Phenological forecasting models and pollen quality of *Vitis vinifera* L.

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Abstract

The aim of this study was to test phenological models for predicting the grapevine budburst and flowering stages using data collected between 1990 to 2014 in two Portuguese wine regions: Vinhos Verdes and Lisbon. Also, pollen quality of 15 cultivars of *Vitis vinifera* L. was analysed in this work. The developed models intended to convey temporal and spatial robustness for grapevine using data collected in the most western viticulture zone of Europe. Three different thermal-time models and nine two-phase models were tested. The development of the models was achieved by an iterative process. The cultivars with the longest time series of phenological data for Arcos de Valdevez (Fernão Pires and Loureiro) and Torres Vedras (Fernão Pires) were used in the estimation. The validation was performed using an independent data-set of different cultivars grown in the Vinhos Verdes and Lisbon wine regions. In the thermal-time models, the UniFORC outperformed the two GDD versions tested, presenting overall higher R^2 (0.75) and lower RMSE (4.55) and MAD (3.60), for *t0* date at budburst date. The best overall two-phase model was the one which combines the Chuine and GDD functions, for the stages of chilling and forcing correspondingly. The results showed that the model's goodness-of-fit indicators performed well, with high R^2 (0.75 to 0.91 for flowering), low RMSE (3 to 6 days for budburst and 2 to 4 days for flowering), and MAD (3 to 4 days for budburst and 2 to 3 days for flowering). The phenological models developed in our study presented high accuracy when applied to several cultivars in different regions and can be used as a predictor tool of budburst and flowering in Portugal. In terms of pollen quality, differences among cultivars in the number of pollen apertures were observed under light microscope. All the cultivars studied showed a higher percentage of tricolporated pollen, however, pollen grains containing one, two or four apertures were also observed. The cultivar Loureiro was the one with the higher percentage of pollen grains with four apertures (3.8%) and Touriga Nacional presented 100% of tricolporated pollen grains. The viability analysis showed that 13 cultivars presented values higher than 50%, with 8 cultivars reaching values above 75%. The pollen germination rates vary greatly for the grapevine cultivars studied, three cultivars show low values of germination (under 14%) in the two media tested, which were Touriga Nacional, Cabernet Franc, and Cabernet Sauvignon while others presented high values of germination like Castelão, Loureiro, Malbec and Petit Verdot. No significant statistical differences between the percentages of germination in the two media studied were found for the majority of cultivars analysed.

Keywords:

Vitis vinifera L.; Phenological Modelling; Crop yield; Pollen; Fertility; Morphology;

Resumo

O objetivo do presente estudo foi analisar modelos fenológicos para prever os estágios de abrolhamento e floração da videira, utilizando dados recolhidos entre 1990 e 2014 em duas regiões vitivinícolas portuguesas: Vinhos Verdes e Lisboa. A qualidade do pólen de 15 castas de *Vitis vinifera* L. foi também analisada. Foram testados três modelos de tempo térmico diferentes, que apenas têm em consideração a acumulação de unidades de calor, e nove modelos de duas fases, que integram a acumulação de unidades de calor e de frio. O desenvolvimento dos modelos foi efetuado por um processo iterativo. As castas com séries temporais mais longas de dados fenológicos para Arcos de Valdevez (Fernão Pires e Loureiro) e Torres Vedras (Fernão Pires) foram utilizadas na estimação do modelo. A validação foi realizada utilizando um conjunto de dados independentes de diferentes castas presentes nas regiões vitivinícolas analisadas (Vinhos Verdes e Lisboa). Nos modelos de tempo térmico, o modelo UniFORC superou as duas versões GDD testadas, apresentando R^2 (0.75), RMSE (4.55) e MAD (3.60) mais elevados, quando a data de início de acumulação de unidades de calor foi definida como a data de abrolhamento. O modelo de duas fases que apresentou melhor performance foi aquele que combinou as funções Chuine e GDD, para as fases de acumulação de unidades de frio e de calor respetivamente. Os resultados dos indicadores de ajuste do modelo mostraram que este funcionou bem, com R^2 elevado (0.75 a 0.91 para a floração), baixo RMSE (3 a 6 dias para o abrolhamento e 2 a 4 dias para a floração) e MAD (3 para 4 dias para o abrolhamento e 2 a 3 dias para a floração). Os modelos fenológicos desenvolvidos neste estudo apresentaram bom ajuste quando aplicados a várias castas em diferentes regiões e podem ser utilizados como "ferramentas" para prever os estados de abrolhamento e floração da videira em Portugal. Em termos de qualidade do pólen, quando analisado ao microscópio ótico, foram detetadas diferenças no número de aberturas nas diferentes castas. Todas as castas estudadas apresentaram elevada percentagem de pólen tricolporado, no entanto, também foram observados grãos de pólen contendo uma, duas ou quatro aberturas. A casta Loureiro foi a maior percentagem de grãos de pólen com quatro aberturas (3.8%) e a Touriga Nacional apresentou 100% de grãos de pólen tricolporados. A análise de viabilidade mostrou que 13 castas apresentaram valores superiores a 50%, com 8 castas atingindo valores acima de 75%. As taxas de germinação de pólen variaram muito para as castas estudadas, sendo que três castas apresentam valores baixos de germinação (menos de 14%) nos dois meios de germinação testados, sendo elas Touriga Nacional, Cabernet Franc e Cabernet Sauvignon, enquanto outras apresentaram altos valores de germinação como Castelão

Loureiro, Malbec e Petit Verdot. Não foram encontradas diferenças estatisticamente significativas entre as percentagens de germinação nos dois meios de germinação para a maioria das castas analisadas.

Palavras-chave:

Vitis vinifera L.; Modelação fenológica; Rendimento das culturas; Pólen; Fertilidade; Morfologia

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List of abbreviations

ANOVA - Analysis of variance;

AIC – Akaike Information Criterion

AVV – Arcos de Valdevez

FEL - Felgueiras

GDD – Growing Degree Days

MAD – Mean Absolute Deviation

R^2 - Coefficient of determination

RMSE – Root Mean Square Error

TOV – Torres Vedras

Dissertation outline

The core of this Dissertation is based on a series of three peer-reviewed Articles complemented with the sections Introduction, state of the arte, general discussion and conclusion. Each section is presented here by stating its research goals and by outlining its relationship with other relevant work.

- Introduction – presents the main problem studied, the reasons that stimulated the achievement of this research, the objectives to be achieved, the hypotheses, the research questions and the structure of the dissertation.
- State of the Art – is based in a brief review on the distribution of the grapevine in the world, on the grapevine (*Vitis vinifera* L.) taxonomy and biological cycle, on the phenology history, scales and modelling and on the pollen morphology and fertility.
- Article 1. Predicting the flowering date of Portuguese grapevine cultivars using temperature-based phenological models: a multi-site approach – Phenological models for predicting the grapevine flowering were tested using data collected in two Portuguese wine regions: Vinhos Verdes and Lisbon for 15 grape cultivars (8 white and 7 red). The developed thermal-time models intended to convey temporal and spatial robustness for grapevine using data collected in the most western viticulture zone of Europe. The revised version of this Article was re-submitted for publication on the International Journal of Biometeorology (Impact factor 2.204, 2nd quartile, of the journal Citation Reports, Web of Science WoS).
- Article 2. Two-phase phenological models to characterise the timing of budburst and flowering of *Vitis vinifera* L. – Two-phase phenological models for predicting the grapevine budburst and flowering were tested using data collected in two Portuguese wine regions: Vinhos Verdes and Lisbon for 15 grape cultivars (8 white and 7 red). The developed two-phase model intended to convey temporal and spatial robustness for grapevine using data collected in Portugal. This Article is under preparation for publication on peer-reviewed journal listed on the journal Citation Reports, Web of Science WoS.
- Article 3. Comparison of pollen quality in *Vitis vinifera* L. cultivars – Pollen quality of 15 cultivars of *Vitis vinifera* L. was studied in this work. Pollen morphology was studied by using an optic microscope, viability was tested by the fluorochromatic reaction and germination was analysed by in vitro assays, using two different media. The underlying idea is that the information content derived from pollen quality can be combined with agronomic to improve grapevine' fruit-set. This

Article is accepted for publication on the Scientia horticultural journal (Impact factor 1.624, 1st quartile journal Citation Reports, Web of Science WoS).

- General Discussion – The main results obtained about the use of phenological modelling to predict *Vitis vinera* L. budburst and flowering using the one phase model based on temperatures during the active growth period and the models that includes also the influence of chilling temperatures during the dormancy period (two-phase model). The results obtained in the analyses of the morphology and fertility of grapevine pollen were also discussed in this section.
- General Conclusion – The main conclusions of all the results obtained about the use of phenological modelling to predict *Vitis vinera* L. budburst and flowering are presented as well as the results obtained in the analyses of the morphology and fertility of grapevine pollen. This section also presents suggestions for future work.

1. Introduction

Since ancient times, wine plays an important role in almost all civilizations. The production of this beverage goes back to the beginning of humanity, in a time at the capacity of transforming grape juice in wine was considered a godsend (Bisson et al. 2002). Grapevines are grown in distinct climate regimes worldwide that provide ideal situations to produce high quality grapes. There are currently between 5.000 and 10.000 varieties of *Vitis vinifera* L. (common grapevine) though only a few are of commercial significance for wine and table grape production.

Vitis vinifera L. is a perennial shrub known as 'vine' predominantly tropical and subtropical, having shoots with tendrils and lobed leaves alternating. It presents great longevity and ability to produce grapes even in the most unfavourable soils conditions, being endowed with a well-developed root system (Pinho 1993).

Temperature and photoperiod are fundamental in influencing grapevine (*Vitis vinifera* L.) phenology (Winkler et al. 1974; Duchene and Schneider 2005; Huglin 1978; Jones and Davis 2000; Jones et al. 2005; Van Leeuwen et al. 2008; Zapata et al. 2015). Understanding how temperature influences the timing of grapevine vegetative and reproductive development and identifying varietal specific differences in phenology is fundamental.

Phenological observations are useful for planning and operating a vineyard but this records are expensive which could explained the limited information on grape phenology. Therefore, phenological models have been developed as an important tool for a cultivar of applications in viticulture, such as: the selection of cultivars to be grown in new areas (Bindi et al. 1997; Gladstones 1992; Jarvis et al. 2017), predict the vulnerability to pest attacks and allow a more efficient planning of harvest and viticulture practices (Williams et al. 1985). These models can also be coupled with climate change scenarios to forecast the impact of climate change on grapevine growth and yield (Cola et al. 2017; Bindi et al. 1997; de Cortazar-Atauri et al. 2009). However, the difficulties in obtaining phenological models which provide accurate predictions on a regional scale prevent them from being exploited to their full potential (Caffarra and Eccel 2010).

Phenological modelling requires four essential steps: data collection, model definition, adjustments of the model to the data using an adapted optimization algorithm that ensures correct convergence and tests of the model hypotheses (Chuine et al. 1999).There are several sources of phenological data such as the airborne pollen (Cunha et al. 2015) and remote sensing images (Cunha et al. 2010), but the phenological observations in ampleographic collections are still the most reliable data mainly for grapevine cultivars studies.

During the last decades, several predictive models of flowering dates based on climate data have been described in the literature (Parker et al. 2011; Chuine et al. 2003). Some models contemplate only the effect of temperature during the active growth period (forcing temperatures) such as Thermal Time model (Cannell and Smith 1983) also named Spring Warming model (Hunter and Lechowicz 1992). But other approaches also include the influence of chilling temperatures as the Alternating model (Murray et al. 1989), the Parallel model (Landsberg 1974; Hänninen 1990; Kramer 1994), the Sequential model (Hänninen 1990; Sarvas 1972; Kramer 1994) and Unified model (Chuine 2000).

The development of site-specific phenological models is important because the plants are adapted to the surrounding environment, and differences in the timing of phenological development are observed (Caffarra and Eccel 2010; Parker et al. 2011; Parker et al. 2013). Therefore, the information derived from these local models contributes to understand the plasticity of a species or of cultivars within the same species to regions with different climatic conditions. Besides that, site-specific models are a useful tool to help winemakers in the management of practices in the vineyard, especially at the flowering level.

The main objectives of this dissertation is to evaluated how phenological events can be estimated using temperature-based phenological models across different grapevine varieties and environments. While the phenology events is known to be related to temperature, our knowledge about the predictive modelling of Portuguese grapevines is still limited. The available long term of climate and phenological data provides an excellent instrument to fill this knowledge gap.

Having theoretically identified some important phenological model based on temperature, the next step is to investigate how to apply them to the Portuguese varieties. Four hypotheses can be tested:

– Can phenological events be reliably estimated in Portuguese grapevines varieties using Thermal Time or Spring Warming models (one-phase model) that only contemplates the effect of the temperatures during the active growth period?

– Does the use of chilling temperatures improve the accuracy of the phenological models?

– What is the most appropriate function (linear or nonlinear) to better explain the temperature action on the phenological development?

– How is the transferability of these models among varieties and ecological conditions?

The pollen quality of different pollen grape varieties were also studied. This research could ultimately lead to an improvement on grape pollination efficiency and,

consequently, on the grape productivity, which can be improved by viticulture practices, providing nutrients according to each variety needs.

2. State of the art

2.1. Taxonomy of *Vitis vinifera* L.

The vines belong to the genus *Vitis* which has been classified within the family variously known as *Vitaceae*, *Vitidaceae* and *Ampelidaceae* (Willis 1973), within the botanical order Rhamnales (Cronquist 1981), which also includes the families *Rhamnaceae* and *Leeaceae*. The *Vitaceae* family includes around 700 species which are, in the majority of the cases, tropical or subtropical and spontaneous in America, Asia and Africa, generally they don't have agronomic value (Böhm 2007).

The *Vitaceae* were divided in two sub-families: one belonging to the North America, Europe and Asian temperate zones and the second one corresponding to the South America, Tropical and Austral Africa, Oceania and Meridional Asia tropical and subtropical zones (Galet 1993). The *Vitaceae* family contains 11 genus and about 480 species (Sampaio 1988).

The genus *Vitis* inlcudes two sub-genera: Euvitis (2n=38 chromossomes) and Muscadinia (2n=40 chromossomes), however triploid or tetraploid cultivars can occur by mutations (Branas 1974).

The species, *Vitis vinifera* L., beloging to the Euvitis sub-genera, is responsible for most of the wine produced nowadays. *Vitis vinifera* L. grapevines were once very widespread throughout Europe, from Scandinavia to North Africa, and for that individual cultivars gradually emerged in different places, reflecting the diversity of existing climatic conditions (Amerine and Wagner 1984). The first forms of wild *Vitis vinifera* L. were dioecious, consisting of both male and female plants, but eventually hermaphroditic forms appeared (Unwin 1991) and nowadays are the dominant type, being propagated vegetatively as well as sexually.

Nearly 24 000 cultivars of vine have been named, of which it is probable that only 5 000 are truly different cultivars (Dry and Gregory 1988). However 150 are widely used, but only 9 cultivars are considered to produce ‘classic’ wines (Viala and Vermorel 1901-10; Robinson 1986).

2.2. Vegetative and reproductive development of grapevine

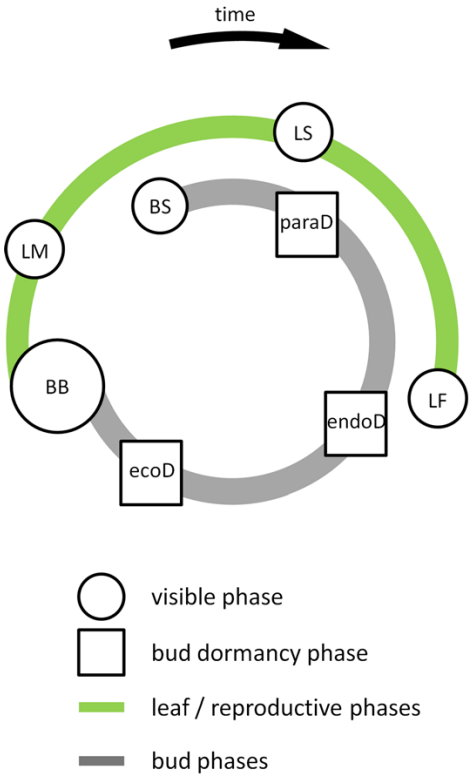
Grapevines, like most other spring flowering perennials, commence forming their flower buds during the preceding season. Flower buds begin to develop in axils of leaf primordia of primary latent buds during late spring and summer before entering a period of dormancy. In response to both shorter day length and cooler temperatures these buds

enter a period of dormancy after which is observed the vegetative and the reproductive growth (Keller 2015). The vegetative growth begins with the break of the dormant buds, passing through different development stages (phenological stages) up to the leaf fall. In the end of the vegetative development the grapevine enters in a dormancy stage where no growth occurs. The reproductive phase starts with the floral initiation (in the spring-summer of the previous year) and formation of new buds until to the development of ripe fruit (which occurs in general at September of the current year).

In cool climates, during the winter, the grapevine does not present visible growth (no increase in the number/volume of organs occurs), being the buds in a state of dormancy or rest (Tromp et al. 2005). In fact, some grapevine parts, such as the shoot tip, cannot tolerate low temperatures and when the plant was exposed to these temperatures for a certain period leafing-out occurs.

Dormancy is based in an interaction between genotype and environment. In this state the organs of the plant are much more resistant to climatic adversities namely low and high temperatures or dryness. So, plants that are capable of establish a dormancy period during unfavourable seasons for their growth are more competitive and more capable of surviving in these conditions comparing to plants that cannot enter this stage (Vegis 1964).

In the grapevine, buds may have three dormancy levels (Sarvas 1974; Lang et al. 1987) as presented in Figure 1: i) ‘para-dormancy’ or ‘summer-dormancy’, where growth is regulated by endogenous factors outside the bud (growth is prevented by hormonal control which is produced from shoots or leaves still growing); ‘endo-dormancy’ or ‘winter-dormancy’ where growth is regulated by physiological factors inside the bud (this inhibition can only be cancelled by low temperature accumulated during a certain extended period); ‘eco-dormancy’ or ‘imposed-dormancy’ where growth is regulated by external environmental factors (Tromp et al. 2005; Andreini et al. 2014).



Code	Phenological phase or stage	Environmental cue
BS	Bud-set	daylength temperatures
paraD	Paradormancy	distal hormonal control
endoD	Endodormancy induction	low temperature, short daylength
	Endodormancy break	low temperature
ecoD	Ecodormancy	warm temperature long daylength
BB	Budburst *Flower / cone emergence*	
LM	Leaf maturation	warm temperature
LS	Leaf senescence	low temperature, short daylength (water stress)
	Fruit / cone growth and maturation	warm T
LF	Leaf fall *Fruit / seed shedding*	

Figure 1 Influence of environmental controls on the timing of leaf and reproductive structures phenophases. Source: Delpierre *et al.*(2016)

With the decreasing of day length, the newly formed buds at the end of the summer may enter a period of inactivity (Caffarra et al. 2011), this phenomena may also occur due to the combination of day length and low temperature at the begging of the autumn (Heidi and Prestrud 2005; Penfield 2008). During this process the leaf and the flower buds of grapevines enter in the endo-dormancy period. Specific accumulation of chilling temperature is necessary to overcome the state of endo-dormancy of the bud in order to budbreak occur (Erez and Lavee 1971; Chuine 2000). Therefore, warmer temperatures during the winter may affect the phenology and cause late budbreak due to a late dormancy break (Viti and Monteleone 1991; Sunley et al. 2006), low percentage of flower budbreak (Viti and Monteleone 1995) and disorganized leafing and blooming, resulting in a high degree of flower bud drop (Erez 2000).

2.3. Plant Phenology

2.3.1. Concept and applications

The history of phenology is very ancient and probably started among the primitive farming societies (Puppi 2007). The great Mediterranean civilisations (Egypt, Mesopotamia), as well as those in Asia (China), have left signs of phenological observations made thousands of years ago (Schwartz 2003).

However, only in 1853 the term "phenology" was proposed for the first time by Charles François Antoine Morren (1807-1858), a Belgian botanist. The term is generally described as the art of observing life cycle phases or activities of plants and animals in their temporal occurrence throughout the year (Lieth 1974). Currently, the notion of phenology comprises the study of the response of recurrent biological events of living beings and their relation to the biotic and abiotic factors (Committee 1972).

Nowadays, phenology provides an important support to the implementation of sustainable crop management providing appropriate dates for some operations like: irrigation, fertilizing, and crop protection and therefore to achieve more stable crop yields and quality (Ruml and Vulić 2005). They are now widely used to predict the impact of the impact of climate change scenarios on crop phenology and vegetation dynamics in different spatial scales.

2.3.2. Phenological scales for the grapevine

The events in the vegetative and reproductive development of the grapevine are characterized by phenological scales some of them presented in the next figures. Baggiolini (1952) proposed a very popular phenological scale composed by sixteen phenophases, between the budburst and the leafs fall, easily identified by observation. This scale was after adjusted by Baggioloni and Baillod (1993) in order to integrate twenty phenophases (Fig. 2).

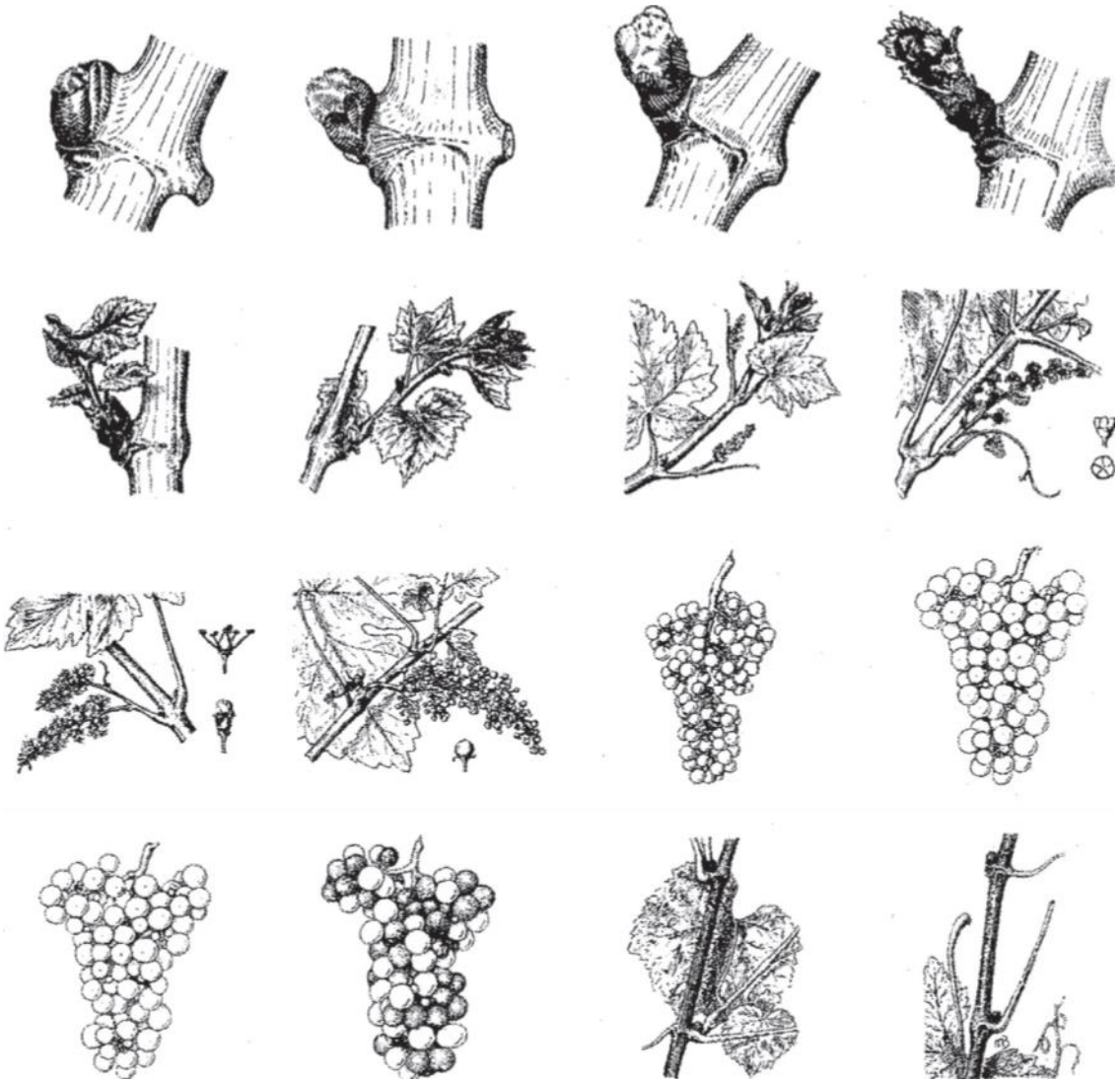


Figure 2 Grapevine growth stages according to Baillod and Baggiolini (1993). Source: Keller (2015).

