Effect of Riboflavin on the Precocious Maturation of Spine Grape Berries (Vitis davidii Foex)

Mingtao Zhu1, Jun Yu1, Sheng Wu, Meijun Wang, and Guoshun Yang2
Horticulture & Landscape College, Hunan Agricultural University, Changsha, Hunan 410128, China; and Hunan Engineering and Technology Research Center for Grape, Changsha, Hunan 410128, China

Abstract. Spine grape (Vitis davidii Foex) is an important wild plant species in South China. To provide economical and environmentally safe ways to promote the precocious maturation of spine grape berries, the effects of riboflavin were investigated. Riboflavin affected the reactive oxygen species metabolism in spine grape berries by increasing superoxide radical production and the hydrogen peroxide content, and it impaired the activities of the antioxidant enzymes superoxide dismutase and catalase. Riboflavin also induced the upregulated expression of maturation-related genes in advance, and the earlier accumulation of anthocyanin and total soluble solids. Phenological observations revealed that the treated grape berries underwent a color-turning stage 9 days earlier than the control, and the maturation stage occurred 7 days earlier than the control. Thus, riboflavin may significantly promote the precocious maturation of spine grape berries.

Materials and Methods

Plant growth and treatment. Six-year-old spine grape vines were used in this study. Twelve vines were grown at the experimental base of Hunan Agricultural University (Changsha, Hunan Province, China). All spine grape vines were subjected to identical pruning and cultivation practices. At 50 d after full bloom (50 DAB), the berries were treated with 0.5 mmol·L⁻¹ riboflavin containing 0.03% (v/v) Tween-80 at noon on a sunny day. Control berries were treated with distilled water containing 0.03% (v/v) Tween-80. Three clusters (20 berries per cluster) per treatment were randomly collected at 0, 10, 20, 30, 40, 50, and 60 d (50, 60, 70, 80, 90, 100, and 110 DAB, respectively) after treatment. The flesh and peel were separated, and the peel was flash-frozen in liquid nitrogen and stored at –80 °C until further processing.

Received for publication 1 May 2019. Accepted for publication 17 June 2019.

We thank Lesley Benyon, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

This work was supported by the National Technology System for Grape Industry (CARS-29-ZP-9) and National key research and development program (2018YFD0201300).

These authors contributed equally.

Corresponding author. E-mail: guoshunyang@aliyun.com

Fig. 1. Effects of riboflavin applications on spine grape berry maturation. Grape berries development stages at 60, 80, and 100 days after full bloom.
Table 1. Effects of riboflavin on the maturation stage of spine grape berries.

| Treatment | Discoloration period (DAB) | Maturation period (DAB) |
|-----------|---------------------------|-------------------------|
| Riboflavin| 58                        | 97                      |
| Control   | 67                        | 104                     |

The discoloration period was defined as when the berries began to change from green to red; the fruit maturation period was defined as when the seeds were completely brown. DAB = days after full bloom.

$H_2O_2$ and $O_2^-$ measurements. The accumulation of $H_2O_2$ and $O_2^-$ in spine grape peels was determined in accordance with the methods of Deng et al. (2014). The $H_2$ O$_2$ content was measured at 560 nm and expressed as nmol.g$^{-1}$ fresh weight (FW), and the $O_2^-$ content was measured at 530 nm and expressed as nmol.min$^{-1}$.g$^{-1}$ FW.

Antioxidant enzyme activity measurements. Total protein was extracted in accordance with the methods of Zaka et al. (2002) with modifications. Briefly, fresh grape peel samples were powdered in liquid nitrogen with a precooled mortar and pestle. The needle powder (0.5 g) was extracted in 5 mL ice-cold extraction buffer (0.1-M Tris–HCl pH 7.5, 0.23-M sucrose, 5% polyvinylpyrrolidone, 1-mM ethylene diamine tetraacetic acid (EDTA), 10-mM KCl, 10-mM MgCl$_2$, and 2.5-mM ascorbic acid). The extract was vortexed and then placed on ice for 20 min. Homogenized samples were then centrifuged at 14,000 g for 15 min at 4 °C, and the resulting supernatants were used for enzyme assays. SOD and CAT activity levels were determined in accordance with the methods of Deng et al. (2014).

