The Combined Effects of Vegetative Stage Corms, Ultra Low Oxygen Cooling Storage and Incubation Time on *Crocus sativus* L.

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**Abstract:** In order to extend the flowering season of *Crocus sativus* L. more time than the methods developed to date and to study their limits, a multivariable trial was carried out with the following factors: vegetative stages of the corm; ultra low oxygen (ULO) cooling storage and incubation time. The main production parameters and the quality saffron were studied. The usage of corms lifted 40 days earlier leaf senescence (V3–40 d) was not technically viable. No benefits were obtained from corms lifted shortly after leaf senescence (V3). Most of the parameters decreased with increasing of storage in ULO chambers for the vegetative stages studied. The corms lifted 20 days earlier leaf senescence (V3–20 d), stored from 0 to 120 days in ULO chambers and incubated 30 and 60 days provided greater number of flowers and more weight of saffron than corms non-incubated. A predictive model was obtained which allows to know the saffron yield in corms lifted in the stage V3–20 d, stored from 0 to 180 days in ULO chambers followed by incubation from 30 to 120 days. The combination of the three factors studied allowed to extend the *Crocus sativus* L. flowering from October to early February with an acceptable saffron yield and a quality similar than saffron grown traditionally.

**Keywords:** predictive model; yield; forced crop; flowering; saffron quality

1. Introduction

Saffron, commercially defined as the dry stigmas of *Crocus sativus* L., is valued for being one of the few spices able to provide colour, taste and flavour to those foods and beverages where it is added. Currently, saffron is highly valued in food and nutraceutical industries, due to its many therapeutic properties [1,2] and because the use of synthetic colorants and flavours is less and less accepted by consumers.

*C. sativus* L. is characterized by a biological cycle with a long pause in summer and an active growth in autumn. Saffron production depends on size of the corms [3]. The flowering of the maternal corm happens from the second half of October to the first half of November. The leaf emergence may occur before, at the same time or after flowering. Next, the vegetative growth of the leaves and roots of the maternal corm begins along with the daughter corms developed (December to February). Next, the gradual senescence of leaves, roots and the maternal corm starts. The transition from vegetative to reproductive development occurs in March [4]. From April to May the corms start their dormant period, although flower differentiation happens during its latency phase (June to August). The optimal
temperature for flower initiation is located between 23–27 °C [5] and the temperature optimum for flower emergence is between 15–17 °C [6,7].

*Crocus sativus* L. is a plant which is cultivated with a low level of mechanization. Most of the operations still carried out manually, so a lot of labour is necessary in a short time, the field work is hard and frequently done under adverse climatic conditions. These disadvantages, along with other social and economic reasons, have caused European saffron production to decrease significantly during the nineties, even disappearing in some areas. Nowadays the production of saffron under greenhouse and the mechanization of some growing operations, such as flower harvesting and separation of the different parts of the flower, are being implemented in *C. sativus* L. cultivation [8–12]. According to the MAPA [13], in Spain there has recently been an increase in the production of this spice, mainly favoured by an increase in its price. These data demonstrate that there is still a great interest in saffron, being this spice highly demanded as active ingredient in food supplements, functional foods, beverages; and in cosmetic formulations and pharmaceutical preparations [14].

Some authors have studied ways to extend the flowering season of *C. sativus* L. through forced production under greenhouse and controlled climate conditions. Benschop [15] stated that corms may be kept at 25 °C for up to eight months and thus retard flowering. However, Molina et al. [6] observed that the flower primordia aborted when the incubation at 25 °C was longer than 150 days. Molina et al. [16] managed to extend their flowering period from early September to mid-December by incubating at 25 °C corms lifted in different vegetative stages.

Molina et al. [5] studied the effect of the low temperature on the flowering of *C. sativus* L. They tested that the storage of corms at 2 °C after flower initiation produces abortion of those flowers already initiated. The more advanced the stage of flower initiation at the beginning of cold-storage, the faster the rate of flower abortion. Storage at freezing temperatures (0 °C or −1 °C) damages the corms, although flowering can be induced in corms stored between 0.5–2 °C. Corms stored in cold before flower initiation form flowers when are stored in incubation conditions at 21–25 °C. The number and size of flowers formed and the yield of saffron per corm depend on the duration and conditions of cold-storage. These parameters decrease gradually with increasing duration of cold-storage; they observed that this decrease is slower when the storage is performed in 1% oxygen than in a normal atmosphere. From these findings they achieved to extend the *Crocus sativus* L. flowering from December to May, but with saffron yield similar to non cold-storage corms only from December to January.

