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THESIS

CELL CYCLE ACTIVITY AND GENE EXPRESSION OF CUCUMBER SEED DURING SEED PRIMING

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Damrongvudhi Onwimol 2014: Cell Cycle Activity and Gene Expression of Cucumber Seed during Seed Priming. Doctor of Philosophy (Agricultural Biotechnology), Major Field: Interdisciplinary Graduate Program. Thesis Advisor: Assistant Professor Thammasak Thongket, Ph.D. 101 pages.

Osmopriming, using of KNO_3 as an osmotic agent, was recognized as one of the methods to enhance germination performance in a laboratory for cucumber seed. Its principle is to initiate germination process to occur to some extent and cease it before radicle emergence taking place. The key factor controlling the effectiveness and success of seed osmopriming is the markers to cease the imbibition process when the desirable germination advancement is achieved. Therefore, the correlation between cell cycle activity and the advancement of seed germination process as well as the physical and biochemical changes during imbibitions periods of germination process and their correlation with the achieved seed quality after priming were studied with the aim to find the effective markers for cucumber seed priming.

Cell cycle activity study during imbibition periods of normal cucumber seed and heat deteriorated cucumber seed revealed that DNA content of (4C+8C)/2C ratio of the seedling radicle tip had a positive correlation with germination advancement by which it increased six hours prior to radicle emergence in normal cucumber seeds. However, it was not for heat deteriorated seeds. The results from the study of physical and biochemical changes during seed imbibitions periods of osmopriming revealed that the imbibition stage at which seed moisture content reached the beginning of phase II of tri-phases of imbibitions gave the best seed quality after priming for both before and after AA treatment. The down regulated expression of *Cucumis sativus EMB-1-like*, involving in desiccation tolerance, in the later stage of phase II of imbibitions coincided with the declining of achieved seed quality after priming indicating the negative effect of excessive imbibition time beyond the beginning of phase II. These results suggest that the seed moisture content at the level reaching the beginning of phase II of imbibition in combination with the expression of the *Cucumis sativus EMB-1-like* can be used as the effective markers to find the suitable time to cease the imbibition process of osmopriming for cucumber seed.

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Student's signature Thesis Advisor's signature

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Damrongvudhi Onwimol

July 2014

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LIST OF ABBREVIATIONS

AA	=	accelerated aging
C	=	DNA content of a holoploid genome with chromosome number n
°C	=	degree Celsius
cDNA	=	complementary DNA
CDKD1	=	cyclin-dependent kinase D-1 gene
cm	=	centimeter
CTAB	=	cetyltrimethylammonium bromide
DAPI	=	4,6-diamidino-2-phenylindole
°	=	degree
ΔCT	=	delta-CT quantification model
DEPC	=	diethyl pyrocarbonate
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
dNTP	=	deoxynucleotide triphosphate
DTT	=	Dithiothreitol
EDTA	=	ethylenediaminetetraacetic acid
EF1α	=	elongation factor 1 alpha gene
EMB-1	=	late embryogenesis abundant 1 gene
g	=	gram
G_0 phase	=	quiescent state of cell cycle
G_1phase	=	first gap phases of cell cycle
G_2phase	=	second gap phases of cell cycle
HAI	=	hours after imbibitions
H_2O_2	=	hydrogen peroxide
KNO_3	=	potassium nitrate
LEA	=	late embryogenesis abundant proteins
LSD	=	least significant difference post hoc tests
′	=	lipda; 1/60 part of a degree in an angle
MaxRE	=	Maximum radicle emergence (%)

LIST OF ABBREVIATIONS (Continued)

MaxF	=	Maximum fungal infected seed (%)
mg	=	milligram
MRET	=	mean radicle emergence time
µL	=	microliter
µm	=	micrometer
µM	=	micromolar
mL	=	milliliter
mm	=	millimeter
mM	=	millmolar
M-MuLV	=	Maloney Marine Leukemia Virus
MPa	=	megapascal
M phase	=	phase of mitosis and cytokinesis of cell cycle
NaCl	=	Sodium chloride
$(NH_4)_2SO_4$	=	ammonium sulfate
%	=	percent
″	=	pilipda; 1/3600 part of a degree in an angle
PVPP	=	polyvinylpolypyrrolidone
ψ_s	=	specific solute potential
ψ_{seed}	=	total water potential of a dry seed
qPCR	=	quantitative reverse transcriptase polymerase chain reaction
RH	=	relative humidity
RNA	=	ribonucleic acid
RNase	=	ribonuclease
s.e.	=	standard error of mean
SMC	=	seed moisture content
S phase	=	phase of DNA replication in cell cycle
t_{50}	=	hours to reach 50% maximum germination
U	=	enzymic activity unit
U_{7525}	=	time between 25% and 75% germination

CELL CYCLE ACTIVITY AND GENE EXPRESSION OF CUCUMBER SEED DURING SEED PRIMING

INTRODUCTION

The first key factor of a successful plantation is a use of good cultivars. In addition, the forwarding of such good cultivars to farmers is important as well. Cucumber is one of the important vegetables in Thailand. It generated revenue for the country, ranked the fourth after watermelon, tomatoes and peppers. Thailand exported about 253 million baht cucumber seeds in 2013 (THASTA, 2013). Therefore, the production of seed quality is important for competitive ability of seed producers. One of the methods that can improve the speed and uniformity of germination is called seed priming. Seed priming is the most important physiological seed enhancement method. Seed priming is a hydration treatment that allows controlled imbibition and induction of the pregerminative metabolism ("activation"), but radicle emergence is prevented (Halmer, 2006). After regaining the moisture again, these primed seeds will germinate in higher speed and more uniformity than unprimed seeds.

However, seed priming can be harmful to the seed lot if the hydration process is too long leading to the emergence of radicle (over-priming incidence) or loss of desiccation tolerance and storability after seed priming (Halmer, 2006). Therefore, the critical step to the success in seed priming is to terminate the hydration process at the appropriate time that provides the satisfy germination advancement without losing desiccation tolerance of that seed lot. In the past, there was an effort to find the appropriate markers, both physicals and biochemical, which are closely related to the germination advancement during the priming process that can be used for controlling the hydration time of seed priming process. It has been reported that the changes of seed moisture content and seed weight as the physical markers have the positive relationship with germination advancement during seed priming process. However, their reliability and precision are rather low. They vary with species, verities and seed lots (Heydecker and Coolbear, 1977). In contrast, the biochemical markers e.g. DNA contents during cell division and the expression of genes related to germination

process and desiccation tolerance were reported to associate with priming progression (Gendreau *et al.*, 2012; Kotak *et al.*, 2007; Sliwinska, 2009). They may give the better reliability and precision for determining the germination advancement during seed priming process.

This study aimed to investigate the relationship of the physical and biochemical changes with the progression of the germination process during as seed priming process. The research finding should give the better understanding that lead to properly control hydration process during seed priming of cucumber in order to obtain high quality seed with acceptable storability.

OBJECTIVES

The ultimate goals of this study was to make an understanding on the physical and biochemical changes during seed priming process of cucumber seed which will lead to the control of the advancement of priming process of cucumber seed. The objectives of this study were as followings:

1) To study the relationship between cell cycle activity at the radicle tip cells of cucumber seed embryo and the advancement of germination process during imbibition period of seed priming.

2) To study the physical and biochemical change and gene expression of cucumber seed during imbibition period of osmopriming process.

LITERATURE REVIEW

1. Cucumber (*Cucumis sativus* L.)

Cucumber is creeping plant of the Cucurbitaceae family. Its diploid number of chromosomes (2n) equals to 14 and it is naturally the cross pollinated crop (Encyclopaedia Britannica, 2013).

F1 hybrid cucumber seed production.

Under natural conditions, most cucurbit cultivars are cross pollinated by insects, mainly bees. However, self-pollination may occur depending on the genotype, environmental conditions, plant population and insect activity. In many cases, for commercial production of cucurbit seed, hives are incorporated in the field to ensure adequate pollination and good yield. This practice is also performed in seed production of open-pollinated seed or hybrids that are not hand pollinated. Heterosis or hybrid vigour has been reported in several cucurbit species. Earliness, yield and fruit quality are some of the most frequent traits influenced by heterosis. Additionally, several disease resistance genes have been introduced in F1 hybrids. Today, most commercial summer squash, cucumber, melon, and watermelon cultivars are F1 hybrids (Robinson, 2000). Some of the reasons for the increased popularity of hybrid cultivars in species of this family are that numerous seeds are produced per fruit and there are a relatively low number of seeds required per hectare for crop establishment. In addition, different techniques to achieve more cost-efficient hybrid seed production have been developed. Among these techniques used for hybrid cucurbit seed production are:

– Hand emasculation and pollination. This technique is frequently used for melon seed production. In this species, andromonoecious lines are common and they must be emasculated and hand pollinated if used as the female parent for producing hybrid seed. This method has also been used for some watermelon and cucumber hybrids. Flower removal or manual removal of male flowers from the female parental line. This method has been used for hybrid seed production of summer squash. It consists of planting the male and female lines in the field, removing the male flowers

from the monoecious female line and then allowing pollination by bees. This method has been replaced by the use of ethephon as a growth regulator that suppresses formation of male flowers in the female line.

– Gynoecy or the use of lines with only female flowers as female parents of hybrids. This method has been used in cucumber. Hybrid seed is produced by growing the gynoecious female line in the same field as the male line producing the pollen. Pollination is performed by bees.

– Use of growth regulators to modify sex expression, ethephon is the most common growth regulator used. This compound is mainly used in commercial hybrid seed production of summer squash and some monoecious cucumber hybrids. Genetic male sterility has been reported in cucumber, melon, squash, and watermelon. However, this principle has not been used extensively. It has importance for winter squash (*C. maxima*) and watermelon hybrid seed production in the United States and China, respectively. When hybrid seeds are produced in the field, an isolation distance of at least 1000 m from other cucumber plants is required. In the case of foundation seed production, isolation should be at least 1500 m. These isolation requirements may be avoided when seeds are produced inside insect-proof structures.

For harvesting, fruits should be harvested manually when they are fully mature and seeds reach their maximum physiological quality. This moment is often determined by the color of the mature fruit, which may vary among cultivars, but usually is a clear brownish color. The maturity of the seeds may be confirmed by cutting the fruit longitudinally and determining that the seeds separate easily from the interior flesh. For gynoecious and parthenocarpic cultivars, acidity of the fruit pulp may also be used as a harvest index. After harvest, seeds are extracted by scraping the open fruit manually or using a crusher and seed extractor (George, 1999). The extracted seeds should be fermented for about one day and then washed.

Currently, the progressions in bioinformatics database are very affects to consider and decision of breeding program. The International Cucurbit Genomics

Initiative (ICuGI) is one of cucumber genome database which have presently established for 3 core missions as follows:

1) Functional Genomics: sequencing of 100,000 ESTs from different melon genotypes and tissues.

2) Mapping: merging the existing melon genetic maps using SSRs as anchor markers.

3) Bioinformatics: development of a webpage for the ICuGI, where certain genomic tools would be available for the cucurbit research community.

The ICuGI is very useful for breeding of cucurbit species. Due to the insights of gene expression related to sex, disease resistance, evolution and biosynthetic pathway can be easily searched *via* website of Cucurbit Genomics Database (Huang *et al.*, 2009).

2. Seed Priming

Seed priming is the most important physiological seed enhancement method. Seed priming is a hydration treatment that allows controlled imbibition and induction of the pregerminative metabolism ("activation"), but radicle emergence is prevented (McDonald, 2000; Schwember and Bradford, 2010). The hydration treatment is stopped before desiccation tolerance is lost (Halmer, 2006; Heydecker and Coolbear, 1977). The critical action is to stop the priming process in the right moment; this time depends on the species and the seed batch. Molecular marker can be used to control the priming process. Priming solutions can be supplemented with plant hormones or beneficial microorganisms. The seeds can be dried back for storage, distribution and planting. Germination speed and uniformity of primed seeds are enhanced (Figure1) and can be interpreted in the way that priming increases seed vigour (short or no "activation" time). A wider temperature range for germination, release of dormancy and faster emergence of uniform seedlings is achieved. This leads to better crop stands and higher yields. A practical drawback of primed seeds is often a decrease in storability and the need for cool storage temperatures.

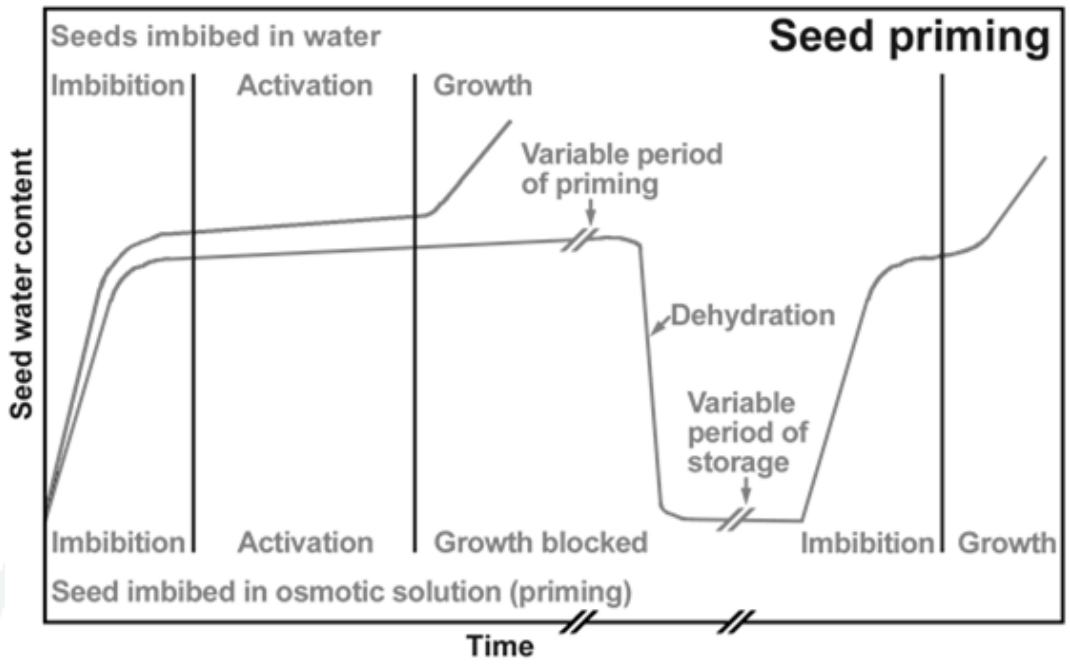

Figure 1 The pattern of water uptake of seed imbibed in water and imbibed in osmotic solution or seed priming.

Source: Bradford and Bewley (2002)

Types of seed priming commonly used (Halmer, 2006):

Osmopriming (osmoconditioning) is the standard priming technique. Seeds are incubated in well aerated solutions with a low water potential, and afterwards washes and dried. The low water potential of the solutions can be achieved by adding osmoticant like mannitol, polyethyleneglycol (PEG) or salts like KCl and KNO_3.

Hydropriming (drum priming) is achieved by continuous or successive addition of a limited amount of water to the seeds. A drum is used for this purpose and the water can also be applied by humid air. 'On-farm steeping' is the cheap and useful technique that is practiced by incubating seeds (cereals, legumes) for a limited time in warm water.

Matrixpriming (matriconditioning) is the incubation of seeds in a solid, insoluble matrix (vermiculite, diatomaceous earth, cross-linked highly water-absorbent polymers) with a limited amount of water. This method confers a slow imbibition.

Pregerminated seeds are only possible with a few species (Bruggink *et al.*, 1999). In contrast to normal priming, seeds are allowed to perform radicle protrusion. This is followed by sorting for specific stages, a treatment that re-induces desiccation tolerance, and drying. The use of pregerminated seeds causes rapid and uniform seedling development.

The hydration treatment is stopped before desiccation tolerance is lost. An important problem is to stop the priming process in the right moment as mention before. Seed quality after this process may be no difference to non-treated seeds, if germination process is stopped too soon after soaking. In other words, if process is stopped too slowly. These seeds may not be stored for long time because the biochemical change of germination process often inversely varied with storage (Soeda *et al.*, 2005). Researchers now are trying to seek the marker that can be used to control the priming process or pinpoint the suitable imbibition period (Halmer, 2006).

3. Markers for the priming process

Presently, markers for the priming process are frequently performed in two aspects (Halmer, 2006), viz:

3.1 Physical markers

Physical markers are usually used for tracking physical changes during the priming process such as the analysis of seed moisture content. This marker is effective, however, the analysis time consuming (about 6 hours for cucumber seeds). Moreover, the weight and seed moisture content (SMC) is a quantitative characteristic and were controlled by many genes. So, high variation of seed weight and SMC among seed lots under common priming treatment can be anticipated. Furthermore, the changes of

weight and moisture content during priming process of alive seed and dead seed are not different.

3.2 Biochemical markers

Currently, molecular markers are being used to indicate the suitable imbibition period in seed priming process. Since the molecular marker outclass the physical markers in many aspects such as shorter analysis phase and more precision because molecular markers can respond to alive seed only. In addition, use of molecular markers will specify at starting point of phenotype or gene by gene analysis. However, the high cost of its analysis is the disadvantage for using these markers. Contemporary reports using molecular markers in multiple formats are (Sliwinska, 2009):

3.2.1 Analysis of estimative nuclear DNA content or cell cycle activity

A mitotic cell division is important during seed germination process because radicle tip cells require such a process to enlarge and push out of the seed coat. This process usually occurs before the process of radicle emergence about 5-10 hours (Figure 2). Seedmen trace the progress of cell division by measuring the amount of DNA (DNA content) in nuclei of radicle tip cell by using the flow cytometry machine (Jing *et al.*, 1999; Liu *et al.*, 1997; Rewers *et al.*, 2009; Sliwinska, 1996; Sliwinska, 2009). DNA content of such tool can be converted to a change of chromosome number index or cell cycle of radicle tip cells which allows the seedmen to specify the precise developmental stage of seeds. This gives possibility to stop the imbibition in the right moment. Another advantage of this tool is short analyzing time since it only takes about 10 minutes per sample. There were many reports of successful use of flow cytometry in seed priming process of many plant varieties such as sugar beet (Sliwinska, 2003) tomatoes (Jing *et al.*, 1999; Liu *et al.*, 1997) and cucumber (Rewers *et al.*, 2009).

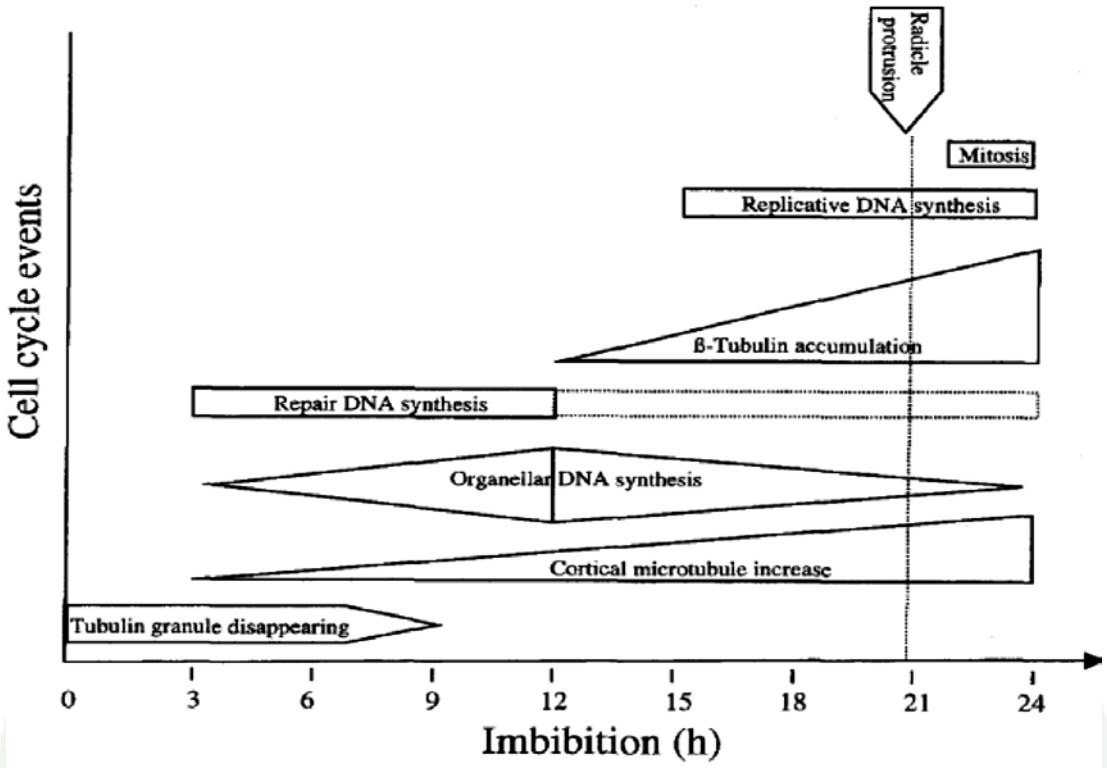

Figure 2 The cell cycle events of radicle tip cells of tomato during different imbibition times.

Source: Jing *et al.* (1999)

3.2.2 Analysis of ethanol production

One risk of priming process is too much of stimulation that is called over primed. When applying in drying process, the over primed seed will be deteriorated more than non-treated seeds. If the deterioration pattern of primed seed has been clarified, seedmen might have stopped the process before -over priming- will be occurred. So, the efficacious procedure, high sensitivity, may identify the suitable imbibition period in a priming process. Kodde *et al.* (2012) offered a fast ethanol assay to detect seed deterioration which examined the loss of mitochondrial membrane integrity. For this reason, this procedure might be used as a marker to pinpoint the suitable imbibition period in a priming process.

3.2.2 Analysis of germinative proteome

It is well known that not only the cell division process is important to germination process but there are several processes that are also important as well (Figure 3). Each process, seed coat degradation, newly synthesized DNA and nutrient mobilization will lead to the final result of germination which is radicle emergence. Genomic, transcriptomic and proteomic changes behind the process can be used as a marker to pinpoint the suitable imbibition period in a priming process. Before any markers get popularity in the seed industry, however, they need to face many challenges such as universalization, accuracy, reproducibility, convenience, and the cost effectiveness. These features should be included in the marker as much as possible (Table 1).

Table 1 Overview of the advantages and disadvantages of each priming marker.

Types of markers	Universalization	Accuracy	Reproducibility	Convenience	Rapidity	Cost effectiveness
Physical markers	moderate	low	moderate	high	moderate	cheap
Biochemical markers						
Cell cycle index	high	high	high	high	high	high
A fast ethanol assay	moderate	moderate	moderate	high	very high	high
Proteomic markers						
Specific protein	high	high	moderate	moderate	moderate	high
Non-specific protein	very high	very high	moderate	moderate	moderate	very high
Transcriptomic markers-mRNA						
Specific gene	very high	very high	high	moderate	moderate	high
Non-specificgene	very high	very high	high	moderate	moderate	very high

Source: Achard *et al.* (2002); Gendreau *et al.* (2008); Job *et al.* (1997); (Kodde *et al.*, 2012; Rewers *et al.*, 2009; Sliwinska, 2009); Soeda *et al.* (2005)

The germinative proteomic markers have been reported by Takatsuka *et al.* (2009). They studied the influence of protein CDKF;1 in Arabidopsis and

suggested that protein CDKF;1 is importantly associated with cell division, cell elongation and endoreduplication in post embryonic development in Arabidopsis seeds. They, proposed that the increase of CDKF;1 can be used as a marker for priming process of Arabidopsis seeds. The possibility of using germinative proteomics as the priming markers were also reported such as *β*-tubulin (Castro *et al.*, 1995), *β*-subunit of 11-S globulin and LEA (late embryogenesis abundant) proteins (Capron *et al.*, 2000; Job *et al.*, 1997) and AUBE1 (binding protein of PI promoter of *RPL21*) (Achard *et al.*, 2002).

