CENTENARY OF THE OIV
45th WORLD CONGRESS OF VINE AND WINE
14-18 OCTOBER 2024 FRANCE-DIJON
OIV

Available on IVES Conference Series vine & wine

DOI : https://doi.org/10.58233/T26uTHoH

Metabolic response of *Vitis vinifera* and interspecific *Vitis* sp. varieties to heat stress, water deficit and combined stress, using a metabolomic approach

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Abstract. As greenhouse gas emissions continue to rise, climate projections indicate an increased likelihood of heat waves and drier conditions in Canada. These changes pose significant challenges to grapevine cultivation, particularly during critical growth stages such as new plantings. Interspecific hybrid grape varieties, developed through different breeding programs that combine *Vitis vinifera* with more robust species like *V. riparia* and *V. labrusca* varieties, are often touted for their potential resilience to environmental stress. As hybrids are initially bred for harsh climates, assumptions are that they possess a higher capacity to withstand environmental stress compared to *V. vinifera* varieties. However, many aspects of this assumption remain to be investigated. To address this gap, we compared the metabolic responses of young *Vitis vinifera* varieties Cabernet franc and Riesling, as well as interspecific *Vitis* hybrids Vidal and Marquette, under heat stress, mild water deficit, and a combination of both, relative to a control. Leaves were sampled after 21 days, extracted using methyl-tert-butyl-ether, methanol and chloroform and analyzed by LC-MS/MS, using an untargeted metabolomics approach. Data were processed using the Compound Discoverer software. Metabolomics analyses revealed over 200 metabolites, including amino acids, phenolics, and sugars, and 50 to 70 metabolites showed significant increases compared to the control condition. Heat stress elicited the most pronounced response across all varieties. On the other side, the combined stress scenario produced contrasting results. For instance, in Cabernet franc, most metabolites were upregulated by heat stress and further increased under combined stress. In contrast, Marquette displayed a dampening effect, as most metabolites upregulated by heat stress showed a lower response under combined stress. These results suggest that Marquette and Vidal might employ different stress mitigation strategies compared to Cabernet franc when facing combined stress. This could indicate a higher level of resilience in these hybrid varieties, a promising finding for adapting to a future marked by climate change.

1. Introduction

Climate significantly impacts plant physiology, with temperature being a major factor directly influencing plants primary and secondary metabolites biosynthesis. The relationship between temperature and many biochemical and biological processes, including enzyme activity and membrane fluidity, is well-documented, highlighting the need to understand these dynamics to ensure crops sustainability and productivity [1, 2]. In Canada, temperatures are expected to rise by 1.5°C to 2.3°C by 2050, with most extreme scenarios predicting up to 6°C by the end of the century [3]. This warming is expected to increase the frequency of heatwaves and drought conditions among others, thus reshaping growing conditions for many crops, especially perennials such as grapevine.

In grapevine, heat stress arises when temperature significantly outruns the upper temperature limit for photosynthesis, which operates optimally from 21°C to 32°C [Jansson]. Indeed, when temperature exceeds 35°C, RuBisCO affinity shifts from CO_2 to O_2, turning night reactions from carbon fixation to photorespiration, and slow down and hindering of photosynthesis. Water stress occurs when the decrease in water availability affects water absorption, further conducting to stomate closure and a break in photosynthesis as well. While grapevine is quite tolerant to mild water stress, the concomitant occurrence of heat conditions may exacerbate the level of stress, conducting to significant impacts on plant physiology and biochemistry.

A large part of research on grapevine physiology has focused on fruit development and quality, particularly regarding the impact of temperature on berry composition

[5, 6], there is a notable gap in understanding how stressors such as heat and water stress, and particularly their combination may affect leaves metabolism. From a physiological and biochemical standpoint, water stress shares some similarities with heat stress: both conduct to an inhibition of photosynthesis and an imbalance in redox potential, leading to oxidative stress. However, in contrast with water stress, high temperature increase fluidity of membranes systems in cells, affecting their functions and permeability. It may also cause conformational changes in protein and enzyme structures also affecting their functions and activity, resulting in reduced metabolic efficiency, and accumulation of biosynthesis intermediates and free radicals [7]. Plant responses to such events through a series of signals resulting on the production of stress metabolites that contribute to alleviate the effect of stress. In grapevine leaf, aromatic amino acids, certain aliphatic amino acids, and phenolic compounds such as tannin have been found to increase in response to heat and/or water stress [6, 8]. Yet, data about leaf response to abiotic stress such as heat and mild water stress is very scarce.

