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Effects of Different Colchicine Concentrations on Latex Amount and Changes in Certain Stomata Characteristics of *Hevea brasiliensis* Muell. Arg. Seedlings *In Vivo*

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ABSTRACT

The effect of colchicine upon certain morphological characteristics of rubber tree (*Hevea brasiliensis*) seedlings aged 13 mth was studied. Different concentrations of 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.3 and 0.5% colchicine together with 1% volume by volume dimethyl sulfoxide (DMSO) were applied once with different application times of 6, 12 and 24 hr. This arrangement was chosen to ascertain the optimal concentration and application time for the induction of polyploid seedlings. The results for the rubber tree seedlings at age 17 mth showed a significant increase in latex amount at concentrations of 0.01 and 0.5% of the mixture. In particular, the concentration of 0.01% was superior to those of the control groups without any change in the latex quality. At colchicine concentrations of 0.01 and 0.08%, the stem diameter difference 5 cm above and below the chemical-treated area was significantly larger than the control. The largest increase was at the concentration of 0.01% which was correlated with the observed latex amount. In all treatments, the number of stomata in the treated plants was significantly greater than those of the control, especially at the concentration of 0.01%, at which the highest number was recorded. The stomatal length was significantly smaller than the control in most treatments including the 0.01% concentration; furthermore, the width of stomata was less, although the difference was not significant and the length-width ratio of the stomata at 0.01% had nearly the same value as the control group.

Flow cytometric determination of the DNA content of the control and the treated rubber seedlings was carried out. The results showed the control seedlings were all diploid with 2 n. Of the treated seedlings, the concentrations of 0.01, 0.04 and 0.5% colchicine gave a similar pattern of aneuploid cells, but also haploid plants appeared at concentrations of 0.08 and 0.3%.

Key words: colchicine, para rubber, latex, *Hevea brasiliensis*, anatomical characteristics

INTRODUCTION

The rubber tree (*Hevea brasilliensis*) is native to Brazil and is an important economic perennial plant in the tropics. It was introduced to Southeast Asia in 1875 and has become very important in Thailand (Ferwerda and Wit, 1969). Therefore it is necessary to improve the production process and the line or variety of the plant for latex production. Rudall (2007) discussed the process whereby some angiosperms produce latex from specialized cells (laticifers) that permeate their

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tissues. In the Euphorbia, laticifers are derived from initial cells in the cotyledonary node of the embryo known as coenocytes. Coenocytic laticifers are termed non-articulated laticifers, but the laticifers of *Hevea braciliensis* undergo cell wall formation while there are repeated cell divisions. Thus they are articulated laticifers. The latiferous tissue of the bark is an organ for the production and storage of latex; as a rubber tree steadily increases in girth, new latex vessels are constantly being added to the bark by the activity of the cambium (Lock, 1913).

Vinod and Meenattoor (1991) reported, that rubber particles are the product of a biochemical pathway taking up a diversion from the catabolic pathway of carbohydrates (sugars). This happens immediately after glycolysis, where acetyl coenzyme A is converted to aceto-acetylcoenzyme A, instead of citric acid, which is usually formed when the catabolism enters the TCA (tricarboxylic acid) cycle. Moreover they considered that the diverted pathway itself is not catabolic, but anabolic, where isoprene monomeres are synthesized. The isoprene synthesis pathway is controlled by specialized enzymes like acetoacetyl CoA acyltransferase and 3-hydroxy-3-methylglutaryl CoA synthetase. Biochemically, all the enzymes are polypeptides and are therefore direct gene products. The isoprene monomeres are synthesized in the laticifer system itself, as the latex is rich in all the enzymes required for the biosynthesis track. However, they stated that the conversion of pyruvate to acetylCoA is distributed inside and outside the mitochondria, which are retained in the laticifers even under the tapping flow.

