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# Comparison of cold hardiness in the leaves of various grape cultivars based on photochemical reflectance index

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## Summary

**We compared cold hardiness with the leaves of 12 grape cultivars, including two of wild species, two of *Vitis vinifera* × *Vitis labrusca*, three INCs (interspecific crosses) and five *Vitis vinifera* L. cultivars using *PRI*-T curves and analysis of variance (ANOVA). The photochemical reflectance index (*PRI*) of most grape leaves decreased linearly when frozen progressively in darkness. The *PRI* of diploid cultivars and blanc cultivars at the standard temperature (*PRI* 4 °C) remained relatively steady during exposure to successively lower temperatures (0, -2, -4 and -6 °C) compared with the polyploid and the red cvs. representing a boundary dividing grape cultivars into tolerant and vulnerable. According to this principle, the cold hardiness of the four species was ranked *Vitis vinifera* L. > INCs = wild species > *Vitis vinifera* × *Vitis labrusca*. $R_{PRI}((PRI\text{-}PRI_{control})/PRI_{control})$ was introduced to divided the cold hardiness of 12 cultivars into three classes. Resistant: $R_{PRI} > 0$; Tolerant: $R_{PRI}$ trended to 0; Vulnerable: $R_{PRI} < 0$. We also observed seasonal differences in the cold hardiness of the cultivars; grape leaves were more vulnerable to cold in fall than in spring.**

K e y w o r d s : photochemical reflectance index; grape; leaves; cold hardiness.

A b b r e v i a t i o n : INCs: interspecific crosses; *PRI*: photochemical reflectance index; $R_{PRI}$: relative *PRI*; $P_n$: net photosynthetic rate; LUE: light use efficiency; *REL*: relative electrolyte leakage; Eu: *V. vinifera* L.; Wi: wild species, species originated from China; E×A: *Vitis vinifera* × *V. labrusca*; IC: interspecific crosses, Ref: reference line.

## Introduction

Grapes can be planted in areas with various climate conditions. The grape-growing area has expanded as a result of global warming (De Orduna 2010, Fraga *et al.* 2015, Ferrise *et al.* 2015, Tóth and Végvári 2015), allowing this crop to be grown at higher latitudes, where plants are more likely to suffer freezing temperature. Many studies have assessed the cold hardiness of grape buds and roots, leading to significant discoveries of practical value (Wample and Bary 1992, Wample *et al.* 1993, Wolf and Cook 1994, Fuller and Telli 1999, Gu 1999; Kovács *et al.* 2003, Ferguson *et al.* 2011). However, few studies have investigated the cold hardiness of grape leaves. The frequency and degree of freezing damage are lower in grape leaves than in buds and roots. Nevertheless, leaves and shoots are seriously damaged during the growing season once exposed to temperature below 0 °C for short periods. This type of injury is difficult to predict and to repair. Therefore, it is important to evaluate the cold hardiness of grape leaves.

Many methods can be used to evaluate the cold hardiness of leaves (Neuner *et al.* 2013, Maughan *et al.* 2015). Methods related to photosynthesis are of the most practical significance, as photosynthesis is the fundamental function of leaves. Nearly all types of stress conditions reduce photosynthetic ability (Taylor *et al.* 1971, Saxby *et al.* 2003, Chaves *et al.* 2009). Most strategies used in cultivation focus on improving photosynthesis or keeping photosynthetic rates steady under stress conditions. Photosynthetic parameters such as $P_n$(net photosynthetic rate) are related to fruit productivity and quality. Therefore, these parameters have been widely used in studies of stress to photosynthesis (Allen and Ort 2001, Neuner *et al.* 2013, Maughan *et al.* 2015). To obtain these parameters, a portable photosynthesis system is usually required. Although accurate, portable photosynthesis systems are not often suitable for agricultural research due to their small scale. It is a time-consuming process to collect enough accurate data for agricultural research using these systems. New technology is therefore needed.

