Flowering Biology and Agroclimatic Requirements of Cider Apple (*Malus Domestica* Borkh.) Cultivars from Asturias in a Context of Climate Change

PhD Thesis

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UNIVERSIDAD DE OVIEDO

SERIDA

UNIVERSIDAD DE OVIEDO

Programa Oficial de Doctorado en Biología Molecular y Celular
Universidad de Oviedo

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Supervised by: **Enrique Dapena de la Fuente**

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Biología de la floración y requerimientos agroclimáticos de cultivares de manzana de sidra (*Malus domestica* Borkh.) de Asturias en un contexto de cambio climático.	Inglés: Flowering biology and agroclimatic requirements of cider apple (*Malus domestica* Borkh.) cultivars from Asturias in a context of climate change.

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RESUMEN (en español)

El manzano (*Malus domestica* Borkh.), al igual que la mayoría de las especies leñosas perennes originarias de climas fríos, pasa los meses de invierno en una fase de letargo invernal o dormancia que le permite sobrevivir a las condiciones climáticas desfavorables (Campoy et al., 2011; Faust et al., 1997; Saure, 1985). Para romper la dormancia deben satisfacerse unos requerimientos climáticos específicos de cada variedad. Una inadecuada salida de la dormancia se traduce en una escasa brotación y una floración irregular que repercute negativamente en la producción (Erez, 2000; Sunley et al., 2006). Debido a que el letargo se caracteriza por la ausencia de cambios fácilmente observables en las yemas u otras estructuras del árbol, la identificación de las fases de acumulación de frío y calor sigue siendo difícil de abordar de forma satisfactoria (Fadón et al., 2020).

Asturias (noroeste de España) tiene una larga tradición en el cultivo de manzano y la producción se sustenta en gran medida en variedades locales de manzana de sidra. Hasta la fecha, las consecuencias negativas de una insuficiente acumulación de frío invernal en las plantaciones comerciales de la región han sido escasas. Sin embargo, existen numerosos estudios científicos que afirman que las temperaturas están aumentando en todo el mundo (Cook et al., 2016) y se espera que estos cambios puedan desencadenar graves consecuencias ambientales y biológicas (IPCC, 2014). Uno de los principales objetivos de esta tesis es conocer si las variedades procedentes de Asturias y actualmente adaptadas a las condiciones ambientales de la zona seguirán siendo adecuadas en el futuro. El conocimiento de los requerimientos de temperatura de las variedades tiene una notable importancia práctica (Fennell, 1999) para científicos y productores, ya que el frío invernal puede disminuir como consecuencia del calentamiento global en muchas zonas del planeta (Luedeling et al., 2011).

La presente tesis doctoral tiene como objetivo conocer los requerimientos agroclimáticos de las variedades locales de manzano, así como la disponibilidad de frio invernal en Asturias en el futuro. Para ello, en primer lugar, la tesis se centra en cómo los cambios en la acumulación de frío y calor han afectado a la fenología de las variedades de manzano en la región. En segundo lugar, este trabajo ha evaluado los impactos del cambio climático en la disponibilidad de frío invernal durante el siglo XXI con el objetivo de elegir las variedades de manzano más adecuadas para la región. En tercer lugar, este trabajo pretende mejorar el conocimiento de varios caracteres relacionados con la floración de una amplia colección de variedades de manzano seleccionadas por el Programa de Investigación de Fruticultura del SERIDA, con el fin de gestionar de una manera más eficaz las variaciones interanuales en la polinización. Esta tesis está organizada en cuatro capítulos que corresponden a cuatro artículos científicos.

A lo largo de un periodo de 41 años, no se observó una disminución significativa en la acumulación de frío invernal a pesar de un aumento de temperatura de 0.30 °C por década. El efecto de adelanto de la floración provocado por un periodo de acumulación de calor más cálido parece haber compensado el efecto de retraso en la floración inducido por un inicio más tardío de la endodormancia. En esta tesis, se determinaron experimental y estadísticamente las necesidades de frío y calor de un conjunto de variedades locales de manzano. Los estudios sugieren que la metodología utilizada para estimar las necesidades agroclimáticas tiene un impacto significativo en los resultados. Sin embargo, ambos enfoques mostraron que la mayoría de las variedades de manzano asturianos tienen unas necesidades de frío medias a altas.

De acuerdo con los escenarios futuros, Asturias no experimentará una disminución elevada del frío invernal a lo largo del siglo XXI. Sin embargo, las proyecciones para un escenario pesimista muestran un riesgo elevado de que variedades con elevadas necesidades de frío no alcancen sus requerimientos de frío después del 2070. Dado que se esperan 72 porciones de frío en el peor escenario, el desarrollo de estrategias de adaptación parece bastante factible para la industria frutícola. Este trabajo afirma que la opción de adaptación más prometedora es hacer coincidir las necesidades agroclimáticas de las variedades con las condiciones climáticas locales.

La mayoría de las variedades de manzano son autoincompatibles, por lo que el periodo de floración, la compatibilidad genética y la viabilidad del polen son de vital importancia para producir rendimientos aceptables. La caracterización fenotípica de la colección de variedades del Programa de Investigación de Fruticultura del SERIDA demostró que existe una gran variabilidad fenotípica en caracteres relacionados con la biología floral. Varias variedades presentan un largo periodo de floración, un elevado nivel de retorno de la floración y producen abundantes cantidades de polen viable. Algunos de los mejores polinizadores son las nuevas obtenciones del programa de mejora ‘X9406-11', 'Perurico' y 'Raxila Dulce', 'Raxona Dulce' y 'Raxona Acida'. En general, se espera que los resultados y métodos desarrollados en esta tesis sean de utilidad en el proceso de obtención de variedades que sigan siendo viables en un futuro más cálido. Además, las metodologías presentadas en este trabajo pueden ser fácilmente implementadas por institutos de mejora genética de frutales de todo el mundo.

RESUMEN (en inglés)

Like most woody perennial species that evolved in cold climates, apple (*Malus domestica* Borkh.) trees spend the winter months in a dormant state that allows them to survive unfavourable weather conditions (Campoy et al., 2011; Faust et al., 1997; Saure, 1985). To break dormancy, cultivar-specific requirements must be fulfilled. Inadequate dormancy release results in poor budbreak and delayed flowering, which negatively impact fruit set (Erez, 2000; Sunley et al., 2006). Since the dormancy phase is characterized by the absence of any easily observable changes in buds or other structures of the plant, the determination of chill and heat accumulation phases remains difficult to address in a satisfactory way (Fadón et al., 2020a).

Asturias (north-western Spain) has a long tradition of apple growing, with local production largely based on traditional cider cultivars. To date, negative consequences of insufficient winter chill accumulation have rarely been observed in commercial apple orchards in the region. However, there is overwhelming scientific evidence that temperatures are rising worldwide (Cook et al., 2016), and these changes are expected to have severe environmental and biological implications (IPCC, 2014). One of the main aims of this thesis is to ascertain if the cultivars that originated and are currently well-adapted to the environmental conditions of Asturias will be still suitable in the future. In this sense, knowledge of the temperature requirements of the cultivars is an issue of practical concern (Fennell, 1999) for scientists and growers, since winter chill may decrease as a consequence of global warming in many areas (Fernandez et al., 2020c; Luedeling et al., 2011).

The present PhD thesis aims to gain insights into the agroclimatic requirements of local apple cultivars and the historic and projected future changes in winter chill in Asturias. For this purpose, first, the thesis focuses on how changes in chill and heat accumulation have affected the phenology of apple cultivars in the region. Second, this research has evaluated the impacts of climate change on the availability of winter chill for the 21st century with the objective of choosing the most suitable apple cultivars to be grown in Asturias. Third, the thesis aims to improve the knowledge of several flowering-related traits of an extensive collection of apple accessions selected by the SERIDA Fruit Research Group in order to better manage interannual variation in pollination outcomes. This thesis is organized into four chapters that correspond to four scientific articles.

Over a period of 41 years, winter chill accumulation did not show a significant decrease despite temperature increases by 0.30 °C per decade. The bloom-advancing effect of a warmer forcing phase appears to have partially overcompensated the bloom-delaying effect of a later onset of the endodormancy phase. In this thesis, chill and heat requirements from a set of apple cultivars were determined experimentally and statistically. The studies suggest that the methodology used for estimating the agroclimatic needs have a significant impact on the results. Nevertheless, both approaches showed that the majority of the Asturian apple cultivars have medium to high chill requirements.

Regarding future scenarios, Asturias will not be severely affected by chill losses through the 21st century. However, the projections for a pessimistic setting show a notable risk of failing to fulfil the chill requirements of high-chill cultivars after 2070. Since high chill accumulation of 72 Chill Portions is still expected even for the worst-case scenario, developing adaptation strategies for the fruit industry should be quite feasible. Matching cultivars with the regional climate conditions appears to be the most promising adaptation option.

Since most apple varieties are self-incompatible, the flowering period, genetic compatibility and pollen viability must be considered in attempts to optimize fruit yields. The characterization of floral and pollen traits demonstrated that the SERIDA apple collection presents large phenotypic variability in floral biology traits. Several cultivars have a long flowering period, an optimal level of return bloom and produce copious amounts of viable pollen. Some of the best pollen donor cultivars are the new releases ‘X9406-11’, ‘Perurico’ and ‘Raxila Dulce’, ‘Raxona Dulce’ and ‘Raxona Acida’. Overall, the results and methods developed in this thesis are expected to be helpful to the local apple breeding program in the development of cultivars that remain viable in a warmer future. The methodologies presented in this work can be easily implemented by tree fruit breeding institutes worldwide

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FORMULARIO RESUMEN DE TESIS POR COMPENDIO

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	SI	**NO**
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Acompaña memoria que incluye		
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Trabajo, Artículo 2

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En caso de compendio de un número de artículos superior a seis, se incorporarán hojas suplementarias conforme a este modelo

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INFORME PARA LA PRESENTACIÓN DE TESIS DOCTORAL COMO COMPENDIO DE PUBLICACIONES

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4.- Informe

La presente Tesis Doctoral incluye 4 artículos científicos, tres de los cuales han sido aceptados para su publicación en revistas científicas incluidas en el *Science Citation Index.* El cuarto capítulo ha sido aceptado para publicación, pero no se había publicado en la fecha de presentación de esta Tesis. Todos los artículos científicos han sido evaluados en una revisión científica por pares, no forman parte de ninguna otra Tesis Doctoral y han sido publicados con posterioridad al inicio de los estudios del doctorando. Por tanto, se considera que esta Tesis Doctoral cumple los requisitos para su publicación como Compendio de Publicaciones.

Oviedo, 24 de enero de 2022

Director de la Tesis Doctoral

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Los estudios presentados en esta Tesis doctoral se han desarrollado en el Programa de Investigación de Fruticultura, del Área de Cultivos Hortofrutícolas y Forestales del Servicio de Investigación y Desarrollo Agroalimentario del Principado de Asturias (SERIDA), en el Instituto de Ciencia de los Cultivos y la Conservación de los Recursos (INRES) de la Universidad de Bonn y en el departamento de Agronomía del Instituto de Ciencias de la Tierra y de la Vida en la Universidad Católica de Lovaina.

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TABLE OF CONTENTS

ABBREVIATIONS AND UNITS

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%	percentage
° C	celsius degree
ANOVA	analysis of variance
a.s.l.	above sea level
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
$CaCl_2$	calcium chloride
CH	Chilling Hour
CIAT	Centro Internacional de Agricultura Tropical
cm	centimetre
CO_2	carbon dioxide
CP	Chill Portion
CR	chill requirement
CU	Chill Unit
CV	coefficient of variation
cv.	cultivar
DM	Dynamic model
DOY	day of the year
e.g.	*exempli gratia*
EPP	effective pollination period
EU	European Union
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
g	gram
GCM	global climate model

GDH	Growing Degree Hours
h	hour
ha	hectare
HR	heat requirement
i.e.	*id est*
IKI	iodine potassium iodide
IO	Carbon iodide
IPCC	Intergovernmental Panel on Climate Change
km	kilometre
l	litre
m	meter
MAPA	Ministerio de Agricultura, Pesca y Alimentación
mg	milligram
mL	millilitre
mm	millimetre
nitrous oxide	N_2O
NOAA	National Oceanic and Atmospheric Administration
PDO	Protected Designation of Origin
pH	potential of hydrogen
PhD	Doctorate of Philosophy
PLS	Partial Least Squares
$PO4^{3-}$	phosphate ion
QTL	quantitative trait loci
RCP	Representative Concentration Pathway
sd	standard deviation
SERIDA	Servicio Regional de Investigación y Desarrollo Agroalimentario

spp.	species *pluralis*
SWC	Safe Winter Chill
TCSA	trunk cross-sectional area
TTC	triphenyl tetrazolium chloride
VIP	variable importance in the projection
W/m^2	watts per square meter
w/v	weight per volumen
µL	microlitre

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. THE CULTIVATED APPLE (*Malus domestica* Borkh.)

1.1. The domesticated apple: origins, botanical characterization and economic importance of the crop

The domesticated apple (*Malus domestica* Borkh.) is a species that belongs to the *Rosaceae* family, genus *Malus.* Regarding the scientific name, some authors consider that the symbol "x" (*Malus x domestica* Borkh.) should be included because this cultivated species is likely the result of an interspecific hybridization (Korban and Skirvin, 1984). The origins of the domesticated apple are still a matter of debate (Cornille et al., 2012). However, it seems clear that the tree is the result of a long evolutionary process and *Malus sieversii*, originated from central Asia, is its wild ancestor and the main contributor to the domesticated apple gene pool (Cornille et al., 2019). Forsline et al. (2003) stated that *Malus domestica* also derived from multiple interspecific hybridizations with other wild apple species such as *Malus baccata*, *Malus orientalis* and *Malus silvestris*. The number of species in the genus *Malus* is still unclear (Pereira-Lorenzo et al., 2009). For example, Harris et al. (2002) reported 55 species whereas Forsline et al. (2003) reported only 27 species.

Most apple cultivars are diploids (2n = 2x = 34) meaning that they have two sets of 17 chromosomes (Velasco et al., 2010) but there is also a significant number of triploid genotypes (Garkava-Gustavsson et al., 2008; Gianfranceschi et al., 1998; Pereira-Lorenzo et al., 2017; Petrisor et al., 2012). Despite of obvious phenotypic differences among thousands of cultivars, some characteristics are similar within the species. The trees are between 3 and 12 meters in height. The leaves are oval in shape and dark green in colour. The tree bears between three to six hermaphroditic flowers with five sepals and petals. At harvest time, apples are usually roundish (between 5 to 10 cm in diameter) and depending on the genotype the fruit can vary widely in colour (red, green or yellow), size, shape, acidity or sugar content (Dapena and Blázquez, 2009; Coque Fuertes et al., 2012).

The apple species has a high degree of adaptability and trees can be grown in wide range of soils. Owing to the large genetic diversity arising from the large secondary contribution, apples can be also commercially produced in many different environments, including cold and tropical regions (Janick, 1997). Around 7500 apple cultivars have been documented worldwide (Watkins, 1984) but only a few cultivars dominate the market. The domesticated apple is the fourth most important fruit crop in terms of production worldwide (after banana, citrus species and melons). Annual average apple production reached 75.8 million metric tons in the growing season 2019/2020 (FAOSTAT, 2021). China, United States of America, Turkey, Poland and India are currently leading the apple production, whereas Spain occupies the twenty second position (FAOSTAT, 2021). Regarding the Spanish apple production, Catalonia represents around 50% of the country's total production, followed by Aragón (19%) and Galicia (10%; MAPA, 2020).

Apples can be classified into three categories based on their use: fresh/dessert, processing/cooking and juice/cider (Merwin et al., 2008). Cider is commonly defined as an alcoholic drink made from fermented juice. Apples used in cider production are normally apples harvested from cultivars grown specifically for cider-making (Mangas and Díaz-Llorente, 2010), although cull apples from the dessert apple industry are sometimes used. It is possible to obtain cider from a single suitable cultivar, but most ciders are a blend of different apple cultivars (Dapena, 1993b; Mangas and Díaz-Llorente, 2010; Merwin et al., 2008). A good blend for producing natural cider (naturally fermented apple juice beverage) is a mix of sweet, sour and bitter apples (Downing, 1989). The final product should have a good balance of acidity, sugar and tannins (Mangas and Díaz-Llorente, 2010). Cider apple cultivars belong like dessert apple trees to the *Malus domestica* species (Chevalier, 1920) and they are not genetically closest to *Malus sylvestris* as initially hypothesized (Cornille et al., 2012). Several studies have developed a classification according to physical-chemical qualities of the fruits in order to choose the most appropriate cultivars for the elaboration of ciders (Dapena, 1996; Lea and Piggott, 2012; Mangas and Díaz-Llorente, 2010). A high proportion of cider apple cultivars are bitter (Pereira-Lorenzo et al., 2009) and the bitterness is due to a high concentrations of polyphenols compounds (Sanoner et al., 1999). The proportion of sour, bitter or sweet cider apple varieties varies between the different growing regions (Dapena, 1993b; Lea and Drilleau, 2003; Picinelli et al., 2000; Williams, 1988).

1.2. Apple germplasm banks and apple breeding programs in the world

The choice of varieties and rootstocks has a crucial importance in the economic profitability of commercial orchards. For hundreds of years, humans have selected the most adequate individuals for breeding purposes. Apple scion (i.e., the fruiting cultivar) breeding programs have focused their efforts, among other traits, on improving fruit yield, fruit quality or developing resistance to pests and diseases.

The high frequency of spontaneous crosses between individuals of *Malus domestica* and other wild relatives from diverse geographic origins has led to a great level of genetic variability in the domesticated apple (Cornille et al., 2014). However, the repeated use of few and genetically related cultivars is gradually reducing the diversity of apple genetic resources (Bramel and Volk, 2019). Several challenges regarding the levels of resistance or tolerance to abiotic and biotic stresses are expected in the future (Bramel and Volk, 2019). The opportunities to successfully address these challenges will depend on the availability of genetic diversity. With the aim of preserving the genetic diversity, numerous germplasm banks (collections of genetic resources) have been established and maintained around the world. The conservation of wild relatives and traditional apple cultivars *in situ* provides an invaluable source of novel genes for apple breeding. Some of the main *Malus* germplasm banks in the world are listed in Table 1. The largest apple repository in Spain is hold by the Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) that currently maintains 800 accessions. Other collections within the Spanish territory are Centro de Investigaciones Agrarias de Mabegondo (407 accessions), Universidad Pública de Navarra (397 accessions), CITA de Aragón (190 accessions), Universidad de Lérida (113 accessions) and Estación Experimental de Aula Dei (54 accessions).

Table 1. Main *Malus* germplasm banks in the world in 2015. The summary only includes *Malus* collection inventories which hold over 350 accessions. Adapted from Bramel and Volk (2019).

Country	Institute	*Malus domestica* accesions	Other *Malus* accesions
Azerbaijan	Genetic Resources Institute of the Azerbaijan National Academy of Sciences	395	3
Belgium	Walloon Agricultural Research Centre (CRA-W)	1600	1
China	The Institute of Pomology, The Chinese Academy of Agricultural Sciences	734	23
Czech Republic	Research and Breeding Institute of Pomology, Holosvousky, Ltd.	635	21
France	National Institute for Agricultural Research (INRA), IRHS	2079	
Germany	Julius Kühn-Institute, Institute for Breeding Research on Fruit Crops	836	49
Hungary	National Agricultural Research and Innovation Centre, Fruitculture Research Institute	1210	
Ireland	Irish Seed Savers Association	200	1
Italy	University of Udine	231	51
Italy	Bologna University, Department of Agricultural Sciences	2500	
Japan	Apple Research Division, NARO Institute of Fruit Tree Science	1100	3
Latvia	Latvia State Institute of Fruit-Growing	644	11
Lithuania	Lithuanian Research Centre for Agriculture and Forestry	561	24
New Zealand	The New Zealand Institute for Plant & Food Research Limited	375	16
Russia	N. I. Vavilov All-Russian Research Institute of Plant Industry RAAS	3467	69

South Africa	Agricultural Research Council (ARC) Infruitec-Nietvoorbij	520	
Sweden	Balsgård, Swedish university of agricultural sciences	370	
Switzerland	Agroscope	1300	
United Kingdom	University of Reading, National Fruit Collection	2174	13
United States	USDA-ARS Plant Genetic Resources Unit, Geneva, NY	1372	59
United States	Temperate Orchard Conservancy	400	

Numerous apple breeding programmes are in progress all over the world. In Europe, Lespinasse et al. (2007) reported at least 32 dessert apple and 3 cider apple breeding programs, including that of SERIDA. Breeding new apple cultivars takes from 15 to 25 years (Sedov, 2014) and breeders should be able to anticipate the future demands from the growers, the market, and the consumers. In addition, major efforts to develop cultivars which can effectively overcome the negative impacts of climate change in many regions are currently an urgent priority.

1.3. The importance of apple farming in Asturias and the genetic resources of the SERIDA apple germplasm bank

Apples are an important socio-economic product in Asturias (northwestern Spain). This region is the largest producer of cider apple in Spain (Torre et al., 2002). Apart from the direct economic benefit for the region, apples represent an emblematic symbol of identity with strong historical roots. In northwestern Spain, the species *Malus sylvestris* is spontaneous and *Malus domestica* was introduced very early (Dapena, 1993a). It is being hypothesized that interspecific hybridization occurred naturally between these two species thousands of years ago. The result of this hybridization is the emergence of a wide array of new apple cultivars. The large varietal diversity of apple makes Asturias a secondary centre of genetic variation (Dapena, 1996; Dapena et al., 2006). Additionally, it has been reported that Asturian natural cider differs in certain respects with French or English ciders (Picinelli et al., 2000). The Asturias region presents a greater number of acid and semi-acid varieties, while in the case of the French

Brittany and England the percentage of bitter and bitter-sweet varieties is higher (Boré and Fleckinger, 1997; Dapena, 1993b; Dapena, 1996, Williams and Copaz, 1988).

Apple orchards are scattered along Asturias although most of the production is concentrated in a region known as “Comarca de la Sidra” (Figure 1). The total planted area represented 10,324 ha in 2010 (INDUROT, 2010) and almost all the apple production is intended for cider-making.

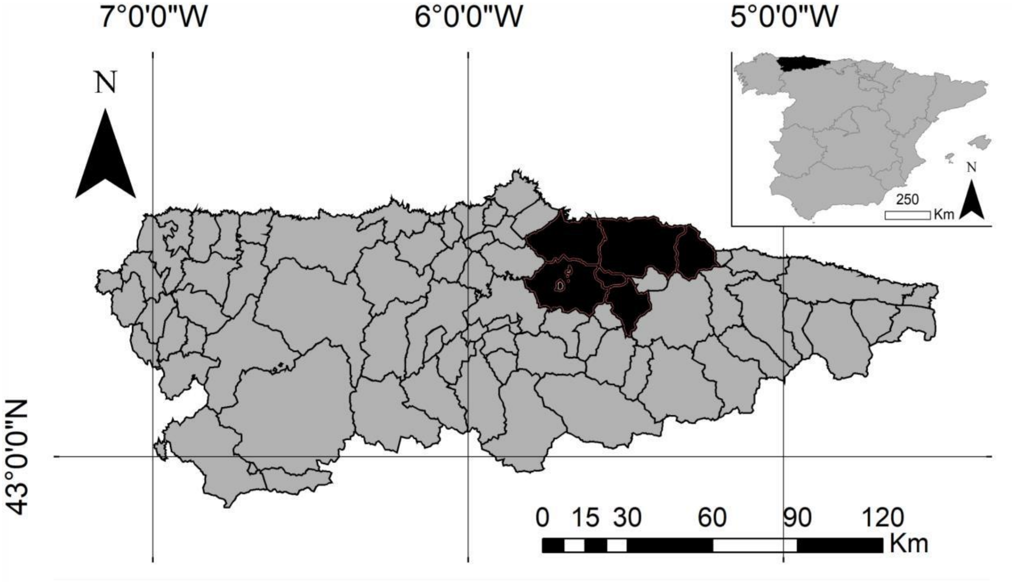

Figure 1. Map of the Asturias region. The inset image shows the location of Asturias within Spain. The larger image shows the municipalities that make up what is known as “Comarca de la Sidra.”

The region has traditionally relied the production on its own well-adapted local cultivars. The former Pomological Station in Villaviciosa established the first collection of varieties in the region in 1956. Since 1990, the Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) established the current apple germplasm bank. The most important exploration of local cultivars was carried out in the period 1995-1997 and currently this apple germplasm collection has around 800 accessions, including 550

Asturian local accessions. Cider apple accessions account for approximately 75% of the collection. Cultivars from other Spanish regions, other countries and different *Malus* species are also conserved.

An agronomic evaluation in terms of the resistance/susceptibility to fungi (scab, powdery mildew, European canker and brown rot), fire blight and aphids (rosy apple aphid and woolly apple aphid) was carried out (Dapena, 1996; Miñarro et al., 2013; Miñarro and Dapena, 2007). Cumulative yield, flowering and ripening periods (Dapena, 1996; Dapena et al., 2013; Dapena and Blázquez, 2009) and other qualitative properties such us physicochemical parameters (juice extraction efficiency, volumetric mass, º Brix, total acidity, pH, total phenolic compounds), fruit composition (sugars, organic acids; Blanco et al., 1992), phenolic composition (Mangas et al., 1999) and volatile components (Arias et al., 2010) were also characterized. This information allowed the research group to have adequate information with regard to the agronomic and technological characteristics of the cultivars and to proceed with the selection of the most interesting ones for local farmers. At the same time, the SERIDA Fruit Research Group has carried out an extensive work in the morphological and molecular characterization of the collection. Previous studies had reported a remarkable genetic variability among the maintained apple accessions (Dapena, 1996; Llamero-García, 2014; Pereira et al., 2017). Aiming to take advantage of this broad gene pool, the SERIDA breeding program was initiated in 1989 using some of the promising candidates to conduct artificial controlled crosses and develop new array of improved cultivars. The main objectives of the local breeding program were: (1) high resistance to diseases and pests (apple scab, European canker, fire blight, rosy apple aphid); (2) regular bearing and (3) organoleptic and technological quality of the fruit (Dapena and Blázquez, 2004). The first selection of varieties was completed at the end of the 1990s and the second selection of varieties concluded in 2016.

Since 2003, a Regulatory Council certifies and protects the cider produced in Asturias under the concept of Protected Designation of Origin “Sidra de Asturias.” The Protected Designation of Origin (PDO) legally protects origin, quality and method of preparation. Initially, only 22 cultivars were covered by the PDO label and all of them were characterised and selected by SERIDA (Dapena and Blázquez, 2009). Currently, there are 76 authorised varieties (Figure 2) and all of them are local cultivars and new cultivars with at least one local cultivars in its parentage. The SERIDA carried out the research work on the characterization, evaluation, breeding and selection of these varieties. In recent years, there is a growing demand of PDO cider and a total of 948 hectares were registered with the Regulatory Council in 2021 (Consejo Regulador de la Denominación de Origen Protegida Sidra de Asturias, personal communication).

Figure 2. Authorised apple varieties for the elaboration of the PDO “Sidra de Asturias.” Source: Consejo Regulador de le Denominación de Origen Protegida “Sidra de Asturias.”

1.4. Agronomic management of apple orchards in Asturias

Orchard management practices are essential to ensure grower profitability. First, the choice of the rootstock is critical for the establishment of new orchards for many reasons including the susceptibility to pest and diseases, vigour and planting distance (Janick and Moore, 1997). Traditionally, apple trees in Asturias have been grown on seedling rootstocks with a tree-density of 100-250 trees/ha (Dapena, 1993a, Dapena et al., 2005). Today, semi-intensive systems have been widely implemented in the region and trees are grown on semi-dwarfing rootstocks (M7, MM106 and MM111) with a tree density between 550 and 750 trees/ha (Dapena, 1993b; Dapena and Blázquez, 1996; Dapena et al., 2002).

Second, the choice of the scion represents a crucial step. From an economic and environmental point of view, the most desirable method for pest and disease control is the selection and breeding of resistant genotypes (Aldwinckle and Lamb, 1981; Cross, 2002; Dapena and Blázquez, 2004). Since average annual rainfall exceeds 1000 mm in Asturias (Dapena and Fernández-Ceballos, 2007), apple trees are normally exposed throughout the year to high levels of relative humidity. Under this environmental conditions, fungal diseases such as apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*) and European canker (*Neonectria ditissima*) are the main phytosanitary problems. Several local cider apple cultivars and all the newly registered varieties by SERIDA have low sensibility to fungal diseases, thus fungicide applications are not usually needed (Dapena, 1996; Dapena et al., 2005; Dapena and Blázquez, 2002). Among harmful arthropods, only the codling moth (*Cydia pomonella*) and the rosy apple aphid (*Dysaphis plantaginea*) can cause a significant damage. In recent years, voles (*Arvicola terrestris*, *Microtus lusitanicus*, *Microtus agrestis*) and apple proliferation (caused by *Candidatus Phytoplasma mali*) represent a potential economic threat.

The harvest season for local cider apple varieties ranged from the end of September until the beginning of December depending on the genotype (Dapena, 1993b, 1996). The ripening process is a key stage that significantly affects the technological quality of apples for cider production. Some biochemical components such as sugars, organic acids, phenolic compounds or pectin levels undergo changes during the fruit ripening process (Blanco et al., 1992; Mangas et al., 1992; Mangas et al., 1999; Mangas and Díaz-Llorente, 2010). DPO apple varieties can be classified into 9 different technological groups according to their total acidity and phenolic compounds (Dapena, 1993b, 1996).

Finally, it is important to highlight that the cider region encounters a historical problem of biennial bearing which means that 45,000 - 50,000 tons/ha of apples are produced in the ‘on’ years while only 10,000 - 16,000 tons/ha are produced in the ‘off’ years. In practice, this means that every two years (‘off’ years), the cider industries need to import apples from other Spanish regions or other countries. However, new releases from the SERIDA breeding program aim to solve to great extent this problem.

2. DORMANCY IN TEMPERATE FRUIT TREES

2.1. Background

Dormancy (i.e., dormant state) in temperate fruit trees is the phase of development between leaf drop in the late autumn or early winter and bud break in spring that allows trees to survive unfavourable winter conditions (Faust et al., 1997). Most of the temperate fruit tree species must overcome a dormant state to resume vegetative growth in the subsequent growing season (Campoy et al., 2011a; Faust et al., 1997; Saure, 1985). The exposure to chilling temperatures for a certain period prevents bud burst after short warm spells during the cold season.

The concept of the chilling requirement (CR) was established to determine the amount of cold after which a fruit tree will break dormancy (Samish and Lavee, 1962). This parameter is genotype-specific and it is considered the key factor in determining the length of the dormancy period (Campoy et al., 2011a; Luedeling, 2012; Saure, 1985). The knowledge of the agro-climatic requirements of a particular variety has a crucial importance for orchard managers from a practical and economical point of view (Fennell, 1999). Large variability in chilling requirements has been reported among cultivars from the same species (Campoy et al., 2012; Fadón et al., 2020b).

The most common approach to explain the transition between the chill and heat phases is the sequential fulfilment of chilling and heat requirements. According to this concept, chill and heat accumulation are independent processes (Erez and Couvillon, 1987). The accumulation of heat only begins after the fulfilment of the chilling requirement and additional chilling has no effect (Darbyshire et al., 2013; Guédon and Legave, 2008; Luedeling et al., 2009a). A different approach considers that a simultaneous combination of chilling and heat accumulation can evoke bud burst. In other words, a trade-off between chill and heat accumulation may compensate to some extent a lack of chill and vice versa, resulting in similar bloom dates (Harrington et al., 2010; Pope et al., 2014).

2.2. Seasonal cycle of dormancy

According to Lang et al. (1987), winter dormancy can be divided into para-, endo- and eco-dormancy stages. During endo-dormancy also known as the “true dormant state,” the buds remain inactive even under favourable temperature conditions as they have not been exposed to enough chilling (Figure 3). Eco-dormancy is mainly modulated by favourable temperature conditions that eventually lead to budburst in spring. Para-dormancy is described as a type of dormancy in which buds remain inactive because of the inhibitory activity caused by other tree structures (e.g., apical dominance; Lang et al. 1987).

Figure 3. Phenological development of apple buds during dormancy. The picture on the left shows dormant buds through endo- and eco- dormancy phases (growth stage 0 of bud development of the international BBCH code for pome fruit; Meier et al., 1994). The picture on the right shows the growth resumption and beginning of bud swelling after eco-dormancy release (growth stage 7 of the BBCH code).

During the dormancy phase of deciduous trees, all physiological processes are suspended or slowed in the bud meristems (Fadón and Rodrigo, 2018; Rohde and Bhalerao, 2007), therefore, flower and vegetative buds do not show any visible sign of growth (Samish, 1954). In apple, Heide and Prestrud (2005) reported that trees are not sensitive to photoperiod and only temperatures control dormancy induction. In short, dormancy release in tree buds relies on the exposure to chilling conditions during endodormancy (chilling phase), followed by warm temperatures during ecodormancy (forcing phase; Figure 4)

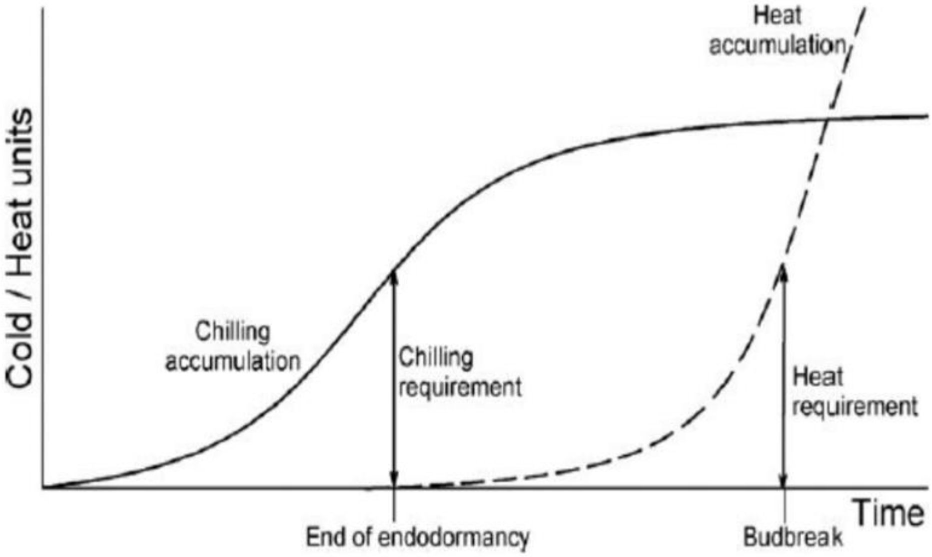

Figure 4. Schematic illustration of the seasonal progression during the dormancy period under the assumption that chill and heat are accumulated sequentially. Adapted from Djaman et al. (2021).

2.3. Quantifying chill and heat accumulation

Various mathematical models have been developed to measure the exposure to effective cold temperatures in deciduous trees (Fishman et al., 1987a,b; Richardson et al., 1974). These models, known as chill models, aim to estimate the chilling requirement (CR) of commercial fruit varieties with the goal of determining their regional suitability as well as forecasting bloom dates (Campoy et al., 2011a; Darbyshire et al., 2013; Luedeling, 2012). Despite major efforts to characterize the biological processes involved in tree dormancy have been undertaken by the scientific community, to date, the currently available chill models are simply based on empirical information and do not include robust biological or physiological parameters (Fadón et al., 2020a; Luedeling, 2012).

All the models for quantifying chill and heat requirements in buds are based on temperature records (Campoy et al., 2011a, Fernandez et al., 2020c; Luedeling, 2012). Traditionally, the Chilling Hours model (Hutchins 1932, as cited by Weinberger, 1950) has been the most commonly used model in horticulture due to its simplicity. This model assumes that temperatures between 0 and 7.2 °C equally contribute to release endodormancy (Figure 5). The Utah model (Richardson et al., 1974) was the first model assigning differential chilling efficiencies to distinct temperature ranges and the first model introducing the concept of "chill negation." According to its mathematical structure, temperatures above 15.9 °C have a negative effect on chill accumulation and the optimum temperatures to accumulate chill range between 2.5 and 12.5 °C (Figure 5).

The Dynamic model (Erez et al., 1990; Fishman et al., 1987a, b) was developed in the Mediterranean climate of Israel and it has emerged as the most complex model. The accumulation of chill is a two-step process mediated by a precursor compound formed due to cold temperatures. This product formed by the first step can be only destroyed by warm conditions. Only once a certain amount of this compound has been accumulated in the plant, this can be transformed into a permanent "chill portion." This metric is the basis for chill quantification and the process is most effective at temperatures around 8 °C (Erez et al., 1990; Fishman et al. 1987a, b; Figure 5).

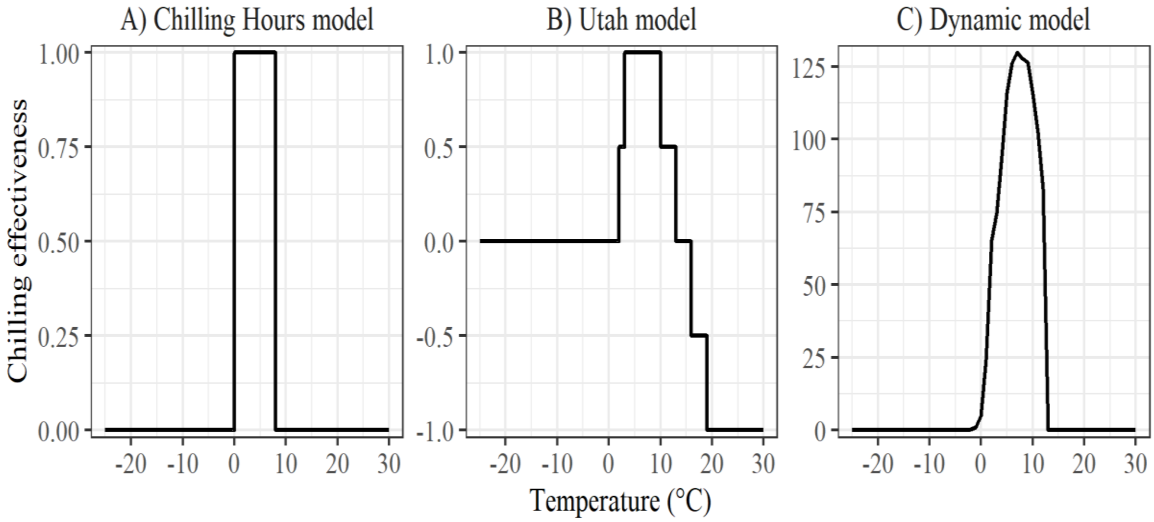

Figure 5. Chill effectiveness of the Chilling Hours model (A), Utah model (B), and Dynamic model (C) for different ranges of hourly temperatures. The model effectiveness in the y-axis is expressed as the specific unit of each model. Since the Dynamic model works as a continuous function, the effectiveness curve for this model shows chill accumulation after 100 continuous hours at the specified temperature (adapted from Fernandez et al., 2020c).

