จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย

กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัย

เรื่อง

# การประยุกต์ใช้วิธีการดีเอ็นเอ ดีเมทิเลชันในการชักนำให้เกิดการเปลี่ยนแปลงลักษณะลำต้น ผลผลิตเมล็ด ปริมาณโปรตีน ปริมาณไขมัน และส่วนประกอบของกรดไขมันในถั่วเหลืองพันธุ์ สจ.5

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**จุฬาลงกรณ์มหาวิทยาลัย**

**ทุนวิจัย**

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# รายงานผลการวิจัย

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

**ชื่อโครงการวิจัย** การประยุกต์ใช้วิธีการดีเอ็นเอ ดีเมทิเลชันในการชักนำให้เกิดการเปลี่ยนแปลงลักษณะลำต้น ผลผลิตเมล็ด ปริมาณโปรตีนและปริมาณไขมันและส่วนประกอบของกรดไขมันในถั่วเหลืองพันธุ์สจ.5

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**เดือนและปีที่ทำวิจัยเสร็จ** สิงหาคม 2542

## บทคัดย่อ

เพื่อประเมินผลการลดกระบวนการเติมหมู่เมทิลโดย 5-azaC ต่อการแสดงออกของลักษณะทั่วไป ปริมาณโปรตีน ปริมาณน้ำมัน และส่วนประกอบของกรดไขมัน โดยใช้ถั่วเหลืองพันธุ์สจ.5 เป็นพืชทดลอง การเพาะเมล็ดบนอาหารสังเคราะห์สูตร MS เสริมด้วย 5-azaC ความเข้มข้น 0, 300, 600, 900, 1200, และ 1500 ไมโครโมลาร์ เป็นเวลา 10 วัน พบว่าต้นกล้าที่ผ่านการให้ 5-azaC มีลักษณะปกติ ยกเว้นความสูงที่ลดลงและความยาวรากที่สั้นลง เมื่อเปรียบเทียบกับชุดควบคุม ซึ่งลักษณะดังกล่าวขึ้นกับความเข้มข้นของ 5-azaC ด้วย จีโนมิก ดีเอ็นเอมีปริมาณการเติมหมู่เมทิลต่ำกว่าในชุดควบคุม การปลูกในแปลงทดลอง โดยการนำเมล็ดมาชักนำด้วยสารละลาย 5-azaC ความเข้มข้น 0, 300, 600, 900, 1200, และ 1500 ไมโครโมลาร์ เป็นเวลา 16 ชั่วโมง พบว่า 5-azaC สามารถชักนำให้เกิดลักษณะต้นสูงและจำนวนข้อมากขึ้น โดยไม่มีผลต่อการเจริญเติบโตอื่นๆ ส่วนการศึกษาผลของ 5-azaC ต่อปริมาณโปรตีนและปริมาณน้ำมัน พบว่า ปริมาณโปรตีนและปริมาณน้ำมันไม่ขึ้นกับความเข้มข้นของ 5-azaC โดยพบว่า 5-azaC ที่ระดับความเข้มข้น 1500 ไมโครโมลาร์ ให้ปริมาณโปรตีนสูงสุด สำหรับ 5-azaC ที่ระดับความเข้มข้น 1200 ไมโครโมลาร์ ให้ปริมาณน้ำมันสูงสุด นอกจากนี้ยังพบว่า 5-azaC ไม่มีผลต่อส่วนประกอบของกรดไขมัน จีโนมิก ดีเอ็นเอที่แยกจากใบที่มีลักษณะที่คัดเลือกไว้ เช่น ลักษณะต้นสูง โปรตีนสูง หรือ น้ำมันสูง มีปริมาณ $m^5C$ ไม่แตกต่างจากชุดควบคุม และผลการศึกษาลักษณะต้นสูง ต้นเตี้ย ปริมาณโปรตีนสูง หรือปริมาณน้ำมันสูง จากประชากรรุ่น $M_0$ ผลปรากฏว่าลักษณะดังกล่าวสามารถถ่ายทอดในประชากรรุ่น $M_1$ ได้

**Project Title** The application of DNA demethylation in alteration of phenotypic, seed production, protein lipid content and fatty acid composition in soybean cultivar SJ.5

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**Year** 1999

**Abstract**

To evaluate the demethylating effects of 5-azaC on the general phenotypes, protein content, oil content and fatty acid composition at the whole plant system, soybean plants (*Glycine max* (L.) Merrill) var. SJ.5 have been submitted to 5-azaC treatment. In *in vitro* culture, seeds were cultured in MS media containing 5-azaC at the concentration of 0, 300, 600, 900, 1200, and 1500 $\mu M$ for 10 days. The 5-azaC-treated seedlings exhibited normal morphology in comparison with the controls except that their height and root length were significantly shorter than that of the controls, and were dose dependent. The isolated genomic DNAs showed hypomethylation as compared with that of the controls. In the field culture, seeds were treated for 16 h with 5-azaC solution at 0, 300, 600, 900, 1200, and 1500 $\mu M$. The treatment induced tall plant and high node number, without apparently affecting any other developmental processes. Studies on the effects of 5-azaC on the protein and oil content revealed that the alteration in the average protein and oil content of treated seeds were dose independent. The highest protein and oil content were derived from the dosage of 1500 $\mu M$ and 1200 $\mu M$, respectively. Furthermore, all fatty acid profiles remained unchange. Genomic DNAs isolated from the selected phenotypes such as tall, high protein and high oil content showed no significant difference in the $m^5C$ content in comparison with DNAs from the control samples. The $M_0$ showed tall, short, high protein and high oil content and exhibited the inheritance of such phenotypes in the $M_1$ plants.

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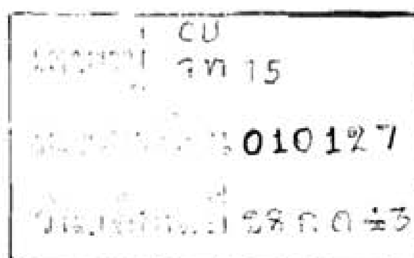

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# ABBREVIATIONS

| | | |
|---|---|---|
| 5-azaC | = | 5-azacytidine |
| A | = | Adenine |
| C | = | Cytosine |
| $C_0$ | = | control plant |
| $C_1$ | = | self-fertilized plant from $C_0$ |
| $CH_3$ | = | methyl group |
| DNA | = | deoxyribonucleic acid |
| EDTA | = | ethylene diamine tetra-acetic acid |
| FA | = | fatty acid |
| FAMEs | = | fatty acid methyl esters |
| g | = | gram |
| $\mu$g | = | microgram |
| $\mu$l | = | microlitre |
| $\mu$M | = | micromolar |
| h | = | hour |
| l | = | litre |
| LA | = | linoleic acid |
| M | = | molar |
| $M_0$ | = | 5-azacytidine treated plant |
| $M_1$ | = | self-fertilized plant from $M_0$ |
| $m^5C$ | = | 5-methylcytosine |
| mg | = | milligram |
| min | = | minute |
| ml | = | milliliter |
| mM | = | millimolar |

| | | |
|---|---|---|
| MUFA | = | monounsaturated fatty acid |
| $^{o}C$ | = | degree Celsius |
| PUFA | = | polyunsaturated fatty acid |
| Q | = | Quanine |
| RNA | = | ribonucleic acid |
| RNase | = | ribonuclease |
| SAFA | = | saturated fatty acid |
| SD | = | standard deviation |
| SE | = | standard error |
| SJ | = | Mae-Jo field crops experiment station |
| T | = | Thymine |
| T-DNA | = | transfer-DNA |

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# CHAPTER I

# INTRODUCTION

## I. Soybean

The soybean, *Glycine max* (L.) Merrill, a member of the family Leguminosae, subfamily Papilionoideae which is one of the world's most important agronomic crops as a source of high oil and protein meal. The average oil content of the soybean is 18 to 22% on a moisture-free basis, it contains 16% saturated fatty acid (palmitic acid; C16:0 and stearic acid; C18:0) and 84% unsaturated fatty acid (oleic acid; C18:1, linoleic acid; C18:2, and linolenic acid; C18:3). The protein content of the soybean varies from 38% to 45% on a moisture-free basis.

The 78% of soybean product is sent to factory for soybean oil production. The 22% soybean is for direct consumption and seed. The example of direct consumption is food, oil, soybean milk, sauce, butter, flour etc. For extracted soybean, there are 15.5% of soybean oil and 77% of meal. The meal is used as raw material in feedstuff industry. The 70% of soybean oil is used for cooking and another 30% is used in other industries such as lubricant, hydraulic oil, or biofuel. Moreover, soybean is the enriched plant for soil due to *Rhizobium japonicum* in soybean root that can fix nitrogen (Nitrogen fixation) from air. Once it decays, soybean and *R. japonicum* increase nitrogen level and other beneficial organics which are beneficial for the next plantation.

When considering planting area and productivity in Thailand, it has been shown that the tendency of gradually decreasing soybean production. The productivity of not more than 400,000 tons from within 2 million rai area is not enough to serve the

full domestic demand. Therefore, import of soybean seed and meal from overseas increases every year with the value up to thousand of millions baht. In 1997, Thailand imported 869,370 tons of soybean seeds which cost 8,611 million baht from USA, Brazil, and Argentina; 1,109,126 tons of meal cost 10,726 million bahts from Brazil and India. Thailand exported merely 226 tons of soybean seeds cost 4.17 million bahts to neighboring countries (information from IT center, Department of Agriculture). Since domestic productivity of soybean could not serve such high demand, the Thai government allowed the import of soybean seeds and meal without limitation on the quantity. The cabinet had finally approved the plan for development of the soybean production (1993-1997) on 21 July 1992. The principle of the new policy had been changed from "Increasing production of soybean to fulfill need within country instead of importing from outside country" to " Strengthen the quality of soybean production and reduce the budget for production per unit". There were 4 plans for operation, research development, production development, transforming development and marketing development.

Regarding to the problem of soybean import from overseas and loss of lots of foreign currency, the problem can be solved by increasing the productivity per rai and improving the soybean seed quality. To increase the productivity, we can do by expanding more plantation area or upgrade the soybean. To improve quality of seed e.g. adjusting quantity and quality of protein and oil, we can improve by manipulation of the following techniques; e.g. plant breeding, induced mutation and genetic engineering.

Classical breeding efforts had traditionally focused on improving oil content. Selected plants had considerably altered seed oil compositions, which indicated that plants could tolerate a wide variation in fatty acid composition of storage lipids. Examples of progress through breeding efforts included the development of rapeseed with a low erucic acid (C22:1) content (canola), which had established rapeseed oil as

a prime edible oil (Stefansson, 1983). Similar efforts had resulted in the reduction of the level of polyunsaturated 18-carbon fatty acids in soybean (Wilcox and Cavins, 1985; Martin and Rinne, 1986; Graef *et al.*, 1988), and linseed oils (Green and Marshal, 1984). Most of the genetic variation in seed lipid fatty acid composition appeared to involve the presence of an allele of a gene that disrupted normal fatty acid metabolism and led to an accumulation of intermediate fatty acid products in the seed storage lipids (Downey, 1987). However, it seemed likely that, because of the inherent limitations of this approach, many other desirable changes in seed oil fatty acid composition might require the direct application of genetic engineering methods. Unfortunately, as in many other aspects of plant biology, the lack of specific information about the biochemistry and regulation of lipid metabolism made it difficult to predict how the introduction of one or a few genes might usefully alter seed lipid synthesis. An additional problem arose from the fact that many of the key enzymes of lipid metabolism were membrane-bound, and attempts to solubilize and purify them from plant sources have not been successful (Lemieux *et al.*, 1990).

Treating soybean seeds with gamma rays, resulted in 3 high oil content lines. Their oil contents ranged from 23.91% to 24.07%. Application of a chemical mutagen (ethylmethane sulphonate, EMS) gave some mutants with high protein content (i.e. more than 45%) and high oil content. Thus, EMS was a very good mutagen for inducing mutations with high protein and oil content in soybean (QIU and GAO, 1986; James and Dooner, 1990).

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Transformation of genetic material into plant tissues including soybean plant had been attempted by several methods; direct chemically induced DNA transfer in clonal cell-lines of wheat (Muller, Lorz, and Lutticke, 1996), electroporation in rice (Dekeyser *et al.*, 1990), microprojectiles in pearl millet (Lambe, Dinant, and Matagne, 1995), or by the more widely used *Agrobacterium*-mediated T-DNA transfers (Chee, Fober, Slightom, 1989). Recently the techniques for manipulating the plant genome

either via direct gene transfer or by use of *Agrobacterium tumefaciens* as a vector had been extended to several agronomically important species, such as tomato, oil rapeseed , rice, maize, pea, soybean and tobacco (Puonti-Kaerlas, Stabel and Eriksson, 1989; Puonti-Kaerlas, Eriksson and Engstrom, 1990; Baldes, Moos, and Geider, 1987; Zhang *et al.*, 1996).

Transgenic followed by classical breeding approaches had been used to alter the degree of desaturation and to reduce or increase the chain length of fatty acids (Knauf, 1987; Ohlrogge, 1994). A major challenge in modifying the composition of plant storage oil was to change the degree of fatty acid desaturation. The first success was an increase in the stearic acid content of canola plant at the expense of the oleic acid ($\Delta$9 C18:1) content. This achieved by down-regulation of the $\Delta$9-stearoyl-ACP desaturase ($\Delta$9 DES) activity by antisense repression (Knutzon *et al.*, 1992).

A complementary DNA(cDNA) encoding $\Delta$9DES from *Brassica rapa* had shown to be expressed in the opposite orientation (antisense) under the control of a napin gene promoter in seeds of rapeseed. The amount of desaturase protein present in these transgenic seeds was drastically reduced, resulting in a decreased formation of C18:1. As a consequence, up to 40% of the fatty acid content was in the form of stearate (Topfer *et a*l., 1995). In another study, an increase in stearic acid content to 11% was achieved by overexpression of a long chain-specific thioesterase (TE) gene from soybean. Transformation of soybean with an additional acyl-ACP thioesterase gene led to reduction of the thioesterase activity and an approximately 2-fold reduction in saturated fatty acid levels in somatic embryos (Yadav *et al*, 1993; Topfer *et al.*, 1995; Ohlrogge, 1994).

Antisense repression of the $\Delta$12-oleate desaturase ($\Delta$12DES) in transgenic rapeseed resulted in an increase in oleic acid up to 83%. Similar results (79% C18:1 as compared with 22% in controls) had been obtained with antisense expression of a $\Delta$

12DES in soybean. Furthermore, the soybean linoleic acid content had increased 10% by antisense suppression of linolate desaturase ($\Delta$15DES) synthesis. All of these results indicated that it might be possible to increase oleic acid content to 90% by seed specific antisense inhibition of the respective desaturase (Topfer *et al.*, 1995). The *Agrobacterium*-mediated gene in soybean transfer would appear to have some advantages because soybean is a dicotyledonous plant species and thus was susceptible to *Agrobacterium* infection (Chee *et al.*, 1989). However, success with this approach had been limited, due to the lack of an efficient transformation system and the inability to regenerate plants from transformed callus or protoplasm (Knauf, 1987; Chee *et al.*, 1989).

These examples provide convincing evidence that plant oil composition can be substantially altered by either inserting or deleting the expression of a single enzyme activity (Topfer *et al.*, 1995). In recent studies, DNA demethylation has been proven to be a new tool for breeding a new plant cultivar with desired phenotype (Sano *et al*, 1989, 1990; Cherdshewasart, *et al.*, 1996). Application of this novel technique for the improvement of soybean production was a challenging approach especially in the recent situation that the genetically modified plant products are rejected or protested by European consumers.

**II 5-azacytidine**

An experimental tool for demethylation is 5-azacytidine (5-azaC), a potent demethylating agent which leads to subsequent hypomethylation of DNA sequence.

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**5-Azacytidine : a chemical tool for demethylation**

If methylation differences play a crucial role in the regulation of differentiation, it should be possible to alter gene expression by demethylating DNA. This has been achieved in a number of case by treating cells with the drug, 5-azacytidine(5-azaC), a potent demethylating agent (Jones, 1984) **(Figure 1)**. This compound was originally developed as a cancer chemotherapeutic agent (Jones, 1985) which is a cytosine analog with a nitrogen atom replacing the carbon atom at position 5 of the pyrimidine ring. When this compound is incorporated into the DNA of dividing cells, and probably binds to the maintenance methylase in an irreversible manner, the enzyme is sequestered and unable to maintain the proper methylation state (Jones and Taylor, 1980) which leads to subsequent hypomethylation of the DNA sequence (Santi *et al.*, 1983; Sabbah, Raise and Tal, 1995). One generation in the presence of the compound is sufficient to cause much of the DNA to become hemimethylated, whereas double-strand demodification can be observed after the next division (Razin and Cedar, 1991).

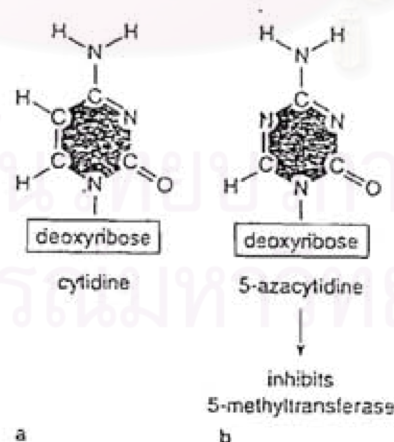


**Figure 1** Cytidine(a) and 5-azacytidine(b) 5-azacytidine irreversibly inhibits 5-methyltransferase, the enzyme that adds methyl groups to the cytosine in DNA (Wolfe, 1993).

The analogue is thought to act by incorporation into the DNA where it functions as a powerful inhibitor of the methylation of newly incorporated cytosine residues (Razin and Riggs, 1980; Jones and Taylor, 1980; Jones, 1986). It is quite clear that the fraudulent nucleotide must be incorporated into DNA in order to inhibit methylation (Jones and Taylor, 198; 1981).The result of this incorporation is a loss of active DNA methyltransferase activity (Taylor and Jones, 1982) which is due to the formation of a tight noncovalent complex (Jones, 1986) or a covalent linkage (Santi, Norment, Garrett, 1984) between the enzyme and 5-azacytosine residues in DNA. The compound is also cytotoxic (Jones, 1985) and can induce chromatid aberrations in *Vicia faba* (Fucik, Michaells, and Rieger, 1970) and morphological mutants in rice, maize, triticale, soybean , flax and *Arabidopsis thaliana* (Sano *et al*, 1989, 1990; Ngampanya, 1996; Heslop-Herrison, 1990; Katoh, Maekawa, Sano, 1993; Fieldes, 1993; Finnegan *et al*., 1996) and can affect differentiation (Jones and Taylor, 1980) in an organism that lacks 5-methylcytosine (Jones, 1985). 5-AzaC also show reactivation efficacy in silencing transgenic seedlings induced by demethylatio (Cherdshewasart, 1991a).

In rice, the predominant phenotypic effect was a decrease in plant height. Rice plants grown from treated seeds were initially short and some retained the "dwarf" stature through maturity. These transmitted the dwarf phenotype to some, but not all, of the first generation progenies, which in turn passed on the dwarf phenotype to the second generation. A generalized, 16% reduction in the methylation of the genomic DNA was induced by 5-azaC in the plants grown from treated rice seed and in both the normal and dwarf progenies of these plants. These studies provide the first indication of a heritable relationship between induced differences in DNA methylation and induced differences in plant growth and development. Also, in maize flax and *Arabidopsis thaliana*, the treatments induced a decrease in plant height and reduction

in the methylation of the genomic DNA (Sano *et al.*, 1989, 1990; Cherdshewasart *et al.* , 1996; Fieldes, 1993 ; Finnegan *et al.*, 1996).

In triticale, the treatments concomitantly induced a number of phenotypic alterations, including an increase in plant height, a decrease in the duration of maturity, and an increase in tillering, and a site-specific alteration in methylation status (Heslop-Herrison, 1990).

In 1993, treating soybean seed with 5-azaC (10-100 $\mu$g/ml) resulted in an increase in the frequency of mutational spots (yellow, dark green and twin)on the leaves (Katoh *et al.*, 1993).

