Identifying new protocol methods in polyploidy research with Cannabis sativa L

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Abstract

The development and critique of an improved experiment and protocol analysis is critical in terms of this research experiment. With little published research in the development of polyploid cannabis, it was crucial that steps were taken to test and report the methods and protocols provided thus far. The development and discovery that *Pisum sativum L.* impeded the ploidy analysis of *Cannabis sativa L*. in flow cytometry when used as an internal standard was recognized. In turn, these findings only reinforced the need to find and test better suited endogenous controls for *Cannabis sativa L.* samples within flow cytometry analysis. The works of producing a more suited protocol for *Cannabis sativa L.* flow cytometry we can better understand the effects of possible secondary metabolites between the plants within flow cytometry as well as help pave the way for future *Cannabis sativa L.* ploidy research.

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Introduction

The adapting agriculture industry has always looked at new ways to better improve the plants we grow today. Methods of plant breeding such as producing polyploid in plants is one such path. The ploidy of the plant influences everything from morphological to biochemical characteristics in the plant, and when utilized, can be a tool to not only increase biodiversity but also develop plants with more desirable traits (Madani et al., 2021). The natural origin of 70-80% of angiosperms such as monocots and dicots, were derived from a polyploid origin, with many still polyploids today (Goldblatt, 1980 as cited in Soltis et al., 2009). However, what are polyploids in plants and what makes them so special?

A polyploid is determined by the presence of more than two complete sets of chromosomes per nucleus or cell (Sattler et al., 2015). Natural polyploids are found in the wild and occur in more than 80% of plant species (Madani et al., 2021). Allopolyploids and Autopolyploids are the two types of polyploid categories. Allopolyploids are produced usually within a hybridization of two separate species with different total homologous pairs. This produces either an unequal chromosome set, or the chromosomes are not homologous among one another, resulting in inhibited meiosis and sterile offspring (Comai, 2005, as cited in Madani et al., 2021). On the other hand, Autopolyploids are created when a cell divides improperly as the chromosomes are doubled but not separated, resulting in all chromosomes derived from the same individual (Madani et al., 2021). The creation of polyploids has been done as far back as the 1930s by Albert Blakeslee and Amos Avery in 1937 (Touchell et al., 2020). Such methods of inducing polyploidy are using mutagens like colchicine. Colchicine is derived from the bulbs of the Autumn crocus (*Colchicum autumnale*) and is commonly used as a gout medication (Touchell et al., 2020). At the point of metaphase in the cell cycle, colchicine creates a colchicine-tubulin

complex by binding to the b-tubulin subunit of the microtubes, disrupting it (Touchell et al., 2020). The resulting autopolyploid cells continue to mitotically divide once the colchicine degrades. Colchicine is usually applied to areas of high cell division such as meristem tissue in diluted solutions. However, to increase the probability of plants developing into homogenous polyploids, many look to somatic embryogenesis or shoot organogenesis regeneration (Touchell et al., 2020). An example, the purple coneflower (*Echinacea purpurea*) only requires a few petioles treated with colchicine to regenerate and develop a brand new polyploid (Nilanthi et al., 2009 as cited in Touchell et al., 2020).

Ploidy manipulation is seen in many of the foods we eat today. Some examples are with seedless fruit from autotetraploid watermelon (Wehner, T.C., 2008 and Compton et al., 1996 as cited in Madani et al., 2021) and even in medicinal oil crops to increase secondary metabolites yields (Crawford et al., 2021). Purple coneflower *(Echinacea Purpurea*) has been studied for its effects on immune modulation, tetraploid produced versions of this medicinal herb have found to yeild more biomass as well as bioactive compounds in comparison to its diploid forms (Xu et al., 2013). Crop breeders have developed polyploids to improve a crops agronomic trait and reduce unwanted attributes such as fertility (Crawford et al., 2021). Polyploids are also used to produce sterile cultivars through the induction of triploidy (Kurtz et al., 2020). However, little research has been done with *Cannabis sativa L.*

