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Regeneration of shoots from immature and mature inflorescences of *Cannabis sativa*

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Abstract

*Cannabis sativa* is usually clonally propagated from plants in the vegetative phase. However, phenotypic traits such as yield and chemical composition can only be assessed in unfertilized plants reaching the end of their life cycle and there are no peer reviewed methods to propagate flowering plants. In this study, immature (3 cultivars) and mature (1 cultivar) floral explants were cultured on thidiazuron and shoot development was observed from both immature and mature explants. This provides the first report of micropropagation from floral tissues in *C. sativa* and will enable plants to be clonally propagated up to the date of harvest.

Keywords

Cannabis, Micropropagation, Shoot regeneration, Reproductive phase, flowers
**Introduction**

*Cannabis sativa* L. has recently gained increased acceptance for medicinal and recreational use in many countries. While phenotypically diverse, medicinal and recreational cultivars are generally determinate, dioecious, outcrossing, annual plants. Plants grown from seed demonstrate high degrees of variability in respect to chemical composition, growth habit, and agronomic traits (de Meijer et al. 2003; Caplan et al. 2017), so clonal propagation is commonly used to maintain uniformity.

Traditionally, this is done using stem cuttings but more recent reports describe methods for in vitro propagation through meristem proliferation and de novo regeneration (Reviewed in Lata et al. 2017). To produce cuttings, mother plants are maintained in the vegetative state under long-day conditions to provide vegetative shoots. While there are some reports of propagation from flowering plants in the gray literature, referred to as “monster cropping”, there are no known peer reviewed reports of propagation from flowering plants.

Early studies attempted to regenerate Cannabis plants from stem, leaf, and petiole explants with limited success (Reviewed in Lata et al., 2017). Low levels of shoot regeneration were obtained from cotyledon, epicotyl, or stem explants and higher levels have been reported from leaf explants. Perhaps the most practical system available for micropropagation uses meristem tips or axillary buds to multiply genetically consistent plants. To date, all reports of in vitro plant regeneration have been from vegetative plant material and may not be applicable to plants in the reproductive phase.

While propagation from vegetative tissues is suitable to multiply plants with known characteristics, many important traits (chemical profile, organoleptic properties, etc.) are only expressed in unfertilized (seedless) plants during the late reproductive phase. This has important implications for selecting new germplasm and establishing large-scale breeding programs, as there are no reliable methods to
propagate them after they have reached full maturity. To circumvent this issue, cuttings can be taken from each plant before inducing flowering and maintained in a vegetative state while the original seedlings are grown to maturity and selected for floral traits. However, this approach is inefficient as it requires each genotype to be maintained in two locations during the selection process, adding significant costs in labour, production space, and operating expenses.

The lack of methods to propagate plants in the reproductive phase also presents issues in the development and large-scale clonal propagation of day-neutral cultivars. These cultivars are not photoperiod sensitive and enter the reproductive phase based on their physiological maturity regardless of day-length. While they offer several agronomic advantages, they cannot be maintained as mother plants and are difficult to clonally propagate. To facilitate more efficient plant breeding and clonal propagation of day-neutral cultivars, a vegetative propagation system using floral tissues would be ideal. The objective of the current study was to evaluate the potential of floral explants for micropropagation of *C. sativa*.

**Materials and Methods**

For this study, 3 high THC accessions of female *C. sativa*, ‘1KG2TF’ (13.4% THC; < 0.07% CBD), ‘S1525’ (22.8% THC; <0.07% CBD), and ‘H5458’ (20.2% THC; <0.07% CBD) were grown from cuttings in a greenhouse at Tweed Farms (CG2TF; Niagara-on-the-lake, Ontario, Canada) or an indoor growth facility at Tweed Inc. (S1525 and H5458; Smiths Falls, ON, Canada). During the early reproductive stage of development (2 - 3.5 weeks post-floral induction), non-senescent inflorescences with green-white carpels were excised from apical and axillary shoots of several individual plants from each cultivar. Bracts and leaves were removed and explants (Figure 1C) and they were surface sterilized with a 1-
minute submersion in ethanol, washed in sterile water for 5 seconds, then again for 45 seconds, then a 10-minute submersion in a solution of 10% v/v commercial bleach followed by three rinses in sterile water for 5 minutes each. Explants were blotted dry on filter paper and temporarily placed in a sealed GA7 culture vessel to prevent desiccation. This was also done using mature inflorescence material from H5458 on the day of harvest (58 days post-floral induction; Figure 1B).

Using a dissecting microscope, the lower flowers of the inflorescences were removed with ultrafine forceps until only 3-5 florets remained on the distal portion (Figure 1C). The stem below the remaining florets was pinched off with forceps and they were cultured in 60x15mm petri plates (Fisherbrand, USA) with four explants per plate and five plates per treatment. The vessels contained 10 ml of semisolid Murashige and Skoog medium (Murashige and Skoog 1962) with MS vitamins (Phytotechnology Laboratories, USA), 3% sucrose, 1 µM Plant Preservative Mixture (Plant Cell Technology, USA), and 2.2 g Phytagel (Sigma-Aldrich, USA). Five concentrations (0, 1, 2, 5, and 10 µM) of Thidiazuron (TDZ; Caisson Labs, USA) were added to the media and it was adjusted to a pH of 5.7 prior to being autoclaved. Cultures were maintained at 23°C under cool white florescent lighting at about 10-30 µmol/m²/s PPFD. Additionally, mature inflorescence explants were also cultured in a thin layer liquid culture (~3mm) using previously described custom vessels (Shukla et al. 2017). The medium used in this treatment was identical to the 10 µM TDZ treatment described above with the exclusion of gelling agent. The explants were cultured for 37 days before they were assessed and photographed. Explants with regenerating shoots from all three cultivars were transferred to a shoot proliferation medium composed of MS salts and vitamins, 3% sucrose, 0.03% activated charcoal, 0.8% agar, 1.86 µM kinetin, and 0.54 µM NAA (prepared as described above) after 60 days. As is typical for C. sativa plants cultured on this medium, most of them developed roots (Figure 1J) and were then transferred to potting mix and acclimatized (Figure I &K) using standard protocols.
Results and Discussion

