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Photoperiodic Response of in vitro *Cannabis sativa* Plants

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Abstract

Most commercial *Cannabis sativa* L. (cannabis) genotypes are short-day plants and cultivators typically use a 12.0 h uninterrupted dark period to induce flowering; however, scientific information is lacking to prove this is the optimal dark period for all genotypes, and cultivar specific photoperiods may increase productivity. Tissue culture can be used for research requiring multiple treatments, proper replication, and in a controlled environment on a smaller scale compared to greenhouse and indoor facilities. To determine whether cannabis explants can flower under varied photoperiods in vitro, explants were grown under one of six photoperiod treatments: 12.0, 13.2, 13.8, 14.4, 15.0, and 16.0 h for four weeks. The percentage of flowering explants was highest under 12.0 and 13.2 h treatments. There were no treatment effects on the fresh weight, final height, or growth index of the explants. The results suggest an uninterrupted dark period of at least 10.8 h (i.e. 13.2 h photoperiod) is needed to induce the flowering of this genotype. In vitro flowering could provide a unique and high throughput approach to study floral/seed development and secondary metabolism in cannabis under highly

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controlled conditions. Further research should determine if this response is the same on a whole plant level.

Introduction

Cannabis sativa L. is an herbaceous, primarily short-day plant that has been used for medicinal and recreational purposes since 2800 B.C. (Farquhar-Smith, 2002). Following decades of prohibition, it is becoming more accepted in today's society for its medicinal effects on pain, inflammation, epilepsy, as well as for recreational use (Small, 2017; Zheng, 2020). As of 2018, the gross domestic product (GDP) of the medicinal and recreational Canadian cannabis industry was valued at approximately $5.7 billion (Statistics Canada, 2020), making it one of the nation's most economically important crops. Since Canada legalized extracts, topicals, tinctures, concentrates, capsules, beverages, and edibles in 2019, the GDP is expected to grow another $2.7 billion (Deloitte, 2019).

Cannabis is considered to be a short-day plant, but there are some auto-flowering genotypes available. Short-day plants require a long, uninterrupted dark period to induce flowering which is considered to be more important than the light period itself (Lumsden and Vince-Prue, 1984). Cultivators have found success with flowering cannabis under a 12.0 h dark period; however, it is unknown if this is optimal for all genotypes. There is a need to determine critical photoperiods for different genotypes since they are dependent latitude of origin (Clarke, 1999; de Meijer and Keizer, 1994). For Thai hemp, critical photoperiods of 11.0 to 12.0 h have been reported (Sengloung et al., 2009), cannabis with French origins had a critical photoperiod between 14.0 and 15.5 h (Struik et al., 2000), Mediterranean hemp between 14.4 and 14.9 h

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(Cosentino et al., 2012), and an unidentified cannabis cultivar between 12.0 and 14.0 h (Clarke, 1999). Some genotypes may benefit from longer photoperiods as it allows for more photosynthesis to take place, therefore increasing plant growth, including height (Farooqi et al., 1999), nodes and stem length (Downs and Borthwick, 1956), and dry weight (Bonner, 1940), and may reduce the time to maturity without sacrificing yield/quality. Given the limited amount of scientific literature on the photoperiodic requirements of cannabis, the optimal dark period has not been determined for a majority of genotypes.

Tissue culture has become an important tool for genetic maintenance and propagation due to its sterility, capability of mass propagation, and preservation of genetics (Feeney and Punja, 2003; Lata et al., 2009). Most cannabis micropropagation is conducted using long-day photoperiods (16.0 to 18.0 h days) and most plants remain in the vegetative state. However, initial observations indicate that some genotypes flower in vitro even under long-day conditions. This demonstrates the capacity for in vitro flower development in cannabis, which is not observed in all species. In vitro flowering and seed set has been proposed in other species as a valuable tool to reduce generation time for applications in plant breeding and to study floral/seed development in a highly controlled environment (Ochatt et al., 2000). Additional aspects specific to cannabis may include the opportunity to study the regulation of secondary metabolites, production of floral tissue for plant regeneration (Piunno et al., 2019), and the potential to rapidly identify the critical photoperiod of specific genotypes.

To date, the occurrence of in vitro flowering has only been observed sporadically and there are no reports indicating if the response to photoperiod is similar to whole plants. Flowering has been demonstrated in other short-day plants in vitro including tobacco (Altamura

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et al., 1991), *Plumbago indica* (Nitsch and Nitsch, 1967), *Cuscuta reflexa* (Baldev, 1962), and *Kalanchoe blossfeldiana* (Dickens and van Staden, 1990). However, the fact that some short-day cannabis genotypes flower under long-day suggests there are other factors at play. In other species, flowering can be in responding to photoperiod, but can also be influenced by various plant growth regulators (Mobini et al., 2015), day/night time temperatures (Adams et al., 2009), and other environmental factors.

The overall hypothesis of this study was that the flowering of cannabis plants grown in vitro can respond to photoperiod. Specific objectives included: 1) to quantify plant response to photoperiod, 2) to investigate the best flowering metric for determining the optimal dark period for inducing flowering, and 3) to determine whether longer photoperiods increase growth of explants. This study also intended to establish a protocol for producing in vitro cannabis flowers for further applications in future cannabis research.

Plant materials

The experiment was conducted in a walk-in tissue culture chamber at the University of Guelph, Guelph, ON, Canada. Established shoot cultures of *Cannabis sativa* L. '802', a high THC genotype (for molecular characterization see Page et al., 2020), was used as a source of explants by taking shoot segments containing at least two nodes (approximately 1.3 cm in length) with the leaves trimmed to approximately half the original size. Four healthy explants of a uniform size were transplanted to the middle of a prepared vessel and equally spaced. The vessels were then tightly sealed with grafting tape.