Among these chill models, the Dynamic model has been widely acknowledged as the most biologically plausible and accurate for temperate fruit trees in mild-winter areas (Campoy et al., 2011a; Fernandez et al., 2020c; Luedeling, 2012; Luedeling et al., 2009a; Ruiz et al., 2007; Zhang and Taylor, 2011). It is important to note that the estimation of chill requirements according to these mathematical models cannot be assumed to be comparable across regions (Luedeling and Brown, 2011) or to remain accurate in a context of global warming (Fernandez et al., 2020c; Luedeling et al., 2011; Luedeling and Brown, 2011).

Regarding the heat quantification, various models were developed for determining the heat requirement (HR) in fruit crops (Anderson et al., 1986; Richardson et al., 1974). The Growing Degree Hours model proposed by Anderson et al. (1986) has emerged as the most commonly used model. This model assigns varying heat accumulation efficiencies to temperatures above a base temperature of 4 °C, with an optimum temperature of 25 °C and a critical temperature threshold of 36 °C.

2.4. Methods for determining cultivar-specific chill and heat requirements

An accurate empirical quantification of chilling and heat requirements in adult trees under field conditions is rather impossible due to buds do not show any visible growth during the dormant state (Dennis, 2003; Fadón et al., 2020a; Samish, 1954). In this sense, several approaches have been applied in temperate fruit trees to experimentally determine the endodormancy release date. Attempting to simulate the behaviour of adult trees, forcing cuttings or potted trees in a growth chamber has been carried out in many dormancy studies (e.g.,Cook and Jacobs, 2000; Cook et al., 2017; Egea et al., 2003; Malagi et al., 2015; Parkes et al., 2020; Prudencio et al., 2018). Forcing one-year-old detached shoots after different periods of chill exposure in the field and monitoring the phenological evolution of the buds has been the preferred choice of many researchers (Dennis, 2003). Nevertheless, the quantification of cultivar-specific CR using one-year-old shoots can result in widely different estimates depending on various aspects such as the type of buds (Campoy et al., 2011b; Erez, 2000), rootstock (Webster, 1995), light (Powell, 1987), altitude (Alburquerque et al., 2008) or experimental design (Dennis, 2003). For example, floral buds generally present a lower chill requirement than vegetative buds (Campoy et al., 2011b; Erez, 2000; Naor et al., 2003). One-year-old apple shoots can have either one type or both types of buds depending on the cultivar and most of the cultivars also exhibit an acrotonic budburst tendency where terminal buds are capable of establishing terminal bud dominance (Cook and Jacobs, 1999).

A number of studies have reported on the CR of apple cultivars after assessing the phenological evolution of the buds under controlled forcing conditions (e.g., El Yaacoubi et al., 2016; Guak and Neilsen, 2013; Hauagge and Cummins, 1991; Parkes et al., 2020). Couvillon and Erez (1985) considered the end of endodormancy when 50% of the buds sprouted after two weeks at 24 °C. Hauggage and Cummins (1991) evaluated three parameters after forcing the shoots with temperatures above 19 °C; percent of terminal budbreak after 21 days of forcing, bud development stage and day on which 50% of the shoots had terminal buds broken. Cook et al. (2017) forced the shoots with a constant temperature of 25 °C and determined the depth of the dormancy as the number of days to 50% budburst. El-Agamy et al. (2000) used a 10 or 50% budbreak as an indicator of endodormancy breaking after 3 weeks of forcing with temperatures ranging from 20 to 25 °C. More recently, Parkes et al. (2020) established the end of the endormancy period when 50% of the buds had reached the green tip stage after 2 weeks of forcing at 25 °C.

The lack of standardization in the methods for forcing buds hampers the comparability of results among varieties and locations and partially explain the apparent incongruences reported in literature when comparing estimates of agro-climatic requirements for the same genotype from different studies (Dennis, 2003). Similarly, Luedeling and Brown (2011) noted the importance of mentioning the location where the agro-climatic needs were determined. For instance, the popular apple variety ‘Granny Smith’ was shown to require 72.8 CP in Australia (Parkes et al., 2020) whereas El Yaacoubi et al. (2016) reported a CR of 59 CP in southern France.

On the other hand, statistical approaches based on long-term observations of flowering stages have been developed to determine the length of the endodormancy phase in deciduous fruit trees (Alonso et al., 2005; Darbyshire et al., 2016; Darbyshire et al., 2017; Funes et al., 2016; Luedeling et al., 2013b; Luedeling and Gassner, 2012).

As an alternative to time-consuming experimental trials, these studies use phenological information to estimate CR statistically. Most of these strategies assume that flowering is the result of the combination of winter chill and spring heat (Darbyshire et al., 2017; Guédon and Legave, 2008; Luedeling et al., 2009a). In recent years, Partial Least Squares (PLS) regression has been applied to delineate chilling and forcing phases in deciduous trees (Benmoussa et al., 2017; Fadón et al., 2021; Luedeling et al., 2013b; Luedeling and Gassner, 2012; Martínez-Lüscher et al., 2017). This approach requires historical temperature and phenology records and for each calendar day of the dormancy season, the PLS regression can identify whether high temperatures tend to delay or advance bloom dates. According to this procedure, CR and HR are accumulated independently but chilling and heat periods can overlap. After delineating the chilling and forcing periods of a particular cultivar, temperature dynamics during both periods can be analysed using common chilling models to determine the variety-specific requirements.

Obtaining new climate-resilient cultivars is one of the major objectives of fruit breeding programs at present. For the successful breeding of improved cultivars, the most important step is the choice of the parents which should be used for crossing. Conventional breeding methods for determining flowering time and agro-climatic requirements are laborious and time-consuming in rosaceous crops. Recent studies in the last decades have found biochemical markers which may indicate the level of dormancy in tree organs (reviewed by Fadón et al., 2020a). Some of these approaches include the study of the phyto-hormonal regulation (Rinne et al., 1994), starch concentration in the flower primordia (Fadón et al., 2018), dynamics of starch and hexoses in branches (Fernandez et al., 2019) and the communication cycles at the cellular level (Rinne et al., 2011). In addition, Falavigna et al. (2015) stated that the regulation of dormancy release is highly heritable, suggesting that regulatory genes can be used as molecular markers for determining the endodormancy breaking. Quantitative trait loci for CR or flowering time have been identified in apple (Liebhard et al., 2003; Van Dyk et al., 2010) and other species in the *Rosaceae* family (Castede et al., 2015; Olukolu et al., 2009; Prudencio et al., 2021). The implementation of marker-assisted selection allows selecting early or late flowering genotypes in the earliest stages of development thus improving the efficiency of fruit tree breeding programs.

2.5. The importance of dormancy in fruit production

The agro-climatic requirements (CR and HR) of fruit tree species should match with the climate conditions of a given place (Luedeling, 2012). In this respect, knowing the climatic requirements of fruit cultivars is especially important for the design of new plantings (Campoy et al. 2011a; Luedeling 2012). With the intention of determining the cultivar suitability in a particular geographical location, Luedeling et al. (2009b) introduced the concept of Safe Winter Chill (SWC) as the amount of winter chill that can be safely expected in 90% of all years. This reasoning incorporated a financial component for an orchard operation and assumed that a failure to fulfil the CR in more than 10% of all years can make orchards economically unfeasible. However, there are few uncertainties concerning the calculation of cultivar-specific chill requirements and its relationship with fruit production in temperate fruit trees. Some studies found economically acceptable yields in growing seasons in which winter chill accumulation dropped below the variety-specific CR reported in literature (Parkes et al., 2020; Pope et al., 2015). Although it appears clear that insufficient chill accumulation during dormancy can result in situations where trees do not completely release dormancy, leading to phenological disorders (Atkinson et al., 2013; Petri and Leite, 2003). Symptoms of an inadequate chilling exposure during winter can vary among tree fruit species (Atkinson, et al., 2013). Some of these physiological symptoms include a delay in anthesis and bud break, erratic bud break (Figure 6), bud abortion, extended flowering period and low-quality pollen (Atkinson et al., 2013; Cook and Jacobs, 2000; Petri and Leite, 2003; Sunley et al., 2006). A combination of these anomalies may result in poor fruit set and, ultimately, a significant reduction in marketable fruit yield (Atkinson et al., 2013). Furthermore, an unsatisfactory fulfilment of the CR during the chilling period negatively affects some fruit quality traits in apple such us fruit weight, size and firmness (El Yaacoubi et al., 2020).

Figure 6. Uneven budburst and flowering in cv. ‘Regona’ on May 20th, 2019 (Villaviciosa, northwestern Spain).

Changes in the environmental conditions are expected to affect the dormancy cycle and, consequently, phenology trends in temperature fruit trees (Campoy et al., 2011a). While global warming is forecast to likely affect the phenological behaviour of fruit and forest trees, the response of a given genotype to climate change can vary greatly across regions (Menzel et al., 2006). Flowering time is probably the most widely used biological indicator of climate change (Martínez-Lüscher et al., 2017; Menzel et al., 2006). Since the life cycle of fruit crops has a strong dependence of temperature (El Yaacoubi et al., 2014), the analysis of historic temperature records alongside long-term phenological records provides valuable information for understanding how climate change is affecting the spring phenology (Menzel et al., 2006).

In apple, the increase of average temperatures in the last decades have resulted either in advances in blooming dates over the years (Chmielewski et al., 2011; Darbyshire et al., 2013; Drepper et al., 2020; Eccel et al., 2009; Grab and Craparo, 2011) or flowering delays (Legave et al., 2015, 2013). The potential impacts of temporal changes in the flowering phenology of fruit trees include asynchrony between anthesis in productive varieties and pollinators (Else and Atkinson, 2010), reduced flowering synchronization between genetically compatible varieties (Funes et al., 2016; Pope et al., 2014) and the risk of spring frost (Blanke and Kunz, 2010; Eccel et al., 2009; Fernandez et al., 2020b).

2.6. Climate change impacts on winter chill

Many scientific studies have confirmed that global warming is happening due to human activity (IPCC, 2014). An increasing level in atmospheric concentrations of anthropogenic greenhouse gases such as carbon dioxide (CO_2), nitrous oxide (N_2O) or Methane (CH_4) has induced an average warming of approximately 1 °C (likely between 0.8 °C and 1.2 °C) above pre-industrial levels in 2017 (IPCC 2018; Figure 7).

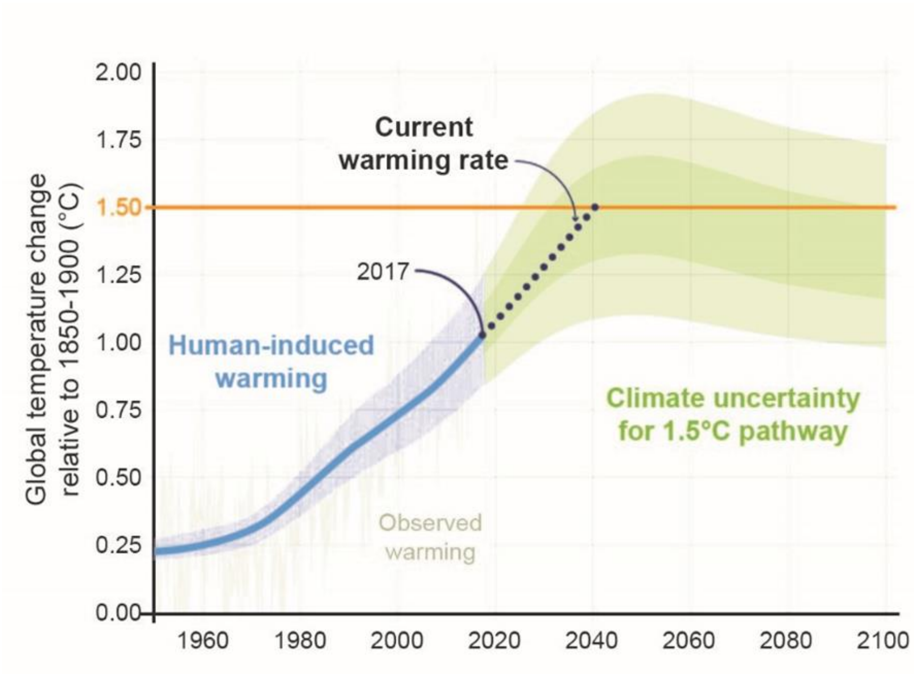

Figure 7. Observed globally averaged temperature (land and ocean) anomalies and predicted changes over the next decades in Celsius degrees relative to pre-industrial levels (1850-1900). Adapted from IPCC (2018).

Regarding future climatic conditions, the Intergovernmental Panel on Climate Change (IPCC) predict significant changes in the mean temperature for the 21st century due to climate change (IPCC, 2014). The pace of global warming will depend on the mitigation measures adopted to tackle climate change. Without significant action, annual mean global temperature is predicted to exceed 1.5 °C between 2030 and 2052 compared to pre-industrial levels (IPCC, 2018).

For climate modelling purposes and to gain a better understanding of the changes in global mean surface temperatures under different scenarios, the IPCC describes four Representative Concentration Pathways (RCPs). An RCP scenario represents the total additional radiative forcing (in W/m^2) expected by the end of the twenty-first century as a result of the concentration of greenhouse gasses in the atmosphere compared to pre-industrial conditions (IPCC, 2014). These pathways include a drastic mitigation scenario (RCP2.6), two intermediate scenarios (RCP4.5 and RCP6.0), and one scenario with very high greenhouse gas concentrations (RCP8.5; IPCC, 2014). The RCP 4.5 scenario predicts that mitigation measures will result in the stabilization of atmospheric greenhouse gas concentrations, while the RCP 8.5 is an extreme scenario which envisages very high emissions until the end of the century (IPCC, 2014).

Regardless of the RCP scenario, significant increments in winter temperatures are likely to compromise the fulfilment of CR necessary to optimally release endodormancy (Benmoussa et al., 2020; Campoy et al., 2011a; Darbyshire et al., 2011; Luedeling, 2012; Luedeling et al., 2011). The amount of winter chill is one of the main limiting factors to produce economically viable yields (Baldocchi and Wong, 2008; Luedeling, 2012). A decline in the availability of winter chill in the medium and long term in many growing regions might restrain the array of commercial varieties or even species that growers can cultivate in a particular region (Luedeling, 2012). Severe production risks on fruit tree species are expected over the course of the twenty-first century in regions characterized by a Mediterranean climate such as Tunisia (Benmousa et al., 2020), Central Valley of California (Luedeling et al., 2011), the Central Valley of Chile (Fernandez et al., 2020b) or northeastern Spain (Rodríguez et al., 2021, 2019). However, it is important to note that the impacts of increasing temperatures on winter chill can differ widely across growing regions. For example, Fernandez et al., (2020c) reported that cool and temperate climate regions may not see a reduction in winter chill and some locations may even gain some chilling as a result of less frequent frost temperatures which supposedly do not contribute to break tree dormancy (Luedeling, 2012).

Apart from the expected changes in winter chill availability, other studies have predicted that climate change might have severe implications in the incidence of crop pests (Garrett et al., 2006). It is also expected to alter the intensity and frequency of extreme climate events. Changes in the total precipitation and rainfall patterns are less certain than temperature trends (IPCC, 2014) although, climate models have predicted that some areas might cope with water stress over the coming decades (Iglesias et al., 2012).

2.7. Adaptation strategies

Climate change and global warming are real threats to the tree fruit industry worldwide (Atkinson et al., 2013; Luedeling, 2012). In some regions, the impacts of climate change on temperate fruit production may be severe and adaptation measures will be required to mitigate the negative effects and to ensure the economic viability of the farms (Benmoussa et al., 2020; Campoy et al., 2011a; Luedeling, 2012). In mild winter regions, the implementation of strategies for adapting orchards to warmer climatic conditions has already become essential.

Some of the cultural practices to reduce the temperature inside the orchard include the use of conventional evaporative cooling generated from overhead irrigation (Erez, 2000) and netting the plots to reduce solar radiation during the warmer hours. The application of dormancy breaking agents (i.e., hydrogen cyanamide), mineral oils or plant growth regulators are often considered to be a feasible method to mitigate the adverse effects of warmer winter temperatures on buds (Campoy et al., 2010; Erez et al., 2006). But this technique does not fully substitute for the chilling requirement (Erez, 2000; Saure, 1985) and most of these compounds represent a high phytotoxicity risk.

A more radical solution to face the challenges of climate change is to move fruit production areas to other locations which are better suited to future climatic conditions.

Farm relocation in response to future warming is not a desired option and many fruit growing regions may be forced to begin a transition to different commercial varieties or crops (Darbyshire et al., 2016; Luedeling, 2012). A successful long-term adaptation strategy to anticipate the climatic changes is the selection and breeding of low chill cultivars with good commercial characteristics. Since the CR is a heritable trait (Campoy et al., 2011a; Falavigna et al., 2015), breeding programs can breed environmentally adapted new cultivars that can meet their CR every year.

3. THE ROLE OF POLLINATION IN FRUIT CROPS

3.1. Background of apple pollination

Pollination is the transfer of pollen grains from the anthers of the male plant to the stigmas of the female plant. This step is essential for the fertilization of the ovules and the development of seeds and fruits (Stephenson, 1981). From the agronomic perspective, fruit set is probably the major limiting factor for the yield of fruit trees and depends to a great extent on the success of the pollination events (Lamp et al., 2001). Besides the fruit set, the shape and weight of marketable fruits in many crops depend on the presence of seeds and thus, the success of pollination (Williams, 1970). Matsumoto et al. (2012) demonstrated that a partial pollination in apple flowers (i.e., not all the ovules were fertilized) led to the development of misshapen fruits. The choice of the pollen source also affects some important commercial characteristics in apple (Brookfield et al., 1996; Garratt et al., 2014; Matsumoto et al., 2012). Around 75% of the crop species rely on animal pollination and the loss of this service may reduce crop yields in fruit trees by 40% (Klein et al., 2007). Insect pollination in agricultural crops is worth €15 Billion in the European Union (Gallai et al., 2009). Apple culture is heavily dependent on insect pollination (Garratt et al., 2014; Ramírez and Davenport, 2013). For example, Garratt et al. (2016) estimated that insects contributed a total of £92 million per annum in the economic gains to producers of four apple varieties in the United Kingdom. Furthermore, pollinators are also shown to enhance fruit quality parameters such us fruit size, shape and mineral content (Buccheri and Di Vaio, 2005; Garibaldi et al., 2013; Garratt et al., 2014).

Apart from the fundamental role of insect pollinators, the pollination success relies on the supply of adequate pollen. This process is influenced by genetic, environmental, physiological and orchard management factors (Free, 1993; Rojo et al., 2015). As most temperate fruit species, apple require cross-pollination between genetically compatible genotypes to set fruit (Broothaerts, 2003). To avoid pollen limitation in commercial orchards, pollen donor trees (i.e., "pollinizers") or cultivar mixtures are required within the same orchard (Free, 1993; Kendall and Solomon, 1973).

3.2. Floral organs and floral biology aspects related to the fertilization process in apple

Large phenotypic variability in flowering biology characteristics has been reported among the nearly 7500 known cultivars of apple (Watkins, 1984). In terms of the morphology of the flowers, there are obvious differences between species of the genus *Malus* and different cultivars of the domesticated apple. The number of flowers per inflorescence in commercial cultivars range between 4 and 7 (Racskó and Miller, 2010). *Malus domestica* flowers have five petals and five sepals (Figure 8). The reproductive part of the flower consists in the male part with around 20 stamens and the female part divided into five styles (Janick, 1997). The ovary has five carpels, each containing two ovules, thus the maximum seed content in one fruit is usually 10 seeds (Jackson, 2003; Janick et al., 1996).

Figure 8. Fully opened flowers in a local cider apple cultivar from Asturias.

Floral traits determine the interactions between plants and pollinators (Junker et al., 2013) affecting the visits from pollinators to the reproductive parts of the flower. Flowers of different apple varieties can vary in size, petal shape, and colour (from white to different shades of pink; Janick et al., 1996). In this sense, flower colour is an important trait since bees cannot see portions of the visible spectrum (McCrea and Levy, 1983). The central flower in each inflorescence and the first one to open, known as king bloom, seems to have the best fruit quality potential thus it is important to ensure the presence of compatible pollen at the beginning of the blooming period.

Jackson (2003) defined anthesis as the period of time during which a flower is open and functional and this concept also refers to the process of anther dehiscence and pollen grains release. The effective pollination period (EPP) is defined as the number of days during which the egg apparatus of a flower can produce a fruit (Williams, 1966). The three main factors determining the EPP (i.e., the longevity of the egg apparatus) are stigma receptivity, pollen tube growth and ovule longevity (Sanzol and Herrero, 2001).

The ability of the stigma to allow pollen germination is the first step for the success of the fertilization process. Pollen germination occurs after a pollen grain contacts the stigma and the germination capacity is highly variable between different genotypes of the same species (Hormaza and Herrero, 1999) or between years for the same genotype (Nikkanen et al., 2000). After the germination of a pollen grain, the pollen tube begins to grow towards the ovule. The longevity of the ovule is also a limiting factor for the fecundity and the subsequent stages including fruit set (Mayer et al., 2011). The duration of EPP can vary greatly in apples ranging from two to nine days depending on the cultivar (Sanzol and Herrero, 2001).

3.3. Pollen germination and pollen viability tests

In fruit breeding programs is important to assess the quality of the pollen before performing artificial pollination experiments. Different terms have been used in literature to define pollen quality. For example, Dafni et al. (2005) distinguished between pollen stainability, fertility, germinability, vigour and viability. There are several tests to determine pollen viability and the results can vary widely among methods. Alexander's stain test (Alexander, 1969) has been one of the most common techniques for testing pollen viability. Other staining procedures which can also distinguish between aborted and non-aborted pollen include IKI (iodine potassium iodide) test (Sulusoglu and Cavusoglu, 2014), fluorescein diacetate test (Shivanna and Rangaswamy, 1992) and TTC (2,3,5-triphenyl tetrazolium chloride) test (Sulusoglu and Cavusoglu, 2014).

Counting germinated pollen grains in a culture medium containing concentrated agar and other sources of exogenous sugars is normally the easiest and widely used method to measure the germination capacity of pollen grains (Dafni and Firmage, 2000; Patel and Mankad, 2014). Different sucrose concentrations ranging from 5 to 30% are normally used in pollen germination studies. It is being reported that high concentrations of sucrose can cause pollen bursting (Jacquemart, 2007; Patel and Mankad, 2014). In addition, the temperature during the incubation time can significantly affect the results (Zlesak et al., 2005).

3.4. Environmental and climatic factors affecting the pollination efficiency

Fruit yield in temperate fruit trees depends on the successful pollination and fertilization processes (Racskó et al., 2007). Even there is strong evidence that genetic characteristics largely determine the efficacy of pollination in fruit trees (Dafni et al., 2005), several studies have reported that environmental, physiological and tree management factors are also decisive for the fertilization process (Free, 1993; Racskó et al., 2007; Rojo et al., 2015; Van Marrewijk, 1993) In fruit crops, unfavourable weather conditions can reduce the success rate of fertilization resulting in low fruit set. Temperatures before and after flowering are also important for successful fruit production (Ramírez and Davenport, 2013).

Pollen germination is directly correlated with temperatures in the 24 hours after the pollen grains contact the stigmatic surface (Williams and Maier, 1977). Low temperatures during full bloom inhibit the germination and pollen tube growth. On contrary, high temperatures inhibit floral induction, reduce pollen viability and cause the desiccation of the stigmatic surface (Van Marrewijk, 1993). Both inadequate temperatures are reported to short the effective pollination period (Sanzol and Herrero, 2001). In addition, the precipitation in the time of induction and differentiation of flowers can reduce the number of pollen grains per anther (Davarynejad et al., 2008).

On the other hand, the level of exposure to winter chill has been shown to influence flower morphology in apple (Couto et al., 2014). Petri and Leite (2003) observed that the of lack of chilling in a particular growing season led to the formation of small flowers and anthers with a reduced number of pollen grains. Couto et al. (2014) reported a smaller number of pollen grains per anther in mild winter regions compared to apple growing regions where chilling requirements are easily fulfilled.

3.5. Floral overlap and genetic compatibility

Floral overlap occurs when flowers in one tree are opened and functional at the same time than flowers in a genetically different tree (Sanzol and Herrero, 2001). The synchronization of the flowering period is the main mechanism facilitating an effective pollen transfer between trees in apple orchards (Free, 1993; Ramírez and Davenport, 2013). It is important to highlight that the flowering date for a given cultivar can fluctuate broadly depending on the geography and climate conditions (Menzel et al., 2006) affecting the blooming synchronization between cultivars previously established in a particular site. Ideally, a combination of early and late-blooming cultivars should be planted alongside a cultivar whose flowering is synchronised with the main cultivar to maximize pollination under environmentally unusual conditions (Delaplane et al., 2000).

Like most species of the Rosaceae family, apple exhibits gametophytic self-incompatibility (Broothaerts, 2003; Matsumoto et al., 2011). The rejection of self-pollen through the failure of pollen growing down the style prevents the production of seeds following self-pollination (Broothaerts, 2003) and favours cross pollination between genetically different individuals. This incompatibility system is controlled by a single polymorphic locus with multiple alleles named S-alleles (Broothaerts, 2003; Matsumoto et al., 2011). Most of the apple cultivars are diploid and therefore they are characterized by two S-alleles (Broothaerts, 2003).

Three main methods are commonly used to determine the compatibility among cultivars. Controlled cross-pollination trials under field conditions have been traditionally conducted to measure fruit set after a specific hand-pollination cross (Hough, 1940). The main disadvantage of this method is that fruit set is highly subjected to environmental conditions. More reliable conclusions can be obtained by using fluorescence microscopy to assess the growth of pollen tubes in the pistil (Jacquemart, 2007). More recently, a genetic approach based on the characterization of S-alleles through molecular analysis allows to precisely determine the S-RNase genotype of a given cultivar (Broothaerts, 2003; Matsumoto et al., 2011). A certain degree of self-fertility has been reported in some dessert apple cultivars such as ‘Golden Delicious’, ‘Idared’ or ‘Elstar’ (De Witte et al., 1995). Nevertheless, the popular variety ‘Golden Delicious’ produce significantly better crops after cross-pollination (Lerner and Hirst, 2002).

3.6. Pollen donor trees and cultivar mixtures

In the design of apple orchards, the selection of cultivars needs to ensure a significant synchronisation of flowering and the presence of fully compatible pollen (Carisio et al., 2020). Besides considering the flowering overlap and the S-genotype, other aspects need to be carefully considered in the configuration of apple plots. For example, pollen quantity and quality must be also taken into account for a successful fruit production (Jacquemart, 2007; Quinet and Jacquemart, 2017).

In commercial apple orchards, either cultivar mixes or pollinizers trees (trees that supplies pollen) are planted in the same orchard. Solid blocks of a single commercial cultivar can be planted alongside ornamental crab apples or wild apple species to act specifically as pollinizers. Some of the advantages of using crabapples are: long flowering period, large amount of flowers, low sensitivity to alternate bearing and optimal pollen characteristics (Lane et al., 1995). Additionally, wild apple relatives take up less space in the orchard than commercial apple varieties thus orchard management may be easier (Church and Williams, 1983). The major disadvantage is that these species does not produce marketable fruit.

The arrangement of the trees within the orchard is important to facilitate cross-pollination throughout the whole orchard block (Garratt et al., 2014; Quinet and Jacquemart, 2017). Quinet and Jacquemart (2017) stated that the efficiency of pollinators decreases when pollinizer and main cultivar trees are planted in different rows. The standard ratio between the pollinating variety and the main variety in apple orchards is often 1:7 (Sheffield et al., 2016). However, the optimal ratio is strongly influenced by the climatic conditions and/or the introduction of managed bees. A recent study by Carisio et al. (2020) reported that the ratio pollinizer/main cv. trees of 1:4 should be adopted to avoid pollen limitation in cv. 'Gala' orchards in northern Italy. On the other hand, several studies have demonstrated the benefits of planting multiple cultivars in the same plot (Didelot et al., 2007). In Asturias, most of the current cider apple orchards are designed to include several cultivars arranged in separate rows with the aim of overlapping the flowering periods among cultivars (Dapena and Blázquez, 1996).

3.7. Alternative pollination methods

In situations where there is a pollination deficit in the orchard either by insufficient pollinators or inadequate pollen-donating trees may be necessary to implement alternative pollination systems. Insect pollination can be enhanced by introducing hives of honeybees or by increasing the number of wild pollinators in the orchard. There are several ways to preserve pollinators-friendly habitats such us planting floral resources or creating artificial nests (Sheffield et al., 2016). Grafting branches from a compatible cultivar into the existing trees or placing floral bouquets during the flowering period are other feasible alternatives for boosting the pollen supply. An innovative approach to improve pollen transfer between cultivars is the use of pollen dispensers in the hive entrance fittings (Quinet et al., 2016a). Finally, artificial pollen applications using an air-blast sprayer can help ensuring an optimal fruit set (Ramírez and Davenport, 2013).

OBJECTIVES AND GENERAL OUTLINE

OBJECTIVES AND GENERAL OUTLINE

The main purpose of this thesis is to gain further insights into the agroclimatic requirements and flowering process of a collection of apple cultivars from the SERIDA germplasm bank in a global context of climate change. Asturias has excellent soil and climatic conditions for apple growing and the region offers a number of advantages over other apple growing regions in Spain. However, changes in the local climate conditions may make necessary to adapt agricultural management strategies over the next decades. In northwestern Spain, chilling has not been considered a limiting factor for the quality of flowering of apple trees but mild winters have become increasingly common in recent years. The analysis of historic chill accumulation trends in a particular region is the first step to understand the current impacts of climate change. Because apple orchards remain productive for many years, it has also become increasingly important to forecast the levels of winter chill under future climate scenarios.

To anticipate future changes and to adjust agricultural practices, local growers are interested in knowing the cultivar-specific chill and heat requirements in order to plant cultivars which can easily fulfil their requirements and avoid the negative consequences in yield and quality. In this thesis, we determined the chilling requirements for breaking dormancy using two approaches and we also tested the performance of chilling models. In addition, changes in environmental conditions might alter flowering time which can potentially affect other important processes such as pollination and fertilization. In this sense, we evaluated the phenotypic variability of floral biology traits among a large collection of genetic resources and determined the cultivars which can better serve as pollen donors in commercial apple orchards.

This thesis is organized in four Chapters according to the specific objectives:

Chapter 1. Agroclimatic requirements and phenological responses to climate change of local apple cultivars in northwestern Spain.

In this chapter, we analysed temperature trends in Asturias over the past 41 years to examine how changes in chill and heat accumulation have affected the phenology of apple cultivars in the region. In particular, this study aims to address the following specific objectives:

1.1. To establish groups of cultivars according to their flowering time.

1.2. To determine the frequency and trend of spring frost events.

1.3. To compare the performance of three mathematical winter chill models under the local climate conditions.

1.4. To analyse how global warming has affected the historic chill accumulation and flowering time of local apple cultivars.

1.5. To statistically delineate the start and end dates of the effective chill and heat accumulation periods and to quantify chill and heat requirements of local apple cultivars.

1.6. To determine the most effective temperatures to overcome dormancy during the chilling and forcing periods.

Chapter 2. Climatic requirements during dormancy in apple trees from northwestern Spain – Global warming may threaten the cultivation of high-chill cultivars.

In this chapter, we experimentally determined the agro-climatic requirements of apple cultivars using a bud forcing method. To assess cultivar suitability in northwestern Spain, we evaluated winter chill availability over the course of the twenty-first century by applying an ensemble of future climate scenarios. The major objectives of this work were:

2.1. To calculate the agro-climatic requirements of ten apple cultivars by implementing a reliable method for forcing shoots under environmentally controlled conditions.

2.2. To compare the precision of chill models under the mild winter conditions of northwestern Spain.

2.3. To test the interaction between chilling and heat requirements and flowering dates.

2.4. To clarify the relationship between chill accumulation and fruit yield under suboptimal chilling conditions.

2.5. To project the impacts of climate change on winter chill for the 21st century under two global warming scenarios.

2.6. To determine the most suitable apple cultivars to be cultivated in Asturias under future climate scenarios and time periods.

Chapter 3. Analysis of the variability of floral and pollen traits in apple cultivars **–** Selecting suitable pollen donors for cider apple orchards.

In this chapter, we aimed to improve our knowledge of the reproductive biology of apple cultivars. We determined the phenological stages and floral and pollen traits in 45 apple cultivars in order to optimize their best use for pollination and also to ascertain if the knowledge of these traits can guide new breeding strategies. This work is complemented by a short communication (**Chapter 4**) and both studies address the following aims**:**

3.1. To contribute to the phenotypic evaluation of flowering biology traits of selected local cultivars and new varieties from the SERIDA breeding program.

3.2. To determine the periods of bloom overlap between cultivars.

3.3. To test the reliability of two easy techniques for determining pollen viability and pollen germination of pollen grains.

3.4. To determine the optimum sucrose concentration for testing the pollen germination of apple cultivars.

3.5. To determine the alternate bearing tendency of a collection of cultivars based on flowering intensity information.

3.6. To identify the cultivars which can better meet the requirements to serve as pollen donors in apple orchards.

3.7. To find out if the quality and quantity of pollen grains in apple cultivars is affected by ‘on’ and ‘off’ years.

Chapter 1

Agroclimatic requirements and phenological responses to climate change of local apple cultivars in northwestern Spain

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Agroclimatic requirements and phenological responses to climate change of local apple cultivars in northwestern Spain

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ABSTRACT

In a global warming context, analyses of historic temperature records are essential to understand the potential impacts of climate change on spring phenology. To estimate flowering trends over recent decades, we analyzed long-term temperature and phenology records of eleven local apple cultivars in Asturias (northwestern Spain) in a temperate oceanic climate. Our results show that, over a period of 30 years, bloom dates of the local cultivars have experienced relatively minor changes, considering that temperatures increased strongly since 1978, by 0.30 °C per decade. An explanation for this weak phenological response to warming may be that these temperature changes only had a small effect on overall chill accumulation, but possibly delayed the onset date of endodormancy, which may have counteracted phenology-advancing effects of warming in spring. At present, chill accumulation in this area is high, at an average of 96 Chill Portions from November to March, which indicates that chill is not currently a limiting factor for the quality of flowering and fruiting in the study area. We used Partial Least Squares (PLS) regression to delineate an effective chilling period between November 12th and February 9th and effective heat accumulation between March 15th and May 4th. While these periods appear plausible, we noticed that this approach was unable to identify well-known differences in chilling requirements among many of the cultivars, with similar chill needs determined for many of them. This observation may be explained by inaccurate expectations about cultivars' climatic needs, by inaccuracy of the chill (and possibly heat) model or, most concerning, by inability of the PLS approach to correctly identify the chilling periods of apple cultivars in this region. Bloom dates were similarly responsive to mean temperature during the chill and the heat accumulation phases, indicating that both processes need to be considered when predicting future phenology.

1. Introduction

Apple (*Malus domestica* Borkh.) trees, like most woody perennial species that evolved in temperate or cold climates, spend the winter months in a dormant state that allows them to survive unfavorable conditions and avoid cold damage (Faust et al., 1997; Saure, 1985; Campoy et al., 2012). To break dormancy, trees undergo two distinct phases that ultimately lead to flowering: endodormancy, during which trees must fulfill cultivar-specific chilling requirements and ecodormancy, when heat requirements need to be satisfied (Lang et al., 1987; Egea et al., 2003). Chilling temperatures are important in fruit production, since they are needed for dormancy release, optimal flowering and satisfactory fruit set (Sunley et al., 2006; Campoy et al., 2011). In apple, the dormancy cycle is only regulated by temperature (Heide and Prestrud, 2005) and a sufficient amount of chill and heat is positively correlated with fruit weight, size and firmness (El Yaacoubi et al., 2020). The amount of chill that is required is cultivar-specific, and large variability has been reported among over 8000 apple cultivars and land races across the world. Nevertheless, most commercial cultivars have high to medium chilling requirements (El Yaacoubi et al., 2016; Parkes et al., 2020).

The apple industry plays a relevant economic and social role in Asturias in northwestern Spain, which contributes about 80 per cent of the total cider production in the country. The bulk of the orchards are composed of several local cultivars, which tend to be well adapted to the agro-climatic conditions of the region. Currently, apple production in

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Asturias relies on cultivars with medium to high chilling requirements (Dapena, 1996; Dapena and Fernández-Ceballos, 2007). So far, consequences of insufficient winter chill accumulation, such as delayed and irregular budburst (Erez, 2000), have rarely been observed in commercial orchards, except for a few occasions in years with particularly mild winters and/or cold early spring.

Global warming may compromise the fulfillment of trees' agroclimatic needs during dormancy (Luedeling and Brown, 2011; Fernandez et al., 2020a). Mean global air temperature increased by 0.74 °C between 1906 and 2005 (IPCC, 2007) and numerous future climate scenarios project major changes in air temperature over the course of the 21 st century (IPCC, 2014). Since plant phenology is strongly influenced by air temperature, long-term phenological observations at specific sites that are combined with meteorological data can provide useful information on plant responses to climate change. In recent decades, advances in spring phenological events have been observed for many tree fruit species in many places (Guédon and Legave, 2008; Legave et al., 2008; Luedeling and Brown, 2011; Darbyshire et al., 2013; El Yaacoubi et al., 2014; Guo et al., 2015; Legave et al., 2015; Yong et al., 2016). These bloom advances are a result of a rise in air temperatures in spring, which has accelerated the fulfillment of heat requirements. However, in some regions, temperature increases in winter appear to have delayed the fulfillment of chilling requirements, sometimes to an extent that could not be compensated by phenology-advancing effects of warming in spring. In such extreme situations, warming during dormancy has been reported to result in delayed bloom dates (Harrington et al., 2010; Campoy et al., 2011; Luedeling et al., 2013a; Legave et al., 2015; Martínez-Lüscher et al., 2017; Bartolini et al., 2019).

