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Green leaf volatiles affect the resveratrol production stimulated by ultraviolet C irradiation in grape leaf discs

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Summary

The effect of green leaf volatiles (GLVs; cis-3-hexenol and trans-2-hexenal) on the resveratrol production stimulated and nonstimulated by ultraviolet C (UV-C) irradiation was elucidated in the leaf discs of 'Delaware' grapes. Each GLV treatment slightly reduced resveratrol levels in the leaf discs. Pretreatment with cis-3-hexenol inhibited the resveratrol accumulation stimulated by UV-C irradiation irrespective of the concentration. On the other hand, pretreatment with trans-2-hexenal at low concentration augmented the levels of resveratrol increased by UV-C irradiation. These results suggest that trans-2-hexenal had a priming effect on the resveratrol accumulation stimulated by UV-C irradiation in grape leaf discs. The highest priming effect of trans-2-hexenal was found at 2.58 µM for 24 h exposure. Exposure times, from 2 to 24 h, of 4.3 µM trans-2-hexenal did not change the priming effect.

K e y w o r d s : green leaf volatiles, grape leaf discs, resveratrol, trans-2-hexenal, UV irradiation.

Introduction

Green leaf volatiles (GLVs), including C6-aldehydes and their alcohols or esters, are found in most terrestrial plants (Hatanaka 1993). GLVs are synthesized first as the aldehyde (*cis*-3-hexenal) from C18 fatty acids by the lipoxygenase pathway and can be converted into the isomer (*trans*-2-hexenal). The corresponding alcohols (*cis*-3-hexenol) and esters are produced through subsequent catalysis of the aldehyde by alcohol dehydrogenase, acetylation or isomerization. GLV synthesis is specifically stimulated by wounding, herbivore damage and microbial attacks (Arimura *et al*. 2009, Croft *et al*. 1993). GLVs are also synthesized after exposure to ozone, high temperature and high light (Beauchamp *et al*. 2005, Loreto *et al*. 2006).

GLVs are physiologically important molecules that mediate the plant defense system. They have bactericidal and fungicidal effects against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Botrytis cinerea* (Kishimoto *et al*. 2008, Nakamura and Hatanaka 2002). GLVs induced by herbivore damage are involved in intra- and interplant signaling, in which GLVs attract predators or parasites of herbivorous insects (Arimura *et al*. 2009). The expression of defense-related genes induced by GLVs has been reported in *Arabidopsis* (Kishimoto *et al*. 2005) and *Citrus* (Gomi *et al*. 2003). Direct and indirect (GLV treatment followed by elicitor treatment) stimulative effects of GLVs on the production of jasmonic acid and sesquiterpenes, which are defense-related compounds, have been observed in corn seedlings (Engelberth *et al*. 2004). In addition, phytoalexin accumulation has been induced by *cis*-3-hexenal and/or *trans*-2-hexenal exposure in artificially wounded cotton balls (Zeringue 1992) and intact leaves of *Arabidopsis* (Kishimoto *et al*. 2006).

The grape phytoalexin is resveratrol (3,5,4′-trihydroxystilbene), and its accumulation is induced not only by pathogen infection but also by abiotic stresses such as ultraviolet (UV) irradiation, wounding, ozone exposure and lime stress (Bavaresco and Fregoni 2001, Bavaresco *et al*. 2005, Shiozaki *et al*. 2013). GLVs have been identified and quantified in grape berries of *Vitis vinifera* (Fan *et al*. 2010, Kalua and Boss 2009) and processed products such as juice and wine (Fischer *et al*. 2000, Iyer *et al*. 2010). However, we have no useful information regarding the effects of GLVs on resveratrol accumulation in grapes.

In the present study, we examined the direct and indirect effects of *cis*-3-hexenol and *trans*-2-hexenal on resveratrol accumulation in the leaf discs of 'Delaware' grapes. UV-C irradiation was used as an elicitor to elucidate their indirect effects. We discovered an indirect effect, namely a priming effect, of *trans*-2-hexenal on the resveratrol accumulation stimulated by UV-C irradiation in the leaf discs.

