

Effect of Colchicine on In Vitro Growth and Ploidicity of Crown Vetiver Plant (*Vetiveria zizanioides* L. Nash)

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Abstract

The goal of this research was to evaluate the effect of colchicine on the formation and growth of shoot from explant crowns by in vitro, as well as to obtain polyploidy vetiver plant (*Vetiveriazizanioides* L. Nash). Induction of polyploidy vetiver plants carried out by culturing explant crowns on MS media supplemented by 2 mg.L⁻¹ BAP and colchicine (0, 30, 60, 90, 120 mg.L⁻¹) for three weeks. Explant and formed shoot regenerated on MS media containing 1 mg.L⁻¹ NAA. The generated plantlets acclimatized on the growing media (cocopeat: husk charcoal: compost = 1: 1: 2). Plant ploidy level of the plants regenerated from treated explant estimated by chromosome counting in root tips. The effect of colchicine on media was able to induce polyploidy in vetiver plants. Five mixoploids were obtained from explant treated colchicine. The vetiver mixoploid plants obtained were 20% and 62.5% from colchicine 60 mg.L⁻¹ and 90 mg.L⁻¹, respectively. The vetiver mixoploid plants consist of diploid (2n=2x=20) and triploid cells (2n=3x=30) or diploid (2n=2x=20) and tetraploid (2n=4x=40). The addition of colchicine in concentrations above 30 mg.L⁻¹ caused inhibition of shoot formation and growth, even a concentration of 120 mg.L⁻¹ caused explant death. However, 30 mg.L⁻¹ colchicine could increase the number of formed shoots, while only 60 mg.L⁻¹ and 90 mg.L⁻¹ could be induced the mixoploidy in the vetiver plant. The results showed that colchicine treatment could increase ploidity in vetiver plants in vitro, but caused inhibition of shoot formation and growth.

Keywords : Chromosome, colchicine, in vitro, polyploid, *Vetiveria zizanioides* L. Nash.

INTRODUCTION

Vetiver (*Vetiveria zizanioides* L. Nash) is a plant belonging to the Graminae group, which often used to produce vetiver oil essential oil. Vetiver oil is used as an ingredient in perfume and soap industry [1]. The world demands of vetiver oil reach 250-300 tons per year. However, Indonesia can only produce 75-200 tons per year from 2014-2017 [2,3].

Polyploid plants have several advantages, including wider leaves, larger stems and stomata diameters, and larger oil glands, which yield more oil production [4] as well as more bioactive compounds [5]. Polyploid plants were also used in the utilization of plant germplasm for high secondary metabolites production [6]. Polyploid *Echinacea purpurea* L. has higher caffeic acid derivatives and alkaloids than its diploid [7].

In vitro polyploid induction can be done by adding colchicine to the media [8,9]. Colchicine binds to tubulin protein to inhibit its polymerization and no spindle thread to form, which plays a critical role in cell division. Chromatids fail to reach the poles and continue into the interphase phase with double the

number of chromosomes [10]. Polyploid induction with colchicine has been successfully carried out in *Pogostemon cablin* [4], vetiver grass [11], and ruzigrass [12]. This low production is caused by the limited availability of superior seeds, so there is a need to improve seedlings' quality and quantity. Therefore tissue culture techniques are widely used to produce superior plants, one of which is the induction of polyploid plants [6].

In vitro induction of polyploid vetiver with colchicine was carried out to determine the effect of colchicine on the growth and formation of shoots and to obtain polyploidy vetiver. This study is important to get superior seedling of vetiver plants with high essential oil content and in large quantities.

RESEARCH METHOD

Plant Material

Vetiver plants were obtained from Sengklek, Pamalayan Village, Bayongbong District, Garut, West Java. The shoots were harvested and surface sterilized with 96% alcohol for 1 minute and 50% bleach (5.25% NaClO) for 20 minutes and rinsed with sterile distilled water for 5 minutes twice. Explant crowns were isolated and cultured on MS media supplemented with 2 mg.L⁻¹ BAP. Formed shoots were multiplied on MS media supplemented with 3 mg.L⁻¹ BAP [13]. In vitro shoots were used as the initial explants.

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Induction of Polyploid with Colchicine *In Vitro*

Explant crown from *in vitro* shoots was isolated and cultured on MS media supplemented with 2mg.L⁻¹ BAP and different concentrations of colchicines (0, 30, 60, 90, and 120 mg.L⁻¹). Each treatment performed with eight replicates. Cultures were incubated at 24 ± 1°C for three weeks in light (600 lux). Percentage of survival explants, the percentage of explants that formed shoots, and the number of shoots formed were observed.