One property of 5-azaC is its ability to alter the differentiated state of certain eukaryotic cells. One example is the formation of twitching muscle cells from non muscle mouse embryo (10T1/2) cell lines, one to two weeks after exposure to the analog (Constantinides *et al*, 1977). The new muscle cells are biochemically and functionally identical to bona fide myocytes and in many instances, remain phenotypically stable in the absence of further drug treatment (Gurdon *et al*, 1984).

In addition to the induction of wide-ranging phenotypic changes which presumably reflects the concerted switching of many genes, 5-azaC has marked effects on the activity of individual selectable gene products. The expression of certain housekeeping genes, such as thymidine kinase, are increased as much as $10^5$-$10^6$ fold following the exposure to the analog (Harris, 1982). Alteration in DNA methylation may be one of the mechanisms regulating the tissue-specific expression can be shown in the tissue transglutaminase gene (Lu and Davies, 1997) and epigenetic control of sexual phenotype in a dioecious plant, *Melandrium album*. The data indicate that female sex suppression in *M. album* XY males is dependent on methylation of specific DNA sequences and can be heritably modified by hypomethylating drugs (Janousek, Siroky, Vyskot, 1996).

Several lines of evidence support the idea that inhibition of DNA methylation is indeed the mechanism of drug action :

1. biologically active doses of 5-azaC are strong inducers of gene expression and potent inhibitors of DNA methylation (Jones, 1986).

2. the effects on cell differentiation are specific for position 5 of cytosine and can be mimicked by other analogues such as pseudoisocytidine and 5-fluorodeoxycytidine which also inhibit methylation and change the differentiated state of the cells (Jones and Taylor, 1980; Jones, 1986).

3. the changes in gene expression are often heritable for many generations in the absence of further drug treatment (Jones, 1985, 1986).

4. the activation frequencies observed are sometimes 5-6 orders of magnitude greater than those expected for the activity of mutagenic agents (Jones, 1985, 1986).

5. the effects of 5-azaC on methylation have been localized to genes which become transcriptionally activated (Jones, 1986).

6. genes which have been inactivated by methylation before introduction into eukaryotic cells can be reactivated by 5-azaC treatment (Simon *et al.*, 1983).

**Aims of the study**

This study was designed to test the hypothesis that 5-azaC can induce hypomethylation in soya seedlings and should resulted in phenotypic changes start from seedling phenotype to biochemical pathway and resulted in alteration of seed production oil and protein contents.

# CHAPTER II

# MATERIALS AND METHODS

## Materials

### A. Plant material

Plant model used throughout this study is soybean (*Glycine max* (L.) Merrill) cv. SJ. 5, kindly supplied by Dr. Jinda Janoon, Field Crop Research Institute, Department of Agriculture and Seed laboratory, Department of Agriculture.

### B. Materials for plant tissue culture

- glass bottles (1.5 x 3.5 in)
- forcep
- petridish
- millipore filter (0.45 micron)

### C. Materials for plant growing

- sand
- soil
- field plot at the Department of Botany, Faculty of Science, Chulalongkorn University (9 x 10 m).

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**D. Materials for DNA extraction**

- pestle and mortar
- Eppendorf tube
- corex tube or any 15 ml centrifuge tube
- ice box
- spatula
- parafilm
- cut-tip
- autopipette

**E. Materials for lipid extraction and fatty acid analysis**

- thimble
- cup

**F. Material for protein extraction**

- digestion tube

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**Chemicals**

**a. Chemicals for plant sterilization**

- 15% Clorox
- Tween 20

**b. Chemicals for plant media preparation**

All chemicals used in MS media (Murashige & Skoog, 1962) were purchased from Sigma Chemical Co., Ltd. (see Appendix)

**c. Chemical for DNA demethylation**

5-azacytidine(4-Amino-1-β-D-ribofuranosyl-5-triazin-2[1H]-one) ($C_8H_{12}N_4O_5$) FW. 244.2 were purchased from Sigma Chemical Co., Ltd.

**d. Chemicals for DNA extraction and DNA methylated base analysis**

- liquid nitrogen (from TIG, Thailand)
- urea extraction buffer
- phenol:chloroform:isoamylalcohol (25:24:1, v/v/v)
- 4.4M ammonium acetate pH 5.2
- iso-propyl alcohol
- TE 10mM
- 70% ethanol
- 20 mg/ml RNase

**e. Chemicals for lipid extraction and fatty acid analysis**

- petroleum ether
- hexane
- methanol:hexane (4:1, v/v)

- acetylchloride
- 6% $K_2CO_3$
- gases (Thonburi-Wattana, Bangkok)

**f. Chemicals for protein determination**

- glycine (standard)
- potassium sulphate and copper sulphate (catalysts)
- concentrated sulfuric acid (98%), N-free
- 4% boric acid
- 40% sodium hydroxide, N-free
- 0.1 N Hydrochloric acid
- mixed indicator (methyl red and bromocresol green)

**Instruments**

The studies were carried out at the Department of Biology, Faculty of Science, the Fats and Oils Research Center (FORC), Department of Transfusion Medicine, Faculty of Allied Health Sciences and Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. The main instruments employed in the experiment are listed below:

- Hot air oven
- Larminar air flow
- Culture room (25°C under 16 h illumination with fluorescent light per day)
- pH meter

- Autoclave
- Eppendorf centrifuge
- Hot plate
- Soxtec system HT22
- HPLC Shimadzu LC-6A system
- GLC 8000 series, Fisons Instrument, Italy
- Spectrophotometer, Shimadzu, Japan
- Magnetic stirrer
- Digestion unit Buchi
- Distillation unit Buchi 315
- Titration unit

**Methods**

**5-Azacytidine treatment and general growing conditions for the seeds** were carried out by two different protocols:

**1. *In vitro* culture**

Soybean seeds were surface sterilized by soaking in a 15% Clorox solution with a few drops of TWEEN 20 for 15 min. followed by several rinses with sterile distilled water. They were cultured in hormone free MS media (See Appendix) containing various 5-azacytidine concentrations (Cherdshewasart, 1991a) under the continuous dark condition for 3 days and then germinated at 25 °C under 16 h illumination with fluorescent light (2800 lux) per day for 7 days. After germinating for 10 day, seedlings were harvested ,plant height and root length were recorded ,cleaned

with sterile distilled water and immediately stored at -70 °C to stabilized the cells for DNA preparation.

**2. Field trial**

Soybean seeds were soaked in 5-azacytidine solution at various concentrations (Cherdshewasart, 1991a) for 16 h under the continuous dark condition and then were extensively washed with water to remove 5-azacytidine. They were grown in the field during May to August 1998. After the first flowering day, the leaves were harvested , cleaned with sterile distilled water and immediately stored at -70°C to stabilize the cells. After mature stage(95-100 days), the seeds were collected counted and weighed and subsequently extracted for the analysis of the protein content, the lipid content and fatty acid profiles.

The plant samples from *in vitro* and field grown were kept at -70°C and then used for the preparation of DNA. To study the levels of DNA methylation after treatment with various 5-azacytidine concentrations at various duration, the DNA was extracted from seedlings and leaves.

**3. The preparation of DNA from plant cells**

The DNA was prepared by The osmotic lysis method (Cherdshewasart, 1991b). Fresh seedlings and matured leaves were collected for genomic DNA preparation. The samples were ground in the presence of liquid nitrogen into fine powder using mortar and pestle. The powder was transferred to a 15-ml corex tube and 6 ml urea extraction buffer and 7 ml phenol:chloroform:isoamylalcohol (25:24:1) were added and homogenized. Then, the tube was capped and shaked until the mixture was

homogeneous ,and stand at room temperature for 15 min. The homogenate was mixed thoroughly and centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was carefully transferred to a new 15-ml centrifuge tube using a 1ml cut-tip. The supernatant was mixed with 1 ml of 4.4M ammonium acetate pH 5.2. The DNA was precipitated with 7 ml isopropanol and resuspended in an appropriate volume of TE. The precipitate was dissolved at room temperature for 15-30 min with occasional shanking. Two $\mu l$ RNase 20 mg/ml was added, mixed and incubated at 37°C for 30 min. The DNA was precipitated again with the aid of 100 $\mu l$ of 4.4M ammonium acetate pH 5.2 and 1.1 ml of isopropanol. The pellet was washed briefly with 500 $\mu l$ 70% ethanol. After the removal of the ethanol, the DNA pellet was allowed to partially dry and then resuspended in an appropriate volume of TE and kept at 4°C.

#### 4. Determination of methylated cytosine

The purified DNA was completely hydrolyzed to individual base with formic acid. The DNA hydrolysates were subjected to methylated cytosine analysis by reversed phase HPLC (Ngernprasirtsiri, Kobayashi, and Akazawa, 1988)

#### 5. The extraction of lipids in soybean seeds

Five g of dried samples in a mash thimble was placed in the middle part of the Soxtec. The wired cup was filled with petroleum ether and attached to a tap. The dried sample in a mash thimble was immersed into boiling petroleum ether and refluxed for 90 min. The thimble was raised and washed with petroleum ether for 45 min. Finally, the solvent from the wired cup was evaporated and the increase in weight was measured.

**6. Fatty acid analysis**

Fatty acid compositions of lipids were determined after being transesterified according to the technique described by Lepage and Roy (1986). Twenty $\mu l$ of lipid was transferred into a leak-proved Teflon lined screw-capped borosilicate test tube and 2 ml of methanol:hexane (4:1,v/v) was immediately added with magnetic stirrer. The saponification of lipids and methylation of liberated fatty acids were performed by using acetylchloride. Two hundred $\mu l$ of acetylchloride was slowly added in the vortexing sample tube, the tube was closed and stirred at 100 °C for 1 h in the stirring dry block. The tube was cooled, 5 ml of 6%$K_2CO_3$ solution was added to stop the reaction and to neutralize the mixture. The mixture was centrifuged to separate the layers. Finally, the hexane upper phase (FAME's inside) was carefully transferred into an Eppendorf tube.

A 1 $\mu l$ aliquot of the hexane upper phase was injected into the 8000 series Gas chromatography with a flame ionization detector. The separation of fatty acids methylesters (FAME's) were performed in a 30 m fused silica capillary column with an internal diameter of 0.32 mm and wall coated with 0.25 $\mu m$, DB-23 P/N 123-2332 (J&W Scientific, USA). Helium was used as carrier gas (1.5 ml/min at 80°C). The split ratio was 10:1.The injection port temperature was 250°C and the detector was set at 300°C. The column temperature was initiated at 80°C and after sample injection the temperature was programmed to 180°C with an increase rate of 10°C/min and held isothermally for 15 min. The second increment was 4°C/min to 220°C. The latter temperature was maintained constant for 15 min.

## 7. The quantitation determination of protein in soybean seeds

Protein content was determined by Kjeldahl procedure. It was divided to 3 parts :digestion, distillation and titration

### Digestion

A sample had to be dried, ground and homogenized. The dried samples were weighed using a balance accurate to 0.1 mg and were quantitatively delivered into the digestion tube. Seven g catalyst (95% $K_2SO_4$ + 5%$CuSO_4$) and 15 ml concentrated sulphuric acid were added to the sample. The tubes were inserted into the digestion unit (370-400 °C). The digestion time is depend on the type of sample, the quantity of the sample and on the digestion mixture (acid and catalyst). In this study, the colour changed to light green in about 1 h. The tubes with the cooled sample were removed from the digestion unit and approximately 50 ml of deionized distilled water was added to the digestion tube.

### Distillation

The receiving flask containing 100 ml of 4% boric acid solution and 2-3 drops of mixing indicator (methyl red and bromocresol green) were filled into a 250 ml flask. Subsequently, the prepared distillation receiver was placed below the distillate outlet and the diluted sample was neutralized. For this purpose, 40% sodium hydroxide was added until the sample shows a distinct change of color to brown or blue. As soon as 100 ml of distillate had been collected, the distillation was then stopped and the

residue from the sample was drained by suction. The final distillate was now ready for titration.

Titration

When using boric acid as receiver, the ammonia was titrated directly with hydrochloric acid. In the case of titration, back titration was done until there is a change of color from green to red.

*Calculation*

$$\% N_2 = \frac{V \times N \times 1400}{E \text{ mg.}}$$

V = Consumption of acid (titration)

N = Normality of the acid

E = Quantity of sample in mg.

$\%$ Protein = $\% N_2$ x conversion factor of soybean

= $\% N_2 \times 5.71$

**8. Genetic analysis of mutants**

The $M_0$ plants were allowed to self-pollinate and the $M_1$ seeds from each $M_0$ plant were individually collected and analyzed for the protein content, the lipid content and fatty acid profiles. The $M_1$ seeds of desired phenotypes were planted to test for the stability and heritability of such phenotypes in the $M_2$ generation during November 1998 - February 1999).

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**9. Statistical analysis**

Data were analyzed as a completely randomized design. All statistical significances were calculated by one-way analysis of variance (ANOVA) by programme SPSS and least square means (LS means) procedures. The significance level was taken to be $\alpha = 0.05$

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# CHAPTER III

# RESULTS

## A. The effects of 5-azacytidine on *in vitro* soybean seedlings

### 1) The effects of 5-azacytidine on the height

The effects of 5-azaC on *in vitro* soybean seedlings were evaluated by the comparison of the maximum, minimum, and mean value $\pm$ SD of height in SJ.5 submitted to 5-azaC treatment at the concentration of 0, 300, 600, 900, 1200, and 1500 $\mu$M for 10 days. The heights were measured. As shown in **Table 1**, the results showed that the seedlings grown from 5-azaC-treated seeds were significantly shorter than the control seedlings. As the treatment concentration increased, there was a significant decrease in the mean stem height. The graphic presentation derived from **Table 1** is shown in **Figures 2 and 3**. The figures showed significant reduction of height with the increasing of 5-azaC concentration. **Figure 4** showed the photograph of dwarf seedlings compared to the control seedlings, which illustrated some of the characteristics scored in **Figures 2 and 3**.

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**Table 1** *In vitro* effects of 5-azaC on the height of the seedlings cultured in MS containing 0, 300, 600, 900, 1200, and 1500 $\mu M$ 5-azaC for 10 days.

| SJ.5 Mo | | 5-azacytidine ($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| height(cm.) | max. | 17.10 | 11.50 | 10.50 | 8.20 | 6.80 | 6.50 |
| | min. | 2.60 | 2.50 | 3.00 | 2.00 | 1.70 | 2.00 |
| | mean | $11.75^{d}$ | $7.63^{c}$ | $6.02^{b}$ | $4.93^{ab}$ | $4.35^{a}$ | $3.41^{a}$ |
| | $\pm$SD | 3.64 | 2.61 | 2.28 | 1.95 | 1.26 | 1.30 |
| | number of plant | 21 | 21 | 21 | 21 | 21 | 21 |
| | no.shorter | 8 | 21 | 21 | 21 | 21 | 21 |

Note : The values are the stem heights (cm) at 10 days and the number of plants that were classified as shorter than that of control.

: The different letters shown as a, b, c, d are significant differences ($p < 0.05$)

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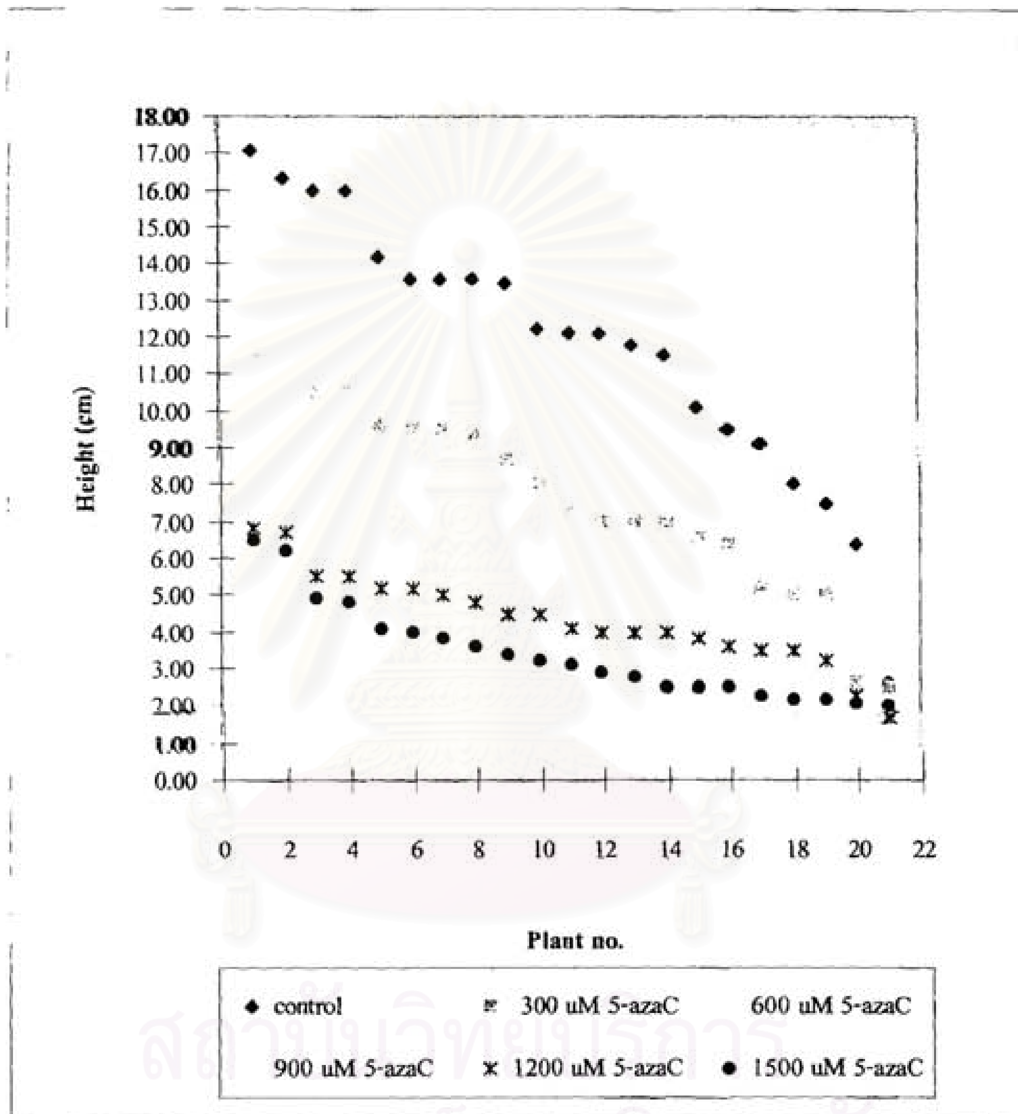


**Figure 2** The height phenotype of the seedlings cultured in MS containing 0-1500 $\mu M$ 5-azaC for 10 days.

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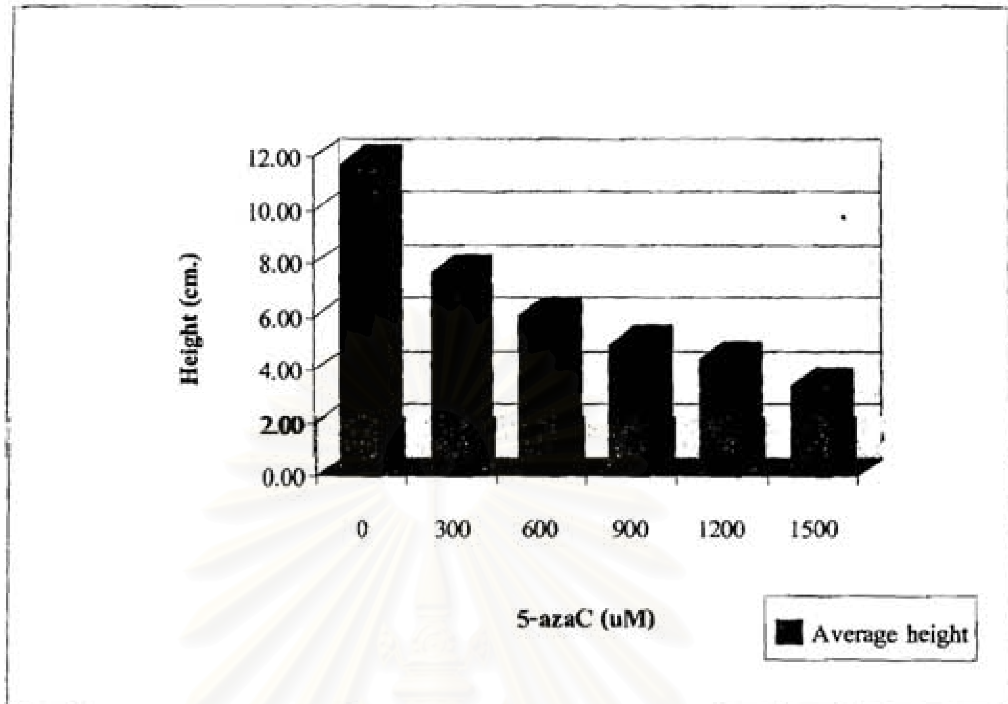


