

Research Paper

Dynamic of bud ecodormancy release in *Vitis vinifera*: Genotypic variation and late frost tolerance traits monitored via chlorophyll fluorescence emission

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ABSTRACT

Early budburst is becoming an increasingly challenging topic in viticulture. Anticipating vegetative resume results in an overall phenological advance, in potential higher risks of late frost and subsequent negative effects on berry quality and overall vine productivity. Phenotypic variation for date of budburst onset (BBCH07) is known in *Vitis vinifera* and potential exploitation of data regarding thermal requests to reach BBCH07 are critical in defining new avenues for viticulture. Nevertheless, reproducible methods are lacking in defining phenological progression in grapevine and further efforts are needed to standardize quantitative dataset associated with early growth stages appearance. In this work, a panel of twenty-one *Vitis vinifera* varieties grown in an experimental vineyard were assessed for early phenological onset (pre-to-post budburst) via visual observation, quantum yield of photosystem II in the dark adapted (F_v/F_m) bud section and growing degree days accumulation over three years. Further experiments were carried out under controlled environmental conditions to evaluate the effect of different simulated late frost on bud viability. Our data proposes F_v/F_m from bud section as a quantitative and reliable tool, although destructive following our pipeline, to monitor early phenological events in grapevine with significant non-linear associations of the F_v/F_m with growing degree days on base 6 °C (GDD_6) and phenology. We observed significant ($p < 0.001$) inter-varietal variation for thermal requests to reach budburst ranking from 140 to 260 GDD_6 although some varieties showed inconsistent data between years (i.e. a plastic response). Late frost damage was associated with phenological progression suggesting a linear and positive correlation between cold injury and de-acclimation from cold hardness up until first leaf appearance. However, monitoring F_v/F_m in selected varieties provided evidence of varietal-specific response to late frost with e.g., Chardonnay and Gewürztraminer showing maintenance of photosystem II activity even at advanced phenological stages. This suggests the presence of preferable acclimation mechanisms to late frost in *Vitis vinifera* that will deserve further investigation. Our data provides a comprehensive analysis of early phenological events in grapevine, providing novel methods of assessment (F_v/F_m), varieties possessing escape strategies (i.e. large thermal accumulation to reach BBCH07) and varieties with putative late frost tolerance even after budburst. Overall, further work is ongoing to define the mechanisms underlying late frost tolerance *per se* and to identify novel varieties with preferable combination of traits.

1. Introduction

Climate change is severely affecting viticulture via an increase in the frequency and the intensity of erratic environmental dynamics (Gambetta and Kurtural, 2021; Xyrafis et al., 2022; Keller, 2010; Van Leeuwen et al., 2019; Van Leeuwen and Darriet, 2016). These combinations of stressors (heat, water limitation, radiative excess) can occur

simultaneously or sequentially (Zandalinas and Mittler, 2022) leading to reduced yield and quality, partially via a modulation in the crop phenological pattern (Cameron et al., 2022). Indeed, the main phenological stages in grapevine (*Vitis vinifera* L.) (i.e. budburst, flowering and veraison) are key steps in the annual life cycle and their onset over the growing season has been associated with direct (Kliewer, 1977; Faralli et al., 2022a,b) and indirect (van Leeuwen and Darriet, 2016) effects on

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yield and berry quality. Although the phenological progression and interval duration is variety-specific, thus under genetic control, the timing of the phenological stages for grapevines can differ between seasons (Tomasi et al., 2011; Jones and Davis, 2000) as significantly driven by environmental conditions (Cameron et al., 2022; Fraga et al., 2012; Santos et al., 2020). In a changing climate, further advancements in the grapevine phenological stage onset are ongoing and expected to be exacerbated due to an increase in early accumulation of thermal units (Jones et al., 2022).

Budburst in grapevine occurs between late winter and early spring and it is a critical stage in yield determination as it falls in periods of high risk of frost that can damage the young, fragile, growing shoot (Leolini et al., 2018). Among environmental variables, air temperature has been shown to be significantly correlated with the onset of budburst as ecodormancy is gradually released via a progressive increase in the underlying seasonal temperature (García de Cortázar-Atauri, et al. 2017; Molitor et al., 2014). Further complexity in budburst onset arises from the evidence of a tight link between the entrance in endodormancy (internal signals repress growth initiation) and the date of budbreak (Shellie et al., 2018). The date of budbreak was also dependent on the timing of grapevine transition from endo- to ecodormancy (environmental signals suppress bud regrowth) (Shellie et al., 2018), potentially due to water-stress feedback from the previous season. In addition, the transition from endo- to ecodormancy needs previous exposure to low temperatures or the application of dormancy release treatments such as cyanamide (Dokoozlian et al., 1995; Pérez et al., 2009; Vergara and Pérez, 2010; Halaly et al., 2011; Londo and Johnson, 2014). These complex interactions are still inadequately understood leading to difficulties in modelling budburst onset. Therefore, selecting, and characterizing *Vitis vinifera* for timing of ecodormancy release or shoot tolerance to late frost is a critical step in providing viticulturists with specific varieties adapted to a changing climate.

Generally, phenological characterization in the field is carried out via visual observation following morphologically based growth scales (e.g., Lorenz et al., 1995). A few studies used chlorophyll fluorescence, and in particular the quantum yield of photosystem II in dark adapted samples (F_v/F_m) as a proxy to evaluate tissues and bud viability after freezing stress and leaf development/age (Jiang et al., 1999; Zulini and Fischer, 2010; Bertamini and Terdaguila, 1995) hence providing a quantitative trait that does not include the operator bias resulting from the visual observation. In Zulini et al. (2010) a specific threshold for bud viability after freezing stress was defined, with F_v/F_m values below 0.5–0.4 associated with a complete failure in growth resume after frost events. Other useful approaches to evaluate bud viability under dynamics of low temperature rely on the use of thermoelectric modules to observe the release of heat following a phase change when water transitions to ice (differential thermal analysis, DTA) (Londo and Kowaleski, 2017). The method has been used to extensively characterize species and genotypic variation for winter cold tolerance in several studies (Londo and Kowaleski, 2017; Mills et al., 2006; North and Kowaleski, 2022), suggesting DTA as a reliable tool to evaluate cold survival, although methods for monitoring quantitatively ecodormancy release dynamics are still lacking. Usually, growth resume in grapevine buds manifests after the occurrence of specific physiological and metabolic events such as an increase in xylem pressure followed by high concentration of phytohormones and sugars in the xylem sap (Meitha et al., 2018) as well as a rise in tissue oxygen status (Signorelli et al., 2020). Meitha et al. (2018) showed a strong regulation of genes linked to photosystems in quiescent buds suggesting the presence of a priming strategy to develop autotrophic metabolism even in the absence of light. This suggests that, well before budburst, an increase in proteins associated with the photosynthetic metabolism are built up in the growing bud. In essence, the F_v/F_m may be used as a quantitative proxy of developmental growth resume (Zulini et al., 2010) as well as for detecting intra-specific phenological progression prior to budburst.

