

Flowering regulation in the facultative biennial *Oenothera biennis* L.: Environmental effects and their relation to growth rate

Raúl Giménez^a, Daniel M. Sorlino^a, Héctor D. Bertero^{b,c}, Edmundo L. Ploschuk^{a,*}

^a Cátedra de Cultivos Industriales, FAUBA, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

^b Cátedra de Producción Vegetal FAUBA, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

^c CONICET, Argentina

ARTICLE INFO

Article history:

Received 3 July 2012

Received in revised form

12 September 2012

Accepted 23 September 2012

Keywords:

Oenothera biennis

Flowering

Photoperiod

Vernalization

Growth rate

High temperature

ABSTRACT

The lifecycle of the facultative biennial oilseed-crop evening primrose (*Oenothera biennis*) is a major constraint for its commercial production under different growing conditions, as a variable proportion of plants fails to flower during the first season and remains as vegetative rosettes (biennial behavior). The aim of this work was to understand how flowering is regulated in this species and to identify the main determinants of its biennial behavior.

Different planting dates and manipulative treatments (seed vernalization, photoperiod extension and fertilization) were employed to analyze if: (i) biennial behavior occurs when obligate requirements for vernalization or photoperiod are not satisfied; and (ii) responses to these environmental cues depend on the size and/or growth rate of rosettes.

Our results indicate that *O. biennis* has an obligate long-day requirement for flowering and a facultative vernalization response. There is no minimum size requirement for vernalization response (as very small seedlings responded to the vernalization treatment) and the rate of development toward flowering under inductive photoperiods was strongly affected by rosette's growth rate. The incidence of high temperatures just before the onset of reproduction is proposed as an inhibitory factor that prevents reproduction under otherwise photo-inductive conditions. This last factor would explain the high incidence of biennial behavior frequently observed in spring/summer sowing in this crop.

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1. Introduction

Evening primrose (*Oenothera biennis* L., Onagraceae) is a facultative biennial plant species native to eastern and central North America and recently domesticated as a specialty oilseed-crop (Lapinskas, 1999; Fieldsend, 2007). Its value lies in its seed oil which contains unusually high levels of gamma-linolenic acid (GLA), an uncommon fatty acid used in pharmaceuticals and nutritional supplements (Hudson, 1984; Russell, 1988; Horrobin, 1992). Although other plant species such as borage (*Borago officinalis* L.) or black-currant (*Ribes nigrum* L.) offer higher GLA concentrations, evening primrose remains the most suitable source of GLA because of its very simple oil profile and its potential for agricultural production (Lapinskas, 1993, 1999; Liu et al., 2003; Ghasemnezhad, 2007).

As a novel crop, evening primrose faces a number of problems for its production on a commercial scale. Among the most

important is the extended lifecycle due to its biennial reproductive behavior (Lapinskas, 1982, 1993; Liu et al., 2003; Ghasemnezhad, 2007). In its biennial form, *O. biennis* is sown in summer or autumn, overwinters as a vegetative rosette, and develops a reproductive stalk in spring (bolting). It flowers and ripens during the following summer and is harvested in autumn, occupying the field during 14 months (Horrobin and Lapinskas, 1993; Lapinskas, 1993). Many hybrids and varieties have been released by breeding programs with shorter life cycles (Lapinskas, 1999; Fieldsend and Morison, 2000; Liu et al., 2003; Kapoor and Nair, 2005; Fieldsend, 2007) that made it possible to sow in spring (spring evening primrose) as an alternative to summer–autumn sowings (winter evening primrose) (Horrobin and Lapinskas, 1993; Ghasemnezhad, 2007). Spring-sown crops have a shorter vegetative phase and total life cycle, without major impact on grain yield or oil quality (Ghasemnezhad and Honermeier, 2008). As a consequence they fit better into crop rotation schemes and present lower weed and diseases control costs (Reeleder, 1994; Fieldsend and Morison, 2000). Despite these advantages summer sowings, or combinations of summer and spring sowings, are still preferred in many production areas (Horrobin and Lapinskas, 1993; Fieldsend and Morison, 2000; Fieldsend, 2004; Król, 2007) because they present a lower uncertainty in yield expectations.

* Corresponding author. Tel.: +54 11 4524 8040/8075.

E-mail addresses: gimenezr@agro.uba.ar (R. Giménez), dsorlino@agro.uba.ar (D.M. Sorlino), bertero@agro.uba.ar (H.D. Bertero), ploschuk@agro.uba.ar (E.L. Ploschuk).

