Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 115 (2007) 144–148
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Influence of carvacrol on survival of *Botrytis cinerea* inoculated in table grapes

Domingo Martínez-Romero [a], Fabián Guillén [a], Juan Miguel Valverde [a], Gloria Bailén [a], Pedro Zapata [a], María Serrano [b], Salvador Castillo [a], Daniel Valero [a,*]

[a] *Department of Food Technology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312, Orihuela, Alicante, Spain*
[b] *Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312, Orihuela, Alicante, Spain*

Received 23 March 2006; received in revised form 10 October 2006; accepted 12 October 2006

Abstract

In this paper, the role of carvacrol vapour atmosphere on *Botrytis cinerea* inoculated in PDA or in grape berries was studied. Four concentrations inside packages were assayed (0.05, 0.2, 0.5 and 1.0 ml l^{-1}). All concentrations inhibited totally the growth of *B. cinerea* in PDA, while in berries the reduction of decayed fruits was significantly greater as carvacrol concentration increased. In addition, the fungal growth (area and volume of infection) was also reduced and dependent on carvacrol concentration. Ethylene and respiration rate (berry physiological parameters) increased drastically in control inoculated-grapes, while these increases were lower as higher were the carvacrol applied doses. The data presented in this work suggest that carvacrol could be used as an innovative tool to control fungal decay during table grape storage, as alternative to the use of synthetic fungicides such as SO_2.
Published by Elsevier B.V.

Keywords: Grapes; *Botrytis cinerea*; Carvacrol; Natural antifungal activity; Postharvest

1. Introduction

Table grape (*Vitis vinifera* L.) is a non-climacteric fruit which shows severe problems during postharvest handling, storage and marketing. As many fruits, grapes have a relatively low pH and thus very sensible to fungal growth (Tournas and Katsoudas, 2005), which could be accelerated by storage conditions, especially high relative humidity. *Botrytis cinerea* causes grey mould even at low temperature, which is considered the most important disease of table grape (Elad et al., 2004). Several means have been used to solve this problem, the most common being the use of SO_2 as synthetic fungicide, but the required high concentration affects berry quality inducing bleaching, accelerated water loss and browning (Carvajal-Millán et al., 2001). Moreover, sulphite residue is another important problem associated with SO_2 fumigations. Thus, consumer's concern for the human well-being and environmental pollution have forced the food industry to search for new strategies as alternative means to control fruit postharvest decay.

Among these new tools, the use of natural antimicrobial compounds such as carvacrol could be a promising strategy. For centuries essential oils have been empirically recognised as antimicrobial, and scientific confirmation has been reviewed recently (Burt, 2004). Thus, carvacrol is the major component (50–86%) of spices such as oregano and thyme (*Thymus* and *Origanum* sp., Kulisic et al., 2004), which have been used from antiquity as food preservatives. Carvacrol is a phenolic compound which its antimicrobial activity has been proved recently against bacteria (Periago et al., 2004), fungi (Daferera et al., 2003) and yeasts (Arora and Kaur, 1999), showing a high potential to improve the shelf life and safety of perishable foods (Holley and Patel, 2005). However, there is little information available about the use of carvacrol in foods, and especially in intact fruits. Thus, pure carvacrol has been used in fish by dipping (Mahmoud et al., 2006) and in vegetable puree (Valero and Giner, 2006).

The main objective of this paper was to study the effect of carvacrol at the vapour phase on the survival of *B. cinerea* in

* Corresponding author. Tel.: +34 96 6749743; fax: +34 96 6749677.
E-mail address: daniel.valero@umh.es (D. Valero).

0168-1605/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2006.10.015

potato dextrose agar (PDA) medium and in artificially inoculated berries. In addition, the physiological response of the fruits (ethylene and respiration rate) during 4 days of storage at 25 °C was also discussed. The results would provide some information about the possible use of carvacrol as natural antifungal compound to avoid grape decay during postharvest storage.

2. Material and methods

2.1. Table grapes and chemical

Fruits (*V. vinifera* L. cv. Autumn Royal) were harvested at commercial ripening stage from a local farm and immediately transferred to the laboratory. Bunches were selected according to homogeneous size, shape, colour, weight and absence of injuries. Then, 375 berries were taken from bunches to perform the treatment in lots of 15 berries in quintuplicate. Another 5 lots of 15 berries were used to analyse the characteristics of the fruits at harvest: colour (L* 30.34±0.53, a* 2.58±0.19, b* −1.81±0.25), respiration rate (31.08±0.37 mg CO_2 kg^{-1} h^{-1}), ethylene production (0.24±0.02 nl g^{-1} h^{-1}), soluble solids (18.01±0.60 °Bx) and titratable acidity (0.38±0.04 g tartaric acid eq. 100 g^{-1}). Carvacrol (99.5% purity) was purchased from Sigma (Sigma-Aldrich, Madrid).

