ABSTRACT

In high-latitude regions, the cold hardiness of buds and canes of grapevine is important for budburst time and yield in the next season. The freezing resistance of buds and canes sampled from six wine grapes currently cultivated in Hokkaido, Japan, all of them grown from autumn to winter, was investigated. A significant difference between the cultivars in their freezing resistance was detected in the buds harvested in winter. In addition, outstanding differences in the low temperature exotherms (LTE) related to the supercooling ability of tissue cells happened in the winter buds, and there is a close relationship between freezing resistance and LTE detected in the winter buds. This suggests that the supercooling ability of tissue cells in winter buds is strongly related to the freezing resistance. However, detailed electron microscopy exposed that the differences in freezing resistance among cultivars appeared in freezing behavior of leaf primordium rather than apical meristem. This indicated that as the water mobility from the bud apical meristem to the spaces around the cane phloem progressed, the slightly dehydrated cells improved the supercooling ability and increased the freezing resistance.

Keywords: Apical meristem; Freezing resistance; Ice formative temperature; Leaf primordia; Seasonal change; Supercooling; Water translocation; Wine grapes

Abbreviation list: DTA: differential thermal analysis; Cryo-SEM: cryo-scanning electron microscopy; HTE: high temperature exotherm; LTE: low temperature exotherm

1. Introduction

Perennial woody plants that can overwinter in extremely cold regions must have high freezing resistance or have a way to avoid lethal intracellular freezing. Sub-zero temperatures can trigger ice formation in intercellular spaces, and then extracellular freezing occurs in plant tissue. When the extracellular freezing behavior accompanied with the osmotic elevation caused by gradual dehydration causes freezing point depression [27], and extra-organ freezing induces the supercooling status in primordium cells [2, 25], intracellular freezing can be avoided. The xylem ray parenchyma cells of typical boreal woody plants respond to sub-zero temperatures with deep supercooling that is accompanied with incomplete desiccation [16]. It is said that the supercooling ability itself is one of the most important traits for woody plants wintering at subzero temperatures in winter, especially for wine grape dormant buds [19].

Hokkaido, in the north of Japan, was not considered to be suited for viticulture (*Vitis vinifera* L.) because of the severe winters with low temperatures and heavy snow. Most cultivars of *V. vinifera* prefer a warm and dry climate, however, some wine grape cultivars bred in Hokkaido have high freezing resistance and can overwinter below zero degrees [22]. Additionally, the region has begun to attract notice as a domestic center of viticulture because of the current tendency towards global warming [10]. However, when the ambient temperature goes down suddenly, wine grapes whose freezing resistance is low can suffer freezing injury even now. The details of the mechanisms for acquiring high freezing resistance have not yet been clarified.

Since the 1970s, in the most severe winter regions, especially in Tokachi, the center of Hokkaido, the unique cultivars, ‘Kiyomi’ which is a bud mutation of ‘Seibel 13053’, ‘Kiyomai’ and ‘Yamasachi’ which were bred from a cross of ‘Kiyomi’ and *Vitis amurensis* Ruprecht were produced as extremely high freeze resistant grapes [15]. The rare cultivars ‘MHAM’ and ‘Rondo’ which are produced in Hokkaido are also known as high freeze resistant grapes with wild grape progenitors [9, 26]. The ‘pure *V. vinifera*’, ‘Muscat Ottonel’ and ‘Zweigeltrebe’ produced in France and Austria, respectively, have relatively high freezing resistance as well [22]. In this study, we assessed the freezing resistance and ice formative temperature of buds and canes and their freezing behavior in slow freezing processes like in nature, using the unique wine grape cultivars produced in Hokkaido.

So far, previous studies speculated with differential thermal analysis (DTA) [19] that the tissue and cell in grape winter dormant buds were equally supercooled and the cane tissue and cell adapted to extra-cellular freezing. However, the supercooling capability changed depending on the cooling rates [24], the magnitude of freezing resistance [23], and permeability barrier formation [12] or partial dehydration under subfreezing temperatures [14]. Moreover, the changes in water content and varietal osmolytes at the buds and canes may affect the freezing tolerance in the process of cold acclimation [3, 13]. Thus, it is not clear what the detailed freezing behavior of wine grape buds and canes tissues is.

First, we researched the freezing resistance and ice formative temperature of buds and canes of six cultivars sampled in winter and autumn using ion leakage analysis and DTA, respectively. Then we chose two cultivars which were different in their freezing resistance, then compared their micro-structural changes before and after slow freezing with a Cryo-SEM technique.

2. Materials and Methods

2.1. Experimental materials

We used fresh vines obtained from 6 cultivars of *Vitis vinifera* or the hybrids from *V. vinifera* and *V. amurensis* (Table 1). Four of them, a white wine cultivar 'Muscat Ottonel (MO)', and red wine cultivars 'Zweigeltrebe (ZR)', 'MHAM (MH)' and 'Rondo (RO)' were sampled from 13 to 15-year-old trees at Tsurunuma Winery (lat. 43°27'45" N, long. 141°48'20" E), Hokkaido Wine co., ltd. The other two, hybrid cultivars 'Kiyomi (KI)' and 'Yamasachi (YA)', were sampled from 10-year-old trees at the orchard of the Field Science Center for the Northern Biosphere at Hokkaido University (lat. 43°04'15" N, long. 141°20'21" E). They were obtained in autumn (3 Oct., 2018 at the winery, 23 Oct., 2018 at the university) and winter (28 Dec., 2017 at the winery, 31 Jan., 2018 at the university). The current vines, including 10 more pieces of buds and internodes (canes) wrapped with vinyl bags, were preserved at 0°C to keep their freshness and freezing resistance status until measurements and all assessments were done. At 4°C, small blocks (3*3*3 mm) were removed from the vines (Figure 1-A) at the node including from buds constructed from the main, second and third buds (Figure 1-B) and internodes and used for experiments. The aggregates of buds were used for the evaluation of freezing

resistance, differential thermal analysis (DTA) and Cryo-SEM observation without distinction of the positions.

2.2. Ion leakage analysis for evaluation of freezing resistance

For the evaluation of freezing resistance of the cells of each tissue block, the extent of freezing injury was assessed in terms of electro-conductivity, as described previously [6]. Samples were cooled with a programmable freezer (Mini-Subzero MC-710, Espec co. ltd., Osaka, Japan). First, 4 (winter samples) or 3 (autumn samples) pieces of buds or cane segments were placed in a 10-mL test tube. They were equilibrated at -3°C for 1 hr. Samples were then cooled at 5°C・hr^{-1} down to the desired temperature from -5°C to -60°C. The samples were taken out of the freezer after holding for 30 min at each temperature. After being cooled to the desired temperatures, all samples were slow-thawed at 4°C for 30 min. Then 4 mL of distilled water was added to the 10-mL test tube and the tubes were shook gently for 4 hr at 100 rpm. The electro-conductivity of all solutions was then measured (1^{st}C) with a conductivity meter (B-173, Horiba, Kyoto). After that, all tubes were boiled for 10 min at 100°C and cooled down, shook again for 1 hr, and then the electro-conductivity (2^{nd}C) was measured a second time.

Other samples were cooled to desired temperatures with control samples that were not cooled (A: 100% survived) and samples which were rapid-cooled in liquid nitrogen and thawed several times (B: 0% survived) as a completely damaged sample. The electro-conductivity of both of these samples was also measured just after soaking the tissues in distilled water (1^{st}A and 1^{st}B) and after boiling (2^{nd}A and 2^{nd}B).