Plant breeding is a method to produce plants with improved characteristics and enhanced productivity compared to the parental material. There are several ways that can be used to improve plant characteristics. The use of chemicals—colchicine for example—is one of these methods. Colchicine is a tricyclic alkaloid substance, derived from the autumn crocus (*Colchicum autumnale*). It is an inhibitor of mitosis by binding to free tubulin with its topolone ring, thus preventing the formation of microtubuli and the mitotic spindle (Metzler, 2003). It disturbs the cell plate formation which divides the cytoplasm into two parts during the cell division process (Klug and Cummings, 2003). The result is a polyploid cell in which the number of chromosomes has doubled. The chemical can enlarge flower size and fruit and seed weight (Tilney-Bassett, 1991; Martelotto *et al*., 2005). Breeders have used this chemical to develop new varieties for many years. Goncalves *et al*. (1983) investigated the use of colchicine in IAN 717 rubber tree clones. The rubber tree clones were treated with a solution of colchicine plus dimethyl sulfaxode (DMSO) to induce polyploidy in plants with 2n = 36 chromosomes. Their results showed morphological variation and a high degree of mixoploidy. Morphologically, the polyploid clones differed from the diploid control. For the current study, rubber seedlings were treated to find out the effect of different concentrations of the chemical on certain growth characteristics. In addition, its effects on the amount of latex released from an artificial wound were studied.

MATERIALS AND METHODS

The experiments were carried out on rubber tree (*Hevea brasiliensis*) seedlings aged 13 mth at the Chumphon College of Agricultural and Technology, Thailand (10°3'58.53"N 99°4'8.80"E) between March 2010 and February 2011. The cultivar RRIM600 was used, which is very common in Thailand. This cultivar is produced by grafting RRIM600 buds onto rootstocks aged 7 mth. The seedlings were purchased at age 9 mth and planted in 17.8 × 17.8 × 33.0 cm plant bags containing soil and palm kernel cake mixture (1:1, volume by volume, v/v). All rubber tree seedlings in the experiment were fertilized with 15-15-15 NPK compound fertilizer by applying 2 g per seedling at 30 d intervals. The plants were

maintained at 50% reduction of solar radiation. The colchicine treatment was introduced after 4 mth, at seedling age 13 mth, so that the same growing conditions could be assured for all seedlings and uniform seedlings could be used.

The colchicine treatment was carried out using different concentrations, with changing application times to an artificial wound at the base of the shoot apical meristem. This was done by soaking the wound in colchicine for a certain time and using aluminum foil to cover the chemical-treated area, so that the chemical was not degraded by light. In addition, only a small part of the plant was affected, so the polyploidy would not occur below the treatment point. The vertical application cut, (length 0.2 cm and depth 0.1 cm), was made with a scalpel. The scalpel had a ring of tape around its tip to assure the same cutting depth for all treated seedlings. A completely randomized factorial experiment was used to study the effects of the colchicine treatment using 26 treatments—24 treatments to cover eight colchicine concentration levels of 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.3 and 0.5% together with 1% v/v dimethyl sulfoxide and three different application times of 6, 12 and 24 hr and two treatments as controls. In one experiment the control group of seedling was wounded artificially with an application time of 6 hr, to check that the wounding did not induce any changes in the plant. The other control group was not treated at all. For all 26 different treatments, there were three replications with three seedlings in each replication.

Seedling survival counts were conducted after 30 d of treatment. Latex was collected from rubber tree seedlings at age 17 mth by wounding artificially with a blade at a height 10 cm above the first application point on the stem. For the tapping, a blade modified from the type used for the application cut was used. The length of the cut was 0.8 cm, the depth was 0.2 cm and the cutting angle was 30° downward from left to right. The tapping was made between 0400 and 0600 hours. The latex amount from the wound at the treated seedlings was compared to the control.

After finishing the experiment, the latex quality at the concentration (0.01%) of the chemical, which was superior to the control groups, was compared to the control by measuring the dry rubber content. In any case, the latex quality was only measured at 6 hr application time of the treatment and in the control, which had not been treated at all. The concentrated rubber content, without bubbles in the sample, was weighed in stainless steel cups on an electric balance. The volume in milliliters of distilled water that equalled the number of grams of the sample was then mixed into the sample. The cup was rotated by hand. Then, 2 mL of acetic acid with a concentration of 2% was added slowly into the cup. Once the latex commenced clotting, the cup with the sample was placed on a steam bath for approximately 10 min until the clotting was completed. The clear serum was separated from the latex clots. Then, each sample was washed with water and pressed by rollers to obtain sheet samples approximately 2 mm thick. The samples were placed in a hot-air oven at 70 °C for 12 hr followed by cooling in a desiccator for 30 minutes and then weighing with the same balance. The percentage of dry rubber content (DRC) was calculated using Equation 1:

$$DRC = (M_1/M_0)100 \quad (1)$$

where M_0 is the weight of concentrated rubber latex in grams and M_1 is the weight of dried rubber sheet in grams.

