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Optimizing cannabis cultivation: an efficient in vitro system for flowering induction

Orly Lavie[1], Kobi Buxdorf[1] and Leor Eshed Williams[1*]

Abstract

Background *Cannabis sativa* L. is a versatile medicinal plant known for its therapeutic properties, derived from its diverse array of secondary metabolites synthesized primarily in female flower organs. Breeding cannabis is challenging due to its dioecious nature, strict regulatory requirements, and the need for photoperiod control to trigger flowering, coupled with highly dispersible pollen that can easily contaminate nearby female flowers. This study aimed to develop a protocol for in vitro flowering in cannabis, investigate factors affecting in vitro flower production, and generate viable in vitro seeds, potentially offering a method for producing sterile cannabinoids or advancing breeding techniques.

Results We show that the life cycle of cannabis can be fully completed in tissue culture; plantlets readily produce inflorescences and viable seeds in vitro. Our findings highlight the superior performance of DKW medium with 2% sucrose in a filtered vessel and emphasize the need for low light intensity during flower induction to optimize production. The improved performance in filtered vessels suggests that plants conduct photosynthesis in vitro, highlighting the need for future investigations into the effects of forced ventilation to refine this system. All tested lines readily developed inflorescences upon induction, with a 100% occurrence rate, including male flowering. We revealed the non-dehiscent trait of in vitro anthers, which is advantageous as it allows for multiple crosses to be conducted in vitro without concerns about cross-contamination.

Conclusion The current work developed and optimized an effective protocol for in vitro flowering and seed production in cannabis, potentially providing a platform for sterile cannabinoid production and an efficient tool for breeding programs. This system allows for the full and consistent control of plant growth conditions year-round, potentially offering the reliable production of sterile molecules suitable for pharmacological use. As a breeding strategy, this method overcomes the complex challenges of breeding cannabis, such as the need for large facilities, by enabling the production of hundreds of lines in a small facility. By offering precise control over factors such as plant growth regulators, light intensity, photoperiod, and temperature, this system also serves as a valuable tool for studying flowering aspects in cannabis.

Keywords In vitro breeding, In vitro seed production, Cannabis cultivars, In vitro photosynthetic capacity, In vitro anther development

Background

Cannabis sativa L., a member of the Cannabaceae family, is classified as an annual dioecious plant species in which male and female flowers are borne on separate unisexual individuals.

Cannabis produces a wide array of specialized secondary metabolites, including more than 120 cannabinoids,

*Correspondence:
Leor Eshed Williams
leor.williams@mail.huji.ac.il
[1] The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Hebrew University of Jerusalem, 76100 Rehovot, Israel

terpenes, and flavonoids [1], which possess therapeutic properties and thus confer recognized medicinal value [2]. The global rise in the cannabis market has significantly boosted the growth of the cannabis industry. This includes intensive breeding efforts aimed at developing elite cannabis cultivars with enhanced cannabinoid profiles tailored to optimize therapeutic efficacy, meet consumer preferences, and improve yield [3]. Cannabinoids and terpenes are synthesized and accumulated in glandular trichomes [4], most densely concentrated on the bracts of female flowers, thus attributing commercial value to the plant. The female cannabis plant exhibits a bushy and robust structure that develops complex inflorescences upon a short photoperiod with numerous small flowers closely packed with high biomass. These inflorescences are primarily located at the tips of the stem and branches and on the leaf axils throughout the plant [5]. Consequently, the cannabinoid profile among inflorescences within plants varies due to differential light exposure and variations in the source-sink relationship. This variability presents a significant challenge, prompting the adoption of various strategies to reduce these inconsistencies. One effective method is regular pruning to enhance light penetration and control plant density [6]. Another approach that has not yet become commercial is using tissue culture systems, like cell suspensions, to mass-produce cannabinoids [7].

The breeding of cannabis is intricate and challenging for several reasons: 1. The induction of flowering requires controlled light regimes, which complicates agricultural practices [8]; 2. In most countries, cannabis is under strict regulation that mandates certain security measures, limits the growing space, and specifies the maximum number of plants; 3. The anemophilous nature of cannabis, relying on wind for pollination, introduces challenges in controlling cross-pollination [9]. A single male plant can produce a vast quantity of pollen grains. This requires strict isolation of male plants to prevent unwanted fertilization of nearby female plants, implementing firm measures that drastically limit the number of crosses feasible in a breeding program [10].

Inducing flowering in tissue culture systems holds promise as a tool to address both issues: reliably producing cannabinoids in a highly controlled environment and enhancing breeding programs by allowing numerous crosses to be conducted in a small facility. In addition, tissue culture systems offer precise control over factors such as plant growth regulators, light intensity, photoperiod, and temperature, making it a valuable tool for studying the elements affecting flower initiation and floral organ development [11].

Plant tissue culture involves cultivating plant cells, tissues, or organs on defined nutrient media under aseptic conditions and controlled environments, occasionally supplemented with growth regulators to induce specific cellular responses [12].

There are numerous reports on in vitro flowering across diverse plant species, with applications ranging from studying floral mechanisms to potential uses as a breeding tool for trait assessment and speeding up developmental timelines [11, 13, 14].

In this study, we developed an effective protocol for inducing flowering in cannabis in vitro. We optimized the media and growing conditions and demonstrated the ability to produce viable seeds. Therefore, this method can serve as a rapid and straightforward approach to breeding programs, a potential method for producing cannabinoids, and a valuable tool for studying the flowering aspects of cannabis.

Results

Experimental setup for in vitro flower induction of *cannabis*

In vitro flowering can serve as a valuable tool for studying specific aspects of flowering in cannabis and for producing important flower components, such as the calyx, to harvest cannabinoids under aseptic conditions. We first followed a standard sterilization protocol to establish our experimental system to introduce explants into tissue culture. We selected stem segment explants from cannabis TA5 cultivar, which was grown in a growth room under an 18/6 h photoperiod (Fig. 1a). Approximately 100 explants were sterilized in a 2% sodium hypochlorite solution (Fig. 1b), then cut into single-node explants, and placed in MS or DKW media as described in the Materials and Methods section, with five single-node sections per vessel (Fig. 1c). The sterilization processes can impose stress on the explants, requiring a recovery period. The stem sections are leafless at this stage, and a few days are required before leaves become apparent. To evaluate the requirement of initial vegetative growth for successful flowering, we compared two photoperiod treatments. Six vessels were subjected to an 18/6 h photoperiod for two weeks (Fig. 1d), before transitioning to a 12/12 h photoperiod to induce flowering (Fig. 1e). Six additional vessels were cultured directly under a 12/12 h photoperiod. The cannabis flower usually has two stigmas (Fig. 1f). To track the number of flowers and their rate of development, we followed the stigma development and counted the number of flowers every few days (Fig. 1g-i). Although the number of flowers developed within 38 days was similar between the two regimes, the plants that were first grown under a vegetative photoperiod appeared more developed. Therefore, we established this regime of two weeks under an 18/6 h cycle before transitioning to flower induction as part of our protocol.

