Effects of Colchicine and Oryzalin on Polyploidy Induction and Production of Capsaicin in *Capsicum frutescens* L.

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ABSTRACT

Polyploidy were induced in *Capsicum frutescens* L. seedlings using colchicine or oryzalin. Seeds were immersed in colchicine at concentrations of 0, 100, 200 and 300 mg/L or oryzalin at concentration of 0, 10, 20 and 30 mg/L under dark conditions at room temperature for 6 h before germination. The results showed that 300 mg/L colchicine could induce the highest rate of polyploidy in *C. frutescens* L. at 70%, while 20 and 30 mg/L oryzalin could induce polyploidy at the same rate of 40%. The stomatal guard cell size and fruit size of polyploid plants were larger than controls, enabling rapid screening of polyploid seedlings. The polyploid plants also produced a larger amount of capsaicin than controls. It was found that fruits from seeds treated with 300 mg/L colchicine produced the largest amount of capsaicin at 4.55 mg/g dry weight compared with controls that produced at 3.32 mg/g dry weight. While, fruits from the seeds treated with 30 mg/L oryzalin produced capsaicin at 4.44 mg/g dry weight compared with controls that produced at 3.25 mg/g dry weight.

Keywords: *Capsicum frutescens* L., colchicine, oryzalin, polyploidy

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INTRODUCTION

Capsicum frutescens L. is a variety of hot chili that is widely grown in Thailand. It is used as spicy ingredient in cooking and also in pharmaceutical purposes. The placenta contains many kinds of secondary metabolites, especially the capsaicinoids. Normally, the major capsaicinoid in chili fruits is capsaicin (Yaldiz *et al.*, 2010; Zewdie and Bosland, 2001). Capsaicin gives spicy taste in chili (Nuñez-Palenius and Ochoa-alejo, 2005). Furthermore, it can induce apoptosis in gastric cancer cells (Chow *et al.*, 2007), used to relieve the chronic pain syndromes, e.g., postherpetic neuralgia, musculoskeletal pain, and bladder dysfunction (Hayman and Kam, 2008).

The production of capsaicin is practically done by extracting from the diploid fruits of Capsicum, which may be less than is desired. One way to increase secondary metabolite production in plants is through the induction of artificial polyploidy (Dhawan and Lavania, 1996). This has been shown to increase the production of secondary metabolites in many plants as compared to their diploid parent, such as the production of artemisinin from *Artemisia annua* (De Jesus-Gonzalez and Weathers, 2003), production of diosgenin from *Dioscorea zingiberensis* (Heping *et al.*, 2008). Artificial polyploidy in plants can be obtained using various methods. Antimicrotubule agents, such as colchicine and oryzalin have been reported to induce polyploid of several plant

species (Rey *et al*., 2002) including *C. anuum* (Pal and Ramanujam, 1939) and *C. frutescens* (Raghuvanshi and Joshi, 1964). These chemicals are used to block metaphase of mitosis in cycling plant cells (Niel and Scherrmann, 2006; Makioka *et al*., 2000). They interfere with the cytoskeleton by inducing microtubule depolymerization, preventing the formation of the mitotic spindle (Caperta *et al*., 2006, Chauvin *et al*., 2003). This reaction results in polyploidy of plant cells.

Colchicine and oryzalin were untilized in this study to induce polyploidy in *C. frutescens* L. and capsaicin production of the polyploidy plants was determined.

MATERIALS AND METHODS

Plant Material Preparation

Capsicum frutescens L. seeds (Lion trademark, Lion Seed Ltd., Part) were used. Seeds were soaked in tap water at room temperature for 30 min. Only seeds that sank to the bottom were collected and germinated on wet tissue paper at room temperature. After 5 days, the germinated seedlings with radicles of 1 mm in length, were collected and used as plant materials.

Polyploid Induction

Twenty germinated seeds were put into 250 ml Erlenmeyer flask containing 100 ml of colchicine at concentrations of 100, 200 and 300 mg/L or oryzalin at 10, 20 and 30 mg/L. While, the control treatment, the germinated seeds were soaked in distilled water and 1% (v/v) DMSO, respectively. The seeds were shaken by orbital shaker at 100 rpm in dark condition at room temperature for 6 h. Then all seeds were washed 3 times with distilled water before they were grown indoor in germinating trays at room temperature. When the seedlings had 2–3 leaves, they were transferred into 2 inch diameter pots and cultivated under shade conditions. The final transplanting to 20 inch diameter pots under greenhouse conditions was done when seedlings had 5–7 leaves

Stomata Guard Cells Measurement

The stomatal guard cell length and width of polyploidy and control plants were measured from three fully developed leaves. The lower epidermis peels were excised from the leaves and placed on a microscope slide and stained with safranine solution for 5 min at room temperature. Measurement of stomatal guard cell size was done under microscope at 1,000× and stomata densities were counted at 400×.