The survival rate was calculated from each of the electro-conductivity values above using the following formula.

$$\textit{The survival rate (\%)} = [1 - (1^{st}C / 2^{nd}C - 1^{st}A / 2^{nd}A) / (1^{st}B / 2^{nd}B - 1^{st}A / 2^{nd}A)] * 100$$

Based on these survival rates, a survival curve was drawn, and from this curve the LT_{50} (lethal temperature of 50% survival) value, which means the temperature where 50 % of the tissue cells survived, was calculated as the freezing resistance. In other words, a lower LT_{50} indicates a higher freezing resistance of the tissue.

2.3. Differential thermal analysis for estimation of ice-formative temperature

Differential thermal analysis (DTA) was conducted according to a method of previous studies [8]. Samples of fresh buds and canes were cut into lengths of about 5 mm and utilized as fresh samples for characterization of low-temperature behavior by DTA. Oven-dried samples, heated at 70 °C for 2 days, were used as a reference. The junction of 36-gauge copper-constantan thermocouples placed in contact with the surface of buds and canes wrapped in parafilm, were placed in a 0.5-mL test tube. A pair of one fresh and one oven-dried sample was placed in each small glass bottle individually and in the programmable freezer described previously. After equilibration at 3°C for 1 hr, the bottles were cooled at $5°C \cdot hr^{-1}$ to desired temperatures between -5°C and -60°C, and the changes were monitored at each temperature. The temperature of freezing events was determined from the difference between the output from the fresh and oven-dried samples with a hybrid recorder (GM10-1JO/MT, GM90PS, Yokogawa Co., Ltd., Tokyo). The profiles shown or described in the text were typical of at least four separate analyses in each case. In many cases of canes, significant heat emissions were detected until about -10°C and were defined as 'HTE (higher temperature exotherms)', which means the freezing of apoplastic water (Figure 1-C). In the case of the buds, 'HTE' could not be detected, instead, small heat emissions were detected below -20°C and were defined as 'LTE (lower thermal exotherms)', which means the breakdown of supercooling and the subsequent occurrence of intracellular freezing (Figure 1-C).

2.4. Cryo-SEM observation for cell behaviors before and after freezing

For observations by electron microscopy, 3-mm trimmed segments of bud or cane were placed tightly with starch paste in a 5-mm metal holder (Figure 1-A). All samples in holders were also cooled slowly as described above, and finally cryofixed from the desired temperature to the temperature of Freon 22 (approx. -160°C), cooled with liquid nitrogen and preserved in liquid nitrogen until observation. Other than the slow-cooled samples, samples not treated with slow cooling were prepared as control. Cryofixed samples were processed for observation in a Cryo-SEM (JSM 6701F, JEOL Co., Ltd., Tokyo) by the method described previously [7]. In brief, a cryofixed sample was transferred to a cold stage, kept at -110°C in the specimen-preparation chamber (Alto2500, Gatan Inc., USA) of the Cryo-SEM, allowed to equilibrate for 10 min, and then fractured. The fracture plane was etched for 5 min at -95°C, replicated by evaporation of gold-palladium for 30 sec. The samples were transferred to the cold stage of a SEM column,

held at -160°C, and the secondary emission image was observed at an accelerating voltage of 5 kV. After observation and photo-image recording by Cryo-SEM, the graphical data of at least three samples per treatment (season, parts of tissue, freezing temperature) were analyzed visually.

2.5. Statistical analysis

Each experiment described above had 3-4 replications and the statistical test for differences among cultivars was done in accordance to the Tukey-Kramer's multiple comparative tests. Only DTA was conducted with 4-18 replications for detecting the exothermal responses from buds and canes.

3. Results

3.1. Freezing resistance of buds and canes

Six cultivars freezing resistances, LT_{50} values that were determined based on the survival curve using the ion leakage method, were evaluated using buds and canes sampled in autumn and winter (Figure 2). The LT_{50} value in winter samples was significantly lower than that in autumn samples in any cultivar and in any parts (buds: $p<0.001$***, canes: $p<0.001$***, student-t test, n=6). In both buds and canes, a significant difference of LT_{50} among cultivars was detected in winter samples but not in autumn samples. In the buds harvested in winter, the LT_{50} of KI, YA, MH and RO cultivars were higher than that of ZR. In winter canes, the LT_{50} of KI and YA cultivars were high, and the LT_{50} of MH and RO were medium, and that of ZR was low. The LT_{50} of MH and ZR cultivars each later compared by electron microscopy were -44.7±1.1 °C vs -27.1±1.8 °C in winter buds, and -39.0±0.7 °C vs -30.9±0.8 °C in winter canes, respectively (MH vs ZR, average value of LT_{50} ± standard error, n = 3).

3.2. Ice formative temperature of buds and canes

Six cultivars ice formative temperature that can be detected by thermocouple as latent heat release by water solidification and be determined using DTA, were evaluated using their buds and canes sampled in winter and autumn (Figure 3). Obviously, the lower temperature exotherms (LTE) of buds tended to be lower than the higher temperature exotherms (HTE) of canes. Significant differences among cultivars were detected in both

LTE of buds and HTE of canes in autumn and winter. Among these differences, the most significant difference was detected in winter buds (Comparisons by mean squares (averaged standard error): winter bud: 281.09 (1.226), winter cane: 29.21 (0.495), autumn bud: 21.36 (0.159), autumn cane: 11.53 (0.262)), the LTE of MH and KI were lowest, and LTE of MO and ZR were highest. The lower LTE means that supercooled intracellular water was frozen at a lower temperature which could be a reason of the high freezing resistance due to high deep supercooling capability. In the case of the autumn bud's LTE, those of MH, RO and MO were lower, and that of KI was higher than the others. In the HTE of winter canes, KI was the lowest and RO was the highest, however in the HTE of autumn canes, MO was lower, and KI, YA and MH were higher than the others.

3.3. Relationship between freezing resistance and Ice formative temperature

Next, the relationships between LT_{50} determined by ion leakage analysis and HTE or LTE temperature observed by DTA were analyzed. The LTE of autumn buds and the HTE of both season canes did not show clear positive relationships, however the LTE of winter buds had a significant positive correlation to LT_{50} at 5% level (Figure 4). From the results described above, some phenomena were suggested as following. (I) There are close relationships between the freezing resistance (LT_{50}) of winter buds and the ice formative temperature (LTE). (II) The relationships did not coincide with the autumn buds and all season's canes. (III) The freezing resistance of ZR was lower than that of KI, YA, RO and MH. In the next step, the freezing behavior of ZR, which is a pure *V. vinifera* spp. and MH which is a hybrid of *V. vinifera* spp. and *V. amurensis* spp. were compared.

3.4. Freezing behavior of buds and canes

The freezing behavior before and after the slow freezing of buds of a ZR cultivar, which was evaluated as having low freezing resistance, was observed with Cryo-SEM (Figure 5A-D). In the apical meristem tissue of winter buds before freezing, cells which were 10 μm in diameter were tightly packed together without any intercellular spaces (Figure 5A). On the surface of the cells in each fraction, the organelles were observed clearly by the cryofixing process to approx. -160°C of Freon 22. At -10°C, at which the freezing damage was not severe in buds of ZR (Figure 2), there was no intracellular freezing in the buds and the structures could be observed clearly even in the process of slow freezing

(Figure 5B). However, after slow freezing to -20°C, slightly shrunken cells were observed throughout the meristematic tissues (Figure 5C). On the other hand, in the slow-frozen leaf primordia at -20°C, the mixtures of severely shrunken cells (arrowheads) and intracellular frozen cells (arrows) with very large extracellular ice crystals (asterisk) were observed (Figure 5D). Hole-like structures on the fracture plane caused by sublimation with deep etching suggests the presence of ice crystals. In these severely shrunken cells, the structures were not clearly observed in the cells even at high magnification.

