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Effects of various winter chilling regimes on flowering quality indicators of Greek olive cultivars

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Abstract

Aims of the present two-year study were to evaluate the feasibility and identify potential drawbacks of the greenhouse/outdoors parallel plant growth methods for investigation of the effects of various winter chilling regimes on flowering quality indicators of four Greek olive cultivars, namely Mastoidis, Amfissis, and Lefkolia Serron (originating from mountainous and colder areas) compared to cv. Koroneiki (grown mainly in plain warm areas). Groups of potted olive plants were either grown outdoors under ambient temperature or transferred into a greenhouse for one, two, or three months during winter in Crete, Greece. During the first year, chilling accumulation deficit caused a marked decrease in the number of inflorescences per plant in all four olive cultivars. In the second year, chilling accumulation deficit had a negative effect on the number of inflorescences per plant in 'Mastoidis' at 3-month greenhouse treatment but not at all in 'Koroneiki'. Chilling deficit caused an overall decrease in the number of flowers per inflorescence in both 'Koroneiki' and 'Mastoidis' as well as in the percentage of morphologically perfect flowers. The width and length of inflorescences were not affected by chilling deficit in both the cultivars. *In vitro* pollen germination was reduced in all greenhouse treatments in 'Koroneiki'; however, this effect was significant only after 3 month, whereas no effect was observed in 'Mastoidis'. The results of the present study may contribute to understanding olive flowering biology and selecting appropriate cultivars for new plantations according to historical meteorological data and predicted climate change scenarios.

Additional key words: climate change, ovary abortion, phenology, pollen germination, vernalization.

Introduction

Olive (*Olea europaea* L.) is one of the most important crops in Mediterranean countries where over a 90 % of global olive production is realized (International Olive Council, http:www.internationaloliveoil.org/). Olive tree is cultivated for the production of olive oil (juice extracted from the fruit by natural means) and for table olives (edible fruit after mild treatment to remove bitter taste and increase preservation time) both consumed and highly appreciated for their nutritional value and health benefits (Accardi *et al*. 2016, Agrawal *et al*. 2017). Additional products of olive tree with an economic and health promoting value are its wood (Santos *et al.* 2017) and leaves (Rahmanian *et al.* 2015), respectively. Last but not least, sustainably managed olive groves may provide significant ecosystem services in the environmentally fragile Mediterranean region (Montanaro *et al*. 2017).

Climate change is expected to have significant impacts in Mediterranean basin agriculture and the environment (Milano *et al*. 2013). Indeed, temperature increase, water deficiency, and extreme climatic events are the main components of the changing climate (Giannakopoulos *et al.* 2009, Kourgialas *et al.* 2019). Crops may thus suffer serious yield reduction as well as plant damage (Koubouris 2018).

For each plant species, there is a temperature range which is suitable for its survival and a narrower temperature range which favors its optimal growth and flowering. Outside this temperature range, plants underperform and may face serious damages, and in an extreme or long term exposure, they may die. For example, an olive tree physiological and reproductive performance may be at risk at temperatures over 35 - 40 °C (Koubouris *et al*. 2009, 2015a,b). Similarly, olive plants face negative changes both at their reproductive and at physiological functions when air temperature drops below -7 and -12 °C, respectively (Pallioti and Bongi 1996, Barranco *et al*. 2005).

Plants often employ a physiological mechanism called dormancy (expressed as vegetative growth arrest and often

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Abbreviations: C - control plants grown outdoors under natural environment; CR - chilling requirement; HSD - honestly significant difference; 1M, 2M, 3M - plants grown in an unheated glasshouse for 1, 2, or 3 winter months.
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leaf dehiscence) to survive during the cold winter period and avoid frost damage (Ramos *et al*. 2018). However, low winter temperatures (*e.g.*, above 1.5 °C and below 16 - 19 °C), may have beneficial effects on plant biological cycle (Cesaraccio *et al*. 2004). In fact, many plant species demand certain exposure to low temperature during winter to overcome dormancy and to be able to develop flowers in the subsequent spring (Campoy *et al*. 2011).

