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Research Paper

Chilling accumulation, dormancy release temperature, and the role of leaves in olive reproductive budburst: Evaluation using shoot explants

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ABSTRACT

Winter rest and release of axillary buds from dormancy have been frequently noted but scarcely studied systematically in the olive tree (*Olea europaea* L). A series of experiments was carried out over three consecutive fall-winter periods, with one-node and three-node leafy and defoliated explants from shoots sampled from non-bearing (OFF) and bearing (ON) trees and forced in a growth chamber at different constant temperatures (12.5, 15, 20 and 30 °C). Buds acquired reproductive budburst capacity after a period of chilling accumulation on the tree for both OFF and ON explants, with percentage of reproductive budburst substantially higher in the OFF explants. Reproductive budburst occurred on explants sampled from early January to the second half of February, with slight variation in dates among the different experiments and seasons. During this period, forcing temperatures and defoliation influenced bud growth and development. Reproductive budburst activity indicated that 12.5 °C forcing temperature added effective chilling and promoted earlier budburst, while 20 °C forcing was only effective in promoting reproductive budburst after buds had accumulated enough natural chilling and 30 °C forcing produced a delay in reproductive budburst. From early sampling dates defoliation promoted vegetative budburst, which was then substituted by reproductive budburst as natural chilling accumulation increased. In histological comparisons of the buds a "shell-zone" of active morphogenetic activity was observed in the third node axils of OFF buds at the start of reproductive budburst capacity, later forming an inflorescence branch by the end of the sampling period. Starch presence shown by staining increased during the natural chilling accumulation period and then progressively disappeared with reproductive budburst. These results indicate dormancy release in the potentially reproductive buds following chilling and prior to inflorescence initiation and development. In addition to the developmental information they provide, the results permit standardizing the protocol to determine the period of dormancy release in olive tree reproductive buds.

1. Introduction

Winter dormancy is an annual developmental phase in deciduous polycarpic plants characterized by total shedding of leaves and generalized dormancy of the buds. In summer, floral induction and differentiation progress until the leaves abscise in autumn, when reproductive and vegetative buds are clearly differentiated with macroscopically visible inflorescences and floral whorls (Saure, 1985; Westwood, 1993). Dormancy allows fruit trees to survive the cold winter and to undergo synchronized budburst once chilling accumulation removes dormancy and spring temperature is favorable to bud growth and development (Campoy et al., 2011; Faust et al., 1997; Saure, 1985; Westwood, 1993).

Olive (*Olea europaea* L.) is an evergreen fruit tree originated and cultivated in the Mediterranean Basin, a region with mild winter temperature, on which fruit-producing inflorescences develop from axillary buds of the leaves on the previous year shoots (Rapoport and Moreno-Alías, 2017). The winter rest period starts when shoot growth ceases in autumn and dormancy becomes generalized in all axillary buds. This period lasts until favorable temperature for bud growth and development returns, and reproductive budburst can occur following sufficient chilling accumulation (Rallo and Cuevas, 2017; Rallo and Martin, 1991).

Abundant crop years (ON years) alternate with low crop years (OFF years) in the olive tree. This well-known biennial bearing fruiting habit (Almeida, 1940; Lavee, 2007; Rallo and Cuevas, 2017) is caused by: a) the reduction of vegetative growth due to assimilate competition by fruiting, and b) the inhibition of the transition to flowering by the

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presence of developing fruits. This inhibition has been associated with permanent changes in leaf chlorogenic acid levels (Lavee et al., 1986) and with the rise of endogenous gibberellins from the seeds (Stutte and Martin, 1986), and hypothesized as a form of "biochemical memory" which affects expression of flowering locus genes (Haberman et al., 2017). Gibberellic acid applied to the tree between anthesis and autumn (Fernandez-Escobar et al., 1992) also inhibited bloom the following year. Thus the number and the fate (vegetative or reproductive) of axillary buds are determined by the presence or absence of developing fruits in the year previous to their sprouting and development.

Up to the present there is no morphological evidence of a floral transition in olive axillary buds during the year of their formation. Comparative anatomical studies before the winter showed differences in node number between buds formed in early or late spring, but in samples from both ON and OFF trees (Ramos, 2000). Internodes of ON-tree buds (potentially vegetative) sampled from July to December also increased length, in contrast to null elongation observed in samples from OFF trees (Fabbri and Alerci, 1999; Ramos, 2000), confirming an earlier arrest of growth in potentially reproductive buds (Rubio-Valdés, 2009). Nevertheless, reproductive axillary buds can only be clearly distinguished from vegetative buds at end-of-winter budburst (Almeida, 1940; De la Rosa et al., 2000; Fabbri and Alerci, 1999; Haberman et al., 2017; Hackett and Hartmann, 1963, 1964, 1967; Hartmann, 1951; Rubio-Valdés, 2009; Troncoso, 1967), and the lack of previous differentiation and visual recognition has contributed to the poor understanding of the role of winter rest in olive reproductive development.

Early studies focused on effective temperatures during winter rest for olive tree reproductive budburst and flowering have suggested a role of low temperature in olive bud floral initiation. Hartmann (1953) and Hartmann and Porlingis (1957) related the number of accumulated hours below 7.2 °C with flowering intensity the next spring. Later, Hackett and Hartmann (1963, 1964, 1967) found that 12.5 °C was an optimal temperature for floral bud differentiation. Other studies concluded that alternating 15/2 °C (maximum/minimum) temperature was more effective than constant 12.5 °C for floral development (Hartmann and Whisler, 1975). Recently genomic evaluation in olive plants has confirmed the accumulation of genes related to flowering in response to exposure to cold temperature below 15 °C (Haberman et al., 2017).

As well as floral initiation, Rallo and Martin (1991) demonstrated a low temperature role in releasing dormancy of potentially reproductive olive tree buds. In those studies using leafy explants from bearing (ON) and not bearing (OFF) trees and natural and controlled-environment chilling accumulation, results demonstrated that 7.2 °C was sufficient to complete chilling requirements, while 12.5 °C provided both chilling requirement fulfillment and adequate temperature for subsequent floral bud growth and differentiation. After the first noted reproductive budburst in 5 January samples, both the percentage of developing floral buds and the rate of their development increased with chilling accumulation (Rallo and Martin, 1991).

More recently, simulation models have been developed to predict flowering time in different sites in Spain, Portugal and Argentina (Aybar et al., 2015; De Melo-Abreu et al., 2004) or budburst time (Cesaraccio et al., 2004), based on the required chilling unit accumulation for dormancy release followed by the thermal time to flowering, or on the accumulation of chill and anti-chill days, respectively.

The accumulated evidence from these different experimental approaches still falls short of clearly describing the processes and factors involved in winter rest and reproductive budburst of olive buds. Furthermore there is no study associating reproductive and vegetative budburst requirements at the macroscopic level with the anatomical changes associated with the onset of floral differentiation. Thus many questions remain to be answered to better comprehend the role of chilling in releasing olive tree potentially reproductive buds from dormancy. What is the calendar of reproductive budburst in relation to effective winter chilling accumulation? What are the factors that influence floral reproductive budburst and when do they act? Once chilling accumulation is sufficient to allow reproductive budburst, what effects are generated in the buds by different temperatures? What is the first anatomical evidence of divergent morphological differentiation between reproductive and vegetative axillary buds and when does it occur? What is the leaf role in those processes? To address these questions a deeper characterization of the olive reproductive budburst process following the onset of winter dormancy is required. For that purpose, a series of experiments with olive woody explants was conducted to: 1) test the effect of natural chilling accumulation, presence of leaves and growth chamber forcing temperatures on reproductive budburst, 2) describe the morphogenetic changes, including bud differentiation and starch content, of axillary buds during winter rest and budburst, and 3) standardize the explant type (size; leafy or defoliated) and forcing temperatures for comparative studies to measure reproductive budburst.

2. Material and methods

2.1. Overview, site and meteorological data

'Manzanilla de Sevilla' adult trees grown under irrigation in the "IFAPA, Centro Alameda del Obispo", Cordoba, Spain (37°51′36.5″N 4°47′53.7′W) were selected as "source trees" on which natural chilling (in the field under actual environmental conditions) occurred. To determine the natural chilling accumulation effect on budburst capacity, shoot explants were sampled from the source trees on successive dates, placed under forcing conditions, and the behavior of their axillary buds monitored. The experiments were carried out in three consecutive autumn-winter seasons during which temperature was recorded daily at the farm meteorological station (Supplementary material Fig. S1). Bearing (ON; low expectation for flowering) and nonbearing (OFF; high expectation for flowering) trees were used as source trees. While following the same general procedure, the experimental protocol was modified over the successive seasons, adapting to the accumulated observations.