For each crop there are several production parameters associated with it that are of great importance in order to evaluate its performance and thus its profitability. Moreover, in the case of *C. sativus* L., it is essential to study the quality of the spice obtained.

Poggi et al. [11] determined the quality of saffron according to ISO 3632 [17] in saffron samples obtained from corms stored under incubation conditions. Many authors have demonstrated that ISO 3632 [17] does not determine properly the content of picrocrocin and safranal [18,19] which are responsible for saffron’s taste and flavour, respectively. In a recent work published by this group, the saffron quality obtained under controlled environmental conditions has been carefully assessed. The study of the effects of growing *C. sativus* L. under two different forcing conditions independently, considering two different vegetative states of corms, on the quality of its obtained saffron has been carried out [20].

In general, predictive models developed for the different types of crops are very useful tools to estimate mainly the different phenological stages, production parameters, and their effect on yield. In the specific case of *C. sativus* L., to our knowledge, no predictive model has been applied so far. Moreover, its cultivation under forcing conditions has been approached in a lesser proportion, so the multivariable combination of three factors (unlike vegetative stages of the corm; different storage time at low temperature and controlled atmosphere; diverse incubation times) as well as its effect on the main production has not been previously studied together.
Therefore, the aim of this work was to test the limits of the methods developed for the cultivation of *C. sativus* L. under forcing conditions and study the possibility of extending their flowering season through the combination of three factors, and determining the effect on the main production parameters and on the saffron quality of the treatments that provided an acceptable yield of saffron.

2. Materials and Methods

2.1. Plant Material

This trial was carried out at the experimental facilities of “Las Tiesas” Albacete, Spain, for two growing seasons (2010–2011 and 2011–2012). The *C. sativus* L. corms were obtained from the company Corporación de Operadores de Azafrán Español, S.L. (Albacete, Spain) and the company Agrícola de Transformación, Manipulación y Comercialización, S.L. (Minaya, Spain). They were lifted before flower formation, in three different vegetative stages: 40 days earlier leaf senescence, the second half of May (V3–40 d); 20 days earlier leaf senescence, the second half of June (V3–20 d); shortly after leaf senescence, the first half of July (V3). The corms were cleaned and graded, and those with an equatorial diameter greater than 35 mm were selected. These corms were dipped in 0.1% prochloraz solution to prevent *Fusarium* and *Penicillum* infestation and were dried immediately using forced airflow. Following, the corms were grouped in batches of 100 units, kept in mesh bags and submitted to different forcing conditions.

2.2. Forcing Conditions

The forcing conditions were chosen according to the results obtained by other authors [5,6].

2.2.1. Cold Storage

The corms were stored in an ultra low oxygen (ULO) cooling chamber in order to retard the growth of buds in the following conditions: 2.5 ± 0.5% O₂ concentration, at 1.0 ± 0.2 °C, with a relative humidity of 70–80% and the ventilation was provided to keep the CO₂ concentration below 600 ppm. The ethylene concentration was also controlled placing Petri dishes of potassium permanganate inside the chamber. The corms were storage during 0, 30, 60, 90, 120, 150 and 180 days, from June to December. Once completed the time in ULO chambers, the corms were kept at incubation conditions.

2.2.2. Incubation Conditions

The corms previously stored in ULO chamber were incubated at 25.0 ± 0.5 °C with a relative humidity of 70 ± 5% and the ventilation was provided to keep the CO₂ concentration below 2500 ppm. The corms were incubated for 0, 30, 60, 90 and 120 days, after their corresponding stay in ULO chambers. Next the corms were placed to flowering.

2.2.3. Flowering Conditions

To force flowering corms each batch of 100 corms was placed in a rigid plastic tray (1.20 m of long, 0.50 m of wide and 0.12 m of height). The corms were covered with a thin vermiculite layer. They were watered (by means of a diffuser) and stored in a flowering room at 18 ± 2 °C under photoperiod of 8 h light and 16 h dark with fluorescent lamps from 2000 to 4000 lux.

2.3. Experimental Design

The experimental design was carried out with the three studied factors: vegetative stage, time in ULO chamber, incubation time and all their possible combinations. For each treatment two set of 100 units were used in each growing season, having two measurements every parameter studied.