Flow Cytometry Analysis

Young leaves from 60 day old plants were collected. Leaf disc approximately 1 cm^2 was chopped with a disposable steel razor blade in 1 ml Otto I buffer (0.1 M citric acid, 0.5% tween-20) to release nuclei. Previously macerated tissues were filtered with 42 µm nylon mesh, and compiled in a polystyrene tube before spinning down at 2,000 rpm for approximately 2 min. The suspension was added to 600 µl Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$, 1 mg/L propidium iodide, 1 mg/L RNase, 2 µl/ml β-mercaptoethanol). Samples were incubated at room temperature in the dark for 30 min. At least 10,000 nuclei were analyzed in each sample. Analyses were performed by using a flow cytometer (The BD FACSCalibur™). The protocol was followed from Otto (1992).

Capsaicin Extraction and Analysis

Ten mature red fruits were collected from each treatment. The seeds were removed. Fruits and placentas were dried at 60 °C in hot air oven about 48 h. Aliquots of 100 mg of the dried chili powder were extracted with 1 ml 95% ethanol in an Eppendorf tube under continuous stirring, 100 rpm, at room temperature for 8 h. Subsequently, the extract was evaporated to completely precipitate at 60 °C in hot air oven. After evaporation, the crude extract (oleoresin) was dissolved in 1 ml of 60% methanol. Finally, the samples were filtered by 0.2 µm membrane (Acrodisc, USA) and used for high-performance liquid chromatography (HPLC) analyses.

Capsaicin was separated by using a Waters HPLC (2690) equipped with a Sunfire C18 column operating at 35°C. One ml of the sample was injected, and separation was achieved with a solvent phase of 6.6% acetic acid in water (phase A) and 70% methanol (phase B) pumped isocratically (73% phase A and 27% phase B) at a flow rate of 1 ml/min. Detection was done at 280 nm. Under these conditions, capsaicin appeared as a peak with a mean retention time of approximately 7.35 min, which corresponded to a standard of capsaicin [(98%; Sigma: M2028 (8–methyl–N–vanillyl–6–nonenamide)]. The protocol was modified from Yaldiz *et al*. (2010).

Statistical Analysis

All data were subjected to analysis of variance (ANOVA). Comparison of mean was performed using LSD test at $P \leq 0.01$.

RESULTS

Polyploidy Induction by Colchicine and Oryzalin

The polyploidy of *C. frutescens* L. seedlings was induced with colchicine and oryzalin. The results clearly showed that polyploidy rate increased with the increasing concentrations of both chemicals, while no polyploidy was observed in controls of both treatments. The colchicine solutions were found to have more effect in inducing polyploidy than oryzalin. The results of colchicine treatment showed that at concentration of 300 mg/L could induce polyploidy at the highest rate of 70%, followed by colchicine at 200 and 100 mg/L that induced at rates of 33 and 20%, respectively (Figure 1). As for oryzalin treatment, 40% of polyploidy rate was accomplished from the treated plants with 20 or 30 mg/L, compared with 10 mg/L that induced at a rate of 22% (Figure 2).