2.2. Experimental procedure and budburst parameters

One- or three-node leafy and defoliated explants were sampled from ON and OFF source trees during three consecutive autumn-winter seasons, forced in growth chambers at different constant temperatures (12.5, 15, 20 and 30 °C), and their axillary buds observed. Shoots of at least nine nodes were cut and immersed in water for transport to the laboratory. Explants approximately 10 cm long were prepared by cutting off the apex and eliminating all leaves and buds from all nodes except the top one or three according with the type of explant (one- or three-node). For the defoliated treatments, the leaves but not the buds were also eliminated in the top one or three nodes. The explants were sprayed with 200 ppm of oxyquinoline sulphate before placing in a container with wet perlite, and all explants covered with a transparent plastic framework that insure relative humidity above 80%. The containers were placed in growth chambers at 12.5, 15, 20 and 30 °C, according to the experiment, with cool white lamps that provide a photon flux density of 200–300 $\mu m/m^2 s^{-1}$. The numbers and bearing condition of the source trees, experimental periods, types of explant, and forcing temperatures of the experiments are indicated in Table 1.

For each sampling date forcing was carried out in randomized blocks with four replications and five explants per replication (Experiment 1) and three replications and four explants per replication (experiments 2 and 3) for each treatment, corresponding with the combination of source tree bearing status (ON or OFF), type of explant (leafy or defoliated, one-node or three- node), and forcing temperature. As olive buds may differentiate to reproductive structures (inflorescences), to vegetative structures (lateral shoots), or remain dormant, budburst was recorded as reproductive or vegetative. Reproductive budburst was considered to occur when bud development

Table 1
Conditions and procedures for the different experiments.

Experiment	Source tree bearing condition	Explant type[a]	Explant sampling times and repetitions (number of blocks, explants/block, buds/block)	Forcing temperature and (period[b])
1	ON, OFF	1-node	Weekly 16 Nov.- 1 March (4 blk, 5 expl, 10 buds)	30 °C (3 wks)
2	ON, OFF	3-node	18 Nov. 18 Dec. 9, 23 Jan. 7, 20 Feb. (3 blk, 4 expl, 24 buds)	12.5 °C (varied[c]) 20 °C (4 wks) 30 °C (3 wks)
3	OFF	3-node	12 Dec. 9, 23 Jan. 6, 20 Feb. (3 blk, 4 expl, 24 buds)	15 °C (5 wks) 20 °C (4 wks) 30 °C (2 wks)

[a] In all experiments, explants with and without (defoliated) leaves were prepared in equal (indicated) number.
[b] Forcing period to obtain maximum budburst depended on experiment, forcing temperature.
[c] Forcing period to obtain maximum budburst at 12.5 °C varied in relation to sampling date (see Figs. 2 and 3).

was between stages 51 and 53 (bud swelling and opening without bracts growing), while vegetative development was recognized as development between stages 03 and 07 (buds separating from the base and external bracts start elongating) according to the scale presented by Sanz-Cortés et al. (2002). Budburst capacity was measured as the occurrence of budburst when buds (on explants) were placed under forcing conditions, and these data were presented as reproductive or total (reproductive plus vegetative). Both buds present at each node were included in the observations.

During the forcing period the percentage of total and reproductive budburst was recorded weekly until maximum reproductive budburst (when the percentage of reproductive budburst reached its maximum value) occurred, after which a further week of observation was carried out to assure there was no additional budburst. The resulting forcing periods varied with respect to sampling date and forcing temperature, and are indicated in Table 1 and Figs. 1–4. Reproductive budburst period was defined as the time from the sampling date immediately before the first significant evidence of reproductive budburst to the first sampling date when maximum budburst occurred.

2.3. Protocol modifications during the successive experiments

The experimental protocol was modified over the successive seasons (Table 1), adapting to the accumulated experimental results shown in the manuscript. Briefly, in experiment 1 one-node explants were used. These were changed to 3-node explants in experiments 2 and 3 to increase the number of total buds without having to increase the explant number, and also to reduce possible effects of explant apex removal by including nodes at varied distance from the apex. Experiments 1 and 2 used both OFF and ON source trees, but only OFF were used in experiment 3 as it became clear that OFF trees provided a consistent source for buds which achieved reproductive budburst, while vegetative budburst was active in the ON trees (Figs. 2 and 3). In experiment 1 weekly sampling started in mid-November and continued throughout the experiment. As the cumulative observations showed minimal reproductive budburst for the fall/early winter period, sampling frequency was reduced to monthly in November and December (experiment 2) and then November sampling eliminated (experiment 3).

For controlled-temperature forcing, 30 °C was initially chosen as a warm temperature to optimize budburst. Additional forcing temperatures of 20 and 12.5 °C were added in experiment 2 as the explant method became regularized. The low 12.5 °C temperature was raised to 15 °C in experiment 3 to reduce chilling during forcing and thus better test the natural chilling accumulation in the source trees (Section 4.2).

2.4. Statistical analysis and calculation of chilling accumulation

For each sampling date average and standard error of the percentage of total and reproductive budburst were calculated and represented graphically. For additional comparisons of explant types and forcing conditions the Kruskal-Wallis non parametric test was used to compare all pairs of means for each sampling date (Supplementary Tables S1–S4). We also calculated the Pearson correlation between vegetative and reproductive budburst during the reproductive budburst period of all experiments (Supplementary material Table S5). Analyses were carried out with Statistix Software Program, version 10 (Statistix, Tallahassee, FL).

Chilling accumulation was determined using thermal units (TU) calculated according to De Melo-Abreu et al. (2004).

2.5. Anatomical observations

Since differentiation is initiated microscopically within the bud, complimentary anatomical observations were carried out to observe any bud differentiation on the source trees during the sampling period, and, with respect to the forcing response, to examine selected key experimental dates or conditions. During experiment 2 additional buds from ON and OFF trees were collected on 17 October, 20 November, 18 December, 9 and 23 January, and 7 and 20 February. Central longitudinal sections were prepared according to the paraffin methodology described by De la Rosa et al. (2000) and stained with toluidine blue (Sakai, 1973) for general structure or IIK (iodine-potassium iodide; Ruzin, 1999) for starch content. The longitudinal sectioning plane was that in which the first, third and fifth leaf primordium nodes (Lp1, Lp3, Lp5) are visible, in relation to the decussate phyllotaxy of the olive tree bud (De la Rosa et al., 2000; Rapoport and Moreno-Alías, 2017). To examine bud anatomical differentiation with respect to the forcing response, on 9 January supplementary explants were also collected and forced during 3 and 9 days at 12.5, 20 and 30 °C, and buds from each date and forcing temperature were processed in a similar manner.

3. Results

3.1. Response to bearing condition, natural chilling accumulation, forcing temperature and defoliation

3.1.1. Experiment 1

As sufficient natural chilling accumulated in the source trees, reproductive budburst could occur in the forced explants. Reproductive budburst of leafy explants started for 4 January samples from OFF trees and 24 January from ON trees, and continued until 15 February and 1 March, respectively (Fig. 1A and C). By the end of the experiment reproductive budburst reached maximum values close to 100% in explants sampled from OFF trees (Fig. 1A) and around 20% in explants sampled from ON trees (Fig. 1C). Differences in reproductive budburst between OFF and ON explants were significant at $P < 0.05$ by the Kruskal-Wallis test for all sampling dates once reproductive budburst had begun (Supplementary material Tables S1–S4). Under forcing vegetative budburst was also promoted, but was replaced by reproductive budburst on successive dates for OFF-tree explants, but only slightly for ON.

Defoliation delayed reproductive development by enhancing early vegetative budburst. Vegetative budburst reached and maintained values close to 100% in defoliated explants in contrast with changing