Reflectance spectrum and remote sensing techniques provide a broad-scale method to assess light use efficiency (LUE) based on photochemical reflectance index (*PRI*). A positive relationship between *PRI* and LUE has been demonstrated (Gamon *et al.* 1992, 1997, Filella *et al.* 1996, Trotter *et al.* 2002, Guo *et al.* 2004, Nakaji *et al.* 2006, Wu *et al.* 2010). Moreover, *PRI* is easier to obtain than photosynthetic parameters. However, no studies focus on using *PRI* to evaluate the cold hardiness of grape leaves.

In this study, we analyzed the impact of freezing temperature on *PRI*, revealing the relationship between *PRI* and frost tolerance in grape leaves, using both *PRI*-T curves and $R_{PRI}$-T curves in freezing temperatures ranging from

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0 to -6 °C. We then evaluated the frost tolerance of 12 grape cultivars. Our analysis of the frost tolerance of grape leaves of different cultivars provides guidelines for cultivar distribution and developing frost-proof cultivation strategies.

## Material and Methods

The study was conducted at the experimental station of Horticultural Science and Engineering College, Shandong Agricultural University, China, in spring and fall, 2014. Twelve cultivars, including two wild species, two *Vitis vinifera* × *V. labrusca* hybrids, three INCs and five *V. vinifera* L. cultivars were subjected to cold hardiness analysis (Tab. 1). Plants were spaced 2 m × 3.5 m (vine × row) apart, watered and fertilized regularly and balance pruned. Pesticide and drip irrigation were provided when necessary.

Sampling: For seasonal cold hardiness tests, leaves of 'Merlot' (*V. vinifera* L.), 'Summer black' (*V. vinifera* × *V. labrusca*), 'Frontenac' (INCs) and 'Shan Bu Tao' (wild species originating in China) were sampled from April 12 to May 2 in spring and on October 4 in autumn. For the other tests, leaves of 12 cultivars were sampled on October 4 in autumn. For spring sampling, the third completely expanded leaves were collected randomly from all vines of all cultivars, with more than 70 leaves sampled per cultivar. For fall sampling, healthy, similarly sized leaves located at the middle position from both sides of the trellis were collected, with more than 30 leaves per cultivar sampled. After collection, the leaves were taken to the laboratory for reflectance spectrum measurement as soon as possible or preserved for a short time at 4 °C.

Leaf disk preparation: Leaves were washed with distilled water to clean their surfaces and dried with filter paper. Then, 70 disks approximately 8 mm in diameter were cut from the leaves with a paper punch. To maintain humidity levels and to balance moisture contents, all leaf disks were soaked in distilled water for 5 min. After the leaf disks were removed from the water and dried their surface with filter paper, they were placed into clean test tubes with stoppers (10 ml in volume, 1.2 cm in diameter, 1.58 mm thick, stored at room temperature before use) using one disk per tube.

Cold treatment: 70 leaf disks were treated in 70 tubes (one piece a tube) submerged in a precooled ethanol bath (75 %, 4 °C) in dark for 10 min. The disks were then cooled step-down at a rate of approximately 0.4 °C per min to the target temperature (2, 0, -2, -4, -6 or -8 °C) and held at that temperature for 10 min. For a particular cultivar, treatment at 4 °C was set as the control treatment.

Measurement of reflectance spectrum: After incubated at the target temperature for 10 min, 10 disks were randomly removed from the ethanol bath. The reflectance spectrum of every leaf disk was measured as soon as possible (within 1 min) using a spectrometer (UNSPEC-SC; PP Systems, USA). Each trial was replicated three times.

Measurement of relative electrolyte leakage (*REL*): Freeze lead to an increase in *REL* (WAN JIKU *et al.* 2015, VERSULES *et al.* 2006). *REL* was measured as soon as reflectance spectrum measurement finished. Disks were placed in 25 mL distilled water at 25 °C for 3 h after which electrical conductance was measured ($E_1$). The samples were then killed completely at 100 °C for 0.5 h and incubated at 25 °C for 24 h and a second electrical conductance was measured ($E_2$).