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Media composition

A previously optimized semi solid tissue culture medium (Page et al., 2020) was used which consisted of 5.32 g/L DKW basal salts and vitamins (Driver and Kuniyuki, 1984; D2470, PhytoTech Laboratories, Shawnee, Kansas, USA), 30 g/L of sucrose, 1 mL/L plant preservative mixture (PPM) (Plant Cell Technology, Washington, DC, USA), and 6 g/L of agar (A360-500, Fisher Chemical, Fair Lawn, New Jersey, USA), adjusted to a pH of 5.7 prior to being autoclaved. About 200 mL of the medium was poured into the bottom of each sterile We-V tissue culture vessel (WeVitro, Guelph, ON, Canada). The medium was autoclaved for 20 minutes at 122^{o}C and 138 kPa.

Growing conditions and experimental design

All explants were grown under a 16.0 h photoperiod for two weeks prior to the implementation of different photoperiod treatments. During this vegetative growth period, the vessels were placed under LED lighting. The LED arrays provided a photosynthetic photon flux density (PPFD) of 19.5 ± 5.5 µmol m^{-2} s^{-1} at explant level and a spectral composition for blue (B; 400-500 nm), green (G; 500-600 nm), and red (R; 600-700 nm) wavebands of B23:G18:R59 (Fig. 1). The peak wavelength and full width at half maximum (FWHM) of the peaks in the blue, green, and red wavebands were 447 nm and 17 nm, 520 nm and 34 nm, and 656 nm and 15 nm, respectively. Spectrum and intensity were measured with a radiometrically-calibrated spectrometer (Flame-S-XR; Ocean Optics, Dunedin, FL) with a cosine corrector attached to a 1.9 m x 400 nm optical fibre.

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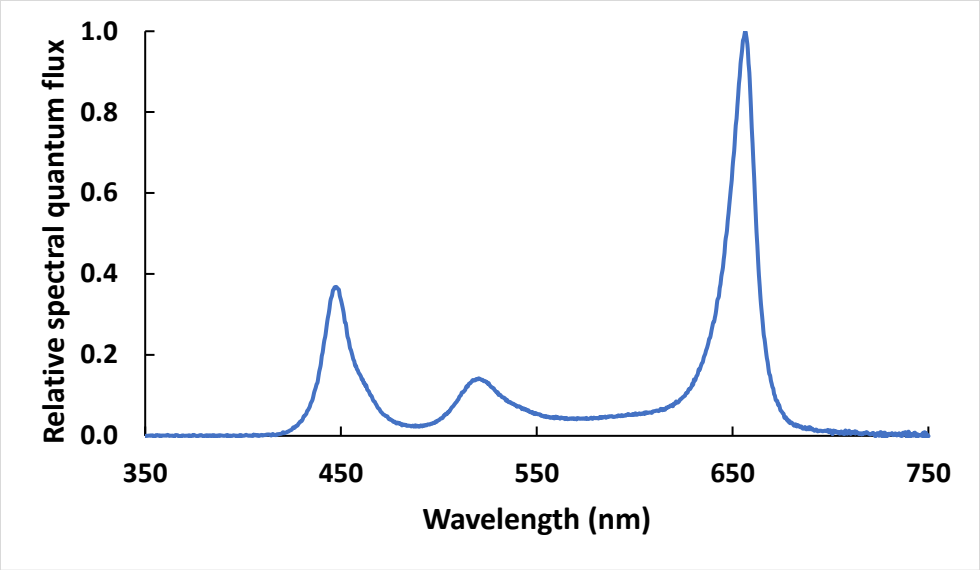

Fig. 1. Relative spectral quantum flux distribution of the light-emitting diode (LED) fixture used during the vegetative stage over the photosynthetically active radiation spectral range (i.e., 400 to 700 nm).

After two weeks, the explants were transferred to one of the six photoperiod treatments: 12.0, 13.2, 13.8, 14.4, 15.0, or 16.0 h, and grown for another four weeks. The environment where the vessels were placed in was maintained at 25 ± 0.9°C (mean ± SD) and a relative humidity of 42 ± 9.2%. Temperature and relative humidity were measured using an external data logger (HOBO UX100-011A; Onset Computer Corporation, Bourne, MA, USA) set to record every minute. For each photoperiod treatment there were four experimental units (i.e., four vessels; four true replicates), with four explants per vessel. The experiment used a completely randomized experimental design; and repeated once over time. Each vessel had its own custom LED array (Fig. 3A) that provided a PPFD of 50.8 ± 1.6 µmol m^{-2} s^{-1} (mean ± SD, n = 12 (pooled for two trials)) at explant height and a spectral composition of blue, green, and red wavebands of B15:G7:R78 (Fig. 2). The peak wavelength and FWHM of the peaks in blue and red wavebands were 456 nm and 18 nm, and 658 nm and 17 nm, respectively. Each

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compartment had a 3D-printed blackout cover which prevented light carryover from nearby treatments while still allowing for air circulation through individual compartments. Panda film (Vivosun, City of Industry, CA, USA) encompassed all compartments as an added precaution to ensure blackout conditions whenever the LEDs were off (Fig. 3B).

