Original Research Article

The effect of colchicine on profiles and contents of withanolides of Ciplukan (*Physalis angulata*) in vitro

Abstract

Colchicine is widely used for artificial polyploidy induction as a strategy to improve secondary metabolites in quantity and quality. This study aims to evaluate the effect of in vitro polyploid induction of *Physalis angulata*, by soaking seeds and cotyledonary nodes in vitro with 0.1% colchicine solution for 24, 48 and 72 hours on withanolides content. The soaking seeds were germinated on MS0 medium, then the cotyledonary nodes were used as shoot induction explants. In another experimental set, cotyledonary nodes were taken from two weeks old in vitro sprouts, soaked in colchicine, and then cultured on shoot induction medium. Cotyledonary nodes from sprouts without colchicine treatment were used as controls. The withanolide content on in vitro regenerated shoots were analyzed two weeks after subculture by HPLC methods. The results showed that a total of 38 withanolides compounds were detected in the retention time 21,6 to 36,8 minutes. Soaking the seeds in colchicine solution was more effective in increasing the withanolide content than the cotyledonary node tissues. However, the exposure time during soaking also affected the amount of withanolide compounds synthesized.

Key words: colchicine, HPLC, in vitro, *Physalis angulata*, withanolide

INTRODUCTION

Physalis spp. is an annual herb belonging to the Solanaceae family. This plant is often found growing wild as a weed in moist and fertile land. Members of the Physalis genus can live in the lowlands to plains with an altitude of about 1,500 m above sea level. In Indonesia, ciplukan plants which are members of the Physalis genus are widely found in several areas such as Java (Sundanese: cecenet or cecendet; Seram: lapinonat), Madura (nyurnyuran or yor-yoran), Bali (Angket, Kepok-kepokan, Keceplokan), Minahasa (Leletokan) and Lombok (Sasak: Dedes).

Ciplukan (*P. angulata*) in addition to producing sweet fruit is also known as a medicinal plant. The medicinal properties are mainly due to the presence of compounds in the withanolide group. Withanolides are a group of polyoxygenated steroids based on the ergostan skeleton and primarily in the aglycone form. Withanolides are mainly synthesized by member of Solanaceae family, including Physalis. There are many withanolides have been isolated from Physalis (Huang et al., 2020).

Efforts to improve the physiological and biochemical characters of medicinal plants lead to an increase in the quantity and quality of secondary metabolites with medicinal properties.

Improvement of this character can be done in in vitro system with artificial polyploidy induction. Polyploidy is a condition in which a cell or organism has more than one pair of chromosomes (diploid). An increase in the number of chromosomes and additional genomic interactions and genetic changes often results in polyploid plants that have superior characters compared to their diploid plants. This makes polyploidization a credible approach for crop improvement (Corneillie et al., 2019).

Medicinal plants with duplicates of the entire chromosome set often have more distinctive characteristics such as a modified phytochemical profile and a higher content of the desired pharmaceutical molecule. Therefore, the induction of chromosome doubling in medicinal plants can have significant economic consequences (Niazian & Nalousi, 2020). Polyploidy induction has been reported to be beneficial for increasing the medicinal value of various medicinal plants, including the tetraploid plant *Sophora tonkinensis* (Wei et al., 2018), *Trollius chinensis* Bunge (Zhang et al., 2016), and *Bletilla striata* (Thunb.) Reichb.f. (Li et al., 2018).

In vitro regeneration systems provide facilities for manipulating ploidy in breeding and developing new plants (Touchell et al., 2020). In vitro ploidy manipulation is usually induced using antimitotic agents such as colchicine, oryzalin and trifluralin. The success of polyploidy induction is influenced not only by the duration of exposure and the concentration of antimitotic agents, but also by the type of tissue, and the interaction with basal media and plant growth regulators. In vitro conditions that are specific to each individual at the taxa, genera, species, or cultivar level, often have specific needs to maximize polyploid induction. Therefore, this study aimed to determine the effect of colchicine treatment under in vitro conditions on the content of withanolides in in vitro regenerated *P. angulata* shoots.