Treated explant and formed shoots were transferred to MS media supplemented with 3 mg.L⁻¹ BAP without colchicine. Cultures incubated at a temperature of 24-25°C, in the light (600 lux) for three weeks.

Shoots that developed after three weeks of culture transferred on MS media with 1 mg.L⁻¹ NAA for rooting. The cultures incubated at a temperature of 24-25°C, in the light (600 lux).

Plantlets were transferred into planting media (cocopeat: husk charcoal: compost = 1:1:2). Four weeks-old vetiver plants analyzed for its ploidy level. The ploidy level of plants was identified by chromosomes counting of the roots' tip.

Preparation of Chromosome and Estimation of Ploidy Level

Root-tips of the regenerated plant were pretreated in a 0.002 M hydroxyquinoline solution for 3 hours and fixed in ethanol-glacial acetic acid solution (3:1) at room temperature for an hour. The fixed root-tips were hydrolyzed in 1 N HCl at 65°C for 3 minutes. The root-tips then soaked for three days in a 2% acetoorcein dye solution. The dyed root-tips was placed on the glass slide and covered with a glass cover and then squashed.

Chromosomes were observed with an Olympus CX31 microscope with 1000x magnification. Ploidy level of the vetiver plants was estimated by chromosomal counting in root-tips. If 2n=20, then it is considered as diploid plantlets, whereas if the chromosomes are 3n=30 or doubles to 4n=40 or even more, then it is considered as polyploid.

Data Analysis

The research design used was a randomized block design with replications as a group. Data were analyzed by one way ANOVA (Analysis of Variance). If there is a significant difference, then it followed by the Duncan test at a confidence interval of 95% (α = 0.05).

RESULT AND DISCUSSION

Effects of Colchicine on Explant Growth and Shoot Formation

Crown explants cultured on MS media without colchicine formed green shoots (Fig. 1A). Meanwhile, addition of colchicine in concentration above 30 mg.L⁻¹ inhibited shoot formation and growth (Fig. 1C, 1D, 1E).

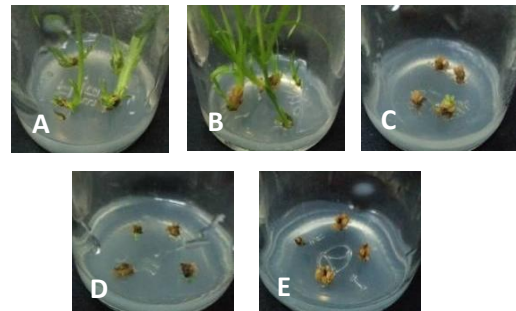


Figure 1. Effects of colchicine addition on the growth of explant crown for three weeks culture. (A). control (without colchicine), (B). 30 mg.L⁻¹, (C). 60 mg.L⁻¹, (D). 90 mg.L⁻¹, (E). 120 mg.L⁻¹

Colchicine's addition to the media affected the percentage of explants' survival, percentage of shoot formation, and the number of shoots. The higher concentration of colchicine on media caused the percentage of explant survival, percentage of shoot formation, and the number of shoot per explant was lower.

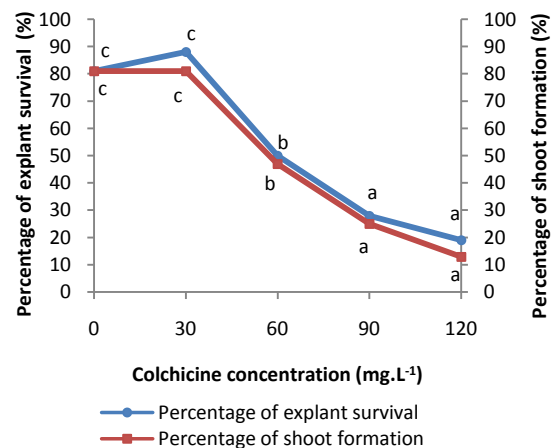


Figure 2. Effect of colchicine on percentage of explant survival and percentage of shoot formation (the same letter in the parameter showed no significance difference in Duncan Test α = 0.05).

In the media without colchicine, the percentage of explants survival and shoots formation was 81%. The addition of 60 mg.L⁻¹ colchicine on media decreased the percentage of explants' survival and shoots formation to 50%

and 47%, respectively. Meanwhile, the percentages of explant survival and shoot formation with the addition of 120 mg.L⁻¹ colchicine were only 19% and 13%. The highest percentage of explant survival (88%), as well as the highest percentage of shoots formation (81%), was recorded on MS media contains 30 mg.L⁻¹ colchicine (Fig. 2).