In this work, a selection of 21 *Vitis vinifera* varieties was monitored

over three years in the field for the onset of budburst. The main objective of this study was to determine, at which value of bud F_v/F_m or growing degree days with specific base temperatures (base of 6 °C or 10 °C; GDD₆ and GDD₁₀ respectively) accumulation, the BBCH 07 phenological phase (i.e. green tip/beginning of budburst according to Lorenz et al., 1995) was reached, hence characterising putative genotypic variation. In addition, we investigated whether there was a correlation between phenology and F_v/F_m values in buds subjected to controlled-environment late frost (i.e. after budburst) therefore hypothesizing putative genotypic variation for late frost tolerance. Our work provides for the first time a three-year investigation with novel quantitative methods in which genotypic variation for budburst and cold tolerance was explored providing evidence of distinct varieties possessing early onset of BBCH07 but still moderate tolerance to late frost.

2. Materials and methods

2.1. Plant material and environmental monitoring

The study was carried out throughout three field seasons: 2021, 2022 and 2023. The plant material used in the study was collected at the experimental vineyard of the Fondazione Edmund Mach. The experimental vineyard is a collection of 24 different European grapevine varieties, trained to a double Guyot: in this work, 21 cultivars of *Vitis vinifera* have been considered, while a sub-set with contrasting thermal requirements was used in 2023 (five varieties). Clones of each variety used is shown in Supplementary Table 1. Although there were differences for rootstocks (that may impose minimal effects on scion phenology, e.g. Sabbatini and Howell (2013)), all varieties were grafted onto *Vitis berlandieri* x *Vitis riparia* hybrids (apart from Schwartzman resulting from a *Vitis rupestris* x *Vitis riparia* hybridization). The row orientation was east-west, the distance on the row was 0,8 m, and 2 m between rows. The vineyard was planted in 2010, and located in the Province of Trento, Italy (46.191031 N, 11.136854 E). During the year 2023, a reduced subset of cultivars selected from 2021 and 2022 data, composed of Chardonnay, Gewürztraminer, Marzemino, Yellow Muscat and Syrah was analysed. Environmental data were monitored all years via a meteorological station located in the campus of the Fondazione Edmund Mach (46.183514 N, 11.120576 E). The environmental data included daily mean, maximum and minimum air temperature (°C), mean relative humidity (%), precipitation (mm) and solar radiation (MJ/m²). The data used in this work (e.g. mean temperature – T^mean) is the average value of data collected every hour (i.e. $n = 24$). Calculation of growing degree days was carried out for both GDD in base 6 °C, 8 °C and 10 °C as follow (in case T^mean was lower than x , we assigned a GDD value of 0):

$$GDD_x = \sum_{01/01}^n T^mean - x$$

where T^mean represents the average daily temperature and x the temperature threshold (i.e. either 6 °C, 8 °C or 10 °C). For this work, we used GDD₆ as an indicator of early thermal accumulation in grapevine as it showed an average higher goodness of fit with BBCH ($R^2 = 0.95$) compared to GDD₈ or GDD₁₀ ($R^2 = 0.90$ and $=0.85$ respectively) (Supplementary Table 2) and as already observed for growing degree days on base 5 °C by García de Cortázar-Atauri et al. (2009). Additionally, we used 6 °C as base temperature since the work carried out by Moncur et al. (1989) highlighted an optimal average base temperature for predicting budburst and first leaf unfolded between several varieties of 3.5 and 7 °C respectively. However, taking in consideration some of the varieties included in our work (e.g. Gewürztraminer, Chardonnay) the average optimal base temperatures shifted to 4.1 and 7.2 °C, therefore much closer to an optimal 6 °C temperature on average for early stages development. Chilling units over the winter period (i.e.

September-December of the previous year) were calculated based on Weinberger (1950).

2.2. Samplings of cane nodes

All samplings were carried out each year from late January to mid April to monitor the early phenological progression of the vines (BBCH00 as dormant bud by up to BBCH15 as 5th leaf unfolded according to Lorenz et al., 1995). Samplings in all years and for all varieties were carried out between 10:00 and 11:00 in the morning. Two fruiting canes and two spurs were pre-pruned at 14 buds and kept vertical up to the end of the experiments. During each sampling, ten nodes with at least 4 cm of remaining apical and basal internode ($n = 10$, position 10 to 12) for each variety were collected in labelled plastic bags while phenology (BBCH) was visually assessed. The sampling was randomly carried out over 140-to-100 plants per variety and avoiding any sampling for canes in which nodes were previously collected. The first and the last block (7 plants) of each row were discarded to avoid edge effect. After sample collection, the material was transferred to the laboratory and subjected to the pre-frost (i.e. control) protocol as detailed in the sections below (2021, 2022 and 2023). During specific samplings, some supplementary nodes ($n = 10$ for each treatment, position 10 to 12) were collected and subjected to a post-frost and/or a recovery treatment (2022 and 2023, sections below). All samples for all analyses were maintained hydrated via keeping them in plastic bags with humid tissues. In Supplementary Figure 1 a summary of the sampling period and treatment applications as well as the working scheme for the three years is reported for each season (pre-frost control, post-frost, recovery).

2.3. Dynamic of ecodormancy release via chlorophyll fluorescence analysis

Buds for each sampled node and for each variety were photographed to subsequently confirm their phenological phase using the BBCH scale. The bud/shoot (depending on BBCH) was subjected to one hour of thermal and dark adaptation at room temperature (20 °C in the dark). This was necessary to avoid potential photoinhibition and therefore bias associated with low temperature (early sampling) and sunlight (late sampling for early budburst varieties). The bud was then sectioned (perpendicularly to the cane in 2021, and in parallel to the cane in the years 2022 and 2023) at room temperature in the dark with a BIZ 700 187 cutter (Paris, France). Measurements were taken using a chlorophyll fluorescence portable system Handy PEA (Hansatech Instrument Ltd., Norfolk, UK) and a modified clip for dark adaptation (removal of the basal part of the clip to allow full illumination on the bud section) (please see Zulini et al., 2010). All samples were dark-adapted before measurement for one hour and care was taken in placing the modified clip (3 mm diameter) onto the bud section. When this was not applicable, the node or the value collected were discarded. The maximum quantum yield of PSII efficiency in dark-adapted samples (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_o)/F_m$, where F_o and F_m represent the minimum and maximum (after a saturation pulse of 3500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) fluorescence, respectively (Bertamini et al., 2021; Murchie and Lawson, 2013). Measurements were carried out in the dark and 20 °C room temperature to avoid light effect on chlorophyll fluorescence values. Samplings (i.e. control treatment) were carried out on: March 8th, 26th and 31st, and April 7th and 15th in 2021 (i.e. $n = 5$ samplings in $n = 10$ replicates); February 8th and 24th, March 4th, 17th, 24th and 31st, April 11th, 15th in 2022 (i.e. $n = 8$ samplings in $n = 10$ replicates); February 13th, 20th and 27th, March 6th, 20th, 27th and 28th and April 3rd, 6th and 17th in 2023 (i.e. $n = 10$ samplings in $n = 10$ replicates). When appropriate, these data were used as pre-frost data (control) in the frost experiments (below).