2.2. Fungus strain and growth condition

The fungus used in this study was *B. cinerea* CECT21000 (Spanish collection of type cultures) and routinely cultured on potato dextrose agar (PDA). The spores of *B. cinerea* were collected and diluted with sterile water till 7500 CFU ml^{-1} and used as stock.

2.3. Experimental design

Two experiments were performed to study the effect of carvacrol on *B. cinerea*, one in vitro with potato dextrose agar (PDA) and other with berries.

2.3.1. In vitro inoculation

Plates with PDA medium culture were inoculated with 50 µl of the above stock (375 spores) diluted previously in 950 µl of sterile water, and then 4 plates were placed in 2-l transparent packages in quintuplicate. Five different treatments were performed by adding 0 (control), 0.1, 0.4, 1 or 2 ml of carvacrol impregnated in four gauzes at four equidistant points, and then packages were hermetically sealed. These amounts gave doses of 0, 0.05, 0.2, 0.5 and 1.0 ml l^{-1} of carvacrol inside packages. Fungal growth was measured by numerating the colonies grown in each plate after 4 and 7 days at 25 °C (read through the transparent lid), and results were the mean±SE.

2.3.2. Berry inoculation

For each berry, an artificial injury (2×2 mm) was practised with sterile lancet, and then 10 µl of the above stock (75 spores) was injected at 2 mm of depth inside the fruit. Fifteen infected berries were distributed in each package on a sterile perforated cardboard to avoid berry movement, and the carvacrol was added as above and with the same concentrations in quintuplicate. After the packages were incubated at 25 °C for 4 days, they were opened and then the following analytical determinations were made: percentage of damaged berries per package, rates of respiration and ethylene production, external diameter and depth of injury. The latest two measurements were made only in berries with symptoms of infection.

2.4. Analytical determinations

2.4.1. Respiration and ethylene production rates

CO_2 and ethylene production were measured by placing the 15 berries of each package in 0.25-l glass jars hermetically sealed with a rubber stopper for 1 h. One ml of the holder atmosphere was withdrawn with a gas syringe, and the ethylene was quantified using a Hewlett-Packard™ model 5890A gas chromatograph (Wilmington, DE) equipped with a flame ionization detector and a 3 m stainless steel column with an inner diameter of 3.5 mm containing activated alumina of 80/100 mesh. The column temperature was 90 °C, and the injector and detector temperatures were 150 °C. Results were the mean± SE of 4 determinations for each package and expressed as nl g^{-1} h^{-1}. For respiration rate determination, another sample of 1 ml of the same atmosphere was withdrawn and CO_2 quantified using a Shimadzu™ 14A gas chromatograph (Kyoto, Japan), with a thermal conductivity detector and a molecular sieve 5A column, 80–100 mesh (Carbosieve SII. Supelco Inc., Bellefonte, USA), of 2 m length and 3 mm i.d. Oven and injector temperature were 50 and 110 °C, respectively. Helium was used as carrier gas at a flow rate of 50 ml min^{-1}. Results were the mean±SE of 4 determinations for each package and expressed as mg CO_2 kg^{-1} h^{-1}.

2.4.2. Damage measurements

Damage was expressed as percentage of fruits with visible fungal infection for each package, and results were the mean± SE. In addition, the incidence of infected wounds was recorded and the effectiveness index (EI) was calculated using the formulae proposed by Neri et al. (2005).

In addition, the following parameters were used to evaluate the severity of fungal damage: external diameter of the infected area and depth infection after cut the berries through the injured zone in two halves longitudinally. Then, damage was expressed as mean±SE of external area (by approximation to a circle) and internal volume (by approximation to half ellipsoid).

Finally, the minimum inhibitory concentration (MIC) of carvacrol was calculated according to Cosentino et al. (1999), as the lowest concentration resulting in a significant decrease (at least 90%) of fungal growth in inoculated berries.