Statistical analysis: *PRI* was calculated as:

$$PRI = \frac{R_{531} - R_{570}}{R_{531} + R_{570}} \qquad E_1$$

$R_{531}$ and $R_{570}$ indicate reflectance at 531 nm and 570 nm. $R_{PRI}$ was calculated as:

$$R_{PRI} = \frac{PRI - C_{PRI}}{C_{PRI}} \qquad E_2$$

$C_{PRI}$ indicates *PRI* at 4°C. *REL* was calculated as:

$$REL = \frac{E_1}{E_2} \qquad E_3$$

After $R_{PRI}$, *REL* were calculated as $E_3$, then *PRI*-T, $R_{PRI}$-T and *REL*-T curves were drawn out for temperature ranges

Table 1

Cultivars used in this study. They were divided into different groups according to their species, berry skin color and ploidy

| Species | Cultivar name | Berry skin color | Ploidy |
|---|---|---|---|
| *Vitis vinifera* L. | Cabernet Sauvignon | Noir | 2N |
| | Merlot | Noir | 2N |
| | Muscat Hamburg | Rose | 2N |
| | Pinot Chardonnay | Blanc | 2N |
| | Riesling | Blanc | 2N |
| *V. vinifera* × *V. labrusca* | Summer Black | Noir | 3N |
| | Kyoho | Noir | 4N |
| INC (Interspecific crossing) | Frontenac | Noir | 2N |
| | Moldova | Noir | 2N |
| | Vidal | Blanc | 2N |
| Wild species | Shan Bu Tao (*Vitis amurensis* Ruprecht) | Noir | 2N |
| | Tangwei Pu Tao (*Vitis davidii* (Romanet du Caillaud) Foex) | Rouge | 2N |

of 4 °C to -8 °C and 0 °C to -6 °C. Correlation analysis was conducted between *PRI* and *REL* at the temperature range 0 to -6 °C. A group of $R_{PRI}$ at 0, -2, -4, -6 °C was collected from each trial and subjected to one way and two way analyses of variance (ANOVA), to compare their levels of cold hardiness with ten replications of each sample. Means separation was performed by Tukey test at a 0.05 significance level. All statistical analyses were carried out using Origin 8.6 or SPSS 21.0.

## Results

Impact of freezing temperatures on *PRI*: We initially constructed *PRI*-T curves to study the impact of freezing temperatures on leaves using *PRI* data from grape leaves of all cultivars at each target temperature. With the decrease of temperature, the *PRI* of grape leaves decreased in a linear manner from 0 to -8 °C, with a linear equation y = -0.00848+0.00107 x (y, *PRI*; x, temperature; $R^2$ = 0.97402) (Fig. 1). Individual *PRI*-T curve could be divided into three parts: 4 to 0 °C, 0 to -6 °C and below -8 °C. Nearly all of the leaf disks of all cultivars were frozen at -8 °C and wilted when exposed to room temperature shortly thereafter, turning brown within a few hours (data not shown). Therefore, -8 °C may be a threshold for keeping leaves alive; all cultivars behaved in a similar manner at temperature below -8 °C. Cold injury usually happened at a temperature below 0 °C, so it is of little significance to study the temperature range 4 to 0 °C.Therefore, the crucial temperature range in which to estimate cold hardiness was determined from 0 to -6 °C, which was used for subsequent analysis.

Impact of freezing temperatures on *REL*: We then constructed a *REL*-T curve to study the impact of freezing temperatures on leaves using *REL* data from grape leaves of all cultivars at each target temperature.

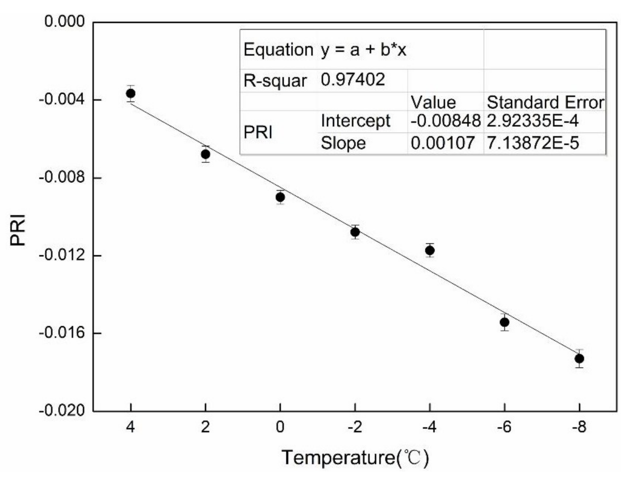


Fig. 1: Impact of freezing temperatures on *PRI* in grape leaf disks. *PRI* data of all 12 cultivars at each target temperature were used to calculate the mean values and the standard error (SE), and then used to construct *PRI*-T curves. Liner fitting was conducted at the temperature range 0-8 °C. The table in the figure was the statistical information of liner fitting, the regression equation, $R^2$ (R-square) were given.

