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Effect of Induced Polyploidy on Some Biochemical Parameters in *Cannabis sativa* L.

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Abstract This study is aimed at testing the efficiency of colchicine on inducing polyploidy in *Cannabis sativa* L. and investigation of effects of polyploidy induction on some primary and secondary metabolites. Shoot tips were treated with three different concentrations of colchicine (0, 0.1, 0.2 %*w*/*v*) for 24 or 48 h. The biggest proportion of the almost coplanar tetraploids (43.33 %) and mixoploids (13.33 %) was obtained from the 24-h treatment in 0.2 and 0.1 %*w*/*v*, respectively. Colchicine with 0.2 % concentration and 48 h duration was more destructive than 24 h. The ploidy levels were screened with flow cytometry. The biochemical analyses showed that reducing sugars, soluble sugars, total protein, and total flavonoids increased significantly in mixoploid plants compared with tetraploid and diploid plants. Tetraploid plants had a higher amount of total proteins, total flavonoids, and starch in comparison with control plants. The results showed that polyploidization could increase the contents of tetrahydrocannabinol in mixoploid plants only, but tetraploid plants had lower amounts of this substance in comparison with diploids. Also, we found such changes in protein concentration in electrophoresis analysis. In overall, our study suggests that tetraploidization could not be useful to produce tetrahydrocannabinol for commercial use, and in this case, mixoploids are more suitable.

Keywords *Cannabis* · Tetraploidy · Mixoploidy · Cannabinoids

Introduction

Cannabis sativa L. belongs to a major group of cultivated medicinal plants. It is an annual plant that grows up to 5 m tall.

Cannabis has been cultivated for more than 4500 years for different purposes, such as fiber, oil, or narcotics.

The oldest use of cannabis (hemp) is for fiber, and later, the protein-rich achenes ("seeds") were used for culinary purposes. Plants yielding the drug were discovered in India, cultivated

Key Message All of the analyzed biochemical parameters except photosynthetic parameters showed significant difference under polyploidy condition. These changes were not suitable for *Cannabis sativa* as a medicinal plant.

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for medicinal purposes as early as 900 B.C. [1]. Hemp cultivation in Iran continued through the 20th century, predominantly for its medicinal properties for whooping cough, as a hypnotic, and a tranquilizer; the dried seed is taken orally as a diuretic. Several therapeutic effects of cannabinoids have been reported in a review by Williamson and Evans [2].

The class of secondary products, unique to the dioecious species *C. sativa* L., includes the terpenophenolic substances known as cannabinoids, which accumulate mainly in the glandular trichomes of the plant [3]. Over 60 cannabinoids are known [4], the most abundant ones being cannabidiol (CBD), Δ^9-tetrahydrocannabinol (THC), and cannabinol (CBN), which are the criteria for distinguishing between the hemp chemotypes (especially Δ^9-THC and CBD and THC/CBD ratio) [5].

Polyploidization is a powerful tool for improving desirable plant characteristics and is an effective breeding method to induce variation [6–9].

Medicinal plants are of great interest in biotechnology study methods, the productive potential of living cells can be used in industrial processes. There can also be profits from the production of materials in agriculture, forestry, horticulture, and medicine.

The purpose of this project is to provide a method for inducing tetraploidy in cannabis and to analyze the effect of ploidy induction on biochemical properties and increase of the plant's medicinal value.

Material and Methods

Polyploidy Induction

Seeds of diploid cannabis were germinated in petri dishes on filter paper at 25 °C and then were sown directly into the perlite medium and placed in the phytotron (16 h/8 h day/night, 34 ±2 °C) and irrigated with 1/2 Hoagland's nutrient three times per week till the onset of flowering.

A factorial experiment, with a control and two concentrations of colchicine 0.1, 0.2 %*W/V* was conducted with two durations of treatment, 24 h (four times treatment with 6-h intervals) and 48 h (four times treatment with 12-h intervals). We used 30 plantlets per each treatment. A drop of colchicine solution (100 μl) was applied manually using a micropipette to newly emerged shoot tip meristems of 1-week-old seedlings.