2.5. Statistical analysis

Data for the physical and physiological parameters were subjected to analysis of variance (one-way ANOVA). Mean comparisons were performed to examine if differences among

treatments were significant at $P<0.05$. All analyses were performed with SPSS software package v. 11.0 for windows. Statistical modelling was performed using linear regression and non-linear regression procedures (SigmaPlot 9.0, 2004 for windows). The fit of the equation was evaluated by the determination of coefficient R^2.

3. Results

3.1. B. cinerea inoculated in vitro

B. cinerea started to grow on PDA after 48 h, and after 4 days at 25 °C control plates had 300 CFU. On the contrary, no mycelium appeared for any of the carvacrol added concentrations (0.05, 0.2, 0.5 or 1.0 ml l^{-1}). To ensure the antifungal effect of carvacrol, plates were incubated 3 days further and the same results were obtained.

3.2. B. cinerea inoculated in grapes

The addition of carvacrol inside the packages led to a reduction of decayed fruits, which was significantly greater as carvacrol concentration increased (Fig. 1). This reduction was fitted to a logarithmic equation with a regression coefficient of $R^2=0.96$ (Table 1). Thus, control packages showed a 95±3% of infected fruits, while percentages of 55±2, 37±2, 18±1 and 6±0.2% were obtained in treated packages for 0.05, 0.2, 0.5 or 1.0 ml l^{-1} carvacrol, respectively. These percentages of damaged berries permitted the calculation of the minimum inhibitory concentration (MIC), which was 1.0 ml carvacrol per litre of package. In addition, the treatment gave an effectiveness index (EI) of 42±2, 61±2, 81±3 and 93±1 for 0.05, 0.2, 0.5 or 1.0 ml l^{-1}, respectively (Fig. 1).

In control berries, the external diameter and depth of infection were 15.7±1.1 and 7.5±0.4 mm, while these parameters were significantly lower in treated berries with efficacy being greater as the carvacrol concentration increased. These results were fitted to a logarithmic equation with a regression coefficients of $R^2=0.87$ and 0.77 for diameter and depth, respectively (Table 1). Thereafter, the external area and internal volume of damage showed similar effects (Fig. 2), that is a reduction of both parameters as carvacrol concentration increased compared to controls (58.0±4.5 mm^2 and 542±57 mm^3, respectively). Similarly, these data were also fitted to logarithmic equation with coefficient regressions of $R^2=0.86$ and 0.83, respectively (Table 1).

Table 1
Equation regressions among different parameters

Concentration of carvacrol (ml l^{-1}) vs	Logarithmic equation	R^2
Percentage of damaged fruit (%)	$y=-1.06\,x+1.84$	0.96
External diameter of damage (mm)	$y=-540\,x+0.81$	0.87
Internal height of damage (mm)	$y=-535\,x+1.07$	0.77
External damaged area (mm^2)	$y=-1121\,x+1.58$	0.86
Internal damaged volume (mm^3)	$y=-1778\,x+2.36$	0.83
Ethylene production (nl g^{-1} h^{-1})	$y=-0.93\,x+0.38$	0.67
Respiration rate (mg kg^{-1} h^{-1})	$y=-731.2\,x+2.25$	0.97
Respiration (mg kg^{-1} h^{-1}) vs	**Logarithmic equation**	R^2
External damaged area (mm^2)	$y=7.61\,x+0.3$	0.99
Internal damaged volume (mm^3)	$y=0.01\,x+0.3$	0.98
Ethylene production (nl g^{-1} h^{-1}) vs	**Linear equation**	R^2
External damaged area (mm^2)	$y=9.02\,x+7.6$	0.80
Internal damaged volume (mm^3)	$y=95.11\,x-11.1$	0.95

3.3. Physiological changes in infected grapes

Ethylene production in infected berries was significantly higher in control fruits than in treated ones (Fig. 3). Thus, after 4 days at 25 °C, control grapes exhibited a ethylene production of 6.00±0.27 nl g^{-1} h^{-1} while a rate of 0.38±0.03 nl g^{-1} h^{-1} for the highest carvacrol concentration (1 ml l^{-1}). Interestingly, this ethylene production was very close to that obtained in recently harvested grapes (0.24±0.02 nl g^{-1} h^{-1}).

