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Research Article

Effect of Thymol and Linalool Fumigation on Postharvest Diseases of Table Grapes

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Abstract Several postharvest diseases of table grapes (*Vitis vinifera*) occur during storage, and gray mold rot is a particularly severe disease because the causal agent, *Botrytis cinerea*, grows at temperatures as low as 0°C. Other postharvest diseases, such as those caused by *Penicillium* spp. and *Aspergillus* spp., also often lead to deterioration in the quality of table grapes after harvest. The use of plant essential oils such as thymol and linalool, to reduce postharvest diseases in several kinds of fruits, including table grapes and oranges, has received much attention in European countries. However, to the best of our knowledge there has been no report of the use of thymol fumigation to control gray mold in table grapes in Korea. Thymol (30 μg/mL) and linalool (120 μg/mL) significantly inhibited mycelial growth and conidia germination of *B. cinerea*. The occurrence rate of gray mold rot of *B. cinerea* and other unknown fungi was significantly reduced by fumigation with 30 μg/mL thymol in several table grape cultivars, such as Campbell early, Muscat Bailey A, Sheridan, and Geobong. In this study, fumigation with 30 μg/mL thymol, had no influence on the sugar content and hardness of grapes, but reduced fungal infection significantly. This suggests that 30 μg/mL thymol could be utilized to reduce deterioration of grapes due to gray mold and other fungal infections during long-term storage.

Keywords *Botrytis cineria*, Fumigation, Linalool, Postharvest diseases, Table grapes, Thymol

Table grapes (*Vitis vinifera*) are harvested from late July to the end of October in Korea. During the harvesting season, the price of table grapes often decreases because of over production. However, the market price may increase during the off-harvest season. Specifically, the demand for high-quality grapes such as Geobong ("Kyoho" in Japan) rapidly increases the price. Therefore, the Farmer's income may increase with appropriate control of postharvest disease. The storage properties of table grapes vary depending on the cultivar. Table grapes are non-climacteric fruits that are highly sensitive to the conditions during postharvest handling, storage, transport, and marketing. Postharvest diseases in table grapes result in severe economic losses to farmers. The decrease in quality is mainly shown by weight loss, color change, and accelerated softening of the fruits. Additionally, table grapes also deteriorate due to rachis browning and the high incidence of berry decay [1-4].

The presence and long survival of conidia of *Botrytis cinerea* and *Penicillium expansum* have been reported in grapes and other fruits [5]. These well-known necrotrophic fungi affect several kinds of horticultural crops. The gray mold caused by *B. cinerea* is known to be the most important disease of table grapes, and uncontrolled infections result in the growth of aerial mycelium spreading rapidly to adjacent berries resulting in a severe loss of quality [6]. In order to overcome this problem, several methods have been developed such as controlled atmosphere storage [2, 7-10], modified atmosphere storage [11], sulfur dioxide (SO_2) treatment, polyethylene film storage, and the use of O_3 during storage [3, 12, 13]. For many years, SO_2 fumigation has been recommended in combination with rapid pre-cooling, followed by storage and transportation at 0°C [14-18]. Other synthetic fungicides such as anilinopyrinidines were also found to be effective against *B. cinerea*, although fungal populations resistant to these chemicals have been reported [19]. However, storage methods are not used widely because of the need for expensive equipment and maintenance. The use of modified atmosphere packaging has been shown to maintain berry quality and to reduce decay in combination with acetic acid, chlorine gas, or a SO_2-commercial generator [20-23]. However, in recent years, table grape consumers have raised concerns about possible SO_2 residues that may elicit an allergic response.

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The attempt to characterize the bioactive properties of essential oils has recently gained much attention in many pharmaceutical and food-processing applications [24]. In foods, the use of essential oils or their individual components, as potential natural preservatives has been reported in cheese [25], bakery products [26], and meat [27], although there is little evidence of a role for them in the control of fruit decay. Essential oils containing high amounts of thymol and carvacrol were reported to possess the highest antioxidant activity [28-32]. In addition, these compounds exhibit other bioactivities, for example, thymol has antiseptic, antibacterial, antifungal, antioxidative, and food preservative properties [33], while carvacrol possesses antifungal properties [34]. Thymol is the major phenolic constituent of thyme oils [35]. Thymol, as a plant essential oil, is generally regarded as a safe compound by the U.S. Food and Drug Administration (FDA) and Environmental Protection Agency (EPA), and was exempt from the requirement of a tolerance by the EPA for application on edible agricultural products (40 CFR Part 180, 6 June 2003) [36]. The mechanism of the antifungal activity of linalool is not known. It is speculated that the enantioselectivity [37] of the compound may regulate the inhibition of β-1-3 glucan or chitin synthesis in the fungal cell wall.