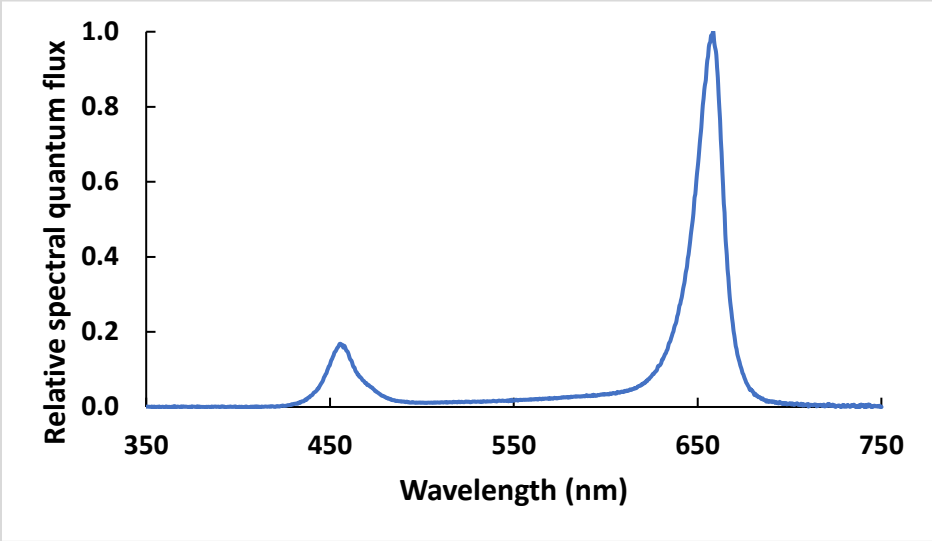

Fig. 2. Relative spectral quantum flux distribution of the custom light-emitting diode (LED) fixtures used for photoperiod treatments over the photosynthetically active radiation spectral range (i.e., 400 to 700 nm).

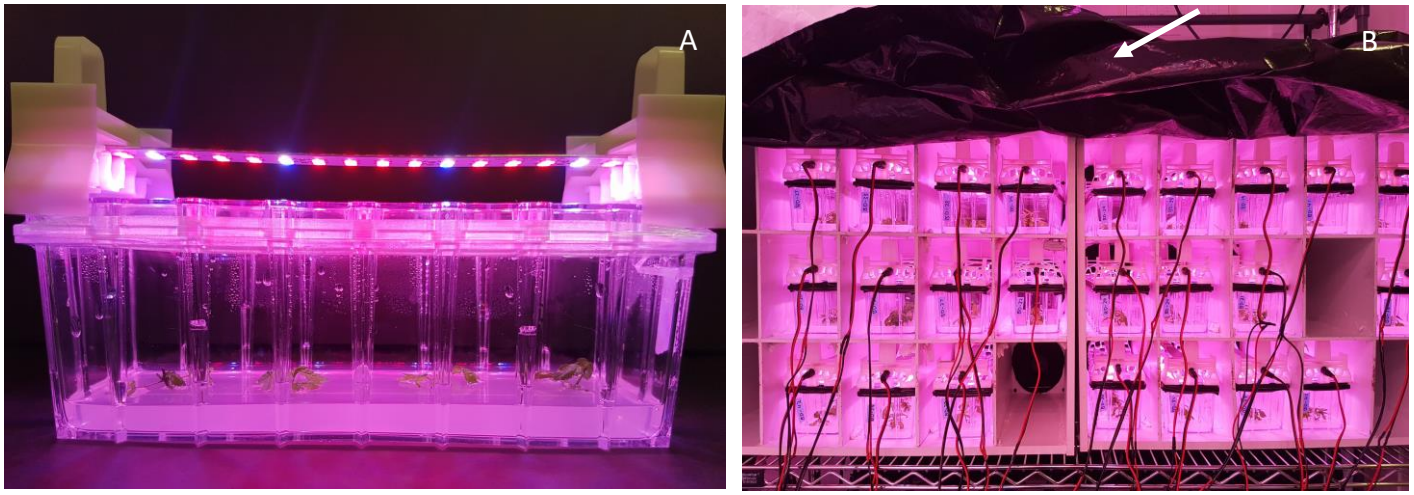

Fig. 3. (A) Tissue culture vessel containing four explants with its own individual LED array. (B) Experimental set-up including the vessels in individual compartments and the panda film, indicated by the white arrow, that encompasses all compartments. Each compartment had its own individual cover to prevent light spillover (not shown).

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150 *Time of flowering and plant growth measurements*

151 During the four-week photoperiod treatment in each of the two repeated experiments,
152 each explant was monitored daily for flower initiation. Flower initiation was determined by the
153 display of pistil formation at the calyx which is a morphological event in female cannabis plants
154 that represents a transition from vegetative to reproductive phase. In this study, pistil
155 formation was deemed a successful flowering event (Fig. 4B and C). Explant height was
156 measured weekly with ImageJ (version 1.52 (100)). Explant height was measured from the
157 media level to the highest point of the explant.

158

159 After the four-week photoperiod treatment, the vessel lids were removed to measure
160 the plant growth attributes of each explant. The height was measured from media level to the
161 highest point of the explant; width 1 was the longest point of explant measured from west to
162 east; and width 2 was the longest point of explant measured from north to south. The growth
163 index was calculated using the following equation: ((Height × Width 1 x Width 2)/300) (Clark
164 and Zheng, 2020). For the final heights, explants were measured with the lid removed as some
165 reached heights above the height of the box (causing lateral growth due to height restrictions).
166 The explants were destructively harvested to obtain aboveground fresh weight. Explants were
167 removed from the medium and all roots (if present) were removed and fresh weight was
168 measured with an analytical balance (Mettler Toledo AE 100; Mettler Toledo, Columbus, OH,
169 USA). Root weight measurements were not recorded since only some explants rooted.