As growing conditions shifts because of climate change, a significant part of current research in horticulture focuses on the development of stress-tolerant and climate-flexible crops [4]. In the recent years, varietal selection in grapevine highly focused on the development of disease and pest resistant/tolerant varieties to reduce pesticides in viticulture [9]. Indeed, most traditional *V. vinifera* varieties are highly sensitive to fungal diseases, and the extensive use of pesticides in wine producing areas became a central issue for the health of workers and that of populations living nearby vineyards [10].

Disease resistant/tolerant varieties are issued from interspecific crosses between different *Vitis* species, including *V. vinifera*, *V. riparia* and *V. labrusca*, among others [9]. Besides their higher tolerance to diseases and pests when compared to traditional *V. vinifera* varieties, and depending on their parents' genetics, interspecific hybrid *Vitis* varieties may carry a significantly higher tolerance than *V. vinifera* varieties to certain abiotic stresses such as cold and frost, suggesting that they could carry an overall wider tolerance to abiotic stresses, including heat and water stress and thus represent a step forward toward climate-flexible vineyards. However, this hypothesis has not been studied yet.

The current study aimed to address these gaps by investigating the effects of heat and water stress, individually and in combination, on the leaf metabolome of two *V. vinifera* varieties (Cabernet franc, Riesling), and two interspecific hybrid *Vitis* varieties (Marquette, Vidal), under controlled conditions. All four varieties are extensively grown in Canada for wine production.

2. Methodology

2.1. Plant Material and Experimental Design

One-year old plants from four varieties including *Vitis vinifera* c.v. Cabernet franc and Riesling, and interspecific hybrid varieties c.v. Vidal (Ugni blanc X Rayon d'Or) and Marquette (MN 1094 X Ravat 262), all grafted on 3309C rootstock were submitted to a completely randomized block design including four treatments (control (CT), heat stress (ST), water stress (SH), and combined heat and water stress (ST x SH)) was repeated on 8 plants per treatments. Treatments were implemented in controlled conditions chambers. Hydric stress was induced through watering plants sufficiently to induce a stem water potential of –10 bars (corresponding to a moderate stress), starting two weeks prior the beginning of the experiment and up to 21 days. Heat stress was induced by exposing plants to daytime temperatures of 38ºC and to night temperature of 35ºC for 21 days.

2.2. Leaf Sampling and Extraction

Mature, fully expanded leaves were sampled on each replicate, 21 days after the beginning of treatments, immediately frozen in liquid nitrogen and stored at –80°C until extractions. Untargeted metabolites were extracted through a biphasic extraction protocol involving methyl-tert-butyl-ether (MTBE), methanol and water. Briefly, 100 mg of ground leaf tissue was extracted in a 3:1 mixture of MTBE and methanol. The organic and aqueous phases were separated using a water-based phase, and the aqueous phase containing polar/semi-polar metabolites was concentrated, reconstituted, filtered, and analysed by LC-MS/MS analysis.

2.3. LC-MS/MS Analysis

Metabolite profiling was performed by liquid chromatography-tandem mass spectrometry on an Orbitrap LC-MS/MS system (Thermo Fisher Scientific). Both positive and negative ionization modes were employed to detect a wide range of compounds, including amino acids, phenolic compounds, and sugars. Data analysis was conducted using Compound Discoverer 3.3 software (Thermo Fisher Scientific). A list of potential compounds was generated taking into account the confidence levels of the Metabolomics Standards Initiative (MSI) criteria from the Metabolomics Society [11]. Initially, only compounds with an MZ cloud (MS/MS) score above 80% were exported. Fish coverage was included for compounds that did not meet the level 2 confidence criteria to compensate for the lack of confidence in identification. Unknown compounds were matched using several databases, including mzCloud for MS/MS data with a mass tolerance of 10 ppm, and ChemSpider and Metabolika for exact mass matching with a $\Delta 5$ ppm tolerance. For each variety, metabolomics data were calculated as $\log_2$ fold changes ($\log_2$ FC) comparisons between treatments (e.g., SH/CT, ST/CT, ST x SH/CT).