2.4. Controlled-environment frost simulation: frost protocol, post-frost analysis and recovery

Nodes collected in the field of each cultivar were stored as above and then randomly placed in a plastic rack. The rack was then positioned in a MIR-154-PE Cooled Incubator (PHC Europe B.V., Nijverheidsweg, Netherlands), at a temperature of -6 °C for 6 h (2022). In the year 2023 a more realistic frost simulation was applied. The cooled incubator was set to expose buds to a dynamic set of temperatures ranging from 0 °C to -4 °C and back to -1 °C for 3 h. This dynamic of late frost application represented a generalized late frost event that occurred in Trentino region in 2017 and was monitored via a weather station located in San Michele all'Adige (Italy; 46.183512 N, 11.120566 E). The frost simulation protocol for 2023 is summarized in Supplementary figure 2. The rack was removed from the cooled incubator at the end of the simulated frost event, then the same protocol of the pre-frost control was applied for fluorescence analysis. All the treatments were applied in dark conditions to avoid light effects. Samplings for this treatment were carried out on March 31st, April 11th and 15th in 2022 (i.e. three samplings in $n = 10$) while in 2023 on April 6th and 17th (i.e. two samplings in $n = 10$). For this analysis, in 2023, only nodes from a sub-set of the 21 varieties were used (Chardonnay, Gewürztraminer, Marzemino, Yellow Muscat, Syrah). The recovery assessment was carried out in 2023 and in the selected sub-set (Chardonnay, Gewürztraminer, Marzemino, Yellow Muscat, Syrah) three hours after stress application. Nodes were photographed, to later assess their phenological stage using the BBCH scale. Samples were then randomly placed in a plastic rack and subjected to the frost cycles. The rack was then retrieved and kept at room temperature (20 °C) in the dark for 8 h. Subsequently, recovery from cold stress was assessed with chlorophyll fluorescence as above. Samplings for this treatment were carried out in April 4th and 17th 2023.

2.5. Osmotic potential analysis

Buds from post-frost protocol in 2023 were rehydrated for 4 h in distilled water to reach full turgor. After thawing, the cell sap was collected using a garlic press and centrifuged for 15 min. Then, supernatant was collected and 50 μl was used for osmotic potential assessment via a freezing point osmometer (Osmomat 3000, Gonotec, Germany) previously calibrated using the 100, 290, 1000 mosmol kg^{-1} standards. After each measurement, the osmometer tip was rinsed using deionized water. Finally, the resulting osmolality (mosmol kg^{-1}) was converted to osmolarity (MPa) using the following formula: ψ_s (MPa) = $-c$ (mosmol kg^{-1}) $\times 2.58 \times 10^{-3}$.

2.6. Statistical analysis

Statistical analyses were carried out with R software (version 4.3.1, R Core Team 2018, PBC, Boston, USA, <http://www.rstudio.com/>). All data were checked for normality and homoscedasticity through visual assessment of distribution and residuals versus fitted values. When skew distribution was present, data were log-transformed. Relationships between variables were assessed following linear or polynomial (quadratic) functions and goodness of fit, equation and significance are provided in the graphs or in tables. Estimation of GDD₆ at which BBCH07 occurred was carried out via linear regression and the equation for each replicate ($n = 10$) was solved to estimate y with a fixed x of 7 (i.e. BBCH07). Data were subjected to two-way ANOVA, one-way ANOVA or ANCOVA depending on factor number. Means separation was carried out via Tukey's test.

3. Results

3.1. In field assessment of the dynamic of ecodormancy release

Environmental monitoring showed significant variation for chilling

accumulation for the different seasons (Fig. 1A). In particular, while the dynamic of accumulation for 2020 and 2021 (i.e. associated with the endodormancy release for the subsequent seasonal ecodormancy build-up in 2021 and 2022 respectively) started around DOY 290, in 2022 chilling accumulation started around DOY 310. The highest chilling unit accumulation was observed in winter 2021, the lowest in winter 2022. Cumulative rainfall was higher for 2021 than 2022 and 2023, with a total of 240, 145 and 150 mm at the end of April, respectively (Fig. 1B). 2022 and 2023 were characterized by slightly higher mean air temperature compared to 2021 (Fig. 1C) although in 2002 the increase in average temperature above 6 °C started later (DOY 50–60) than 2021 and 2023

The dynamic of cumulative GDD₆ provided evidence of significant differences in thermal unit accumulation between the three field seasons (Fig. 2A). While in all years, the initiation of thermal units' accumulation was very similar, a significant higher GDD₆ build-up was evident for 2023 compared to 2021 and 2022 and in particular starting from end of February (DOY 60–70). On the contrary, similar GDD₆ accumulation was observed between 2022 and 2021 although slightly higher cumulative GDD₆ was observed in March 2021 compared to 2022. BBCH progression between years and for all the varieties tested (i.e. the mean of BBCH occurrence mediated for all the genetic material) was quicker in 2023 for most of the phenological stages assessed when compared to 2021 and 2022. On average, BBCH07 was reached in DOY 109 in 2021, DOY 101 in 2022 and DOY 93 in 2023 (Fig. 2B).

As expected, there was a significant association between GDD₆ accumulation and BBCH onset on average for years and varieties (Fig. 3A). This relationship was significantly ($p < 0.001$) explained by a quadratic function with positive, yet minimal, slope suggesting a near-

linear phenological response at increasing cumulative GDD₆. For characterizing the GDD₆ at which onset of BBCH07 occurs (sections below), a linear model (linear regression) was used assuming minimal effect of the curvature, mainly driven by the slow BBCH dynamic at low GDD₆. Thermal accumulation in the bud/shoot was significantly associated with F_v/F_m value analysed in the bud/shoot section ($p < 0.001$, Fig. 3B) although the relationship reaches a plateau around GDD₆ 150–200. Similar relationship and for average values (i.e. varieties and years) was observed between the quantum yield of PSII in the bud section and the BBCH ($p < 0.001$, Fig. 1C). Similarly to the F_v/F_m to GDD₆ association, the F_v/F_m to BBCH association plateaued at around BBCH07 (budburst).

Monitoring the dynamic of ecodormancy release in 21 varieties of *Vitis vinifera* via chlorophyll fluorescence, provided evidence of varietal-specific dynamic of PSII efficiency progression (Supplementary Fig. 3). For all the varieties, the relationship between F_v/F_m with BBCH and GDD₆ was quadratic ($p < 0.001$, Fig. 4). Indeed, the goodness of fitness (R^2) was 0.80–0.85 for all relationships. Overall, variation in baseline F_v/F_m (i.e., at GDD₆ close to 0) was observed between varieties (Fig. 4, contrasting varieties) associated with steeper F_v/F_m dynamics under developing GDD₆ and/or BBCH (Supplementary Table 3, e.g., Chardonnay, Marzemino, Syrah). Overall, the F_v/F_m value at which BBCH07 was observed in all varieties, ranked from 0.66 of Pinot blanc to 0.79 of Lagrein. Significant ($p < 0.001$) inter-annual effect was observed for F_v/F_m values and for each variety when GDD₆ or BBCH were treated as covariates (Supplementary Table 3).