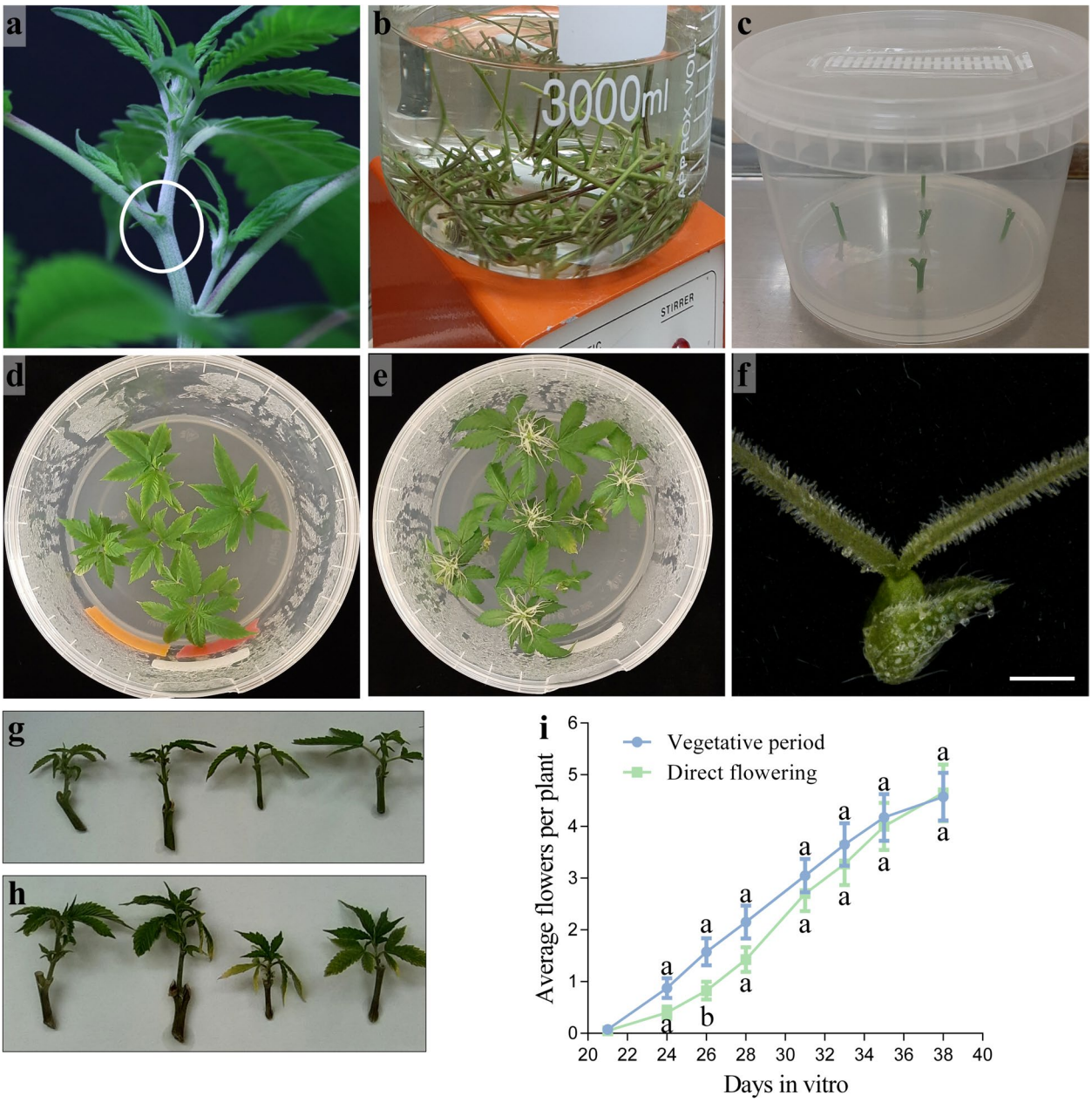

Fig. 1 Tissue culture experimental system for flower induction in cannabis. **a** Stem segment explants, cut from indoor TA5 plants growing under 18/6 h light/dark photoperiod, were introduced into tissue culture (a white circle marks a node segment). **b** Stem section explants were surface sterilized in a 2% sodium hypochlorite solution. **c** The stems were separated into single-node segments, and five explants were cultured in each vessel. **d** Developed plants at two weeks under an 18/6 h light/dark photoperiod. **e** Cannabis flowers developed under a 12/12 h cycle to promote flowering. Images were taken three weeks into the flowering photoperiod. **f** A close-up of an in vitro cannabis flower (bar = 100μm). **g** and **h** Comparison between two photoperiod regimes. Node segments were introduced to tissue culture on the same date. **g** Vessels were immediately cultured under a flower-inductive photoperiod (12/12 h). **h** Vessels were cultured for two weeks under an 18/6 h cycle before switching to 12/12 h. Images were taken two weeks after introducing to tissue culture. **i** Average number of flowers per plant under two photoperiod regimes, with counts taken at specified times after introducing to tissue culture. The average flower number was calculated for five plants per vessel, presented as mean ± SE per plant for eight vessels for each treatment (n = 8); different letters represent a significant difference at a $p < 0.05$ using the Student's *t*-test

Media optimization for enhanced in vitro flower induction

Cannabis is known to exhibit a high rate of hyperhydricity in tissue culture systems, a phenomenon that varies depending on the genotype [15]. Several methods can be employed, to address this issue, including incorporating activated charcoal into the growth medium [16]. To determine the best media for inducing flowering in vitro in cannabis and to assess the tissue's response to activated charcoal, we tested two media commonly used for stem explants: MS and DKW-based media (see Materials and Methods), with or without activated charcoal. Adding activated charcoal to the DKW medium significantly reduced the number of flowers compared to the DKW medium alone but did not affect the MS medium (Fig. 2a). These findings indicate that activated charcoal does not benefit cannabis flowering in tissue culture. Activated charcoal is known to absorb plant growth regulators and other organic supplements [16]. It might be that the presence of activated charcoal reduced the availability of important substances to the plant, thereby reducing flowering.

Statistical tests comparing the performance of plants in the two media without activated charcoal showed that plants in the DKW medium exhibited higher flower production than those in the MS medium at three-time points (Fig. 2a, marked by an asterisk (*)).

In tissue culture systems, sugar serves two primary roles: as an energy source [17] and as a signal for flower induction [18]. The common sucrose concentration in tissue culture for shoot growth ranges between 1 and 4% [19, 20]. To examine the impact of different sucrose concentrations on flowering, four sucrose levels (1% to 4%) were compared in the MS medium (Fig. 2b) and two concentrations (2% and 3%) in the DKW medium (Fig. 2c). However, there were no significant differences in flowering across the concentrations, except between the 2% and 1% sucrose concentrations in the MS medium at three-time points, where a decrease was observed at the 1% sucrose treatment.

Besides sucrose, hormones can also play a role in increasing the number of flowers. Cytokinins, for instance, can influence meristem size, which may enhance flower production [21]. Additionally, cytokinins have been shown to facilitate the transition from vegetative to floral meristems in tissue culture [13]. The 6-Benzylaminopurine (6-BA) cytokinin is particularly recognized as a highly effective inducer of floral induction in vitro [22, 23]. To test the effect of 6-BA on the in vitro flowering of cannabis, we added either 2 mg/L of 6-BA solely during the vegetative phase to promote a larger meristem or 1 mg/L and 5 mg/L of 6-BA during the flowering phase to assess its impact on floral induction. Surprisingly, 6-BA did not enhance flower production; instead, it resulted in a significant decrease (Fig. 2d). This effect might be attributed to our basic DKW medium, which contains adenine hemisulfate (see Materials and Methods), a precursor of cytokinins, resulting in excessively high concentrations of cytokinin.

Other elements that could affect flower numbers, such as phosphate in the media or lower temperature during vegetative growth to increase meristem size, didn't perform better (Fig. S1 and S2).