With the decrease of temperature, the *REL* of grape leaves changed little at 4 to 0 °C , however increased in a linear manner at the crucial temperature range (0 to -6 °C), with a linear equation y = 27.06933-0.52438 x (y, *REL*; x, temperature; $R^2$ = -0.99694) (Fig. 2). Correlation between *PRI* and *REL* at the crucial temperature range (0 to -6 °C) was analyzed at the 0.01 level. Results showed that, *PRI* was significantly negatively correlated to *REL* (r = -0.993$^{**}$),

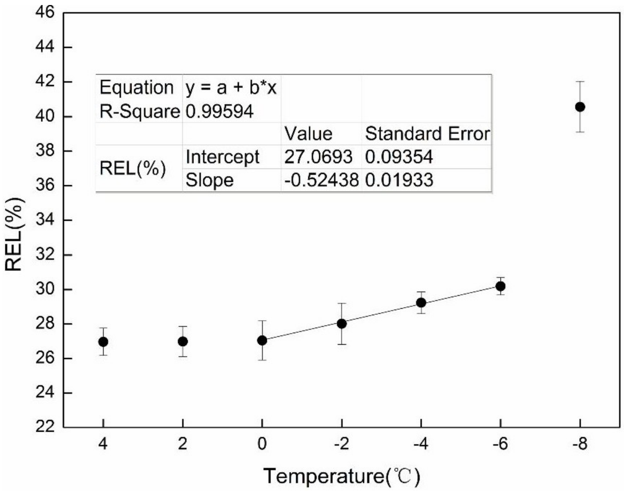


Fig. 2: Impact of the decreasing freezing temperature on *REL* in grape leaf disks. *REL* data of all 12 cultivars at each target temperature were used to caculate the mean values and the standard error (SE), and then used to construct *REL*-T curves. Liner fitting was conducted at the temperature range 0-6 °C. The table in the figure was the statistical information of liner fitting, the regression equation, $R^2$ (R-square) were given.

which proved that it is practicable to use *PRI* as a parameter in testing the impact of low temperature on grape leaves.

Identifying cold tolerant cultivars: The cultivars were classified into two groups based on their ploidy, with great differences in genetic background, using *PRI*-T curves to identify the features of cold tolerant and vulnerable cultivars. To compare the degree of decrease in *PRI* for all cultivars at the same level, data were then transformed to $R_{PRI}$ using the equation $R_{PRI} = (PRI-C_{PRI})/C_{PRI}$ ($C_{PRI}$, *PRI* at 4 °C). When we compared the $R_{PRI}$-T curves of diploid and polyploid cultivars, dramatic differences were observed (Fig. 3). The *PRI* of polyploid cultivars decreased significantly ($R_{PRI} < 0$) at each target temperature compared with the control (α = 0.05). However, the *PRI* of diploid cultivars remained steady, at normal and controlled levels ($R_{PRI} = 0$, α = 0.05). Similar results were obtained when the cultivars were classified based on berry skin color. The *PRI* of red cultivars decreased significantly with the decrease of temperature, whereas there was little change in *PRI* for the white cultivars. These results suggest that the ability for cultivars to maintain steady *PRI* values at control levels (*PRI* at 4 °C) when leaves are exposed to freezing temperature from 0 to -6 °C indicates that they are cold tolerant, as opposed to cold vulnerable.