Eichhorn and Lorenz (1997) developed a scale composed by twenty two phenological stages which present a numerical codification, and its more detailed than the Baggiolini one. This scale was after modified by Coombe (1995) and was named “Modified Eichhorn-Lorenz System”. This last scale is represented by fourty seven phenophases, which are divided into eight principal phases and a detailed set of intermediate phases (Fig. 3).

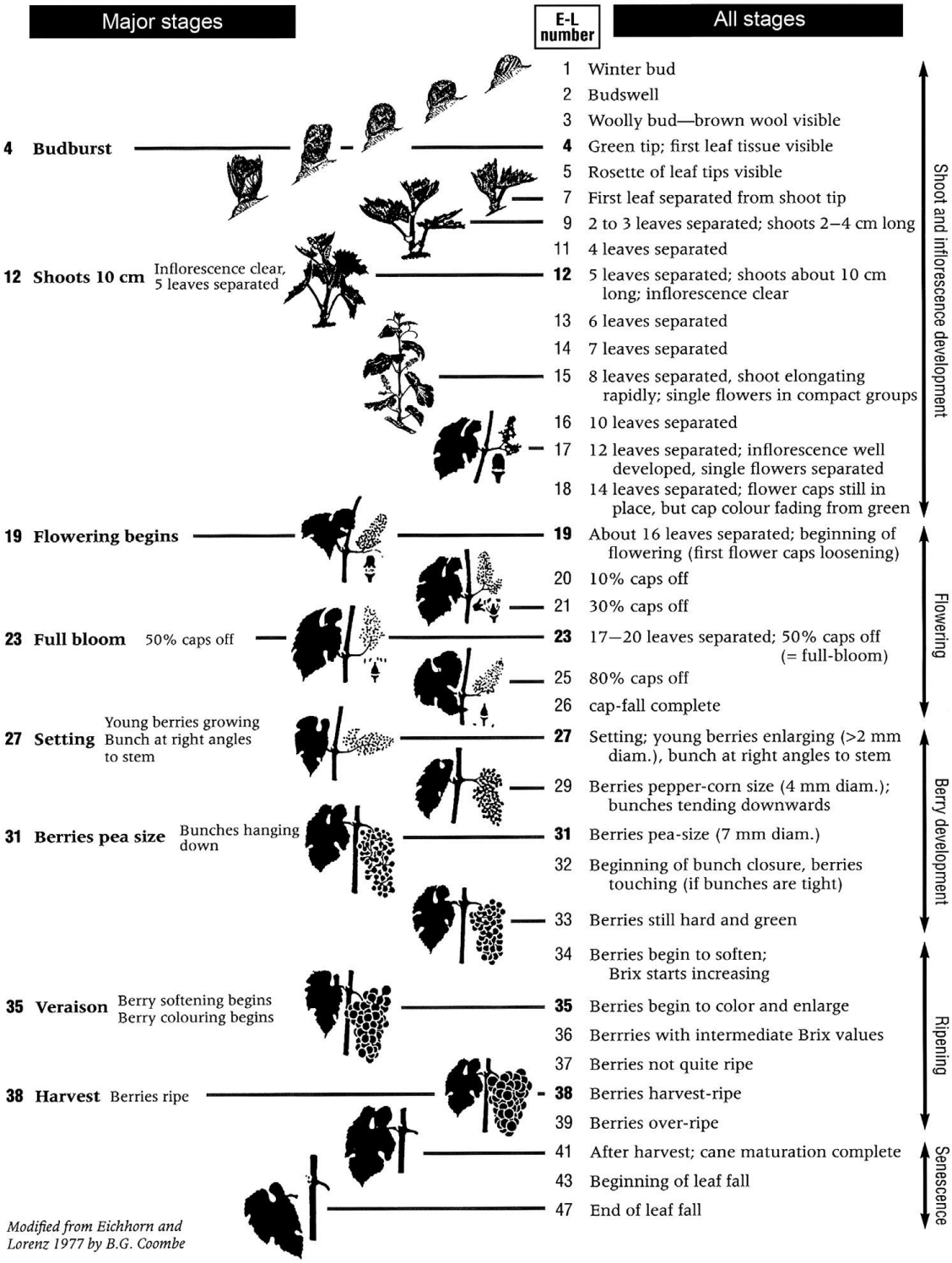


Figure 3 Grapevine growth stages according to the modified scale of Eichhorn and Lorenz by B.G. Coombe (Coombe 1995).

Lorenz (1995) proposed another phenological scale based in an adaptation of BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) for the grapevine. This classification system was originally established to describe the cereals development (Zadocks et al. 1974). The new scale, arose from the adaptation of the

BBCH system, is composed by forty seven principal stages complemented by a multiple number of intermediate micro states (Lorenz et al. 1995; Schwartz 2003).

The developmental phenological events of each plant are subject to variations in timing, even for same variety, according to the variability of the environmental conditions in the vineyard. In some grape varieties there may be a great difference between the date when the first individual and the last individual from the same population reach a given phenological stage. So is necessary to establish a criterion to define when the observed population transitions to a new phenophase. Usually it is defined as the day when 50% of the population reaches a particular phenological stage.

In order to facilitate the field observation of plant phenology with adequate temporal sampling, it may involve the use of remote methods for measuring the transition between stages, such as the use of multiflash cameras for image acquiring along the vineyard (Crimmins and Crimmins 2008), the use of vegetation reflectance indices (Reed et al. 1994; White et al. 2009; Rodrigues et al. 2013; Cunha et al. 2010) or the record of pollen emissions (Cunha et al. 2015; Ribeiro et al. 2006).

2.3.3.Phenological Modelling

Groups of phenological models

The development of phenological models based on temperature probably started with René-Antoine Ferchault de Réaumur (1735), an academic of the Sciences in Paris. Réaumur proposed that crop flowering in cereals occurs when the sum of the environmental temperature from sowing date reaches a certain value. He developed a mathematical model based on temperature – “model of thermic summations”, representing this relationship. In 1950, the French naturalist M. Adanson modified the model developed by Réaumur, introducing the concept of thermal threshold, where the thermal summation is calculated excluding temperatures below 0 ºC. This last model, with some changes and variants, has been widely and successfully applied till today to make phenologial predictions based on temperature (Puppi 2007).

Nowadays, the interest in phenological modelling continues to increase, especially for forecasting the impact of climatic change scenarios on crop and, ecosystems modelling (Puppi 2007).

The phenological dynamic is determined by complex interactions between genetic and environmental factors. Air temperature can be a dominant factor controlling the timing of phenological phases (Hunter and Lechowicz 1992; Galan et al. 2001; Ruml and Vulić 2005). Also, photoperiod, as well as rainfall and solar radiation have influence on the plant development (Friedel et al. 1993). Some studies showed that the impact of the soil temperature, water content, soil type and nutrient supply could be significant

(Wielgolaski 1999). However, is believed that the influence of soil on plant development is usually smaller than the influence of climate (Wielgolaski 2001).

Currently, two groups of phenological models were developed for perennial crops. The first group includes the Thermal Time or Spring Warming models that only contemplates the effect of the temperatures (forcing temperatures) during the active growth period (one-phase model) (Hunter and Lechowicz 1992; Cannell and Smith 1983; Robertson 1968).

The second group also includes the impact of chilling temperatures during the dormancy phase (two-phase model) and contemplates the following models represented on Figure 4: i) Sequential Model (Hänninen 1990; Richardson et al. 1974; Hänninen 1987) which assumes that the effect of forcing temperatures cannot be effective unless chilling requirements have already been fulfilled; ii) Parallel Model (Landsberg 1974; Hänninen 1987, 1990) which assumes that forcing temperatures can be active concomitant with the time spent for chilling conditions and they are not fully active as long as full chilling is not reached; iii) Alternating Model (Murray et al. 1989; Cannell and Smith 1983; Kramer 1994) which assumes a negative exponential relationship between the sum of forcing units required for completion of a growth phase and the sum of chilling units received (Fig. 4);

Other empirical models were proposed to simulate the impact of temperature during the dormancy phase on phenology events: iv) Unified Model (Chuine 2000); v) Deepening Rest (Kobayashi et al. 1982) based on two general functions that describe the relationship between temperature and the rates of chilling and forcing development; vi) Four Phases Model (Vegis 1964; Hänninen 1990) assumes three phases of dormancy (pre-rest, true-rest, and post-rest) before the phase of quiescence. This is formalized by an increasing temperature threshold for forcing during pre-rest and a decreasing temperature threshold for forcing post-rest, and buds cannot respond to forcing temperature at all during true rest.

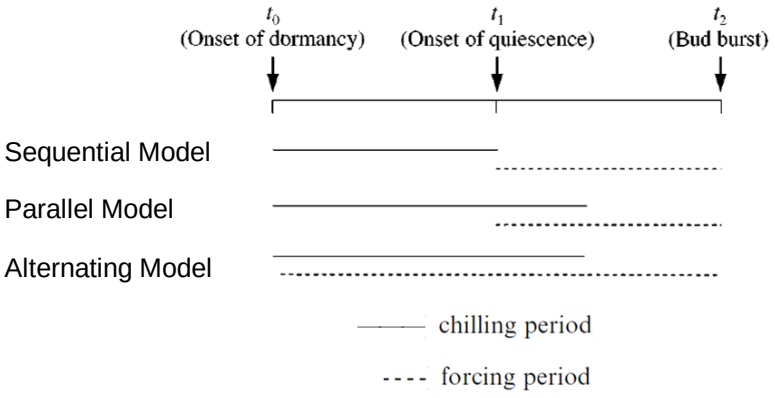


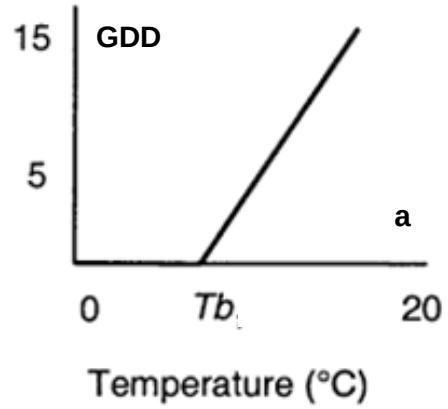
Figure 4 The three different assumptions concerning the period when growth is affected by forcing and chilling temperatures: (————) chilling period; (----------) forcing period. Figure adapted from (Chuine 2000).

The Thermal-time models are based on the principle that a given phenological stage occurs on the day (*ts*) when a critical value of the state of forcing temperatures (*Sf*), denoted by *F** has been reached (Eq. 1):

$$S_f\ (t_s) = \sum_{t_0}^{ts} Rf(x_t) \geq F^* \ (1)$$

The *Sf* is described as a sum of the daily rate of forcing (*Rf*), which is a function of temperature (*xt* is the daily mean temperature), and starts at *t0* (day of the year, DOY).

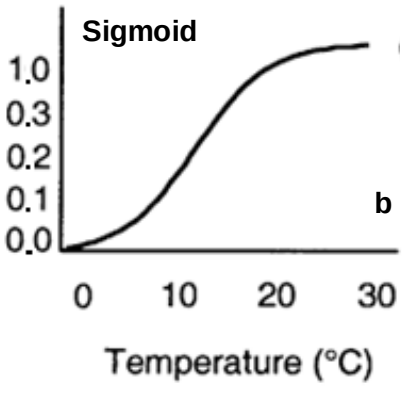
The developmental responses to temperature during the growth phase have been described by various types of functions, some examples can be seen in Figure 5. Forcing units have commonly been formulated either as degree-days (Fig. 5a; C°.Day^{-1}) or as in the following functions (Fig. 5b,c).



$$R_f(x_t) = \begin{cases} 0\ if\ x_t < Tb \\ x_t - Tb\ if\ x_t \geq Tb \end{cases} \ (a)$$

x_t - daily mean temperature

Tb - base temperature above which the thermal summation is calculated

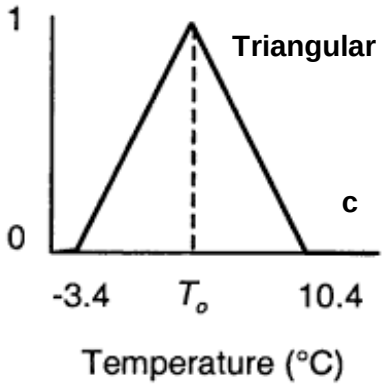


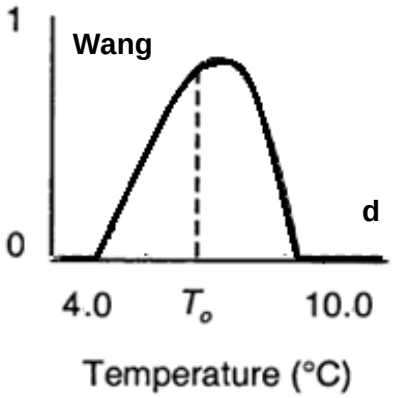
$$R_f = \begin{cases} 0\ if\ x_t < 0 \\ \dfrac{1}{1 + e^{d(x_t - e)}}\ if\ x_t \geq 0 \end{cases} \ (b)$$

x_t - daily mean temperature

d - (<0) defines the sharpness of the response curve

e - (>0) is the mid-response temperature





$$R_f(x_t) = \begin{cases} 0 \; if \; x_t << Tmin \\ \dfrac{x_t - Tmin}{Topt - Tmin} \; if \; Tmin < x_t << Topt \\ \dfrac{x_t - Tmax}{Topt - Tmax} if \; Topt < x_t < Tmax \\ 0 \; if \; x_t >> Tmax \end{cases} (c)$$

$$R_f(x_t) = Max \left[2(x_t - Tmin)^{\alpha}(Topt - Tmin)^{\alpha} - \frac{(x_t - Tmin)^{2\alpha}}{(Topt - Tmin)^{2\alpha}}, 0 \right] (d)$$

x_t - daily mean temperature

Tmin - minimum temperature above which the thermal summation is calculated

Topt - optimal temperature from which the amount of units of heat accumulated start to decrease

Tmax - maximum temperature from which no thermal summation occurs

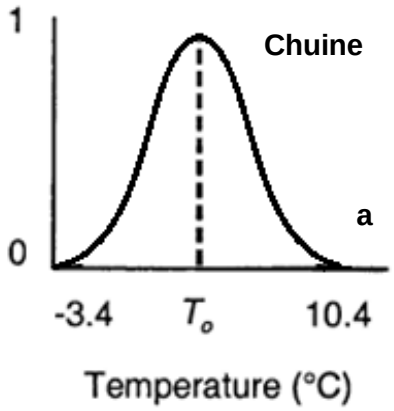
$$\alpha = \ln(2) / \ln\left(\frac{Tmax - Tmin}{Topt - Tmin}\right)$$

Figure 5 Representation of different responses to temperature used to calculate forcing units. The temperature values in the graphic are only for example. Adapted from: Chuine *et al.* (2003).

The two-phase models, which also integrate the dormancy phase, assume that a critical value of the state of chilling (*Sc*), denoted by *C** (Eq. 2) must be reached to break dormancy (*td*), with the daily rate of chilling (*Rc*) calcuted by different functions.

$$S_c\,(t_d) = \sum_{t_0}^{td} Rc(x_t) \geq C^* (2)$$

The developmental responses to temperature during the dormancy phase have been described by various types of functions, some examples can be seen in Figure 5c and Figure 6.



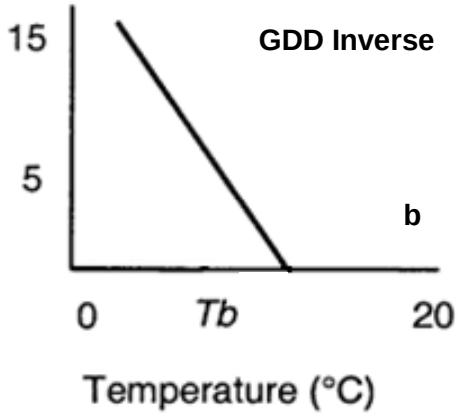
$$Rc(x_t) = \frac{1}{\left(1 + e^{[a(x_t - c)^2 + b(x_t - c)]}\right)} \quad (a)$$

x_t - daily mean temperature

a – width of the window over which the function is not null, a ∈ [0,10]

b – sharpness of the response curve and its asymmetry, b ∈ [-30,15]

c – mid-response value when *b* is close to zero, and a boundary to the temperature range over which chilling units accumulate, when *b* strongly differs from zero



$$R_C(x_t) = \begin{cases} Tb - x_t \; if \; x_t < Tb \\ 0 \; if \; x_t \geq Tb \end{cases} \quad (b)$$

xt - daily mean temperature

Tb - base temperature above which the thermal summation is not calculated

Figure 6 Representation of different responses to temperature used to calculate dormancy units. The temperature values in the graphic are only for example. Adapted from: Chuine *et al.* (2003).

Model parametrization

The phenological models parametrization could be fitted using different algorithms model fit as presented in Cox, Gibbons *et. al.* (2006). In this work the developed models are fitted by minimizing a least-squares function in the parameters space using a simulated annealing method (Chuine et al. 1999). The Metropolis algorithm (Metropolis et al. 1953) was used to fit the models and explores the parameter space around the global optimum found at the end of its search. This algorithm enables a more effective searching of the parameter space, which proceeds through a "random walk" tending to gradually decrease the residual sum of squares (Chuine et al. 1999, 1998; Press et al. 1989). The parameter space is explored roughly at first by "large steps" between one set of parameter values and the next, and then in detail with a smaller step size around the relative or absolute minimum found. A set of parameters that increases the residual sum of squares is rejected: the probability of rejecting it is higher at the initial stages of the fitting procedure, when there is also a higher probability that the minimum

found is a relative one. The Metropolis algorithm gradually decreases its “temperature”, so decreasing the probability of rejecting a change that increases the sum of squares. By decreasing the temperature very slowly, the convergence towards the absolute minimum will be improved, decreasing the probability of the algorithm falling into one local minima (Chuine et al. 1999).

2.4. Pollen morphology and fertility

Palynology is the science that studies the morphology of pollen grains and spores, as well as their dispersion and applications (Erdtman 1969). Pollen grains are the male gametophyte produced in the anthers of higher plants, and its main role is to intervene in the sexual reproduction of spermatophytes (Pérez et al. 2007). Pollen grains of most plants live for only a few hours to a few days after leaving the anther (Dafni and Firmage 2000), and successful ones never touch the ground.

2.4.1. Pollen morphology

The pollen grain is coated by a wall denominated sporoderm, generally stratified/layered and composed by two different layers. The inner layer, intine, has a pectocellulose nature and its function is to protect the pollen cell content. The outer layer, exine, is composed by sporopollenin being very resistant to decay. This layer is composed by an outer sculptured sexine and an inner unsculptured nexine. The nexine is homogeneous and continuos, and can be divided up to 2 layers called nexin I and II. Sexine is composed by a set of radially-directed rods supporting a roof-like structure (tectum), which may be partially perforated, flat, ornamented (with spikes and other protrusions) or completely absent (Abreu 1984; Erdtman 1969; Wodehouse 1935). Rods supporting the tectum are known as columella (which are usually simple, but may be branched), and rods not supporting anything but standing vertically on the nexine are called baculla (Sugandha 2016; Ribeiro 2008; Hesse 2009).

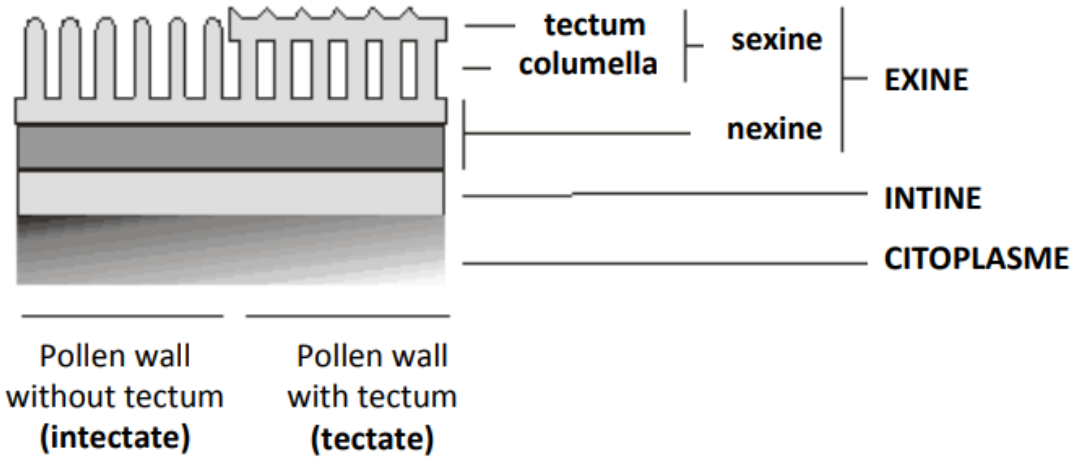


Figure 7 Stratification of the pollen wall. Adapted: Smith and Galán (2017)

In most pollen grains, the exine is interrupted by apertures, from where the pollen tube will emerge at germination (Lersten 2008).

Pollen morphology can be used for taxonomic characterization enabling a more accurate evaluation of morphological characteristics (Soares et al. 2013).

Pollen polymorphism is a widespread phenomenon among the higher plants including different grapevine species and cultivars (Cargnello et al. 1980; Dzyuba et al. 2006; Gallardo et al. 2009; Maria et al. 1994). Generally, the pollen grains of *Vitis vinifera* L. present a sub-spherical to triangular shape due to the presence of three furrows with three pores (tricolporated form) (Abreu et al. 2006; Alva et al. 2015) (Fig. 8). The presence of atypical pollen showing bicolporated, acolporate, collapsed or shriveled morphology can be related with the irregular productivity presented by some grapevine cultivars (Abreu et al. 2006; Caporali et al. 2003; Lombardo et al. 1978).