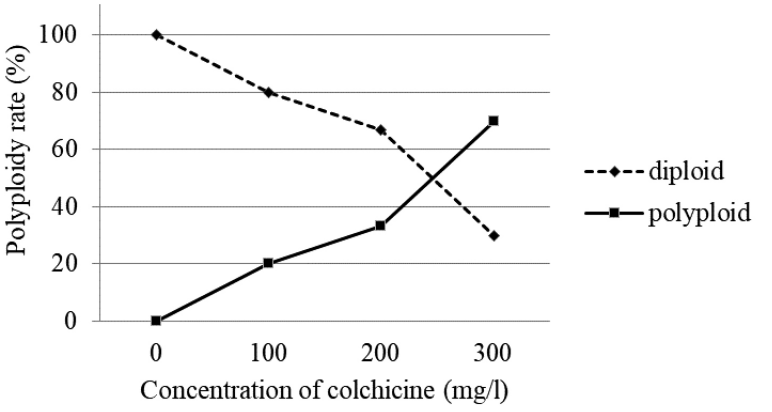


Figure 1 Polyploidy rate in *C. frutescens* L. induced by colchicine

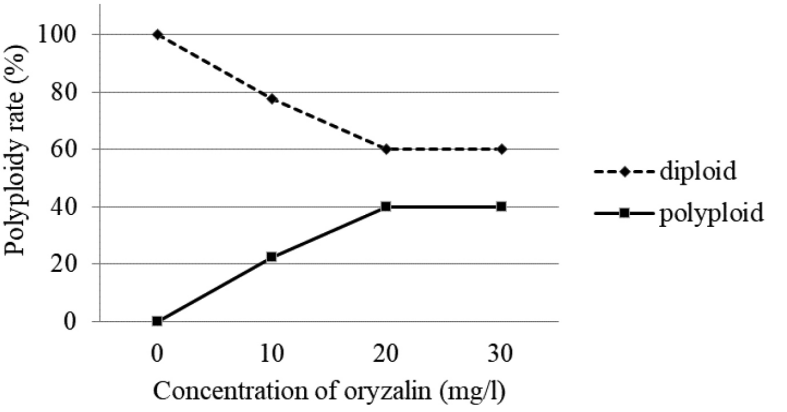
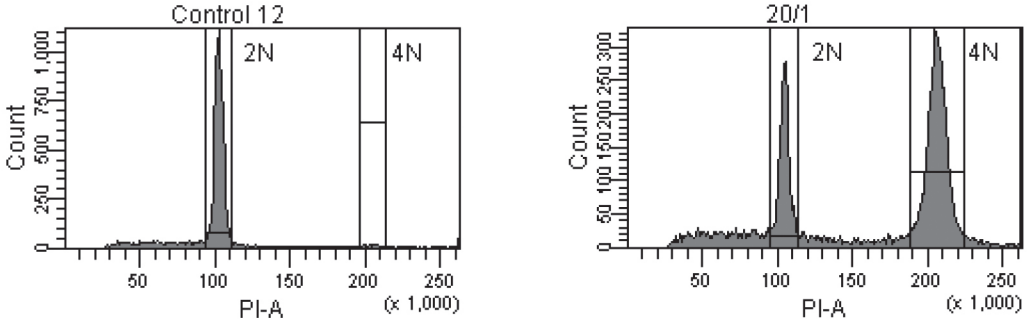


Figure 2 Polyploidy rate in *C. frutescens* L. induced by oryzalin.

Flow Cytometry Analysis

The results of flow cytometric analysis showed that all polyploid plants were mixoploid. The ploidy level of flow cytometric analysis showed the G1 peak of 2n appeared on channel 100, whereas the G1 peak of the 4n was on channels 200 (Figure 3). Percentage analysis of diploid and polyploid cells in mixoploid showed that percentage of tetraploid cells increased with the increasing of colchicine and oryzalin concentrations (Table 1 and Table 2).



(a) (b)

Figure 3 Histogram of the relative fluorescence intensity of nuclei isolated from the leaves of diploid and polyploid *C. frutescens* L. (a) The control diploid plant of *C. frutescens* L. and (b) a polyploidy plant of *C. frutescens* L.

Table 1 Flow cytometry data of polyploidy *C. frutescens* L. induced by colchicine

Colchicine (mg/L)	Diploid cells[1/] (%)	Tetraploid cells [1/] (%)
Control	78.90 ± 3.54[a]	1.44 ± 0.30[a]
100	66.20 ± 3.68[b]	34.90 ± 2.00[b]
200	26.78 ± 1.66[c]	40.70 ± 1.59[b]
300	23.34 ± 1.47[d]	43.34 ± 1.78[b]
F-test	**	**
C.V.	5.71	5.19

[1/] Data represented mean ± S.D. of five independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

Table 2 Flow cytometry data of polyploidy *C. frutescens* L. induced by oryzalin

Oryzalin (mg/L)	Diploid cells[1/] (%)	Tetraploid cells [1/] (%)
Control	78.42 ± 4.67[a]	1.40 ± 0.19[a]
10	65.46 ± 4.29[b]	35.96 ± 2.98[b]
20	24.27 ± 2.01[c]	42.06 ± 2.09[b]
30	25.24 ± 2.13[c]	41.24 ± 1.95[b]
F-test	**	**
C.V.	6.29	7.52