METHODS

Explant preparation

Seeds were obtained from the ripe fruit of the Physalis plant which grows wild in the corn fields of the South Malang area. After the seeds were dried, they were then sterilized in a 20% commercial bleach solution for 15 minutes and rinsed with sterile distilled water for 5 minutes three times. The sterilized seeds were ready to be germinated and used for colchicine treatment.

Colchicine application in in vitro systems

Colchicine application was carried out on two different types of tissue, namely 1) seeds that will germinate to produce seedling as explant sources and 2) cotyledon node explants derived from in vitro seedling that were ready to be cultured on shoot induction medium.

Soaking the seeds in a colchicine solution. Seeds that have been surface sterilized were soaked in 0.1% colchicine solution for 24, 48 and 72 hours. Seeds without soaking in colchicine solution were used as control. In vitro seed germination was undertaken in a water medium solidified with agar without adding nutrients and plant growth regulators (PGR). Each treatment in colchicine was repeated five times (culture bottles). Cotyledon nodes from two weeks old seedling of control and colchicine treatment were subcultured into shoot induction medium (MS + BAP 2 mg/L + IAA 0.05 mg/L). Two weeks later the regenerated shoots clumps were separated and subcultured into the same medium for shoot multiplication. The multiplicated shoots were ready to be analyzed for withanolides profile and content using the HPLC method.

Soaking cotyledon node explants in colchicine solution. The process of seed germination to produce seedling was the same as described in the colchicine treatment of seeds. Sprouted Cotyledonary nodes were excised from 2 weeks old seedling and then soaked in 0.1% colchicine. The exposure time and number of repetitions in colchicine treatment were like seeds treatment. Subsequently, shoot induction and identification of withanolide compounds were carried out the same as in the seed soaking treatment.

Analysis of withanolide content by HPLC method

Analysis of withanolide profiles begins with phytochemical screening and identification of withanolide compounds using LC-MS (Mastuti et al., 2021).

Preparation of in vitro shoot. Shoot cultures were prepared for HPLC analysis consisted of seven types of samples, namely control shoots, shoots derived from soaking seeds for 24, 48 and 72 hours and shoots derived from soaking the cotyledon nodes for 24, 48 and 72 hours. In vitro shoots of 0.1 g were ground with mortar and pestle, then 10 mL of methanol p.a was added, stirred until homogeneous and allowed to stand for 30 minutes. The solution was sonicated with a sonicator (Laboratory Ultrasonic Homogenizer sino sonics) for 45 minutes at room temperature, filtered using a vacuum filter (Pyrex vacuum filter) and then concentrated using a rotary evaporator. The concentrated extract was redissolved with 4 mL of methanol, then homogenized with a sonicator for 45 minutes at room temperature. The solution was concentrated using a rotary evaporator. The concentrated extract was redissolved with 10 mL of methanol, then filtered to obtain the sample solution filtrate.

Measuring withanolide levels by HPLC. HPLC analysis refers to (Cao et al., 2015) with modifications and carried out with the SPD M20-Photo Diode Array Detector. Samples were analyzed at 35°C in Shim-pack VP ODS 5 Column DSm, 150 x 4.6 mm. The mobile phase using the isocratic method uses acetonitrile and water in a ratio of 60:40. The sample solution filtrate and the mobile phase (eluent) were filtered with a Polytetrafluoroethylene (PTFE) membrane and degassing was carried out. The sample solution was filtered again with a cellulose nitrate membrane. The standard compound withanolid used is the standard library based on the results of the analysis by LC-MS (Shimadzu). The analysis was carried out at a wavelength of 215 nm with a flow rate of 1 mL/min for 50 minutes. The withanolid content was calculated based on the standard curve obtained from the internal standard through the LC-MS/HPLC (Shimadzu) program using Labsolution software. The results of running HPLC for each sample were in the form of a chromatogram, retention time (minutes) for each peak, and the names of the compounds withanolids for each peak and the levels of withanolids per fresh weight (FW).