The relationship between GDD₆ and phenology was further explored to estimate via linear regression the GDD₆ required to reach BBCH07 in each variety and for each year of assessment. Significant variation was overall observed ($p < 0.001$) between varieties on average (Fig. 5A).

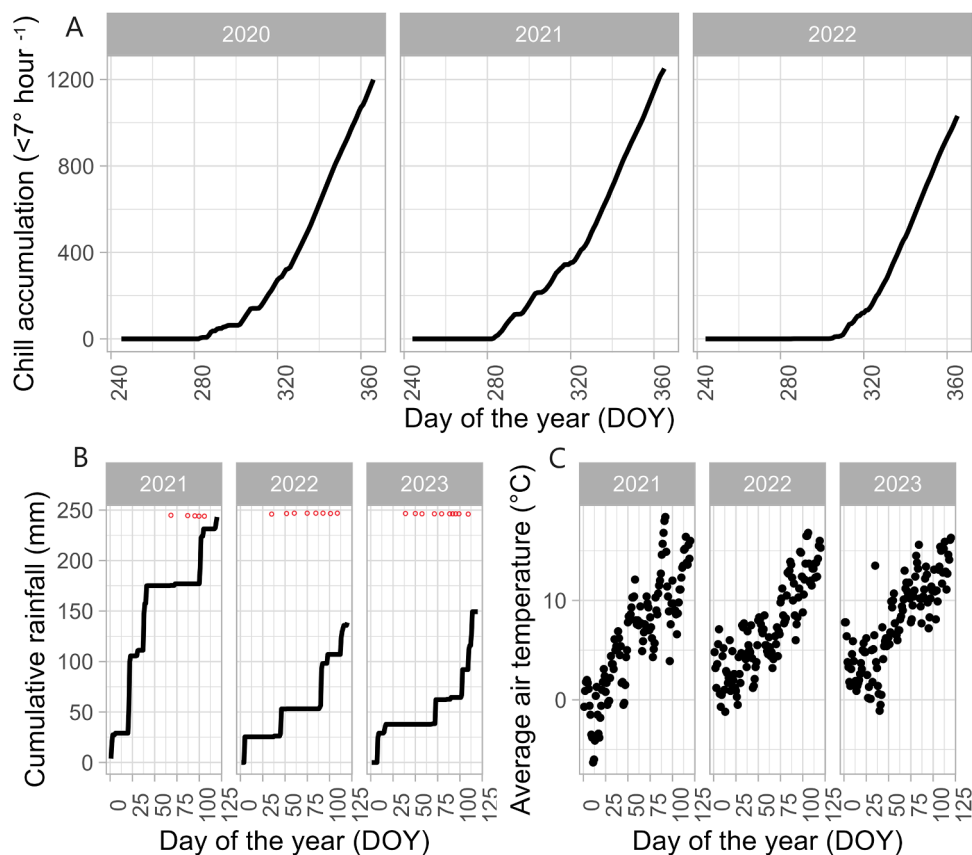


Fig. 1. Development of hourly chilling unit accumulation (A) over winter (September–December) assessed as temperature below 7 °C and for the year preceding the analysis. In B, cumulative rainfall (mm) is shown for the three seasons and over the period of sampling (January–April). Red dots represent the sampling date as described in materials and methods (but expressed in day of the year, DOY). In C, the daily average temperature is shown for the three seasons and over the period of sampling (January–April).

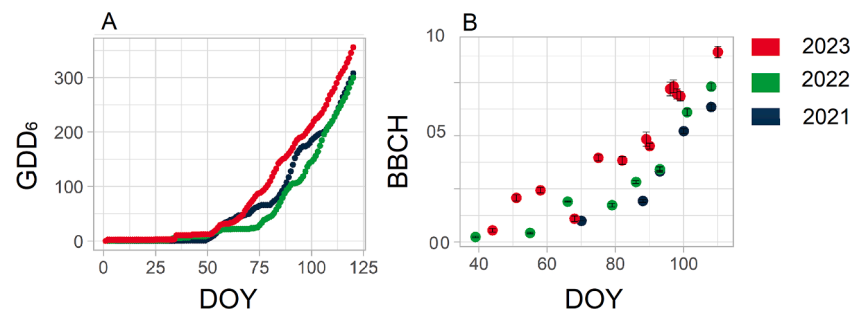


Fig. 2. Cumulative growing degree days in base 6 (GDD_6) accumulation for the 2021, 2022 and 2023 seasons (A) and relative BBCH progression for the same years from day of the year (DOY) 0 to 120 (B). Points for B are average values and error bars represent standard error of the mean (SEM).

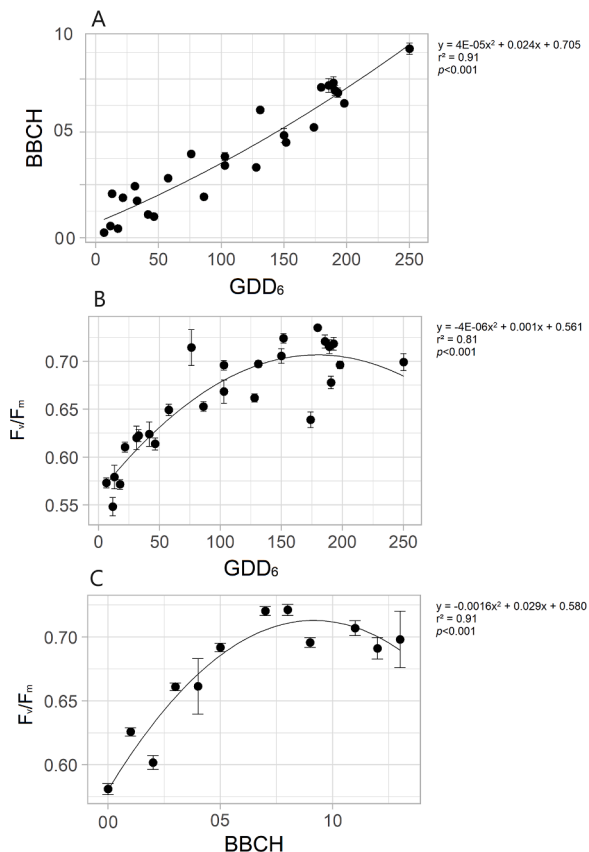


Fig. 3. Relationship between GDD_6 and phenological onset assessed via BBCH scale (A). In B) and C) the relationship between F_v/F_m with GDD_6 and BBCH07 respectively is shown. Points for C to E are average values (years and variety). Error bars represent standard error of the mean (SEM). The curve was fitted with a quadratic function while goodness of fit, significant and equation of the fitted function is shown in the graph.