[1/] Data represented mean ± S.D. of five independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

Effects of Colchicine and Oryzalin on Stomata Guard Cell Size

Measurement of stomata size and density is essential for initial screening the ploidy level of large quantities of plants (Yang *et al*., 2006). In this study, the average length and width of polyploid stomata guard cells were larger than diploid. Colchicine at 300 mg/L induced the biggest stomata guard cell length and width at 33 and 26.01 µm, respectively, compared with the controls that was 25 and 18.89 µm, respectively (Table 3 and Figure 4). On the other hand, induction of polyploidy by oryzalin also gave the same results in stomata guard cell size as colchicine. Oryzalin at the highest tested concentration of 30 mg/L, could induce the biggest stomata length and width at 32.50 and 25.25 µm, respectively. While, the length and width of stomata in controls were 25 and 19.45 µm, respectively (Table 4 and Figure 5).

Table 3 Effects of colchicine on stomata guard cell size and density in *C. frutescens* L.

Colchicine (mg/L)	Guard cell width [1/] (µm)	Guard cell length [1/] (µm)	Guard cell density [1/] (cell/mm^2)
Control	18.89 ± 1.02[a]	25.00 ± 1.07[a]	29.90 ± 0.97[a]
100	25.82 ± 1.17[b]	32.50 ± 1.11[b]	21.60 ± 1.57[b]
200	25.87 ± 1.01[b]	33.50 ± 1.09[b]	21.50 ± 1.28[b]
300	26.01 ± 1.13[b]	33.00 ± 1.15[b]	20.85 ± 1.04[b]
F-test	**	**	**
C.V.	7.03	3.86	5.27

[1/] Data represented mean ± S.D. of twenty independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

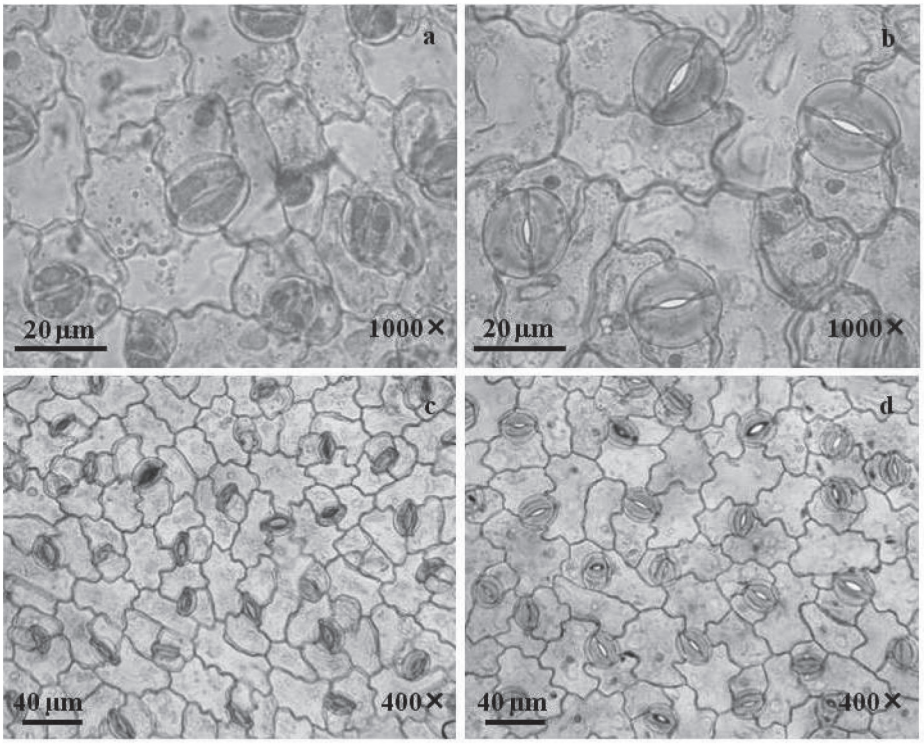


Figure 4 Stomata guard cell size and density of polyploidy *C. frutescens* L. induced by chochicine (a = control size, b = polyploidy size, c = control density, d = polyploidy density)

Table 4 Effects of oryzalin on stomata guard cell size and density in *C. frutescens* L.