The decrease in survival percentage of explants was caused by the toxic effects of colchicine. Colchicine, a highly poisonous alkaloid, originally extracted from *Colchicum autumnale*, is used in medicine, especially for the treatment of gout. Colchicine is a mutagenic chemical compound that can inhibit mitotic activity due to the binding of colchicine to tubulin and the nuclear spindle activity, thereby causing cell death [14]. Concentration above 0.1% of colchicine significantly decreased the percentage of survival explant and shoot regeneration in *Humulus lupulus* [15] and *Paulownia tomentosa* [16]. A high concentration of colchicine (above 0.4%) in *Pyrus communis* L. triggered cell death due to its toxic effect [17].

Total shoots and number of shoots per explant on the control media were 14 and 4 respectively on media. The addition of 60 mg.L⁻¹ and 120 mg.L⁻¹ colchicine on culture media reduced the number of shoot up to 50-85% (Fig. 3). The highest total shoot (17) and the number of shoot per explant (4) was observed on MS media contains 30 mg.L⁻¹ colchicine.

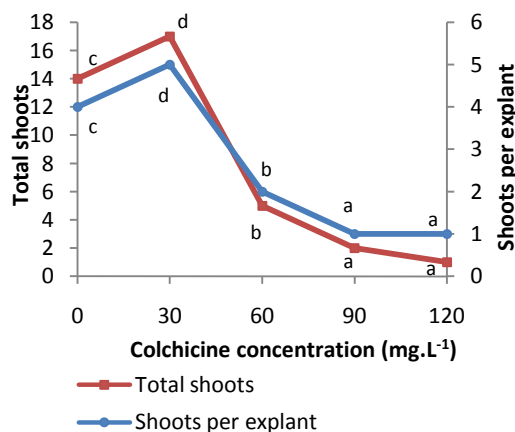


Figure 3. Effect of colchicine addition on media on the total shoots and number of shoots per explant (the same letter in the parameter showed no significance difference in Duncan Test $\alpha = 0.05$).

The reduction of shoot formation in the treatment with colchicine has a negative side effect, due to the use of antimitotic agents for inducing polyploidy in plants. Extreme genetic

redundancy and somatic instability after chromosome doubling affect cell proliferation, regeneration, and shoot elongation. The previous result also showed that the regeneration rate in *Rhododendron* was highly lethal and have aberrations like reduced growth rate. In addition to doubling meristematic cells, high concentration could reduce the overall vigor of the plant [18]. In Marigold, explants were reduced significantly from 87.83% on 0.001% colchicine to 27.26% on 0.05% colchicines. Colchicine inhibited the formation of spindle fibers, resulted in polyploid cells. Colchicine, as an antimitotic agent, binds to plant cell tubulin dimmers, causing depolymerization of microtubules, thus disrupting the cell cycle [19].

Plantlet Regeneration and Acclimatization

The shoots were able to form plantlets after colchicine treatment on MS media with 1 mg.L⁻¹ NAA addition (Fig. 4A). The regenerated plantlets were dark green and large (Fig. 4B). Plantlet that successfully regenerated on media without colchicine was 23 plantlets. Addition of 30, 60, and 90 mg.L⁻¹ colchicine on media result in 20, 5, and 19 plantlets respectively (Table 1); no plantlet obtained at 120 mg.L⁻¹.

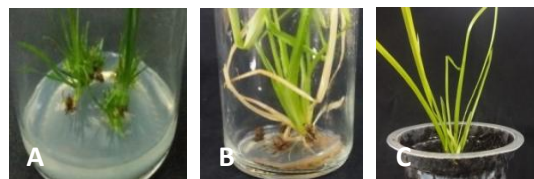


Figure 4. Plantlet regeneration and acclimatization from explant grown on media containing colchicines. (A). Shoot, (B). Plantlet, (C). Plant

Table 1. Effects of colchicine at different concentration on plantlet regeneration and acclimatization

Colchicine (mg.L ⁻¹)	Number of plantlets	Number of plants	Acclimatization success (%)
0	23	20	87
30	20	18	90
60	5	5	100
90	19	18	95
120	-	-	-

Four weeks after transferring the plantlet to the greenhouse, the plant of vetiver has a rather large, green leaves (Fig. 4C). The acclimatization success rate of the plantlet was 87% -100%. Plant obtained from control media were 20 plants. Whereas, the number of plants obtained from colchicine treatment at 30, 60, and 90 mg.L⁻¹ were 18, 5, and 18 plants, respectively (Table 1).