Comparing the cold hardiness of various cultivars and species: The cold hardiness of the 12 cultivars examined in this study was divided into three

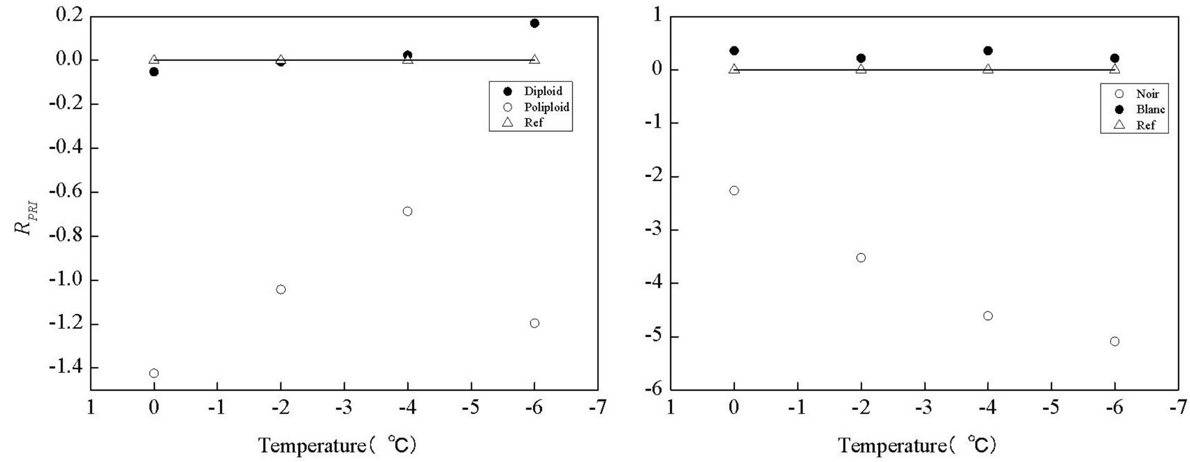


Fig. 3: Different $R_{PRI}$-T curves produced using 12 cultivars divided into two groups with great differences in genetic background based on their ploidy (left) and berry skin color (right). α = 0.05. Ref: Reference ($PRI_{control}$ - $PRI_{control}$) / $PRI_{control}$ = 0.

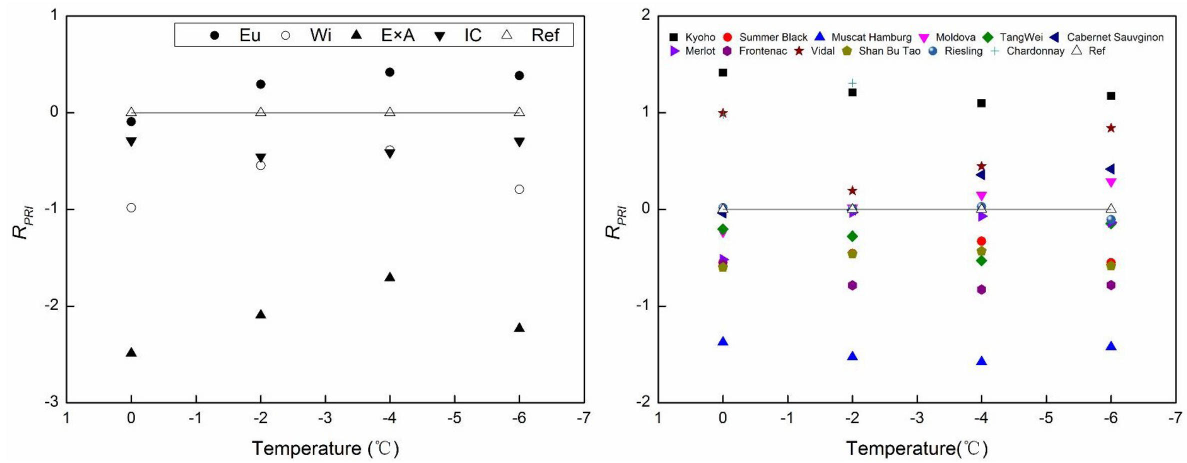


Fig. 4: The cold hardiness of 4 species and 12 cultivars were examined by $R_{PRI}$ under freezing treatment. Eu: *V. vinifera* L.; Wi: wild species; E×A: *Vitis vinifera* × *V. labrusca*; IC: interspecific crosses.

