HortScience 58(9):1018–1022. 2023. https://doi.org/10.21273/HORTSCI17169-23

# In Vivo and In Vitro Chromosome Doubling of ‘I3’ Hemp

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*Keywords.* *Cannabis sativa*, in vivo, in vitro, polyploid, colchicine, oryzalin

***Abstract.*** ***Cannabis sativa*** **L. is a diploid (2*x*) herbaceous plant that provides a wide variety of products such as essential oils, fiber, and medicine. Hemp was defined in the 2018 Farm Bill as a Cannabis plant with a delta-9 tetrahydrocannabinol concentration of not more than 0.3% on a dry-weight basis. Polyploidy is frequently used in plant breeding to manipulate vigor, reproductive fertility, and biochemistry. By inducing polyploidy/chromosome doubling, we may increase the compounds of interest, principally CBD (cannabidiol), produced by hemp. The purpose of this experiment was to evaluate the efficacy of different treatments of colchicine and oryzalin applied in vivo and in vitro to induce polyploidy in ‘I3’ hemp. After treating vegetative cuttings with colchicine or oryzalin, we had a 31% survival rate. Of the 85 survivors, we recovered two tetraploids: one from the 12-h 0.05% colchicine treatment group and the other from the 12-h 0.2% colchicine treatment group. For the in vitro portion of the experiment, the 12-h 50-μM oryzalin treatment yielded one tetraploid and the 36 h 50 μM oryzalin treatment yielded one cytochimera (mixoploid). The relative efficiency of some treatments showed potential for a simple method to induce tetraploids in clonal hemp for breeding.**

*Cannabis sativa* is an herbaceous plant that originated in Central Asia and has been used by humans for thousands of years due to its industrial (Karche and Singh 2019), ornamental (Hesami et al. 2022a), nutritional (Krüger et al. 2022), medicinal, and recreational (Hesami et al. 2022b) potentials. From regulatory and application perspectives, cannabis plants are categorized based on the level of Δ9-tetrahydrocannabinol (THC), one of the most important phytocannabinoids (Kovalchuk et al. 2020). Plants are generally classified and regulated as industrial hemp if it contains less than 0.3% THC in the dried flower (this level varies by country) or drug-type with more than this threshold (Hesami et al. 2020). *Cannabis sativa* is propagated by seed (Potter 2009), by vegetative stem cuttings (McLeod et al. 2022), or micropropagation (Lata et al. 2017). In 2018, industrial hemp was legalized at the federal level in the United States (US Department of Agriculture 2018). Since then, the industrial hemp industry has been expanding rapidly, as hemp can be used for textile (Vandepitte et al. 2020), food (Shen et al. 2021), paper (Temirel et al. 2021), and more. Conventional breeding processes are effective when attempting to create new cultivars with superior qualities (Hesami et al. 2021). However, these processes are time-consuming, and they require several generations before the cultivar is stable. One strategy breeders use is to double the chromosomes of the plant, which is called inducing polyploidy (Mansouri and Bagheri 2017).

Polyploidy is a condition in which cells that are normally diploid acquire one or more additional sets of chromosomes. Polyploidy can be induced using mitotic spindle inhibitors (Parsons et al. 2019), such as colchicine (Mansouri and Bagheri 2017) or oryzalin (Contreras and Hoskins 2020). Polyploidy is considered a valuable tool for the genetic improvement of crop plants (Crawford et al. 2021) because the results from chromosome doubling often lead to the discovery of desirable traits (Niazian and Nalousi 2020). Some desirable traits that come from polyploidization include increased heterozygosity and hybrid vigor. The extra chromosomes can also serve as a buffer for deleterious mutations (Fox et al. 2020). Furthermore, plants with odd ploidy levels (e.g., triploids) commonly have reduced fertility, which would be a benefit for hemp producers in regions where pollination with high THC pollen is undesirable.

