounds. The fruits were then stored at 3.5 °C ± 0.5 °C (Mohammadi et al., 2015a) for 21 days to determine the effects of EOs under storage conditions. Before postharvest storage, fruits were kept at 25 °C for 2 h to initiate infection (Xu et al., 2021).

As mentioned above, fruit quality characteristics (weight loss, soluble solids concentration-SSC, pH and ascorbic acid-AA) and disease severity were measured with 3 days interval. The 0-5 scale was used to assess disease severity (DS) of each fruit (Huang et al., 2011). In this scale, 0 means no infection, where 1, 2, 3, 4 and 5 means < 20%, 20.1% to 40%, 40.1% to 60%, 60.1% to 80% and > 80.1% rotted area, respectively. Weight loss (%) was determined for each replication by measuring the initial and final weights and calculation the loss with the standard ratio method. SSC of each fruit was then measured with a hand refractomer as % Brix. A pH meter was then used to determine the pH of each fruit. Lastly, the AA (mg 100 g-1) of each fruit was determined by titrating the fruit juice with iodine solution (Skinner 1997).

Data analysis

The data of each mycelial growth inhibition (%), disease severity and fruit quality characteristic were all summed in Microsoft Excel by calculating the means and standard deviation of each treatment. These descriptive statistics were used to prepare the figures and tables for better presentation of the data. Statistical comparison of the means was then carried with Tukey's test after ANOVA at 5% significance level. The SPSS 22.0 software was used for statistical analysis.

Results and Discussion

Antifungal activity of essential oils

The influence of the tested treatments on the colony formations (diameter-cm) of two isolates of *B. cinerea* are given in Table 1. It is clear from the results that the colony diameter increased during the incubation period and reached to maximum in the 7th day of incubation.

Table 1. Influence of *L. angustifolia* and *T. spicata* essential oils, incorporated into the PDA media, on the colony diameter (cm) of mycelial growth of *B. cinerea* isolates (M1-5 and M3-5) during 7 days of incubation

Treatments	3 days	5 days	7 days	3 days	5 days	7 days
	***L. angustifolia* on M1-5 isolate**			***T. spicata* on M1-5 isolate**		
EO (0.25 mL L^{-1})	3.99 b	6.69 c	8.03 b	5.08 a	8.34 a	8.44 a
EO (0.50 mL L^{-1})	2.99 c	6.14 c	7.46 c	4.04 b	7.71 bc	8.50 a
EO (1.00 mL L^{-1})	0.40 d	2.89 d	6.68 c	3.93 b	6.86 d	7.63 b
EO (2.00 mL L^{-1})	0.49 d	0.50 e	0.64 d	1.24 c	4.01 e	7.08 c
Control-1 (sterile water)	4.49 a	7.33 b	8.50 a	4.49 ab	7.33 c	8.50 a
Control-2 (70% ethanol)	4.56 a	7.99 a	8.50 a	4.56 ab	7.99 ab	8.50 a
Control-3 (fungicide)	0.50 d	0.50 e	0.50 d	0.50 c	0.50 e	0.50 d
	***L. angustifolia* on M3-5 isolate**			***T. spicata* on M3-5 isolate**		
EO (0.25 mL L^{-1})	4.63 c	8.26 a	8.50 a	5.49 b	8.50 a	8.50 a
EO (0.50 mL L^{-1})	4.65 c	8.50 a	8.50 a	2.98 d	7.89 b	8.50 a
EO (1.00 mL L^{-1})	2.89 d	7.45 b	8.50 a	4.03 c	8.00 b	8.26 b
EO (2.00 mL L^{-1})	1.55 e	4.48 c	8.50 a	1.18 e	2.80 c	4.15 c
Control-1 (sterile water)	5.68 b	8.50 a	8.50 a	5.68 b	8.50 a	8.50 a
Control-2 (70% ethanol)	6.10 a	8.50 a	8.50 a	6.10 a	8.50 a	8.50 a
Control-3 (fungicide)	0.50 f	0.50 d	0.50 b	0.50 f	0.50 c	0.50 d

Different letters next to the values at each incubation day for different oils and isolates, indicates significant difference according to Tukey's HSD test ($p \leq 0.05$).