Gallardo *et al.* (2001) studied the dynamics of non-specific proteins during germination of Arabidopsis seed by using 2-D gel electrophoresis and protein identification by MALDI-TOF. They found a number of proteins that had already been identified (1,325 species) during germination process of Arabidopsis. Therefore, researchers proposed the possibility of use of protein pattern for considering as an efficient marker.

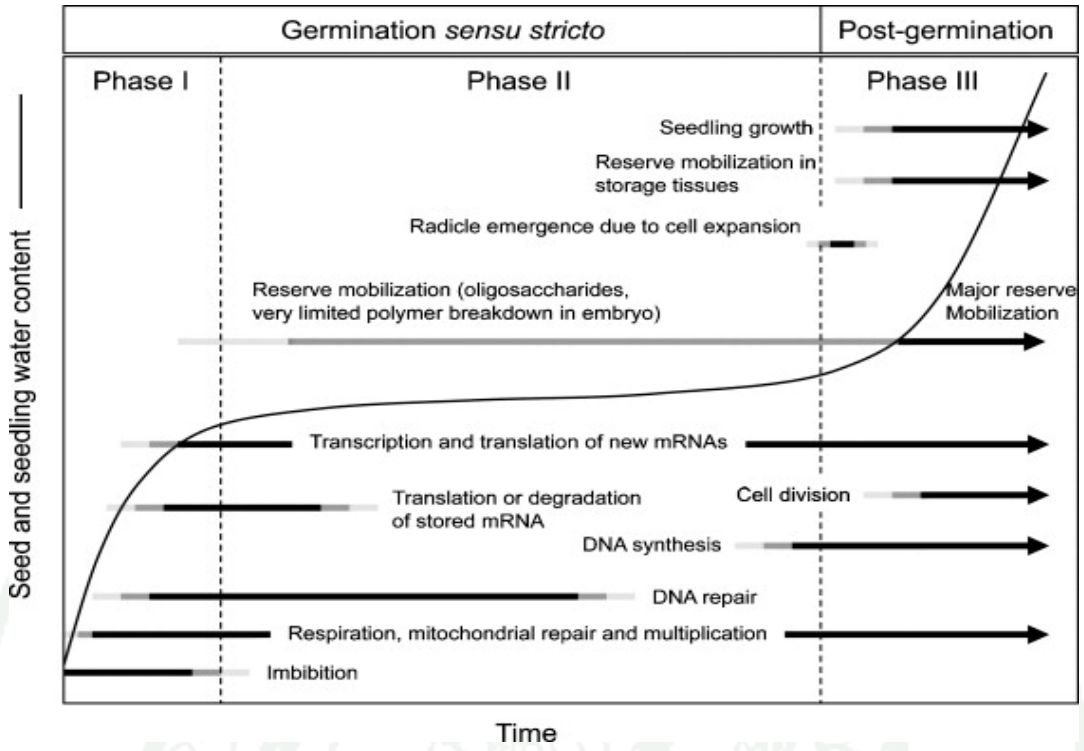

Figure 3 The biochemical changes in seeds during germination process.

Source: (Varier *et al.*, 2010)

3.2.3 Analysis of germinative transcriptome

The mRNA expression associated with the process of germination as a seed priming marker was widely used among seed researchers after A.D. 2005. The mRNA is the first level that reflects the expression of gene. The germinative transcriptomic approach, by using mRNA expression, is similar to the germinative proteomic approach as mentioned above. Two techniques for gene expression analysis; semi-or quantitative PCR and cDNA microarray were currently used by different groups of researchers (Gendreau *et al.*, 2008; Soeda *et al.*, 2005). It is noted that latter research commonly focus on the gene associated cyclin-dependent kinase (Cdk). Cyclin-dependent kinase activity closely involves in seed germination. The cyclin-dependent kinase activity would be occured before the cell cycle activity (see cell cycle activity in section 3.2.1). So, a study of the cell cycle by using flow cytometry can inform only the result of increase of DNA content while the study of Cdk will provide

information prior to increase of DNA content. The Cdk is an operator which will cause an increase or decrease of the DNA content as shown in Figure 4.

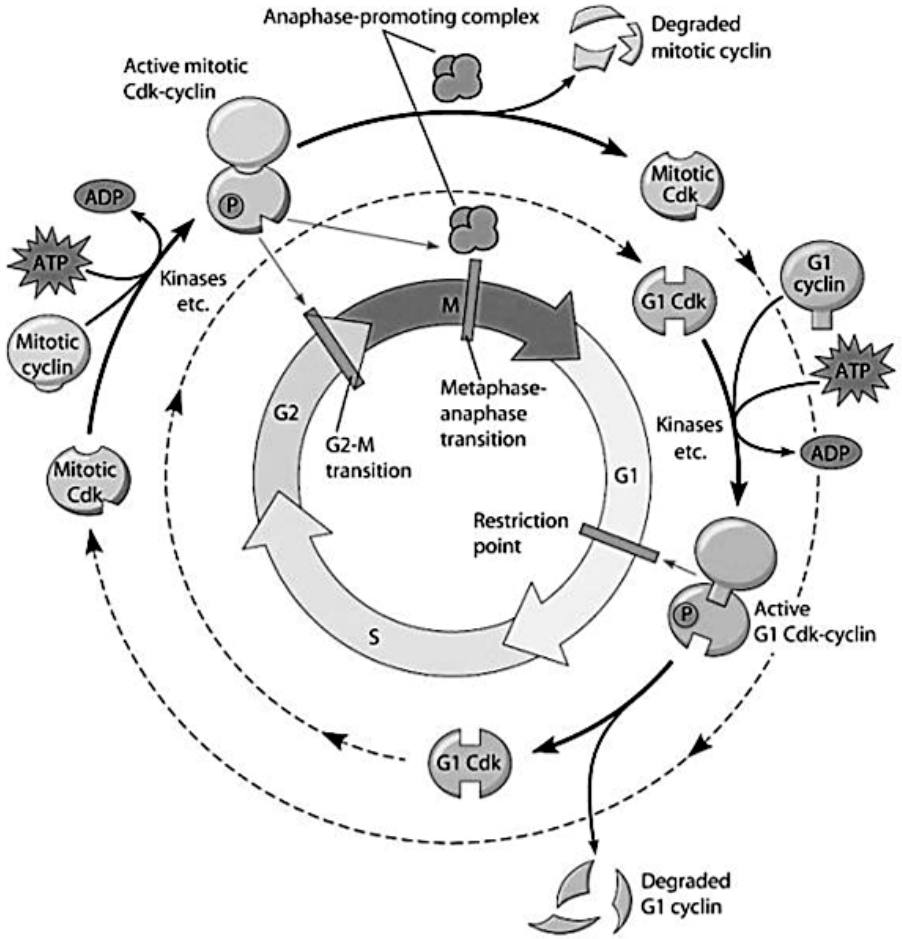

Figure 4 Relationship of cyclin-dependent kinase (Cdk) on the cell cycle in a eukaryotic cell.

Source: Inźe and Veylder (2006)

Internal processes that control seed storability is called desiccation tolerance process which is often inversely related to the germination capability (Boubriak *et al.*, 2007). Many studies have revealed the effect of desiccation tolerance on the seed storability. The expression of gene related desiccation tolerance associated with seed quality after priming was strongly concerned (Schwember and Bradford, 2005). Water potential (ψ) in dry seeds which are in equilibrium with 20% relative humidity can be as

low as -200 MPa. (Walters and Koster, 2007). In such a low humidity condition, seeds need some mechanisms to maintain energy and keep important molecules such as the conformational changes in the DNA. The change of DNA conformation will change into a B-form or synthesis LEA proteins to protect DNA from dryness including reduction of cell respiration. The expression of desiccation tolerance gene in dry seeds will be reduced when seeds obtain suitable conditions that can stimulate the germination process to take place.

MATERIALS AND METHODS

To be able to understand relationship between physical and molecular change during cucumber seed priming, this research is divided into two experiments:

1) Cell cycle activity of embryo radicle tip of cucumber seed during imbibition process.

1.1 Seed source

Seeds of cucumber (*Cucumis sativus* L.), obtained from Thai Seed and Agriculture Co. Ltd., Bangkok, were used in this experiment. The seeds had been kept at 5 ± 1°C, in 74% relative humidity (RH), for nine months subsequent to harvesting in July 2010 from a commercial seed production field situated at Suphanburi province, Thailand (14° 45'N, 100° 04'E). Prior to the experiment, the seeds were graded to include only seeds of 16 - 25 mg. Seeds were surface sterilized in 0.6% sodium hypochlorite for 20 minutes and subsequently washed with sterile water. Sterilized seeds were air-dried on a thin layer of filter paper to 8% moisture and kept at 5 ± 1°C prior to ageing treatments.

1.2 Seed vigour modification

Seed samples were subjected to three ageing treatments; control (untreated treatment), heating with warm air at 45 and 50°C. Seeds were placed on the surface of screen trays in 0.43 l polyethylene boxes, 11 × 11 × 6.5 cm, with 40 mL of saturated NaCl (UNIVAR) solution at the bottom of each box to create an environment of 74 - 75% RH. Each plastic box was subsequently incubated at either 45 or 50°C for 96 hours (memmert paraffin-ovens Model UNE 400PA). After the ageing treatments, the SMC was determined at 130°C for 1 hour and a standard germination test was conducted to verify the vigour difference among seed is subjected to difference aging treatments.

1.2.1 Seed moisture testing

SMC was determined at high constant temperature oven method (ISTA, 2012). Two replicates of 4.5 ± 0.5 g were dried in a hot air oven at 130–133°C for 1 hour ± 3 minutes then returned to room temperature for 30 minutes. The SMC was expressed in a percentage of the seed fresh weight (FW) by the following equation:

$$\text{SMC (percentage of fresh weight basis)} = \frac{\text{FW}-\text{DW}}{\text{FW}} \times 100\%$$

1.2.2 Germination test

The germination test was carried out immediately after the ageing treatments, in accordance with ISTA's rules for seed testing (ISTA, 2012), except only that one hundred seeds were used per treatment (four replicates of25 seeds each). Seeds were placed on top of moistened blotter papers in transparent 12 × 12 × 9 cm polyethylene boxes. Each box contained one replication (25 seeds) of each treatment. The boxes were placed in a germination chamber set at 25°C. Radicle emergence (about 2 mm in length) and germination (normal seedling) were determined at 24 hour intervals for eight days. Seedling quality was evaluated in accordance with the ISTA rules for seed testing. The mean emergence time (MET) and mean germination time (MGT) were calculated based on the following equation (Ellis and Roberts, 1978):

$$\text{MET or MRET (days)} = \Sigma(nD) / \Sigma n$$

Where n denotes the number of seeds showing radicle emergence (for MET) or number of normal seedling (for MRET) on day D and D is the number of days from the commencement of the germination test.

The remaining seeds were stored at 45% RH at room temperature prior to subjecting to imbibition process and DNA content analysis with flow cytometry.

1.3 Imbibition treatment

Every seed samples were imbibed in 20 mL deionized water in 9 cm diameter Petri dishes which were covered with plastic film to prevent evaporation and kept at 25 ± 1°C in the dark for two days. During the imbibition period, at the intervals of six hours, two samples from each ageing treatment were taken. One sample was used for SMC determination by using high constant temperature oven method, while the other was analyzed for DNA content by using a flow cytometer. The length of time to radicle emergence (2 mm in length) was also recorded.

1.4 DNA content analysis

A sample of 1-2 mm of the embryo radicle tip was chopped and used for flow cytometry (Figure 5). Samples were prepared as described by Koroleva *et al.* (2004). The radicle tip was dissected with a sharp razor blade in a plastic Petri dish and thawed by gentle agitation in 1 mL of Partec lysis buffer. After the addition of 1.5 mL of Partec staining buffer, the suspension was filtered through a 30-µM nylon mesh filter and analyzed on a Partec PAS II flow cytometer (Partec, Münster, Germany).

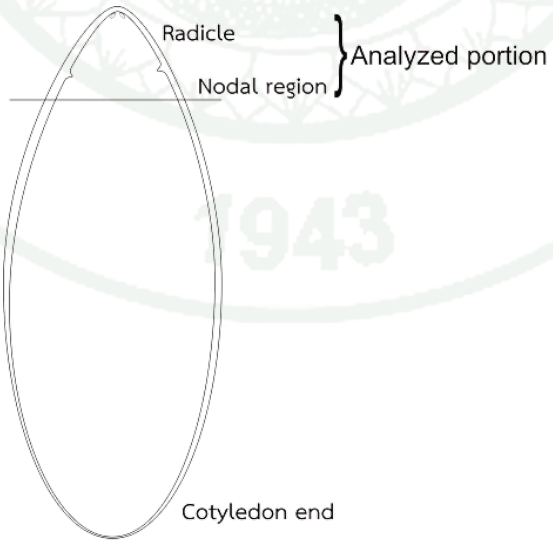

Figure 5 The position of seed to be cut in order to analyze the DNA content by using flow cytometer.

Analyzes were performed on four replicates, with a linear amplification of the signal. For each sample, 4,000-6,000 nuclei were analyzed. Histograms were evaluated with the application of the DPAC v.2.2 software. The proportion of nuclei of dissimilar DNA contents, the (4C + 8C) / 2C ratios as well as the mean C-value, the mean ploidy, were calculated according the proposed equation of Barow and Jovtchev (2007):

$$\text{mean C-value} = \frac{(2\times n2c)+(4\times n4c)+(8\times n8c)}{(n2c)+(n4c)+(n8c)}$$

Wheren 2C, n4C and n8C is number of nuclei with DNA content equal to 2C, 4C and 8C, respectively.C is a DNA content of a holoploid genome with chromosome number n, irrespective of the degree of plant ploidy.

1.4 Statistical analysis

Single-factor ANOVA (fixed effect model) and least significant difference (LSD) *post hoc* tests were performed on the results at a significance level of $P \leq 0.05$. The percentage data from the germination tests, the ratio data of DNA contents, the mean C-value, and the (4C + 8C) / 2C ratio, were subjected to analysis of variance after angular transformation (transformed by arcsine, untransformed values are shown in the table to facilitate comparison).

2) Physical and biochemical changes and gene expression of cucumber seed during imbibition period of osmopriming process.

2.1 Seed source

Two lots of cucumberF1 hybrid seed, CU-50 and CU-51 obtained from Chia Tai Seeds Co. Ltd. were used in this experiment. CU-50 was produced in 2007, while CU-51 was produced in 2008 at the commercial seed production field (18°16′12″N, 99°37′30″SE). Both seed lots had been stored at 5 ± 1°C in 35–40 %RH until they were used in this experiment. After receiving the seeds, the germination test was conducted to assess their quality. Both seed lots were equal in percentage radicle emergence of 100% but CU-51 had higher seed vigour than CU-50 as indicated by germination time and mean

germination time (Appendix Table A1). Therefore in this experiment, CU-50 represented low vigour seed lot while CU-51 represented high vigour seed lot. Before the experiment began, the seeds were graded to include only seeds that weighed between 20-40 mg. Seeds were surface sterilized in 0.6% sodium hypochlorite for 10 minutes and subsequently washed with sterile water for 10 minutes. Sterilized seeds were air-dried on a thin layer of filter paper approximate five hours thereafter SMC were reduced to 15% and were stored at 5 ± 1°C in darkness prior to exposure to priming processes.

2.2 Osmopriming process

Two osmopriming processes, rapid and slow osmopriming were used in this experiment by using the osmotic solutions with two osmotic potential. Two concentrations of KNO_3, 8.42 and 190.47 mM, were used to provide the osmotic potential with ψ_s of -0.05 and -0.8 MPa, respectively. During osmopriming process, seeds were contained in 19.2 × 28 × 5.7 cm transparent polystyrene boxes, and incubated in a dark cabinet at 20°C (5 mL of solution·g^{-1} of seed). For the rapid osmopriming process, both seed lots were primed in ψ_s of -0.05 MPa at 20°C using 8.42 mM KNO_3 (according to the Van't Hoff equation and were verified using a vapor pressure osmometer, Wescor model 5600, calibrated against NaCl standards). For slow osmopriming process, seed was primed in ψ_s of -0.8 MPa at 20°C by using 190.47 mM KNO_3. Seeds from each lot were soaked in the KNO_3 solution until the 50% of seed population had radicle emergence. During osmopriming process, the sample was withdrawn at 5 and 10 hours for rapid and slow osmopriming process, respectively, for physical, biochemical and gene expression analysis.

2.3 Analysis of physical, biochemical changes during osmopriming process

Analysis of physical and biochemical changes during osmopriming process were conducted at 5 hour intervals for the rapid osmopriming and at 10 hour intervals for slow osmopriming until radicle emergence of 50% of sample population was realized.

2.3.1 SMC

The seed moisture changing was investigated as the physical quality change during osmopriming process. SMC was determined by the high constant

temperature oven method similar to 1.2.1 (ISTA, 2012). In brief, two replicates of 4.5 ± 0.5 g seed sample were dried in 130–133°C for 1 hour ± 3 minutes then returned to room temperature for 30 minutes. The SMC, fresh weight basis, was expressed as a percentage of the seed fresh weight.

2.3.2 Radicle emergence

Cumulative radicle emergence, about 2 mm in length, were evaluated at 5 hour intervals for the rapid osmopriming and at 10 hour intervals for slow osmopriming until finished soaking (radicle emergence of 50% of sample population).

2.3.3 Ethanol production

The headspace ethanol of seed sample produced during heated incubation condition was determined by a fast ethanol assay to indicate seed deterioration during priming process. Two replicates of each sample were used for measuring ethanol production from partially imbibed seeds in 57°C for 4 hours using the ethanol assay which were described by Kodde *et al.* (2012) except only that water was added to achieve the desired percentage seed moisture of 30%FW. The amount of added water was computed based on the initial seed moisture content of 9.5%FW by estimate.

2.2.4 Molecular changes: quantitative RT-PCRs (qPCR)

Total RNA isolation following qPCR analysis to verify the relative quantification of expression for *Cucumis sativus CDKD1-like* and *Cucumis sativus EMB-1-like* by using *Cucumis sativus EF1α -like* as a reference genes was performed.

Pestles, mortars and all glassware used in the total RNA isolation were kept overnight at 200°C, whereas plastic ware and solutions were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved to inactivate RNases.

After giving samples from each time point for each seed lot and osmopriming method combination, excessive solution was forsaken and once rinse with DEPC-treated water. Two milligrams of seed samples were immediately submerged in 10

mL of RNA preservation medium (4M $(NH_4)_2SO_4$, 10 mM EDTA, and 0.1 M Mes free acid, pH 4.6), after that, seed was cross-sectioned to be less than 0.5 cm and stored at 4°C overnight for allowing the solution to thoroughly penetrate the tissue. Excessive supernatant were removed and stored samples at -80°C until total RNA was isolated. For the isolation of total RNA, a commercial kit of GeneJET Plant RNA Purification (Thermo Fisher Scientific Inc., Mini Kit 50 preps, cat# K0801) was used according to the manual from the company. Modified manual by which the polyvinylpolypyrrolidone (PVPP, 60mg/ml) and Dithiothreitol (DTT, 40 mM) were added to the extraction buffer to inactivate phenolic compounds present in the seed coat. Total RNA was treated with DNase I (Thermo Fisher Scientific Inc.) to remove any traces of genomic DNA according to the manufacturer's instructions before the first-strand cDNA synthesis was performed. The first-strand cDNA synthesis was performed using oligo$(dT)_{18}$ primers (Thermo Fisher Scientific Inc.) and 200 U of Maloney Marine Leukemia Virus (M-MuLV) reverse transcriptase (Thermo Fisher Scientific Inc.) for 30 minutes at 37°C. Successful synthesis of first-strand cDNA was confirmed by the presence of PCR amplification product using a primer pair (5′-ACTGTGCTGTCCTCATTATTG-3′ and 5′-AGGGTGAAAGCAAGAAGAGC-3′) designed to amplify an exon sequence of a gene encoding the EF1α (Wan *et al.*, 2010), while a touchdown PCR were used for these conditions (Korbie and Mattick, 2008). The qPCRs were carried out in a total volume of 20 µL containing 10.0 µL of 2× FastStart Essential DNA Probes Master (Roche Applied Science), 0.5 µL of TaqMan® probe (0.25 µM of final concentration in reaction), 0.5 µL (10 µM) of respective primer pairs (Appendix Table E1), 2.0 µL of a single-strand cDNA template from samples and 6.5 µL of PCR grade water. The thermal conditions for real-time PCR were 95.0°C for 10 minutes (heat activation) followed by 45 cycles of 95.0°C for 10 seconds and at 55.0°C for 10 seconds, while ramping rates were equivalent to $4.4°C \cdot S^{-1}$ and $2.2°C \cdot S^{-1}$ for cycles of 95.0°C and 55.0°C, respectively. All reactions were performed in triplicate in Multiplate™ Low-Profile 96-Well (Bio-Rad, cat# MLL-9651) using the Mastercycler®eprealplex (Eppendorf). Quantification cycles (CT) values were selected and analyzed using the realplex software. The relative expression of each target gene was calculated using the $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008).

2.4 Radicle emergence behaviour after priming process

The radicle emergence analysis was conducted at 20°C in darkness with four replicates. Radicle emergence seed (2 mm in length and look healthy), fungal infected seed and unemergence seed (neither radicle emergence nor fungal infected seed) (Figure 6) were determined at 6 hour intervals for 84 hours. GERMINATOR software (Joosen *et al.*, 2010), a curve-fitting program designed for the analysis of radicle emergence data from a four-parameter Hill function by iteration, was used to calculate radicle emergence indices e.g. maximum radicle emergence (MaxRE, %), mean radicle emergence time (MRET; hours),radicle emergence time (t_{50}, hours), uniformity (T75 – T25; U_{7525}), and maximum fungal infected seed (MaxF, %). Accelerated aging (AA) test, incubating seed at 45°C in 75%RH for 96 hours, was used to age the seed equivalent to 6 month old in the storage (Demir and Mavi, 2008).

2.5 Statistical analysis

The ANOVA (fixed effect model) of physical and biochemical changes and seed quality after priming for both before and after AA treatment of each imbibition times was conducted under the Completely Randomized Design for n each of priming technique and seed vigour level combination. The least significant difference (LSD) and Duncan's New Multiple Range Test (DMRT) were used to differentiate statistical difference among means at a significance level of $P \leq 0.05$. The data of the germination tests, such as MaxRE and MaxF, were transformed by angular transformation method (arcsine method) before being subjected to the analysis of variance.