However, dormancy release seems to be affected also by other factors aside temperature. Water deficit during the cold period of the year was found to substitute chilling effect for breaking dormancy in Peru where precipitation is mainly realized during the warm period of the year (Connor and Ferreres 2005). On the other hand, the hypothesis that increased precipitation shortens the dormancy period in fruit trees was also suggested (Sfakiotakis 1993) implying that vernalization mechanisms may differ between plant species.

The chilling requirement (CR; duration of plant exposure to effective chilling temperatures) may differ quantitatively as well as qualitatively (temperature and plant phenological stage) between plant species, varieties, and growing regions (Li *et al*. 2016).

The olive tree develops flowers during spring on shoots that have been formed during the previous vegetative period. Several factors, such as a genotype, duration of winter chilling previous year's fruit load, nutritional and water balance, as well as other crop management practices and environmental components, affect flowering abundance and quality (Fabbri and Benelli 2000). For example, it has been reported that olive trees growing at a temperature constantly higher than 15.5 °C do not produce any flowers (Hartmann and Porlinglis 1957). Indeed, the failure of olive plantations to flower and yield fruit, when established in areas with warm winter, confirms that olive tree requires chilling for dormancy release (De Melo-Abreu *et al*. 2004). In a recent study, olive flower induction was shown to be regulated by the expression of the *Flowering locus T* gene (Haberman *et al*. 2017).

Reasonably, not all olive cultivars have similar chilling demands (Gabaldon-Leal *et al*. 2017). For example, cv. Leccino was considered to have higher needs in chilling accumulation compared to Arbequina and Frantoio (Aybar *et al*. 2015). Similarly, some olive cultivars, such as Sevillano and Ascolana are considered to have a very high CR (Kailis and Harris 2007), whereas in North African countries, local olive cultivars grow and produce sufficient yields under hot environmental conditions (Acila *et al*. 2017). There is also a debate on the efficiency of different temperatures for chilling accumulation and break of dormancy, *e.g.*, 7 °C was initially considered as optimal (Hartman and Porlingis 1957), but a higher temperature of 12.5 °C was suggested more recently (Ramos *et al*. 2018).

Climate change predictions related to temperature increase in the Mediterranean region estimate that chilling accumulation units will be reduced for some of the most important crops such as peach, apple, grape, and olive (Perez Lopez *et al*. 2012). Since olive is grown in many areas with highly variable climate and winter temperature range, caution should be paid to select appropriate cultivars that meet CR and produce sufficient fruit yields.

Aims of the present study were: *1*) to evaluate the feasibility and identify potential drawbacks of the greenhouse/outdoors parallel plant growth as a method for chilling accumulation studies and *2*) to investigate the effects of various winter chilling regimes on flowering quality indicators of Greek olive cultivars naturally grown or cultivated in different environments.

Materials and methods

Plants and growth conditions: The present two-year study was implemented in the Institute of Olive Tree, Subtropical Crops and Viticulture in Chania, Greece (latitude 35°; longitude 23°; altitude 28 m a.s.l.). During the first year, a preliminary trial with four olive cultivars was implemented. Eighty (two-year-old) potted plants of cultivars Mastoidis (grown in southern Greece), Amfissis (grown in central Greece), Lefkolia Serron (grown in northern Greece), and Koroneiki (grown mainly in plain areas with warm climate and produces satisfactory yields even in the hot climate of North African countries). They were separated in 5-item groups. All inflorescences were removed during spring to avoid alternate bearing effect and plants were routinely cultured until autumn in an open nursery. At the end of November, three groups of plants for each cultivar were transferred in an unheated glasshouse with a north-south orientation for 1, 2, or 3 winter months and one group of plants for each cultivar was grown outdoors under natural environmental conditions (Fig. 1 Suppl.) to simulate winter temperature variation and to study its effect on subsequent flowering. Treatments are named as 1M, 2M, 3M, and C, throughout the paper.