To summarize, our analysis indicates that DKW media without activated charcoal and 6-BA is most effective. Since there are no significant differences between 2 and 3% sucrose, and 2% is more economical and potentially reduces contamination, we will continue using 2% sucrose.

Evaluating photosynthesis and light response in tissue-cultured *cannabis*

Our results, showing no significant difference between 2 and 3% sucrose concentrations, raise the question of whether the plants in the vessel conduct photosynthesis. To address this, we first examined the presence of stomata, which is essential for gas exchange. Scanning electron microscopy analysis of abaxial fan leaves from plants growing in tissue culture and growth room reveals that leaves in tissue culture develop stomata similar to those in growth room plants (Fig. 3a). To compare stomatal density, we employed the nail polish method and found that leaves grown in tissue culture possess significantly fewer stomata (average of 35 per $0.15mm^2$) compared to those from growth room plants (average of 45 per $0.15mm^2$) (Fig. 3b–c). However, the normal appearance of the stomata of leaves from tissue culture suggests that the plants are capable of photosynthesis. Subsequently, we tested the plants' capacity for photosynthesis by cultivating stem nodes in a medium devoid of carbon energy sources. Since photosynthesis requires gas exchange, we used two types of vessels: one hermetically sealed and the other equipped with a gas-permeable filter. Both were placed under 71 ± 14 μmol· m^{-2}·s^{-1} light in an 18/6 h photoperiod.

Plants cultured in media without sucrose appeared pale and less developed than those grown with 2% sucrose, although the ones in the filtered vessel exhibited better growth (Fig. 3d–e, Fig. S3). To quantify this, we measured the chlorophyll content and found that plants grown with 2% sucrose exhibited higher chlorophyll content in both vessels compared to those grown without a carbon source (Fig. 3f). However, plants cultivated in a filtered vessel, with or without sucrose, exhibited significantly higher chlorophyll content compared to their equivalents in a sealed box, implying that gas exchange allows in vitro

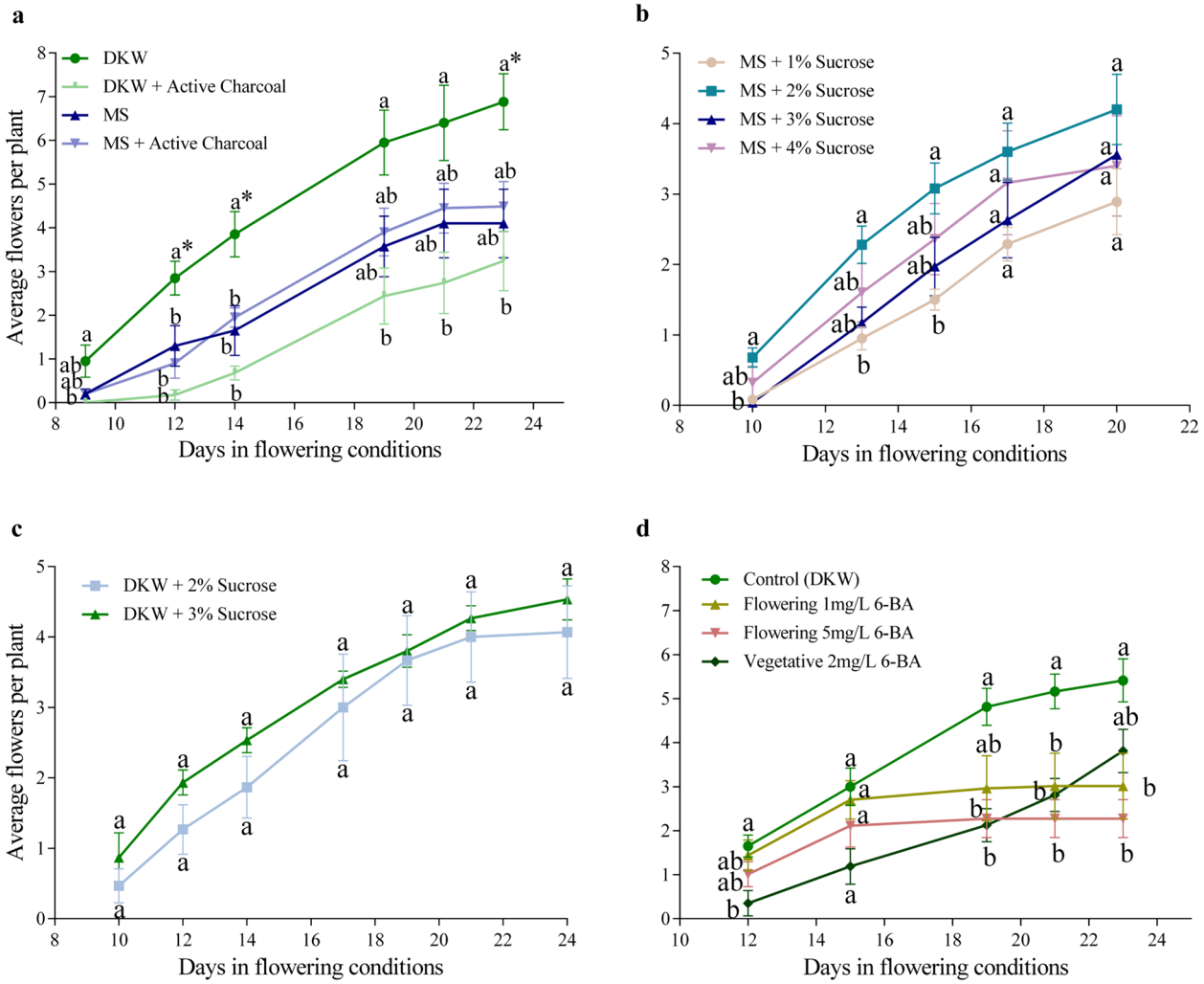

Fig. 2 Flowering of cannabis under various media treatments. TA5 cannabis explants were cultured under an 18/6 h light/dark cycle for two weeks and then transferred to a 12/12 h flowering photoperiod. **a** Evaluation of the effect of activated charcoal in DKW medium compared to MS medium. The addition of activated charcoal significantly decreased flower production in the DKW medium and did not affect MS. Average flower numbers, presented as mean ± SE per plant, were calculated for five plants per vessel across four vessels for each treatment (n = 4). Different letters represent significant differences at $p < 0.05$ using the Tukey HSD test. Asterisks indicate significant differences between DKW and MS at $p < 0.05$ using the Student's t-test. **b** Impact of sucrose concentration in MS medium on flowering. Explants were cultured in MS medium, supplemented with four different concentrations of sucrose, across both vegetative and flowering photoperiods. Average flower numbers, presented as mean ± SE, were calculated for five plants per vessel across five vessels for each treatment (n = 5). Different letters represent significant differences at $p < 0.05$ using the Tukey HSD test. **c** The effect of sucrose concentration in DKW media on flowering. Average flower numbers, presented as mean ± SE, were calculated for five plants per vessel across three vessels for each treatment (n = 3); at *p*-value < 0.05 using Student *t*-test. **d** The effect of 6-Benzylaminopurine (6-BA) on cannabis flowering. 6-BA was added either during the vegetative phase only or during the flowering regime, as indicated in the index. Average flower numbers, presented as mean ± SE, were calculated for five plants per vessel across four vessels for each treatment (n = 4). Different letters represent significant differences at $p < 0.05$ using the Tukey HSD test

plants to carry out photosynthesis. As a result, we have incorporated the use of a filtered box into our protocol.