Several models have been proposed for quantifying chill and heat accumulation. The most common concept of the dormancy season stipulates that chilling and heat requirements are fulfilled sequentially (Guédon and Legave, 2008; Luedeling et al., 2009; Darbyshire et al., 2013), but some recent studies have proposed more complex concepts that include an overlapping phase of both agroclimatic stimuli (Pope et al., 2014), or the possibility that budbreak can be triggered by various combinations of chill and heat accumulation (Harrington et al., 2010). To measure the accumulation of winter chill in deciduous trees, various models have been developed: the Chilling Hours Model (Hutchins 1932, as cited by Weinberger, 1950), the Utah Model (Richardson et al., 1974) and the Dynamic Model (Fishman et al., 1987a, b). For quantifying heat accumulation, the Growing Degree Hours Model (Anderson et al., 1986) is the most widely used model.

Since buds do not exhibit easily observable changes during dormancy, delineation of the chill and heat accumulation has long remained elusive, especially where no controlled experiments could be undertaken. In recent years, Partial Least Squares (PLS) regression has been used to overcome this limitation (Luedeling and Gassner, 2012). This statistical approach requires long-term temperature and phenology records. For each calendar day of the dormancy season, PLS regression can identify whether high temperatures tend to delay or advance bloom dates. In many climatic settings, this information can then be used to delineate the endormancy phase, when high temperatures should delay budbreak, and the ecodormancy phase, when high temperatures should result in advanced phenology.

The analysis of historic chill accumulation trends is a decisive step towards a better understanding of the impacts of climate change in a particular region. In a context of global warming, chill trend estimations can be very sensitive to the choice of chill model (Luedeling et al., 2009; Fernandez et al., 2020b). In the particular case of Asturias, the potential impacts of climate change on locally available winter chill may include changes in the timing of phenological events, which may have implications for agricultural management. Significant warming during the chilling phase could reduce the number of suitable cultivars for cider production in the region. Another important factor to consider is the possibility of increased frequency of adverse weather events such as late spring frosts, which can be associated with shifts in budbreak dates. While late damaging frosts have traditionally been rare in the study region, advances in spring phenology may lead to earlier appearance of advanced flowering stages, which are more frost-sensitive than fully dormant buds (Westwood, 1999). Anticipating future production risks related to the dormancy season would be facilitated by accurate knowledge of the flowering times of each local cultivar. Reliable characterization of chilling and heat requirements is also important for adapting agricultural practices to possible new constraints, as well as for the design of new orchards and as guidance for future breeding strategies.

To provide information for risk assessment and strategic decisions on the composition of future orchards, we pursued two objectives. First, we analyzed temperature trends in Asturias over the past 41 years to examine how changes in chill and heat accumulation have affected the phenology of apple cultivars in northwestern Spain. Second, we determined the start and end dates of the effective chill and heat accumulation periods to quantify chill and heat requirements of local apple cultivars using Partial Least Squares (PLS) analysis.

2. Materials and methods

2.1. Study area

The study was carried out at Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) in Villaviciosa, Asturias, northwestern Spain (43.46 °N, 5.43 °W, 10 m above sea level) (Fig. 1). Villaviciosa is located in an area known as "Comarca de la Sidra", the most important cider apple growing region in Spain. The climate in this region can be defined as temperate and humid oceanic climate. Temperatures are mild in winter, summers are not dry or very hot and the annual rainfall is fairly evenly distributed over the year with an average annual rainfall around 1100 mm.

2.2. Climate data and trends

Daily minimum and maximum temperatures were collected from the weather station of the SERIDA research institute situated just next to the experimental orchards. Sporadic gaps in the meteorological data were filled with information from the nearest weather station (Gijón, 43.54 °N; 5.62 °W, 30 m a.s.l. and 17 km away). These data were bias-corrected (by -1.37 and +0.35 °C for daily minimum and maximum temperatures, respectively) based on an analysis of all days for which both stations had data.

Annual temperature trends were analyzed over a 41-year period (1978–2019), including detailed analysis of temperature variation between October and May, the period that includes all dormancy-related processes for fruit trees in this region. Additionally, the total numbers of frost days (air temperature below 0 °C) between November and March and between March and May were identified for each year.

2.3. Phenological observations

Phenology data were collected for eleven Asturian cider apple cultivars. The dataset contains observations for two time periods: 1987–1996 and 2004–2019 (no observations were available in 2017). Trees were monitored twice a week, and flowering dates were recorded when trees reached the F2 stage (full bloom) according to Fleckinger (1945), which corresponds to stage 65 (~50 % of flowers open) of the BBCH code. Phenology was monitored in three experimental orchards located within a radius of less than 1 km, all of which were managed with the same agricultural practices.

The set of local cultivars we investigated varied widely in terms of flowering time. All of them are recognized as local cultivars by a "Protected Designation of Origin" quality label (Dapena and Blázquez, 2009). The cultivars we examined are: 'Clara', 'Coloradona', 'Perezosa', 'De la Riega', 'Verdialona', 'Blanquina', 'Teorica', 'Xuanina', 'Collaos',

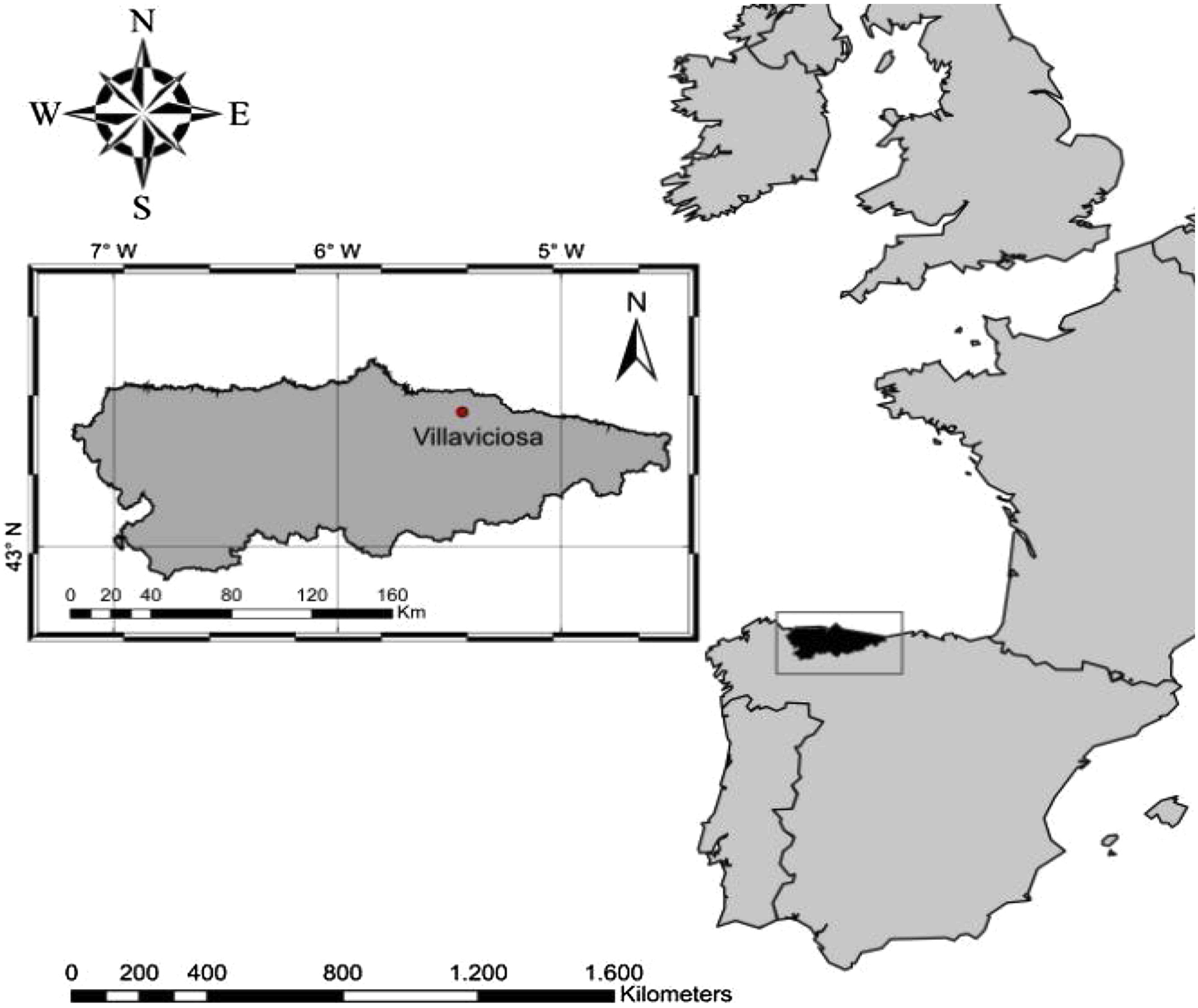

Fig. 1. Map of the study area. The red dot in the inset image shows the location of the weather station used in the study. The larger image shows the location of the Asturias region within Spain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

'Perico 'and 'Raxao'.

2.4. Chill and heat accumulation models

Chill and heat models require hourly temperature data. Hourly records were constructed from daily minimum and maximum temperature records based on geographic latitude (Spencer, 1971; Almorox et al., 2005) using procedures proposed by Linvill (1990), which are included in the chillR package (Luedeling, 2019) for the R programming language (R Core Team, 2020).

Daily chill accumulation was calculated according to three chill models. The Chilling Hours Model (Hutchins 1932, as cited by Weinberger, 1950) is the simplest model, but it does not perform well in mild and warm areas (Dennis, 2003). The Utah Model (Richardson et al., 1974), which assigns varying chilling efficiencies to several distinct temperature ranges, has also shown problems in mild climates, where it appears to overestimate the chill-negating effect of warm temperatures (Campoy et al., 2011). The Dynamic Model (Fishman et al., 1987a, b), the most recent and most complex of the commonly used models, has been widely acknowledged as the most accurate for mild-winter climates (e.g., Ruiz et al., 2007; Luedeling et al., 2009; Zhang and Taylor, 2011; Campoy et al., 2013; Parkes et al., 2020). In this model, chill is accumulated by a two-step process, in which only the intermediate product formed by the first step can be destroyed by warm conditions. Daily heat accumulation was calculated according to the Growing Degree Hours Model (Anderson et al., 1986), a model that assigns varying heat accumulation efficiencies to temperatures above a base temperature of 4 °C, with an optimum temperature of 25 °C and a critical temperature of 36 °C.

2.5. Identification of the chilling and forcing periods

Chill and heat accumulation were determined by applying Partial Least Squares Regression (PLS) (Luedeling and Gassner, 2012) using full bloom dates observed during the 2004–2019 period. The analysis was implemented using the chillR package (version 0.70.21) (Luedeling, 2019). The onset and the end of the chilling and forcing periods were based on the two major outputs of the analysis: the variable importance in the projection (VIP) statistic, calculated for each independent variable, and the standardized coefficients of the model. As in previous studies, a VIP value of 0.8 was selected as a cut-off for considering coefficients for particular days important (Wold et al., 2001; Luedeling et al., 2013a). A negative coefficient for daily chill or heat accumulation on a particular day of the year indicates that high rates of chill or heat accumulation on that particular date are correlated to an early bloom date. We based the PLS analysis on chill quantified with the Dynamic Model, defining the beginning of the chilling phase as the first date of a pronounced period with consistently negative standardized coefficients and VIP values above 0.8. The onset of the forcing phase was determined using the same criteria, and the median bloom date was established as the end of the heat phase. Cultivar-specific chill and heat requirements were calculated as the mean accumulation of the respective model between the two dates defining the corresponding phase for each year. We estimated uncertainty by computing the standard deviation of chill or heat accumulated during these phases. In order to represent temperature responses of the set of eleven apple cultivars to mean temperatures during the chilling and forcing phases identified by PLS regression, bloom dates were plotted in relation to mean temperatures during both periods using the Kriging interpolation technique (Luedeling et al., 2013a).

2.6. Statistical analysis

Trends in air temperature series, historic chill levels and flowering records were determined using the non-parametric Mann–Kendall test (Mann, 1945; Kendall, 1975). Kendall's Tau coefficient (τ) and a critical p value of 0.05 were used to detect significant time series trends from 1978 to 2019. Flowering records from the studied cultivars were analyzed by hierarchical cluster analysis using the average linkage (between groups) method. PLS regression analysis was performed using version 0.70.21 of the chillR package (Luedeling, 2019). Chill and heat accumulation dynamics over the past decades were studied using a running mean function (Luedeling and Gassner, 2012; Luedeling et al., 2013a). All analyses were run in the R programming environment (R Development Core and Team, 2020; version 3.6.3).

3. Results

3.1. Temperature trend and variability

Annual and seasonal temperature trends were analyzed using meteorological data collected in Villaviciosa during the period 1978–2019. This location has a mean daily temperature of 13.35 °C, with mean daily minimum and maximum temperatures of 8.62 °C and 18.34 °C, respectively (Table A1 in the supplementary materials). Over the past 41 years, the average daily temperature increased significantly ($\tau = 0.48$, $p < 0.001$) by 1.21 °C, at a rate of 0.30 °C per decade. The warmest year was 2014 (14.3 °C) and the coldest mean annual temperature was recorded in 1980 (12.4 °C). From October to May, the period which involves the physiological processes of relevance for the flowering time of fruit trees in the study region, mean daily minimum, mean and maximum temperatures were 6.35 °C, 11.14 °C and 16.39 °C, respectively (Fig. 2). Warming trends during this period were similar to trends for the whole year, with the mean daily temperature registering an increase by 1.16 °C (+0.29 °C/decade). Temperature rise was more pronounced for the minimum temperature (+1.25 °C in total, at +0.31 °C/decade; $p < 0.001$) than for the maximum temperature (+0.56 °C in total, at +0.14 °C/decade, $p = 0.12$). The strongest positive trend was found for January (+0.41 °C/decade, $p = 0.013$), the month with the coldest mean temperature (8.24 °C).

Flowering of apple trees is generally observed in Asturias in April and May. In terms of air temperature, the spring phase commonly occurs from February to April at mid-latitudes of the Northern Hemisphere (Chmielewski and Rotzer, 2001). In this spring phase, we found an increase in mean temperature by 0.29 °C/decade, with the strongest warming trend observed in April (+0.43 °C/decade).

Annual mean temperature anomalies, compared to the mean of the entire record we analyzed, showed a clear warming signature, with years that were cooler than the long-term mean being a rare occurrence after 1996 (and then just slightly cooler than the mean; Fig. 3).

The long-term temperature record indicated a significant decrease in the annual number of frost days between November and March during the 1978–2019 period, showing a decline by 2.5 days per decade since 1978 ($\tau = -0.24$ and $p = 0.03$). Few frost days were identified between March and May (average of 0.9 days per year). Approximately 60 % of the years did not experience any frost events between March and May. In particular, during the flowering months of most cultivars, frost risk has historically been low, with no frost events detected in May, and only two days in April, over the entire study period.

3.2. Flowering trends and phenological changes in local apple cultivars

The average flowering date of apple trees in Villaviciosa spanned a period of 30 days, ranging between April 21 st (in 2011) and May 21 st (in 2004). The least variation was found in the earliest flowering cultivar 'Clara' (23 days) and the widest spread in the late flowering cultivar

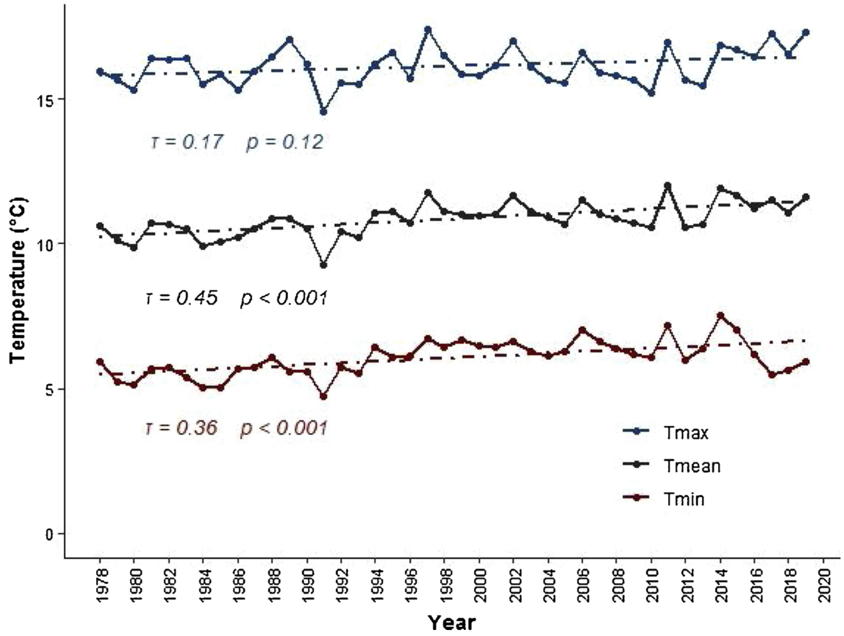

Fig. 2. Trends of annual means of daily minimum, mean and maximum temperatures recorded at Villaviciosa during the phenological season of fruit tree species (October-May) from 1978 to 2019. Kendall's rank correlation coefficient (τ), as well as the probability of the observed results occurring in the absence of a temporal trend (p) are indicated for each time series.

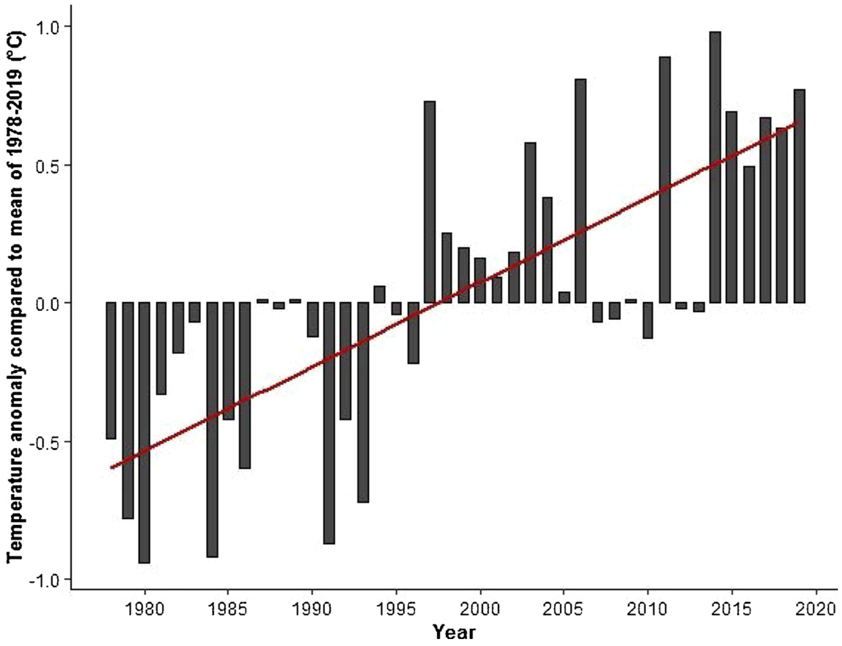

Fig. 3. Anomalies in mean annual air temperature in Villaviciosa, compared to the 1978-2019 average. The red line represents the result of linear regression analysis of the annual anomalies over time. Anomaly stands for the temperature deviation in degrees Celsius relative to the standard period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

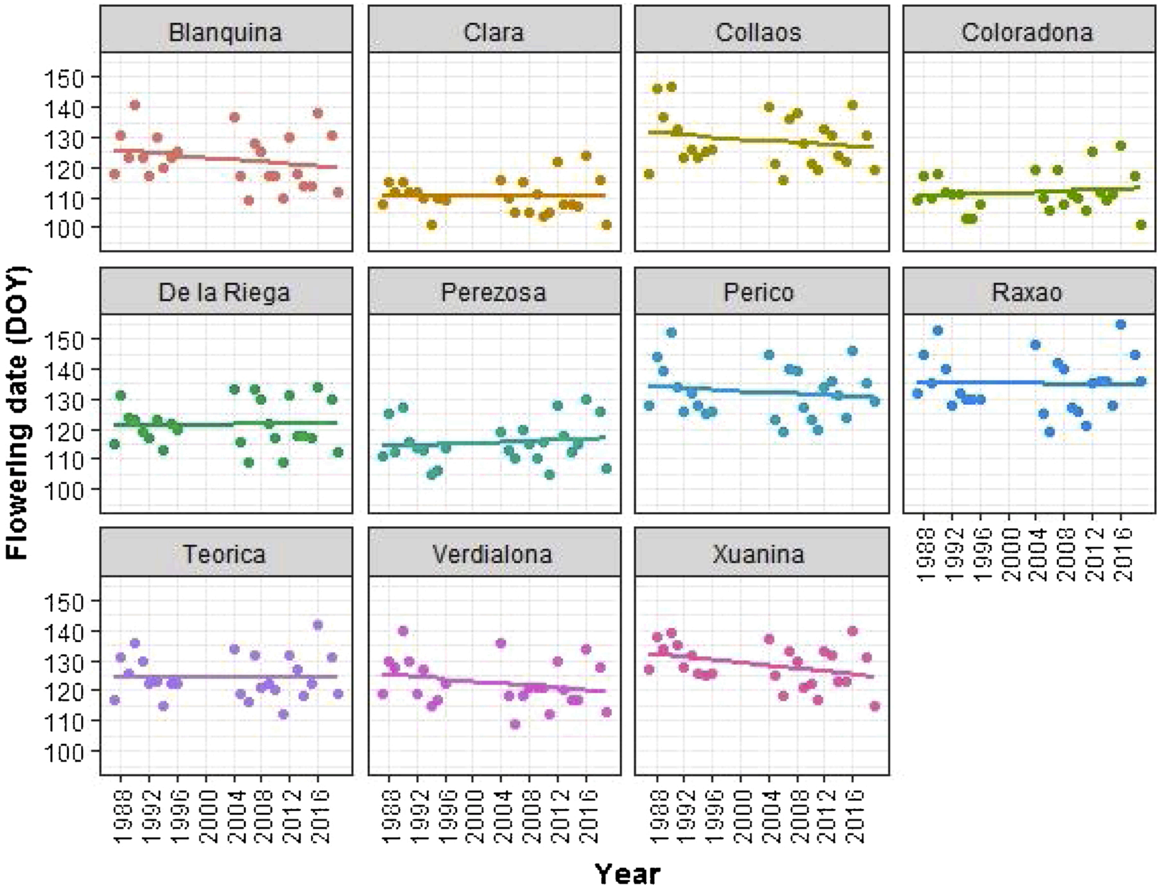

Fig. 4. Flowering date (DOY = day of the year) of 11 apple cultivars in Asturias, Spain, between 1987-1996 and 2004-2019 (with 2017 missing), with trends visualized by linear regression.

'Raxao' (36 days) (Fig. A1 in the supplementary materials). The cluster analysis revealed five distinct groups according to the flowering time (Fig. A2 in the supplementary materials). Based on this analysis, the local apple cultivars can be grouped into five flowering groups: an early-flowering group formed by 'Clara' and 'Coloradona', an intermediate-flowering group ('Perezosa'), an intermediate/late-flowering group ('Verdialona', 'De la Riega 'and 'Blanquina'), a late-flowering group ('Xuanina', 'Collaos 'and 'Teorica') and a very late-flowering group ('Perico 'and 'Raxao'). The recent warming did not lead to pronounced variation in flowering dates over the study period (Fig. 4). Mean flowering dates across all cultivars in the study region showed a slight advancing trend (−0.13 days/year), which was not statistically significant ($\tau = -0.12$, $p = 0.44$). The predominant trend among these cultivars is a moderate advancement of flowering (Fig. 4). The changes in temperature led to slight delays in flowering in the three cultivars with the earliest bloom dates (i.e. 'Clara', 'Coloradona' and 'Perezosa') and slight bloom advances in the other study cultivars. It should be noted that for all cultivars except 'Xuanina', the possibility that there was no trend in the dataset could not be statistically excluded ($p > 0.05$).

3.3. Historic chill and heat trends

We calculated chill accumulation from November to the end of March for each winter season and evaluated the results for trends. We chose November 1 st as the start date, since this time corresponds roughly to the accumulation of the first Chill Portions.

Even though all the models indicated a decline in winter chill over the past 41 years, change trends differed across the three models (Fig. 5). The Chilling Hours (CH) model showed the most severe decline (-5.7 CH/year; $\tau = -0.24$; $p = 0.03$) followed by the Utah model (−1.91 CU/year; $\tau = -0.1$; $p = 0.36$). The Dynamic model, which measures chill in Chill Portions (CP), indicated only negligible changes in winter chill levels (−0.04 CP/year; $\tau = -0.03$; $p = 0.45$). Across all winter months, January accounted for the greatest chill accumulation (21.8 CP), followed closely by December (20.6 CP). The coldest winter on record was the 1990/1991 season, with a mean temperature of 7.64 °C. This cold winter registered the highest total winter chill according to the Chilling Hours Model. Interestingly, neither the Dynamic Model nor the Utah Model were in agreement with this assessment, and they also did not identify the warmest winter as the one with the lowest chill accumulation.

While overall chill accumulation remained relatively stable, we observed a gradual trend towards later onset of endodormancy. In consequence, chill accumulated during the first fifteen days of November, as quantified by the Dynamic Model, decreased by 0.51 CP/decade ($\tau = -0.31$; $p = 0.006$). For the whole month of November, chill accumulation experienced a similar rate of decline (−1.13 CP/decade; $\tau = -0.28$; $p = 0.009$). A particularly large decline was observed since the 2000s, and chill accumulation in November only exceeded 5 CP in four years since the beginning of the current century.

We calculated heat accumulation following the Growing Degree Hours (GDH) Model for the months of February, March and April. The average flowering date for the set of local cultivars was May 4th. Experimental work performed by forcing buds in a temperature-controlled environment suggests that some of the cultivars completed their endodormancy phase in February (Delgado et al., in preparation). Heat accumulation significantly increased by an average of 52.5 GDH per year ($\tau = 0.27$; $p = 0.01$). This increase was stronger between 1980 and 2009, with a rate of 63.3 GDH per year, than during the following decade, when heat accumulation appeared fairly stable. It is important to highlight that heat increases in April (27.8 GDH/year; $\tau = 0.27$; $p = 0.01$) contributed most strongly to the overall rise in heat accumulation.

3.4. Chilling and forcing periods for apple cultivars in the study region

Chilling and forcing periods were delineated by PLS regression. While we conducted this analysis for all three chill models, we only report results for the Dynamic Model, which has been found to be more

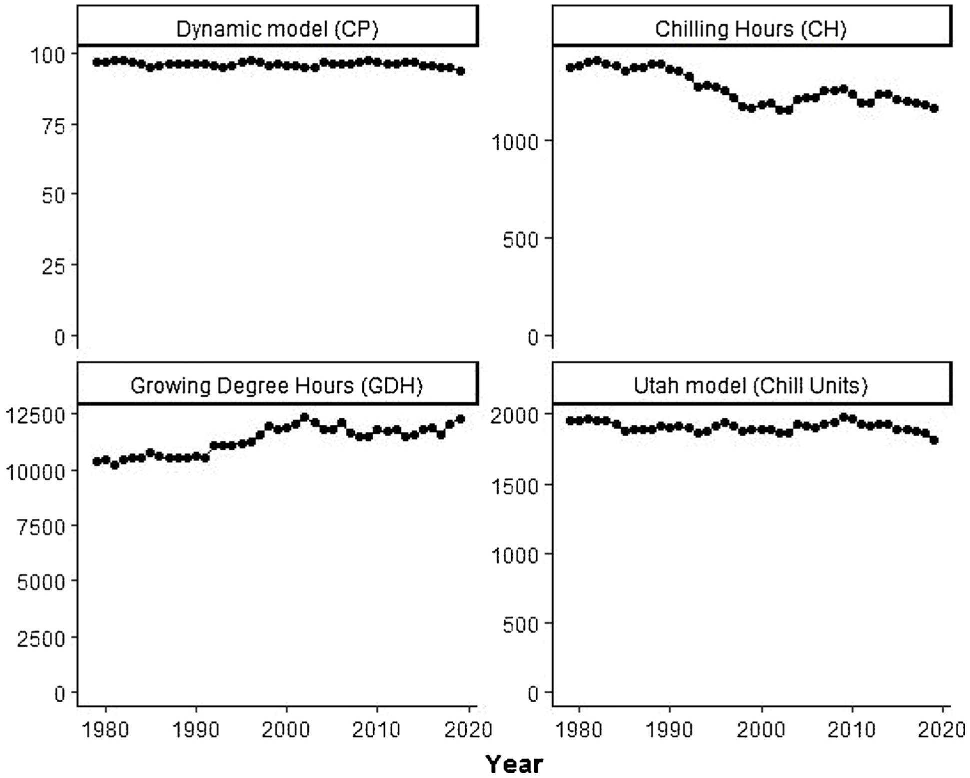

Fig. 5. Chill accumulation, calculated in Chill Portions (CP), Chilling Hours (CH) and Chill Units (CU), during the dormant season (November 1 st – March 31 st), and heat accumulation calculated in Growing Degree Hours (GDH) from February 1 st to April 30th, in Asturias, Spain, between 1978 and 2019.

appropriate than the other models in mild winter regions (Luedeling, 2012; Luedeling and Brown, 2011; Guo et al., 2015). Using phenological observations from 2004 to 2019, effective chilling and forcing periods for apple trees in the SERIDA institute occurred from November 12th to February 9th (88 days) and from March 15th to May 4th (50 days), respectively (Fig. 6). Chilling and forcing phases were delineated very clearly, with the large majority of days during the chilling phase and every single day during the forcing phase showing negative model coefficients and a VIP score above 0.8. These two criteria indicate significant effects of the rates of chill and heat accumulation, respectively, on flowering dates, but variation in VIP scores and model coefficients during both periods imply that not every date of these periods has an equally strong effect. The greatest responsiveness to chill was found between mid-December and mid-January, and the greatest responsiveness to heat occurred during the first half of April.

The delineation of chilling and warming phases differed slightly between the models selected to obtain the outputs. We did not find evidence of a significant period of overlap between chilling and forcing periods in this region, where winters are moderately cold and springs are mild. According to the delineation of the chilling period obtained by applying the Dynamic Model, the average chill accumulation during the identified period was 809 CH, 1128 CU or 59.5 CP, respectively. For all three models, the year with the lowest chill accumulation was 2015/2016, yet the highest-chill year varied according to the model. The average heat accumulation was 8647 GDH. Interannual variability was greater for heat accumulation (17 %) than for chill (7 % with the Dynamic Model).

For the purpose of distinguishing the effects of air temperature during chilling and forcing periods identified by the PLS procedure, flowering records of the study cultivars were plotted in relation to mean temperatures during both periods (Fig. 7). Based on the graphical presentation, we observed approximately diagonal contour lines, which means that the local apple cultivars showed similar sensitivity to temperatures during both periods. Hence, advances in spring phenology in the whole set of local apple cultivars may arise from cooler temperatures during the chilling period and/or warmer temperatures during the forcing period.

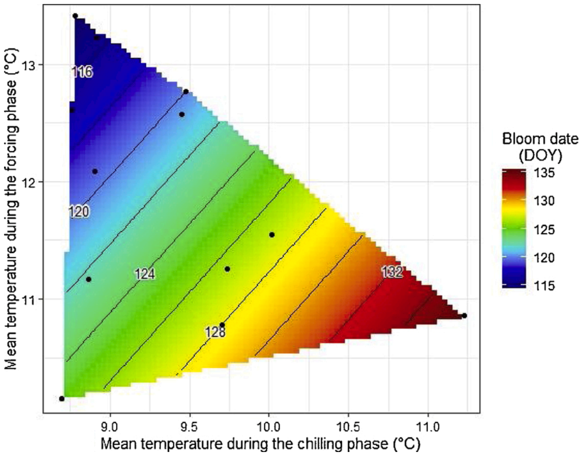

Fig. 7. Response of the average bloom date of eleven apple cultivars to mean temperatures during the chilling and forcing periods delineated by PLS regression (November 12th – February 9th and March 15th – May 4th, respectively). Black dots represent observed apple flowering dates and colors and contour lines indicate the timing of flowering dates expressed in Julian dates (days of the year; DOY). The color spectrum represents a gradient between early and late flowering dates.

3.5. *Chill and heat requirements of local apple cultivars*

Chilling and forcing periods were delineated for each cultivar (Fig. 8), and the specific requirements were calculated according to chill and heat accumulation during the effective periods (Table 1). The cultivars 'Perezosa', 'Verdialona', 'Xuanina', 'Clara' and 'Coloradona' showed some periods with high VIP values and negative coefficients before the dates chosen as the beginning of the chilling phase (Fig. 8). These days at the beginning of October were discarded for biological reasons, since trees were still bearing fruit at that time.

The length of the chilling period ranged from 89 days ('Collaos' and 'Verdialona') to 108 days ('Perico' and 'Raxao'). The end of the chilling period was quite similar for all of the cultivars ranging between

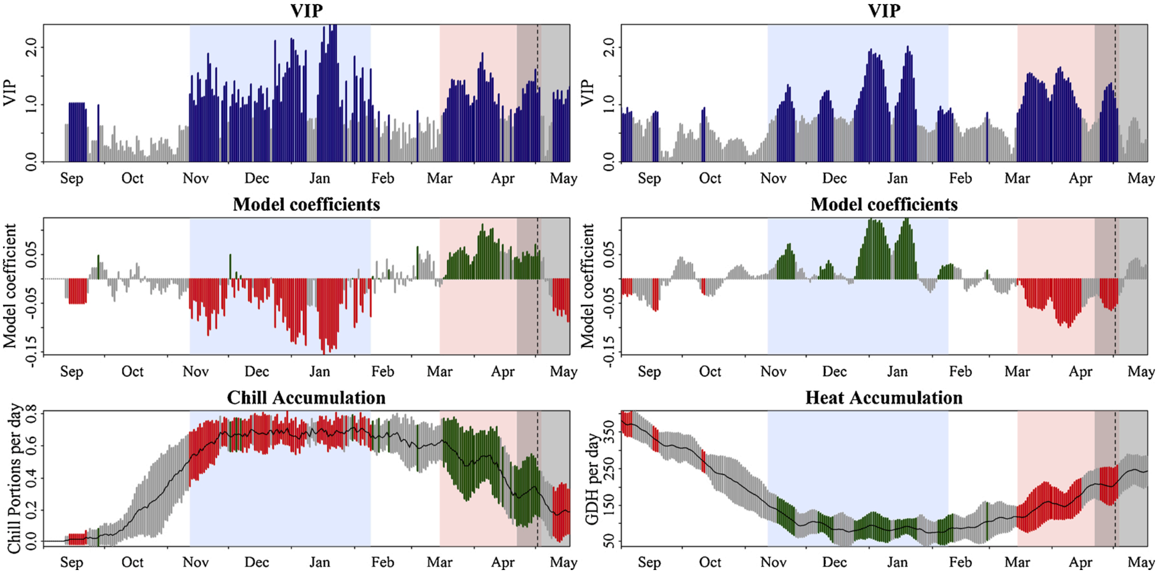

Fig. 6. Results of the Partial Least Squares (PLS) regression analysis for bloom of local apple cultivars in Villaviciosa, northwestern Spain, between 2004-2019, using the Dynamic Model and the GDH Model for chill and heat accumulation, respectively. Blue bars in the top row indicate that VIP scores are above 0.8. Red bars mark negative model coefficients, which represent an important correlation between flowering and daily chill and heat accumulation. GDH stands for Growing Degree Hours; CP for Chill Portions; VIP for Variable Importance in the Projection. Blue shading indicates the chilling phase, red shading represents the forcing phase, grey shading shows the range of bloom dates, with the dashed lines marking median flowering dates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

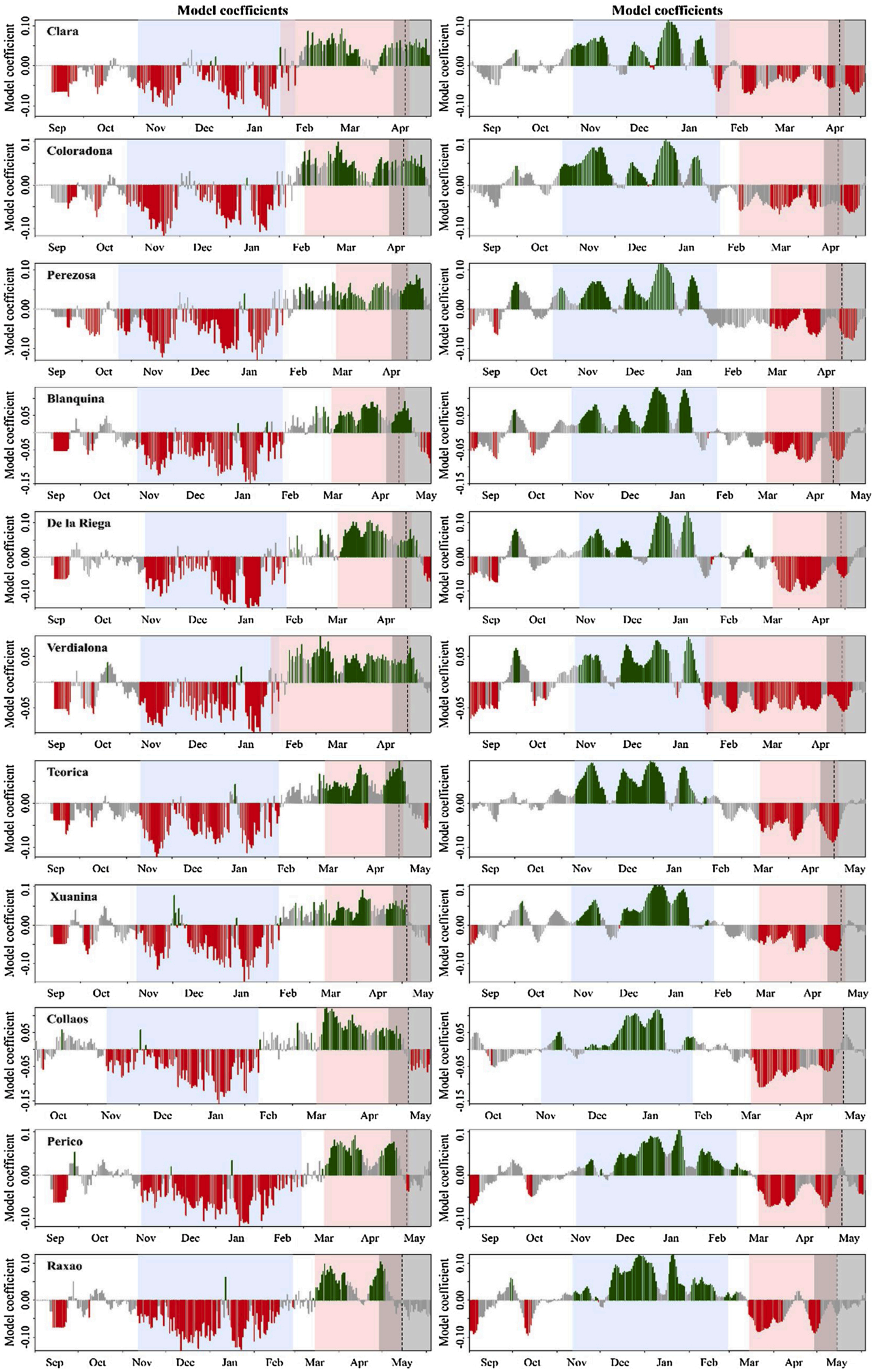

Fig. 8. Model coefficients of Partial Least Squares (PLS) regression between daily chill and heat accumulation and flowering dates of eleven local apple cultivars in Villaviciosa, northwestern Spain between 2004-2019, using the Dynamic Model and the GDH Model for chill and heat accumulation, respectively. See caption of Fig. 6 for more details.