Data analysis

The levels of withanolide compounds were tabulated based on the type of tissue (seeds and cotyledonary nodes) and the exposure time. The percentage of withanolide compounds based on their concentration and the average increase or decrease in withanolide levels was calculated by MS Excel 365. Changes in the levels of withanolide compounds in shoots in vitro without and with colchicine treatment were calculated by the following formula:

$$Xn_i = \left(\frac{bn_i - a_i}{a_i}\right) \times 100\%$$

Notes: a= levels of withanolide in in vitro shoots control (without immersion in 0.1% colchicine solution); b = concentration of withanolide in in vitro shoots soaked in 0.1% colchicine, n = exposure time 24, 48 and 72 hours, X = change in concentration of withanolide compounds, i = type of withanolide compound 1, 2, …, n.

RESULTS AND DISCUSSION

Withanolide intensity in in vitro *P. angulata* shoot derived from seeds treated with colchicine

All seeds soaked in 0.1% colchicine at various eaxposure times were able to regenerate shoots. Chromatographic analysis using the HPLC method identified 38 peaks of withanolide compounds on shoot derived from seeds which were soaked or not soaked in 0.1% colchicine (Figure 1). The retention time to elicit withanolide compounds was from 21,6 to 36,8 minutes. Shoots derived from seeds soaked in 0.1% colchicine at all duration times showed higher intensity of absorbances (milli Absorbance-unit) (mAU) rather than control. The seeds that are not soaked in colchicine regenerated shoots which had the lowest intensity of absorbance (Figure 1A). Meanwhile, seeds soaked in colchicine for 24 hours (Figure 1B) and 48 hours (Figure 1C) produced shoots that had higher intensity of absorbance. However, the intensity of absorbance decreased when the soaking seeds was prolonged up to 72 hours (Figure 1D). Shoots derived from the seeds after soaking in 0.1% colchicine for 48 h produced the highest intensity of absorbance compared to the control and other exposure time.

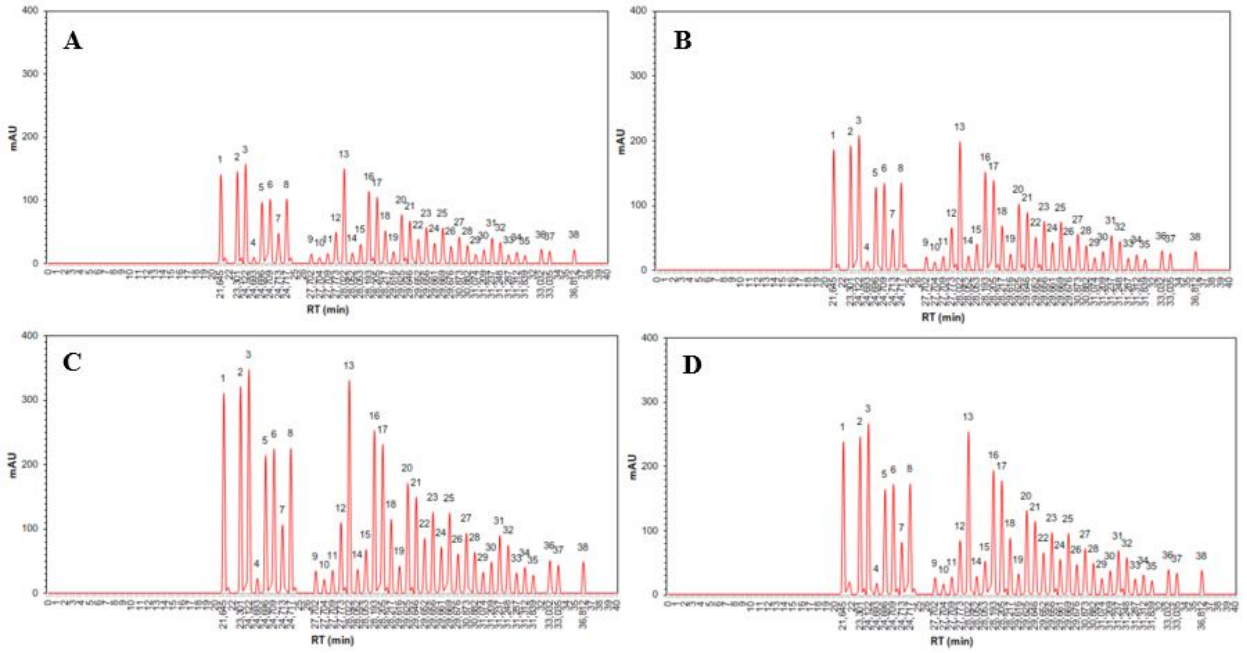


Figure 1. Chromatograms profile of withanolide compounds on Physalis in vitro shoots derived from soaking the seeds in 0.1% colchicine solution using the HPLC method. A. without soaking (control), B. soaking for 24 h, C. for 48 h, D. for 72 h.