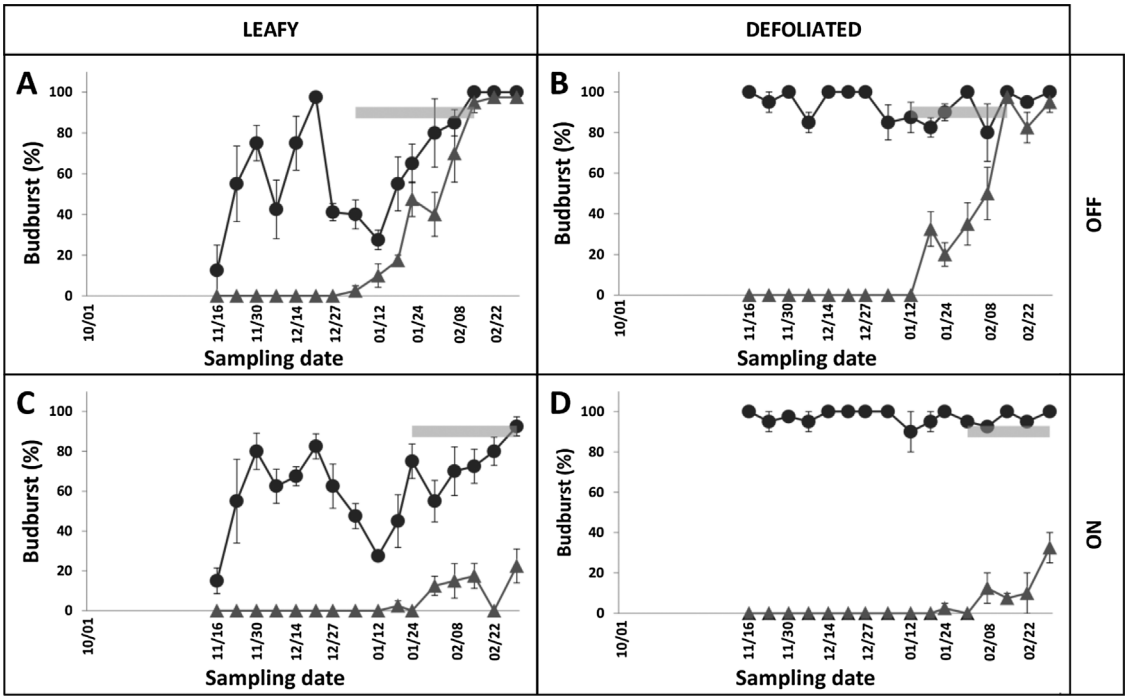

Fig. 1. Total (circles; vegetative + reproductive) and reproductive (triangles) budburst in leafy (left, A and C) and defoliated (right, B and D) one-node explants from shoots sampled from OFF (top) and ON (bottom) Trees 16 November – 7 March, and forced during 3 weeks at 30 °C. Budburst% based on 40 buds/date (2 buds/node, 1 node/explant, 5 explants/rep, 4 reps). Broad horizontal bar = Reproductive Budburst Period. Vertical bars = ± SE. Experiment 1.

values over sampling times in leafy explants (Fig. 1A vs. B). Reproductive budburst onset was also slightly delayed in the ON-tree explants relative to the OFF-tree explants, starting by late- and mid-January respectively. Then, however, the maximum reproductive budburst values and the sampling dates on which they were reached were similar between the defoliated and leafy OFF-tree explants (Fig. 1A and B) on one hand, and the defoliated and leafy ON-tree explants (Fig. 1C and D) on the other. Vegetative and reproductive budburst correlated negatively for all four types of explants (Fig. 1A–D), with respective significance of $P < 0.0036$, $P < 0.0001$, $P < 0.004$ and $P < 0.0000$ (Supplementary Material Table S5).

3.1.2. Experiment 2 (Figs. 2 and 3)

At all forcing temperatures (12.5, 20 and 30 °C), reproductive budburst increased with successive sampling date in both leafy and defoliated explants from OFF trees, reaching maximum values from 70 to above 90% (Fig. 2). For the leafy explants, reproductive budburst from OFF trees at 12.5 °C occurred for all sampling dates (from 17 October to 20 February), at 20 °C occurred for samples from 9 January to 20 February, while at 30 °C it occurred only between 7 and 20 February. This different timing of reproductive budburst expression in relation to forcing conditions is reflected in differences shown by the Kruskal-Wallis test among daily values (Supplementary material Table S2).

In all explants from ON trees, reproductive budburst was null for most sampling dates or very low on the last sampling date (Fig. 3). Accordingly no significant differences for reproductive budburst were found by the Kruskal-Wallis test in this treatment, (Supplementary material Table S3).

Defoliation influenced vegetative budburst in the explants from both OFF (Fig. 2) and ON (Fig. 3) trees throughout most of the sampling period, increasing vegetative budburst and/or delaying reproductive budburst, and presenting a notable difference between total and reproductive budburst. OFF-tree explant reproductive budburst was delayed by defoliation until the end of February, when high maximum reproductive capacity was reached in both explant types and at all three forcing temperatures (Fig. 2).

The time under forcing for explants to reach maximum budburst was consistently 3–4 weeks for 20 °C forcing and 2–3 weeks for 30 °C (Table 1). In contrast the time required to reach maximum budburst under 12.5 °C forcing decreased from 18 to 4 weeks with the sampling time (Figs. Figure 2A and B; Figure 3A and B).

3.1.3. Experiment 3 (Fig. 4)

In this experiment, using explants from only OFF trees, sampling from 12 December to 20 February, and forcing at 15, 20 and 30 °C, budburst once more increased with sampling time at all forcing temperatures. At 15 °C forcing reproductive budburst was observed after the 12 December sampling, and at 20 and 30 °C after the 9 January sampling. Reproductive budburst increase with respect to sampling date was initially more abrupt at 15 °C forcing and more gradual at 20 and 30 °C, until reaching maximum (> 60%) at the last sampling date (20 February) in all cases (Fig. 4). Corresponding with these patterns, differences ($P < 0.05$) were shown by the Kruskal-Wallis test among daily values for 9 and 23 January and 6 February samples, but not at 12 December and 20 February (Supplementary Material Table S4).

Defoliation also increased total budburst during the sampling period, with vegetative budburst decreasing as reproductive budburst increased (Fig. 4). Vegetative and reproductive budburst for defoliated explants were negative and significantly correlated ($P < 0.004$, $P < 0.0012$ and $P < 0.009$) at the three forcing temperatures. (Supplementary Material Table S5).

3.2. Chilling accumulation for reproductive budburst onset and for attainment of maximum budburst

For all of the OFF-tree leafy explant trials the dates and corresponding chilling units for the reproductive budburst onset and the attainment of maximum budburst are summarized in Table 2. The dates for the onset of reproductive budburst varied notably among experiments and among the forcing temperatures used to test bud reproductive capacity. Consistently, though, early onset dates were