Oryzalin (mg/L)	Guard cell width [1/] (µm)	Guard cell length [1/] (µm)	Guard cell density [1/] (cell/mm²)
Control	19.45 ± 0.79[a]	25.00 ± 1.18[a]	29.90 ± 0.97[a]
10	24.00 ± 1.17[b]	32.60 ± 1.23[b]	21.60 ± 1.57[b]
20	25.20 ± 1.19[b]	32.55 ± 1.09[b]	21.50 ± 1.28[b]
30	25.25 ± 1.21[b]	32.50 ± 1.15[b]	20.85 ± 1.04[b]
F-test	**	**	**
C.V.	6.90	4.06	5.27

[1/] Data represented mean ± S.D. of twenty independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

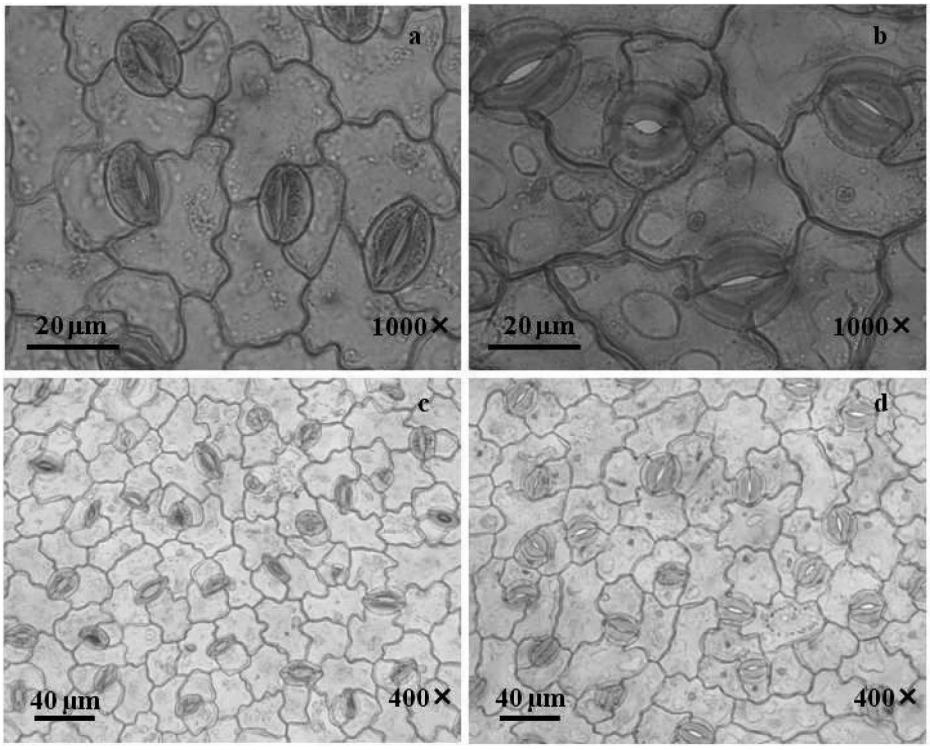


Figure 5 Stomata guard cell size and density of polyploidy *C. frutescens* L. induced by oryzalin (a = control size, b = polyploidy size, c = control density, d = polyploidy density)

Effects of Colchicine and Oryzalin on Fruit Size

Fruit characters have been found to vary with ploidy level. Polyploid fruits had more length and diameter than diploid fruits. Colchicine and oryzalin at the lowest tested concentration could induce the biggest fruits than at higher concentrations and also control. The polyploidy fruits induced with 100 mg/L colchicine had fruit length and diameter of 5.03 and 0.78 cm, respectively. While in controls, fruit length and diameter were 3.99 and 0.63 cm, respectively (Table 5 and Figure 6a). The same results were also found in polyploidy fruits induced with 10 mg/L oryzalin. They were 4.50 and 0.37 cm in fruit length and diameter, respectively, compared with controls of 3.48 and 0.34 cm, respectively (Table 6 and Figure 6b).