Bolting failure has been reported as an important productive constraint in spring evening primrose that can determine the success or failure of the crop (Lotti et al., 1984; Lapinskas, 1999; Deng et al., 2001; Król, 2007) and mainly explains the lower yield stability of this management option. A variable proportion of plants in a crop may not initiate reproduction in spring, remaining as vegetative rosettes until harvest time in autumn. As these plants would neither bolt nor flower until the following spring (i.e. they behave as biennials), they do not contribute to seed yield (Król, 2007). Knowledge of factors involved in the transition from vegetative growth to reproductive development in *O. biennis* is important not only to explain its annual or biennial behavior in different environments, but also to determine the feasibility of the crop in areas where it has not been grown before.

Although several flowering control factors have been proposed, an integrated analysis of how reproductive behavior of *O. biennis* is controlled is still lacking. Biennial behavior could be explained as the consequence of the dissatisfaction of an obligate requirement for one or more developmental factors, like vernalization or long photoperiods (Chouard, 1960; Picard, 1967; Reekie and Reekie, 1991; Lapinskas, 1993; Liu et al., 2003). Besides this, there is evidence that factors usually associated with growth could also be involved (Kromer and Gross, 1987; Roy et al., 1993; Lapinskas, 1999; Król and Berbeć, 2004; Król, 2007). Several authors agree that facultative biennial plant species must reach a minimum critical size to trigger flower initiation in response to environmental stimuli (Werner, 1975; Gross, 1981; Kachi and Hirose, 1983; Klinkhamer et al., 1987). In this way, environments that restrict growth would promote biennial behavior by not allowing the plant to attain the critical size before inductive conditions occur (Kachi and Hirose, 1985; Kagaya et al., 2009).

The aim of this work was to identify the main determinants of biennial behavior in *O. biennis* by analyzing the effect of vernalization, photoperiod and their interactions with plant growth under different growing conditions. Two hypotheses were tested: (H1) biennial behavior occurs when obligate requirements for vernalization or photoperiod are not satisfied; and (H2) response to these environmental cues depends on the size and/or growth rate of rosettes.

2. Material and methods

2.1. General

Field experiments were performed at the Faculty of Agronomy of the University of Buenos Aires, Argentina (34°35'S, 58°29'W) between years 2005 and 2010. These experiments were conducted in pots under semi-controlled field conditions in which different planting dates (PD) were employed to explore a broad range of photo-thermal environments (Table 1). In every PD (save for those including a vernalization treatment described in the next section) seedlings were grown in germination trays (40 ml plastic boxes) containing a peat-based substratum (Dynamics 1®), in a greenhouse. When seedlings were considered to be large enough to withstand field conditions (between 3 and 5 leaves), they were transplanted outdoors to 7 l pots (one plant per pot) containing a mixture of local soil and sand (5:4). In the field, pots were arranged in plots with 3 replicates (plots) per treatment. The number of pots per replicate was variable (see Table 1) with a distance between plants of 0.3 m. Plants were hand-weeded and watered as needed. Cipermetrine 25% (20 cm³ hl⁻¹) and Zineb 70% (20 g hl⁻¹) were applied every 15 days to prevent pest and fungal diseases. Maximum and minimum daily temperatures were obtained from an automatic meteorological station located 200 m from the experimental site, and daylength was calculated from U.S.

Naval Observatory data (www.usno.navy.mil), considering natural daylength plus civil twilights. *O. biennis* seeds were provided by Marisol Berti Diaz (University of Concepcion, Chile) and derive from a population provided by Kings (currently Technology Crops Ltd., UK) after several cycles of multiplication in central Chile.

2.2. Vernalization experiments

Planting dates **a**, **b** and **f** (Table 1), included a seed-vernalization treatment to determine if a period of low temperature is an obligate requirement for *O.* flowering, and to evaluate if the species can be vernalized at this stage. Seed vernalization treatments (**Vn+**) consisted in keeping pre-germinated seeds in a 5 °C growth chamber for 5 weeks. **Vn+** seeds were pre-germinated in a 20/30 °C growth chamber and once germinated, buried in plastic trays (17 × 12 × 5 cm) with sterilized moistened sand and placed in the 5 °C growth chamber. Non-vernalized treatments (**Vn-**) started a week before the end of the **Vn+** treatment. **Vn-** seeds were pre-germinated and buried in similar plastic trays but maintained in a 20/30 °C growth chamber until plants reached the same stage of development as **Vn+** (hypocotyl length of 0.5–1.5 cm). Seedlings from both treatments were then transplanted to germination trays, and maintained under greenhouse conditions (8 weeks) until transplant to pots in the field under natural photoperiods. In planting date **a** (late winter), a greenhouse conditioned to keep temperature above 10 °C, was used to minimize the risk of natural vernalization. A completely randomized design was used in these experiments.