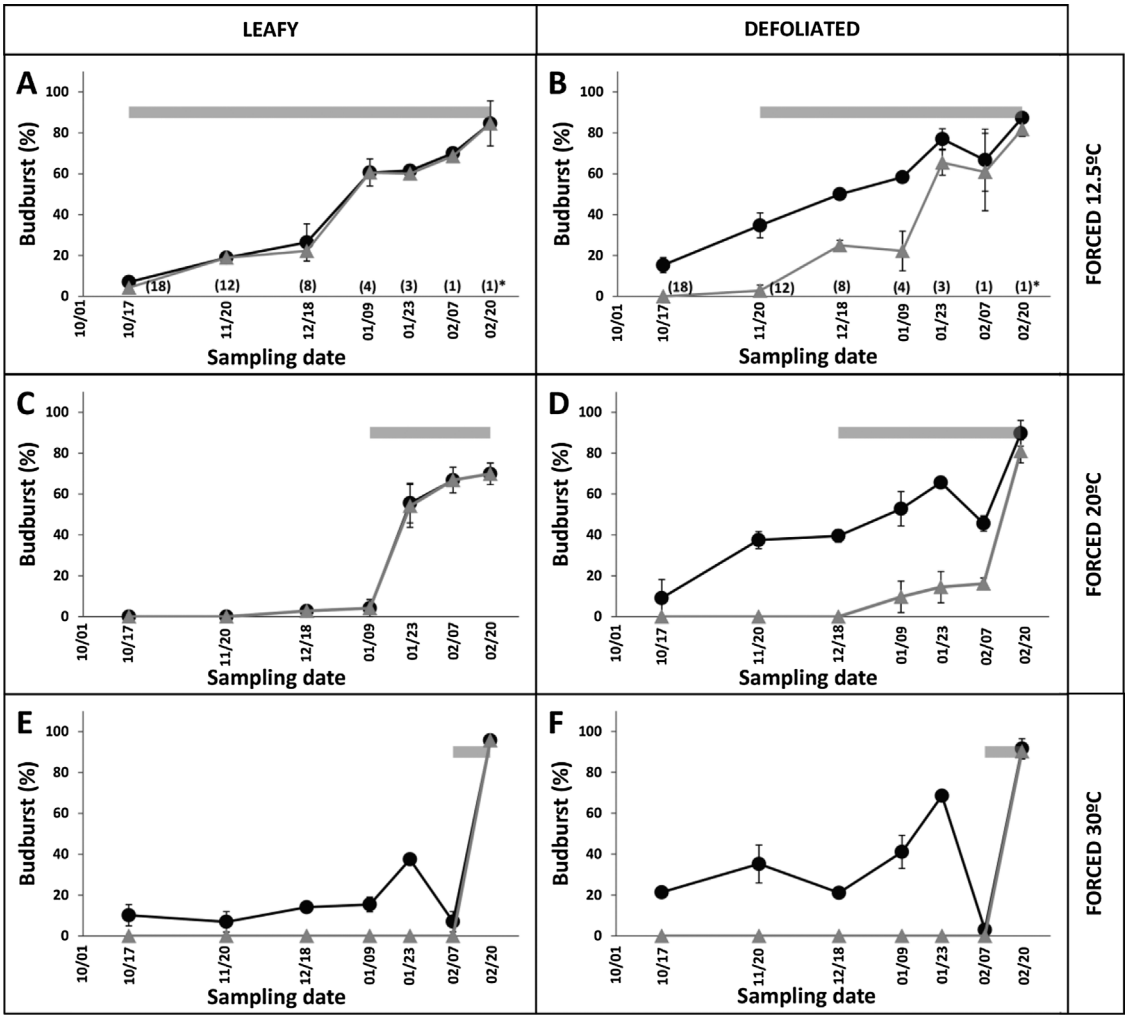

Fig. 2. Total (circles; vegetative + reproductive) and reproductive (triangles) budburst in leafy (left) and defoliated (right) three-node explants from shoots sampled from OFF Trees 17 December − 20 February, and forced at different temperatures until maximum budburst Forcing temperatures and periods: 12.5 °C for (highly) variable weeks depending on sampling date (A, B), 20 °C for 3–4 weeks (C, D), and 30 °C for 2–3 weeks (E, F). Budburst% based on 72 buds/date (2 buds/node, 3 nodes/explant, 4 explants/rep, 3 reps). Broad horizontal bar = Reproductive Budburst Period. Vertical bars = ± SE. (x)* = weeks under forcing until maximum budburst for each sampling date at 12.5 °C. Experiment 2; see also Fig. 3 (ON trees).

associated with low forcing temperature, later ones with higher forcing temperatures. Natural chilling units associated with reproductive budburst onset also varied, although they were similar for the 20 °C forcing temperature in both experiments 2 and 3. In contrast, maximum budburst occurred at similar times and with similar natural chilling for all forcing temperatures in experiments 2 and 3. Experiment 1 diverged from results of the other experiments at the same 30 °C forcing temperature, in showing earlier dates and lower chilling units for both initial and maximum reproductive budburst.

3.3. Anatomical observations of bud structure and development

From October to December, buds from previously OFF and ON trees showed similar structure consisting of either four or five nodes, each node containing two leaf primordia, and no visible changes were noted during this period (Fig. 5). In both the four- and five-node buds, the first- and third-node leaf primordia (Lp1, 3) have elongated. In the four-node buds the apex presented a slightly rounded flat appearance (Fig. 5A). Structure of the five-node buds was similar to that of the four-node buds, with the addition of two lateral buttresses on the shoot apex representing the initiation of the fifth node (Fig. 5B).

On 9 January buds from previously OFF trees showed visible activity, readily apparent as the development of a broadening ‘shell-zone’ of organized cell division in the axils of the third node (Fig. 6A). By 7 February inflorescence branch development was fully progressing in those axils, as well as in the bud apex (Fig. 6C). In contrast, no such changes, nor any others, were observed in buds from previously ON trees (Fig. 6B and D). Starch grains are densely visible in the 9 January OFF-tree buds, and present in lesser quantity on 7 February (Fig. 7A and C), differing with the minor amounts in the corresponding ON-tree buds (Fig. 7B and D).

OFF-tree buds collected from the tree on 9 January forced three days at 20 °C remain closed, with the most external primordium pair (Lp1) curved over the terminal node and no apparent external changes (Fig. 8A). Internally, however, the third node “shell-zone” has begun to produce a primordial branch (Fig. 8C). Conversely, the ON-tree bud external primordia have opened slightly and started to elongate (Fig. 8B), but their third node axils remain inactive (Fig. 8D).

The reproductive development of the four-node buds, in which the fifth node will be reproductive, can be observed in Fig. 10 Four-node buds from OFF-tree explants collected on 9 January and forced 3 days at 20 °C show branch primordium initiation in the third-node axils (Fig. 9A), similar to that of five-node buds which underwent the same conditions (Fig. 8C). Moreover, differentiation of the new fifth node starts, characterized by flattening of the bud apex to form a plateau (Fig. 9A). After 9 days at 20 °C forcing temperature the apex has started

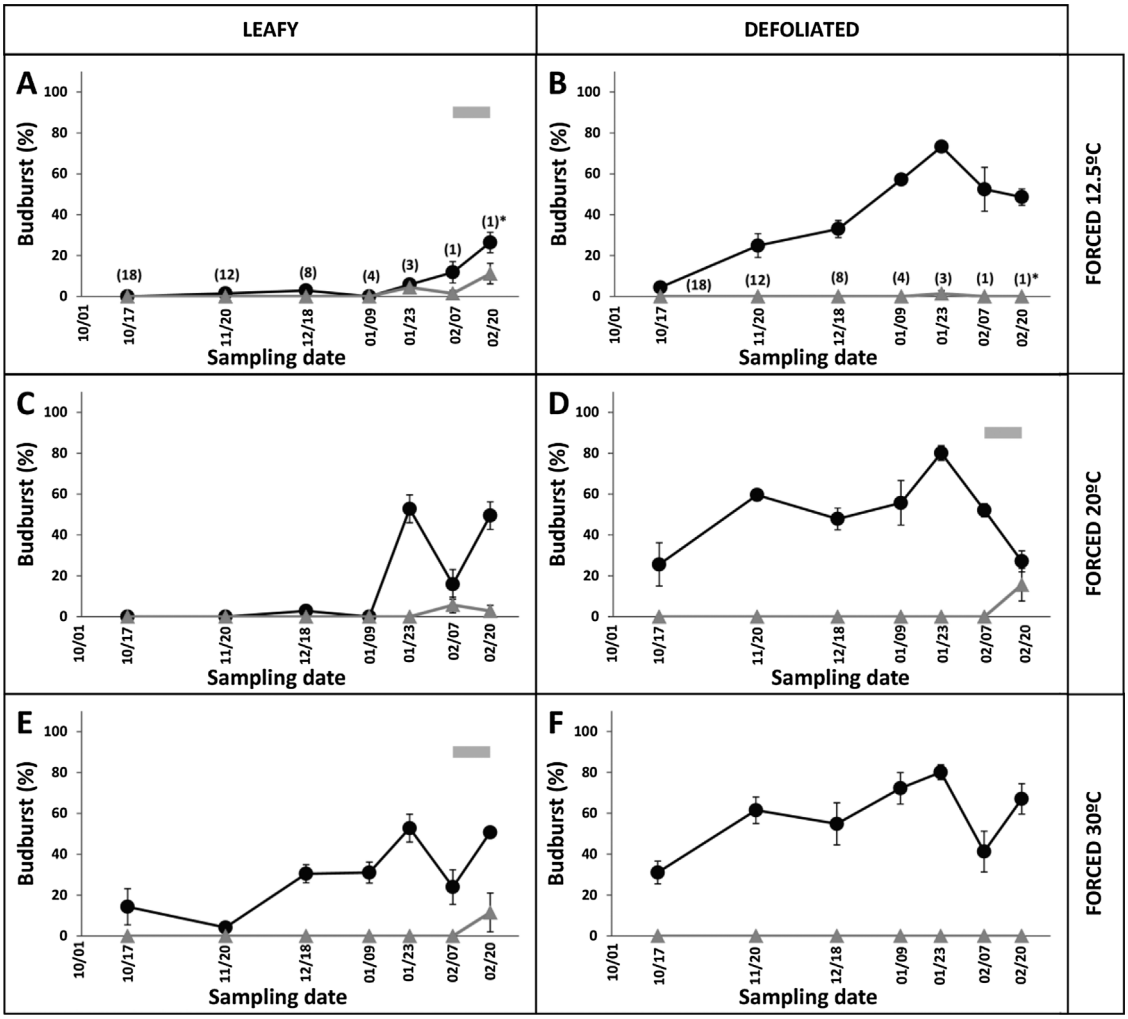

Fig. 3. Total (circles; vegetative + reproductive) and reproductive (triangles) budburst in leafy (left) and defoliated (right) three-node explants from ON Trees 17 December – 20 February, and forced at different temperatures until maximum budburst. Forcing temperatures and periods: 12.5 °C for (highly) variable weeks depending on sampling date (A, B), 20 °C for 3–4 weeks (C, D), and 30 °C for 2–3 weeks (E, F). Budburst% based on 72 buds/date (2 buds/node, 3 nodes/explant, 4 explants/rep, 3 reps). Broad horizontal bar = Reproductive Budburst Period. Vertical bars = ± SE. (x)* = weeks under forcing until maximum budburst for each sampling date at 12.5 °C. Experiment 2; see also Fig. 2 (OFF trees).

to form inflorescence branches (Fig. 9B), demonstrating a different morphogenetic pattern from that which occurs when the fifth node was formed in a still undifferentiated bud (Fig. 5B).