During the second year, the experiment was repeated for 'Koroneiki' and 'Mastoidis' based on the results of the first year (abundant and stable flowering in the control plants in contrast with an intrinsic poorer and unstable flowering in 'Amfissis' and 'Lefkolia Serron', which might interfere with the effect of chilling accumulation on flowering - details in the Discussion). Forty (three-year-old) potted plants were separated in five-item groups and steps described in the previous paragraph were followed. A thermograph was employed for monitoring the air temperature inside the glasshouse and the adjacent meteorological station for the ambient temperature outdoors. A gap in outdoors temperature record was observed around the year change due to a temporal failure in the meteorological station which was then fixed. A simplified method to assess chilling accumulation was devised; for each treatment and cultivar, the total number of hours below 16 °C during the study period was recorded. The maximum threshold temperature of 16 °C was selected according to Aguilera *et al*. (2014) since above this limit, no chilling accumulation is realized. In the present study, plants grown outdoors under natural environmental conditions received 1 835 h below 16 °C during the three winter months (Fig. 2 Suppl.). Plants grown in the glasshouse for 1, 2, or 3 winter months received 1 663, 1497, and 1 214 h below 16 °C, respectively.

Phenology and floral quality measurements: During the preliminary trial of the first year, in April, we determined the percentage of plants with complete absence of flowering and for the flowered plants, the number of inflorescences per plant. In the main experiment of the second year, in the following spring, flowering phenology was recorded according to Sanz-Cortes *et al*. (2002) as well as qualitative and quantitative flowering quality indicators, namely: *a*) the percentage of plants with total absence of flowering, *b*) for the flowering plants, the number of inflorescences per plant, *c*) the number of flowers/inflorescence, *d*) the percentage of morphologically perfect flowers (with pistil and stamen), *e*) the length of inflorescence, and *f*) the width of inflorescence. Finally, a destructive sampling of inflorescence was implemented for subsequent laboratory measurements.

***In vitro* pollen germination:** In order to assess pollen performance of plants grown at various winter chilling regimes, pollen was collected from freshly opened flowers and subsequently incubated at room temperature (~ 25 °C) in the dark in a growth chamber (*Kottermann 2770, D3162*; Hanigsen, Germany) for 24 h before counting pollen germination and pollen tube length according to Koubouris *et al*. (2015b). Throughout the experiment, pollen was cultured on a solid medium consisting of 0.8 % (m/v) agar, 15 % (m/v) sucrose, 1.64 mM boric acid, and 60 mg dm^{-3} tetracycline hydrochloride according to Koubouris *et al*. (2009). Pollen germination was evaluated on 4 Petri dish fields containing over 50 pollen grains for each treatment.

Statistical analysis: Data were analyzed using *SPSS* (*SPSS Inc.*, Chicago, USA) and were subjected to one-way analysis of variance. Significantly different means were calculated at $P \leq 0.05$ using the Tukey honestly significant difference test.

Results

During the first year, reduced winter chilling caused a marked decrease in the number of inflorescences per plant in all four olive cultivars (Fig. 1*A*). These results are statistically significant ($P < 0.05$) for 'Koroneiki' (3M), 'Mastoidis' (2M+3M) and 'Lefkolia Serron'(2M) treatments. In the case of 'Amfissis', there were no statistically significant differences in spite of the total absence of flowers in treatments 2M+3M, potentially due to high variations among plants (Fig. 1*A*). In 'Koroneiki', plants without flowers were observed only in 3M, in 'Mastoidis' in all greenhouse treatments whereas in 'Lefkolia Serron'and 'Amfissis' in all four treatments (Fig. 1*B*).