2.3. Photoperiod and fertilization experiments

Artificial daylength extensions were used in some PDs (Table 1) to analyze the effect of photoperiod under contrasting growing conditions. Natural photoperiod (**PP_N**) was extended to a constant 18 h-daylength (**PP₁₈**) by using a combination of lamps (two 45 W incandescent bulbs and one 30 W fluorescent tube per plot, adding ≈0.43 MJ per hour of photoperiod extension) programmed to automatically turn on and off by an electronic timer (from 0500 h to dawn, and from dusk to 2200 h). In planting date **c**, an additional photoperiod level (15 h per day, **PP₁₅**) and a fertilization treatment (**N**) were included to increase variability in plant growth and to explore photoperiod by fertilization interactions. A complete randomized design was used for the photoperiod experiments, and a factorial split-plot design was employed for the photoperiod × fertilization one, using photoperiod as the main plot (3 levels: **PP₁₈**, **PP₁₅** and **PP_N**) and fertilization as subplots (2 levels: **N+**, fertilized and **N-**, without fertilization). **N+** consisted of the application of 2 g per pot of a granular NPK fertilizer (20–20–20), 10 d prior to transplant. **N** was also applied in summer planting date **e**, but no daylength extension was used in that experiment.

2.4. Phenology and growth determinations

Flower beginning time (**FBT**) was defined as the time from transplant to the beginning of flowering and was registered in every individual plant for each PD. The proportion of plants that flowered (at least one open flower in the main stem) before the second growing season (i.e. the second spring–summer period in a plant lifecycle) was used to estimate the % of annual plants (**%An**); otherwise plants were considered as biennials. The onset of stem elongation (**OSE**), which precedes flowering was recognized as the first macroscopic sign of the reproductive status and included in the analysis for planting dates **c** to **m**. A plant was registered at **OSE** when its stem reached 1 cm in length, determined by fitting non-linear functions of plant height against time, with height measured on a 3–4 day-interval. Estimations of rosette area (**RA**, cm²) were performed twice a week from digital photographs taken from above

Table 1

Description of the experiments performed in this work. **PD**: planting date identification, **Mean Temp.**: mean temperature from planting to the first sign of a reproductive behavior in each experiment/treatment (**OSE₍₁₎**), **Vn days**: sum of days with mean temperatures below 10 °C between planting in germination trays and **OSE₍₁₎**, **N**: number of pots employed per plot (replicate). Treatments: **Vn**: seed vernalization; **N**: NPK fertilization; **PP**: photoperiod extension. All treatments included its respective controls, except in **PD g**, in which only photoperiod-extended plants were employed (**PP₁₈**). Variables measured: **%An**: proportion of annual plants; **FBT**: time to flowering beginning; **OSE**: onset of stem elongation; **GR**: rosette's growth rate (includes measurements of rosette's area **RA**).

PD	Outdoors planting date	Treatments	Onset of treatment	Variables measured	Mean Temp.	Vn days	N
a	07 September 2005	Vn	16 June	%An; FBT	16.4	5	16
b	09 December 2005	Vn	06 September	%An; FBT	23.3	0	16
c	13 October 2006	N; PP	13 October; 17 October	%An; FBT; OSE	19.3	14	10
d	21 May, 2007	PP	19 June	%An; FBT; OSE; GR	10.1	29	18
e	10 January 2008	N	01 January	%An; FBT	25.0	0	12
f	29 January 2008	Vn; PP	26 November; 1 February	%An; FBT; OSE; GR	24.8	0	8
g	11 July 2008	PP	16 July	%An; FBT; OSE; GR	14.1	14	20
h	06 August 2008	PP	11 August	%An; FBT; OSE; GR	13.9	10	32
i	11 November 2008	PP	11 November	%An; FBT; OSE; GR	23.5	0	20
j	20 November 2008	PP	21 November	%An; FBT; OSE	24.2	0	16
k	05 February 2009	PP	14 February	%An; FBT; OSE; GR	24.6	0	20
l	18 March 2010	PP	24 March	%An; FBT; OSE; GR	21.3	0	21
m	08 July 2010	PP	09 July	%An; FBT; OSE	9.5	25	7

the plants, and analyzed with an image-processing software (UTH-SCSA Image Tool 3.00). Growth rate (**GR**, $\text{cm}^2 \text{d}^{-1}$) was calculated for each plant as the ratio of the difference between two consecutive **RA** measurements and the time interval between them.

2.5. Data analysis

Two-tailed unpaired t-tests were performed to evaluate differences ($p < 0.05$) in **%An** and **FBT** between **Vn+** and **Vn-** (planting dates **a**, **b** and **f**) or **PP₁₈** and **PP_N** (planting dates **c** to **m**). **%An** data were analyzed following arcsine transformation (Little and Hills, 1975). In **PD c**, a two-way ANOVA was employed to quantify the effects of photoperiod, fertilization and their interaction on **%An** and duration of two developmental phases of annual plants: planting to **OSE** and **OSE** to flowering. Additionally, linear and non-linear regression analyses were employed on annual plants data from **PP₁₈** treatments in different **PD** to determine if the rate of development toward **OSE** (**DR_{PP-OSE}**) under photo-inductive conditions is modulated by the size (**RA_{pp}**, rosette area at the onset of the photoperiod treatment) or growth rate (**GR_{pp}**, mean growth rate of rosettes during the photoperiod treatment) of rosettes. **GR_{pp}** was calculated for every plant as the mean **GR** between the onset of the photoperiod treatment and that of reproduction (**OSE₍₁₎**, the date when the first **PP₁₈** plant in the experiment reached the **OSE** status). The period before **OSE₍₁₎** was chosen to exclude reproductive effects on **GR** of the most developmentally advanced plants within a treatment.