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Fig. 4. Cannabis explants depicting (A) vegetative growth, (B) pistil emerging from calyx, (C) close-up of pistil emergence, (D) trichomes on perigonal bract, (E) developed flower, and (F) explants at the end of the four-week photoperiod treatments.

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196 *Statistical analysis*

197 Data were analyzed using a generalized linear mixed model in Statistical Analysis
198 Software University Edition (SAS, version: university.cny.sas.com@sas:university-
199 6p.2/6p.2.a70b47b86698-1-1**,** SAS Institute Inc., Cary, NC, USA). Two trials were performed, and
200 the different variances were accounted for by using rep and rep*treatment. A Tukey-Kramer's
201 test at the 95% significance level was used to determine whether there were differences among
202 photoperiod treatments. When there were no statistical differences among the two trials, the
203 data were combined. Otherwise, the data were analyzed as two separate experiments (i.e.,
204 days to first flower). The residuals were checked and transformed using a lognormal
205 distribution to satisfy the assumptions.

206

207 **Results**

208 *Flower initiation*

209 The percentage of plants that flowered was highest in the 12.0 and 13.2 h photoperiod
210 treatments with 76 ± 11% and 72 ± 9%, respectively, with no difference between the two
211 treatments. As the photoperiods got longer, the percentage of plants flowered decreased, with
212 22 ± 10% and 11 ± 7% of explants in the 13.8 and 14.4 h treatment, respectively. Minimal
213 flower events occurred in the 15.0 and 16.0 h treatments (with < 3 %) (Fig. 5). The first day to
214 flower occurred after six days under the 12.0 and 13.8 h photoperiods in trial 1, but occurred
215 after three days under 13.2 h in trial 2. Floral initiation reached 25% and 50% quickest under
216 the 12.0 h photoperiod, followed by the 13.2 h photoperiod in both trials (Table 1). Explants
217 under 13.8, 14.4, 15.0, and 16.0 h did not reach 50% flowering in both trials.

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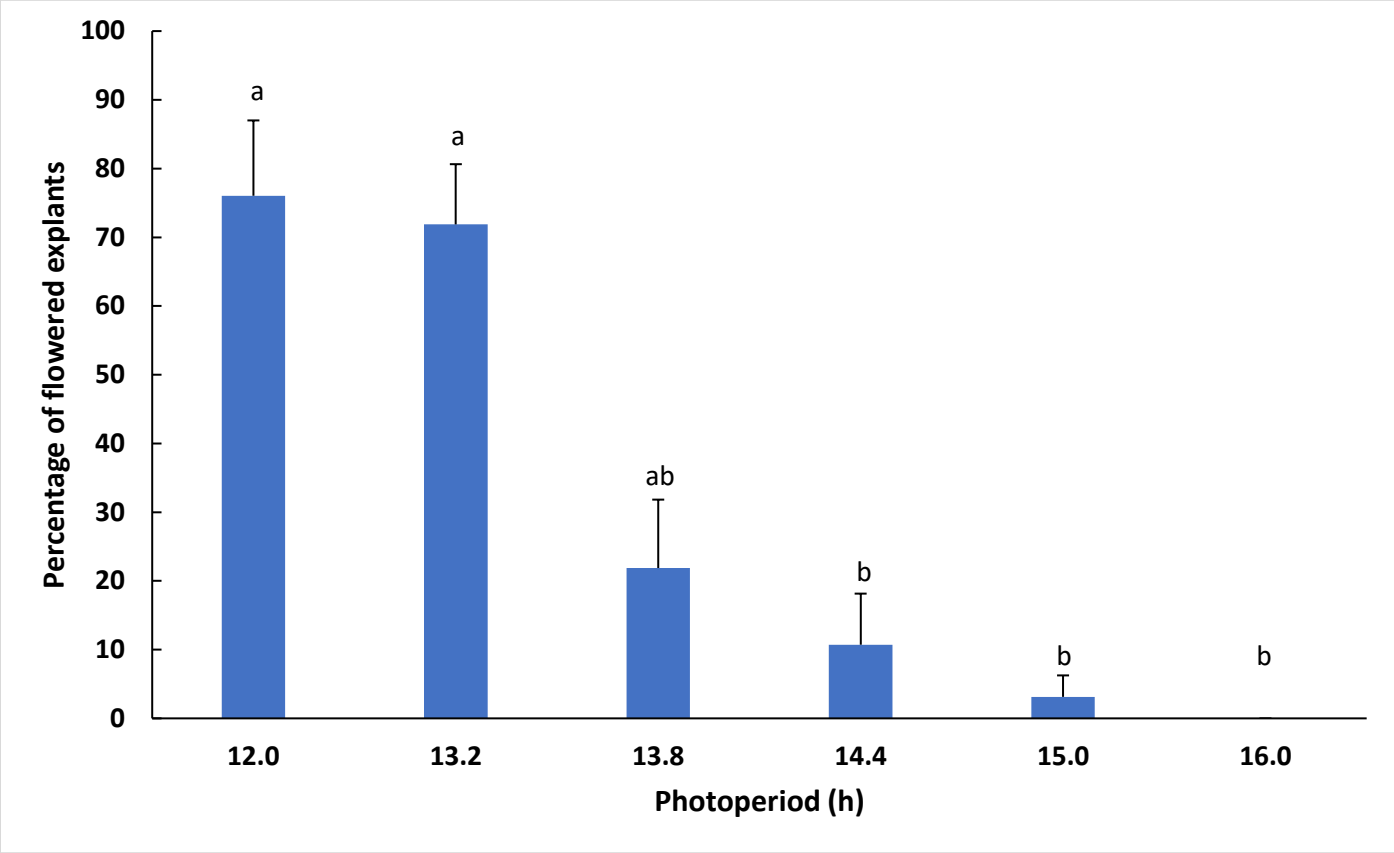

Fig. 5. Percentage explants with flower initiated under 12.0, 13.2, 13.8, 14.4, 15.0, and 16.0 h photoperiods. Data are means ± SE (n = 8). Bars bearing different letter depicts significant differences at $P < 0.05$ using Tukey-Kramer multiple comparisons test.