In Korea, postharvest treatment is not yet in the case of table grapes. There are some storage containers used for delayed distribution during the postharvest season, where the table grapes are maintained at 0.5 ± 1°C. If table grapes are maintained without any significant loss by fungal decay with postharvest treatments, farmers could get better profit through delaying the market period by cold chamber storage during the overproduction season. The objectives of the present study were to determine the inhibitory concentration of thymol on mycelial growth and conidia germination of postharvest pathogens. This study was extended to investigate the effect of thymol on the development of postharvest disease in table grapes.

MATERIALS AND METHODS

The effect of different concentrations of thymol and linalool on inhibition of *Botrytis cinerea*. Mycelial plugs (3 mm in diameter) of *B. cinerea* KB isolate from cv. Campbell early, and GB isolate from cv. Geobong were cultured on potato dextrose agar (PDA). In these experiments, 30~480 μg/mL essential oil was dropped into filter paper disks (3 mm in diameter) (Advantec No. 50405692; Toyo Roshi Kaisha Ltd., Tokyo, Japan) on the cover plate. The plates were then incubated upside down at 24°C until the mycelium covered the entire control plate. The growth was determined by measuring the diameter of mycelium from the center of the plate. To study the effect of thymol (minimum 99.5%; Sigma-Aldrich, St. Louis, MO, USA) and linalool (minimum 97%; Sigma-Aldrich) on conidia germination, approximately 100 conidia were spread on each PDA and thymol or linalool treatment was given in the same manner.

Comparison of the effects of thymol and linalool on fungal growth inhibition. Mycelial plugs (3 mm in diameter) of *B. cinerea* KB and GB were transferred onto PDA. The plates were stored upside down after thymol (30 μg/mL), linalool (120 μg/mL) (Sigma-Aldrich) were dropped onto paper disks on the cover plate. The plates were incubated at 24°C until the mycelium completely covered the control plate, as determined by the diameter of the mycelium.

Inoculum preparation of *Botrytis cinearea*. *B. cinerea* (GB and KB) were grown under darkness for 3 days and UV (200~290 nm) for 1 day, followed by 4 days of incubation on V-8 juice agar at room temperature (20 ± 2°C). Next, conidia obtained from the plates were suspended in 0.5% tween 20 at the concentration of 1.4×10^7 conidia/mL.

Inoculation of table grape cultivars. The grape berries were cut from the branch using sterilized experimental scissors, without any wound to the surface of berries. The cut berries were inoculated with conidia suspension (10^6 conidia/mL) by immersing the base of the berry, where the stalk was attached, in conidial suspensions.

Thymol fumigation of *B. cinerea*-inoculated and naturally infested table grapes inside plastic containers. Plastic 2.1 L containers (large size ziploc; Korea Johnson Co. Ltd., Seoul, Korea) were rinsed with 70% ethanol. Campbell early, Muscat Bailey A (MBA), Sheridan, and Geobong (Kyoho in Japanese) varieties were used. Thirty berries of cv. Geobong and 40 berries of each of the remaining cultivars were used to fill the plastic containers. The edges of the plastic containers were greased before the lids were replaced to make sure each was airtight after thymol (30 μg/mL) was dropped onto filter papers (Whatman 42 cat. No. 1055; Whatman International Ltd., Kent, UK). Grapes were stored for 7 days inside a plastic container.

The thymol disks were taken out 7 days after fumigation and the plastic containers of grapes were stored for 30 days in a refrigerator set at 1 ± 1°C. The berries of Campbell early and Geobong cv. in the plastic containers were further incubated at 24°C for 7~30 days after storage to determine fungal infection.