Material and Methods

G L V a n d U V - C t r e a t m e n t s : Mature leaves of 'Delaware' grapes, which were grown in a research field at Osaka Prefecture University, were sampled from the first to fifth nodes (counting up from the first bearing nodes) at the end of July 2010. Twelve leaf discs (Ø 20 mm) were prepared from one leaf sample with a cork borer and placed on wetted filter paper in six Petri dishes (one to two discs per dish from the one leaf) with the abaxial side upward. Sixty leaf discs (six dishes) were exposed to GLVs. *cis*-3-Hexenol (MW: 100.16, sg: 0.848 $g \cdot mL^{-1}$) and *trans*-2-hexenal (MW: 98.14, sg: 0.846 $g \cdot mL^{-1}$) were used as the GLVs (Fig. 1). GLVs were applied to three filter papers (4 cm square) that were adhered with Vaseline to the inside of the lid of a tightly closed container after being diluted with dimethyl sulfoxide (DMSO). Each GLV was applied at a volume of 0, 0.5, 1.5 and 4.5 $\mu L \cdot L^{-1}$ to the

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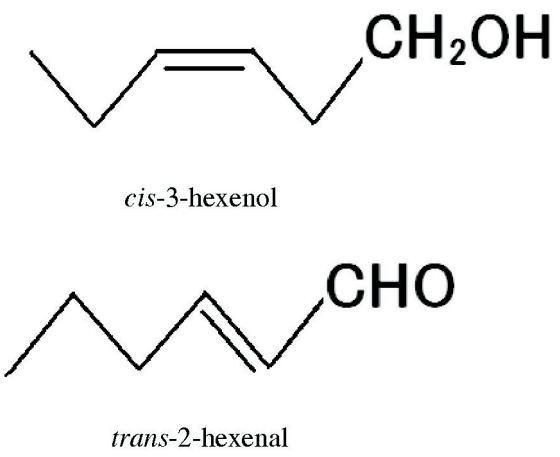

Fig. 1: Green leaf volatile compounds used in this study.

container, which corresponded to 0, 4.2, 12.7 and 38.1 µM, respectively, in the gas phase for *cis*-3-hexenol, and 0, 4.3, 12.9 and 38.8 µM, respectively, for *trans*-2-hexenal. For controls, DMSO only was applied to the filter paper. The leaf discs were exposed to GLVs in the dark at 25 °C for 24 h. The leaf discs on three of the six dishes were irradiated with UV-C (0.42 mW/cm^2) for 5 min and the rest of the samples were not irradiated. The leaf discs in the control, GLV exposure and GLV + UV-C treatments were frozen with liquid nitrogen after being kept at room temperature for 24 h.

The effect of *trans*-2-hexenal pretreatment on the resveratrol accumulation in 'Delaware' leaf discs was further confirmed by using the leaf discs prepared at the end of May 2011. A comparison of the concentration and exposure time was conducted with 'Delaware' leaf discs prepared at the beginning of June 2011. The *trans*-2-hexenal effect for 24 h exposure was compared for concentrations of 0.86, 2.58, 4.30, 6.03 and 8.62 µM. The effect of 4.3 µM *trans*-2-hexenal was compared for exposure times of 2, 6, 12 and 24 h.

trans-resveratrol analysis: *trans*-Resveratrol was extracted from the leaf discs in the present study by boiling them in distilled water. A significant linear positive correlation was observed between the concentration of resveratrol from the boiling extraction and the common method (extraction by MeOH and purification with column chromatography [SHIOZAKI *et al*. 2013]) ($P < 0.05$, $R^2 = 0.96$).