Table 1. The number of days it took to see the first flower in any cannabis explant, average days to flower, days for 25% and 50% plants to flower under each photoperiod treatment for trials 1 and 2.

	Trial 1				Trial 2			
Photoperiod (h)	Average days to flower[z]	Days to first flower	Days to 25% flowering	Days to 50% flowering	Average days to flower	Days to first flower	Days to 25% flowering	Days to 50% flowering
12.0	11.6	6	8	13	17.6	10	11	19
13.2	14.2	7	11	19	18.8	3	17	22
13.8	6	6	> 28	> 28	21.3	4	24	> 28
14.4	> 28[y]	> 28	> 28	> 28	15.3	5	> 28	> 28
15.0	12	12	> 28	> 28	> 28	> 28	> 28	> 28
16.0	> 28	> 28	> 28	> 28	> 28	> 28	> 28	> 28

[z]The average days to flower initiation was calculated by averaging the number of days it took for each explant to flower under each photoperiod treatment for each individual trial. This value only accounted for flowered explants.

[y]Greater than 28 days (> 28) indicates no flowering events occurred in that treatment.

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Fresh weight

The aboveground fresh weight of explants was not significantly affected by the varied photoperiod treatments and had an average of 0.4383 ± 0.0550 g. There were large variances and the fresh weights ranged from 0.0818 g to 1.8112 g (Fig. 6).

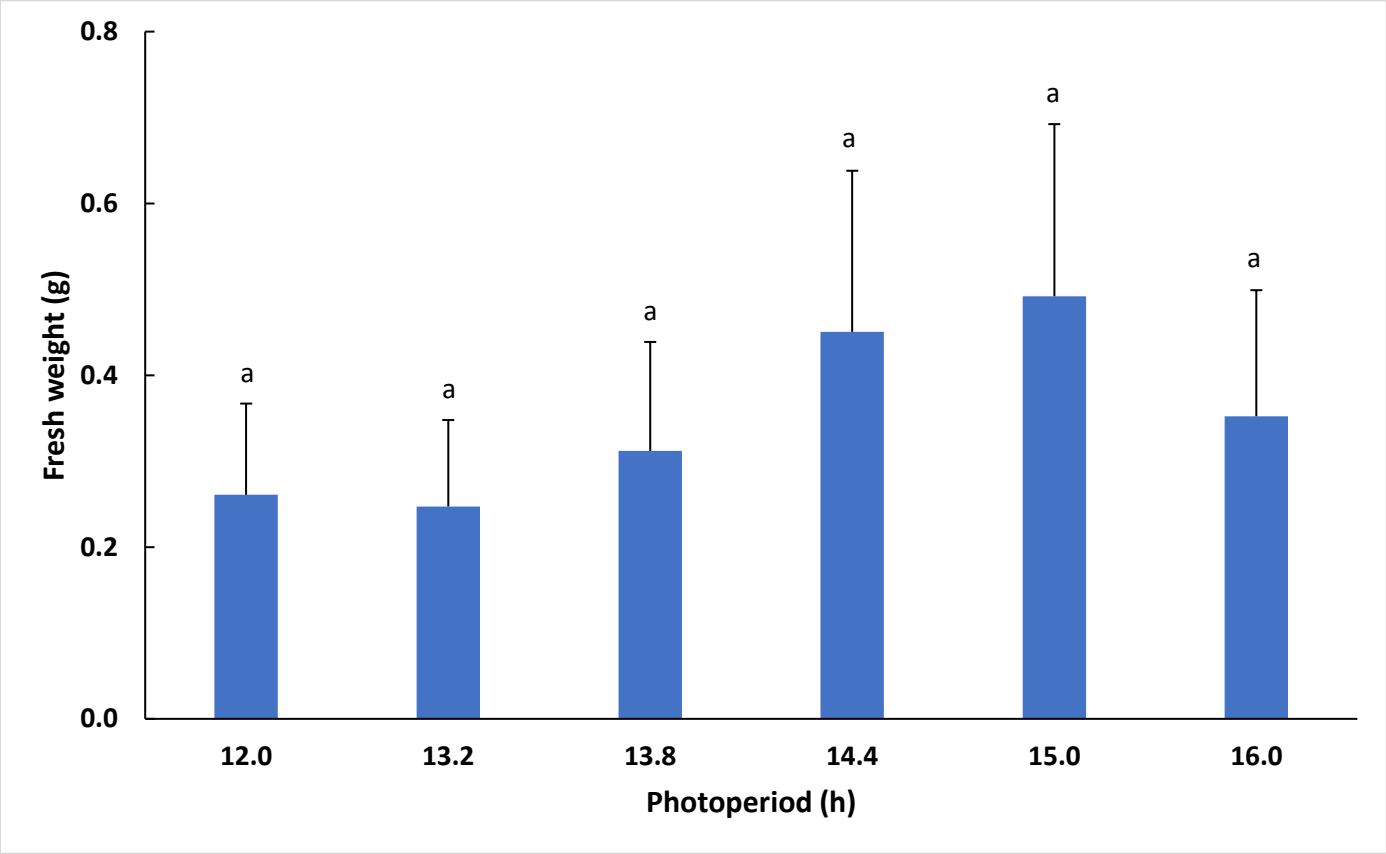

Fig. 6. Aboveground fresh weight of cannabis explants under 12.0, 13.2, 13.8, 14.4, 15.0, and 16.0 h photoperiods. Data are means ± SE (n = 8). Bars bearing different letter depicts significant differences at $P < 0.05$ using Tukey-Kramer multiple comparisons test.