Three main groups of GDD_6 requirements were detected, in particular: low GDD_6 requirement to reach BBCH07 (i.e. early varieties) such as Nosiola, Nebbiolo, Marzemino, Chardonnay and Teroldego (Fig. 5A, low heat requirement). This group requires an average of 140–150 GDD_6 to reach budburst. On the contrary, there was a group of varieties with opposite behaviour characterized by high GDD_6 requirements to reach BBCH07 that were Merlot, Incrocio Manzoni, Lagrein, Rebo, Syrah, Moscat rose, Schiava, Pinot blanc, Pinot gris and Riesling that showed a thermal requirement for reaching BBCH07 ranking from 210 to 250 on average (Fig. 5A, high heat requirement). The remaining varieties were characterized by a thermal requirement between 160 and 200 GDD_6 to reach BBCH07 (Fig. 5A, intermediate heat requirement). However, factor Year was significant ($p < 0.001$) suggesting the existence of an

inter-annual variation (Fig. 5B). Indeed, significant differences were observed in each year for some varieties, with for instance Chardonnay, Riesling, Gewürztraminer, Lagrein, Incrocio Manzoni, Muscat blanc, Yellow muscat and Pinot gris showing high ($p < 0.001$) plasticity to GDD_6 accumulation hence reaching BBCH07 at different GDD_6 values over two-to-three years. On the contrary, some varieties such as Syrah, Teroldego, Sauvignon, Pinot noir, Cabernet franc did not show variation in GDD_6 requirements between years of assessment.

3.2. Controlled late frost application and genotypic variation for late frost tolerance

The late frost simulation in cane nodes in 2022 showed significant variation between varieties, mainly associated with post-frost analysis of the F_v/F_m . Overall, applying -6°C over three specific dates in 2022 showed significant treatment effect ($p < 0.001$) with a general reduction in F_v/F_m compared to the control pre-frost treatments that was significant for early budburst genotypes since 31 March 2022 (e.g. Marzemino and Teroldego) (Fig. 6A). However, from 11th April 2022, most of the varieties reduced drastically F_v/F_m and compared to the control pre-frost treatments below bud viability maintenance (i.e. below 0.5–0.4 as proposed by Zulini et al., 2010) with only the late budburst genotypes (i.e. Merlot, Lagrein, Syrah, Pinot blanc, Pinot noir) partially maintaining bud viability (Fig. 6B). Indeed, under low temperature conditions, there was a clear association between F_v/F_m (on average over three frosts applications) and BBCH of the bud-shoot, although specific varieties (i.e. Gewürztraminer) were capable at maintaining higher F_v/F_m even at advanced phenological patterns (e.g. close to BBCH07) (Fig. 6C).

Late frost simulation events in cane buds sampled in the field in 2023 for a sub-set of genotypes based on their phenological onset confirmed significant variation between varieties, mainly associated with post-frost analysis of the F_v/F_m (Fig. 7A). Overall, applying a dynamic reduction in ambient temperature showed significant effect ($p < 0.001$) compared to the control pre-frost treatments. On average, the reduction in F_v/F_m was below the viability threshold only for Marzemino, followed by a minimal recovery dynamic. The relationship between F_v/F_m and BBCH confirmed the maintenance of high F_v/F_m for advanced phenology in Gewürztraminer and in 2023 also for Chardonnay (Fig. 7B). Indeed, analysis of the reduction in F_v/F_m at increasing phenology revealed varietal-specific trends with Marzemino being highly susceptible to frost events at early phenological stages (i.e. before budburst) while Syrah, although particularly sensitive, maintained unaffected F_v/F_m values only up to BBCH05 followed by a sharp drop starting from budburst (Fig. 7C). Chardonnay and Gewürztraminer, however, maintained high F_v/F_m even at post-budburst phenological stages (i.e. up to BBCH12). No significant differences were observed for osmotic potential in pre and post frost shoots (all BBCH above 07) although a tendency in more negative values were observed for Syrah and Gewürztraminer (Fig. 7D, $p = 0.254$).

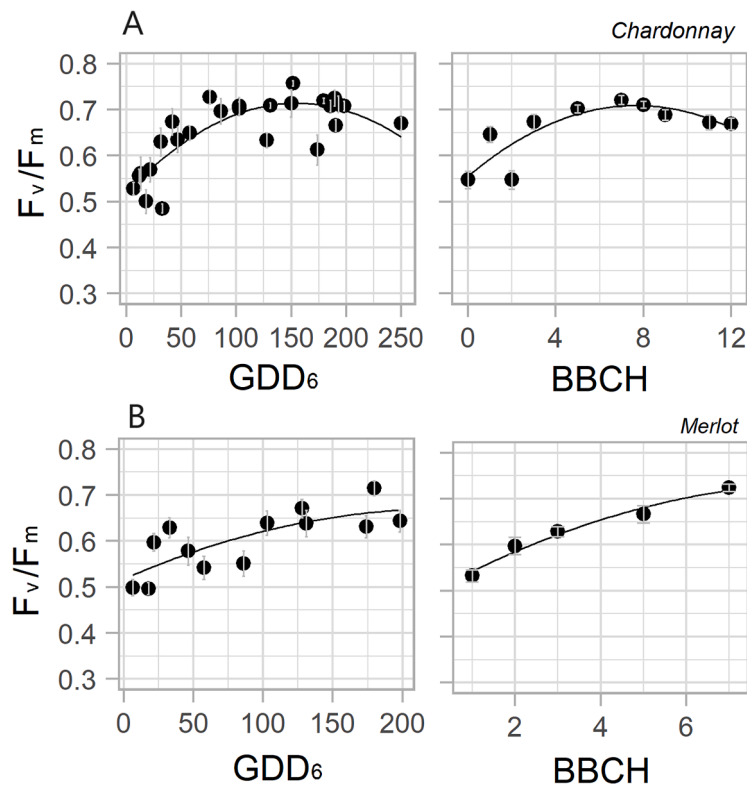


Fig. 4. Example of phenotypic variation for dynamic quantum yield of photosystem II in dark adapted material (F_v/F_m) for the bud-shoot of two contrasting varieties (Chardonnay, A and Merlot, B) at increasing GDD_6 and/or BBCH. Points are means over two-to-three years ($n = 30-40$) and error bars represent standard error of the mean (SEM). Fitting represents quadratic functions for all the graphs while relative equations, goodness of fit and significance are shown in Supplementary Fig. 3 and Supplementary Table 3 for each of the variety tested in this work.