To study stomatal changes, a fully expanded fourth leaf from the apex of a seedling aged 18 mth from each *treatment* was sampled for stomatal counts, together with its length and width measurement. The data were compared to the control. Epidermal peels were taken from the *lower surface* of the leaves using transparent adhesive tape, which was then mounted on a microscope slide. Three samples were taken from

each treatment in the mid-lamina region. The area surrounding or nearby large veins was avoided. The stomatal size (with guard cells) from each sample was measured with an ocular micrometer under the microscope. The stomata were counted in three randomly chosen microscopic fields. Each field consisted of 0.123 mm^2 at 600× magnification. The number of stomata per field was converted to the number of stomata per square millimeter. The stomatal length/width ratio (SLW) was calculated using Equation 2:

$$SLW = SL/SW \quad (2)$$

where SL is the length of the stomata and SW is the width of the stomata.

Figure 1 shows a photomicrograph of a stomata derived from this research and an diagram of where the length and width measurements were taken.

The stem diameter measurement involved using a vernier caliper to measure 5 cm above and below the area treated with the chemical. The measured value of the lower section was subtracted from the upper one. Two diameter measurements were taken at right angles to each other at the same height of the stem and the average was used as the measurement of stem diameter (West, 2009).

Analysis of variance (ANOVA) was used to analyze the significance of all collected data. Fisher's least significant difference was used as *a post hoc test* to *compare* treatment *means, e*xcept for the latex quality, where only ANOVA was used. In all tests $P < 0.05$ was considered significant.

After one month, at the end of the experiment, fresh foliar tissue samples of the second leaf were taken from each seedling. The ploidy level of these samples was determined using flow cytometry. From the sample, 50 mg of healthy fresh leaves were chopped with a sharp razor blade and stained with 500 µL ice-cold CyStain UV Ploidy in a plastic Petri dish. The CyStain UV Ploidy contained 4',6-diamidino-2-phenylindole (DAPI) which binds at AT-rich regions of DNA. The solution obtained was filtered with a Partec CellTrics disposable filter to eliminate cell debris. Then, the samples were analyzed using a Partec PAII laser flow cytometer (Partec GmbH, Münster, Germany) equipped with a 100 W Osram HBO 100/2 mercury arc lamp, a TK420 dichroic mirror, a GG 435 longpass filter and a device to measure blue emissions. Histograms were analyzed using the Partec FloMax software (Partec GmbH, Münster, Germany), which determines the peak

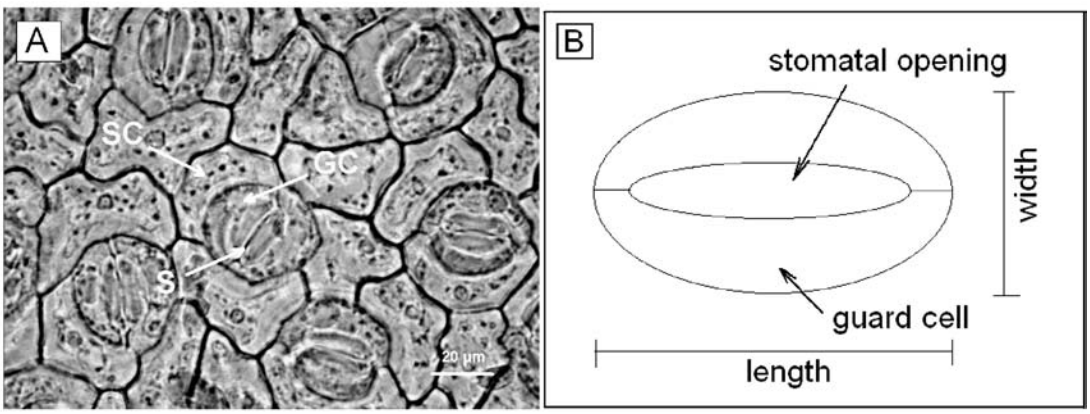

Figure 1 (A) Photomicrograph (600×) of the leaf epidermis of *Hevea brasiliensis* seedling showing stomatal complexes (S) with guard cells (GC) and subsidiary cells (SC), after colchicine treatment at a concentration of 0.08% for 24 hr; (B) Diagram of a stoma indicating the positions used to measure the length and the width.