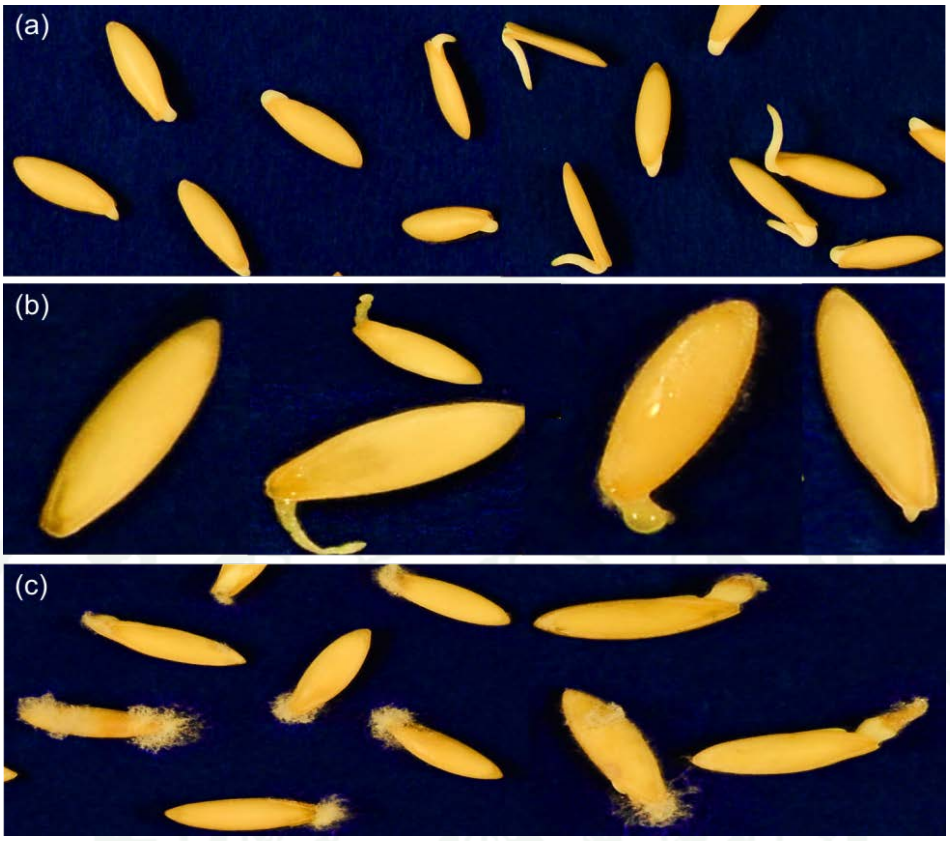

Figure 6 The illustrations of (a) radicle emergence seed, (b) unemergence seed and (c) fungal infected seed d in this experiment.

Place and Duration

The experiments were carried out in the Seed laboratory, Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen and The Laboratory of Plant Cell, Tissue and Transformation Kasetsart University, Kamphaeng Saen Campus, Kamphaeng Saen, Nakhon Pathom, Thailand, from August 2010 to September 2013.

RESULTS AND DISCUSSION

The study of the physical and molecular change during cucumber seed priming, which is divided into two experimentals: 1) Cell cycle activity of embryo radicle tip of cucumber seed during imbibition process and 2) Physical and biochemical changes and gene expression of cucumber seed during imbibition period of osmopriming process. The results were as follows.

1) Cell cycle activity of embryo radicle tip of cucumber seed during imbibition process.

1.1 SMC and seed germination after seed vigour modification

There was no significant difference in SMC after ageing among cucumber seeds subjected to different ageing treatments. The SMC ranged between 9.1 and 9.4% on a fresh weight basis. No significant differences were found in the germination or radicle emergence percentages among the cucumber seeds subjected to different ageing treatments. However, highly significant differences were found in MGT and MET. The MGT of cucumber seeds subjected to ageing treatment at 50°C was significantly increased whilst the MGT of control and 45°C-aged seeds were not significant difference. The MET of 50°C-aged seeds was 2 days while it was approximately 1 day for the control seeds (Table 2).

Time to first radicle emergence was delayed as the degree of ageing increased; time to radicle emergence of control seeds was 24 hours followed by 30 and 36 hours for 45°Cand 50°C-aged seeds, respectively (Figure 7).

Table 2 Effects of heat treatments on germination and radicle emergence of cucumber seeds.

Heat treatment conditions	Germination		Radicle emergence	
	Germination (%)	Mean germination time (days)	Radicle emergence (%)	Mean emergence time (days)
control	94.0 ± 0.1 [a]	4.1 ± 0.1 [a]	97.0 ± 0.1 [a]	1.1 ± 0.0 [a]
45°C 96 hours	92.0 ± 0.1	4.0 ± 0.1	98.0 ± 0.1	1.4 ± 0.0
50°C 96 hours	89.0 ± 0.0	4.5 ± 0.1	100.0 ± 0.0	2.0 ± 0.0
$LSD_{0.05}$	0.7597	0.0002	0.3782	<0.0001

[a] Values are means ± s.e. of n determinations.

Figure 7 First seed radicle emergence time (hours) during imbibition of cucumber seeds in deionized water in the dark at 25°C for 48 hours. Seeds were either untreated (control) or had been aged at 45°C and 74% RH for 96 hours or at 50°C and 74% RH for 96 hours. The arrows indicate radicle emergence (2 mm in length).

1.2 Cell cycle activity after ageing treatments

Most of the arrested cells (G_0 phase) in the radicle tip of dry cucumber seed contained 2C DNA (Figure 8). During imbibition, changes in the 2C, 4C and 8C DNA contents in the radicle tip cells for cucumber seeds subjected to different ageing conditions were observed. In the control seeds, the 2C DNA content was found to increase from approximately 64% at the arresting stage to 100% within six hours of imbibition. The proportion of 4C and 8C DNA increased as an indication of cell cycle transition from G_1 to S and S to G_2 during 18-30 hours of imbibition. The 2C DNA content was further increased after 36 hours indicating transition to the G_1 phase of the subsequent cell cycle. The progression of the cell cycle was slow in cucumber seeds subjected to ageing treatments. The extent of the delay increased with the severity of the ageing treatment. For the 45°C-aged seeds, after entering into the G_1phase at six hours of imbibition, the cells remained in that phase for up to 18 hours of imbibition, six hours longer than the control seeds, before moving to the S phase. Moreover, up to 48 hours of imbibition were necessary before the cells moved into the G_1phase of the second sequence of the cell cycle. The prolonged delay in the cell cycle was also found in seeds subjected to more severe deterioration (50°C). Twelve hours of imbibition were required (six hours more than both the control and the 45°C-aged seeds) for radicle tips cells to enter the first G_1 cell cycle. The next phase transition from the G_1 to the S phase was also delayed in the 50°C-aged seeds. After the transition from the G_1phase to the S phase, the cells were arrested at the S phase for 18 hours before progressing to the S/G_2 or G_2phase after 48 hours of imbibition. This resulted in the failure to observe the second sequence of the cell cycle in 50°C-aged seeds within the 48 hours imbibition time (Figure 8).

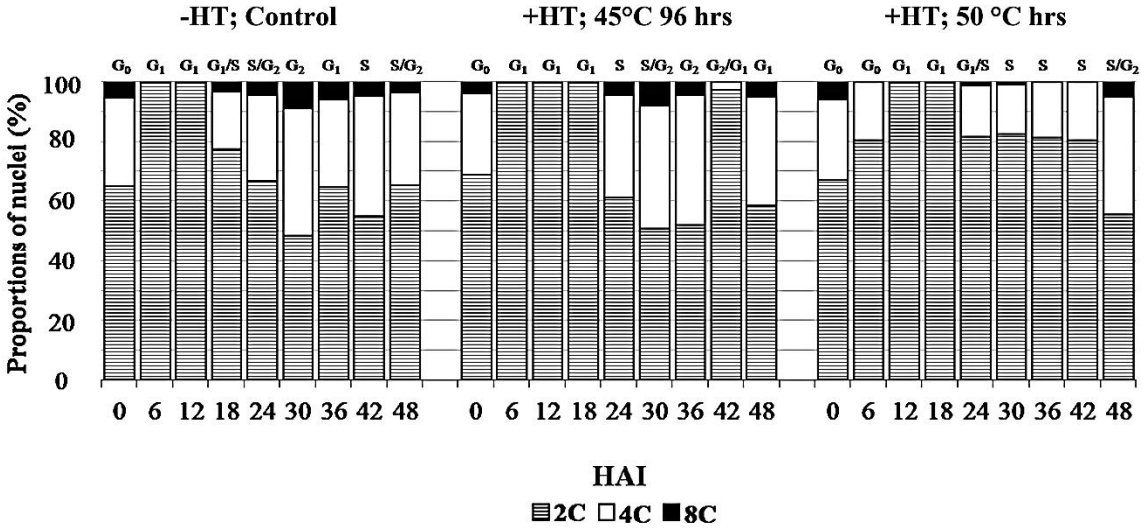

Figure 8 Proportions of 2C, 4C and 8C DNA in the nuclei of embryo radicle tips of cucumber seeds during imbibition in deionized water at 25°C in the dark for 48 hours. Upper case letters refer to the expected synchronized cell cycle profile after imbibition (G_0, resting or quiescent state; G_1, post-mitotic phase; G_2, premitotic phase, and S, synthesis phase during which DNA is replicated).

The changes in SMC of cucumber seeds not subjected to an ageing treatment were in accordance with the conventional tri-phase of water uptake (figure 9A). Control seed MC increased rapidly within six hours of imbibition and remained stable between 6 and 24 hours of imbibitions, followed by additional increase after visible radicle emergence at 24 hours. The changes in MC during imbibition of 45°C- and 50°C aged seeds were altered compared with control seeds (Figure 9B and 9C). After a rapid increase within six hours of imbibition, the MC remained unchanged for the rest of the imbibition time despite radicle emergence, which took place at 30 and 36 hours after imbibition, for 45°C- and 50°C-aged seeds, respectively.

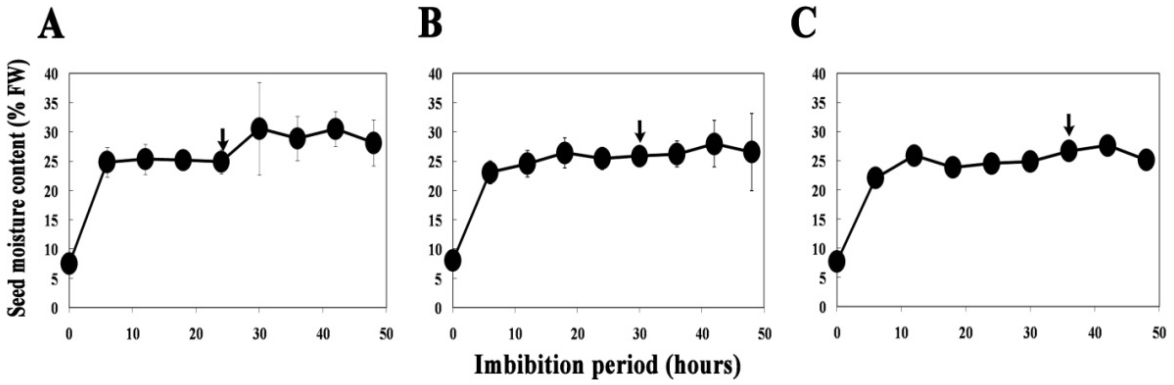

Figure 9 Moisture contents of cucumber seeds during imbibition. A = control seeds; B = seeds aged at 45°C, 74% RH for 96 hours; C = seeds aged at 50°C 74% RH for 96 hours. The arrows indicate radicle emergence. Error bars denote the s.d. (n = 10); missing error bars indicate that they are smaller than the symbols.

The (4C + 8C) / 2C ratio changes of control seeds and of 45°C-aged seeds during imbibition revealed a similar trend (Figure 10A, B). After reach to phase II of the water uptake process, the ratios increased gradually and reached the maximum value that signifies the cell cycle transition from G_1 to S and S to G_2, respectively. A coincident relationship between radicle emergence and the progression of the cell cycle for both control and 45°C-aged seeds was evident. Radicle emergence occurred at the later stages of the S phase, six hours before the (4C + 8C) / 2C ratio attained the maximum value. In contrast, radicle emergence of the 50°C-aged seeds occurred without a noticeable increases in the (4C + 8C) / 2C ratio (Figure 10C). The trend of mean C-values during imbibition of cucumber seeds subjected to diverse ageing treatments were very much in accordance with their (4C + 8C) / 2C ratio, except for the 45°C-aged seeds where the peak of the mean C-value was observed at 30 hours of imbibition, the time of its radicle emergence, whereas that of the (4C + 8C) / 2C ratio was observed at 36 hours, 6 hours after radicle emergence (Figure 10B). The mean C-value of radicle tip cells confirmed that cell cycle activity was arrested during 6-18 hours of imbibition for the seeds aged at 45°C (Figure 10B) as well as during 6-18 hours and 24-42 hours of imbibition for the seeds aged at 50°C (Figure 10C).

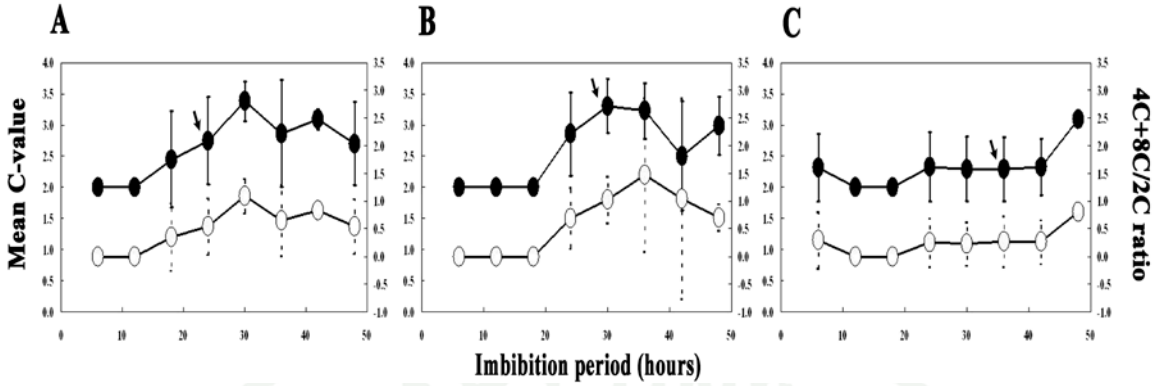

Figure 10 Mean C–values (●) and (4C+8C)/2C ratio (○) in radicle tips of cucumber during germination. A =control seeds; B = seeds aged at 45°C, 74% RH for 96 hours; C = seeds aged at 50°C 74% RH for 96 hours. The arrows indicate radicle emergence. Error bars denote the s.d. (n = 4); missing error bars indicate that they are smaller than the symbols.

The germination results indicated that, although ageing at 45 or 50°C and 74% RH for 96 hours did not significantly affect the total percentage germination or percentage radicle emergence of cucumber seed lots, it significantly delayed radicle emergence and germination. Mean germination time is used as a measure of seed vigour (ISTA, 1995) and the increase in germination time indicates the occurrence of seed deterioration (Finch-Savage *et al.*, 2004; Pourhadian and Khajehpour, 2010). Hence, although we do not have a field emergence test result, it is clear that the vigour of the aged cucumber seed lots had declined and the seeds had entered the early stage of deterioration. The longer the delay in radicle emergence and germination time, the higher the extent of deterioration occurred. A similar phenomenon was observed when cauliflower seed lots with different vigour levels were primed (Powell *et al.*, 2000). Cucumber is a unique polyploidy species with nuclei ploidy levels between 4C and 16C in dry mature seeds (Jing *et al.*, 1999; Sliwinska *et al.*, 2009). However, in the present experiment the radicle tip cells rarely contained DNA in excess of 8C. This may have been due to the effects of the DNA binding dye (4,6-diamidino-2-phenylindole, DAPI), which binds to only the minor groove of DNA helix, and the application of a linear amplification of the signal in the current experiment. Similar results were found by Zhang *et al.* (2009)who stained DNA in cucumber radicle cells with DAPI DNA staining dye and applied linear amplification of the signal revealing no DNA contents in excess of 8C. The results from the cell cycle analysis indicated that the delay of radicle emergence in cucumber seeds subjected to ageing treatments was probably caused

by the transient arrest of the cell cycle. The extent of this arrest was dependent on the severity of the ageing treatments. Seeds which were subjected to mild deterioration (45°C) were found to arrest in the G_1phase before progressing through the G_1 / S checkpoint to the next S phase. As for the seeds subjected to more severe ageing conditions (50°C), cell cycle arrest was observed in two phases; at G_1phase during 6-18 hours of imbibition and at the subsequent S phase during 24-42 hours of imbibition. Jang *et al.*(2005) reported that the phase at which the cell cycle is arrested depends on the point at which cells experience stress. In the present experiment, dry mature cucumber seeds were subjected to stress treatments after removal from storage. For seeds subjected to ageing treatments, cell cycles were found to first arrest in the G_1phase of the first cell cycle subsequent to rehydration. In seeds subjected to more severe deterioration at 50°C an additional cell cycle arrest in the subsequent S phase was observed. The stable (4C + 8C) / 2C ratio and the mean C-value during 6-18 and 24-42 hours of imbibition (figure 10C) confirmed the cell cycle block at both the G_1 and S phases for the 50°C-aged seeds. Overall, the flow cytometry results are verification that ageing treatments at high temperature (45-50°C) and high relative humidity (74% RH) cause significant deterioration to the cells of dry cucumber seeds. At higher temperature, the damage to the cells appears to be more pronounced. After rehydration, damaged cells were found to possibly undergo cell repair during the arrest of the cell cycle at the closest checkpoint prior to the continuation of the regular cell cycle activity.

The increase of the (4C + 8C) / 2C ratio during germination indicates an increase of the G_2 cell population in the radicle tip cells before radicle emergence in many species including barley (Gendreau *et al.*, 2008), maize (Sánchez *et al.*, 2005), and sugarbeet (Sliwinska, 2000). This indicates that cell cycle activity in the rehydrated seed is initiated prior to radicle elongation. In the current experiment, for seeds not subjected to ageing conditions (control) or subjected to mild levels of deterioration (ageing at 45°C), radicle emergence occurred six hours prior to the attainment of the maximum values for the (4C + 8C) / 2C ratio. This correlation agrees with reports of (Sliwinska, 2009)who proposed the G_2 / G_1 ratio or (4C + 8C + 16) / 2C ratio might be used as a marker of the advancement of germination / priming of cucumber seeds. However, we noted that seeds subjected to more severe ageing conditions (50°C) did not show an increased (4C + 8C) / 2C ratio prior to radicle emergence (Figure 10C), since radicle tip cells were arrested in the S phase (Figure 8). This result indicates that the progression of the cell cycle of aged seeds during germination is divergent to that of non-aged seed. The use of the (4C + 8C) / 2C ratio and

the mean-C value as a marker for germination advancement might not be effective for the cucumber seed lots that undergo deterioration or have been subjected to unfavourable conditions even though there is no noticeable germination reduction. The results from the present experiment suggest that the seed physiological age must be taken into consideration prior to application of the cell cycle parameter as an efficient marker of the progression of germination during seed priming.

2) Physical and biochemical changes and seed quality of cucumber during imbibition period of osmopriming process.

Storability after priming process is vital to commercial seed production in the seed industry and primed seeds must be stored for some extent before they will be sold. In this study, two cucumber seed lots with different seed vigour were subjected to two osmopriming processes (rapid and slow priming). The physical and biochemical changes of the seed during different imbibition period were investigated. The relationship between seed quality after osmopriming and physical and biochemical changes were determined.

2.1 Physical and biochemical changes during slow osmopriming and its quality after priming process

2.1.1 SMC

The SMC change during imbibition period of CU-51, the high vigour cucumber seed lot, subjected to slow priming followed the SMC scenario of tri-phase of imbibitions. The transition between phase I and II occurred at hour 10 of imbibitions (Figure 11). The increase of SMC as the mark of phase transition from II to III was not visible although thirty percentage of seed population had reached the radicle emergence stage and the imbibitions process was terminated.

The SMC changing during imbibition period of CU-50, the low vigour cucumber seed lot, subjected to slow osmopriming followed the SMC scenario of tri-phase of imbibitions except that there was a significantly decreased in SMC, instead of being steady, during the transition of phase I to II. There was no transition of SMC form Phase II to III due to the imbibition process was terminated prior to Phase III taking place (Figure 11).

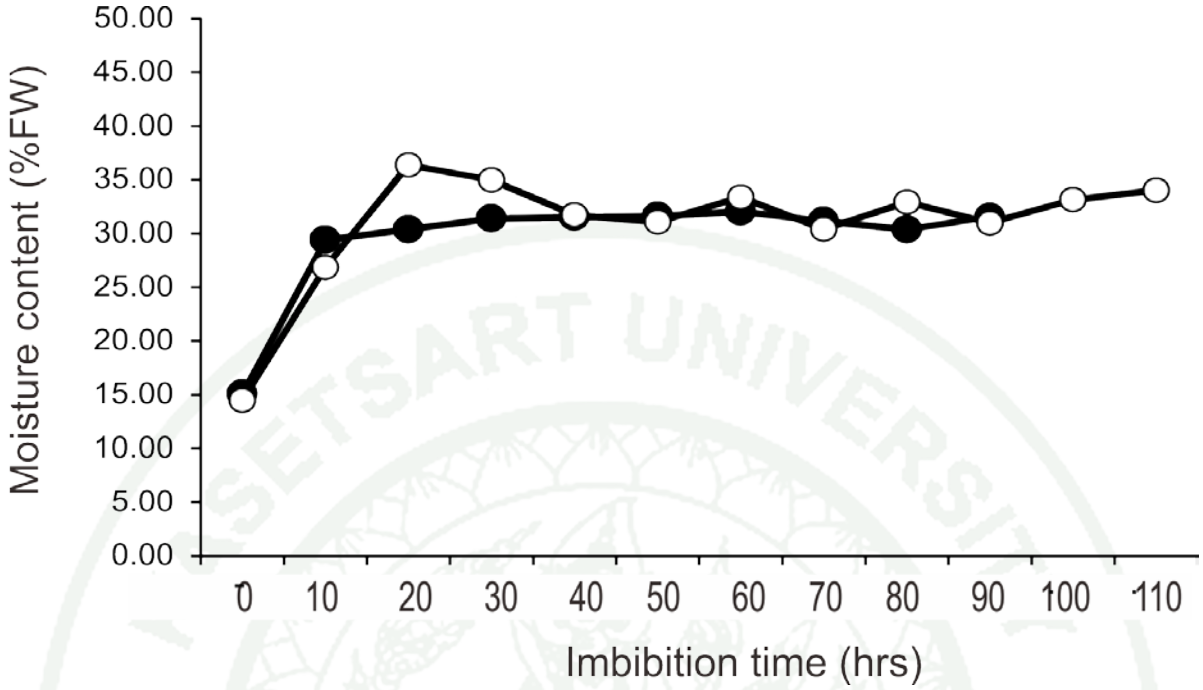

Figure 11 Seed moisture content during imbibition period of the high and low vigour cucumber seed lot,CU-50 and CU-51 respectively, subjected to slow osmopriming process (ψ_s of -0.8 MPa of KNO_3 at 20°C).The high vigour seed lot is represented by lines with closed circle and the low vigour seed lot is by lines with open circle. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.1.2 Radicle emergence during imbibition

The radicle emergence of CU-51, the high vigour cucumber seed lot, during imbibition periods of slow osmopriming was visible at the hour 50 and the cumulative radicle emergence significantly increased as the imbibitions times increase and was up to 60.5% when the imbibitions process was terminated after the hour 90 (Figure 12 and Appendix Table B1).

The radicle emergence of CU-50, the low vigour cucumber seed lot, during imbibition periods of slow osmopriming process began at hour 70 and significantly increased as the imbibition time increased reaching the highest value of 47.8% at hour 110 (Figure 12 and Appendix Table B2).