The light intensity in ex-vitro-grown plants (Fig. 4a), directly affects photosynthesis, with increased light levels enhancing photosynthetic activity until reaching a saturation point [24]. Light also impacts morphogenesis and the induction of flowering [25]. To test the effect of increasing light intensity on in vitro cannabis flowering, we cultured plants under an 18/6 h cycle for three weeks at two different light intensities measured at plant height: low (71 ± 14 µmol· m^{-2}·s^{-1}) and high (173 ± 20 µmol· m^{-2}·s^{-1}). We then transferred the plants to a flowering photoperiod (12/12 h) and redistributed them into the two light intensities, resulting in four treatment groups

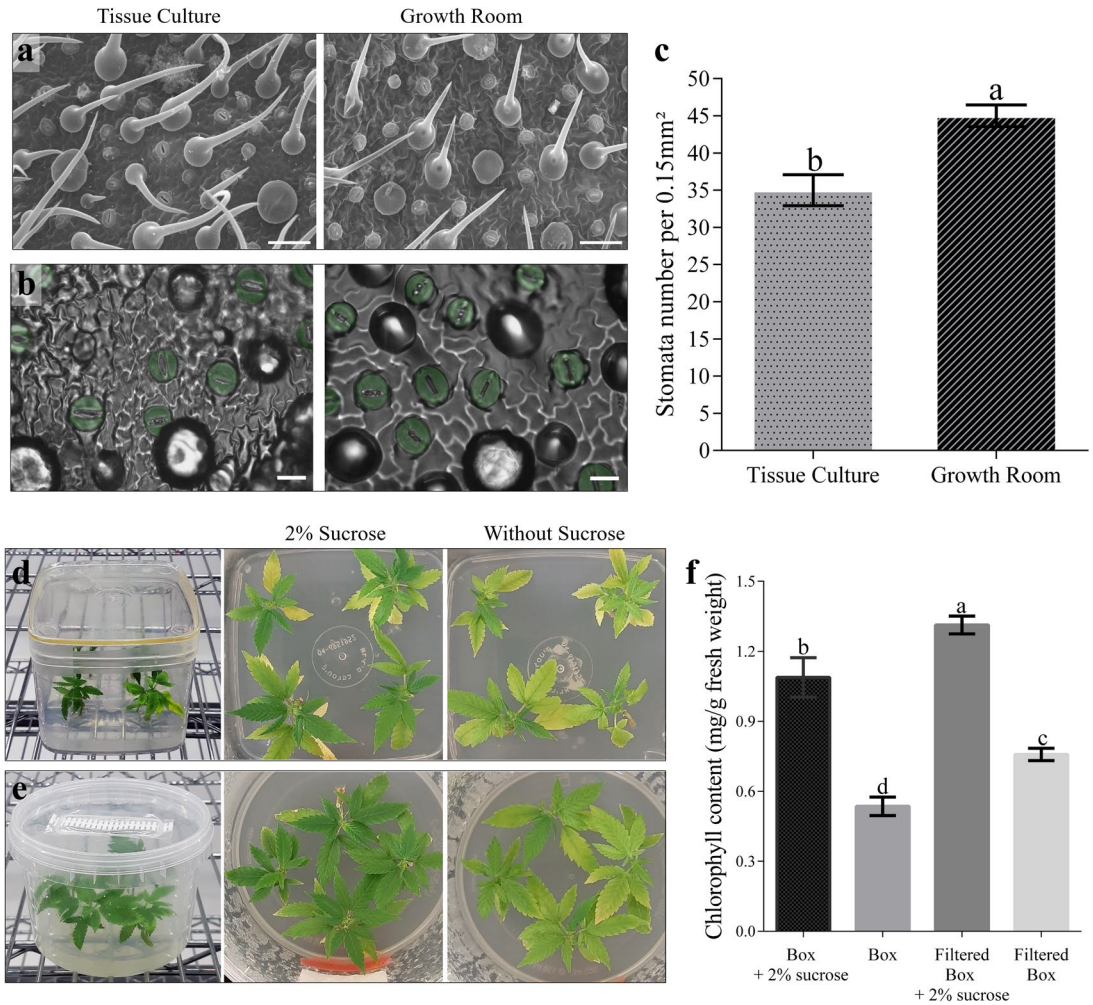

Fig. 3 Stomatal abundance and chlorophyll content in cannabis leaves in tissue culture. **a-c** Comparison of TA5 cannabis stomata on the abaxial side of the fan leaves from tissue culture and growth room. **a** Scanning electron microscopy (SEM) image shows a similar stomata structure between the tissue culture and growth room. Bar = 50 µm. **b**, **c**. Stomata counting on abaxial fan leaves from tissue culture and growth room, using a light microscope (see Materials and Methods; bar = 20 µm). The number of stomata measured in 0.15 mm^2 is presented as mean ± SE (n = 10), and different letters indicate significant differences at $p < 0.05$, using the Student's t-test. **d-f** Cannabis growth and chlorophyll content analysis in tissue culture without supplemented sugar. TA5 cannabis plants were cultivated in tissue culture either in a box (**d**) or a filtered box (**e**), with or without added sucrose. **f** Average chlorophyll content (measured in mg/g fresh weight) was calculated for two fresh leaves (number 3 and 4 from the apex) from each plant (a total of eight leaves per vessel). Leaves were weighed, and chlorophyll was extracted. Chlorophyll content was calculated according to spectrophotometric measurements at 645 and 663 nm (see Materials and Methods). Data are presented as mean ± SE for six vessels with four plants per vessel for each treatment (n = 6), and different letters represent a significant difference at $p < 0.05$ using the Tukey HSD test

based on changes in light intensity: Low to Low, Low to High, High to Low, and High to High. Plants exposed to low light intensity during the flowering-promoting photoperiod produced significantly more flowers (Fig. 4b-d) with a final average of 9.3 (Low to Low) and 9.9 (High to Low) flowers per plant and appeared to have longer stigmas compared to those exposed to high intensity (Fig. 4e). Plants under high light intensity during the 12/12 h cycle had a final average of 7.3 flowers per plant, regardless of the light intensity during the vegetative phase. Notably, the selected light intensity was significantly lower than what is typically used in growth rooms (Fig. 4a), demonstrating that tissue-cultured plants are more susceptible to damage from high-intensity light.

Variability and response of *cannabis* cultivars to in vitro flowering

Cannabis cultivars exhibit significant variability in inflorescence traits, including flowering time, architecture, number of flowers, flower size, and compaction