position, coefficient of variation, and the relative ploidy index of the samples.

RESULTS

Rubber seedlings were treated once with colchicine concentrations of 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.3 and 0.5% for 6, 12 and 24 hr. *The latex was collected* at 0400 to 0600 hours. The temperature range was 22.8–23.1 °C and the relative humidity range was 87–92% during the operational time. The results showed a significant increase in the amount of latex at colchicine concentrations of 0.01 and 0.5%. The concentration of 0.01% showed an increase in the latex amount for all application times. At 0.5%, a significantly larger amount of latex was collected at an application time of 6 hr. In addition, at 0.3%, the latex amount was higher than in the control, although not significantly higher. The concentration dose of 0.01% was superior to those of the control group. However, at 0.02, 0.04, 0.06, 0.08 and 0.1% colchicine concentrations, the latex amount was less, but not significantly different. The application time affected all treatments in the same manner (Table 1).

Latex quality in the new mutant seedlings, indicated by the dry rubber content, was not significantly different. The percentages of average dry rubber content from the collected concentrated latex were 38.81% at a concentration of 0.01% and 39.12% in the control groups (Table 2).

Table 1 Effect of colchicine concentration on latex amount of *Hevea brasiliensis* seedlings at different application times.

Colchicine concentration (%)	Latex amount (g)			Mean
	6 hr	12 hr	24 hr	
0.01	0.24	0.23	0.24	0.24^{d}
0.02	0.14	0.12	0.14	0.14^{a}
0.04	0.12	0.12	0.15	0.13^{a}
0.06	0.19	0.15	0.14	0.16^{ab}
0.08	0.16	0.15	0.16	0.15^{ab}
0.1	0.13	0.11	0.13	0.12^{a}
0.3	0.19	0.19	0.18	0.19^{bc}
0.5	0.23	0.18	0.19	0.20^{c}
Control 1	0.16	nd	nd	0.16^{ab}
Control 2	0.16	nd	nd	0.16^{ab}

Values followed by different superscript letters within a column differ significantly ($P < 0.05$).
nd = no experiment at the application time.

Table 2 Effect of colchicine concentration on average dry rubber content of *Hevea brasiliensis* seedlings at 6 hr application time.

Colchicine concentration (%)	Average dry rubber content (%)
0.01	38.81^{a}
Control	39.12^{a}

Values followed by different superscript letters within a column differ significantly ($P < 0.05$).

Table 3 shows the effect of colchicine application on the difference in stem diameter. At colchicine concentrations of 0.01 and 0.08%, the figures were significantly larger than those of the control. At concentrations of 0.02 and 0.06%, the difference in stem diameter was larger, but not significantly. The largest figure was at a concentration of 0.01%. At concentrations of 0.1, 0.3 and 0.5%, the figures were less than those of the control but not significantly different. The application time in all treatments did not affect the amount, except for the concentration of 0.08% at a treatment of 24 hr (Table 3) that resulted in the largest difference in stem diameter compared to the means over all application times.

According to Table 4, the numbers of stomata per square millimeter in all treatments were significantly larger than those of the control, with the highest numbers found at concentrations of 0.01 % (12 hr) and 0.02% (6 hr). The concentration of 0.01% colchicine resulted in the highest numbers of stomata over all application times.