The production parameters that show the influence of different forcing conditions on the *Crocus sativus* L. production are: flowering corms (FC), weight of saffron per corm (WSC), number of flowers per corm (Nº FC), unit weight of dry stigma (UWDS) and flowering time (FP). These parameters
were measured through a calibrated analytical balance (Gram Precision, Series ST, Barcelona, Spain). Moreover, the quality of the obtained saffron from the treatments which provided an acceptable yield was evaluated, according to ISO 3632 [17] and using an HPLC-DAD method [21], for the purpose of being compared with the traditionally grown saffron.

The stigmas obtained were dehydrated in an oven without convection (Cortem, Selecta, Barcelona, Spain) on a silk sieve with a diameter of 15 cm at 110 ± 2 °C for 40 min.

The first growing season (2010–2011) was a preliminary study, carried out for establishing vegetative stage of corms and ULO and incubation times used during the second growing season.

The experimental design and the measured variables are summarized below (Table 1).

**Table 1.** Experimental design and measured variables for the growing season 2011–2012.

| Samples | Vegetative Stage | ULO Cooling Chamber Time (Days) | Incubation Time (Days) | Flowering Conditions | Production Parameters | Analytical Determination |
|---------|------------------|---------------------------------|------------------------|----------------------|-----------------------|-------------------------|
| 2 repetitions 100 corms/treatment | V3–20 d and V3 | 0, 30, 60, 90, 120, 150 and 180 | 0, 30, 60, 90 and 120 | 18 ± 2 °C; 8 h/16 h photoperiod (light/dark) | FC, WSC, N° FC, UWDS and FP | Quality saffron (ISO 3632:2011 and HPLC-DAD [21])
| And all their possible combinations |

V3: shortly after leaf senescence; V3–20 d: 20 days earlier leaf senescence; FC: flowering corms; WSC: weight of saffron per corm; N° FC: number of flowers per corm; UWDS: unit weight of dry stigma; and FP: flowering time.

### 2.4. Saffron Extraction Preparation and Solvents

The saffron aqueous extracts were prepared following ISO 3632 [17]. A total of 500 mg of powdered saffron, previously passed through a sieve of 0.5 mm pore diameter, was placed in a 1 L volumetric flask and 900 mL of Milli-Q water as added. The solution was stirred using a magnetic stir bar at 1000 rpm for one hour while being kept away from light. The flask was filled to the 1 L mark, and the solution was homogenized via agitation. The solution was filtered through a filter made of hydrophilic polytetrafluoroethylene (PTFE) with a pore size of 0.45 µm (Millipore, Bedford, MA, USA). Acetonitrile was obtained from Panreac (Barcelona, Spain). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

### 2.5. Spectrophotometric Analysis

The UV-vis of the saffron extracts after proper dilution (1:10, v/v) were monitored by scanning from 190 to 700 nm using a Lambda 25 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) with UV WinLab 2.85.04 software (Perkin-Elmer). Colouring strength ($A_{1\% 1\ cm}^{440}$ nm), $A_{1\% 1\ cm}^{257}$ nm, and $A_{1\% 1\ cm}^{330}$ nm were determined according to ISO 3632 [17]. All of the analyses were performed in duplicate.

### 2.6. Moisture and Volatile Matter Content

Determination of moisture and volatile matter content of saffron was carried out according to ISO 3632 [17], slightly amended; using 250 mg instead of 2500 mg for each treatment and in duplicate.

### 2.7. HPLC-DAD Analysis

Twenty microliters of each sample (saffron extracts) were injected into a 1200 HPLC chromatography system (Agilent, Palo Alto, CA, USA) equipped with a 150 mm × 4.6 mm i.d., 5 µm Luna C18 column (Phenomenex, Le Pecq Cedex, France) that was equilibrated at 30 °C. The eluents were water (A) and acetonitrile (B) with the following gradient: 20% B, 0–5 min; 20–80% B, 5–15 min; and 80% B, 15–20 min. The flow rate was 0.8 mL/min. The DAD detector (Hewlett Packard, Waldbronn, Germany) was set at 250, 330 and 440 nm for picrocrocin, safranal, and crocetin ester detection, respectively. All of the analyses were performed in duplicate, and two measurements were taken for each replicate. The identification and quantification of $trans$-4-GG-crocetin esters, $trans$-3-Gg...
crocetin esters, cis-4-GG-crocetin esters, cis-3-Gg crocetin esters, picrocrocin, and safranal were carried out as found in the previous work [21]. The other crocetin esters were identified as outlined in a previous paper [22] and the quantification as an approximation was carried out with the trans-crocetins esters with one or two glucoses being quantified with the calibration curve of trans-3-Gg-crocetin ester and the crocetin esters with five glucoses were quantified by the calibration curve of trans-4-GG crocetin ester. The same approximation was followed for the cis isomers. All of the analyses were performed in duplicate.