Table 5 Effect of colchicine on *C. frutescens* L. fruit size

Colchicine (mg/L)	Fruit length [1/] (cm)	Fruit diameter [1/] (cm)
Control	3.99 ± 0.23[a]	0.63 ± 0.07[a]
100	5.03 ± 0.27[b]	0.78 ± 0.06[b]
200	4.96 ± 0.24[b]	0.78 ± 0.07[b]
300	4.18 ± 0.19[a]	0.78 ± 0.03[b]
F-test	**	**
C.V.	5.13	7.87

[1/] Data represented mean ± S.D. of twenty independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

Table 6 Effect of oryzalin on *C. frutescens* L. fruit size

Oryzalin (mg/L)	Fruit length [1/] (cm)	Fruit diameter [1/] (cm)
Control	3.48 ± 0.23[a]	0.34 ± 0.04[a]
10	4.50 ± 0.27[b]	0.37 ± 0.08[ab]
20	4.44 ± 0.32[b]	0.54 ± 0.07[c]
30	3.88 ± 0.20[a]	0.41 ± 0.07[b]
F-test	**	**
C.V.	6.47	15.74

[1/] Data represented mean ± S.D. of twenty independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

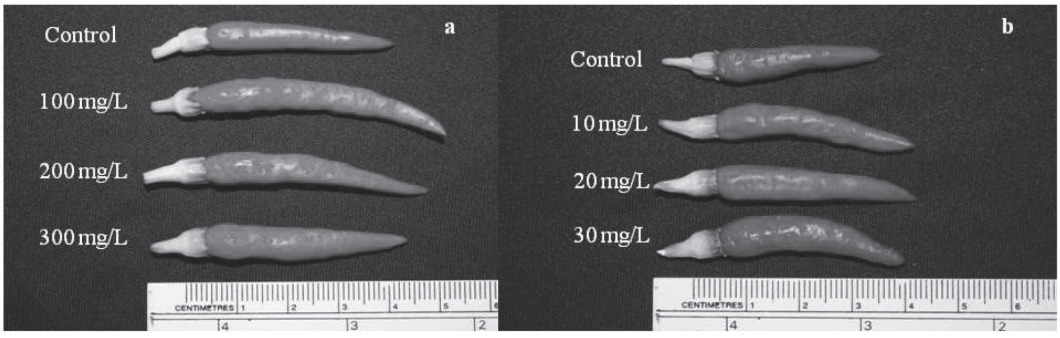


Figure 6 Effects of (a) colchicine and (b) oryzalin on *C. frutescens* L. fruits

Capsaicin Analysis by HPLC

The results from HPLC clearly showed that the retention time of capsaicin chromatogram for the capsaicin standard and the samples were at 7.35 minutes (Figure 7a and 7b). The results of capsaicin quantity showed that polyploidy fruits contained more capsaicin than controls. Both colchicine and oryzalin at high concentrations could induce more amount of capsaicin than controls. In addition, colchicine had more advantage for increasing capsaicin amount than oryzalin. It was found that colchicine at 300 mg/L could induce the highest amount of capsaicin at 4.55 mg/g dry weight, followed by 200 and 100 mg/L colchicine that induced capsaicin at 4.01 and 3.40 mg/g dry weight, respectively, compared with the controls that produced only 3.32 mg/g dry weight (Table 7). Polyploidy fruits induced by oryzalin, at 10, 20 and 30 mg/L produced capsaicin at 3.20, 3.96 and 4.44 mg/g dry weight, respectively, compared with the controls that produced only 3.25 mg/g dry weight (Table 8).

Table 7 Effect of colchicine on capsaicin production in *C. frutescens* L. fruit

Colchicine (mg/L)	Capsaicin [1/] (mg/g dry weight)
Control	3.32 ± 0.06[a]
100	3.40 ± 0.03[a]
200	4.01 ± 0.12[b]
300	4.55 ± 0.04[c]
F-test	**
C.V.	1.78

[1/] Data represented mean ± S.D. of twenty independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

Table 8 Effect of oryzalin on capsaicin production in *C. frutescens* L. fruit

Oryzalin (mg/L)	Capsaicin [1/] (mg/g dry weight)
Control	3.25 ± 0.09[a]
10	3.20 ± 0.10[a]
20	3.96 ± 0.14[b]
30	4.44 ± 0.10[c]
F-test	**
C.V.	2.89

[1/] Data represented mean ± S.D. of twenty independent replicates. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD