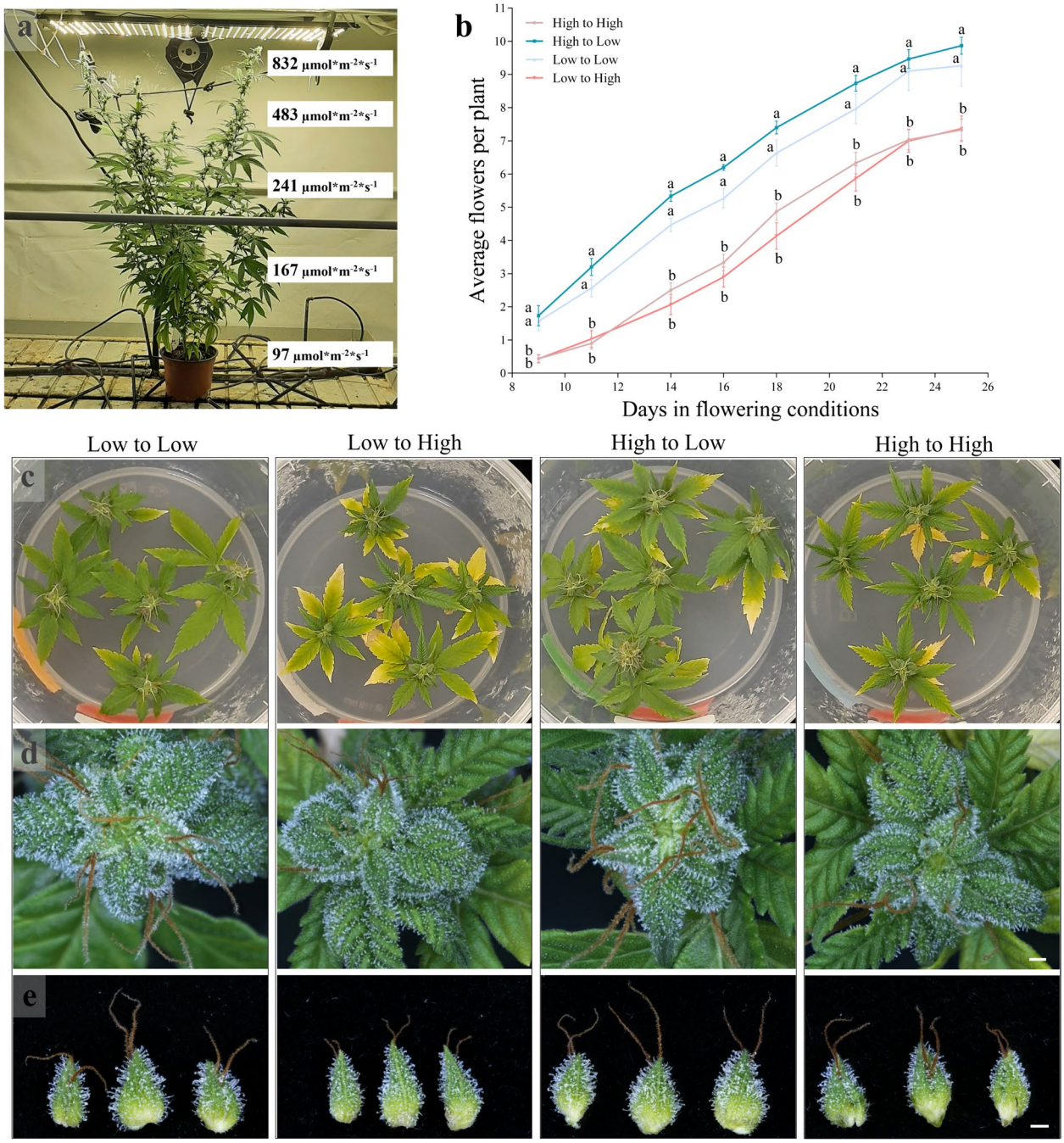

Fig. 4 Cannabis flowering under two different light intensities. TA5 cannabis explants were subjected to two light-intensity treatments during three weeks of vegetative growth (18/6 h light/dark): Low (71 ± 14 µmol·m^{-2}·s^{-1}) and High (173 ± 20 µmol· m^{-2}·s^{-1}). Subsequently, they were transferred to a 12/12 h cycle to induce flowering and reassigned to the two light intensities, resulting in four treatments (Low to Low, Low to High, High to Low, and High to High). **a** Light intensity at various distances from the light source along the plant height in the growth room. **b** Comparison of flowering responses among light-intensity treatments. Average flower numbers, presented as mean ± SE per plant, were calculated across six vessels, with five plants per vessel, for each treatment (n = 6); different letters represent significant differences at $p < 0.05$ using Tukey HSD test. Plants exposed to low light intensity during flowering-promoting photoperiod produced significantly more flowers than those exposed to high light intensity. **c** Cannabis flowering in tissue culture vessel. The light intensities are indicated. Images were taken on day 25 after flower induction. **d** Close-up of cannabis inflorescences for each of the above treatments, and **e** close-up of detached cannabis flowers. Higher light intensity during the flowering period appears to cause shorter stigmas. Images d and e were captured using a stereomicroscope (bar = 100µm)

[5, 26–28]. To assess the response of other cultivars to in vitro flowering, we selected two additional cultivars, Sky 1 and Magic 9, that exhibit distinct inflorescence phenotypes when grown in a growth room and introduced them to tissue culture. Following flower induction, we identified three distinct phenotypic differences: number of flowers, stigma length, and stigma browning (Fig. 5, Fig. S4, and Fig. S5). In all cultivars, flowers began to appear on day 9. The Sky 1 cultivar produced significantly more flowers than the Magic 9 and TA5 cultivars (Fig. 5a), displaying a notable difference from its growth room equivalent (Fig. 5b). The TA5 stigmas appeared to be shorter than those of Sky 1 and Magic 9 and changed their color to brown before those of the other cultivars (Fig. 5c-e), suggesting that TA5's receptivity declines more quickly. This is consistent with the TA5 stigma observed in the growth room plants.

This experiment demonstrates that different cultivars behave differently in tissue culture, and their inflorescence characteristics do not always match their growth room phenotype, suggesting an interaction between genetic background and in vitro growth conditions.

In vitro pollination and seed development in *cannabis*

To assess the potential of in vitro flowering as a method for rapid breeding, we examined the ability of male plants to produce viable pollen and female plants to develop viable seeds. To this end, we cultured male cannabis plants (Bt cultivar) and female plants (cultivar Sky 1) for two weeks under an 18/6 h cycle before transferring them to a 12/12 h flowering photoperiod.

After 18 days, all plants produced flowers and were ready for pollination (Fig. 6a–d). Since the anther did not dehisce and release the pollen, we manually pinched it with a needle and tapped it over vessels containing female plants. All female inflorescent (25 plants) developed seeds in vitro (Fig. 6e) with an average of 4.41 ± 0.36 seeds per plant. However, only 2.44 ± 0.17 seeds per plant germinated (mean ± SE calculated per vessel, with five plants per vessel) (Table S1). In vitro hybrid plants were grown from seeds to fully developed plants (Fig. 6f–h).

Altogether, eight weeks were needed, from introducing the stem segment to tissue culture to producing viable seeds, highlighting the in vitro flowering system as an excellent method for fast and practical breeding.

Discussion

Plants in tissue cultures are grown under controlled conditions that differ significantly from the natural environment. These optimized conditions enhance growth but can also induce stress due to the lack of fluctuations throughout the day and the lack of natural cues, leading to altered physiological responses. However, this platform allows us to fine-tune factors such as nutrient composition, light intensity, and temperature, thereby enhancing growth and development.

In this study, we focused on determining the optimal conditions for the in vitro flowering of cannabis. This approach holds immense potential for the sterile production of secondary metabolites free from pathogens like *Botrytis cinerea*, with high consistency and the likely capability to control the cannabinoid content. Given the complexity of breeding in cannabis, it also provides a valuable strategy for breeding programs, especially when resources are limited.

Using our protocol, we cultivated five plants per vessel and arranged the cultures on a shelf measuring 45 cm in width and 1 m in length, accommodating a total of 32 vessels in a 4 by 8 configuration (Fig. S6). This setup allowed for the growth of 160 plants per shelf. By stacking four shelves vertically in a standard growth room, we demonstrated the capacity to grow a total of 640 plants on a 1-m length floor area, highlighting the substantial potential of this method.

However, it is important to note this approach's limitations, including the potentially lower yield of synthesized cannabinoids compared to traditional cultivation and the possible need for genotype-specific media development [29].