**Figure 3** The response of *in vitro* mean stem height to the treatments of increasing of 5-azaC concentrations.

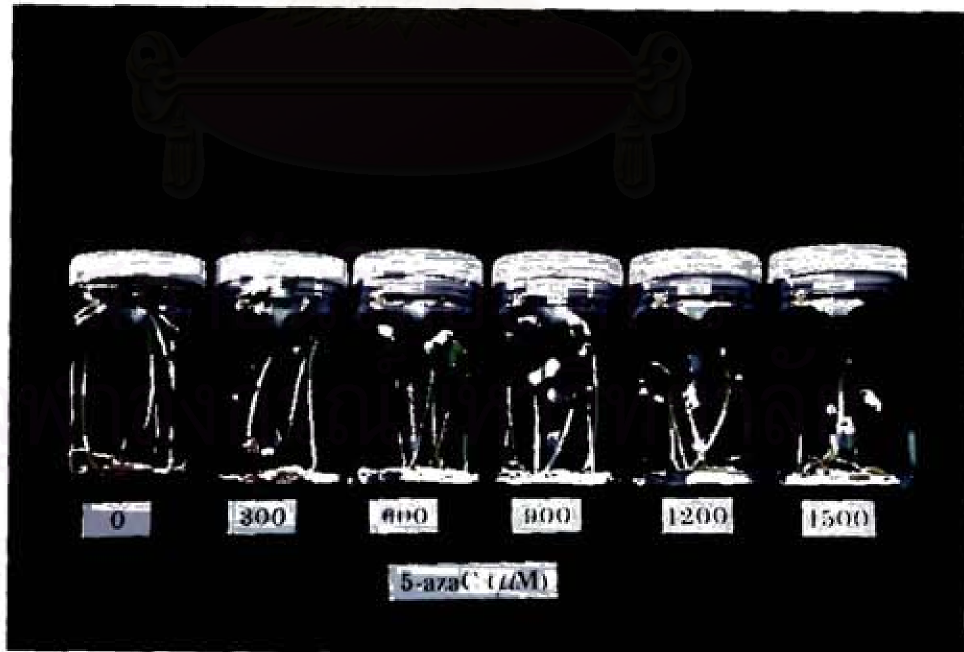


**Figure 4** The phenotype of the dwarf compared to the control seedlings (leftmost).

**2) The effects of 5-azacytidine on the root length**

The effects of 5-azaC on *in vitro* soybean seedlings were also evaluated by the comparison of the maximum, minimum and mean value $\pm$ SD of root length in SJ.5 submitted to 5-azaC treatment at the concentration of 0, 300, 600, 900, 1200, and 1500 $\mu M$ for 10 days (**Table 2**). The data showed that root length of seedlings grown from 5-azaC treated seeds were significantly shorter than that of the control seedlings. As the treatment concentration increased, there was a significant decrease in the root length. As shown in **Figures 5 and 6**, the figure showed significant reduction of the root length with the increasing of 5-azaC concentrations. **Figure 7** showed root phenotype of treated seedlings compared to the control seedlings.

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**Table 2** *In vitro* effects of 5-azaC on the root length of the seedlings cultured in MS containing 0, 300, 600, 900, 1200, and 1500 $\mu M$ 5-azaC for 10 days.

| SJ.5 Mo | | 5-azacytidine ($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| root length(cm.) | max. | 17.80 | 10.10 | 9.70 | 7.20 | 6.20 | 5.00 |
| | min. | 8.40 | 2.70 | 2.40 | 2.20 | 2.30 | 0.50 |
| | mean | $13.20^{d}$ | $7.64^{c}$ | $6.25^{c}$ | $4.60^{b}$ | $4.34^{ab}$ | $2.84^{a}$ |
| | ± SD | 2.02 | 1.89 | 1.72 | 1.37 | 1.04 | 1.23 |
| | number of plant | 21 | 21 | 21 | 21 | 21 | 21 |
| | no. shorter | 10 | 21 | 21 | 21 | 21 | 21 |

Note: The values are the root length (cm.) at 10 days and the number of plants that were classified as shorter than that of control.

: The different letters shown as a, b, c, d are significant differences ($p < 0.05$)

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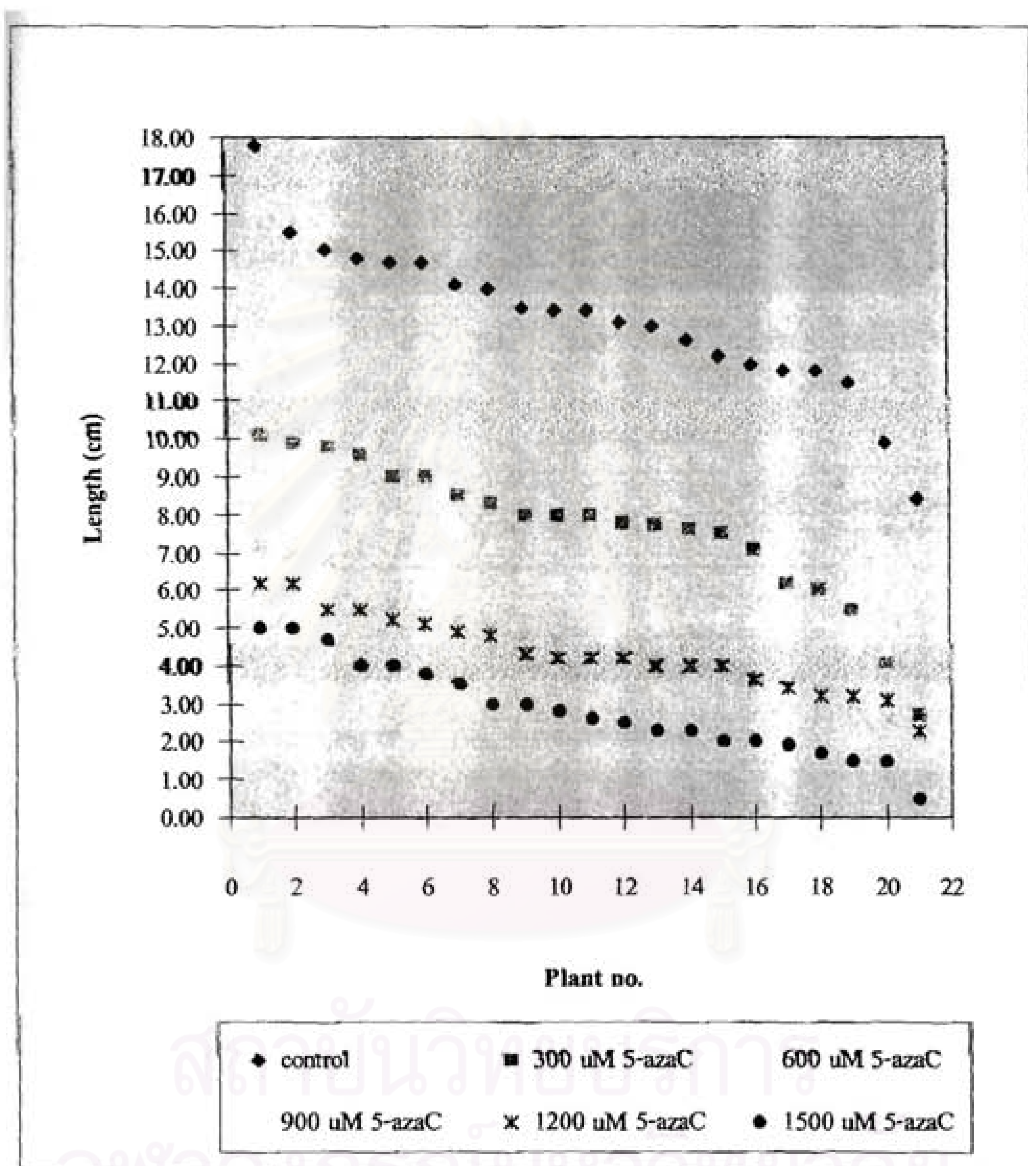


**Figure 5** The root phenotype of the seedlings cultured in MS containing 0-1500 $\mu M$ 5-azaC for 10 days.

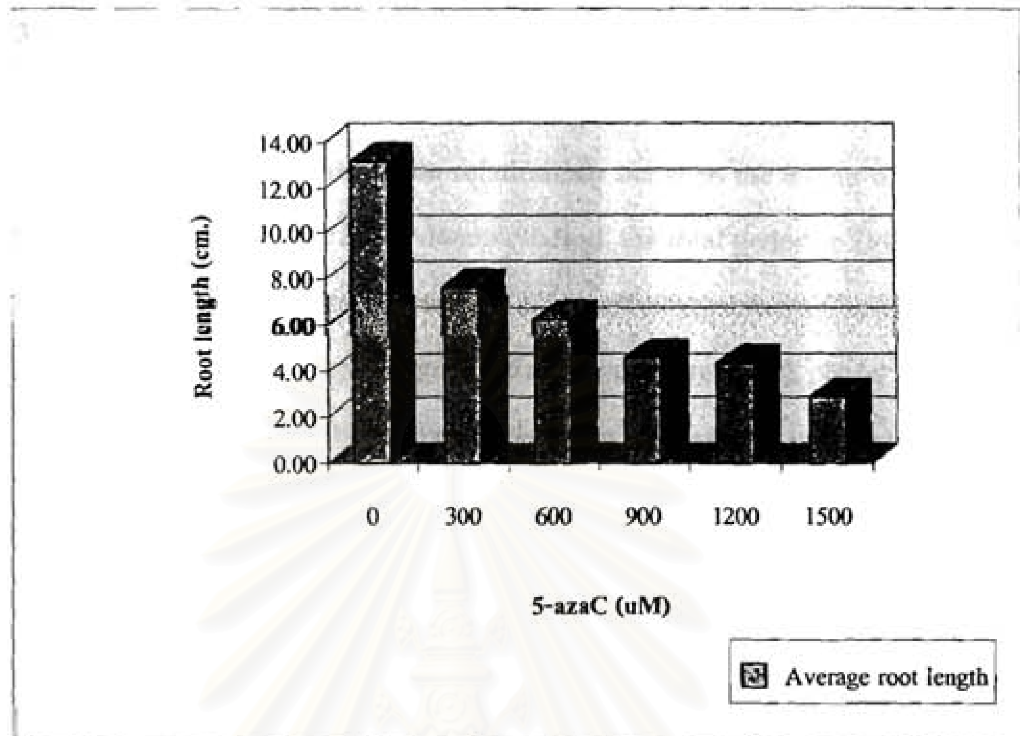


**Figure 6** The response of *in vitro* mean root length to the treatments of increasing of 5-azaC concentrations.

**Figure 7** The root phenotypic change *in vitro* of 5-azaC treated compared to the control seedlings (leftmost)

### 3) Methylated cytosine analysis

In order to determine the relationship between the extent of 5-azaC treatment and the level of genomic DNA demethylation, the total genomic DNA was hydrolyzed into purine and pyrimidine bases and the 5-methylcytosine ($m^5C$) content, expressed as percent of total C and $m^5C$, were determined by reversed phase HPLC analysis as shown in **Table 3**. Their characteristics were defined in **Table 4**. The results suggested that 5-azaC induced demethylation of genomic DNA and consequently a reduction in plant stem length and root length as shown in **Figure 8.**

**Table 3** The effects of different 5-azaC concentrations on the genomic DNA methylation of soybean seedlings.

| DNA source | Percentage of | | | $m^5C$/total C (%) |
|---|---|---|---|---|
| | Total C | C | $m^5C$ | |
| Control | $16.60 \pm 0.12^{b}$ | $12.27 \pm 0.04$ | $4.33 \pm 0.10^{bc}$ | $26.08 \pm 0.41^{bc}$ |
| 300 $\mu M$ 5-azaC treated | $16.63 \pm 0.07^{b}$ | $12.26 \pm 0.02$ | $4.37 \pm 0.04^{bc}$ | $26.28 \pm 0.18^{c}$ |
| 600 $\mu M$ 5-azaC treated | $16.67 \pm 0.08^{b}$ | $12.27 \pm 0.01$ | $4.40 \pm 0.08^{c}$ | $26.39 \pm 0.32^{c}$ |
| 900 $\mu M$ 5-azaC treated | $16.57 \pm 0.02^{ab}$ | $12.30 \pm 0.01$ | $4.27 \pm 0.03^{ab}$ | $25.75 \pm 0.16^{ab}$ |
| 1200 $\mu M$ 5-azaC treated | $16.55 \pm 0.03^{ab}$ | $12.29 \pm 0.02$ | $4.26 \pm 0.01^{ab}$ | $25.74 \pm 0.03^{ab}$ |
| 1500 $\mu M$ 5-azaC treated | $16.47 \pm 0.01^{a}$ | $12.26 \pm 0.04$ | $4.22 \pm 0.02^{a}$ | $25.60 \pm 0.15^{a}$ |

Note : The results are expressed as mean $\pm$ SD. of three determinations.

: The different letters shown in the same column as a, b, c are significant differences ($p < 0.05$)

**Table 4** The relationship between phenotypic changes and the $m^5C$ content of the seedlings cultured in MS containing 0,300,600, 900, 1200, and 1500 $\mu M$ 5-azaC for 10 days.

| | 5-azaC ($\mu M$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 300 | 600 | 900 | 1200 | 1500 |
| mean height(cm) | $11.75^{d}$ | $7.63^{c}$ | $6.02^{b}$ | $4.93^{ab}$ | $4.35^{a}$ | $3.41^{a}$ |
| ± SD | 3.64 | 2.61 | 2.28 | 1.95 | 1.26 | 1.30 |
| mean root length (cm) | $13.20^{d}$ | $7.64^{c}$ | $6.25^{c}$ | $4.60^{b}$ | $4.34^{ab}$ | $2.84^{a}$ |
| ± SD | 2.02 | 1.89 | 1.72 | 1.37 | 1.04 | 1.23 |
| % $m^5C$ | $26.08^{bc}$ | $26.28^{c}$ | $26.39^{c}$ | $25.75^{ab}$ | $25.74^{ab}$ | $25.60^{a}$ |
| ± SD | 0.41 | 0.18 | 0.32 | 0.16 | 0.03 | 0.15 |

The different letters shown in the same row as a, b, c are significant differences ($p < 0.05$)

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**Figure 8** The effects of 5-azaC on the height and root length *in vitro* for 10 days.

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**B. <u>The effects of 5-azaC on the mature soybean plants (Mo)</u>**

From the comparison of the maximum, minimum and mean value ± SD of the phenotypes, protein content, lipid content, fatty acid composition, and level of DNA methylation of 5-azaC treated versus control plants among all 5-azaC treated doses at the mature period revealed the followings.

**1) The effects of 5-azaC on the phenotypes**

The data of the height were shown in **Table 5** and **Figures 9 and 10**. The results showed that the plant height was dramatically increased with the increasing 5-azaC concentrations up to 1200 $\mu$M compared with the control plants. However, the plant development seemed to be unaffected by 5-azaC with no difference in the pod number, seed number, and seed weight (**Tables 6-9,** respectively). **Figure 11** showed pod numbers of 5-azaC-treated plants compared to the control plants.

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**Table 5** The effects of 0, 300, 600, 900, 1,200, and 1,500 $\mu M$ 5-azaC on the height of the Mo.

| SJ.5 Mo | | 5-azacytidine($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| height(cm.) | max. | 104 | 97 | 100 | 120 | 121 | 100 |
| | min. | 53 | 60 | 58 | 69 | 65 | 58 |
| | mean | $75^{c}$ | $79^{bc}$ | $75^{c}$ | $88^{ab}$ | $94^{a}$ | $88^{ab}$ |
| | ± SD | 16.44 | 13.11 | 11.22 | 14.59 | 16.44 | 12.76 |
| | number of plant | 14 | 14 | 14 | 15 | 13 | 10 |

The different letters shown as a, b, c are significant differences ($p < 0.05$)

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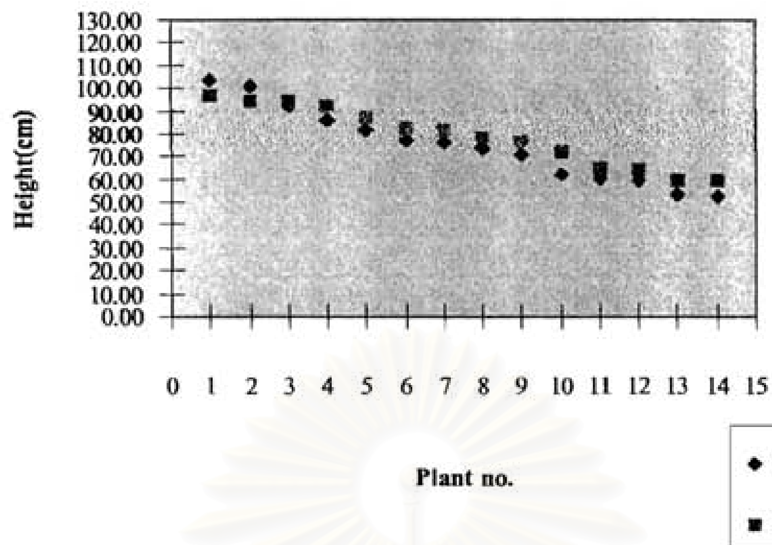
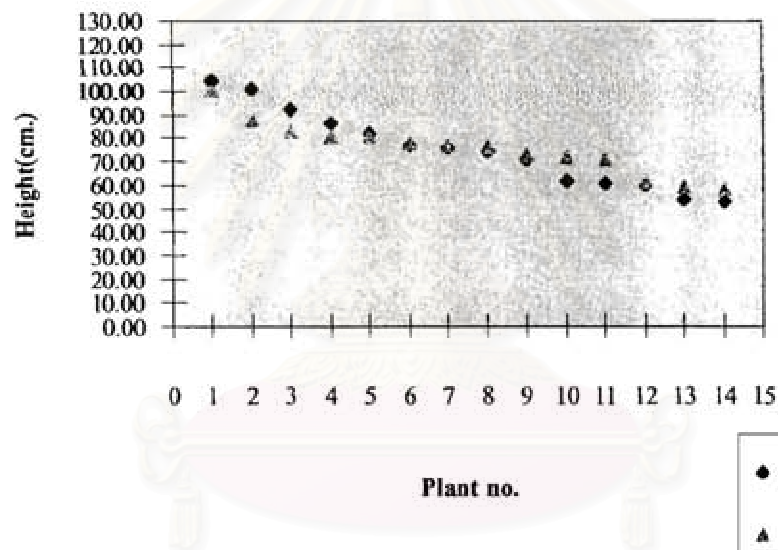

130.00
120.00
110.00
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Height(cm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Plant no.
Control
300 uM 5-azaC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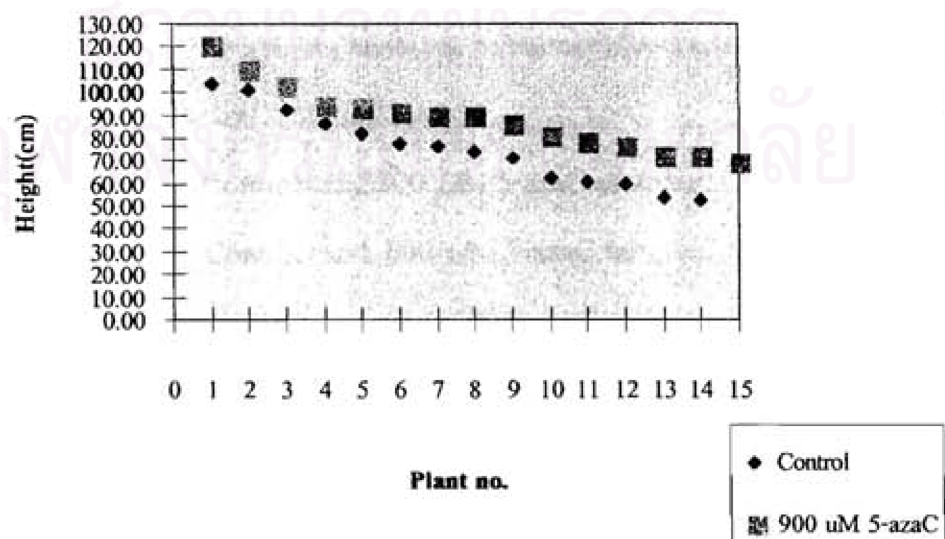
120.00
110.00
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Height(cm.)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Plant no.
Control
600 uM 5-azaC