Frozen leaf samples were ground to a fine powder in liquid nitrogen with a mortar and pestle. A 0.3 g sample of leaf powder was boiled in 50 mL of distilled water for 30 min. After cooling in air, the sample was filtered and adjusted to 50 mL with distilled water. The sample was partitioned against an equal volume of hexane to remove lipids and chlorophyll, and the pH of the aqueous sample was adjusted to 8.0-8.5 with 0.1 N NaOH. *trans*-Resveratrol in the aqueous sample was extracted three times using an ethyl acetate equivalent to the water phase volume. The combined ethyl acetate extract was reduced to dryness *in vacuo*. The dry samples containing *trans*-resveratrol were redissolved in 1 mL ethyl acetate and centrifuged at 1,800 g for 5 min. Twenty microliters of the supernatant was then analyzed by HPLC equipped with a PDA detector (SPD-M20A, Shimadzu, Kyoto, Japan). *trans*-resveratrol in the sample was quantified at a wavelength of 306 nm and identified by comparing the photoabsorption spectrum from 200 to 400 nm with that of standard *trans*-resveratrol (Sigma-Aldrich Japan, Tokyo, Japan).

Statistical analysis: The data were analyzed by analysis of variance and means were compared by Fisher's PLSD test, with significance set at $P < 0.05$, with StatView 5.0 (SAS Institute Inc.).

Results and Discussion

In the experiment conducted in 2010, *trans*-resveratrol levels in the leaf discs of the control, in which neither GLV exposure nor UV-C irradiation treatments were used, was 3.1-3.3 µg·gfw (Fig. 2). UV-C irradiation increased the resveratrol levels by 4-6 times that of the control. *cis*-3-Hexenol significantly reduced the resveratrol levels 24 h after its exposure alone and significantly inhibited the accumulation of resveratrol induced by UV-C irradiation irrespec-

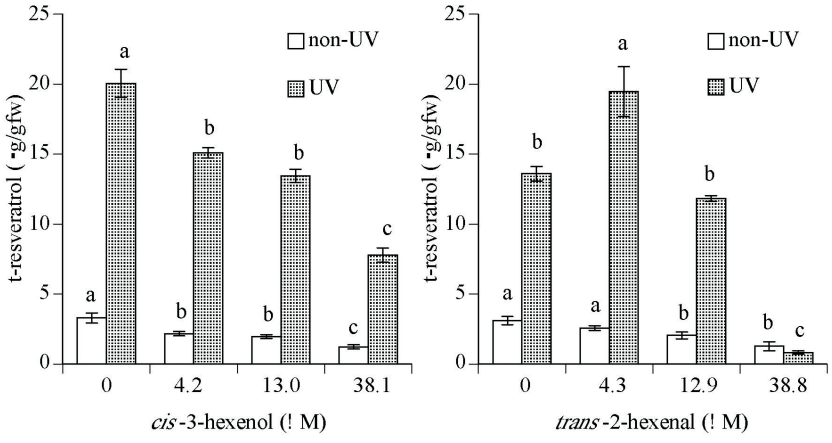

Fig. 2: Effects of *cis*-3-hexenol (left) and *trans*-2-hexenal (right) on resveratrol accumulation in leaf discs of 'Delaware' grapes (2010). Different letters indicate significant differences between GLV concentration within each UV treatment at $P < 0.05$ (Fisher's PLSD test).

tive of the concentration. *trans*-2-Hexenal exposure alone at its highest concentration (38.8 µM) significantly reduced the resveratrol levels. The resveratrol level in the leaf discs pretreated with *trans*-2-hexenal at 12.9 µM was similar to that in the UV-C irradiation treatment alone. Pretreatment with *trans*-2-hexenal at 38.8 µM completely nullified the effect of UV-C on resveratrol accumulation. Surprisingly, pretreatment with *trans*-2-hexenal at 4.3 µM made the leaf discs sensitive to the resveratrol accumulation induced by UV-C; the resveratrol level was about 1.4 times higher than that of leaf discs treated with UV-C alone. Results of the *trans*-2-hexenal pretreatment were also confirmed by the experiment conducted in 2011 (Fig. 3). The *trans*-resveratrol level in the UV-C irradiation alone treatment was 53.5 µg/gfw, whereas pretreatment of 4.3 µM *trans*-2-hexenal augmented the level by 118.3 µg/gfw. The resveratrol levels of the leaf discs after *trans*-2-hexenal treatment and UV-C irradiation were higher in the experiment of 2011, in which the leaf materials were collected at the end of May, than that in the experiment of 2010, in which the materials were collected at the end of July. Pool et al. (1981) reported that younger and older leaves of *Vitis rupestris* and *V. riparia* showed less resveratrol productivity than the mid-age leaves. The leaves used in 2011 were probably the age that is higher in resveratrol productivity than that in 2010.