2.8. Statistical Analysis

Evaluation of the statistical significance of differences was performed using analysis of variance (ANOVA) and Duncan test to find the significance difference between mean values of the samples with the aid of the SPSS 24.0 for Windows statistical program (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of the Forcing Conditions on the Production Parameters

The vegetative stages of the corms chosen to be evaluated in this work would have to be previous to the stage of flower formation, because Molina et al. [5] checked that corms storage at 2 °C after flower initiation provide the abortion of those flowers already initiated. So, in this study the corms were lifted in three different vegetative stages: V3–40 d, V3–20 and V3. During the first growing season was showed the non-technical viability of the corms lifted V3–40 days. In this vegetative stage the presence of green leaves make difficult the extraction, cleaning and grading mechanized of the corms, so these operations should be made manually. For this reason, the vegetative stage V3–40 d was not carried out in the second growing season.

In Table 2 can be seen the mean value for each parameter analyzed and their significance level for the growing season 2011–2012. In this table is shown the main effects and the interactions between the factors studied (vegetative stages, time in ULO chamber and time in incubation conditions). In Table S1 are shown the results for the growing season 2010–2011 (Supplementary Material). For the season 2011–2012 the corms lifted in V3 did not flower when the storage time in ULO chamber was higher than 120 days, as can be seen in the Figures 1–4.

The interactions between the three studied factors on the production parameters are presented in the Figures 1–4. In Table 3 can be seen the slope in absolute value, intercept and correlation coefficient (R²) of the adjusted lines for these representations.

Table 2. Mean value of the parameters analysed and level of significant differences for the studied factors and their interactions, season 2011–2012.

| Parameter                        | Mean | Error | Main Effects | Interactions between Factors |
|----------------------------------|------|-------|--------------|-----------------------------|
|                                  |      |       | VS ULO INC    | VS*INC ULO*INC VS*ULO*INC |
| Flowering Corms (Units)          | 86.8 | ±0.25 | *** ***       | NS NS NS NS NS NS NS NS |
| Flowering Period (mg)            | 12.6 | ±0.32 | *** *** ***   | *** *** *** *** *** *** *** |
| Number of Flowers per Corm (Units) | 1.4  | ±0.02 | *** *** ***   | *** *** *** *** *** *** *** |
| Unit Weight of Dry Stigma (mg)   | 6.9  | ±0.09 | *** *** ***   | *** *** *** *** *** *** *** |
| Weight of Dry Stigma per corm (mg) | 10.1 | ±0.14 | *** *** ***   | *** *** *** *** *** *** *** |

VS: vegetative stage; ULO: days in ULO chamber; INC: days in incubation conditions. One-way analysis of variance (ANOVA) for each parameter and for the interactions studied, significant difference at * 0.01 < p < 0.05, at ** 0.001 < p < 0.01, and at *** p < 0.001, according to Duncan test 1.
Figure 1. Graph of the weight of saffron per corm (mg) for the different incubation times during the ULO storage, for the different vegetative stages studied (V3–20 d and V3).

Figure 2. Cont.
Figure 2. Graph of the number of flowers per corm (unit) for different incubation times during the ULO storage, for the different vegetative stages studied (V3–20 d and V3).

Figure 3. Graph of the unit weight of dry stigma (mg) for different incubation times during the ULO storage, for the different vegetative stages studied (V3–20 d and V3).
Figure 4. Graph of the flowering time (days) for different incubation times during the ULO storage, for the different vegetative stages studied (V3–20 d and V3).

Figure 1 shows the representation of WSC for the different incubation times, during the ULO storage. In the vegetative stage V3–20 d the saffron yield decreased linearly as the time in ULO increased:

\[
\text{WSC (mg per corm)} = m \times D + n
\]  

where \(m\) is the slope, \(D\) is ULO time in days and \(n\) is the intercept.