130.00
120.00
110.00
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Height(cm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Plant no.
Control
900 uM 5-azaC

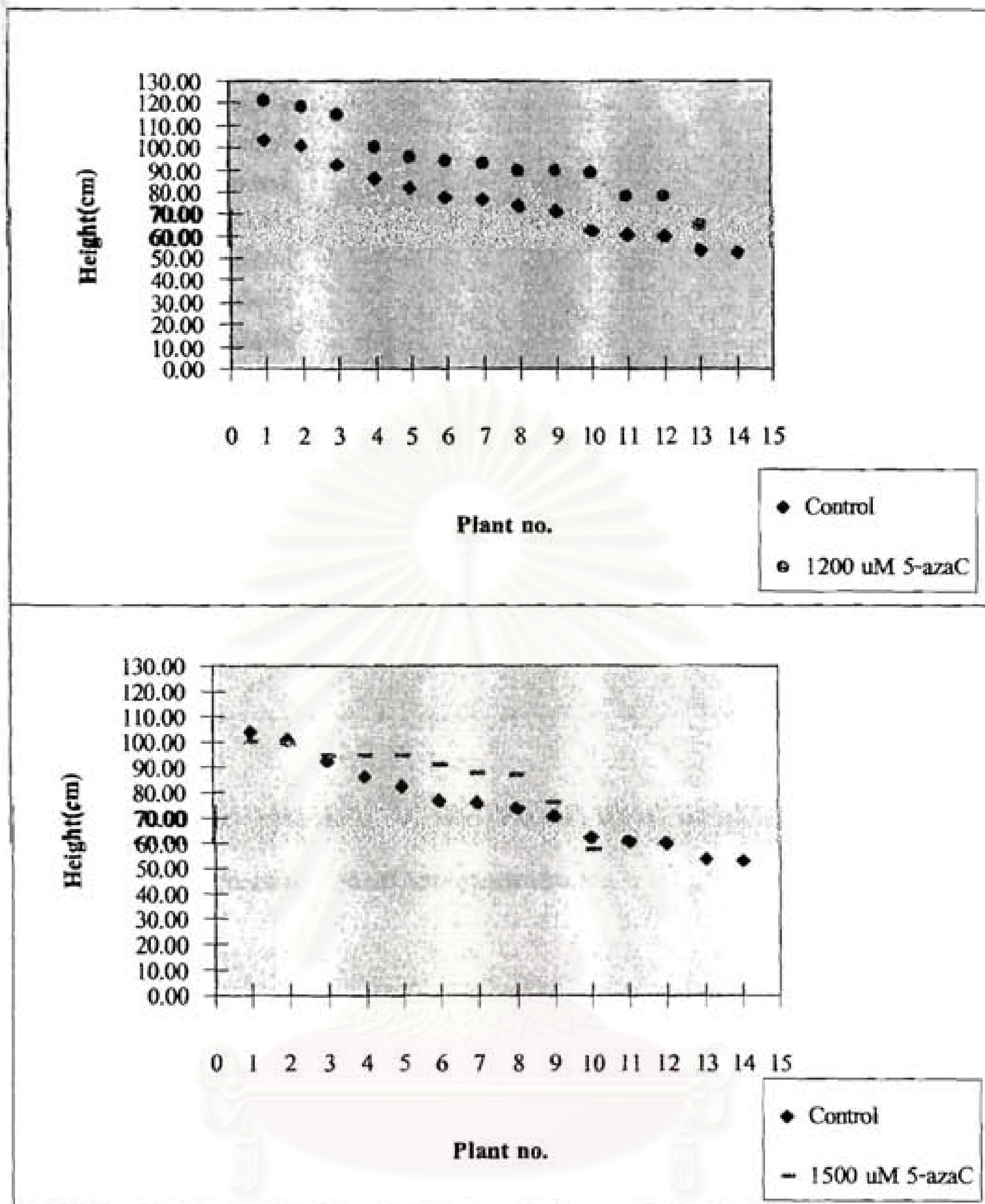



**Figure 9** A-E. The height phenotype of mature plants soaked in 0-1500 $\mu$M 5-azaC solution for 16 h.

**A** Controls and 300 $\mu$M 5-azaC samples;

**B** Controls and 600 $\mu$M 5-azaC samples;

**C** Controls and 900 $\mu$M 5-azaC samples;

**D** Controls and 1200 $\mu$M 5-azaC samples;

**E** Controls and 1500 $\mu$M 5-azaC samples

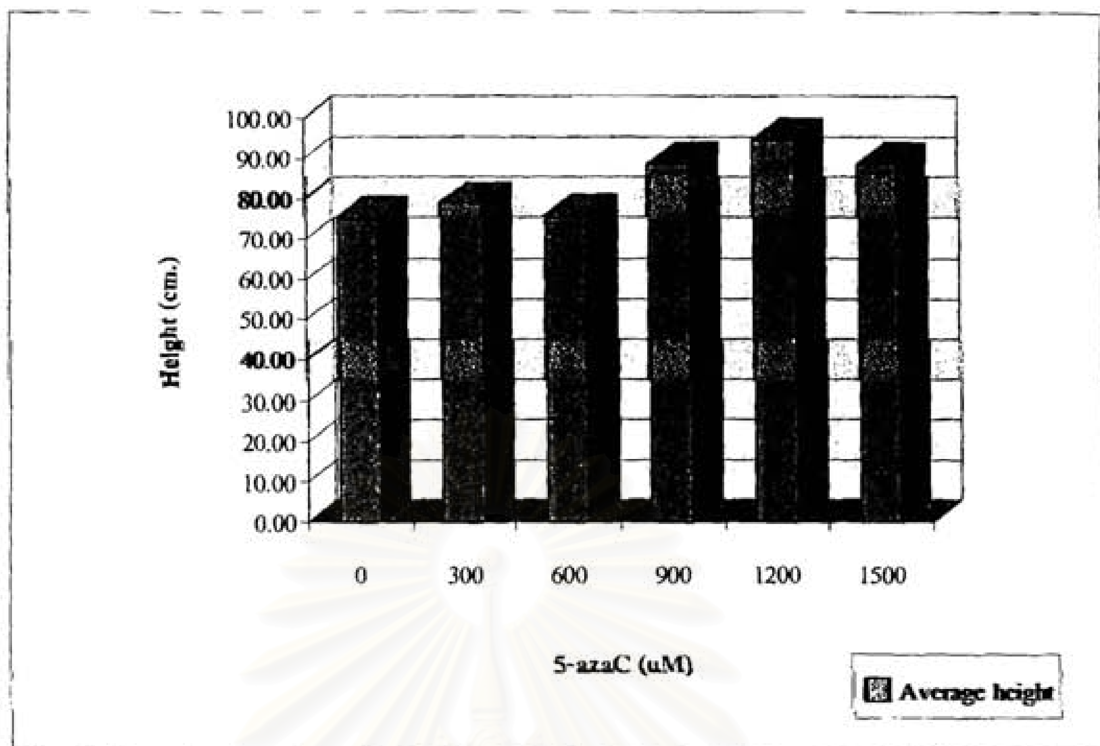



**Figure 10** The response of the mean stem height to the treatment of increasing 5-azaC concentrations.

**Table 6** The effects of 0, 300, 600, 900, 1,200, and 1,500 $\mu M$ 5-azaC on the pod number of the Mo

| SJ.5 Mo | | 5-azacytidine($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| | max. | 265 | 317 | 249 | 310 | 258 | 267 |
| | min. | 44 | 76 | 83 | 89 | 68 | 113 |
| **pod number** | **mean** | **134** | **170** | **152** | **168** | **142** | **184** |
| | ±SD | 74.84 | 69.59 | 50.34 | 68.51 | 52.02 | 54.28 |
| | number of plant | 14 | 14 | 14 | 15 | 13 | 10 |

**Table 7** The effects of 0, 300, 600, 900, 1,200, and 1,500 $\mu M$ 5-azaC on the seed number of the Mo

| SJ.5 Mo | | 5-azacytidine($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| seed number | max. | 479 | 615 | 502 | 594 | 467 | 509 |
| | min. | 64 | 130 | 143 | 163 | 142 | 231 |
| | mean | 240 | 309 | 279 | 311 | 261 | 315 |
| | ±SD | 134.16 | 131.75 | 97.52 | 139.55 | 90.80 | 94.56 |
| | number of plant | 14 | 14 | 14 | 15 | 13 | 10 |

**Table 8** The effects of 0, 300, 600, 900, 1,200, and 1,500 $\mu M$ 5-azaC on the total weight seeds of the Mo

| SJ.5 Mo | | 5-azacytidine($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| total wt.(g) | max. | 59.99 | 68.22 | 60.81 | 78.51 | 69.6 | 56.70 |
| | min. | 8.03 | 16.78 | 17.46 | 18.83 | 10.3 | 23.13 |
| | mean | 29.98 | 38.02 | 34.30 | 39.54 | 31.77 | 41.90 |
| | ±SD | 19.05 | 15.89 | 14.07 | 20.03 | 13.81 | 11.45 |
| | number of plant | 14 | 14 | 14 | 15 | 13 | 10 |

**Table 9** The effects of 0, 300, 600, 900, 1,200, and 1,500 $\mu M$ 5-azaC on the 100 seeds weight of the Mo

| SJ.5 Mo | | 5-azacytidine($\mu M$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| 100 seeds wt.(g) | max. | 14.63 | 14.50 | 13.94 | 15.62 | 14.29 | 20.06 |
| | min. | 9.34 | 9.87 | 8.80 | 8.55 | 7.25 | 8.26 |
| | mean | 12.08 | 12.30 | 12.13 | 12.61 | 12.01 | 13.46 |
| | ±SD | 1.76 | 1.61 | 1.72 | 1.79 | 2.13 | 2.99 |
| | **number of plant** | **14** | **14** | **14** | **15** | **13** | **10** |

**Figure 11** The dwarf and high pod number SJ.5 Mo compared to the control plant (leftmost)

**2) The effects of 5-azaC on the protein content**

The effects of 5-azaC on the protein content in seeds from the $M_0$ and $C_0$ plants were evaluated by the comparison of mean value of the controls, mean value $\pm$ 2SD and mean value $\pm$ 3SD of the controls in SJ.5 submitted to 5-azaC treatments of 0-1500 $\mu M$. As shown in **Table 10** and **Figures 12 and 13**, the average protein content of treated seeds (except 1200 $\mu M$) were higher than that of the control seeds. However, this difference was not statistical significant. The highest protein content was derived from the dosage of 1500 $\mu M$, the lowest protein content was derived from the dosage of 1200 $\mu M$ as shown in **Figure 14**. The probability of occurrence of high protein content mutation from 5-azaC treated plants is summarized in **Table 11**.

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**Table 10** The effect of 5-azaC on the protein content in seeds of the $M_0$ soaked with 5-azaC for 16 h

| SJ.5 Mo | | 5-azacytidine ($\mu$M) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| Protein(%) | max. | 41.17 | 42.52 | 41.78 | 45.19 | 39.94 | 43.19 |
| | min. | 33.46 | 37.29 | 37.46 | 34.72 | 32.73 | 36.19 |
| | mean | 37.12 | 39.45 | 38.70 | 39.41 | 36.56 | 39.46 |
| | ± SD | 2.40 | 2.22 | 1.51 | 2.99 | 2.23 | 2.54 |
| | number of plant | 7 | 7 | 7 | 11 | 7 | 7 |

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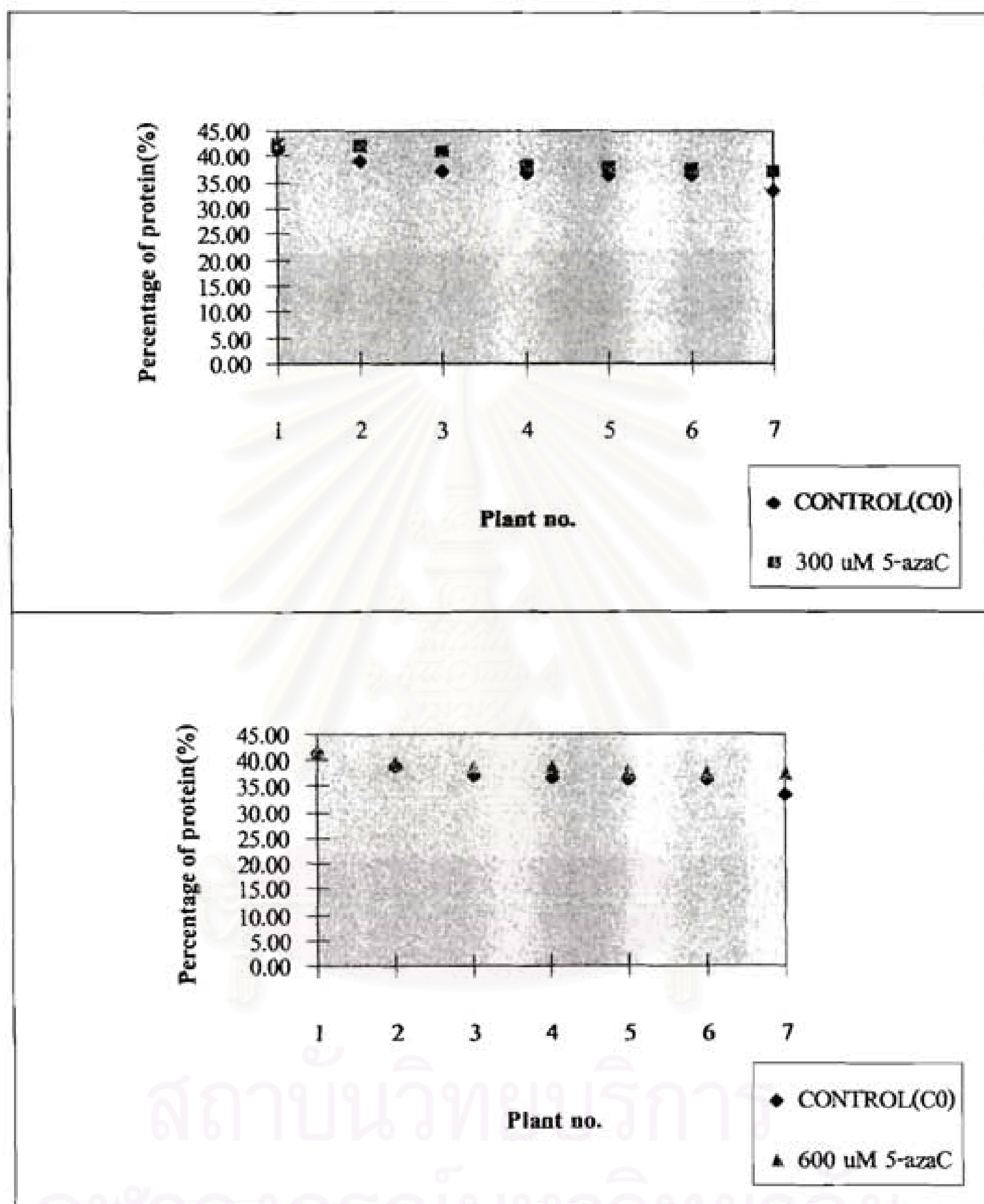
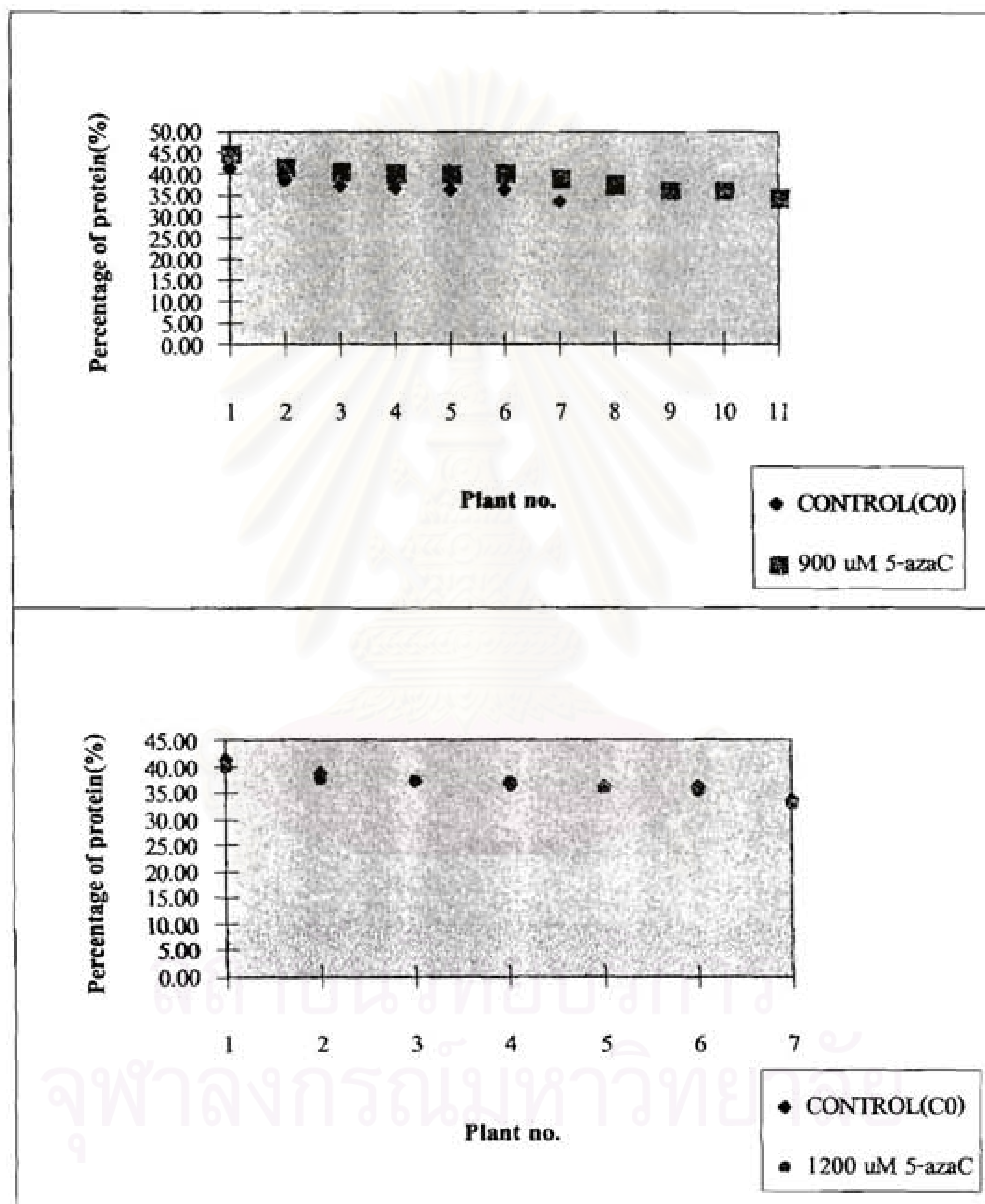

Percentage of protein(%)
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1
2
3
4
5
6
7
Plant no.
CONTROL(C0)
300 uM 5-azaC
Percentage of protein(%)
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1
2
3
4
5
6
7
Plant no.
CONTROL(C0)
600 uM 5-azaC



Percentage of protein(%)
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1
2
3
4
5
6
7
8
9
10
11
Plant no.
CONTROL(C0)
900 uM 5-azaC
Percentage of protein(%)
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1
2
3
4
5
6
7
Plant no.
CONTROL(C0)
1200 uM 5-azaC