Flow Cytometric Analysis

Flow cytometric analysis of nuclear DNA content was performed on isolated nuclei. Ploidy level of plants was analyzed by flow cytometry (Partec PA, Germany) with arc-UV lamp conducted according to the Gu et al. procedure [10]. Fifty days after treatment, pieces with a size of 0.5 cm^2 were obtained from leaves of tetraploid and diploid plants. An amount of 400 ml of nuclear extraction buffer (solution A device kit) was poured on them, and with a sharp blade way to prevent crushing the tissue, leaf sections were torn as well. The resulting solution was passed through the filter apparatus, and 1600 ml of nuclear stain solution DAPI (solution B kit) was added to it after a minute to count devices. At least, 5000 cells per sample volume (typically measured by the peaks) were obtained and were interpreted by software Mode Fit. Before harvest, flow cytometric analyses of samples were repeated to prove ploidy level. After stable and genetically pure polyploids (autotetraploids in this case) were realized, we started to study the impact of ploidy induction on various parameters.

Phytochemical Measurement

The third leaves of 2-month-old seedlings were used for all analyses. Lichtenthaler assay [11] was used to measure the total chlorophyll and carotenoids.

Soluble sugar content of samples were determined with anthrone reagent based on Roe method [12]. Somogy method was used to measure the amount of reducing sugars in leaves and roots [13].

For extraction and measurement of starch, samples were homogenized in hot 80 % ethanol to remove soluble sugars, and starch was extracted by perchloric acid. Hot acidic hydrolysis starch into glucose. The glucose molecules dehydrated and produced hydroxymethyl furfural. This combination creates green color by anthrone reagent. Sample absorbance in dark green to light green was read at 630 nm. Glucose values were obtained using a standard curve of glucose, and starch content values obtained for the factor 0/9 was multiplied [14]. Estimation of cellulose was done with anthrone reagent based on Updegroff method [15]. Wagner [16] and Krizek [17] assays were used for measurement of total anthocyanin and flavonoids, respectively. The total protein of fresh leaves and roots of plants was determined according to Bradford [18].

Assessment of elements in plant tissue was done by ICP-OES. Half a gram of dried sample (or equivalent) and 5 ml of concentrated nitric acid were added to a 50-ml Folin digestion tube. The mixture was heated to 120–130 °C for 14–16 h and is then treated with hydrogen peroxide. After digestion, the samples were diluted to 50 ml with distilled water. This solution was analyzed by ICP-OES. For each sample, the elements of Ca, K, Mg, P, and S were measured.

The third leaves (with almost 7 cm length) of 2-month-old plants and male and female flowers from flowering plants were used for cannabinoid measurement. All of the samples were dried in room temperature in darkness. A sample material (50 mg) was placed in a test tube with 1 ml chloroform. Sonication was applied for 15 min. After filtration, the solvent was evaporated to dryness, and the residue was dissolved in 0.5 ml methanol. Chromatographic separations of cannabinoids were performed as described by Rustichelli et al. [19]. Cannabinoid peaks were identified by cannabinoid standards (THC and CBD).

Statistical Analysis

The experiment was arranged in a completely randomized design with three treatments and 30 replicates. For microscopic examination of diploid and tetraploid plants, five replications were employed and three replicates were used for the measurement of biochemical compounds. SPSS software was employed for statistical analysis, and graphs were plotted by Excel software. Means were compared using Duncan's multiple range tests at $P<0.05$.

Results

Polyploidy Induction

Seedlings of *C. sativa* were allowed to develop to the two true-leaf stages, at which point, a solution of colchicine (0, 0.1, 0.2 %) was applied to the apical growing tip, as described in "Material and Methods". The total surviving seedlings across time reduced with increase in colchicine concentration. Survival rate was 100, 89.96, and 73.33 % in 0, 0.1, and 0.2 % colchicine treatments, respectively. Colchicine with 0.2 % concentration decreased the number

of surviving with the increase of treatment time. All of the elicited treatments caused a polyploidy response as mixoploids or tetraploids in treated plants.

The optimal condition for tetraploid induction was 24 h, 0.2 % colchicine treatment, which resulted in a 73.33 % survival rate and 43.33 % tetraploid induction. The group of plants with two ploidy levels (diploid) and tetraploid in the same tissue (which means poly-polarization has not occurred in all cells of the treated tissues) was produced from 0.1 % colchicine-treated apical meristem samples for 24 h. The highest percentage of mixoploids was 13.33 % (Table 1).

Flow Cytometric Analysis

The fluorescence intensity of diploid and tetraploid nuclei was determined by flow cytometry. The tetraploid plants showed a peak at the position of channel 51.47, whereas the diploid plants showed a peak at channel 25.35. This result demonstrated that the DNA content in tetraploid plant cells was almost two times of that in diploid cells (Fig. 1). The chimera plants showed two peaks at the position of channels 22.67 and 48.91 (Fig. 1).