Cannabis sativa L. such as hemp or marijuana is a member of the *Canabaceae* family. The plants are dioecious with staminate heterogametic male plants and pistillate homogametic female plants (Moliterni et al., 2004 as cited in Kurtz et al., 2020). Cannabis has been cultivated for over 4500 years for fiber, oil and narcotic usage (Bagheri & Mansouri, 2014) making it a model candidate for polyploidy research. The influences polyploidy could have on biomass and

secondary metabolite yields in the cannabis fiber, oil, and medicinal industry is huge. Thus, there is a need to develop and critique the procedure and protocol methods of polyploid generation in *Cannabis sativa L.*

An initial experiment was conducted based on a previous paper (Bagheri & Mansouri, 2014) to develop polyploid cannabis through different concentrations of colchicine in solution and determine if induced polyploidy played any role in lethality of male hemp plants. However, the results provided from the flow cytometer were unsatisfactory and quite poor. It was later discovered that the *Pisum sativum L.* internal standard used within the protocol for the flow cytometer analysis had secondary metabolites that negatively affected the Cannabis ploidy results. The 110ul sample was composed of the two species in an 80:30 v/v ratio of Cannabis to *Pisum*. Inferring that even at a lower concentration, the secondary metabolites of the *Pisum* still had enough power to mask the Cannabis ploidy results in the flow cytometer. It was hypothesized that the lower the concentration of *Pisum* and higher the concentration of Cannabis in the flow cytometer samples would result in a better Cannabis ploidy result. If this hypothesis is supported, the development of an improved protocol for Cannabis experiments using flow cytometry will better the future in Cannabis polyploid research.

Materials and Methods

Preparation

The experiment was designed using three treatments and a control of colchicine application to young 1-inch plantlets. Each treatment/control contained three replications, with 18 plants per replication and a combined total of 216 plants. *Cannabis sativa* L. hemp seeds were provided by Dr. Tim Sharbel. Seeds were sown in sunshine mix number 4, in a 2.54 x 2.54” x 5.08

centimeters plastic seedling pot. Seeds were left under a humidity dome with 18 hour day length cycle at 24 degree Celsius during the day and 21 degree Celsius during the night using PHILIPS F32T8/TL930 Deluxe soft White grow light. After 5 days the seedlings were all roughly 2.54 centimeters tall. The seedlings were then organized into replication trays of 18 individuals per tray by random block design, with two treatment sets per humidity dome and watering tray.

Procedure

Three treatments of colchicine concentration were prepared before application. Colchicine treatments were prepared from a stock solution; concentrations of 0.2% for treatment 1, 0.5% for treatment 2, and 0.8% for treatment 3. Control plants were prepared with a negative control solution of deionized water. Each solution included a drop of dish soap as a surfactant agent.

The application process was conducted using a micropipette set to 100ul, collecting, and applying the correct treatment solution to the apical meristem of the hemp seedlings. Based on the randomized block layout of the treatment trays, caution was used to maintain no cross contamination of pipette tips to different treatment or control solutions. After application seedlings were given a dark period of 24 hours, and then continued a daylength schedule of 18 hour days at 24 degree Celsius during the day and 21 degree Celsius during the night. Plants were monitored Monday, Wednesday, Friday and watered using deionized water provided by the university tissue culture laboratory.