Table 5 shows the stomatal length. At concentrations of 0.01, 0.06 and 0.5%, the lengths were significantly smaller than those of the control. The other treatments showed no significant difference to the control, except for the dose at 0.02%, where the length was greater (but not significantly so) than in the control group. Table 5 also shows the width of the stomata. At 0.01, 0.02, 0.04 and 0.06%, the width was less than in the control group, with the width at 0.06% being significantly shorter. The length-width ratio of the stomata is shown in Table 6. The values at concentrations of 0.1 and 0.5% were significantly smaller than in the control group, but at 0.01% somewhat equal to the control. The largest L/W ratio, was found at the concentration of 0.04%, although it was not significant compared to the control.

Figure 2 presents the DNA content of the control and treated rubber seedling leaves. The treated plants differed in the nuclear DNA content from those of the control, with the control peaks located on channel 200 with a very small negligible peak at channel 400. The control seedling is thus predominantly diploid with 2n. The samples with colchicine concentrations of 0.01, 0.04 and 0.5% gave a similar pattern of aneuploid cells. However,

Table 3 Effect of colchicine concentration on the average difference in the stem diameter 5 cm above and below the chemical-treated area at different application times.

Colchicine concentration (%)	Difference in the stem diameter (cm)			Mean
	6 hr	12 hr	24 hr	
0.01	0.17	0.18	0.18	0.18^{c}
0.02	0.06	0.09	0.08	0.08^{ab}
0.04	0.09	0.13	0.09	0.10^{b}
0.06	0.14	0.08	0.06	0.09^{ab}
0.08	0.11	0.14	0.24	0.16^{c}
0.1	0.07	0.03	0.01	0.04^{a}
0.3	0.06	0.03	0.02	0.04^{a}
0.5	0.06	0.03	0.06	0.05^{ab}
Control 1	0.07	nd	nd	0.07^{ab}
Control 2	0.06	nd	nd	0.06^{ab}

Values followed by different letters within a column differ significantly ($P < 0.05$).
nd = no experiment at the application time.

the morphological characters of both polyploidy and mosaics. However, their girth, growth vigor and dry rubber content were inferior to those of their parent diploids. The trunks of most polyploid trees showed protrusions and depressions to some extent. Several studies have shown an increase in the mortality of many colchicine-treated plants and their survival decreased as the chemical

Table 4 Effect of colchicine concentration on numbers of the stomata of *Hevea brasiliensis* seedlings at different application times.

Colchicine concentration (%)	Average number of stomata			Mean
	6 hr	12 hr	24 hr	
0.01	289.98	311.65	293.59	298.40^{f}
0.02	311.65	284.55	290.88	295.69^{ef}
0.04	258.36	280.94	281.84	273.71^{cde}
0.06	299.91	288.17	278.23	288.77^{def}
0.08	296.30	280.94	282.87	280.04^{cdef}
0.1	272.81	267.39	252.94	264.38^{bc}
0.3	255.65	247.52	248.42	250.53^{b}
0.5	273.71	271.91	264.68	270.10^{bcd}
Control 1	222.22	nd	nd	222.22^{a}
Control 2	224.03	nd	nd	224.03^{a}

The average number of stomata per 600× microscopic field was converted to the average number of stomata per square millimeter.

Values followed by different superscript letters within a column differ significantly ($P < 0.05$).

nd = no experiment at the application time.

Table 5 Effect of colchicine concentration on length and width of stomata of *Hevea brasiliensis* seedlings at different application times.

Colchicine concentration (%)	Length of stomata (μm)			Mean	Width of stomata (μm)			Mean
	6 hr	12 hr	24 hr		6 hr	12 hr	24 hr	
0.01	24.74	23.89	23.98	24.20^{ab}	16.67	17.50	17.22	17.13^{ab}
0.02	26.20	24.82	25.74	25.59^{c}	17.96	17.13	17.04	17.38^{abc}
0.04	24.82	24.91	25.83	25.19^{bc}	17.41	16.67	17.04	17.04^{ab}
0.06	24.07	23.72	24.91	24.24^{ab}	16.85	16.85	16.11	16.61^{a}
0.08	24.35	25.09	25.00	24.82^{bc}	17.13	17.96	18.80	17.96^{bc}
0.1	25.56	24.72	24.26	24.85^{bc}	18.24	19.35	19.35	18.98^{d}
0.3	25.00	25.37	25.28	25.22^{bc}	16.94	19.07	18.89	18.30^{cd}
0.5	23.82	23.06	23.06	23.31^{a}	18.89	17.50	17.50	17.96^{bc}
Control 1	25.46	nd	nd	25.46^{c}	17.96	nd	nd	17.96^{bc}
Control 2	25.37	nd	nd	25.37^{c}	17.69	nd	nd	17.69^{bc}

Values followed by different superscript letters within a column differ significantly ($P < 0.05$).

nd = no experiment at the application time.