Table 1
Bloom dates, chilling and forcing periods of 11 local apple cultivars in Villaviciosa (northwestern Spain) between 2004 and 2019. The range of bloom dates represents the difference (in days) between the earliest and the latest full bloom date for each cultivar in our dataset.

Cultivars	Bloom date				Chilling period			Forcing period		
	First	Last	Median	Range (Days)	Start	End	No. of days	Start	End	No. of days
'Clara'	11-Apr	4-May	21-Apr	23	4-Nov	9-Feb	97	31-Jan	21-Apr	80
'Coloradona'	11-Apr	7-May	23-Apr	26	29-Oct	5-Feb	99	17-Feb	23-Apr	65
'Perezosa'	15-Apr	10-May	26-Apr	25	24-Oct	5-Feb	104	11-Mar	26-Apr	46
'Verdialona'	19-Apr	16-May	1-May	27	8-Nov	5-Feb	89	31-Jan	1-May	90
'Blanquina'	19-Apr	18-May	1-May	29	7-Nov	10-Feb	95	14-Mar	1-May	48
'De la Riega'	19-Apr	14-May	2-May	25	11-Nov	10-Feb	91	15-Mar	2-May	48
'Teorica'	22-Apr	26-May	4-May	34	4-Nov	10-Feb	92	13-Mar	4-May	52
'Xuanina'	25-Apr	20-May	7-May	25	7-Nov	9-Feb	94	11-Mar	7-May	57
'Collaos'	26-Apr	20-May	8-May	24	12-Nov	9-Feb	89	15-Mar	8-May	54
'Perico'	29-Apr	26-May	11-May	27	12-Nov	28-Feb	108	15-Mar	11-May	57
'Raxao'	29-Apr	4-Jun	15-May	36	12-Nov	28-Feb	108	15-Mar	15-May	61

February 5th and February 10th, with the exception of 'Perico' and 'Raxao', which finished their chilling phase on February 28th. The onset of the forcing phase was similar for most of the cultivars and only differed significantly for 'Clara', 'Coloradona' and 'Verdialona'. The length of the forcing phase varied more strongly than the chilling phase, but this variation was largely driven by variation in flowering dates rather than by the onset of ecodormancy.

For most cultivars, the forcing period started after the end of the chilling period. The only cultivars for which we detected a small overlap between the chilling and forcing phases were 'Clara' (9 days) and 'Verdialona' (6 days). For all other cultivars, the gap between chill and heat accumulation phases among the different cultivars lasted between 7 and 20 % of the period between the first day of the chilling phase and the last day of the forcing phase in each cultivar.

Chilling requirements (CR) for endodormancy release did not vary significantly among most of the cultivars (Table 2). 'Verdialona' had the lowest CR (59.4 CP), whereas 'Perico' and 'Raxao' had the highest CR (72.7 CP). Heat requirements (HR) ranged between 7,326 GDH (for 'Perezosa') and 11,917 GDH (for 'Verdialona'), indicating that HR were slightly more variable among cultivars than CR.

Table 2
Cultivar-specific CR and HR (± standard deviation) for local apple cultivars in Asturias, Spain. Chill and heat requirements were estimated with the Chilling Hours (CH), Utah (CU), Dynamic (CP) and GDH models (GDH). Chilling-Forcing overlap (%) is the percentage of time of the total period between the beginning of chilling phase and the end of forcing phase with an overlap between phases.

Cultivars	Chill requirements			Heat requirements (GDH)	Chilling-Forcing overlap (%)
	CP	CU	CH		
'Clara'	63.9 ± 5.2	1,296 ± 149	842 ± 123	9,921 ± 1,554	5 %
'Coloradona'	63.5 ± 5.5	1,263 ± 157	815 ± 120	8,909 ± 1,406	–
'Perezosa'	64.4 ± 6.2	1,267 ± 173	826 ± 126	7,326 ± 1,182	–
'Verdialona'	59.4 ± 4.7	1,213 ± 132	789 ± 111	11,917 ± 1,799	3 %
'Blanquina'	63.3 ± 5.0	1,292 ± 142	847 ± 122	7,940 ± 1,369	–
'De la Riega'	61.3 ± 4.5	1,260 ± 135	830 ± 116	8,022 ± 1,419	–
'Teorica'	61.7 ± 4.5	1,266 ± 135	833 ± 117	8,646 ± 1,479	–
'Xuanina'	62.6 ± 5.1	1,278 ± 143	836 ± 120	9,570 ± 1,547	–
'Collaos'	60.3 ± 4.5	1,240 ± 135	818 ± 113	9,585 ± 1,495	–
'Perico'	72.7 ± 5.1	1,495 ± 167	1,005 ± 148	10,156 ± 1,594	–
'Raxao'	72.7 ± 5.1	1,495 ± 167	1,005 ± 148	11,111 ± 1,657	–

4. Discussion

4.1. Temperature response of bloom dates and chill and heat accumulation

The observed temperature changes have important consequences for apple cultivation, because temperature is the primary driver of phenological development (Walther et al., 2002; Chmielewski et al., 2004). Temperatures at SERIDA (Villaviciosa) have been increasing at a faster rate (+0.30 °C) than the mean global land surface temperature, which has only risen by approximately 0.18 °C per decade since 1981 (NOAA, 2019, Global Climate Summary). Our analysis also revealed that the pace of temperature increase was faster for minimum temperatures than for maximum temperatures, confirming earlier reports that have indicated greater sensitivity to climate change for the lowest than for the highest temperatures of the day (Luedeling et al., 2009). The average temperature during the phenological season in Asturias showed a positive trend of +0.29 °C/decade since 1978. Nevertheless, the resulting increase by 1.16 °C for temperatures between November and March during this 41-year period has not led to a statistically significant reduction in winter chill accumulation. Even though winters in Villaviciosa have not been particularly cold during recent decades (mean temperature of 8.5 °C between 1978 and 2019), chill accumulation was high, at an average of 96 CP per year. This observation indicates that current winter conditions at this location are favorable for chill accumulation (according to the Dynamic Model) and have apparently been cool enough for recent warming to have no negative impacts on this agroclimatic metric.

A major driver of the timing of phenological events in temperate regions of the Northern Hemisphere is variability in mean air temperature from February to April (Chmielewski and Rotzer, 2001). In Asturias, air temperature during these three months increased by approximately 1.16 °C over the past four decades, with the most remarkable warming, by 1.71 °C, occurring in April. Temperature during this month, which immediately precedes full bloom in most of the local cultivars, has been identified as the strongest driver of flowering time (Lu et al., 2006). Significantly warmer mean temperatures in the month responsible for almost half of the heat accumulation have thus led to a faster fulfillment of HR, which particularly impacted late-flowering cultivars.

Declining winter chill has been reported for several regions (Baldocchi and Wong, 2008; Luedeling et al., 2009; Atkinson et al., 2013), and the relationship between winter chill and spring events in the context of global warming has been widely studied (e.g., Luedeling and Brown, 2011; Campoy et al., 2011; Luedeling, 2012; Bartolini et al., 2019). For apple trees, previous reports have reported advances in spring events over the past few decades in France (Legave et al., 2008), Germany (Chmielewski et al., 2004), Japan (Fujisawa and Kobayashi, 2010), northern Italy (Eccel et al., 2009), Australia (Darbyshire et al., 2013), China (Yong et al., 2016) and Belgium (Drepper et al., 2020). Only a few authors found a delay in flowering dates in mild winter areas, resulting from a delayed onset of the dormant season, which subsequently delayed winter events and the fulfillment of CR and HR (Legave et al., 2013, 2015; Guo et al., 2019).

It is important to note that the impacts of global warming vary across countries and regions. In central Italy, for instance, Bartolini et al. (2019) found a significant reduction in chill unit accumulation, with a loss of a third of the initial accumulation over approximately the same forty-year period as considered in our study.

In Asturias, the lack of a clear shift in mean bloom dates over the past 30 years likely resulted from a later onset of chill accumulation, a longer time to accumulate enough chill to meet cultivar-specific chilling requirements and a shorter duration of the forcing phase. A delay in the accumulation of the first Chill Portions due to a significant temperature increase in November may have contributed to later fulfilment of cultivar-specific CR, especially in high-chill cultivars. In this context, and assuming a sequential transition between phases, the ecodormancy phase may have been shortened in recent years, but local cultivars may have reached their heat needs faster than before as a result of the marked warming trend observed in April. The occurrence of the highest year-to-year variation in the latest-blooming cultivar may have resulted from the combination of high chill and heat requirements. Cultivars with high CR and HR values (as appears to be the case for ‘Raxao’) are particularly sensitive to year-to-year variation in climatic conditions and may thus exhibit a wide range of bloom dates. On the other hand, winter chill is not a limiting factor in early-blooming cultivars. This may explain the low variation in flowering dates found in’ Clara’, which is largely a result of different heat levels during the ecodormacy phase.

The lack of a strong phenological response to warming also indicates that local apple cultivars in Asturias are well adapted to the particular climatic setting of this region and resilient to some variation and change in winter temperatures. Overall, a tendency towards advancing flowering dates was found for most of the study cultivars, whereas early-blooming cultivars tended towards later bloom dates. This is in agreement with previous reports that early-blooming species and cultivars are particularly prone to showing delayed flowering in response to warming (Doi et al., 2008).

Our results of only minor phenology changes in response to warming have potential agronomic implications. Hazardous spring frost events, which may increase in frequency due to flowering advances, do not seem to present a major risk in Asturias, since the number of frost days in April is currently small and appears to be decreasing further. Temperature increases in spring may have favorable effects on apple cultivation, in particular during flowering, when temperature may positively affect pollen quality, pollen viability and pollinator activity.

4.2. Temperature response phases and chill and heat requirements

Through the selection of a reasonably long series of flowering records, PLS regression allowed clear identification of the days of the year when the accumulation of chill or heat had a significant impact on flowering dates. Luedeling et al. (2013b) stated that delineation of the phases is clearer in areas where freezing temperatures are rare, an observation that is confirmed by our analysis, in which all cultivars showed almost uninterrupted periods of negative model coefficients. Several authors have shown that in a cool winter location, where chilling requirements are easily satisfied and variability in chill accumulation does not greatly influence flowering dates, the PLS approach can be a useful tool to determine the chilling and forcing periods (Guo et al., 2014; Darbyshire et al., 2017). Using this statistical approach, other authors have reported an overlap between the chilling and forcing periods in *Prunus* spp. (Guo et al., 2015; Benmoussa et al., 2017a; Martínez-Lüscher et al., 2017) and apple (Guo et al., 2019). Under the fairly mild winters of northwestern Spain, we did not find an overlap between phases for the set of eleven apple cultivars. For the period between February 9th and March 15th, we did not identify a consistent pattern of negative coefficients. We suspect that the PLS procedure is unable to clearly assign these days to one of the phases, as they can be part of the endormancy phase in some years and be associated with the ecodormancy phase in others. It also seems possible that, in some years, chilling requirements have already been fulfilled at this time for the bulk of the local cultivars, but temperatures during this early part of the forcing period are too cool to have a significant impact on spring phenology. On the other hand, the beginning and the end of the chilling period fell on the exact same days for several of the cultivars, resulting in similar or even identical estimates of their chilling requirements. The studied cultivars were selected in the study region, and to date, they are only cultivated in northern Spain, so that no estimates of their chill requirements are available from other locations. The calculated chill requirements between 59 and 73 CP are consistent with the designation of these local cultivars as medium-high chill cultivars (Dapena, 1996). Given the wide range of flowering dates across the eleven apple cultivars, with a maximum difference in mean bloom dates of 30 days, we are somewhat surprised by the relatively low variation in estimated chilling requirements across the eleven cultivars. Such similar estimates across multiple cultivars, also determined with the PLS approach, have been reported previously for almonds and pistachios in Tunisia (Benmoussa et al., 2017a, b). It is of course possible that cultivars selected in the same region have similar agroclimatic needs. On the other hand, the delineation of chilling and forcing phases may also be responsive to typical local temperature dynamics, with temperatures usually rising or dropping at certain times, which may produce PLS coefficient patterns that are not directly related to tree physiology. To resolve this question, comparisons of statistically and experimentally derived cultivar-specific chilling requirements should be undertaken.

In general, the delineated chilling and forcing periods, as well as the estimated chill and heat requirements, appear plausible, especially when based on the Dynamic Model, which has been found superior to alternative models, particularly in warm environments (Luedeling and Brown, 2011). We note, however, that we used the Dynamic Model with predefined parameters that were initially obtained from experimental work with peach (Fishman et al., 1987a, b). These parameters should ideally be calibrated for apple, or even for each cultivar, as recently suggested for apricot by Egea et al. (2021).

Compared to the chilling period, the heat accumulation phase varied strongly in length across the 11 cultivars, lasting between 46 and 90 days. Assuming that the climatic requirements we derived are accurate, this finding indicates that variation in bloom dates among these locally selected cultivars derives primarily from genetic differences in heat requirements, while chill needs are relatively similar.

Several studies have presented CR estimates for apple cultivars, with most of them based on laboratory experiments, where shoots were forced in a growth chamber (e.g., Hauagge and Cummins, 1991; Guak and Neilsen, 2013; El Yaacoubi et al., 2016; Parkes et al., 2020). Only a few analyses so far have estimated CR and HR using the PLS procedures (Darbyshire et al., 2017; Díez-Palet et al., 2019; El Yaacoubi et al., 2020). Comparisons between experimentally and statistically derived CR estimated in mild winter regions showed considerable differences, with PLS regression consistently reporting lower requirements for the same cultivar and geographical location. For example, in southern Australia, the cultivar-specific chilling requirements for the cultivar ‘Cripps Pink ‘varied from 52 CP according to the PLS regression analysis (Darbyshire et al., 2017) to 73 CP using a forced bud method (Parkes et al., 2020). Similar variation was found for the cultivar ‘Gala ‘in northern Morocco where El Yaacoubi et al. (2016) reported 61 CP in a controlled environment experiment, contrasting with 44 CP found by applying the PLS approach (El Yaacoubi et al., 2020).

Of particular interest for the present analysis is a study by Díez-Palet et al. (2019), who evaluated apple cultivars in Girona (northeastern Spain). Similar to our results, they reported the presence of gaps between chilling and forcing phases, as well as only small differences in CR among apple cultivars (Díez-Palet et al., 2019). The beginning of the chilling period at our study site on November 12th seems to be in accordance with previous studies on apple in the Mediterranean climates of southern France (El Yaacoubi et al., 2014) and northeastern Spain (Díez-Palet et al., 2019). Studies from colder regions, in contrast, have reported earlier onset dates, as well as later end dates for the chilling phase. Guo et al. (2019) identified a chilling period between September 24th and February 19th in northwestern China, and the chilling phase was found to last until the last week of February in Belgium (Drepper et al., 2020). Díez-Palet et al. (2019) identified a similar onset of the chilling phase in dessert apple cultivars, although the length of the period was shorter than in our study, possibly because the specific chilling requirements of dessert apple cultivars are lower.

The bloom timing responses to temperature vary considerably across regions (Menzel et al., 2006). In warm regions, bloom dates have been reported not to be very responsive to temperatures during the forcing phase (Benmoussa et al., 2017b). In colder locations, in contrast, it is conditions during the chilling phase temperatures that barely affect bloom dates (Guo et al., 2015; Martínez-Lüscher et al., 2017). The tree responses to temperature during the delineated phases obtained by applying the PLS procedure indicate that for apple cultivars grown in a typical oceanic climate location, where temperatures are mild all year round, bloom dates are controlled in equal measure by temperatures during both the chilling and the forcing phases. In such locations, both processes need to be equally considered for developing climate-resilient apple cultivars.

5. Conclusions

Our results represent an advance in assessing the possible influence of climate change on apple phenology in mild humid climates such as that of northwestern Spain. Under local climate conditions, winter chill accumulation did not show a significant decrease despite temperature increases by 0.30 °C per decade since 1978. Our results indicate that local apple cultivars have shown a high degree of phenotypic plasticity to respond to gradual changes in the environmental conditions. However, their resilience to warming winters appears to vary across cultivars. The early-blooming cultivars showed a slight tendency towards a flowering delay, whereas slight advances in flowering dates were generally observed in intermediate/late and late-blooming cultivars. This information suggests that the local apple breeding program should prioritize the use of locally maintained germplasm in the process of obtaining environmentally adapted new cultivars. Finally, our results confirm that the amount of winter chill available in coastal areas in northern Spain has not been significantly affected by recent warming. An average chill accumulation of 96 CP implies that a transition to cultivars with lower chilling requirements and/or geographical shifts to other suitable cultivation areas is not currently an urgent priority.

Author's contributions

AD and ED designed the study. ED collected phenology and meteorological data. AD performed the analysis with input from EL, JAE and ED. AD wrote the manuscript and all authors contributed to interpretation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2021.110093.

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Chapter 2

Climatic requirements during dormancy in apple trees from northwestern Spain – Global warming may threaten the cultivation of high-chill cultivars

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Climatic requirements during dormancy in apple trees from northwestern Spain – Global warming may threaten the cultivation of high-chill cultivars

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Fruit breeding

ABSTRACT

Winter chill is expected to decrease in many mild-winter regions under future climatic conditions. Reliable estimates of the chill requirements (CR) of fruit trees are essential for assessing the current suitability of cultivars and potential climate change impacts on fruit production. We determined chill and heat requirements of ten apple cultivars in northwestern Spain using a bud-forcing method. CR ranged from 59 (‘Granny Smith’) to 90 (‘Regona’) Chill Portions (CP) according to the Dynamic Model. These results indicate that international dessert apple cultivars such as ‘Elstar’ and ‘Granny Smith’ have clearly lower CR than the studied local cultivars. The agro-climatic needs of the traditional apple cultivars are aligned with the historical climate conditions in the region. To assess future apple cultivation in northwestern Spain, we evaluated winter chill availability over the course of the twenty-first century by applying an ensemble of future climate scenarios. Relative to the past, projected winter chill might decline by between 9 and 12 CP under an intermediate global warming scenario and by between 9 and 24 CP under a pessimistic scenario. Despite relatively minor changes, the viability of some local apple cultivars may be jeopardized by their high CR. Results suggest that even a moderate decline in future winter chill, relative to fairly high levels observed in the past, can threaten the economic sustainability of fruit tree orchards composed of high-chill genotypes. Strategies such as growing low- to moderate-chill cultivars may be critical for sustaining future apple production in the region. Our findings can help guide new breeding strategies aiming to develop climate-resilient cultivars adapted to future environmental conditions.

1. Introduction

Dormancy is a physiological mechanism that allows temperate trees to cease growth and remain inactive during unfavourable climatic conditions in order to protect sensitive tissue (Faust et al., 1997). From dormancy onset to flowering time, trees experience complex physiological modifications triggered by cold and warm temperatures (Luedeling et al., 2013; Malagi et al., 2015). According to Lang et al. (1987), the dormant period can be divided into two sub-stages: endodormancy, usually known as the true dormant state and regulated by cold temperatures; and ecodormancy, which is mainly modulated by favourable environmental conditions that eventually evoke bud burst. To overcome dormancy and resume growth in the following season, temperate fruit trees must fulfil a genotype-specific chill requirement (CR) between late autumn and early spring (Campoy et al., 2011a; Erez, 2000; Luedeling, 2012). In mild winter climates, insufficient chill accumulation during dormancy can lead to numerous phenological disorders such as uneven vegetative development, bud abortion, a delayed and extended flowering period, poor fruit set and, ultimately, a significant reduction in final yield (Atkinson et al., 2013; Petri and Leite, 2003; Sunley et al., 2006). In order to explain the process of winter chill accumulation in deciduous fruit trees, various models have been developed. Traditionally, the Chilling Hours Model (Hutchins, 1932, as cited by Weinberger, 1950) has been used to quantify chill accumulation. The Utah Model (Richardson et al., 1974) provides good results in cold climates but has not performed well in mild-winter regions (Dennis, 2003). Finally, the Dynamic Model (Erez et al., 1990; Fishman et al., 1987a, 1987b) is recognized to date as the most adequate model for mild-winter areas (e.g. Campoy et al., 2013; Luedeling et al., 2009; Parkes et al., 2020; Ruiz et al., 2007). Regarding the quantification of heat, Growing Degree Hours (GDH) are estimated using the Growing Degree Hours Model proposed by Anderson et al. (1986). Large genotypic variation in chill requirements has been reported among temperate fruit trees. Knowing the chilling requirement of a specific cultivar has important practical

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and economic implications (Fennell, 1999).

The European Apple Inventory (Watkins, 1984) lists around ten thousand cultivars of apple (*Malus domestica* Borkh.). In comparison with other fruit species, most apple cultivars show high to medium chilling requirements (El Yaacoubi et al., 2016; Hauagge and Cummins, 1991; Parkes et al., 2020), mostly because the wild relatives of the domesticated apple originated from central and inner Asia where winters are very cold (Forsline et al., 2003; Ignatov and Bodishevskaya, 2011). However, several breeding programs have released cultivars with lower CR in order to extend the cultivation of this species to warmer climates. Although few studies have reported on the CR of different apple cultivars, the methodologies employed for the determination of the trees' climatic needs vary widely, hampering the comparability of results among studies (El Yaacoubi et al., 2016; Funes et al., 2016; Guak and Neilsen, 2013; Hauagge and Cummins, 1991; Parkes et al., 2020). Two main tests are often used to experimentally determine the chilling requirements in temperate fruit trees after a certain period under controlled conditions: measurement of the weight of floral primordia (Tabuenca, 1964) and forcing of detached shoots under warm conditions (e.g. Cook and Jacobs, 2000; Cook et al., 2017; Malagi et al., 2015; Parkes et al., 2020; Prudencio et al., 2018). Forcing long cuttings with numerous buds from the previous year and monitoring their phenological evolution has been the preferred choice in many studies (Dennis, 2003). On the other hand, statistical approaches based on long-term phenological datasets have also been used to determine the length of the endodormancy phase (Alonso et al., 2005; Darbyshire et al., 2016; Funes et al., 2016; Luedeling et al., 2021, 2013; Luedeling and Gassner, 2012). The dynamics of dormancy release in apple trees cultivated in Oceanic climate regions have rarely been studied. Asturias, a coastal location in North-West Spain, has an Oceanic climate, subtype Atlantic, according to Martín-Vide and Olcina (2001), with mild winters in the fruit production areas (Delgado et al., 2021). Cider apples have a high economic value in Asturias representing approximately 20% of the total production of Spain (MAPA, 2019). Apart from the direct impact on the growers' economy, the cider industry also has industrial, cultural and social relevance in the region. The Servicio Regional de Investigación y Desarrollo Agroalimentario de Asturias (SERIDA) maintains an apple germplasm bank with 800 accessions, out of which 525 are Asturian apple accessions. Most of the cultivars grown in the region during the past 30 years were selected by the institute and included under a "Protected Designation of Origin" quality label (Dapena and Blázquez, 2009). To avoid early flowering after warm spells during winter, approximately 86% of the locally maintained apple cultivars exhibit intermediate to very late flowering times (Dapena, 1996).

Significant changes in mean temperature are predicted for the 21st century due to climate change (IPCC, 2014). For future scenarios, the Intergovernmental Panel on Climate Change (IPCC) describes four Representative Concentration Pathways (RCPs) of atmospheric greenhouse gas concentrations (IPCC, 2014). These pathways include an intermediate scenario (RCP4.5) as well as a pessimistic scenario with very high greenhouse gas concentrations (RCP8.5; IPCC, 2014). The pace of global warming depends on the mitigation measures implemented to tackle climate change. Without significant action, annual mean global temperature increase is likely to exceed 1.5 °C between 2030 and 2052 compared to pre-industrial levels (IPCC, 2018). Global changes in environmental conditions will potentially affect phenology trends in temperate fruit trees (Menzel et al., 2006), and higher winter temperatures are expected to compromise the fulfilment of CR necessary to break endodormancy (Campoy et al., 2011a; Darbyshire et al., 2011; Luedeling et al., 2009, 2011; Luedeling, 2012). The impacts of increasing temperatures on winter chill can vary across different growing regions (Fernandez et al., 2020c; Luedeling et al., 2011). In some regions, the impacts of climate change on temperate fruit production may be severe, and adaptation measures will be required in order to ensure the economic viability of farms (Benmoussa et al., 2017; Campoy et al., 2011a; Luedeling et al., 2011). Projected climate change impacts on winter chill accumulation in apple trees have been studied in warm-winter climates such as Australia (Darbyshire et al., 2014; Parkes et al., 2020) and northeastern Spain (Funes et al., 2016; Rodríguez et al., 2021). In such locations, some of the widely grown apple varieties are expected to be unsuitable due to both the medium/high chill needs of most of the commercial varieties and the severe decline in winter chill expected for the second part of the 21st century.

In a recent study, Delgado et al. (2021) analysed phenological and temperature trends in Asturias over a 41-year period (1978–2019). This work showed that a temperature rise of 0.30 °C/decade did not imply a significant reduction in winter chill accumulation (in Chill Portions), indicating that cultivar-specific CR have been easily satisfied in the past. However, the rate of greenhouse gas emissions can change over the coming decades, affecting the rate of temperature increases, which may lead to conditions that fall outside the effective range defined for chill accumulation according to different chill models (Fernandez et al., 2020c). The local cultivars traditionally planted in the Asturias region are phenotypically adapted to their area of origin, which may explain observations of weak phenological responses to warming conditions (Delgado et al., 2021). However, the phenotypic plasticity to respond to the increase in global temperatures inevitably has limits (Donnelly et al., 2012), and some consequences of insufficient winter chill, such as delayed and irregular bud burst, were observed in a small number of cultivars in recent growing seasons (Consejo Regulador de la Denominación de Origen Protegida Sidra de Asturias, personal communication). Climatic conditions projected for the future may threaten the viability of the local cultivars, making adaptation strategies essential for ensuring orchard viability in a warming future. For this reason, precise characterization of the chill and heat requirements of each individual genotype is needed to support farmers in identifying adequate sets of cultivars that allow them to adapt their orchards to future climate conditions.

The main goals of this study were (1) to calculate the agro-climatic requirements of ten apple cultivars by implementing a reliable method for forcing shoots under environmentally controlled conditions and compare the precision and reliability of chill models under the mild-winter conditions of northwestern Spain; (2) to evaluate the impacts of climate change on winter chill for the 21st century for an ensemble of future climate scenarios; and (3) to generate a portfolio of the most suitable apple cultivars to be cultivated in the Asturias region under future climate scenarios.

2. Materials and methods

2.1. Site description

We conducted our experiments during two consecutive growing seasons (i.e. 2018/2019 and 2019/2020) in two experimental orchards of the Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) in Villaviciosa, Asturias, North-West Spain (43° 28′ N, 5° 26′ W, 10 m above sea level). The weather conditions in the study site show an average annual temperature of 13.4 °C (Delgado et al., 2021) and average annual rainfall of about 1100 mm (Dapena and Fernández-Ceballos, 2007). The soil of the experimental plots was balanced and fertile, with a loamy texture, a pH ranging from 6.8 to 7.2 and an organic matter content between 2.7 and 2.8%.

2.2. Plant material

The plant material we used in this study included eight Asturian cider and two international dessert apple cultivars, which cover a wide range of flowering times (Table A1 in the supplementary materials). The local cultivars were 'Collaos', 'De la Riega', 'Regona', 'Solarina', 'Xuanina', 'Blanquina', 'Limón Montés' and 'Perico'. The cultivars 'Elstar' and 'Granny Smith' were selected as international references.

For the forcing experiment, we used 11-year-old apple trees as shoot donors for the Asturian cultivars. For 'Elstar' and 'Granny Smith', shoots were collected from 21-year-old trees. For the phenological observations under field conditions, we monitored 10-year-old trees grafted onto 'M7' rootstock for the local cultivars and 18-year-old trees grafted onto 'Pi 80' rootstock in the case of the dessert apple cultivars. All experimental trees were trained in a central leader system, drip irrigated and managed under organic cultural practices. Regarding the alternate bearing cycle of the experimental trees, the 2018/2019 season was an 'on' year, whereas the 2019/2020 season was an 'off' year.

2.3. Experimental design

Hourly temperature data were recorded in the orchard using data loggers (EL-USB-502 loggers, Lascar Instruments, UK) inside a miniature Stevenson's screen, positioned 1.5 m above the ground. The initial date for chill accumulation was set to November 1st, since the accumulation of the first Chill Portion was identified on November 2nd for the 2018/2019 season and on November 4th for the 2019/2020 season. Since the Dynamic Model is self-regulating, with chill accumulation only starting when weather conditions are suitable for chill accumulation, choosing November 1st as the start date seemed reasonable under our climatic conditions.

Samples of one-year-old shoots, with similar vigour and length, were taken from the central part of the trees at different times. For each cultivar, we collected six shoots with a minimum of 16 buds (about 45 cm in length and 1 cm in diameter) every 3–4 days. We sampled shoots from the beginning of January until the last cultivar fulfilled its CR, representing a total of 24 dates for the 2018/2019 season and 20 dates for the 2019/2020 season. After collection, we placed the shoots in a solution of sucrose (3% w/v) and aluminium sulphate (1% w/v) inside a growth chamber with constant environmental conditions (22 ± 1 °C, photoperiod of 16/8 h under white fluorescent tubes and a relative humidity of 85 ± 9%). Conditions were similar to those described by Campoy et al. (2013) and Fernandez et al. (2019). To avoid bud desiccation, shoots received one minute of overhead water mist three times a day. The base of the shoots was cut by 1 cm once a week, and the sucrose solution was replaced after 10 days.

2.4. Determination of chilling and heat requirements

Buds in the growth chamber were assessed twice a week, and the endodormancy release date was established when green tip (visible signs of green expanding leaves), equivalent to growth stage 07 of bud development of the international BBCH code for pome fruit (Meier et al., 1994), was observed on a minimum of 50% of the buds on a minimum of three shoots after 19 days in the forcing chamber. Winter chill was estimated according to the Dynamic Model (Erez et al., 1990; Fishman et al., 1987a, 1987b). However, for the sake of comparison with previous studies, we also included the Chilling Hours Model (Hutchins, 1932, as cited by Weinberger, 1950) as well as the Utah Model (Richardson et al., 1974).

For all cultivars, the heat requirement (HR) for blooming was estimated by the number of Growing Degree Hours, following the model proposed by Anderson et al. (1986), accumulated between the endodormancy release date (estimated through the forcing experiment) and the date when full bloom was recorded in the orchard. We monitored trees in the orchard twice a week and determined the flowering date as the moment when trees had reached stage 65 of the BBCH code (full bloom, approximately 50% of flowers open; Meier et al., 1994).

2.5. Flowering density and productivity evaluation

Flowering density and productivity were evaluated in the same trees that were assessed for estimating heat requirements. To measure flowering intensity and productivity, we defined an ordinal scale with 6 levels: i) 0 – no flowering or production, ii) 1 – very low flowering or production (2–10% flower or fruit coverage on the tree), iii) 2 – low flowering or production (11–30%), iv) 3 – medium flowering or production (31–60%), v) 4 – high flowering or production (61–80%), and vi) 5 – very high flowering or production (> 81%). We evaluated three replicates per cultivar and determined the mean among them.

2.6. Historic and future climate conditions for apple production in Asturias

For assessing historic and future conditions, we implemented the methods described by Benmoussa et al. (2020) and Fernandez et al. (2020b), with some modifications. We used daily minimum and maximum temperature data collected by a weather station in Villaviciosa from 1978 to 2020. Hourly temperature records were constructed and gaps in the series were bias-corrected using procedures described by Delgado et al. (2021).

Additionally, we generated historic weather scenarios based on the data collected between 1978 and 2020. These scenarios represent the plausible weather conditions likely to be observed at a particular point in time given the climatic conditions of that period. Using the recorded data for calibration, we applied the RMAWGEN weather generator (Cordano and Eccel, 2014) to produce 100 years of synthetic weather (minimum and maximum temperature estimates) representing the conditions of a particular year. This process was applied for 8 points in time, representing conditions of the years 1977, 1983, 1989, 1995, 2001, 2007, 2013, and 2019. This approach allowed us to generate distributions of plausible values rather than relying on a single data point for each season.

Future climate projections for this location were obtained from the ClimateWizard database maintained by the International Center for Tropical Agriculture (CIAT) via an application programming interface (https://github.com/CIATDAPA/climate_wizard_api). This database contains projections by 15 global climate models (GCM; Table A2 in the supplementary materials). Similar to the procedure described above, we used these projections for calibrating the weather generator to produce 100 years of synthetic weather data representing likely conditions projected by the respective climate model. Future temperature scenarios for the 21st century were produced for two Representative Concentration Pathways (RCP4.5 and RCP8.5; IPCC, 2014) for three periods (2020–2040, 2041–2070 and 2071–2100). Their central years, 2030, 2055, and 2085, represent near, intermediate, and far future periods, respectively. In total, we generated 90 future scenarios. The RCP4.5 scenario assumes that mitigation measures will result in the stabilization of atmospheric greenhouse gas concentrations starting around 2040, while the RCP8.5 is a fairly extreme scenario with very high emissions until the end of the century (IPCC, 2014).

2.7. Winter chill estimation and cultivar suitability assessment under future climate scenarios

Winter chill was calculated for all years on record, for all historic scenarios and for each combination of RCP, time horizon and climate model. We defined the winter season to be between November 1st and March 31st in all scenarios. Since the weather generator only provides data for daily minimum and maximum temperature, we derived hourly records from daily extremes based on geographic latitude using procedures proposed by Linvill (1990). For this assessment, we only used the Dynamic Model (Erez et al., 1990; Fishman et al., 1987a, 1987b), as this model has emerged as the most adequate currently available for estimating chill accumulation in deciduous fruit trees from a number of model comparison studies in mild as well as temperate winter regions (e.g. Campoy et al., 2013; Fernandez et al., 2020a, 2020b, 2020c; Luedeling et al., 2011; Luedeling and Brown, 2011; Ruiz et al., 2018; Zhang and Taylor, 2011). To assess the possible impacts of global warming on the cultivation of the most important apple cultivars in the study region and provide a risk analysis, we calculated the probability of CR fulfilment according to the different combinations of RCP scenario, time horizon, and GCM. We defined this probability as the share of years (out of 100) for which the estimated chill availability is likely to exceed the CR. To this end, we used the CR estimated through forcing experiments and compared it to the likely chill estimated for each of the 100 synthetic years per scenario.

2.8. Data processing, analyses, and visualization of results

All analyses were implemented in the R programming environment (R Development Core and Team, 2020; version 3.6.3). For agro-climatic analyses, we used the chillR package (version 0.70.24) for R (Luedeling, 2019). For visualizing the results, we used the ggplot2 library (Wickham, 2011). Spearman's rank correlation analysis was used to determine the relationship between agro-climatic requirements, days of chill accumulation, days of heat accumulation and flowering dates. Spearman's correlation coefficients were calculated using individual values (year and cultivar) for each variable.

3. Results

3.1. Seasonal chill and heat accumulation under field conditions

The average temperature between November 1st and March 31st was 10.06 °C in 2018/2019 and 10.67 °C in 2019/2020. Chill accumulated during the 2018/2019 season was higher (1428 CU and 1073 CH) than in 2019/2020 (1282 CU and 818 CH) according to the Utah and Chilling Hours Models, respectively. Using the Dynamic Model, in contrast, we estimated slightly higher values for the second season (88.4 CP) compared to the first one (87.7 CP). Regarding heat accumulation between February 1st and April 30th, we estimated higher values in the 2019/2020 season (17,880 GDH) than in the 2018/2019 season (15,894 GDH).

3.2. Chilling requirements for breaking dormancy in apple cultivars

The duration of the endodormancy phase ranged from 96 to 160 days ('Granny Smith' and 'Regona', respectively; Table 1). The average duration of chill accumulation for the set of apple cultivars was 126 days in 2018/2019 and 139 days in 2019/2020. Differences between seasons were large in the cultivars 'Perico', 'Solarina' and 'Limón Montés' and small in cv. 'Granny Smith' (Table 1). The lowest chill accumulation was found in the two foreign cultivars 'Granny Smith' and 'Elstar'. These cultivars finished their endodormancy phase after accumulating 59.3 CP and 1006 CU ('Granny Smith') and 65.6 CP and 1096 CU ('Elstar'), respectively. 'Regona' exhibited the highest CR in our study and released dormancy after the fulfilment of 90.2 CP and 1351 CU. Local Asturian cultivars showed a wide range of CR, ranging from 72.2 to 90.2 CP. Almost identical CP estimates were obtained for the cultivars 'Collaos', 'Limón Montés' and 'Solarina'.

3.3. Flowering dates and heat requirements for flowering in apple cultivars

Full bloom was first observed in the variety 'Elstar', 30 days earlier than in the latest flowering cultivar 'Limón Montés' (Table 2). The mean flowering date for the set of cultivars was April 26th for the 2018/2019 season and 9 days later (i.e. on May 4th) for the 2019/2020 season. The largest variation between years (18 days) was found for the cultivar 'Collaos' and the lowest variation (1 day) for the cultivar 'Regona'. The highest HR was identified for the cultivar 'Granny Smith' (13,174 GDH) and the lowest for the local cultivar 'Regona' (6512 GDH). Half of the cultivars showed a heat requirement ranging from 10,000 to 12,000 GDH (Table 2).