Final height

The final height of the explants was not affected by the photoperiod treatments and had an average of 2.9 ± 0.16 cm, ranging from 1.7 cm to 6.0 cm (Fig. 7).

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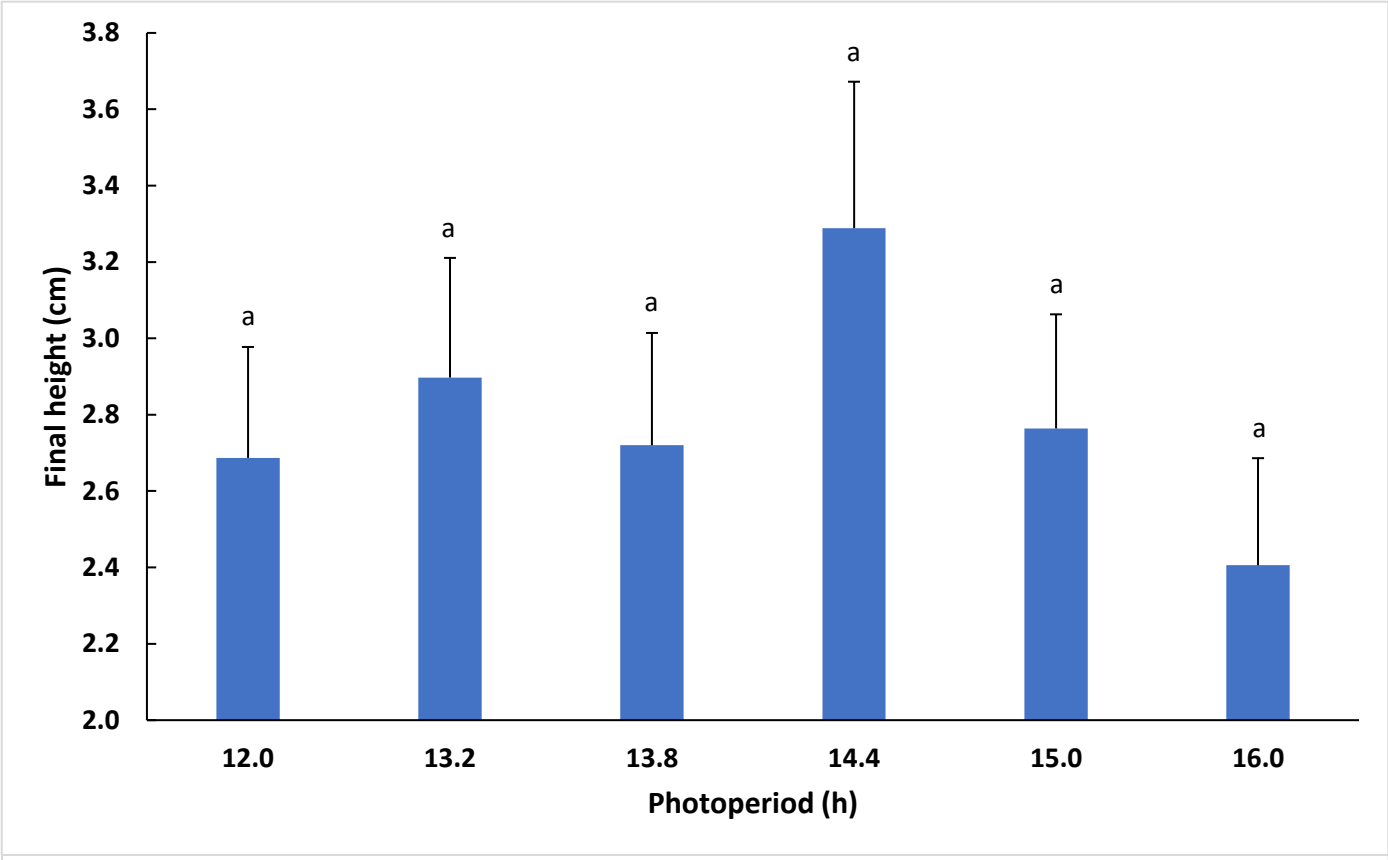

Fig. 7. Final height of cannabis explants under 12.0, 13.2, 13.8, 14.4, 15.0, and 16.0 h photoperiods. Data are means ± SE (n = 8). Bars bearing different letter depicts significant differences at $P < 0.05$ using Tukey-Kramer multiple comparisons test.

Growth index

There was no treatment effect on the growth index and which had an average of 0.36 ± 0.07. There was large variability and the growth index ranged from 0.05 to 2.16 (Fig. 8).