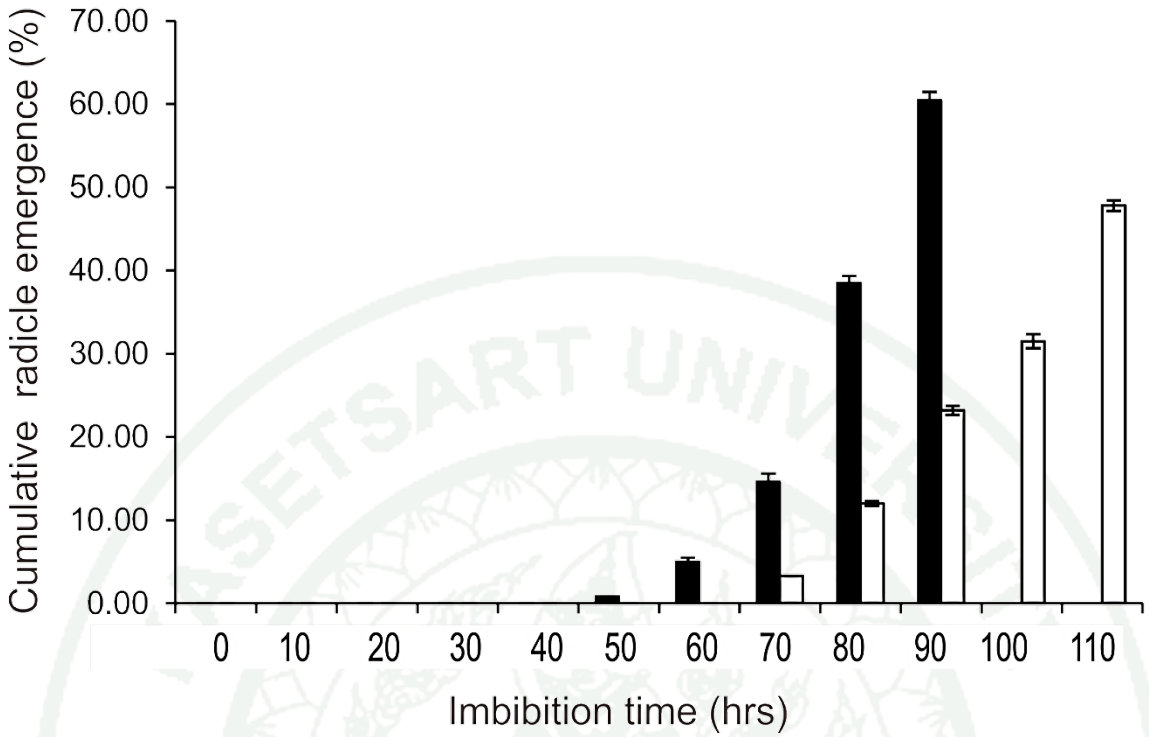

Figure 12 Cumulative radicle emergence of high and low vigour cucumber seed lot, CU-50 and CU-51 respectively, subjected to slow osmopriming process (ψ_s of -0.8 MPa of KNO_3 at 20°C). The high vigour seed lot is represented by black bars and the low vigour seed lot is by white bars. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.1.3 Headspace ethanol

There was no significant difference in the ethanol content among different imbibitions times produced by primed seed of CU-51, the high vigour seed lot, with slow priming. The maximum headspace ethanol production of 178.5 5μg·L^{-1} of primed seed was found at hour 70 compared to 25 μg·L^{-1} of the control seed (unprimed) (Figure13 and Appendix Table B1).

The significant differences in the ethanol content production was found when the CU-50, the low vigour cucumber seed lot, was subjected to slow priming. The significant increase of ethanol production was found after the seeds were imbibed for 40 hours onward although the significant drop of ethanol production were found at hour 60 and 70 (Figure 13 and Appendix Table B1).

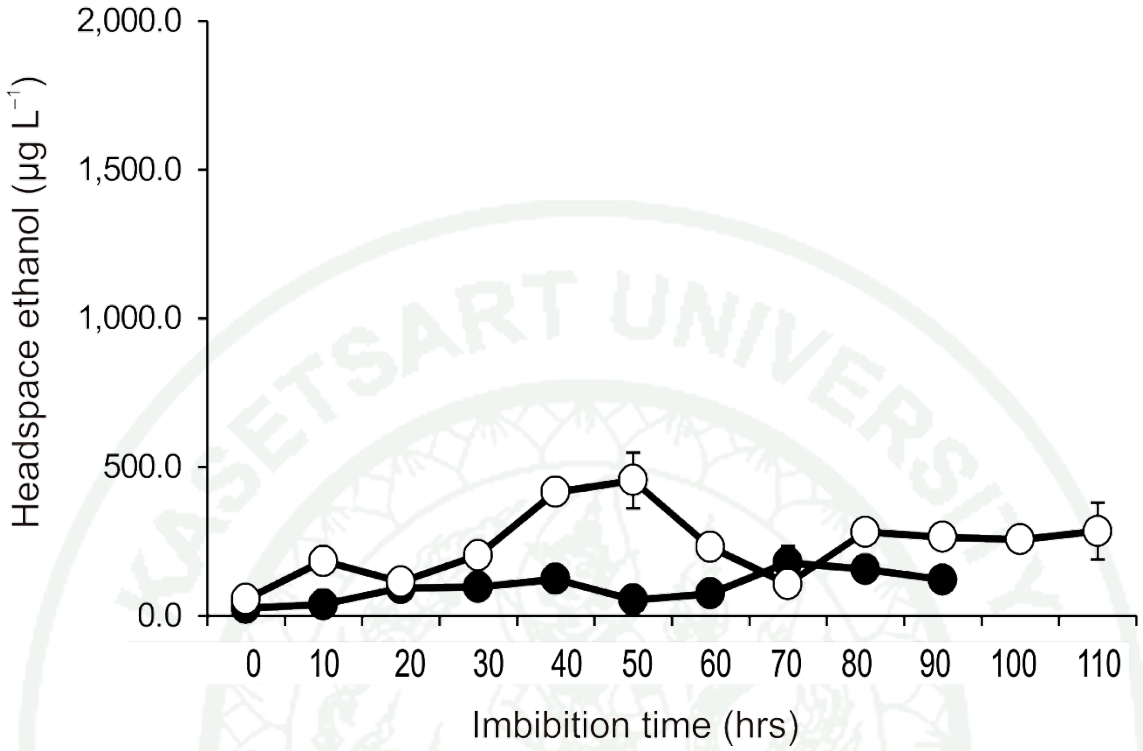

Figure 13 Headspace ethanol production of high and low vigour cucumber seed lot, CU-50 and CU-51 respectively, subjected to slow osmopriming process (ψ_s of -0.8 MPa of KNO_3 at 20°C). The high vigour seed lot is represented by lines with closed circle and the low vigour seed lot is by lines with open circle. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.1.4 Gene expression

The expression of *Cucumis sativus CDKD1-like* of CU-51 seed during various imbibition times of slow osmopriming process were more pronounced and up-regulated while that of *EMB-1-like* was less pronounced and down-regulated as the imbibitions time increased. The significant increase in the expression of *CDKD1-like* gene occurred at hour 70 while the significant decrease of the expression of *EMB-1* was found at hour 20 onward (Figure 14).

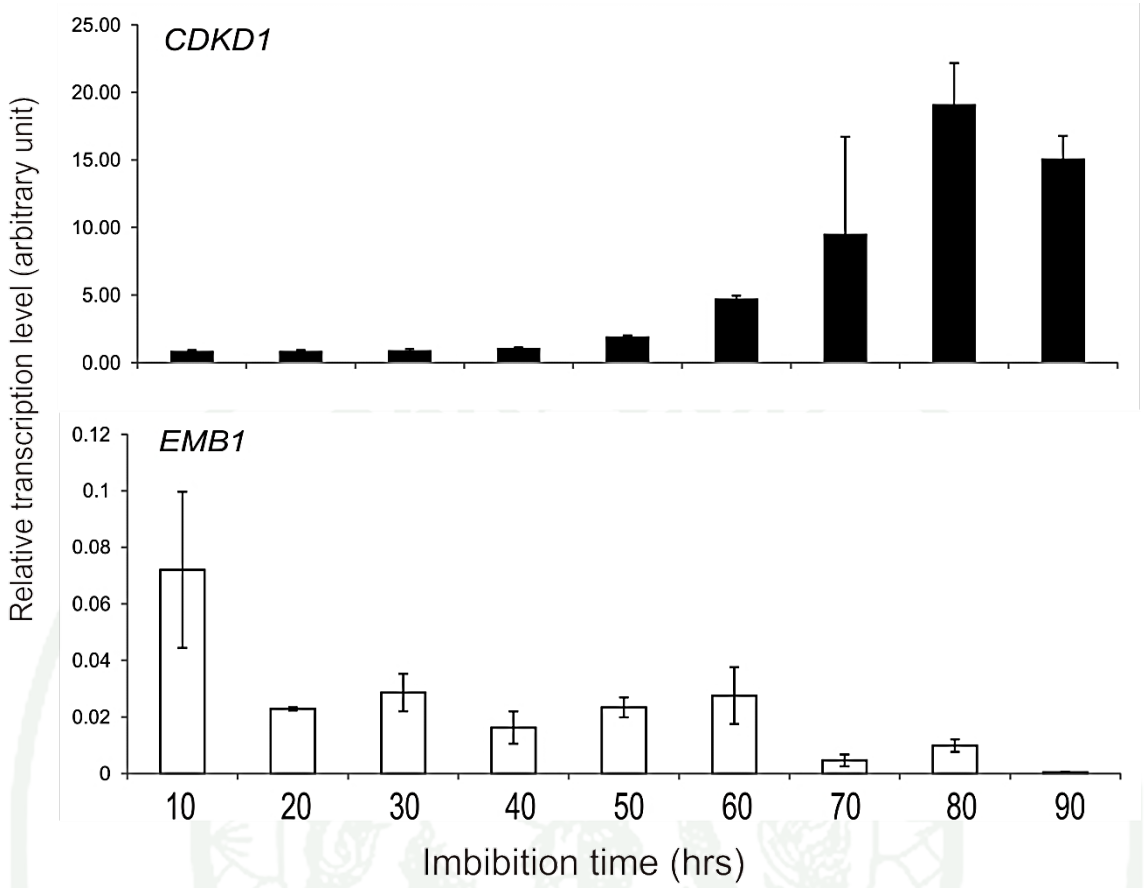

Figure 14 The expression for *Cucumis sativus CDKD1-like* and *Cucumis sativus EMB-1-like* of high vigour cucumber seed lot, CU-51, subjected to slow osmopriming process (ψ_s of -0.8 MPa of KNO_3 at 20°C) with various imbibitions times. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

The expression levels of *Cucumis sativus CDKD1-like* of CU 50, the low vigour seed was more than C*ucumis sativus EMB-1-like* about 20-fold. The expression of *Cucumis sativus CDKD1-like* would be gradually up-regulated over time and the maximum of mean expression levels of *Cucumis sativus CDKD1-like* was at hour 70. In contrast to that of high vigor seed lot, the expression of *Cucumis sativus EMB1-like* during the imbibition period was very low in the beginning of imbibition period before it increased and reached the highest value at hour 90 (Figure 15).

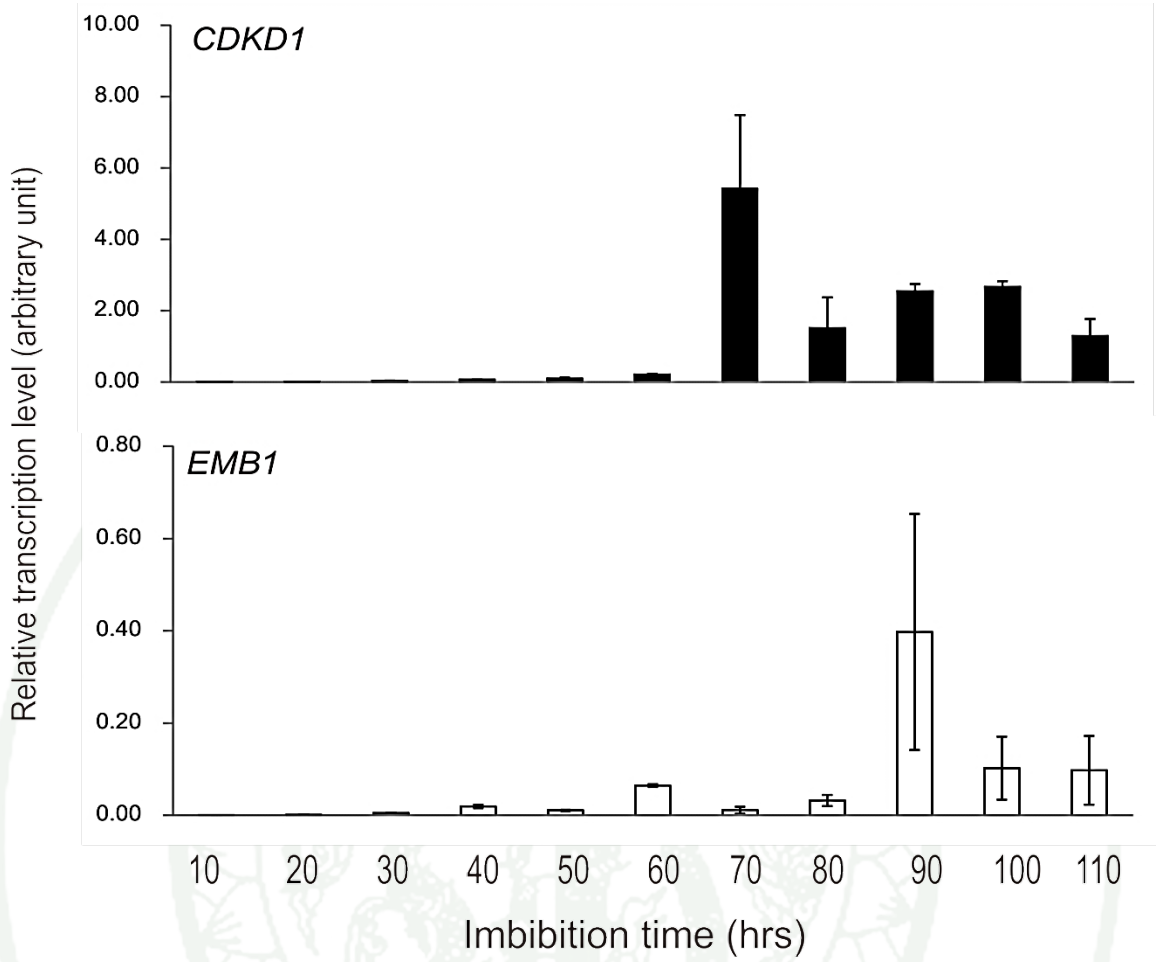

Figure 15 The expression for *Cucumis sativus CDKD1-like* and *Cucumis sativus EMB-1-like* of low vigour cucumber seed lot, CU-50, subjected to slow osmopriming process (ψ_s of -0.8 MPa of KNO_3 at 20°C) with various imbibitions times. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.1.5 Seed quality after priming

1) Before AA-test

There were significant differences in radicle emergence behaviors among CU-51, the high vigour cucumber seed lot, primed with different imbibitions times while non-significant difference in number of fungal infected seed among different imbibitions time was found. The short soaking time of 10-20 hours showed the promising imbibition treatment since (Table 3). It gave the highest MaxRE of 95% and 94%, respectively as compared to 83% of control treatment (Table 3). The speed of radicle

emergence as expressed by t_{50} and MRET and uniformity expressed by U_{7525} were improved the most from short soaking time of osmopriming. The t_{50}, MRET and U_{7525}at hour 10 were 21.39 and 21.33 and 3.91 hours compared to 32.45, 32.41 and 8.8 hours of unprimed seed, respectively.

The radicle emergence behaviors of CU-50, the low initial vigour cucumber seed lot, subjected to slow osmopriming with various imbibitions time were shown in Table 4. The MaxRE of primed seed for all soaking periods did not improved when compared to that of unprimed seed (control). However, when the speed of radicle emergence and uniformity of radicle emergence were concerned, priming with short soaking times between 10-30 hours gave the best radicle emergence performance. The t_{50} and MRET values were significant highest and the U_{7525} was lowest when the imbibition times were between 10-30 HAI.

2) After AA-test

The radicle emergence behaviors of CU-51, the high vigour cucumber seed lot, after slow priming followed by the AA treatment were shown in Table 5. After AA treatment, the priming with short soaking time of 10-20 hours still gave the best seed quality when MaxRE was concerned. It also gave the significantly highest speed of radicle emergence when T_{50} and MRET were concerned, though after AA treatment, the U_{7525} among imbibition time was no longer significantly different.

The radicle emergence behaviors of CU-50, low vigour cucumber seed lot, subjected to slow osmopriming with various imbibition times followed by AA treatment were shown in Table 6. The seed quality after AA treatment was in accordance with the quality prior to AA treatment. There was no-significant difference in MaxRE between unprimed seed and primed seed with short imbibition time of 10 hours. The MaxRE after ageing decreased as the imbibition times increased. Priming with short imbibitions time of 10 hours gave the highest speed of radicle emergence in term of t_{50} and MRET after AA treatment.

Table 3 Radicle emergence behaviors of CU-51, the high vigour cucumber seed lot, as affected by slow osmopriming process.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours) [a]	Mean radicle emergence time (MRET; hours) [a]	Uniformity (T_{75} - T_{25}; U_{7525}; hours) [a]	Maximum fungal infected seed (MaxF, %) [a]
0 hr. (control)	83^{c}	32.47^{a}	33.41^{a}	8.80^{ab}	8.0
10 hours	95^{a}	21.24^{d}	21.21^{d}	4.05^{cd}	7.0
20 hours	94^{ab}	21.54^{d}	21.45^{cd}	3.76^{cd}	2.0
30 hours	85^{bc}	21.44^{d}	21.01^{d}	2.91^{d}	9.0
40 hours	89^{abc}	22.57^{cd}	22.90^{bcd}	5.58^{bcd}	7.0
50 hours	81^{c}	23.12^{cd}	23.17^{bcd}	6.64^{bc}	13.0
60 hours	83^{c}	24.61^{c}	25.02^{bc}	5.78^{bcd}	10.0
70 hours	93^{abc}	24.17^{cd}	25.12^{bc}	8.19^{ab}	2.0
80 hours	90^{abc}	25.39^{c}	26.11^{b}	7.19^{bc}	1.0
90 hours	83^{c}	29.12^{b}	30.37^{a}	11.15^{a}	3.0
Pr>F	0.0054	<.0001	<.0001	0.0004	0.0640
CV (%)	4.1422	7.6988	9.3232	35.7263	89.1186

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Table 4 Radicle emergence behaviors of CU-50, the low vigour cucumber seed lot, as affected by slow osmopriming process.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours) [a]	Mean radicle emergence time (MRET; hours) [a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours) [a]	Maximum fungal infected seed (MaxF, %) [a]
0 hr. (control)	96.0^{a}	27.35^{bcd}	27.74^{bcd}	5.38^{bcd}	0.0^{d}
10 hours	86.0^{ab}	22.99^{ef}	23.06^{e}	4.31^{cd}	5.0^{bcd}
20 hours	62.0^{ed}	24.46^{def}	25.18^{ed}	7.36^{abcd}	30.0^{a}
30 hours	86.0^{ab}	22.86^{f}	22.73^{e}	3.45^{d}	7.0^{bc}
40 hours	79.0^{bc}	25.00^{def}	25.59^{ed}	6.71^{abcd}	8 .0^{bc}
50 hours	82.0^{ab}	25.51^{def}	26.21^{ed}	7.21^{abcd}	5 .0^{bcd}
60 hours	62.0^{e}	29.77^{abc}	30.78^{abc}	9.49^{ab}	9.0^{b}
70 hours	65.0^{cde}	31.45^{a}	32.11^{a}	8.47^{abc}	5 .0^{bcd}
80 hours	79.0^{bc}	30.65^{ab}	31.65^{ab}	9.72^{a}	1.0^{cd}
90 hours	74.0^{bcd}	26.68^{cde}	27.41^{cd}	7.24^{abcd}	4.0^{bcd}
100hours	66.0^{cde}	29.98^{abc}	30.81^{abc}	7.86^{abc}	2 .0^{bcd}
110hours	63.0^{ed}	30.93^{ab}	31.86^{a}	9.30^{ab}	0.0d
Pr>F	<.0001	<.0001	<.0001	0.0193	<.0001
CV (%)	7.1199	8.5615	9.0915	35.1561	75.3175

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Table 5 Radicle emergence behaviors of CU-51, the high vigour cucumber seed lot, as affected by slow osmopriming process followed by accelerated aging treatment.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours)[a]	Mean radicle emergence time (MRET; hours)[a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours)[a]	Maximum fungal infected seed (MaxF, %)[a]
0 hr. (control)	87.0^{ab}	32.21^{bcd}	34.27^{bc}	14.61	2.0
10 hours	92.0^{a}	30.52^{cd}	32.19^{cd}	12.91	0.0
20 hours	91.0^{a}	30.89^{cd}	31.89^{cd}	10.29	0.0
30 hours	82.0^{abc}	33.33^{bcd}	34.45^{bc}	10.97	1.0
40 hours	88.0^{ab}	28.44^{d}	28.26^{a}	5.56	1.0
50 hours	70.0^{d}	33.43^{bcd}	34.83^{bc}	11.19	1.0
60 hours	82.0^{abc}	36.16^{b}	36.53^{bc}	7.56	0.0
70 hours	78.0^{bcd}	41.05^{a}	42.16^{a}	11.77	0.0
80 hours	78.0^{bcd}	35.44^{bc}	36.31^{bc}	10.10	2.0
90 hours	72.0^{cd}	37.20^{ab}	38.31^{ab}	10.43	1.0
Pr>F	<.0001	<.0001	<.0001	0.1145	0.5243
CV (%)	3.9322	9.1498	9.0375	36.2233	243.4322

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Table 6 Radicle emergence behaviors of CU-50, the low vigour cucumber seed lot, as affected by slow osmopriming process followed by accelerated aging treatment.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours)[a]	Mean radicle emergence time (MRET; hours)[a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours)[a]	Maximum fungal infected seed (MaxF, %)[a]
0 hr. (control)	100.00[a]	28.34[ed]	29.23[cd]	9.61[cd]	0.0
10 hours	81.00[abc]	26.43[e]	26.81[d]	7.55[d]	0.0
20 hours	83.00[ab]	30.89[cde]	33.91[bc]	18.18[ab]	4.0
30 hours	77.00[bc]	33.56[bcd]	35.79[b]	15.51[abc]	2.0
40 hours	83.00[ab]	32.24[bcde]	34.06[bc]	13.57[abcd]	1.0
50 hours	69.00[bc]	35.75[bc]	37.30[b]	12.48[bcd]	0.0
60 hours	69.00[bc]	35.75[bc]	37.30[b]	12.48[bcd]	0.0
70 hours	80.00[bc]	35.96[bc]	39.10[b]	20.71[a]	2.0
80 hours	64.00[bc]	45.24[a]	47.32[a]	18.08[ab]	0.0
90 hours	69.00[bc]	34.27[bcd]	36.34[b]	14.78[abcd]	0.0
100 hours	64.00[c]	43.87[a]	45.84[a]	17.13[ab]	0.0
110 hours	78.00[bc]	37.62[b]	39.20[b]	13.67[abcd]	0.0
Pr>F	0.0061	<.0001	<.0001	0.0105	0.0689
CV (%)	7.7313	10.8543	10.6839	31.2062	228.6156

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

For the overall results of slow osmopriming, it was evident that the imbibition period of 10-20 HAI gave the best seed quality after-slow osmopriming for high vigour cucumber seed lot, CU-51. In that period, the expression of *Cucumis sativus EMB-1-like*is, the desiccation tolerance gene and SMC were only the parameters that significantly changes. However, the use only of SMC to pinpoint suitable imbibition period might not be reliable because it is influenced by many metabolic processes in which may bring about a high variability and low precision. In contrast, the change of *Cucumis sativus EMB-1-like* gene expression would be more effective marker since it can indicate the early stage of desiccation tolerance loosing of primed seed.