The fungicide treatment was noted to have very high influence on the prevention of the colony formation in both isolates. The EOs were then observed to have varying significant influence on the colony formation. The EO of *L. angustifolia* reduced the colony formation of M1-5 isolate only at its high concentrations, while the same concentration had no effect on the other isolate, M3-5. On the other hand, the EO of *T. spicata* had very low influence on the M1-5 isolate, while its highest dose was noted to have significant influence on the prevention of the M3-5 isolate. The formation of the colonies and their visual appearance was also showed in Figure 1.

The results about the mycelial growth inhibition (%) of the treatments are presented in Figure 2. The results clearly presented that the low to high doses of *L. angustifolia* essential oil had low to high influence on the mycelial growth inhibition of the M1-5 isolate, but no effect on M3-5. The highest dose of *L. angustifolia* (2.00 mL L^{-1}) was found to have more than 90% of mycelial growth inhibition on M1-5. The results for *T. spicata* essential oil were found to be quite different. This EO had higher influence on the M3-5 isolate and lower on the M1-5 isolate. The highest concentration of *T. spicata* (2.00 mL L^{-1}) provided more than 50% of mycelial growth inhibition on the M3-5 isolate.

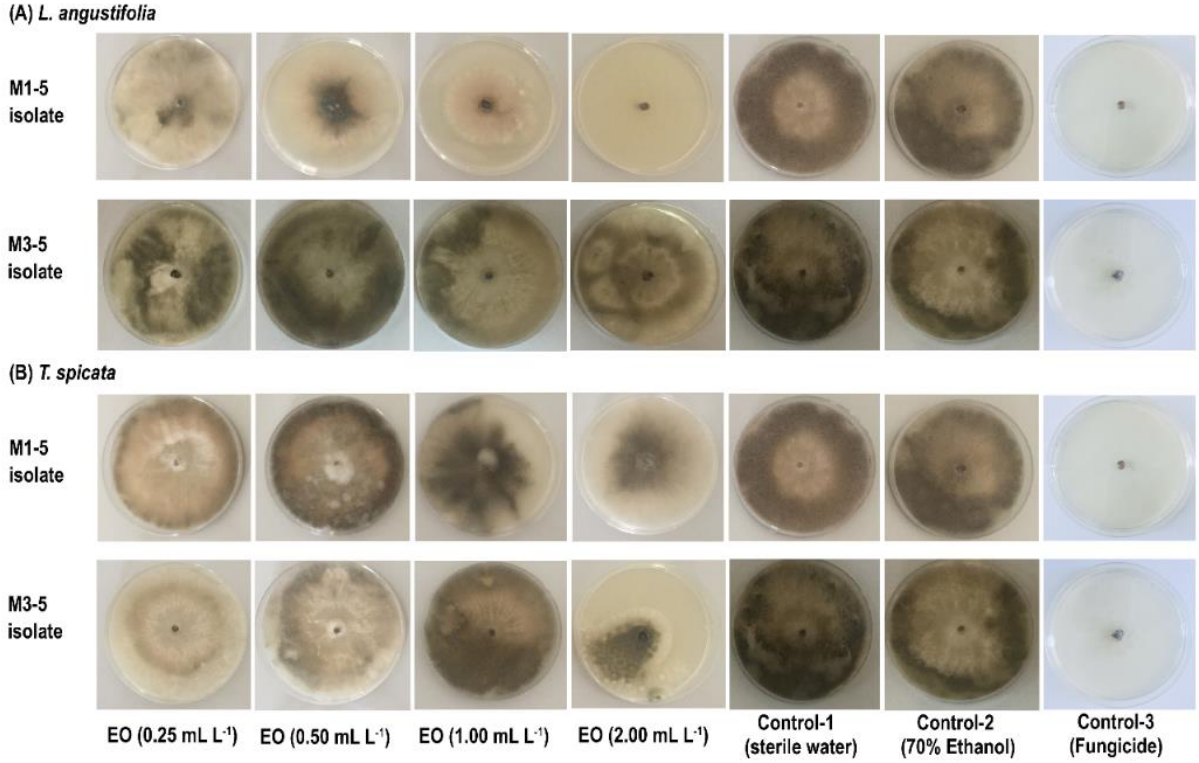

Figure 1. Visual appearance of mycelial growth of two isolates of *B. cinerea* on PDA growing media, as affected by different application doses of (A) *L. angustifolia* and (B) *T. spicata* essential oils, after 7 days of incubation.

Results of present study were found to be in accordance with several studies which reported high influence of essential oils on the mycelial growth of different fungi (Gumus, 2010; Stupar et al., 2014; Kordali et al., 2016; Kahramanoğlu, 2019). The success of these EOs can be associated with their diverse chemical compositions, i.e. eucalyptol and linalool for *L. angustifolia* (Karadağ et al., 2021) and carvacrol and γ-terpinene for *T. spicata* (Sengun et al., 2021). In a similar study, *L. angustifolia* with a high concentrations (37.61%) of linalool was noted to have significant influence on the growth of *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium* sp., *Trichoderma