2.4. Data Analysis

Statistical significance ($P \leq 0.05$) per group ratio was calculated by a one-way ANOVA model with Tukey as post-hoc test. Adjusted *p*-value was calculated using

Benjamini-Hochberg correction for the false-discovery rate. A hierarchical clustering heatmap analysis was conducted on compounds identified with a high level of confidence, focusing on those that exhibited significant changes in at least one group ratio, to visualize the metabolic response patterns across grapevine varieties and treatments.

3. Results and discussion

Like most plants, grapevine has developed a variety of coordinated responses to maintain homeostasis and cope with abiotic stresses such as water deficit and heat stress [11]. These stresses, increasingly prevalent due to climate change, can significantly impact vine performance, with further consequences on berry quality. Stomatal closure, resulting in decreased stomatal conductance and leaf transpiration, is a well-known stress response primarily associated with water stress. However, stomatal responses to heat stress can be more complex, as plants may need to balance water conservation with leaf cooling through transpiration [12, 13].

Our study examined the responses of two *V. vinifera* varieties (Cabernet franc, Riesling) and two interspecific hybrids (Marquette, Vidal) to heat stress (ST), water stress (SH), and their combination (ST x SH). Initial physiological measurements revealed variety-specific responses in stomatal conductance and transpiration rates under different stress conditions (data not shown). As expected, heat stress generally increased stomatal conductance and transpiration rates, while water stress decreased them. Stomatal conductance was significantly reduced in Marquette, Riesling and Vidal when heat was combined to water stress (data not shown), suggesting that preserving water was prioritized over cooling the leaf in these varieties. Furthermore, these physiological changes confirmed the effectiveness of our experimental treatments in inducing stress conditions in our potted grapevine, providing a solid ground for subsequent metabolomic analysis.

3.1. Metabolic Changes Associated with Heat Stress

A comprehensive analysis of the leaf metabolome of the four studied varieties was conducted and led to the identification of over 200 metabolites, including amino acids, organic acids, polyphenols, and sugars, hence providing a detailed picture of the metabolic changes occurring in response to the induced stress. Among these compounds, 50 to 70 metabolites showed significant changes compared to the control. We focused our analysis on these compounds, presenting them as hierarchical clustering heatmaps (Figures 1 and 2). This visualization highlights the variety-specific metabolic adaptations to these abiotic stresses, demonstrating how young grapevines modulate their biochemistry to cope with environmental challenges.

The response of *V. vinifera* Cabernet franc to heat stress (ST) was among the strongest one among the studied varieties, as a total of 50 compounds from various chemical classes, including amino acids, organic acids, polyphenols, and sugars were upregulated by 1.9 to 15.7 log_2 FC (Figure 1). In Riesling, 41 metabolites from similar chemical families were upregulated by 0.2 to 16.4 log_2 FC.

The response of interspecific hybrid varieties Marquette and Vidal was different than that of *V. vinifera* varieties in the number of compounds that were upregulated, but the intensity of upregulation (e.g. log_2 FC) was similar (Figure 2). For instance, Marquette leaf only upregulated 30 compounds (3.5 to 15.3 log_2 FC) whereas Vidal upregulated only 26 compounds (3.1 to 15.4 log_2 FC).