Fig. 10 presents buds collected on 9 January from ON and OFF trees and forced 9 days at 30 °C. In the ON-tree buds the leaf primordia and bud axis have elongated substantially (Fig. 10A), concomitant with vegetative growth, and axillary bud primordia start to develop at the third node level (Fig. 10B). In OFF-tree buds, no further development has occurred during the 30 °C forcing, as buds remain closed with no visible elongation, and the third node axils remain in the "shell-zone" status (Fig. 10C), identical to that observed in buds collected from the Trees 9 January (Fig. 6A).

4. Discussion

4.1. Experimental overview of winter rest and dormancy release in olive tree reproductive buds

Winter rest and chilling requirements for the release of axillary buds from dormancy have been little studied in olive in comparison to other fruit trees. For instance, a search from 1900 to date (end of 2016) in the Web of Science (WoS) on bud dormancy indicates 437, 415 and 25 articles on apple, peach and olive trees, respectively. A similar search on chilling requirements indicates 237, 416 and 24 articles, respectively. The low incidence of problems associated with dormancy and chilling requirements in the olive tree's traditional Mediterranean growing area must be responsible for the few studies in this species. However absence and asynchronous flowering in new olive growing areas in North and South America (Aybar et al., 2015; Castillo-Llanque et al., 2014; Hartmann, 1951; Rapoport, 2014; Rubio-Valdés, 2009) are very likely related to insufficient chilling during winter rest. Also, the global warming forecasted in the Mediterranean Basin (Ponti et al., 2014) may affect the performance of autochthonous cultivars of this area in the future. Therefore the role of bud dormancy and chilling requirements in the olive reproductive biennial cycle merits further study.

The size and morphological complexity of adult fruit trees clearly limits the possibilities of systematically testing temperature effects on different physiological and developmental processes. Since the middle of the past century explant forcing has been successfully used to determine the chilling requirements for release of deciduous fruit tree flower buds from dormancy, including the standardization of experimental units and conditions (Andreini et al., 2008; Campoy et al., 2011; Charrier et al., 2011; Erez and Lavee, 1971; Samish, 1954; Tabuenca, 1964; Walser et al., 1981; Weinberger, 1950). In a perennial evergreen species such as the olive tree it provides the additional advantage of preserving the presence and function of the leaves, enabling relatively lengthy periods under controlled environment in growth chambers and

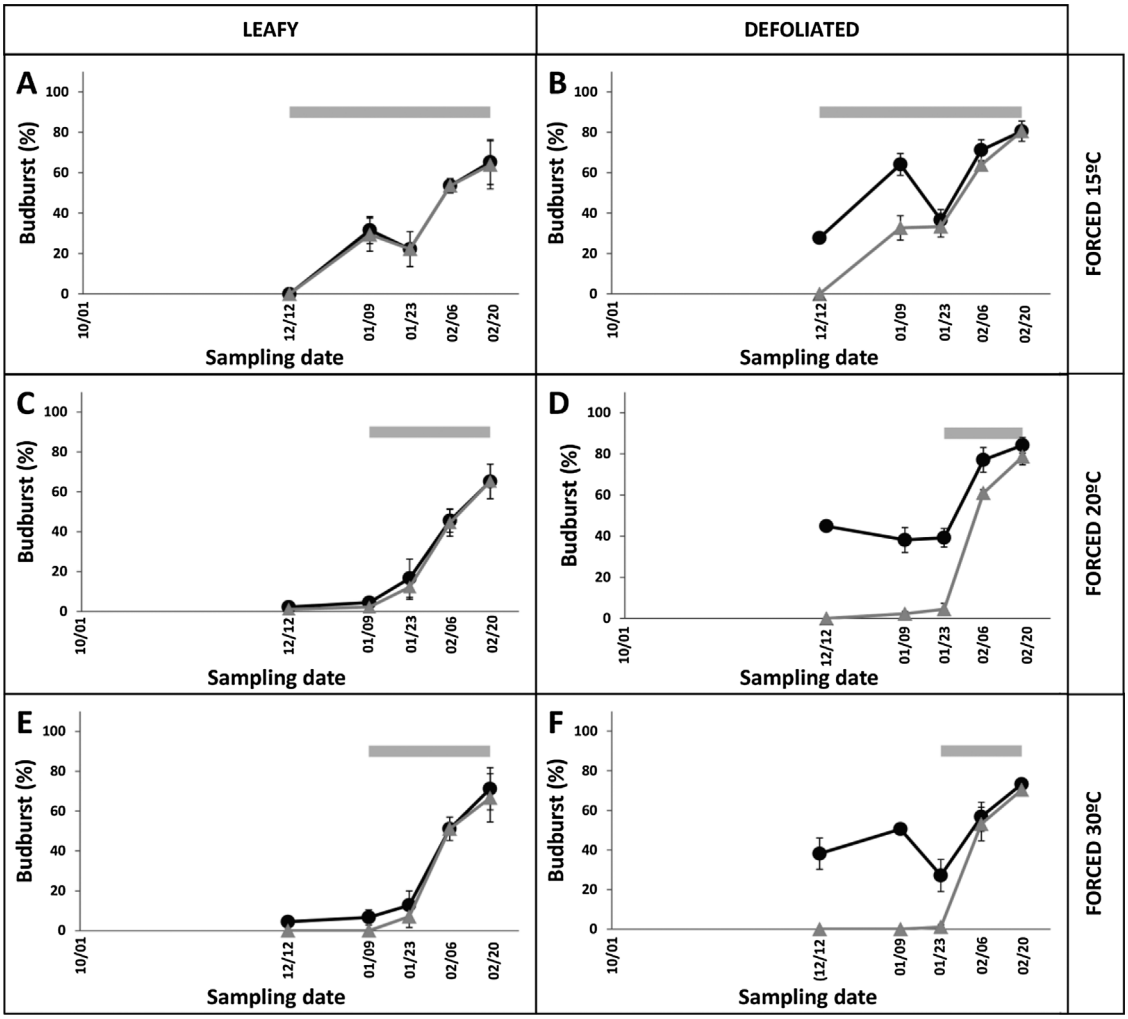

Fig. 4. Total (circles; vegetative + reproductive) and reproductive (triangles) budburst in leafy (left) and defoliated (right) three-node explants from shoots sampled from OFF Trees 12 December – 20 February, and forced at different temperatures until maximum budburst. Forcing temperatures and periods: 15 °C for 2–5 weeks (top – A, B), 20 °C for 2–4 weeks (middle – C, D), and 30 °C for 2 weeks (bottom – E, F). Budburst% based on 72 buds/date (2 buds/node, 3 nodes/explant, 4 explants/rep, 3 reps). Broad horizontal bar = Reproductive Budburst Period. Vertical bars = ± SE. Experiment 3.

in greenhouses under mist (Rallo et al., 1994; Rallo and Martin, 1991). Such standardization of experimental conditions remains a highly useful tool for testing both classical and new analytical procedures, including predictive models or molecular analyses.

4.2. Chilling accumulation releases potential reproductive buds from dormancy

In deciduous fruit trees, chilling is the factor determining the release of winter-dormant buds from dormancy. Once chilling requirements are completed, the buds acquire growth capacity, and will sprout under favorable temperature (Campoy et al., 2011; Chandler et al., 1937; Lang, 1987; Saure, 1985; Westwood, 1993). Previously in olive trees it has also been shown that progressively increasing budburst in shoot explants is related to chilling accumulation during winter (Rallo et al., 1994; Rallo and Martin, 1991). In the current experiments the total amount of flowering (*i.e.* reproductive budburst) depended on tree bearing status, with maximum reproductive budburst consistently over 65% in OFF-tree explants but under 30% in ON-tree buds (Figs. 1–3). That behavior concurs with the reported inhibitory role of developing fruits in olive tree floral induction (Almeida, 1940; Fernández-Escobar et al., 1992; Lavee et al., 1986; Navarro et al., 1990). Independent of tree bearing status, however, chilling accumulation progressively promoted dormancy release in all experiments, including all explant types (one-node and three-node, leafy and defoliated) forced at 12.5, 15, 20 and 30 °C (Figs. 1–4), consistent with the proposed role of chilling to release olive reproductive buds from dormancy (Rallo and Martin, 1991).

The positive effect of chilling accumulation on dormancy release and subsequent reproductive budburst was noted not only in the increased budburst as sampling date progressed, as described above, but also in the response to forcing at low temperature. Thus when OFF-tree leafy explants were forced at 12.5 °C reproductive budburst occurred in much earlier samples than with 20 °C forcing (Fig. 2). Those early samples, however, required much longer forcing times than the later dates (Fig. 2A, numbers in parentheses) and longer time than at 20 °C (Table 1). This behavior can be explained by additional chilling being provided by the low temperature during forcing at early dates when source-tree chilling was insufficient for dormancy release; the additional chilling under forcing required more time. In the same manner OFF-tree leafy explant budburst of the 9 January samples under 15° forcing, prior to those at 20° and 30 °C forcing (Fig. 4), is also evidence of chilling accumulation during forcing. These observations concur with the identification of 12.5 °C as a temperature effective for chilling accumulation (Rallo and Martin, 1991), while the stronger chilling effect at 12.5 °C and the lesser but still existent effect at 15 °C is consistent with the 15.9 °C olive cold-temperature response threshold proposed by De Melo-Abreu et al. (2004).