viride* and *Bipolaris spicifera*. The antifungal activity of *L. angustifolia* was noted to vary among concentrations and isolates and was noted to have similar effects with the EOs of *Origanum vulgare* and *Rosmarinus officinalis* (Stupar et al., 2014). The antifungal activity of *T. spicata* was also previously noted for different fungi. Gumus (2010) reported that the EO of *T. spicata* reduce the activity of *A. parasiticus* (NRRL 465 and NRRL 2999).

Impacts of volatile essential oils on disease severity

Figure 3 shows the effects of vapor contact application of *L. angustifolia* and *T. spicata* EOs on the disease severity, caused by two isolates of *B. cinerea*, on cv. Camarosa strawberry fruits. Results clearly showed that both EOs have significant influence on the prevention of disease severity, where this impact is higher than un-treated controls, but lower than the fungicide application.

Although the impacts are lower than the fungicide treatment, the disease severity of two isolates treated with EOs, especially with *T. spicata*, was noted to be acceptable in 12 days of storage. The *T. spicata* EO treatment reduced the disease severity score of M1-5 isolate from 4.1 to 1.5 at the 12th day of storage, which at the same time provided better control of disease severity of M3-5 isolate (reduced from 4.2 to 1.2). Slightly lower impact was observed from the *L. angustifolia* which provided a score of 1.8 on M1-5 and 1.4 on M3-5. Several studies suggested that the disease severity or decay at strawberry fruits generally starts after 4-6 days of storage at 4 °C (Mohammadi et al., 2015b; Kahramanoğlu, 2019).

Camarosa is a highly perishable strawberry cultivar and 12 days is a very good success in storage without or with acceptable level of disease severity (Kahramanoğlu 2019). Current results are in agreement with some previous studies, which reported moderate-to-high antifungal activity for *L. angustifolia* (Stupar et al., 2014) and *T. spicata* against (Gumus 2010) different fungi. Several studies (Fadli et al., 2012) recommended that the antifungal activity of essential oils can be due to their ability to disrupt cell membrane of the fungi, ability to cause a loss of integrity in cell wall and ability to prevent the respiration (Fadli et al., 2012).