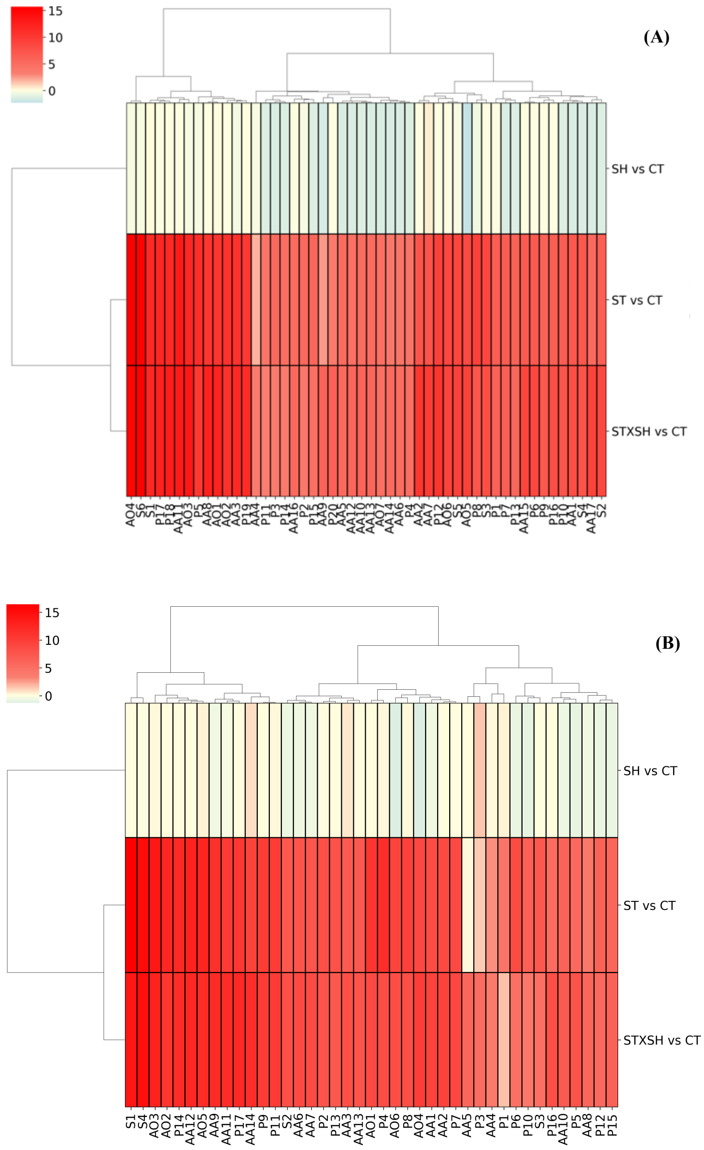
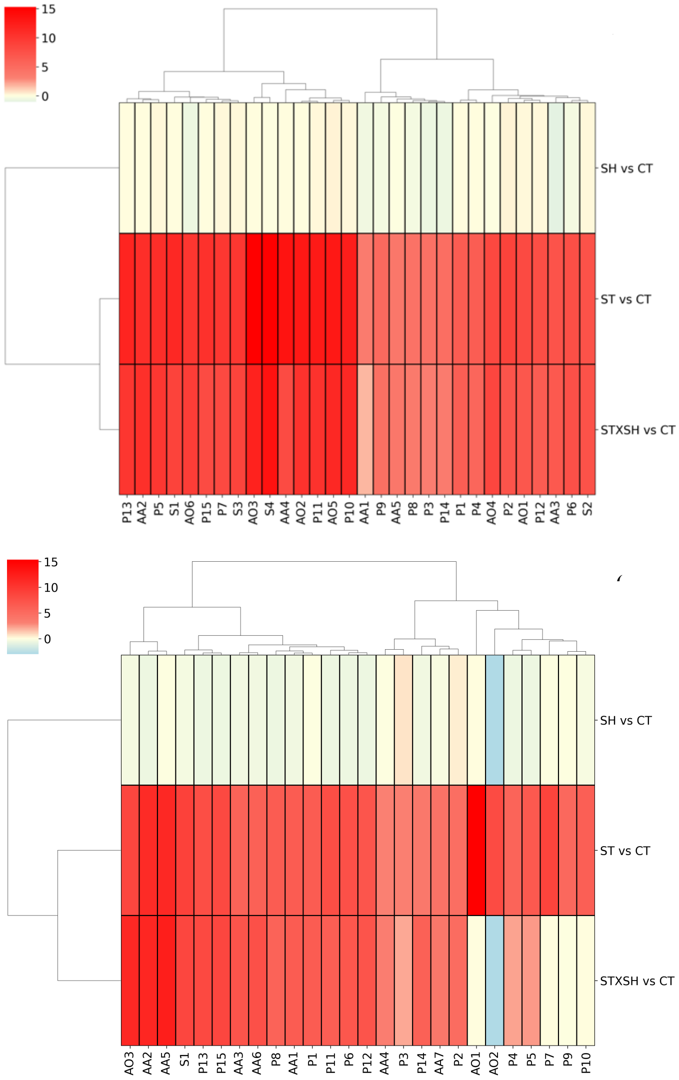


Figure 1. Heatmap of Log_2 Fold Change Values Across Three Pairwise Comparisons of the Leaf Metabolome of *V. vinifera* c.v. Cabernet franc (A) and Riesling (B) Plants Submitted to Three Stress Conditions, Compared to Control: SH vs CT (Water Stress vs Control), ST vs CT (Thermal Stress vs Control), and ST x SH vs CT (Combined Thermal and Water Stress vs Control). Key Metabolites Include Amino Acids (AA), Organic Acids (AO), Polyphenols (P), and Sugars (S).