When we introduced the stem segments to tissue culture, the flowering time and the number of flowers produced were comparable, regardless of whether the plants were immediately subjected to a flowering-promoting photoperiod or first experienced a vegetative phase. We propose that since the day length cue is perceived in leaves to initiate the cascade that activates the flowering

(See figure on next page.)

Fig. 5 Cannabis cultivars grown in vitro exhibit differences in flowering patterns. Cannabis explants from three cultivars (TA5, Magic 9, and Sky 1) were cultured under vegetative conditions (18/6 h light/dark) for 17 days and then transferred to a 12/12 h cycle to induce flowering. **a** Comparison of in vitro flowering responses of different cannabis cultivars. Average flower numbers, presented as mean ± SE per plant, were calculated for five plants per vessel across six vessels for each treatment (n = 6). Different letters indicate significant differences at $p < 0.05$, as determined by the Tukey HSD test. **b** Four-week-old inflorescences of the cannabis cultivars in the growth room. **c** Cannabis cultivars after four weeks under flowering induction. **d** Close-up of the in vitro inflorescences. **e** Close-up of detached in vitro female flower. TA5 stigmas appeared to be shorter than those of Sky 1 and Magic 9, and they were the first to change color to brown both in the growth room and in tissue cultures; Images d and e were captured using a stereomicroscope (bar = 100μm)

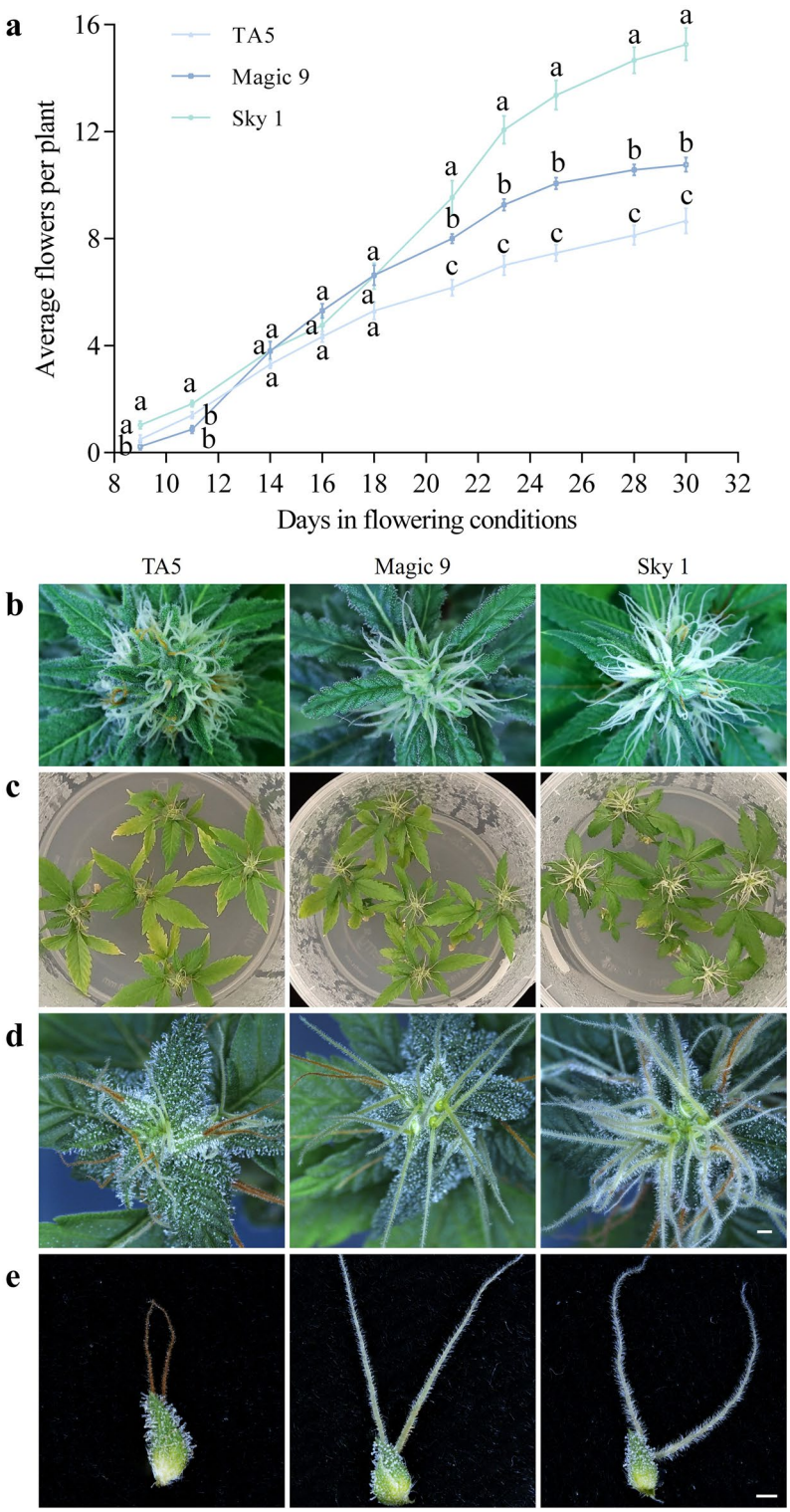

Fig. 5 (See legend on previous page.)

machinery [30], a stem node without leaves cannot sense day length and thus cannot induce the transition of the meristem to an inflorescence meristem. Therefore, a few days are needed to produce leaves, even under a short-day photoperiod, before flower meristems can form. This resulted in similar timing and numbers of flowers between the two regimes.