3. Results

3.1. Planting date effects

The different environments provided by planting dates highly impacted on the type of reproductive behavior (annual or biennial) of *O. biennis*, and the length of the pre-flowering phase of annual plants (Fig. 1). No biennial plants were registered in May to August **PDs** (late autumn to mid-winter). Biennial behavior was increasingly evident in late-winter to summer **PDs** as an abrupt decrease in **%An**. In summer **PDs** almost none of the plants flowered during the first growing season. The length of the pre-flowering phase (**FBT**) of annual plants shortened as **PD** was delayed from May (178 d) to December (55 d). Differences in lifecycle length between plants of different reproductive behavior can be inferred from the January planting date (**PD f**), when biennial plants flowered more than 200 days later than annual plants.

3.2. Vernalization effect

Seed-vernalization treatment (**Vn+**) increased **%An**, and accelerated the rate of development toward flowering in both planting dates **a** and **b** (late winter and spring, respectively), but not in planting date **f** (mid summer), where none of the plants exposed to natural daylength flowered in the first growing season, regardless of their vernalization treatment (Fig. 2). In **PD a** vernalized plants (**Vn+**) flowered 4.5 days before non-vernalized plants (**Vn-**, $p < 0.05$) and the proportion of annual plants increased from 82% in **Vn-** to 94% in **Vn+** ($p < 0.05$). A similar pattern was observed in **PD b** in which vernalization tended to increase **%An** (from 34% to 52% for **Vn-** and **Vn+**, respectively, $p < 0.1$) and to shorten **FBT** in 2.6 days ($p = 0.13$). Compared to **PD a**, **PD b** presented a lower **%An** and a shorter **FBT**, in accordance with the general response to planting date variation observed in Fig. 1.

3.3. Photoperiod effect

Daylength extension had a major impact in determining the reproductive behavior of *O. biennis*. In summer **PDs** in which annual behavior did not naturally occur (Fig. 1) **PP₁₈** treatment increased **%An** to 83.3%, 96.7% and 98.4% for **PD f**, **k** and **l**, respectively but

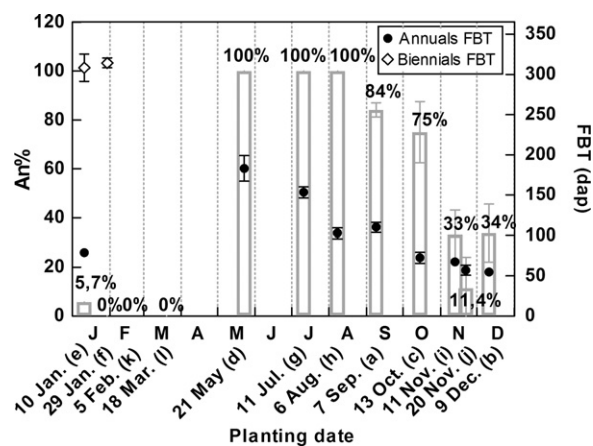


Fig. 1. Proportion of annual plants (**%An**; bars) and flowering beginning time (**FBT**; days after outdoor planting; points) of plants of *Oenothera biennis* from different planting dates. Closed symbols represent mean **FBT** of annual plants and open symbols represent **FBT** of plants with biennial behavior (only for two planting dates). Letters between brackets indicate planting date identification as presented in Table 1. Vertical segments in each bar/symbol represent the standard error of the means.

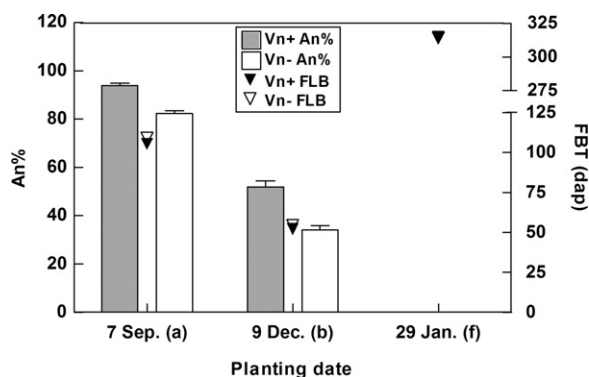


Fig. 2. Proportion of annual plants (%An; bars) and flowering beginning time (FBT; days after outdoor planting; triangles) of seed-vernalized (filled bars; closed triangles) and non-vernalized (open bars; open triangles) *Oenothera biennis* plants from different outdoors planting dates. Letters between brackets indicate planting date identification as presented in Table 1. Vertical segments in each bar/symbol represent the standard error of the means.