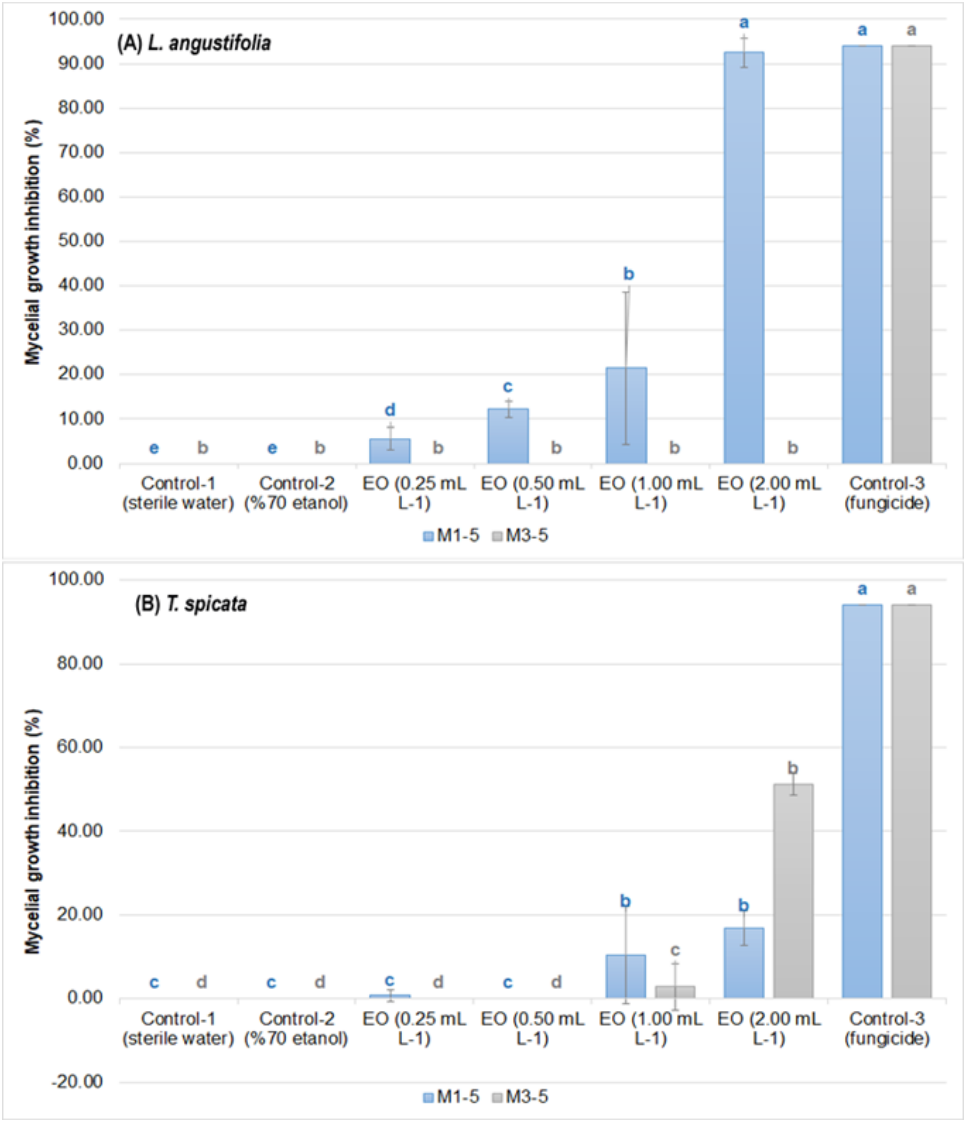

Figure 2. Mycelial growth inhibition of *B. cinerea* isolates (M1-5 and M3-5) after 7 days of incubation, caused by the (A) *L. angustifolia* and (B) *T. spicata* essential oils added on PDA. Different letters above the columns of each isolates separately indicates significant difference among the treatments according to Tukey's HSD test ($p \leq 0.05$).