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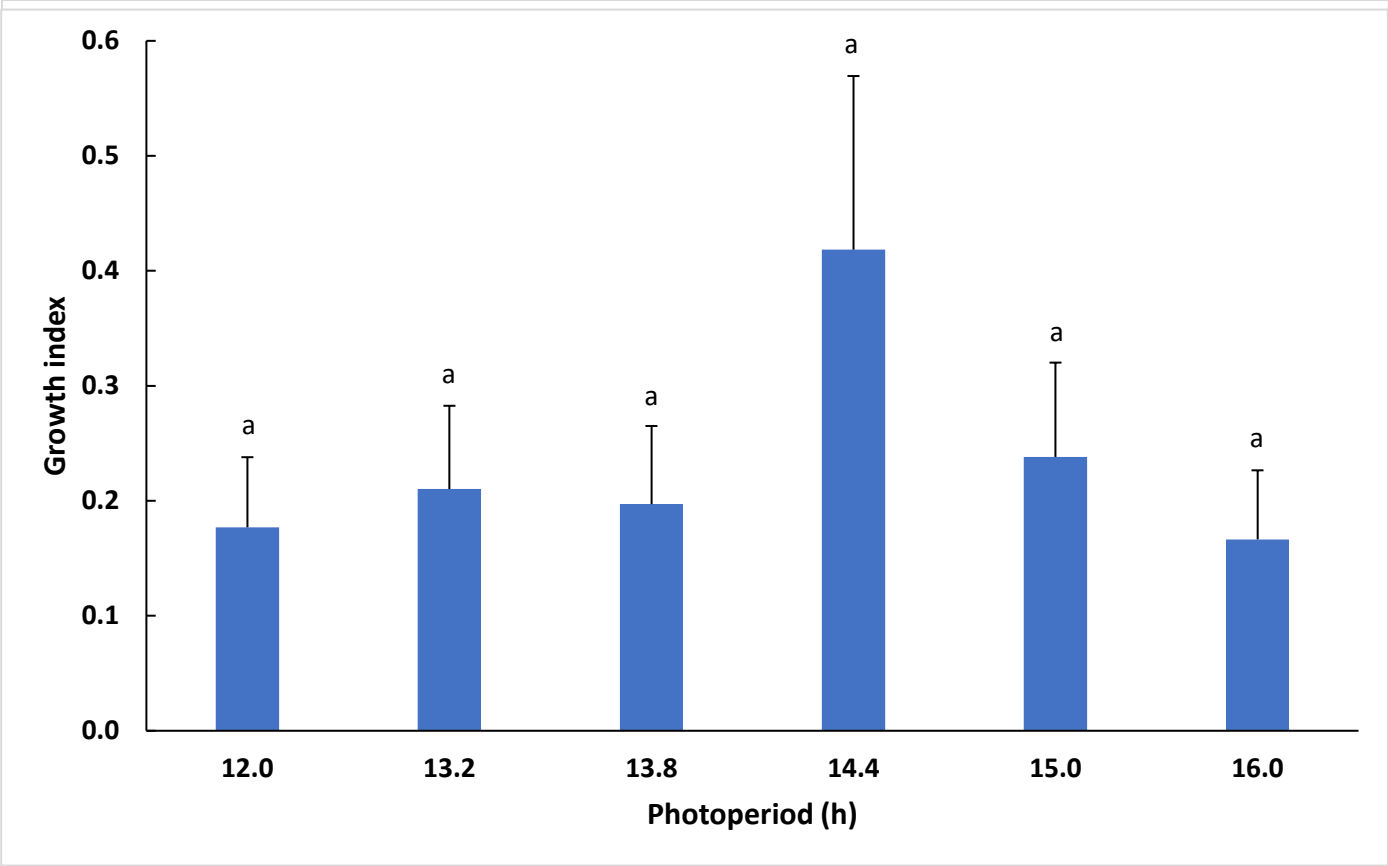

Fig. 8. Growth index of cannabis explants under 12.0, 13.2, 13.8, 14.4, 15.0, and 16.0 h photoperiods. Data are means ± SE (n = 8). Bars bearing different letter depicts significant differences at $P < 0.05$ using Tukey-Kramer multiple comparisons test.

Discussion

Flowering and photoperiod

The six photoperiod treatments were designed to have an initial 10% increase from 12.0 to 13.2 h, then a 5% incremental increase from 13.2 h onward. The 12.0 and 13.2 h photoperiods had the highest percentage (74%) of flowered explants which quickly dropped to only 22% in the 13.8 h treatment, and much less when the photoperiod further increased and eventually reached 0%. This particular parameter was the most reliable flowering metric which has also been used for other plant species such as strawberries (Vince-Prue and Guttridge,

1973), ornamentals (Craig and Runkle, 2013), and in vitro tomatoes (Dielen et al., 2001). Hemp studies using whole plants have reported similar results with higher percentages of flowering when grown under less than 14.0 h photoperiods, while plants under 17.0 and 20.0 h remained vegetative (Borthwick and Scully, 1954). It was also reported that outdoor-grown hemp under longer photoperiods had delayed flowering; however, 48.3% and 47.5% still flowered under 16.0 and 19.0 h, respectively (Borthwick and Scully, 1954). Although there was a high percentage of flowering under the 16.0 and 19.0 h photoperiods, some hemp genotypes are day-neutral and do not rely entirely on the photoperiod to induce flowering. The explants in the current study responded similarly to hemp grown in greenhouse and outdoor production which suggests that explants may respond similarly on a whole plant level; however, this needs to be further validated.