Shoot survival in *Paphiopedilum villosum* decreased as the concentration of colchicine

increased [20]. Furthermore, there was no significant difference between diploid and higher ploidy for the length or width of leaves in *Hebe* sp. [21]. The acclimatization success rate of plantlet *Artemisia annua* reached up to 65.89%. Mixoploid clones (84%) and tetraploid clones (82.67%) from colchicine treatment showed a higher success rate than diploid clones (61.68%) [22]. The acclimatization of *Eriobotrya japonica* tetraploid plantlet showed slow growth and the resulted leaves were wider than diploid plants [23].

Ploidy Level Identification

Ploidy level of colchicine induced plants was determined by chromosomes counting in root-tips. Control media produced diploid plants (Fig. 5A). The addition of colchicine on media was able to induce mixoploidy, which is characterized by two or more types of ploidy in one individual plant. There was diploid ($2n=2x=20$), triploid ($2n=3x=30$) or diploid ($2n=2x=20$), tetraploid ($2n=4x=40$) (Fig. 5B).

The addition of colchicine to the culture medium induces cell polyploidy through chromosome doubling. Polyploidy is the condition of having more than two sets of chromosomes. Polyploidy can be achieved by chromosome doubling leads to changes in gene dosage that causes chromosomal rearrangements. Unreduced gametes transmit genetic diversity considered as effective alternatives for somatic chromosome doubling [24].

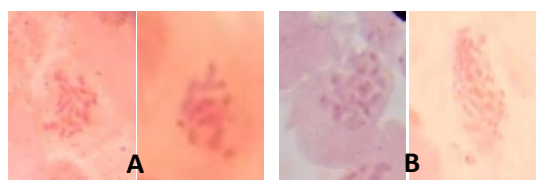


Fig 5. Vetiver cell chromosomes as a result of colchicine treatment by in vitro. (A) Diploid, (B) Mixoploid.

Addition of colchicine on media induced polyploidy of regenerated plantlet. Plant regenerated from explant treated colchicine of 30 mg.L^{-1} showed diploid level. Five mixoploids obtained from explant treated colchicine. One mixoploid (20%) obtained from the 60 mg.L^{-1} colchicine, and five mixoploids (62.5%) obtained by 90 mg.L^{-1} colchicine (Table 2).

Mixoploid is a condition in which the tissue composed of cells with different ploidy levels. Mixoploid shows that it was not all cells in the tissue exposed to colchicine compound changes in the amount chromosome. Mixoploidy is often associated with the occurrence of polyploidy,

hybridization, chemical, and in the same case, it is genetically controlled [25].

Table 2. Effect of colchicine at different concentration on polyploid induction in *Vetiveria zizanioides*

Colchicine (mg.L ⁻¹)	Number of plant	Number of plants with observed roots	Plants ploidy (%)	
			Diploid	Mixoploid
0	20	5	100	0
30	18	7	100	0
60	5	5	80	20
90	18	8	37.5	62.5

Colchicine was a compound that used to induce polyploidy artificially. Colchicine as antimitotic compound, worked by duplicating the number of chromosomes, so that polyploid trait formed [23]. Colchicine induced polyploidy due to its ability to binds to tubulin proteins to inhibited polymerization of tubulin and no spindle threads, which plays a crucial role in cell division, so that chromatid separation couldn't happen [10]. Addition of 0.01 % of colchicine could induced polyploidy in *Bacopa monnieri* [26] and mixoploidy in *Physalis peruviana* L. [27]. Furthermore, addition of 0.1 % colchicine in *Trollius chinensis* thought to be optimal to induce the polyploid plants [28].

CONCLUSION

Colchicine on media affected the shoot formation and growth of crown explants and induced the polyploidy of generated plantlets. The colchicine concentration above 30 mg.L^{-1} on media inhibited shoot formation and growth. However, 30 mg.L^{-1} colchicine can increase the number of the formed shoot.

The addition of 60 mg.L^{-1} and 90 mg.L^{-1} colchicine on media induced polyploidy in vetiver was 20% and 62.5%, respectively. Mixoploid vetiver plant consist of diploid ($2n=2x=20$) and triploid ($2n=3x=30$) cells or diploid ($2n=2x=20$) and tetraploid ($2n=4x=40$) cells.

ACKNOWLEDGMENTS

Part of this research was funded by the Higher Education Applied Research Project, Directorate of Research and Community Service, Directorate General of Research and Development Strengthening, Ministry of Research, Technology, and Higher Education.

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