In case of naturally infested table grapes in the experiment, only two cultivars of Campbell early and Geobong were used due to the availability of grape cultivars produced, and the table grapes were stored for 60 days after fumigation. However, all other treatments were consistent with those used to inoculate table grapes with *B. cinerea*.

Linalool fumigation of naturally infested grapes inside plastic containers. Thirty berries taken from cultivars Campbell early, Sheridan, and MBA, were placed inside the plastic containers as described previously. The grapes were treated with 120 μg/mL linalool fumigation for 7 days and stored at 1 ± 1°C for 30 days. Each treatment was replicated

four times. Linalool fumigation of grapes inoculated with *B. cinerea* (KB and GB) could not be conducted because the table grapes were out of season during the experimental period. Furthermore, 120 μg/mL linalool resulted in a strong odor during the experiment, so was not chosen for use in the further experiment investigating the storage of table grapes for 60 days.

Statistical analysis. All data were analyzed using one-way analysis of variance (ANOVA), and *t*-test and Fisher's least significant difference method were applied to determine differences among the means with significance set at $p \leq 0.05$. Data were analyzed using Sigma Stat ver. 2.0 (Jandel Engineering Ltd., Beds, UK). Data were arcsine transformed when percentages were analyzed [38].

RESULTS

The effect of different concentrations of thymol and linalool on fungal inhibition. *B. cinerea* (GB), 30 μg/mL thymol and 120 μg/mL linalool were able to completely inhibit mycelial growth. Mycelial growth was significantly reduced by fumigation with 30 μg/mL thymol and 120 μg/mL linalool, compared with 9 cm of growth (in diameter) and 71.4% of conidial germination of the control plate (*t*-test, $p = 0.05$). Ethanol had a small inhibitory effect on mycelial growth and conidia germination compared to the control (Table 1).

The effects of thymol and linalool on mycelial growth and conidia germination in *B. cinerea* (KB), showed almost the same trend as that observed in *B. cinerea* (GB) in terms of fungal inhibition (Tables 1 and 2).

Table 1. Effect of thymol and linalool on mycelial growth and conidia germination of *Botrytis cinerea* (KB) isolated from table grape, cv. Campbell early

Essential oil	Conc[a]. (μg/mL)	*B. cinerea* (KB)	
		Mycelial growth[b] (cm)	Conidia germination (%)
Linalool	Control	9.0 ± 0.00a[c]	86.7a
	EtOH	9.0 ± 0.00a	53.3b
	30	5.1 ± 0.07b	26.6c
	60	4.2 ± 0.54c	0d
	120	0d	0d
	240	0d	0d
	480	0d	0d
Thymol	Control	8.5 ± 0.55a	86.7a
	EtOH	7.1 ± 0.39b	26.7b
	30	0c	0c
	60	0c	0c
	120	0c	0c
	240	0c	0c
	480	0c	0c

[a]The concentration of ethanol as a dilution reagent was 70%.
[b]Values are presented as mean ± standard error of the mean.
[c]Means followed by same letters are not significantly different among different concentrations within columns of each treatment (least significant difference, $p = 0.05$).

Table 2. Effect of thymol and linalool on mycelial growth of two isolates of *Botrytis cinerea*

Fungus	Treatment (μg/mL)	Potato dextrose agar (mycelial growth in diameter)[a]
Isolate from Campbell early	Control	9.0 ± 0.00a[b]
	Thymol 20	0.3 ± 0.14b
	Thymol 30	0b
	Linalool 120	0b
Isolated from Geobong	Control	9.0 ± 0.00a
	Thymol 20	0b
	Thymol 30	0b
	Linalool 120	0.3 ± 0.14b

[a]Values are presented as mean ± standard error of the mean.
[b]Means followed by same letters are not significantly different among treatments within columns of each isolate (least significant difference, $p = 0.05$).

Comparison of the inhibitory effects of thymol and linalool on inhibition of fungal growth. Linalool at 120 μg/mL showed better control against mycelial growth and conidia germination, but this concentration resulted in a strong odor. Due to this, thymol at 30 μg/mL was subsequently used to further control the Gray mold rot of table grapes in further experiments. This is because it was evident that 30 μg/mL thymol and 120 μg/mL linalool provided good protection against mycelial growth and conidia germination. The effect of 30 μg/mL thymol on mycelial growth was determined to be optimal following PDA analysis. *B. cinerea* KB and GB exhibited some growth at 20 μg/mL thymol, but growth was inhibited at 30 μg/ml (Table 2).