Withanolide intensity in in vitro *P. angulata* shoot derived from cotyledonary nodes treated with colchicine

Cotyledonary node explants that immersed in 0.1% colchicine also showed capability of shoot regeneration. HPLC analysis was identified the similar number and types of withanolides with the same retention time. However, they showed a different trend of intensity of absorbance (mAU). Shoots derived from soaking cotyledonary nodes for 24 hours (Figure 2B) produced the highest intensity of absorbance of withanolides compounds compared to control (Figure 2A) and soaking the cotyledonary nodes for 48 h (Figure 2C) and 72 h (Figure 2D). In addition, the cotyledonary nodes soaked for 72 hours even had lower intensity of absorbance (mAU) of withanolides compounds than the control without soaking in colchicine.

These results indicated that immersion of seeds and cotyledon nodes in 0.1% colchicine for several exposures had no effect on the profile of the synthesized whitanolides. The number and type of withanolides detected in control and treatment shoots did not differ. However, the different of exposure time affected the intensity of the absorbance of the withanolides compounds.

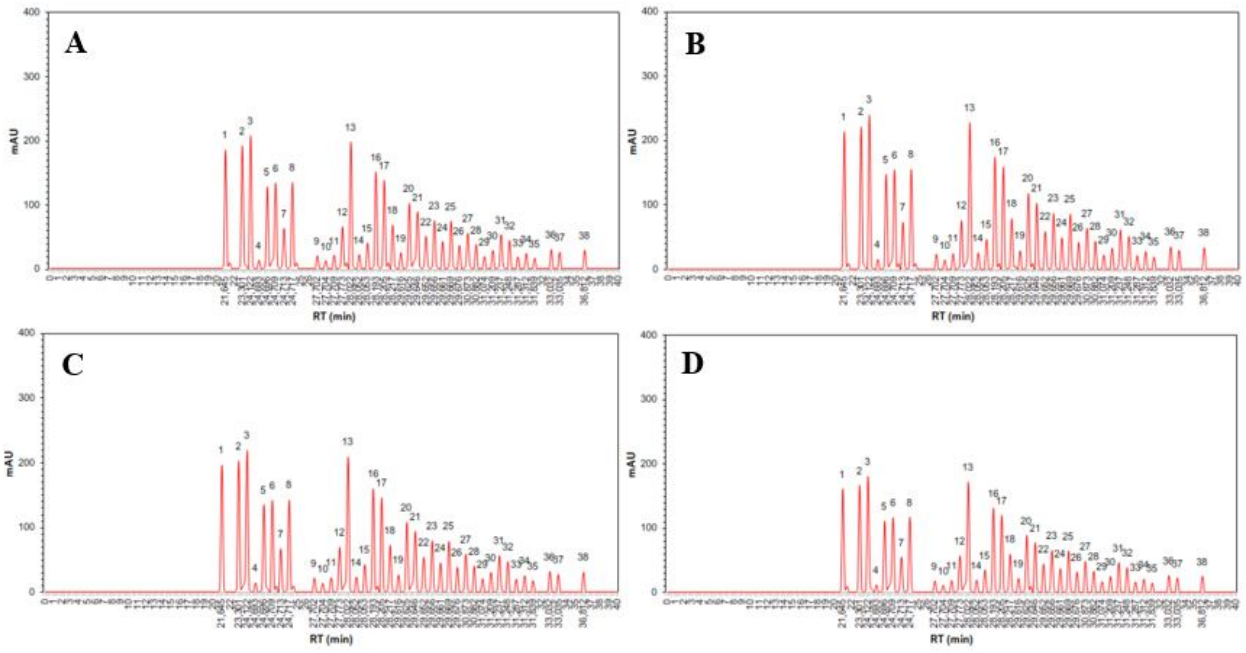


Figure 2. Chromatograms profile of withanolide compounds on Physalis in vitro shoots derived from soaking cotyledonary node explants in 0.1% colchicine solution using the HPLC method. A. without soaking (control), B. soaking for 24 h, C. for 48 h, D. for 72 h.