The freezing behavior before and after the slow freezing of buds of a MH cultivar, which was evaluated as having high freezing resistance, was also observed (Figure 5E-H). In the apical meristem tissue of winter buds, cells of the same size of a ZR cultivar were arranged closely before freezing (Figure 5E). At -20°C at which the freezing damage was little, some organelles could be recognized even after slow freezing (Figure 5F). However, after slow freezing to -40°C, shrunken cells were observed throughout the meristematic tissues (Figure 5G). At the same temperature of slow-frozen leaf primordia, the mixtures of severely shrunken cells (arrowheads) and cells with intracellular freezing (arrows) with huge extracellular ice crystals (asterisk) were observed (Figure 5H).

The freezing behavior before and after the slow freezing of canes of a MH cultivar were also observed (Figure 6). The behaviors of ZR were the same of MH cultivars (data not shown). In the tissue of winter canes before the slow freezing, cells of each specific tissue were arranged in an orderly fashion including the vascular bundles, phloem and xylem (Figure 6A). At -15°C at which the freezing damage was not severe in winter canes, intercellular ice formation could be observed near phloem tubes and cortex tissues (asterisks) (Figure 6B). Intracellularly formed ice with smooth surface showed exceeding the original cell size (surrounded area with dotted lines, Figure 6C). The same structures could be observed in the winter canes of ZR cultivars in the process of slow freezing to -15°C (Figure 6D). In contrast to winter canes, in the slow-frozen autumn canes at -15°C, ice crystals were not observed around the phloem tubes and cortex (Figure 6E). In these samples at -15°C, cells with intracellular freezing were observed in the cortex (Figure 6F).

4. Discussion

4.1. The gaps for freezing resistance, LT_{50} and LTE

253 Many previous studies suggested that the key factor for the freezing resistance of
254 grapevine wintering buds was explained by the magnitude of supercooling ability in three
255 (primary/secondary/tertiary) buds [17]. Especially the freezing tolerance of primary buds
256 has a great influence for the grapevine activity after wintering [1]. This study also showed
257 that the freezing resistance of winter buds has a close relationship to LTE, the breaking
258 point of supercooling (Figure 4). However, the supercooling breaking temperatures did
259 not coincide with LT_{50}, meaning the regular differences in temperature within the
260 relationship, LTE minus 20 degrees, was nearly equal to LT_{50}, in spite of the different
261 genetic backgrounds. That means that LT_{50} was smaller than LTE in winter, nevertheless
262 they were almost the same in autumn.
263 Two possibilities can be considered for that. Firstly, the results may indicate that there
264 are seasonal differences in the responsiveness against intracellular ice formation and the
265 death of the buds, the instantial response in autumn and the tardier response in winter.
266 The grape wintering buds were constructed with apical meristems, primordia, layer of
267 bracts, hairs, and lignified scales (Figure 1-B). In the Cryo-SEM observation, the
268 tendency of supercooling of primordium seemed to be different between autumn and
269 winter (Figure 5-D and 5-H). Lethal intracellular freezing was also observed in
270 primordium cells but not in apical meristems of winter buds. In any case, the LTE can be
271 linked to the lethal damage of a grapevine buds so that the preceding studies showed a
272 causal association between the grape wintering bud's freezing tolerance and LTE [1, 19].
273 These showed that supercooling ability of the primordium cells of grape buds may change
274 drastically with cold acclimation.
275 The differences in methods for evaluation of the survivability of buds can be looked at as
276 a second reason for the gaps between them. Previous research used visual checks of
277 tissue's browning for the evaluation of survivability [1, 19], and we adopted ion leakage
278 analysis for checking freezing resistance that can be measurable for cell survivability
279 directly on that time. There may exist a time gap for checking the viability status of
280 samples between this and preceding studies. These results may indicate that the tissue
281 cells of some grape winter buds but not all died with supercooling breaking, and then the
282 dead tissue areas may expand gradually. However, in this study the buds suffered lethal
283 damage when it generated LTE as the preceding studies showed.
284 The differences in freezing resistance between winter and autumn buds were significant,
285 and the differences in cultivars could be shown in winter buds (Figure 2). These results

286 may represent that the freezing resistance increases as it gets close to winter, and the
287 native capability for increasing their freezing resistance may be different among cultivars.
288 In spite of the difference of LT_{50} or LTE among cultivars, the freezing behavior of apical
289 meristem cells at subzero-temperatures lower than each LTE, where we see *faint*
290 *shrinkage*, seemed to be basically the same between ZR and MH cultivars (Figure 5C and
291 5G). Moreover, the severely shrunken cells and the intracellular freezing cells, which are
292 of potentially dead cells, were observed in primordia of both cultivars (Figure 5D and
293 5H). The volume of the primordia including the bud scales are comparatively larger than
294 the meristem tissues (Figure 1B), therefore, the LT_{50} resulted from the ion leakage
295 analysis, possibly LTE also could be affected by the primordia. Generally, in the case of
296 grapevines, one or more LTE peaks were detected from the bud primordia freezes [4],
297 with a few exceptions that LTE is not detected with the highest freezing resistance of *V.*
298 *riparia* [23]. The meristems of the primary bud are more susceptible to freezing injury
299 than the other buds [17], so the tolerance must influence that of the whole bud [1].
300 However, the primordium may be more susceptible than the meristems as bud tissue at
301 the cellular level at the same dormant status are weak against severe deformation caused
302 by extracellular freezing and intracellular freezing by supercooling breaking occurs easily
303 there. The aspect of intracellular freezing observed by Cryo-SEM represents the lethal
304 status [16]. The deformation by extracellular freezing also must be a cause of the cell
305 death if the deformation of cell walls during slow freezing was severe to cause close
306 apposition of plasma membranes which might induce the malfunction of membranes and
307 organelles [11]. The differences in the susceptibility between meristems and primordium
308 might be one of the causes of inconsistence between LTE and LT_{50} of buds.
309
310 *4.2. Water translocation, one of the mechanisms of grapevine wintering*
311 The freezing resistance of the buds was higher in winter than that in autumn (Figure 2).
312 And in the case of the winter buds, the supercooled apical meristematic cells after slow
313 freezing showed evidence of dehydration in all samples by Cryo-SEM observation
314 (Figure 5). Moreover, the putative ice crystal like structure near phloem and cortex in the
315 surface of the cane's fraction could be observed in winter but not in autumn (Figure 6).
316 These results may indicate that due to the flowing water drain from the apical meristems
317 to canes in winter, dehydrated meristematic cells may change to a more ideal state for
318 supercooling. It is known as common in the seasonal changes of northern wintering