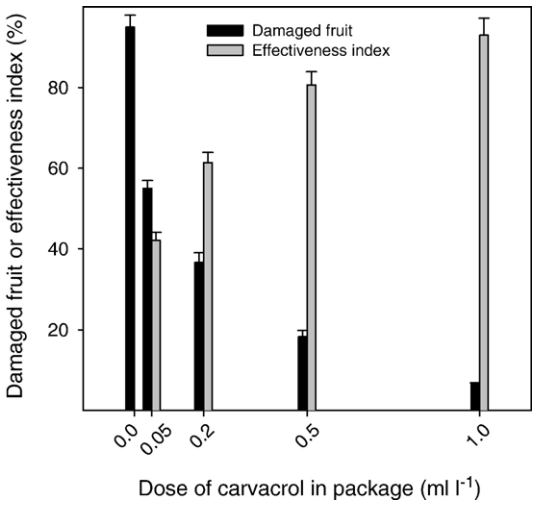

Fig. 1. Percentage of damaged fruits and effectiveness index (EI) in *B. cinerea* inoculated berries at different doses of carvacrol.

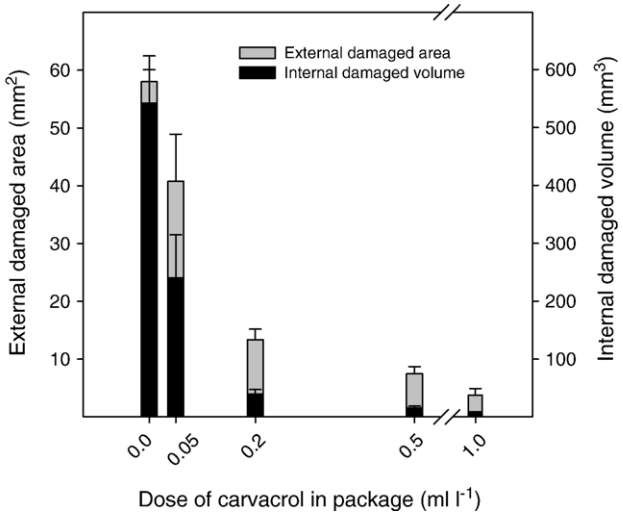

Fig. 2. Effect of carvacrol on external and internal damage in berries by *B. cinerea*.

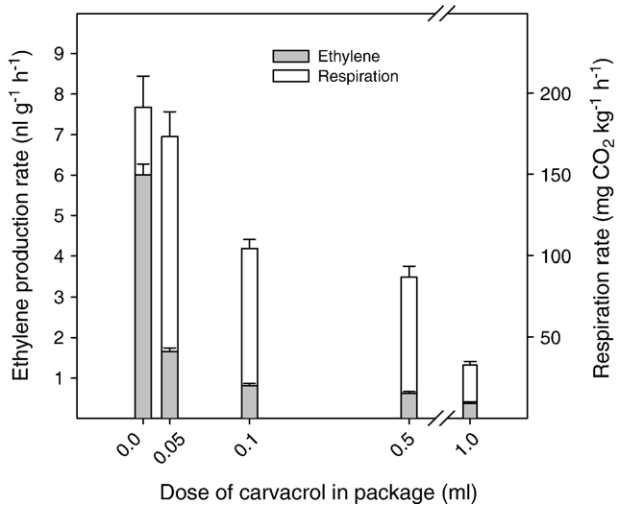

Fig. 3. Influence of carvacrol on respiration rate and ethylene production in *B. cinerea* inoculated berries.

On the other hand, the respiration rate at harvest (31.08± 0.37 mg CO_2 kg^{-1} h^{-1}) increased drastically (191.10±19.30 mg CO_2 kg^{-1} h^{-1}) in inoculated control berries after 4 days at 25 °C, the increase being significantly lower as carvacrol dose increased (Fig. 3). Moreover, for the highest carvacrol dose (1 ml l^{-1}) no significant changes in respiration rate were detected from that obtained at harvest.

The analysis of the data revealed that ethylene production was poorly correlated (R^2=0.34) to carvacrol concentration with a linear equation, this regression being increased (R^2=0.67) when data were fitted to logarithmic equation. However, the correlation between ethylene production and both external area and internal volume was linearly correlated (R^2=0.80 and 0.95, respectively). In addition, the correlation between respiration rate and carvacrol concentration, and external area and internal volume was always logarithmically correlated with higher regression coefficients (Table 1).