Flow Cytometry preparation and protocol

The first flow cytometry prep and analysis was conducted 19 days post application of the colchicine treatment solutions and the second prep and analysis was conducted 23 days post application. Flow cytometry samples were developed using Dr. Martin Mau’s protocol on High

Throughput Flow Cytometry on leaf tissue. One square centimeter of leaf tissue was collected from each of the hemp seedlings; some treated seedlings developed a callus-like material at their apical meristem which was used if no leaf tissue was yet produced. If the plant was deceased at the time of tissue collection, the dead leaf tissue was not used. The leaf tissue was put into separate wells of a 0.5ml 96-well plate (Bio-One, Cat#82051-472) and put on ice. Each well was provided a 5mm Zirkonia-Yttra coated bead with 100ul of nuclei extraction buffer (CyStain UV precise P automate nuclei extraction and DNA staining kit, Cat# 05-5002-a, Sysmex) and sealed with foil (Peel Seal Cat# KBS-0601-001, LGC Genomics) and a heat sealer (ALPS 50 V, Cat# AB-1443A, Thermo Fisher Scientific). The well plates samples were then homogenized using a Geno grinder (2010 Geno/Grinder, SPEX SamplePrep, Metuchen, NJ, USA) for 25 seconds at 1600 rpm. These leaf samples were then centrifuged for 30 seconds at 1000rpm. Using a 200ul orifice tip (FisherScientific, Cat.#: 02-707-134), samples were filtered through a 96-well filter plate (AcroPrep Advance 350, 30-40 µm PP/PE, Cat# 8027, PALL Corporation, New York, USA) into a 96-well 300ul elution plate (96-Well Microplates, Greiner Bio-One, Cat#82050-636, VWR, Radnor, PA, USA).

To develop an internal control, 12 Frozen Pea (*Pisum sativum* L.) leaves were used and chopped in a petri dish and razor blade with 2ml of nuclei extraction buffer (CyStain UV precise P automate nuclei extraction buffer; Partec GmbH). Once pea leaf tissue was homogenized, it was supplemented with an extra 4ml of nuclei extraction buffer (CyStain UV precise P automate nuclei extraction buffer) and filtered into a sterile 15ml sample tube using a CellTrics™ filter with 30um nylon mesh.

Using a micropipette, a 80:30 v/v of 110ul mixture of sample cannabis leaf tissue to internal standard pea leaf tissue was created and added to a 96-well 300ul measurement plate

supplemented with 140ul DAPI staining suspension (CyStain UV precise P automate nuclei extraction and DNA staining kit, Cat# 05-5002-a, Sysmex) per well. The samples were then analysed for ploidy and FCSS (Flow Cytometry Seed Screen; see Aliyu et al., 2010) using a flow cytometer (CytoFLEX) with a plate loader (BeckmanCoulter, Indianapolis, IN, USA) and a 405 nm (Violet) laser. The samples were then configured and analyzed using version 2.5 CytExpert Software.

Flow cytometry Investigating procedure

Results of the flow cytometry for many samples were inconclusive, thus a step was taken to better improve this experiment by investigating the protocol itself. Control cannabis leaf tissue was collected and 1 square cm was chopped in a petri dish using a razor blade and tissue was supplemented with 110ul of nuclei extraction buffer and DTT (Dithiothreitol) under the flow hood. The same was done with *Pisum* leaf tissue. Both these samples were collected separately and filtered through a CellTrics™ filter with 30um nylon mesh into a 500ul microfuge tube. Both these samples were applied separately into a 96-well 300ul measurement plate supplemented with 140ul DAPI staining suspension (CyStain UV precise P automate nuclei extraction and DNA staining kit, Cat# 05-5002-a, Sysmex) per well. This process of leaf chopping and filtering was repeated three more times, and instead Cannabis and *Pisum* samples were combined in a 110ul mixture at 80:30, 80:20, and 80:10 ratios respectively, supplemented again with 140 DAPI staining suspension, and run through the flow cytometer (CytoFLEX) with a plate loader (BeckmanCoulter, Indianapolis, IN, USA) and a 405 nm (Violet) laser. The samples were then configured and analyzed using version 2.5 CytExpert Software.

Results

Applied treatments of different colchicine solutions in comparison to the developed mortality rate of Cannabis seedlings was analysed. It was discovered that an increase in % colchicine concentration applied developed an increase in the mortality rate of the Cannabis seedlings.