Table 2
Reproductive budburst dates and thermal (chilling) units according to source tree calendar, tested in leafy OFF-tree explants forced at different temperatures.

Experiment	Forcing Temperature[a]	Reproductive. Budburst Onset[b]		Reproductive Budburst Maximum[c]	
		Date	Chilling units	Date	Chilling units
1	30 °C	5 Jan.	378.0	15 Feb.	874.3
2	12.5 °C	8 Oct.	0	20 Feb.	1049.8
	20 °C	10 Jan.	669.3	20 Feb.	1049.8
	30 °C	8 Feb.	959.5	20 Feb.	1049.8
3	15 °C	13 Dec.	344.1	20 Feb.	1070.4
	20 °C	10 Jan.	675.1	20 Feb.	1070.4
	30 °C	10 Jan.	675.1	20 Feb.	1070.4

[a] Forcing temperatures 12.5, 15, 20 and 30 °C in experiments 1, 2, and 3 (See Figs. Figure 1A; Figure 2A, C, E; and Figure 4A, C, E).

[b] First explant sampling date for which reproductive budburst was observed under forcing, and natural chilling units on that date calculated according to De Melo-Abreu et al. (2004).

[c] First explant sampling date for which maximum budburst under forcing was reached, and natural chilling units on that date calculated according to De Melo-Abreu et al.

4.3. High temperature delays the onset of reproductive budburst, possibly by nullifying chilling accumulation

With respect to sampling date, reproductive budburst in OFF-tree leafy explants forced at 20 °C and 30 °C began later with respect to sampling date than at 12.5 °C (Fig. 2) and 15 °C (Fig. 4). These patterns suggest that 30 °C and perhaps 20 °C may partially reverse chilling accumulation, as suggested previously in olive (Badr and Hartmann, 1971; Hackett and Hartmann, 1967). Chilling accumulation annulment by moderate temperatures above a critical threshold has been recognized and considered an important factor in models estimating chilling requirements for overcoming winter rest in deciduous fruit trees (Erez et al., 1979; Erez and Couvillon, 1987; Erez and Lavee, 1971; Fishman et al., 1987; Gilreath and Buchanan, 1981; Richardson et al., 1974; Shaltout et al., 1983) and in olive (Aybar et al., 2015; Cesaraccio et al., 2004; De Melo-Abreu et al., 2004).

In interpreting the later onset of budburst at higher temperatures it is difficult to separate the effect of supplementary chilling accumulation at the lower temperatures, as described in section 4.2. Furthermore, at all forcing temperatures studied in the comparative experiments, maximum budburst of 60–80% was achieved by the final sampling date (Figs. 2 and 4). However strong evidence for the inhibition of budburst even though adequate chilling has taken place is shown by the anatomical sections: following 9 January sampling and 9 days forcing at 30 °C, the third-node axils persist in shell-zone configuration (Fig. 10C), in contrast to developing a lateral inflorescence structure, already apparent at 3 days forcing at 20 °C (Fig. 8C). Genomic evaluation in olive plants has revealed that reducing chilling accumulation by shortening the cold temperature period could also reduce the accumulation of genes related to flowering (Haberman et al., 2017).

4.4. Reproductive differentiation in the buds with respect to natural winter chilling accumulation and forcing

Prior to end-of-winter budburst, no differentiation of reproductive structures was observed, in line with the many previous reports (Almeida, 1940; De la Rosa et al., 2000; Fabbri and Alerci, 1999; Hackett and Hartmann, 1963, 1964, 1967; Hartmann, 1951; Rubio-Valdés, 2009; Troncoso, 1967) and recent observations by Haberman et al. (2017). From October to December, all buds from both ON and OFF trees maintained a similar structure, consisting of a short central axis with four or five nodes, each containing two oppositely positioned leaf primordia (Fig. 5). Consistent with the decussate phyllotaxy of the olive tree, alternating nodes 1, 3 and 5 and their respective axils are observed in the central longitudinal sections of the appropriate plane, whereas nodes 2 and 4, oriented in the perpendicular plane, are observed in the serial sections (De la Rosa et al., 2000; Rapoport and Moreno-Álias, 2017). In the four-node buds, leaf primordia of nodes 1 (Lp1) and 3 (Lp3) were visible and the apex presented a smooth, slightly rounded appearance (Fig. 5A). In five-node buds the fifth (Lp5) pair of leaf primordia is also present, showing varying degrees of formation (Fig. 5B).

Our study of the source-tree buds showed readily visible structural differences between OFF and ON-tree buds consisting in cell multiplication to form a shell zone in the third-node (Lp3) axil of OFF- but not in the corresponding ON-tree buds (Fig. 6A and B). This was observed in buds from the source trees on 9 January, the initial sample date for which reproductive budburst occurred in most of the experimental conditions, evidencing the acquisition of chilling requirements. By 7 February the development of the inflorescence lateral branch in that axil had clearly progressed in the OFF-tree samples (Fig. 6C), while the ON-tree buds continued with no changes in the third-node axil (Fig. 6D). On 7 February the OFF-tree bud apex also demonstrated significant reproductive differentiation. These early changes in the third-node axil, followed by apical branching, were also observed by De la Rosa et al. (2000), whereas others (Fabbri and Alerci, 1999; Haberman et al., 2017; Troncoso, 1967) focused on apical dome differentiation.

In addition to structural differentiation the 9 January OFF-tree buds also showed high starch content, much of which was depleted by 7 February. In contrast, ON-tree buds showed little starch and no change during the same period (Fig. 7). Starch is a carbohydrate stored during winter rest in different plants organs and afterwards used for active growth. Increased starch content during chilling accumulation and the progressive decrease in synchrony with bud break and inflorescence

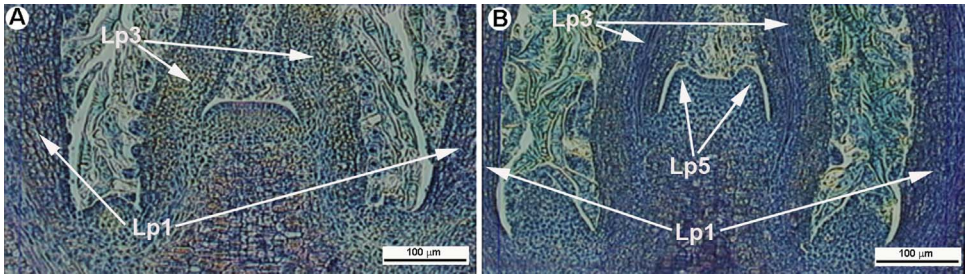

Fig. 5. Morphological structure of representative olive tree four- (A) and five- (B) node buds in central longitudinal sections stained with toluidine blue, as observed from October to December, in both ON and OFF trees. A. In four-node buds the first (Lp1) and third (Lp3) pairs of decussate leaf primordia are visible and the apex has a smooth, slightly rounded appearance. B. In five-node buds the fifth (Lp5) pair of leaf primordia is also visible as two newly formed buttresses at the sides of the apical zone. In both the four- and five-node buds, the first- and third-node leaf primordia and their corresponding axils are visible in the presented sectioning plane. The second and fourth nodes (and respective axils) are not visible as they lie in a perpendicular plane in relation to the presented section.