Phytochemical Measurement

Results of the comparison of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids between tetraploid and diploid plants showed no significant differences. In general, an increase in the ploidy level of cannabis was not associated with an increase in chlorophyll content (Table 2).

Anthocyanin accumulation declined in the leaves of tetraploid and mixoploid plants compared with diploid plants (Table 2). But there were no significant differences between mixoploid and tetraploid plants in this case. The average amount of total flavonoids in mixoploid plants was nearly twice as that in diploid plants. Also, the total flavonoid content increased significantly in tetraploid plants compared to the diploid plants (Table 2). Mixoploid and tetraploid plants showed a significant reduction of 53 and 80% in the cellulose content of stem tissue compared to control plants, respectively. Also, the tetraploid plants have less cellulose than the other two samples (Table 2).

The leaves of mixoploid and tetraploid plants contained higher amounts of starch in comparison with control plants (Table 2). The most content of starch was observed in mixoploid plants.

Table 1 Effects of colchicine concentration and duration of treatment on polyploidy induction in cannabis plants (significant at 0/05 %)

Colchicine concentration (%*w*/*v*)	Duration of treatment (hours)	No. of seedlings treated	Survival rate (%)	No. of tetraploids obtained (%)	No. of mixoploids obtained (%)
0	24	30	100a	0	0
	48	30	100a	0	0
0.1	24	30	89.96b	0	4(13.33)
	48	30	89.96b	0	2(6.66)
0.2	24	30	73.33c	13(43.33)	0
	48	30	63.33d	8(26.62)	0

Means followed by the same letter on the line do not differ by the Duncan test at probability %5

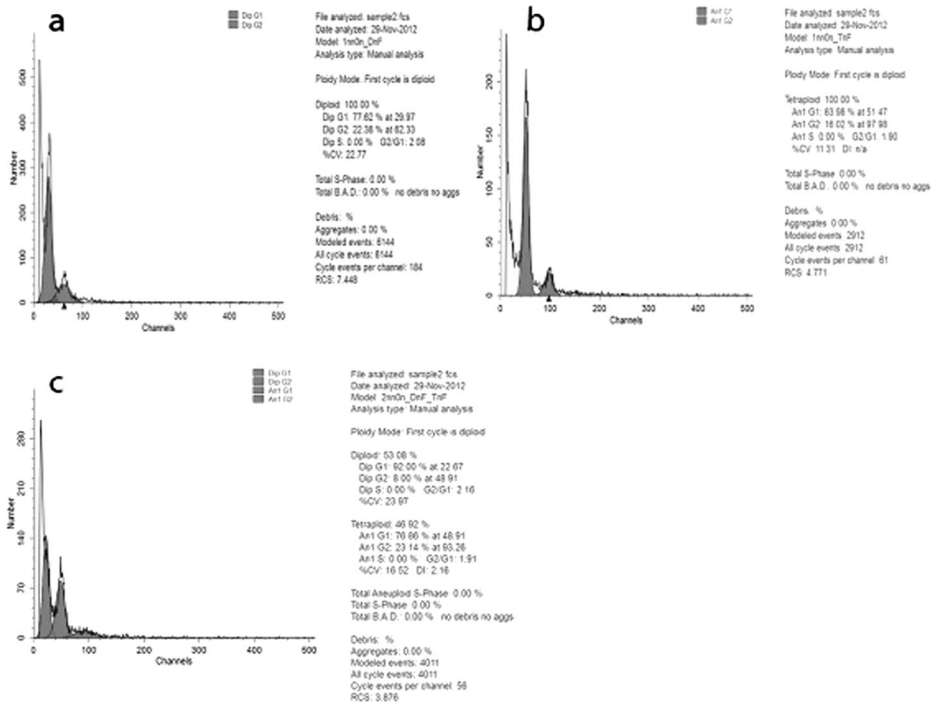

Fig. 1 Flow cytometric analysis of surviving individuals of *Cannabis sativa* L. 50 days after colchicine treatment to apical meristems of seedlings. **a** Diploid plant (control). **b** Induced tetraploid plant. **c** Induced mixoploid plant (i.e., containing diploid and tetraploid cells)

Putative tetraploid plants showed significant decrease in the amount of soluble sugars than diploid plants while the soluble sugar content in leaves of mixoploids showed a significant increase (about twice). The changes in soluble sugars in roots were similar to the leaves so that the amount of sugar in the roots of tetraploid plants showed a 41 % reduction, compared to the diploid plants. The highest soluble sugar content was found in the leaves and roots of mixoploid plants (Table 3).