Looking at Table 1, with the negative control, the application of no colchicine but instead a water mixed with a surfactant, there was a 98.15% survival rate 21 days post treatment and 85.19% survival rate 42 days post treatment, making a decrease in survival for the negative control -1.85% 21 days post treatment and -14.81% 42 days post treatment respectively. A colchicine solution treatment of 0.2% developed a 79.63% survival rate 21 days post treatment and a 51.85% survival rate 42 days post treatment. Moving forward, a 0.5% colchicine treatment developed a 42.59% survival rate 21 days post treatment and a 22.22% survival rate 42 days post treatment. Finally, at 21 days post treatment the 0.8% colchicine treatment had a 31.48% survival rate and 3.70% survival rate 42 days post treatment. The decrease in survivability 21 days post treatment of -20.37% in 0.2% colchicine, -57.41% in 0.5% colchicine, and -68.52% in 0.8% colchicine is interesting. It was examined that 42-day post treatment the decrease in survival of 0.2% colchicine was -48.15%, -77.78% for 0.5% colchicine, and -96.3% for 0.8% colchicine. The downward trajectory of survival probability of the upper limits of each treatment is important to note, as well as how they differ between each other, it can be seen that with 21 day post treatment there is a survival percentage upper limit of 0. 999033 to 0. 456937 shifting from a control (0% colchicine) to treatment 3 (0.8% colchicine). In comparison to 42 days post treatment where there is survival percentage upper limit of 0. 929422 to 0.138398 shifting from a control (0% colchicine) to treatment 3 (0.8% colchicine), with an additional note to the steeper trajectory of survival percentage 42 day post colchicine treatment.

Table 1: % survival of Cannabis plants at time of sample collection for flow cytometry analysis for the specific colchicine treatments

Treatment	% Colchicine solution	Number of samples at time of application	Number of samples at time of 1st flow cytometry analysis	Survival Probability	0.95 Confidence Interval	
					Lower Limit	Upper limit
Control	0	54	53	0.981481	0.888162	0.999033
T1	0.2	54	43	0.796296	0.66076	0.889255
T2	0.5	54	23	0.425926	0.295029	0.567329
T3	0.8	54	17	0.314815	0.199058	0.456937

Treatment	% Colchicine solution	Number of samples at time of application	Number of samples at time of 2nd flow cytometry analysis	Survival Probability	0.95 Confidence Interval	
					Lower Limit	Upper limit
Control	0	54	46	0.851852	0.723268	0.929422
T1	0.2	54	28	0.518519	0.379811	0.654601
T2	0.5	54	12	0.222222	0.124812	0.359464
T3	0.8	54	2	0.037037	0.006441	0.138398

NOTE: % Colchicine solution applied 10/05/2022, 1st Flow cytometry analysis done on 10/26/2022, 2nd Flow cytometry analysis done on 11/16/2022

Statistics were done through the Kaplan-Meier Survival Probability Estimates

The lower and upper limits of the 95% confidence intervals are calculated according to the efficient-score method (corrected for continuity) described by Robert Newcombe (1998), based on the procedure outlined by E. B. Wilson (1927)

Reference (Kaplan & Meier, 1958, Newcombe, 1998, Wilson, 1927) as cited in Lowry, R. (n.d.). Statistical computation web site. VassarStats. Retrieved March 13, 2023, from http://vassarstats.net/

After running the Cannabis leaf tissue through the flow cytometer the first and second time, it was felt something was interfering in developing quality results. At first glance it was thought that samples were not submerged in enough ice or that the time taken for preparation was too significant.

Figure 2 depicts the results found after an examination on what would happen if flow cytometry analysis was done on each Cannabis control and Pisum by themselves, followed by tests to see a decreased amount of the Pisum internal standard with a constant amount Cannabis.

To note, a different staining agent DTT (Dithiothreitol) was used instead of DAPI (CyStain UV precise P automate nuclei extraction and DNA staining kit, Cat# 05-5002-a, Sysmex) to also see if this played additional role in negatively influencing the previous results.