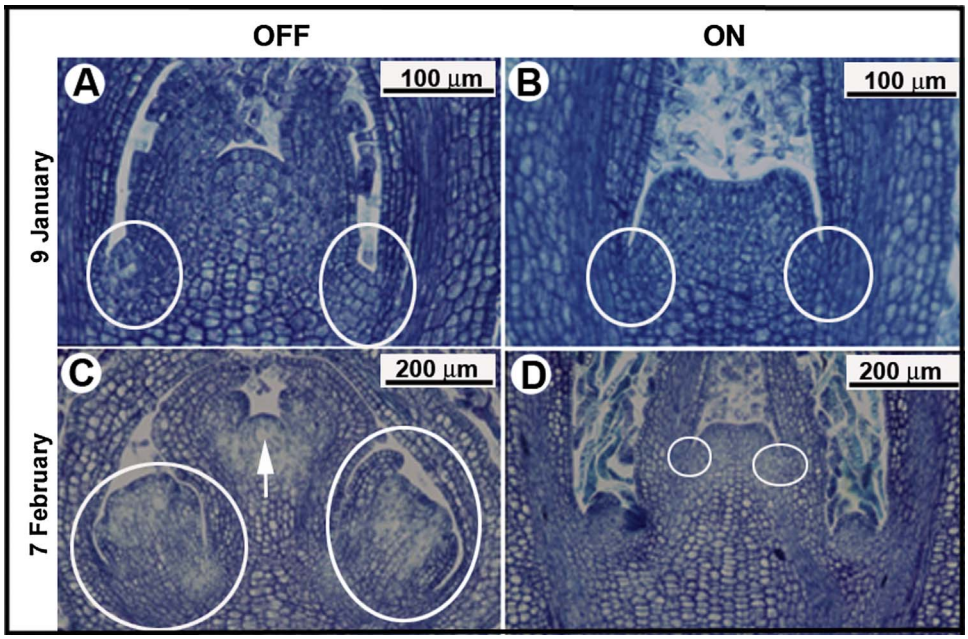

Fig. 6. Olive bud third-node axils (circles) for previous OFF (left) and ON (right) trees on 9 January (top) and 7 February (bottom), observed in central longitudinal sections stained with toluidine blue. On 9 January (A), buds from previous OFF trees start to develop a "shell-zone" of cell division activity in this axil, while on 7 February (C) an inflorescence branch is already developing, and the bud apex (arrow) is also undergoing lateral organ differentiation. In buds from previous ON trees (B and D) no morphological changes are visible.

growth agree with previous observations in different fruit species (Fadón, 2015; Felker et al., 1983; Feng et al., 2013; Lavee, 1973) and in olive (De la Rosa et al., 2000).

Continued reproductive differentiation of the 9 January OFF-tree explants during forcing confirmed the acquired capacity for reproductive budburst observed in the explant experiments. When forced at 20 °C, a lateral primordium immediately developed in 3 days in the third-node axil (Figs. Figure 8C and Figure 9A). In the four-node buds, apical fifth-node differentiation formed reproductive lateral structures (Fig. 9B), in contrast to new leaf primordia formation before winter chilling took place (Fig. 5B). ON-tree explant buds, in contrast, showed elongated leaf primordia after 20 and 30 °C forcing (Figs. Figure 8B and Figure 10A, respectively), representative of the onset of vegetative budburst, and vegetative axillary bud primordia in the third node after 9 days forcing at 30 °C.

4.5. The role of the leaves: a possible case of paradormancy

During olive tree shoot growth, bud formation halts in close association with the cessation of growth in the subtending leaf and the bud remains as a dormant structure (Rubio-Valdés, 2009). Removal of the shoot apex (decapitation) and defoliation of the explants promoted vegetative budburst of the undifferentiated buds until the start of reproductive budburst under favorable temperatures following the completion of chilling accumulation. These results indicate the inhibition of bud growth by the subtending leaves from the onset of bud dormancy throughout the entire shoot growing season, until maximum budburst capacity is reached, suggesting the hypothesis of a joint effect of paradormancy (imposed by the leaf) and endodormancy (imposed directly on the bud) (Lang, 1987). Thus the subtending leaves could play a role in maintaining dormancy from the time of bud formation, similar to the suggested action of the bud scales apple (Fulford, 1966).

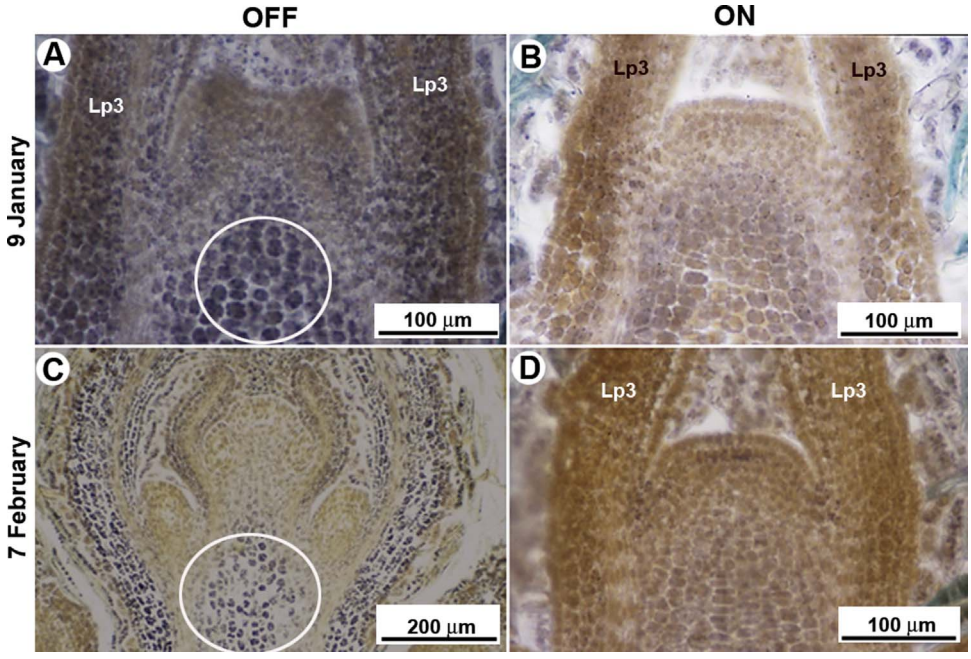

Fig. 7. Starch content in olive bud central axis from previous OFF (left) and ON (right) trees on 9 January (top) and 7 February (bottom), as stained with IIK. On 9 January (A), buds from previous OFF trees show many dark blue-black staining starch grains in the central zone of the bud axis (circle), while on 7 February (C), the starch appears to have decreased (circle). In buds from previous ON trees (B and D), null or scarce starch grains are visible on both dates. Lp3–third-node leaf primordia.

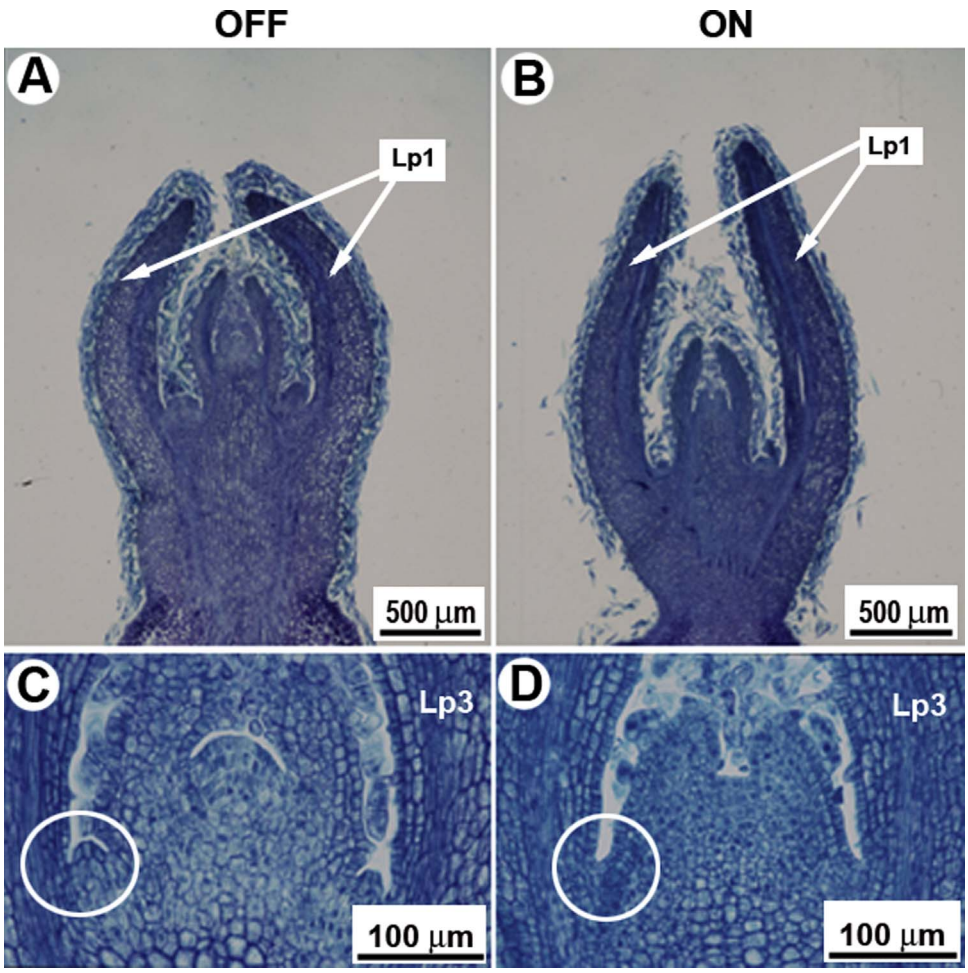

Fig. 8. Growth and differentiation of five-node olive buds on explants collected 9 January from previous OFF (left) and ON (right) source trees and forced 3 days at 20 °C, observed in central transverse sections stained with toluidine blue (upper, whole buds; lower, axils of third-node primordia). Leaf primordia from OFF-tree buds (A) show no visible changes, while leaf primordia from ON-tree buds (B) start to elongate, particularly the most external leaf primordia (Lp1). In OFF trees (C), a primordium starts to form at the third-node axils (circle), while no visible change occurs in the ON-tree buds (D) at the axils of the third-node leaf primordia (circle).