Table 2

Cold hardiness classification of 12 grape cultivars according to $R_{PRI}$ from high to low

| Classification | Cultivars | Species | $R_{PRI}$ |
|---|---|---|---|
| Resistant | Pinot Chardonnay | *Vitis vinifera* L. | 1.570 ± 0.291 |
| | Kyoho | *V. vinifera* × *V.labrusca* | 1.224 ± 0.068 |
| Tolerant | Vidal | INC | 0.618 ± 0.183 |
| | Cabernet Sauvignon | *Vitis vinifera* L. | 0.184 ± 0.119 |
| | Moldova | INC | 0.056 ± 0.111 |
| | Riesling | *Vitis vinifera* L. | -0.013 ± 0.031 |
| | Merlot | *Vitis vinifera* L. | -0.188 ± 0.112 |
| | Tang Wei Pu Tao | Wild specie | -0.288 ± 0.084 |
| | Summer Black | *V. vinifera* × *V. labrusca* | -0.469 ± 0.053 |
| | Shan Bu Tao | *Vitis.amurensis* Ruprecht | -0.517 ± 0.043 |
| Vulnerable | Frontenac | INC | -0.740 ± 0.059 |
| | Muscat Hamburg | *Vitis vinifera* L. | -1.472 ± 0.047 |

classes based on their $R_{PRI}$ (Fig. 4, Tab. 2) under freezing treatment, as follows. 1) Resistant: The *PRI* of cultivars in this class were markedly higher than that of the control, $R_{PRI} > 0$ ($p < 0.05$), indicating that they are highly cold tolerant and widely adapted to cold temperature, including 'Pinot' 'Chardonnay' > 'Kyoho'. 2) Tolerant: The *PRI* of cultivars in this class changed little compared with the control, and there were no significant differences ($p < 0.05$) between experimental groups and the control, as analyzed by ANOVA. Therefore, $R_{PRI}$ trended to 0, indicating that these cultivars retain a relatively stable status when exposed to a short period of freezing temperatures, including 'Vidal' = 'Cabernet Sauvignon' = 'Moldova' = 'Riesling' = 'Merlot' = 'Tang Wei Pu Tao' > 'Summer Black' = 'Shan Bu Tao'. 3) ulnerable: $R_{PRI} < 0$ ($p < 0.05$). The *PRI* in these cultivars decreased dramatically as the temperature decreased, reflecting inhibited or damaged photosynthesis induced by freezing temperatures; this class is the most vulnerable to cold, including 'Frontenac' = 'Muscat Hamburg'. Overall, of the four grape species examined in the study, the species with the high to low cold hardiness were *V. vinifera* L. > INCs = wild species > *V. vinifera* × *V. labrusca* ($\alpha = 0.05$).

Seasonal variation in cold hardiness: We also evaluated the cold hardiness of the leaves of different cultivars in different seasons. Specifically, we analyzed the $R_{PRI}$-T curves from four cultivars, including 'Shan Bu Tao' (*V. amurensis* Ruprecht), 'Summer black' (*V. vinifera* × *V. labrusca*), 'Frontenac' (INC) and 'Merlot' (*V. vinifera* L.) in spring and fall. We conducted a paired samples test of $R_{PRI}$ in spring and fall at a 0.05 significance level. The results indicate that the cold hardiness of grape leaves varied greatly in spring and fall; the leaves were more vulnerable to cold in fall than in spring (Fig. 5).