Table 6 Effect of colchicine concentration on L/W ratio of stomata of *Hevea brasiliensis* seedlings at different application times.

Colchicine concentration (%)	L/W ratio of stomata			Mean
	6 hr	12 hr	24 hr	
0.01	1.51	1.38	1.42	1.44^{bcd}
0.02	1.46	1.48	1.53	1.49^{cd}
0.04	1.44	1.53	1.55	1.51^{d}
0.06	1.45	1.42	1.58	1.49^{cd}
0.08	1.44	1.42	1.36	1.41^{bc}
0.1	1.42	1.29	1.27	1.33^{a}
0.3	1.50	1.34	1.35	1.40^{ab}
0.5	1.28	1.34	1.34	1.32^{a}
Control 1	1.44	nd	nd	1.44^{bcd}
Control 2	1.43	nd	nd	1.43^{bcd}

Values followed by different superscript letters within a column differ significantly ($P < 0.05$).
nd = no experiment at the application time.

Table 7 Peaks and ploidy at flow cytometric determination according to Figure 2.

Colchicine concentration (%)	Mean of peak	Ploidy (n) indicated by channel number	Chromosome content
Control	200.52	diploid (2n)	2n = 36, n = 18
0.01	161.87	aneuploid between 1n and 2n	n > 18
0.04	142.05	aneuploid between 1n and 2n	n > 18
0.08	126.86	haploid (1n)	n > 18
0.30	116.69	haploid (1n)	n > 18
0.50	134.63	aneuploid between 1n and 2n	n > 18

at concentrations of 0.08 and 0.3%, a relatively high frequency of haploid cells (that is, cells with 1 n DNA contents) occurred. No polyploidy was found in any treatment, though clearly some chromosome doubling could be observed. Table 7 shows a summary of the flow cytometry readouts presented in Figure 2.

DISCUSSION

This experiment was performed to obtain rubber plants delivering larger latex yields than those observed in normal plants. The grafted shoot tips of *Hevea brasiliensis* RRIM600 rubber seedlings were treated once for 6, 12 and 24 hr with aqueous solutions of colchicine at concentrations of 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.3 and 0.5%. Colchicine is an alkaloid chemical that induces polyploidy in plants by disrupting the cell plate formation during cellular division, so that the plant cell does not divide into two cells. This produces a single cell with a nucleus having twice the chromosome number. The colchicine application in this research was expected to induce polyploidy in diploid plants with 2n = 36 chromosomes, which is equivalent to two sets of chromosomes. However, as previous research has shown, the colchicine application has also some negative side effects. Youjun *et al*. (2003) reported that their colchicine-treated clones were polyploids with