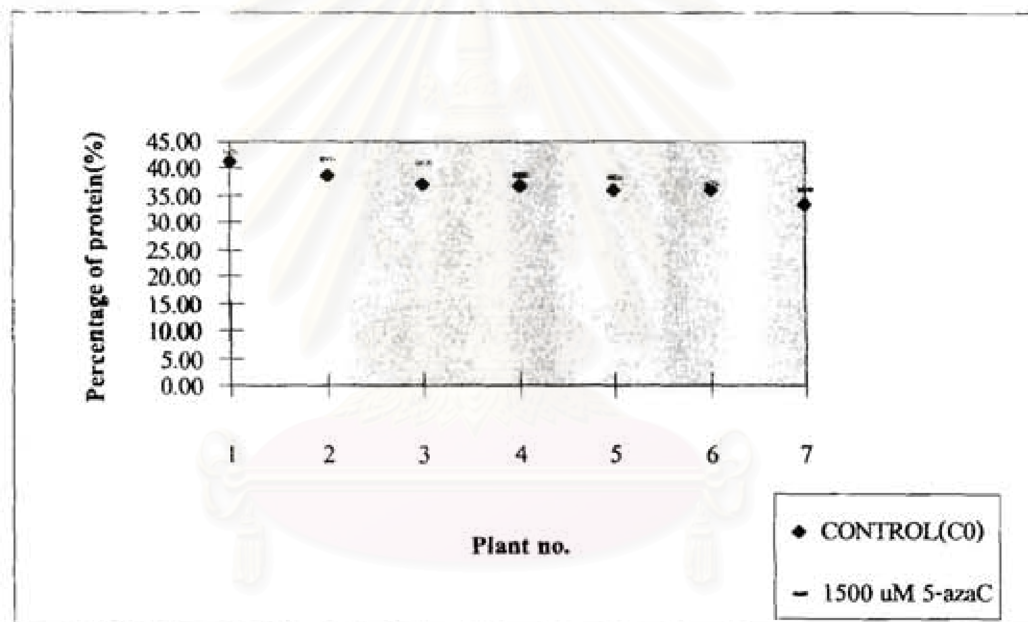



**Figure 12** A-E. The protein content of the $M_0$ induced by 5-azaC for 16 h.

**A** Controls and 300 $\mu M$ 5-azaC samples;

**B** Controls and 600 $\mu M$ 5-azaC samples;

**C** Controls and 900 $\mu M$ 5-azaC samples;

**D** Controls and 1200 $\mu M$ 5-azaC samples;

**E** Controls and 1500 $\mu M$ 5-azaC samples

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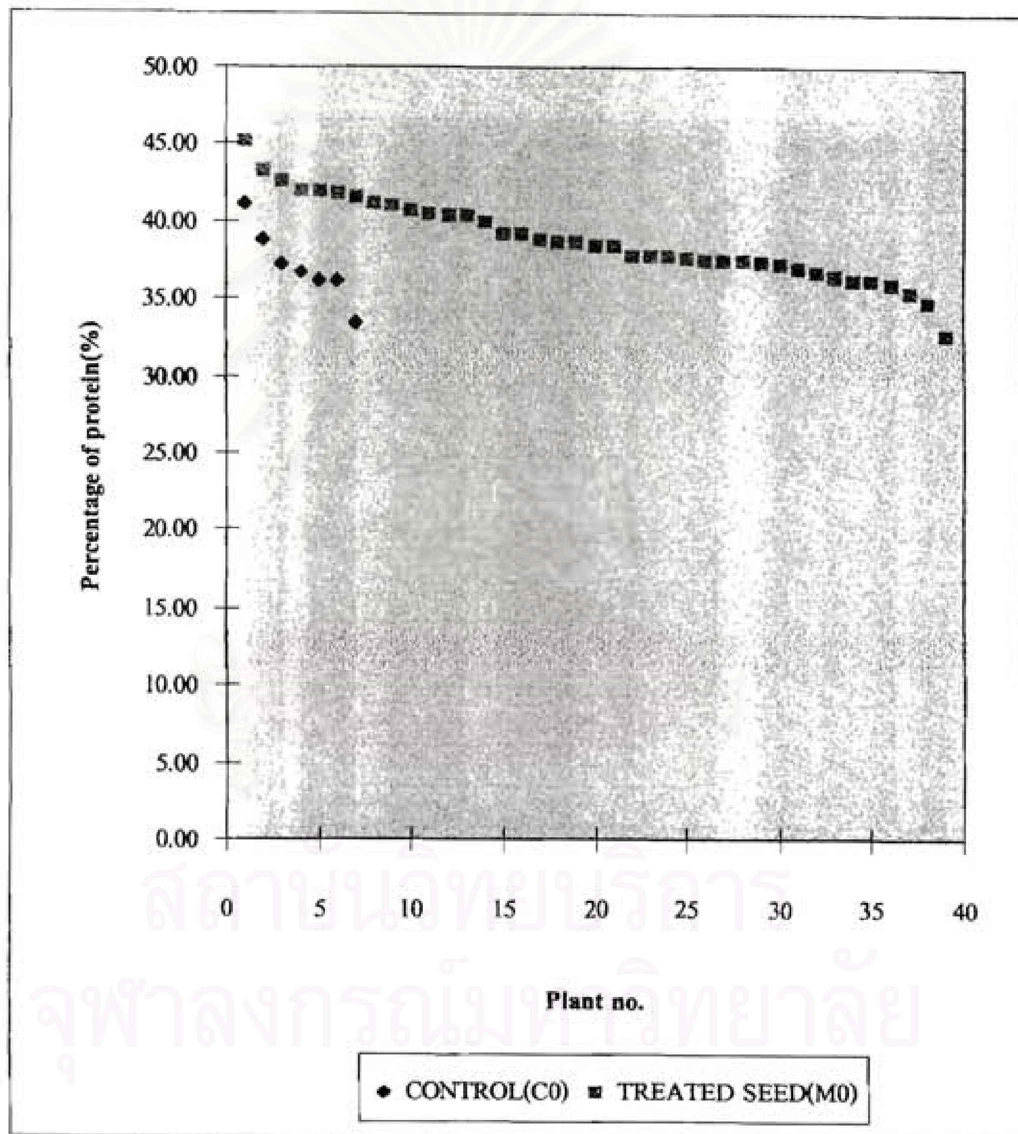


**Figure 13** The protein content of the all treated $M_0$ and control samples.

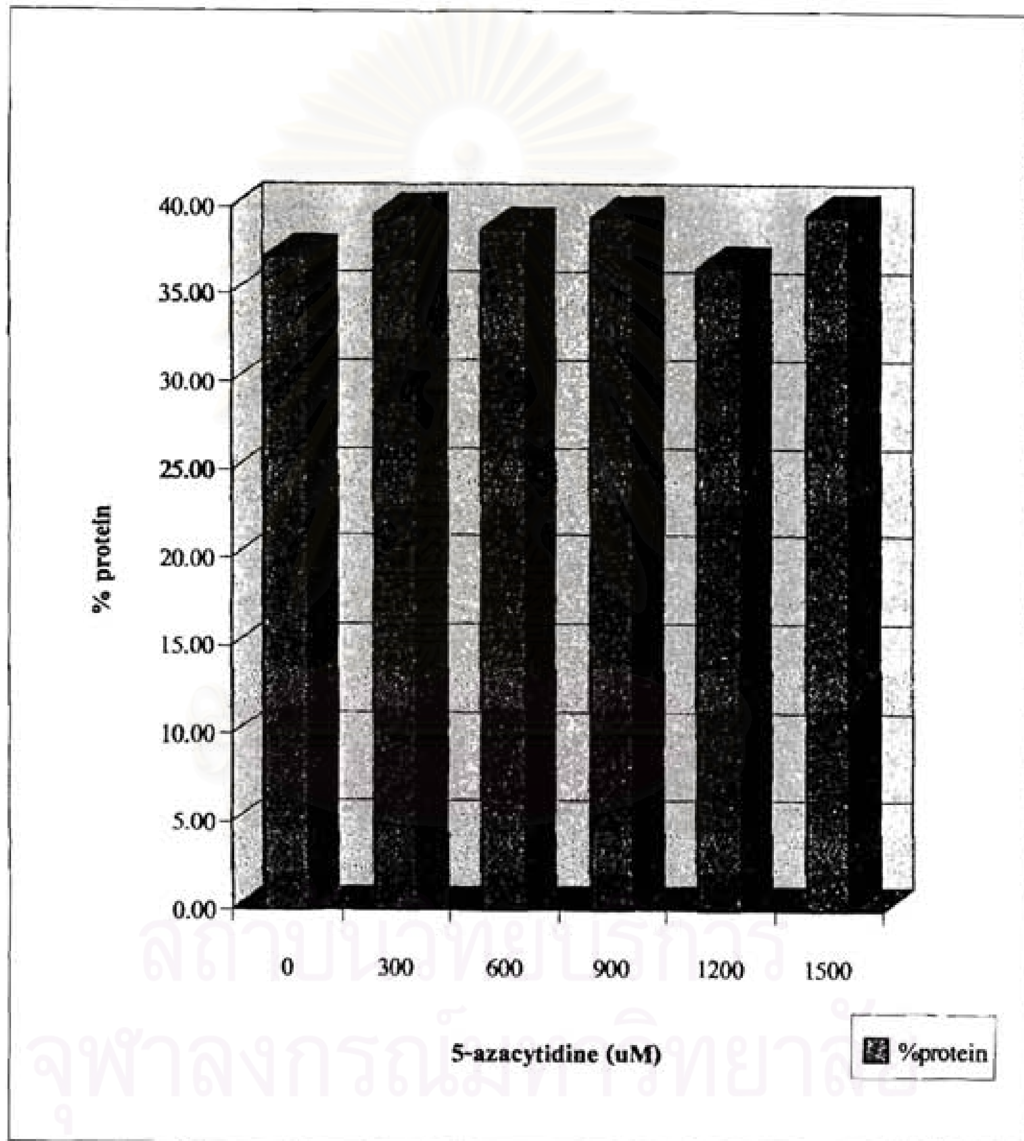


**Figure 14** The effects of 5-azaC on the protein content

**Table 11** The rate of occurrence of soybeans phenotypic change with high protein content induced by 5-azaC treatment.

| 5-azaC ($\mu M$) | No. of selections | Higher than check+SD[a] | | Two times SD[a] | | Three times SD[a] | | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | | Line | % | Line | % | Line | % | Line | % |
| 300 | 7 | 3 | 42.86 | 2 | 28.57 | 0 | 0 | 2 | 28.57 |
| 600 | 7 | 1 | 14.28 | 0 | 0 | 0 | 0 | 0 | 0 |
| 900 | 11 | 6 | 54.54 | 2 | 18.18 | 1 | 9.09 | 3 | 27.27 |
| 1200 | 7 | 1 | 14.28 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1500 | 7 | 3 | 42.86 | 1 | 14.28 | 0 | 0 | 1 | 14.28 |
| Total | 39 | 14 | 35.90 | 5 | 12.82 | 1 | 2.56 | 6 | 15.38 |

SD[a] : standard deviation

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**3) The effects of 5-azaC on the oil content**

The effects of 5-azaC on the oil content in seeds from the $M_0$ and $C_0$ plants were evaluated by the comparison of the mean value of the controls, mean value $\pm$ 2SD and the mean value $\pm$ 3SD of the controls in SJ.5 submitted to 5-azaC treatments of 0-1500 $\mu$M as shown in **Tables 12 and 13**.The results of the oil content were summarized in **Table 12** and **Figures 15 and 16.** The data indicated that the average oil content of treated seeds were higher than that of the control seeds and showed statistical significant. As shown in **Figure 17**, the highest oil content was derived from the dosage of 1200 $\mu$M and the lowest oil content was derived from the dosage of 300 $\mu$M. **Table** 13 showed the probability of occurrence of high oil content mutation from 5-azaC treated plant. The effects of 5-azaC on the relation of the protein and oil content were shown in **Figure 18**.

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**Table 12** The effects of 5-azaC on the oil content

| SJ.5 Mo | | 5-azacytidine ($\mu$M) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 300 | 600 | 900 | 1200 | 1500 |
| Oil (%) | max. | 18.26 | 18.47 | 20.74 | 21.04 | 22.84 | 19.84 |
| | min. | 15.34 | 14.9 | 15.56 | 15.04 | 18.38 | 16.12 |
| | mean | $17.11^{b}$ | $17.09^{b}$ | $18.62^{ab}$ | $17.46^{b}$ | $20.38^{a}$ | $18.35^{ab}$ |
| | ± SD | 1.12 | 1.38 | 1.94 | 2.24 | 1.65 | 1.71 |
| | number of plant | 5 | 5 | 5 | 5 | 5 | 5 |

The different letters shown as a, b are significant differences ($p < 0.05$)

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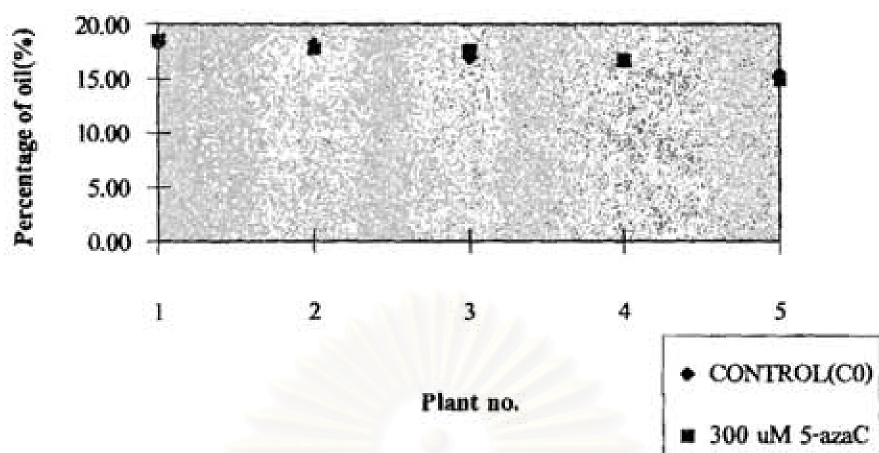
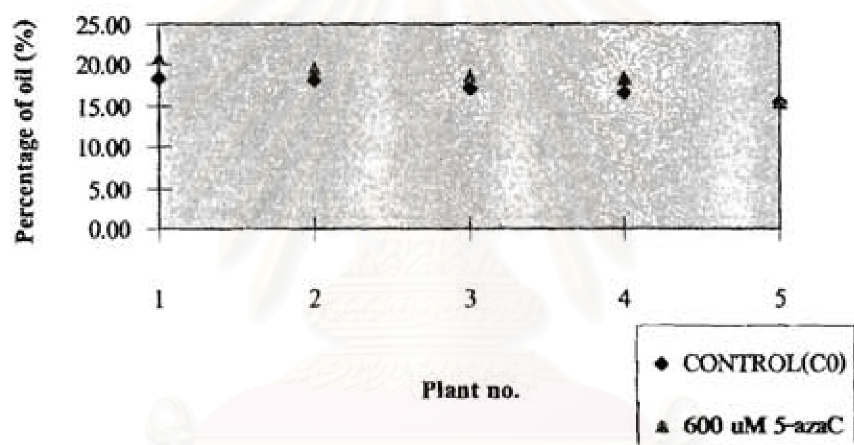

Percentage of oil(%)
20.00
15.00
10.00
5.00
0.00
1
2
3
4
5
Plant no.
CONTROL(C0)
300 uM 5-azaC


Percentage of oil (%)
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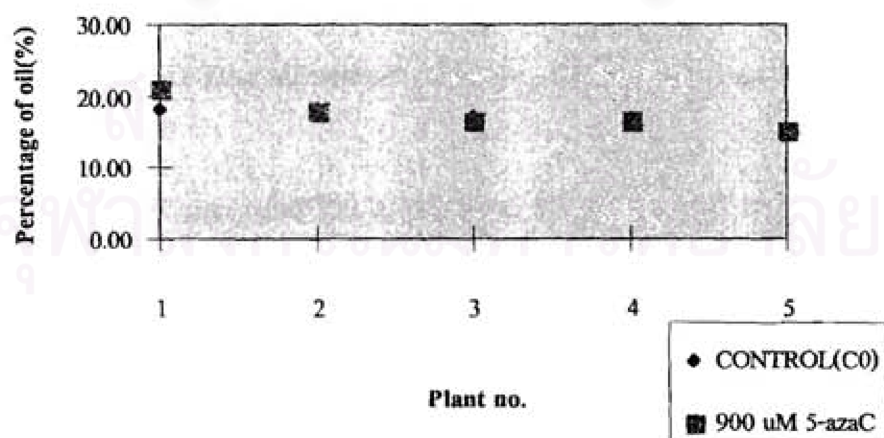
15.00
10.00
5.00
0.00
1
2
3
4
5
Plant no.
CONTROL(C0)
600 uM 5-azaC


Percentage of oil(%)
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10.00
0.00
1
2
3
4
5
Plant no.
CONTROL(C0)
900 uM 5-azaC