Prior experiments have successfully induced polyploidy in cannabis (Crawford et al. 2021). Parsons et al. (2019) induced polyploidy in cannabis to produce tetraploid plants. As a result, the tetraploid fan leaves were larger, trichome density was increased by 40%, and the cannabidiol (CBD) content increased by 9% (Parsons et al. 2019). In previous cannabis polyploidization experiments, both colchicine and oryzalin (Habibi et al. 2022) have been used to induce polyploidy, but the problem is that there is not a consistent concentration or treatment length used throughout the experiments. As such, there is more work to understand optimal treatment(s) for hemp broadly and for specific cultivars. The goal of this study was to find optimal methods to induce whole genome duplication in ‘I3’ hemp using both in vivo and in vitro methods.

Received for publication 24 Mar 2023. Accepted for publication 13 Jun 2023.

Published online 11 Aug 2023.

We gratefully acknowledge Oregon CBD for providing the funding for this research.

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## Materials and Methods

### Plant material

Plants of the cannabinoid-free cultivar I3 were maintained in a climate-controlled glasshouse with a 24-h photoperiod supplied by 400-W high-pressure sodium lamps (Sun System, Vancouver, WA, USA) with a mean canopy light intensity of 750 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Stock plants were potted at the beginning of October in 18.9-L containers. The containers were filled with a soilless potting mix (Metro-Mix; Sun Gro Horticulture, Agawam, MA, USA) and perlite (Supreme Perlite Co., Portland, OR, USA; 2:1 by volume) and incorporated with 67.5 g of 18N–2.6P–9.1K controlled-release fertilizer (Harrell’s, Lakeland, FL, USA) per 2 $ft^3$ of soilless potting mix (Metro-Mix). The stock plants were fertilized weekly with a water-soluble 20N–8.7P–16.6K general-purpose fertilizer (Jack’s Professional; JR Peters, Allentown, PA, USA) at 100-ppm concentration that was measured by a water-powered, nonelectric chemical injector (Dosatron International, Clearwater, FL, USA).

For the in vivo portion of the experiment, a mixture of tip, subterminal, and basal cuttings were collected at ~1400 HR from stock plants 2 months after potting. All cuttings were ~8 cm and had one or two fully expanded leaves.

At the same time, 40 single-node segments ~0.5 cm in length containing two axillary buds (hemp has opposite leaves) were collected at ~0700 HR as explants for in vitro polyploidization treatments.

### Inducing polyploidy

*In vivo treatments.* After the cuttings were taken, they were immediately moved from the greenhouse to the laboratory to be treated. A total of 270 cuttings were fully submerged in either colchicine (Sigma-Aldrich, St. Louis, MO, USA) or oryzalin (Surflan AS; United Phosphorus, Trenton, NJ, USA) solutions. The colchicine concentrations were 0% (control), 0.02%, 0.05%, 0.1%, 0.2%. The oryzalin (Surflan) concentrations used were 0% (control), 0.002%, 0.005%, 0.01%, 0.02%. One percent dimethyl sulfoxide (DMSO; Sigma-Aldrich) (v/v) was added to all treatments to improve cellular penetration, and all treatments had their pH adjusted to 5.7. Nine cuttings were placed into each 250-mL beaker filled with 200 mL of respective treatment solution and placed on a rotary shaker at 100 rpm for 3, 6, or 12 h.

After cuttings were removed from their treatments, they were rinsed under running

tap water for ~5 s, and then set into 50-cell trays filled with soilless potting mix (Sunshine-Mix; Sun Gro Horticulture) and perlite (Supreme Perlite Co.; 2:1 by volume) and incorporated with 67.5 g of 18N–2.6P–9.1K controlled-release fertilizer (Harrell's, Lakeland, FL, USA) per 2 $ft^3$ of soilless potting mix (Sunshine mix) and rooted under humidity domes according to McLeod et al. (2022). The cuttings were rooted indoors under T5 lights (59 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with 24-h photoperiod. After rooting, the cuttings were transplanted into 2-7/8-in × 5-1/2-in band pots (Anderson Die and Manufacturing Inc., Portland, OR, USA) filled with soilless potting mix (Metro Mix) and perlite (2:1 by volume) and moved into a glasshouse under the conditions described above. Rooted cuttings were grown in the glasshouse for three weeks, until there was sufficient plant material for ploidy analysis. The survival rate of the vegetative cuttings was recorded 3 weeks after the treatments were applied.