Colchicine effect on withanolides profile of in vitro shoot of *P. angulata*

All types of 38 peaks compounds isolated from *P. angulata* shoots in vitro can be classified into 12 groups (Table 1). The diversity of withanolides showed that physalin and withangulatin compounds have the most types, as many as 9. Meanwhile, the only one type of withanolides were phyasnolid, withaferin, withanone and withaphysalin.

Table 1. Diversity of withanolide compounds isolated from *P. angulata* shoots in vitro

No	Group	Number of types	Type
1	hidroksiwithanolide	3	18-hidroksiwithanolid D 28-hidroksiwithanolid 4β-hidroksiwithanolid E
2	dihidrowithanolide	2	2,3-dihidrowithanolid E** dihidrowithanolid E
3	phyperunolide	2	C, D
4	physagulin	4	D, L, M, N
5	physalin	9	A, B, D, E, F, G, H, I, J
6	Physanolid	1	A
7	withaferin	1	A
8	withangulatin	9	A, B, C, D, E, F, G, H, I
9	withanolide	4	A, B, D, E
10	Withanone	1	Withanone
11	withaphysalin	1	A*
12	withaphysanolide	1	A
TOTAL		38	

The contents of withanolide compounds in the shoots of *P. angulata* in vitro without colchicine treatment ranged from 6.64 - 242.90 ug/g which the lowest and highest concentration was 2,3 dihydrowithanolide E and withaphysalin A, respectively (Table 1). Soaking seeds and cotyledonary nodes in colchicine generally regenerated shoots in vitro with increasing withanolide content. However, the pattern of increasing in the two of them is different. Soaking the seeds for 24 hours increased the production of withanolides the least. Soaking for 48 hours can produce compounds withanolides about five times more. However, the addition of exposure time up to 72 hours increased withanolides compounds only three times more than soaking for 24 hours. This shows that soaking seeds in 0.1% colchicine solution for 48 hours is the optimal time to produce the highest levels of withanolides.

On the other hand, the highest increasing in the production of withanolides occurred when the cotyledonary nodes were soaked for 24 hours. The longer the soaking, the less withanolide levels increased. Even at 72 hours of soaking, the levels of withanolide were less than the control, which was indicated by a negative value. This indicates that soaking the cotyledonary node for a long-time result in impaired withanolide synthesis.

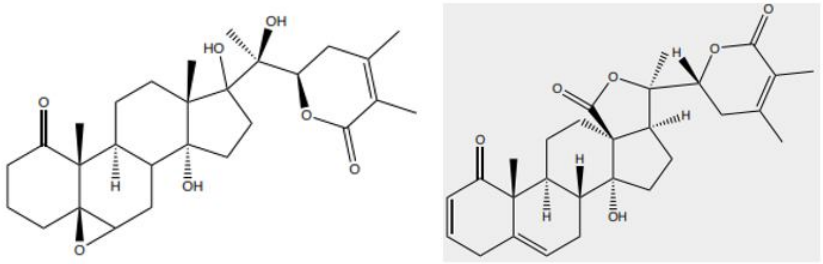
Table 2. Effect of colchicine treatment on change of withanolides content

Peak No	Retention time (min)	Whitanolide compounds	Control (ug/g DW)	Increasing of Withanolide content in *P. angulata* shoots in vitro (µg/g DW)					
				Soaking **seeds** in 0.1% colchicine solution (hours)[1)]			Soaking **cotyledonary node** explants in 0.1% colchicine solution (hours)[2)]		
				24	48	72	24	48	72
1	21.645	withaphysanolide A	216.45	16.15	91.42	47.53	36.00	21.93	-2.84
2	23.301	withanolide B	223.40	16.12	91.25	47.45	35.94	21.89	-2.84
3	24.122	withaphysalin A*	242.90	16.10	91.07	47.36	35.87	21.85	-2.84