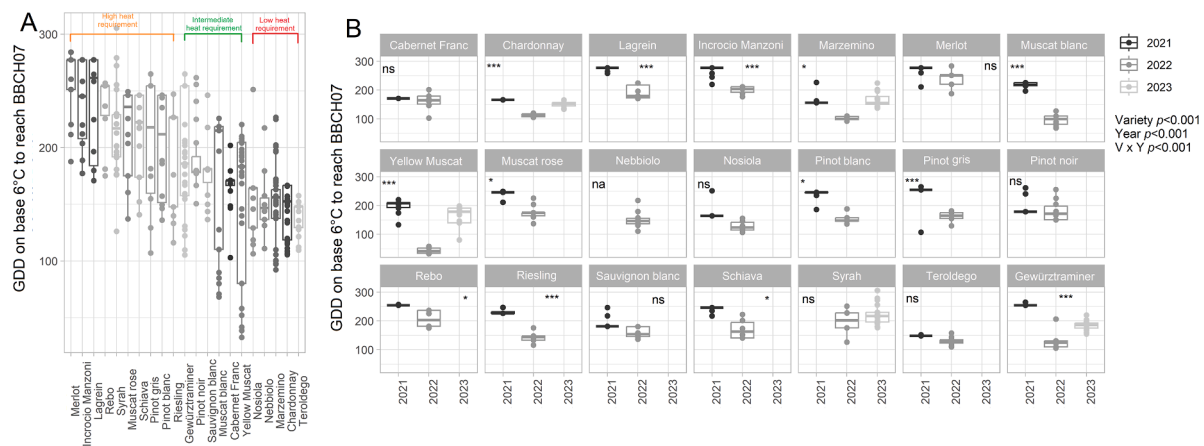


Fig. 5. A) Growing degree days on base 6 °C (GDD_6) accumulation to reach BBCH07 on 21 *Vitis vinifera* varieties over two-to-three field seasons. Data were estimated via linear regression and subsequently the linear equation was solved for BBCH07 ($n = 10$ per year, $p < 0.001$). The three main groups of varieties (high, intermediate, and low heat requirement to reach BBCH07) are highlighted in the figure. B) Growing degree days on base 6 °C (GDD_6) accumulation to reach BBCH07 on 21 *Vitis vinifera* varieties and defined for each year. Asterisks represent significant differences between years and for each variety following one-way ANOVA (***) $p < 0.001$, * $p < 0.05$, ns $p > 0.05$, na interaction not applicable). In the graphs, points represent raw data while horizontal lines within boxes indicate the median, and boxes indicate the upper and lower quartiles. Whiskers indicate the ranges of the minimum and maximum values. Data were analysed with one-way ANOVA and p-values are shown in the graph.

4. Discussion

4.1. Chlorophyll fluorescence can be used as a quantitative method to assess ecodormancy release in *Vitis vinifera*

In agriculture, phenological progression for a given species represents the ability to grow a crop within the defined climatic regime. The

knowledge of the onset for specific phenological stages in grapevine is advantageous as management and chemical practices can be applied at optimum times (Jones and Davis, 2000). Several methods for monitoring phenology are at the moment in use for agricultural purposes: (1) operator visual observations usually conducted on a plant-to-plant scale (Lancashire et al., 1991; Koch et al., 2009; Denny et al., 2014; Nordt et al., 2021), (2) drone or in-situ continuous measurements, which are

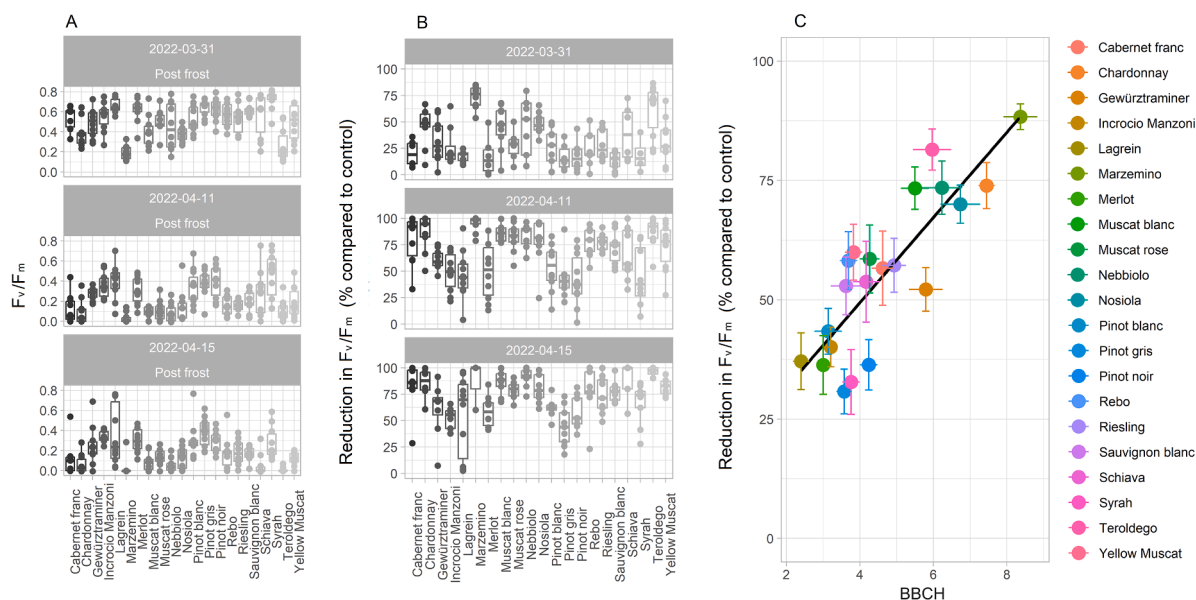


Fig. 6. A) Quantum yield of photosystem II (F_v/F_m) in sections of dark-adapted bud-shoot for 21 *Vitis vinifera* varieties subjected to a simulated late frost condition ($-6\text{ }^\circ\text{C}$ for 6 h) in controlled environmental conditions ($p < 0.001$ for the three experiments and post frost F_v/F_m). B) Reduction (%) in F_v/F_m values following frost conditions and compared to control pre-frost values ($p < 0.001$ for the three experiments). In the graphs, points represent raw data while horizontal lines within boxes indicate the median, and boxes indicate the upper and lower quartiles. Whiskers indicate the ranges of the minimum and maximum values. Data were analysed with one-way ANOVA. Data are from field season 2022 and data were taken before and after stress application. C) Association between reduction in F_v/F_m and BBCH on average (i.e. including all the three frost applications) for all the 21 varieties tested.