*In vitro treatments.* Explant cuttings were surface sterilized for 1 min in 70% ethanol, then soaked in a 0.1% plant preservative mixture (PPM; Plant Cell Technology Inc., Washington, DC, USA) for 30 min. Explants were then surface-disinfected in a solution of 2% sodium hypochlorite (Bi-Mart bleach, Eugene, OR, USA; 5% sodium hypochlorite) and 0.1% Tween (VWR Life Science, Radnor, PA, USA; v/v) for 10 m, during which time they were transferred into a laminar flow hood. Following sterilization, explants were rinsed in sterile water at least three times until no suds remained. Sterilized explants were inoculated on 50 mL of solid, sterile tissue culture medium as described by Halstead et al. (2022), using glucose as the carbon source and Driver–Kuniyuki–Walnut (DKW) medium (D2470, PhytoTech Laboratories, Lenexa, KS, USA) as the nutrient source, in 372-mL culture vessels (C2100, PhytoTech Laboratories). The explants were cultured for 5 weeks in the initial medium. Forty new nodal segments containing axillary buds were subcultured from initial plantlets in culture tubes containing the same media as described earlier.

The 50-µM oryzalin treatment was prepared by dissolving oryzalin in 100% ethanol and DMSO (Sigma-Aldrich) for a final concentration of 2.5% ethanol + 1% DMSO (v/v), pH 5.7. After 1 week, treatments were applied to all 40 tubes of one subcultured plantlet per tube representing an experimental unit. Treatments included a control (DKW medium + 2.5% ethanol and 1% DMSO) and the 50-µM oryzalin treatment. The solution was filter sterilized with a 0.45-µM polytetrafluoroethylene syringe filter before being diluted to the final 50-µM oryzalin concentration and applied to the explants, as described in Contreras and Meneghelli (2016). The treatments were applied by pouring 10 mL of solution into each tube, ensuring that all explants were fully submerged (Fig. 3). Treated explants were placed on a rotary shaker for either 12 or 36 h at 100 rpm. After treatment, the liquid solutions were poured out of the culture tubes, all cuttings were rinsed in a 0.1% PPM solution and then transferred to fresh media (as described earlier) in new culture tubes. Plants were grown in 372-mL culture vessels (C2100, PhytoTech Laboratories) in a growth chamber at 25 ± 2 °C with a 16-h photoperiod at ~21 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Survival was recorded after 1 week based on tissue culture, with green tissue recorded as a surviving plant and brown tissue recorded as a nonsurvived plant. After 8 weeks, plants with sufficient leaf tissue were evaluated using flow cytometry.

### Flow cytometric analysis

Ploidy analysis was conducted according to Schulze and Contreras (2017) with the modification that a Quantum P flow cytometer (Quantum Analysis GmbH, Münster, Germany) was used. All surviving cuttings and explants were evaluated using *Pisum sativum* 'Citrad' (2C = 8.76 pg) as the internal standard for genome size estimation. The leaf tissue from the treated plants was used to run flow cytometry, and two repeats of each plant were screened. If a plant had multiple shoots, a leaf tissue sample from each individual shoot of the plant was screened. If a tested plant showed that both diploid and tetraploid cells were present, it was defined as a mixoploid.

### Data analysis

Data were analyzed using and Microsoft Excel (Version 2209, Build 16.0.15629.20200) by preparing scatter plots and fitting trend lines to the data. Single cuttings and explants in each treatment served as experimental units.

## Results and Discussion

*In vivo.* Of 270 cuttings propagated, 85 cuttings (31%) rooted successfully (Table 1). Of the 85 survivors, 22 of the cuttings showed polyploid peaks on their flow cytometry histograms (26% polyploid induction), including 20 $2x + 4x$ cytochimeras (Fig. 1B) and two tetraploids (Fig. 1A).