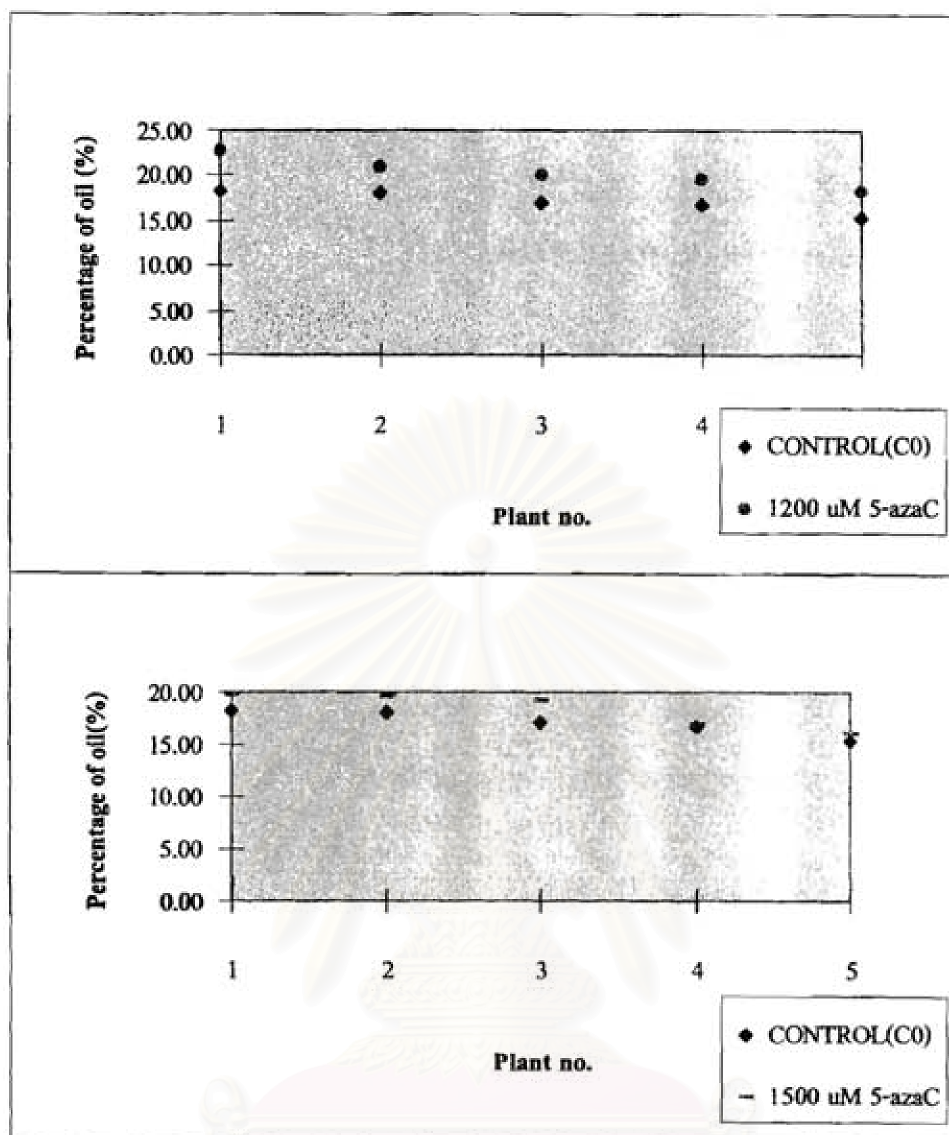


**Figure 15** A-E. Tthe oil content of the $M_0$ induced by 5-azaC for 16 h.

**A** Controls and 300 $\mu M$ 5-azaC samples;

**B** Controls and 600 $\mu M$ 5-azaC samples;

**C** Controls and 900 $\mu M$ 5-azaC samples;

**D** Controls and 1200 $\mu M$ 5-azaC samples;

**E** Controls and 1500 $\mu M$ 5-azaC samples

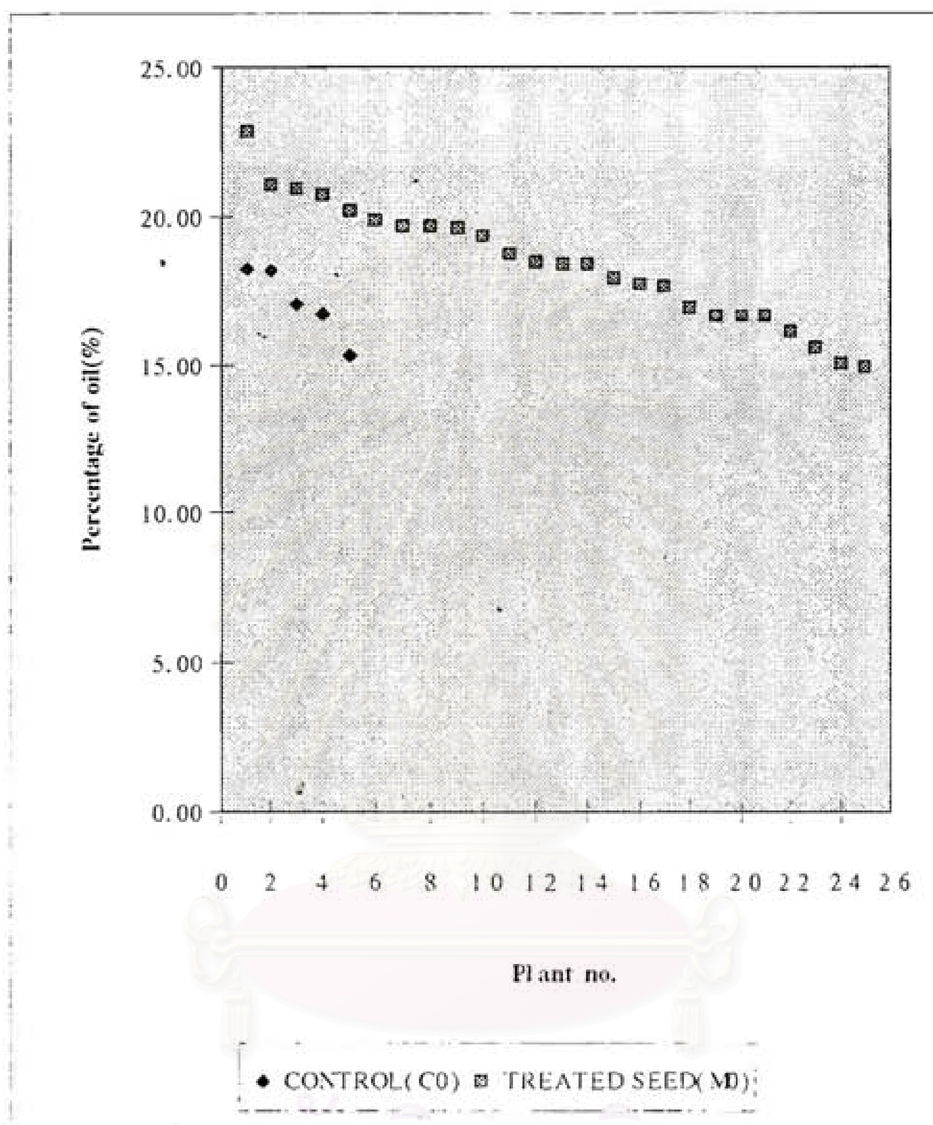


**Figure 16** The oil content of the all treated $M_0$ and control sample

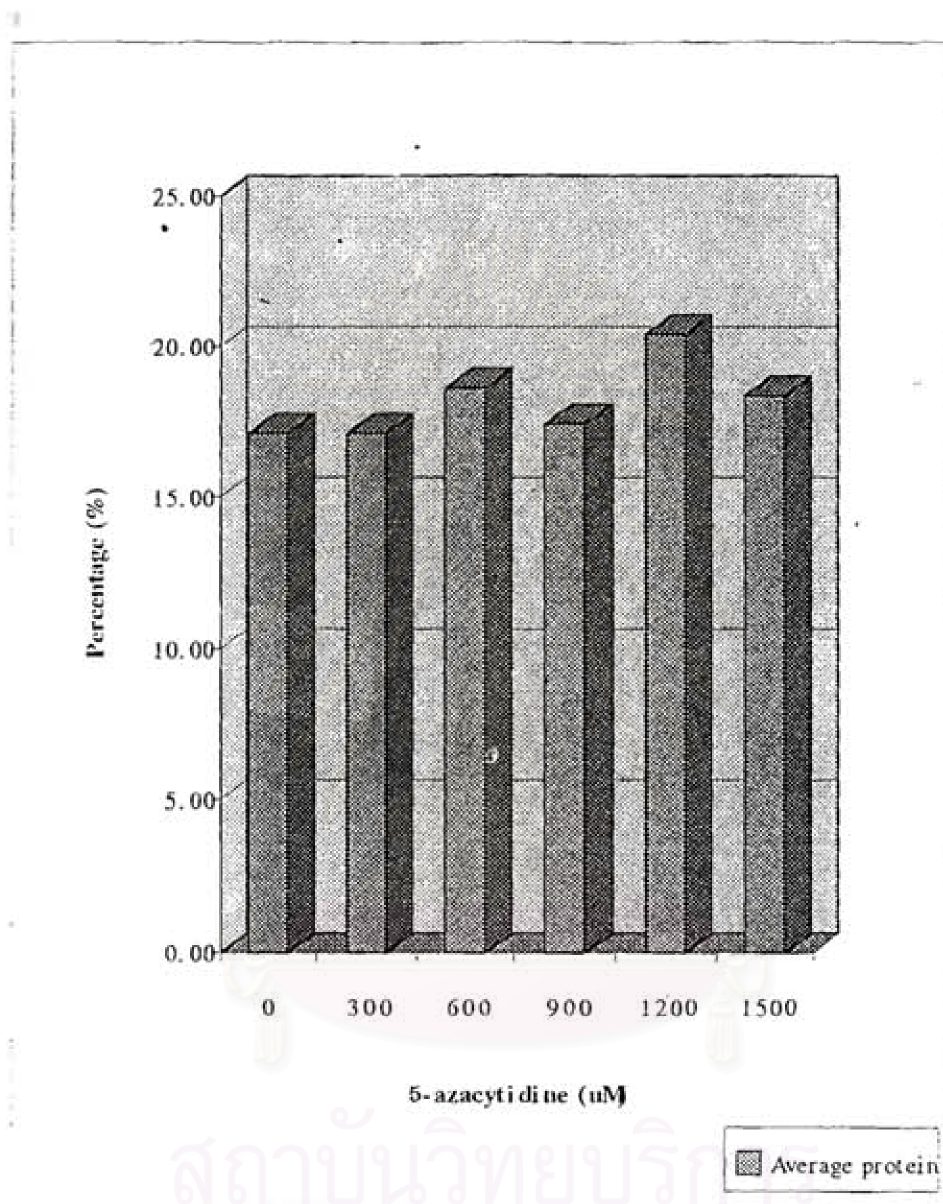


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**Figure 17** The effects of 5-azaC on the oil content

**Table 13** The rate of occurrence of soybeans phynotypic change with high oil content induced by 5-azaC treatment

| 5-azaC ($\mu$M) | No. of selections | Higher than check+SD[a] | | Two times SD[a] | | Three times SD[a] | | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | | Line | % | Line | % | Line | % | Line | % |
| 300 | 5 | 1 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| 600 | 5 | 4 | 80 | 2 | 40 | 1 | 20 | 3 | 60 |
| 900 | 5 | 1 | 20 | 1 | 20 | 1 | 20 | 2 | 40 |
| 1200 | 5 | 5 | 100 | 4 | 80 | 2 | 40 | 6 | 120 |
| 1500 | 5 | 3 | 60 | 2 | 40 | 0 | 0 | 2 | 40 |
| Total | 25 | 14 | 56 | 9 | 36 | 4 | 16 | 13 | 52 |

SD[a] : standard deviation

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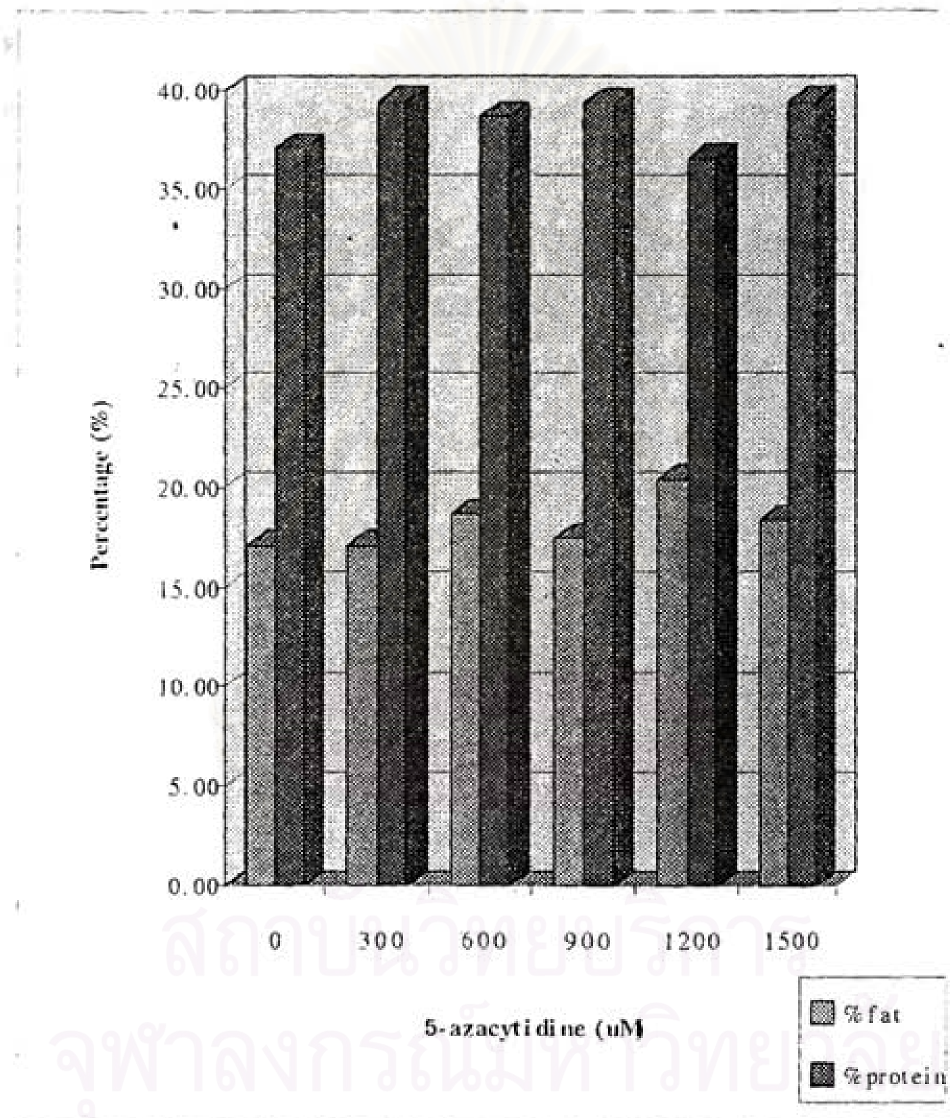


**Figure 18** The effects of 5-azaC upon the relation of the oil and protein

**4) The effects of 5-azaC on the fatty acid compositions**

The effects of 5-azaC on the fatty acid composition in seeds from the $M_0$ and $C_0$ plants were evaluated by the comparison of mean value of the controls in SJ.5 submitted to 5-azaC treatments of 0-1500 $\mu M$ as shown in **Tables 14 and 15.**The compositions of soybean fatty acids of the plants grown from treated seeds and control seeds were shown in **Tables 14 and** 15. The data indicate that all fatty acids remained unchange with increasing 5-azaC concentrations. The percentage of fatty acid content of the C16:0, C18:0, C18:1, C18:2 and C18:3 of the seeds from treated and control plants were shown in **Figure 19.** The figure showed no significant alteration of soybean fatty acid content at various 5-azaC concentrations. The results suggested that 5-azaC did not affect to fatty acids content in soybean seeds cv. SJ.5.

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**Table 14** The composition of saturated and unsaturated fatty acid in g/100 g of the total soybean seed fatty acid of the treated and control plants with various 5-azaC concentration for 16 h.

| Seed fatty acid | 5-azaC concentration | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 μM | 300 μM | 600 μM | 900 μM | 1200 μM | 1500 μM | p-value |
| SAFA | 15.00 ± 0.58 | 15.48 ± 0.80 | 15.24 ± 0.50 | 14.76 ± 0.17 | 15.16 ± 1.02 | 15.10 ± 0.43 | 0.72 |
| MUFA | 22.31 ± 2.84 | 19.41 ± 2.77 | 20.76 ± 2.99 | 22.00 ± 3.73 | 19.50 ± 5.48 | 19.36 ± 2.55 | 0.72 |
| PUFA | 62.95 ± 3.01 | 65.11 ± 2.25 | 63.99 ± 2.65 | 63.24 ± 3.62 | 65.34 ± 4.89 | 65.54 ± 2.70 | 0.80 |
| Sat/Unsat. | 0.18 ± 0.01 | 0.18 ± 0.01 | 0.18 ± 0.01 | 0.17 ± 0.00 | 0.18 ± 0.01 | 0.18 ± 0.01 | 0.69 |
| C18:1/C18:2 | 0.26 ± 0.02 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.26 ± 0.02 | 0.25 ± 0.01 | 0.93 |
| C18:2/C18:3 | 10.46 ± 0.45 | 10.28 ± 0.30 | 10.96 ± 0.94 | 10.75 ± 0.96 | 10.39 ± 0.34 | 10.50 ± 0.66 | 0.72 |

The results are expressed as Mean ± SD of four determinations.

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**Table 15** The fatty acid profiles in g/100g total FA of soybean after the soaking with 5-azaC at the concentration of 0, 300, 600, 900, 1200, and 1500 μM

| Fatty acid | 5-azacytidine | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 μM | 300 μM | 600 μM | 900 μM | 1200 μM | 1500 μM | P-value |
| C16:0 | 11.71 ± 0.58 | 12.05 ± 0.30 | 11.92 ± 0.29 | 11.55 ± 0.33 | 11.39 ± 0.54 | 11.73 ± 0.43 | 0.33 |
| C18:0 | 3.29 ± 0.19 | 3.43 ± 0.64 | 3.32 ± 0.41 | 3.20 ± 0.22 | 3.77 ± 0.48 | 3.37 ± 0.28 | 0.47 |
| C18:1 n-9 | 20.13 ± 2.76 | 18.01 ± 2.80 | 19.27 ± 2.82 | 20.38 ± 3.72 | 18.05 ± 5.33 | 17.88 ± 2.58 | 0.79 |
| C18:1 n-7 | 1.62 ± 0.17 | 1.40 ± 0.18 | 1.49 ± 0.17 | 1.62 ± 0.06 | 1.45 ± 0.21 | 1.48 ± 0.13 | 0.32 |
| C18:1 total | 22.06 ± 2.80 | 19.41 ± 2.77 | 20.76 ± 2.99 | 22.00 ± 3.73 | 19.50 ± 5.48 | 19.36 ± 2.55 | 0.75 |
| C18:2 | 57.44 ± 2.64 | 59.33 ± 2.04 | 58.62 ± 2.42 | 57.82 ± 3.27 | 59.60 ± 4.44 | 59.82 ± 2.30 | 0.82 |
| C18:3 | 5.50 ± 0.42 | 5.77 ± 0.26 | 5.38 ± 0.50 | 5.42 ± 0.61 | 5.74 ± 0.48 | 5.72 ± 0.52 | 0.73 |

The results are expressed as mean ± SD of four determinations.

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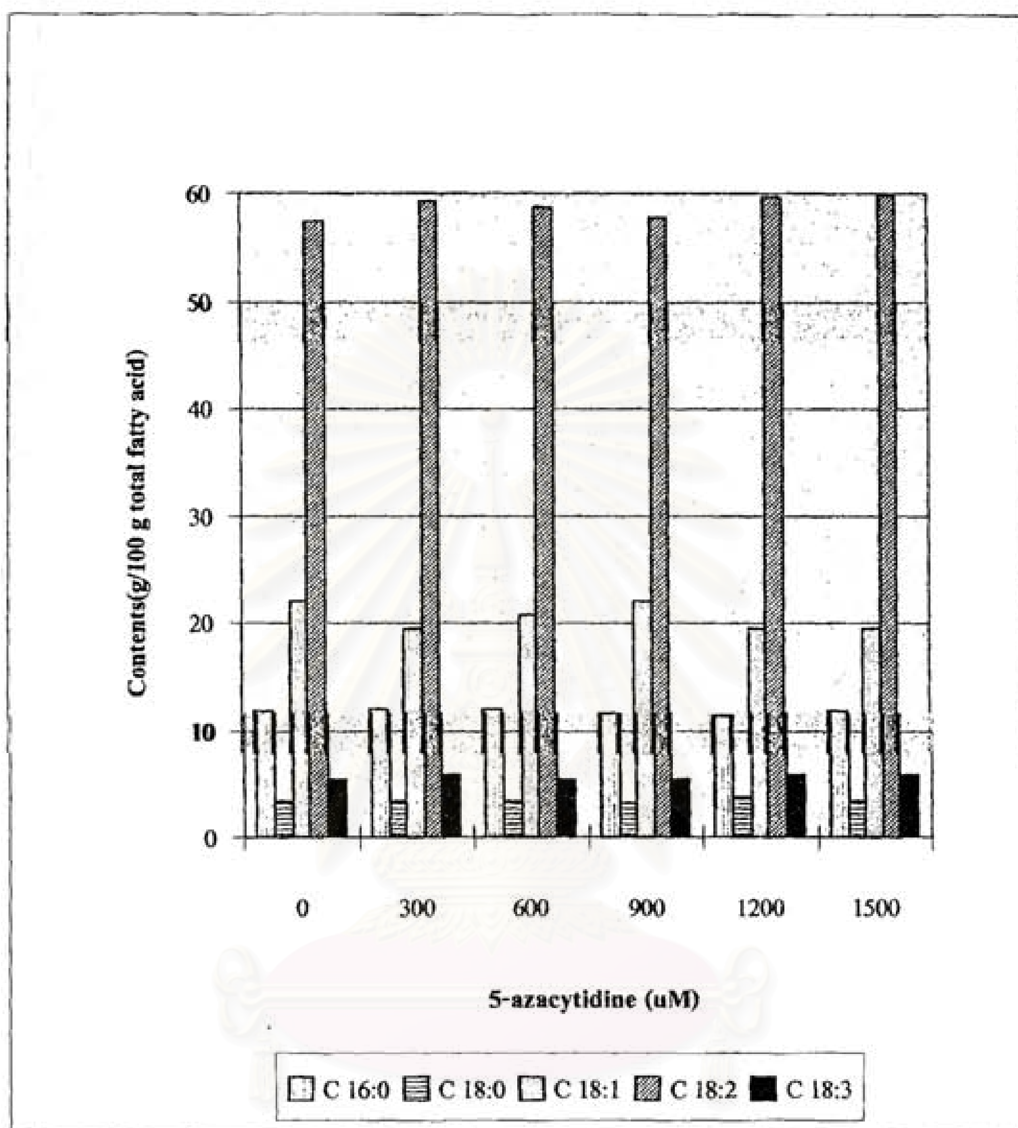


**Figure 19** Comparison of fatty acid content in percentage between C16:0, C18:0, C18:1, C18:2, and C18:3 found in soybean seeds from treated and control plants.

**5) Methylated cytosine analysis**

In order to determine the relationship between 5-azaC dosage and the level of genomic DNA demethylation, the total genomic DNA was extracted from treated and control plants and submitted to the reversed phase HPLC analysis. The percentage of $m^5C$ in the control and 5-azaC-treated plants are shown in **Table 16**. When compared with the control plants, the 5-azaC-treated plants showed some significant difference in the $m^5C$ content. The second point shown in **Table 16**, indicating that 5-azaC resulted in hypomethylated of the 5-azaC-treated plants without any dosage dependent.