Determination of anthocyanin and total soluble solids (TSS) content. The extraction of anthocyanin from grape peel was performed in accordance with the methods of Liang et al. (2011) with modifications. Briefly, fresh grape peel samples were powdered in liquid nitrogen. Then, 0.5 g powdered samples were placed in 1.5-mL ice-cold extraction buffer (2.28:70, formic acid:water:methanol). The extract was vortexed for 10 min. Then, the extracts were centrifuged at 14,000 g for 10 min at 4 °C, and the resulting supernatants were used for total anthocyanin assays. Total anthocyanins were determined using a pH differential method described by Cheng and Breen (1991). Results were expressed as mg.g$^{-1}$ FW. The TSS contents of the flesh was determined using a digital refractometer (PR-1; Atago, Tokyo, Japan) and expressed as a percentage (%).

Expression analysis. Total RNA was extracted from spine grape peel using a TIANGEN RNA prep Pure Plant Kit (Tiangen Biotech, Beijing, China). First-strand complementary DNA (cDNA) was synthesized from 1 mg of total RNA using a PrimeScript RT reagent kit (TaKaRa Bio-technology Co. Ltd., Dalian, China). The quantitative real-time polymerase chain reaction (PCR) amplifications were carried out in triplicate in 96-well plates, having 20 mL total volumes per well, using SYBR Green PCR Master Mix (TaKaRa Bio-technology Co. Ltd., in an Applied Biosystems, Foster City, CA). The primer sequences specific for the amplification of the cDNA fragments of PAL and LOX are listed by Trouvelot et al. (2008). The expression levels were calculated as $2^{-\Delta\Delta C_t}$ and normalized to the Ct value of VvActin (Sun et al., 2010).

Results

Effects of riboflavin applications on the maturation stage of spine grape berries. In this experiment, we took photos of spine grape clusters at 60, 80, and 100 DAB (Fig. 1). At 60 DAB, the treated fruit had already entered the coloring period, whereas the control had not. At 80 DAB, in the riboflavin-treated clusters, the berries were mostly red, and a few were even purple, whereas in the control cluster, the berries were mostly light red and a small number of berries was still green. At 100 DAB, the treated cluster was completely purple, whereas the control was still red. As shown in Table 1, the coloring rate of riboflavin-treated grape berries occurred 9 d earlier than the control, and the fruit maturation date was $\approx$7 d earlier than the control.

Effects of riboflavin treatments on total anthocyanin and TSS contents. To determine whether the riboflavin treatment promoted the precocious maturation of spine grapes, total anthocyanin and TSS contents, as the main criteria for evaluating the maturity of grape fruit, were measured (Fig. 2). As is shown in Fig. 2A, the total anthocyanin contents in peels of grapes treated with riboflavin were greater than that of the control group from 60 DAB to 100 DAB, but there were no significant differences between treatments at 110 DAB. Similarly, the riboflavin treatment of fruit promoted an early increase in the TSS contents in grape fruit, from 60 DAB to 100 DAB, and the TSS contents of grape fruit were significantly greater than in the control.

Effects of riboflavin treatments on antioxidant enzyme activity levels. The activities of antioxidant enzymes, including SOD and CAT, were measured in spine grape berries (Fig. 4). In riboflavin-treated berries, the SOD activity remained relatively low until 70 DAB, and peaked at 80 DAB, which was earlier than the control but the peak was lower. The SOD activity was significantly lower after the riboflavin treatment from 60 DAB to 110 DAB compared with the control berries (Fig. 4A). Similarly, riboflavin significantly restrained the CAT activity in grape berries (Fig. 4B).