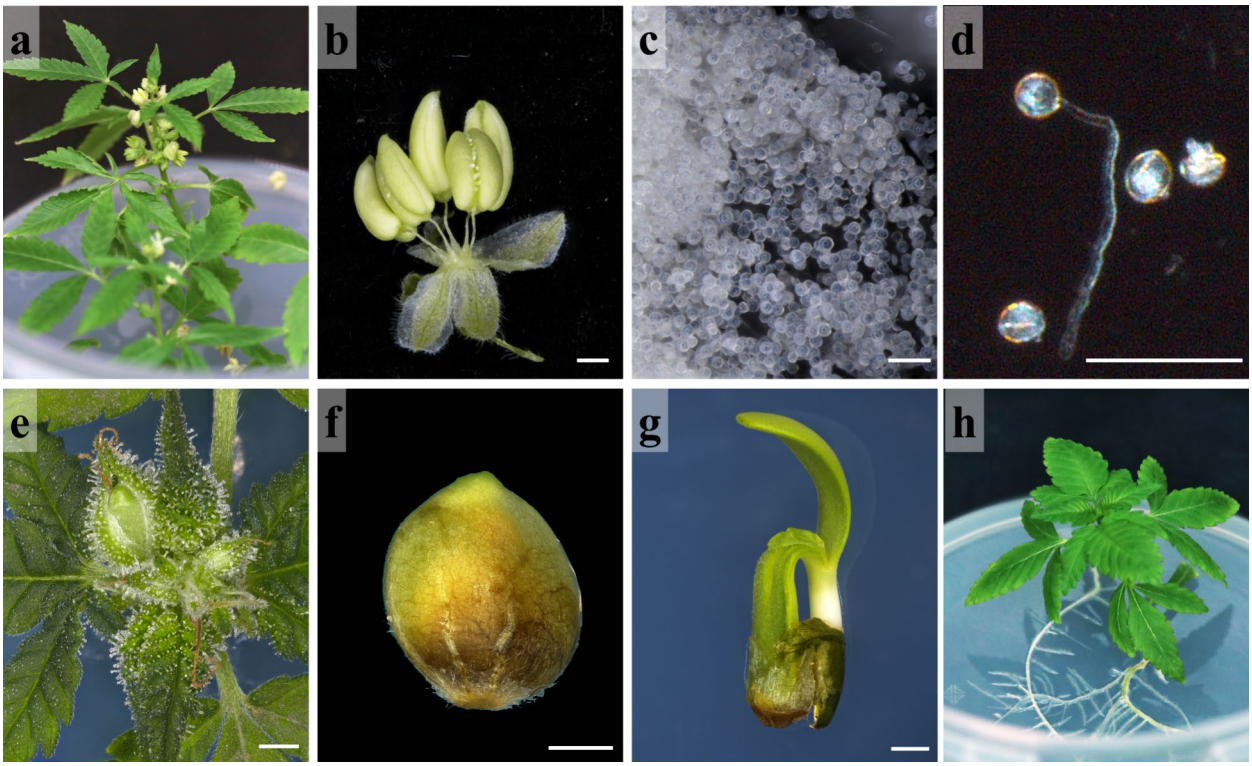

Fig. 6 Cannabis fertilization in vitro. **a** Male cannabis explant (cultivar Bt) flowering in vitro. **b** Cannabis male flower developed in vitro. **c** Pollen extracted from an in vitro anther. **d** In vitro germination of the extracted pollen. **e** In vitro inflorescence with developing seeds. In vitro-produced pollen was used to fertilize in vitro female plants (Sky 1 cultivar). **f** In vitro cannabis seed three weeks post-pollination **g** Germination of the hybrid seed in vitro. **h** The in vitro hybrid cannabis plant. Images b-g were captured using a stereomicroscope. Bar: b = 1000µm, c,d = 10µm, e,f,g = 100µm

In *Arabidopsis thaliana*, leaf perception of day length induces the expression of the *FLOWERING LOCUS T* (*FT*) gene, known as florigen, which is transported through the phloem to the shoot apical meristem to promote flowering [31, 32]. In short-day plants, the mechanism of flowering induction is still being elucidated, but the process appears to be conserved [33]. Genes homologous to *FT* were identified in many short-day plant species [30, 34], including *CsFT* in cannabis [35].

Our results, showing normal stomata development in tissue culture and high chlorophyll content with improved growth in filtered vessels with and without a carbon source, suggest that cannabis shoots can perform photosynthesis in tissue culture. However, the moderate plant growth in a sugar-free medium indicates that the plant cannot be fully autotrophic under our growth conditions with passive ventilation, likely due to limited CO_2. This is supported by the CO_2 level measured within a filtered vessel containing plants, showing values between 150 and 250 mmol/mol during the photoperiod, depending on the number of filters, while the CO_2 concentration in the room was maintained at 350–400 mmol/mol [36]. It would be interesting to test the cannabis performance under a forced ventilation system in the future.

In tissue culture, sucrose in the media provides the necessary carbon for energy and growth, with standard concentrations generally ranging from 2 to 3% (w/v). As we did not observe a significant difference between these concentrations, we selected the 2% for our protocol, which offers several potential advantages. Low sucrose levels can enhance photosynthetic activity, partly by stimulating Rubisco activity, which often leads to increased plant vigor [37–39]. Low sucrose level also reduces the risk of microbial contamination [32] and minimizes the phenomenon of hyperhydricity in sensitive cultivars [40]. Excess sucrose can cause metabolic imbalances that affect the synthesis of secondary metabolites [41]. Furthermore, using the lowest effective concentration is cost-effective for large-scale or long-term experiments.

Light intensity can promote mixotrophic growth, which relies on both sucrose in the media and photosynthesis. Our results, however, show that increasing the light intensity during the flowering-promoting photoperiod led to significantly fewer flowers. The appearance of yellow leaves when high-intensity light is applied (Fig.

S7) suggests that the plants might be experiencing light-induced stress.

In outdoor plants, prolonged exposure to excessive light can lead to photoinhibition, reducing photosynthetic capacity and causing chlorophyll degradation. The light intensity applied in tissue culture is considerably lower than that of outdoor environments[42] because most of the energy comes from the sucrose in the media and because high light intensity can induce stress.

Plants in tissue culture are placed in a closed box without buffer elements like wind, shading, and light fluctuations throughout the day. This may intensify the effects of high light and might cause increased temperature and humidity. Additionally, tissue cultures often consist of young tissues in early growth stages, making them more susceptible to environmental stresses due to the lack of protective structures like a thick cuticle or well-developed cell walls [43].

It might be that the lack of a phyllosphere microbiome due to sterile conditions in tissue cultures reduces plant fitness and increases susceptibility to light-induced stress. Microorganisms can produce protective compounds, such as pigments and antioxidants [44], that absorb excess light, and they can also form a physical barrier that reduces the amount of light reaching the leaf surface [45], thereby preventing potential damage from excessive light exposure.

In our experiment, when low light intensity was applied during the 12/12 h cycle, the plants produced more flowers regardless of the intensity during the vegetative phase. We propose that during the initial period under the flowering induction regime, the plants have time to recover before the transition to inflorescence meristem. During this phase, the newly developing leaves grow under low light and become robust, supporting future inflorescence meristem and flower development.

Microflowering for rapid breeding

Seed production in vitro remains relatively unexplored, with only a few studies reported. In orchids, tomatoes, and wheat, in vitro flowering is proposed to accelerate breeding programs by shortening the life cycle [20, 46, 47]. In peas, it is suggested as a method to synchronize flowering in distant varieties, thereby facilitating fertilization and seed production [48]. Here, we highlight this method as an approach to overcoming the complexities of breeding in cannabis.

In cannabis, the individual female and male flowers that developed in vitro exhibited phenotypes similar to those of growth room flowers. However, the anthers did not burst and release pollen. Typically, anthers open under low relative humidity (RH), whereas high RH can delay or prevent this process [49]. This suggests that the high humidity levels within the culture vessel likely kept the cannabis anthers closed. In addition, pollen grains are typically partially desiccated upon dispersal, and before they germinate, they must rehydrate by absorbing water from the stigma on which they land [50]. It might be that losing and gaining back moisture is crucial for pollen grain germination. Therefore, RH in the flower's surrounding environment can decrease the viability of the pollen from these flowers. Nevertheless, in our setting, although the anther did not dehisce naturally, it released pollen with our assistance, which proved to be viable according to our germination test and the successful fertilization.

The average number of female flowers produced was six, with an average of 4.4 seeds developing per inflorescence/plant. Low pollen grain viability might have led to incomplete fertilization, preventing some flowers from producing seeds. Another possibility is that the plant's small size could not support extensive seed development. Out of the collected seeds, only an average of 2.4 seeds per plant germinated (Table S1). However, achieving one successful succession per generation is sufficient for a breeding program aimed at hybrid seed production and generating numerous homozygous plants to test various combinations.

Conclusions

This study has successfully established a method for in vitro flowering induction in cannabis, providing a valuable tool for both research and practical applications. Our findings highlight the superior performance of DKW medium with 2% sucrose in a filtered vessel and emphasize the need for low light intensity during flower induction to optimize production. The improved performance in filtered vessels suggests that plants conduct photosynthesis in vitro, underscoring the need for future investigations into the effects of forced ventilation to refine this system. We show that this method applies to several cultivars and to male flowering as well. Additionally, we use this system to produce viable seeds in vitro. Altogether, this method offers a robust framework for cannabis research and cultivation, with potential applications in sterile cannabinoid production and efficient breeding strategies.