In the second year, chilling accumulation deficit had a negative effect ($P < 0.05$) on the number of inflorescences per plant in 'Mastoidis' at 3M but not at all in 'Koroneiki' (Fig. 2*A*). The incidence of plants with a complete absence of flowers was observed in 1M and 2M in 'Koroneiki' and 1M and 3M in 'Mastoidis', whereas all outdoors grown plants successfully formed flowers in both cultivars (Fig. 2*B*).

Chilling deficit through maintaining plants inside the greenhouse for various time intervals of winter caused an earlier inflorescence buds swelling compared to the control in 'Koroneiki' (Fig. 3). However, in the case of 'Mastoidis', the effect was variable, and we observed no clear trend. In the end, the date of flower opening was not affected by the treatments but mostly by the cultivar.

Chilling deficit caused an overall decrease in the number of flowers per inflorescence in both cultivars; however, this effect was statistically significant ($P < 0.05$) only in the case of 1M in 'Mastoidis' (Fig. 4*A*). The percentage of morphologically perfect flowers (with pistil and stamen) was significantly reduced ($P < 0.05$) in 'Koroneiki' at 3M (Fig. 4*B*). In the case of 'Mastoidis', a reduction was observed at 2M and 3M but it was statistically significant ($P < 0.05$) only at 2M. Inflorescence width and length were not affected by chilling deficit in both cultivars (Fig. 4*C*,*D*). *In vitro* pollen germination was reduced in all greenhouse treatments in 'Koroneiki', however, this

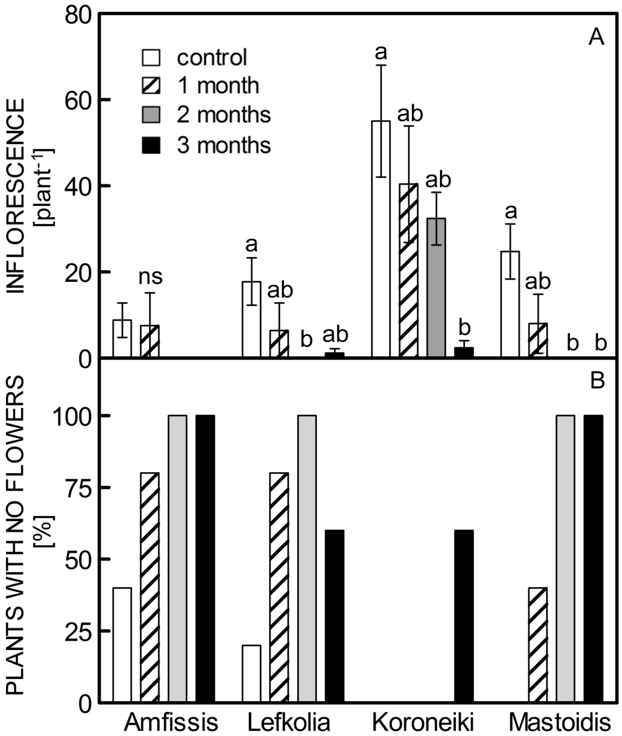

Fig. 1. Effect of winter chilling deficit (potted plants transferred for 1, 2, or 3 months in a greenhouse) during the first year on the number of inflorescences per plant (*A*) and the percentage of plants with total absence of flowers (*B*) in olive cultivars Amfissis, Lefkolia Serron, Koroneiki, and Mastoidis. Means ± SEs, *n* = 5, significantly different means marked with different letters in *A* were calculated at $P \leq 0.05$ using the Tukey honestly significant difference test. Statistical analysis was not feasible for *B*.