Primarily it was observed, without any mixing of the two plant tissues, the Cannabis control provided a prominent diploid peak (as seen in Figure 1.A). The Pisum sample also produced a developed diploid peak (as seen in Figure 1.B). As represented in Table 2, both these independent sample measured peaks of 13cm for cannabis and 9.8cm for *Pisum*. Secondary observations noted that with the mixed sample of 80:30 v/v Cannabis to *Pisum*, the cannabis peak drops significantly to 1.5cm, while the *Pisum* peak remains relatively unchanged at 8cm (as depicted in Figure 1.C and stated in Table 2). The mixed sample of 80:20 v/v Cannabis to *Pisum*, developed a slightly higher Cannabis peak measurement of 2.7cm as well as a slightly higher *Pisum* peak of 11.4cm (as depicted in Figure 1.D and stated in Table 2). Finally, the mixed sample of 80:10 v/v Cannabis to *Pisum*, developed two relatively equal small peaks. The Cannabis peak measured 1.6cm and the *Pisum* peak measured 3.4cm (as depicted in Figure 1.E and stated in Table 2). In Table 2 it was calculated that the ratio of size in the mixed samples of 80:30 v/v Cannabis to *Pisum* was 1:5.3 centimeters Cannabis to *Pisum*, 80:20 v/v Cannabis to *Pisum* was 1:4.2 centimeters Cannabis to *Pisum*, and 1:2.125 centimeters Cannabis to *Pisum* for 80:10 v/v Cannabis to *Pisum.*