Major upregulated compounds were similar among varieties and mainly included amino acids such as glutamic acid and threonine, organic acids such as furoic, citric and gluconic acids, sugars such as trehalose, and polyphenols such as catechin, quercetin and rutin. Most of these metabolites are known to contribute to plant's tolerance to abiotic stress, making these finding consistent with previous studies emphasizing the role of specific metabolites in protecting young plants from heat-induced damages [14].



Marquette (A) and Vidal (B) Plants Submitted to Three Stress Conditions, Compared to Control: SH vs CT (Water Stress vs Control), ST vs CT (Thermal Stress vs Control), and ST x SH vs CT (Combined Thermal and Water Stress vs Control). Key Metabolites Include Amino Acids (AA), Organic Acids (AO), Polyphenols (P), and Sugars (S).

3.2. Metabolic Changes Associated with Water Stress

Water stress (SH) induced in our experimental grapevines was quite mild but still affected stomatal conductance and leaf transpiration, as described earlier, hence suggesting that photosynthesis was significantly slowed down. The leaf metabolome of water stress *V. vinifera* varieties showed mild responses, with most identified metabolites being slightly down-regulated by 0 to –3 $\log_2$ FC in Cabernet franc and Riesling, and close to no up-regulated metabolites. A similar response was observed in both interspecific hybrid varieties Marquette and Vidal.

The moderate impact and tendency towards the down-regulation (not significant for most compounds) of metabolite biosynthesis under water stress (SH) suggest that young plants may adopt a conservative strategy in these conditions, including a reduction in metabolic activity. This response is particularly relevant for young plants that may have limited energy reserves as well as physiological capacity to cope with abiotic stress, which is particularly relevant when photosynthesis is paused.

3.3. Metabolic Changes Associated with Combined Heat and Water Stress

In environmental condition, the combination of abiotic stress, or their subsequent occurrence during plant's growth cycle, is prevailing, making it hard to isolate the effect of a particular stress, or the combination of two stress. Moreover, abiotic stress may have additive effects (e.g. drought and soil salinity) whereas others may have little impact over each other. In the current study, the addition of heat stress to plants that were already under water deficit (ST X SH) showed significant impacts on leaf metabolome.

In *V. vinifera* Cabernet franc, a slightly higher upregulation was observed in the combined stress conditions compared to heat stress alone, for the majority of the 50 compounds identified (38 out of 50; Figure 1). However, this difference is subtle, with the absolute difference in $\log_2$ FC typically less than 2, except for eight compounds that showed more pronounced changes.

A similar response was observed in Riesling, although more compounds were down-regulated in response to the combined stress conditions. Indeed, a balanced distribution of metabolic changes was observed in this variety, with 23 compounds showing amplification of the upregulation and 18 exhibiting an attenuation of the upregulation when compared to heat stress alone. This balance indicates that the response is not uniform across all metabolites.

While Cabernet franc showed a slight intensification of certain metabolic responses under combined stress, Marquette exhibited a consistent attenuation of the expression of most compounds when compared to heat stress alone. Indeed, 29 out of 30 compounds upregulated under heat stress were slightly reduced under combined stress, suggesting a more conservative response as stress intensify. This pattern was particularly pronounced for six compounds that showed a more substantial reduction in upregulation (difference in $|\log 2\ FC| > 2$). These include organic acids such as malic and gluconic acids (e.g., AO2 and AO3, respectively) and polyphenols such as quercetin-3β-D-glucoside (P11) and trifolin (P15), and sugars such as fructose (S1).