3.4. Correlation between chill and heat requirements, bloom dates, and flowering intensity

The analysis indicated a high correlation (Spearman's correlation coefficient; $\rho = 0.96$) between CR quantified with the Utah and Chilling Hours Models. Correlations of both metrics with the CR according to the Dynamic Model were considerably weaker (Fig. 1). A high correlation was found between bloom dates and cultivar-specific CR using the Dynamic Model ($\rho = 0.77$), but the correlation was significantly lower when applying the Utah and Chilling Hours Models (Fig. 1). The relationship between chilling requirements (in Chill Portions) and heat requirements was negative with a moderate coefficient ($\rho = -0.53$). On the other hand, heat requirements and bloom dates showed a negligible correlation ($\rho = 0.04$). No correlation was observed between flowering intensity and cultivar-specific CR in Chill Portions ($\rho = 0.008$).

Table 1

Chilling requirements of 10 apple cultivars in Asturias (northwestern Spain). Chilling requirements were estimated with the Dynamic (Chill Portions – CP), Utah (Chill Units – CU) and Chilling Hours (Chilling Hours – CH) models.

Cultivar	Year	Dormancy breaking date	Chill fulfilment (days)	Mean	Dynamic Model (CP)	Mean	Utah Model (CU)	Mean	Chilling Hours Model (CH)	Mean
Granny Smith	2019	05-Feb	96	96	61.2	59.3	1102	1006	680	614
	2020	04-Feb	95		57.3		910		548	
Elstar	2019	12-Feb	103	106	64.6	65.6	1172	1096	729	674
	2020	18-Feb	109		66.5		1019		618	
De la Riega	2019	22-Feb	113	118	69.6	72.2	1234	1162	851	774
	2020	03-Mar	123		74.5		1090		696	
Blanquina	2019	08-Mar	127	132	75.6	78.4	1278	1225	959	855
	2020	17-Mar	137		81.1		1172		750	
Xuanina	2019	08-Mar	127	136	75.6	79.9	1278	1239	959	855
	2020	24-Mar	144		84.2		1199		750	
Collaos	2019	24-Mar	138	145	80.7	84.6	1347	1304	1003	911
	2020	01-Apr	152		88.4		1260		818	
Perico	2019	12-Mar	131	142	76.6	82.5	1285	1273	964	891
	2020	01-Apr	152		88.4		1260		818	
Limón Montés	2019	15-Mar	134	144	78.5	84.0	1315	1296	977	904
	2020	03-Apr	154		89.4		1277		830	
Solarina	2019	19-Mar	138	148	80.7	85.2	1347	1311	1003	921
	2020	07-Apr	158		89.7		1275		839	
Regona	2019	05-Apr	155	160	89.7	90.2	1456	1351	1125	982
	2020	13-Apr	164		90.7		1245		839	

Table 2
Flowering date (F2 stage, full bloom) and heat requirements between dormancy release and flowering of 10 apple cultivars in Asturias (northwestern Spain) according to the Growing Degree Hours Model (GDH). The flowering intensity and productivity in the 2018/2019 and 2019/2020 seasons are expressed on a scale from 0 to 5.

Cultivar	Year	Flowering date (F2)	Heat fulfillment (days)	GDH	Mean	Flowering intensity (0–5)	Productivity (0–5)
Granny Smith	2019	15-Apr	69	11,909	13,174	2.0	2.0
	2020	20-Apr	76	14,349		2.5	2.3
Elstar	2019	11-Apr	58	10,090	10,649	2.0	1.5
	2020	16-Apr	58	11,208		2.0	2.0
De la Riega	2019	22-Apr	59	11,210	11,770	3.0	3.0
	2020	30-Apr	58	12,328		3.5	3.0
Blanquina	2019	16-Apr	39	7051	8438	0	0
	2020	30-Apr	44	9824		4.3	4.0
Xuanina	2019	25-Apr	48	9034	9690	3.5	3.2
	2020	07-May	44	10,345		3.0	2.7
Collaos	2019	30-Apr	42	8654	10,543	2.7	2.5
	2020	18-May	47	12,052		2.3	2.0
Perico	2019	09-May	58	11,655	11,375	4.0	3.3
	2020	14-May	43	11,094		2.5	1.2
Limón Montés	2019	09-May	55	11,201	11,249	3.5	2.7
	2020	18-May	40	11,297		1.7	1.5
Solarina	2019	02-May	44	8871	7897	0.5	0.5
	2020	04-May	26	6923		4.5	3.7
Regona	2019	06-May	31	6959	6512	0.5	0.5
	2020	07-May	23	6065		3.0	2.7

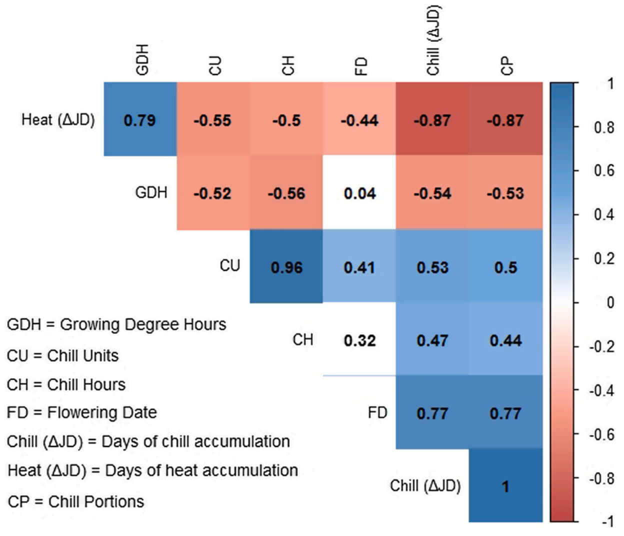

Fig. 1. Spearman's correlation coefficients (ρ) between chill requirement (CR; Chill Units, Chill Portions and Chill Hours), heat requirement (HR; Growing Degree Hours), days of chill accumulation, days of heat accumulation and flowering dates of 10 apple cultivars in Asturias (northwestern Spain). The color gradient shows the strength of the correlation. Correlations that were not significant at $p < 0.05$ are represented by white boxes.

3.5. *Future winter chill*

Model results showed a reduction in winter chill levels by 2030, 2055 and 2085 in both RCP scenarios compared with the historic chill accumulation (Fig. 2). The models forecast broadly similar projections for the three time horizons in the RCP4.5 scenario. Average chill accumulation by 2030, 2055 and 2085 was projected at 87.3, 84.1 and 86.3 CP, respectively. These estimates represent relatively minor changes, between 9 and 12% less winter chill, relative to the 1978–2019 baseline. For the RCP8.5 scenario, expected losses are 9 CP by 2030, 14 CP by 2055, and 24 CP by 2085. The strongest decline in winter chill was found by the end of the twenty-first century with a reduction by one quarter of the available winter chill.

3.6. *CR fulfillment in past and future climates*

Based on the estimates of the cultivar-specific CR (measured in Chill Portions; summarized in Table 1), we determined how often this agro-climatic requirement was fulfilled between 1978 and 2020 and whether it is likely to be fulfilled in the future. In the past, cultivar-specific CR were met every year during the study period for most cultivars except for 'Limón Montés', 'Solarina', 'Perico', and 'Regona' (Fig. 3). In two years during the 42-year period, the cultivars 'Perico' and 'Solarina' did not meet their specific CR and only in one growing season over the entire period the cv. 'Limón Montés' did not reach its specific CR. The cultivar 'Regona' was not able to fulfill its CR in approximately 13% of the years.

Regarding future scenarios, our results suggest that some cultivars may not be able to fulfill their chill requirement in the near future, although our estimates of seasonal chill accumulation for this time slice showed only a small decline relative to the past. According to the projections, CR needed for breaking dormancy would not be reached in more than half of all years for the cultivars 'Limón Montés', 'Collaos', 'Solarina' and 'Regona' under the RCP4.5 scenario by 2020–2040. By the same time horizon and RCP scenario, our estimates show that between 4 and 2 out of 10 years may be challenging in terms of chill accumulation for cultivars such as 'Perico', 'Xuanina', and 'Blanquina'. The cultivars 'Granny Smith', 'Elstar', and 'De la Riega' are expected to meet their CR regardless of time horizon under this moderate-warming scenario.

Under an RCP8.5 scenario and in particular after 2040, local cultivars are expected to frequently fail to fulfill chilling requirements. By 2055 only the local cultivars 'Blanquina', 'De la Riega', and 'Xuanina' are projected to receive enough chill in more than 50% of all years. Prospects appear alarming for the other local cultivars, as they may be exposed to inadequate chill levels in between 60 and 90% of all years. By the end of the century (2071–2100) under the high-warming RCP scenario only the two foreign apple cultivars and the local cultivar 'De la Riega' would satisfy their CR in at least half of all years.

4. Discussion

4.1. *Protocol for quantifying the agro-climatic requirements of apple cultivars*

Estimates of cultivar-specific CR differ considerably depending on the location, experimental design, and mathematical model (Dennis,

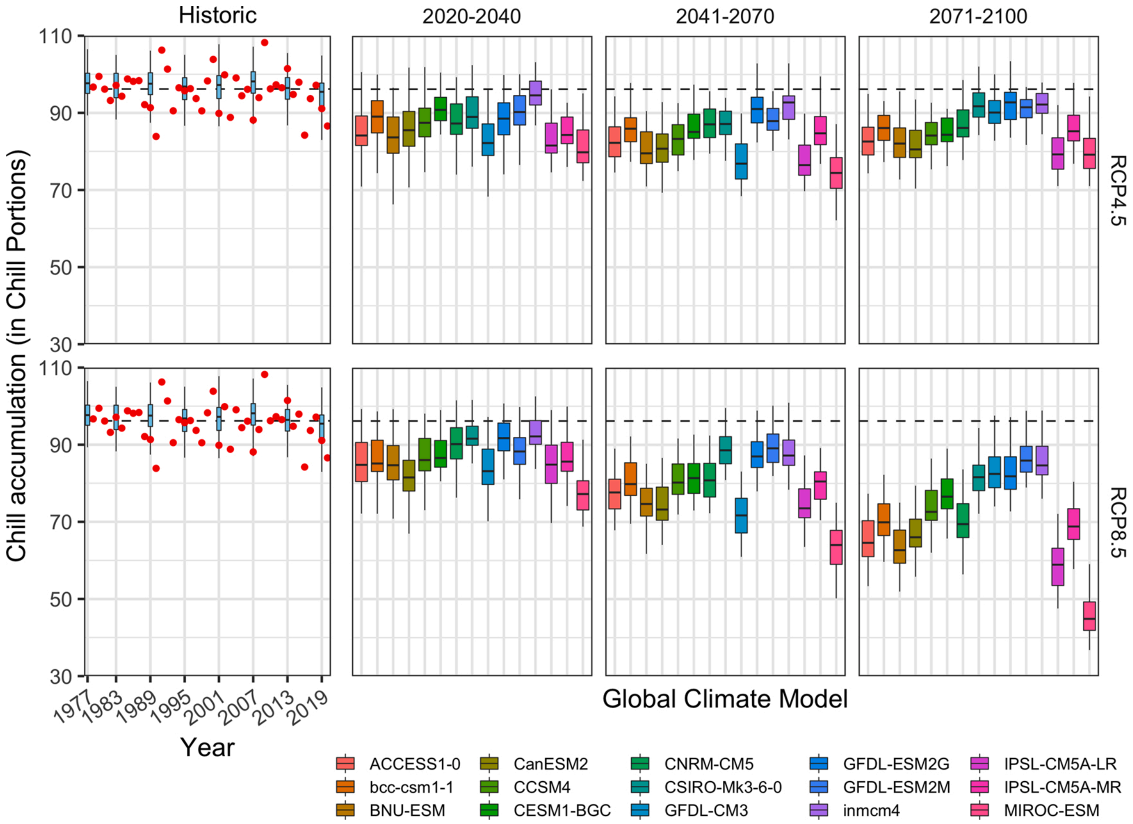

Fig. 2. Historic and projected future chill distributions (calculated in Chill Portions) during the dormant season (November 1st to March 31st) in Villaviciosa (northwestern Spain). In the historic panels (left), the red dots indicate actual chill accumulation for each season, whereas blue boxplots show the chill distribution across 100 simulated winters in historic temperature scenarios (representing typical conditions in 1977, 1983, 1989, 1995, 2001, 2007, 2013 and 2019). In the other six panels, boxplots illustrate the expected chill distribution according to 15 GCMs by three time horizons (2030, 2055 and 2085) under two greenhouse gas concentration scenarios (RCP4.5 and RCP8.5). Each boxplot illustrates chill accumulation for 100 simulated winter seasons for each of 15 climate models with the edges of the boxes indicating the 25th and 75th percentiles. The dashed line represents the median historic chill accumulation between 1978 and 2020.

2003). In our study, both experimental seasons showed significantly lower chill and higher heat than the average accumulation between 1978 and 2019, which was quantified at 96 CP and 11,354 GDH, respectively (Delgado et al., 2021). In the context of increasingly warmer winters and springs, the range of environmental conditions observed during both growing seasons may allow obtaining agro-climatic estimations that will remain valid under climatic conditions expected in the study region in the near future.

We found relatively small differences in CR for the cultivars ‘Elstar’ and ‘Granny Smith’ compared to previously published results. Hauagge and Cummins (1991) reported that the cultivar ‘Elstar’ needed 1027 CU according to the Utah Model to overcome endodormancy in New York State, whereas we calculated a slightly larger estimate (1096 CU) in our study site. Greater variation for the same cultivar was found in western Germany, where Fernandez et al. (2020a) reported 50 CP, contrasting with a higher CR of 65.6 CP obtained in our study. The variety ‘Granny Smith’ was shown to require between 1049 (Hauagge and Cummins, 1991) and 1239 CU (Parkes et al., 2020) according to the Utah Model or between 59 CP (El Yaacoubi et al., 2016) and 72.8 CP (Parkes et al., 2020) according to the Dynamic Model. We estimated the CR for this cultivar to be lower (59.3 CP) than what was found in Australia (Parkes et al., 2020), but our results were well aligned with findings from southern France (El Yaacoubi et al., 2016). Most efforts to quantify the climatic needs of dormant apple trees have focused on determining the CR, since this trait is often a limiting factor for fruit production in mild winter regions. Information about cultivar-specific heat requirements in apple is rather scarce in comparison with chill requirements. El Yaacoubi et al. (2016) reported 9264 GDH for the cultivar ‘Granny Smith’, a value that is considerably lower than the one obtained in our study (13,174 GDH). This discrepancy may be partially explained by differences in the methods used for estimating agro-climatic requirements, as well as by the poor interchangeability of models among regions (Fernandez et al., 2020c; Luedeling and Brown, 2011; Viti et al., 2010).

4.2. Relationship between agro-climatic requirements, flowering dates, and flowering intensity

‘Blanquina’ and ‘De la Riega’ showed advancements in mean bloom dates of 8 and 3 days, respectively, when compared to the average flowering date between 2004 and 2019 (Delgado et al., 2021). On the other hand, the late-flowering cultivars ‘Perico’ and ‘Collaos’ flowered one and two days later than usual. This information suggests that cultivars with lower CR were less affected by warming, whereas the cultivars with higher CR showed a lengthening of the period needed to overcome endodormancy.

Luedeling and Brown (2011) noted the importance of stating the location where estimates of a tree’s agro-climatic needs were determined. Most studies in mild winter climates have reported that the flowering time in temperate fruit trees is mainly influenced by CR, with a much smaller contribution from HR (Alburquerque et al., 2008; Campoy et al., 2012; Egea et al., 2003; Maulión et al., 2014; Ruiz et al., 2007, 2018). Our results suggest that also under the Oceanic climate of Asturias, the flowering date of apple cultivars is primarily the consequence of differences in CR with a minor contribution of HR. Likewise, several authors have reported that genotypes with low CR flower earlier than genotypes with high CR (Alburquerque et al., 2008; Egea et al., 2003; Maulión et al., 2014). This information is partially in accordance with our results, even though the order of flowering across cultivars with

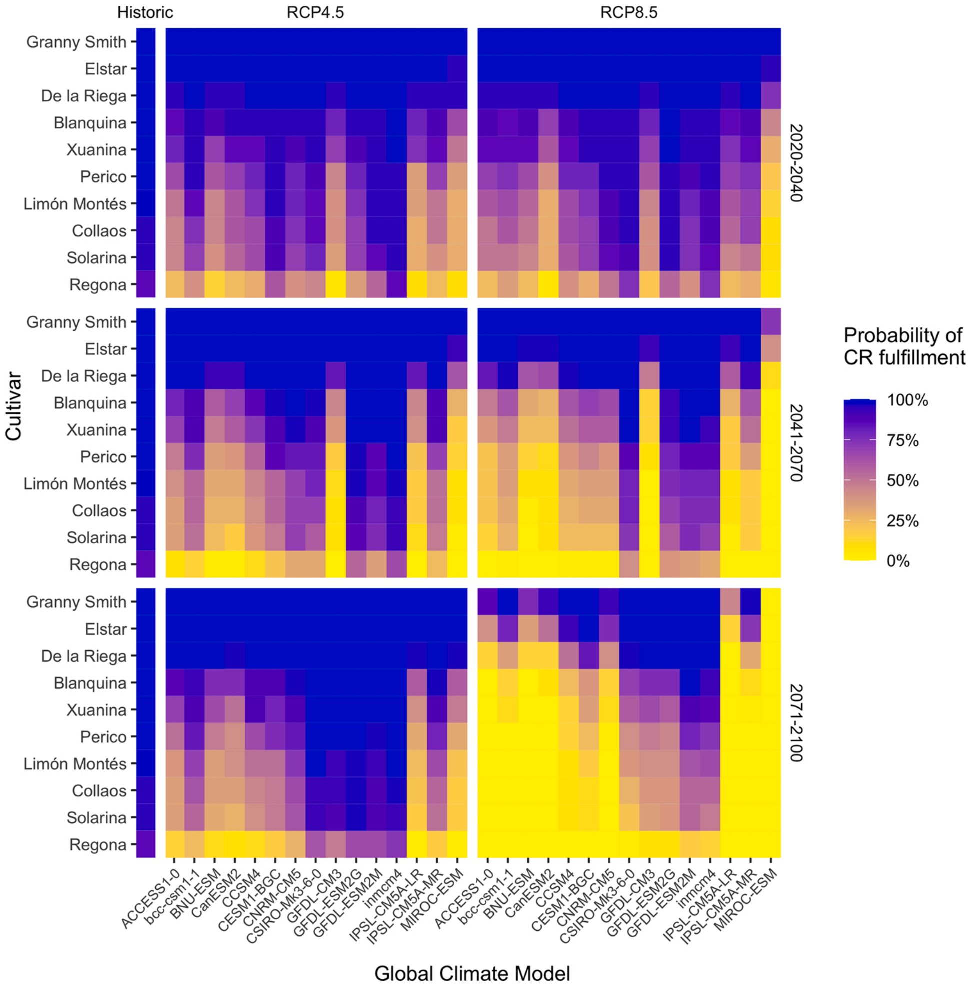

Fig. 3. Probability of satisfying the estimated cultivar-specific chill requirement (calculated as Chill Portions, see Table 1) of 10 apple cultivars in Villaviciosa (northwestern Spain) for two RCP scenarios (RCP4.5 and RCP8.5), for four time horizons (historic, 2030, 2055 and 2085) and 15 global climate models.

relatively similar CR can change depending on HR estimates, as appears to be the case with ‘De la Riega’ and ‘Blanquina’. The average full bloom date of the cultivar that showed the highest chill requirement was also observed a few days earlier than in the two other genotypes. Some authors have reported that cultivars with high CR showed lower HR to reach bloom (Campoy et al., 2011b, 2012; Gao et al., 2012; Ruiz et al., 2007). This information is partially in agreement with our results, as the highest HR was found in the cultivar with the lowest CR (‘Granny Smith’) and the lowest estimate of HR was reported in the cultivar with the highest chill needs (‘Regona’). Ruiz et al. (2007) suggested that higher HR found in cultivars with low CR could be explained by less effective heat accumulation in the first days after overcoming the endodormancy phase in comparison with days later in the season. Sparks (2005) indicated that high-chill genotypes in cold regions may have developed an adaptive survival mechanism to complete the fruiting cycle faster, explaining to a large extent the lower HR shown by these genotypes. Finally, some authors reported that distinct genotypes within the same species have different chilling and heat requirements depending on their climate of origin (Charrier et al., 2011). In this study, the lowest CR was found in the two foreign cultivars (‘Granny Smith’ originated in Australia and ‘Elstar’ was bred in the Netherlands; Noiton and Alspach, 1996). Cultivars that originated in Asturias seem to be well adapted to the local environmental conditions, featuring high chill requirements.

No correlation was found between the cultivar-specific CR and the observed flowering intensity. In apple trees, the genotype is the main cause of biennial bearing (Hanke et al., 2007). In the case of Asturian apple cultivars, flowering intensity and by extension yield are highly influenced by the biennial bearing pattern of each local cultivar (Dapena, 1996; Dapena and Blázquez, 2009). This genetic trait makes drawing conclusions regarding production losses associated with a lack of winter chill difficult. In the case of cv. ‘Regona’, which showed uneven budbreak during two successive experimental seasons, the yield for both the ‘on’ and ‘off’ years was similar to previous records from the same orchard block (data not shown). For this cultivar, we determined a CR slightly higher than the seasonal chill accumulation between November and March, suggesting that trees may still have responded to some extent to late chilling conditions during April. A delay in the fulfillment of the CR caused by an unusually extended endodormancy period may have led to erratic budbreak and a lower number of leaves in this cultivar. Our results, however, might indicate that only severe chill insufficiencies or consecutive seasons with suboptimal chilling conditions can produce a significant reduction in fruit yield.

4.3. Chilling requirement fulfillment of apple cultivars under future climate scenarios and implications for the local apple industry

Our results confirm findings from previous studies suggesting that CRs have been easily satisfied in Asturian apple orchards (Dapena, 1996; Delgado et al., 2021). The estimated CR are aligned with historically observed chill over the past four decades, and only the cultivar ‘Regona’ experienced a lack of winter chill in approximately one in eight years.

To evaluate the suitability of species and cultivars for a particular location under future scenarios, Luedeling et al. (2009) defined the concept of Safe Winter Chill as the winter chill that can be expected to be exceeded in 90% of all years. This assumes that failure to fulfill CR in more than 10% of all years can make orchards economically unviable. Applying similar reasoning to our results, all scenarios where the cultivar-specific CR is met in >90% of the years can be interpreted as “safe” and all others as “unsafe” However, some considerations need to be addressed to confirm the suitability of some crops or cultivars for a particular region. First, the choice of method for estimating the CR has a decisive influence on CR estimates (Dennis, 2003), and the defined protocol might not be accurate enough to detect the transition to the ecodormancy phase, resulting in an overestimation of CR. Second, several authors have suggested a partial compensation between chill and heat in deciduous trees (Darbyshire et al., 2016; Harrington et al., 2010; Kaufmann and Blanke, 2019). Finally, some studies reported inconsistencies in this kind of analysis, as they found economically acceptable yields in seasons when winter chill accumulation dropped below the estimated CR of the cultivars (Parkes et al., 2020; Pope et al., 2014). All these findings stress that large knowledge gaps remain regarding the underlying physiology of the dormancy process (Campoy et al., 2011a; Fadón et al., 2020; Luedeling, 2012). Despite some uncertainties, the methodology presented in this work provides the local fruit research institute with the necessary tools to deliver appropriate recommendations on future plantings.

Our results are consistent with previous analyses assessing the impacts of global warming on chill accumulation. In Mediterranean climate areas, studies have predicted severe impacts of insufficient chill accumulation on fruit tree species (Benmoussa et al., 2020; Fernandez et al., 2020b; Luedeling et al., 2011). Our analyses of future winter chill in coastal locations of Asturias indicate that the use of some popular apple landraces is threatened by the chill losses expected throughout the 21st century. Under an intermediate greenhouse gas concentration scenario (RCP4.5), several local cultivars may still receive sufficient chill to reach their CR most of the time. Assuming that CR must be met completely, the choice of local Asturian cultivars that are almost completely safe will be limited to cultivars such as ‘De la Riega’ and ‘Blanquina’ under this scenario. Under the RCP8.5 scenario, projections show a notable risk of failing to fulfill chill requirements of local high-chill cultivars after 2070. Only cultivars with a similar behaviour to the non-native apple cultivars ‘Granny Smith’ and ‘Elstar’ would remain viable in this high-warming environment. Of particular concern is the viability of the cultivar ‘Regona’, which is already showing some symptoms of chill deficiencies, with further limitations arising from chill losses expected for both RCPs and all time slices.

The silver lining of the projected changes in winter chill is that apple production is expected to remain viable from 2020 to 2070, as the region is expected to maintain a substantial amount of chill under both RCP scenarios. Similarly, future chill is expected to exceed 72 CP even in the worst-case scenario (between 2071 and 2100), hence a transition to cultivars with lower CR can be easily implemented in the region. However, replacement of high-chill cultivars must be made considering a wide range of agro-climatic aspects (Campoy et al., 2019), such as frost risk, water availability, and other environmental conditions, which play a substantial role in tree physiology and fruit quality. Likewise, the particularities of the local fruit industry must also be considered. While low-chill apple varieties are already available, and global breeding programs are currently working on the development of varieties that are phenotypically adapted to mild winter climates, it is important to note that apples intended for cider-making require particular technological and organoleptic features, and not all kinds of apples are suitable for this purpose (Merwin et al., 2008). Furthermore, the sale of cider under the ‘Protected Designation of Origin’ label, which implies the use of 100% Asturian apples, has achieved strong growth in the past decade. The strength of this brand might be compromised if the consolidated cider industry cannot ensure sufficient supply of apples of the registered local cultivars, which they use as part of the blend.

The first step in the process of breeding high-quality cider apple cultivars with lower CR is the identification of suitable germplasm. Owing to the large population of local apple cultivars maintained at SERIDA (around 525 accessions), among which cultivars with lower CR are available (Dapena, 1996), it seems preferable to choose among these local genetic resources than importing plant material from foreign breeding programs. Delgado et al. (2021) stated that Asturian apple cultivars showed a weak phenological response to the gradual rise of temperature, so locally maintained germplasm may have adaptation benefits. Because the situation does not appear to be particularly alarming in the next 20 years in terms of the fulfillment of CR, the local apple breeding program has some time to adjust to future climate prospects and develop a new array of climate-resilient cultivars that are suited for the region. Nevertheless, considering the lengthy timescales associated with the traditional breeding approach in pome fruit species, precise characterization of chill and heat requirements may become increasingly important in the coming years as critical information for choosing the most suitable genotypes to be used in future crosses. Some work in this direction has been initiated, with some of the newly registered cultivars obtained by the SERIDA breeding program expected to have similar or lower CR than ‘De la Riega’ based on pedigree information and flowering behaviour. A further adaptation option in the study region is to increase cultivation in the interior valleys of the region and at higher elevations where winters are cooler. In Asturias, apple orchards are often located close to the coast, but due to the varied topography of the region, with a wide elevation gradient, shifting apple production to cooler areas might be possible to some extent.

Other fruit growing regions have begun a transition to different crops that are better suited to future climatic conditions, but this conversion should be the last option in Asturias, where apples and cider have strong cultural roots (Torre et al., 2002). In our opinion, the most efficient adjustment for apple farms in order to remain profitable and avoid the adverse consequences of future warming is the identification and breeding of environmentally adapted cultivars that can meet their CR every year.

5. Conclusions

Our results indicate that the viability of high-chill apple genotypes in a typical temperate Oceanic climate might be jeopardized under future environmental conditions. Even though we predicted only moderate reductions in future winter chill compared to the past, some local high-chill cultivars may fail to meet their chill requirements in future decades. Despite projections showing a decrease in winter chill availability in all scenarios, fruit tree production does not seem particularly threatened. Since 72 CP are expected even for the worst-case scenarios, local cultivars with low or moderate chill requirements are expected to remain productive in the future despite the projected declines. With these levels of winter chill, developing adaptation strategies should be quite feasible for farmers and orchard managers. A crucial ingredient for such adaptation strategies is a better understanding of the agro-climatic needs of apple cultivars and the relationship between chill accumulation and fruit yield under warmer scenarios, which can support breeding programs in selecting cultivars with adequate chilling requirements.

CRediT authorship contribution statement

Alvaro Delgado collected the plant material and phenological data. **Enrique Dapena** contributed to the experimental design and set up of the facilities necessary for its development. **Eike Luedeling** developed the code for future projections of winter chill. **Alvaro Delgado** performed the analysis with inputs from **Eike Luedeling**, **Eduardo Fernandez** and **Enrique Dapena**. **Eduardo Fernandez** produced the figures. **Alvaro Delgado** wrote the manuscript and all authors contributed to interpretation of the results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eja.2021.126374.

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Chapter 3

Analysis of the Variability of Floral and Pollen Traits in Apple Cultivars — Selecting Suitable Pollen Donors for Cider Apple Orchards

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Article

Analysis of the Variability of Floral and Pollen Traits in Apple Cultivars—Selecting Suitable Pollen Donors for Cider Apple Orchards

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Abstract: Most apple trees (*Malus domestica* Borkh.) are self-incompatible and fruit yield depends on cross-pollination between genetically compatible cultivars with synchronous flowering. Flowering intensity can vary strongly among years due to the biennial bearing habit of the cultivars. The knowledge of the phenological stages and floral and pollen characteristics is essential to select suitable pollen donors. We evaluated the phenotypic variability of flowering-related traits (i.e., flowering phenology, flowering intensity, pollen production and pollen quality) in 45 apple cultivars over two successive flowering seasons. Large phenotypic variability was found among the studied cultivars indicating that the local germplasm collection provides a good source of genetic and phenotypic diversity. However, low correlations were observed between floral biology traits and, consequently, the improvement in one trait seems not to affect other traits. Some of the cultivars such as 'Perurico' and 'Raxila Dulce' regularly produced copious amounts of high-quality pollen which can improve the pollen load dispersion leading to a most effective pollination process. We did not identify statistically significant correlations between pollen attributes and the biennial bearing phenomenon. The large variation in bloom dates from year-to-year observed under a typical Oceanic climate makes it advisable to combine cultivars in new plantings.

Keywords: *Malus domestica*; blooming time; pollen quality; pollen production; 'on' and 'off' years; phenotypic variation

1. Introduction

Pollination is a key event in plant reproduction which consists in the transfer of pollen grains from anthers to stigmas in order to fertilize the ovules and begin the development of seeds and fruits [1]. Apple (*Malus domestica* Borkh.) is known as a gametophytic self-incompatible flowering tree [2] and yield relies on the successful cross-pollination between at least two cross-compatible cultivars which flower synchronously [3,4].

The quantity and quality of apple production is significantly affected by the effectiveness of the pollination process [5,6]. Apart from the fundamental requirement for fruit set and tree productivity, the presence of different pollen sources has a favorable impact on fruit quality parameters such as size, shape and organic matter [5,7–10]. Apple trees are heavily dependent on insect pollination and the contribution of pollinators is also essential for obtaining consistent yields [3,5,6].

To avoid pollen limitation in commercial orchards, either pollen donor trees (i.e., "pollinizers") or cultivar mixtures are planted in the same orchard [11,12]. Garratt et al.

[5] and Quinet and Jacquemart [13] stated that the improvement in the density and arrangement of pollinating cultivars in the orchard promotes the share of pollinators carrying viable pollen, eventually enhancing fruit and seed set.

Flowering is one of the main processes leading to crop productivity [14] in fruit trees, therefore, their phenotypic study has practical implications for breeding purposes. To the extent that fruit yield and quality is primarily the result of the flower quantity and quality [15], the modification and improvement of relevant floral biology traits is expected to play a major role in the process of obtaining a more sustainable productivity. Since apple orchards require several years before new plantings become profitable, the selection of cultivars is vitally important. For the characterization of pollen donor plants, also known as pollinizers, it is essential to evaluate a variety of phenotypic traits such as floral overlap with productive varieties, bloom intensity, pollen production and pollen quality. Flowering intensity and pollen production are important traits to measure the pollination ability of any cultivar due to that a higher number of pollen grains can improve the success of ovule fertilization and, consequently, the fruit set of commercial trees [16]. The production of pollen is cultivar-specific [17] and it is primarily determined by the pollen quantity per anther. Additionally, the quality of the pollen characterized by pollen viability and/or pollen germination must also be considered with the goal of ensuring economically acceptable yields. Pollen germination studies on apple trees have shown wide variability for the same variety depending on the trees' location and the laboratory assessment [4], but it has been proven that triploid cultivars exhibit lower pollen germination rates than diploid cultivars [18,19].

Apples are an economically important agricultural product in Asturias. This region, situated in north-western Spain, has a long tradition in cider-making and inherently linked to this tradition there is a high diversity of local apple cultivars. The Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) currently maintains 800 apple accessions, of which approximately 500 are local accessions [20]. Over the last three decades, the bulk of the cider apple orchards planted in Asturias are composed of between four and six different cultivars. These cultivars are part of the 22 cultivars initially included in the quality label "Protected Designation of Origin (PDO) Asturian Cider." Recently, the PDO regulatory board endorsed 54 new apple varieties, including 18 new varieties selected by the SERIDA breeding program, which are expected to be planted in Asturias and other regions over the next few years. Most of the cider apple orchards in this region are planted on semi-dwarfing rootstocks with a tree density of around 650 trees/ha. This system contrasts with high-density plantings of many apple growing regions where solid monovarietal blocks were widely implemented. In a recent study, Martínez-Sastre et al. [21] did not find a deficit of pollinators in most of the Asturian apple orchards. Hence, the maximum level of pollination in these farms may potentially be achieved by ensuring the presence of large quantities of compatible pollen and facilitating cross-pollination accomplished by pollinators [5,13,22].

On the other hand, the study region relies on local cider cultivars which exhibit different degrees of biennial or alternate bearing [23,24]. Biennial bearing is a common problem in apple trees where the excessive number of fruits in 'on' years trigger a competition between fruit formation and flower initiation, resulting in low or non-existent yields in 'off' years [25–27]. At the regional level, this situation causes problems in the supply of apples to the cider industries in 'off' years and increases the risks of disruptions in the commercialization during 'on' years. Mazzeo et al. [28] showed that olive trees produce less pollen grains but with a significantly higher viability in 'off' years with the aim to maximize the fertilization of the ovules. The relationship between the quantity and quality of pollen grains affected by the 'on' and 'off' year has not been well studied in apples.

The main aim of this study was to characterize and describe the variability of some flowering-related traits among 45 apple cultivars in order to optimize their use for pollination purposes and also to ascertain if the knowledge of these floral and pollen characteristics can support new breeding strategies. More specifically, using a range of replicated field and laboratory experiments, this study determined: (1) periods of bloom overlap between cultivars; (2) flowering intensity and pollen production and (3) viability and germination of pollen grains. This work also raised the following questions: (i) is the quality and number of pollen grains affected in an 'off' year with respect to an 'on' year in apple trees? and (ii) which cultivars can better meet the pollen donor requirements?

2. Materials and Methods

2.1. Site Description and Plant Material

The study was carried out in Villaviciosa, Asturias, north-western Spain (43° 28′ N, 5° 26′ W, 10 m above sea level). Forty-five apple cultivars (Table S1) were evaluated in three nearby experimental plots belonging to SERIDA (within a radius of approximately 150 m). In 2018 and 2019, twenty-two cultivars included in the quality label "Protected Designation of Origin Asturian Cider" since 2002 [23] were evaluated in a single block of three trees of each cultivar within a 10-year-old orchard. In 2019 and 2020, we observed a block of two trees for each cultivar in a 9-year-old orchard comprising twenty-one cultivars from the SERIDA apple breeding program (eighteen cider apple cultivars and three dessert apple cultivars). Fourteen of these cultivars were recently registered as protected varieties and the remaining seven are currently in the process of being registered. The dessert apple cultivar 'Granny Smith' and the crab apple *Malus floribunda* Siebold ex Van Houtte clone 821 grown in an 18-year-old repository orchard were included as international references. All plots consisted of semi-dwarf trees grown on vertical axis. Trees were grafted onto M.7 rootstock and their planting distance was 5.5 m × 2.5 m, apart from *Malus floribunda* 821 and 'Granny Smith' trees which were grafted onto PI.80 rootstock and planted with a distance of 4.75 m × 1.75 m. The research orchards have similar soil conditions, are managed under organic certification standards and trees are normally pruned every year. Each cultivar was evaluated over a period of two successive flowering seasons, including both an 'on' and an 'off' year except for *Malus floribunda* 821, 'Blanquina', 'Meana' and 'Verdialona' which were assessed only in one season.

2.2. Flowering Dates, Flowering Duration and Bloom Synchronization

Flowering phenology observations were carried out twice a week according to the international BBCH code for pome fruit [29] from green tip (stage 53) until petal fall (stage 67). Flowering time was established as the moment when trees reached stage 65 of the BBCH (full bloom, approximately 50% of flowers open). The duration of flowering was calculated as the period (days) between the phenological growth stage 61 of the BBCH code (first bloom, approximately 10% of flowers open) and petal fall. Bloom synchronization was determined based on the overlapping periods between cultivars.

2.3. Flowering Intensity and Pollen Production

The total number of inflorescences on each tree for all cultivars was counted using a tally counter. Accurate counts get more complicated after the flowers start to open [30], consequently, the total number of inflorescences for each cultivar was recorded between the phenological stages 61 and 65 of the BBCH code [29]. This approach was taken instead of counting the number of inflorescences on typical branches [31] due to the selected cultivars exhibiting pronounced differences in vigor, tree architecture and fruit-bearing habits [23,24]. The trunk circumference was measured at 40 cm height from the ground, approximately 20 cm above the graft union, and density of blossoms was converted to flowers per square centimeter of trunk cross-sectional area (TCSA) [32]. The number of flowers per inflorescence was evaluated by counting the number of flowers per inflorescence in 10 typical inflorescences per tree in the studied block. The number of anthers per flower in each cultivar was determined by counting the number of anthers in ten flowers randomly picked at balloon stage. With the intention of determining the number of pollen grains in one flower, one sample of 20 anthers from each cultivar was prepared by placing all the anthers in a 2 ml Eppendorf tube and letting them to dehisce in a growth chamber at 21 °C for 48 h. One milliliter of aqueous eosin solution (C.I. + $PO4^{3-}$) was added to the dried anthers and samples were shaken on Vortex for 30 s just before 0.2 mL from the solution was applied to a Malassez hemocytometer (adapted from Bieniasz et al. [33]). Two counts from the same tube were performed with the Malassez hemocytometer. Pollen grains were counted using a Nikon Eclipse 50i compound microscope at 10× magnification (Figure S1a) and the number of pollen grains in one anther was calculated by dividing the total amount of pollen grains in 20 anthers by 20.