Effects of riboflavin on PAL and LOX gene expression levels. To understand the mechanisms involved in the riboflavin-induced precocious maturation of spine grapes, the expression of PAL, as the starting gene of the anthocyanin biosynthetic pathway, and that of LOX, as the main gene associated with fruit maturation and softening, were analyzed (Fig. 5). After the riboflavin treatment, the expression patterns of
PAL (Fig. 5A) and LOX (Fig. 5B) genes were similar, and there were no significant differences between the first stage before treatment and the last stage after treatment. However, from 60 DAB to 90 DAB, both PAL and LOX genes showed upregulated expression levels in both treated and control berries, but the expression levels of PAL and LOX genes in treated berries were significantly greater than in the control. At 100 DAB, PAL and LOX genes were downregulated owing to the maturation of the treated berries. However, the expression level of PAL and LOX in control peaks at 100 DAB, but the peak is later and lower than the riboflavin-treated berries.

**Discussion**

The use of precocious maturation-related cultivation technology is of great significance because it can alter the supply period of grape fruit, which will benefit fruit farmers. To promote the precocious maturation of grape fruit, various methods have been tested, such as hormones, light, temperature, and ROS (Klee and Giovannoni, 2011; Qin et al., 2012; Tian et al., 2013). Riboflavin is a photosensitizer, which can lead to the generation of ROS, such as O$_2^-$ and H$_2$O$_2$ (Deng et al., 2014). In previous research, we compared the effects of different concentrations of riboflavin on the promotion of fruit maturation and found that 0.5 mmol·L$^{-1}$ of riboflavin solution had the best effect. In this study, riboflavin was applied to spine grape berries at 50 DAB to promote the precocious maturation of the berries. The riboflavin-treated grape berries entered the color-turning stage 9 d earlier than the control, and the maturation stage occurred $\approx$7 d earlier than in the control (Table 1, Fig. 1). Our data suggest that riboflavin-mediated ROS production affects the maturation period of spine grape berries.

Total anthocyanin and TSS accumulations are well-known signs of grape berry maturation (Koshita et al., 2011). Here, earlier and quicker rates of increase in total anthocyanin (Fig. 2A) and TSS (Fig. 2B) were observed in riboflavin-treated berries compared with the control, indicating the maturation-related priming effect of riboflavin after treatment. This finding is in agreement with early reports that the accumulation of ROS accelerated fruit senescence (Jimenez et al., 2002; Qin et al., 2009; Tian et al., 2013). In addition, when the treated and control berries were all mature at 110 DAB, there were no significant differences in anthocyanin and TSS contents, which indicated that riboflavin has little effect on anthocyanin and soluble solid contents of grape berry.

Fruit maturation is an oxidative phenomenon accompanied by a pronounced increase in ROS, particularly O$_2^-$ and H$_2$O$_2$, accumulation (Tian et al., 2013; Warm and Laties, 1982). Riboflavin is involved in peroxidation, which affects the production of ROS. Taheri and Tarighi (2011) reported that the oxidative burst was induced in sugar beet after they were treated with riboflavin. Li et al. (2012) also found a similar result in experiments conducted on pear fruit. H$_2$O$_2$ and O$_2^-$, as important ROS, which are induced by riboflavin treatment obviously, so only these two ROS were detected in our study. Here, riboflavin treatments resulted in earlier peak values of O$_2^-$ (Fig. 2A) and H$_2$O$_2$ (Fig. 2B) in berries and increased their contents compared with the control. These results corroborated that O$_2^-$ and H$_2$O$_2$ are important forms of ROS and essential to the induction of fruit maturation (Tian et al., 2013). To further investigate the role of riboflavin in promoting grape berry maturation, the activities of SOD, a key enzyme that dismutates O$_2^-$ into H$_2$O$_2$, and CAT, a key enzyme that degrades H$_2$O$_2$ into water and
oxygen (Boubakri et al., 2013), were measured in riboflavin-treated grape berry peels. Riboflavin impaired SOD (Fig. 3A) and CAT activity levels (Fig. 3B). Similarly, Deng et al. (2014) found that riboflavin impaired antioxidant enzyme activities, which may be related to high content of ROS produced by the degradation of riboflavin.