** = significant difference at $P \leq 0.01$

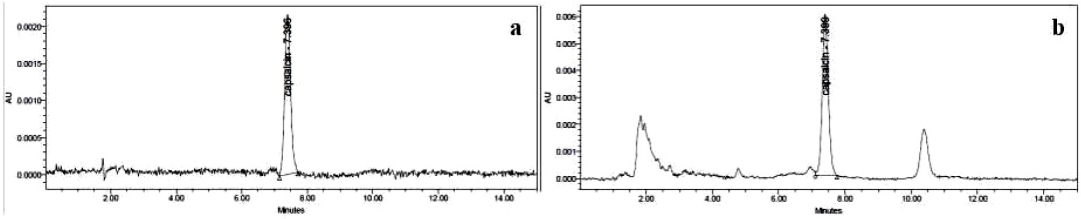


Figure 7 Chromatogram of capsaicin separation by HPLC from (a) capsaicin standard and (b) fruit samples

DISCUSSION

Polyploidy Induction by Colchicine and Oryzalin

In this study, we observed that increasing colchicine and oryzalin concentrations could increase polyploidy rate in *C. frutescens* L. Our results agreed with the work of Lehrer *et al*. (2008) who exposed *Berberis thunbergii* var. *atropurpurea* seeds to colchicine and oryzalin and found that the highest colchicine and oryzalin concentrations of 2% and 0.02% respectively could induce polyploidy seedlings. The successful induction was also reported in *Paulownia tomentosa* (Tang *et al*., 2010), *Dioscorea zingiberensis* (Zhang *et al*., 2010b) and *Miscanthus sinensis* (Petersen *et al*., 2003).

Flow Cytometry Analysis

The flow cytometry identified and screen ploidy level more accurate and faster than conventional methods, such as chromosome counting or stomata length measurements (Nguyen *et al*., 2003). These results also agreed with the studies in *Capsicum frutescens* L. (Raghuvanshi and Joshi, 1964), *Citrullus lanatus* (Jaskani *et al*., 2005), *Colophospermum mopane* (Rubuluza *et al*., 2007) and *Solanum lycopersicum* (Praca *et al*., 2009).

Effects of Colchicine and Oryzalin on Stomata Guard Cell Size

On the initial screen for polyploid, an increase in the size of the stomata guard cells and a reduced density of stomata per unit leaf–area could be used to eliminate much of the diploid background population. Stomata size and density were reported to effectively indicate ploidy levels in *Astragalus membranaceus* (Chen and Gao, 2007), *Alocasia micholitziana* (Thao *et al*., 2003) and *Zizyphus jujube* Mill. Cv. Zhanhua (Gu *et al*., 2005). The method used to measure stomata size and density are simple almost non-destructive and does not require expensive equipments.

Effects of Colchicine and Oryzalin on Fruit Size

In general, the polyploid plants have larger cells size and organs than in diploid plants because polyploidy plant cells have more amount of DNA content and cytoplasm volume that cause the bigger in nuclear size, plant cells and organs than diploid plant cells (Sugimoto-Shirasu and Roberts, 2003). In this study supported by the experiment of Zhang *et al*. (2010a) showed that the diameter and the length tetraploid fruit of *Cucumis melo* were bigger than diploid fruit, diameter and length of tetraploid fruit were 12.93 and 13.37 cm compared to 11.60 and 11.77 cm, respectively in diploid fruit. Similary, Kataoka *et al*. (2010) reported that width and length of tetraploid fruits of *Actinidia arguta* bigger than that of diploid, the result showed that width and length of tetraploid fruits about 2.4 and 2.7 cm compared to diploid fruits of about 1.8 and 2.3 cm, respectively.

Capsaicin Analysis by HPLC

In general, polyploid plants produce more secondary metabolite than diploid plant because the multiplication of chromosome has been proposed to increase metabolic activity and gene expression (Lavania, 2005). This finding agreed with the results from Xing *et al*. (2011), who reported that the quantities of vindolin, catharantine and

vinblastine in tetraploid *catharanthus roseus* (L.) G. Don were higher than diploid. Similary, Banyai *et al*. (2010) found that quantity of Artemisinin in tetraploid *Artemisia annua* L. was higher than diploid.

CONCLUSION

The results presented here demonstrate that the induction of polyploidy in *Capsicum frutescens* L. by colchicine was more effectively than oryzalin. The size of the polyploid fruit was bigger than diploid fruit and the polyploid fruits can produce more capsaicin than diploid.

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