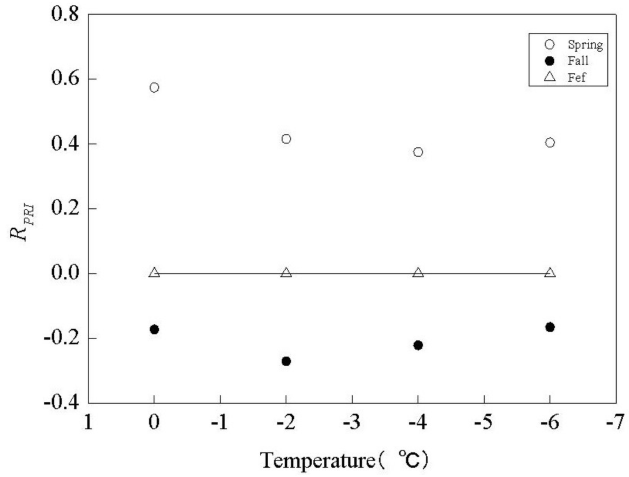


Fig. 5: Cold hardiness of the leaves in different seasons by $R_{PRI}$ under freezing treatment. $R_{PRI}$ data from leaves of 'Merlot', 'Summer black', 'Frontenac' and 'Shan Bu Tao' in both spring and fall at each target temperature were used to caculate the mean values and the standard error (SE),and then were used to constructed the $R_{PRI}$ -T curves.

## Discussion

It has been known for decades that *PRI* is positively related to LUE (Filella *et al.* 1996, Trotter *et al.* 2002, Guo and Trotter 2004, Nakaji *et al.* 2006, Wu *et al.* 2010). However, this parameter should not be used directly to estimate cold hardiness in grape germplasm resources, as little work has been done to examine the reaction of *PRI* to low temperature. In the present study, we found that *PRI* tended to decrease in a liner manner as a response to freezing temperature decrease (Fig. 1). Moreover, *PRI* was negatively correlated to *REL,* which proved that it is practicable to use *PRI* in testing injury of freezing temperature on grape leaves. However, the relationship between *PRI* and cold hardiness is still unclear. During the process of temperature decreasing, at least two parameters (speed of decrease, degree of decrease) can be used as indicators to estimate the cold hardiness of leaves; we do not currently know which parameter is more useful. The characters of plants are fundamentally controlled by their genetic background. Generally speaking, great differences in genetic background usually lead to great differences in characters. To identify the relationship between cold hardiness, decrease in *PRI* in freezing temperatures and genetic background, we divided 12 grape cultivars into two classes according to their ploidy. Berry skin color was also taken as a standard of classification, as blanc cultivars usually behave better in cool climates than red cultures. Our results suggest that the ability of cultivars to maintain steady *PRI* at control levels (*PRI* at 4 °C) when leaves are exposed to freezing temperature from 0 to -6 °C represents a boundary dividing cultivars into the cold tolerant and the vulnerable. This rule was demonstrated by ploidy and berry skin color tests (Fig. 3). Based on the principle of $R_{PRI}$, the cold hardiness of 12 cultivars was divided into three classes (Tab. 2). Although the mechanism underlying the decrease in *PRI* during freezing is currently unclear, we developed a convenient method to estimate the frost tolerance of grape cultivars. This method will be quite useful for determining the distribution boundary of a cultivar or species via remote sensing.

Generally speaking, freezing usually occurs twice a year in temperate zones in growing seasons: first in spring and the other in fall. To fully elucidate the frost tolerance of grape leaves, we conducted tests during both seasons. We found that leaves were of higher cold tolerance in spring than in fall (Fig. 5). This phenomenon is of vital importance. Buds and vines mature in autumn, during which nutrients (*i.e.*, photosynthates) are required. If leaves frozen early in autumn, fewer nutrients will be provided to buds and vines from leaves, leading to incomplete maturity of these tissues. More vines may therefore become frozen and die in winter, possibly leading to a failure in production in the following year. Our findings suggest that more attention should be paid to prevent leaves from early frost in fall to help buds and vines mature.

## Conclusion

In this study, leaf *PRI* was used to assess the cold hardiness of various grape cultivars. We propose using $R_{PRI}$ as an indicator to estimate the cold hardiness of grape cultivars: $R_{PRI} > 0$ ($p < 0.05$) indicates high cold hardiness and wide temperature adaption of cultivars; $R_{PRI} = 0$ ($p < 0.05$) indicates that the cultivars can maintain a relatively stable status when exposed to short periods of freezing temperatures; $R_{PRI} < 0$ ($p < 0.05$) indicates cultivars that are most vulnerable to cold. The cold hardiness of cultivars varied from species and seasons. The cold hardiness of the four species examined in this study was ranked as *V. vinifera* L. > INC = wild species > *V. vinifera* × *V. labrusca.* Leaves were more cold tolerant in spring than in fall.

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