Survival ranged from 11% for the 0.1%, 6-h colchicine treatment to 89% for the 0.2%, 3-h colchicine treatments. Schulze and Contreras (2017) experimented with in vivo chromosome doubling on Portuguese Cherry-laurel and observed a range of survival among treatments. Similar to our study, colchicine applied at 0.2% resulted in the highest survival among treatments using seedlings instead of stem cuttings. They also used a different application method in which they treated exposed meristems directly using a semisolid drop vs. our method of submerging the entire stem cutting, which likely had an impact on survival.

Mansouri and Bagheri (2017) performed an in vivo polyploid experiment on cannabis.

Table 1. Response of 'I3' hemp stem cuttings after in vivo exposure to colchicine and Surflan for 3, 6, and 12 at various concentrations by submerging the stem cuttings into liquid treatment mixtures. The data are presented as the number of cuttings in each treatment, the percent survival per treatment, the percent of $2x + 4x$ cytochimeras per number of survivors per treatment, and the percent of tetraploids ($4x$) per number of survivors per treatment. Eight weeks after treatments, survival and ploidy was evaluated to determine number of cytochimeras and tetraploids.

| Treatments | No. of treated cuttings | Survival[i] | $2x + 4x$ cytochimera per survivor | $4x$ per survivor |
|---|---|---|---|---|
| Colchicine 0% 3 h | 9 | 78% | 0% | 0% |
| Colchicine 0.02% 3 h | 9 | 100% | 0% | 0% |
| Colchicine 0.05% 3 h | 9 | 67% | 50% | 0% |
| Colchicine 0.1% 3 h | 9 | 67% | 50% | 0% |
| Colchicine 0.2% 3 h | 9 | 89% | 38% | 0% |
| Surflan 0% 3 h | 9 | 56% | 0% | 0% |
| Surflan 0.002% 3 h | 9 | 11% | 0% | 0% |
| Surflan 0.005% 3 h | 9 | 67% | 0% | 0% |
| Surflan 0.01% 3 h | 9 | 0% | 0% | 0% |
| Surflan 0.02% 3 h | 9 | 0% | 0% | 0% |
| Colchicine 0% 6 h | 9 | 56% | 0% | 0% |
| Colchicine 0.02% 6 h | 9 | 22% | 0% | 0% |
| Colchicine 0.05% 6 h | 9 | 22% | 0% | 0% |
| Colchicine 0.1% 6 h | 9 | 11% | 100% | 0% |
| Colchicine 0.2% 6 h | 9 | 33% | 100% | 0% |
| Surflan 0% 6 h | 9 | 33% | 0% | 0% |
| Surflan 0.002% 6 h | 9 | 22% | 50% | 0% |
| Surflan 0.005% 6 h | 9 | 0% | 0% | 0% |
| Surflan 0.01% 6 h | 9 | 0% | 0% | 0% |
| Surflan 0.02% 6 h | 9 | 0% | 0% | 0% |
| Colchicine 0% 12 h | 9 | 33% | 0% | 0% |
| Colchicine 0.02% 12 h | 9 | 11% | 0% | 0% |
| Colchicine 0.05% 12 h | 9 | 78% | 29% | 14% |
| Colchicine 0.1% 12 h | 9 | 0% | 0% | 0% |
| Colchicine 0.2% 12 h | 9 | 33% | 33% | 33% |
| Surflan 0% 12 h | 9 | 22% | 50% | 0% |
| Surflan 0.002% 12 h | 9 | 33% | 67% | 0% |
| Surflan 0.005% 12 h | 9 | 0% | 0% | 0% |
| Surflan 0.01% 12 h | 9 | 0% | 0% | 0% |
| Surflan 0.02% 12 h | 9 | 0% | 0% | 0% |

[i] Cuttings had sufficient leaf tissue for flow cytometry 8 weeks after propagation.