**PAL** is the starting gene of anthocyanin synthetic pathway, and its expression level is closely related to anthocyanin accumulation. Enhancing the expression of **PAL** can promote the synthesis of anthocyanins (Qzeki et al., 2010; Wang et al., 2005). Taheri and Tarighi (2010) reported that riboflavin strongly elicited the expression of **PAL** and **LOX** genes in rice, and they found a similar pattern in sugar beet (2011). Here, we found that riboflavin could promote the accumulation of anthocyanin in the treated berries earlier than in the control group (Fig. 1A). Moreover, during the 60–90 DAB period, the **PAL** gene expression (Fig. 5A) and the total anthocyanin content were greater than those in the control group. **PAL** gene expression increases at the early ripening stages in fruit, but diminishes at the end of ripening (Pombo et al., 2011). At 100 DAB, the treated fruit were fully mature, total anthocyanin accumulation was completed, and **PAL** gene expression was down-regulated. However, the control group was not fully mature yet; therefore, the **PAL** gene was up-regulated (Fig. 5A) and the total anthocyanin accumulation was still occurring (Fig. 2A). Thus, the expression level of the **PAL** gene in fruit treated at this point was lower than that of the control group, but the total anthocyanin content was greater than that of the control group.

**LOX** plays an important role in plant growth, development, and maturation, as well as resistance to mechanical damage, disease, and insect infection (Heitz et al., 1997; Marcelle, 1991). In higher plants, **LOX** is associated with the synthesis of ethylene, jasmonic acid, and abscisic acid, which contribute to fruit ripening (Parry, 1991). We found similar results (Fig. 5B). Before ripening, the expression of the **LOX** gene in riboflavin-treated fruit was greater than that of the control. In line with the **PAL** gene expression pattern, the expression level of the **LOX** gene decreased in the later stages of fruit ripening (Lin et al., 2018). Consequently, we found that the expression of the **LOX** gene in treated fruit was lower than that in the control at 100 DAB.

**Conclusion**

In conclusion, our study showed the ability of riboflavin to promote the precocious maturation of spine grape. Riboflavin induces maturation responses, including **O**₂⁻ and **H**₂**O**₂ accumulation, decreased antioxidant enzyme activity, the upregulated expression of maturation-related genes in advance, and the earlier accumulation of anthocyanin and TSS. We also showed the effects of riboflavin treatments on the col-
Sun, L., M. Zhang, J. Ren, J.X. Qi, G.J. Zhang, and P. Leng. 2010. Reciprocity between abscisic acid and ethylene at the onset of berry ripening and after harvest. BMC Plant Biol. 10:257–268.

Taheri, P. and S. Tarighi. 2011. A survey on basal resistance and riboflavin-induced defense responses of sugar beet against *Rhizoctonia solani*. J. Plant Physiol. 168:1114–1122.

Taheri, S. and S. Tarighi. 2010. Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropa-noid pathway. J. Plant Physiol. 167:201–208.

Tian, S., G. Qin, and B. Li. 2013. Reactive oxygen species involved in regulating fruit senescence and fungal pathogenicity. Plant Mol. Biol. 82:593–602.

Tian, S.P., A.L. Jiang, Y. Xu, and Y.S. Wang. 2004. Responses of physiology and quality of sweet cherry fruit to different atmospheres in storage. Food Chem. 87:43–49.

Trouvelot, S., A.L. Vamier, M. Allègre, L. Mercier, F. Baillieu, C. Arnould, V. Gianinazzi-Pearson, O. Klarzynski, J.M. Joubert, A. Joubert, and X. Daire. 2008. A beta-1,3 glucan sulfate induces resistance in grapevine against plasmopara viticola through priming of defense responses, including HR-like cell death. Mol. Plant Microbe Interact. 21:232–243.

Warm, E. and G.G. Laties. 1982. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. Phytochemistry 1:7–31.

Zaka, R., C. Vandecasteele, and M. Misset. 2002. Effects of low chronic doses of ionizing radiation on antioxidant enzymes and G6PDH activities in *Stipa capillata* (Poaceae). J. Expt. Bot. 53:1979–1987.