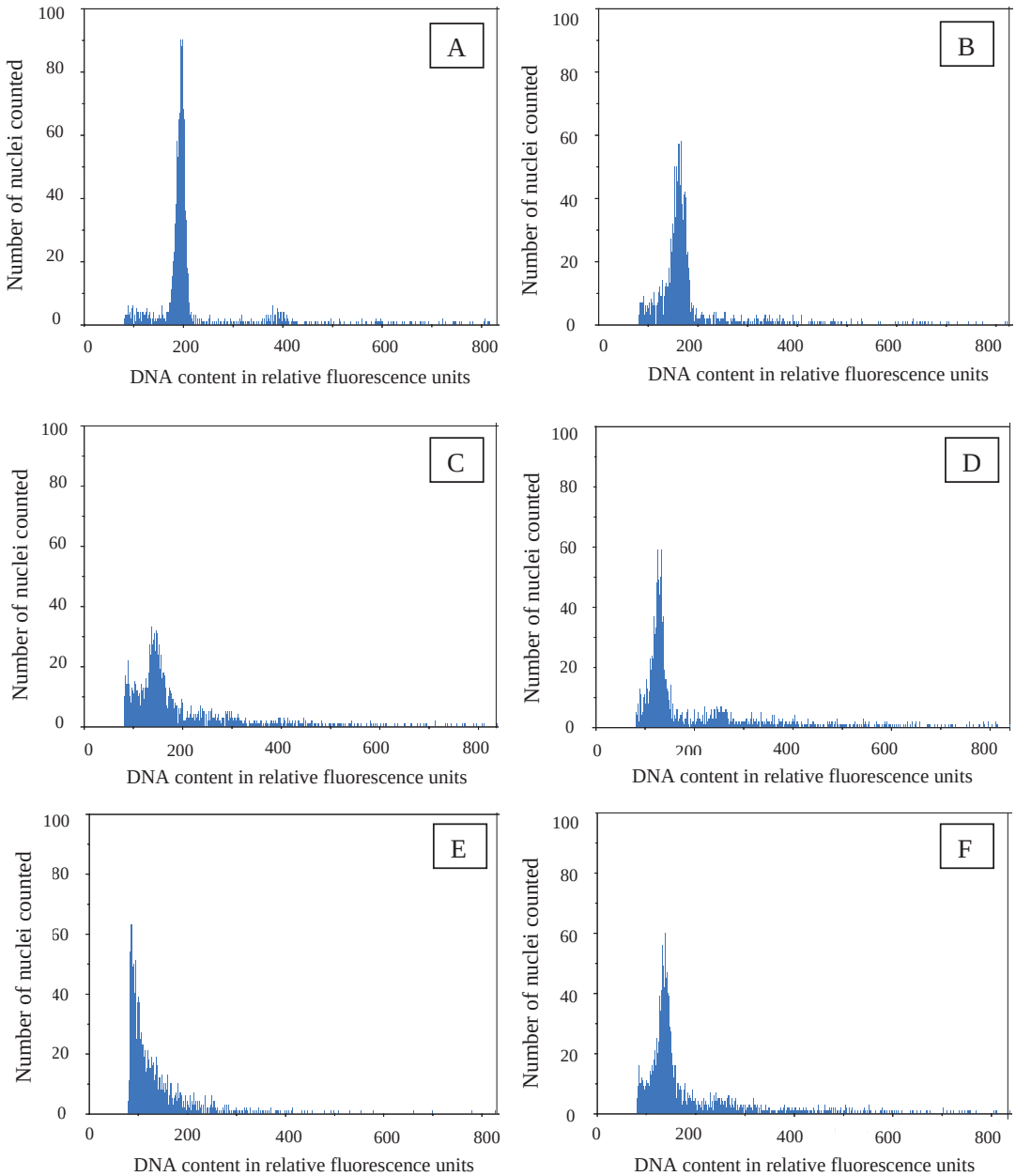

Figure 2 Flow cytometric histogram of DNA content of 4',6-diamidino-2-phenylindole-stained nuclei isolated from rubber plant leaf tissue of: (A) Control; (B) Seedlings treated with 0.01% colchicine; (C) Seedlings treated with 0.04% colchicine; (D) Seedlings treated with 0.08% colchicine; (E) Seedlings treated with 0.3% colchicine; (F) Seedlings treated with 0.5% colchicine. The x-axis shows the DNA content in relative fluorescence units. The y-axis shows the number of nuclei counted per histogram channel.