Vidal's response to combined stress (ST x SH) presented distinct characteristics. Unlike Cabernet franc, which showed a slight amplification of metabolic response under combined stress, and Marquette, which displayed a slight attenuation of its response when compared to heat stress alone, Vidal adopted an intermediate strategy, exhibiting an amplification of the upregulation of 12 compounds under combined stress (although mostly less than 1.5 log_2 FC), while 14 show attenuation. Some metabolites that were upregulated under heat stress maintained similar levels under combined stress, while others showed varied responses.

Of interest, among varieties that modulated their metabolome in response to combined stress, certain compounds were specifically and highly up-regulated when compared to heat stress alone, including proline in Cabernet franc, tryptophan, caffeic acid and resveratrol in Riesling, and resveratrol in Vidal. The significant upregulation of protective osmolytes such as proline indicates a strategic allocation of metabolic resources to maintain cellular integrity and function [15]. Additionally, the increase in polyphenols under stress conditions highlights the need to mitigate oxidative stress associated with heat stress [16].

The nuanced responses observed among varieties reinforce the idea of a fine-tuned, potentially additive effect when both stresses were present, rather than a purely synergistic one. It also shows that young grapevine plants can modulate their physiological responses to manage the challenges posed by simultaneous heat and water stress, likely balancing the need for stress protection with energy conservation.

3.4. Are interspecific hybrids *Vitis* varieties more resilient than *V. vinifera* varieties?

Studied varieties showed multifaceted metabolic adjustment when confronted to heat and water stress, and combined stress. These data underscores grapevine's capacity to adapt to changing environmental conditions, particularly in the context of climate change. Yet, the response of interspecific hybrid varieties was of lower intensity in terms of biochemical diversity (e.g. number of up-regulated compounds), suggesting a more straightforward approach to stress management. They also exhibited a more modest response to combined stress when compared to *V. vinifera*, suggesting that these conditions were impacting them to a lesser extent.

Marquette's unique strategy when faced with combined stresses, characterized by a more consistent attenuation of the leaf metabolome, could reflect a greater resilience to combined environmental stress when compared to Cabernet franc. This adaptive capacity could be a major asset for this hybrid variety under future, more severe climatic conditions. This metabolic plasticity aligns with previous studies showing that grapevine varieties can exhibit distinct responses to abiotic stresses [17]. The attenuation of amino acid accumulation under combined stress might indicate a more efficient nitrogen metabolism or a shift in resource allocation strategies [18]. Similarly, the modulation of polyphenol production could be part of a fine-tuned antioxidant response that balances stress protection with energy conservation [19].

Marquette's ability to adjust its metabolic responses under different stress conditions could be attributed to its hybrid genotype, potentially benefiting from diverse genetic backgrounds that confer enhanced stress tolerance. This metabolic flexibility might allow Marquette to maintain better plant functions under challenging environmental conditions, potentially translating to improved vine health and grape quality in stressful seasons.

4. Conclusion

The leaf metabolome of *V. vinifera* varieties Cabernet franc and Riesling and interspecific hybrid varieties Marquette and Vidal revealed distinct responses to heat stress, water stress, and their combination, highlighting the complex metabolic strategies employed by different varieties to cope with abiotic stressors.

Among the studied varieties showed significant metabolic changes under heat stress along with up-regulation of similar compounds, indicating shared mechanisms of heat tolerance across grapevine species. Water stress alone generally induced a moderate to not significant shift in leaf metabolome, generally tending toward a down-regulation of metabolites, suggesting a conservative strategy to preserve carbon reserves under those conditions. Combined stress responses varied by variety: Cabernet franc exhibited a slight intensification of metabolic responses whereas Marquette showed a tendency towards attenuation.

Interspecific hybrid varieties (Marquette and Vidal) response was generally weaker than that of V. vinifera, suggesting these varieties were less perturbated by stress conditions, and that they could potentially have an increase resilience to abiotic stress.

Specific metabolites, particularly amino and organic acids, and polyphenols, emerged as key players in stress response across varieties, though their regulation differed among cultivars. These variety-specific metabolic signatures provide valuable insights into grapevine stress adaptation mechanisms and that more resilient varieties such as interspecific hybrids may be characterized by a weaker response to stressful conditions, which complies with the idea that one of the main attributes of resilient plant is to avoid being perturbated by variations in environmental conditions.

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