A comparison of the concentration of *trans*-2-hexenal

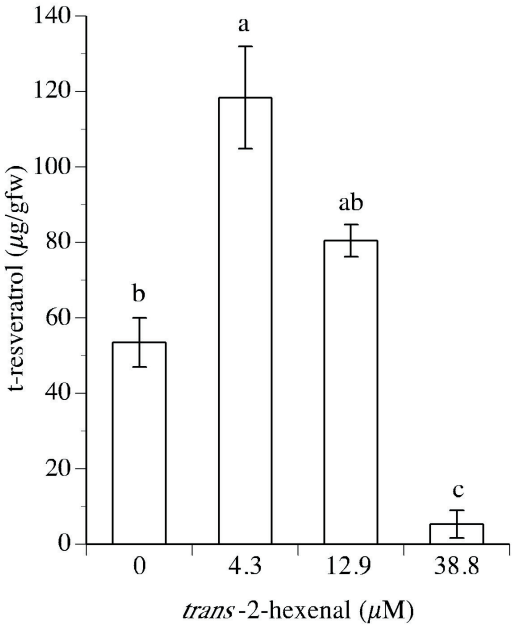

Fig. 3: Effect of pretreatment with *trans*-2-hexenal on subsequent UV-C-induced resveratrol accumulation in leaf discs of 'Delaware' grapes (2011). Different letters indicate significant differences at $P < 0.05$ (Fisher's PLSD test).

pretreated for 24 h showed that 2.58 µM of *trans*-2-hexenal was most effective (Fig. 4). There were no significant differences in the resveratrol levels between 2, 6, 12 and 24 h pretreatment time (Fig. 5). These results indicate that the effective concentration of *trans*-2-hexenal ranges from 2.58 to 4.3 µM and that the pretreatment effect of *trans*-2-hexenal upon resveratrol accumulation in our experimental conditions could be observed after exposure for 2 h.

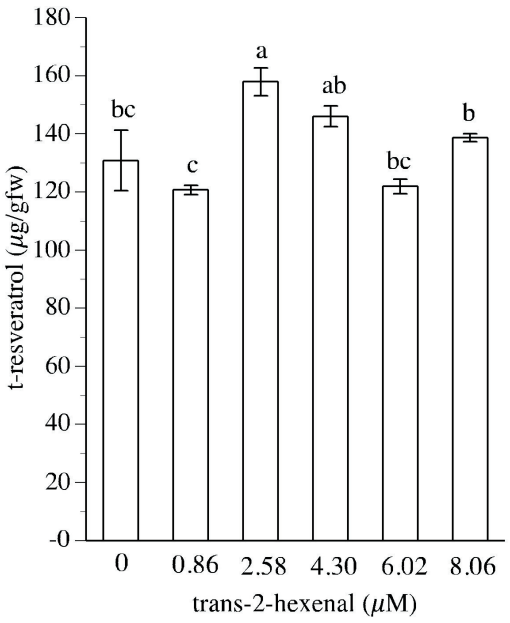

Fig. 4: Comparison of the concentration of *trans*-2-hexenal on subsequent UV-C-induced resveratrol accumulation in leaf discs of 'Delaware' grapes (2011). Different letters indicate significant differences at $P < 0.05$ (Fisher's PLSD test).