Peak No	Retention time (min)	Whitanolide compounds	Control (ug/g DW)	Increasing of Withanolide content in *P. angulata* shoots in vitro (µg/g DW)					
				Soaking **seeds** in 0.1% colchicine solution (hours)[1)]			Soaking **cotyledonary node** explants in 0.1% colchicine solution (hours)[2)]		
				24	48	72	24	48	72
4	24.693	withanone	7.89	31.56	188.45	97.75	71.10	44.40	-4.27
5	24.696	withanolide A	145.67	16.45	93.28	48.50	36.67	22.36	-2.87
6	24.709	withaferin A	154.14	16.36	92.72	48.21	36.47	22.23	-2.86
7	24.713	withanolide D	68.39	17.44	99.52	51.73	38.93	23.81	-2.96
8	24.717	withangulatin F	154.97	16.38	92.88	48.29	36.53	22.27	-2.86
9	27.702	18-hydroxywithanolide D	15.98	24.10	141.47	73.44	54.10	33.52	-3.58
10	27.704	2.3-dihydrowithanolide E**	6.64	35.71	214.58	111.27	80.55	50.45	-4.66
11	27.709	withanolide E	17.08	23.23	136.00	70.60	52.12	32.25	-3.50
12	27.773	dihydrowithanolide E	71.00	17.40	99.27	51.60	38.84	23.75	-2.96
13	28.022	physanolide A	231.83	16.13	91.26	47.46	35.94	21.90	-2.84
14	28.052	28-hydroxywithanolide	17.68	23.32	136.53	70.88	52.31	32.38	-3.51
15	28.053	4β-hydroxywithanolide E	40.04	18.82	108.24	56.24	42.08	25.83	-3.09
16	28.193	physalin B	174.73	16.33	92.52	48.11	36.40	22.19	-2.86
17	28.205	withangulatin B	158.57	16.39	92.89	48.30	36.53	22.27	-2.86
18	28.217	withangulatin E	74.83	17.29	98.61	51.26	38.60	23.60	-2.95
19	29.616	phyperunolide D	21.41	21.78	126.84	65.87	48.81	30.13	-3.36
20	29.625	physalin A	115.78	16.67	94.66	49.22	37.17	22.68	-2.89
21	29.646	withangulatin I	99.14	16.88	96.02	49.92	37.66	23.00	-2.91
22	29.652	physalin F	52.93	18.08	103.54	53.81	40.38	24.74	-3.02
23	29.656	physalin G	82.18	17.25	98.31	51.10	38.49	23.53	-2.94
24	29.661	physalin J	42.80	18.75	107.80	56.01	41.92	25.72	-3.08
25	29.669	withangulatin A	81.13	17.17	97.82	50.85	38.31	23.41	-2.93
26	29.676	physagulin M	35.52	19.33	111.46	57.91	43.25	26.57	-3.14
27	30.873	withangulatin C	58.79	17.79	101.76	52.89	39.74	24.33	-2.99
28	30.882	withangulatin H	37.52	19.02	109.47	56.88	42.53	26.11	-3.11
29	31.074	withangulatin G	14.55	24.80	145.86	75.71	55.69	34.54	-3.64
30	31.209	phyperunolide C	26.17	20.73	120.25	62.45	46.43	28.61	-3.27
31	31.237	physalin I	56.51	17.86	102.16	53.10	39.88	24.42	-3.00
32	31.248	physagulin N	44.92	18.61	106.87	55.53	41.59	25.51	-3.07
33	31.287	physalin D	14.04	24.80	145.90	75.73	55.71	34.55	-3.64
34	31.312	physalin E	20.40	22.14	129.12	67.04	49.63	30.66	-3.40
35	31.839	withangulatin D	11.27	27.53	163.06	84.61	61.91	38.52	-3.90
36	33.032	physagulin L	28.06	20.06	116.01	60.26	44.89	27.63	-3.20
37	33.035	physalin H	22.48	21.47	124.88	64.85	48.10	29.68	-3.33
38	36.812	physagulin D	26.57	20.60	119.45	62.04	46.14	28.42	-3.25