2.4. Evaluation of Pollen Quality

Pollen quality was evaluated using two different parameters, pollen viability and pollen germination. A total of 30 flowers at balloon stage from all the trees in the experimental block were sampled and anthers were removed. Pollen from these anthers was left to dehisce in Petri dishes at 21 °C for 48 h. Pollen viability was assessed using Iodine Potassium Iodide (IKI) staining technique (1 g potassium iodide (KI) + 0.5 g iodine (I) dissolved in 100 mL distilled water). Pollen was spread in a microscope slide, mixed with 120 μL of the solution and covered with a coverslip. Counts were made using a Nikon Eclipse 50i compound microscope ten minutes after pollen was placed on IKI solution. One hundred pollen grains in four different areas of the same preparation were observed. Pollen grains stained dark brown were scored as viable and unstained grains were counted as non-viable (Figure S1b). Pollen viability tests were not conducted in 2018. On the same day as pollen viability tests were carried out, pollen from the same Petri dishes was used to determine the germination of pollen grains. Pollen germination was evaluated *in vitro* by dusting the pollen on concentrated agar (1.5%) containing 10% sucrose and boric acid (240 mg/L). After 24 h at 21 °C, pollen germination was measured using an Olympus SZX12 stereomicroscope (Olympus Corporation, Tokyo, Japan). Four different subsamples of one hundred pollen grains from the same agar plate were observed and pollen germination percentage was calculated. Pollen grains were considered germinated when the length of a pollen tube exceeded its diameter ([34]; Figure S1c).

2.5. Alternate Bearing Tendency and Its Influence on Pollen Properties

Return bloom was calculated according to the number of inflorescences/TCSA cm^2 on two successive growing seasons [35]. The growing season with the largest number of inflorescences was considered the 'on' year and this information was used to determine if there is a correlation between pollen quantity and quality in an 'off' year with respect to an 'on' year. If at least one of the trees in the studied block showed a different biennial bearing pattern, data from this cultivar was discarded for further analysis. Hence, twenty-four cultivars which showed a clear biennial bearing pattern during these two seasons were selected for the analysis.

2.6. Statistical Analysis

Prior to all statistical analyses, the assumption of normality was investigated using the Shapiro–Wilk test. Pollen germination and pollen viability rates were transformed into arcsine square root values. Duration of flowering was analyzed after log 10 transformation. One-way ANOVA was used to investigate the differences among cultivars and also to determine changes in pollen properties between 'on' and 'off' years. Means were compared with Duncan's Multiple Range Test at $p < 0.05$. Correlation coefficients between floral and pollen characteristics were determined using Pearson's correlation coefficient using individual values (year and cultivar) for each variable. All analyses and figures were produced in the R programming environment (version 4.0.4; [36]).

3. Results

3.1. Phenological Observations, Duration of Flowering and Flowering Overlap between Cultivars

Full bloom was first observed in *Malus floribunda* 821 on March 24th. Excluding this early flowering crab apple species, the two-year mean bloom date ranged from April 13th (‘Colorá Amarga’) to May 20th (‘Raxao’). The mean bloom date for the set of apple cultivars was recorded on May 8th (season 2017/2018), April 23rd (season 2018/2019) and April 30th (season 2019/2020). Pronounced year-to-year variation in flowering dates was observed between flowering seasons for most of the evaluated cultivars, although the order of flowering among cultivars was similar in both years (Figure S2). Approximately 40% of the cultivars exhibited similar flowering time and flowered within the same week. Among the newly registered cultivars (Table S1), we found a lower proportion of late-flowering cultivars, and only the cultivar ‘Raxarega’ belonged to the very late-flowering group (Figure S2).

The average duration of flowering ranged between 7 days in cultivars ‘Verdialona’ and ‘X9406-11’ and 16 days in cv. ‘Raxona Ácida’. The cultivars ‘Raxona Ácida’ and ‘Raxona Dulce’ exhibited a mean blooming duration of 16 days with also a slight variation between years in the length of the period. For the set of studied cultivars, bloom duration varied from 10 to 15 days in 2017/2018, from 8 to 20 days in 2018/2019 and from 7 to 14 days in 2019/2020. Less than one quarter of the cultivars showed a two-year average duration shorter than 10 days (Table 1).

Table 1. Flowering time, flowering duration, flowering intensity (number of inflorescences/cm^2 of trunk cross-sectional area) and floral characterization of 44 domesticated apple cultivars and *Malus floribunda* 821 in Villaviciosa (north-western Spain) in two successive years (i.e., 2018–2019 or 2019–2020). Data from two successive flowering seasons was pooled to obtain a unique value for each cultivar and variable. Two-year mean values are accompanied by the standard deviation. ANOVA results using the year and cultivar as factors are shown.

Cultivar	Flowering Date	Duration of Flowering	Inflorescences/TCSA (cm^2)	Number of Flowers/Inflorescence	Number of Anthers/Flower
Blanquina	28-Apr	12 ± 2.83	4.88 ± 3.13	5.7 ± 0.59	18.4 ± 1.58
Carrió	02-May	10.5 ± 0.71	5.22 ± 7.54	5.7 ± 0.54	19.05 ± 0.95
Clara	18-Apr	11.5 ± 3.53	8.99 ± 2.20	6.08 ± 0.63	17.6 ± 1.43
Collaos	04-May	10.5 ± 0.70	10.35 ± 4.50	5.46 ± 0.65	18.2 ± 1.16
Cladurina	17-Apr	12.5 ± 2.12	13.2 ± 7.55	5.75 ± 0.63	18.15 ± 1.72
Cladurina Amargo-Ácida	15-Apr	12.5 ± 0.71	8.3 ± 9.71	5.45 ± 0.69	18.55 ± 1.66
Colladina	07-May	10.5 ± 0.70	19.2 ± 4.81	5.83 ± 0.66	19.1 ± 1.52
Colorá Amarga	13-Apr	12 ± 2.83	7.9 ± 2.93	5.57 ± 0.50	18.6 ± 0.99
Coloradona	18-Apr	11.5 ± 2.12	10.99 ± 2.47	5.97 ± 0.50	18.45 ± 1.32
De la Riega	01-May	9.5 ± 3.53	12.27 ± 10.74	5.42 ± 0.76	19.65 ± 1.53
Durcolorá	18-Apr	12.5 ± 1.41	17.7 ± 9.90	5.55 ± 0.69	17.3 ± 1.15
Durona de Tresali	08-May	10.5 ± 0.71	4.45 ± 6.52	4.17 ± 0.77	19.67 ± 0.82
Ernestina	01-May	8.5 ± 2.12	8.36 ± 9.89	5.52 ± 0.68	19.2 ± 1.55
Fuentes	06-May	10 ± 0	5.93 ± 6.71	5.68 ± 0.69	19.25 ± 1.17
Granny Smith	17-Apr	7.5 ± 0.71	6.18 ± 1.26	5.73 ± 0.51	19.15 ± 1.07
Limón Montés	13-May	10 ± 0	6.78 ± 4.06	5.75 ± 0.58	19.05 ± 1.28
Malus floribunda 821	24-Mar	11	27.67 ± 17.62	7.15 ± 0.67	20.8 ± 1.48
Meana	10-May	14	7.98 ± 4.69	5.7 ± 0.69	19.4 ± 1.18
Panquerina	02-May	12 ± 2.82	8.25 ± 7.40	5.3 ± 0.77	19.1 ± 1.41
Perezosa	25-Apr	9.5 ± 0.71	8.22 ± 7.27	5.8 ± 0.71	19.6 ± 1.05
Perico	11-May	12.5 ± 2.12	9.20 ± 7.23	5.63 ± 0.54	18.75 ± 0.97
Perurico	26-Apr	9 ± 1.41	15.21 ± 10.99	5.73 ± 0.66	18.8 ± 1.06
Perurico Precoz	27-Apr	10.5 ± 4.95	13.95 ± 13.11	5.68 ± 0.63	17.85 ± 1.31
Prieta	13-May	12 ± 2.83	10.00 ± 8.20	5.77 ± 0.50	18.55 ± 1.28
Raxao	20-May	8.5 ± 2.12	7.8 ± 5.56	5 ± 0.58	19.15 ± 1.19
Raxarega	13-May	12 ± 2.83	6.93 ± 3.81	5.43 ± 0.49	18.05 ± 1.15
Raxila Ácida	28-Apr	9 ± 1.41	7.2 ± 4.02	5.95 ± 0.51	18.55 ± 1.36
Raxila Dulce	18-Apr	12.5 ± 2.12	12.09 ± 4.11	6.25 ± 0.44	19.05 ± 1.09
Raxila Rayada	18-Apr	9 ± 2.82	20.3 ± 2.69	5.78 ± 0.55	19.15 ± 0.88
Raxina Ácida	01-May	12 ± 2.80	18.5 ± 10.39	5.53 ± 0.66	19.05 ± 0.94
Raxina Amarga	27-Apr	12.5 ± 3.5	11.9 ± 1.73	4.88 ± 0.58	18.55 ± 0.88

Raxina Dulce	29-Apr	9 ± 2.83	5.9 ± 2.91	5.1 ± 0.52	19.05 ± 1.10
Raxina Marelo	28-Apr	11.5 ± 0.71	11.19 ± 4.81	5.05 ± 0.39	18.05 ± 1.87
Raxona Ácida	05-May	16 ± 7.07	15.9 ± 9.69	5.4 ± 0.50	19 ± 1.03
Raxona Dulce	03-May	15.5 ± 2.12	12.13 ± 5.14	5.58 ± 0.61	18 ± 1.30
Regona	08-May	10.5 ± 3.54	7.31 ± 8.22	5.65 ± 0.64	19.75 ± 1.52
Rosadona	17-Apr	11 ± 0	13.8 ± 4.48	5.95 ± 0.58	19.65 ± 0.93
San Roqueña	01-May	11 ± 1.41	11.45 ± 11.09	5.28 ± 0.76	16.7 ± 2.00
Solarina	06-May	11 ± 0	6 ± 5.64	5.45 ± 0.58	18.45 ± 1.36
Teorica	04-May	10.5 ± 4.95	4.8 ± 3.71	5.64 ± 0.64	18.15 ± 1.23
Verdialona	07-May	7	3.96 ± 5.37	5.2 ± 0.72	19.5 ± 1.17
X9406-49	22-Apr	12 ± 2.83	19.14 ± 13.11	5.43 ± 0.64	18.15 ± 1.56
X9406-57	17-Apr	12.5 ± 2.12	12.3 ± 7.98	5.25 ± 0.59	18.7 ± 1.39
X9406-11	20-Apr	7 ± 0	17.5 ± 8.24	5.6 ± 0.50	17.7 ± 1.52
Xuanina	02-May	10.5 ± 4.95	10.94 ± 5.06	5 ± 0.68	18.9 ± 0.91
ANOVA					
Cultivar		$F_{44,41} = 1.14$, $p = 0.35$	$F_{44,162} = 2.86$, $p < 0.001$	$F_{44,1693} = 15.78$, $p < 0.001$	$F_{44,815} = 1.79$, $p = 0.035$
Year		$F_{1,41} = 4.8$, $p = 0.02$	$F_{1,162} = 4.98$, $p = 0.028$	$F_{1,1693} = 4.30$, $p = 0.014$	$F_{1,815} = 0.74$, $p = 0.48$

3.2. Phenotypic Evaluation of Floral and Pollen Traits

The number of flowers per inflorescence varied among cultivars (Table 1). The mean number across the studied cultivars was 5.6. The largest and the lowest mean number of flowers per inflorescence were found in *Malus floribunda* 821 (7.2) and 'Durona de Tresali' (4.2), respectively. Large variability was observed especially for the number of blossom clusters/TCSA (cm^2) (Table 1). The cultivar 'Raxila Rayada' showed the highest two-year average value (20.3) whereas the minimum number was recorded in 'Verdialona' (4.1). The number of anthers per flower ranged from a minimum of 16.7 ('San Roqueña') to a maximum of 20.8 (*Malus Floribunda* 821), with most of the cultivars having between 18 and 20 anthers per flower (Table 1). The number of pollen grains per anther showed a wide variability among cultivars ($F_{44,127} = 2.29$, $p = 0.005$; Figure 1) and this value ranged from 1319 in 'Cladurina Amargo-Ácida' to 8056 in 'Raxila Dulce'.

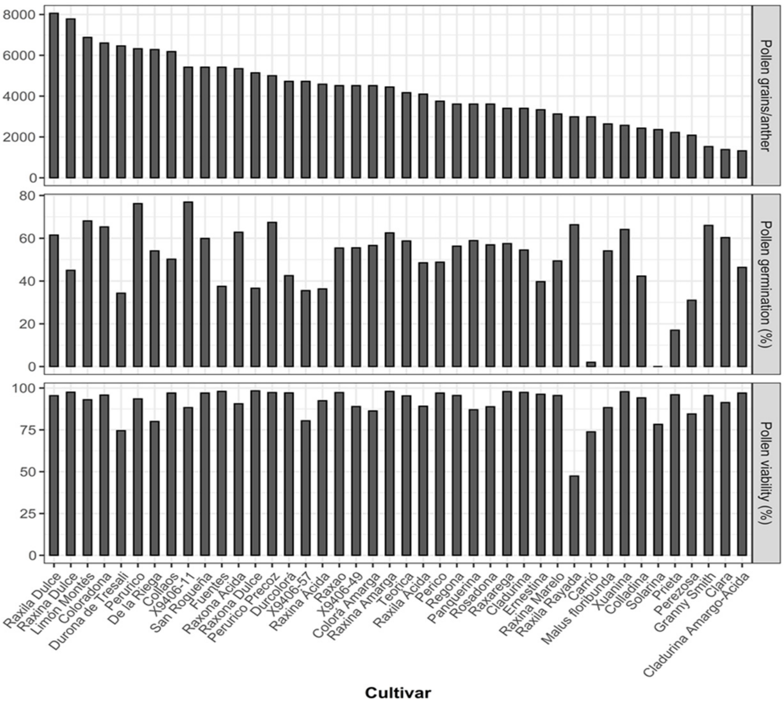

Figure 1. Means of pollen grains per anther, pollen germination (%) and pollen viability (%) of 41 apple cultivars in Villaviciosa (north-western Spain) across two successive years (i.e., 2018–2019 or 2019–2020).

All cultivars with the only exception of ‘Raxila Rayada’ showed a pollen viability value above 70% (Figure 1). The highest and the lowest pollen viability estimates were found in ‘Raxona Dulce’ (98.3%) and ‘Raxila Rayada’ (47.4%), respectively. Pollen germination differed considerably among cultivars ($F_{44,256} = 18.90$, $p < 0.001$; Figure 1). The lowest mean germination rates were detected in ‘Solarina’ and ‘Carrió’ which are triploid cultivars (Dapena, personal communication). Only considering diploid cultivars, the maximum germination percentage (76.9%) was found in the accession ‘X9406-11’ whereas the lowest value was observed in cv. ′Prieta’ (17%). Pollen germination and viability percentages in both years were similar for each cultivar, although a greater level of variation among cultivars was found in pollen germination (coefficient of variation; CV = 34.5) than pollen viability estimates (CV = 12.3; Figure 1). Pollen viability was higher than the pollen germination in all but one of the studied cultivars.

3.3. Effects of Biennial Bearing on Floral and Pollen Traits

Important differences in the density of blossoms between two successive growing seasons were observed among the examined apple cultivars. The number of inflorescences/TCSA cm^2 in an ‘off’ year was generally lower in the traditional apple cultivars than in the new releases obtained by the breeding program. Among the twenty-four cultivars used for this study, the return bloom percentages ranged from 0.4 (‘Ernestina’) to 84.2 (‘Raxina Amarga’; Table S2).

Biennial bearing pattern showed a statistically significant effect on the duration of flowering ($F_{1,23} = 8.11$, $p = 0.009$). The average flowering duration in ′on′ and ′off′ years were 11.8 and 9.8 days, respectively. We did not find statistically significant differences between ‘on’ and ‘off’ years for the other flowering traits analyzed in this work. Pollen viability, pollen germination and pollen production showed almost the same values during both seasons (Figure 2). Although no general effect of biennial bearing was observed on these traits, significant differences were identified for cv. ‘Limón Montés’ which showed higher values during the ‘off’ year for pollen germination ($F_{1,7} = 25.5$, $p < 0.001$) and pollen production ($F_{1,3} = 13.03$, $p = 0.011$).

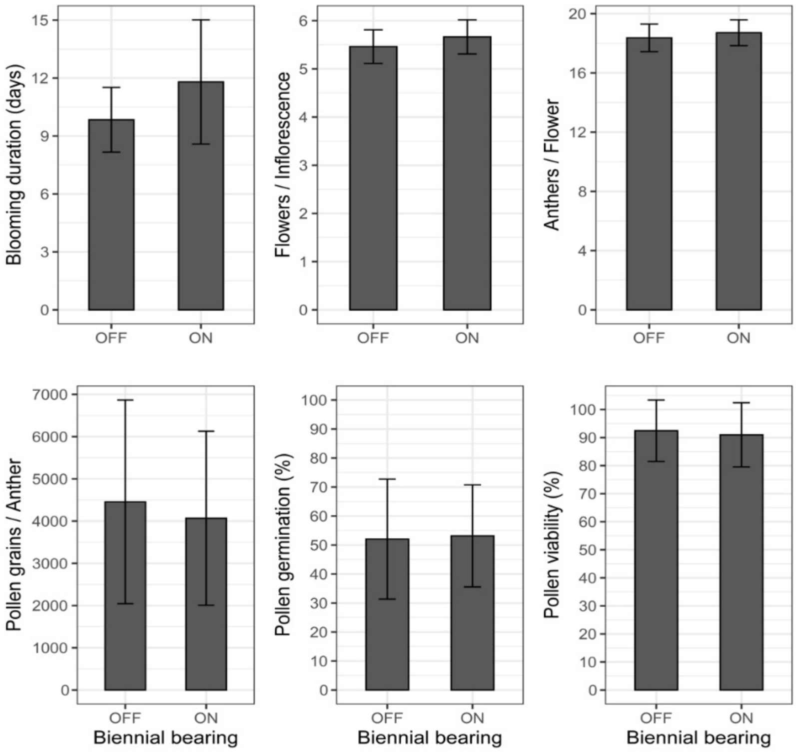

Figure 2. Effects of biennial bearing on floral and pollen biology attributes among a set of apple cultivars in Villaviciosa (north-western Spain). The biennial bearing effect is expressed as the difference in the mean values between the 'on' and 'off' years for six flowering-related traits. The vertical line shows the standard error of the mean.

3.4. Correlations between Floral and Pollen Traits

Among two successive seasons of phenotypic observations, Pearson´s correlation coefficients indicated that most of the studied traits were poorly correlated to each other (Figure 3). A moderate to low positive correlation was observed between pollen germination and pollen grains per anther (Pearson's correlation coefficient; $r = 0.32$, $p = 0.01$). A negative correlation between flowering dates (day of the year) and the number of flowers per inflorescence was also found ($r = -0.36$, $p = 0.004$). A weak positive correlation was observed between bloom dates and pollen viability ($r = 0.3$, $p = 0.015$) and between the number of inflorescences/cm^2 of trunk cross-sectional area and the number of flowers per inflorescence ($r = 0.28$, $p = 0.021$). There were not significant correlations among the other variables at $p < 0.05$ (Figure 3).

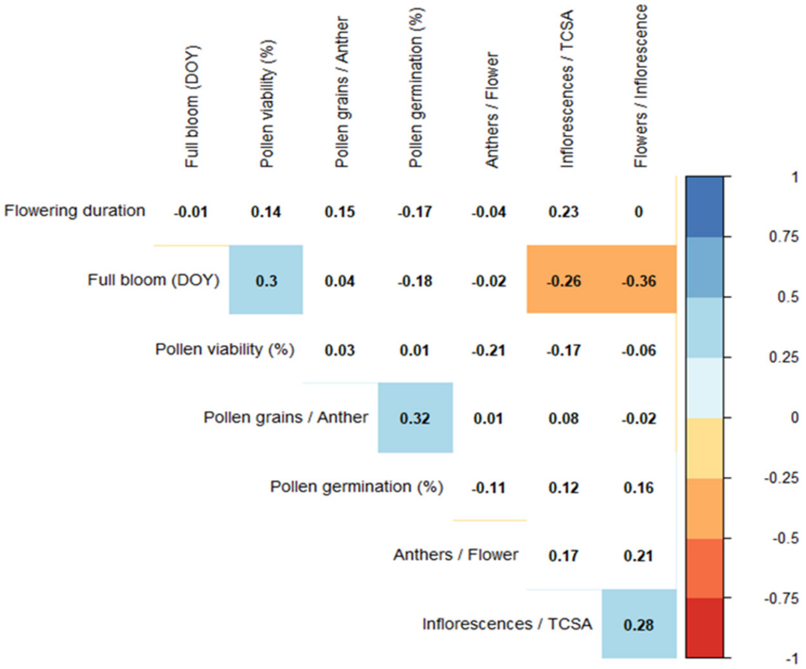

Figure 3. Pearson's correlation coefficients between floral and pollen characteristics used to characterize 45 apple cultivars in Villaviciosa (north-western Spain). The color gradient shows the strength of the correlation. Correlations that were not significant at $p < 0.05$ are represented by white boxes.

3.5. Evaluation of Apple Cultivars as Pollen Donors in Apple Orchards

Cultivars with medium-high biennial bearing tendency (less than 12 inflorescences/cm^2 of trunk cross-sectional area) were excluded, hence 14 cultivars were chosen as suitable pollinizers (Figure 4). According to this analysis, the cultivars 'X9406-11', 'Perurico' and 'Raxila Dulce' have the best attributes to be used as pollen-donating trees but other cultivars such as 'Raxona Ácida', 'Perurico Precoz', 'De la Riega' and 'Raxila Rayada' seem also to have great potential.

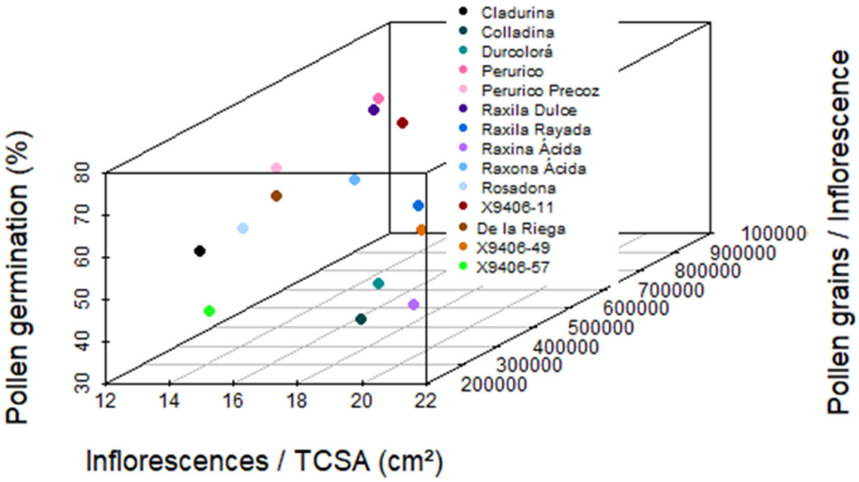

Figure 4. Three-dimensional scatterplot combining 3 variables (i.e., pollen germination (%), number of inflorescences/cm^2 of trunk cross-sectional area (TCSA)and number of pollen grains per inflorescence) for 14 apple cultivars in Villaviciosa (north-western Spain). The solid points stand for the means across the two-year data.

4. Discussion

4.1. Floral and Pollen Characteristics of Local Apple Cultivars

Blooming time and flowering duration for the same cultivar can vary considerably over years at the same location [37]. The duration of bloom is affected by temperatures and rainfall during this time [38]. Most of the previously published studies reported that the apple flowering period normally lasts between 10 and 17 days [39–41]. Across all cultivars and growing seasons, we found an average duration of 11 days, in line with a previous study in the same region by Dapena [26] who reported an average flowering time of 11.6 days across 104 Asturian cultivars. Flowering overlap is an essential prerequisite to ensure cross-pollination between trees in apple orchards [4,42]. According to our data, the possibilities of matching the flowering period of two or more cultivars are numerous owing to the broad range of flowering dates of the cultivars approved by the PDO appellation. Pollination can be more delicate in early flowering cultivars but some degree of bloom overlap between these cultivars occurred during both seasons. The only cultivar that might have had problems receiving pollen from other cultivars is the cv. 'Raxao'. This very late-flowering cultivar flowered one week later than the immediately preceding cultivar. We hypothesized that this is a direct consequence of the very high chill and heat requirements exhibited by this cultivar [43] and the suboptimal winter chill conditions observed during these two seasons [44]. Apart from the coincidence in flowering dates, the duration of the bloom period is especially relevant since a long-lasting flowering period can lead to a more stable year-to-year fruit set. In general, the average duration of flowering seems adequate, although a quarter of the studied cultivars showed a two-year average shorter than 10 days in our region. These cultivars seem to have a relatively short period of pollen-receptive flowers and this situation might imply placing in the same orchard block other cultivars featuring optimal flowering-related characteristics to enhance cross-pollination. The cultivars ´Raxona Ácida' and 'Raxona Dulce' exhibited a long blooming duration of 16 days. These cultivars can potentially be used to increase flowering overlap or even to pollinate at the same time cultivars from different flowering groups.

Minor differences between cultivars with respect to the number of flowers per inflorescence and the number of anthers per flower were found in our work. Regarding the number of anthers per flower, Church et al. [31] reported that dessert apple cultivars normally have a maximum of 20 anthers which is in agreement with our observations.

The analysis of pollen production has agronomic implications in fruit crops [45]. To achieve the best balance between pollen supply and demand in the orchard, it is important to choose cultivars which can guarantee a high production of viable pollen every year. Additionally, the ability of the insects to pollinate effectively depends on the amount of pollen transported on their bodies and the number of pollen grains that they deposit on the stigmas [46,47]. Planting trees that produce a large quantity of high-quality pollen is expected to increase the pollen loads on insects, and this aspect has a direct effect on tree productivity and fruit quality [13,22]. Our results regarding the number of pollen grains per anther are, in general, consistent with previous reports since we found on average 4348 pollen grains per anther and 81,403 pollen grains per flower for the whole set of cultivars, but large variability among cultivars was observed. Dixin and Fuyon [48] found an average of 8428 pollen grains per anther in several apple cultivars. Other studies reported between 40,800 pollen grains per flower in cv. 'Jerseymac' [49] to 123,750 in the crab apple *Malus floribunda* (Javid et al., 2019). Pollen from fruit trees is often considered viable if the germination percentage value is >25% [50]. Among diploid cultivars, a germination percentage lower than 25% was only observed in cv. 'Fuentes' in 2018 and cultivars 'Prieta' and 'Raxina Ácida' in 2019, suggesting an adequate fertilization process for the large majority of cultivars. Moreover, our results regarding the pollen properties in apple trees support the ones described by Javid and Rather [51]. These authors found that the average pollen viability and pollen germination percentages across a set of varieties in India were 95.5% and 56%, respectively. Using a 10% sucrose medium, Albuquerque Junior et al. [52] observed pollen germination rates ranging between 59.6 and 73.2%, which are similar than the ones obtained in our work.

Although floral and pollen biology characteristics are variety-specific, some discrepancies with previous studies for the same variety are most likely the result of environmental and agronomic conditions [45,53,54]. Flower induction occurs in the year preceding bloom and floral and vegetative buds need to overcome a dormancy phase throughout the winter [55,56]. Since cultivar-specific chilling requirements for breaking dormancy must be fully satisfied [56,57], insufficient chill accumulation has a detrimental impact on the quality of blooming [58,59]. In Villaviciosa (north-western Spain), chill accumulation in seasons 2018/2019 and 2019/2020 was lower than the average chill accumulation from 1978 to 2019 [43] and it is possible that some cultivars did not reach their cultivar-specific chill requirement [44]. It is important to note that despite the high degree of phenotypic plasticity in response to temperature warming shown by some of these cultivars [43], large year-to-year fluctuations in flowering dates under our typical Oceanic climate conditions were observed. Because new plantings remain productive for many years, several cultivars will be advised for commercial orchards in north-western Spain to maximize flowering overlap and to avoid pollen limitations.

Since the start of the SERIDA apple breeding program, one of the main goals was to obtain regular-bearing cultivars. The sensitivity to alternate bearing of the Asturian local cultivars had been assessed on fruit yield [23,24]. The classifications based on yield can be sometimes misinterpreted as a result of multiple factors from peak of flowering until harvest time which can affect fruit production [60]. Most of the newly registered apple cultivars obtained by the SERIDA breeding program from crosses between local cultivars and other varieties showed a low tendency to alternate bearing based on flowering intensity data compared to the local germplasm.

4.2. Evaluation of Cultivar Suitability as Pollen Donors for the Local Apple Industry

Pollen yield per flower, flowering duration, pollen viability and return bloom are the main aspects explaining the potential of a particular cultivar as a pollen donor. Information regarding these flowering-related characteristics among a wide list of local cultivars and new varieties demonstrated that not all cultivars are adequate in terms of their pollinating potential. The results showed that several cultivars produced copious amounts of viable pollen in combination with an optimal level of return bloom without the implementation of thinning treatments. If blooming periods are similar, some of the best pollen-donating cultivars are the new cultivars 'X9406-11', 'Perurico' and 'Raxila Dulce'. In addition, 'Raxona Dulce' and 'Raxona Acida' also feature good pollen properties and a particularly long flowering period. *Malus floribunda* 821 met all the previously mentioned criteria but this species blooms very early in the study region, thus flowering synchronization with other cultivars is highly unlikely.

In Asturias, most of the current cider apple orchards are designed including several traditional cultivars [26]. In this work, we provide some insights regarding the best pollen donors for the local apple industry. Several cultivars meet the requirements to act specifically as pollinizers in the hypothetical case of the implementation of single-cultivar orchards. The presence of different cultivars presumably ensures pollen availability but also brings additional horticultural management challenges. Some of these challenges include specific pruning, plant protection and harvesting [61,62], as well as the choice of the adequate planting distance. However, considering the particularities of the local industry, we believe that the advantages of planting various cultivars outweigh the disadvantages. First, it has been reported that the presence of more than one variety in the same orchard helps to prevent the spread of diseases [63]. Secondly, the diversity of pollen can also increase the amount of pollen deposited on the stigmas and at the same time, increase the chance of receiving pollen from genetically compatible donors [64]. Another aspect to consider is that local cider industries normally prefer working with a combination of cultivars belonging to different technological groups according to their total acidity and phenolic content [23]. Finally, monovarietal orchards are highly sensitive to environmental conditions, thus orchards with multiple cultivars can minimize the negative impacts on productivity due to adverse weather conditions by diversifying the risks. Contrary to the classical approach of using exclusively pollinating varieties to supply pollen to the main variety, we found among our collection a number of cultivars showing at the same time excellent agronomic and fruit qualities and good performance as pollen donors. These pollinating cultivars have a long flowering period, low sensitivity to alternate bearing, produce a good supply of high-quality pollen and apples are also marketable for cider-making.

Further research should focus on pollen donor tree density and their distribution within the orchard, as well as the effect of different pollen sources on fruit yield and quality. In addition, more investigation is needed for new releases to determine cross-compatibility among cultivars. Some of the recently registered cultivars are genetically related so it can be expected that some of them do not cross-pollinate each other well (Delgado et al., in preparation). The characterization of the S-genotype of the traditional cultivars and the new releases can be particularly useful to increase the number of potential pollen donor cultivars for the dessert apple industry. Quinet et al. [65] stated that some old cultivars have rare S-genotypes, mainly those that were not used in breeding programs. Since most of the widely grown apple varieties are genetically related [66], the discovery of rare alleles among our collection may further increase the interest of some of these cultivars as pollinizers.

4.3. Correlations between Floral and Pollen Traits and Implications for Fruit Breeding Programs

Agronomic characterization in a wide range of phenotypic traits has been thoroughly carried out at SERIDA but the genetic variability among cultivars in terms of floral biology aspects had not previously been studied in detail. Breeding new apple cultivars requires a long time and involves many steps with the selection of the most suitable parents as the first step [67]. In the case of the studied apple cultivars, no obvious correlations were found between floral biology factors. Therefore, one parameter cannot be used to predict another one or, in other words, the change in one floral trait does not elicit statistically significant changes in other traits. Nonetheless, few weak statistically significant correlations were found among the studied traits. A positive correlation between pollen germination and pollen grains per anther may indicate that a higher production of pollen in a particular year positively affects the germination of pollen grains. A negative correlation between the flowering date (day of the year) and the number of flowers per inflorescence appears to be an indication of a lower number of flowers per inflorescence in late-flowering cultivars. A positive correlation between bloom dates and pollen viability may suggest that presumably better environmental conditions prior to flowering in intermediate and late-blooming cultivars positively affect pollen development.

However, due to the great phenotypic diversity found in the apple collection, some specific characteristics can be useful for breeding purposes. For example, particularly relevant from the perspective of fruit breeding institutes is the level of return bloom. Some cultivars developed by the local breeding program such as 'Raxina Marelo', 'Raxila Rayada′, 'Colladina' and 'Raxina Amarga' showed percentages of return bloom above 70%. Because the flowering pattern appears to be more cultivar-dependent than fruiting [68], this information can be used to breed regular-bearing cultivars. Likewise, the cultivar 'Durona de Tresali' bore a low number of flowers per inflorescence (4.2) in comparison with most of the cultivars which showed on average between 5.5 and 6 flowers. This genetic characteristic may potentially reduce the need for flower or fruit thinning. Furthermore, the identification of a broad range of flowering dates among the local and recently registered cultivars is expected to be helpful to design new crosses. Crossing some of these cultivars can lead to the development of new releases that flower during a specific period, providing some practical advantages, such as avoiding frost damage or facilitating cross-pollination with other cultivars.

On the other hand, there is scarce information related to the correlations of floral biology aspects in temperate fruit trees. Regarding the interaction between pollen quality and flowering dates, Ruiz et al. [69] found a significant negative correlation between pollen germination and flowering time in apricot varieties in south-eastern Spain, suggesting that late-flowering cultivars showed lower pollen quality than early-flowering cultivars in this species. On the contrary, we found that environmental conditions in our study site seem more favorable for late-flowering cultivars.

4.4. Floral Biology Traits and Quality of Pollen Grains Affected by 'On' and 'Off' Years

Significant differences between the 'on' and 'off' year were only found for the duration of flowering. Cultivars in an 'off' year showed a shorter duration of flowering than cultivars in an 'on' year. It seems reasonable to expect that a smaller number of inflorescences in an ′off′ year can affect the length of the blooming in apple trees. Gallota et al. [70] found a significant difference for the number of pollen grains per anther between two successive years in apricot cultivars, although these authors observed that trees were able to produce the same amount of pollen grains per flower each year. In olive trees, Mazzeo et al. [28] and Methamem et al. [71] showed that trees produced fewer pollen grains but increased their values of pollen viability and germination during 'off' years to maximize ovule fertilization. Contrary to these results in olive groves, we predicted a similar efficiency in the rate of fertilization among a collection of apple cultivars. A significant trend toward both higher amount of pollen grains per anther and higher viability rates during an 'off' year was only observed for cv. 'Limón Montés'. This cultivar may have developed some adaptation mechanisms and its presence in the orchard may lead to a more stable production in the surrounding cultivars.

Nonetheless, further experimentation on the relationship between biennial bearing and flowering traits across a larger number of growing seasons, as well as a larger sample size may help to generate more conclusive findings regarding the aspects involved in the flowering behavior of this important fruit species.

5. Conclusions

Large phenotypic variability in floral biology traits was found in the set of apple cultivars even though most of the cultivars were selected in the study region and therefore have the same geographical origin. Our study covering two successive flowering seasons highlights some remarkable differences among apple cultivars in terms of flowering intensity and pollen biology attributes. Our estimations of pollen viability and germination seem sufficient for an optimal fertilization process but large differences among cultivars were found regarding the production of pollen and the number of flowers per square centimeter of trunk cross-sectional area. Despite the floral biology parameters demonstrate genetic variability and thus breeding potential, our results also indicated that the characterization of floral and pollen characteristics appears to have limited applicability in apple breeding programs. The lack of obvious correlations between traits suggests that the studied traits need to be evaluated separately since traits cannot be used to predict each other. On the other hand, significant differences in pollen quality and quantity were not observed in the 'off' year with respect to the 'on' year in apple trees, suggesting a similar efficiency of the fertilization process during two successive flowering seasons. Finally, this work is expected to be useful for local growers in north-western Spain who want to decide the cultivar selection for new apple orchards. Cultivar mixtures will be advised for new plantings due to the large year-to-year fluctuations in flowering dates observed under an Oceanic climate.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/agronomy11091717/s1, Table S1: Reported parentage and flowering time (full bloom) of 45 apple cultivars in Villaviciosa (north-western Spain), Figure S1: Pollen production, pollen viability and pollen germination of apple pollen grains, Table S2: Percentage of inflorescences in an 'on' year which flowered again in an 'off' year among a list of 24 apple cultivars, Figure S2: Flowering period of 44 apple (*Malus domestica* Borkh.) cultivars in two successive flowering seasons (i.e., 2018–2019 or 2019–2020) in Villaviciosa (north-western Spain).

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Chapter 4

Optimum sucrose concentration for testing the pollen germination of apple cultivars

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Optimum sucrose concentration for testing the pollen germination of apple cultivars

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Abstract

The germination of pollen is a key factor in the fertilization success of self-incompatible flowering trees such as apple (*Malus domestica* Borkh.). Breeding programs have a particular interest in evaluating the germinability of pollens from the male parents before performing controlled crosses. *In vitro* pollen germination is often considered a simple reliable method to determine the exact amount of viable pollen but the composition of the artificial medium has important implications. In this study, we evaluated the optimal concentration of sucrose (5, 10, 15 and 20%) in the agar-plate medium by testing the germination capacity of 8 apple cultivars. Over the concentrations tested, 15% sucrose solution gave the best results followed by 20%. The percentage of germination significantly varied among cultivars. In the 15% sucrose concentration, the highest percentage was found in 'Perurico' (95%) and the lowest in 'Raxina Dulce' (66%). These levels are sufficient for an effective fertilisation. Furthermore, the germination of pollen was not affected by the flowering date of the cultivars.

Keywords: *Malus domestica*, pollen quality, sucrose, agarose medium, breeding.