Thymol fumigation of the *B. cinerea*-inoculated table grapes inside plastic containers. Fungal infection of *B. cinerea* from inoculated table grapes stored without thymol treatment for 30 days at 1 ± 1°C were 4.4%, 13.8%, 1.9%, and 2.5% for Campbell early, MBA, Sheridan, and Geobong, respectively. In contrast, thymol treatment at a final concentration of 30 μg/mL resulted in a significant reduction of fungal infection of *B. cinerea*, which were 1.3%, 5.6%, 0%, and 1.7% for Campbell early, MBA, Sheridan, and Geobong cvs., respectively. An unknown fungal infection that showed only white hyphae was also identified during storage. The Geobong cultivar exhibited severe infection by unknown species in the absence of thymol treatment, which was 64.2%, followed by 39.4% of MBA, and 10.5% of Campbell early. However, thymol significantly reduced fungal infection to 29.2%, 39.4%, and 10.5% for Geobong, MBA, and Campbell early, respectively. However, the unknown fungal infection in 19.2% of Sheridan grapes treated with thymol, was not reduced compared to that (19.4%) of the control. *Penicillium* infections of the

Table 3. Effect of thymol on the postharvest disease incidence from the *Botrytis cinerea*-inoculated table grapes stored for 30 days after 7 days of thymol treatment

Cultivar	Treatment[a]	Fungal infection (%)[b]			
		B. cinerea	*Penicillium* spp.	Unknown spp.[c]	Total
Campbell early	Control	4.4 ± 1.18a[c]	0a	10.5 ± 1.32a	14.9a
	Thymol	1.3 ± 0.29b	0a	0b	1.3b
MBA	Control	13.8 ± 0.65a	4.4 ± 0.48a	39.4 ± 2.90a	57.6a
	Thymol	5.6 ± 0.63b	0b	4.2 ± 0.75b	9.8b
Sheridan	Control	1.9 ± 0.48a	0.6 ± 0.25a	19.4 ± 1.18a	21.9a
	Thymol	0b	0b	19.2 ± 1.68b	19.2b
Geobong	Control	2.5 ± 0.48a	10.8 ± 1.03a	64.2 ± 1.93a	77.5a
	Thymol	1.7 ± 0.29b	0b	29.2 ± 1.75b	30.9b

[a]Experiment was conducted using plastic containers, size of 2.1 L (Large size ziploc, KOREA JOHNSON Co. Ltd., Seoul). The berries were treated with 30 μg/mL of thymol. There were 5 replications per treatment and each replication had 40 berries for Campbell early and 30 berries for Geobong, respectively.

[b]Values are presented as mean ± standard error of the mean.

[c]Most of unkown fungi grown as mycelium only on berries turned out to be *B. cinerea* (approximately nine of 10 fungi) when the fungi were grown on potato dextrose agar at 25°C.

[d]Means followed by same letters are not significantly different between treatments within cultivars of each column (*t*-test, $p = 0.05$).

control were 0% of Campbell early, 0.6% of Sheridan, 4.4% of MBA, and 10.8% of Geobong. However, no disease developed following thymol treatment (Table 3).

Thymol significantly reduced postharvest fungal infection. However, overall, it had no significant effect on sugar content and hardness except fincv. Geobong (*t*-test, $p = 0.05$). The sugar content of untreated grapes ranged from 15~18° Brix at 30 days after storage, and from 14~17° Brix for thymol treated grapes. Hardness of the untreated and thymol treated grapes ranged from 0.4~0.9 kg and from 0.7~0.8 kg, respectively, depending on the cultivar.

Thymol fumigation of naturally infested table grapes inside a plastic container. Significant reductions of postharvest disease development were observed in the naturally infested thymol-treated grapes stored for 60 days. In this condition, 0% fungal infection occurred. However, the thymol-treatment control plates were infected with *B. cinerea* at 0.6%, unknown fungi 3.7%, and *Penicillium* spp. 3.0% from cv. Campbell early, respectively, and as *B. cinerea* at 18.3%, unknown fungi 26.7% and *Penicillium* spp. 0.8% from cv. Geobong, respectively. These table grapes were harvested in early September 2006 (Table 4).