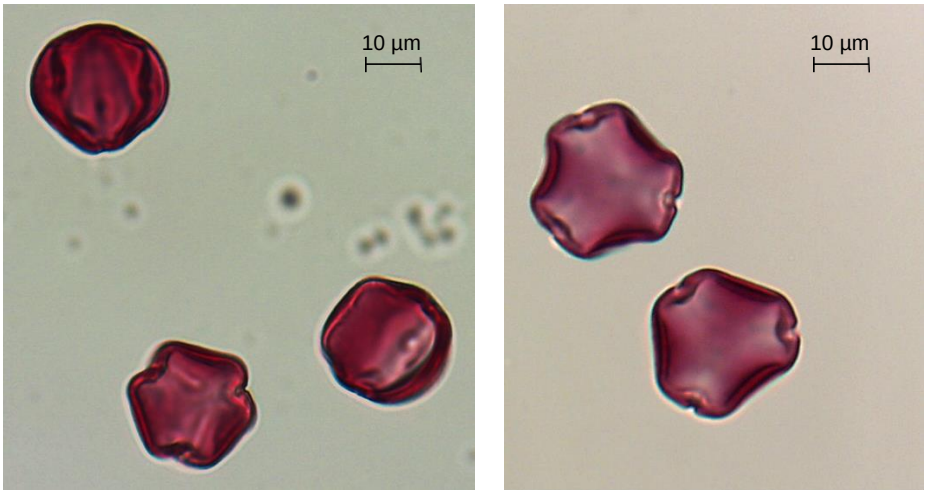


Figure 8 Acetolyzed pollen of *Vitis vinifera* L. analyzed under light microscopy. Tricolporated pollen observed from different perspectives.

2.4.2. Fertility

Pollen fertility is the result of a combination of different traits such as the viability and germination ability in favourable conditions. The first one is evaluated when the pollen grain is mature and the second one through the formation and growth of the pollen tube *in vitro* conditions (Stanley and Linskens 1974). It can be influenced by genetic, environmental (temperature and humidity) and agronomic factors and is closely related to a greater or lesser ability to produce fruit and consequently higher or lower yields.

The evaluation of pollen fertility is important in the study of various aspects related with plant productivity such as the storage potential of pollen grains for controlled pollination, the evaluation of intra- and inter-cultivar incompatibility or the clonal selection and genetic breeding trials (Dafni and Firmage 2000). In fact, comparison of inter-cultivar pollen fertility allows us to determine the cultivars more appropriate for a given region and their potential for genetic improvement or clonal selection. The methodologies used to characterize pollen fertility can be very diverse and include staining techniques, *in vitro*, germination tests or in natural conditions, and the evaluation of the percentage of fertilisation and fruiting (Stanley and Linskens 1974).

The pollen viability can be evaluated by several methods that allow to test several biochemical and morphological characteristics in order to ascertain the integrity of the pollen grain. Among the methods used are: i) staining techniques for the pollen cytoplasmic constituents (methylene blue or propionic carmine); ii) evaluation of the enzymatic activity (tetrazolium test and Baker's reagent to verify dehydrogenase activity; benzidine test to test the peroxidase activity); iii) fluorochromatic reaction that evaluate the integrity of the pollen grain plasmalemma (fluorescein diacetate reaction - FCR) (Dafni and Firmage 2000; Heslopharrison et al. 1984).

The evaluation of pollen germination ability is usually performed under *in vitro* conditions (Fig. 9). For this it is necessary to prepare a medium with similar composition to the stigma so that the pollen grain germinates. Generally *in vitro* pollen germination requires hydration, a source of carbohydrates and boron. Additionally, the germination medium can also include other components such as calcium, magnesium and potassium (Brewbaker and Kwack 1963; Fan et al. 2001). All these elements can be combined at different concentrations according to plant species tested (Feijo et al. 1995; Linskens 1964). Hydration is essential to the pollen germination since at the time of dehiscence the pollen is released dehydrated into the atmosphere, suffering rehydration on the stigma (Wilsen and Hepler 2007). Sucrose is generally used as the source of carbohydrates, to maintain the osmotic pressure of the medium and also as a substrate for pollen grain metabolism, whereas boron plays an important role in the growth of the

pollen tube (Stanley and Linskens 1974). Furthermore, incubation temperature and time are also important parameters to control (Kelen and Demirtas 2003; Fu et al. 2005).

Figure 9 *In vitro* germination of pollen analysed under light microscopy (A) and close-up of a germinated pollen grain, being observed the developed pollen tube (B).

2. Article 1 “Predicting the flowering date of Portuguese grapevine varieties using temperature-based phenological models: a multi-site approach”

International Journal of Biometeorology

Predicting the flowering date of Portuguese grapevine varieties using temperature-based phenological models: a multi-site approach.

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Abstract:	Phenological models for predicting the grapevine flowering timing were tested using data collected between 1990-2014 in two Portuguese wine regions: Vinhos Verdes and Lisbon for 15 grape varieties (8 white and 7 red). The developed models were used to convey temporal and spatial robustness using phenological data collected in the most western viticulture zone of Europe. Three different models were tested for flowering estimation: Growing Degree Days - GDD model, GDD Triangular and UniFORC. Estimation of the models was performed using two grape varieties, present in both regions and based on an iterative generalization process. Three dates were tested for the beginning of the accumulation of heat units (t0 date): budburst date, 1st January and 1st September. The best overall date was budburst. Furthermore, for each model parameters it was estimated an intermediate range of values common for the studied regions. These values were further optimized in order to obtain one model that could be used for a diverse range of grape varieties in both wine regions. An external validation was performed using an independent data-set from 13 grape varieties (7 red and 6 white). The results showed a high R2 (0.59-0.89), low RMSE (3-7 days) and MAD (2-6 days). The UniFORC model overall performed slightly better than the two GDD models tested, presenting overall higher R2 (0.75) and lower RMSE (4.55) and MAD (3.60). The phenological models developed in our study presented good accuracy when applied to several varieties in different regions and can be used as a predictor tool of flowering date in Portugal.

Predicting the flowering date of Portuguese grapevine varieties using temperature-based phenological models: a multi-site approach

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Abstract

Phenological models for predicting the grapevine flowering timing were tested using data collected between 1990-2014 in two Portuguese wine regions: Vinhos Verdes and Lisbon for 15 grape varieties (8 white and 7 red). The developed models were used to convey temporal and spatial robustness using phenological data collected in the most western viticulture zone of Europe. Three different models were tested for flowering estimation: Growing Degree Days - GDD model, GDD Triangular and UniFORC. Estimation of the models was performed using two grape varieties, present in both regions and based on an iterative generalization process. Three dates were tested for the beginning of the accumulation of heat units (t_0 date): budburst date, 1st January and 1st September. The best overall date was budburst. Furthermore, for each model parameters it was estimated an intermediate range of values common for the studied regions. These values were further optimized in order to obtain one model that could be used for a diverse range of grape varieties in both wine regions. An external validation was performed using an independent data-set from 13 grape varieties (7 red and 6 white). The results showed a high R^2 (0.59-0.89), low RMSE (3-7 days) and MAD (2-6 days). The UniFORC model overall performed slightly better than the two GDD models tested, presenting overall higher R^2 (0.75) and lower RMSE (4.55) and MAD (3.60). The phenological models developed in our study presented good accuracy when applied to several varieties in different regions and can be used as a predictor tool of flowering date in Portugal.

Keywords: phenology, grapevine flowering, thermal models, optimization algorithm, simulated annealing

1. Introduction

The study of typical vegetation cycles and their connection to climate, called vegetation phenology, plays a prominent role in local, regional and global agricultural and ecosystems simulation models (Chuine 2000; Jones 2003; Running and Hunt 1993). The knowledge of the annual timing of phenophases and their variability can help decision making in crop management which leads finally to higher and more stable crop yields and quality as well as to an improved food quality (Chmielewski et al. 2013). Phenology monitoring and forecasting is an important support tool to the implementation of cropping schedules, the organization of crop rotation, and catch cropping (Chmielewski et al. 2013). It is also important to support crop management operations like: irrigation, fertilizing, and crop protection (Ruml and Vulić 2005). Phenological observations are also useful to define the growing season length,

evaluate the risk of frost damage and to make forecasts of plant development and harvest dates (Chmielewski et al. 2013).

In viticulture phenological modelling is important for a variety of applications, such as the more efficient planning of harvest and viticultural practices (Williams et al. 1985), predicting the vulnerability to pest attacks and the selection of cultivars in new areas (Gladstones 1992; Bindi et al. 1997; Orlandi and Mancini 1999; Jarvis et al. 2017). The models can be coupled with climate change scenarios projecting the impact of climate change on grapevine (Cola et al. 2017). However, the difficulties in obtaining phenological models which provide accurate predictions on a local and regional scale prevent them from being exploited to their full potential (Caffarra and Eccel 2010).

Phenology modelling requires four essential steps: data collection, model definition, adjustments of the model to the data using an adapted optimization algorithm that ensures correct convergence and tests of the model hypotheses (Chuine et al. 1999). Data collection includes observations of meteorological parameters, being the environmental temperature the variable commonly used, photoperiod and phenological stages. Phenological data can be derived from remote sensing images (Cunha et al. 2010), or airborne pollen (Cunha et al. 2015), but the observations in phenological collections are still the most reliable data mainly for grapevine studies.

The *Vitis vinifera* L. vegetative cycle begins at budburst and finishes at leaf colouring and fall in the end of the growth phase, when the development of the plant ceases and the dormancy stage begins (Pouget 1972; Chaos et al. 2007). Temperature and photoperiod are fundamental in influencing grapevine phenological dynamics (Winkler et al. 1974; Huglin 1978; Jones and Davis 2000; Duchene and Schneider 2005; Jones et al. 2005; Van Leeuwen et al. 2008; Zapata et al. 2015). Temperature affects the cell metabolism, carbon accumulation and other biochemical processes (Tanino et al. 2010). The variation in the photoperiod, including long nights, affects the phytochromes (photoreceptors), which control signal transduction in order to regulate shoot growth (Tanino et al. 2010; Chaos et al. 2007; Olsen 2010; Victor et al. 2010). Although, grapevine phenology is therefore correlated with local conditions and genetics (varietal choice) (Gladstones 1992, van Leeuwen et al. 2004, van Leeuwen et al. 2008), it was showed that temperature is the predominant influence on development over photoperiod and light intensity (Buttrose 1969). In fact, photoperiod is unlikely to influence inter-annual variation of phenology in a single place since it does not vary from one year to another (Chuine et al. 1999).

Understanding how temperature influences the timing of grapevine vegetative and reproductive development and identifying varietal specific differences in phenology is fundamental. In fact, when these developmental phases are well-adapted to the local conditions, the grapes at harvest may acquire a desired combination of parameters such as sugar, acidity, aromatic and phenolic compounds or other desired qualities to produce wine with high quality (Jones and Davis 2000; Jones et al. 2005; Jones 2006; Van Leeuwen et al. 2008). Furthermore, the phenological models coupled with climate scenarios, could play an important role in predicting the triggering of grapevine phenological stages that could help avoid stress and mitigate projected changes in climate (Cunha and Richter 2016).

Different phenological models for predicting the dates of flowering have been described in the literature (e. g. Parker et al. 2011; Chuine et al. 2003). Some models examine only the effect of the temperatures during the active growth period (forcing temperatures) such as Thermal Time model (Cannell

and Smith 1983) also named Spring Warming model (Hunter and Lechowicz 1992a; Hunter and Lechowicz 1992b). Other modelling approaches include also the influence of chilling temperatures such as the Alternating model (Murray et al. 1989), the Parallel model (Landsberg 1974; Hänninen 1990; Kramer 1994), the Sequential model (Sarvas 1972; Hänninen 1990; Kramer 1994) and Unified model (Chuine 2000).

In phenological modelling, the development of site-specific models is important because the plants are adapted to the surrounding environment, and differences in the timing of phenological development are observed (Caffarra and Eccel 2010; Parker et al. 2011; Parker et al. 2013). Therefore, the information derived from these site-specific models contributes to understand the plasticity of a species or of varieties within the same species to regions with different climatic conditions. In addition, site-specific models are a useful tool to help winemakers in the management of practices in the vineyard, especially at the flowering date since it is a good indicator of the harvest date. Nonetheless, it is also important to develop a unified model of multi-regional application to describe and predict phenological behaviour on a special scale (Parker et al. 2011).

Therefore, the main objective of this study was to test and validate a multi-year, multi-variety and multi-site temperature-based phenological models of flowering date using data collected in Portugal, the most western viticulture zone of Europe.

The phenological models were calibrated and validated using a dataset collected in two Portuguese wine regions and contains a large number of autochthonous varieties concentrated in a small area. The best models were chosen in terms of efficiency relative to its complexity. The developed models intended to convey temporal and spatial robustness for the species *Vitis vinifera* L in Portugal.

2. Materials and methods

2.1. Study area

The research was carried out in two Portuguese wine regions: Vinhos Verdes and Lisbon (Fig. 1). The phenological dataset in the Vinhos Verdes region was collected in two test sites, one located in Arcos de Valdevez (AVV) in the experimental field of the Amândio Galhano Wine Station research (41º48'57"N, 8º25'35"W, 70 m a.s.l) and the second one recorded in Felgueiras (FEL) in the Quinta de Sergude Experimental Station of Fruticulture (41º16´N 8º05´W, 110 m a.s.l.), located about 50 km south of AVV test site. In the Lisbon wine region, the phenological dataset was collected in Quinta dos Almoínha (39º01'46''N, 9º01'35''W, 99 m a.s.l) belonging to the Portuguese 'Instituto Nacional de Investigação Agrária e Veterinária' (INIAV) located in Dois Portos, Torres Vedras (TOV).

The wine regions studied differ greatly in terms of weather, soils, grape varieties and vine-growing systems. In both wine regions, the most significant climatic feature is an irregular annual rainfall level distributed throughout the year, mainly concentrated in winter and spring. Air temperature increases inversely to precipitation: winters are cool and wet, and summers are hot and dry (Cunha et al. 2015).

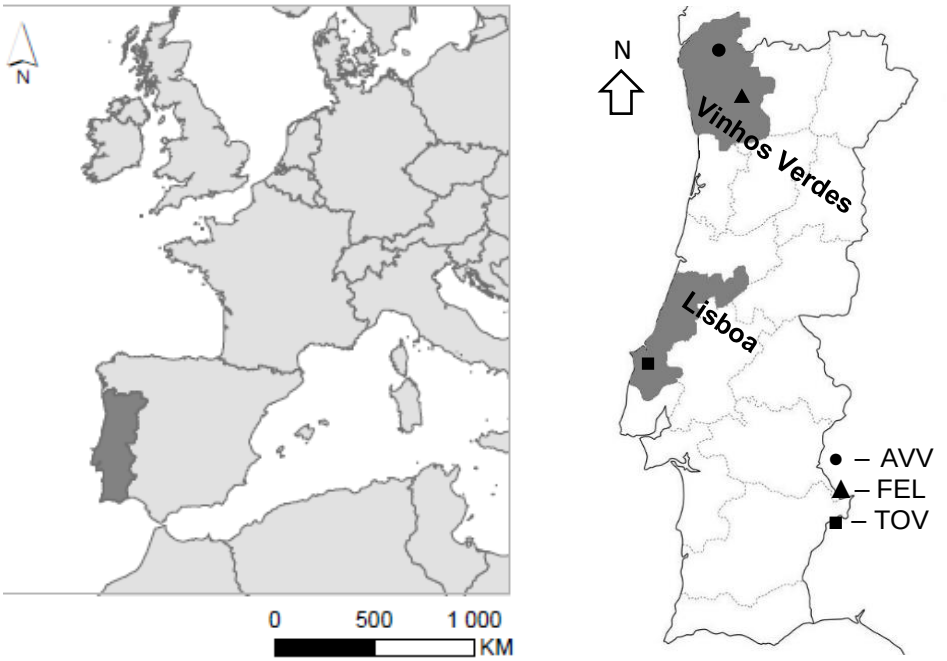


Figure 1 Map of Portugal with the studied wine regions represented and the location of the sites analysed in this work.

2.2. Temperature and phenological observations

The meteorological data were recorded in weather stations located near the field (< 5 km) used for the phenological observations. Daily observations of maximum and minimum temperatures, were used to calculate the daily mean temperature (*Tm*, ºC). The Vinhos Verdes region typically shows annual mean temperatures of about 15 °C and Lisbon region of 17 °C. The Torres Vedras site (TOV) experienced warmer temperatures (mean, minimum and maximum) for the period from 1st of January to Flowering (Table 1), while Arcos de Valdevez (AVV) registered lower temperatures during this period. In TOV, the mean temperature during the period between budburst and flowering was about 2.3 ºC higher than the mean temperature in the same period in AVV and 1.6 ºC in Felgueiras (FEL) (Table 1).

During the growing season the average temperature is about 17 ºC in AVV and 20 ºC in TOV, and according to the climate maturity grouping (Jones 2006), the growing season can be classified as "Intermediate" and "Warm", respectively for AVV and TOV wine regions (Table 1).

Table 1 Summary of average, minimum and maximum temperatures for each studied region for the period from 1st January to the budburst date, from 1st January date to the flowering date and from budburst to flowering from 1990 to 2014.

Temperature (Cº)	1st January - Budburst	1st January - Flowering	Budburst-Flowering	Growing Season
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Arcos de Valdevez (AVV)				
Mean	10.4 ± 2.8	12.0 ± 3.5	14.1 ± 3.2	16.6 ± 0.78
Minimum	6.3 ± 3.3	7.6 ± 3.4	9.4 ± 2.8	11.6 ± 0.63
Maximum	14.5 ± 3.5	16.4 ± 4.4	18.8 ± 4.3	21.5 ± 1.09
Torres Vedras (TOV)				
Mean	12.8 ± 2.4	14.5 ± 3.1	16.5 ± 2.6	19.3 ± 0.90

Minimum	9.6 ± 2.6	10.9 ± 2.9	12.6 ± 2.2	15.1 ± 0.61
Maximum	16.0 ± 2.7	18.0 ± 3.8	20.3 ± 3.6	23.4 ± 1.23
Felgueiras (FEL)				
Mean	11.7 ± 2.9	12.8 ± 3.3	14.9 ± 2.9	16.6 ± 0.78
Minimum	7.8 ± 3.4	8.9 ± 3.6	11.0 ± 2.9	11.6 ± 0.63
Maximum	15.5 ± 3.3	16.6 ± 3.8	18.7 ± 3.7	21.5 ± 1.09

SD: standard deviation

This study is based on long-term grapevine phenological observations for the dates of budburst and flowering collected in each region. Time-series were collected between 1990 and 2014 (periods ranged between 8 to 25 years according to the regions) for 15 different grape varieties (8 white and 7 red) (Table 2). Phenological observations were collected, at field levels per grape variety, using the Baggiollini phenological scale (Baggiolini 1952) every 3 to 4 days in AVV and TOV and 6 to 7 days in FEL. Phenological observations were registered according to the ''Organisation Internationale de la Vigne et du Vin'' (OIV) phenophase descriptors (OIV 2009). A given phenological stage was reached, and date recorded when the event occurred in 50 % of the plants for each grape variety, at the stages 'C: budbreak' and 'I: flowering' of Baggiolini Scale, which corresponds to the stages 'EL 07 Beginning of bud burst: green shoot tips just visible' and 'EL 65 Full flowering: 50% of flowerhoods fallen' respectively according to the Eichhorn and Lorenz (E-L) system (Lorenz et al., 1995).

2.3. Modelling grapevine phenology

In this work, different phenological models were tested and validated for predicting the grapevine flowering dates, at the variety level. Several long-term series of phenological data of different grapevine varieties were collected in Portuguese wine regions (Vinhos Verdes and Lisbon) and used in the development of the models. The varieties were compared within the same modelling framework and the models were applied to different locations in order to achieve a single model with fixed optimized parameters that could accurately forecast multi-variety and multi-site grapevine flowering dates.

The models were developed using the program PMP 5.5 (Phenology Modeling Platform) (Chuine et al. 2003).

2.3.1 Phenological model

Three different phenological models were tested: Spring Warming (Growing Degree Days - GDD model), Spring Warming modified (designated GDD triangular) and UniFORC (Chuine et al. 2003).

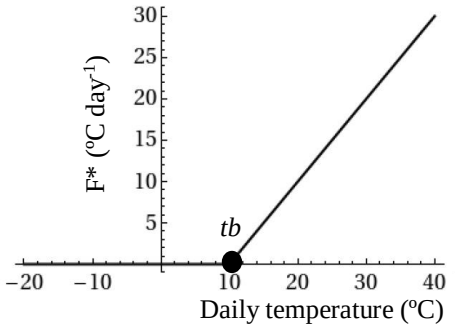
These models consider only the action of forcing temperatures and they assume that a given phenological stage occurs on the day (t_s) when a critical value of the state of forcing temperatures (S_f), denoted by F^* has been reached (Eq. 1):

$$S_f\ (t_s) = \sum_{t_0}^{ts} Rf(x_t) \geq F^* \quad (1)$$

The S_f is described as a sum of the daily rate of forcing (R_f), which is a function of temperature (x_t is the daily mean temperature). In this work three models were used for estimate the R_f:

-Growing Degree Day model

The Growing Degree Day (GDD – ºC day^{-1}) approach (Fig. 2; Eq.2) includes three parameters, t_0, T_b and F^*, where *Tb* corresponds to a base temperature above which the number of forcing units is calculated (van der Schoot and Rinne 2011).

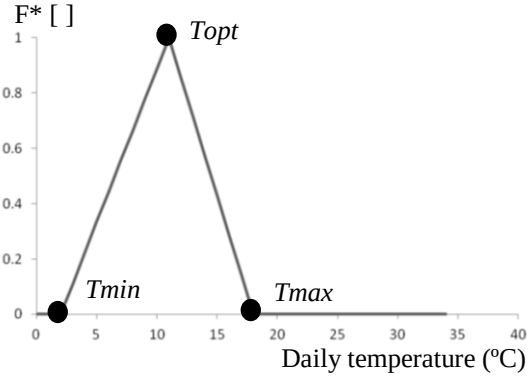


$$R_f = GDD(x_t) = \begin{cases} 0 \; if \; x_t < Tb \\ x_t - Tb \; if \; x_t \geq Tb \end{cases} \quad (2)$$

Figure 2 Graphic representation of Growing Degree Days (GDD) approach. *F** is the number of forcing units, x_t is the daily mean temperature and *Tb* is the base temperature above which the thermal summation is calculated. (The *Tb* of 10ºC is used as an example for model representation).

-GDD Triangular model

The GDD triangular approach includes four model parameters to be estimated the t_0, *Tmin*, *Topt*, *Tmax* and *F**(Fig.3, Eq.3). This approach is based on ratios of temperatures, hence the *F** takes values between 0 and 1.

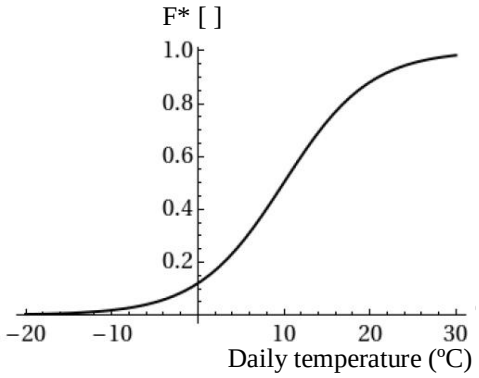


$$R_f(x_t) = \begin{cases} 0 \; if \; x_t << Tmin \\ \dfrac{x_t - Tmin}{Topt - Tmin} \; if \; Tmin < x_t << Topt \\ \dfrac{x_t - Tmax}{Topt - Tmax} \; if \; Topt < x_t < Tmax \\ 0 \; if \; x_t >> Tmax \end{cases} \quad (3)$$

Figure 3 Graphic representation of Growing Degree Days model modified with the triangular function. *F** is the forcing units, x_t is the daily mean temperature, *Tmin* is the minimum temperature above which the thermal summation is calculated, *Topt* is the optimal temperature from which the amount of units of heat accumulated start to decrease and *Tmax* is the maximum temperature from which no thermal summation occurs.