From 30 to 120 days in incubation conditions the slopes of the adjusted lines (Table 3) decreased progressively, so the saffron yield diminished slower as the ULO time got more increase. The corms stored in ULO chamber from 0 to 120 days, followed by 30 and 60 days at incubation conditions, provided saffron yield higher than that obtained from corms non-incubated.

For the vegetative stages V3 the saffron yield obtained was significantly less than that reached from corms lifted in V3–20 d, because a great number of flower buds aborted. The effect of ULO storage was similar to V3–20 d, though the slopes of the adjusted lines for the different incubation times were less than in V3–20 d, so the saffron yield were more regular than V3–20 d. The incubation did not offer positive effect for most of the studied conditions.
Table 3. Slope, intercept, and correlation coefficient ($R^2$) of the curves adjusted for each studied parameter.

| Parameter                           | VS | INC (days) | Slope (m) | Intercept (n) | $R^2$ |
|-------------------------------------|----|------------|-----------|---------------|-------|
| **Weight of saffron per corm**      |    |            |           |               |       |
| V3–20 d                             | 0  | −0.179     | 27.385    | 0.892         |       |
|                                     | 30 | −0.220     | 35.779    | 0.939         |       |
|                                     | 60 | −0.189     | 32.331    | 0.937         |       |
|                                     | 90 | −0.129     | 22.328    | 0.951         |       |
|                                     | 120| −0.103     | 17.599    | 0.963         |       |
| V3                                  | 0  | −0.107     | 12.288    | 0.848         |       |
|                                     | 30 | −0.137     | 13.953    | 0.865         |       |
|                                     | 60 | −0.095     | 9.800     | 0.900         |       |
|                                     | 90 | −0.093     | 9.887     | 0.942         |       |
|                                     | 120| −0.122     | 12.585    | 0.900         |       |
| **Number of flowers per corm**      |    |            |           |               |       |
| V3–20 d                             | 0  | −0.019     | 3.036     | 0.884         |       |
|                                     | 30 | −0.024     | 4.232     | 0.980         |       |
|                                     | 60 | −0.021     | 3.786     | 0.953         |       |
|                                     | 90 | −0.018     | 3.212     | 0.911         |       |
|                                     | 120| −0.018     | 3.045     | 0.982         |       |
| V3                                  | 0  | −0.010     | 1.374     | 0.788         |       |
|                                     | 30 | −0.013     | 1.433     | 0.960         |       |
|                                     | 60 | −0.012     | 1.342     | 0.918         |       |
|                                     | 90 | −0.013     | 1.407     | 0.917         |       |
|                                     | 120| −0.016     | 1.692     | 0.907         |       |
| **Unit weight of dry stigma**       |    |            |           |               |       |
| V3–20 d                             | 0  | −0.062     | 10.829    | 0.931         |       |
|                                     | 30 | −0.022     | 8.721     | 0.721         |       |
|                                     | 60 | −0.008     | 8.439     | 0.310         |       |
|                                     | 90 | 0.014      | 6.064     | 0.196         |       |
|                                     | 120| 0.002      | 5.997     | 0.063         |       |
| V3                                  | 0  | −0.058     | 9.891     | 0.981         |       |
|                                     | 30 | −0.084     | 11.039    | 0.936         |       |
|                                     | 60 | −0.008     | 6.838     | 0.050         |       |
|                                     | 90 | −0.073     | 8.229     | 0.891         |       |
|                                     | 120| 0.014      | 6.578     | 0.130         |       |
| **Flowering time**                  |    |            |           |               |       |
| V3–20 d                             | 0  | −0.089     | 15.536    | 0.656         |       |
|                                     | 30 | −0.059     | 14.304    | 0.668         |       |
|                                     | 60 | −0.044     | 12.964    | 0.652         |       |
|                                     | 90 | −0.037     | 9.732     | 0.740         |       |
|                                     | 120| −0.029     | 7.411     | 0.651         |       |
| V3                                  | 0  | −0.095     | 14.100    | 0.823         |       |
|                                     | 30 | −0.105     | 14.000    | 0.887         |       |
|                                     | 60 | −0.093     | 11.400    | 0.958         |       |
|                                     | 90 | −0.098     | 11.000    | 0.733         |       |
|                                     | 120| −0.093     | 11.300    | 0.873         |       |

VS: vegetative stage; INC: time in incubation conditions.