was not sufficient to reduce the high incidence of biennial behavior commonly observed in late-spring PDs (i.e. PDs i and j), suggesting that other factor/s could be involved in flower induction under those conditions (Fig. 3). Like for seed-vernalization responses, PP₁₈ affected not only %An but also reduced FBT of annual plants. Differences between photoperiod treatments were reduced as PD was delayed toward the summer and the difference between natural and extended photoperiod decreased.

3.4. Photoperiod × fertilization interactions

The photoperiod × fertilization experiment performed in PD c (early spring) showed that, in addition to vernalization and daylength, mineral nutrition has a role in *O. biennis* development (Fig. 4). %An increased significantly from 75 ± 7% to more than 95% in response to photoperiod extension ($p < 0.05$), NPK fertilization ($p = 0.016$; Fig. 4A) or the combination of both. FBT was also modified by photoperiod and NPK fertilization, but their effect differed (Fig. 4B). On the one hand, photoperiod extension anticipated flowering in a similar way in PP₁₅ and PP₁₈, by shortening the length of two developmental sub-phases: planting to OSE (5 days shorter in PP₁₅ and PP₁₈ versus PP_N, $p < 0.0001$) and OSE to flowering (8 days shorter in photoperiod-extended treatments, $p < 0.0001$). However,

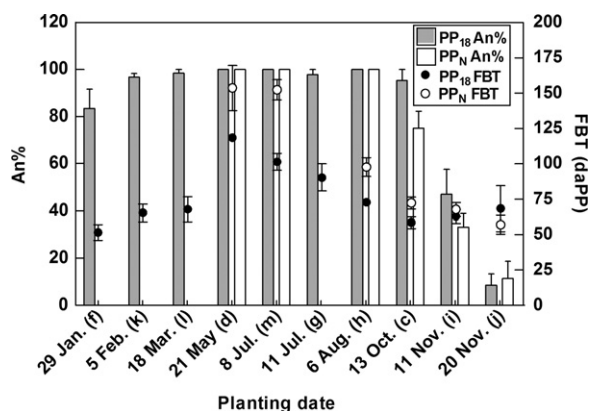


Fig. 3. Proportion of annual plants (An%, bars) and time to flowering beginning of annual plants (FBT, days after onset of photoperiod treatment; circles) in a 18-h photoperiod-extended treatment (PP₁₈, filled bars and circles) and a natural daylength treatment (PP_N, open bars and circles) in different planting dates (identification letter between brackets). In planting date g, only photoperiod-extended (PP₁₈) plants were employed, without a control level (PP_N). Vertical segments in each bar/symbol represent the standard error of the means.

mineral fertilization only shortened the length of the former sub-phase (5 days shorter than non-fertilized treatments, $p < 0.0001$) but not that of the latter ($p > 0.05$). In PD e, N treatment did not have a significant effect neither on %An nor on FBT as arising from the fact that only 2 plants in the experiment (from N- treatment) presented annual behavior (data not shown).

3.5. Growth-development relationship

DR_{pp-ose} was positively related to GR_{pp} of photoperiod-extended plants, but this relationship was stronger when data from experiments exposed to warmer temperatures (i.e. higher than 22–23 °C, according to Table 1) were excluded from the analysis (Fig. 5A). Under cool growing conditions (PDs d, g, h and i), DR_{pp-ose} responded linearly to increasing GR_{pp} up to 5 cm² d⁻¹, with a diminishing response at higher GR_{pp} values. Plants exploring warmer growing conditions (PDs f, i and k) presented lower DR_{pp-ose} values at a given GR_{pp}, suggesting supraoptimal temperature conditions for development. No relationship was found between DR_{pp-ose} and RA_{pp} when all PP₁₈ data were analyzed together ($r^2 = 0.05$; data not shown). However, a linear association between GR_{pp} and RA_{pp} was found when each PD was analyzed by separate (Fig. 5B), so RA_{pp} would partially determine DR_{pp-ose}, through its effect on the subsequent growth rate of rosettes.