Notes: *withanolides compounds with the highest content; ** withanolide compounds with the lowest content

The results show that colchicine treatment in different tissues affected the levels of withanolide content. Colchicine treatment in seed tissue was able to provide higher changes in withanolide levels than cotyledonary node tissue (Table 2). Among the 38 withanolides detected by HPLC 2.3-dihydrowithanolide E was synthesized in the highest amount, while withaphysalin A (Figure 3) was synthesized in the least amount. This condition consistently occurred in both control and treatment shoots.



2.3-dihydrowithanolide E Withaphysalin A

Figure 3. The highest and the lowest withanolide compounds

In this study, seed germination and shoot regeneration were not inhibited by colchicine treatment. According to (Manzoor et al., 2019), colchicine treatment of seeds is the most effective method to produce tetraploids. Colchicine treatment of seeds had little effect on germination (Kobayashi et al., 2008). Smaller seeds absorb more colchicine solution in a shorter time (surface area to volume ratio). However, colchicine treatment of 0.05 – 0.1% on soybean seeds reduced the germination rate (Mangena, 2020). According to (Sattler et al., 2016), plant tissues or organs have different sensitivity to antimitotic agents. The difference in the effect of colchicine treatment on different types of explants was because the survival rate of the explants was influenced by the permeability of the explant tissue and the ability to transport antimitotic agents (Allum et al., 2007).

Variations in soaking time also affected the content of withanolide compounds synthesized in shoots from soaking seeds and cotyledonary nodes. In vitro shoots from seeds that were soaked for 48 hours produced the highest levels of withanolides, which ranged from 16.31 – 250.16 ug/g DW. The addition of soaking time up to 72 h decreased the withanolide content (9.45 – 143.98 ug/g DW) although it was still higher than the withanolide content in shoots from cotyledonary nodes that were soaked for 24 h (4.43 – 68.5 ug/g DW).

Ploidy manipulation using chemical compounds has been widely carried out in vitro. The use of colchicine to induce polyploidy can increase the potential for secondary metabolite production. It has been widely used to produce important compounds in medicinal plants (Bernard et al., 2012). It was also explained that the induction of polyploidy can affect the physiological and biochemical processes of plants and affect the biosynthetic pathways of primary and secondary metabolites. This change is due to a reduction in the ratio of membrane to the amount of chromatin which leads to an increase in the contact between the chromatin material and the nuclear membrane and an increase in gene activity for each cell. The volume and proliferation rate of tetraploid versus diploid cells were 2 and 1.5, respectively. This is very beneficial in cellular production processes associated with cell surface metabolic activities.

Modification of ploidy is a strategy to increase metabolite production in plants because it can change the quantitative and qualitative patterns of secondary metabolite production (Madani et al., 2021). Medicinal plants undergoing chromosomal duplication often have a more distinctive phytochemical profile with a higher content of the desired pharmaceutical molecule. Therefore, the induction of medicinal plant chromosome doubling can have significant economic consequences (Niazian & Nalousi, 2020).

The increase in the range of colchicine concentrations in the initial seed treatment was not always accompanied by an increase in the secondary compounds produced. This trend was also

observed in anthocyanin content and glycyrrhizic acid in callus tissue produced from Licorice seeds (Bernard et al., 2012). According to (Lavania, 2005) this is due to the effect of chromosomal doubling is not always identical for all genes but depends on the genotype and species although it affects genomic activity. This is because colchicine not only affects cell division but spreads through cells, disrupting cellular mechanisms and causing toxicity at high concentrations. Colchicine has an impact on the viscosity of the cytoplasm so that cells cannot function normally (Suliman & Asander, 2019).

Conclusion

In vitro colchicine treatment was proven to increase the production of *P. angulata* withanolides. However, the production of withanolides is also influenced by the type of tissue and the length of immersion in the colchicine solution. The results of this study indicate that the in vitro system has the potential to be used to support the availability of herbal medicinal ingredients.

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