-UniFORC model

The UniForc model includes four parameters t_0, *d*, *e*, *F** to be fitted, with *d*<0 and *e*>0 (Fig.4, Eq. 4). The parameter *d* (<0) defines the sharpness of the response curve and *e* (>0) is the mid-response temperature. The rate of forcing *F** is an exponential function that takes values between 0 and 1.



$$R_f = \begin{cases} 0 \; if \; x_t < 0 \\ \dfrac{1}{1 + e^{d(x_t - e)}} \; if \; x_t \geq 0 \end{cases} \quad (4)$$

Figure 4 Graphic representation of UniFORC model (Eq 4). *F** is forcing units, x_t is the daily mean temperature, *d* defines the sharpness of the response curve and *e* is the mid-response temperature.

2.3.2 Model Estimation

In our modeling strategy, the timing of flowering was estimated in a two-phase approach. The varieties with the longest time series in Arcos de Valdevez (Fernão Pires, n=25, and Loureiro, n=25) and in Torres Vedras (Fernão Pires, n=25) were used in this process.

In a first phase, estimation of the models was based on an iterative generalization process where three dates were tested for the beginning of the accumulation of heat units (t_0 date): budburst date, 1st January, and 1st September. This allowed selecting the best overall t_0 date. Furthermore for each tested model was found an intermediate range of values for the estimated parameter common for the three regions.

In a second phase, for each model the intermediate values of the estimated parameters were iteratively optimized (the T_b for the GDD model, the *Tmin*, *Topt* and *Tmax* in GDD Triangular model and *e* in UniFORC model) in order to obtain one model with fixed parameters that can be used for the estimation of the flowering time in a diverse range of grape varieties.

Model parameters were fitted using the Metropolis algorithm (Metropolis et al. 1953), known to avoid local extrema of any kind of function while searching the solution for an optimization problem.

2.3.3 Model validation

To evaluate the prediction accuracy of the estimated models, an external validation was performed using an independent dataset consisting of several time-series ranging from 8 to 25 years (398 observations), from 13 grape varieties (7 red and 6 white) in the three test sites (AVV, TOV and FEL) from two wine regions (Table 2). This allows selecting the most precise model to predict the timing of grapevine flowering based on an inter-regional assessment of the model accuracy.

Therefore, in agreement with the previous parameterization in the GDD model, the T_b was fixed and *F** was fitted; in the GDD Triangular model the *Tmin*, *Topt*, *Tmax* value were fixed and the *F** was fitted; in the UniFORC model the value of *e* was fixed and the *d* and *F** were fitted.

2.3.4 Goodness-of-fit indicators

The estimation and the selection of the best model during the validation process was based on the model fit by coefficient of determination (R^2, Eq.5), the Mean Absolute Deviation (MAD, Eq.6) and on the Root Mean Square Error (RMSE, Eq.7) between predicted and observed values calculated based on the following equations:

$$R^2 = \frac{(SStot - SSres)}{SStot} \quad (5)$$

$$RMSE = \sqrt{\frac{SSres}{n}} \quad (6)$$

$$MAD = \frac{\sum_{i=1}^{n} |Xobs_i - Xpre_i|}{n} \quad (7)$$

Where *SStot* is the Total Sum of Squares, *SSres* is the Residual Sum of Squares, *n* the number of observations and *Xobsi* and *Xprei* are, respectively, the observed values and predicted values.

The average difference between predicted and observed flowering date above the upper quartile ($>Q_{75}$) was also used in the selection of the best model in the estimation process.

For the best-fitted model, a linear regression through the origin between predicted and observed flowering dates was performed. The hypothesis is that the concordance line of this regression passes through the origin and has a coefficient of regression (*b0*) of unity, indicating the absence of bias and showing no significant differences between predicted and observed flowering date. An analysis of frequencies of the differences between predicted and observed flowering dates was also performed for each test site.

3. Results

3.1 Regional phenological time series

The analysis of long-term grapevine phenological observations in three regions of Portugal (Table 2) showed that there is a marked inter-annual variation of both budburst and flowering phenological dates among grape varieties.

Table 2 Average and standard deviation of the budburst and flowering dates for the grape varieties studied and the period between these two phenological stages (Budburst-Flowering). The days of the year (DOY) presented for each period were calculated from 1st of January (and this day was considered day 1).

Variety	Years (nº)				Budburst	Flowering	Budburst-Flowering
	AVV	TOV	FEL	Total	DOY	DOY	Nº of days
White varieties							
Alvarinho	25	20	-	45	79.6 ± 7.0	145.7 ± 8.7	66.1 ± 8.3
Avesso	8	-	-	8	76.6 ± 4.9	144.3 ± 8.6	67.3 ± 7.2
Azal	8	-	-	8	85.5 ± 5.9	150.8 ± 8.5	65.0 ± 8.4
Chasselas	8	25	-	33	76.1 ± 8.5	143.2 ± 8.8	67.2 ± 7.6
Fernão Pires	25	25	-	50	75.3 ± 7.3	143.9 ± 9.3	68.6 ± 8.3
Loureiro	25	20	15	60	84.4 ± 9.9	148.4 ± 9.3	64.0 ± 10.0
Pedernã	17	-	15	32	94.5 ± 12.1	154.1 ± 9.9	59.6 ± 11.6
Trajadura	25	-	15	40	93.5 ± 9.8	153.2 ± 9.4	59.7 ± 11.0
Red varieties							
Aragonez	-	21	-	21	81.1 ± 9.6	144.7 ± 8.8	63.6 ± 8.6
Baga	-	20	-	20	82.7 ± 8.0	145.0 ± 9.5	62.3 ± 7.4
Castelão	8	25	-	33	74.3 ± 8.3	141.3 ± 8.5	67.1 ± 7.6
Espadeiro tinto	25	-	14	39	93.6 ± 10.3	153.0 ± 9.4	59.4 ± 10.4
Padeiro Basto	25	14	-	39	89.0 ± 7.6	151.4 ± 7.5	62.4 ± 8.6

Touriga Nacional	-	20	-	20	76.6 ± 7.7	140.8 ± 9.8	64.1 ± 7.8
Vinhão	25	-	-	25	93.5 ± 6.8	154.4 ± 8.8	60.9 ± 9.7

The budburst dates vary from DOY (Day Of the Year) 74 (March 15th for Castelão) to DOY 95 (April 05th for Pedernã) and the flowering occurs between DOY 141 (May 21st for Castelão e Touriga Nacional) and DOY 154 (June 03rd for Vinhão and Pedernã) and the period between budburst and flowering has an average duration of 59 (Pedernã, Trajadura and Espadeiro Tinto) to 67 days (Avesso, Chasselas and Castelão) (Table 2).

The average budburst date in Vinhos Verdes was DOY 88 (March 29th) and in Lisbon was DOY 78 (March 19th). In Vinhos Verdes region, the AVV average budburst date was more early than the FEL (DOY 86 – March 27th – and DOY 99 – April 9th – respectively). The same tendency was observed for the average flowering date, which was more late in Vinhos Verdes (DOY 151 – May 31st) compared to Lisbon (DOY 143 – May 23rd). In Vinhos Verdes, the AVV average flowering date was earlier than the FEL (DOY 150 – May 30th – and DOY 155 – June 04th – respectively).

A descriptive statistical analysis and an inter-varieties comparison for the budburst and flowering dates were performed (Table 2). The varieties studied were compared for the occurrence of budburst and flowering date and based on Lopes et al. (2008) and Robinson et al. (2012) were classified as early varieties (Avesso, Chasselas, Fernão Pires, Castelão and Touriga Nacional) and late varieties (Pedernã, Trajadura, Espadeiro Tinto and Vinhão) (Table 2).

3.2 Modelling grapevine phenology

3.2.1 Model estimation

In the iterative process, estimation of the models was tested for three forced t_0 dates: budburst date, 1st January and 1st September. The differences between the tested t_0 dates were very small in terms of efficiency within and between each type of model (Table 4), with none of the models performing significantly better. Nonetheless, in each model, the budburst date allowed achieving the highest R^2 and lowest RMSE and MAD. It was also observed that the average difference between predicted and observed dates above the upper quartile were smaller (differences of 1 and 2 days).

Furthermore, the models considering t_0 as the budburst date presented similar intermediate range of values for the estimated parameter, which was not observed for the others. These results allowed to select the budburst as the best overall t_0 date to be used in the next steps of the optimization process.

Table 3 Summary of the estimation process results for the three models studied. The lines in bold correspond to the t_0 date selected.

Date for accumulation of heat units (t_0)	Fernão Pires								Loureiro			
	AVV				TOV				AVV			
	R^2	RMSE	MAD	$>Q_{75}$	R^2	RMSE	MAD	$>Q_{75}$	R^2	RMSE	MAD	$>Q_{75}$
Growing Degree Days (GDD)												
1st January	0.81	4.24	3.49	6.88	0.74	4.32	3.49	6.98	0.70	4.71	3.95	7.73
1st September	0.82	4.15	3.54	6.63	0.74	4.32	3.50	6.95	0.71	4.65	3.86	7.73
Budburst	**0.87**	**3.09**	**2.56**	**4.97**	**0.71**	**3.99**	**3.38**	**6.58**	**0.80**	**4.12**	**3.24**	**6.72**
Growing Degree Days (GDD) Triangular												
1st January	0.82	4.15	3.52	6.67	0.75	4.22	3.39	7.17	0.70	4.76	3.96	8.00
1st September	0.82	4.08	3.37	6.75	0.74	4.31	3.54	7.18	0.70	4.69	3.83	7.90

Budburst	**0.87**	**3.07**	**2.49**	**4.95**	**0.72**	**3.91**	**3.28**	**6.23**	**0.80**	**4.10**	**3.23**	**6.67**
UniFORC												
1st January	0.82	4.08	3.41	6.38	0.65	5.03	3.41	7.93	0.72	4.54	3.78	7.55
1st September	0.82	4.06	3.45	6.42	0.65	4.93	3.48	7.78	0.71	4.56	3.70	7.70
Budburst	**0.86**	**3.18**	**2.54**	**5.00**	**0.65**	**4.41**	**3.39**	**7.73**	**0.78**	**4.28**	**3.27**	**7.13**

$>Q_{75}$ are the values of average difference between predicted and observed flowering dates above the upper quartile

Using the observations corresponding to the varieties Fernão Pires in AVV region, Fernão Pires in TOV region and Loureiro in AVV region and the budburst date as t_0, it was observed that the estimated parameters converge to the following values, respectively (Table 5, values fitted): i) GDD model the values of *Tb* were 7.89, 9.13 and 8.37 ºC; ii) GDD Triangular model the values of *Tmin* were 7.90, 8.96 and 8.37 ºC, the values of *Topt* were 23.01, 22.89 and 22.39 ºC and for the *Tmax* were 30.70, 29.77 and 29.06 ºC; iii) UniFORC model the values of *e* were 16.22, 14.77 and 16.34 ºC. Based on these results, in a second iteration process the model's parameters were constrained to a fixed range of values: i) GDD model *Tb* assumed values between 7 and 10 ºC; ii) GDD Triangular model *Tmin* assumed values ranging between 8 and 9 ºC, *Tmax* between 29 and 31 ºC and *Topt* the value of 23 ºC since the temperatures in the fitted model converged consistently to this value; iii) UniFORC model the parameter *e* assumed values between 14 and 16 ºC. In this second iteration step, as observed in the decision process to select the t_0 date, the efficiency of the estimated models was quite similar. Therefore, the choice of the best parameter set for each model was based on the lowest average difference between predicted and observed dates above the upper quartile (Table 5).

In the GDD model the T_b was fixed at 8 ºC and in the GDD Triangular model *Tmin* at 8 ºC, *Topt* at 23 ºC, *Tmax* at 31 ºC and the *F** was fitted. In the UniFORC model the value of *e* was fixed at 16 ºC and the *d* and *F** were fitted (Table 5).

Table 4 Summary of the results from the estimation process second phase where t_0 date was budburst and for each model an intermediate set of values of the estimated parameters were optimized. The lines in bold correspond to the parameters and respective models selected. The $>Q_{75}$ column are the values of average difference between predicted and observed dates above the upper quartile.

Iteration step	Fernão Pires AVV				Fernão Pires TOV				Loureiro AVV			
	Parameters	R^2	RMSE	$>Q_{75}$	Parameters	R^2	RMSE	$>Q_{75}$	Parameters	R^2	RMSE	$>Q_{75}$
Growing Degree Days (GDD) - T_b												
Fitted	7.89	0.87	3.09	4,97	9.13	0.71	3.99	6.58	8.37	0.80	4.12	6.72
1	7	0.86	3.20	5,10	7	0.69	4.17	6.83	7	0.78	4.28	7.47
2	**8**	**0.86**	**3.09**	**4.92**	**8**	**0.70**	**4.05**	**6.73**	**8**	**0.80**	**4.13**	**6.92**
3	9	0.83	3.47	5.42	9	0.71	4.17	6.62	9	0.78	4.28	7.10
4	10	0.83	3.47	6.90	10	0.7	4.05	6.48	10	0.74	4.72	8.35
Growing Degree Days (GDD) Triangular – Tmin, Topt, Tmax												
Fitted	7.90; 23.01; 30.70	0.87	3.07	4.97	8.96; 22.89; 29.77	0.72	3.91	6.22	8.37; 22.39; 29.06	0.80	4.10	6.75
1	8; 23; 29	0.78	3.93	4.98	8; 23; 29	0.72	3.96	6.48	8; 23; 29	0.80	4.12	6.88
2	8; 23; 30	0.87	3.07	4.95	8; 23; 30	0.72	3.97	6.50	8; 23; 30	0.80	4.12	6.88
3	**8; 23; 31**	**0.87**	**3.07**	**4.97**	**8; 23; 31**	**0.72**	**3.97**	**6.47**	**8; 23; 31**	**0.80**	**4.11**	**6.87**
4	9; 23; 29	0.83	3.44	5.43	9; 23; 29	0.72	3.91	6.27	9; 23; 29	0.79	4.20	7.07
5	9; 23; 30	0.83	3.45	5.43	9; 23; 30	0.72	3.91	6.33	9; 23; 30	0.79	4.20	7.07
6	9; 23; 31	0.83	3.45	5.43	9; 23; 31	0.72	3.91	6.33	9; 23; 31	0.79	4.20	7.05
UniFORC – d: fitted, *e*												
Fitted	16.22	0.86	3.18	5.00	14.77	0.65	4.41	6.90	16.34	0.78	4.28	6.98
1	14	0.85	3.27	5.48	14	0.66	4.34	7.23	14	0.78	4.32	7.10
2	15	0.86	3.09	5.08	15	0.70	4.05	6.53	15	0.79	4.26	6.92
3	**16**	**0.87**	**3.01**	**4.88**	**16**	**0.72**	**3.96**	**6.22**	**16**	**0.79**	**4.24**	**6.97**

$>Q_{75}$ are the values of average difference between predicted and observed flowering dates above the upper quartile

3.2.2 Model validation

In Table 6 are presented the results of the estimation and external validation with the independent dataset of the flowering dates from 15 grapevine varieties. The overall mean R^2, RMSE and MAD values obtained in the estimation and in the validation steps for each tested model were similar: i) GDD model R^2: 0.79 vs 0.75, RMSE: 4 days and MAD: 3 vs 4 days; ii) GDD Triangular model R^2: 0.80 vs 0.75, RMSE: 4 days and MAD: 3 vs 4 days; iii) UniFORC model R^2: 0.79 vs 0.77, RMSE 4 days and MAD: 3 days (Table 6). The same pattern was observed for white vs red varieties and for earlier vs later varieties.

Table 5 Goodness-of-fit indicators of the model estimation and validation process using the dataset for 15 grapevine varieties in three regions (AVV, TOV and FEL).

	Grape Varieties	GDD T_b=8			GDD Triangular *Tmin*=8.0, *Topt*=23, *Tmax*=31			UniFORC D=fitted; *e*=16.0				
		RMSE	R^2	MAD	RMSE	R^2	MAD	RMSE	R^2	MAD	R^2[1:1]	*b0*[1:1]
Arcos de Valdevez (AVV)	Alvarinho	3.41	0.83	2.90	3.42	0.83	2.93	3.30	0.84	2.73	0.84	1.01
	Avesso	3.66	0.70	2.99	3.66	0.70	3.01	3.27	0.76	2.44	0.77	0.99
	Azal	3.67	0.78	2.95	3.67	0.78	2.86	3.32	0.82	2.68	0.82	1.00
	Chasselas	3.42	0.77	3.23	3.39	0.78	3.20	3.11	0.81	2.35	0.82	0.99
	Fernão Pires*	3.09	0.86	2.53	3.07	0.87	2.49	3.01	0.87	2.48	0.86	1.00
	Loureiro*	4.13	0.80	3.20	4.11	0.80	3.20	4.24	0.79	3.34	0.79	1.00
	Pedernã	4.02	0.84	3.04	4.01	0.84	2.99	3.38	0.89	2.95	0.88	1.00
	Trajadura	4.48	0.84	3.28	4.50	0.84	3.33	4.24	0.86	3.20	0.86	1.00
	Castelão	4.11	0.59	3.49	4.11	0.59	3.48	3.34	0.73	2.38	0.73	1.00
	Espadeiro tinto	3.40	0.83	2.73	3.37	0.83	2.68	3.33	0.83	2.63	0.83	1.00
	Padeiro Basto	4.06	0.78	3.13	4.04	0.78	3.11	4.15	0.77	3.16	0.77	0.99
	Vinhão	4.23	0.74	3.62	4.21	0.74	3.51	4.78	0.74	3.34	0.75	1.00
	Mean	3.81	0.78	3.09	3.8	0.78	3.07	3.62	0.81	2.81	0.81	1.00
Torres Vedras (TOV)	Alvarinho	3.64	0.78	3.12	3.67	0.78	3.13	3.65	0.78	2.85	0.78	1.00
	Chasselas	3.84	0.74	3.25	3.88	0.74	3.23	3.84	0.74	3.25	0.72	1.00
	Fernão Pires*	4.05	0.70	3.36	3.97	0.72	3.23	3.95	0.71	3.36	0.70	1.00
	Loureiro	3.88	0.79	3.25	3.92	0.79	3.31	3.85	0.79	3.27	0.78	1.00
	Aragonez	3.81	0.79	3.18	3.82	0.79	3.18	3.63	0.81	3.11	0.79	1.00
	Baga	3.99	0.70	3.22	3.82	0.72	3.03	3.85	0.72	3.15	0.71	1.00
	Castelão	3.79	0.76	2.99	3.85	0.75	3.05	3.81	0.76	3.08	0.75	1.00
	Padeiro Basto	3.72	0.78	2.98	3.79	0.77	3.04	3.10	0.85	2.36	0.79	1.00
	Touriga Nacional	3.24	0.82	2.62	3.28	0.81	2.63	3.17	0.83	2.54	0.82	1.00
	Mean	3.77	0.76	3.11	3.78	0.76	3.09	3.65	0.78	3.00	0.76	1.00
Felgueiras (FEL)	Loureiro	6.32	0.67	4.95	6.35	0.67	5.14	6.41	0.66	5.01	0.66	1.00
	Pedernã	6.56	0.67	5.64	6.73	0.65	5.95	6.33	0.69	4.73	0.67	1.01
	Trajadura	5.78	0.64	4.37	5.84	0.64	4.59	5.77	0.64	4.13	0.62	1.00
	Espadeiro tinto	6.34	0.73	5.70	6.58	0.71	5.90	6.36	0.73	5.38	0.71	1.00
	Mean	6.25	0.68	5.17	6.38	0.67	5.39	6.37	0.67	4.99	0.67	1.00
Mean	Overall	4.61	0.74	3.79	4.65	0.74	3.85	4.55	0.75	3.60	0.75	1.00
	Estimation	3.76	0.79	3.03	3.72	0.80	2.97	3.73	0.79	3.06	0.78	1.00

Validation	4.33	0.75	3.56	4.36	0.75	3.59	4.19	0.77	3.29	0.76	1.00
Validation White	4.26	0.76	3.47	4.28	0.76	3.51	4.11	0.78	3.25	0.77	1.00
Validation Red	4.07	0.75	3.37	4.09	0.75	3.36	3.95	0.78	3.11	0.77	1.00

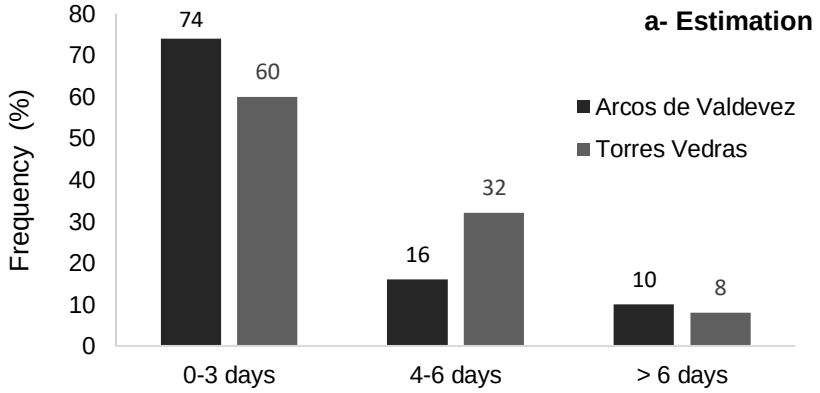
* Variety used in the model's estimation.
R^2[1:1] and *b0*[1:1] are, respectively, the coefficients of determination and the coefficient of regression through the origin between predicted and observed values for the UniFORC model.

In fact, the only variety with greatest differences was Castelão in AVV for the GDD and GDD Triangular models with a R^2 value of 0.59 and RMSE of 4.11 while for the UniFORC model the statistical indicators were similar to those found for the other varieties. This can be justified by the small number of observations for this variety when compared to the others (only 8 years).

3.2 Model selection

Along the estimation and validation steps the three phenological models tested presented minimal differences in terms of efficiency in forecasting the flowering dates, with equal or very close R^2 values and MAD differences less than a fraction of one day between the models. However, the UniFORC model (e=16ºC), when compared with the two GDD versions tested, presented overall a slightly higher R^2 (0.75), particularly in the validation step in AVV region for the varieties Castelão (0.59 vs 0.73), Avesso (0.70 vs 0.76), Azal (0.78 vs 0.82) and Pedernã (0.84 vs 0.89) and in TOV region for Padeiro Basto (0.78 vs 0.85). Also it was the model with the lower overall RMSE (4.55) and MAD (3.60) (Table 6) and the one with the best performance in the validation process with a mean R^2 of 0.77, RMSE of 4.19 days and MAD of 3.29 days. This model showed the best results for the white and red varieties tested in terms of R^2 (0.78), RMSE (4.11 vs 3.95) and MAD (3.25 vs 3.11).

Figure 5 presents the differences in the number of days between predicted and observed flowering dates attained by the UniFORC model plotted against the frequency of occurrence. Of the 75 observations used in the model estimation (Loureiro, AVV and Fernão Pires, AVV and TOV), about three-quarters of the cases had differences between predicted and observed flowering date below 3 days (Fig. 5a). In the model validation, using 398 observations from 13 grapevine varieties in 3 regions (AVV, TOV and FEL), the differences between predicted and observed values were lower than 3 days in 74% of the cases in AVV and TOV and 49% for FEL (Fig.5B).