In this study a saffron yield $\geq 10$ mg per corm was considered profitable, because is weight of one stigma, yields less than one flower per corm were not considered acceptable. These yields were obtained from corms lifted in the stage V3–20 d, stored up to 60 days in ULO chambers followed by incubation conditions from 0 to 120 days. Figure 5 shows the calendar of flowering for the forcing conditions which provided profitable saffron yield. In these conditions the saffron yield mean was 23 mg per corm, ranged from 36 to 10 mg of saffron per corm.
For the vegetative stage V3–20 d the slopes obtained in the Equation (1), for the different incubation times (Table 3), were represented vs. the incubation time, except time 0 days. The points were adjusted linearly with $R^2 = 0.975$ (Figure 6A), showing that the slopes were a linear function of the incubation time. The intercepts were also represented vs. the incubation time, displaying the same linear behaviour that the slopes, with $R^2 = 0.966$ (Figure 6B). Replacing these functions in the Equation (1), a model prediction equation for the saffron yield was obtained according to ULO and incubation times:

$$
WSC \text{ (mg per corm)} = 0.0014 \times D \times E - 0.2624 \times D - 0.2151 \times E + 43.1450 \quad (2)
$$

Where $D$ is ULO time in days and $E$ is incubation time in days. This model would be valid for corms in the stage V3–20 d, from 0 to 180 days in ULO conditions and incubated from 30 to 120 days.

Figure 2 represents the N° FC for different incubation times during the ULO storage. The behavior of this parameter was similar to WSC. The corms lifted in V3–20 d supplied a number of flowers per corm significantly greater than those lifted in V3. This parameter decreased as the time in ULO chamber increased. The corms lifted in V3–20 d and incubated during 30 and 60 days produced N° FC higher than the corms non-incubated.

The unit weight of dry stigma for different incubation times during the ULO storage is represented in Figure 3. This parameter was not showed a clear tendency with the storage time for both studied vegetative stages. In most cases decreased as the ULO time increased, being this effect clearer in corms incubated during short times.
Figure 4 shows the representation of the FP for the different incubation times during the ULO storage. For the vegetative stage V3–20 d the results obtained did not follow linear behaviour. From 0 to 60 days in incubation conditions the flowering time increased as the ULO time until 60 days. Then, it diminished as the ULO time increased. For long incubation periods, from 90 to 120 days, the flowering period decreased as the time in ULO conditions increased. The effect of the incubation time did not show clear tendency.

For the vegetative stage V3 the flowering time were significantly lower than in the previous vegetative stage. This parameter decreased as the time in ULO increased. The effect of the incubation time was not showed a clear tendency.

The combination of the three factors studied allowed to extending the flowering period of *Crocus sativus* L. from October to May. The flowering was extended until May from corms lifted in V3–20 d, stored 180 days in ULO chamber and incubated 120 days.

The date of entry of the corms under flowering conditions for the different treatments which provided profitable saffron yield in the stage V3–20 d, was as follows (Figure 5): 0 ULO and 0 Incubation (11 July), 0 ULO and 30 Incubation (10 August), 0 ULO and 60 Incubation (9 September), 0 ULO and 90 Incubation (9 October), and 0 ULO and 120 Incubation (8 November); 30 ULO and 0 Incubation (10 August), 30 ULO and 30 Incubation (9 September), 30 ULO and 60 Incubation (9 October), 30 ULO and 90 Incubation (8 November), and 30 ULO and 120 Incubation (8 December); 60 ULO and 0 Incubation (9 October), 60 ULO and 30 Incubation (9 October), 60 ULO and 60 Incubation (8 November), 60 ULO and 90 Incubation (8 December), and 60 ULO and 120 Incubation (7 January).

3.2. Effect of the Forcing Conditions on the Saffron Quality

The ISO 3632 [17] is the standard more used internationally to determine the saffron quality and its price; therefore, the quality according to this standard was determined in the saffron samples obtained under forcing conditions which provided an acceptable yield.