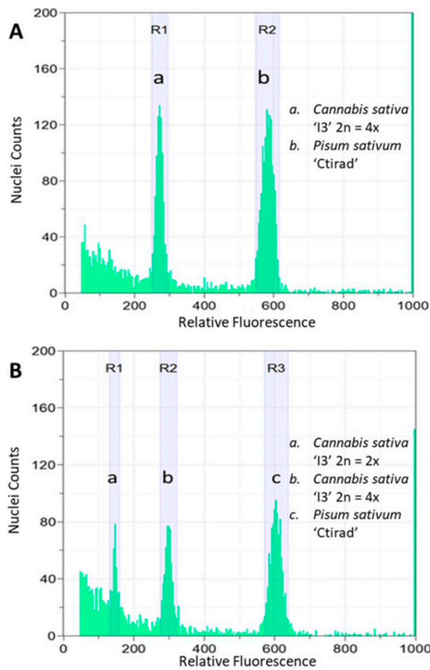


Fig. 1. Flow cytometric output of 'I3' hemp and the resulting induced polyploids after treatments. (**A**) A tetraploid 'I3' hemp histogram; 2n = 4*x* and the *Pisum sativum* 'Ctirad' internal standard. (**B**) A cytochimera 'I3' hemp histogram; 2n = 2*x*, 2n = 4*x*, and the *Pisum sativum* 'Ctirad' internal standard.

They used a method like Schulze and Contreras (2017), where they applied drops of colchicine to the apical meristem of growing seedlings. The results showed that 0.2% of colchicine for 24 h was the most efficient for production of polyploid plants. They used seedlings instead of vegetative stem cuttings, and drops were applied to the apical meristem vs. the whole cutting being submerged in the treatment for our experiment. However, these results show that polyploidy was induced in hemp using a 24 h treatment, which was twice as long as my longest treatment duration. This is evidence that we could have had a different result if we treated our cuttings for a longer period, as the Mansouri and Bagheri (2017) experiment treated their plants for 24 and 48 h instead of 3, 6, and 12 h.

The majority (82%) of polyploid cuttings came from colchicine treatments, and the remaining 18% from Surflan treatments. Both tetraploids were the results of colchicine treatments, one coming from a 12-h, 0.2% treatment and the other from a 12-h, 0.05% treatment. Kurtz et al. (2020) also successfully produced tetraploids using a 12-h, 0.05% colchicine treatment in their experiment; this treatment worked on five cultivars. However, they used pregerminated seeds instead of stem cuttings for their experiment, which could influence the survival and number of tetraploid plants. Kurtz et al. (2020) treated pregerminated seeds of five hemp cultivars with 0.05% colchicine for 12 h. This resulted in emergence percentages ranging from 78% to 100% for the five cultivars, as well as confirmed tetraploid plants for each of the cultivars. Their experiment shows evidence that using pregerminated seedlings may be an efficient alternative to vegetative stem cuttings when inducing polyploidy in hemp.

There was not an obvious correlation between colchicine concentrations applied and stem-cutting survival (Fig. 2A) or percent cytochimeras among survivors (Fig. 2B). We expected survival to decline with increasing concentrations and polyploids to increase. This was the case in the Schulze and Contreras (2017) experiment on *Prunus laurocerasus* and *Prunus lusitanica*. When colchicine was applied to *P. lusitanica* at 0.2%, there was a 98.8% survival rate and two cytochimera were recorded. When the treatment was increased to 0.8% colchicine, survival dropped down to 60.7%, but there were four cytochimera. Similar findings have been observed in other crops such as *Watsonia lepida* (Ascough et al. 2008) and *Lychnis senno* (Chen et al. 2006) where survival decreases but polyploid induction increases as the mitotic spindle inhibitor concentrations are increased. Between treatment lengths, the 3-h treatment group averaged the highest overall survival. For colchicine concentrations, one of 18 polyploids came from the 0.01% concentration treatment (6%), six of 18 came from the 0.05% concentration treatment (33%), three of 18 came from the 0.1% concentration (17%), and eight of 18 polyploids came from the 0.2% concentration group (44%). One tetraploid resulted from the 12-h, 0.2% concentration treatment, and the other from a 12-h, 0.05% concentration treatment (Table 1). The majority of our polyploids came from 0.2% colchicine treatments, which was our highest concentration. Lelakes (1960) performed a cannabis polyploidy experiment and had the most success inducing polyploids using a 3 h 0.5% colchicine treatment. This treatment concentration was more than double our highest treatment concentration and was still successful. Lelakes' data show that higher colchicine concentrations can be used to induce polyploidy in hemp. However, like other experiments referenced, Lelakes used a different method of colchicine application. Lelakes immersed seedlings in colchicine at different stages of growth, whereas our experiment submerged vegetative stem cuttings right after they were cut from stock plants. The differences in these two methods could have affected survival and number of tetraploid plants.