3.6. High temperature effect

The prevalence of biennial behavior in late-spring planting dates, even when Vn+ (PD b) or PP₁₈ (PDs i and j) treatments were applied, led us to consider the *ad-hoc* hypothesis that other factors could be preventing the transition to the reproductive phase (OSE) in those conditions. As every plant that reached the OSE status flowered in a short time during the same season, we explored whether environmental conditions prior to OSE have a bearing on the reproductive behavior of *O. biennis*. Accordingly, %An seems to strongly depend on the time of the year in which OSE started (OSE₍₁₎); when OSE occurred in a time window between November and February, there was a proportion of plants that behaved as biennials, even under extended photoperiods (Fig. 6A). This time window coincides with the warmest season of the year, suggesting that high temperatures may have an inhibitory effect on flowering initiation of *O. biennis*. Thus, %An remained practically at 100% in those experiments where the onset of reproduction (i.e. an arbitrary chosen period of 15 days starting at OSE₍₁₎) coincided with a mean maximum temperature (T_{max}) lower than 27 °C, but declined abruptly when this temperature threshold was surpassed (Fig. 6B).

4. Discussion

As reported in previous studies, planting date had a major impact on the reproductive behavior of *O. biennis* (Roy et al., 1993; Król, 2007). Under non-manipulated field conditions, annual behavior prevailed in early planting dates (autumn to late winter) and the proportion of biennial plants increased as planting date was delayed from late-winter to summer (Fig. 1).

Although biennial behavior in late sown *O. biennis* (i.e. spring–summer) had previously been attributed to a non-satisfied absolute cold requirement (Chouard, 1960; Picard, 1967; Grignac, 1988; Reekie, 1997; Berti et al., 2006), the evidence from this study does not support this premise. A high proportion of annual plants occurred in Vn- of PD a and a considerable amount of Vn- plants also behaved as annuals in PD b, both exploring temperatures usually assumed as non-vernalizing (>10 °C) (Fig. 2), although some degree of natural vernalization cannot be discarded in PD a (Table 1). The absence of an absolute vernalization requirement is also shown in summer PDs f and i, where photoperiod extensions

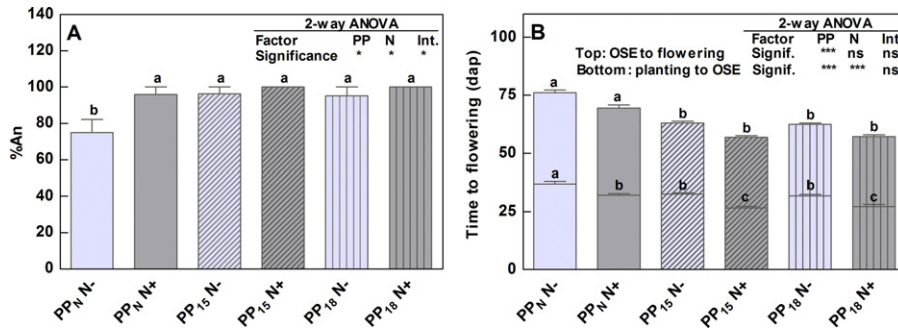


Fig. 4. (A) Proportion of annual plants (%An) and (B) Duration of pre-flowering phase in a photoperiod × fertilization experiment of *Oenothera biennis* (planting date c). In (B) pre-flowering phase is divided in two sub-phases: from planting to OSE (bottom bars) and from OSE to flowering (top bars). Significance of the factors effect (PP: photoperiod; N: fertilization; Int.: interaction between PP and N) from the two-way ANOVA is expressed as * ($p < 0.05$), *** ($p < 0.0001$) or ns (not significant, $p > 0.05$). Different letters indicate significant differences (Tukey, $p < 0.05$) among treatments. Vertical segments in each bar represent the standard error of the means.

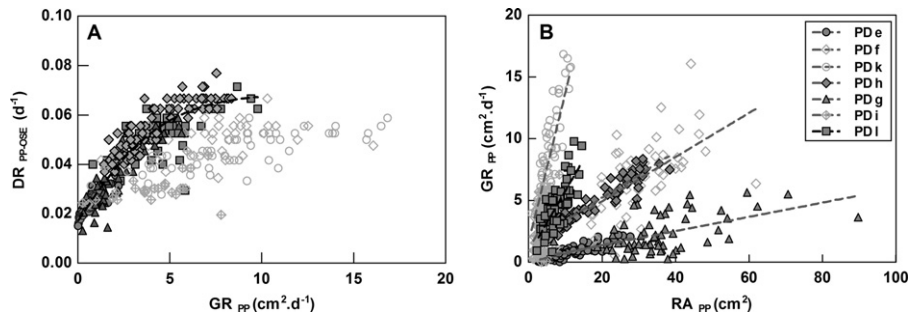


Fig. 5. (A) Relationship between the rate of development from the beginning of PP_{18} treatment to the onset of stem elongation (DR_{PP-OSE}) and the mean growth rate during the PP_{18} treatment before the onset of reproduction (GR_{PP}); (B) Growth rate during PP_{18} treatment as a function of rosette area at the onset of photoperiod treatment (RA_{PP}). Each symbol represents an individual plant. Different symbols represent different planting dates (PD). Filled symbols correspond to PDs exploring cool growing conditions (mean temperature below 22 °C), open symbols correspond to PDs exploring warm growing conditions (mean temperature higher than 23 °C) during the vegetative phase. The segmented line in (A) represent a non-linear regression fit (Boltzmann sigmoid; $r^2 = 0.89$, $n = 267$) for planting dates with cool growing conditions; segmented lines in (B) represent linear regression fits for different PDs.