319 woody plants [5]. Looking at the cold resistant woody species ‘larch’, most intercellular
320 and extracellular water from bud cells translocate to ice crystals in extracellular spaces
321 just under crowns and scales, and keeps the status of ‘extra-organ freezing’ [2]. In contrast,
322 no water movement from the bud to the stem or scales occurred during freezing in the
323 woody species ‘green alder’ [20]. In this study, there are no intercellular spaces for
324 extracellular ice growth to reserve the water molecules in the adjacent buds under the bud
325 crowns in grapevines (Figure 1B). Instead, the space for the water is reserved near the
326 cortex and phloem in the canes in winter (Figure 6). Water molecules may be stored in
327 the ice at adjacent buds where it freezes and thaws in winter and spring temporarily, and
328 may be available for budburst in the next season. The possibility that canes may act as an
329 ‘ice sink’ has been reported. In that experiment, buds that were treated with an oil-coating
330 technique inhibiting the partial dehydration, showed lower supercooling capability under
331 subfreezing temperatures [14]. Moreover, the ice propagation to buds from canes is
332 prevented with the barriers made of pectin just under the bud of *Vitis* plants [12]. However,
333 a previous study reported that marked dehydration of the whole cane took place during
334 the cold acclimation process [13] so further detailed study of water translocation between
335 buds and canes is necessary.
336 The absence of water near phloem and cortex observed specifically in autumn samples of
337 the cane (Figure 6E) was not caused by ice sublimation from the etching experiment.
338 Because cells with shallow etching could be observed in the intracellular freezing cortex
339 cells in autumn cane fraction (Figure 6F). This result suggested that the water exists inside
340 cells in the cane.
341
342 *4.3. The effects of genetic background of V. amurensis*
343 The Cryo-SEM observations were focused on the cultivars, ZR and MH because of
344 limited time. The difference in freezing behavior was detected in the supercooling ability
345 of leaf primordia. In Japan, a grapevine cultivar, ‘MHAM’ is cultivated only in Hokkaido
346 in northern Japan. ‘MHAM (Muscat Hamburg Amurensis) ’ has genetic backgrounds of
347 Muscat cultivars, *V. amurensis*, *V. coignetiae* and unidentified wild grapes [9], and the
348 phenotypes may affect the deep supercooling ability of leaf primordia. An experimental
349 cultivar ‘Rondo’ whose seed parent is ‘Zarya Severa’ including a line of *V. amurensis*
350 [26] was not observed with microscopy in this study, however, the freezing resistance of
351 winter buds was significantly higher than that of ZR. It is not clear what the effects of the

genetic background of *V. amurensis* is on the freezing resistance and freezing behavior of buds and canes in this study. Recently, a proteomic study revealed that some specific metabolic changes which increased phenylpropanoid biosynthesis of *V. amurensis* in the process of cold acclimation can contribute to its excellent cold hardiness [18]. To clarify the mechanisms of the freezing resistance of Hokkaido grapevine cultivars, the tissue and cell freezing behavior of other cultivars including Kiyomi, Kiyomai and Yamasachi described in the introduction, have to be thoroughly investigated.

5. Conclusion

This study revealed that significant differences in the freezing resistance of lateral buds after cold acclimation were detected among several grapevine cultivars cultivated in northern Japan. The differences in the freezing resistance of cultivars may be affected by the susceptibility against slow freezing of the primordial tissues rather than that of the apical meristematic tissues. The meristematic tissue cells seemed to be 'moderately dehydrated' during slow freezing, however, convincing evidence for the destination of the water translocation could not be identified except for the spaces nearby phloem and cortex in the cane. The space acting like an '*ice sink*' [21] observed in this study may be the first case of the extra-organ freezing of grapevine buds and canes. The water content of buds, especially in early autumn, has a stronger correlation with the freezing tolerance of grapevines [3]. Further studies on the changes in osmoregulants and the translocation of water during cold acclimation and deacclimation are necessary to clarify the wintering mechanism of northern grapevines.

Acknowledgements

This research was supported by grants from the Robust research program 'Exploitation of forcing culture system for nursery stock of wine grapes' of agriculture, forestry and fisheries, Hokkaido University. We would like to extend our appreciation to Farm manager Mr. K. Saitoh and N. Imamura of Tsurunuma Winery, Hokkaido Wine co., ltd. for providing helpful suggestions and preparation of the many parts of experimental

samples. We would like to thank Dr. M. Yasui and Dr. T. Ito of the electron microscope laboratory and research faculty of agriculture at Hokkaido University for assisting with observation using Cryo-SEM, and Mr. H. Nakano of the Field Science Center for the Northern Biosphere at Hokkaido University for assisting with maintenance of the experimental field.

Declaration of competing interest

All authors declare there are no conflicts of interest that would prejudice the impartiality of this scientific work.

References

[1] I. Dami, L. Shouxin, Z. Yi, Evaluation of Primary Bud Freezing Tolerance of Twenty-three Winegrape Cultivars New to the Eastern United States, Am. J. Enol. Viticult. 67 (2016) 139-145. https://doi.org/10.5344/ajev.2015.15047.

[2] K. Endoh, C. Kuwabara, K. Arakawa, S. Fujikawa, Consideration of the reasons why dormant buds of trees have evolved extraorgan freezing as an adaptation for winter survival, Environ. Exp. Bot 106 (2014) 52-59. https://doi.org/10.1016/j.envexpbot.2014.02.008.

[3] A. Ershadi, R. Karimi, K. Naderi Mahdei. Freezing tolerance and its relationship with soluble carbohydrates, proline and water content in 12 grapevine cultivars. Acta Physiol. Plant. 38 (2016) 2.
doi: 10.1007/s11738-015-2021-6.

[4] Y.A. Fennel, Freezing tolerance and injury in grapevines, J. Crop Improv. 10 (1-2) (2004) 201-235. https://doi.org/10.1300/J411v10n01_09.

[5] S. Fujikawa, Y. Jitsuyama, K. Kuroda, Determination of the role of cold acclimation-induced diverse changes in plant cells from the viewpoint of avoidance of freezing injury, J. Plant Res. 112 (1999) 237-244. https://doi.org/10.1007/PL00013880.

[6] S. Fujikawa, K. Miura, Plasma membrane ultrastructural changes caused by mechanical stress in the formation of extracellular ice as a primary cause of slow freezing injury in fruit-bodies of basidiomycetes (*Lyophyllum ulmarium* Fr. KuÈ hner), Cryobiology 23 (1986) 371-382. https://doi.org/10.1016/0011-2240(86)90042-8.

418 [7] S. Fujikawa, Freeze-fracture techniques, In: *Electron Microscopy in Biology, A*
419 *Practical Approach*, Harris, J. R. (ed.). IRL Press, Oxford (1991) pp. 173-201.
420 [8] S. Fujikawa, Seasonal ultrastructural alterations in the plasma membrane produced by
421 slow freezing in cortical tissues of mulberry (*Morus bombycis* Koids. cv. Goroji),
422 Trees 8 (1994) 288-296. https://doi.org/10.1007/BF00202673.
423 [9] N. Goto-Yamamoto, M. Numata, J. Furukawa, Characterization of Hokujun
424 (Beichun) and Muscat Hamburg-Amurensis (MH-AM) by Simple Sequence Repeat
425 (SSR) Analysis, J. ASEV Jpn. 24 (1) (2013) 4-8. (English abstract)
426 https://iss.ndl.go.jp/books/R100000002-I000000099553-00.
427 [10] T. Hirota, T. Yamazaki, Y. Yasui, J. Furukawa, K. Niwa, M. Nemoto, T. Hamazaki,
428 S. Shimoda, H. Kanno, Z. Nishio, Climate change favors viticulture and wine
429 production in Hokkaido, Japan. - An example of Pinot Noir cultivation since 1998-,
430 Climate in Biosphere 17 (2017) 34-45. (English abstract)
431 [11] Y. Jitsuyama, T. Suzuki, T. Harada, S. Fujikawa, Ultrastructural study on mechanism
432 of increased freezing tolerance due to extracellular glucose in cabbage leaf cells,
433 Cryoletters 18 (1997) 33-44. http://hdl.handle.net/2115/64526.
434 [12] S.K. Jones, D.B. McKersie, J. Paroschy, Prevention of ice propagation by
435 permeability barriers in bud axes of *Vitis vinifera*, Can. J. Bot. 78 (1) (2000) 3-9.
436 https://doi.org/10.1139/b99-137.
437 [13] R. Karimi, Cold hardiness evaluation of 20 commercial table grape (*Vitis vinifera*
438 L.) Cultivars. Int. J. Fruit Sci. 20 (3) (2020) 433-450.
439 DOI:10.1080/15538362.2019.1651242
440 [14] J. Kasuga, Y. Tsumura, D. Kondoh, Y. Jitsuyama, R. Horiuchi, K. Arakawa, Cryo-
441 scanning electron microscopy reveals that supercooling of overwintering buds of
442 freezing-resistant interspecific hybrid grape 'Yamasachi' is accompanied by partial
443 dehydration, J. Plant Physiol. 253 (2020) 153248.
444 https://doi.org/10.1016/j.jplph.2020.153248.
445 [15] K. Koyama, H. Kamigakiuchi, K. Iwashita, R. Mochioka, N. Goto-Yamamoto,
446 Polyphenolic diversity and characterization in the red–purple berries of East Asian
447 wild *Vitis* species, Phytochemistry 134 (2017) 78–86.
448 [16] K. Kuroda, J. Kasuga, K. Arakawa, S. Fujikawa, Xylem ray parenchyma cells in
449 boreal hardwood species respond to subfreezing temperatures by deep supercooling