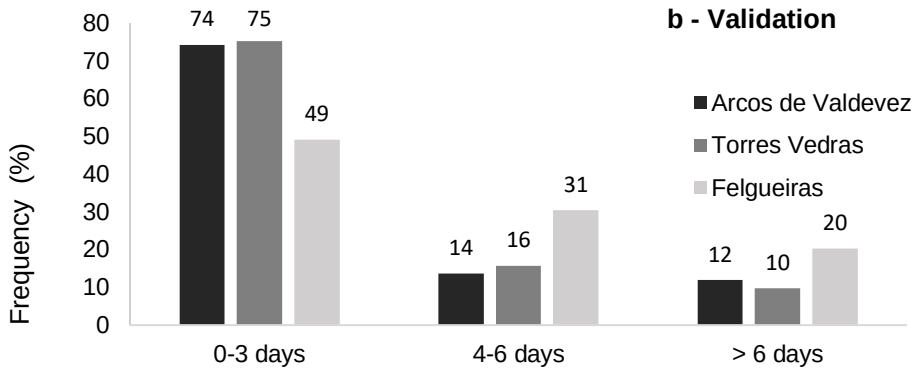
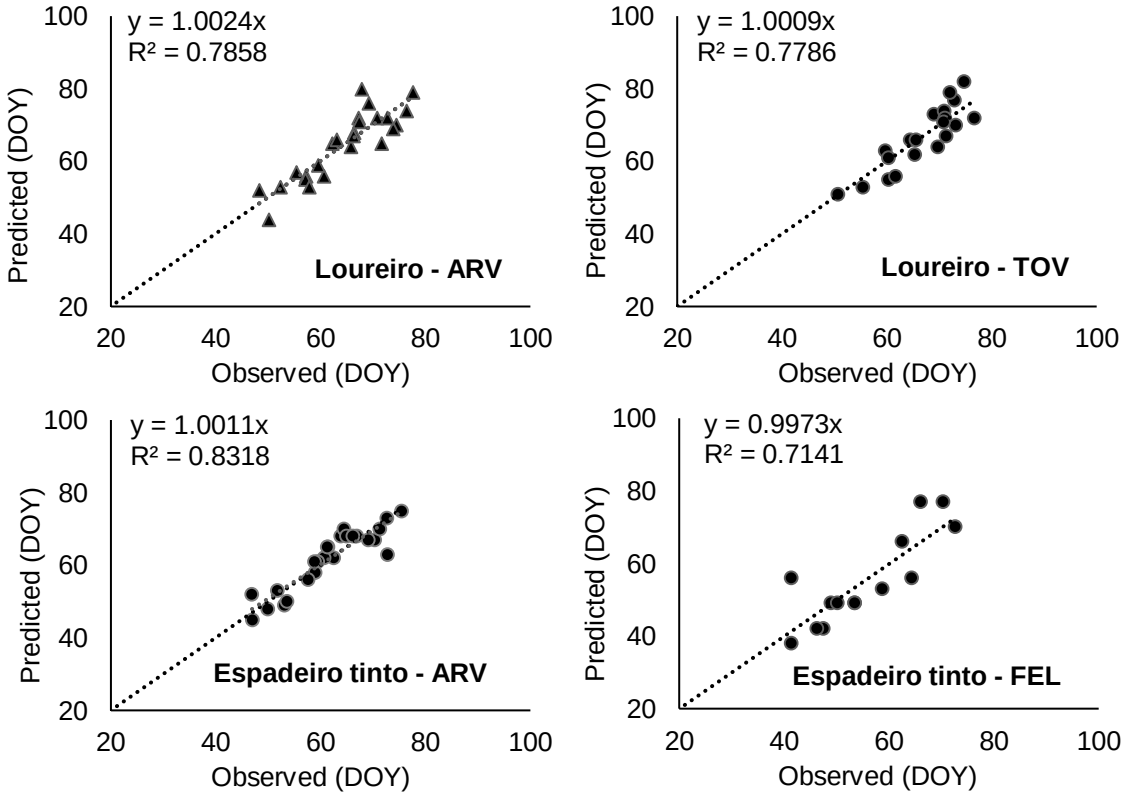


Figure 5 Frequency in percentage of difference in days between predicted and observed flowering date for Loureiro, AVV, and Fernão Pires, AVV and TOV (A), used in the UniFORC model estimation (5a) and for UniFORC validation (5b) with 13 grapevine varieties in 3 regions (AVV, TOV and FEL).

306 The results of the linear regression through the origin between predicted and observed flowering
307 dates for the UniFORC model are presented in Table 6. Figure 6 highlights the results for the variety
308 Loureiro in AVV (which was used in the estimation process) and TOV regions and for the variety Espadeiro
309 tinto in AVV and FEL. Overall the regression coefficients (*b0*) are close to 1.0 in both estimation (average
310 *b0*=1.0) and validation (average *b0*=1.0) indicating, therefore, the absence of bias.



311 Figure 6 Predicted and observed dates of flowering for two varieties using the UniFORC model. Closed
312 triangles (▲) represent data used for the model estimation and closed circles (●) represent data used for the
313 model validation.

314 **4. Discussion**

315 Three temperature-based phenological models were estimated and validated to predict the timing
316 of flowering using a long phenological dataset for a large number of grapevine varieties grown in two
317 Portuguese wine regions (Vinhos Verdes and Lisbon).
318 The parameterization of the models was achieved by an iterative process. In the first estimation
319 phase, the varieties with the longest time series of phenological data for AVV (Fernão Pires and Loureiro)
320 and TOV (Fernão Pires) were used in order to determine for each model type - GDD, GDD Triangular and
321 UniFORC – the fixed parameter estimates that better predict the flowering date. As such, the temperatures
322 chosen to incorporate into these models were those that best fit the different data series. Therefore, the base
323 temperature (T_b) for the GDD model was established at 8 ºC. In the GDD Triangular model the minimum
324 (*Tmin*), optimal (*Topt*) and maximum (*Tmax*) temperature were fixed at 8 ºC, 23 ºC and 31 ºC, respectively.
325 Several base temperatures have been mentioned in the literature as the optimum threshold above which
326 temperature summation is efficient for growth. In *Vitis vinifera* L., the 10 ºC has been suggested by several
327 authors for the base/minimum temperature (Winkler et al. 1974; Huglin and Schneider 1986; Carbonneau
328 et al. 1992; Riou 1994). Parker et al. (2011), proposed a general temperature-based phenological model for
329 vineyard flowering using data from France, Italy, Switzerland and Greece where a base temperature of 0
330 °C was found to give best fit. For instance, in Portugal Fraga et al. (2016) proposed a based temperature of
331 9.2 ºC for Fernão Pires and 8.9 ºC for Castelão. The grapevine phenotypic plasticity can explain this range
332 of base temperatures, with the value being influenced not only by intrinsic factors of the plant but also by
333 the local environment (Chuine & Cour 1999). In our study it was observed that the efficiency gain when a
334 range of *Tb* between 7 and 10 ºC were tested was small.
335 The interval between 25 ºC and 35 ºC was considered the optimal range of temperature for
336 photosynthesis and temperatures above 35 °C appear to have a negative effect on photosynthetic processes
337 (e.g. Greer and Weedon 2012). In fact, the *Topt* assumed in our study (23 ºC) is close to the optimum
338 temperature (25 ºC) proposed by Caffara and Eccel (2010) for grapevine phenological development.
339 In our study the date for the beginning of the accumulation of heat units (t_0) was fixed as the
340 budburst date. During the study period this phenological stage occurred on average during March (March
341 29th in Vinhos Verdes and March 19th in Lisbon), which is not far for the value of 60 days proposed by
342 Parker et al. (2011) as the initial date for the thermal summation. The period between budburst and
343 flowering is shorter (from 59 to 67 days) compared with the other periods tested (1st of September –
344 Flowering and 1st of January – Flowering) which implies the existence of less meteorological and biological
345 variability that influence the timing of flowering. This would consequently increase the accuracy of the
346 phenological model. On the other hand the inter annual variability of budburst date reflects the prevailing
347 conditions of chilling accumulation during the dormancy phase and indirectly incorporates this period into
348 the model. Indeed it would be interesting to ascertain if the optimization of a two-phase model considering
349 the successive stages of dormancy and growth, using an Alternating, Parallel or Sequential approach, could
350 increase the accuracy of the models. Nonetheless, the model developed considering the budburst as the start
351 date for the thermal accumulation provide a tool for the wine producers to incorporate the local-specific
352 variability of the budburst dates to predict the date of flowering.
353 In a second phase of the process, a validation was performed by testing the three estimated models
354 with fixed parameters to 13 varieties grown in the Vinhos Verdes (AVV and FEL) and Lisbon (TOV) wine

355 regions. Therefore, the models (GDD, GDD Triangular and UniFORC) were compared using 473
356 observations of flowering date. Overall, the accuracy in forecasting the flowering dates is very similar
357 between the three models tested for all varieties, with high R^2 and low RMSE and MAD lower than 4 days
358 in AVV and TOV and lower than 6 days in FEL. This similarity between the models can indicate that the
359 parameter estimates allow a similar explicability of the flowering date, varying only the type of response
360 function of the plant to temperature. Nevertheless, the UniFORC model was the one presenting the overall
361 best results, with a R^2 higher than 0.75 in 67% and 65% of the cases, respectively, for the estimation and
362 validation data sets, which indicate that most of the observed annual flowering dates were explained by this
363 model. In addition, from the biological point of view the phenological development of most plant species
364 is continuous and non-linear (Chuine et al. 2003) and the UniFORC model represents this type of
365 physiological response of the plant to ambient temperature, even though having two parameters (F^* and d)
366 that have to be fitted.
367 With the UniFORC model the MAD was on average 2.81 days in AVV and 3.00 days in TOV. In
368 FEL the average MAD was 4.99 days. In our study, the interval of days between phenological observations
369 in the field was in the case of AVV and TOV 3/4 days and in FEL 6/7 days. Therefore, the errors obtained
370 are lower than the frequency of field observations.
371 Shorter intervals between field phenological observations can improve the quality of data and
372 consequently the precision of the models to predict the flowering date. Daily field observations can be made
373 using new technologies such as the use of satellite remote sensing and digital imaging systems (e.g. using
374 multiple flashes camera for image acquisition) (INCREASE 2013).
375 In our study the number of phenological observations is unbalanced between sites and across
376 varieties, and between estimation and validation dataset. In fact, the longest time-series (25 years) were
377 used in the estimation process that allows to account for great inter-annual variability. In the validation
378 phase more variable time series, with 8 to 25 years, were used. However, the statistical indicators of the
379 series with different observations are very similar, which corroborates the robustness of the results.
380 **5. Conclusion**
381 In this paper we show how grapevine flowering date can be estimated using temperature-based
382 phenological models across different genotypes (grape varieties) and environments (wine regions) in
383 Portugal. Three models were tested GDD, GDD triangular and UniFORC and the accuracy for predicting
384 grapevine flowering date was very similar between them. A UniFORC model with fixed optimized
385 parameters starting from the budburst date presented the best overall fit.
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3. Article 2 “Two-phase phenological models to characterise the timing of budburst and flowering of *Vitis vinifera* L.”

Two-phase phenological models to predict the timing of budburstand flowering of *Vitis vinifera* L.

PAPER IN PREPARATION – WORKING PAPER

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Abstract

Knowledge on the dates of specific stages of crop development can be particularly important in grapevine helping the planning, organization and timely execution of several agricultural practices. The aim of this study was to test two-phase phenological models, which include chilling and forcing temperatures for predicting the grapevine budburst and flowering stages using data collected between 1990 to 2014 in two Portuguese wine regions: Vinhos Verdes and Lisbon. Nine models were tested where different functions for the chilling and for the heat accumulation were combined. Estimation of the models was performed using 2 grape cultivars, present in both regions, based on an iterative generalization process for one forced *t0* date: 1st September. The best overall model was the one which combines the Chuine and GDD functions, for the stages of chilling and forcing correspondingly. The values of model parameters were further optimized in order to obtain one model that could be used for a diverse range of grape cultivars. An external validation was performed using an independent data-set from three grape cultivars. The results showed that the model's goodness-of-fit indicators performed well, with high R^2 (0.31 to 0.67 for budburst and 0.75 to 0.91 for flowering), low RMSE (3 to 6 days for budburst and 2 to 4 days for flowering), and were able to predict the budburst and flowering dates with an average error of 4 days and 3 days respectively. The phenological models developed in our study presented high accuracy when applied to several cultivars in different regions and can be used as a predictor tool of budburst and flowering in Portugal.

Keywords: phenology, grapevine flowering, grapevine budburst, two-phase models, optimization algorithm, simulated annealing

1. Introduction (under preparation)

Vegetation phenology refers to the seasonal timing of recurring biological events, the causes of their timing, their relationship to biotic and abiotic forces, and the interrelations among phases of the same or different species (Lieth 1974). Knowledge on expected phenology dates can be useful in many scientific disciplines from climate change, biodiversity, agriculture and forestry. The timing of phenological events and their variability can provide valuable data for planning, organizing and timely execution of certain standard and special (preventive and protective) agricultural activities that require advanced information on the dates of specific stages of crop development (Ruml and Vulić 2005). This is particularly important with deciduous plants such as grapevine, where predicting phenological dates is of economic and technical concern.

The grapevine biological cycle is biannual and it's composed of the vegetative and the reproductive cycles. The buds formed in the first year give rise to shoots carrying fruit in the second year (Keller 2015). In the northern hemisphere, the vegetative cycle, begins with the break

of the dormant buds in late winter, continues with the grapevine passing through different development stages and finishes with the leafing-out in autumn. At this point, the plant enters a dormancy stage where no growth occurs. In the reproductive cycle, the floral initiation begins in the previous year during spring, which after a dormant period in winter continues its growth until the development of fruit (September). Therefore, perennial crops like the grapevine remain alive throughout successive winters, during which periods they are in a state characterized by the absence of any increase in the number/volume of organs even under suitable environmental condition – winter dormancy or rest (Tromp et al. 2005).

Dormancy is a complex physiological process strongly dependent on the interaction between genotype and environment. The organs of the plants which are in this state are much more resistant to climate adversities such as low and high temperatures and dryness. So, plants that are capable of establishing a dormancy period before the beginning of unfavorable seasons to their growth are more competitive and more capable of surviving in this conditions comparing to plants that cannot enter this stage (Vegis 1964).

In the grapevine, buds may exist at three different dormancy levels (Sarvas 1974; Lang et al. 1987): i) 'para-dormancy' or 'summer-dormancy', where growth is regulated by endogenous plant factors outside the bud (growth is prevented by factors which are produced from shoots or leaves that are still growing); 'endo-dormancy' or 'winter-dormancy' where growth is regulated by physiological factors inside the bud (this inhibition can only be cancelled by low temperature accumulated during a certain extended period); 'eco-dormancy' or 'imposed-dormancy' where growth is regulated by external environmental factors like short photoperiod or extremely low temperatures (Tromp et al. 2005; Andreini et al. 2014).

With the decreasing of day length, newly formed buds at the end of the summer may enter a period of inactivity (Caffarra et al. 2011), this phenomenon may also occur due to the combination of day length and low temperature at the begging of the autumn (Heidi and Prestrud 2005; Penfield 2008). This process causes the leaf and the flower buds of grapevines to enter in the endo-dormancy period. Specific accumulation of chilling temperature is necessary to overcome the state of endo-dormancy of the bud in order to budbreak occur (Chuine 2000; Erez and Lavee 1971). Therefore, warmer temperatures during the winter may affect the phenology and cause late bud break due to a late dormancy break (Viti and Monteleone 1991; Sunley et al. 2006), low percentage of flowering budbreak (Viti and Monteleone 1995) and prolonged leafing and blooming periods (Erez 2000).

Currently, two groups of phenological models have been developed for perennial crops. The first group includes the Thermal Time or Spring Warming models that only contemplates the effect of the temperatures (forcing temperatures) during the active growth period (one-phase model) (Hunter and Lechowicz 1992; Cannell and Smith 1983a; Robertson 1968).

The second group also includes the impact of chilling temperatures during the dormancy phase (two-phase model) and contemplates the following models: i) Sequential Model (Hänninen 1990b; Richardson et al. 1974; Hänninen 1987) which assumes that the effect of forcing temperatures cannot be effective unless chilling requirements have already been fulfilled; ii) Parallel Model (Landsberg 1974; Hänninen 1987, 1990b) which assumes that forcing temperatures can be active concomitant with the time spent for chilling conditions and they are not fully active as long as full chilling is not reached; iii) Alternating Model (Murray et al. 1989; Cannell and Smith 1983a; Kramer 1994) which assumes a negative exponential relationship between the sum of forcing units required for completion of a growth phase and the sum of chilling units received.

Portugal is characterized by large inter-annual variation on the dates of occurrence of the grapevine phenological stages. Despite this instability in the phenological dates in Portugal, and the adverse effects for all the contributors involved in wine industry, there is no accurate, validate

and scientifically based operational predictive model for phenological events. Predicting phenological dates is important for a variety of applications, such as the more efficient planning of viticultural practices and harvest decisions (Williams and Penfield 1985), predicting the vulnerability to pest attacks and the selection of cultivars in new areas (Gladstones 1992; Bindi et al. 1997; Orlandi and Mancini 1999; Jarvis et al. 2017). Also, phenological models can be coupled with climate change scenarios projecting the impact of climate change on grapevine (Cola et al. 2017).

In the past few years, a number of thermal based models (which take into account the action of forcing units in the growing period) generally based on the growing degree days GDD (Cannell and Smith 1983b), has been tested for predicting phenological dates using regional data (Lopes et al. 2008; Fraga et al. 2016; Oliveira 1998). However, model improvement through nonlinear functions of response to temperature such as sigmoidal (Hänninen 1990b) and triangular (Hänninen 1990a) functions and the influence of chilling on the occurrence date of the different phenological stages have not been tested yet.

Therefore, the main goal of this work is to test the two-phase models capable of predicting the timing of budburst and flowering of grapevine using external data (data not used to adjust the model) collected in Portugal and to compare models estimated in two different Portuguese regions in order to evaluate the differentiation between cultivars.

Nine models were tested with Portuguese grapevine from two regions and result from a combination of three different types of hypothesis: i) does the coupling of chilling and forcing functions improve the accuracy of the thermal based model? ii) what is the most appropriate function (linear or nonlinear) to better explain the temperature action on the phenological development? iii) how is the transferability of these models among varieties and ecological conditions? The experimental design was complemented in order to test each model development hypothesis independently. Adjustments of the models and tests were performed using the dates of budburst and flowering of Portuguese grapevine cultivars located in two wine demarcated regions.

2. Material and Methods

2.1. Metereological and phenological data

The research was carried out using metereological and phenological data from two Portuguese wine regions: Vinhos Verdes and Lisbon region (Figure 1). These regions differ in terms of weather, soils, grape cultivars and vine-growing systems. The weather in these regions is characterized by an irregular annual rainfall level distributed throughout the year, mainly concentrated in winter and spring, and by an inverse increase in air temperature relatively to precipitation. In fact, winters are cool and wet, and summers are hot and dry (Cunha et al. 2015).

Phenological data was collected in Vinhos Verdes region in Arcos de Valdevez – AVV (Minho, Northwestern part) in the experimental field of the Amândio Galhano Wine Station research (41º48'57"N, 8º25'35"W, 70 m a.s.l) and in Lisbon wine region in Torres Vedras – TOV (wester-central part of Portugal) in Quinta dos Almoínha (39º01'46''N, 9º01'35''W, 99 m a.s.l.; Fig. 1) belonging to the Portuguese 'Instituto Nacional de Investigação Agrária e Veterinária' (INIAV) located in Dois Portos, Torres Vedras. The AVV test site is the one that is further to the north, and is distant from TOV about 300 km.

Long-term series of grapevine phenological observations of the dates of budburst and flowering of different grape cultivars were used. Observations were performed at field, using the Baggiollini phenological scale (Baggiolini 1952). A given phenological stage was reached, and date recorded when the event occurred in 50 % of the plants under observation for each grape variety, at the stages 'C: budbreak' and 'I: flowering' of Baggiolini Scale, which corresponds to the stages 'EL 07 Beginning of budburst: green shoot tips just visible' and 'EL 65 Full flowering: 50% of flowerhoods fallen' respectively according to the Eichhorn and Lorenz (E-L) system

(Lorenz et al., 1995). The observations were performed every 7 days in the budburst stage and 3 to 4 days in the flowering stage. Data were registered according to the ‘‘Organisation Internationale de la Vigne et du Vin’’ (OIV) phenophase descriptors (OIV 2009).

Time-series were collected between 1990 and 2014 (periods ranged from 8 to 25 years according to the regions) for three white cultivars – Chasselas (CHA), Fernão Pires (FP), Loureiro (LOU) – and one red cultivar – Padeiro Basto (PB).

Meteorological data used for model development was recorded in a weather station located near (< 5 km) the field where the phenological observations were performed. Daily observations of maximum and minimum temperatures were registered and used to calculate the daily mean temperature. The AVV region typically shows annual mean temperatures of about 15 °C and TOV of 17 °C. The site with warmer temperatures (mean, minimum and maximum) for the period from 1st of January to Flowering was Torre Vedras (TOV), while Arcos de Valdevez (AVV) was the one with lower temperatures during this period (Table 1).

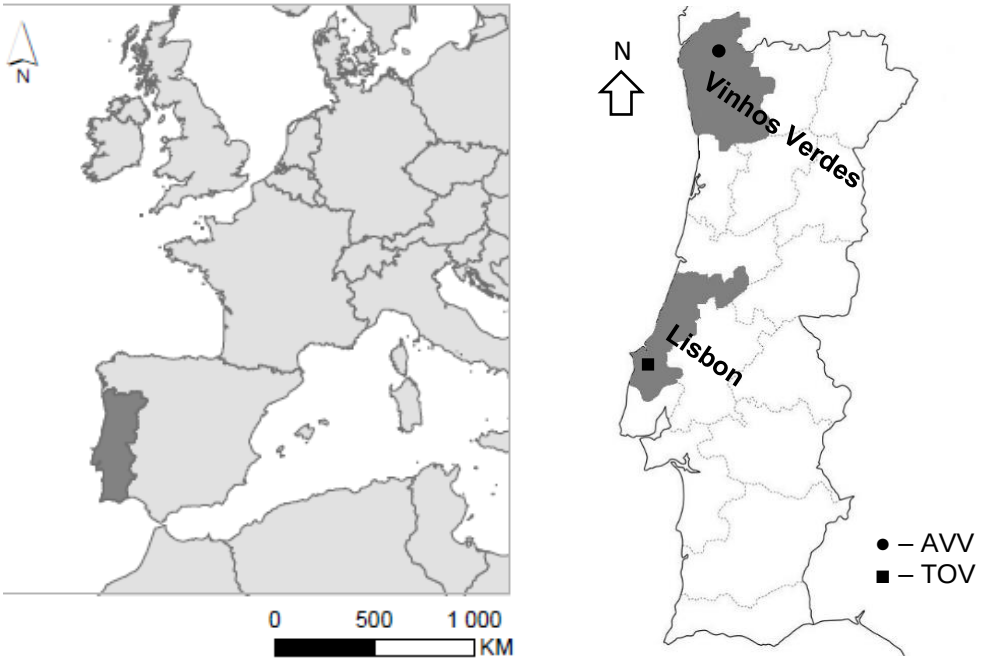

Figure 10 Map of Portugal with the wine regions where the research was carried out are represented as well as the location of test sites analyzed in this work.