4. Discussion

In this work, carvacrol (2-*p*-cimenol or 5-isopropyl-2-methylphenol) was highly effective in reducing the growth of *B. cinerea* in both PDA and berry. However, efficiency was higher in PDA than in grapes, since no fungal growth in PDA was obtained for any of the assayed carvacrol concentrations, while in grape a 97% inhibition was obtained only for the highest dose of carvacrol. There is not much literature dealing on carvacrol and *Botrytis*, but evidence exists of the differential effectiveness of carvacrol on microbial growth depending on the culture medium (Schwämmle et al., 2001; Neri et al., 2005). It is interesting to point out that carvacrol inhibited totally the spore germination and mycelium development in PDA. In this sense, carvacrol has been recognised as one of the most potent monoterpenoid with antifungal activity against *Botrytis* (Tsao and Zhou, 2000). In addition, carvacrol was highly effective in reducing the spore germination and mycelium growth of *B. cinerea* inoculated in grapes, although the concentration to achieve over a 90% of inhibition was 1 ml l^{-1}, which was considered as the MIC for table grapes. This is in agreement with previous reports, in which greater concentration of essential oil was needed in food systems than in vitro to gain the same efficacy (Smid and Gorris, 1999; Burt, 2004). The mechanism of action of carvacrol against fungi is not well-known, but the general hypothesis is focused on membrane and cell wall damages with morphological deformation, collapse and deterioration of the conidia and/or hyphae (Zambonelli et al., 2004; Neri et al., 2005).

The inoculation of control berries with *B. cinerea* induced softening and watering of the infected zone as well as mycelium growth. These symptoms were reduced as carvacrol concentration increased inside the packages, demonstrated by the lower external diameter, internal height, external area and internal volume of infection. The effectiveness of carvacrol on alleviating this damage was higher at external than internal level, since carvacrol was applied as vapour and then the primary contact zone was the berry epidermis. This effect is in agreement with the higher correlation obtained between carvacrol concentration and external diameter of the damaged area than internal volume damage (Table 1).

Some physiological parameters of table grapes, such as ethylene production and respiration rate, revealed that control berries exhibited the highest rates compared to treated grapes. Since table grape is considered a non-climacteric fruit, and thus its ripening process is not associated to increases in ethylene production (Martínez-Romero et al., 2003), the observed increase might be a consequence of the fungal growth. In fact, there was a linear correlation between ethylene and damage, and thus the fungus was responsible for the majority of ethylene production, apart of the basal level typical of non-climacteric fruits. Accordingly, it has been reported that *B. cinerea* produced greater amounts of ethylene as the concentration of conidia inoculated in vitro or in the climacteric tomato fruit increased (Cristescu et al., 2002).

The respiration rate was clearly affected by carvacrol concentration and dimension of infection, which was corroborated by the high correlation found for these parameters (Table 1). Control berries exhibited the highest respiration rate compared to treated fruits, which could be attributed to the alleviation of damage severity by carvacrol. There is evidence supporting the relationship between respiration rate and fungal decay in fruits. Thus, the increase in respiration rate preceded the appearance of rots in grapes during storage (Bower, 2001). Although any numeration on fungi viable counts was performed in this work, carvacrol would reduced the viable counts of fungi as has been observed for its isomer thymol in 'Crimson' table grapes (Valverde et al., 2005). Moreover, the alleviation of damage by carvacrol would permit the reduction in respiration rate thorough maintenance of tissue integrity, since damaged tissue in apricot exhibited higher respiration rate than non-damaged fruits (Martínez-Romero et al., 2002). Finally, the well-known antioxidant activity of carvacrol (Ruberto and Baratta, 2000; Ponce et al., 2004) might probably reduce the fruit metabolism, although the intrinsic mechanism is still unclear.

In conclusion, carvacrol was an effective tool to control fungal decay in grapes by reducing the *B. cinerea* decay. The

data presented in this work suggest that the use of carvacrol could be an innovative and useful tool as alternative to the use of synthetic fungicides such as SO_2 to avoid decay in table grapes during storage.

The next step for commercial application should be to analyse the effect of carvacrol on sensorial properties of grapes. Additionally, the combination of essential oils with other means of preservation, such as modified atmosphere packaging would provide useful information for active packaging development.

Acknowledgements

This work has been co-funded by the Spanish Ministry of Science and Technology through project AGL2003-03257/ALI and the European Commission with FEDER funds.

References

Arora, D.S., Kaur, J., 1999. Antimicrobial activity of spices. International Journal of Antimicrobial Agents 12, 257–262.

Bower, J.H., 2001. The relationship between respiration rate and storage life of fresh produce. PhD Thesis, University of Western Sydney, Richmond, Australia.

Burt, S., 2004. Essential oils: their antibacterial properties and potential application in food—a review. International Journal of Food Microbiology 94, 223–253.