Table 2 Effects of polyploidy levels on photosynthetic pigments, anthocyanin, flavonoids, and starch in leaves and cellulose in the stem of cannabis plants

	2×	2×+4×	4×
Chlorophyll a (mg g^{-1} FW)	9.841±0.18a	9.8±0.18a	10.203±0.18a
Chlorophyll b (mg g^{-1} FW)	4.799±0.18a	4.6±0.23a	5.196±0.23a
Total chlorophyll (mg g^{-1} FW)	14.64±0.24a	14.40±0.37a	15.1±0.78a
Carotenoids (mg g^{-1} FW)	2.4±0.2a	2.11±0.19a	2.3±0.15a
Anthocyanin (μM g^{-1} FW)	33.5±1.5a	17.31±0.92b	16.34±1.03b
Flavonoids (mM g^{-1} FW)	0.9±0.022c	1.65±0.07a	1.13±0.03b
Cellulose (mg g^{-1} FW)	3.6±0.23a	1.7±0.17b	0.6±0.08c
Starch (mg g^{-1} FW)	31.52±1.11c	67.85±1.65a	50.18±2.4b

Data are means±SE. The different letters in the same row indicated significant difference ($P \leq 0.05$) by Duncan's test

Table 3 Effects of polyploidy levels on soluble sugars, reducing sugars, and protein in the leaves and roots in cannabis plants

	2×		2×+4×		4×	
	Leaf	Root	Leaf	Root	Leaf	Root
Soluble sugar (mg g^{-1} FW)	24.29±0.41b	19.64±0.32c	46.47±0.67a	25.49±0.07b	18.35±0.84d	11.8±0.25e
Reducing sugars (mg g^{-1} FW)	16.74±0.07b	8.49±0.19e	22.84±0.14a	10.15±0.34d	12.74±0.21c	6.98±0.75f
Protein (mg g^{-1} FW)	74.83±3c	34.5±2.5d	237.33±5a	34±2.4d	144.83±4b	34.9±3d

Data are means±SE. The different letters in the same row indicated significant difference ($P \leq 0.05$) by Duncan's test

The induction of tetraploidy in cannabis had a significant effect on the reduction level of reducing sugars in the plant. The amount of these compounds in the leaves and roots of tetraploid plants decreased by 24.14 and 17.78 % than that in the control samples, respectively. On the other hand, significant increase of reducing sugar levels was observed in the leaves and roots of mixoploid plants in comparison with control and tetraploid plants (Table 3).

The total protein content in leaves of mixoploid plants showed the greatest increase in comparison with tetraploid and diploid plants. The amount of protein in leaves of tetraploid plants showed a two-fold increase. The protein content of roots did not show any significant change between different ploidy levels (Table 3).

The results of the measurement of five elements, calcium, potassium, magnesium, phosphorus, and sulfur in the leaves and roots of tetraploid and diploid plants of cannabis are represented in Table 4. Tetraploid plants had higher concentration of calcium in leaves and roots in comparison to diploid plants. Unlike diploid plants, the amount of calcium did not show a significant difference between the root and shoot in tetraploid plants. The amount of potassium showed a significant increase in the roots of tetraploid plants in comparison to diploids, but it was similar in leaves of tetraploid and diploid plants. Polyploidy was ineffective on magnesium concentration and its distribution between leaves and roots. Phosphorus concentration was higher in the leaves of the tetraploid plants than in the same organ of diploid plants. The root of tetraploid plants had a lower concentration of phosphorus in comparison to diploid plants. Polyploidy caused a decrease in sulfur content of leaves and an increase in the amount of this ion in roots in comparison with the same organs in diploid plants.

Table 4 Effect of ploidy levels on element concentrations in the leaves and roots of cannabis plants. The data are based on grams per 100 g dry weight

Ploidy level		Ca	K	Mg	P	S
Diploid	Leaf	0.22±0.018b	1.7±0.32a	0.31±0.037a	0.27±0.037bc	0.41±0.076b
	Root	0.12±0.051c	0.6±0.14c	0.20±0.024b	0.31±0.042b	0.30±0.061c
Tetraploid	Leaf	0.43±0.014a	1.5±0.13a	0.31±0.045a	0.57±0.037a	0.20±0.023d
	Root	0.4±0.011a	1.1±0.072b	0.19±0.057b	0.21±0.011c	0.73±0.039a