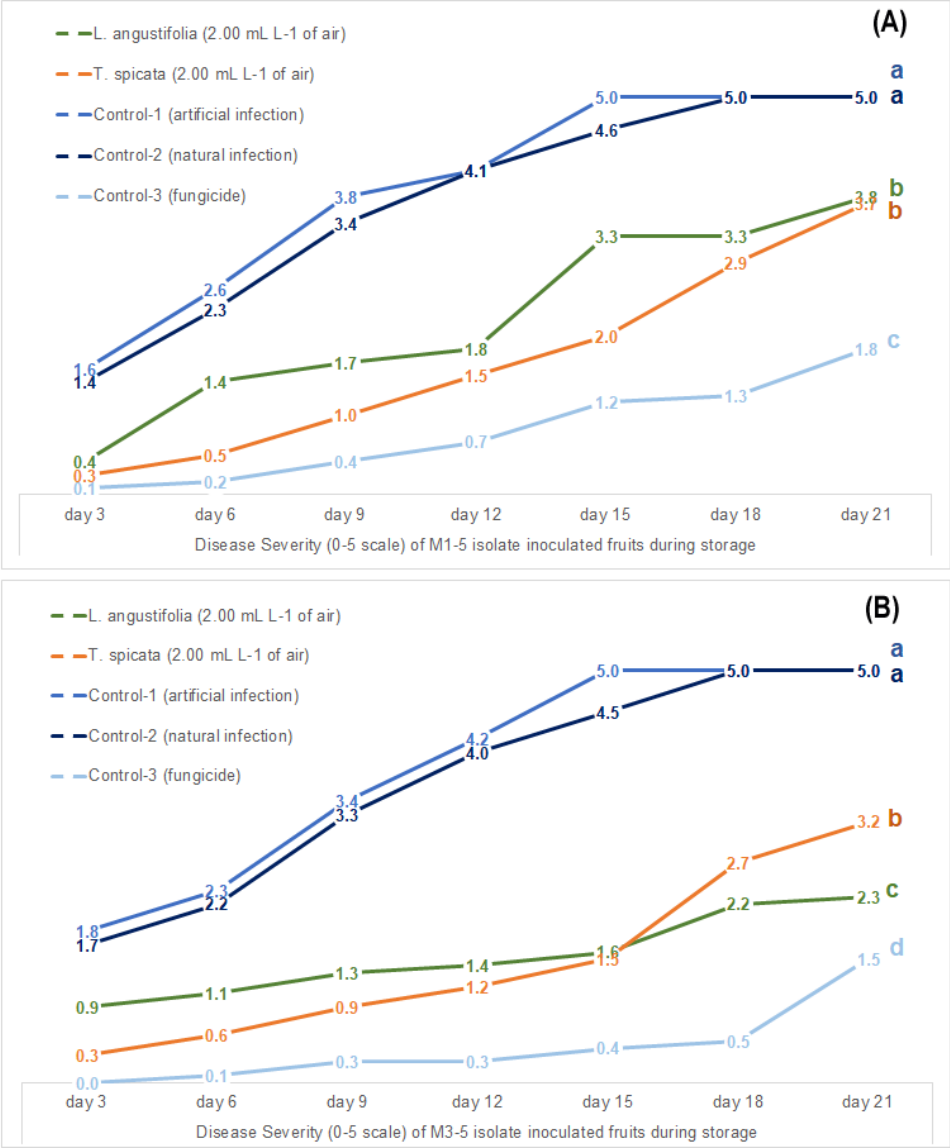

Figure 3. Disease severity of strawberry fruits during 21 days of storage as affected by vapor application of *L. angustifolia* and *T. spicata* essential oils after inoculation with two isolates (A) M1-5 and (B) M3-5 of *B. cinerea*. Different letters on the right side of lines indicates significant difference (by comparing the final day) among the treatments according to Tukey's HSD test ($p \leq 0.05$).