Carvajal-Millán, E., Carvallo, T., Orozco, J.A., Martínez, MA., Tapia, I., Guerrero, VM., Rascon-Chu, A., Llamas, J., Gardea, A.A., 2001. Polyphenol oxidase activity, color changes, and dehydration in table grape rachis during development and storage as affected by *n*-(2-chloro-4-pyridyl)-*n*-phenylurea. Journal of Agricultural and Food Chemistry 49, 946–951.

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., Palmas, F., 1999. In vitro antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Letter in Applied Microbiology 29, 130–135.

Cristescu, S.M., De Martinis, D., Hekkert, S.L., Parker, D.H., Harren, F.J.M., 2002. Ethylene production by *Botrytis cinerea in vitro* and in tomatoes. Applied Environmental Microbiology 68, 5342–5350.

Daferera, D.J., Ziogas, B.N., Polissiou, M.C., 2003. The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subs. *michiganensis*. Crop Protection 22, 39–44.

Elad, Y., Williamson, B., Tudzynsky, P., Delen, N., 2004. *Botrytis*: Biology, Pathology and Control. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Holley, R.A., Patel, D., 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Review Food Microbiology 22, 273–292.

Kulisic, T., Radonic, A., Katalinic, V., Milos, M., 2004. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chemistry 85, 633–640.

Mahmoud, B.S.M., Yamazaki, K., Miyashita, K., Kawai, Y., Shin, I.S., Suzuki, T., 2006. Preservative effect of combined treatment with electrolyzed NaCl solutions and essential oil compounds on carp fillets during convectional air-drying. International Journal of Food Microbiology 106, 331–337.

Martínez-Romero, Serrano, M., Carbonell, A., Burgos, L., Riquelme, F., Valero, D., 2002. Effect of postharvest putrescine treatment on extending shelf life and reducing mechanical damage in apricot. Journal of Food Science 67, 1706–1712.

Martínez-Romero, D., Guillén, F., Castillo, S., Valero, D., Serrano, M., 2003. Modified atmosphere packaging maintains quality of table grapes. Journal of Food Science 68, 1838–1843.

Neri, F., Mari, M., Brigati, S., 2005. Control of *Penicillum expansum* by plant volatile compounds. Plant Pathology 55, 1–6.

Periago, P.M., Delgado, B., Fernández, P.S., Palop, A., 2004. Use of carvacrol and cymene to control growth and viability of *Listeria monocytogenes* cells and predictions of survivors using frequency distribution functions. Journal of Food Protection 67, 1408–1416.

Ponce, A.G., del Valle, C.E., Roura, S.I., 2004. Natural essential oils as reducing agents of peroxidase activity in leafy vegetables. Lebensmittel Wissenschaft und Technology 37, 199–204.

Ruberto, G., Baratta, M.T., 2000. Antioxidant activity of selected essential oil components in two lipid model systems. Food Chemistry 69, 167–174.

Schwämmle, D., Winkelhausen, E., Kuzmanova, S., Steiner, W., 2001. Isolation of carvacrol assimilating microorganisms. Food Technology and Biotechnology 39, 341–345.

Smid, E.J., Gorris, L.G.M., 1999. Natural antimicrobials for food preservation. In: Rahman, M.S. (Ed.), Handbook of Food Preservation. Marcel Dekker, New York, pp. 285–308.

Tournas, V.H., Katsoudas, E., 2005. Mould and yeast flora in fresh berries, grapes and citrus fruits. International Journal of Food Microbiology 105, 11–17.

Tsao, R., Zhou, T., 2000. Antifungal activity of monoterpenoid against postharvest pathogens *Botrytis cinerea* and *Monilinia fructicola*. Journal of Essential Oil Research 12, 113–121.

Valero, M., Giner, M.J., 2006. Effects of antimicrobial components of essential oils on growth of *Bacillus cereus* INRA L2104 in and the sensory qualities of carrot broth. International Journal of Food Microbiology 106, 90–94.

Valverde, J.M., Guillén, F., Martínez-Romero, D., Castillo, S., Serrano, M., Valero, D., 2005. Improvement of table grapes quality and safety by the combination of modified atmosphere packaging (MAP) and eugenol, menthol or thymol. Journal of Agricultural and Food Chemistry 53, 7458–7464.

Zambonelli, A., D'Aulerio, A.Z., Severi, A., Bemvenuti, S., Maggi, L., Bianchi, A., 2004. Chemical composition and fungicidal activity of commercial essential oils of *Thymus vulgaris* L. Journal of Essential Oil Research 16, 69–74.