(PP_{18}) were enough to achieve high percentages of annual plants without vernalization. As $Vn+$ treatment had an effect in flowering promotion and increased %An in PDs a and b, vernalization would be a facultative rather than an obligate requirement for flowering in this species.

As regards to photoperiod effects, our results clearly indicate that this species is an obligate long-day plant, in agreement with previous references (Chouard, 1960; Vince-Prue, 1975; Liu et al., 2003) and with our first hypothesis. This response explains why in summer PDs (f, k, l) none of the plants exploring decreasing photoperiods (PP_N) flowered in the first growing season while most of photoperiod-extended plants (PP_{18}) did so (Fig. 3). In early PDs (autumn–winter, d, m, h) photoperiod extension had a quantitative

effect on time to flowering beginning. Thus, both PP_{18} and PP_N achieved 100% of annual plants, while PP_{18} flowered earlier.

Although an obligate photoperiod response could explain the qualitative effect of daylength extension in summer PDs and the quantitative effect in autumn–winter ones, it does not explain the high proportion of biennial plants in spring PDs c, i and j (Fig. 3), in which photoinductive conditions are satisfied for both PP_{18} and PP_N treatments. As PD was delayed from August to November, the proportion of annual PP_N plants decreased abruptly from 100 to 10%, and this tendency could not be reverted by the PP_{18} treatment. Therefore, other conditions besides long photoperiods should be met to trigger the flowering response. Our findings suggest that a period of high temperatures just before OSE may act as an

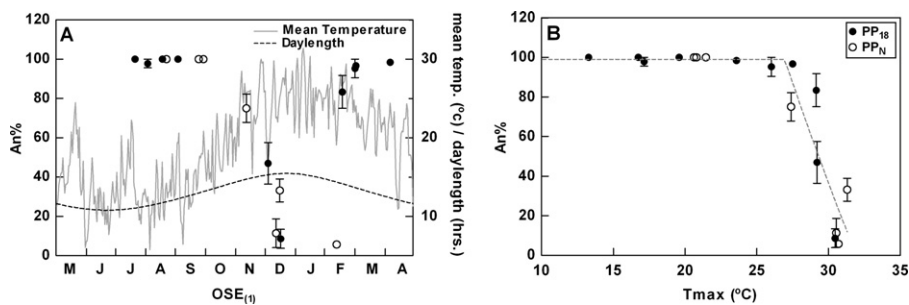


Fig. 6. (A) Proportion of annual plants (An%) for experiments exploring natural (PP_N , empty symbols) or 18-h (PP_{18} , filled symbols) photoperiods, as a function of (a) the date in which reproduction started ($OSE_{(1)}$), and (b) the mean maximum temperature (°C) of a 15-day period starting at $OSE_{(1)}$ (T_{max}). Full and segmented lines in (A) show illustratively the annual dynamics of mean temperature and daylength for the year 2008, respectively. Segmented line in (B) represents a bi-linear function fit (plateau followed by a one phase decay, $r^2 = 0.84$). Vertical segments in each symbol represent the standard error of the mean.

inhibitory factor that would prevent reproduction under otherwise photo-inductive conditions (Fig. 6). For different PDs, we could successfully relate %An to the mean maximum temperature (T_{max}) of an arbitrary 15 d-time window starting at $OSE_{(1)}$ (when only one or few plants in a treatment reached the reproductive status, while the remaining were still defining whether they will flower – annuals- or not – biennials-); the proportion of annual plants decreased abruptly as T_{max} increased above a threshold of around 27 °C (Fig. 6B). Once reproduction is initiated (plants reached the OSE stage), high temperatures would not suppress flowering as arising from the fact that all plants that reached OSE flowered shortly after in our experiments, no matter the environmental conditions. Inhibition of reproduction by high temperatures was also reported for another facultative biennial, *Eustoma grandiflorum* (Ohkawa et al., 1991; Harbaugh, 1992, 2007).