450 that is accompanied by incomplete desiccation, Plant Physiol. 131 (2003) 736-744.
451 https://doi.org/10.1104/pp.011601.
452 [17] E.R. Lee, J.G. Warren, V.L. Gusta, eds. Biological Ice Nucleation and Its
453 Application, APS Press (1995) 184.
454 [18] V. F. Masocha, Q. Li, Z. Zhu, F. Chai, X. Sun, Z. Wang, L. Yang, Q. Wang, F. Xin,
455 Proteomic variation in Vitis amurensis and V. vinifera buds during cold acclimation,
456 Sci. Hort. 263 (2020) 109143.
457 https://doi.org/10.1016/j.scienta.2019.109143
458 [19] J.L. Mills, C.J. Ferguson, M. Keller, Cold-hardiness evaluation of grapevine buds
459 and cane tissue, Am. J. Enol. Viticult. 57 (2006) 194-200.
460 [20] G. Neuner, K. Monitzer, D. Kaplenig, J. Ingruber, Deep supercooling enabled by
461 surface impregnation with lipophilic substances explains the survival of
462 overwintering buds at extreme freezing, Plant, Cell Environ. (2019) 1-10.
463 https://doi.org/10.1111/pce.13545.
464 [21] G. Neuner, K. Monitzer, D. Kaplenig, J. Ingruber, Frost Survival Mechanism of
465 Vegetative Buds in Temperate Trees: Deep Supercooling and Extraorgan Freezing
466 vs. Ice Tolerance, Front. Plant Sci. 10, 537 (2019) 1-13.
467 https://doi.org/10.3389/fpls.2019.00537.
468 [22] Organization of the Department of Agriculture, the Hokkaido Government.
469 Guidebook for introduction of Winegrapes (edition2), Bureau for Promotion of
470 Agricultural Production, Agricultural Production Promotion Division (2018) 1-29.
471 http://www.pref.hokkaido.lg.jp/ns/nsk/kaju/jyouzouyoubudounotebiki1.pdf (access
472 at 12th Mar. 2020)
473 [23] P. Pierquet, C. Stushnoff, Relationship of low temperature exotherms to cold injury
474 in *Vitis riparia* Michx. Am. J. Enol. Viticult. 31 (1980) 1-6.
475 [24] H.A. Quamme, Use of thermal analysis to measure freezing resistance of grape
476 buds, Can. J. Plant Sci. 66 (1986) 945-952. https://doi.org/10.4141/cjps86-116.
477 [25] A. Sakai, Freezing avoidance mechanism of primordial shoots of conifer buds,
478 Plant Cell Physiol. 20 (1979) 1381-1390.
479 https://doi.org/10.1093/oxfordjournals.pcp.a075937.
480 [26] A. Wojdyło, J. Samoticha, P. Nowicka, J. Chmielewska, Characterisation of (poly)
481 phenolic constituents of two interspecific red hybrids of Rondo and Regent (*Vitis*

482 *vinifera*) by LC–PDA–ESI-MS QTof, Food Chem. 239 (2018) pp. 94–101.
483 https://doi.org/10.1016/j.foodchem.2017.06.077.
484 [27] Z. Xin, J. Browse, Cold comfort farm: The acclimation of plants to freezing
485 temperatures, Plant Cell Environ. 23 (2000) 893–902. https://doi.org/10.1046/j.1365-
486 3040.2000.00611.x.
487

488 **Figure legends**

489

490 **Figure 1. A-C**. Diagrams for explanations of experimental samples and methods. **A** A
491 typical grape vine and preparing the samples for observation with a Cryo-SEM. **B**
492 Stereoscopic microscope image of a winter bud section of *V. spp.* 'Zweigeltrebe'. Primary
493 bud, Secondary bud, Tertiary bud include apical meristems, primordia, bracts, hairs and
494 lignified scales. Scale bar represents 1mm. **C** A typical profile of the (DTA) differential
495 thermal analysis of the winter bud and cane of *V. Spp.* 'MHAM'. Orange: bud. Blue: cane.
496 Arrowheads indicate HTE (high temperature exotherm) or LTE (low temperature
497 exotherm). ΔT represents difference between the output from the fresh and oven-dried
498 samples.

499

500 **Figure 2.** Changes in freezing resistance of bud (left) and cane (right) tissues of 6 grape
501 vine cultivars sampled at winter (□) or autumn (■) seasons. LT_{50} represent freezing
502 resistance evaluated by ion leakage measurement, the smaller minus value meant the
503 higher resistance. The box and whisker plot display data as shown in small panel in figure.
504 The boxes denote the interquartile range, whereas data falling within 1.5 times the value
505 of the interquartile range are represented by two T-bars (the lower and upper extremes).
506 Small circles indicate outliers (data between 1.5-3x the interquartile range). The
507 horizontal line and 'x' within the box show the median and the average values from 4
508 (winter samples) or 3 (autumn samples) replications, respectively. Different letters beside
509 average indicate significant differences among cultivars in same season at a 5% level
510 (Tukey-Kramer's test).

511

512 **Figure 3.** Changes in temperature exotherms of bud (left) and cane (right) tissues of 6
513 grape vine cultivars sampled at winter (□) or autumn (■) seasons. HTE (high
514 temperature exotherm) in canes and LTE (low temperature exotherm) in buds represent
515 heat of ice solidification in tissue cells evaluated by DTA (differential thermal analysis),
516 the smaller minus value meant the higher resistance. The box and whisker plot display
517 data as shown in the small panel in the figure. The boxes denote the interquartile range,
518 whereas data falling within 1.5 times the value of the interquartile range are represented
519 by two T-bars (the lower and upper extremes). Small circles indicate outliers (data
520 between 1.5-3x the interquartile range). The horizontal line and 'x' within the box show