Data are means±SE. The different letters in the same row indicated significant difference ($P \leq 0.05$) by Duncan's test

The amount of THC increased only in mixoploid vegetative plants (Table 5). CBD content increased in mixoploid and tetraploid leaves in vegetative stage plants. The most increase was observed in mixoploid plants (Table 5). THC content showed a significant decrease in the male and female flowers of tetraploid plants in productive stage (Table 5). The leaves of male tetraploid plants had lower content of THC, but the ones of female plants showed no difference (Table 5). CBD levels in the male and female flowers of tetraploid plants did not show significant differences compared with diploid plants (Table 5). In reproductive leaves of female tetraploid plants, CBD content significantly increased compared with the reproductive leaves of diploid plants. CBD was similar in the leaves of male tetraploid and diploid plants (Table 5).

The results of a qualitative study of proteins in leaves and roots of tetraploid, diploid, and mixoploid plants suggest that a new band with a molecular weight of 68 kDa is added in the leaves and roots of a tetraploid plant sample. Rubisco (ribulose-1, 5-biphosphate carboxylase/oxygenase) is a major soluble protein in leaves. The large subunit of the native enzyme with a molecular weight of 55 kDa [20] was the major component of the leaf proteins and was present with quantitative variation in all samples. However, a band with a molecular weight of 175 kDa was highlighted in polyploid leaf and tetraploid root samples compared to the diploid ones (Fig. 2).

Discussion

The results showed that chlorophyll content does not change with polyploidy induction in cannabis plants. Although, the result of a study conducted by Xu et al. [21] found discrepancies, and they reported that with the increase in the ploidy level of *Juncus effusus*, the amount of chlorophyll a and chlorophyll b significantly decreased. However, more research on diploid and tetraploid plants of *Urginea indica* showed that chlorophyll does not change [22]. Therefore, it can be concluded that the amount of chlorophyll can also be influenced by studied species. It is observed that despite no changes in photosynthetic pigments and soluble and reducing sugar content were lowered in tetraploid plants.

The results of electrophoresis showed a decrease in rubisco enzyme in tetraploid plants. It can be a reason for reduction of sugar content in plants. Also, in our anatomical study of cross-sections of leaves, we found that polyploid cannabis contained a lower air space in the leaf tissue (unpublished) that it can cause tolerance increase in CO_2 diffusion, resulting in reduction of CO_2 fixation and carbohydrate content. Jaskani et al. [23] reported that the total sugar

Table 5 Effect of ploidy levels on THC and CBD content in the leaves of vegetative cannabis plants and in the leaves and flowers of productive plants

	2×		4×		2×+4×	
	THC	CBD	THC	CBD	THC	CBD
Vegetative leaf (mg g^{-1} DW)	5.329±0.5e	16±1c	5.08±0.69e	19±0.9b	10.573±0.2d	25.8±1.5a
Male leaf (mg g^{-1} DW)	4.212±0.4c	21.6±2.5a	1.801±0.3d	25±3a	–	–
Female leaf (mg g^{-1} DW)	0.725±0.1c	15.4±1.5b	0.662±0.09c	69±3a	–	–
Male flower (mg g^{-1} DW)	0.662±0.1b	14.2±1.2a	0.396±0.05b	15±0.9a	–	–
Female flower (mg g^{-1} DW)	1.303±0.1b	15.3±1.5a	0.86±0.12c	16.5±1a	–	–

Data are means±SE. The different letters in the same row indicated significant difference ($P \leq 0.05$) by Duncan's test and *t* test

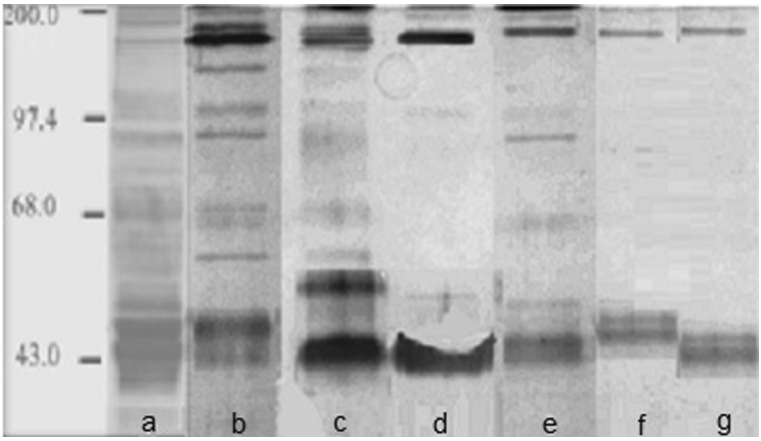

Fig. 2 Electrophoretic leaf and root protein profiles of induced diploid plant of cannabis *sativa* L. marker (*a*). Tetraploid leaves (*b*). Diploid leaves (*c*). Mixoploid leaves (*d*). Tetraploid roots (*e*). Diploid roots (*f*). Mixoploid roots (*g*)