2.2 Physical and biochemical changes during rapid osmopriming and its quality after priming process

2.3.1 SMC

The SMC changing of CU-51, the high initial vigour cucumber seed lot, during imbibitions periods of rapid osmopriming followed the SMC scenario of tri-phase of imbibitions (Figure 16). The Phase I of imbibitions began between 0 to 10 hours after soaking where the SMC reached the highest value of 35% FW at hour 10 before transferring to Phase II by the SMC slightly, but significantly, decreased and continued being steady until the end of imbibition time of at hour 40.

The SMC change of CU-50, the low vigour cucumber seed lot, during imbibitions periods of rapid osmopriming followed the SMC scenario of tri-phase of imbibitions by which the transition of Phase I to II occurred at hour 5 (Figure 16).

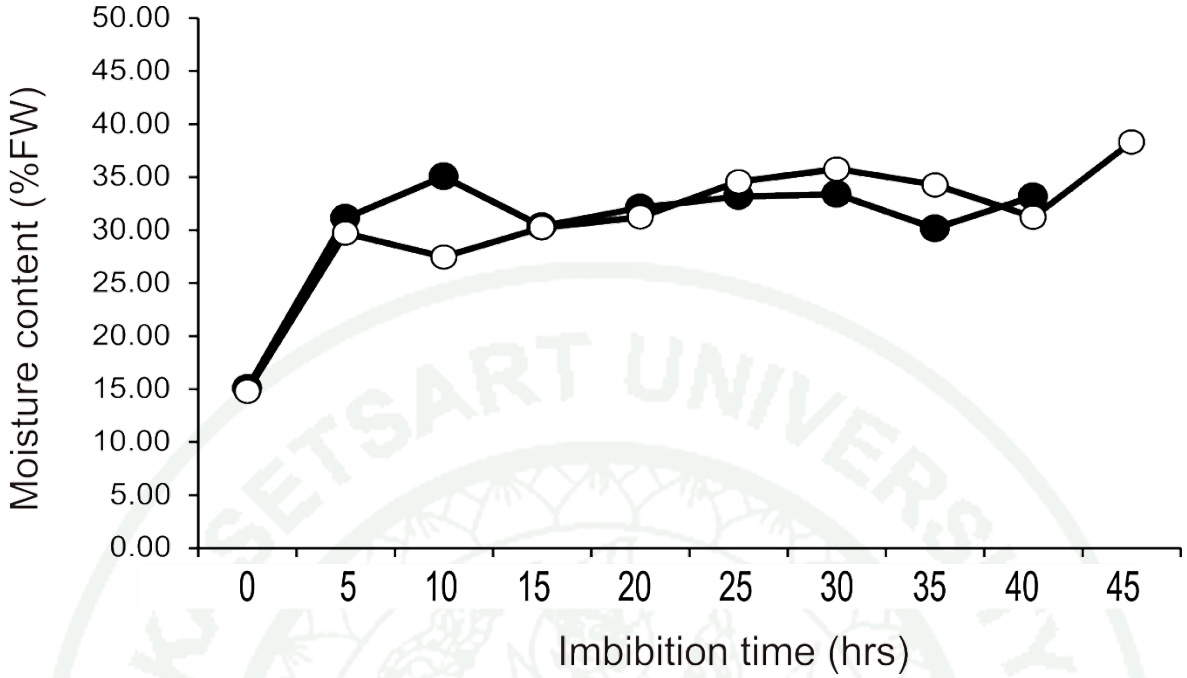

Figure 16 Seed moisture content during imbibition period of the high and low vigour cucumber seed lot, CU-50 and CU-51 respectively, subjected to rapid osmopriming process (ψ_s of -0.05MPa of KNO_3 at 20°C). The high vigour seed lot is represented by lines with closed circle and the low vigour seed lot by lines with open circle. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.3.2 Radicle emergence during imbibitions

The radicle emergence of CU-51, the high vigour cucumber seed lot, subjected to rapid osmopriming with various imbibitions time was visible at hour 30 and reached the highest value of 59.9% at hour 40 (Figure 17 and Appendix Table B3).

The radicle emergence of CU-50, the low vigour cucumber seed lot, subjected to rapid osmopriming with various imbibitions time was visible at hour 30 and reached the highest value of 50.9% at hour 45 (Figure 17 and Appendix Table B4).

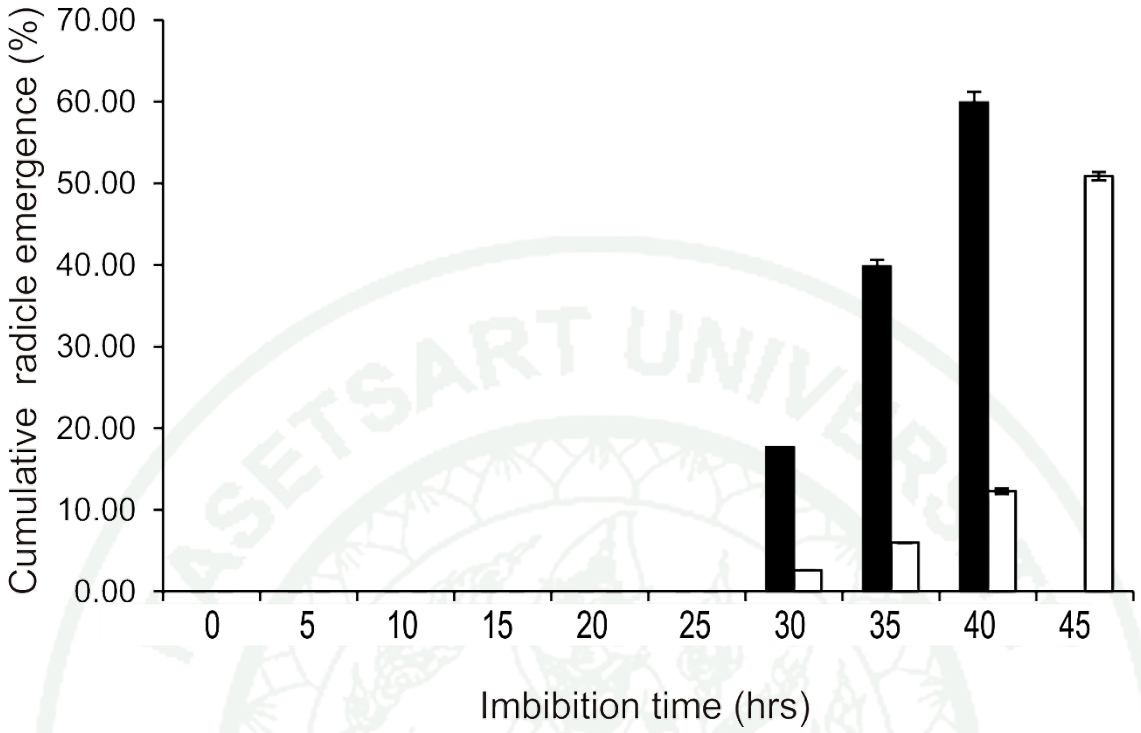

Figure 17 Cumulative radicle emergence of high and low vigour cucumber seed lot, CU-50 and CU-51 respectively, subjected to slow osmopriming process (ψ_s of -0.05MPa of KNO_3 at 20°C). The high vigour seed lot is represented in black bars and the low vigour seed lot is in white bars. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.3.3 Headspace ethanol

Fluctuations of methanol production from CU-51, the high vigour cucumber seed lot, subjected to rapid osmopriming with various imbibition times was found (Figure 18 and Appendix Table B3). The ethanol production of primed seeds significantly increased during imbibitions time were between 0-10 hours and between 25-30 hours and significantly decreased when they were between 10-25 hours and between 30-40 hours.

Fluctuations of methanol production from CU-50, the low vigour cucumber seed lot, subjected to rapid osmopriming with various imbibition times was found (Figure 18 and Appendix Table B4). The ethanol production of primed seeds

significantly increased during imbibitions time were between 25-30 hours and significantly decreased when they were between 35-40 hours.

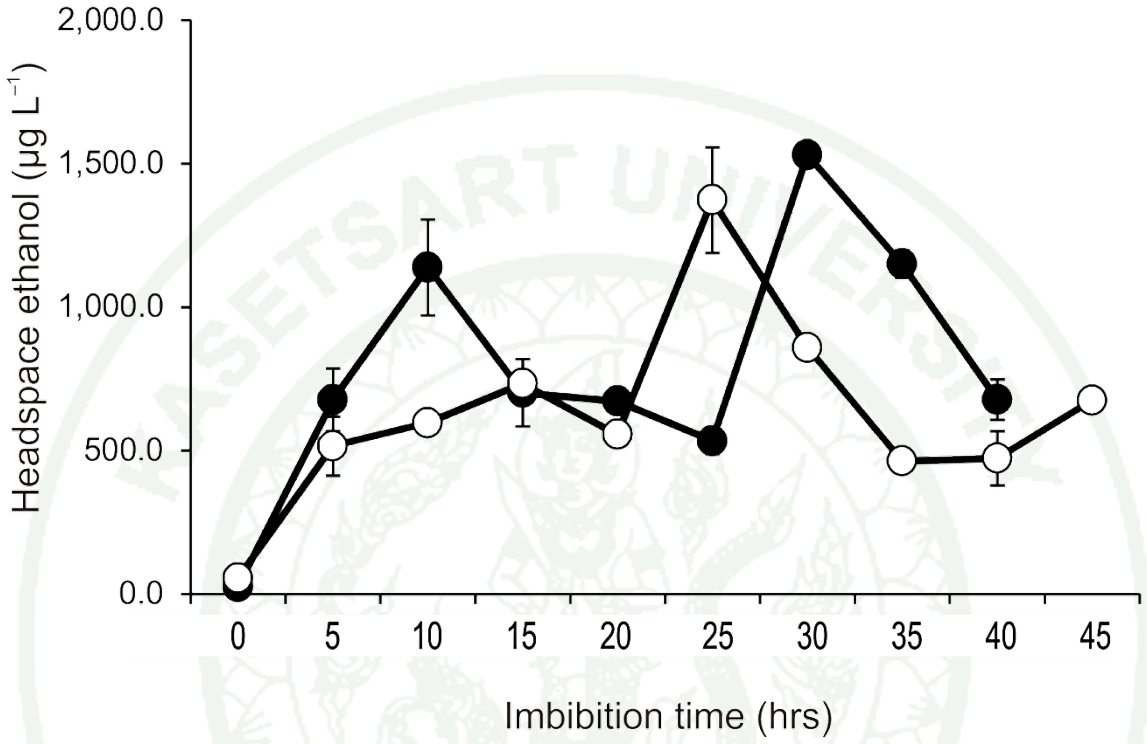

Figure 18 Headspace ethanol production of the high and the low vigour cucumber seed lot CU-50 and CU-51 respectively, subjected to rapid osmopriming process (ψ_s of -0.05MPa of KNO_3 at 20°C). The high vigour seed lot is represented in lines with closed circle and the low vigour seed lot is in lines with open circle. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.3.4 Gene expression

There was non-significant difference in the expression of *Cucumis sativus CDKD1-like* from CU-51, the high vigour cucumber seed lot, among various imbibitions times during rapid osmopriming process. However, the significant down-regulate expression of *Cucumis sativus EMB-1-like* was found after 10 hours of imbibitions onward (Figure 19).

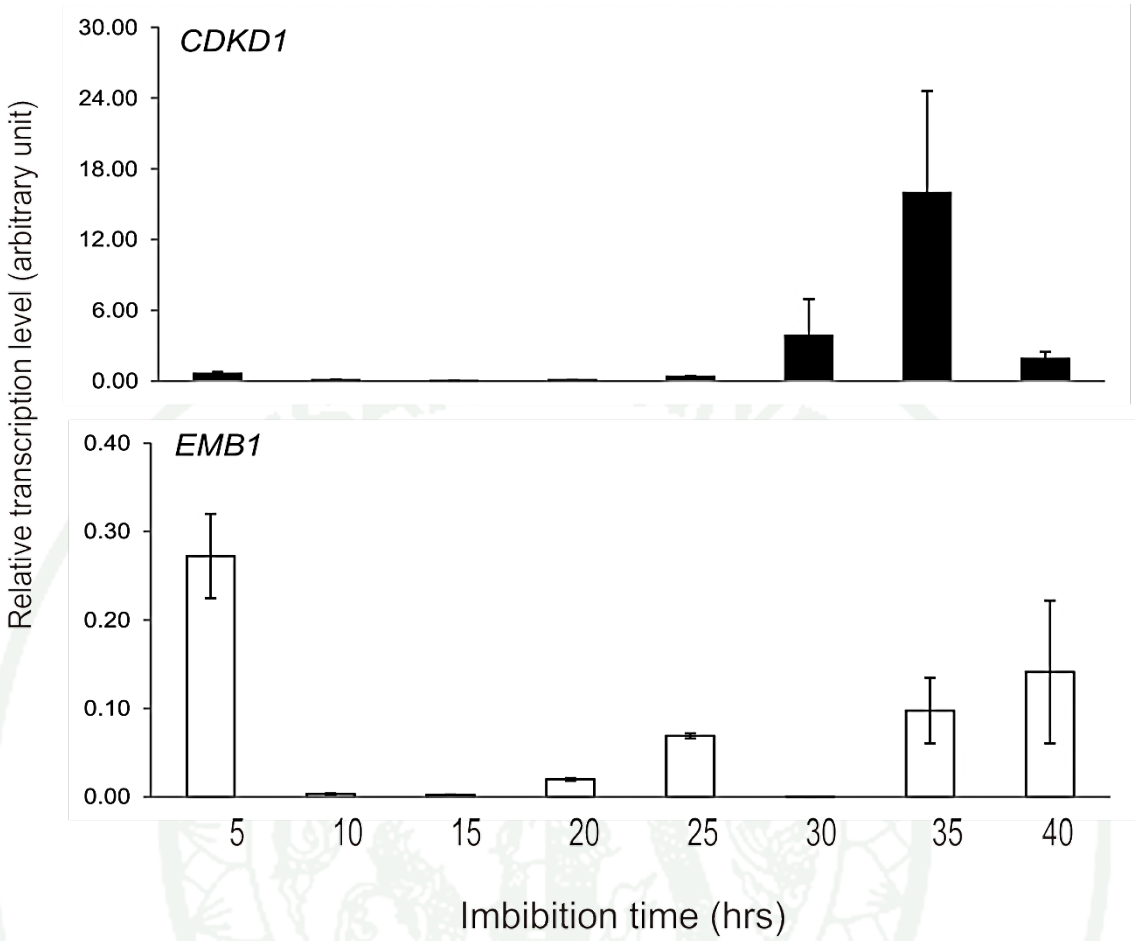

Figure 19 The expression for *Cucumis sativus CDKD1-like* and *Cucumis sativus EMB-1-like* of high vigour cucumber seed lot, CU-51, subjected to rapid osmopriming process (ψs of -0.05 MPa of KNO_3 at 20°C) with various imbibitions times. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

There was non-significant difference in the expression of *Cucumis sativus EMB-1-like* from CU-50, the low vigour cucumber seed lot, among various imbibitions times during rapid osmopriming process. However, the significant up-regulate expression of *Cucumis sativus CDKD1-like* was found at hour 20 (Figure 20).

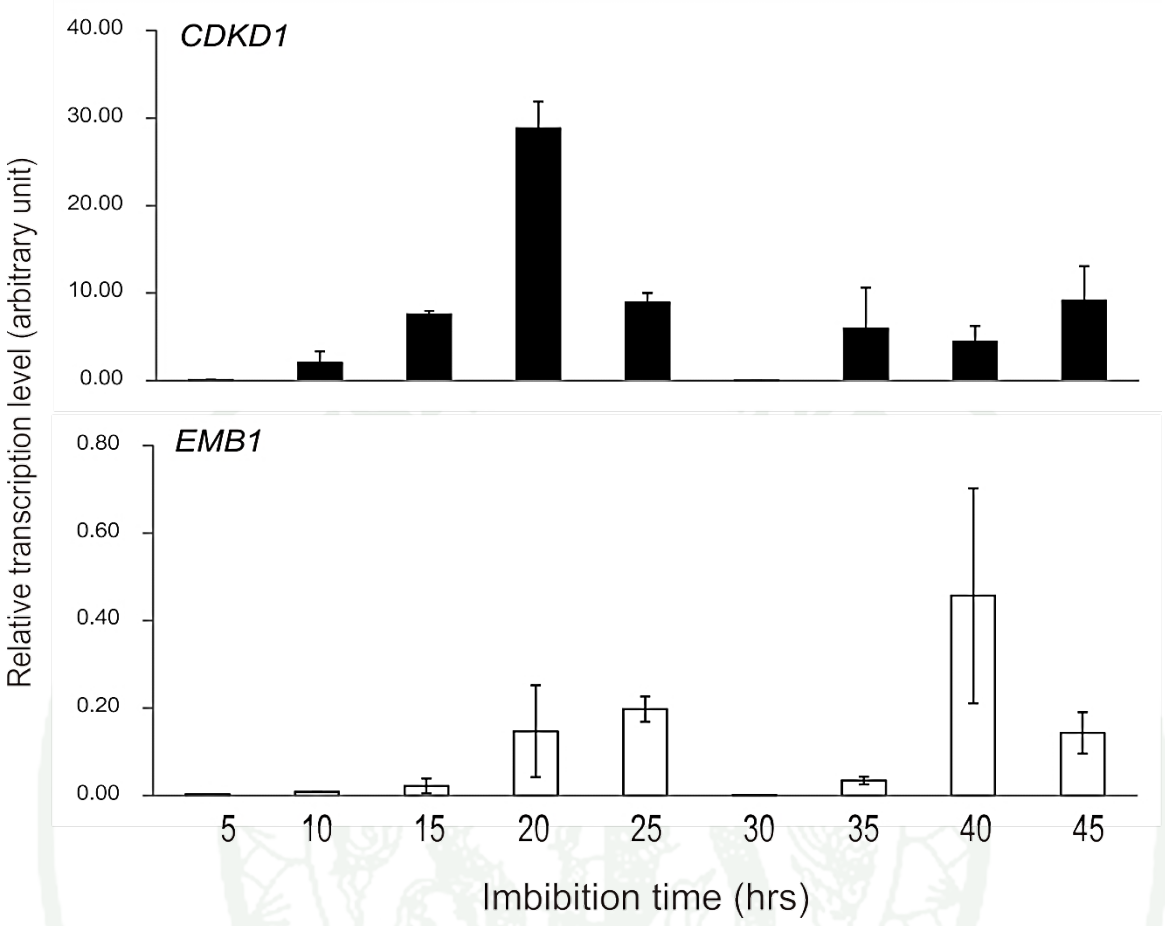

Figure 20 The expression for *Cucumis sativus CDKD1-like* and *Cucumis sativus EMB-1-like* of low vigour cucumber seed lot, CU-50, subjected to rapid osmopriming process (ψs of -0.05 MPa of KNO_3 at 20°C) with various imbibitions times. Error bars denote the s.e. (n = 4); missing error bars indicate that they are smaller than the symbols.

2.3.5 Seed quality after priming

1) Before AA-test

For radicle emergence behavior of CU-51, the high vigour seed lot, there were no any imbibition period that gave the seed quality better than control. The imbibition period of 5 hour was only soaking time giving the germination result and could be analyzed by GERMINATOR software. MaxRE and MaxF of imbibition period of 5 HAI were 70.0 % and 28.0%, whilst, those of control were 84.0 % and 12%, respectively. Not only the reduction of maximum radicle emergence and increment of maximum fungal

infected seed were found, but also the low homogeneity. U_{7525} of them were 11.8 hours comparing to 4.4 hours of control. However, primed seeds from imbibition period of 5 hours had high speed of radicle emergence more than control. Its t_{50} and MRET were 12.1 and 15.0 hours comparing to 32.6 and 33.1 hours of control. The rest of imbibition times gave non sigmoid curve of radicle emergence data which could not be analyzed by the software resulting in unavailable result as shown in Table 7.

For radicle emergence behavior of CU-50, the low vigour seed lot, there was no any imbibition period that had quality better than control (Table 8). There was no imbibition period that GERMINATOR software could analyze in this treatment except control.

2) After AA-test

For the seed quality after priming process and AA-treatment of CU-51, the high vigour seed lot, all radicle emergence parameter results were in accordance with the results found before the AA treatment (Table 9). MaxRE, t_{50}, and MRET were less than control while MaxF and U_{7525} were still more than control. MaxRE and t_{50} were 66.0% and 28.67 hours comparing to 95.0% and 32.5hours for control, respectively.

For the quality after priming process and AA- treatment of CU-50, the low vigour seed lot, all radicle emergence parameter results were in accordance with the results found before the AA treatment (Table 10). There was no any imbibition period that had quality better than control.

Table 7 Radicle emergence behaviors of CU-51, the high vigour cucumber seed lot, as affected by rapid osmopriming process.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours) [a]	Mean radicle emergence time (MRET; hours) [a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours) [a]	Maximum fungal infected seed (MaxF, %) [a]
0 hr. (control)	84.0[a]	32.6[a]	33.1[a]	4.40[a]	12.0[d]
5 hours	70.0[b]	12.1[b]	15.0[b]	11.80[b]	28.0[c]
10 hours	N/A	N/A	N/A	N/A	96.0[a]
15 hours	N/A	N/A	N/A	N/A	99.0[a]
20 hours	N/A	N/A	N/A	N/A	94.0[a]
25 hours	N/A	N/A	N/A	N/A	72.0[b]
30 hours	N/A	N/A	N/A	N/A	90.0[a]
35 hours	N/A	N/A	N/A	N/A	91.0[a]
40 hours	N/A	N/A	N/A	N/A	85.0[ab]
Pr>F	<.0001	<.0001	<.0001	<.0001	<.0001
CV (%)	18.5164	24.3834	21.9243	50.7929	7.5849

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Table 8 Radicle emergence behaviors of CU-50, the low vigour cucumber seed lot, affected by rapid osmopriming process.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours) [a]	Mean radicle emergence time (MRET; hours) [a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours) [a]	Maximum fungal infected seed (MaxF, %) [a]
0 hr. (control)	88.0[a]	36.10[a]	26.4[a]	5.00[a]	1.0[c]
5 hours	N/A	N/A	N/A	N/A	66.0[b]
10 hours	N/A	N/A	N/A	N/A	68.0[b]
15 hours	N/A	N/A	N/A	N/A	97.0[c]
20 hours	N/A	N/A	N/A	N/A	89.0[c]
25 hours	N/A	N/A	N/A	N/A	87.0[c]
30 hours	N/A	N/A	N/A	N/A	78.0[c]
35 hours	N/A	N/A	N/A	N/A	94.0[c]
40 hours	N/A	N/A	N/A	N/A	96.0[c]
45 hours	N/A	N/A	N/A	N/A	97.0[c]
Pr>F	<.0001	<.0001	<.0001	<.0001	<.0001
CV (%)	0.0000	30.9023	32.3375	72.0510	6.2900

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Table 9 Radicle emergence behaviors of CU-51, the high vigour cucumber seed lot, affected by rapid osmopriming process followed by accelerated aging treatment.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours)[a]	Mean radicle emergence time (MRET; hours)[a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours)[a]	Maximum fungal infected seed (MaxF, %)[a]
0 hr. (control)	95.0[a]	32.46[a]	34.48[a]	14.45[a]	0.0[d]
5 hours	66.0[b]	28.67[b]	30.65[a]	17.55[a]	24.0[c]
10 hours	N/A	N/A	N/A	N/A	65.0[ab]
15 hours	N/A	N/A	N/A	N/A	93.0[a]
20 hours	N/A	N/A	N/A	N/A	61.0[b]
25 hours	N/A	N/A	N/A	N/A	64.0[ab]
30 hours	N/A	N/A	N/A	N/A	63.0[ab]
35 hours	N/A	N/A	N/A	N/A	77.0[ab]
40 hours	N/A	N/A	N/A	N/A	69.0[ab]
Pr>F	<.0001	<.0001	<.0001	<.0001	<.0001
CV (%)	13.9332	20.4850	37.1798	113.0025	16.4444

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Table 10 Radicle emergence behaviors of CU-50, the low vigour cucumber seed lot, after rapid osmopriming process accelerated aging treatment.