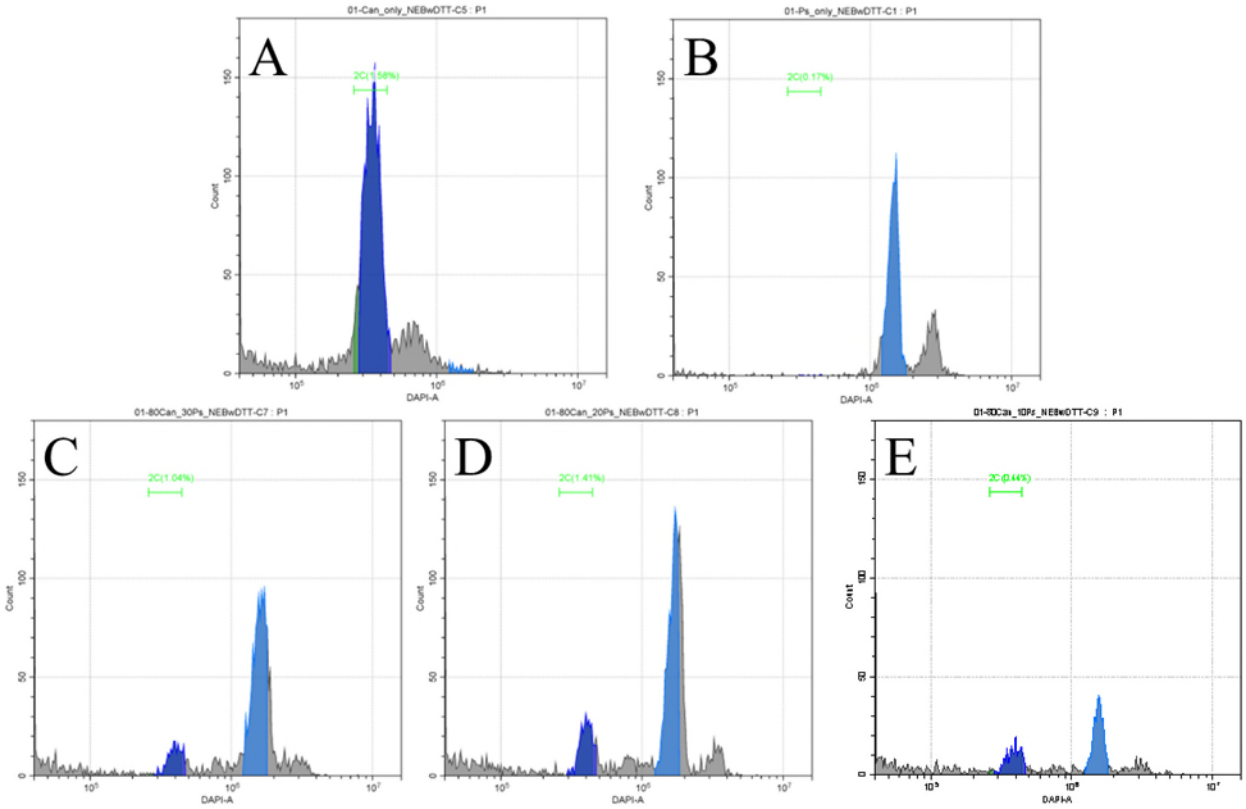

Figure 1: Flow cytometric histograms depicting (A) diploid Cannabis profile, (B) diploid *Pisum* profile, (C) ratio of 80:30 v/v Cannabis to Pisum, (D) ratio of 80:20 v/v Cannabis to Pisum, (E) ratio of 80:10 v/v Cannabis to Pisum. Note: Y-value range (0-180), X-value domain (40000-16000000), all histograms in logarithmic scale

Table 2: Ratio of peak heights of histograms of Cannabis vs *Pisum* depicted in Figure 2 in centimeters.

	Cannabis only	*Pisum* only	80:30 v/v Cannabis:*Pisum*	80:20 v/v Cannabis:*Pisum*	80:10 v/v Cannabis:*Pisum*
Peak Height of Cannabis	13	-	1.5	2.7	1.6
Peak height of *Pisum*	-	9.8	8	11.4	3.4
Ratio Cannabis:*Pisum*	13:1	9.8:1	1:5.3	1:4.2	1:2.125

Note: all histograms were measured at a scale of 15x15.5cm,

P-value of 0.054197167 with a 0.95 confidence interval, no significance

Discussion

It is important to note the difference in decreasing trajectories of % survival of 21 vs 42 days post treatment as described in Table 1, as factors such as time could influence the affects of colchicine post treatment. This could be an aspect to identify in future research with colchicine application on hemp plants and its lasting affects over time.

The development and critique of an improved experiment and protocol analysis is critical in terms of this research experiment. The test to see if a lower concentration of *Pisum* and higher concentration of Cannabis in the flow cytometer samples would support the hypothesis of improving Cannabis flow cytometer results. With a decrease in concentration of *Pisum,* the Cannabis ploidy peaks still were underdeveloped. The best result analysed was taken from the mixed sample of 80:10 v/v Cannabis to *Pisum,* with the Cannabis only reaching roughly ½ the size or 1:2.125 centimeters of the *Pisum* peak with 8 times the amount of Cannabis to *Pisum* in the flow cytometry sample.

With little published research in the development of polyploid cannabis, it was crucial that these steps were taken to test and report the methods and protocols provided thus far. The negative interaction between the samples of Cannabis and Pisum tissue provided in flow cytometry was unfortunate, as it has been used as an internal standard in other flow cytometry experiments prior such as determining genome size for plants such as coconut (*Cocos nucifera* L.) (Freitas Neto et al., 2016). However only reference beads such as (UV bright 3 m, Quantum Analysis GmbH, Münster, Germany) (Crawford et al., 2021). Radish (*Raphanus sativa*) ‘Saxa” (Parsons et al., 2019) were found to be used as an internal standard in hemp. Such issues of *Pisum sativum* affecting the Cannabis flow cytometry results could be due to its secondary metabolites.

Conclusion

Testing both the efficiency and influence of reference beads and other plant species as internal standards on the flow cytometry analysis on hemp should be conducted in future experiments. A controlled genome of test plants utilizing hemp clones could in turn also help sort through and better determine the effects of secondary metabolites on hemp flow cytometry results.

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