content was comparable in diploid and tetraploid fruits of watermelon line. Unlike our results, Grange et al. [24] observed higher total sugar content in triploid than in diploid fruit of watermelon. Increasing the amount of starch can also be a reason to reduce the amount of sugar in the tetraploid cannabis plant. It may be because of the created disorders in triosephosphate translocation from chloroplast to cytosol under these circumstances.

Many studies have shown that flavonoid accumulation provides protection against mechanism environmental factors and are involved in many aspects of plant growth, including pathogen resistance, pigment production, and protection against ultraviolet radiation, which contributes to the growth of pollen and seed coat development [25]. Anthocyanins are flavonoid pigments responsible for the red to purple colors in fruits and flowers [26]. They belong to the group of polyphenols with a flavonoid skeleton of $C_3C_6C_3$. Anthocyanins have been accepted as medicines in many countries [27]. This material may be nutritional value of foods by preventing oxidation of lipids and proteins. Thus, investigation on differentiations of flavonoids, anthocyanins, and other secondary metabolites in created polyploidy plants is both ecologically and physiologically important.

According to the results, polyploidy has different effects of flavonoids and anthocyanins. Polyploidy increased the amount of flavonoids but decreased the amount of anthocyanins. How polyploidy affects the biosynthesis of flavonoids and anthocyanins, more research is needed in this case. We did not find any report on flavonoids and anthocyanin changes in other polyploid plants.

So far, slight studies have been done on the effects of polyploidy on the uptake and transport of main elements. Elements such as nitrogen and phosphorus, which are used to build nucleic acids, may directly be involved in a larger genome [28]. The result of measuring elements indicated elevated levels of calcium in both leaves and the roots of tetraploid plants that show increased uptake of this major element. Potassium is an essential element in maintaining plant cell turgor pressure. Increase of potassium in roots of tetraploid plants could be due to stimulation of potassium uptake. Phosphorus content decreased in roots while increased in the leaves. The reason for which that polyploidy has an impact on increasing the transfer of this element from the roots to the shoot because polyploid shoots need more phosphorus for nucleic acid synthesis. Transfer of P from the roots to the shoots increased in

diploid plants. Conversely, phosphorus and sulfur content in leaves decreased. This represents the effect of polyploidy on the ion transport from root to shoot. Polyploidy induction leads to a reduction of 53 % in the cellulose content. Cell surface to volume ratio shows decreases in polyploid plants than in diploid plants, due to the reduced number of cells and the increased volume of cells. Thus, a decrease in cellulose content was acceptable. Tetraploids have greatly reduced cellulose content in secondary cell walls. This decrease in cellulose is correlated with alterations in the physical properties of the wall.

The main psychoactive component of cannabis is THC and CBD. Overall, cannabis contains a high proportion of THC/CBD use for medicinal purposes. The unheated forms such as THCA-A (THC-acid-A) and CBDA have fewer side effects [29]. The active ingredients are not always increased in upper ploidy levels and, in some cases, even reduced active ingredients may be due to the suppression of some genes in diploid plants in effect of polyploidy. This has metabolic metabolism regulating the biosynthesis of active ingredients that they can cause problems. In this study, the ratio of THC/CBD in tetraploids was reduced compared with diploids. However, THC levels in mixoploid plants showed an increase when compared with diploid plants. The morphological study revealed that the number of trichomes in tetraploid plants was reduced (unpublished) that could be the reason for the decline on cannabinoids in this plant. Similarly, with increasing ploidy level of the *Mentha spicata* essential oils declined. Also, in the purple foxglove, glycosidic material is partially reduced [30]. Given the strong and consistent elevation in the amount of leaf starch, flavonoids and anthocyanins, reducing and soluble sugars, proteins, flowers and leaf THC in mixoploid is achieved as a result. A closer examination of the metabolic pathway in mixoploid plants to identify the most valuable medicinal and economic plants should be placed in a breeding program.

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