From the Surflan treatments we recovered two mixoploids from the 0.002%, 12-h treatment group; one mixoploid from the 0.002%, 6-h treatment group; and one from the 0%, 12-h control group. In Fig. 2C, there is a negative correlation between the Surflan concentration and the survival of 'I3' stem cuttings, the survival of stem cuttings decreases as the Surflan concentration increases. The same can be observed in relation to the cytochimera per number of survivors (Fig. 2d), where cytochimeras decrease as the concentration increases.

Using 12-h colchicine treatments at 0.05% and 0.2% concentrations, we were able to successfully double the diploid 'I3' hemp genome using in vivo techniques. None of the Surflan treatments were successful in yielding homogeneous tetraploids, but we did recover four mixoploids that have been shown to be useful in breeding if the LII histogenic layer is tetraploid (Olsen et al. 2006); however, we did not identify the ploidy levels of constituent histogenic layers. The 3- and 6-h treatment lengths were successful in producing mixoploids, but no tetraploids were produced from these treatments.

*In vitro.* Thirty-five percent of the explants displayed green tissue 1 week after treatment: 7.5% of survivors came from the 12-h control group; 12.5% came from the 36-h control group; 12.5% came from the 12-h, 50-μM oryzalin group; and 2.5% came from the 36-h, 50-μM oryzalin treatment. The explants treated for the in vitro part of this experiment are shown in Fig. 3. Four of the 14 surviving explants developed enough leaf tissue for flow cytometric analysis after 8 weeks (29%). These included two 12-h control treatments, one 50-μM oryzalin 12-h treatment, and one 50-μM oryzalin 36-h treatment. Of these four, the 12-h, 50-μM oryzalin treatment yielded a tetraploid plant, and the 36-h, 50-μM oryzalin treatment yielded a cytochimera (mixoploid) plant (Table 2). The other two evaluated plants from the control group were diploids.

The data show low survival numbers in the in vitro experiment. This could be due to the subculturing method used in combination with the genotype. Subcultured cuttings were not vigorous and had frail, thin stems. Furthermore, plantlets in the initial culture were not vigorous, producing few leaves and nodes. The low vigor of the subculture cuttings is likely the cause of the poor survival outcomes observed. Subcultures were employed in this case to ensure plant sterility so that posttreatment survival could be evaluated with other factors impacting survival counts as controlled as possible. However, this approach had the opposite of the intended effect, and it is difficult to evaluate whether plant survival was due to a treatment effect, both because of the small sample size and the varying but generally poor vigor of the subcultured cuttings. Further experiments to evaluate the effects of these treatments on subcultured cuttings would do well to use a hemp genotype known to perform vigorously in a tissue culture setting. Otherwise, based on the results of this trial, subculturing may not be a reliable method to produce vigorous cuttings. Although subculturing can provide some control of external factors, it is not necessary for the evaluation of ploidy manipulation treatments in hemp tissue culture, as demonstrated by Parsons et al. (2019), who successfully treated axillary bud explants with oryzalin and eventually grew the cuttings into full plants transitioned back into the greenhouse. In hindsight, we may have been able to achieve the desired