During the growing season the average temperature is about 17 ºC in AVV and 19 ºC in TOV (Table 1), and according to the climate maturity grouping (Jones 2006), the growing season can be classified as “Intermediate” and “Warm”, respectively for AVV and TOV wine regions.

Table 1 Summary of average and standard deviation of the minimum and maximum temperatures for each studied region for the period from 1st January to the budburst date, from 1st January date to the flowering date from 1990 to 2014.

Temperature (Cº)	1st January – Budburst	1st January – Flowering	Budburst – Flowering	Growing Season
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Arcos de Valdevez (AVV)				
Mean	10.4 ± 2.8	12.0 ± 3.5	14.1 ± 3.2	16.6 ± 0.78
Minimum	6.3 ± 3.3	7.6 ± 3.4	9.4 ± 2.8	11.6 ± 0.63
Maximum	14.5 ± 3.5	16.4 ± 4.4	18.8 ± 4.3	21.5 ± 1.09
Torres Vedras (TOV)				
Mean	12.8 ± 2.4	14.5 ± 3.1	16.5 ± 2.6	19.3 ± 0.90
Minimum	9.6 ± 2.6	10.9 ± 2.9	12.6 ± 2.2	15.1 ± 0.61
Maximum	16.0 ± 2.7	18.0 ± 3.8	20.3 ± 3.6	23.4 ± 1.23

SD: standard deviation

2.2. Model development and validation

2.2.1.Preliminary study and model selection

Several two-phase phenological models were tested and validated for predicting the grapevine budburst and flowering dates for the four grape varieties under study. These models are derived from the Sequential model (Sarvas 1974; Hänninen 1990b; Kramer 1994; Hänninen 1987), which assumes that the effect of forcing temperatures cannot be effective unless chilling requirements have already been fulfilled (Chuine et al. 1998).

Two-phase models, integrate the dormancy phase and assume that a critical value of the state of chilling (*Sc*), denoted *C** (Eq. 1) must be reached to break dormancy (*td*), with the daily rate of chilling (*Rc*) calculated by different chilling functions that are temperature dependent. At this point, a sum of forcing units can start to accumulate until reaching a critical value of the state if forcing (*Sf*), denoted *F**, as described by Eq. 2. The *Sf* is described as a sum of the daily rate of forcing (*Rf*), which is a function of temperature (*xt* is the daily mean temperature), and starts at *t0* (day of the year, DOY) and ends at flowering (*ts*).

$$S_c\ (t_d) = \sum_{t_0}^{td} Rc(x_t) \geq C^*\ (1)$$

$$S_f\ (t_s) = \sum_{t_0}^{ts} Rf(x_t) \geq F^*\ (2)$$

The grapevine developmental responses to temperature during the dormancy and growth phase could be described by different types of functions (Table 2).

Table 2 Representation of different functions of responses to temperature used to calculate chilling and forcing units.

Chilling functions	*Forcing functions*
• GDD Inverse $R_C(x_t) = \begin{cases} Tb - x_t \; if \; x_t < Tb \\ 0 \; if \; x_t \geq Tb \end{cases} \; (a)$ *xt* - daily mean temperature *Tb* - base temperature above which the thermal summation is not calculated	• GDD $R_f(x_t) = \begin{cases} 0 \; if \; x_t < Tb \\ x_t - Tb \; if \; x_t \geq Tb \end{cases} \; (b)$ x_t - daily mean temperature *Tb* - base temperature above which the thermal summation is calculated
• Triangular $R_f(x_t) = \begin{cases} 0 \; if \; x_t << Tmin \\ \frac{x_t - Tmin}{Topt - Tmin} \; if \; Tmin < x_t << Topt \\ \frac{x_t - Tmax}{Topt - Tmax} if \; Topt < x_t < Tmax \\ 0 \; if \; x_t >> Tmax \end{cases} \; (c)$ x_t - daily mean temperature *Tmin* - minimum temperature above which the thermal summation is calculated *Topt* - optimal temperature from which the amount of units of heat accumulated start to decrease *Tmax* - maximum temperature from which no thermal summation occurs	• Wang $R_f(x_t) = Max \left[2(x_t - Tmin)^{\alpha}(Topt - Tmin)^{\alpha} - \frac{(x_t - Tmin)^{2\alpha}}{(Topt - Tmin)^{2\alpha}}, 0 \right] (d)$ x_t - daily mean temperature *Tmin* - minimum temperature above which the thermal summation is calculated *Topt* - optimal temperature from which the amount of units of heat accumulated start to decrease *Tmax* - maximum temperature from which no thermal summation occurs $\alpha = \ln(2) / \ln\left(\frac{Tmax - Tmin}{Topt - Tmin}\right)$
• Chuine $Rc(x_t) = \frac{1}{\left(1 + e^{[a(x_t - c)^2 + b(x_t - c)]}\right)} \; (e)$ x_t - daily mean temperature *a* – width of the window over which the function is not null, a ∈ [0,10] *b* – sharpness of the response curve and its asymmetry, b ∈ [-30,15] *c* – mid-response value when *b* is close to zero, and a boundary to the temperature range over which chilling units accumulate, when *b* strongly differs from zero	• Sigmoid $R_f = \begin{cases} 0 \; if \; x_t < 0 \\ \frac{1}{1 + e^{d(x_t - e)}} \; if \; x_t \geq 0 \end{cases} \; (f)$ x_t - daily mean temperature d - (<0) defines the sharpness of the response curve e - (>0) is the mid-response temperature

2.2.2. Model estimation

In our modeling strategy, the timing of budburst and flowering were estimated in a two-phase approach. In a first phase, estimation of the models was based on an iterative generalization process for nine two-phase models (Table 3) with one fixed *t0* date (1st September). This allowed selecting the best overall combination of functions to develop a final model. In a second phase, the selected model was simulated with an intermediate range of values for parameters estimation, in order to obtain one model that could be applied for a diverse range of grape cultivars for the timing of budburst/flowering. The cultivars with the longest phenological and metereological time series (25 years) in Arcos de Valdevez (Fernão Pires and Loureiro) and in Torres Vedras (Fernão Pires) were used in this steps.

The models adjustments optimization was performed by minimizing the least-squares function in the parameters space using a simulated annealing method (Chuine et al. 1999) of Metropolis algorithm (Metropolis et al. 1953). This algorithm is used to fit the model parameters and explore the parameters space around the global optimum found at the end of its search step.

The models were developed using the program PMP 5.5 (Phenology Modeling Platform) (Chuine et al. 2003).

Table 3 Combinations of different functions of chilling and forcing action, which form nine two-phase phenological models tested in the estimation process.

Chilling function	*Forcing function*	*Acronym*
Chuine	GDD	Ch_GDD
	Sigmoid	Ch_Sig
	Wang	Ch_Wang
GDD Inverse	GDD	GDD_iGDD
	Sigmoid	GDDi_Sig
	Wang	GDD_Wang
Triangular	GDD	Tri_GDD
	Sigmoid	Tri_Sig
	Wang	Tri_Wang

2.2.3.Model validation

The models estimated based on the AVV and TOV dataset (Fernão Pires, AVV e TOV, and Loureiro, AVV) were validated on independent datasets (92 observations of several time-series ranging from 8 to 25 years, 3 grape cultivars (Chasselas, Loureiro and Padeiro Basto) and two regions (AVV and TOV). This allowed selecting the most reliable model to predict the timing of grapevine budburst and flowering based on an inter-regional assessment of the model accuracy.

The estimation and the selection of the best model during the validation process were based on the Mean Absolute Deviation (MAD), maximum error (Max. error) and on the Root Mean Square Error (RMSE) between observed and predicted values. These indicators are expressed in the same unit as the data, where the lowest value is associated with the best model. Another indicators of model fit were the coefficient of determination (R^2) and the Akaike Information Criterion (AIC).

3. Results and Discussion

3.1. Analysis of phenological time series

A descriptive statistical analysis was performed for the budburst and flowering dates of the grapevine phenological observations performed in the two regions of Portugal (Table 4). The analysis showed that there was an inter-annual variation of both budburst and flowering phenological dates among grape cultivars. Overall, the budburst dates vary from DOY (Day Of the Year) 75 (Fernão Pires) to DOY 89 (Padeiro Basto) and the flowering occurs between DOY 143 (Chasselas) and DOY 151 (Padeiro Basto) (Table 4). In general, TOV is erlier than the AVV region for the four varieties analysed.

Padeiro Basto presented the latest budburst date in both AVV and TOV regions (DOY 90 and 87 respectively, which corresponds to 31th and 28th March). Comparing the budburst date of the three white varieties the most late one was Loureiro in both regions (DOY 86 – 27th March – in AVV and DOY 77 – 18th March – in TOV).

The same tendency was observed in flowering, where Padeiro Basto showed the latest budburst date in both AVV and TOV regions (DOY 153 and 148 respectively, which corresponds

to 2nd June and 28th May). For the three white varieties analysed, the one with the latest flowering date was Loureiro in AVV and TOV regions (DOY 150 – 30th May – and DOY 144 – 24th May).

Table 4 Statistical analyses of the budburst and flowering dates for the four grape cultivars studied studied in the two regions of Portugal, Torres Vedras (TOV) and Arcos de Valdevez (AVV). The days of the year presented in this table for each period were calculated from 1st of January (considered as day 1). The data was collected between 1990-2014.

Region	Chasselas		Fernão Pires		Loureiro		P.Basto	
	obs	Mean ± SD	obs	Mean ± SD	obs	Mean ± SD	obs	Mean ± SD
Budburst								
AVV	8	80.7 ± 5.5	25	74.6 ± 6.1	25	85.8 ± 6.4	25	90.2 ± 6.5
TOV	25	74.6 ± 8.6	25	76.0 ± 8.2	20	77.1 ± 9.4	14	86.9 ± 8.7
Overall	33	76.1 ± 8.5	50	75.3 ± 7.3	45	84.4 ± 9.9	39	89.0 ± 7.6
Flowering								
AVV	8	147.4 ± 8.0	25	145.1 ± 9.7	25	150.4 ± 8.6	25	153.2 ± 7.7
TOV	25	141.9 ± 8.4	25	142.6 ± 8.5	20	143.8 ± 8.8	14	148.1 ± 5.5
Overall	33	143.2 ± 8.8	50	143.2 ± 8.8	45	148.4 ± 9.3	39	151.4 ± 7.5

3.2. Modelling grapevine phenology

3.2.1.Model selection

The nine models proposed in Table 3 to estimate the budburst and flowering dates were selected considering, in a first phase, the fixed common t_0 date of 1st September to start chilling accumulation. The remain model parameters were fitted, using the observations corresponding to the cultivars Fernão Pires (FP) in AVV region, Fernão Pires in TOV region and Loureiro (LOU) in AVV region.

Table 5 and table 6 present the parametrization and the goodness-of-fit indicators for the nine models studied respectively for the budburst and flowering stage. The models where the accumulation of the chilling units was represented by the Triangular function and the GDD Inverse function presented low biological coherence and these models were not used for further analysis of model selection. The Triangular function assumes that the accumulation of chilling units can occur after the 20ºC (*Tmax* generally vary from 30 to 40 ºC) and the in the GDD Inverse function, the accumulation of chilling units only ends after 35 ºC, which have low biological adherence (Table 5 and 6).

Also, all the three combinations based on the Chuine models (Chuine/GDD; Chuine/Sigmoid; Chuine/Wang) when compared with the other models based on the GDD inverse and Triangular functions, presented consistently better results for the goodness-of-fit indicators for both budbreak and flowering (Table 5 and 6).

The model with the best performance was the Ch_GDD which presents higher R^2 (0.57 to 0.68 for budburst and 0.74 to 0.83 for flowering), lower RMSE (3 to 5 days for budburst and 4 days for flowering), maximum error (8 to 15 days for budburst and 8 days for flowering), AIC (74 to 96 for budburst and 82 to 85 for flowering) and MAD (3 to 4 days for budburst and 3 to 4 days for flowering) (Table 5 and 6). Also, this model presented, in general the lower value for the AIC indicator.

Table 5 Summary of the estimation process results for the nine models studied for the budburst stage. The lines in bold correspond to the parameters and respective models selected.

Model	Model parameters								Goodness-of fit				
(variety x region)	Chiling				Forcing				R^2	RMSE	AIC	MAD	Max error
Chuine/GDD	***a***	***b***	***c***	***C****	***Tb***	**--**	**--**	***F****					
FP_AVV	**0.93**	**-24.11**	**-3.82**	**146.05**	**2.45**	**--**	**--**	**355.19**	**0.68**	**3.44**	**73.84**	**2.53**	**7.90**
LOU_AVV	**0.83**	**-25.91**	**-4.99**	**94.91**	**0.01**	**--**	**--**	**1125.92**	**0.58**	**4.16**	**83.30**	**3.57**	**8.20**
FP_TOV	**0.46**	**10.60**	**29.02**	**114.78**	**5.00**	**--**	**--**	**631.72**	**0.57**	**5.36**	**95.93**	**4.08**	**15.10**
Chuine/Sigmoid	*a*	*b*	*c*	*C**	*d*	*e*	--	*F**					
FP_AVV	0.71	13.39	22.09	147.17	-1.54	8.00	--	41.17	0.53	5.60	83.28	2.90	10.60
LOU_AVV	1.03	-22.87	4.05	142.95	-0.46	7.37	--	47.43	0.53	4.40	88.11	3.32	8.80
FP_TOV	0.56	-12.80	6.00	143.98	-0.56	11.18	--	36.22	0.56	5.42	96.51	3.92	15.40
Chuine/Wang	*a*	*b*	*c*	*C**	*Topt*	*Tmin*	*Tmax*	*F**					
FP_AVV	0.38	-8.69	0.02	145.03	17.42	4.79	36.29	34.67	0.65	3.61	80.12	2.85	8.70
LOU_AVV	0.92	-24.81	-0.85	96.49	28.63	-20.19	38.82	35.89	0.57	4.21	98.79	3.60	8.20
FP_TOV	0.51	11.64	29.80	114.84	23.98	-4.65	31.60	34.03	0.59	5.24	87.87	4.18	16.20
GDD Inverse/GDD	*Tb*	--	--	*C**	*Tb*	--	--	*F**					
FP_AVV	37.10	--	--	3479.11	3.47	--	--	332.15	0.57	3.99	77.21	3.24	9.30
LOU_AVV	38.93	--	--	2285.70	0.70	--	--	1032.13	0.44	4.81	86.49	3.78	10.10
FP_TOV	39.95	--	--	2235.98	5.79	--	--	650.95	0.58	5.30	91.40	3.84	11.50
GDD inverse/Sigmoid	*Tb*	--	--	*C**	*d*	*e*	--	*F**					
FP_AVV	37.48	--	--	3566.72	-1.58	7.38	--	39.81	0.46	4.47	84.88	3.75	9.40
LOU_AVV	34.76	--	--	3037.44	-0.40	8.56	--	41.24	0.19	5.78	97.76	4.68	12.70
FP_TOV	37.20	--	--	3053.02	-1.10	11.37	--	37.99	0.42	6.24	101.55	4.82	14.30
GDD Inverse/Wang	*Tb*	--	--	*C**	*Topt*	*Tmin*	*Tmax*	*F**					
FP_AVV	37.96	--	--	3589.32	21.39	5.64	49.91	31.03	0.57	4.01	81.46	3.39	9.60
LOU_AVV	38.80	--	--	2300.04	29.50	-11.44	41.86	37.18	0.44	4.83	90.77	3.76	9.90
FP_TOV	38.45	--	--	3154.30	19.73	2.16	26.04	29.74	0.49	5.84	100.21	4.45	12.30
Triangular/GDD	*Topt*	*Tmin*	*Tmax*	*C**	*Tb*	--	--	*F**					
FP_AVV	13.32	-38.83	38.78	130.96	2.45	--	--	372.96	0.71	3.29	71.59	2.64	8.20
LOU_AVV	20.17	-39.32	40.07	101.71	0.44	--	--	899.69	0.50	4.55	87.74	3.77	7.90
FP_TOV	7.89	-9.78	44.43	73.75	5.99	--	--	630.40	0.58	5.30	95.39	3.78	14.10
Triangular/Sigmoid	*Topt*	*Tmin*	*Tmax*	*C**	*d*	*e*	--	*F**					
FP_AVV	13.10	-20.25	44.71	130.98	-40.00	6.88		41.98	0.67	3.51	70.29	3.59	9.10
LOU_AVV	19.02	-21.76	43.52	130.24	-37.66	8.45		41.91	0.59	4.11	84.67	3.40	11.00
FP_TOV	17.38	-12.71	49.97	130.33	-40.00	10.79		41.11	0.59	5.25	99.07	3.40	9.10
Triangular/Wang	*Topt*	*Tmin*	*Tmax*	*C**	*Topt*	*Tmin*	*Tmax*	*F**					
FP_AVV	13.52	-19.71	41.92	128.15	19.50	5.50	46.14	33.52	0.71	3.30	75.68	2.80	4.70
LOU_AVV	20.03	-39.20	36.21	127.31	18.87	5.00	48.77	46.89	0.53	4.43	90.40	3.70	7.80
FP_TOV	8.73	-17.86	40.98	69.30	25.38	-29.94	31.48	32.91	0.60	5.21	98.49	3.76	14.50

Table 6 Summary of the estimation process results for the nine models studied for the flowering stage. The lines in bold correspond to the parameters and respective models selected.

Model	Model parameters								Goodness-of fit				
(variety x region)	Chiling				Forcing				R^2	RMSE	AIC	MAD	Max error
Chuine/GDD	***a***	***b***	***c***	***C****	***Tb***	--	--	***F****					
FP_AVV	**1.38**	**-27.85**	**1.13**	**172.50**	**7.77**	--	--	**474.81**	**0.83**	**4.02**	**81.53**	**3.32**	**8.40**
LOU_AVV	**0.91**	**-23.93**	**-4.34**	**202.90**	**8.02**	--	--	**384.01**	**0.75**	**4.35**	**84.94**	**3.33**	**8.20**
FP_TOV	**1.10**	**-23.27**	**8.69**	**173.51**	**9.62**	--	--	**530.81**	**0.74**	**4.30**	**85.47**	**3.55**	**8.10**
Chuine/Sigmoid	*a*	*b*	*c*	*C**	*d*	*e*	--	*F**					
FP_AVV	1.16	-25.37	0.60	169.87	-0.26	16.91	--	26.78	0.84	3.91	82.13	3.20	7.40
LOU_AVV	1.18	-22.92	2.25	210.09	-0.32	15.26	--	23.67	0.77	4.12	84.83	3.09	10.80
FP_TOV	2.53	-27.37	5.23	88.24	-0.20	17.25	--	47.64	0.71	4.55	89.67	4.26	9.30
Chuine/Wang	*a*	*b*	*c*	*C**	*Topt*	*Tmin*	*Tmax*	*F**					
FP_AVV	0.50	11.61	22.27	175.00	25.97	0.55	33.74	27.82	0.84	3.88	83.74	3.21	6.80
LOU_AVV	0.65	-11.36	3.79	192.94	21.47	-20.43	25.38	28.56	0.74	4.42	90.32	3.56	10.00
FP_TOV	0.11	4.00	29.76	143.97	25.71	-27.80	31.88	52.25	0.74	4.33	89.22	3.56	8.50
GDD Inverse/GDD	*Tb*	--	--	*C**	*Tb*	--	--	*F**					
FP_AVV	36.90	--	--	4278.00	8.33	--	--	428.45	0.81	4.25	80.31	3.38	8.70
LOU_AVV	37.79	--	--	4602.07	7.09	--	--	568.09	0.64	5.14	89.83	3.39	10.10
FP_TOV	37.08	--	--	2684.55	6.98	--	--	1003.67	0.72	4.43	78.67	3.64	8.90
GDD inverse/Sigmoid	*Tb*	--	--	*C**	*d*	*e*	--	*F**					
FP_AVV	38.68	--	--	2324.76	-0.24	18.83	--	28.57	0.82	4.09	80.49	3.56	7.90
LOU_AVV	38.17	--	--	2248.20	-0.19	20.06	--	32.84	0.63	5.23	92.71	4.26	13.50
FP_TOV	36.84	--	--	2352.94	-0.25	16.15	--	57.71	0.73	4.43	84.42	3.54	10.90
GDD Inverse/Wang	*Tb*	--	--	*C**	*Topt*	*Tmin*	*Tmax*	*F**					
FP_AVV	36.75	--	--	3543.90	21.71	-27.09	26.07	41.87	0.80	4.30	84.90	3.37	9.50
LOU_AVV	38.26	--	--	4686.60	25.27	-3.16	33.09	35.35	0.64	5.17	94.15	3.84	9.90
FP_TOV	39.00	--	--	3294.52	26.45	1.99	35.65	52.86	0.73	4.37	85.72	3.73	9.30
Triangular/GDD	*Topt*	*Tmin*	*Tmax*	*C**	*Tb*	--	--	*F**					
FP_AVV	21.70	-20.34	22.26	132.35	7.76	--	--	493.12	0.82	4.07	82.20	3.32	8.60
LOU_AVV	21.65	-24.28	21.68	169.40	8.54	--	--	303.88	0.79	3.98	81.06	3.40	9.80
FP_TOV	29.28	-30.26	46.13	136.39	8.31	--	--	646.53	0.73	4.39	85.98	3.46	8.40
Triangular/Sigmoid	*Topt*	*Tmin*	*Tmax*	*C**	*d*	*e*	--	*F**					
FP_AVV	17.97	-23.79	39.93	169.20	-0.27	16.93	--	21.17	0.80	4.33	87.06	3.59	9.10
LOU_AVV	16.45	-18.30	40.41	187.05	-0.31	16.21	--	19.31	0.78	4.03	83.70	3.48	9.80
FP_TOV	18.05	0.82	48.23	181.92	-36.21	16.39	--	19.63	0.72	4.45	88.66	3.45	9.60
Triangular/Wang	*Topt*	*Tmin*	*Tmax*	*C**	*Topt*	*Tmin*	*Tmax*	*F**					
FP_AVV	15.68	-24.22	39.32	192.92	22.27	-25.97	25.39	13.44	0.83	4.03	85.64	3.26	10.40
LOU_AVV	16.38	-35.86	36.50	194.29	28.97	9.43	45.61	19.42	0.77	4.12	86.81	3.46	11.10
FP_TOV	23.94	-34.14	46.30	190.81	24.61	10.00	30.80	16.37	0.65	5.00	96.44	4.20	9.70

After this first approach, based on the previous results, an iteration process was performed for the Ch_GDD combination. The model's parameters were a constraint in the Chuine function but continued fitted in the GDD function (Table 7) in order to obtain a more generalized model for different cultivars and regions (Table 8).

3.2.2.Model validation

Table 9 presents the results of the external validation of the Ch_GDD model with the independent dataset from 3 grapevine cultivars (Loureiro in TOV, Padeiro Basto and Chasselas in AVV and TOV). The model's goodness-of-fit indicators presented a R^2 varying from 0.31 to 0.66 for budburst and 0.75 to 0.91 for flowering, low RMSE (4 to 6 days for budburst and 2 to 4 days for flowering), and MAD (3 to 4 days for budburst and 2 to 3 days for flowering) (Table 9). In fact, the only cultivar with the greatest difference in R^2 was Padeiro Basto in AVV for budburst with an R^2 value of 0.31, although the values of the other model's goodness-of-fit indicators are similar to the ones obtained for the other varieties (Table 9).