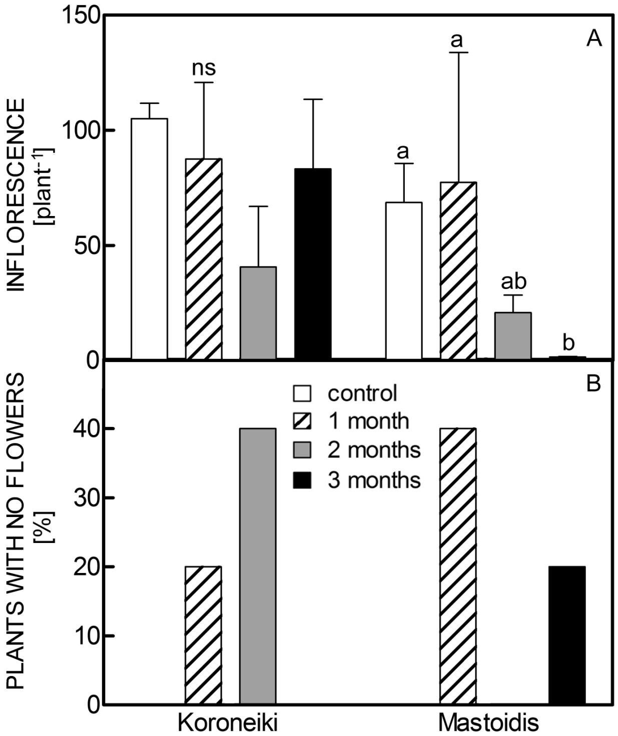

Fig. 2. Effect of winter chilling deficit (potted plants transferred for 1, 2 or 3 months in a greenhouse) during the second year on the number of inflorescences per plant (*A*) and the percentage of plants with total absence of flowers (*B*) in olive cultivars Koroneiki and Mastoidis. Means ± SEs, $n = 5$, significantly different means marked by different letters in *A* were calculated at $P \leq 0.05$ using the Tukey honestly significant difference test. Statistical analysis was not feasible for *B*.

effect was significant ($P < 0.05$) only at 3M, whereas no significant effect was observed in 'Mastoidis' (Fig. 5).

Discussion

Olive is the major tree crop in Greece and it is cultivated in a vast variety of microclimatic conditions as, for example, the hot, arid southern part of the country on the island Crete up to the rather cold northwestern areas such as Alexandroupoli near borders with Turkey and Bulgaria. In spite of the germplasm richness available in Greece, estimated around 100 cultivars, basic genetic and morphological data are available for less than 40 of them (Koubouris *et al*. 2019), and chilling requirements have been estimated only for 'Koroneiki' in the environmental conditions of Andalusia, Spain (Gabaldón-Leal *et al*. 2017).

Plants grown in the glasshouse for 1, 2, or 3 winter months were exposed to reduced chilling hours of -9 % (M1), -18 % (M2) and -34 % (M3), compared to the control. There seems to be differentiation among treatments achieved by our experimental design. Also, the temperature fluctuation inside the greenhouse followed the trend of ambient conditions observed outdoors providing a more realistic environment for chilling accumulation

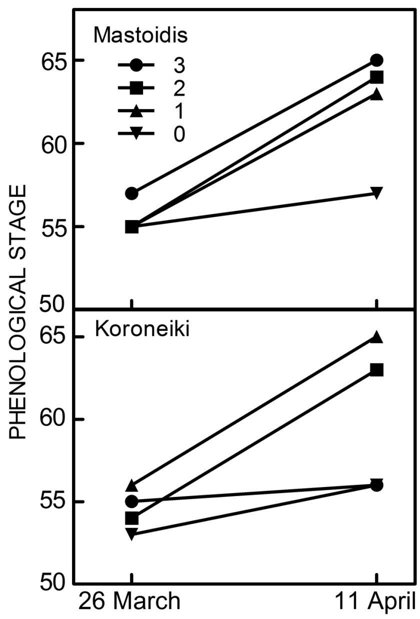

Fig. 3. Effect of winter chilling deficit (potted plants transferred for 1, 2 or 3 months to a greenhouse) during the second year on flowering phenology in olive cultivars Koroneiki and Mastoidis based on the scale of Sanz-Cortes *et al*. (2002) ($n = 5$).

studies compared to studies at growth chambers where the temperature is set to be almost stable throughout the day (Ramos *et al*. 2018). When even a higher accuracy in environmental conditions is desired, the suggested strategy is to employ open-top chambers with artificial temperature control as successfully tested by Benlloch-Gonzalez *et al*. (2018). Another available method for chilling accumulation studies would be sampling explants and forcing them in growth chambers under the desired temperature regimes (Ramos *et al*. 2018). In any case, it should be noted that variation of day/night temperatures plays a role in chilling accumulation efficiency since maintaining plants in a constant temperature of 7 °C results in minimal flowering (Denney and McEachern 1983).