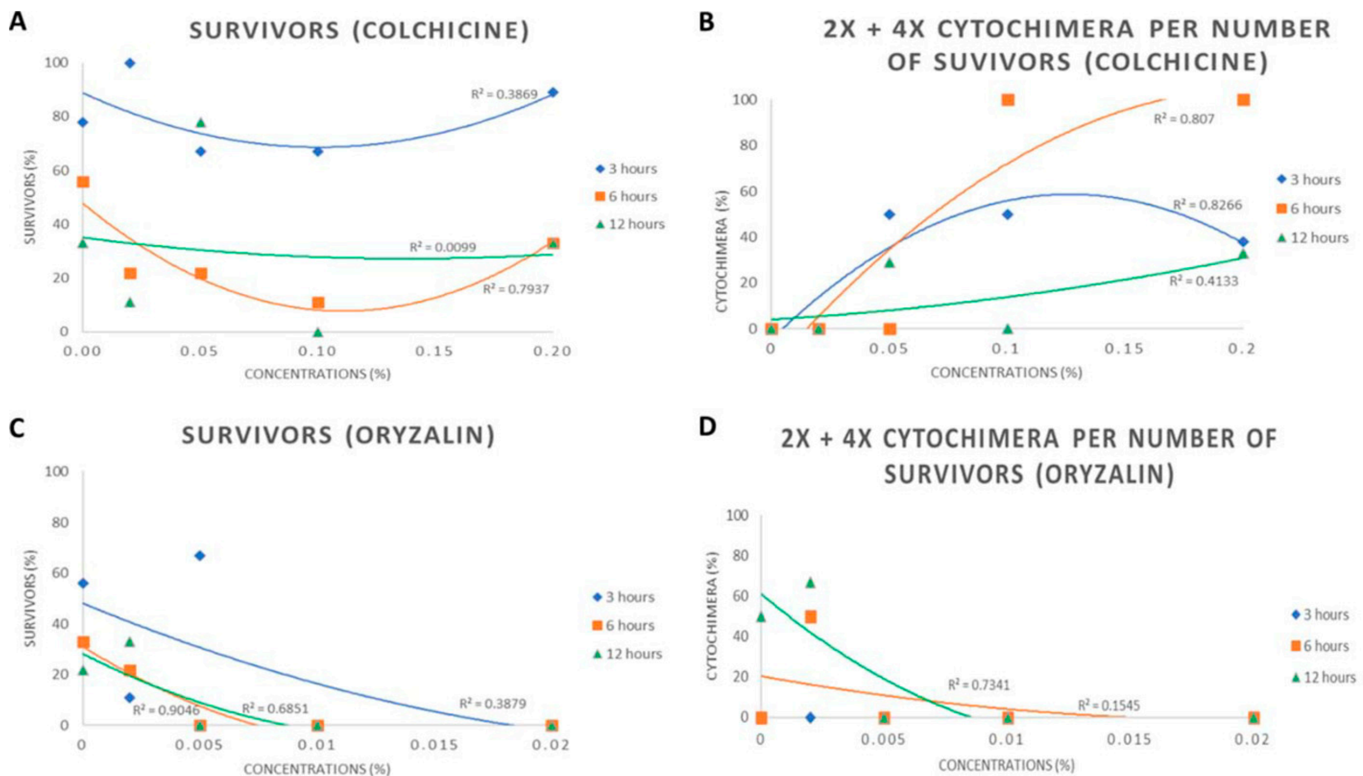


Fig. 2. Response of in vivo 'I3' hemp to exposure of 0%, 0.02%, 0.05%, 0.1%, or 0.2% of colchicine (**A** and **B**) for 3, 6, or 12 h; 0%, 0.002%, 0.005%, 0.01%, or 0.02% of oryzalin (Surflan) (**C** and **D**) for 3, 6, or 12 h. Data are expressed as the percent of stem cuttings that survived or were found to be cytochimeras. (**A**) Survival percentage of stem cuttings exposed to various colchicine concentrations for 3 h ($y = 1995.2x^2 - 401.1x + 88.771$; $R^2 = 0.3869$), 6 h ($y = 412.55x^2 - 113.75x + 35.053$; $R^2 = 0.0099$), or 12 h ($y = 3261.9x^2 - 722.49x + 47.753$; $R^2 = 0.7937$). (**B**) Percentage of surviving stem cuttings that were cytochimeras (2x + 4x) following exposure to various colchicine concentrations for 3 h ($y = -3948.9x^2 + 999.02x - 4.5481$; $R^2 = 0.8266$), 6 h ($y = -2813.9x^2 + 1171x - 16.883$; $R^2 = 0.807$), or 12 h ($y = 359.31x^2 + 62.186x + 3.9968$; $R^2 = 0.4133$). (**C**) Survival percentage of stem cuttings exposed to various Surflan concentrations for 3 h ($y = 65065x^2 - 3817.1x + 48.163$; $R^2 = 0.3879$), 6 h ($y = 212381x^2 - 5765.2x + 31.193$; $R^2 = 0.9046$), or 12 h ($y = 161429x^2 - 4637.1x + 28.236$; $R^2 = 0.6851$). (**D**) Percentage of surviving stem cuttings that were cytochimeras (2x + 4x) following exposure to various Surflan concentrations for 3 h (no cuttings were found to be composed solely of 2x + 4x tissue after 3 h of Surflan exposure), 6 h ($y = 54113x^2 - 2164.5x + 20.292$; $R^2 = 0.1545$), or 12 h ($y = 358225x^2 - 10193x + 60.925$; $R^2 = 0.7341$).