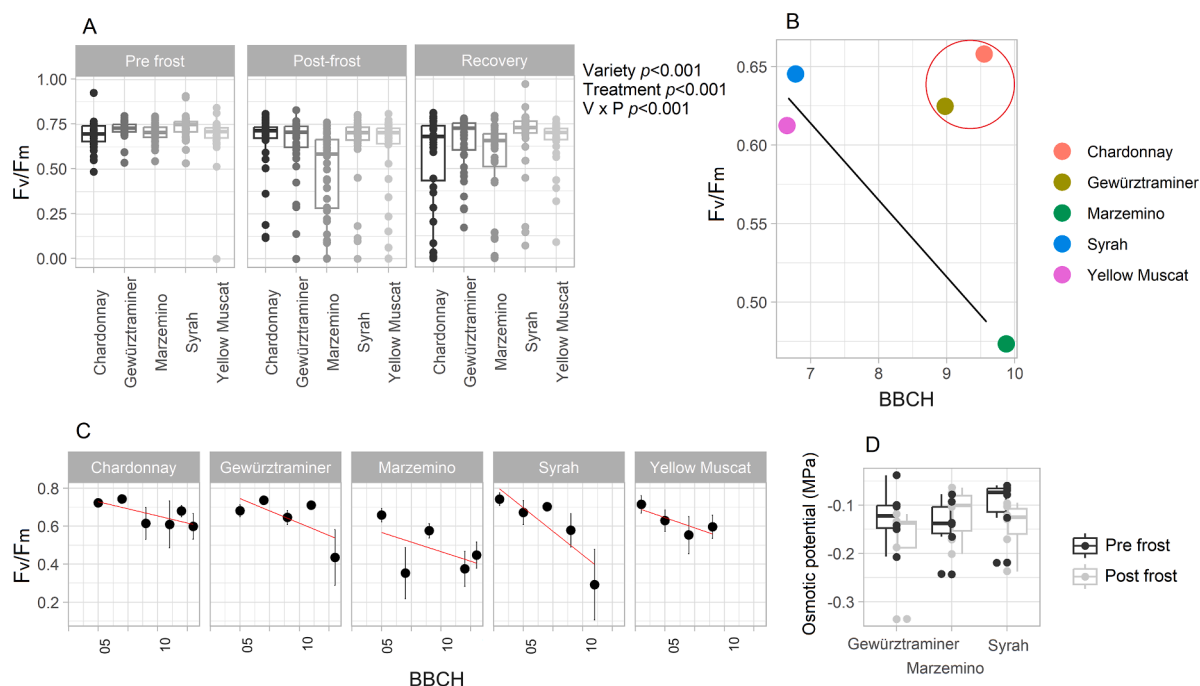


Fig. 7. A) Quantum yield of photosystem II (F_v/F_m) in sections of dark-adapted bud-shoot for five *Vitis vinifera* varieties subjected to a simulated late frost event under controlled environmental conditions. Data were taken before (control treatment), immediately after and three hours after the stress application. In the graphs, points represent raw data while horizontal lines within boxes indicate the median, and boxes indicate the upper and lower quartiles. Whiskers indicate the ranges of the minimum and maximum values. Data were analysed with two-way ANOVA and p-values are shown in the graph. Data are from field season 2023 and analysis were carried out before and after stress application on the 6th and 17th of April. B) Association between F_v/F_m and BBCH on average (i.e. including all frost applications) for all the 5 varieties tested in 2023. Red circle represents variety that maintain high F_v/F_m at increasing BBCH. C) Dynamic of F_v/F_m reduction under a frost event in nodes at different phenological stages. Data are means ($n = 10$) and lines are fitted with linear regression while error bars represent the standard error of the mean (SEM). D) Osmotic potential of shoot sap in three contrasting varieties and collected in pre and post frost application ($n = 5-6$). Data were analysed with two-way ANOVA and p-values are shown in the graph or in the text.

carried out on a regional to local scale (Zorer, 2022; Richardson et al., 2009; Brown et al., 2016; Richardson, 2018), and (3) satellite remote sensing which is applied on regional to global scales (Cleland et al., 2007; Richardson et al., 2013). While 2) and 3) resulted in attractive approaches with potential for spatial evaluation and large-scale analysis, the issue remains concerning quantitative methods for plant-to-plant phenological assessment. Indeed, phenological evaluation via visual observation remain largely in use (Cameron et al., 2022; Tomasi et al., 2011), potentially because easy and straightforward yet exposed to biases from operator interpretations and subjectivity in relation to within-phase estimation. Methods for a robust phenological assessment should be quick, reliable, non-destructive and quantitative (Zorer, 2022) and while 2) and 3) may fall within this description, they lack (so far) of “in-plant” specificity (e.g., grapevine apical dominance assessment in a fruiting cane may not be feasible with a drone via i.e., RGB, to our knowledge). In our work, we evaluated a quantitative protocol (i.e., chlorophyll fluorescence) for monitoring budburst that, however, requires the dissection of the bud (i.e. destructive) and time for dark-adaptation and in-field sampling. Overall, while the proposed approach is an attractive method for experimental work, at this stage the lack of throughput limits the methodology for large screening or even farm-level analysis. Further work is needed to characterize the possibility to integrate other imaging approaches (e.g. chlorophyll fluorescence or hyperspectral imaging) that may combine quantitative assessment with spatial tissue evaluation e.g. dormant bud as well as secondary dormant buds viability. This analysis, that can only be replaced by in-vivo microscopy (that lack of a quantitative source and is still destructive) (Ferrara and Mazzeo, 2021), revealed high specificity for distinct phenological onset and physiological processes. First, due to the significant association with GDD₆ and BBCH under similar environmental conditions, we propose the possibility to use the fluorescence emission from the bud section as a putatively proxy of pre-BBCH07 ecodormancy release. Yet, phenotypic variation for F_v/F_m value at BBCH07 was observed suggesting 1) the need to consider a potential varietal effect on the fluorescence emission but also that 2) the differences in F_v/F_m between varieties at BBCH07 can be putatively associated with different capacity of photosynthetic protein build up at the time of budburst. Second, the reduction in F_v/F_m after budburst seen in all varieties and the relative variation for pre-BBCH07 F_v/F_m value suggests that young early-autotrophic shoots/leaves, by developing light capture ability earlier than CO₂ assimilation capacity (Sperdouli and Moustakas, 2012), requires photoprotective mechanisms (non-photochemical quenching) and therefore energy dissipation capacity to avoid possible photodamage to the PSII. Indeed, this transient reduction in F_v/F_m may indicate that the transition between heterotrophy and autotrophy varies between varieties potentially via prioritizing either resource allocation even after budburst for shoot growth (low F_v/F_m at BBCH07) or photosynthetic CO₂ assimilation autotrophy (high F_v/F_m at BBCH07). In essence, this evaluation may help both modelling or defining underlying mechanisms associated with ecodormancy release and therefore weighing management approaches applied with the aim of postponing budburst (i.e., late pruning) (Poni et al., 2022). Further work may help dissecting additional processes in relation to early phenological progression in *Vitis vinifera*.

4.2. Thermal accumulation to reach budburst differs between varieties while showing environmental driven plasticity: new opportunities for defining frost tolerance via escape strategies?

Budburst is a key phenological stage for grapevine with large site and cultivar variability (Tomasi et al., 2011; Jones and Davis, 2000; Cameron et al., 2022). Indeed, BBCH07-09 defines the beginning of the growth cycle and any delay or anticipation in this stage impacts the whole cycle with significant effects on flowering time, ripening period and harvest date (Cameron et al., 2022; Gambetta and Kurtural, 2021; Xyrafis et al., 2022; Keller, 2010; Van Leeuwen et al. 2019). It is not

casual that three out of four autochthonous varieties belonging to Trentino Alto-Adige region (Teroldego, Nosiola, Marzemino) were also reaching BBCH07 with very low thermal requirements. This may corroborate the common local concept in which low GDD₆ requirement to reach BBCH07 in a generally cold environment (i.e. prior to the sudden and generalized increase in average air temperature due to climate change) may have been considered a preferable trait potentially associated with specific oenological aims such as: i) capacity of resume the vegetative period as soon as the long winter ends and ii) quick phenological progression with the aim of reaching a desirable °Brix at harvest in a relatively cool summer and with adequate crop load. At these climatic trends, however, these varieties are constantly threatened by late frosts as early phenological progressions following a warmer winter and anticipated GDD accumulation means that the young shoot is primarily growing in periods of high risk of low nighttime-to-dawn temperature (Poni et al., 2022).