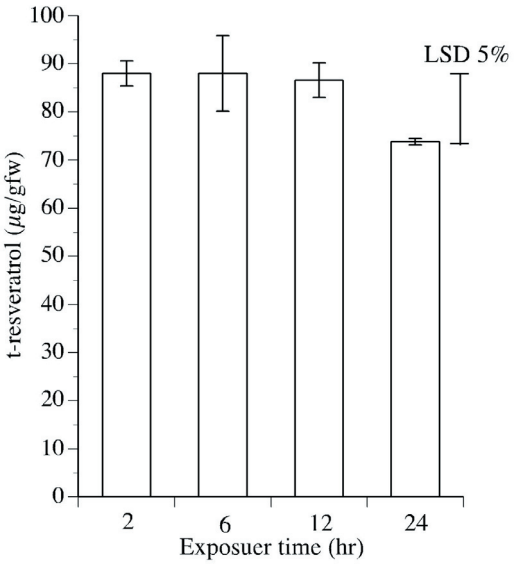

Fig. 5: Comparison of the exposure time of *trans*-2-hexenal on subsequent UV-C-induced resveratrol accumulation in leaf discs of 'Delaware' grapes (2011). Vertical bar indicates least significant difference at $P < 0.05$.

These results clearly demonstrate that *trans*-2-hexenal, but not *cis*-3-hexenol, primes UV-C-induced resveratrol accumulation in grape leaf discs at the restricted concentration of 2.58–4.3 µM, although *trans*-2-hexenal alone did not affect resveratrol accumulation. Effects of this GLV seem to be peculiar to phytoalexin production in grape leaf discs. *trans*-2-Hexenal has been shown to directly induce phytoalexin accumulation in artificially wounded cotton balls (Zeringue 1992) and in intact leaves of *Arabidopsis* (Kishimoto *et al*. 2006). Priming (elicitors: wounding and crude regurgitant) as well as elicitor (the direct effect on

the intact materials) effects of GLVs have been observed in the production of jasmonic acid and sesquiterpenes, which are defense-related compounds acting against herbivores and pathogens in corn seedlings (Engelberth *et al*. 2004). In this corn experiment, *cis*-3-hexenol was also effective on the accumulation of jasmonic acid and sesquiterpenes. According to the molecular structure, *trans*-2-hexenal has a higher chemical reactivity than *cis*-3-hexenol because *trans*-2-hexenal is a reactive electrophilic species with an α,β-unsaturated carbonyl moiety. However, the effects of *cis*-3-hexenol and *trans*-2-hexenal seem to depend on the plant species. The effect of *cis*-3-hexenol on the induction of defense gene expression was comparable with that of *trans*-2-hexenal in *Arabidopsis* (Kishimoto *et al*. 2005). On the other hand, in *Citrus*, fewer defense genes were induced by *cis*-3-hexenol compared with those induced by *trans*-2-hexenal (Gomi *et al*. 2003).

When we discuss the effect of *trans*-2-hexenal on resveratrol accumulation, it is necessary to consider the influence of wounding carefully. This is because the edges of leaf discs can be regarded as wounded leaves. Wounding can stimulate not only GLV emissions but also phytoalexin accumulation in a wide range of plants (Arimura *et al*. 2009, De Bruxelles and Roberts 2001).

The major volatile released upon mechanical wounding of *Arabidopsis* leaves was GLV ester (*cis*-3-hexenyl acetate), although the volatile first emitted was the aldehyde; emission of *cis*-3-hexenal reached a peak 30-45 s after damage and decreased at steady levels approximately 8 min later (D'Auria *et al*. 2007). In *Zea mays*, GLV ester (*cis*-3-hexenyl acetate) was reported to be ineffective in triggering defense responses (Farag *et al*. 2005). In the present study, GLVs induced by damage in the preparation of leaf discs might be ineffective analogues for resveratrol accumulation. Further, GLV emission levels with respect to concentration and duration might not be sufficient to explain the effects noted in this study.