Results of present study are also valuable in terms of their application type. Essential oils have high ability to volatilize and are not soluble in water. Thus, the application of EOs as coating or film is difficult in practice and these characteristics may reduce their activities (Carvalho et al., 2016). In present study, the EOs, during the *in vivo* studies, had been applied as vapor contact and found to have significant influence on the mycelial growth of *B. cinerea*. Most of the available studies in the published literature (Pavinatto et al., 2020) suggest the contact application or incorporated application of EOs with coatings or films; therefore current results are important in terms of their characteristics of application. The vaporized application of EOs had also been reported to have less or no impact on the sensory quality of fruits, which make it as an important alternative (Velázquez-Nuñez et al., 2013). Current results are not novel for the science (in terms of their application method) which was tested and recommended by several studies for controlling different fungi (Velázquez-Nuñez et al., 2013; Paris et al., 2020), but novel for the EOs of *L. angustifolia* and *T. spicata* against *B. cinerea*. A closely related study by Mpho et al., (2013) tested the combined effects of vapor of lemongrass oil (100 µL) and modified atmosphere packaging (MAP) in avocado fruits and suggested better performance in the combined treatment for controlling the *C. gloeosporioides*. MAP is a very important technique for improving postharvest quality and nutritional parameters of fruits and vegetables (Kurubas et al., 2019). Discussion of current results made it possible to conclude that the combination of vapor application of EOs with packaging techniques (i.e. MAP), could be more effective in controlling fungi and improving the storability of fruits, including strawberry.

Impacts of volatile essential oils on fruit quality

The impacts of vapor application of the *L. angustifolia* and *T. spicata* essential oils on different fruit quality parameters (weight loss, AA, SSC and pH) were also tested in current study. The results are presented in Figure 4.