Thymol treatment did not influence sugar content and hardness (*t*-test, $p = 0.05$). Sugar content and hardness of non-inoculated thymol-treated Geobong grapes stored for 60 days was each 13.1° Brix and 0.8 kg, respectively, and 13.4° Brix and 0.8 kg for non-treated grapes, respectively. The sugar content and hardness of non-inoculated Campbell early grapes was each 13.2° Brix and 0.7 kg, respectively, in non-treated control, and 13.0° Brix and 0.7 kg, respectively, following thymol treatment.

Linalool fumigation of naturally infested table grapes inside plastic containers. The incidence of *B. cinerea*, *Penicillium* spp. and unknown spp. infections were 20.8%,

Table 4. Postharvest disease incidence of naturally infested table grapes stored for 60 days after treated with 7 days of thymol fumigation

Cultivar	Treatment[a]	Fungal infection (%)[b]			
		Botrytis cinerea	*Penicillium* spp.	Unknown spp.[c]	Total[b]
Campbell early	Control	0.6a[d]	3a	3.7a	7.3a
	Thymol	0a	0b	0b	0b
Geobong	Control	18.3a	0.8a	26.7a	45.8a
	Thymol	0b	0b	0b	0b

[a]Experiment was conducted using plastic containers, size of 2.1 L (Large size ziploc, KOREA JOHNSON Co. Ltd., Seoul). The grape was treated with 30 μg/mL of thymol. The disease incidence rate by different fungi after 2 months of storage in the refrigerator set at 2 ± 1°C. There were 5 replications per treatment and each replication had 40 berries for Campbell early and 30 berries for Geobong.

[b]When each berry is infected with different kinds of fungi, the infection rate for each fungi was determined.

[c]Most of unkown fungi grown as mycelium only on berries turned out to be *B. cinerea* (approximately nine of 10 fungi) when the fungi were grown on potato dextrose agar at 25°C.

[d]Means followed by same letters are not significantly different between treatments within cultivars (*t*-test, $p = 0.05$).

Table 5. Postharvest disease occurrence of the naturally infested table grape stored for 30 days after 7 days of linalool treatment

Cultivar	Treatment[a] (μg/mL)	Fungal infection (%)[b]			
		Botrytis cinerea	*Penicillium* spp.	Unknown spp.[c]	Total
Campbell early	Control	20.8 ± 3.0a[d]	0.8 ± 0.25a	14.2 ± 1.9a	35.8a
	Linalool	3.3 ± 1.0b	0b	0b	3.3b
MBA	Control	44.2 ± 2.7a	0a	4.2 ± 1.3	48.4a
	Linalool	0b	0a	4.2 ± 0.5	4.2b
Sheridan	Control	20.0 ± 0.8a	0a	31.7 ± 1.3a	51.7a
	Linalool	0b	0a	19.2 ± 2.8b	19.2b

[a]Experiment was conducted using plastic containers, size of 2.1 L (Large size ziploc, KOREA JOHNSON Co. Ltd., Seoul). There were 5 replications per treatment and each replication had 40 berries for Campbell early and 30 berries for Geobong.
[b]Values are presented as mean ± standard error of the mean.
[c]Most of unkown fungi grown as mycelium only on berries turned out to be *B. cinerea* (approximately nine of 10 fungi) when the fungi were grown on potato dextrose agar at 25°C.
[d]Means followed by same letters are not significantly different between treatments within cultivars of each column (*t*-test, $p = 0.05$).

0.8%, and 14.2% in Campbell early, respectively; 44.2%, 0%, 4.2% in MBA, respectively, and 20%, 0%, 31.7%, in Sheridan, respectively, in naturally infested grapes at 30 days after storage. In contrast, the disease incidence rate of the grapes fumigated with linalool at 120 μg/mL decreased remarkably. The incidence rates of *B. cinerea*, *Penicillium* spp. and unknown spp. infections were 3.3%, 0%, 0% in Campbell early, 0%, 0%, 4.2% in MBA, and 0%, 0%, 19.2% in Sheridan, respectively, at 30 days of storage after fumigation with 120 μg/mL linalool (Table 5).