Table 4 shows the mean value for the ISO 3632 [17] parameters analysed and Duncan test results for these forcing conditions. All the analysed samples belonged to commercial category I and the following values were obtained: moisture and volatile matter content, 10.1–3.4%; *A*$_{1\, cm}$ 257 nm, 52.68–40.32 nm; *A*$_{1\, cm}$ 330 nm, 52.68–40.32 nm.

| ISO 3232:2011 (Parameter ± SD) | ULO (Days) | Incubation Time (Days) | ANOVA 1 |
|--------------------------------|------------|------------------------|---------|
|                                | 0          | 30                     | 60      | 90      | 120     |
| Colouring Strength (A$_{1\, cm}$ 440 nm) | 0          | 291.01 ± 0.92          | 262.83 ± 1.00 | 251.28 ± 0.54 | 262.00 ± 3.25 | 216.24 ± 0.89 | *** |
|                                | 30         | 243.49 ± 0.38          | 258.24 ± 1.52 | 260.67 ± 1.33 | 256.93 ± 1.77 | 221.65 ± 0.77 | *** |
|                                | 60         | 224.13 ± 0.82          | 244.04 ± 2.13 | 273.26 ± 0.67 | 206.79 ± 0.62 | 232.79 ± 0.06 | *** |
| ANOVA 2                         | 0          | ***                   | ***      | ***      | ***      |
|                                | 30         | ***                   | ***      | ***      | ***      |
|                                | 60         | ***                   | ***      | ***      | ***      |
| A$_{1\, cm}$ 257 nm             | 0          | 110.66 ± 0.30          | 98.18 ± 0.06 | 101.93 ± 1.11 | 110.55 ± 2.09 | 92.14 ± 0.09 | *** |
|                                | 30         | 100.39 ± 0.16          | 99.41 ± 0.30 | 107.36 ± 0.07 | 102.90 ± 0.08 | 83.03 ± 0.10 | *** |
|                                | 60         | 91.80 ± 0.23          | 104.43 ± 0.95 | 100.42 ± 1.59 | 81.54 ± 0.07 | 89.99 ± 0.04 | *** |
| ANOVA 2                         | 0          | ***                   | ***      | ***      | ***      |
|                                | 30         | ***                   | ***      | ***      | ***      |
|                                | 60         | ***                   | ***      | ***      | ***      |
| A$_{1\, cm}$ 330 nm             | 0          | 90.23 ± 0.19          | 43.27 ± 0.14 | 48.36 ± 0.61 | 52.68 ± 3.20 | 40.80 ± 0.17 | *** |
|                                | 30         | 45.57 ± 0.23          | 45.46 ± 0.31 | 46.93 ± 0.18 | 48.93 ± 0.37 | 36.22 ± 0.03 | *** |
|                                | 60         | 41.56 ± 0.10          | 49.78 ± 0.47 | 40.82 ± 0.12 | 43.10 ± 0.75 | 40.32 ± 0.08 | *** |
| ANOVA 2                         | 0          | ***                   | ***      | ***      | ***      |
|                                | 30         | ***                   | ***      | ***      | ***      |
|                                | 60         | ***                   | ***      | ***      | ***      |

1 Values are the mean of two extracts examined in duplicate (2 × 2n); SD= standard deviation. One-way analysis of variance (ANOVA) for each column and row is included (significant difference at *p* < 0.01, at *p* < 0.001, according to Duncan test 2). $a$, $b$, $c$, $d$, $e$, different letters within columns indicate significant differences at *p* < 0.05, according to Duncan test 2; $a$, $b$, $c$, $d$, $e$, different letters within rows indicate significant differences at *p* < 0.05, according to Duncan test 2. *** p < 0.001.
When the corms were not stored in ULO chambers the incubation did not produce a positive effect on most of the ISO parameters analysed. However, the corm storage in ULO chambers 30 and 60 days increased the colouring strength after 30 and 60 days in incubation conditions.

The main metabolites that define the quality of saffron are total crocetins esters, picrocrocin and safranal; the concentration of these parameters was determined. Figure 7 shows the effect of the different forcing conditions on the main saffron metabolites. The total crocin esters ranged from 23.80 to 17.98 mg/100 mg of saffron; picrocrocin oscillated from 20.60 to 9.16 mg/100 mg of saffron; safranal oscillated from 1.15 to 0.42 mg/100 mg of saffron.