The mean critical chilling requirements (C^*) for the tested grapevine cultivars is 124 $\pm$ 33 for budburst and 173 $\pm$ 29 for flowering date. Botelho, Pavanello *et al.* (2007), reported for the critical chilling requirements of Cabernet Sauvignon values around 336 h at temperatures below 6 ºC to attain budburst (which was fulfilled in about 2 weeks). In our study, the estimated chilling function considers temperatures above 10 ºC until 20 ºC also to be effective. In fact, a premise applied in our study was the beginning of the accumulation of chilling units (*t0*) starts from September 1st and in this month it is frequently observed for the studied regions, particularly in TOV, mean daily temperature between 10 ºC and 20 ºC. Similar results on the impact of mild temperatures on chilling requirements were also found by Caffarra and Eccel (2010) for grapevine and Heide (Heide 2003) for *Betula pubescens*. As grapevine endodormancy is usually broken by temperatures below 10 to 12 ºC (Pouget 1968; Dokoozlian 1999) it can be argued that mild temperatures in the autumn contribute to early dormancy (and subsequent release), while warm temperatures above 20ºC delay it. Therefore, the contribution of mild and warm temperature for chilling requirements highlights the dynamic of the modelled system and possibility points to a constant component in the rate of phenological development, as all temperatures contribute to development during all dormancy stages (either by contributing to fulfilment of critical chilling requirements, or by decreasing critical forcing requirements, or by increasing the state of forcing) during all dormancy stages (Caffarra and Eccel 2010).

The mean critical forcing requirements (F^*) for the tested grapevine is 478 for budburst and 754 for flowering, however the values of F^* are very variable between different varieties and regions mainly for the budburst. It is possible that varieties may differ in their rate of development between stages and their sensitivity to a given temperature may change as a function of this rate as reported by several authors (Pouget 1966, Buttrose 1969, Moncur et al. 1989, Calo et al. 1994). One limitation of the Sequential model approach presented is that varieties can be at different stages during the developmental cycle when the thermal summation begins and their individual rate of development and temperature sensitivity would not be accounted for. Further work will be necessary to refine the F^* values for each variety by combining all records available.

Figure 2 presents the differences in the number of days between observed and predicted budburst and flowering dates by the selected model plotted against the frequency of occurrence. For the 75 observations used for model estimation (Loureiro, AVV and Fernão Pires, AVV and TOV), about 92% of cases had differences between observed and predicted budburst/flowering date below 8 days (Fig. 2a, 2A). In the model validation, using 92 observations from 3 grapevine cultivars in 2 regions (AVV and TOV), the differences between observed and predicted values were lower than 8 days in approximately 84% of the cases in the case of budbusrt and 95% in the case of flowering (Fig.2b, 2B).

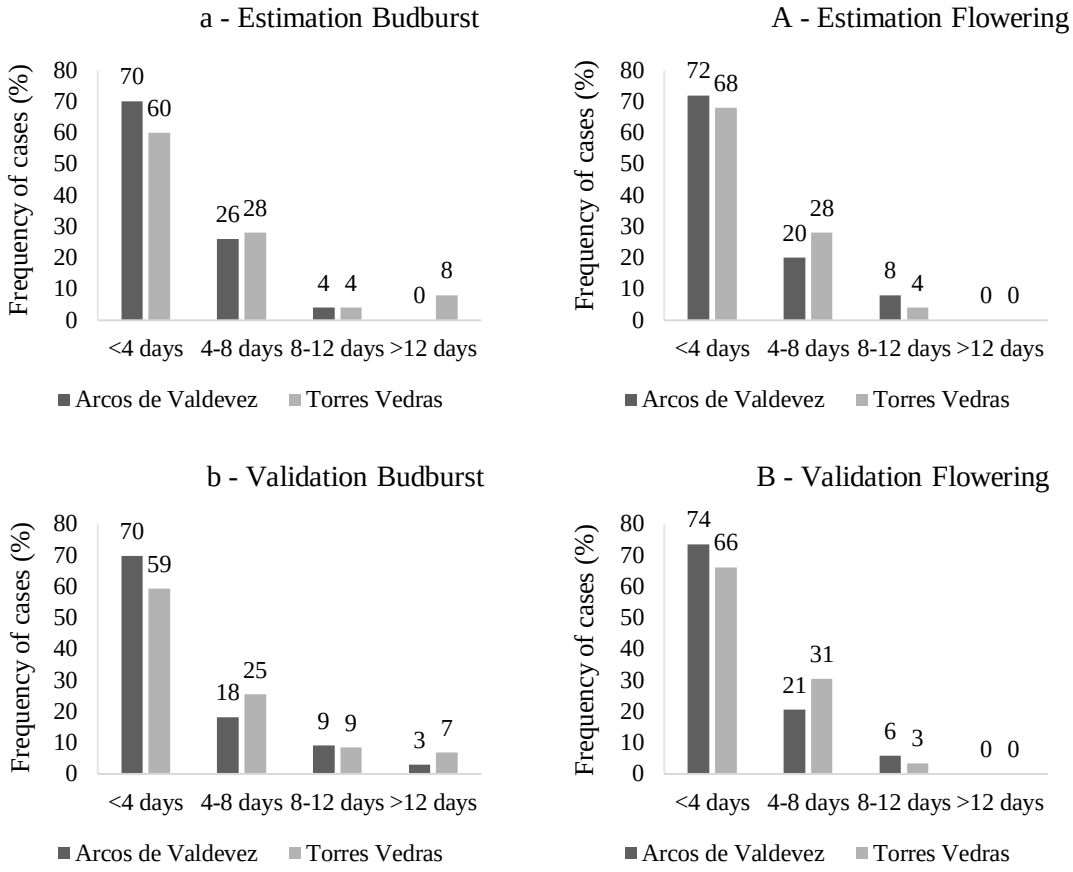

Figure 11 Frequency of difference in days between observed and predicted values for Loureiro, AVV, and Fernão Pires, AVV and TOV, used for model estimation (2a, 2A) and for model validation (2b, 2B) with 3 grape cultivars in 2 regions (AVV and TOV).

The results of the linear regression through the origin between observed and predicted budburst/flowering dates for the developed model are presented in Table 10 and 11. Overall the regression coefficients (*b0*) was close to 1.0 in both estimation (average *b0*=1.0) and validation (average *b0*=1.0) indicating, therefore, the absence of bias.

The results showed that chilling influences grapevine development. The action of chilling can be determinate by applying two-phase models. The advantages of this type of models compared with the thermal-time ones (models which only consider the action of forcing units) is its application for the installation of new vineyards since it doesn't requires information about the phenological stages that precedes the budburst or flowering and it can be used in climate change scenarios, providing information that can help in the selection of new or alternative cultivars suitable to the future temperature regimes.

Table 7 Summary of the range of values used on the iteration process of model estimation.

Phenological	Chuine			GDD
Stage	a	b	c	*Tb*
Budburst	[0,2]	[-28,11]	[-5,30]	Fitted
Flowering	[0,2]	[-28,-20]	[-5,9]	Fitted

Table 8 Summary of the parametrization process results for the Ch_GDD model studied.

Varieties			Chuine		GDD		Goodness-of-fit indicators						
Regions	a	b	c	C*	*Tb*	F*	R^2	RMSE	Max. error	AIC	MAD	R^2[1:1]	*b0*[1:1]
Budburst													
FP AVV	0.98	-23.00	-1.26	144.57	2.55	366.12	0.67	3.50	7.70	74.64	2.64	0.67	1.00
FP TOV	0.72	-24.37	4.04	114.03	5.29	609.56	0.58	5.34	14.70	95.76	3.96	0.58	1.00
LOU AVV	0.89	-23.55	-0.50	96.86	0.25	1080.31	0.57	4.21	7.30	83.85	3.52	0.57	1.00
Flowering													
FP AVV	0.88	-20.83	-1.14	175.92	7.73	479.91	0.83	3.98	8.30	81.07	3.33	0.83	1.00
FP TOV	0.73	-20.28	9.00	168.00	8.39	654.47	0.76	4.14	10.2	83.06	3.09	0.76	1.00
LOU AVV	1.04	-20.07	2.50	202.10	7.19	428.96	0.75	4.33	10.6	85.35	3.34	0.76	1.00

R^2[1:1] and *b0*[1:1] are, respectively, the coefficients of determination and the coefficient of regression through the origin between predicted and observed values.

Table 9 Goodness-of-fit indicators of the model validation process using the dataset for 3 grape cultivars in 2 regions (AVV and TOV).

	Chuine			GDD			Goodness-of-fit indicators						
	a	b	c	*C**	*Tb*	*F**	R^2	RMSE	M. error	AIC	MAD	R^2[1:1]	*b0*[1:1]
Budburst													
LOU TOV	0.58	-23.04	-4.11	100.86	6.60	591.59	0.55	6.34	18.40	85.90	4.24	0.55	1.00
CHA AVV	1.57	-23.69	6.12	173.05	2.31	64.36	0.62	3.87	6.40	33.64	3.48	0.55	1.00
CHA TOV	0.82	-23.66	1.43	114.17	5.43	571.43	0.53	5.92	9.50	100.89	3.41	0.53	1.00
PB AVV	1.08	-23.43	4.51	169.92	0.07	438.91	0.31	5.39	12.60	96.24	2.94	0.31	1.00
PB TOV	1.15	-23.04	-0.39	75.11	5.08	725.81	0.66	5.05	8.60	57.32	4.11	0.66	1.00
Flowering													
LOU TOV	0.65	-20.61	4.20	131.84	5.14	1263.53	0.75	4.42	10.50	71.40	3.36	0.75	1.00
CHA AVV	0.88	-20.09	-3.12	199.41	7.75	347.18	0.91	2.42	4.50	26.13	2.13	0.91	1.00
CHA TOV	0.87	-27.58	-1.52	144.25	6.98	920.15	0.79	3.82	8.50	79.08	2.82	0.79	1.00
PB AVV	0.65	-20.56	2.71	217.54	8.79	317.78	0.75	3.82	8.70	78.95	2.19	0.73	1.00
PB TOV	0.87	-27.58	-1.52	144.25	6.98	920.15	0.79	3.82	4.80	57.68	2.52	0.73	1.00

Varieties Loureiro (LOU), Chasselas (CHA) and Padeiro Bastos (PB) from the regions of Torres Vedras (TOV) and Vinhos Verdes test site of Arcos_Valdevez (AVV).

R^2[1:1] and *b0*[1:1] are, respectively, the coefficients of determination and the coefficient of regression through the origin between predicted and observed values.

4. Conclusion

The aim of this study was to develop a two-phase models capable of predicting the timing of budburst and flowering in different *Vitis vinifera* L. cultivars. A preliminary study and model selection suggested the type of model to be used for each of these phenophases and indicated a set of measures that would prevent overfitting during model estimation, besides obtaining a more physiologically meaningful process-based model. The resulting model, which combine the Chuine (for the stage of chilling) and the GDD (for the stage of forcing) functions, was estimated and validated for different grapevine cultivars and can be considered to be the best model tested for predicting grapevine budburst and flowering date. The developed models could be used to plan agricultural practices in the short term and to predict the impact of climate change on viticulture in the long term.

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4. Article 3 “Comparison of pollen quality in *Vitis vinifera* L. cultivars”

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Research Paper

Comparison of pollen quality in *Vitis vinifera* L. cultivars

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ABSTRACT

Pollen quality of 15 cultivars of *Vitis vinifera* L. was studied in this work. Pollen viability was tested by the fluorochromatic reaction and germination was analyzed by *in vitro* assays, using two different media. Differences among cultivars in the number of pollen apertures were observed under light microscope. All the cultivars studied showed a higher percentage of tricolporated pollen, however, pollen grains containing one, two or four apertures were also observed. The cultivar Loureiro was the one with the higher percentage of pollen grains with four apertures (3.8%) and Touriga Nacional presented 100% of tricolporated pollen grains. The viability analysis showed that 13 cultivars presented values higher than 50%, with 8 cultivars reaching values above 75%. The pollen germination rates vary greatly for the grapevine cultivars studied, three cultivars show low values of germination (under 14%) in the two media tested, which were Touriga Nacional, Cabernet Franc, and Cabernet Sauvignon while others presented high values of germination like Castelão, Loureiro, Malbec and Petit Verdot. No significant statistical differences between the percentages of germination in the two media studied were found for the majority of cultivars analyzed.

1. Introduction

Grapevine is one of the most cultivated and economically important fruit crops. It is the number one perennial crop, with more than seven million hectares planted, ranging from 50° N, through the tropics, to 43° S, in all continents except Antarctica. Although grapevines grow from temperate to tropical regions, most vineyards are planted in temperate climates regions, with the most concentrated vineyard area occurring in Europe (OIV, 2014). Grapes can be used for multiple purposes but winemaking has the highest economic value.

Grape production is related to pollen fertility which depends on the viability and germination potential of pollen (Lombardo et al., 1978). Pollen contains the male gametophyte and it is produced in the anthers of the higher plants, being its main role the sexual reproduction of spermatophytes (Pérez et al., 2007).

Pollen polymorphism is a widespread phenomenon among the higher plants including different grapevine species and cultivars (Cargnello et al., 1980; Dzyuba et al., 2006; Gallardo et al., 2009; Maria et al., 1994). Generally, the pollen grains of *Vitis vinifera* L. present a sub-spherical to triangular shape due to the presence of three furrows with three apertures (furrows with pores − tricolporated form) (Abreu et al., 2006; Alva et al., 2015). The irregular productivity presented by some grapevine cultivars may be related to the presence of atypical pollen with bicolporated, acolporate, collapsed or shriveled morphology (Abreu et al., 2006; Caporali et al., 2003; Lombardo et al., 1978). However it can also be due to other factors such as adverse environmental conditions, to incompatible pollen–pistil interaction (recognition system), to anomalous development of the ovule, to the life period of the embryo sac, and to several other physiological factors of the plants such as nutritional and phytopathological conditions (Carraro et al., 1979; Keller and Koblet, 1994; Keller et al., 2003). The presence of hermaphrodite flowers, one of the most important traits developed during the grapevine domestication, has been suggested to be the result of a mutation, and the development of acolporate pollen could be some reminiscence of their earlier dioecious lineages (Alva et al., 2015).

Pollen quality, often designated as pollen fertility, is the result of a combination of different traits such as the viability of the mature pollen and the germination ability through the formation and growth of the pollen tube *in vitro* conditions (Stanley and Linskens, 1974). They can be influenced by genetic, environmental (temperature and humidity) and agronomic factors.

The evaluation of pollen quality is important in several aspects, namely in the study of the storage potential of pollen grains for controlled pollination, in the evaluation of intra- and inter-cultivar incompatibility, in the clonal selection and genetic breeding trials (Dafni

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and Firmage, 2000) as well as to define some agronomic practices to improve pollen fertility (Caliskan et al., 2017; Novara et al., 2017; Sabir, 2015). Furthermore, some studies show that if pollen germination ratio is equal or superior to 30% the cultivars could be used as a pollinator (Fidan, 1975), which may be important in the selection of cultivars for vineyard plots.

This study aims to increase the knowledge on pollen quality of several grapevine cultivars with a different number of pollen apertures by the evaluation of their viability and germination rates in two nutritional media. This information can be used to support sustainable agronomic decisions in order to improve pollen quality providing appropriate nutrients as well as the selection of grape cultivars avoiding irregular productivity.

2. Materials and methods

2.1. Plant material

The pollen was collected from the ampelographic collection of Quinta dos Almoínha (39°01′46″N, 9°01′35″W, 99 m a.s.l) belonging to the 'Instituto Nacional de Investigação Agrária e Veterinária' (INIAV), located in the Lisbon wine region, Dois Portos, Torres Vedras. The vineyard has 2 ha and 724 accessions or botanical clones, the vines are trained on a vertical trellis at a plant density of about 3200 vines per hectare and grated onto SO4 rootstock. The soil is classified as calcic fluvisol (eutric) (FAO, 2006). Fifteen grapevine cultivars were studied in this work, some are mainly cultivated in Portugal (white cultivars: Fernão Pires and Loureiro and red cultivars: Castelão, Touriga Franca e Touriga Nacional) and others are spread worldwide (white cultivars: Colombard, Sauvignon, Sémilllon and Ugni Blanc and red cultivars: Cárménère, Cabernet Franc, Cabernet Sauvignon, Malbec, Merlot, Petit Verdot) (Anderson and Aryal, 2013). All the cultivars present androgynous flowers, produce seeded berries and are used for winemaking. The cultivars Castelão, Petit Verdot, Touriga Franca, Touriga Nacional are reported to present problems in fruit set (Dupraz and Spring, 2011; Eiras-Dias and Loureiro, 2016; Kerridge and Antcliff, 1999; Reynier, 2016; Sousa et al., 2007).

For each cultivar, several inflorescences of different vine stocks were randomly picked to Petri dishes at the stage 'EL 65 Full flowering: 50% of flowerhoods fallen' according to the Eichhorn and Lorenz (E-L) system (Lorenz et al., 1995). The collected samples were promptly isolated to avoid possible contamination with pollen of other cultivars and dried at 27 °C. After two days, the anthers were gently crushed and the pollen thus released was passed through different grades of sieves to obtain pure pollen. Pollen samples were stored at −20° C.

2.2. Pollen apertures

The number of pollen apertures was studied on acetolyzed pollen samples (Erdtman, 1969). This method had as purpose the destruction of the cytoplasmic content facilitating the later observation.

The microscopy observations were based on five fields per sample (each one containing 100 pollen grains) and the pollen grains were counted separately according to the number of apertures. These results were expressed in percentage.

2.3. Pollen viability

The pollen viability was tested by a fluorochromatic reaction with fluorescein diacetate (FDA) using a Leica microscope equipped with a mercury lamp of 50 W. The pollen grains were suspended in FDA 2%, during 5 min in obscurity. The FDA after entering the pollen grain is subsequently converted, by intracellular enzymes, into fluorescein which is a polar and fluorescent molecule. If the pollen vegetative cell membrane is intact, the fluorescein accumulates temporarily inside the pollen inducing fluorescence (Shivanna and Heslop-Harrison, 1981). So, pollen grains that appear with strong fluorescence are viable, while non-fluorescence pollen grains are non-viable (Heslop-Harrison, 1992). The viability was calculated counting three fields per sample, each one containing 100 pollen grains and the results expressed in percentage.

2.4. Pollen germination

In vitro pollen germination rate was assessed using two germination media. The medium 1 was composed by 100 ppm of boric acid, 20% of sucrose, and 0.6% of agar. The medium 2 was supplemented with 300 ppm of calcium nitrate. Pollen samples were germinated at 25 °C in the dark for 48 h. In order to calculate the germination rate, three fields per sample (each one containing 100 pollen grains) were counted in two moments – after 24 h and 48 h – using a light microscope (Leica DMLB). The pollen grains were considered as germinated when the length of their tube is greater than the pollen diameter (Nepi and Franchi, 2000).

2.5. Statistical analysis

Statistical analysis included a *t*-test to determine the effects of the both germination media studied on the percentage of pollen germination for each grape cultivar.

A one-way ANOVA followed by a *post-hoc* Duncan test was performed in order to determine the significant statistical differences between the percentage of viability and of germination among grape cultivars. A P value < 0.05 was considered to be significant.

3. Results

3.1. Pollen apertures

Fig. 1 presents the diversity in the number of pollen apertures observed among the studied grapevine cultivars. All the cultivars studied showed more than 95% of tricolporated pollen being of 100% in the Touriga Nacional cultivar (Table 1). In all 15 cultivars analyzed, none of them presented acolporated pollen grains and only 2 cultivars (Loureiro and Cabernet Sauvignon) contain pollen grains with only one aperture. Loureiro is the cultivar with the major percentage of pollen grains with four apertures (3.8%). The highest frequency (4.4%) of

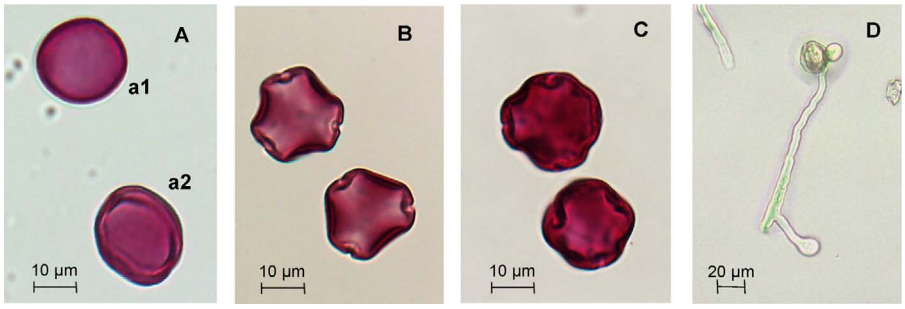

Fig. 1. A,B,C – Acetolyzed pollen of Vitis vinifera L. cultivars analyzed under light microscopy: A – Cabernet Sauvignon with one aperture (a1) and with two apertures (a2); B – Tricolporated pollen of Petit Verdot; C – Carménére with four apertures. D – *in vitro* germination of pollen analyzed under light microscopy.

Table 1
Frequency of occurrence of the number of pollen apertures among the grapevine cultivars studied.

	Percentage of pollen apertures				
Cultivar	No apertures	1 aperture	2 apertures	3 apertures	4 apertures
Carménère	–	–	1.4	97.0	1.6
Castelão	–	–	0.2	99.6	0.2
Cabernet Franc	–	–	1.8	97.8	0.4
Cabernet Sauvignon	–	0.2	1.4	98.4	–
Colombard	–	–	0.2	99.8	–
Fernão Pires	–	–	1.2	98.8	–
Loureiro	–	0.2	1.0	95.0	3.8
Malbec	–	–	0.6	98.8	0.6
Merlot	–	–	4.4	95.2	0.4
Petit Verdot	–	–	0.8	98.6	0.6
Sauvignon	–	–	1.0	99.0	–
Sémillon	–	–	1.2	98.8	–
Touriga Franca	–	–	0.4	99.6	–
Touriga Nacional	–	–	–	100	–
Ugni Blanc	–	–	0.8	99.2	–

pollen with two apertures occurred in the cultivar Merlot.

3.2. Pollen viability and germination

Fig. 2 and Table 2 presents the results of the pollen viability and germination for the 15 cultivars studied. The viability assays show great significant statistical differences ($P < 0.000$) among grape cultivars (Fig. 2). The lowest viability rate was observed in the Touriga Nacional (19.3%), while Colombard pollen showed the highest viability (99.3%). The other 13 cultivars presented values of viability rate higher than 50% being higher than 75% for eight of them.

The germination rate show great significant statistical variability ($P < 0.00$) among grape cultivars (Table 2). The germination rate is significantly lower (< 14%) in both media for three cultivars: Touriga Nacional (< 7%), Cabernet Franc (< 4%) and Cabernet Sauvignon (< 12%). Castelão, Loureiro, Malbec and Petit Verdot pollen showed germination rates higher than 40% in both media (Table 2).

The medium supplemented with calcium nitrate (medium 2) significantly increased the germination rates after 48 h in the cultivars Colombard and Touriga Franca. The Cabernet Sauvignon pollen presented a germination rate of 12% in medium 1 (48 h), but its germination was inhibited in medium 2. For the other 12 cultivars tested, the between-media differences in germination rates were not statistically significant (Table 2).