In a previous study, grape skins were found to accumulate resveratrol after being wounded with a needle, although the resveratrol levels induced by such wounds were significantly lower than those in the skins subsequently infected by *Botrytis cinerea* (Bavaresco *et al*. 1997). Resveratrol accumulation induced by wounding was at a level of one-hundredth of that induced by UV irradiation in peanut leaves, which produce resveratrol as the phytoalexin, as do grapes (Chung *et al*. 2003). In the present study, we detected about 3 µg/gfw resveratrol in the control leaf discs 48 h after preparation (Fig. 2). However, resveratrol could not be measured in leaf discs of 'Cabernet Sauvignon' grapes just after preparation, but reached levels of 1-8 µg/gfw after infection with *Plasmopara viticola* (van Zeller de Macedo Basto Gonçalves *et al*. 2011). In our experimental condition, there was a possibility that the resveratrol accumulation was already stimulated by wounding in the preparation of the leaf discs. However, the elicitor effect on resveratrol accumulation was probably considerably lower than that caused by pathogen infection and UV-C irradiation mentioned in the above experiments with grape skins and peanut plants. Zeringue (1992) stated that wounding can induce phytoalexin production and is necessary for *trans*-2-hexenal to function as an active elicitor for phytoalexin production in cotton plants. However, *trans*-2-hexenal exposure alone lacked the stimulative effect on resveratrol accumulation in spite of the preliminary stimulation of resveratrol synthesis by wounding. This suggests that *trans*-2-hexenal has no direct effect on the resveratrol synthesis pathway in grape leaves. *trans*-2-Hexenal might prepare grape leaves, by an as yet unknown mechanism, to respond sensitively to subsequent intense elicitors.

This study provides basic information about the effects of green leaf volatiles on resveratrol accumulation in grapes by using leaf discs. Priming effect of *trans*-2-hexenal on resveratrol accumulation should be corroborated by further investigation with intact leaves that can exclude the influence of wounding. In addition, the elucidation of the effect of green leaf volatiles not only in leaves but also in other organs such as berries, in which the skins have heigh resveratrol productivity, seems necessary to fully understand the effect of green leaf volatiles on resveratrol accumulation in grapes.

References

Arimura, G.; Matsui, K.; Takabayashi, J.; 2009: Chemical and molecular ecology of herbivore-induced plant volatiles: Proximate factors and their ultimate functions. Plant Cell Physiol. **50**, 911-923.

Bavaresco, L.; Civardi, S.; Pezzutto, S.; Vezzulli, S.; Ferrari, F.; 2005: Grape production, technological parameters, and stilbenic compounds as affected by lime-induced chlorosis. Vitis **44**, 63-65.

Bavaresco, L.; Fregoni, C.; 2001: Physiological role and molecular aspects of grapevine stilbenic compounds. In: K. Roubelakis-Angelakis (Ed.): Molecular bology & biothechnology of the grapevine, 153-182. Kluwer, Dordrecht, The Netherlands.

Bavaresco, L.; Petegolli, D.; Cantù, E.; Fregoni, M.; Chiusa, G.; Trevisan, M.; 1997: Elicitation and accumulation of stilbene phytoalexins in grapevine berries infected by *Botrytis cinerea*. Vitis, **36**, 77-83.

Beauchamp, J.; Wisthaler, A.; Hansel, A.; Kleist, E.; Miebach, M.; Ninemets, U.; Schurr, U.; Wildt, J.; 2005: Ozone induced emission of biogenic VOC from tobacco: relationships between ozone uptake and emission of LOX products. Plant Cell Envi. **28**, 1334-1343.

Chung, I. M.; Park, M. R.; Chun, J. C.; Yun, S. J.; 2003: Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic stresses and hormones in peanut plants. Plant Sci. **164**, 103-109.

Croft, K. P. C.; Juttner, F.; Slusarenko, A. J.; 1993: Volatile products of lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant. Physiol. **101**, 13-24.

D'Auria, J. C.; Pichersky, E.; Schaub, A.; Hansel, A.; Gershenzon, J.; 2007: Characterization of a BAHD acyltransferase responsible for producing the green leaf volatile (Z)-3-hexen-1-yl- acetate in *Arabidopsis thaliana*. Plant J. **49**, 194-207.

De Bruxelles, G. L.; Roberts, M. R.; 2001: Signal regulating multiple responses to wounding and herbivores. Crit. Rev. Plant Sci. **20,** 487-521.