Imbibition period (hours)	Maximum radicle emergence (MaxRE, %)[a]	Radicle emergence time (t_{50}; hours)[a]	Mean radicle emergence time (MRET; hours)[a]	Uniformity ($T_{75} - T_{25}$; U_{7525}; hours)[a]	Maximum fungal infected seed (MaxF, %)[a]
0 hr. (control)	99.0[a]	26.30[a]	26.20[a]	5.90[a]	0.0[d]
5 hours	N/A	N/A	N/A	N/A	50.0[c]
10 hours	N/A	N/A	N/A	N/A	58.0[bc]
15 hours	N/A	N/A	N/A	N/A	59.0[bc]
20 hours	N/A	N/A	N/A	N/A	80.0[ab]
25 hours	N/A	N/A	N/A	N/A	64.0[abc]
30 hours	N/A	N/A	N/A	N/A	73.0[abc]
35 hours	N/A	N/A	N/A	N/A	86.0[a]
40 hours	N/A	N/A	N/A	N/A	70.0[abc]
45 hours	N/A	N/A	N/A	N/A	50.0[c]
Pr>F	<.0001	<.0001	<.0001	<.0001	<.0001
CV (%)	3.1782	12.6142	15.0203	47.4795	12.9886

[a] Values are means of 4 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

The result from the first experiment indicated that DNA content of cucumber radicle tip during imbibitions has an association with radicle emergence by which an increment of (4C + 8C) / 2C ratio will occurred around 6 hours prior to radicle emergence. Therefore it may be used as a biochemical marker to indicate the priming advancement before radicle emergence taking place. However, the correlation between priming advancement and quality of primed seed is needed to be determined. In the second experiment, the correlation between the physical and chemical changes during priming process and the quality of prime seeds is investigated. Furthermore, primed seeds were also subjected to AA treatment to investigate effect of priming treatments on seed vigour which has a positive correlation with seed storability.

In this second experiment, the osmopriming technique was used to determine the physical and biochemical changes during imbibition periods of priming process. Potassium Nitrate (KNO_3) was used as an osmoticant because it give no toxic to cucumber seeds (Demir and Oztokat, 2003; Golezanik and Esmaeilpour, 2008; Nascimento and Aragão, 2004; Nerson *et al.*, 1985; Welbaum and Bradford, 1991). Two concentrations of KNO_3 solution; 8.42 and 190.47 mM, were used to provide the solution with different ψ_s in order to observe the effect of imbibition speeds on the physical and biochemical changes and the quality of primed seeds. Two cucumber seed lots with different vigour levels were included in the experiment to investigate seed vigour effect on the physical and biochemical changes and on the seed quality during imbibitions periods of priming process and after AA treatment.

The seed quality analysis of primed cucumber seed revealed that the rapid osmopriming gave significantly poor seed quality after priming comparing to unprimed seeds for both seed vigour levels. The rapid osmopriming may cause injury associate with rapid water uptake to cucumber seeds resulting in poor germination performance for both before and after AA treatment. The imbibition injury can take place when dry seeds are soaked in relatively high ψ_s solution (Bewley *et al.*, 2013). In this second experiment, cucumber seed sample had been stored at 5 ± 1°C and 35–40 %RH condition for five years having SMC of 6.9 %FW before they were subjected to rapid priming process using ψ_s - 0.05 MPa KNO_3 solution. In contrast, the slow osmopriming of high vigour seed lot for short imbibition times (10 hours) gave the best seed quality after priming as well after AA treatment. Since, the seed quality after AA treatment has a positive correlation with seed

storability (Demir and Mavi, 2008). Therefore, the AA treatment result of primed seed found in this experiment should indicate the promising storability of primed seed by slow priming with the short imbibition time.

In this study, some physical and biochemical changes during imbibition periods of osmopriming process were determined to investigate the correlation of the changes and the quality of primed seed. Time to the first radicle emergence showed no positive correlation with seed quality during imbibitions periods. It varied with imbibition speed and seed vigour levels. In rapid osmopriming, first radicle emergence was found at hour 30 of imbibitions regardless seed vigour levels while in slow osmopiming, it was found at hour 50 for CU-51, the high vigour seed lot, and at hour 70 for CU-50, the low vigour seed lot. The late radicle emergence of low vigour cucumber seed was in agree with the result found in the first experiment when radicle emergence of low vigour seed delayed due to the arrest of cell cycle activity during imbibition. In another words, time to first radicle emergence parameter could not be used as marker for priming process since it took place when the quality of primed seed had already dropped in some extent. Although, the difference in ethanol production of primed seed between slow and rapid osmopriming was noticeable by which rapid osmopriming caused primed seed to produce higher ethanol production than slow osmopriming, there was no correlation between ethanol production and seed quality during imbibition periods. Therefore, the ethanol content could not be used as marker for cucumber seed priming as well.

The SMC changes during imbibition periods of all priming treatments combination between imbibitions speed and seed vigour levels were o followed the SMC scenario of tri-phase of imbibitions. The transition of phase I to II took place when SMC were between 30-35% at 5 and 10 hour of imbibitions period for rapid and slow osmopriming, respectively for both CU 51, the high vigour seed lot, and CU-50, the low vigour seed lot. Hence, the results indicated that time to phase I to II changing did not vary with seed vigour levels since water movement into seed during phase I of imbibitions is forced by the water potential difference between seed and environment regardless the seed viability (Bewley *et al.*, 2013). In contrast, it varied with imbibitions speed which is influenced by ψ_s of soaking solution used. When the seed quality and SMC changes during imbibition period of osmopriming were concerned, it revealed that the highest quality of primed cucumber seeds were obtained when the SMC was at the beginning of phase I of tri-phase of

imbibitions. This finding suggested that the end of phase I or the beginning of phase II of tri-phase of imbibitions may be the appropriate stage to terminate imbibition process for priming of cucumber seed in order to obtain the highest germination performance as well as storability. However, time to reach the end of phase I or the beginning of Phase II can vary with ψ_s of imbibition solution as above mentioned and possibly with plant varieties. Therefore, stage of imbibitions indicated by SMC may not be the most effective marker for cucumber seed priming.

In this experiment, the expression of *Cucumis sativus EMB-1-like,* the desiccant tolerance involving gene, showed the association with the quality of primed seeds while it was not for *Cucumis sativus CDKD1-like*. The expression of *Cucumis sativus EMB-1-like* of CU-51, the high vigour seed lot, took place at 5 and 10 hour of imbibitions for both rapid and slow osmpriming processes before it significantly down-regulated thereafter. From SMC and seed quality change results during imbibiion periods mentioned above, the imbibition time that the expression of *Cucumis sativus EMB-1-like* still existed coincided with the beginning stage of Phase II of triphase of imbibitions for cucumber seed by which the quality of primed seed were maximum. This finding could explain quality loss of primed cucumber seeds found in this experiment when they were imbibed in the KNO_3 solution longer than 5 and 10 hour for rapid and slow osmopriming, respectively. Since *Cucumis sativus EMB-1-like* involves in the desiccant tolerance of seeds and after imbibition periods, primed seeds will be dried back to moisture level safe for storage. It has been well documented that the keeping desiccation tolerance can maintain the longevity in primed seeds (Bruggink *et al.*, 1999; Schwember and Bradford, 2005). In contrast, releasing seeds from the desiccation tolerance would lead to reduce the self-defense mechanism from fungi of cucumber seed (Oliver, 2007) leading to poor germination performance. This results support the suggestion mentioned earlier that in cucumber seed priming, the imbibition process must be terminated when SMC reached the beginning of phase II of imbibitions since, at that stage, the desiccation tolerance of seed still exist. In conclusion, the correlation between the expression of *Cucumis sativus EMB-1-like* and quality of primed cucumber seeds during imbibitions periods had shown to be consistency with seed vigour levels. Therefore, it may be used as a possible marker for cucumber seed priming. However, the easy and fast assay to analyze gene expression should be developed.

Lastly, the finding of time to terminate imbibition period at the early stage of phase II of triphase of imbibition for cucumber seed priming may be in contradiction to the general understanding in the past that the longer the imbibitions periods, but without radicle emergence, the better quality of primed seed. This might be because of species difference. Cucumber seed is the fast germinating seed therefore it might not need as long period of time to stimulate germination process during seed priming process as the slow germinating seed like hot chili seeds. Nevertheless, further studies are needed for better understanding.

CONCLUSIONS

The cell cycle activity during imbibition period and physiological and biochemical changes during osmopriming process of cucumber seeds were explicitly revealed in this study. Based on our results, the conclusions can be drawn as follows:

1) During imbibition period, the high vigour cucumber seed underwent normal cell cycle activity and the DNA content of (4C+8C)/2C ratio significantly increased 6 hours before radicle emergence.

2) There was an arrest of cell cycle activity found in heat deteriorated cucumber seeds resulting in the delay of radicle emergence in the extent of deterioration severity and the radicle emergence took place without the increment of (4C+3C)/2C DNA content ratio.

3) The positive correlation between the cell cycle activity at the radicle tip cells of cucumber seed embryo and the germination advancement during imbibition process was only found for high vigour cucumber seed lot, but not for low vigour seed lot or seed lot that underwent deterioration.

4) The imbibition of high vigour cucumber seed in KNO_3 solution with ψ_s -0.05 MPa at 20 °C (slow osmopriming) until the SMC reached the early stage of phase II of the tri-phase of imbibitions gave the highest seed quality after priming.

5) The down-regulate expression of *Cucumis sativus EMB-1-like*, the desiccant tolerance involving gene, coincided with the declining of seed quality in the later stage of phase II of tri-phase of imbibition during osmopriming process.

6) The changing of SMC and the expression of *Cucumis sativus EMB-1-like* during the imbibition period of slow osmopiming of high vigour cucumber seed were highly associated with seed quality after priming.

7) There was no relationship between ethanol production of cucumber seed during imbibition periods of osmopriming process and the seed quality after priming of seed.

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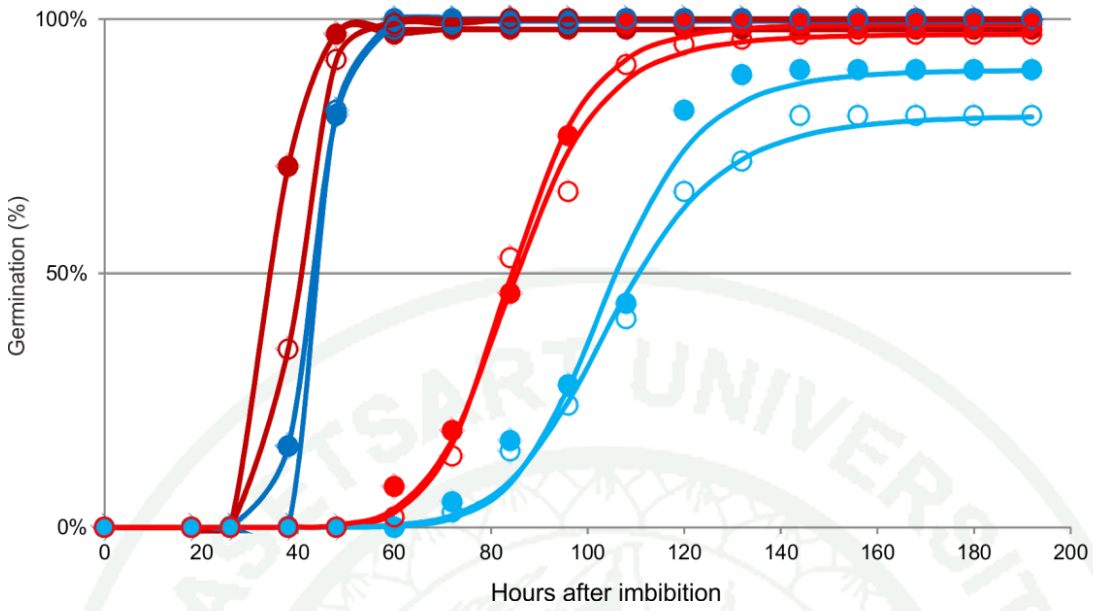
APPENDICES

Appendix A

Preliminary experiment: Germination behavior of CU-50 and CU-51



Appendix Figure A1 Germination curve fitting to the four parameter Hill function of cumulative germination of the rapid and the slow osmopriming process in CU-50 and CU-51 using GERMINATOR package. The first biological replications are represented in lines with closed circle and the second biological replications are in lines with open circle. The black red lines illustrate cumulative germination percentage of rapid osmopriming process, whereas the red lines represent slow osmopriming process with CU-51. Dark blue lines illustrate cumulative germination percentage of rapid osmopriming process, whereas the red lines are slow osmopriming process with CU-50. The values are means of five replicates.

Appendix Table A1 Germination parameters of CU-50 and CU-51 before they were subjected to osmopriming process.[a]

Seed lots	Germination parameters[b]				
	Maximum germination (%)	Maximum normal seedlings (%)	Germination time (t_{50}; hours)[c]	Mean germination time (MGT; hours)	Uniformity (T75 – T25; U_{7525}, hours)[d]
CU-50 (produced in 2007)	100 ± 0.0	88 ± 2.1	32.5 ± 0.6	28.7 ± 0.6	4.8 ± 0.2
CU-51 (produced in 2008)	100 ± 0.0	97.5 ± 0.3	22.4 ± 0.1	19.9 ± 0.1	4.4 ± 0.1

a Values are means ± s.e. of four determinations.

b Germination test at 25°C in dark.

c Hours to reach 50% maximum germination.

d Time between 25% and 75% germination.

Appendix Table A2 Parameters characterizing seed germination curves of five replicates of the rapid and the slow osmopriming process in CU-50 and CU-51 using GERMINATOR package (see also Appendix Figure A1).

Seed lots	Priming processes	Biological replications	Time to 50 percentage germination (hours)	Correlation coefficient for the fitted curve (r^2)
CU-50	rapid osmopriming	1	43.0 ± 0.5	1.00
(produced in 2007)		2	46.0 ± 0.2	0.99
	slow osmopriming	1	106.3 ± 1.6	1.00
		2	110.2 ± 2.1	1.00
CU-51	rapid osmopriming	1	35.7 ± 1.2	1.00
(produced in 2008)		2	39.8 ± 0.3	0.99
	slow osmopriming	1	84.2 ± 2.1	1.00
		2	85.7 ± 1.9	0.99

Appendix B

Raw data of physical and biochemical change during imbibition period of osmopriming process

Appendix Table B1 Raw data of physical and biochemical change during imbibition period of high initial seed vigour during slow osmopriming process.

Imbibition period (hours)	Cumulative radicle emergence (%)[a]	Moisture content (% FW)[b]	Relative transcription level (arbitrary unit) [c]		Headspace ethanol (μg L^{-1}) [b]
			Cucumis sativus CDKD1-like	*Cucumis sativus EMB-1-like*	
0 hr. (control)	0.0[e]	15.07[e]	-	-	25.0
10 hours	0.0[e]	29.40[d]	0.860[b]	0.072[a]	37.5
20 hours	0.0[e]	30.37[cd]	0.861[b]	0.023[b]	92.0
30 hours	0.0[e]	31.39[bcd]	0.914[b]	0.029[b]	96.0
40 hours	0.0[e]	31.51[ab]	1.094[b]	0.016[b]	122.5
50 hours	0.8[e]	31.61[abc]	1.945[b]	0.023[b]	53.0
60 hours	5.0[d]	32.03[a]	4.757[b]	0.028[b]	73.5
70 hours	14.6[c]	31.16[bcd]	9.537[ab]	0.005[b]	178.5
80 hours	38.5[b]	30.36[cd]	19.130[a]	0.010[b]	156.0
90 hours	60.5[a]	31.54[ab]	15.103[a]	0.000[b]	120.5
Pr>F	<.0001	<.0001	0.0035	0.0359	0.2173
CV (%)	6.2570	1.0098	93.5435	96.00403	57.3416

[a] Values are means of 8 replicates from 2 biological replications.

[b] Values are means of 2 replicates.

[c] Values are means of 3 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Appendix Table B2 Raw data of physical and biochemical change during imbibition period of low initial seed vigour during slow osmopriming process.

Imbibition period (hours)	Cumulative radicle emergence (%)[a]	Moisture content (% FW)[b]	Relative transcription level (arbitrary unit) [c]		Headspace ethanol (μg L^{-1}) [b]
			Cucumis sativus CDKD1-like	*Cucumis sativus EMB-1-like*	
0 hr. (control)	0.0[f]	14.77[d]	-	-	54.5[d]
10 hours	0.0[f]	30.92[c]	0.002[b]	0.000	183.5[cd]
20 hours	0.0[f]	40.14[b]	0.005[b]	0.001	113.0[cd]
30 hours	0.0[f]	47.20[a]	0.034[b]	0.005	202.0[cd]
40 hours	0.0[f]	31.77[cb]	0.070[b]	0.019	415.5[ab]
50 hours	0.0[f]	33.00[b]	0.098[b]	0.011	455.0[a]
60 hours	0.0[f]	32.33[b]	0.215[b]	0.065	230.0[bcd]
70 hours	3.3[e]	30.38[cb]	5.423[a]	0.011	105.5[cd]
80 hours	12.0[d]	32.92[cb]	1.516[b]	0.032	281.5[abc]
90 hours	23.2[c]	28.82[cb]	2.541[b]	0.397	264.0[abc]
100 hours	31.5[b]	34.28[cb]	2.672[b]	0.102	256.0[bc]
110 hours	47.8[a]	34.40b	1.298[b]	0.098	284.0[abc]
Pr>F	<.0001	<.0001	0.0027	0.2937	0.0107
CV (%)	11.0030	4.4406	116.3631	261.7899	34.7493

[a] Values are means of 8 replicates from 2 biological replications.

[b] Values are means of 2 replicates.

[c] Values are means of 3 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Appendix Table B3 Raw data of physical and biochemical change during imbibition period of high initial seed vigour during rapid osmopriming process.

Imbibition period (hours)	Cumulative radicle emergence (%)[a]	Moisture content (% FW)[b]	Relative transcription level (arbitrary unit) [c]		Headspace ethanol (μg L^{-1}) [b]
			Cucumis sativus CDKD1-like	*Cucumis sativus EMB-1-like*	
0 hr. (control)	0.0[d]	15.07[d]	-	-	25.0[c]
5 hours	0.0[d]	31.12[b]	0.641	0.272[a]	676.5[d]
10 hours	0.0[d]	35.04[a]	0.078	0.004[b]	1138.0[a]
15 hours	0.0[d]	30.335[c]	0.010	0.002[b]	701.5[d]
20 hours	0.0[d]	32.10[b]	0.074	0.020[b]	671.5[d]
25 hours	0.0[d]	33.17[ab]	0.377	0.069[b]	533.0[d]
30 hours	17.7[c]	33.37[b]	3.831	0.000[b]	1530.5[a]
35 hours	39.8[b]	30.14[bc]	15.966	0.098[b]	1150.0[a]
40 hours	59.9[a]	33.16[ab]	1.899	0.141[b]	677.5[d]
Pr>F	<.0001	<.0001	0.1416	0.0049	0.0004
CV (%)	5.9929	1.6673	241.5381	99.7789	21.1485

[a] Values are means of 8 replicates from 2 biological replications.

[b] Values are means of 2 replicates.

[c] Values are means of 3 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Appendix Table B4 Raw data of physical and biochemical change during imbibition period of low initial seed vigour during rapid osmopriming process.

Imbibition period (hours)	Cumulative radicle emergence (%)[a]	Moisture content (% FW)[b]	Relative transcription level (arbitrary unit) [c]		Headspace ethanol (μg L^{-1}) [b]
			Cucumis sativus CDKD1-like	*Cucumis sativus EMB-1-like*	
0 hr. (control)	0.0[e]	14.77[d]	-	-	54.5[d]
5 hours	0.0[e]	29.69[b]	0.085[b]	0.003	515.5[bc]
10 hours	0.0[e]	27.45[c]	2.067[b]	0.008	596.0[bc]
15 hours	0.0[e]	30.22[b]	7.581[b]	0.022	733.0[bc]
20 hours	0.0[e]	31.22[b]	28.832[a]	0.147	555.5[bc]
25 hours	0.0[e]	34.56[b]	8.920[b]	0.198	1373.0[a]
30 hours	2.6[d]	35.74[ab]	0.009[b]	0.001	859.0[b]
35 hours	6.0[c]	34.25[b]	5.971[b]	0.034	463.0[c]
40 hours	12.3[b]	31.20[b]	4.443[b]	0.457	473.0[c]
45 hours	50.9[a]	38.29[a]	9.157[b]	0.143	674.0[bc]
Pr>F	<.0001	<.0001	<.0001	0.1474	0.0006
CV (%)	12.8800	2.2715	68.73863	172.047	23.9815

[a] Values are means of 8 replicates from 2 biological replications.

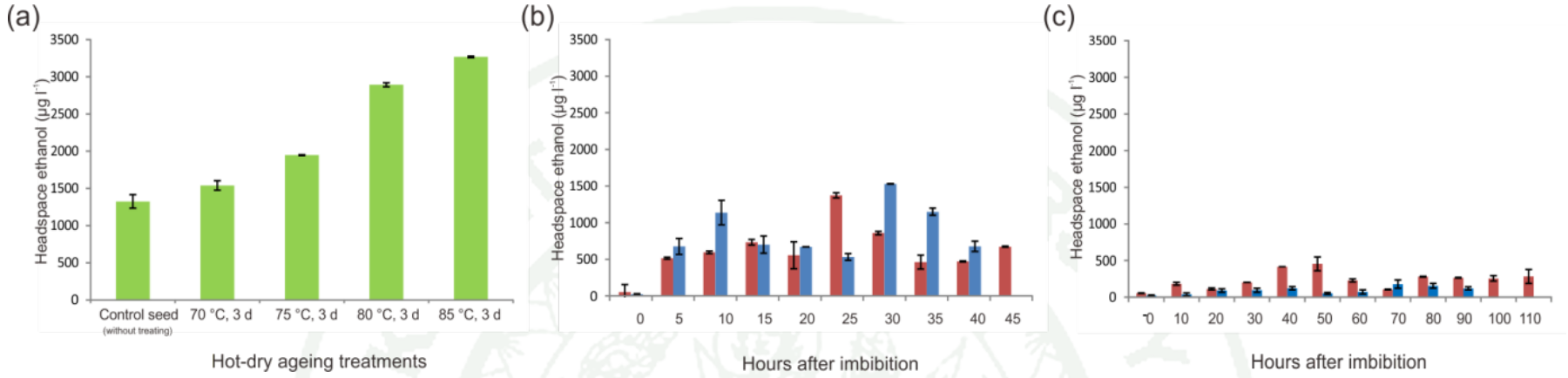
[b] Values are means of 2 replicates.