Afterwards, when enough chilling is accumulated to release dormancy, reproductive budburst capacity recovers and, the developing inflorescences would then be fed rather than restrained by the subtending leaves as was suggested by sequential shoot defoliation during winter and spring (Rallo and Martin, 1991).

4.6. *Standardizing explant studies to quantify chilling requirements for the release of olive reproductive buds from dormancy*

The experimental limitations due to the size and morphological complexity of adult fruit trees requires a simplified system such as explants for *in vivo* testing of chilling and dormancy release requirements under controlled conditions. Although reproductive budburst increased with progressively greater amounts of chilling accumulation in all experiments, varied budburst behavior under the different experimental conditions indicates the value of standardizing experimental procedures for olive dormancy release studies and furthermore provides suggestions for their optimization. Standardizing explant characteristics and forcing conditions for determining the chilling requirements in comparative studies, particularly for cultivar and genotype evaluation, has been recommended for deciduous fruit trees (Atkinson et al., 2013; Campoy et al., 2011; Dennis, 2003; Saure, 1985).

The low and irregular percentage of maximum reproductive budburst in ON-tree explants and its frequently associated high standard

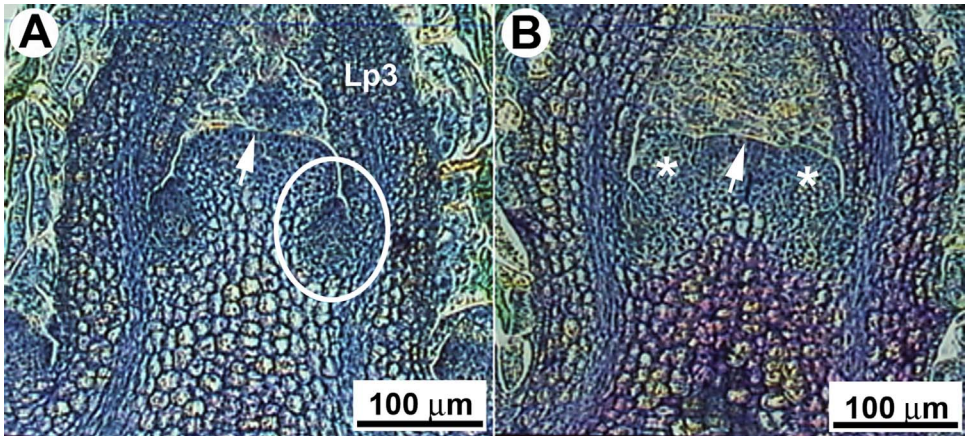

Fig. 9. Morphological development in four-node buds from explants collected 9 January from an previous OFF tree, after forcing at 20 °C during 3 (left) and 9 (right) days, observed in the central longitudinal sections stained with toluidine blue. After 3 days forcing (A), a lateral primordium (circle) is visible at the third-node axil, and the apex (arrow) forms a smooth plateau. After 9 days forcing (B), the apex comprises a central (arrow) and two lateral (stars) zones, initiating a new reproductive fifth node, which is substantially different from the fifth-node formation of a not-yet reproductively differentiating bud. (Fig. 6B).

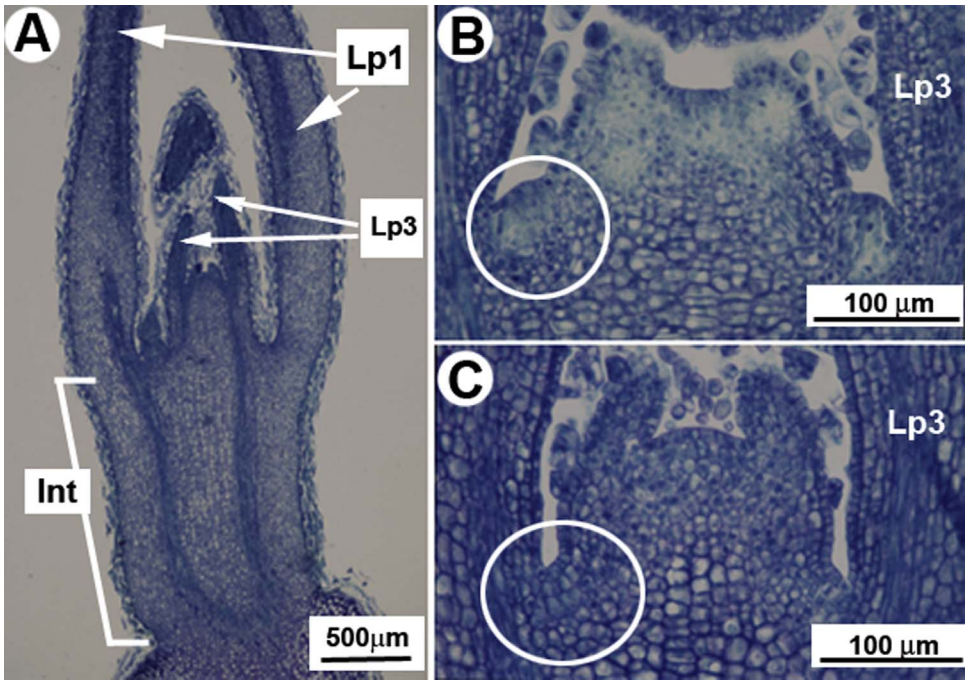

Fig. 10. Growth and differentiation of five-node olive buds collected 9 January from previous ON (A and B) and OFF (C) trees and forced 9 days at 30 °C, observed in central transverse sections stained with toluidine blue. First and third leaf primordia (Lp1 and Lp3) and basal internode (Int) in buds from ON trees (A) show notable elongation, and the slight start of lateral bud primordia at the Lp3 axil (B; circle). In buds from OFF trees (C), the third-node axils (circle) remain with the initial "shell-zone" cellular organization typical for the 9 January sampling date (Fig. 7A).

errors, a pattern also observed in previous studies (Rallo and Martin, 1991; Rubio-Valdés, 2009), indicate that explants from OFF trees represent a superior option for experiments to determine olive tree dormancy release and chilling requirements. Regarding number of explant nodes, three-node explants not only provide more buds for observation than one-node explants, but also help dissipate possible effects of explant apex removal on bud dormancy (Dun et al., 2006) by including nodes at varied distance from the apex. With defoliation, even though the absence of the subtending leaves can help elucidate the physiological interaction of the leaves and buds, it can interfere with dormancy mechanisms by promoting or allowing vegetative budburst before chilling accumulation is completed. Finally, the use of three-node leafy explants from OFF source trees is validated by the close and repetitive correspondence of progressive dormancy release and reproductive budburst with chilling accumulation.

The forcing temperatures showed different budburst responses, likely because they not only permitted budburst in relation to the bud's acquired the capacity to do so, but also further influenced that capacity by either additional chilling accumulation (Section 4.2) or chilling negation (Section 4.3). Among the temperatures used, 20 °C is the best choice for standardization, as it most consistently produced continuous and progressive dormancy release of the potentially reproductive buds once chilling requirements were fulfilled in the source trees (Figs. 2 and 4). Furthermore it is above the proposed threshold of effective temperatures for chilling accumulation (De Melo-Abreu et al., 2004) and did not delay the onset of reproductive differentiation as did 30 °C (Fig. 10C). Forcing at 12.5 °C, and to a lesser extent 15 °C, resulted in an exceptionally long budburst period (see Section 4.2), due to the double action of first providing chilling and then permitting growth once chilling requirements were fulfilled. Apart from temperature the controlled climatic conditions we used for forcing, that is relative humidity close to saturation (RH > 80%), neutral photoperiod (12/12 h, day/night), and photon flux density of 200–300 μmol $m^{-2} s^{-1}$, were adequate.

5. Conclusions

Our results represent a) the confirmation that chilling plays an essential role in releasing axillary potential reproductive buds from dormancy, b) evidence of favorable and unfavorable overlapping ranges of temperature for chilling accumulation/negation, and for reproductive budburst, c) the first indication of the role of leaves in the maintenance of axillary bud dormancy during winter rest, and d) the early anatomical evidence of olive reproductive bud initiation consisting of a "shell zone" of cell division in the bud third-node axil at the onset of reproductive budburst. We also suggest standard procedures for using woody explants to study the factors which determine axillary reproductive bud release from dormancy and to quantifying chilling requirements in olive genotypes, providing a highly useful tool for both classical and modern analytical approaches, including predictive models and genomic analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.scienta.2017.11.003.

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