521 the median and the average values from 4-18 replications, respectively. Different large or
522 small letters above the bars indicate significant differences among cultivars in same
523 tissues and season at a 5% level (Tukey-Kramer's test).
524
525 **Figure 4.** Relationships between temperature exotherms of winter bud tissues and the
526 freezing resistances. Each circle represents the means from 4 replications and bars show
527 the standard errors. Regression analysis shows significance at 5% level (*), using
528 Peason's correlation coefficient test. The formulae and R^2 values were showed in all cases.
529
530 **Figure 5. A-H.** Cryo-SEM images of bud and primordia cells in *V. spp.* 'Zweigeltrebe'
531 （A-D）and 'MHAM'（E-H）. **A** Winter apical meristem cells before cooling, showing
532 control structures of 'Zweigeltrebe'. **B** Cells in winter apical meristem after slow cooling
533 to -10°C. **C** Dehydrated cells in winter apical meristem after slow cooling to -20°C,
534 showing deformation of cells. **D** Various cells in winter leaf primordium after slow
535 cooling to -20°C, showing severe deformed cells and intracellular freezing cells. Arrows
536 and Arrowheads showed intracellular freezing and extracellular freezing cells,
537 respectively. Asterisks showed extracellular ice. **E** Winter apical meristem cells before
538 cooling, showing control structures of 'MHAM'. **F** Cells in winter apical meristem after
539 slow cooling to -20°C. **G** Dehydrated cells in winter apical meristem after slow cooling
540 to -40°C, showing deformation of cells. **H** Various cells in winter leaf primordium after
541 slow cooling to -40°C, showing severe deformed cells and intracellular freezing cells.
542 Arrows and Arrowheads showed intracellular freezing and extracellular freezing cells,
543 respectively. Asterisks showed extracellular ices. Scale bars represent 10 μm.
544
545 **Figure 6 A-F**. Cryo-SEM images of cane tissue cells in *V. spp.* 'MHAM' (A-C, E-F) and
546 'Zweigeltrebe' (D). **A** A half of profile in winter cane tissue cells before cooling,
547 showing control structures. Scale bar represents 1mm. **B** Similar angle view of **A** in
548 winter cane after slow cooling to -15°C, showing icy appearance near phloem tissues.
549 Asterisks showed ice crystals. Scale bar represents 100 μm. **C** Close up of **B**, showing
550 extracellular ice. The area surrounding the dotted line represents ice crystals. Scale bar
551 represents 10 μm. **D** Winter cane after slow cooling to -15°C, showing icy appearance
552 near phloem tissues of 'Zweigeltrebe'. Scale bar represents 10 μm. **E** Similar angle view
553 of **A** in autumn cane after slow cooling to -15°C, not showing icy appearance near phloem

554 tissues. Scale bar represents 100 μm. **F** Autumn cane after slow cooling to -15°C,
555 intracellular freezing cells in the cortex. Scale bar represents 10 μm. Ep: Epidermis, Co:
556 Cortex, Ph: Phloem, Xy: Xylem, Pi: Pith.
557

558 **Table**

559

560 Table 1. Experimental samples of 6 cultivars of *Vitis spp*.

Cultivar (Abbreviation)	Scientific name and Origin	Country/Region
Muscat Ottonel* (MO)	*V. vinifera* (‘Chasselas’ x ‘Muscat de Saumur’)	France
Zweigeltrebe (ZR)	*V. vinifera* (‘Saint Laurent’ x ‘Blaufränkisch’)	Austria
MHAM (MH)	*V. amurensis x V. vinifera* L. ‘Muscat Hamburg’	China
Rondo (RO)	*V. vinifera* (‘Zarya Severa**’ x ‘Saint Laurent’)	Germany
Kiyomi (KI)	Bud mutation of *V. vinifera* ‘Seibel 13053’	Japan/ Hokkaido
Yamasachi (YA)	*V. amurensis x V. vinifera* ‘Kiyomi’	Japan/ Hokkaido

561 *As regards all other cultivars except for ‘Muscat Ottonel (for white wine)’ are red grape varieties used for red wine.

562 ** ‘Zarya Severa originated from *V. amurensis* and *V. vinifera* L. ‘Malingre Précoce’.

563

Figures

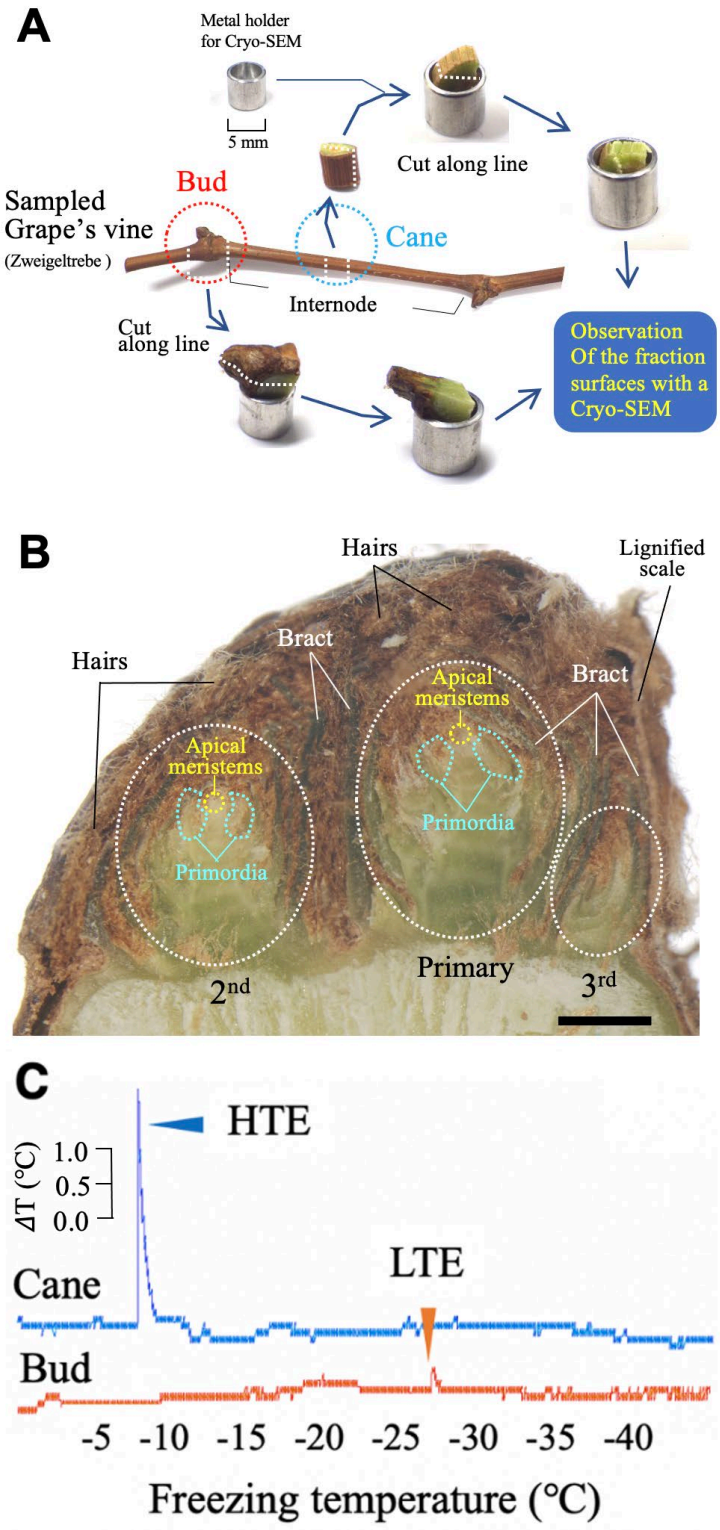

Figure 1.