INTRODUCTION

The domesticated apple is the fourth most important fruit crop in terms of production worldwide (FAOSTAT, 2021). In Europe, there are currently 42 apple breeding programs focusing their efforts on developing varieties with desirable traits (Byrne, 2012). During the last 30 years, the apple breeding program of Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) have conducted inter-variety controlled crosses to develop new varieties in two periods: 1989-2001 and 2016-2021. During the most recent period, an average of 12 crosses per year were carried out, which translated into more than 3300 flowers hand-pollinated each flowering season. Furthermore, climate change may accelerate the development of new cultivars adapted to the new climatic conditions, making necessary to increase the number of controlled hybridizations between varieties in the following years for breeding purposes. Since the flowering time of the cultivars maintained by the SERIDA apple repository can vary up to 4 weeks (Dapena, 1996; Delgado et al., 2021a), hand-pollination crosses cannot be conducted directly in the field and pollen needs to be collected and dried up beforehand.

The development of a reliable procedure for determining the quality of the pollen of the cultivars which are going to be used as male parents have important implications in breeding programs and pollination trials. *In vitro* germination (or "pollen germinability") tests have been widely used to determine the viability of pollen in fruit crops (Bieniasz et al., 2017; Dafni et al., 2005; Luo et al., 2020). Ungerminated pollen grains or those showing poor pollen tube growth are expected to be ineffective for the fertilization of the ovules (Mayer et al., 2011), therefore the germinability of the pollen should be ideally checked before performing controlled hybridizations.

The composition of the medium used for *in vitro* pollen germination tests can significantly affect pollen metabolism (Dafni and Firmage, 2000; Patel and Mankad, 2014; Taylor and Hepler, 1997) which may have an influence on the results obtained. It has also been reported that the optimum medium is specific for each fruit tree species (Dafni and Firmage, 2000; Liu et al., 2013). Figueiredo et al. (2013) stated that boric acid is essential for the germination of pollen grains in the culture medium. Additionally, the presence of exogenous sugars like sucrose in the medium provides osmotic conditions and nutrition for the metabolism of the pollen (Patel and Mankad, 2014). For this reason, the concentration of this component should be studied for successful *in vitro* germination. The main aims of this study were to investigate the optimum sucrose concentration for testing the pollen germination of apple cultivars using the agar-plate method and to evaluate if there are differences between genotypes depending on the sucrose concentration.

MATERIALS AND METHODS

The impact of sucrose concentration on *in vitro* pollen germination was determined in eight apple cultivars spanning a wide range of flowering time in the study region. The cultivars used in this work were: 'Perurico', 'Perurico Precoz', 'Raxila Dulce', 'Raxina Amarga', 'Raxina Dulce', 'Raxona Ácida', 'Raxona Dulce' and 'Regona'. The experiments were conducted in the SERIDA experimental orchards in Villaviciosa (Asturias, north-western Spain) in 2020. Growing conditions and orchard characteristics were described in detail by Delgado et al. (2021b). A total of 60 flowers at balloon stage from each cultivar were collected when trees in the experimental block reached the stage 65 of the BBCH code (full bloom, approximately 50% of flowers open; Meier et al., 1994). Flowers were carried out to the laboratory and anthers were removed. Pollen was left to dehisce in Petri dishes at 21 °C for 48 h. The most suitable sucrose concentration for the solid agarose-medium (1.5% agar and boric acid (240 mg/L)) was evaluated by testing four sucrose concentrations (5, 10, 15 and 20%). Pollen germination was evaluated by dusting the dehisced pollen over the Petri dishes, containing approximately 25 mL of the medium, and incubated them at a constant temperature of 21 °C for 24 hours. After the incubation period, the germination percentage for each sucrose concentration was calculated by counting four replicates per cultivar. One hundred pollen grains in each preparation were observed using an Olympus SZX12 stereomicroscope (Olympus Corporation, Tokyo, Japan) and pollen grains were considered germinated when the length of a pollen tube was longer than its diameter (Hedhly et al., 2005). Germination percentages were normalized by arcsine-square root transformation prior to analysis and means were compared among treatments using the analyses of variance (ANOVA). Post-hoc analyses were performed using Tukey's tests to determine the differences among concentrations and cultivars. All analyses and figures were developed in the R programming environment (version 4.0.4; R Core Team, 2021).

RESULTS AND DISCUSSION

The sucrose concentration had a significant effect on pollen germination ($F_{3,28}$ = 45.32, P < 0.001). Pollen germination rates increased up to 15% sucrose concentration and decreased at 20% (Figure 1). The highest percentage of germination for the set of studied cultivars was observed in 15% sucrose concentration (85%) followed by 20% concentration (70%). The difference between 5 and 10% sucrose concentration was not statistically significant. The highest pollen germination rates for all cultivars except cv. ´Raxina Dulce´ were obtained from the 15% sucrose concentration plates (Table 1). This only exception indicates a general effect rather than a genotype dependent effect, suggesting that a 15% sucrose concentration should be used to assess batches of pollen from apple breeding programs.

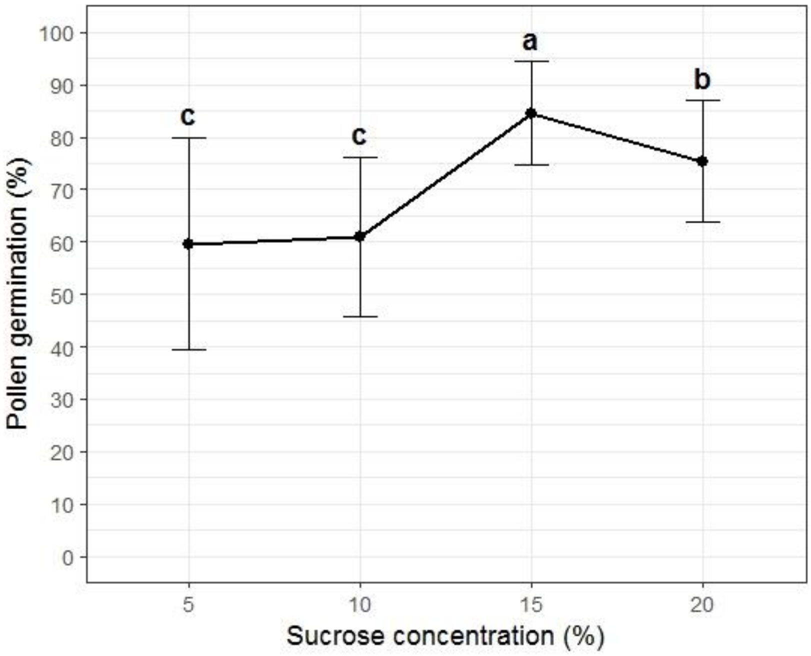

Figure 1. Mean germination percentage *in vitro* of pollen grains of eight apple cultivars subjected to different values of sucrose of the agarose culture medium. The vertical lines on each point indicate the standard deviation. Different letters represent statistically significant differences at $p < 0.05$.

It is important to note that pollen bursting was not observed under any of the sucrose concentrations examined. The osmotic pressure in pollen cell walls seemed adequate even with the highest concentration tested in this experiment (20%). The mean pollen germination percentages across all sucrose concentrations significantly differed among genotypes ($F_{7,\ 117}$ = 17.92, P < 0.001; Table 1). Our results show a high level of variation in pollen germination values across the set of local cultivars and new releases from the local breeding program. Across all tested concentrations, the highest pollen germination rates were obtained in cultivars ´Raxona Ácida´ and 'Perurico' whereas the lowest was observed in cv. ´Raxina Dulce´ (Table 1). When only considering the samples from the medium containing a 15% sucrose concentration, which appears to be the optimal, all cultivars but 'Raxina Dulce' showed a percentage of germination above 75%. From an agronomic perspective, it is widely known that pollen germination greatly affects fruit set in temperate fruit trees. According to Santos et al. (1996), pollen is considered viable if the germination percentage exceeds 25%. Results indicated that the pollens from all the analysed cultivars were suitable for an optimal fertilization process as previously reported by Delgado et al. (2021b) for the same cultivars.

Table 1. The effect of sucrose concentration on pollen germination (%) in eight apple cultivars. Different letters represent statistically significant differences at $p < 0.05$.

Cultivar	**Sucrose concentration (%)**				
	5	**10**	**15**	**20**	**Mean**
Perurico	82 ± 5.2	77 ± 7.7	95 ± 2.5	80 ± 6.6	83 ± 5.2 ab
Perurico Precoz	74 ± 7.1	68 ± 10.8	85 ± 5.3	83 ± 8.7	77 ± 10.2bc
Raxila Dulce	25 ± 3	66 ± 11.9	92 ± 1.8	78 ± 11.6	65 ± 27.1 de
Raxina Amarga	63 ± 7.1	60 ± 3.3	86 ± 3.5	60 ± 4	67 ± 11.9 de
Raxina Dulce	43 ± 2	36 ± 9.6	66 ± 12	67 ± 6.1	53 ± 16.4f
Raxona Ácida	83 ± 6.4	73 ± 8.9	92 ± 4	90 ± 2.9	84 ± 9.5 a
Raxona Dulce	50 ± 7.5	46 ± 7.2	78 ± 3.8	68 ± 7.2	60 ± 14.6ef

Regona	57 ± 12.9	60 ± 6.7	85 ± 3.4	79 ± 10.8	70 ± 14.7 cd
Mean	60 ± 20.2 c	61 ± 15.3 c	85 ± 9.8 a	75 ± 11.6 b	70 ± 18.1

On the other hand, we did not find a relationship between full bloom dates (Table 2) and germination rates using the optimal sucrose concentration (coefficient of determination = 0.098). This appears to be an indication that there is no effect of the flowering date on pollen viability in contrast to Hedhly et al. (2005) and Ruiz and Egea (2008) who reported a significant correlation in apricot cultivars.

Table 2. Full bloom date expressed as the day of the year (DOY) of 8 apple cultivars in Villaviciosa (north-western Spain) in 2020.

Cultivar	**Flowering date (DOY)**
Raxila Dulce	111
Perurico	121
Raxina Amarga	121
Raxina Dulce	125
Regona	126
Perurico Precoz	128
Raxona Dulce	131
Raxona Ácida	132

Previously published studies assessing *in vitro* pollen germination showed disagreements over the most suitable sucrose concentration for apple cultivars. Eti et al. (1998) reported the highest germination average across three apple varieties in the 20% sucrose concentration. Other authors obtained the highest germination rate with 10% sucrose solution (Bist, 1985; Chauhan et al., 2008; Kumar, 1996) in contrast with Sharma et al. (2005) and Öztürk (2005) who recommended the 15% sucrose solution as the optimum concentration which is in conformity with the findings of the present study.

Similar variability across studies regarding the optimal sucrose concentration has been also reported in other fruit tree species of the Rosacea family. In line with our results, Asma et al. (2008) evaluated a collection of apricot cultivars and obtained the highest germination percentage from the 15% sucrose treatment followed by 20% and 10%. In sweet cherry cultivars, Beyhan and Karakaş (2009) reported better germination percentages on a media supplemented with 20% sucrose solution. In peach cultivars, Milutinović et al. (2006) found that most of the cultivars showed the highest percentage of germination in a 15% sucrose solution. Bayazit et al. (2011) found that several almond species exhibited the highest rate in a medium containing 10%, although some *Prunus dulcis* genotypes showed higher germination percentages in the 20% sucrose solution. This variability across studies implied that there are some knowledge gaps regarding the mechanisms by which pollens from different species or cultivars of the same species require a cultivar-specific sucrose concentration.

Finally, it is worth noting that pollen performance shows a high variability from year to year depending on the environmental conditions (Delph et al., 1997). This is mainly due to the fact that pollen germination is significantly affected by the temperatures in the 24 h following its deposition on stigmas (Williams and Maier, 1977). The erratic year-to-year fluctuations in weather conditions during flowering time in north-western Spain (Delgado et al., 2021b) suggest the need for additional research on the effects of temperature and relative humidity on pollen germination.

CONCLUSIONS

In vitro pollen germination is a simple method to test pollen viability but the presence of sucrose in the medium plays an essential role in the estimates. The highest germination percentage was found in the culture medium containing 15% sucrose, suggesting that this concentration should be used by apple scientists who want to accurately evaluate the viability of pollen grains before conducting cross-pollination assays. Fruit breeders are continually searching for plant material which can be useful to achieve new breeding goals. The exchange of pollen between breeders wishing to cross genotypes from different locations is nowadays a relatively common practice. In this respect, the outputs of this study are expected to be useful to apple breeders or researchers who want to conduct pollination trials and to those who need to test pollen germinability after a shipment or a period of storage.

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GENERAL DISCUSSION

GENERAL DISCUSSION

1. Seasonal chill and heat accumulation and chill model selection

Winter climate conditions in the study site from 1978 to 2019 showed a clear tendency to become progressively milder. The mean daily temperature between November and March in the municipality of Villaviciosa was 9.33 °C. During these months, the mean daily temperature increased at a rate of 0.26 °C per decade and the number of days with temperatures below 0 °C showed a decline of 2.5 days per decade. Total chill accumulation was calculated under field conditions using the Dynamic, Utah and Chill Hours models. Under our regional climatic conditions, the Dynamic model showed a negligible decrease in winter chill availability whereas both the Utah model and, in particular, the Chilling Hours model showed a marked decline over a 41-year period. Our results are in accordance with previous reports by Fernandez et al. (2020c) and Luedeling and Brown (2011) who demonstrated that the Dynamic model is the least sensitive to temperature warming in mild winter regions. The results are also consistent with previous studies highlighting that the Dynamic model is the best available option for quantifying winter chill accumulation in mild winter climates (e.g., Campoy et al., 2013; Fernandez et al., 2020c; Luedeling and Brown, 2011; Ruiz et al., 2018; Zhang and Taylor, 2011).

Over the study period, the apple growing region of “Concejo de Villaviciosa” has maintained a high level of winter chill according to the Dynamic model. An average chill accumulation of 96 CP suggests that the temperatures observed in the study site matched to a great extent the chill-effective range proposed in the Dynamic model. Additionally, we hypothesized that a reduction in the number of days with freezing temperatures in winter, which are not effective for chilling accumulation according to the Dynamic model, might have led to minor increases in chill accumulation and this situation might have partially overcompensated a likely effect of temperature warming on other days of the season.

Regarding the heat accumulation in spring, a strong increase was found between 1980 and 2009 according to the Growing Degree Hours model developed by Anderson et al. (1986). Over the whole period, we detected an average rise of 52.5 GDH per year, with more than a half of this value explained by the strong warming trend observed in April (0.43 °C). Despite the fact that temperature increases were observed in all months, the mean temperature in February and March is still moderately low in the area of Villaviciosa. Thus, although there is a quantification of GDH, the occurrence of temperatures below the lowest threshold for heat accumulation is relatively common during these two months.

2. Determination of chilling and heat requirements of apple cultivars

An accurate knowledge of the agro-climatic requirements for flowering is a crucial prerequisite for selecting cultivars adapted to local climatic conditions and to choose parents for controlled crosses in breeding programs. The variability in published chill requirements is considered one of the biggest problems facing the fruit industry and breeding programs nowadays (Dennis, 2003; Luedeling, 2012). In this work, we found substantial differences in the chill and heat requirements estimates obtained for the same cultivar and location using two different approaches. We first evaluated the adequacy of the Partial Least Square regression methodology (Luedeling et al., 2013b; Luedeling and Gassner, 2012) due to the SERIDA Fruit Research Group has the availability of long-term phenological datasets of dozens of cultivars. The PLS regression analysis seems adequate in the delineation of the effective chilling and heat accumulation phases under a temperate oceanic climate, although it has shown some limitations in determining cultivar-specific chill requirements across different genotypes. Whereas the varietal climatic requirements appear plausible, a low variation of 8 CP between ‘Clara’ and ‘Raxao’, two cultivars whose historic flowering dates differed an average of 25 days might suggest that this approach delivered similar chill needs across most of the cultivars. Previous studies suggested that a minimum of between 15 and 20 years of flowering records are needed for producing valuable PLS results (Luedeling et al., 2013a; Luedeling and Gassner, 2012). In this work, dormancy-related climatic requirements were obtained with only 14 experimental seasons, consequently the outputs might potentially be improved by increasing the number of annual records in the following growing seasons. However, a recent study by Fernandez et al. (2021a) contradicted this statement and stated that excessive temperature variation may hamper the delineation of the chilling and forcing phases.

To reduce the uncertainties with regard the accuracy of the statistically derived chilling requirements, we sampled one-year old shoots throughout the winter period and forced them under controlled conditions to determine the end of the endodormancy phase. Under artificial forcing conditions, the CR varied from 59 (‘Granny Smith’) to 90 (‘Regona’) Chill Portions. Local cultivars exhibited a broad range of chilling requirements and differences among them seem more plausible according to their flowering dates. In addition, we found relatively small differences in the CR for the international dessert apple varieties analysed in this work compared to previous studies using similar experimental methods (El Yaacoubi et al., 2016; Hauagge and Cummins,1991; Parkes et al., 2020).

It is important to note that large variation in the determination of the agro-climatic requirements for the same cultivar was found between both approaches. The cultivars used in both experiments were ‘De la Riega’, ‘Blanquina’, ‘Xuanina’, ‘Perico’ and ‘Collaos’. The least variation between approaches according to the Dynamic model was found in cv. ‘Perico’ (9.8 CP) and the highest in cv. ‘Collaos’ (24.3 CP). Except for cv. ‘Collaos’, the other cultivars were ranked in the same position according to their specific chill requirement when both methodologies were compared. To our knowledge there are only few published studies reporting experimentally and statistically the CR and HR for a given cultivar in the same location. In a recent study, Fernandez et al. (2021a) developed a procedure for forcing potted trees to different artificial climatic conditions within the same season and delineating the dormancy phases through PLS regression. These authors compared the estimations of this strategy to the ones obtained by applying the experimental methodology of forcing shoots through winter (Fernandez et al., 2020a) and they found that the PLS procedure provided more comparable estimations for cv. ‘Elstar’ regarding the CR than the HR. In contrast to these results, we found less variability between both approaches for the HR. For example, cultivar ‘Xuanina’ was shown to require 9570 (PLS regression analysis) and 9660 (bud forcing method) GDH to reach full bloom. What seems clear in both approaches is that Asturian local cultivars are generally medium to high chill cultivars. Moreover, the chill requirements of the local apple cultivars under study were high when compared to those published for popular apple varieties in other studies (El Yaacoubi et al., 2016; Funes et al., 2016; Parkes et al., 2020).

The results of the works that I have presented in this thesis provides information of a few cultivars which represent a large percentage of the area harvested in the region and they are currently essential for the cider industries. However, there are now 76 varieties approved by the Regulatory Council of the DPO Sidra de Asturias. These cultivars were selected or characterized by SERIDA and include 18 new releases from the breeding program. Most of these new releases have in their pedigree at least a local cultivar and in some cases another cultivar originated or bred in other countries. Therefore, further work should involve the estimation of cultivar-specific agroclimatic requirements of a larger number of genotypes in order to assess their adaptability in a context of global warming. It is important to note that other early-flowering cultivars from the PDO such as ‘Clara’ or ‘Coloradona’ were not included in the trials. These cultivars and several new releases from the breeding program such as ‘Colorá Amarga’, ‘Cladurina Amargo-Ácida’ or ‘Rosadona’ are expected to have low-medium chill requirements.

On the other hand, the estimations were obtained using a common protocol in dormancy-related studies for forcing shoots under environmentally controlled conditions. However, some remarks regarding the specific forced bud method developed in this study need to be better discussed. The forcing period of 19 days was slightly adapted from previous studies as several criteria had been applied in *Malus* spp. to determine the time of endodormancy release after assessing the phenological development of buds (e.g., Cook et al., 2017; Finetto, 2013; Hauagge and Cummins, 1991; Parkes et al., 2020). In all cases, the period of heat forcing is longer than that of *Prunus* spp. (e.g., Campoy et al., 2013; Egea et al., 2003; Prudencio et al., 2018; Ruiz et al., 2007), possibly because of the higher CR exhibited by most apple varieties (Hauagge and Cummins, 1991; Parkes et al., 2020). Hauagge and Cummins (1991) evaluated the percentage of bud break in terminal buds after 21 days of forcing conditions. More recently, Parkes et al. (2020) determined the CR of apple varieties defining the end of the endodormancy phase as the time when 50% of the buds reached the green tip stage after 2 weeks of forcing. The latter authors reported severe inconsistencies between the outputs of the cultivar-specific CRs determined experimentally and the observed productivity in Australian apple orchards. They also indicated that forcing the experimental shoots for a period exceeding two weeks together with a lower budbreak threshold can capture an earlier shift into ecodormancy. In our opinion shorter periods than 19 days were not suitable because they may lead to an overestimation of CR and possibly an underestimation of HR. Based on our experience from previous years, the decision of choosing a 50 percent budbreak threshold implies ignoring the variability in the percentage of budbreak among the shoots sampled the same day by averaging. Campoy et al. (2019) suggested that CR estimations based on 50% bud break might underestimate the number flowering buds require to achieve optimal yields in commercial sweet cherry orchards where many flowers are required. To avoid this effect, we believe that the decision of establishing the end of the endodormancy phase as the moment when a minimum of 50% of the buds on a minimum of 50% of the shoots reached the green tip stage allows us to assume that in apple orchards, where flower or fruit thinning is normally recommended, the procedure developed in this study can guarantee that the estimations of agro-climatic needs lead to commercially competitive yields.

3. Agroclimatic requirements and phenological response of apple cultivars to temperature warming in Asturias

According to the results of forcing shoots in a controlled environment, the flowering time of apple cultivars in northwestern Spain is driven mainly by the chilling requirements. Our findings in northwestern Spain are somewhat surprising since the chill accumulation from November to March can be considered high in this location. In this context, it could be expected that chilling requirements are satisfied early and the flowering time is thus mainly determined by the heat requirements as previously suggested in cold winter areas such as north-eastern Spain (Alonso et al., 2005). This fact can be partially explained by the slow heat accumulation under a typical Oceanic climate in February and March and more likely by the high varietal CR of the local cultivars.

On the other hand, recent warming has affected tree phenology worldwide, but the phenological response depends on the species and geographical area (Shi et al., 2017). The results of the work I have presented in this thesis reveal that the blooming dates of local apple cultivars in Villaviciosa have experienced minor changes despite the mean temperature increased by 0.30 °C per decade since 1978. However, the cultivar´s responsiveness to temperature changes varied among the studied apple cultivars. Early flowering cultivars showed a slight tendency towards later bloom dates, whereas intermediate/late and late-blooming cultivars showed an advancing trend. A tendency towards advancing bloom dates has been reported as the most likely response to changes in regional climates (Chmielewski and Rötzer, 2001; Guédon and Legave, 2008; Legave and Clauzel, 2005) but phenological delays caused by a later endodormancy release has been also reported in species such as sweet cherry (Fadón et al., 2021), pistachio (Elloumi et al., 2013) and apple (Legave et al., 2013).

In general, Asturian apple landraces showed a high degree of phenotypic plasticity, which may explain weak phenological responses to warming conditions. This could suggest that local apple cultivars in Asturias are, to date, well adapted to the climatic conditions of the region. While the rising of average temperatures is affecting all regions over the globe, the responses to warming can differ strongly among regions for the same cultivar. Some of the local cultivars are currently being planted in other areas of north Spain and regions featuring completely different climatic conditions such as New York State (United States), where winters are much cooler than northwestern Spain. Campoy (2009) reported a great specificity in the adaptation of the apricot cultivars to each area. Hence, further research on the phenological behaviour of the same apple cultivars under different climatic conditions may help to develop a better understanding of the adaptive phenotypic plasticity in response to climate change of apple landraces.

4. Prospects of decreasing winter chill in Asturias throughout the 21st century

Significant chill losses are expected in large parts of the globe as a consequence of the increase in air temperature caused by higher atmospheric carbon dioxide concentrations (IPCC, 2018). The effect of future warming on chill accumulation levels depends on the region and the pace of the temperature increases (Luedeling et al., 2011). In Asturias, Taboada et al. (2011) predicted an increase of 0.88 °C (0.62 – 1.1 °C) in the region between 2010-2039 considering a A1B scenario (IPCC, 2007). A previously published study by Dapena and Fernandez-Ceballos (2007) reported that the average annual temperature in Asturias increased by 1.3 °C, at a rate of 0.43 °C per decade from 1976 to 2005. In this report, the authors found a significantly warmer trend than the results presented in this thesis (0.30 °C per decade), but this divergence can be mainly explained by two reasons. First, this work analysed the temperature records from 7 meteorological stations in Asturias including 4 stations situated in the interior valleys of the region and further from the ocean. The effect of large water bodies could have slowed down the increase in the average temperature of the 3 municipalities located near the sea, as is the case of Villaviciosa. The second possible reason is a likely stabilization of the temperature rise in the last 15 years as this work found during the spring months in Villaviciosa.

Winter chill has been forecasted to decrease in many of the most important growing regions for deciduous trees (Baldocchi and Wong, 2008; Darbyshire et al., 2011; Fernandez et al., 2020b; Fernandez et al., 2021b; Luedeling et al., 2011; Rodríguez et al., 2019). The work I showed in Chapter 2 predicts a slight decrease in chill availability over the course of the 21st century in northwestern Spain. Relative to the past, projected winter chill might decline by between 9 and 12 CP under an intermediate warming scenario and by between 9 and 24 CP under a pessimistic scenario. Apart from the greenhouse gas emission scenarios, the decline in winter chill varied with the choice of the Global Climate Model (GCM). Overall, GCMs projected similar values for the RCP4.5 scenario with differences between model projections becoming more evident for the pessimistic scenario, especially for the 2071-2100 period. MIROC-ESM (Watanabe et al., 2011) forecasted by far the strongest decline in chill accumulation whereas GFDL-ESM2G and GFDL-ESM2M (Delworth et al., 2006) showed the less severe impact of global warming in winter chill availability. The GCMs are useful tools for understanding the long-term impacts of climate predictions but it is not fully clear which mathematical representation can better predict changes in environmental conditions for each region. In such cases, the use of ensemble analyses in which all of the options are represented in the results seems the most appropriate approach.

Our results are in line with other studies that proved chill losses in mild winter climates (Darbyshire et al., 2016; Fernandez et al., 2021b; Fernandez et al., 2020c; Luedeling et al., 2011). However, our results suggest that winter chill levels in northwestern Spain will not be affected by climate change as strongly as most Mediterranean climate areas. By the end of the 21st century, the results of Chapter 2 indicate reductions of only about 12% for the intermediate scenario and 25% for the pessimistic scenario. Fernandez et al. (2020c) presented an exhaustive assessment of future chill levels in several locations according to the Dynamic model. These authors found a decrease trend in winter chill of 45% in Central Chile and 63% in some areas of Tunisia under the most severe warming scenario.

In a recent study, Rodriguez et al. (2019) projected the safe winter chill in peninsular Spain and the Balearic Islands using high-resolution observational gridded temperature data. This assessment projected a general decrease in safe winter chill across the country and the impact of climate change on chill availability is expected to be more severe in the south of Spain and Mediterranean coastal areas. Areas from important fruit growing regions such as Murcia or Comunidad Valenciana could suffer important chill losses in the near future, and they might be expose to low chill levels ranging from 25 to 50 CP. According to this study, for the 2071–2100 period and for the RCP8.5 setting, these regions might not surpass 25 CP most of the years. The results presented in this thesis are coincident with this work, since we have confirmed that the level of winter chill in the north of Spain is expected to be high as a consequence of the current cooler conditions in this part of the country. Nevertheless, Rodriguez et al. (2019) reported a winter chill reduction ranging from 45 to 30 CP for our study area under a RCP8.5 scenario for the 2071–2100 period. The discrepancy between the outputs from this approach and the analysis of the temperature data from a specific meteorological station seems large since a decline of 24 CP was found for the same pessimistic scenario and time slice in our study. The difference between both analyses for a pessimistic scenario might suggest that wider datasets using gridded temperature information might fail in capturing microclimatic conditions of a specific place. In conclusion, adaptation for tree fruit cultivation and especially apples appear to be much difficult in other parts of the world and Spain than what we found in this study in the Asturias region.

5. Cultivar selection to match future climate conditions

Climate change is expected to have a significant impact on future agricultural systems and adaptation will be necessary in many locations (Eitzinger et al., 2010). The selection of appropriate cultivars for a given area is crucial for reaching sustainable production levels. However, it is important to point out that the methodology used to determine the agroclimatic requirements has a decisive impact on the regional suitability assessments of fruit species (Campoy et al., 2019; Luedeling, 2012).

Historically, the risk of insufficient chill accumulation in Villaviciosa has been mainly restricted to a small number of cultivars with high chill requirements in very anomalous growing seasons (Dapena, 1996). Chapter 2 evidenced that local apple cultivars met their specific CR from 1978 to 2020 and only cv. ‘Regona’ was not able to fulfil its CR in more than 10% of all years. According to the future winter chill availability, the situation does not appear to be particularly worrying in the region in the near future in terms of the fulfilment of CRs. Hence, the already planted orchards should not experience perceptible consequences in productivity during their life span. However, the results I show in this thesis suggest that under global warming conditions, meeting the CR to break dormancy of some high-chill cider apple cultivars might become increasingly challenging, especially under pessimistic climate scenarios. In this sense, the selection of appropriate cultivars must be valid for few decades ahead since typical cider apple orchards in the region can be productive for at least 30 years which implies that climatic conditions must be suitable for them over a similar period. Moreover, in the current transition from extensive to semi-intensive or intensive production systems where the investments in new plantings are considerably higher is very important to have the assurance that the new plots will reach the potential maximum yield throughout the entire lifespan of the orchard.

In Spain, the influence of global warming on apple trees has only been studied in the northeast of the country (Funes et al., 2016; Rodríguez et al., 2021, 2019). Rodríguez et al. (2021) suggested that most of the dessert apple varieties might have difficulties in fulfilling their chilling requirements under the RCP8.5 scenario in the largest apple production areas of Catalonia (northeastern Spain). This statement is in accordance with Funes et al. (2016) who reported that warmer winters might challenge the fulfilment of chilling requirements in future periods in the same area. Both studies evaluated the viability of some of the most popular dessert apple varieties such as 'Fuji', 'Granny Smith' and 'Golden Delicious' and found that temperature warming in winter could compromise the viability of these varieties. According to literature, these varieties may require between 50 to 77 CP to overcome dormancy (El Yaacoubi et al., 2020, 2016; Erez, 2000; Funes et al., 2016; Parkes et al., 2020). Even the variety with the lowest chilling requirement ('Golden Delicious') might be jeopardized in this important production area under severe emissions scenarios. In this sense, it is important to note that all these important varieties could easily satisfy their specific climatic requirements in Asturias in both low and high emissions scenarios, provided of course the reported CR estimations are precise.

To anticipate changes, the new selections must be ideally able to fulfil their chill requirements above 90% of all years under future climate conditions (Luedeling et al., 2009a). The long-term goal of the apple breeding program will be to develop a new array of cultivars that have a CR lower than 72 CP (worst case scenario in the far future). In the short and medium-term, cultivars featuring a CR lower or equal than 84 CP should be obtained or selected. The lengthy timescales associated with the traditional breeding methodologies in apple, which are normally longer than 15 years from the selection of the appropriate progenitors until the cultivars are registered and released to industry (Sedov, 2014) may imply that important decisions must be made in the coming years. In the process of breeding cultivars with lower CRs, progenitors with low or medium chilling requirements must be used. In Chapter 1, I reported that late frost events in April and May have decreased compared to the past, hence genotypes with lower chill requirements that potentially bloom early might not be especially susceptible to early-season frost damage. Some work in this direction was initiated and several of the recently registered cultivars obtained by the SERIDA breeding program are expected to have similar or lower CR than ‘De la Riega’ (72.2 CP) based on flowering dates information (see Chapter 3).

6. Consequences of climate change for the local apple industry

A future reduction of winter chill in Asturias might probably lead to adverse consequences of insufficient winter chill in some apple cultivars. First, it is important to underline that a significant reduction in tree productivity associated to suboptimal chilling conditions was not observed in the set of studied cultivars. Only cv. ‘Regona’ showed some typical symptoms of chill deficiencies in the SERIDA experimental plots and other orchards in the region in 2019. However, a slight deficiency of winter chill did not have an impact on final crop yield in the SERIDA blocks. Even though a reduction in tree orchard productivity was not found, the typical symptoms of chill insufficiencies during dormancy such as delayed and irregular bud break were obvious in some trees. Uneven budbreak in the same tree and between trees can complicate some cultural practices. For example, thinning flowers and fruitlets to reduce the alternate bearing of local cultivars will become more difficult if there are multiple phenological stages on the same tree.

The predicted changes in environmental conditions might lead to physiological and biological changes in apple trees and this situation might have socio-economic impacts in the region. Sugiura et al. (2013) reported some effects of global warming for apples in Japan and predicted that ‘Fuji’ apples will ripen earlier and consequently they might lose some of their organoleptically properties. To our knowledge, the impacts of warmer summers in some key fruit characteristics that define the quality of the cider such as sugars, organic acids or phenolic composition have not been studied in-depth in previous studies. At present, the cider industry relies on late-ripening apple cultivars which are normally harvested between early-October and early-December (Dapena, 1996; Dapena and Blazquez, 2009; Mangas and Díaz-Llorente, 2010). While extreme hot temperatures in summer might not be a common problem under the Oceanic climate of northwestern Spain (Taboada et al., 2011), a moderate temperature increase in spring and summer will increase heat availability and this might speed up the fruit maturation, affecting the harvest season start date in most cultivars and therefore affecting the logistics and scheduling of the transformation industries. However, earlier ripening dates could have a positive effect in the climatic conditions during the harvesting period of late ripening cultivars which tend to be adverse.

In addition, climate change is likely to change the precipitation regimes in many regions (Xie et al., 2015). Another threat arising from the increase in summer temperatures is the high risk of water deficit in the trees. It is certainly possible to reach a scenario in which apple orchards in the region might end up in a transition to irrigation systems if the rainfall regime also follows a downward trend as it was reported by Dapena and Fernandez-Ceballos (2007). Another possible negative effect from a changing climate might affect the interaction of apple trees with common and novel pests and diseases but the consequences are not easy to predict.

On the other hand, Li et al. (2020) analysed some major climatic factors in Northwest China and indicated that the positive effects of climate change on apples during the next century will be greater than the negative effects in this province. Likewise, it is possible that climate change might bring new opportunities to the fruit industry of Asturias. The projected new agroclimatic conditions may potentially open the door to the cultivation of other fruit species. A significant level of winter chill during winter together with a marked trend towards warmer springs in which the risk of damaging frost events seems not to pose a significant threat may allow to exploit the cultivation of other tree fruit species such as citrus fruit, avocado, apricot or almond. Finally, it is important to highlight that the results of this thesis are primarily focused on the seasonal chill accumulation and the satisfaction of chill requirements. Cultivar replacements and the introduction of new species need to consider multiple agronomic factors to avoid maladaptation (Campoy et al., 2019).

7. Adaptation measures to changes in winter conditions

At present, the coastal locations of Asturias have enough winter chill even for high-chill cultivars but forecasted changes for the intermediate and far future suggest a notable risk for chill fulfilment of high-chill cultivars. The information I presented in this thesis reveals that some of the cultivars will need to be gradually replaced by cultivars that require lower chill to overcome dormancy. Notwithstanding some shortcomings of the methodologies developed in this thesis, the results of these works are expected to be valuable to anticipate changes and to guide the new strategies for the local apple industry during the first half of the 21st century.

Adaptation measures need to ensure the future economic sustainability of the farms. There are several management strategies to compensate insufficient winter chill in established fruit orchards, but the implementation of new horticultural practices requires substantial investments. Short-term adjustments in pome fruit orchards are very costly, especially for the Asturian fruit industry where the profits of most of the farms are low. The application of rest-breaking chemicals to artificially break dormancy demonstrated that is a likely viable adaptation option for certain situations (Elloumi et al., 2013; Erez et al., 2006). However, local growers are not familiar with such rest-breaking substances, and at present, most of the apple farms are low-input cropping systems.

A possible medium-term adaptation strategy is the choice of the rootstock. Some authors reported that the rootstock might improve bud break of tree fruit cultivars planted in warmer climates (Webster, 1995) and therefore can be an effective climate change adaptation strategy. In Asturias, a high proportion of the surface is still planted with trees grown on seedling rootstocks. If clonal apple rootstocks can reduce the requirement of chill, albeit on a small scale, traditional orchards growing high-chill cultivars might need move to semi-intensive systems.

A further long-term adaptation option in Asturias is to increase cultivation in pre-coastal valleys at higher elevations where winters are cooler and buds can accumulate enough chill. Previous studies found a positive relationship between chill accumulation and altitude (Alburquerque et al., 2008). Asturias is one of the most mountainous regions in Europe, but farm relocation depends on many factors, such as the competition with other agricultural activities (i.e., extensive cattle breeding) and the availability of suitable plots with adequate topography and soil conditions in comparation to the traditional cultivation areas. In conclusion, long-term adaptation strategies to face the negative impacts of climate change are the preferred alternative and we believe that the most efficient option is the careful selection and breeding of cultivars with moderate CRs that will be adapted to the new environmental conditions in the traditional apple growing areas.

Finally, this thesis may not be ended without calling for action on climate change mitigation efforts. Ambitious efforts to reduce CO_2 must be made in the name of the climate protection to limit the warming rate within the 1.5 °C (IPPC, 2018) and avoid the RCP8.5 or equivalent scenarios. Overall, the results I presented in this thesis coincide with earlier studies which found that adaptation to secure temperate fruit production would be much more feasible for moderate warming scenarios than for pessimistic scenarios (Barrio et al., 2021; Fernandez et al., 2020b; Fernandez et al., 2021b; Luedeling, 2012; Luedeling et al., 2011).

8. Variability of floral and pollen traits in apple cultivars from the SERIDA collection

The SERIDA Fruit Research Group has gathered precise information on tree vigour, susceptibility to pests and diseases and fruit characteristics in a large number of local cultivars (Dapena, 1996; Dapena and Blázquez, 2006). Nevertheless, the characterization of floral and pollen traits had not previously been undertaken. In-depth characterization of these traits can be relevant for genetic improvement (Costes and Gion, 2015). In this thesis, we found a large phenotypic variability in floral biology traits among a comprehensive set of apple cultivars. Some of the cultivars of the PDO label “Denominación de Origen Protegida Sidra de Asturias” are triploid and produce sterile pollen. Hence, the use of the cultivars ‘Solarina’ and ‘Carrió’ as a paternal progenitor will pose problems due to its low pollen germinability. Most diploid cultivars showed good pollen viability (>75%) and germination (>30%) rates, suggesting an adequate fertilization process for almost the entire set of genotypes in two successive seasons.