Concerning our second hypothesis we found that, in agreement with it, the time to reproductive growth initiation (OSE) under inductive photoperiods (PP₁₈) depended quantitatively on rosette's growth rate (GR_{pp}) (Fig. 5A). Under inductive photoperiods and mild temperatures, the higher the GR_{pp}, the sooner the plant reached the OSE status. This relationship would also explain why in PD c, fertilized plants (N+) flowered earlier than non-fertilized ones (N-; Fig. 4B). The quantitative effect of plant growth rate on rate of development toward OSE could also explain the qualitative effect of N+ on *O. biennis* reproductive behavior (i.e. the proportion of plants with annual behavior, %An; Fig. 4A). Factors that hasten flower transition, like vernalization or photoperiod, but also growth factors such as mineral fertilization (N+), may exert a positive effect on %An, by allowing flower induction to occur under adequate conditions for flower transition. As in nature photo-inductive daylengths and inhibiting high temperatures may temporally overlap (Fig. 6A), the success of a crop will depend on its possibility to place the onset of reproduction as early as possible in the spring–summer season, when both photoperiod and temperature are favorable to reproduction. If because of late sowing dates or poor growing conditions (low soil fertility, lack of rainfall, very high plant densities, etc.) flower induction is delayed toward summer, plants will be exposed to inhibiting high temperatures and behave as biennials.

The evidence collected in this work provides an alternative explanation for the influence of rosette size and/or growth factors on *O. biennis* reproductive behavior. Biennial behavior registered in late sown crops (Roy et al., 1993; Król, 2007) or crops grown under restricting growing conditions (Kromer and Gross, 1987; Roy et al., 1993; Small and Catling, 1999; Król and Berbeć, 2004; Król, 2007) would not be the consequence of the non attainment of a critical size for reproduction *sensu* Werner (1975) and Gross (1981), but the result of delaying flower induction until environmental conditions that inhibit reproduction. Contrary to previous reports on this (Chouard, 1960) and other facultative biennial plant species (Werner, 1975; Baskin and Baskin, 1979; Klinkhamer et al., 1987; Prins et al., 1990), we found no evidence of a critical size for vernalization, as even pre-germinated seeds could respond to the Vn+ treatment by anticipating flowering time (Fig. 2).

In addition, a threshold size for photoperiod response was not evident: rosettes as small as 40 cm² where observed at OSE state in PD d, while very large rosettes (RA > 600 cm²) of PP₁₈ treatment behaved as biennials in PDs f and i (data not shown). Moreover, when PP₁₈ data from different PDs were analyzed together, RA_{pp} was not as a good predictor of rosette development (data not shown) as GR_{pp} (Fig. 5A). The fact that RA_{pp} partly determines the subsequent GR_{pp} of rosettes, in a different manner for different growing conditions (Fig. 5B), may explain why previous reports on this (Gross, 1981) and presumably other facultative biennials (Kachi and Hirose, 1983, 1985; De Jong et al., 1986; Lacey, 1986; Rees and Rose, 2002) succeeded in predicting flowering behavior

from size measurements taken in a given environment, and why the hypothetical threshold size of a species vary across different growing conditions (Wesselingh et al., 1993, 1997; De Jong et al., 1998).

5. Conclusion

The control of flowering in *O. biennis* seems to rely on the effect of two main environmental cues: long photoperiod, which is an obligatory requirement for reproduction; and high temperatures blocking flowering under otherwise inductive photoperiodic conditions. As in nature these cues partially overlap, the reproductive behavior of this species strongly depends on when reproduction initiates: as long as plants are exposed to photo-inductive conditions and cool temperatures, they will flower. Since the probability of meeting these conditions is higher early in the spring–summer season, every factor that hastens the rate of development toward reproduction would indirectly promote annual behavior. Besides vernalization, growth rate has a bearing on *O. biennis* rate of development so, in addition to common developmental factors, growth factors can also influence reproductive behavior of this species.

Acknowledgements

We thank Marisol Berti Diaz for providing the genetic material employed in this work, and Sofia Güell and Luis Pedace for technical assistance in the experiments.

This work was supported by a grant from the University of Buenos Aires (UBACYT G008 and W219).

Appendix A. List of abbreviations

H1	Hypothesis 1
H2	Hypothesis 2
PD	planting date
Vn+	seed vernalization treatment
Vn-	non vernalized treatment
PP _N	natural photoperiod treatment
PP ₁₅	15-h photoperiod treatment
PP ₁₈	18-h photoperiod treatment
N+	fertilized treatment
N-	non fertilized treatment
OSE	onset of stem elongation in each plant
OSE ₍₁₎	day when the first plant of a treatment reaches OSE
FBT	time from transplant to flowering beginning (first open flower in each plant)
%An	proportion of annual plants in a treatment
RA	rosette area (cm ²)
RA _{pp}	rosette area at the onset of the photoperiod treatment (cm ²)
GR	rosette growth rate (cm ² d ⁻¹)
GR _{pp}	rosette growth rate from the onset of the photoperiod treatment until OSE ₍₁₎ (cm ² d ⁻¹)
DR _{pp-ose}	rate of development from the onset of the photoperiod treatment to OSE (d ⁻¹)
Tmax	mean maximum temperature (°C) of a 15 d-time window starting at OSE ₍₁₎

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