[c] Values are means of 3 replicates.

Values for the certain parameter marked with the same letter are not significantly different at $Pr \leq 0.05$ (DMRT)

Appendix C

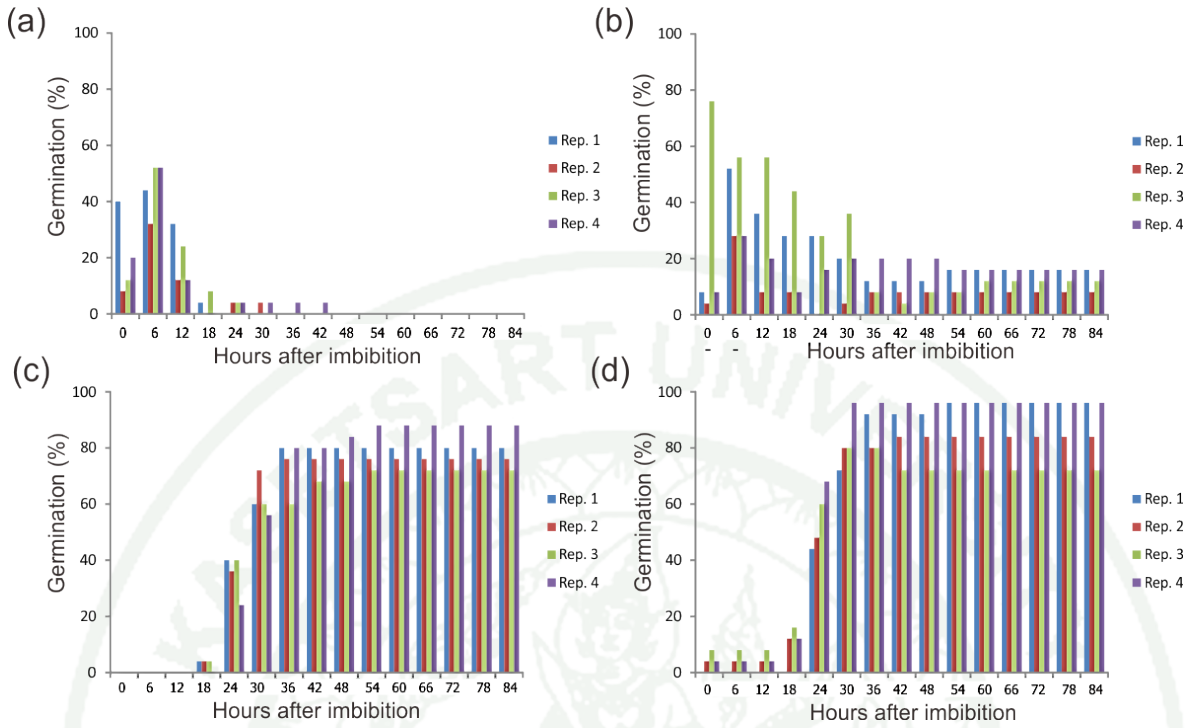
Preliminary experiment: A fast ethanol assay



Appendix Figure C1 The successful condition to classify the deterioration of hot-dried treated seed (a) but cannot be applied to check the deterioration of seeds in each of imbibition period in this experiment (b and c). Figure (a), preliminary experimental studies, cucumber seeds were subjected to four hot dry ageing treatments at 70°C, 75°C, 80°C, and 85°C for three days then after that headspace ethanol was measured by using a condition of the incubating at 57°C for 5 hours with desired seed moisture percentage about 30%SMC. This experiment, headspace ethanol was measured after incubation at 57°C for 5 hours of rapid osmopriming process (b) and slow osmopriming process (c). CU-50 was illustrated in red bar, whilst CU-51 was the blue bar (mean of three replicates ± s.e.).

Appendix D

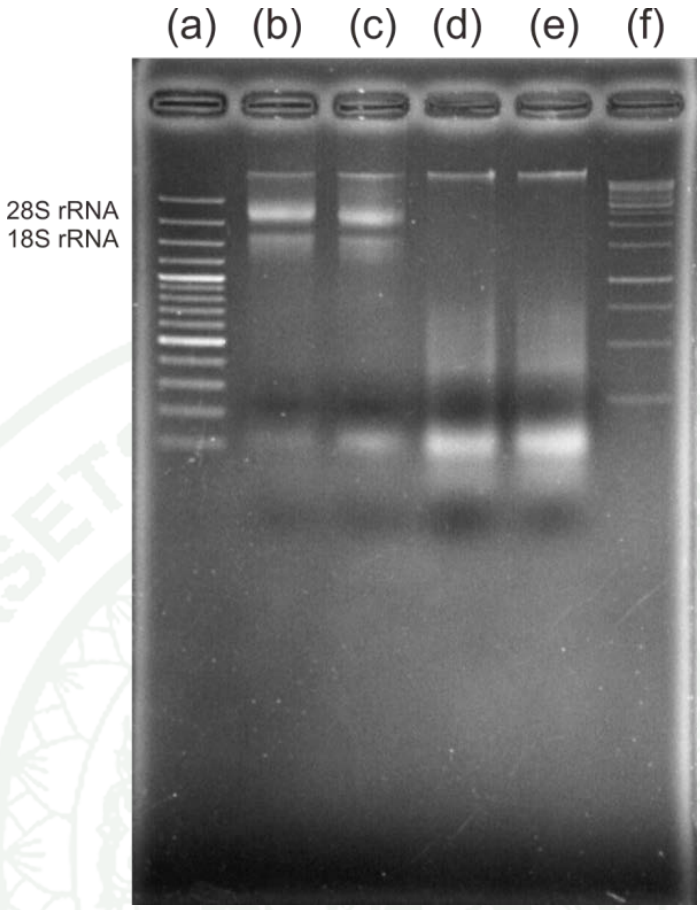
GERMINATOR package



Appendix Figure D1 Comparisons of cumulative radicle emergence percentage between imbibition periods of 40 hours after imbibition. The rapid osmopriming process (ψ_s of -0.05 MPa of KNO_3 at 20°C) performing with CU-50 (a) and CU-51 (b). The slow osmopriming process (ψ_s of -0.8 MPa of KNO_3 at 20°C) performing with CU-50 (c) and CU-51 (d).

Appendix E

RNA integrity and primer sequences used for real time PCR amplification



Appendix Figure E1 RNA integrity deriving from 4 days old seedlings (b and c) and dry seeds (d and e, 15.07 ± 0.03% of SMC) of CU-51. Lane a and f showed GeneRuler 100 bp plus DNA ladder. Total RNA was isolated after preserving in RNA preservation medium by using modified rapid CTAB protocol that described by Gambino *et al.* (2008), but the difference only added cetyltrimethylammonium bromide (CTAB) and phenol blocker polyvinylpyrrolidone (PVP-40) up to 6% of extraction buffer. For checking total RNA integrity, 1 µg total RNA was subjected to gel electrophoresis on 1.5% agarose gel containing 1% formaldehyde, stained with ethidium bromide and visualised using a UV transilluminator, GeneFlash (Imgen Technologies).

Appendix Table E1 Primer sequences used for real time PCR amplification.

Names	Types	Sequence (5'->3')	Amplicon size (base pairs)	Genbank accession number
Cucumis sativus CDKD1-like	Forward primer	GGTACTAAGCAATATGGTTC	97	XM_004137283.1
	Reverse primer	CACTTGAACCCTGAAGAA		
	TaqMan probe	AGTGGATGTTTGGGCAGCAG		
Cucumis sativus EMB-1-like	Forward primer	ATGTCGTCGGAGCAGGAA	103	XM_004148563.1
	Reverse primer	GCTCCTGAGCTTCAAGACTTT		
	TaqMan probe	TGGCCCTGGCGTCGAGTTTACC		
Cucumis sativus EF1α -like	Forward primer	AGCACGCTCTTCTTGCTTTC	95	EF446145.1
	Reverse primer	CCTTGCCTTGGAGTATTTGG		
	TaqMan probe	TGCAACAAGATGGATGCCACCA		

Appendix F

Publications

Onwimol, D., Chanprame, S. and Thongket, T. (2012), *Seed Sci. & Technol.*, **40**, 238-247

Arrest of cell cycle associated with delayed radicle emergence in deteriorated cucumber seed

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Summary

The objective of this experiment was to study germination parameters and cell cycle activity of radical tip cells during imbibition of cucumber seeds using flow cytometry. Samples from a uniform cucumber seed lot were subjected to one of two ageing treatments prior to imbibition; non-aged seeds were used as a control. Radicle emergence was delayed in seeds subjected to an ageing treatment. Control seeds exhibited normal cell cycle activity, with radicle emergence at 24 hours of imbibition, whereas the cell cycles of the deteriorated seeds were slower and radicle emergence delayed. The length of the delay was related to the severity of the ageing treatment. A single arrest at the G_1 phase was found during 6-18 hours of imbibition in seeds that had been treated for 96 hours at 45°C, and two arrests at both G_1 and S phases were found during 6-18 hours and 24-42 hours for seeds that had been treated for 96 hours at 50°C. This suggests that the initial physiological age of the seeds needs to be taken into consideration prior to the application of flow cytometry for monitoring cucumber seed germination advancement during the seed priming process.

Introduction

Seed priming – imbibing seed to initiate germination, followed by dehydration to halt germination prior to radical protrusion – enhances seed quality. Upon planting, primed seeds germinate (radical protrusion) faster and with greater uniformity compared with non-primed seeds. The efficiency of seed priming depends on the extent of advancement of the germination process (Lanteri *et al.*, 1996). Since it is difficult to identify the stage of germination by morphological examination, reliable physical or biochemical markers to determine the stage of germination during seed priming could improve the efficiency of the process. Sliwinska *et al.* (2009) proposed that cell cycle activity in seed somatic cells could be a cellular marker to detect both seed maturity and progression of the germination process, and hence might be used in both seed production and seed priming. The normal

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cell cycle mode is characterised by a round of DNA replication (S phase) followed by mitosis and cytokinesis (M phase). Two gap phases (G_1 and G_2) usually separate the S and M phases. In a mature dry seed most of the nuclei are in the G_0 / G_1 phase of cell cycle (G_0 referred to as the quiescent state) (Inźe and de Veylder, 2006; Sliwinska, 2006). However, in embryo somatic cells of some species, the nuclei can undergo endoreduplication, a process in which cell nuclei conduct repeated rounds of DNA replication without cell division that results in the presence of cells with dissimilar ploidy levels within an organ (Nagl, 1976; Sliwinska *et al*., 2009). The ratio of different ploidy levels in seed embryos was reported to correlate with the progression of germination in several studies (da Silva *et al*., 2008; Gendreau *et al*., 2008). Rewers *et al*. (2009) found that the time course of the advancement of germination at the stage of germination *sensu stricto* in cucumber seed was highly correlated with the (4C + 8C + 16C) / 2C ratio, or G_2 / G_1 ratio, of nuclei. It has thus been recommended that the patterns of G_2 / G_1 ratio can be used as markers of advancement of germination in several polysomatic species, including cucumber (Rewers *et al*., 2009).

Factors such as genotype, initial seed vigour, environmental stress and chemical toxins potentially affect cell cycle progression (Lanteri *et al*., 1996; van Pijlen *et al*., 1996). Jang *et al*. (2005) reported that heat and H_2O_2 caused deceleration of DNA synthesis and allowed time for cell repair in tobacco BY-2 cells. However, little is known about the effects of temperature and relative humidity in reducing cell cycle activity. In tropical and subtropical countries, seed may deteriorate rapidly due to high temperatures and / or high relative humidity. Climatic conditions in the field or poor storage conditions can cause seed deterioration without a noticeable drop in germination. Hence, the cell cycle behaviour of seeds that have deteriorated due to weather conditions in the field, stress during seed development or damage during processing, needs to be investigated and taken into consideration prior to its application as a reliable marker to indicate germination progression during seed priming.

The objective of this study was to monitor the changes in cell cycle progression in cucumber radical tip cells during germination of cucumber seeds that have diverse deterioration levels after exposure to high-temperature ageing treatments. The endoreduplication level and seed moisture content throughout the germination process were also studied.

Materials and methods

Plant materials

Seeds of cucumber (*Cucumis sativus* L.), obtained from Thai Seed and Agriculture Co. Ltd., Bangkok, were used in this experiment. The seeds had been stored at 5 ± 1°C for nine months subsequent to harvesting in July 2010 from a commercial seed production field situated at Suphanburi province, Thailand (14° 45'N, 100° 04'E). Prior to the experiment, the seeds were graded to include only seeds of 16 - 25 mg. Seeds were surface sterilised in 0.6% sodium hypochlorite for 20 minutes and subsequently washed with sterile water. Sterilised seeds were air-dried on a thin layer of filter paper to 8% moisture and stored at 5 ± 1°C prior to exposure to ageing treatments.

Heat deterioration treatment

Seed samples were subjected to three ageing treatments; control (no treatment), 45 and 50°C. Seeds were placed on the surface of screen trays in 0.43 l plastic boxes with 40 mL of saturated NaCl solution at the bottom of each box to create an environment of 74% relative humidity (RH). Each plastic box was subsequently incubated at either 45 or 50°C for 96 hours. After the ageing treatments, the seed moisture content was measured by the high constant temperature oven method (ISTA, 2011) and a standard germination test was conducted. The remaining seeds were stored at 45% RH at room temperature prior to imbibition and DNA content analysis with flow cytometry.

Germination test

Germination testing was carried out immediately after the ageing treatments, in accordance with ISTA (2011), except only 100 seeds were used per treatment (four replicates of 25 seeds each). Seeds were placed on top of moistened blotter papers in transparent 12 × 12 × 9 cm polyethylene boxes. Each box contained one replication (25 seeds) of a single treatment. The boxes were placed in a germination chamber set at 25°C. Radicle emergence (about 2 mm in length) and germination (normal seedling) were determined at 24 hour intervals for eight days. Seedling quality was evaluated in accordance with the ISTA rules.

The mean emergence time (MET) and mean germination time (MGT) were calculated based on the following equation (Ellis and Roberts, 1978):

$$\text{MET or MGT, days} = \Sigma(nD) / \Sigma n$$

Where n denotes the number of seeds showing radicle emergence (for MET) or which germinated (for MGT) on day D and D is the number of days from the commencement of the germination test.

Imbibition treatment

Seeds were imbibed in 20 mL deionised water in 9 cm diameter Petri dishes which were covered with plastic film to prevent evaporation and kept at 25 ± 1°C in the dark for two days. During the imbibition period, at intervals of six hours, two samples from each ageing treatment were taken. One sample was used for seed moisture content determination (high constant temperature oven method), while the other sample was analysed for DNA content with a flow cytometer. The length of time to radicle emergence (2 mm in length) was also recorded.

DNA content analysis

A sample of 1-2 mm of the embryo radicle tip was chopped and used for flow cytometry. Samples were prepared as described by Koroleva *et al.* (2004). The radicle tip was dissected with a sharp razor blade in a plastic Petri dish and thawed by gentle agitation in 1 ml of Partec lysis buffer. After the addition of 1.5 ml of Partec staining buffer, the suspension was filtered through a 30-μM nylon mesh filter and analysed on a Partec PAS II flow cytometer (Partec, Münster, Germany). Analyses were performed on four

replicates, with a linear amplification of the signal. For each sample, 4,000-6,000 nuclei were analysed. Histograms were evaluated with the application of the DPAC v.2.2 software. The proportion of nuclei of dissimilar DNA contents, the mean C-value, the mean ploidy (Barow and Jovtchev, 2007), as well as the (4C + 8C) / 2C ratios were calculated.

Statistical analysis

Single-factor ANOVA (fixed effect model) and least significant difference (LSD) *post hoc* tests were performed on the results at a significance level of $P \leq 0.05$. The percentage data from the germination tests, the ratio data of DNA contents, the mean C-value, and the (4C + 8C) / 2C ratio, were subjected to analysis of variance after angular transformation (transformed by arcsine, $\sqrt{x/100}$; untransformed values are shown in the table to facilitate comparison).

Results

Seed moisture content and Seed germination after ageing treatments

There was no significant difference in seed moisture content after ageing among cucumber seeds subjected to different ageing treatments, ranging between 9.1 and 9.4% on a fresh weight basis (data not shown). No significant differences were found in the germination or radicle emergence percentages among the cucumber seeds subjected to different ageing treatments. However, highly significant differences were found in MGT and MET. The MGT of cucumber seeds subjected to ageing at 50°C was significantly increased whilst the MGT of control and 45°C-aged seeds were similar. The MET of 50°C-aged seeds was 2 days while it was approximately 1 day for the control seeds (table 1). That is to say that the MET of 50°C-aged seeds was significantly increased in comparison with the control treatment.

Time to (first) radicle emergence

The time of radicle emergence was delayed as the degree of ageing increased; time to radicle emergence of control seeds was 24 hours followed by 30 and 36 hours for 45°C- and 50°C-aged seeds, respectively (figure 1).

Table 1. Effects of ageing treatments on germination and radicle emergence of cucumber seeds[a].

Ageing treatment	Germination (%)	Mean germination time (days)	Radicle emergence (%)	Mean emergence time (days)
control	94	4.1 ± 0.05	97	1.1 ± 0.02
45°C 96 hours	92	4.0 ± 0.05	98	1.4 ± 0.03
50°C 96 hours	89	4.5 ± 0.07	100	2.0 ± 0.03
$LSD_{0.05}$	0.7597	0.0002	0.3782	<0.0001

[a]Values are means ± s.e. of four determinations.

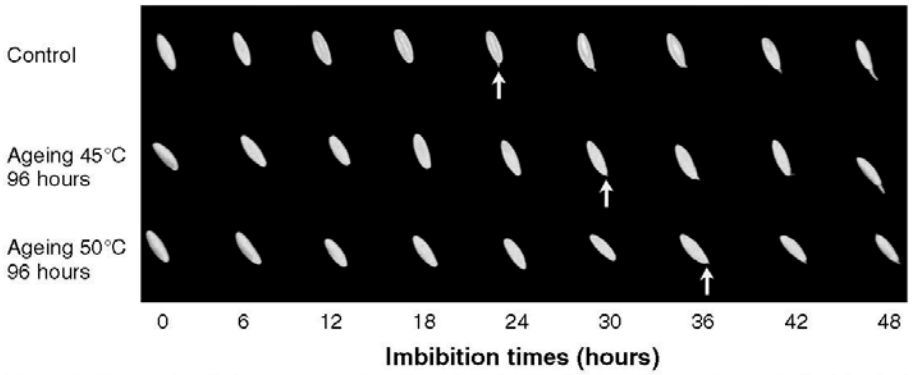

Figure 1. First seed radicle emergence time (hours) during imbibition of cucumber seeds in deionised water in the dark at 25°C for 48 hours. Seeds were either untreated (control) or had been aged at 45°C and 74% RH for 96 hours or at 50°C and 74% RH for 96 hours. The arrows indicate radicle emergence (2 mm in length).

Flow cytometry

Most of the arrested cells (G_0 phase) in the radicle tip of dry cucumber seed contained 2C DNA (figure 2). During imbibition, changes in the 2C, 4C and 8C DNA contents in the radicle tip cells for cucumber seeds subjected to different ageing conditions were observed. In the control seeds, the 2C DNA content was found to increase from approximately 64% at the arresting stage to 100% within six hours of imbibition. The proportion of 4C and 8C DNA increased, an indication of cell cycle transition from G_1 to S and S to G_2 during 18-30 hours of imbibition. The 2C DNA content was further increased after 36 hours indicating transition to the G_1 phase of the subsequent cell cycle. The progression of the cell cycle was slowed in cucumber seeds subjected to ageing treatments. The extent of the delay increased with the severity of the ageing treatment. For the 45°C-aged seeds, after emergence into the G_1 phase at six hours of imbibition, the cells remained in that phase for up to 18 hours of imbibition, six hours longer than the control seeds, before the transition to the S phase. Moreover, up to 48 hours of imbibition were necessary before the cells moved into the G_1 phase of the second sequence of the cell cycle. The prolonged delay in the cell cycle was also found in seeds subjected to more severe deterioration (50°C). Twelve hours of imbibition were required (six hours more than both the control and the 45°C-aged seeds) for radical tips cells to enter the first G_1 cell cycle. The next phase transition from the G_1 to the S phase was also delayed in the 50°C-aged seeds. After the transition from the G_1 phase to the S phase, the cells were arrested at the S phase for 18 hours before progressing to the S/G_2 or G_2 phase after 48 hours of imbibition. This resulted in the failure to observe the second sequence of the cell cycle in 50°C-aged seeds within the 48 hours imbibition time (figure 2).

Seed moisture content

The changes in seed moisture content (MC) of cucumber seeds not subjected to an ageing treatment were in accordance with the conventional tri-phase of water uptake (figure 3A). Control seed MC seeds increased rapidly within six hours of imbibition and remained

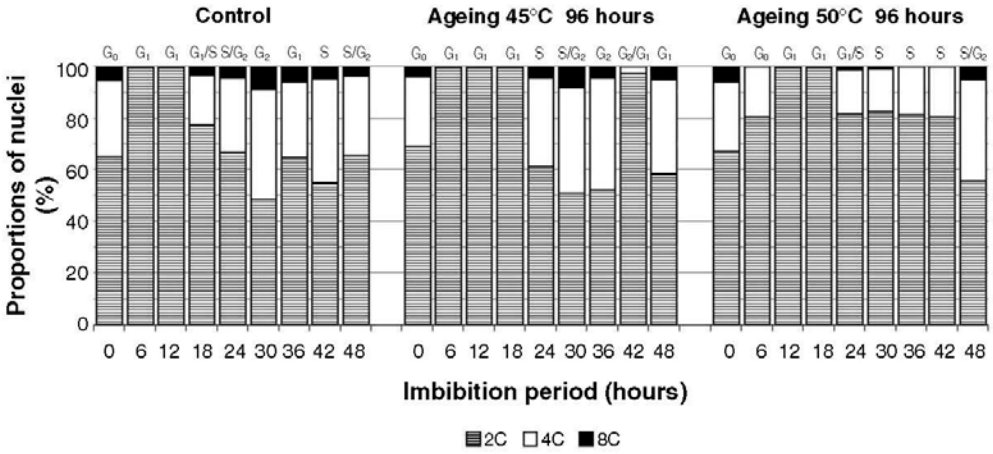

Figure 2. Proportions of 2C, 4C and 8C DNA in the nuclei of embryo radicle tips of cucumber seeds during imbibition in deionised water at 25°C in the dark for 48 hours. Upper case letters refer to the expected synchronised cell cycle profile after imbibition (G_0, resting or quiescent state; G_1, post-mitotic phase; G_2, pre-mitotic phase, and S, synthesis phase during which DNA is replicated).