In order to determine the relationship between phenotypic changes and the level of genomic DNA demethylation, DNA was isolated from plants of short and tall counterparts, and also from mature leaves of the control plants as shown in **Table 17**. When compared with the control plants, the DNA methylation patterns showed no significant difference in the $m^5C$ content between talls and shorts plants.

DNA from plants of high protein , high oil and also from mature leaves of control plants were shown in **Table 17**. When compared with the control plants, DNA methylation patterns showed no significant difference in the $m^5C$ content between high protein and high oil plants.

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**Table 16** The $m^5C$ content (mole%) of DNAs from control and 5-azaC- treated $M_0$ soybean plants.

| DNA source | Percentage of | | | $m^5C$/total C (%) |
|---|---|---|---|---|
| | Total C | C | $m^5C$ | |
| Control | 16.51 ± 0.04 | 12.21 ± 0.06 | 4.30 ± 0.05 | 26.02 ± 0.31[b] |
| 300 μM 5-azaC treated | 16.48 ± 0.00 | 12.27 ± 0.01 | 4.22 ± 0.01 | 25.58 ± 0.05[a] |
| 600 μM 5-azaC treated | 16.51 ± 0.04 | 12.27 ± 0.04 | 4.24 ± 0.04 | 25.59 ± 0.04[a] |
| 900 μM 5-azaC treated | 16.51 ± 0.07 | 12.25 ± 0.05 | 4.26 ± 0.03 | 25.80 ± 0.10[ab] |
| 1200 μM 5-azaC treated | 16.54 ± 0.04 | 12.28 ± 0.05 | 4.26 ± 0.01 | 25.72 ± 0.11[ab] |
| 1500 μM 5-azaC treated | 16.50 ± 0.04 | 12.24 ± 0.04 | 4.25 ± 0.03 | 25.78 ± 0.18[ab] |

Note : The results are expressed as mean of three determinations.

: The different letters shown as a, b are significant differences ($p < 0.05$)

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**Table 17** The $m^5C$ content (mole%) of DNAs from selected phenotypes of the $M_0$

| | Total C | C | $m^5C$ | $m^5C$/totalC (%) |
|---|---|---|---|---|
| control plants | 16.55 | 12.21 | 4.34 | 26.22 |
| | 16.52 | 12.28 | 4.24 | 25.67 |
| | 16.46 | 12.15 | 4.31 | 26.18 |
| **mean** | **16.51** | **12.21** | **4.30** | **26.02** |
| tall plants | 16.43 | 12.21 | 4.22 | 25.68 |
| | 16.60 | 12.32 | 4.28 | 25.78 |
| | 16.51 | 12.25 | 4.26 | 25.80 |
| **mean** | **16.51** | **12.26** | **4.25** | **25.75** |
| short plants | 16.50 | 12.22 | 4.28 | 25.59 |
| | 16.48 | 12.27 | 4.21 | 25.55 |
| | 16.51 | 12.21 | 4.30 | 26.04 |
| **mean** | **16.50** | **12.23** | **4.26** | **25.73** |
| high protein plants | 16.60 | 12.32 | 4.28 | 25.78 |
| | 16.46 | 12.22 | 4.24 | 25.76 |
| | 16.48 | 12.26 | 4.22 | 25.61 |
| **mean** | **16.51** | **12.27** | **4.25** | **25.72** |
| high oil plants | 16.55 | 12.31 | 4.24 | 25.62 |
| | 16.57 | 12.32 | 4.25 | 25.65 |
| | 16.50 | 12.24 | 4.26 | 25.82 |
| **mean** | **16.54** | **12.29** | **4.25** | **25.70** |

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**C. Subsequent effects of the 5-azaC treatments in the $M_1$**

To determine whether the phenotypic changes induced by 5-azaC were heritable in the absence of further 5-azaC treatment, the growth of self-fertilized progenies of 5-azaC-treated and control parental plants were examined.

**1) The effects of 5-azaC on the phenotype**

The selected plants based on increasing and decreasing in height were studied. The segregation of height phenotype in the $M_1$ are shown in **Table 18** and their characterization are shown in **Tables 19 and 20**.

1.1 The $M_1$ generation from a typical 5-azaC-induced tall, segregated into 6 normal and 5 tall plants as shown in **Table 18 and Figure 20**. Besides, their phenotypes were defined in **Table 19**. The results showed that the progeny from the tall plants were significantly taller than the control plants. Moreover, the leaf size of the progeny from the tall plants were significantly shorter than that of the control plants and the 100 seeds weight of the progeny from the tall plants were significantly less than that of the control plants.

1.2 As shown in **Table 18 and Figure 21**, the $M_1$ generation from a typical 5-azaC-induced short, segregated into 4 normal and 7 short plants. Moreover, their phenotypes of the $M_1$ generation from a typical 5-azaC-induced short phenotype were defined in **Table 20**. The results show that the progeny from the short plants were significantly shorter than the control plants. Besides, the leaf size of the progeny from the short plants were significantly larger and shorter than that of the control plants.

**Table 18** The segregation of height phenotype in the $M_1$

| $M_0$ plants | | $M_1$ plants | |
|---|---|---|---|
| Phenotype | No. used | Progeny produced by $M_0$ | mean height (cm.) |
| tall | 11 | 6, normal | 54.00 ± 0.89 |
| | | 5, tall | 58.00 ± 1.87 |
| short | 11 | 4, normal | 50.75 ± 2.75 |
| | | 7, short | 40.14 ± 4.60 |
| control | 9 | 9, normal | 51.88 ± 3.41 |

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Table 19 Characterization of the $M_1$ generation from a typical 5-azaC-induced tall phenotype.

| | height(cm) | | flowering day | | width of leaves (cm.) | | length of leaves (cm.) | | pod number | | length of pod (cm.) | | 100 seeds wt.(g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ |
| max. | 59 | 61 | 35 | 34 | 8.62 | 7.98 | 14.03 | 12.52 | 122 | 166 | 3.31 | 3.29 | 16.21 | 13.26 |
| min. | 47 | 53 | 33 | 33 | 5.87 | 6.02 | 11.80 | 9.57 | 76 | 48 | 3.03 | 3.14 | 13.82 | 12.19 |
| mean | 51.89 | 55.82* | 34 | 34 | 6.84 | 6.93 | 12.71 | 10.98* | 94 | 98 | 3.17 | 3.20 | 15.28 | 12.62* |
| ±SD | 3.41 | 2.48 | 0.60 | 0.52 | 0.77 | 0.58 | 0.68 | 0.94 | 21.40 | 37.20 | 0.11 | 0.06 | 0.97 | 0.44 |
| no.of plant | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 |

The symbol as * is significant difference from the control ($p < 0.05$)

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**Figure 20** The $M_1$ generation from 5-azaC-induced tall compared to the control plants (leftmost) , scale: 15 cm.

Table 20 Characterization of the $M_1$ generation from a typical 5-azaC-induced short phenotype.

| | height(cm) | | flowering day | | width of leaves (cm.) | | length of leaves (cm.) | | pod number | | length of pod (cm.) | | 100 seeds wt.(g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ | $C_1$ | $M_1$ |
| max. | 59 | 54 | 35 | 37 | 8.62 | 9.00 | 14.03 | 13.30 | 122 | 115 | 3.31 | 3.18 | 16.21 | 16.23 |
| min. | 47 | 32 | 33 | 33 | 5.87 | 6.45 | 11.80 | 9.75 | 76 | 83 | 3.03 | 2.98 | 13.82 | 14.08 |
| mean | 51.89 | 44.00* | 34 | 35 | 6.84 | 7.74* | 12.71 | 11.65* | 94 | 101 | 3.17 | 3.10 | 15.28 | 15.27 |
| ±SD | 3.41 | 6.60 | 0.60 | 1.10 | 0.77 | 0.85 | 0.68 | 1.17 | 21.4 | 11.70 | 0.11 | 0.09 | 0.97 | 0.87 |
| no.of plant | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 | 9 | 11 |

The symbol as * is significant difference from the control ($p < 0.05$)

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**Figure 21** The $M_1$ generation from 5-azaC-induced short compared to the control plants (leftmost), scale : 15 cm.

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**2) The effects of 5-azaC on the protein content**

The phenotypes of the $M_1$ generation from a typical 5-azaC-induced highest protein content were selected and defined in **Table 21.** The results show that the protein content of the progeny from the highest protein plants were significantly higher than that of the control plants. Therefore, the high protein plant induced by 5-azaC was heritable at least up to the second generation.

**Table 21** Subsequent effects of 5-azaC on the protein content of seeds from 5-azaC-induced high protein plant

| Protein content (%) | $C_1$ | $M_1$ |
|---|---|---|
| max. | 39.20 | 42.10 |
| min. | 36.53 | 39.28 |
| mean | 37.88 | 40.57* |
| ± SD | 1.02 | 1.05 |
| no. of plant | 5 | 5 |

The symbol as * is significant difference from the control.

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**3) The effects of 5-azaC on the percentage of the oil content**

The phenotypes of the $M_1$ generation from a typical 5-azaC-induced highest oil content were selected and defined in **Table 22.** The results showed that the oil content of the progeny from the highest oil plants were not significantly higher than that of the control plants.

**Table 22** Subsequent effects of 5-azaC on the oil content of seeds from 5-azaC-induced high oil plant

| Oil content (%) | $C_1$ | $M_1$ |
|---|---|---|
| max. | 22.62 | 23.17 |
| min. | 20.38 | 21.54 |
| mean | 21.56 | 22.16 |
| ±SD | 0.81 | 0.88 |
| no. of plant | 5 | 5 |

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**4) The effects of 5-azaC on the fatty acid composition**

The $M_1$ seeds from a induced high linoleic acid content plant were selected, their fatty acid composition were determined and presented in **Table 23.** The results show that oil from their $M_1$ seeds showed significant modifications of stearic acid content when compared with oil from the control plants. Nevertheless, genetic analysis indicates that it was not inheritable.

**Table 23** Overall fatty acid composition of the $C_1$ and $M_1$

| Fatty acid | $C_1$ | $M_1$ |
|---|---|---|
| C16:0 | 12.38 ± 0.41 | 12.21 ± 0.43 |
| C18:0 | 3.93 ± 0.13 | 3.71 ± 0.15* |
| C18:1 n-9 | 15.74 ± 0.49 | 16.15 ± 0.41 |
| C18:1 n-7 | 1.34 ± 0.09 | 1.38 ± 0.08 |
| C18:1 total | 17.08 ± 0.50 | 17.54 ± 0.47 |
| C18:2 | 59.58 ± 0.74 | 59.83 ± 0.31 |
| C18:3 | 7.03 ± 0.26 | 6.72 ± 0.19 |

The symbol as * is significant difference from the control

**5) Methylated cytosine analysis**

To determine whether the demethylation induced by 5-azaC treatment was heritable, the $m^5C$ content of DNA samples from the controls, the tall, short, high protein, high oil progenies of 5-azaC-treated parent were examined as shown in **Table 24.** When compared with the control plants, the DNA methylation patterns showed no significant difference in the $m^5C$ content between selected phenotypes.

**Table 24** The $m^5C$ content (mole %) of DNAs from selected phenotypes of the $M_1$

| DNA source | Percentage of | | | $m^5C$/total C (%) |
|---|---|---|---|---|
| | Total C | C | $m^5C$ | |
| control | 16.56 $\pm$ 0.02 | 12.30 $\pm$ 0.05 | 4.26 $\pm$ 0.06 | 25.73 $\pm$ 0.33 |
| tall plants | 16.47 $\pm$ 0.03 | 12.23 $\pm$ 0.11 | 4.25 $\pm$ 0.09 | 25.78 $\pm$ 0.58 |
| short plants | 16.57 $\pm$ 0.04 | 12.31 $\pm$ 0.04 | 4.26 $\pm$ 0.02 | 25.74 $\pm$ 0.09 |
| high protein plants | 16.52 $\pm$ 0.03 | 12.31 $\pm$ 0.06 | 4.21 $\pm$ 0.06 | 25.50 $\pm$ 0.38 |
| high oil plants | 16.55 $\pm$ 0.06 | 12.29 $\pm$ 0.02 | 4.27 $\pm$ 0.06 | 25.77 $\pm$ 0.24 |

Note : The results are expressed as mean $\pm$ SD. of three determinations.

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# CHAPTER IV

# DISCUSSION

## A. The effects of 5-azacytidine on *in vitro* seedlings

### 1) The effects of 5-azaC on the phenotypes

When seeds were treated with various 5-azaC concentrations in hormone free MS media for 10 days, the root length and plant height analyses during seedling growth demonstrated that the growth rates were directly affected in the 5-azaC-treated seedlings in such a way that the growth rates were decreased with the increasing 5-azaC concentrations. The plant height was also proportionally decreased with the increasing 5-azaC concentrations. For example, the mean stem heights were 11.75 $\pm$ 3.64 cm in control seedlings (n = 21) and 3.41 $\pm$ 1.30 cm in 1500 $\mu$M 5-azaC-treated seedlings (n = 21), representing a 71% reduction in plant height induced by 5-azaC (**Table 1**). Furthermore, root length was also proportionally decreased with the increasing 5-azaC concentration. For example, the mean root lengths were 13.20 $\pm$ 2.02 cm in control seedlings (n = 21) and 2.84 $\pm$ 1.23 cm in 1500 $\mu$M 5-azaC-treated seedlings (n = 21), representing a 78% reduction in root length induced by 5-azaC (**Table 2**). As the treatment concentrations increased, there was a significant decreased in the mean stem height and root length but the plant development seemed to be unaffected by 5-azaC with no difference in the number of leaves or tillers. Besides, the 5-azaC-treated seedlings appeared to be uniform in height and root length throughout the entire growing period and the within-group variances were consistently lower than the variances of the control seedlings. The SE of height and root length means can be seen from **Tables 1 and 2**, respectively. These results indicated that 5-azaC might exhibit some effect at the DNA level, possibly at least altering the expression of

particular gene(s) involved in plant growth, and direct effects on DNA replication, protein synthesis or cell division as found in japonica rice treated with 5-azacytidine (Sano *et al.*, 1989; 1990). Our results confirmed that such effect could be happened in soya, the dicotyledonous plant with the direct corelated between hypomethylated DNA and such novel phenotype. In each treatment, the viability of seedlings was 100%, indicating that 5-azaC did not influence plant survival as shown in **Table 1**. On further growth, some of these dwarf seedlings became revertants, whereas the dwarf phenotypes was still maintained in the rest of the treated plant until maturity (data not shown). It might be assumed that some of the genes involved in early plant growth and development might have been affected in all treated seeds, since nearly all seedlings showed dwarf phenotype after treatment. However, as many of these dwarf seedlings attained the tall phenotype on further growth, they might have undergone a certain level of remethylation, by *de novo* methyltransferases, at some of the loci involved in plant growth. The effects of 5-azaC on plant growth in soybean were very similar to those reported in maize, rice, flax, and *Chenopodium rubrum* (Sano *et al.*, 1989, 1990; Cherdshewasart *et al.*, 1996; Fieldes, 1994; Albrechtova *et al.*, 1994).

In maize (*Zea mays*, cv. honey bantum), seeds were treated for 16 h with 0.3 mM 5-azaC. At maturity, a 28% reduction in the total stem length of treated plants was observed in comparison with the controls. The genomic DNA isolated from an 5-azaC-treated plant, showed approximately a 8% reduction in $m^5C$ content ( Sano *et al.*, 1989).

In rice (*Oryza sativa* L.), germinated seeds were treated with 0.3 mM 5-azaC for 3 days. The predominant phenotypic effect was a decrease in plant height. Rice plants grown from treated seeds were initially short and some retained the "dwarf" phenotype. The dwarf phenotype was inherited in some of the first generation progenies, which in turn passed on the dwarf phenotype to the second generation. The genomic DNA isolated from mature leaves of 5-azaC-treated seeds showed

approximately a 16% reduction in the $m^5C$ content in comparison with DNA from the control samples (Sano *et al.*, 1990).

In flax (*Linum usitatissimum*) , seeds were treated with 0-1.5 mM 5-azaC for 24 h. which resulted in induction of a reduction in the height of the plants grown from treated seed and also induced a marked decrease in the flowering age (Fieldes, 1994).

In *Chenopodium rubrum*, the growth pattern was altered following the treatment with $10^{-4}$-$10^{-9}$ M 5-azaC. The plants had developmental abnormalities, including enlarged apical meristem, stimulated formation and growth of axillary buds, and inhibited stem growth (Albrechtova *et al.*, 1994).

In conclusion, our finding demonstrated cleary on the concerrence of the demethylating treatment by 5-azacytidine and the induction of novel dwarf and short root phenotype.

**2) Demethylation of seedling genomic DNA by 5-azaC treatment**

The percentage of $m^5C$ in the control and 5-azaC-treated soybean seedlings were shown in **Tables 3 and 4**. The data showed 26.08% and 25.60% of the total cytosines in the DNAs from the control and 1500 $\mu M$ 5-azaC-treated plants, respectively, corresponding to a 0.38% reduction of $m^5C$ in 5-azaC-induced dwarf plants. When compared with the control seedlings, the 5-azaC-treated seedlings showed significant difference in the $m^5C$ content (**Table 3**). As shown in **Table 4**, the absolute levels of genomic demethylation and height , root length varied among experiments. Treatments with 5-azaC concentration up to 900 $\mu M$ caused a reduction in plant stem length and root length and a reduction of genomic methylation to 0.33% of control seedlings. Furthermore, with 5-azaC concentrations as high as 1500 $\mu M$ , it was found that a decrease in the $m^5C$ content and a reduction in plant stem length and root length were more pronounced than those observed at the dosage of 900 $\mu M$.

The results from the experiments with 5-azaC suggested that the demethylation of certain gene(s) involved in plant growth might be the cause of the induced dwarfism and 5-azaC might act at one particular site to reduce methylation and subsequently induced the dwarf phenotype (Sano *et al.*, 1989).

For the induction of dwarfism and undermethylation of genomic DNAs, 1500 $\mu M$ 5-azaC treatment resulted in a higher level of induction than 300-1200 $\mu M$ 5-azaC treatments. These differences in the levels of 5-azaC required for induction might be due to cell line-specific differences in the sensitivity of the methylation to 5-azaC. For 5-azaC induction on genomic DNA demethylation, the drug must first be incorporated into DNA during cell division. *In vitro* seedlings, only a small percentage of the cell population might be dividing at any particular time. Since 5-azaC was unstable in aqueous solution (light and temperature sensitivities), higher concentrations might be required to maintain an effective concentration over the long periods of time required for incorporation of the drug into the DNA of cell (Klass *et al*, 1989).

The percentage of $m^5C$ was decreased significantly in the treated seedlings but the alteration of $m^5C$ level was less than that of the *Zea mays*, cv. honey bantum and *Oryza sativa* L. treated with the same demethylating agent. Such differences may derived from the organization of the genome which might be the difference between the species or doses and time of exposure to 5-azaC.

In conclusion, our finding demonstrated clearly the concerrence of DNA hypomethylation induced by the demethylating agent 5-azacytidine and the induction of novel dwarf and short root phenotype.

**B. The effects of 5-azaC on the mature soybean plants** ($M_0$)

**1) The effects of 5-azaC on the phenotypes**

At the maturity, plant height was increased with the increasing 5-azaC concentrations up to 1200 $\mu M$ (94.46 $\pm$ 16.44 cm) and was reduced with the increasing 5-azaC concentration up to 1500 $\mu M$ (88.40 $\pm$ 12.76 cm) compared with control plants (75.21 $\pm$ 16.44 cm) as shown in **Table 5**. However, plant development seemed to have been unaffected by 5-azaC with no difference in the flowering day, leaf size, side branching, pod number, length of pod, seed number, and seed weight (**Table 6-9**). Nearly all the mean values of the treated samples were higher than the mean values of control samples.. The effects of 5-azaC on plant growth (height) in this experiment resembled the effects in triticale (Heslop-Herrison, 1990) but paralleled the effects in rice, maize and soybean (*in vitro*).

In triticale, the treatments induced a number of phenotypic alterations, including an increase in plant height, a decrease in the age to maturity and an increase in tillering , and a site-specific alteration in methylation status (Heslop-Herrison, 1990).