The percentage of plants with an absolute absence of flowers provides another very interesting insight regarding genotypic response to a chilling deficit. Due to the presence of plants with no flowering even in the control plants during the first year, 'Lefkolia Serron' and 'Amfissis' were considered to have intrinsic poor flowering and thus excluded from the second year experiment. This finding might also be an indication of high CR of these olive cultivars, which was not fulfilled even in the plants grown outdoors in the mild winter conditions of Crete. Therefore, in future trials, a higher chilling accumulation should be achieved in the control plants, either by selecting a site with lower temperature regime or by artificially regulating environmental conditions. During the second year of the present study, the incidence of plants with no flowers varied between cultivars but being zero in the control plants of both cultivars ('Koroneiki' and 'Mastoidis') provides a quality assurance indicator for the experiment. Growing olive plants constantly over 16 °C in a greenhouse results in a complete absence of inflorescence in other

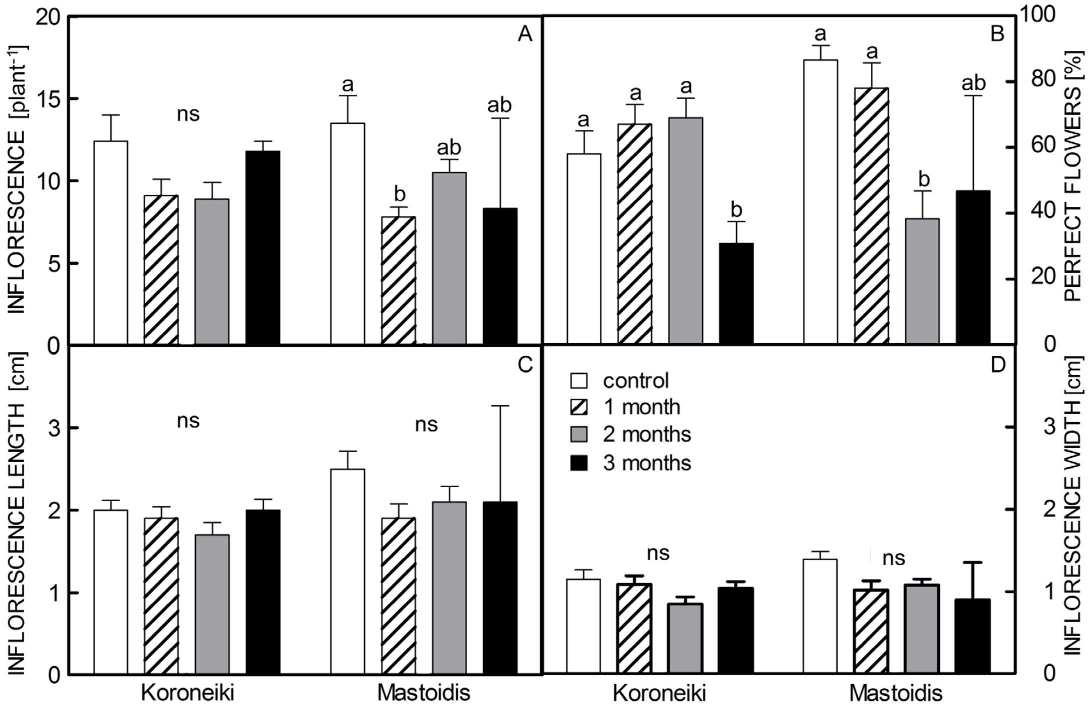

Fig. 4. Effect of winter chilling deficit (potted plants transferred for 1, 2 or 3 months in a greenhouse) during the second year on the number of flowers per inflorescence (*A; n* = 20), the percentage of morphologically perfect flowers with pistils and stamens (*B*; *n* = 100), inflorescence length (*C*; *n* = 20), and inflorescence width (*D; n* = 20) in olive cultivars Koroneiki and Mastoidis. Means ± SEs, significantly different means marked by different letters were calculated at $P \leq 0.05$ using the Tukey honestly significant difference test.