4. Discussion

The fifteen cultivars studied showed a percentage of tricolporated pollen grains equal or superior to 95%. However, with the exception of Touriga Nacional, all the other cultivars exhibited a diversity in the number of pollen apertures, being the pollen with two or four apertures the most abundant. The presence of pollen with one aperture was only observed in two cultivars. Previous studies reported the presence of considerable amount of acolporated pollen grains in the Loureiro cultivar (Abreu et al., 2006), Carménère and Merlot (Alva et al., 2015) but this occurrence was not detected in our study. According to these authors, the acolporated pollen was not able to germinate *in vitro*, suggesting that it is not functional (Abreu et al., 2006; Alva et al., 2015).

The incidence of atypical pollen (mostly of the acolporate type) has also been observed in other grapevine cultivars like Mourisco, Picolit Giallo, Moscato Rosa, Ceresa and Bicane, being characterized by their low fruit productivity (Carraro et al., 1981; Lombardo et al., 1978).

In our study, thirteen cultivars presented values of pollen viability higher than 50%, and eight cultivars show values higher than 75%. The pollen germination rates ranged between 6.7–60.0%, which is in line to those found for other grapevine cultivars in two studies: one including the cultivars Burdur dimriti, Sariemin, Tilki kuyrugu, Razaki, Buzgulu, Siyah buzgulu and Siyah gemre with values varying from 23.8 to 80.8% (Kelen and Demirtas, 2003) and other including the cultivars Gamay, Chardonnay, Pinot Noir, Bogazkere, Okuzgozu, Clairette, Cinsaut, Emir, Papaz Karasi, Alicante Bouschet, Riesling, Kalecik Karasi, Semillon,

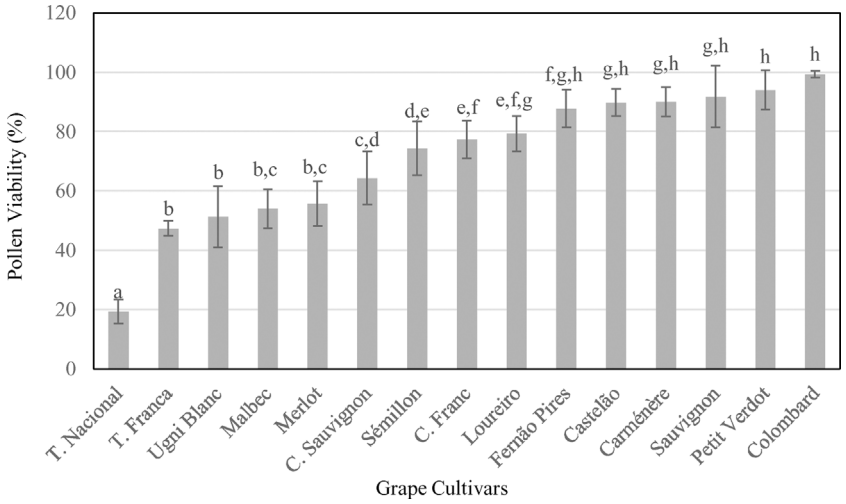

Fig. 2. Differences on the percentage of pollen viability, for the studied grapevine cultivars. Each vertical bar represents mean ± standard error. A significant difference between the cultivars was determined by comparing means using Duncan post-hoc test ($P < 0.05$). Different letters show significant difference between the cultivars.

Table 2
Pollen germination rate (mean and standard error) in different media for the studied grapevine cultivars.

	Germination (%)				
	Medium 1		Medium 2		
Cultivar	24 h	48 h	24 h	48 h	*P* value*
Carménère	11.7 ± 10.7	21.7 ± 2.9^{a,b,c}	21.7 ± 5.1	21.7 ± 5.1^{d}	0.929
Castelão	58.0 ± 5.6	58.3 ± 6.7^{e,f}	31.0 ± 4.6	31.0 ± 4.6^{d,e}	0.090
C. Franc	3.0 ± 3.0	11.3 ± 7.1^{a}	3.7 ± 2.3	4.3 ± 1.2^{a,b,c}	0.090
C. Sauvignon	7.0 ± 3.6	11.7 ± 7.6^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.039
Colombard	8.7 ± 8.1	16.7 ± 3.8^{a,b}	19.7 ± 12.2	31.0 ± 7.0^{d,e}	0.036
Fernão Pires	34.3 ± 6.0	42.7 ± 16.2^{d,e}	21.7 ± 11.0	27.3 ± 3.1^{d,e}	0.182
Loureiro	46.7 ± 14.6	60.0 ± 10.0^{f}	14.3 ± 11.7	40.3 ± 11.6^{e,f}	0.090
Malbec	45.7 ± 15.5	47.0 ± 15.0^{d,e,f}	35.0 ± 18.0	48.0 ± 9.2^{f}	0.830
Merlot	13.3 ± 4.9	35.7 ± 14.0^{c,d}	12.3 ± 4.7	28.7 ± 11.8^{d,e}	0.545
Petit Verdot	16.0 ± 1.0	43.7 ± 3.2^{d,e,f}	16.3 ± 11.9	49.3 ± 16.0^{f}	0.580
Sauvignon	35.7 ± 13.6	39.7 ± 4.2^{d}	29.3 ± 5.1	41.0 ± 9.2^{e,f}	0.830
Sémillon	33.0 ± 6.6	33.3 ± 18.1^{b,c,d}	15.3 ± 5.0	18.3 ± 11.0^{c,d}	0.288
T. Franca	11.0 ± 2.6	19.7 ± 4.0^{a,b,c}	20.0 ± 3.6	30.3 ± 2.5^{d,e}	0.018
T. Nacional	1.3 ± 1.5	6.7 ± 2.5^{a}	1.7 ± 2.9	2.0 ± 3.5^{a,b}	0.132
Ugni Blanc	3.3 ± 2.1	7.7 ± 2.1^{a}	8.0 ± 1.0	14.0 ± 5.3^{b,c,d}	0.266
Test F	10.041		9.469		
(sig.)**	0.000		0.000		

* A significant difference between the two media measured at 48 h was determined by comparing means using *t*-test ($P < 0.05$).
** Statistical significance of the Fischer test in the ANOVA. A significant difference between the cultivars was determined by comparing means using Duncan *post-hoc* test ($P < 0.05$). Within columns, different uppercase letters show significant difference between the cultivars.

Trakya Ilkeren, Yalova Incisi, Muscat Ottonel, Hafizali,Italya, Hamburg Misketi, Tekirdag Cekirdeksizi, 2B-56, Kozak Beyazi and Cabernet Sauvignon with values varying from 19.7 to 80.9% Korkutal et al., 2004). Touriga Nacional pollen showed very low percentages of germination in both media (values under 7%), which can be related to problems in fruit set (like abortion of flowers) that frequently occur in this cultivar (Castro et al., 2015).

The *in vitro* pollen germination aims to simulate the *in vivo* conditions. In laboratory, several parameters must be optimized for each species such as the germination media, temperature, and humidity conditions in order to obtain a percentage of germination as close as possible to the real germination potential (Pinillos and Cuevas, 2008; Shivanna and Johri, 1985). Carreno et al. (2009) studied the influence of some compounds and temperature on *in vitro* pollen germination capability and its preservation for some grapevine cultivars. The optimum temperature for pollen germination is dependent on the genotype, but in general ranged 25–30 °C, as the ones tested in our study. In addition, the composition of the media tested in our experiments is similar to the one used in other studies for cultivars of *Vitis vinifera* L. (Abreu et al., 2006; Alva et al., 2015; Korkutal et al., 2004).

This work evaluated the effect of boron and calcium for pollen germination. The boron is essential for germination and pollen tube growth (Cheng and Rerkasem, 1993; Feijo et al., 1995; Huang et al., 2000; Iwai et al., 2006; Obermeyer and Blatt, 1995; Stanley and Linskens, 1974; Taylor and Hepler, 1997; Wang et al., 2003). So, boron deficiency can inhibit reproductive growth by affecting pollen germination, pollen tube growth, fruit set and seed formation (Blevins and Lukaszewski, 1998; Dell and Huang, 1997; Tanaka and Fujiwara, 2008) and can also promote pollen tube anomalies such as the swelling at the tube tip or tube bursting (Acar et al., 2010; Holdaway-Clarke and Hepler, 2003; Wang et al., 2003; Yang et al., 1999). The calcium is known to stimulate the growth of pollen tube, increasing pollen germination (Koncalova et al., 1975). In our study, the calcium supplement was beneficial for the germination of Cabernet Sauvignon, Colombard and Touriga Franca pollen. These results are in line with Sabir (2015) that reported a significant increase in pollen germination rates.

Sucrose, like other exogenous sugars, is essential for providing osmotic environment and nutrition to *in vitro* germination (Malik et al., 1982). Additionally, pollen bursting is observed in *in vitro* conditions where there is a lack of concentration of sucrose (Baloch et al., 2001). Media with boric acid and sucrose stimulates germination and tube development because boron makes a complex with sugar promoting a better translocation than the non-borate, non-ionized sugar molecules (Gauch and Dugger, 1953; Sidhu and Malik, 1986; Vasil, 1964). The percentage of sucrose used in this study is similar to the one used in other studies for some cultivars of grapevine (Abreu et al., 2006; Kelen and Demirtas, 2003).

5. Conclusion

In the present study, all the cultivars studied showed a percentage of tricolporated pollen equal or superior to 95% with Touriga Nacional presenting 100%. The pollen with four apertures was the most abundant atypical one but pollen with one or two apertures were also observed.

The pollen viability ranged from 19.3 to 99.3% and the germination rates vary greatly for the grapevine cultivars studied. Three cultivars showed low values of germination (Touriga Nacional, Cabernet Franc and Cabernet Sauvignon) while Castelão, Loureiro, Malbec and Petit Verdot presented higher values.

The results suggest that pollen quality differed among the cultivars studied, and this may have an impact on pollination efficiency and, consequently, on the grape productivity, which can be improved by viticulture practices, providing nutrients according to each cultivar needs.

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5. General Discussion

5.1. Phenological modelling

A process-based model is the mathematical (and normally computer-based) representation of one or several processes characterizing the behaviour of well-delimited biological systems of fundamental or economical interest (Buck-Sorlin 2013). When this behaviour is known or assumed the process-based model is usually developed using available information (suitable dataset). Its parameters can be estimated through optimisation of the model fit (Cox et al. 2006).

The models present in this work were developed in order to describe the relationship between temperature and phenology dates. They were estimated and validated for predicting the timing of budburst and flowering using a long phenological dataset for a large number of grape cultivars grown in two Portuguese wine regions: Vinhos Verdes and Lisbon.

In this work, two types of phenological models were tested. The first type includes the Thermal Time or Spring Warming models (one-phase model) that only contemplates the effect of the temperatures during the active growth period (forcing temperatures) (Hunter and Lechowicz 1992; Cannell and Smith 1983; Robertson 1968). The second type includes also the influence of chilling temperatures integrating the dormancy phase (two-phase model) and the Sequential Model was chosen to be tested (Hänninen 1990; Richardson et al. 1974; Hänninen 1987)

The development of both thermal-based and two-phase models were achieved by an iterative process. The cultivars with the longest time series of phenological data for AVV (Fernão Pires and Loureiro) and TOV (Fernão Pires) were used in the estimation. The validation of both model types were performed using an independent data-set of different cultivars grown in the Vinhos Verdes and Lisbon wine regions. Overall, it was observed high internal validities (R^2) of the models developed for forecast the budburst and flowering dates (for both thermal-time and two-phase models), describing very well the phenological dataset variability.

The thermal-based and two-phase models were presented in two companion scientific Articles designated, respectively, as “Article 1” and “Article 2”.

In Article 1, it was tested and validate three multi-year, multi-variety and multi-site thermal-time phenological models (GDD, GDD Triangular and UniFORC) using 473 observations of the flowering date. In the estimation process it was selected, from the three *t0* dates tested (dates from when the sum of daily rate of forcing – *Rf* – begins), the budburst date (previous phenological stage) as the best overall fitted.

In our data series the average budburst date occurs at March, which is in line with this results found by Parker (2011) that showed the optimal values for the base temperature and start day for the thermal summation for predicting the *Vitis vinifera* L. flowering and veraison were *t0* at 60 DOY (approximately March 1st) and *Tb* value of 0°C for the Northern hemisphere.

In the degree-days models, the minimum temperature above which the thermal summation is calculated (*Tb, Tmin*) was established at 8 ºC that is a value in line with the optimal range of temperatures found in the bibliography for the grapevine phenological development (Winkler et al. 1974; Huglin and Schneider 1986; Carbonneau et al. 1992; Riou 1994; Fraga et al. 2016). The optimal temperature (*Topt*) for heat accumulation was fixed at 23 ºC and the maximum (*Tmax*) as 31 ºC. In fact it is reported that temperatures between 25 ºC and 35 ºC are considered optimal for photosynthesis while temperatures above 35 °C appear to have a negative effect on photosynthetic activity (Caffarra and Eccel 2010; Greer and Weedon 2012).

Overall, the three thermal-time models performed well for all cultivars, with high R^2 and low RMSE and MAD lower than 4 days in AVV and TOV and lower than 6 days in FEL. These results agree with the observations made in the field that in the case of AVV and TOV were carried out every 3/4 days and in the FEL every 6/7 days.

Comparing the three models tested, the UniFORC model performed better than the GDD ones, presenting a R^2 higher than 0.75 in 67% and 65% of the cases for the estimation and validation data sets, respectively. Generally, in AVV and TOV, the RMSE was on average 4 days an in FEL was 5 days. These results indicate that most of the inter-annual variability observed in the *Vitis vinifera* L. flowering dates were explained by this model. So, the results show that the time of flowering can be predicted with a good precision based solely on the effect of temperature during the active growth period (forcing temperatures), similarly to the results obtained by Parker *et al*. (2011)

In Article 2, it was tested nine two-phase phenological models for predicting the budburst and flowering dates of *Vitis vinifera* L. the date to begin the accumulation of chilling units (*t0*) was considered the 1st of September of the previous year while the remain parameters were fitted. The developed models resulted from different combinations of functions used to characterize the stage of chilling and the stage of forcing (accumulation of chill and heat units). The models where the accumulation of the chilling units was represented by the Triangular function and the GDD Inverse presented low biological coherence compared with the ones including the Chuine function. In fact, all the two-phase models tested using the Chuine function combined with the GDD, Sigmoid and Wang functions to calculate the accumulation of heat units presented good

values of R^2, RMSE, maximum error, AIC and MAD for the budbreak and flowering stages. The Chuine/GDD model was the best performing one during the estimation process, present higher R^2 (0.57-0.68 for budburst and 0.74-0.83 for flowering) and lower RMSE (3-5 days for budburst and 4 days for flowering), maximum error (8-15 days for budburst and 8 days for flowering), AIC (74-96 for budburst and 82- 85 for flowering) and MAD (3-4 days for budburst and for flowering) in the majority of the cases. During the external validation with the independent dataset this model's goodness-of-fit indicators performed well, with high R^2 (0.75-0.91 for flowering), low RMSE (4-6 days for budburst and 2-4 days for flowering), and MAD (3-4 days for budburst and 2-3 days for flowering). These results agree with the observations made in the field that in the case of budburst was carried out every 7 days and for flowering every 3/4 days.

In our study, the critical chilling requirements for the tested grapevine cultivars varied between 124 $\pm$ 33 for budburst and between 172 $\pm$ 31 for flowering. For Cabernet Sauvignon the values reported for the critical chilling requirements were about 336 h at temperatures below 6 ºC to attain budburst (which was fulfilled in about 2 weeks)(Botelho et al. 2007). In our study, the estimated chilling function considers temperatures above 10 ºC until 20 ºC also to be effective. In fact, a premise applied in our study was the beginning of the accumulation of chilling units (*t0*) starts from September 1st and in this month it is frequently observed for the studied regions, particularly in TOV, mean daily temperature between 10 ºC and 20 ºC. Similar results were found by Caffara and Eccel (2010) for grapevine and Heide for *Betula pubescens*. As endodormancy is usually broken by temperatures below 10 to 12 ºC (Pouget 1968; Dokoozlian 1999) it can be argued that mild temperatures in the autumn contribute to early dormancy (and subsequent release), while warm temperatures above 20ºC delay it.

Also, it highlights the dynamicity of the modelled system and possibility points to a constant component in the rate of phenological development, as all temperatures contribute to development during all dormancy stages (either by contributing to fulfilment of critical chilling requirements, or by decreasing critical forcing requirements, or by increasing the state of forcing) during all dormancy stages (Caffarra and Eccel 2010). The results of the linear regression through the origin between observed and predicted budburst / flowering dates, for the one and two phase models that presented a better performance, show that, overall, the regression coefficients (*b0*) was close to 1.0 in both estimation and validation steps indicating, therefore, the absence of bias.

Comparing the two types of models studied, the thermal-time model developed comprises differences in phenological timing for a wide range of cultivars compared within the same modelling framework, it is simple to use by winemakers, can be applied

to the vineyard level, can be integrated in agricultural management software and it is reported to forecast accurately the flowering dates in temperate climate up to 2050 (Chuine et al. 2016). However, this model requires previous budburst data in order to forecast the flowering stage and therefore when used within a framework of climate warming scenarios they need to be use with caution if climatic conditions become too warm compared to the training climatic dataset. In such case, it seems preferable to use two-phase models (Chuine et al. 2016). The advantages of the two-phase models estimated in our study compared with the thermal-time one is its application for the installation of new vineyards since it doesn't requires information about the phenological stages that precedes the budburst or flowering and it can be used in climate change scenarios, providing information that can help in the selection of new or alternative cultivars suitable to the future temperature regimes.

The classification of budburst and flowering that have been presented provide a characterization of the varietal response (*C** and *F** value) for these stages. This currently provides an extensive understanding of the intra-specific variability for the timing of each stage for a range of cultivars. In fact, when the grapevine developmental phases are well-adapted to the local conditions, the grapes at harvest may acquire a desired combination of parameters such as sugar, acidity, aromatic and phenolic compounds or other desired qualities to produce wine with high quality (Jones and Davis 2000; Jones et al. 2005; Jones 2006; Van Leeuwen et al. 2008).

5.2. Pollen morphology and fertility

Pollen morphology and quality of 15 cultivars of *Vitis vinifera* L. were studied in this work.

Differences among cultivars in the number of pollen apertures were observed under light microscope. Pollen grains are surrounded by an extremely resistant wall interrupted in places by apertures that play a key role in reproduction since the pollen tube growth is initiated at these sites (Albert et al. 2010). All the cultivars studied showed a higher percentage of tricolporated pollen, however, pollen grains containing one, two or four apertures were also observed. The cultivar Loureiro was the one with the higher percentage of pollen grains with four apertures (3.8%) and Touriga Nacional presented 100% of tricolporated pollen grains.

Pollen quality is important for growers and breeders. The characterization and viability of pollen grains are useful tools to guide crosses in breeding programs (Soares et al. 2013). In our study, pollen viability was tested by the fluorochromatic reaction and our results showed that 13 cultivars presented values higher than 50%, with 8 cultivars reaching values above 75%.

The *in vitro* germination was assayed, using two different solid media (one composed by boric acid, sucrose, and agar and other supplemented with calcium nitrate). Since the *in vitro* pollen germination aims to simulate the *in vivo* conditions it is necessary to optimize in the laboratory, several parameters for each species (media composition, temperature, and humidity conditions) (Pinillos and Cuevas 2008; Shivanna and Johri 1985). In the literature, for some grapevine cultivars, the optimum temperature for pollen germination ranged from 25 to 30°C (2009), similar to the ones tested in our study. In addition, the composition of the media tested in our experiments is similar to the one used in other studies for cultivars of *Vitis vinifera* L. (Abreu et al. 2006; Alva et al. 2015; Korkutal et al. 2004).

The pollen germination rates vary greatly for the grapevine cultivars studied (6.7 to 60.0%), three cultivars show low values of germination (under 14%) in the two media tested, which were Touriga Nacional, Cabernet Franc, and Cabernet Sauvignon while others presented high values of germination like Castelão, Loureiro, Malbec and Petit Verdot. For the majority of cultivars analysed, no significant statistical differences were found between the percentages of germination in the two media studied.

Our results are in line to those found for other grapevine cultivars in two studies. For Burdur dimriti, Sariemin, Tilki kuyrugu, Razaki, Buzgulu, Siyah buzgulu and Siyah gemre germination percentages varied from 23.8 to 80.8% (Kelen and Demirtas 2003) while for the cultivars Gamay, Chardonnay, Pinot Noir, Bogazkere, Okuzgozu, Clairette, Cinsaut, Emir, Papaz Karasi, Alicante Bouschet, Riesling, Kalecik Karasi, Semillon, Trakya Ilkeren, Yalova Incisi, Muscat Ottonel, Hafizali,Italya, Hamburg Misketi, Tekirdag Cekirdeksizi, 2B-56, Kozak Beyazi and Cabernet Sauvignon values varied from 19.7 to 80.9% (Korkutal et al. 2004).

Touriga Nacional pollen showed very low percentages of germination in both media (values under 7%), which can be related to problems in fruit set (like abortion of flowers) that frequently occur in this cultivar (Castro et al. 2015).

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6. General Conclusion and perspectives

6.1. Conclusion

Phenology modelling involves four essential steps: data collection, model definition, adjustments of the model to the data using an adapted optimization algorithm that ensures correct convergence and tests of the model hypotheses. There are several sources of phenological data such as the airborne pollen and observations in phenological collections.

In this work, we study how grapevine budburst and flowering date can be estimated using temperature-based phenological models across different genotypes and environments. The UniFORC model calibrated for the Portuguese grapevine cultivars, in “Article 1”, can be considered the best model tested for predicting grapevine flowering date, outperforming the standard GDD models. Its simplicity makes it easy to use and its implementation to predict the timing of flowering for different grapevine cultivars and regions.

Furthermore, we also study how grapevine budburst and flowering date can be estimated using two-phase phenological models across different grape cultivars and wine regions, in “Article 2”. The model composed by the Chuine (for the stage of chilling) and the GDD (for the stage of forcing) functions, was considered the best model calibrated predicting the budburst and flowering date for the Portuguese grapevine cultivars studied.

The developed models provided good results, with high accuracy, enabling their direct application by wine industries to support vineyard management decisions.

In the present work, the pollen morphology and fertility of fifteen grapevine cultivars were also studied. The percentage of pollen grains with three apertures (tricolporated) was equal or superior to 95%, with Touriga Nacional presenting 100%. Although, pollen grains with one or two apertures were observed, the pollen grains with four apertures were the most abundant atypical type.

The pollen viability ranged from 19.3 to 99.3% and the germination rates vary greatly for the grapevine cultivars studied (6.7 to 60%). Three cultivars showed low values of germination (under 14% for Touriga Nacional, Cabernet Franc and Cabernet Sauvignon) while Castelão, Loureiro, Malbec and Petit Verdot presented higher values (superior to 40%).

These results suggest that pollen quality differed among the cultivars studied, and this may have an impact on pollination efficiency and, consequently, on the grape

productivity, which can be improved by viticulture practices, providing nutrients according to specific needs of each cultivar.

6.2. Perspectives

Based on the results obtained in this work, there are some perspectives of improvement and development that can be done.

Portugal is one of the countries with a larger set of grapevine cultivars, and most of them have never been involved before in phenological modeling studies. It would, therefore, be important to give continuity to the work done and to test the models developed to other cultivars, as well as to other wine regions.

It would also be important the integration of other environmental parameters in the phenological models to increase its accuracy since the temperature was unable to explain the total of the observed variability in budburst and flowering dates.

It would be interesting to apply the developed models to climate change scenarios, in order to give a greater understanding of how different grapevine cultivars would adapt and the suitability for successful production in Portuguese wine regions.

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