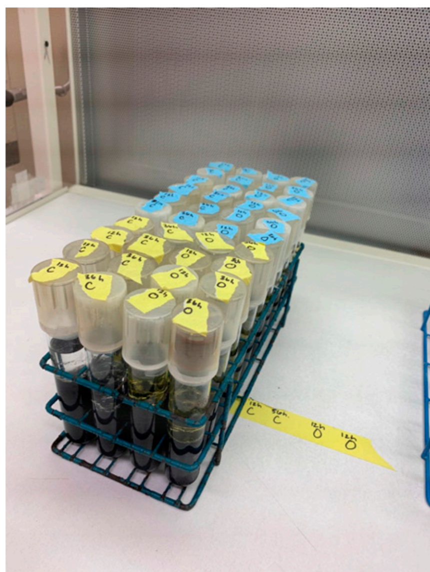

Fig. 3. The 40 explants being treated for the in vitro portion of the experiment, right before they were moved to the rotary shaker. The explants were treated by having 10 mL of treatment solution poured on top of them, which fully submerged all explants. Each culture tube contained one explant and served as an experimental unit.

vigorous growth in our explants if we used the "hedging" technique described by Murphy and Adelberg (2021) in their in vitro cannabis experiment instead of subculturing.

Parsons et al. (2019) performed one of their in vitro polyploid experiment trials using 20-, 40-, and 60-μM as the oryzalin treatment concentrations. They had the highest number of tetraploids result from the 20- and 40-μM treatments. In our in vitro experiment, there was only one treatment concentration (50 μM) besides the control. Their experiment was successful in producing multiple tetraploids at both 20- and 40-μM oryzalin treatment concentrations, so it is possible that we could have had better results if we used a lower oryzalin concentration on our explants.

Most explants did not have enough plant material to sample for flow cytometry; this could be due to the allotted growth period. Parsons et al. (2019) stated that in their experiment, sterile shoots emerged after 1 to 5 months. We only allowed the explants 8 weeks to grow before starting flow cytometry. If the explants were allowed a longer growth period, there may have been more leaf tissue produced that would have allowed us to test more plant.

In conclusion, we were able to double 'I3' hemp chromosomes successfully in both in vivo and in vitro environments by using different mitotic spindle inhibitors at various concentrations and treatment durations. We were limited by time constraints when attempting to evaluate the survival of the in vitro explants but were still able to induce polyploidy within this group. Further optimization is possible to create a more efficient protocol for inducing polyploids, such as evaluating how well our methods work when

Table 2. In vitro treatments and quantity of ploidy altered 'I3' hemp explants.

| Concn (Oryzalin), μM | Time, h | No. treated | Survivors[i] | 2*x* + 4*x* | 4*x* |
|---|---|---|---|---|---|
| 0 | 12 | 10 | 3 | 0 | 0 |
| 0 | 36 | 10 | 5 | 0 | 0 |
| 50 | 12 | 10 | 5 | 0 | 1 |
| 50 | 36 | 10 | 1 | 1 | 0 |

[i] Explants displayed green tissue 1 week after treatment.

dealing with other cultivars, as well as evaluating whether using the "hedging" technique would have resulted in more vigorous growth in the explants which could have raised our survival rate. However, for generating polyploids of individual genotypes to be used in interploidy crossing to generate triploids, our methods may prove useful to other breeders.

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