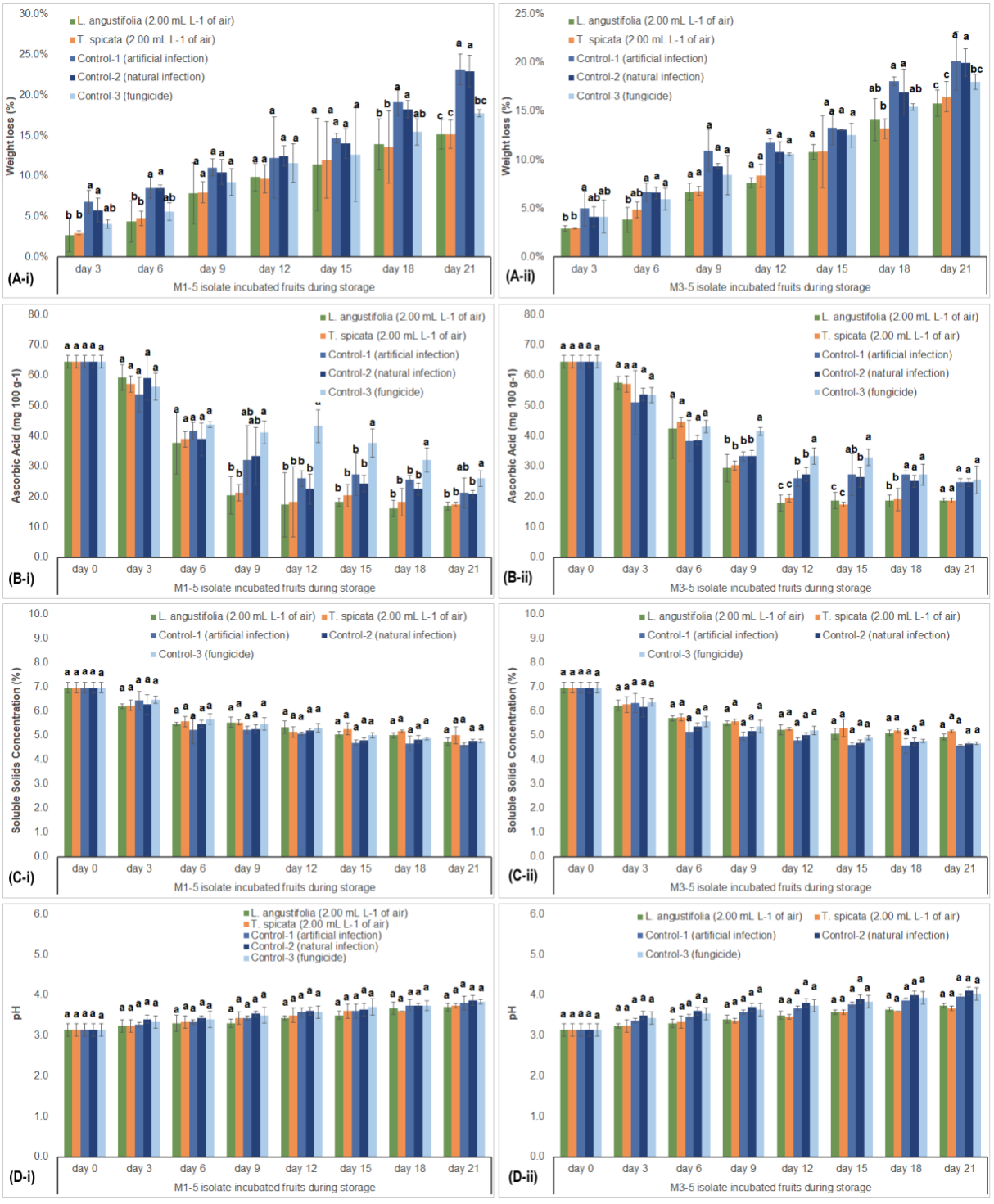

Figure 4. (A) Weight loss, (B) AA, (C) SSC and (D) pH of strawberry fruits during 21 days of storage as affected by vapor application of *L. angustifolia* and *T. spicata* essential oils after inoculation with two isolates (i) M1-5 and (ii) M3-5 of *B. cinerea*. Different letters above the columns at each measurement point indicates significant difference among the treatments according to Tukey's HSD test ($p \leq 0.05$).

It was observed that the reduction disease severity and weight loss of the fruits have a significant relationship. The treatments which were noted to provide better performance in controlling the disease severity were also found to reduce the weight loss of fruits. Similar impacts were noted on both isolates of *B. cinerea* (Figure 4A). In a similar work, it was noted that another EO, belonging to the lemongrass,

provides good performance in maintaining the weight of strawberry fruits when it is combined with chitosan edible coating (Khalifa et al., 2016). The ascorbic acid (AA) content of the fruits decreased during storage. This result is in accordance with the reports of Atress et al., (2010) and Kahramanoğlu (2009). The treatments were noted to have no significant impact on the AA content of the fruits (Figure 4B). The results for SSC was noted to be similar with the AA contents of the fruits. It was decreased during the storage and no impact was noted for the treatments (Figure 4C). Contrary to SSC and TA values, the fruit pH was noted to have an increasing trend during storage. The treatments were again noted to have no significant influence on the pH (Figure 4D). Even though the EOs had very minor impact on the fruit quality, the prevention of the weight loss is an important result for the study. The direct application of EO had been noted to provide similar positive impacts on the fruit quality which was associated with some physiological changes in fruits, such as inducing the synthesis of several enzymes (PPO, SOD, CAT and POD) (Kahramanoğlu et al., 2020; Wan et al., 2021).

Conclusion

The essential oils of *L. angustifolia* and *T. spicata* had been found to have significant influence on the prevention of mycelial growth of *B. cinerea*. The results are novel in terms of the direct vapor contact application of the oils into fruit packaging. Besides to that, it is well-known that packaging of fruits with special materials allowing modification of the inner atmosphere (reducing oxygen and increasing carbon dioxide) is highly beneficial for improving storability of the fruits. Therefore, it is thought that the combination of such materials together with direct vapor application of essential oils would have better performance. However, further studies are required to clarify their effects, especially in combination with different fruit packaging materials.

Compliance with Ethical Standards

Conflict of interest

Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

İ.K.: Conceptualization, Methodology, Data curation, Writing - original draft; **T.G.K.**: Conceptualization, Methodology, Resources, Investigation; **A.U.B.**: Methodology, Resources, Investigation; **R.G.**: Conceptualization, Methodology, Resources, Investigation, Writing - review & editing; **H.A.**: Investigation.

Ethical approval

Authors declare no requirements for any ethical approval.

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Data availability

All data were summed and presenten in the paper. The complete list of raw data is available upon request.

Consent for publication

We, as the authors of present paper, give our consent for the publication of this paper in the International Journal of Agriculture Environment and Food Sciences.

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