The sugar content and hardness was not affected by 120 μg/mL of linalool except for MBA (*t*-test, $p = 0.05$). The sugar contents of the untreated grapes were 12.1° Brix, 17.4° Brix, and 14.6° Brix for Campbell early, MBA and Sheridan, respectively. Hardness of the untreated grapes was 0.6 kg, 0.6 kg, and 0.9 kg for linalool Campbell early, MBA and Sheridan, respectively.

DISCUSSION

It is widely accepted that conidia on the surface of grape berries, and the mycelia of *B. cinerea* developed from diseased berries, are potential inoculum for postharvest *Botrytis* decay during the storage of table grapes. Decay damage is also caused by latent infections, probably arising at bloom, as well as by surface conidia [39].

Thymol could significantly inhibit mycelial growth and conidia germination at 30 μg/mL, and this effect was similar to that observed at 120 μg/mL linalool, indicating that thymol fumigation could be a very successful treatment to reduce postharvest fungal infection of table grapes. A sulfur dioxide (SO_2) releasing pad is used commercially to control postharvest disease, in which SO_2 acts as an oxidant of the surface molecule of the infecting microorganism. Considering that SO_2 residues may remain in widely used agricultural products; thymol could be an excellent alternative to SO_2 in the control of postharvest disease in agricultural products. The concentration of linalool (120 μg/mL) used in this study resulted in residual odor much stronger than that from thymol (30 μg/mL).

The antifungal activity of thymol against *B. cinerea* was observed in the present studies, both *in vitro* and *in vivo*. The growth of the isolates from both Campbell early and Geobong, that is, *B. cinerea* KB and GB, were significantly inhibited with the essential oils of 30 μg/mL thymol and 120 μg/mL linalool. It was obvious that thymol at 30 μg/mL was more effective at inhibiting fungal growth compared to linalool, and so thymol fumigation was further studied for the control of postharvest disease in our study, over a longer storage period of 60 days. The inhibitory effect of thymol on mycelial growth and conidial germination was significant on PDA treated with 250 μg/mL thymol dissolved in dimethyl sulfoxide, resulting in 48.2% reduction in the rate of mycelial growth and 2% of conidial germination of *B. cinerea* [40]. However, our study showed that 30 μg/mL thymol could completely inhibit mycelial growth and conidial germination. This difference might be because the method to treat *B. cinerea* with thymol in previous studies was different, such as the way in which thymol was incorporated into PDA [40]. In the present study, *B. cinerea* was exposed to thymol fumigation dissolved in 70% ethanol. Furthermore, the authors also reported that thymol was more effective in fungal inhibition than was linalool [40]; a result that was similar to that observed in the present study. In addition, thymol could inhibit mycelial growth and conidial germination of other fungi such as *Rhizoctonia solani*, *Alternaria mali*, *Phytophthora capsici* [40]. It was also reported that thymol could inhibit radial growth of *Penicillium digitatum* by 100% compared to the control at 200 μg/mL [41].

In this study, thymol fumigation significantly reduced *B. cinerea* infection on naturally infested or artificially inoculated grapes with conidia following 30 or 60 days of storage at 1 ± 1°C. Total fungal infection caused by *B. cinerea* and other fungi was often higher in cv. Geobong than that in cv. Campbell early, except in one experiment, implying that Campbell early might be more resistant to *B.*

cinerea and other fungal infections. Liu *et al.* [42] reported that fumigation with thymol at 30 μg/mL reduced the incidence of *Botrytis* rot from 35% in untreated cherry fruit to 0.5%. Furthermore, 50 μg/mL thymol in an aqueous solution significantly reduced the infection rate to 15.5% in strawberry fruits from 43.5% in the untreated control, when vaporized inside the container [32]. Considering that the postharvest fungal infection of MBA and Sheridan cultivars was higher than that of Campbell early, those table grape cultivars were speculated to be more susceptible to total fungal infection like cv. Geobong.

The data presented in this study suggest that the use of thymol inside storage containers can effectively reduce fungal decay caused by *B. cinerea*, *Penicillium* spp., and other fungi in table grapes during long-term cold storage. Thymol could be commercialized for the treatment of postharvest disease of table grapes, and possibly other agricultural products, considering that thymol can be purchased at just $14.00 per 1 kg [36].

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