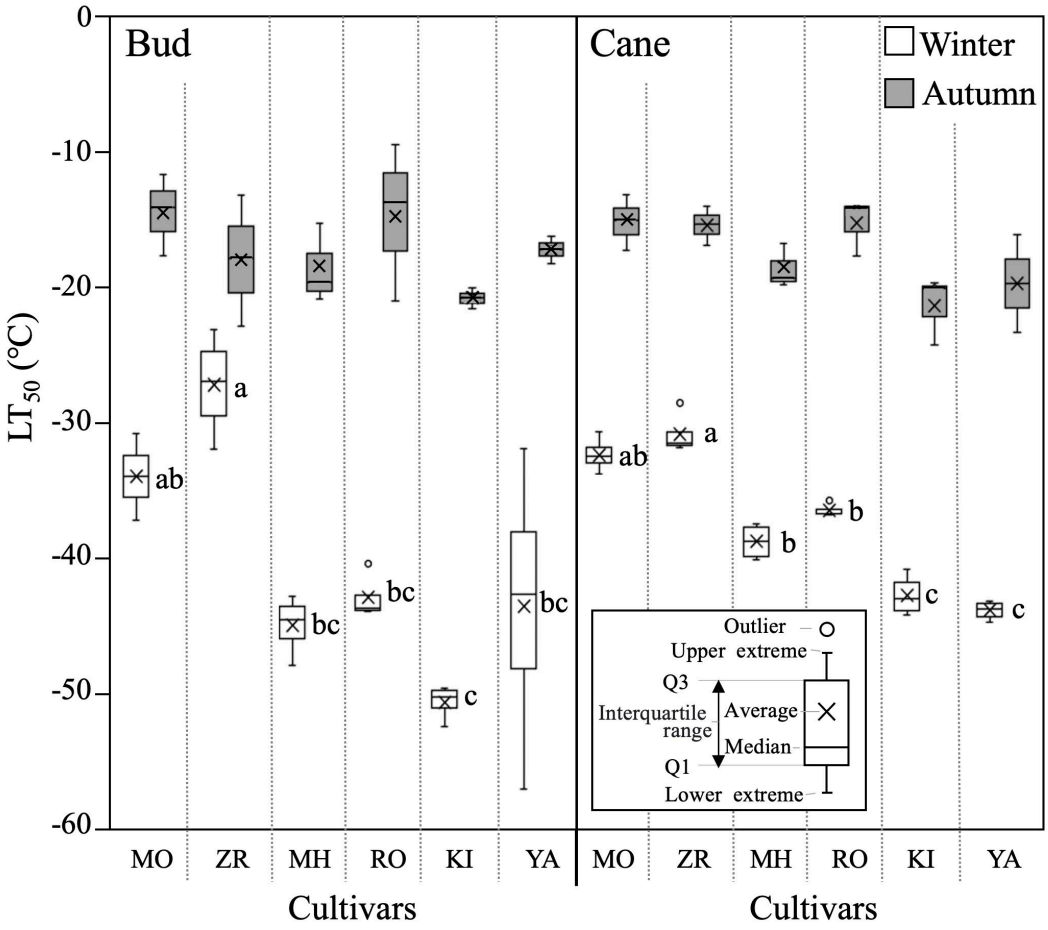
Bud
Cane
Winter
Autumn
LT50 (°C)
0
-10
-20
-30
-40
-50
-60
ab
a
bc
bc
c
bc
ab
a
b
b
c
c
Outlier
Upper extreme
Q3
Interquartile range
Average
Median
Q1
Lower extreme
MO
ZR
MH
RO
KI
YA
Cultivars

Figure 2.

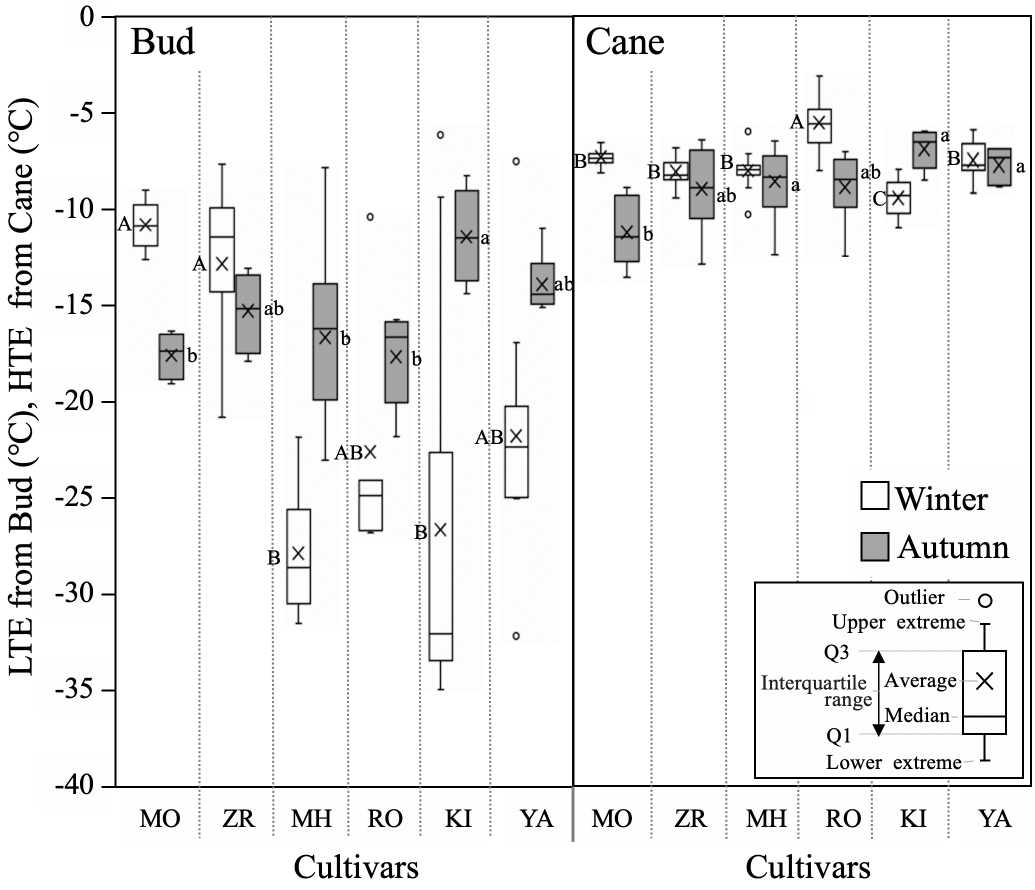
Bud
Cane
LTE from Bud (°C), HTE from Cane (°C)
0
-5
-10
-15
-20
-25
-30
-35
-40
MO
ZR
MH
RO
KI
YA
Cultivars
Winter
Autumn
Outlier
Upper extreme
Q3
Interquartile range
Average
Median
Q1
Lower extreme

Figure 3.

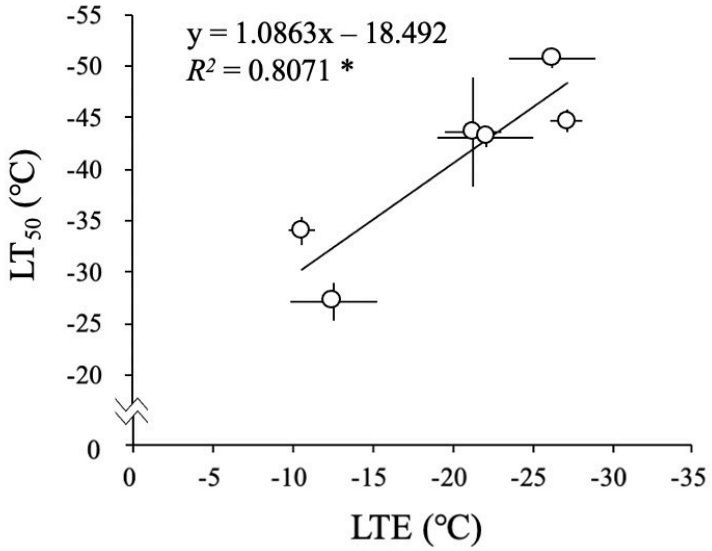
y = 1.0863x – 18.492
R^2 = 0.8071 *
LT_{50} (°C)
-55
-50
-45
-40
-35
-30
-25
-20
0
0
-5
-10
-15
-20
-25
-30
-35
LTE (°C)

Figure 4.