The time to first flower initiation and the average day to flower initiation were different between the two trials. For example, under the two successful photoperiod treatments (12.0 and 13.2 h), the average days to the first flower initiation for all the flowered explants were 12-14 days in trial 1 and 18-19 days in trial 2. The reason for the discrepancy is not known, but could be due to different physiological conditions of the starting material despite all attempts to maintain uniform conditions and materials. However, it is within the range of the commonly observed flowering time in commercial drug-type cannabis production facilities (S. Golem, personal communication). This flowering metric is not that reliable as it can be misleading. For example, under the 15.0 h photoperiod treatment, the observed first explant to flower was at day 12, but there were less than 5% of the explants flowered during the whole trial. Flowering under the 15.0 h photoperiod may have been a result of environmental factors rather than a

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photoperiod response, which is consistent with earlier observations of some short-day genotypes sporadically flowering under long-day conditions in vitro. Environmental factors such as: low red to far-red ratio in cool-season grain legumes (Croser et al., 2016), higher temperatures later in the growing cycle in chrysanthemums (Carvalho et al., 2005), and exogenous growth regulators in vegetable crops (Franklin et al., 2000; Sheeja and Mandal, 2003) have reportedly enhanced floral development in terms of time and quantity. Although these factors were not present in the current study (i.e., exogenous growth regulators), there is evidence to show that flowering can occur in vitro with photoperiod not being the only driver.

Of all the photoperiod treatments, only the 12.0 and 13.2 h photoperiods reached 50% flowering, which took 13-19 days in trial 1 and 19-22 days in trial 2. The time to reach 50% flowering can be used to get an idea of the length of time to grow the explants in vitro, but should not be used to determine the flowering metric since only two of six photoperiods reached 50% flowering. Using the time it takes to reach 50% flowering would take significantly less time than growing whole plants in a greenhouse or indoors. To put this into perspective, hemp genotypes grown in a controlled environment reached 50% floral initiation 20-27 days under a 12.0 h photoperiod, but took 50-55 days under a 16.0 h photoperiod, after sowing (Lisson et al., 2000). Other hemp studies show that after sowing, plants grown under less than 13 h 40 min took 33-34 days to exhibit first male flower and 45-46 days to 50% flowering. With the same cultivar, it took 50 days to exhibit first male flower and 71 days for 50% of plants to reach flowering when grown under a photoperiod of 14 h 40 min (Hall et al., 2014). By using tissue culture, we could complete an entire trial in 36 days (with successful treatments reaching

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353 50% flowering) while having several photoperiods at one time to determine cannabis plants
354 response to photoperiod.
355 Our results indicate that this cannabis genotype needs a photoperiod no longer than
356 13.8 h (i.e., 10.2 h uninterrupted dark period) per day, and to be safe it should be less than 13.2
357 h (i.e., 10.8 h uninterrupted dark period), to induce flowering based on the percentage of
358 explants flowered. The best indicator for determining the optimal photoperiod should be based
359 on the percentage of flowering since it is accurate and not misleading to cultivators. However,
360 whether a photoperiod less than 13.8 h is successful for the whole plant is unknown. Future
361 research should use whole plants to determine the critical photoperiod, the uninterrupted dark
362 period, needed for flower initiation for this genotype. If the results from whole plants match
363 the results from explants, then using explants from tissue culture would be a quick and easy
364 method for determining the critical photoperiod for different cannabis genotypes.
365
366 *Growth and photoperiod*
367 Longer photoperiods usually result in more photosynthesis and plant growth (Kozai et
368 al., 1995; Kurilčik et al., 2008); however, to ensure floral initiation of cannabis plants, a majority
369 of cannabis cultivators are growing plants under a 12.0 h photoperiod. For greenhouse
370 cultivation, this means the cultivators need to block the sunlight a few hours a day during long
371 summer days which can be a waste of natural sunlight. For indoor cultivation, this could reduce
372 the effectiveness of plants using growth resources. The third objective of this study was to
373 investigate whether longer photoperiods can increase plant growth while also inducing
374 flowering. Our results showed that plants under shorter photoperiods (≤ 13.8 h) were usually

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smaller than those under longer photoperiods; however, there was no photoperiod treatment effect on any of the measured explant growth attributes (i.e., final height, fresh weight, and growth index). While the lack of treatment effects could be caused by the variability among explant growth and insufficient replication, it should also be highlighted that in vitro plantlets are mixotrophic and do not rely on light as their sole carbon source. Due to the relatively low light intensities and supplemental sucrose used in this study, the relative impact of extra light due to longer photoperiods is likely to be less pronounced than would be expected in a photoautotrophic system.

Another source of error that made growth rates similar among treatments is the sporadic development of roots on some explants. The presence of roots can affect the uptake of nutrients and plantlets in this study that developed roots were generally more vigorous. Since rooting only occurred on some explants and did not appear to be related to photoperiod, this may have added a source of variation that would have masked any treatment effect.

Our results show that for this genotype of cannabis, photoperiod did not significantly influence growth, but did induce floral development. Further investigation using larger replication may help determine if photoperiod influences plant growth rates in vitro. Regardless, due to the mixotrophic nature of traditional tissue culture, this is likely to be substantially different than what would be observed in a greenhouse or growth room. Further studies implementing a photoautotrophic tissue culture system may help to further elucidate these relationships, but ultimately whole plant studies are needed.

Conclusion

In conclusion, the results of this study demonstrated that explants of cannabis genotype '802' can be induced to flower when the photoperiod is at or shorter than 13.2 h per day, or more correctly, at or longer than 10.8 h uninterrupted dark period per day. The percentage of flowering explants is the best indicator for photoperiod determination tests among the other metrics such as times for the days to first, 25%, 50% floral initiation as it provides a more accurate representation of how the explants responded under the varied photoperiod treatments. Due the large variations in size and growth of explants generated from tissue culture, it may not be a reliable method to determine the plant growth in response to photoperiod. Future research need to use whole plants to determine the critical photoperiod for flower initiation for this genotype. With further investigation, the use of tissue culture can be used by cultivators to save time and space when researching the photoperiod specificity of their genotypes to help optimize production as well as establishing an in vitro system to study floral/seed development, develop in vitro breeding platforms, and investigate the regulation of secondary metabolism under highly controlled conditions.

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