As mentioned above, the height of soybean plant (*in vivo*) did not correlate with the height of the treated plants from which they were derived. The difference of plant phenotype may be due to : Firstly, the differences may derived from difference in doses and time of exposure to 5-azaC or experimental condition. Secondly, due to the fact that plant growth is under polygenic controlled and DNA demethylation presumably occurs randomly among cells. Each gene and not all sequences are demethylated to the same extent, so different cells or genes will receive different demethylation patterns (Sano *et al.*, 1990). In addition, demethylated sites may be remethylated after a certain number of cell divisions and/or generations by *de novo* methyltransferase (Sano *et al.*, 1990). Finally, DNA methyltransferases in soybean

may be very active enzymes so 5-azaC may slightly affect or unaffect to genes that regulated plant growth or plant development.

**2) The effects of 5-azaC on the protein content**

As shown in **Table 10** and **Figures 12 and 13,** 5-azaC can induce high protein content mutations in $M_0$ lines treated with 5-azaC. The protein content ranged from 32.73% (1200 $\mu$M) to 45.19% (900 $\mu$M), with the highest value being 8.07% more than that of the control and the lowest value 4.39% less than the control. An average protein content of treated seeds were fluctuated with the increasing 5-azaC concentration, but no significant difference in the average protein content. The highest protein content was derived from the dosage of 300 $\mu$M (39.45% $\pm$ 2.22%) and the lowest protein content was derived from the dosage of 1200 $\mu$M (36.56 $\pm$ 2.23%) compared with control (37.12% $\pm$ 2.40%) as shown in **Figure 14**. The figure showed no significant alteration of protein at various 5-azaC concentrations.

**Table 11** shows the probabilities of occurrence of high protein content mutations from the 5-azaC treated variety was 15.38% for $M_0$. All four dose treatments (except for 1200 $\mu$M) induced high protein content mutations. As for induction of high protein content after 5-azaC treatment, 12.82% and 2.56% of seeds from $M_0$ exceeded the standard deviation of control by two times and three times, respectively. These results suggested that 5-azaC might slightly affect the genes that regulated protein metabolism or seed development.

An average protein content of treated seeds were fluctuated with the increasing 5-azaC concentrations. Because the level of 5-azaC required for optimal induction might be due to cell line or gene-specific differences in sensitivity of the methylation to 5-azaC. In addition, fluctuation of the protein content may occur from doses and short duration of exposure to 5-azaC because the long periods of time may be required for incorporation of the 5-azaC into the DNA of cell to alter gene expression. Besides,

due to the fact that 5-azaC had random hypomethylating effects and not all sequences are demethylated to the same extent, so the protein content of treated seed were fluctuated with the 5-azaC treatment. Finally, seeds were the late plant differentiation so demethylated sites may be remethylated after a certain number of cell division by *de novo* methyltransferase (Sano *et al*., 1990).

Due to our clearly demonstrate that 5-azaC could exhibit high protein soya plant. This finding is benefit to agricultural sector. It opens a chance to apply this protocol for soya plant improvement for such purpose. Certainly its high protein seeds will be benefit also for the animal feed industry.

**3) The effects of 5-azaC on the oil content**

As shown in **Table 12** and **Figure17**, 5-azaC can induce high oil content mutations. The frequency of high oil content mutations in $M_0$ lines treated with 5-azaC were ranged from 14.90% to 22.84%, with the highest value being 5.73% more than that of the control and the lowest value 2.21% less than that of the control. An average oil content of treated seeds were fluctuated with the increasing 5-azaC concentrations. The highest oil content was derived from the dosage of 1200 $\mu M$ (20.38% $\pm$ 1.65%) and the lowest oil content was derived from the dosage of 300 $\mu M$ (17.09% $\pm$ 1.38%) compared with control (17.11% $\pm$ 1.12%) as shown in **Figure 17.** The figure showed significant alteration of oil content at various 5-azaC concentrations ($p < 0.05$).

**Table** 13 showed that the probabilities of occurrence of high oil content mutations from the 5-azaC treated was 52% for $M_0$. All dose treatments induced high oil content mutations. As for induction of high oil content after 5-azaC treatment, 36% and 16% of seeds from $M_0$ exceeded the standard deviation of control by two times and three times, respectively.

In conclusion, our finding demonstrated clearly that DNA hypomethylation induced by 5-azaC treatment could exhibit a novel high oil content phenotype which would be benefit to the soya oil production industry.

**4) The effects of 5-azaC on the fatty acids composition**

A collection of mutants of *Glycine max* with altered seed lipid composition was isolated by determining the fatty acid composition from 20 seed samples. After lipid was subjected into fatty acid methylation and FAMEs were quantitated. The compositions of fatty acids in soybean seed at the pre- and post- 5-azaC treatment with various concentrations were shown in **Table 14**. The data demonstrated that the fatty acid compositions of treated seeds were fluctuated with the increasing 5-azaC concentrations. In addition, the fatty acid composition of treated soybean seeds had slightly increased in the amount of 18:2 and 18:3 fatty acids and a reduced level of 18:1 fatty acids when compared to the wild type, but not statistical significant. The decreased accumulation of 18:1 was accompanied by increased levels of 18:2 and 18:3. As shown in **Table 15**, the values was considered as total saturated fatty acid (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). **Table 15** clearly expressed that the treated soybean seed showed slightly reduced level of MUFA and slightly increased level of PUFA compared with the control seed, but not significant difference in statistics. The results were likely to demonstrate the fatty acid composition as shown in **Table 14**. The graphic presentation derived from **Table 14** was shown in **Figure 19**. The figure shows no significant alteration of soybean fatty acid at various 5-azaC concentrations. These results suggested that the 5-azaC may slightly affect to genes that regulated lipid metabolism, seed development ,similar to the effects of 5-azaC on the protein content.

Because of the inherent limitations of this approach, many other desirable change in seed oil fatty acid composition may require the directed application of

genetic engineering methods. Unfortunately, as in many other aspects of plant biology, the lack of specific information about the biochemistry and regulation of lipid metabolism makes it difficult to predict how the introduction of one or a few genes might usefully alter seed lipid synthesis.

Eventhough the fat content could be elevated by 5-azaC treatment, but the fatty acid composition is far more response to such application.

**5) Demethylation of genomic DNA by 5-azaC treatment**

Seeds were treated with various 5-azaC concentrations for 16 h, and cultivated for 3 months. Genomic DNAs were extracted from the short, tall, high protein, and high oil plants as well as from the controls. **Table 16** showed that the percentage of $m^5C$ in DNA were 26.02% and 25.78% from the control and 1500 $\mu M$ 5-azaC-treated plants, respectively, corresponding to a 0.24% reduction of $m^5C$ in 1500 $\mu M$ 5-azaC-treated plants. When compared with the control plants, the 5-azaC-treated plants showed some significant difference in the $m^5C$ content. This slightly hypomethylated change was not related to the phenotypic change such as the height.

**Table 17** summarized these experimental data, which showed that, 26.02%, 25.75% and 25.73% of the total cytosines in DNAs from control, 5-azaC-induced short and 5-azaC- induced tall plants were methylated, respectively, corresponding to a 0.27% reduction of $m^5C$ in 5-azaC-induced tall plants and a 0.29% reduction of $m^5C$ in 5-azaC-induced short plants. When compared with the control plants, the 5-azaC-induced tall and short plants showed no significant difference in the $m^5C$ content.

As shown in **Table 17**, 26.02% , 25.72%, 25.70% of the total cytosines in DNAs from control, induced high protein, and induced high oil plants, respectively, corresponding to a 0.30% reduction of $m^5C$ in induced high protein plants and a 0.32 % reduction of $m^5C$ in induced high oil plants. When compared with control plants,

the 5-azaC-induced high protein and high oil plants showed no significant difference in the $m^5C$ content.

These results indicated that the genes involved in plant growth, protein and lipid metabolism may have been slightly affected in all treated seeds by 5-azaC treatment. Because this experiment was focused on the late stage of plant growth such as protein content, oil content, and fatty acid in the soybean seeds. DNA from treated plants may have undergone a certain level of remethylation, by *de novo* methyltransferase(s) after a number of cell division or time of exposure to 5-azaC not enough for incorporation of the 5-azaC into the DNA of cell (Sano *et al*, 1990). Finally, DNA methyltransferases in soybean seed may be very active enzymes so 5-azaC slightly affect on the level of DNA methylation but 5-azaC can affect on the phenotypic variation such as height.

**C. <u>Subsequent effects of the 5-azaC treatments in the $M_1$</u>**

To determine whether the phenotypic changes induced by 5-azaC were inheritable in the absence of further 5-azaC treatment, the growth of self-fertilized progenies 5-azaC-treated and control parental plants were examined. These seeds were planted in the field (November 1998-February 1999), their final stem lengths were measured at maturity.

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**1) The effects of 5-azaC on the phenotype**

**Table 18** showed the mean heights for the tall and short treated plants used to generate the $M_1$ generation. The $C_1$ plants from an control parental line showed an average stem length of $51.89 \pm 3.41$ cm (n = 9).

The $M_1$ generation, derived from a treated parental line that had been talled by the treatment with an average stem length of $55.82 \pm 2.48$ cm (n = 11). When

compared with the control, the progeny from the tall plants were significantly taller than the control plants. However, the $M_1$ generation (n = 11) from a typical 5-azaC-induced tall, segregated into 6 normal (54 $\pm$ 0.89 cm) and 5 tall (58 $\pm$ 1.87 cm) plants as shown in **Table 18**. The phenotypes of these $C_1$ and tall $M_1$ plants were shown in **Figure 20. Table 19** showed that the leaf size of the progeny from the tall plants were significantly shorter than that of the control plants. For example, the mean length of leaves were 12.71 $\pm$ 0.68 cm in the control plants and 10.98 $\pm$ 0.94 cm in the progeny from the tall plants and the 100 seeds weight of the progeny from the tall plants (12.62 $\pm$ 0.44 g) were significantly less than that of the control plants (15.28 $\pm$ 0.97 g).

The $M_1$ generation, derived from a treated parental line that had been shorted by treatment with an average stem length of 44 $\pm$ 6.60 cm (n = 11). When compared with the control, the progeny from the short plants were significantly shorter than the control plants. However, the $M_1$ generation (n = 11) from a typical 5-azaC-induced short, segregated into 5 normal (50.75 $\pm$ 2.75 cm) and 7 short (40.14 $\pm$ 4.60 cm) plants. The phenotypes of these $C_1$ and short $M_1$ plants are shown in **Figure 19. Table 20** shows that the leaf size of the progeny from the short plants were significantly larger and shorter than that of the control plants. For example, the mean width x length of leaves in the control plants and in the progeny from the short plants were 6.84 x 12.71 cm and 7.74 x 11.65 cm, respectively.

Based on the mean height, the progeny from the short plants were significantly shorter than the control plants and the progeny from the tall plants were significantly taller than the control plants.

**2) The effects of 5-azaC on the protein content**

The $M_1$ generation from a typical 5-azaC induced highest protein content plant (45.19%) were selected and defined in **Table 21**. The frequency of high protein content in the $M_1$ generation treated with 5-azaC were ranged from 39.28% to 42.10%,

with the highest value being 4.22% more than that of the control (37.88%) and all average protein content of the $M_1$ generation were higher than that of the control. Besides, the data show that the average protein content of $M_1$ were significantly higher than that of the control plants. The situation being similar to that of the selected $M_0$ line, where the highest value was 8.07% more and the lowest value was 4.39% less than the control. The variation in genetics also showed that the protein content increased in $M_0$ more than in $M_1$. Though the increase in $M_1$ was low, it was still higher than that of the control, indicating the existence of high protein content lines. While, the average protein content of $C_1$ was the same as that of the $C_0$. This study indicated that the selected 5-azaC induced high protein were inheritable.

In conclusion, our experiment confirmed that 5-azaC even randomly induced hypomethylation, but such high protein phenotype is some part stable inherited to the progeny which will give a chance for plant breeder to take benefit from this novel technique.

**3) The effects of 5-azaC on the lipid content**

The $M_1$ generation from a typical 5-azaC induced highest oil content plant (22.84%) were selected and defined in **Table 22**. The frequency of high oil content in the $M_1$ generation treated with 5-azaC were ranged from 21.54% to 23.17%, with the highest value being 1.61% more than that of the control (21.56%) and all average oil content of the $M_1$ generation were higher than that of the control. Although, the data showed that the average oil content of $M_1$ were no significantly higher than that of the control plants. The situation being similar to that of the selected $M_0$ line, where the highest value was 5.73% more and the lowest value was 2.21% less than the control. The variation in genetics also showed that the oil content increased in $M_1$ more than in $M_0$ and it was still higher than that of the control, indicating the existence of high oil

content lines. This study indicated that the selected 5-azaC-induced high oil were certainly inheritable and thus should also benefit to the plant breeders.

**4) Subsequent effects of 5-azaC on the fatty acid composition**

In order to examine the inheritance of the altered fatty acid composition in the various lines with abnormal fatty acid composition (increased linoleic acid). The $M_1$ seeds were planted, grown to maturity and the fatty acid composition of samples of the $M_1$ seeds was determined and presented in **Table 23**.

As shown in **Table 23**, the $M_1$ was found to exhibit slightly increase in stearic acid content in the seed oil compared with the original variety. Nevertheless, genetic analysis indicates that it was not inheritable. This result may be due to : the oil content of soybean seeds was a polygenic and responsible to environmental effects that occur during plant development ($M_0$ generation were grown in the field during May to August 1998 and $M_1$ generation were grown during November 1998 to February 1999).

This finding confirm that to induce any alteration in the soya fatty acid composition is really impossible by the application of the demethylation process.

**5) Methylated cytosine analysis**

Genomic DNAs were extracted from the selected phenotypes as well as from the controls. **Table 24** showed that the percentage of $m^5C$ in DNA were 25.73%, 25.78%, 25.74%, 25.50% and 25.77% from the control, the tall, the short, high protein, high oil plants, respectively. When compared with the control plants, the selected phenotypes showed no significant difference in the $m^5C$ content. In the $M_2$ generation, all plants showed the same $m^5C$ levels as their treated and control parents.

# CHAPTER V

# SUMMARY AND CONCLUSION

The effects of 5-azaC treatment on plant growth, protein content, oil content and fatty acid composition in the soybean seeds are summarized as follows.

## A. The effects of 5-azaC on *in vitro* soybean seedlings

1. 5-AzaC did not influence plant survival; the viability of seedlings in each treatment was 100%
2. The plant height of 5-azaC-treated seedlings was proportionally decreased with the increasing 5-azaC concentration with the maximum value of 71% reduction in plant height in the dosage of 1500 $\mu$M.
3. The root length of 5-azaC-treated seedlings was also proportionally decreased with the increasing 5-azaC concentration with the maximum value of 78% reduction in root length in the dosage of 1500 $\mu$M.
4. The plant development seemed to be unaffected by 5-azaC treatment with no difference in the number of leaves or tillers.
5. 5-AzaC induced demethylation of genomic DNAs of the seedlings with the maximum value of 1.69% reduction in the $m^5C$ content.

**In conclusion**, the plant height and root length of 5-azaC-treated seedlings were 5-azaC dose dependent and related to the hypomethylation status.

**B. The effects of 5-azaC on the mature soybean plants ($M_0$)**

1. 5-AzaC treatment induced tall plant and high node number, without apparently affecting any developmental processes.
2. The average protein and oil content of 5-azaC-treated seeds were dose independent, the highest protein content and the highest oil content were derived from the dosage of 1500 $\mu$M and 1200$\mu$M 5-azaC, respectively.
3. Fatty acid profiles remained unchange with 5-azaC treatment.
4. Genomic DNA isolated from the selected phenotype such as tall, high protein or high oil content showed no significant difference in the $m^5C$ content in comparison with DNA from the control samples.

**In conclusion**, the 5-azaC induced tall phenotype, high protein or high oil content which were dose independent and not related to the hypomethylation status.

**C. Subsequent effects of 5-azaC on the $M_1$**

1. The $M_1$ generation from a typical 5-azaC-induced tall plant were significantly taller than the control plants. However, the progeny from the tall plant, segregated into 6 normal and 5 tall plants.
2. The $M_1$ generation from a typical 5-azaC-induced short plant were significantly shorter than the control plants. However, the progeny from the short plant, segregated into 5 normal and 7 short plants.
3. The variation in genetics showed that the protein content is higher in the $M_0$ than in the $M_1$, which $M_1$ was low, but still higher than that of the control, this indicating the existence of high protein content line.
4. The variation in genetics showed that the oil content is higher in the $M_1$ than in the $M_0$, and it was still higher than that of the control, this indicating

the existence of high oil content line.

5. Genomic DNA isolated from the selected phenotype such as tall, high protein or high oil content showed no significant difference in the $m^5C$ content in comparison with DNA from the control samples.

**In conclusion**, the selected 5-azaC induced phenotypes were inheritable.

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# APPENDIX

## Chemical preparation

1) MS media (Murashige and Skoog, 1962)

| | | |
|---|---|---|
| Macronutrients | | |
| $KNO_3$ | 1,900 | mg/l |
| $NH_4NO_3$ | 1,650 | mg/l |
| $CaCl_2.2H_2O$ | 440 | mg/l |
| $MgSO_4.7H_2O$ | 370 | mg/l |
| $KH_2PO_4$ | 170 | mg/l |
| Micronutrients | | |
| $MnSO_4.4H_2O$ | 22.30 | mg/l |
| $ZnSO_4.4H_2O$ | 8.60 | mg/l |
| $H_3BO_3$ | 6.20 | mg/l |
| KI | 0.83 | mg/l |
| $Na_2MoO_4.2H_2O$ | 0.25 | mg/l |
| $CuSO_4.5H_2O$ | 0.025 | mg/l |
| $CoCl_2.6H_2O$ | 0.025 | mg/l |
| Fe EDTA | | |
| $Na_2EDTA$ | 37.30 | mg/l |
| $FeSO_4.7H_2O$ | 27.80 | mg/l |
| Vitamin | | |
| Nicotinic acid | 0.25 | mg/l |
| Pyridoxine HCl | 0.25 | mg/l |

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| | | |
|---|---|---|
| Thiamine HCl | 0.05 | mg/l |
| Glycine | 1 | mg/l |
| Agar | 8 | g/l |
| Sucrose | 30 | g/l |
| pH 5.6 | | |

2) 50mM 5-azacytidine

5-azacytidine 0.1221 g are dissolved in deionized water. Adjust the volume of the solution to 10 ml and sterilize by filtration, kept at -20°C.

3) 5M NaCl

NaCl 292.2 g are dissolved in distilled water. Adjust the volume of the solution to 1 liter and sterilize by autoclaving.

4) 0.5M $Na_2EDTA$ pH 8.0

$Na_2EDTA$ 186.1 g are dissolved in 800 ml of distilled water. Adjust the pH to 8.0 with 20 g of NaOH. Adjust the volume of the solution to 1 liter and sterilize by autoclaving.

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5) 1M Tris-HCl pH 8.0

Tris-base 121.1 g are dissolved in 800 ml of distilled water. Adjust the pH to 8.0 with conc. HCl. Adjust the volume of the solution to 1 liter and sterilize by autoclaving.

6) 4.4M ammonium acetate pH 5.2

Add 105 ml deionized water to a 500 ml beaker and add 50 ml glacial acetic acid ,stir with a magnetic stirrer while adding drops of 45 ml ammonium hydroxide.

7) TE buffer pH 8.0

Ten ml of 1M Tris-HCl pH 8.0 and 2 ml of 0.5M $Na_2EDTA$ pH 8.0 are dissolved in deionized water and adjusted the volume of the solution to 1 liter.

8) 20% Sarkosyl

N-Lauryl sarkosyl 20 g are dissolved in deionized water. Adjust the volume of the solution to 100 ml.

9) Urea extraction buffer (total volume 400 ml)

| | | |
|---|---|---|
| Urea | 168 | g |
| 5M NaCl | 25 | ml |
| 1M Tris-HCl pH 8.0 | 20 | ml |
| 0.5M $Na_2EDTA$ pH 8.0 | 16 | ml |
| 20% Sarkosyl | 20 | ml |

These chemicals are dissolved in deionized water. Adjust the volume of the solution to 400 ml (don't autoclave).

10) Mixing indicator

Dissolve 20 mg of methyl-red and 100 mg of bromocresol-green in 100 ml of ethanol.

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