Our data characterized two specific dynamics associated with varietal variation. First, a large variability for GDD₆ accumulation to reach BBCH07 exists within the varieties selected (Fig. 3A). This information is critical to potentially guide viticulturists in selecting varieties with high thermal requirements in vineyard or portion of the vineyard with specific risks of late frosts (e.g., where physical barrier exacerbates thermal inversion) (Poni et al., 2022). This choice should be coupled with additional information and quantitative data about varietal, but also intra-varietal variability as shown for other specific traits in grapevine associated with stress tolerance (e.g. Bertamini et al., 2021; Faralli et al., 2022a,b; Faralli et al., 2023). The latter (i.e. intra-varietal variation), if significant, may provide homogeneous genetic material for a specific oenological aim without impacting farm varietal choice. Second, we highlighted significant plasticity in some varieties for GDD₆ accumulation requirements to reach BBCH07 (Fig. 3B). This preliminary result firstly suggests that, as proposed by Shellie et al. (2018), asynchronization of the dormant and active bud states may occur following erratic environmental conditions. Chilling units' accumulation over winter as well as winter-to-spring cumulative rainfall and air temperature may additionally explain part of the inter-annual variability observed. For instance, although in 2022 the chilling units build up was delayed and cumulatively lower compared to the previous years (suggesting a potential reduced need for cold hardiness de-acclimation, hence lower GDD requirements to reach BBCH07 (North and Kovaleski, 2022), the lack of spring rainfall may have partially hampered early phenological onset, stabilizing thermal requests for BBCH07 to similar values compared to 2021. We provided phenotypic variation for potential plasticity to dormancy x environment response, with Syrah, Teroldego, Sauvignon, Pinot noir, Cabernet franc showing similar GDD₆ requirements over years to reach budburst. Although the physiological and biochemical basis underlying these trends are unknown, the consistency in GDD₆ requirement of these varieties make them more suitable for monitoring or predicting late frost damages or, more in general, budburst date.

4.3. Frost tolerance per se vs phenologically driven escape strategies: determine the interactive response to select adapted varieties?

Late frost is becoming an increasing problem in viticulture (Poni et al., 2022). The increase in frequency in these erratic phenomena is associated with warmer winter-to-spring seasonality that can anticipated budburst, hence leading to higher risk of late frost in periods where freezing temperatures are likely to occur (Lavalle et al., 2009). Although frost damages during and closely after budbreak may be compensated by secondary buds, generally yield is significantly reduced (Del Zozzo et al. 2022). In our work the dynamic of phenology with bud-shoot sensitivity to frost was evident: simulated late frost applied after BBCH09 on average implies reduction in F_v/F_m values (below the viability threshold of 0.5–0.4 as proposed in Zulini et al., 2010) in all the varieties. Our work confirms the negative link between cold hardiness

de-acclimation processes (and subsequent ecodormancy release) and freeze sensitivity of the growing buds. A few reports provided evidence of the absence of any acclimation dynamic for young shoot to low temperature (Fuller and Telli, 1999; Probesting et al., 1980) due to the loss of supercooling ability. In these studies, actively growing shoot (i.e. after BBCH09) failed to survive following a temperature treatment below $-2.5\text{ }^{\circ}\text{C}$ although buds in winter can survive much lower temperatures. Our work provides, for the first time to our knowledge, genotypic variation for post-budburst frost tolerance: Chardonnay and Gewürztraminer, were characterized by a F_v/F_m maintenance following a simulated late frost suggesting the presence of putative cold acclimation and/or late frost tolerance. A previous study by Fuller and Telli (1999) observed significant phenological earliness in budbreak for Siegrebbe compared to Madeleine Angevine, although no differences in cold tolerance *per se* were detected. However, a few American *Vitis* species (e.g. *Vitis riparia*), are known to possess higher cold tolerance in endo-to-ecodormant buds when compared to *Vitis vinifera* mainly via an early accumulation of osmolytes in tissues such as raffinose (Grant and Dami, 2015; Franks, 1985). Several biophysical mechanisms have been proposed for e.g. raffinose-induced cold tolerance in buds during winter, including decreasing the osmotic potential, depressing the freezing point of cell water (Burke et al. 1976), and providing a structure-preserving effect upon binding to proteins and membranes (Grant and Dami, 2015; Santarius, 1973; Lineberger and Steponkus, 1980). Therefore, it is possible to hypothesize differences in water content during spring or in molecules that affect the concentration and osmotic pressure of the cell sap, and hence the ability of the plant organ to supercool, as potential mechanisms underlying the varietal sensitivity after BBCH09 to late frost found in our work (Valle, 2002). However, in our study, only minimal trends were observed for osmotic potential between contrasting varieties suggesting that the concentration of specific osmolytes (e.g. the ratio between different sugars) may be more relevant rather than their total concentration *per se*. In addition, the sampling method (i.e. nodes collected from the field) as well as frost dynamics (i.e. the intensity and duration of the simulated frost) may have masked some of the variation expected, in terms of osmolyte accumulation, under natural field conditions. Although we were partially unable to dissect between tolerance and avoidance strategies for the varieties possessing high F_v/F_m ratio under simulated late frost, our data suggest that some *Vitis vinifera* varieties may display a combination of the proposed mechanisms. Further work should be carried out to better assess the mechanisms underlying the observed trends.

5. Conclusions

The evaluation of early phenological events is a critical step for adapting viticulture to climate change. Linking quantitative data (i.e. BBCH07, F_v/F_m) to specific models (e.g. GDD₆) can be key in defining novel varieties with superior adaptation in a given environment. Our work provides for the first time 1) a proof of concept in the use of chlorophyll fluorescence to detect early phenological onset and cold stress tolerance in grapevine bud/shoot; 2) a comprehensive evaluation of phenotypic variation for thermal requirements to reach BBCH07 as well as varieties exhibiting inter-annual plasticity (different GDD₆ in different years) or elasticity (similar GDD₆ in different years); 3) possible varieties exhibiting specific tolerance or acclimation to low temperature even after BBCH07 although the putative mechanisms should be further investigated. Overall, this work lay the foundation for additional exploration of phenotypic variability in early phenological progression in *Vitis vinifera*, suggesting the need of the assessment of the mechanistic basis underlying the observed differences between varieties.

CRedit authorship contribution statement

Michele Faralli: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation,

Formal analysis, Data curation, Conceptualization. **Samuel Martintoni:** Methodology, Investigation. **Francesco Dotti Giberti:** Investigation. **Massimo Bertamini:** Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Michele Faralli reports administrative support was provided by University of Trento. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.scienta.2024.113169](https://doi.org/10.1016/j.scienta.2024.113169).

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