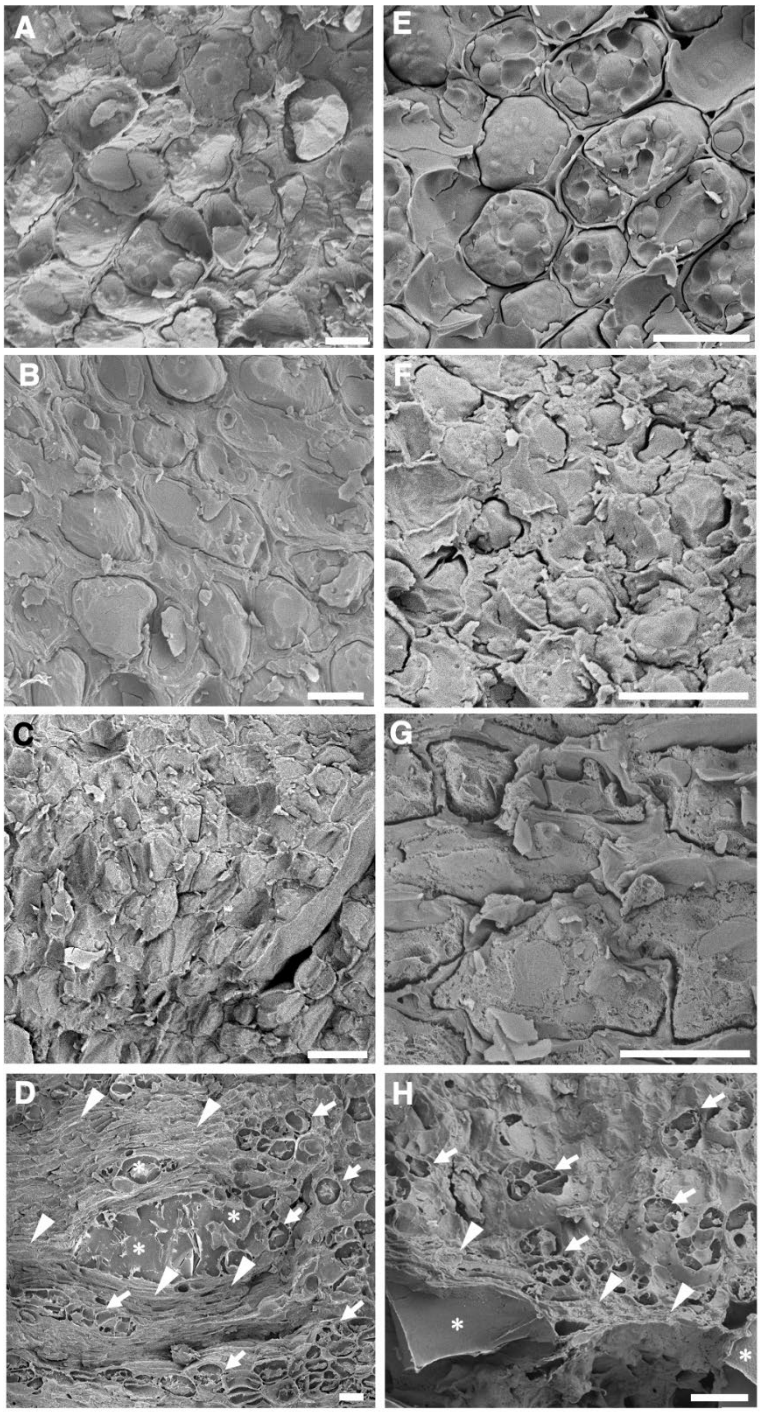

Figure 5.

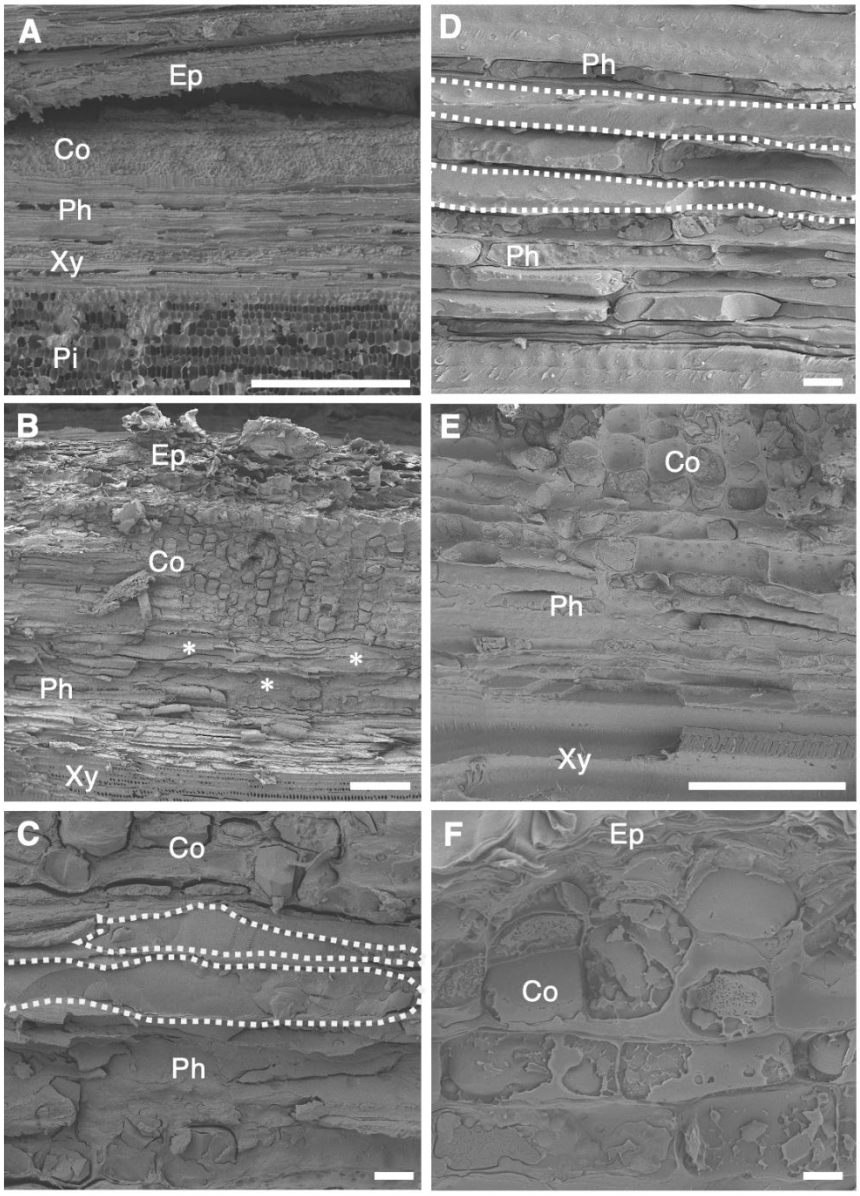

Figure 6.