According to Kemp and Wertheim (1999), the overlapping flowering period in apple orchards can be considered short if the gap between the full bloom date of two varieties exceeds 6 days. Meeting this requirement seems very feasible in new plantings due to the wide range of flowering dates of the cultivars approved by the PDO appellation. Within the collection studied, we found a number of cultivars showing a good “double-purpose” performance. Several cultivars produce a high amount of viable pollen, have a long flowering period and exhibit low alternate bearing indexes. Additionally, these cultivars have good agronomic and technological properties for cider-making (Dapena, in preparation).

On the other hand, some inter-annual variation for the flowering related traits examined in this work was observed between two successive growing seasons, indicating an influence of the environmental conditions. In this respect, year-to-year variation in chill and heat accumulation may lead to shifts in flowering dates, affecting the degree of flowering overlap between cultivars. Although the order of flowering was fairly similar for most of the cultivars between both experimental seasons. Findings from

Chapter 2 suggest that bloom overlap should continue to be monitored over the next decades as changes in chill and/or heat levels might affect the bloom synchronization previously established for the cultivars in the region. Additionally, local adaptability is especially important regarding the flowering behaviour, since, for instance, several apple pollinizers in England were not suitable in the United States because their flowering times were not synchronized with the main variety (Kang et al., 2002). In addition, Kwon et al. (2015) and Church et al. (1983) reported that some crab apple species, typically used as pollinizers in intensive apple orchard, are susceptible to some diseases and pests and a reservoir of aphid pests. Cultivars selected by the SERIDA breeding program are resistant or low susceptible to diseases and pests including tree-damaging aphids (Dapena and Blázquez, 2004). In this way, the criterion of planting exclusively cultivars from the PDO appellation in which a precise phenotypic characterization under local climatic conditions has been conducted for many years by the SERIDA Fruit Research Group reinforces the idea of avoiding the introduction of foreign apple cultivars in the region.

The studies presented in this thesis also shed light on the pollinating potential of several widely used apple cultivars in the region. Although we believe that there is still room for improvement in this field to increase the profitability of cider apple farms, we hypothesize that the alternate bearing tendency of some local cultivars under the current management practices limit the potential gains of optimising the pollination practices to enhance productivity in these cultivars. One of the main aims of the SERIDA apple breeding program has been the development of regular bearing cultivars. In Chapter 3, we proved that the flowering density varied strongly among the cultivars studied, suggesting that the return bloom is highly genotype dependent in apple. Moreover, most of the recently developed varieties from the SERIDA breeding program have a low tendency to biennial bearing based on return bloom data.

On another note, the highest pollen dispersal in apple orchards occurs within a range of between 35 and 86 m (Kron et al., 2001). Bees are the most important pollen vectors in Asturian apple orchards (Martínez-Sastre et al., 2020). A high density of apple orchards and isolated apple trees is common in some municipalities of Asturias. There is no scientific evidence, but it is possible that pollination can be positively affected by the abundant sources of cross compatible pollen from the neighbouring apple fields, taking into account the cultivar mixtures can greatly vary between growers. Contrary to the results obtained in olive trees (Mazzeo et al., 2014), pollen viability and pollen production did not decrease in ‘on’ years in apple, suggesting that the trees are able to feed and develop a large number of pollens grains every year which can potentially bring economic advantages to local growers.

Finally, regarding the methods for determining pollen quality, we did not identify a correlation between pollen viability and pollen germination. As previous studies reported, the stain test might not be able to accurately differentiate between fresh pollen and aborted pollen. Hence, this work recommends an *in vitro* culture medium containing 15% sucrose.

9. Characterization, conservation and use of apple genetic resources

The SERIDA Fruit Research Group has selected local cultivars and developed new cultivars suitable to the current regional climate. These cultivars are recommended due to their resistance or low susceptibility to pests and diseases, productivity and juice quality. Moreover, the selected local cultivars are free of royalties and licences for growers. Varieties developed in other parts of the world can be adapted to the growing conditions of Asturias in terms of the fulfilment of chilling and heat requirements. However, a potential replacement requires a wider risk assessment and for example, some popular apple varieties are very susceptible to fungal diseases in the region. Inamahoro (2020) reported that low-chill cultivars are known for poor resistance to diseases. In line with EU Green Deal development strategy, disease resistant cultivars are vitally important for a sustainable production. In this sense, SERIDA cultivars are tolerant to fungi which makes the use of fungicides normally unnecessary.

Although it is not a specific goal of this thesis, we want to emphasize the importance of the conservation of the genetic diversity to support the development of new varieties. In this thesis, we identified a large phenotypic and genetic diversity among a collection of local cultivars. To prevent the genetic erosion and extinction of many local cultivars, the SERIDA has maintained an important collection of local cultivars and its local gene bank currently preserve *ex situ* a population of 550 local accessions. These resources are very useful to obtain cultivars well adapted to local agricultural conditions as they evolved in the region, and they might have developed mechanisms for adaptation to local climatic conditions. The phenotypic evaluation of large germplasm collections is costly and time-consuming, but this information provides a valuable asset to identify genetic diversity for breeding. For example, Guerriero et al. (2010) assessed the flowering dates of 229 apricot varieties maintained in a germplasm collection in Venturina (Italy) during an exceptionally warm winter. These authors found that many genotypes from northern climates showed a poor flowering pattern whereas cultivars from Italy exhibited a more abundant blooming this year. Similarly to this trial in Italy, the SERIDA Fruit Research Group has collected phenological information of a large number of local cultivars since 1986 which means that a large pool of valuable genetic material can be exploited in the coming years.

CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

1. Under the climate conditions of northwestern Spain, winter chill accumulation did not show a significant decrease despite temperature increases by 0.30 °C per decade since 1978.

2. A delay in the onset date of endodormancy may have counteracted the phenology-advancing effects of a warmer forcing phase in spring, so that overall bloom dates showed slight advances. The results also indicate that local apple cultivars have shown a high degree of phenotypic plasticity to respond to gradual changes in environmental conditions. However, their resilience to warming winters appears to vary across cultivars.

3. The choice of the method for estimating chill and heat requirements has a significant effect on the cultivar-specific estimates. Both statistically and experimentally derived chilling requirements of apple cultivars showed that Asturian apple cultivars are generally medium to high-chill cultivars.

4. The Dynamic model showed the least variation in estimating the cultivar-specific chill requirements across seasons, which suggests that this is the most adequate model for explaining the chill accumulation process in the mild Oceanic climate of northwestern Spain.

5. A decreasing trend in winter chill availability throughout the 21st century was found regardless of the greenhouse gas emission scenario. Relative to the past, projected winter chill might decline by between 9 and 12 CP under an intermediate global warming scenario and by between 9 and 24 CP under a pessimistic scenario.

6. Chill requirements have been easily satisfied in Asturian apple cultivars in the last four decades. However, our results indicate that high-chill apple genotypes might fail to meet their chill requirements in future decades.

7. Apple production does not seem particularly threatened, since 72 CP are expected even for the worst-case scenario. Local cultivars with low or moderate chill

requirements should be able to easily overcome the dormancy phase and remain productive.

8. Results confirm that the SERIDA apple collection presents large phenotypic variability in floral biology traits. Several cultivars in the collection and new cultivar releases from the breeding program have excellent qualities as pollen donors.

9. The weak correlations between floral and pollen traits suggests that these important characteristics in fruit production need to be evaluated separately since traits cannot be used to predict each other.

10. Cultivar mixtures are advised for new plantings to reduce the possible negative effects of a lack of flowering synchronization depending on the environmental conditions.

CONCLUSIONES

1. En las condiciones climáticas del noroeste de España, la acumulación de frío invernal no mostró un descenso significativo a pesar del aumento de la temperatura en 0.30 °C por década desde 1978.

2. Un retraso en la fecha de inicio de la endodormancia puede haber minimizado los efectos de avance fenológico inducido por el incremento de temperatura en primavera durante la fase de acumulación de calor, de modo que las fechas de floración globales mostraron ligeros avances. Los resultados también indican que las variedades locales de manzano han mostrado un alto grado de plasticidad fenotípica en respuesta a los cambios graduales en las condiciones ambientales. Sin embargo, su adaptación al aumento de las temperaturas en invierno parece variar según las variedades.

3. La elección del método para estimar las necesidades de frío y calor tiene un importante efecto sobre la determinación de los requerimientos agroclimáticos específicos de cada variedad. Las necesidades de frío de las variedades de manzano

obtenidas tanto estadística como experimentalmente mostraron que las variedades asturianas presentan unos requerimientos de frío de medios a altos.

4. El modelo Dinámico presentó la menor variabilidad en las estimaciones de las necesidades de frío específicas de las variedades entre los diferentes años de cultivo, lo que sugiere que este es el modelo más adecuado para explicar el proceso de acumulación de frío en el clima oceánico templado del noroeste de España.

5. Se encontró una tendencia descendente en la disponibilidad de frío invernal a lo largo del siglo XXI, independientemente del escenario de emisiones de gases de efecto invernadero. En relación con la acumulación de frío histórica, el frío invernal podría disminuir entre 9 y 12 CP bajo un escenario de calentamiento global intermedio y entre 9 y 24 CP bajo un escenario pesimista.

6. Las necesidades de frío han sido fácilmente satisfechas en las variedades de manzano asturianas en las últimas cuatro décadas. Sin embargo, nuestros resultados indican que los genotipos de manzano con altos requerimientos de frío podrían no satisfacer sus requerimientos de frío en décadas futuras.

7. La producción de manzana no parece especialmente amenazada, ya que se esperan 72 CP incluso para el peor escenario. Las variedades locales con requerimientos de frío bajos o moderados deberían ser capaces de superar fácilmente la fase de dormancia y seguir siendo productivas.

8. Los resultados confirman que la colección de variedades de manzano del SERIDA presenta una gran variabilidad fenotípica en los caracteres relacionados con la biología floral. Varias variedades locales de la colección y nuevas obtenciones del programa de mejora tienen excelentes cualidades como polinizadores.

9. Las débiles correlaciones entre caracteres relacionados con la biología floral sugieren que estas importantes características para la producción de fruta deben ser

evaluadas por separado, ya que los caracteres no pueden ser utilizados para predecirse mutuamente.

10. Se aconseja combinaciones de variedades en las nuevas plantaciones para reducir los posibles efectos negativos de la ausencia de sincronización de la floración dependiendo de las condiciones ambientales.

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ANNEXES

Annex Chapter 1

Supplementary materials for Chapter 1

Published in Scientia Horticulturae on 13 March 2021

https://doi.org/10.1016/j.scienta.2021.110093

This document provides additional information for Chapter 1 “Agroclimatic requirements and phenological responses to climate change of local apple cultivars in northwestern Spain.”

Table A1. Trends in daily mean, maximum and minimum temperature collected in Villaviciosa (NW Spain) during 41 years (1978–2019). The significance of changes for the annual temperature and the temperature between October and May was estimated by the Mann-Kendall test.

Month/Period	Daily temperature	Mean temperature (°C)	Temperature slope (°C/decade)
October	Average	14.40	0.40
	Maximum	20.06	0.43
	Minimum	9.52	0.29
November	Average	10.84	0.30
	Maximum	16.24	-0.12
	Minimum	6.19	0.49
December	Average	8.74	0.13
	Maximum	14.44	0.03
	Minimum	4.04	0.03
January	Average	8.24	0.41
	Maximum	13.81	0.43
	Minimum	3.55	0.29
February	Average	8.51	0.22
	Maximum	14.05	-0.05
	Minimum	3.61	0.18
March	Average	10.31	0.23
	Maximum	15.54	0.08
	Minimum	5.30	0.26
April	Average	11.63	0.43
	Maximum	16.33	0.44
	Minimum	6.81	0.33
May	Average	14.25	0.27
	Maximum	18.67	0.32
	Minimum	9.47	0.34

October- May	Average	11.14	0.29***
	Maximum	16.39	0.14
	Minimum	6.35	0.31***
1978-2019	Average	13.35	0.30***
	Maximum	18.34	0.21**
	Minimum	8.62	0.29***

*p < 0.05. **p < 0.01. ***P < 0.001.

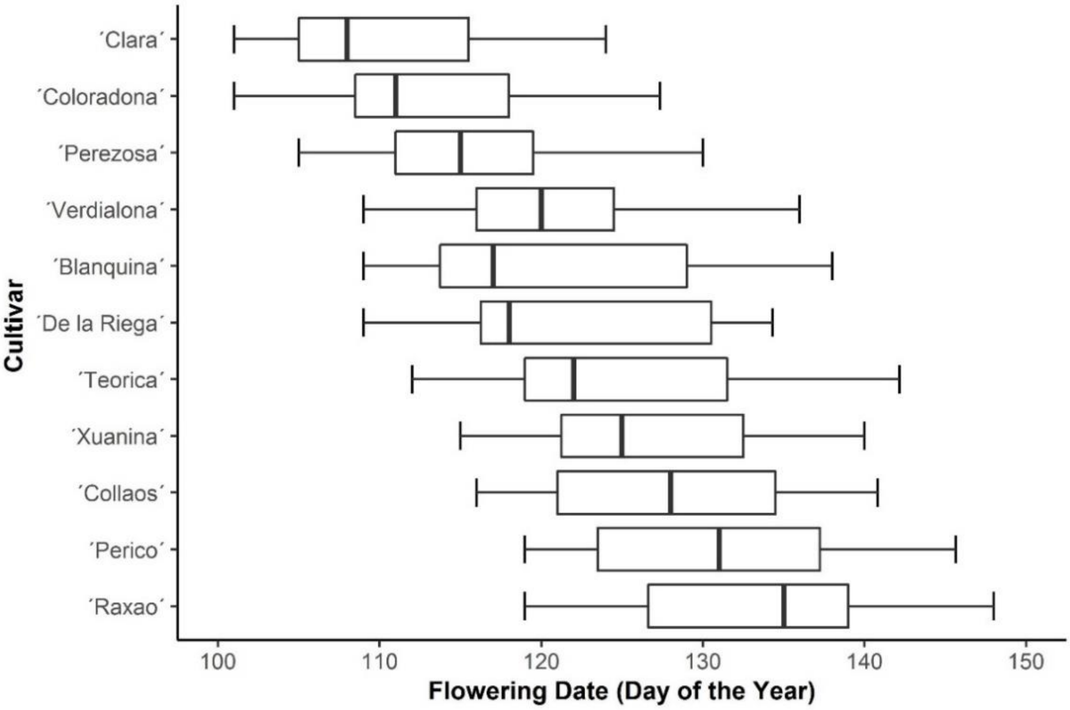

Figure A1. Distribution of flowering date records (day of the year) of 11 apple cultivars from 2004 to 2019 (with 2017 missing). The band inside the box is the median and ends of the whiskers represent variability.

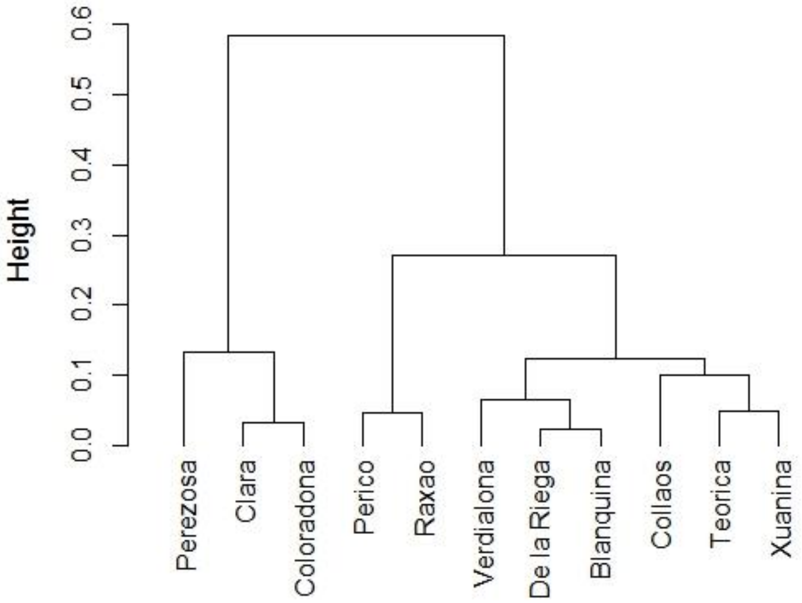

Figure A2. Dendrogram of hierarchical clustering of 11 apple cultivars based on flowering records from 2004 to 2019 (with 2017 missing).

Annex Chapter 2

Supplementary materials for Chapter 2

Published in European Journal of Agronomy on 19 August 2021

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This document provides additional information for Chapter 2. Briefly, Table A1 shows information about the origin of the cultivars used in the study, as well as some phenotypic traits such as flowering and harvest time, and fruit yield. In Table A2, we show information regarding the Global Climate Models we used in the study to forecast future chill levels in Asturias (northwestern Spain) as well as the main source of the respective models. Figure A1 shows the graphical abstract submitted to the journal.

Table A1. Reported parentage, country of origin and phenotypic characterization of 10 apple (*Malus domestica* Borkh.) cultivars in Villaviciosa (northwestern Spain).

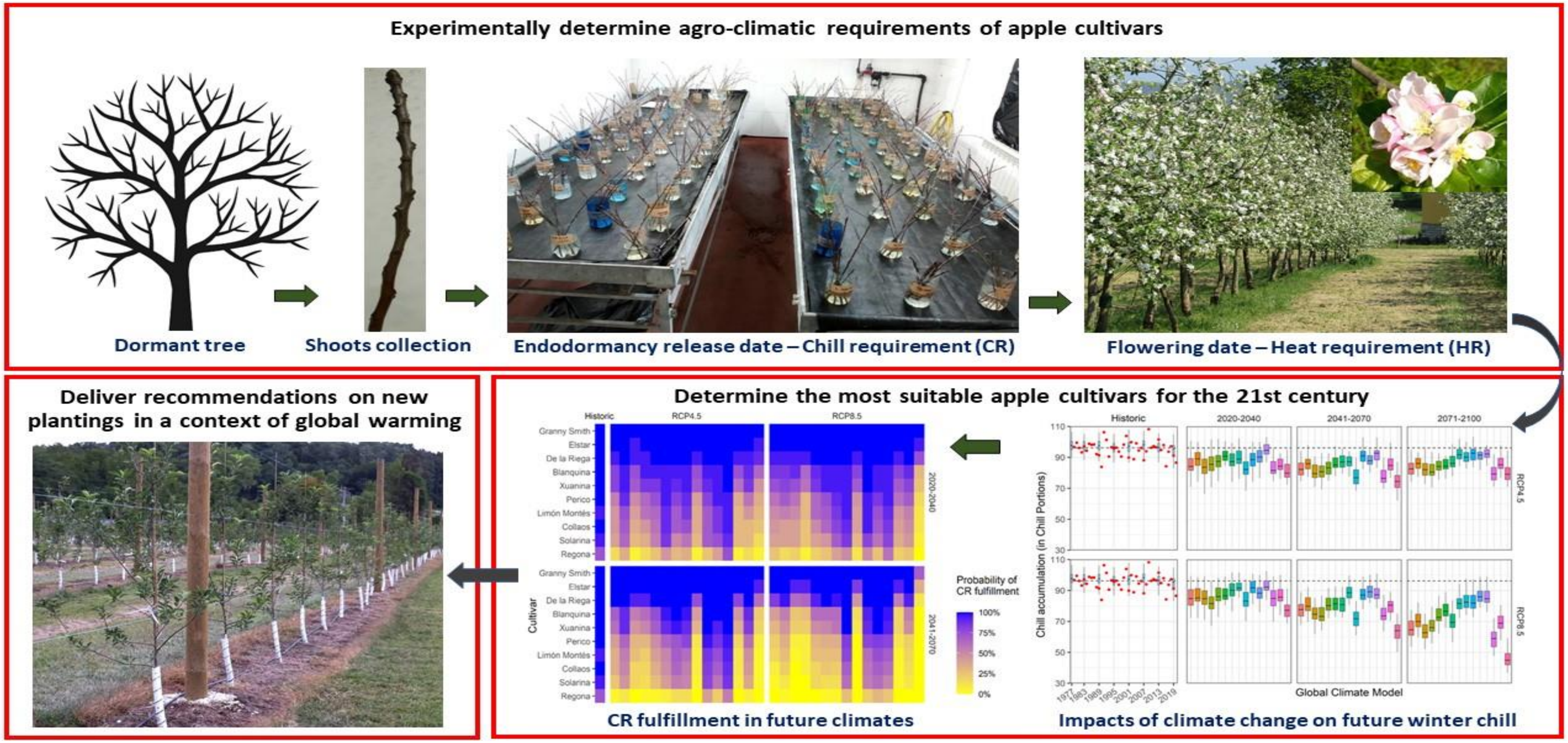
Cultivar	Pedigree	Origin	Flowering time	Harvest time	Yield	Biennial bearing	Categorization System
Granny Smith	Bred from ´French Crab´	Australia	Early	Third decade of October	>25 t/ha	Low	Sharp
Elstar	Golden Delicious x Ingrid Marie	The Netherlands	Early	Second to third decade of September	>25 t/ha	Low	Semisharp
De la Riega	Unknown	Asturias (Spain)	Intermediate/late	End of October/ first decade of November	>25 t/ha	High	Semisharp
Perico	Unknown	Asturias (Spain)	Very late	Second decade of November	>25 t/ha	High	Semisharp
Regona	Unknown	Asturias (Spain)	Very late	Second decade of November	>25 t/ha	High	Sharp-bitter
Solarina	Unknown	Asturias (Spain)	Late	Second to third decade of October	>35 t/ha	High	Semisharp
Xuanina	Unknown	Asturias (Spain)	Late	End of October/ first decade of November	>35 t/ha	Low-medium	Sharp
Blanquina	Unknown	Asturias (Spain)	Intermediate/late	Second decade of October	>25 t/ha	High	Sharp
Collaos	Unknown	Asturias (Spain)	Late	Second to third decade of November	>30 t/ha	Medium	Semisharp
Limón Montes	Unknown	Asturias (Spain)	Very late	First to second decade of November	>25 t/ha	Medium	Sharp

The information shown in this table was obtained from trees established in the apple germplasm collection at Servicio Regional de Investigación y Desarrollo Agroalimentario (Villaviciosa, NW Spain). Each cultivar had three replicates in a unique block design. More information about the local apple cultivars can be found in Dapena and Blázquez (2009).

Table A2. Global climate models used for future temperature projections in Villaviciosa (northwestern Spain). Adapted from del Barrio et al. (2021).

Organization	Model name	Abbreviation	Reference and/or link
Beijing Climate Center	Climate System Model 1.1	bcc-csm1-1	Wu (2012) http://forecast.bcccsm.ncc-cma.net/web/channel-43.htm
Geophysical Fluid Dynamics Laboratory	Earth System Models 2G	GFDL-ESM2G	Delworth et al. (2006) https://www.gfdl.noaa.gov/earth-system-model/
	Earth System Models 2M	GFDL-ESM2M	
	Climate Model 3	GFDL-CM3	Donner et al. (2011)
Institute of Numerical Mathematics	Climate Model Version 4	inmcm4	Volodin et al. (2010)
Institute Pierre - Simon Laplace	Climate Model 5A Low Resolution	IPSL-CM5A-LR	https://cmc.ipsl.fr/ipsl-climate-models/ipsl-cm5/
	Climate Model 5A Mid Resolution	IPSL-CM5A-MR	
Community Climate System Model	Version 4	CCSM4	http://www.cesm.ucar.edu/models/ccsm4.0/
Community Earth System Model	Version 1 – BioGeoChemical model enabled	CESM1-BGC	Lindsay et al. (2014)
Beijing Normal University	Earth System Model	BNU-ESM	Ji et al. (2014)
Canadian Earth System	Version 2	CanESM2	Chylek et al. (2011)
Model for Interdisciplinary Research On Climate	Earth System Model	MIROC-ESM	Watanabe et al. (2011)
Centre National de Recherches Météorologiques	Climate Model 5	CNRM-CM5	http://www.umr cnrm.fr/spip.php?article126&lang=en
Australian Community Climate and Earth	System Simulator 1.0	ACCESS1-0	Bi et al. (2013)
Commonwealth Scientific and Industrial Research Organisation	Mark3.6.0	CSIRO-Mk3-6-0	Rotstayn et al. (2010)

Figure A1. Graphical abstract of the paper “Climatic requirements during dormancy in apple trees from northwestern Spain – Global warming may threaten the cultivation of high-chill cultivars.”



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Annex Chapter 3

Supplementary materials for Chapter 3

Published in Agronomy on 13 March 2021

https://doi.org/10.3390/agronomy11091717

This document provides additional information for Chapter 3 “Analysis of the Variability of Floral and Pollen Traits in Apple Cultivars—Selecting Suitable Pollen Donors for Cider Apple Orchards.”

Table S1. Reported parentage and flowering time (full bloom) of 45 apple genotypes in Villaviciosa (north-western Spain).

Cultivar	Pedigree	Flowering time
Cladurina*	Durón Arroes x Clarina	Intermediate
Cladurina Amargo-Ácida*	Durón Arroes x Clarina	Intermediate
Colladina*	Collaos x Florina	Intermediate/late
Colorá Amarga**	Coloradona x H6419	Early/intermediate
Durcolorá**	Durón Arroes x Coloradona	Intermediate/late
Perurico*	Perico x H232	Intermediate/late
Perurico Precoz*	Perico x H233	Intermediate/late
Raxarega*	Raxao x H232	Very late
Raxila Ácida*	Raxao x Priscila	Intermediate/late
Raxila Dulce**	Raxao x Priscila	Intermediate
Raxila Rayada*	Raxao x Priscila	Intermediate/late
Raxina Ácida*	Raxao x Florina	Late to very late
Raxina Amarga*	Raxao x Florina	Intermediate/late
Raxina Dulce*	Raxao x Florina	Late
Raxina Marelo*	Raxao x Florina	Intermediate/late
Raxona Ácida*	Raxao x H232	Late
Raxona Dulce**	Raxao x H232	Late
Rosadona*	Coloradona x H6419	Intermediate
X9406-11**	Reineta Encarnada x H2310	Intermediate/late
X9406-49**	Reineta Encarnada x H2310	Intermediate/late
X9406-57**	Reineta Encarnada x H2310	Early/intermediate
Blanquina	Unknown (local cultivar)	Intermediate
Carrió	Unknown (local cultivar)	Late
Clarina (also known as 'Clara')	Unknown (local cultivar)	Early/intermediate
Collaos	Unknown (local cultivar)	Late
Coloradona	Unknown (local cultivar)	Early/intermediate
De la Riega	Unknown (local cultivar)	Intermediate/late

Durona de Tresali	Unknown (local cultivar)	Late/very late
Ernestina	Unknown (local cultivar)	Late
Fuentes	Unknown (local cultivar)	Late/very late
Limón Montés	Unknown (local cultivar)	Very late
Meana	Unknown (local cultivar)	Late
Panquerina	Unknown (local cultivar)	Late
Perezosa	Unknown (local cultivar)	Intermediate
Perico	Unknown (local cultivar)	Very late
Prieta	Unknown (local cultivar)	Very late
Raxao	Unknown (local cultivar)	Extra-late
Regona	Unknown (local cultivar)	Very late
San Roqueña	Unknown (local cultivar)	Late
Solarina	Unknown (local cultivar)	Late/very Late
Teorica	Unknown (local cultivar)	Late
Verdialona	Unknown (local cultivar)	Intermediate/late
Xuanina	Unknown (local cultivar)	Late
Granny Smith	Bred from ´French Crab´	Intermediate
Malus floribunda 821	Clone 821 of *Malus floribunda*	Very early

*Cultivars obtained by the SERIDA breeding program already protected.

**Plant material from the SERIDA breeding program in the process of registration and protection.

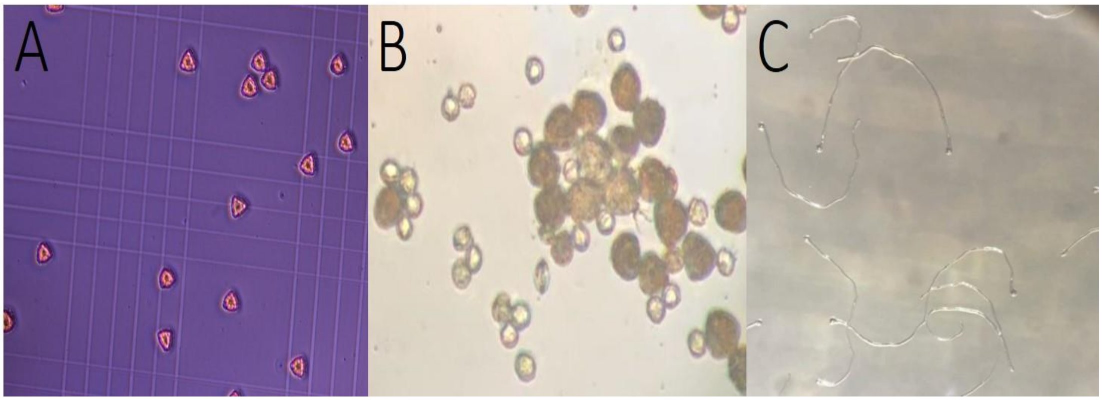


Figure S1. Pollen production, pollen viability and pollen germination of apple pollen grains. (A) Apple pollen grains stained with aqueous eosin solution in a Malassez hemocytometer (10× magnification). (B) Pollen viability test of apple pollen grains using IKI stain (10× magnification). Pollen grains stained dark are scored as viable and unstained grains are counted as non-viable. (C) *In Vitro* pollen germination in concentrated agar (1.5%) containing 10% sucrose and boric acid (240 mg/L) after 24 hours at 21°C. Pollen grains were considered germinated when the length of a pollen tube exceeded its diameter.

Table S2. Percentage of total number of inflorescences in an 'on' year which flowered again in an 'off' year among a list of 24 apple cultivars.

Cultivar	Return bloom (%)
Ernestina	0.4
Solarina	0.6
Regona	1.2
Cladurina Amargo-Ácida	9.4
Prieta	18.5
San Roqueña	20.3
Perurico	23.2
Perurico Precoz	31.6
Raxarega	36.4
Raxona Ácida	39.1
Limón Montes	41.2
Raxina Dulce	42.4
Durcolorá	43.2
Raxila Ácida	44.0
X9406-11	50.5
Raxina Ácida	55.7
X9406-49	60.1
Raxila Dulce	61.2
Colorá Amarga	66.7
Raxina Marelo	70.0
Granny Smith	79.9
Raxila Rayada	80.6
Colladina	81.4
Raxina Amarga	84.2

Figure S2. Flowering period of 44 apple (*Malus domestica* Borkh.) cultivars in two successive flowering seasons (i.e., 2018–2019 or 2019-2020) in Villaviciosa (north-western Spain). F1, F2 and G stand for the date of first bloom, full bloom and petal fall, respectively. The solid colour bars indicate the blooming duration for each flowering season. Symbol “*” stands for missing phenological data.

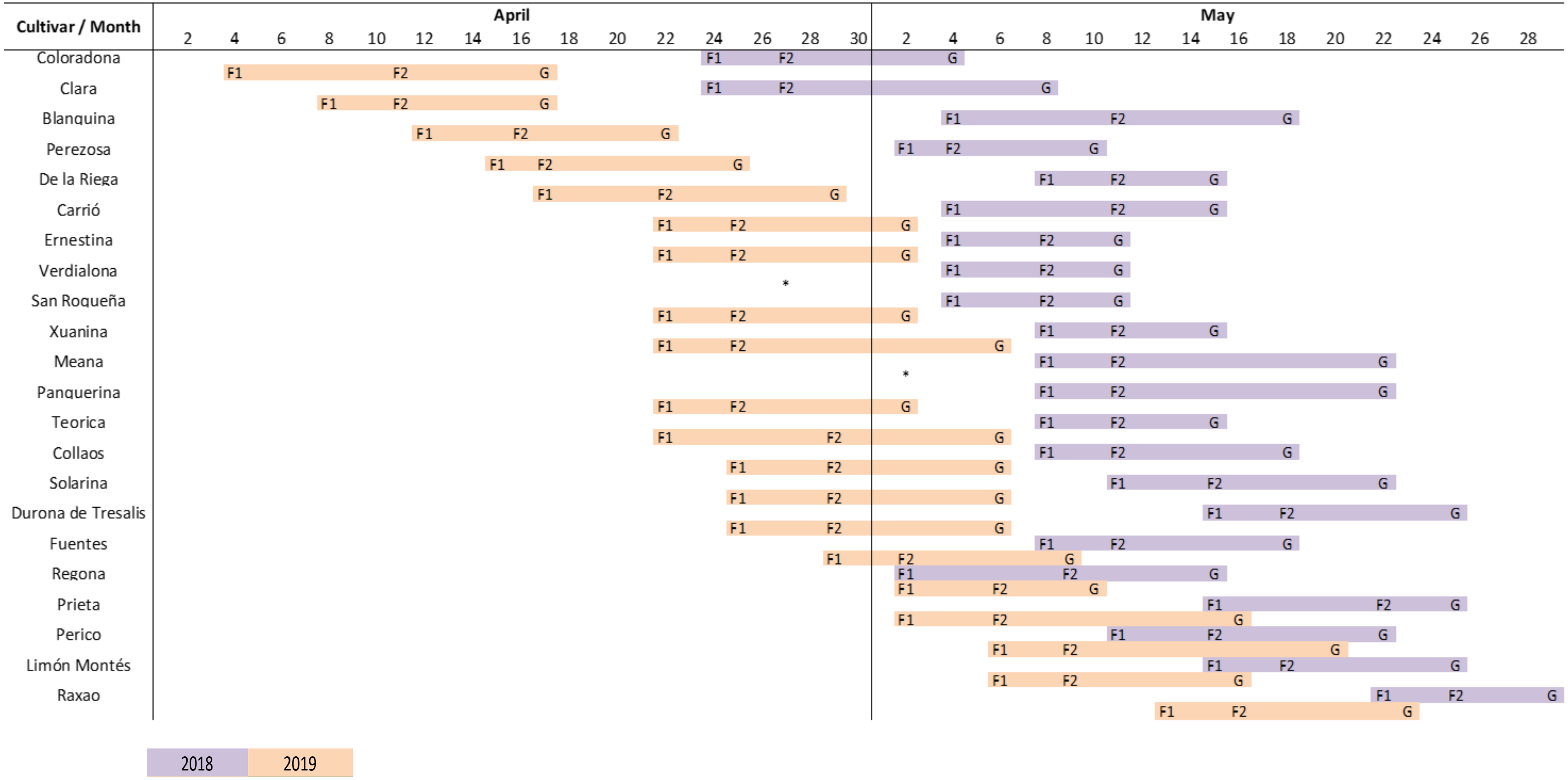
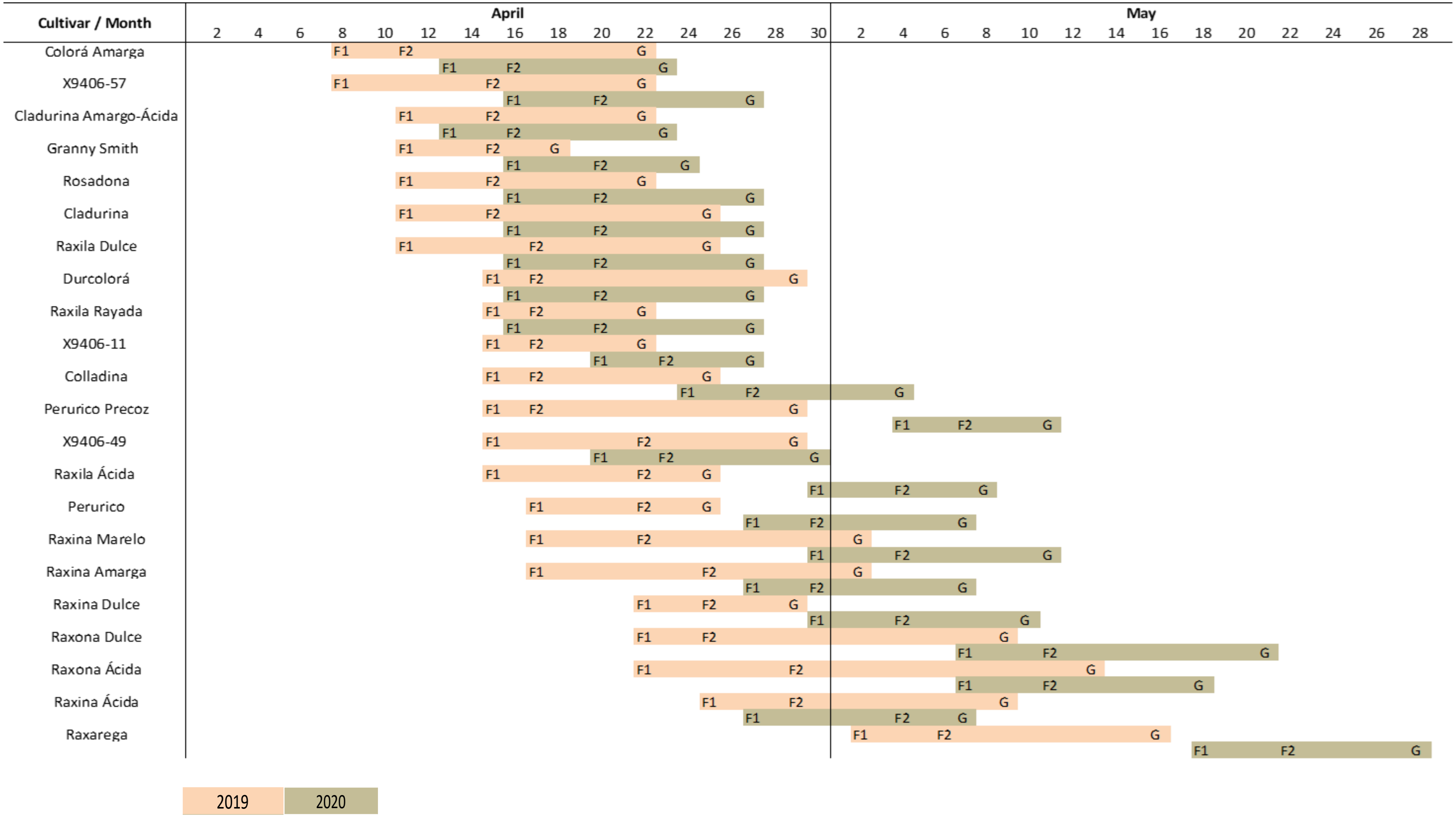


Figure S2. Continued.



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