Engelberth, J.; Alborn, H. T.; Schmelz, E. A.; Tumlinson, J. H.; 2004: Airborn signals prime plants against insect herbivore attack. Proc. Natl. Acad. Sci. USA **101**, 1781-1785.

Fan, W.; Xu, Y.; Jiang, W.; Li, J.; 2010: Identification and quantification of impact aroma compounds in 4 nonfloral *Vitis vinifera* varieties grapes. J. Food. Sci. **75**, S81-S88.

Farag, M. A.; Fokar, M.; Abd, H.; Zhang, H.; Allen, R.D.; Paré, P. W.; 2005: (Z)-3-Hexenol induces defense genes and downstream metabolites in maize. Planta, **220**, 900-909.

Fischer, U.; Strasser, M.; Gutzler, K.; 2000: Impact of fermentation

technology on the phenolic and volatile composition of German red wines. Int. J. Food Sci. Tech. **35**, 81-94.

Gomi, K.; Yamasaki, Y.; Yamamoto, H.; Akimitsu, K.; 2003: Characterization of a hydroperoxidelyase gene and effect of C_6-volatiles on expression of genes of the oxylipin metabolism in Citrus. J. Plant Physiol. **160**, 1219-1231.

Hatanaka, A.; 1993: The biogeneration of green odour by green leaves. Phytochem. **34**, 1201-1218.

Iyer, M. M.; Sacks, G. L.; Padilla-Zakour, O.I.; 2010: Impact of harvesting and processing conditions on green leaf volatile development and phenolics in Concord grape juice. J. Food. Sci. **75**, C297-C304.

Kalua, C. M.; Boss, P. K.; 2009: Evolution of volatile compounds during the development of Cabernet Sauvignon grapes (*Vitis vinifera* L.) J. Agric. Food Chem. **57**, 3818-3830.

Kishimoto, K.; Matusi, K.; Ozawa, R.; Takabayashi, J.; 2005: Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. Plant Cell Physiol. **46**, 1093-1102.

Kishimoto, K.; Matusi, K.; Ozawa, R.; Takabayashi, J.; 2006: Components of C6-aldehyde-induced resistance in *Arabidopsis thaliana* against a necrotrophic fungal pathogen, *Botrytis cinerea*. Plant Sci. **170**, 715-723.

Kishimoto, K.; Matusi, K.; Ozawa, R.; Takabayashi, J.; 2008: Direct fungicidal activities of C6-aldehydes are important constituents for defense responses in *Arabidopsis* against *Botrytis cinerea*. Phytochem. **69**, 2127-2132.

Loreto, F.; Barta, C.; Brilli, F.; Nogues, I.; 2006: On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. Plant Cell Envi. **29,** 1820-1828.

Nakamura, S.; Hatanaka, A.; 2002: Green-leaf-derived C6-aroma compounds with potent antibacterial action that act on both gram-negative and gram-positive bacteria. J. Agric. Food Chem. **50,** 7639-7644.

Pool, R. M.; Creasy, L. L.; Frackelton, A. S.; 1981: Resveratrol and the viniferins, their application to screening for disease resistance in grape breeding programs. Vitis **20**, 136-145.

Shiozaki, S.; Nakamura, T.; Ogata, T.; 2013: Resveratrol productivity of wild grapes native to Japan: *Vitis ficifolia* var. *lobata* and *V. ficifolia* var. *ganebu*. Am. J. Enol. Vitic. **64**, 163-168.

Van Zeller De Macedo Basto Gonçalves, M. I.; Bavaresco, L.; Civardi, S.; Ferrari, F.; 2011: Interaction between *Plasmopara viticola* infection and stilbene synthesis in leaves and berries of ten 'Cabernet Sauvignon' clones. Vitis **50,** 119-122.

Zringue, H. J.; 1992: Effects of C_6-C_{10} alkenals and alkanals on eliciting a defence response in the developing cotton boll. Phytochem. **31**, 2305-2308.

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