concentration increased (Takamura and Miyajima, 1996; Beck *et al*., 2003; Nair, 2004; Rubuluza *et al*., 2007). Many trials resulted in weak-looking and diseased plants after applying the chemical (Takamura and Miyajima, 1996; Beck *et al*., 2003; Nair, 2004), which was not the case in the results obtained from the current experiment. This might have been due to the fact that after the pre-tests, the harming concentrations (which resulted in less vital plants showing browning necrosis around the shoot tip) were not applied in the current experiment. Another explanation might be that actually no polyploidy was induced. This fact was also proven by the flow cytometer determination of the DNA contents from the treated rubber seedlings, which showed that no polyploidy was induced. Only one peak was shown at channel 200, coding for diploidy in the control group. In the treated groups, the peaks were around channel 200 or even smaller, indicating that after the treatment, the seedlings were still diploid. After the application with concentrations of 0.01, 0.04 and 0.5%, aneuploid cells occurred. Aneuploidy is an abnormal number of chromosomes, where an extra or missing chromosome can introduce a genetic disorder; this happens during cell division and only single chromosomes are affected, not the whole genome (Sitte *et al*., 2002). It could also be possible that centromeres interact unequally with the mitotic spindle, causing chromosome loss (Ravi and Simon, 2010). But how then can the changes in latex yield, stem diameter and stomata characteristics be explained? It seems that only the chromosome coding for the polypeptides in the latex and the number of stomata have been affected. Perhaps the increased latex amount, which was significant at concentrations of 0.01% and 0.5%, is connected with the stem diameter, which was greater than those of the original diploid seedlings. This could have resulted from more latex vessels being added to the bark (Lock, 1913). However, there were also reductions in the stem diameter at concentrations of 0.1, 0.3 and 0.5% although these was not significantly different from the control. This counters the theory that an increase in stem diameter will lead to a gain in the latex yield. This might only be effective at the lowest concentration dose. At the highest tested dose (where the stem diameter was not significantly different from the control group), perhaps the latex-producing cells were affected by the treatment with the chemical. The latex amount in the other treatments was about the same as in the control, though there was no change in quality. At the treatment dose of 0.3%, the plants seemed to have changed to haploid plants. The haploid mutant was also observed in sorghum after colchicine treatment (Simantel and Ross, 1964) and also colchicine-induced haploids and aneuhaploids were reported in *Solanum chacoense* Bitt. (Hermsen, 1969) The lack of polyploidy could also have resulted from the short application times applied in the current research. According to Glendon and Staden (2008), who tested *Watsonia lepida* under colchicine treatment, the best survival (84%) was found at 24 hr with the lowest concentration, but no polyploid plants were found, nor did a 48 hr treatment produce any polyploids. At an application time of 72 hr, mixoploidy occurred. A review of the literature indicated that the usual application time of colchicine is much longer and there are other possibilities which have to be considered. Perhaps the time of the treatment using seedlings aged 13 mth (6 mth after grafting) has to be taken into account. The literature review indicated that in other research where colchicine was used, it was usually applied to microspores or pollen (Hansen and Andersen, 1998), the roots (Barlow, 1969) and seeds (Chieko *et al*., 2009). An explanation for not finding polyploid cells in the current experiment could also be the fact that samples were taken from the second fully developed leaf, which is usually already determined in the apical meristem. Thus, it may be less prone to modifications induced by colchicine. Another reason could be that the apical meristem was not treated because it was

too small, as only a small cut was used to apply the colchicine. Perhaps a colchicine application around the whole shoot tip is necessary to induce more changes.

The results for the study on the stomata showed that the stomata were not necessarily larger in size than those of normal plants—both larger and smaller stomata were found. At 0.04%, there was a resultant increase (though not significantly) in the L/W ratio, while at 0.1 and 0.5%, the L/W ratio decreased significantly. The number of stomata changed with the treatment dose and application time, but it increased significantly in all treatments. Larger numbers per area were obtained over all treatment doses and application times compared to the control groups. Thus, while the findings of the current research did not result in polyploidy, other benefits occurred. The lowest applied concentration resulted in a higher latex yield, a significantly larger stem diameter and more stomata with a larger L/W ratio. It could be possible to potentially extend centromere-mediated genome elimination to produce haploid rubber plants which are valuable for breeding programs by reducing the ploidy of the plant. The haploid plants would still have the same or improved traits, but would be less prone to diseases and plant pathogens than the polyploid ones and could be used to install new breeding lines. Therefore, this approach could be used to genetically modify the rubber tree according to future needs. Nevertheless, more studies on the new mutant seedlings are recommended, such as on their resistance to diseases. It is clear that the right colchicine concentration is important because the outcome of the conducted experiments differed markedly depending on the chosen concentration and the application time. With the exception of 0.01%, there was no concentration at which all the surveyed factors increased. Clarification of all the questions that arise requires more research using the same experimental conditions with additional treatments and also much longer application times.

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