cultivars (Hackett and Hartman 1967). A reduced number of flowers per inflorescence and a reduced percentage of perfect flowers due to chilling deficit observed in the present work are in agreement with previous studies in other olive cultivars, *e.g.*, absence or asynchronous flowering in new olive growing areas (Castillo-Llanque *et al*. 2014, Aybar *et al*. 2015) as well as a reduced number of inflorescence and an increased ovary abortion (Deng *et al*. 1988) may be related to chilling accumulation deficit of the grown cultivars as reported in Ramos *et al*. (2018). However, during springtime, when CR fulfillment has been completed and inflorescence approach anthesis, low-temperature incidents may have negative impacts on flowering and fruit set of olive (Koubouris *et al*. 2010a).

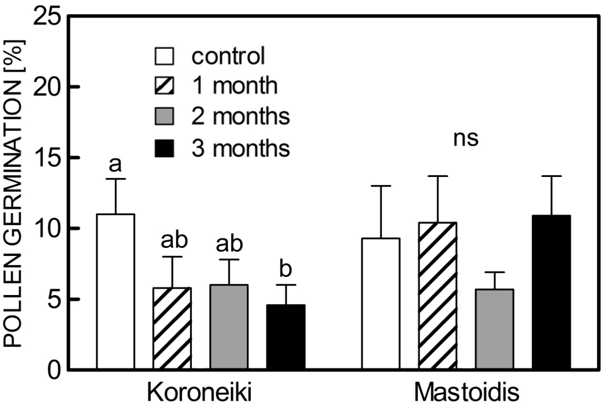

Fig. 5. Effect of winter chilling deficit (potted plants transferred for 1, 2 or 3 months in a greenhouse) during the second year on *in vitro* pollen germination from olive cultivars Koroneiki and Mastoidis. Means ± SEs, *n* = 200, significantly different means marked by different letters were calculated at $P \leq 0.05$ using the Tukey honestly significant difference test.

In the present study, the effect of chilling deficit reached by maintaining plants inside the greenhouse for various time intervals of winter caused an earlier inflorescence buds swelling in 'Koroneiki', probably due to higher thermal units accumulation compared to the control plants grown outdoors, following chilling accumulation demand fulfillment as also observed in multiple sites of Spain, Italy, and Tunisia by Aguilera *et al*. (2014). However, in the end, the date of flowering was not affected by the chilling treatments but only depended on the cultivar as was also previously demonstrated for these cultivars (Koubouris *et al*. 2010b). This is also supported by the fact that from 1st March and onward, all plants were grown outdoors in identical environmental conditions and management practices. It was therefore reasonable that inflorescence length and width were similar in all treatments of each cultivar since these features are mainly regulated by thermal accumulation, water and nutrition balance, and other factors rather than CR (Cuevas *et al*. 1994, Acebedo *et al*. 2000, Rapoport *et al*. 2012, Aguilera *et al*. 2014). Similarly, minor differences observed here in pollen germination after different treatments are unlikely to be related to the chilling deficit but probably should be further studied in future experiments.

Some important effects of winter chilling deficit on flowering quality indicators of major Greek olive cultivars were elucidated in the present study. Multiyear and multisite trials with enriched germplasm coupled with flowering-related gene expression analysis (Haberman *et al*. 2017) are in progress to provide precious knowledge on olive flowering biology and contribute to selecting appropriate cultivars for new plantations according to historical meteorological data and predicted climate change scenarios.

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