Most explants on basal MS medium increased marginally in size and remained green but had no further development (Figure 1G) with the exception of one explant of S1525 where a single shoot was observed. All treatments containing TDZ induced shoot growth in 2 of 3 cultivars tested (Figure 1E&F), while the tissue of immature explants from H5458 appeared bleached in all treatments (including control); perhaps suggesting that this cultivar was more sensitive to the surface disinfection protocol. An analysis of variance (ANOVA) was conducted for both responding cultivars using JMP 14.1.0. but the model effect was only significant for cv. S1525 using a p-value of 0.05 (p-value = 0.0077). Following the ANOVA a regression analysis was conducted for the response to TDZ concentrations for cv. S1525. However, it should be noted that despite the lack of significance for the response of cv. 1KG2TF (due to the sporadic response among explants) shoots were only produced in the presence of TDZ and further evaluation is needed to assess this genotype’s response. The response of both cultivars, along with the R² value for cv. S1525, are presented in Figure 2.

While immature floral explants from H5458 did not regenerate shoots, mature inflorescences of this cultivar developed small, green shoot-like structures after 18 days. These structures continued to develop shoots in the 1 and 10 µM TDZ treatments. Compared to the immature inflorescences from 1KG2TF and S1525, these explants took longer to develop shoots, fewer explants responded, and further development was slower. Ultimately, only one elongated shoot was recovered (Figure 1H) from a mature inflorescence explant, originating from a thin layer liquid culture system (We Vitro inc.; Guelph, Canada).

Several studies have reported in vitro propagation of Cannabis sativa through meristem proliferation using nodal cultures and de novo regeneration from various vegetative explants (Reviewed in Lata et al., 2017). While callus has been initiated from C. sativa flowers (Raharjo et al. 2006), the cultures failed to
develop shoots or somatic embryos, making this the first known report of shoot regeneration from floral tissues in this species.

Shoot development was successfully induced in response to all concentrations of TDZ that were tested for cv. S1525 and 1KG2TF. TDZ has been shown to be among the most effective PGRs for shoot proliferation and de novo regeneration in Cannabis (Reviewed in Lata et al, 2017). In this study, it appears that the regeneration is occurring from existing meristematic tissue, but this was not specifically determined and some callusing was observed. Shoots induced by TDZ from leaf explants in other studies successfully developed into plants that were chemically and genetically similar to the parent plants (Reviewed in Lata et al., 2017). While this does not necessarily apply to the current system, the regenerated shoots developed into vigorous plants with no obvious abnormalities and THC/CBD levels within expected ranges (S1524 = 22.8% SE +/- .87; 1KG2TF = 13.4% SE +/- 0.05; H5458 = 20.2% SE +/- 0.97; CBD < 0.07 for all cultivars).

This study established shoot cultures from floral explants and was successful using source plants in the early and late reproductive phases. The shoots regenerated from floral explants rooted, acclimatized, and grew into phenotypically normal plants (Figure 1L&M). To the best of our knowledge, this is the first report of shoot regeneration or plant propagation from C. sativa in the reproductive phase. These techniques will enable plants to be clonally propagated up to the day of harvest, which has not previously been possible. While further work is needed to refine the protocol, it will help improve the efficiency of C. sativa breeding programs and clonal propagation of day-neutral cultivars.
References


Figure Captions

**Figure 1:** Stages of establishment of in vitro culture of *Cannabis sativa* from inflorescence tissues. (A) Immature inflorescence of *C. sativa* cv. 1KG2TF growing in the greenhouse. (B) Mature inflorescence of *C. sativa* cv. H5458 growing in an indoor growth room (C) Day 1 explant of approximately 5 florets from distal portion of the inflorescence. (D) 15-day old cultures of inflorescence explant on 2µM TDZ. (E) Shoot and leaf growth after 37 days of growth on 1µM TDZ. (F) Cluster of shoots and leaf growth after 37 days of growth at 10µM TDZ. (G) Inflorescence explant on control media after 37 days of growth. (H) Shoot emergence from a mature inflorescence at 50 days. (I) Acclimatized plant derived from mature inflorescence explant 21 days after transfer to potting mix. (J) Shoot and root development from inflorescence explants on shoot growth medium. (K) Acclimatized plantlet derived from inflorescence explant. (L & M) Plants regenerated from floral explants growing in the production facility approximately 4 weeks post floral induction.

**Figure 2:** Average number of shoot clusters formed from immature inflorescence explants of S1525 (●) and 1KG2TF (▲) in response to various levels of TDZ. Observations were taken from responding explants after 66 days in culture. Bars represent the standard error of the mean. Please note that the analysis of variance was only significant for S1525 so a regression line was not calculated for 1KG2TF.
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\[ y = 0.3704x + 0.4499 \]

\[ R^2 = 0.270582 \]