Methods

Plant material

Cannabis sativa L. plants from cultivars TA5, Sky 1, Magic 9 (female cultivars), and Bt (male plant) (our private lines) were used in this study. The plants were grown in a growth room in 1-L pots with 'Bental 11' planting soil (Tuff Substrates), regularly irrigated with tap water, and fertilized with BOUNTY1 (N.P.K. 4:2:6, Zalmanson

Dshanim). The plants were grown at 26°C (measured at plants' canopy) under Metal halide 600W light bulbs (UPLUX, USA) in a long-day photoperiod of 18/6 h light/dark cycle to sustain vegetative growth.

Tissue culture

TA5 plants in their vegetative phase were introduced to tissue culture by cutting stem segments. The explants were sterilized by submerging and stirring them in a 2% sodium hypochlorite solution containing 0.1% (w/w) Triton X-100 for eight minutes, followed by three rinses with sterilized water. The segments were sliced into single-node explants and transferred into vessels containing the appropriate media. Two basal media were used in this study: 1. Murashige and Skoog (MS) [51] consist of 4.4 g/L MS including Nitsch vitamins (M0256, Duchefa), 3% sucrose (w/v), and 0.5 g/L MES. 2. Driver and Kuniyaki Walnut (DKW) [52] containing 5.6 g/L DKW/JUGLANS basal salt mixture including vitamins (D0247, Duchefa), 3% sucrose (w/v), 80 mg/L adenine hemisulfate (A0908, Duchefa) and 0.5 g/L MES. The pH of the media was adjusted to 5.8 and 0.8% (w/v) plant agar (P1101, Duchefa) was used as the gelling agent. The media were sterilized by autoclaving at 121°C for 20 min and poured into either a ventilated round polypropylene micro box (O118/80 + OD118, SacO2) with a volume of 65 ml or into a closed 10 × 10 × 10 cm square polycarbonate box (Fig. 3d) with a volume of 84 ml. Five stem explants were cultured in each vessel for two weeks under an 18/6 h light/dark photoperiod to promote vegetative growth. The plants were then transferred to fresh media and placed under a 12/12 h light/dark cycle to induce flowering and re-cultured in fresh media every two weeks. Both growth conditions were set at a temperature of 25°C. Flowers were counted using a stereomicroscope every several days.

Fertilization: To conduct an in vitro pollination procedure, male flowers were carefully removed from the plant and placed on a sterile Petri dish, followed by pinching with a sterile needle. In the laminar flow cabinet, the anthers were gently tapped using forceps to distribute pollen onto the stigma.

Media optimization

For cytokinin effect, plants were cultured on DKW medium for two weeks in vegetative conditions (18/6 h) and then cultured under flowering-promoting photoperiod (12/12 h) in three media: control (DKW medium + 80 mg adenine), 1 mg/L 6-Benzylaminopurine (6-BA) (B001, Caisson) and 5 mg/L 6-BA. In addition, one group was cultured on DKW with 2 mg/L 6-BA medium during the vegetative phase and re-cultured to control DKW medium when transferred to flowering induction.

For the activated charcoal effect, 0.5 g/L activated charcoal (C9157, Sigma) was added to DKW or MS media during both the vegetative phase and flowering-promoting photoperiod.

Stomata counting analysis

A silicone impression material (Elite HD+, Zhermack) was used to create molds of abaxial fan leaves. Next, patterns from those molds were taken using nail polish and checked under a light microscope. From each leaf, stomata were counted in three areas (0.15 mm^2 each) along the main vascular bundles (Fig. S8).

Chlorophyll extraction and measurements

Chlorophyll content was assessed on plants cultured as follows: first, all stem explants were cultured on DKW medium with 2% sucrose in a ventilated micro box (four explants per micro box) for three weeks under a long-day photoperiod (18/6 h). Next, they were divided into four treatments: DKW medium with or without 2% sucrose in a ventilated micro box and DKW medium with or without 2% sucrose in a closed 10 × 10 × 10 cm square polycarbonate box. Plants were grown for four weeks under the same conditions as before without media renewal. For chlorophyll measurements, two fresh leaves (numbers 3 and 4 from the top) from each plant (a total of eight leaves per vessel) were taken for extraction with 25ml of 80% acetone. Chlorophyll was measured according to Arnon 1949 [53]. The leaves were weighed before extraction to determine their fresh weight, and spectrophotometric measurements at 645 and 663 nm were conducted using the Infinite M Plex (Tecan). The chlorophyll concentration was calculated using the formula: $((20.2 \times A_{645}) + (8.02 \times A_{663})) * V / (1000 \times W)$, where V is the solution volume and W is the weight of the leaves.

Light intensity experiments

Explants were introduced to culture as described. During the vegetative phase (18/6 h), vessels were divided into two light-intensity treatments: Low (71 ± 14 $\mu mol \cdot m^{-2} s^{-1}$) and High ($173 \pm 20$ $\mu mol \cdot m^{-2} s^{-1}$), measured at the plant height level. After three weeks, plants from each light treatment were re-cultured in fresh media and placed under a flowering-promoting photoperiod (12/12 h) at the two different light intensities, resulting in a total of four treatments: Low to Low, Low to High, High to Low, and High to High. Light intensity was measured using a LI-180 Spectrometer (LI-COR).

Pollen germination

Pollen germination was assessed following the protocol of Flajšman et al., 2021 [54]. The germination medium consisted of 170 g/L sucrose, 0.1 g/L H_3BO_3,

0.432 g/L $Ca(NO_3)_2 * 4H_2O$. The pH was adjusted to 7.0, and 0.7% (w/v) plant agar was used as the gelling agent. The medium was sterilized by autoclaving before being poured into Petri dishes. Male flowers were gently tapped to distribute pollen across the plate. Subsequently, the Petri dishes were incubated in the dark at room temperature for 24 h. Images were taken using a Nikon SMZ-18 stereo zoom microscope (Nikon).

Scanning *electron* microscopy

Scanning electron microscopy (SEM) was conducted following the method described previously [55]. Briefly, fresh fan leaves from plants grown in tissue culture or growth room were initially fixed in 100% methanol for 10 min. Subsequently, they were rinsed three times for 30 min in 100% ethanol, dried using a K850 critical point dryer, and then mounted on stubs. The specimens were further coated with a thin layer (2 nm) of gold–palladium using a Q150T ES (Quorum Technologies Ltd, Lewes, UK) for SEM imaging in a JSM-IT100 SEM (Jeol Ltd, Tokyo, Japan).

Experimental design and statistical analysis

The experiment utilized a completely randomized design, with a specified number of replicates (designated as n = number of vessels), each comprising the average of five explants cultured within a single vessel. The data were analyzed using JMP software (SAS Institute, Cary, NC, USA). Means comparison was conducted using analysis of variance (ANOVA) with post-hoc Tukey–Kramer honest significant difference (HSD) test (for multiple comparisons) or Student's t-test (for one comparison) at $\alpha = 0.05$ significance level and the data are represented as mean ± SE (standard error). Treatments showing statistically significant difference are indicated by different letters.

Supplementary Information

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Additional file 1.

Author contributions

LEW, OL, and KB: Conceptualization; LEW and OL: design of the work; OL: conducted the experiments; LEW and OL: Writing Original Draft Preparation; LEW and OL: Writing Review and Editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

KB and LEW are listed as inventors on a PCT Patent Application PCT Patent Application No. PCT/IL2022/050007.

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