**Figure 7.** Effect of the different forcing conditions on the main saffron metabolites. X, y, z, different letters within ULO time mean significant differences (p < 0.05); a, b, c, d, e, different letters within incubation time mean significant differences (p < 0.05).
As can be seen in Figure 7, when the corms not were stored in ULO chamber and were incubated for 30 days, a positive effect on the total crocetin esters, together with a decrease in the amount of picrocrocin and safranal was observed; this effect was also shown in saffron obtained from corms stored in ULO chamber 30 and 60 days followed by incubation for 30 and 60 days respectively.

4. Discussion

ULO storage showed a negative effect on most of the parameters, which decreased significantly as the time in ULO chamber increased. However, a cold-storage up to 60 days provided acceptable saffron yields. Molina et al. [5] found saffron with similar yield to non-forced corms from corms lifted after leaf-withering, stored at 2 °C in 1% oxygen for 70 days and incubated until 120 days. These results are similar to the obtained in this study.

The incubation displayed positive effect on the saffron yield and the number of flowers per corm. The corms incubated during 30 and 60 days had saffron yield and number of flowers per corm greater than corm non-incubated. Molina et al. [16] showed that the incubation improved the development of flower buds. They observed that the formation of a maximum number of flowers per corm happed in corms incubated at 25 °C over 50 days. These results were in accordance with our study.

The unit weight of dry stigma (UWDS) was lower than that obtained by Molina et al. [5]. This fact can be due to some differences in studied conditions during flowering period between both works, for example the vegetative stage of the lifted corms (V3 vs. V3–20 d), the relative humidity (85 ± 2% vs. 70 ± 5%), and the concentration of the CO₂ (400 ppm vs. 2500 ppm). On the other hand, the number of flowers per corms was higher than the observed by them.

This was the first time that a predictive model is obtained, which allows to know the saffron yield in corms lifted in the stage V3–20 d, stored from 0 to 180 days in ULO chambers followed by incubation from 30 to 120 days. This model is useful for C. sativus L. growers and it could encourage its growing under forcing conditions.

Poggi et al. [11] determined the quality of saffron obtained from corms stored in incubation conditions according to ISO 3632; they observed an improvement in the saffron quality obtained from incubated corms, though the times of incubation for which these results were obtained were not specified. In this study, when the corms were not stored in ULO chambers the incubation did not produce a positive effect on the most of the parameter ISO analysed, but an improvement in the colouring strength was observed in corms previously stored in ULO chamber and incubated for 30 and 60 days.

The ISO 3632:2011 parameters and the main metabolites of saffron obtained from Crocus sativus L. grown traditionally has been determined by numerous authors [19,23–25]. The quality of saffron obtained under forcing conditions has been determined for the first time, both using the standard ISO 3632 and by HPLC-DAD analysis, in a work of this group [20]. Taking into account the papers mentioned above, the obtained values of the ISO parameters and the amounts of the main compounds of saffron obtained from corms under all studied forcing conditions were within the range reported for these authors.

The corms not stored in ULO chambers and incubated during, 30, 60 and 90 days had a total crocetin esters significantly higher than non-incubated corms, being the highest found in corms incubated for 30 days, in this moment, along with the increase of crocetin esters, a decrease in picrocrocin and safranal was observed. This effect was also shown in saffron obtained from corms stored in ULO 30 and 60 days followed by incubation for 30 and 60 days, respectively.

5. Conclusions

In conclusion, the corms of Crocus sativus L. lifted 20 days before leaf senescence may remain for up to 60 days stored in an ULO chamber, followed by incubation until 120 days at 25 °C, and flowering at 18 ± 2 °C. These conditions allowed to obtain saffron from October to February with profitable yield and saffron quality similar as saffron grown traditionally.
The flowering of *Crocus sativus* L. could be delayed until May by increasing the time spent in the ULO chamber, but the yield obtained would not be profitable.

For the first time a predictive model, which allows us to know the saffron yield in forcing conditions, has been obtained. This model is a very useful tool for saffron producers, and it could encourage the *Crocus sativus* L. growing under forcing conditions.

The increase of crocetin esters concentration, together with a decrease in picrocrocin and safranal, in some of the studied forcing conditions, could be due to a change in the route of biosynthesis of the main saffron metabolites. Although this effect was repeated at different treatments, more studies are needed to be confirmed.

**Supplementary Materials**: The following are available online at [http://www.mdpi.com/2073-4395/10/11/1775/s1](http://www.mdpi.com/2073-4395/10/11/1775/s1), Table S1: Mean value of the parameters analysed and level of significant differences for the studied factors and their interactions, season 2010–2011.

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