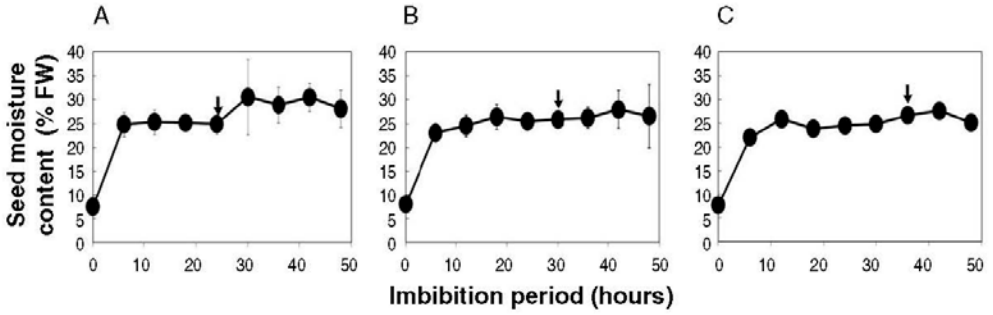

Figure 3. Moisture contents of cucumber seeds during imbibition. A = control seeds; B = seeds aged at 45°C, 74% RH for 96 hours; C = seeds aged at 50°C 74% RH for 96 hours. The arrows indicate radicle emergence. Error bars denote the s.d. (n = 10); missing error bars indicate that they are smaller than the symbols.

stable between 6 and 24 hours of imbibitions, followed by additional increase after visible radicle emergence at 24 hours. The changes in MC during imbibition of 45°C- and 50°C-aged seeds were altered compared with control seeds (figure 3B and 3C). After a rapid increase within six hours of imbibition, the MC remained unchanged for the rest of the imbibition time despite radical emergence, which took place at 30 and 36 hours after imbibition, for 45°C- and 50°C-aged seeds, respectively.

The (4C + 8C) / 2C ratio changes of control seeds and of 45°C-aged seeds during imbibition revealed a similar trend (figure 4A, B). After emergence into phase II of the water uptake process, the ratios increased gradually and reached the maximum value that signifies the cell cycle transition from G_1 to S and S to G_2, respectively. A coincident relationship between radicle emergence and the progression of the cell cycle for both

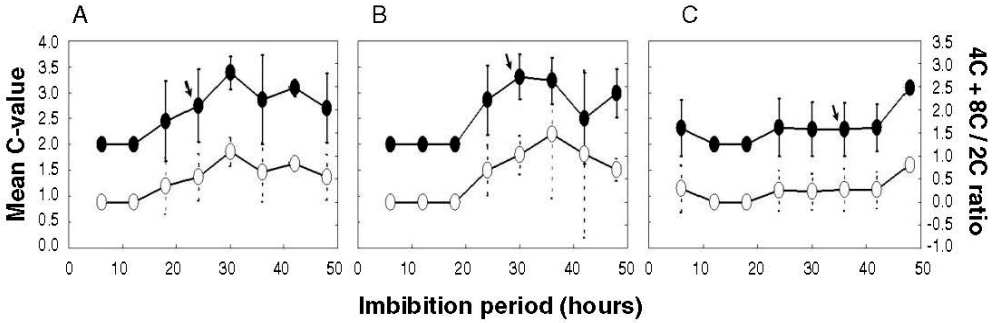

Figure 4. Mean C–values (●) and (4C+8C)/2C ratio (○) in radicle tips of cucumber during germination. A = control seeds; B = seeds aged at 45°C, 74% RH for 96 hours; C = seeds aged at 50°C 74% RH for 96 hours. The arrows indicate radicle emergence. Error bars denote the s.d. (n = 4); missing error bars indicate that they are smaller than the symbols.

control and 45°C-aged seeds was evident. Radicle emergence occurred at the later stages of the S phase, six hours before the (4C + 8C) / 2C ratio attained the maximum value. In contrast, radicle emergence of the 50°C-aged seeds occurred without a noticeable increases in the (4C + 8C) / 2C ratio (figure 4C).

The trend of mean C-values during imbibition of cucumber seeds subjected to diverse ageing treatments were very much in accordance with their (4C + 8C) / 2C ratio, except for the 45°C-aged seeds where the peak of the mean C-value was observed at 30 hours of imbibition, the time of its radicle emergence, whereas that of the (4C + 8C) / 2C ratio was observed at 36 hours, 6 hours after radicle emergence (figure 4B). The mean C-value of radicle tip cells confirmed that cell cycle activity was arrested during 6-18 hours of imbibiton for the seeds aged at 45°C (figure 4B) as well as during 6-18 hours and 24-42 hours of imbibition for the seeds aged at 50°C (figure 4C).

Discussion

The germination results indicate that, although ageing at 45 or 50°C and 74% RH for 96 hours does not significantly affect the total percentage germination or percentage radical emergence of cucumber seed lots, it significantly delayed radicle emergence and germination. Mean germination time is used as a measure of seed vigour (ISTA, 1995) and the increase in germination time indicates the occurrence of seed deterioration (Finch-Savage *et al.*, 2004; Pourhadian and Khajehpour, 2010). Hence, although we do not have a field emergence test result, it is clear that the vigour of the aged cucumber seed lots had declined and the seeds had entered the early stage of deterioration. The longer the delay in radical emergence and germination time, the higher the extent of deterioration that has occurred. A similar phenomenon was observed when cauliflower seed lots with different vigour levels were primed (Powell *et al.*, 2000).

Cucumber is a unique polyploidy species with nuclei ploidy levels between 4C and 16C in dry mature seeds (Sliwinska *et al.*, 2009; Jing *et al.*, 1999). However, in the present

experiment the radicle tip cells rarely contained DNA in excess of 8C. This may have been due to the effects of the DNA binding dye (4,6-diamidino-2-phenylindole, DAPI), which binds to only the minor groove of DNA helix, and the application of a linear amplification of the signal in the current experiment. Similar results were found by Zhang *et al.* (2009) who stained DNA in cucumber radicle cells with DAPI DNA staining dye and applied linear amplification of the signal revealing no DNA contents in excess of 8C.

The results from the cell cycle analysis indicate that the delay of radicle emergence in cucumber seeds subjected to ageing treatments is probably caused by the transient arrest of the cell cycle. The extent of this arrest is dependent on the severity of the ageing treatments. Seeds which were subjected to mild deterioration (45°C) were found to arrest in the G_1 phase before progressing through the G_1 / S checkpoint to the next S phase. As for the seeds subjected to more severe ageing conditions (50°C), cell cycle arrest was observed in two phases; at G_1 phase during 6-18 hours of imbibition and at the subsequent S phase during 24-42 hours of imbibition. Jang *et al.* (2005) reported that the phase at which the cell cycle is arrested depends on the point at which cells experience stress. In the present experiment, dry mature cucumber seeds were subjected to stress treatments after removal from storage. For seeds subjected to ageing treatments, cell cycles were found to first arrest in the G_1 phase of the first cell cycle subsequent to rehydration. In seeds subjected to more severe deterioration at 50°C an additional cell cycle arrest in the subsequent S phase was observed. The stable (4C + 8C) / 2C ratio and the mean C-value during 6-18 and 24-42 hours of imbibition (figure 4C) confirmed the cell cycle block at both the G_1 and S phases for the 50°C-aged seeds. Overall, the flow cytometry results are verification that ageing treatments at high temperature (45-50°C) and high relative humidity (74% RH) cause significant deterioration to the cells of dry cucumber seeds. At higher temperature, the damage to the cells appears to be more pronounced. After rehydration, damaged cells were found to possibly undergo cell repair during the arrest of the cell cycle at the closest checkpoint prior to the continuation of the regular cell cycle activity.

The increase of the (4C + 8C) / 2C ratio during germination indicates an increase of the G_2 cell population in the radicle tip cells before radicle emergence in many species including barley (Gendreau *et al.*, 2008), maize (Sánchez *et al.*, 2005), and sugarbeet (Sliwinska, 2000). This indicates that cell cycle activity in the rehydrated seed is initiated prior to radicle elongation. In the current experiment, for seeds not subjected to ageing conditions (control) or subjected to mild levels of deterioration (ageing at 45°C), radicle emergence occurred six hours prior to the attainment of the maximum values for the (4C + 8C) / 2C ratio. This correlation agrees with reports of Sliwinska *et al.* (2009) who proposed the G_2 / G_1 ratio or (4C + 8C + 16) / 2C ratio might be used as a marker of the advancement of germination / priming of cucumber seeds. However, we noted that seeds subjected to more severe ageing conditions (50°C) did not show an increased (4C + 8C) / 2C ratio prior to radicle emergence (figure 4C), since radicle tip cells were arrested in the S phase (figure 2). This result indicates that the progression of the cell cycle of aged seeds during germination is divergent to that of non-aged seed. The use of the (4C + 8C) / 2C ratio and the mean-C value as a marker for germination advancement might not be effective for the cucumber seed lots that undergo deterioration or have been subjected to unfavourable conditions even though there is no noticeable germination

reduction. The results from the present experiment suggest that the seed physiological age must be taken into consideration prior to application of the cell cycle parameter as an efficient marker of the progression of germination during seed priming.

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Hybridity Test of Cucumbers *via* Ultrathin Layer Isoelectric Focusing Technique by Using Water as the Extraction Buffer

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ABSTRACT

Ultrathin layer isoelectric focusing (UTLIEF) technique using water as the extraction buffer is cheap, fast and reliable technique for varietal identification in cucumber. However, the application for hybridity testing has not been revealed yet. Therefore, in this study, the possibility of application of UTLIEF technique for testing the hybridity of 10 F1 hybrid seeds and their respective parental lines were evaluated. It was found that nine out of ten pairs of parental lines can be differentiated by the difference in combination of male and female marker bands while eight out of ten F1 hybrid seed were verified using only male marker bands in this UTLIEF technique using water as the protein extract solvent and gel pH range of 2-11. More male marker bands (MMB) were found than female marker bands (FMB). Most of polymorphic cucumber seed protein bands were focused in the pH range of 6-10. The result indicated that the UTLIEF technique using water as the extraction buffer and the gel pH gradient of 2-11 is of valued to be used for hybridity test in the commercial cucumber hybrid seed production.

Key words: cucumber, seed storage protein, electrophoresis, isoelectric point, water

INTRODUCTION

Cucumber is one of the economically important vegetable crop in the world. Currently, in most of vegetable seed companies, new F1 hybrid cultivars have been

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continuously produced to meet the requirements of consumers which are changeable over short period of time. Hence, the cultivar verification and hybridity tests are their inevitable works in the quality control of breeding and seed production programs. The conventional grow-out test is recognized as time, labor and cost consuming and environment dependent (Cooke, 1995; McDonald, 1998). The DNA fingerprint technique, the most potential for varietal differentiation, is economically too expensive for large-scale and routine commercial seed testing work the cheaper isozyme analysis has many restricts including specificity of plant tissues and analytical environment sensitivity (Cooke, 1995; Dou *et al*., 2012).

In the recent year, the ultrathin-layer isoelectric focusing (UTLIEF) technique using thin polyacrylamide gel of 0.15 mm was reported as cheap, fast and reliable technique for varietal identification and hybridity test in several crops (Van den Berg, 1990; Yan, 2013b). The technique is based on the possibility that seed storage proteins of different varieties are difference in molecular weight, size, and electrical charge. Proteins with difference electrical charges have different isoelectric point (pI) values and can be separated in the isoelectric focusing gel electrophoresis. Using appropriate protein extract buffer and gel pH gradient, the polymorphic of protein band patterns among seed proteins from different varieties (electrophoretograms) can be found during protein separation in the isoelectric focusing gel and used to verify the varieties. The success of UTLIEF technique for varietal identification was reported for tomato (Wang *et al*., 2000) and cucumber (Onwimol *et al*., 2010; Tu *et al*., 2012). In hybridity test, marker proteins from male and female parent lines are both present in hybrid seed and can be used to verify the hybridity of their respective parents. In the International Seed Testing Association (ISTA) Rules of Seed Testing, the UTLIEF technique is accepted as hybridity test for maize and sunflower (ISTA, 2012). The success of this technique for hybridity test was also found in other crops, e.g. rice (Yan *et al*., 2008; Zhao *et al*., 2005), and pumpkin (Yan, 2013a).

In cucumber hybridity test, Tu *et al*. (2012) successfully found the male marker bands by using the 2-chroloethanol as the seed proteins extract solvent. This indicated the polymorphic of cucumber seed proteins extracted were prolamin, the aqueous alcohol soluble proteins. However, this extraction buffer is costly, toxic to human, harmful to the environment and most of all, the import is restricted and not commercially available in Thailand. In 2010, Onwimol *et al*. reported the polymorphic of albumin proteins between F1 hybrid cucumber varieties by using water as the seed protein extract solvent and gel pH gradient of 2-11. Water is the cheap, safe and environmental-friendly solvent. If water is also able to differentiate male and female marker proteins in cucumber hybridity test, it would be a promising protein extract solvent for varietal identification and hybridity test for cucumber *via* UTLIEF technique. Hence, the objective of this study was to determine the possibility and efficiency of the UTLIEF technique using water as the seed protein extract solvent and pH gradient of 2-11 for testing of hybridity of local cucumber hybrid varieties.

MATERIALS AND METHODS

Seed samples

Ten cucumber (*Cucumis sativus* L.) parental lines and their corresponding F1 hybrid seeds (denoted CS01 to CS10), obtained from Thai Seed and Agriculture Co. Ltd., Bangkok, Thailand, were used in this experiment. The seed production of the parental lines and the hybrid followed the description of Robinson (2000) with hand emasculation and pollination techniques. The seed field situated at 14° 45'N, 100° 04'E. After harvesting from a commercial seed production field, the seeds had been stored at 5±1°C in darkness until seed protein extraction was conducted.

Protein extraction

Extraction of seed storage proteins was conducted as described in Onwimol *et al.* (2010). The distilled water was used as a protein extraction solvent. One gram of seeds of F1 hybrid and its parental lines were randomly selected by a seed divider. Seed was crushed in 1.5 ml microcentrifuge tube using sterilized metal beads and a grinding mill. The distilled water at the ratio of 5:2 (v/w) was then added and left at 25±2 °C for two hours before centrifuging at 10,000 rpm for five minutes. The supernatant was transferred to a new tube and again centrifuged at 10,000 rpm for five minutes-then supernatant was used for UTLIEF.

Ultrathin layer isoelectric focusing (UTLIEF)

The UTLIEF gels were prepared according to the flap technique described in section 8.8.5, chapter 8: Species and Variety Testing, of International Rules for Seed Testing (ISTA, 2012). The polymerization solution master mixed for 10 gels was prepared by mixing 50.0 ml acrylamide (6.8%T) with 1.60 g taurine, 50.0 µl N N N'N'-tetramethylenediamine (TEMED) and 350 µl of 20% (w/v) ammonium peroxydisulphate. A gel pH gradient was generated by adding 4.40 ml of 2-11ampholytes (SinuLyte™) into the polymerization solution. An aliquot of 6.5 ml polymerization solution was dropped on the Gel-Grip™ film (240 x 180 x 0.12 mm, Sinus, Germany). The cover glass plate with two adhesive tapes (0.15 mm in thickness) stick on both long sides as spacers was gently placed on top to evenly spread the aliquot over the film and allow it to polymerize for one hour at 25±2°C.

The iso-electro focusing (IEF) was carried out on an IEF-SYS™ horizontal electrophoresis unit (Scie-Plus™). The unit was connected to a cooling apparatus to cool the gel surface down to 8 °C. The anodal electrode was placed on the top and cathodal electrode was on the bottom of gel. The anode solution contained 0.33% (w/v) L-aspartic acid and 0.37% (w/v) L-glutamic acid while the cathode solution contained 0.47% (w/v) L-arginine, 0.36% (w/v) L-lysine and 12 (v/v) ethylenediamine. An application strip was placed on top of

gel surface at about 5 mm away down from the anode strip. The aliquot of 20 µl of each seed protein samples after diluted with distilled water to the dilution of 8:12 (v/v) were loaded into the respective wells of an application strip. The IEF was carried out for 90 minutes at 150 V/cm. The electricity was supplied by a power supply unit (Consort E833) which provided the current of 5 mA, 15W and 1,500 V.

After focusing, the gel that sticked on the surface of the Gel-Grip™ was fixed in 12% (W/V) trichloroacetic acid for 20 minutes, then stained in Coomassie Brilliant Blue solution (0.015% (w/v) Coomassie R250, 0.045% (w/v), Coomassie G250, 11% (v/v) acetic acid, 18% (v/v) ethanol and 71% (w/v) water) for 15 minutes and destained in the destaining solution (30% (v/v) ethanol, 5% acetic acid and 65% (v/v) water) for 10 minutes. After rinsing with water, the gel was air dried overnight at room temperature. The dried gel that stick on the surface of Gel-Grip™ was then covered with transparent adhesive film before interpretation of the electrophoretograms of seed protein would be evaluated following instruction of section 8.8.7.4 in International Rules for Seed Testing (ISTA, 2012).

RESULTS

The UTLIEF electrophoretograms were analyzed for evaluation of hybridity test. At least one unique protein band must be found in male protein band pattern (male marker band; MMB) and appear in the protein band pattern of respective hybrid seed thus its hybridity can be verified. The self-pollinated seed is verified for any individual hybrid seed that shares the common protein band pattern with its respective female parental line without any MMB. The out-crossing seed is verified for any individual hybrid seed that contains any alien protein band which is not belong to either respective male and female parental line.

None of self-pollinated seed and out-crossing seed was found in any F1 hybrid seed electrophoretogram. The number of MMB and female marker band (FMB) found in the ten pairs of male and female parental lines (CS01-CS10) tested in this study are shown in Table 1.

Table 1. Male and female marker bands that are found in each line of cucumber hybridity test *via* UTLIEF using water as protein extraction buffer.

Lines	CS01	CS02	CS03	CS04	CS05	CS06	CS07	CS08	CS09	CS10
Male marker bands (MMB)	1	2	0	1	1	2	1	1	2	0
Female marker bands (FMB)	0	1	1	4	1	1	1	1	0	0

Six of them contain both MMBs and FMBs and three contain either MMBs or FMB. These can be concluded that polymorphic protein bands between male and female parental lines are found in nine pairs except only CS10. Regarding the MMBs, they exist in eight male parental lines namely; CS01, CS02, CS04, CS05, CS06, CS07, CS08, and CS09. Five of them namely CS01, CS04, CS05, CS07, and CS08 contained only one MMB (Figure 1) while three namely CS02, CS06 and CS09 contained two MMBs (Figure 2). Only CS03 and CS10 parental lines that their protein band patterns contained none of MMB when water was used as protein extraction solution. However, the hybridity test of CS03 was successfully accomplished in the later retest of UTLIEF using phosphate buffer solution as the protein extract solvent (data not shown). In addition, the most polymorphic bands were also found in the high pH range around 6-10. The example of UTLIEF electrophoretograms are shown in Figure 1 and 2.

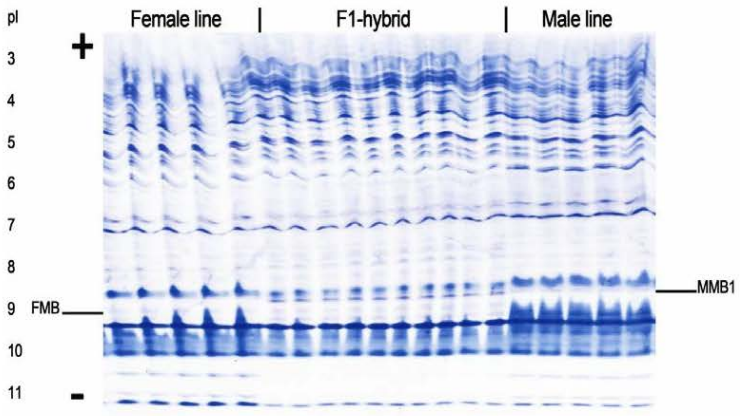

Figure 1. The UTLIEF electrophoretogram of water extracted seed proteins of CS05 cross carried out on gel with pH gradient of 2-11 (FMB = female marker band, MMB = male marker band, + = anode, - = cathode).

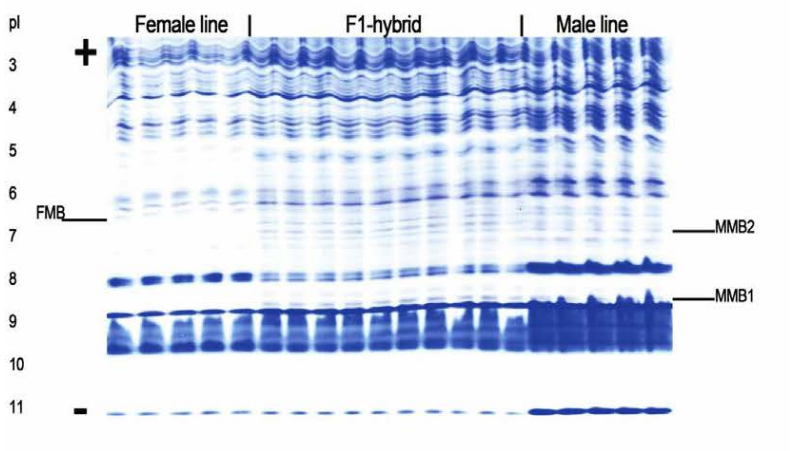

Figure 2. The UTLIEF electrophoretogram of water extracted seed proteins of CS02 cross carried out on gel with pH gradient of 2-11 (FMB = female marker band, MMB = male marker band, + = anode, - = cathode).

DISCUSSION

The major protein in cucumber seed is the salt-soluble protein globulins (Vickery *et al*., 1941). Interestingly, the result from this study showed that the polymorphic of albumin proteins, the minor and water soluble storage protein in cucumber seed, between the male and female parent lines can be found in nine crosses or 90% of tested crosses. Albumins is mostly found in food reserving tissues parts in seeds such as endosperm in monocotyledon or cotyledon in dicotyledonous species (Shimada *et al*., 2003). Degtyarenko *et al*. (1986) reported the success of varietal identification of 20 cucumber varieties by using electrophoresis to separate albumin fractions. This result confirms the high efficiency of water as the protein extract solvent to discriminate cucumber seed proteins reported by Onwimol *et al*. (2010). Only one cross, CS10, that the difference in albumins between their parental lines was not found indicating the genetically different proteins, if there is any, should belong to other protein groups and the different extract solvents is required as reported by Tu *et al*. (2012) and Yan (2013a). Eight pairs crosses of F1 hybrid seeds and their parent lines or 80% of samples in this study were successfully identified by this technique using water as the protein extract solvent. This result shows the possibility and efficiency of water as the protein extract solvent for cucumber hybridity test *via* UTLIEF technique. Water is the most convenient, safe, and cheapest solvent compare with the other solvents. Therefore, it should be the first choice of protein extract solvent when the cucumber hybridity test is conducted using UTLIEF technique.

In this study, the marker proteins were found to be focused in the gel pH range of 6-10. This gel pH range found in agreement with Tu *et al.* (2012) who reported that polymorphic protein bands of cucurbit seeds occurred at gel pH of 6-9 in varietal identification using UTLIEF technique. Considering porous size of polyacrylamide gels and position of pI, these polymorphic seed proteins in cucumber should be 24–205 kilo Daltons and were acidic proteins (Biosciences, 1999). The successful attempt to verify hybridity of CS03 with their parental lines by changing solvent from water to phosphate buffer solution as the protein extract solvent before carrying out UTLIEF in this study indicated that the polymorphic of proteins of this cross might be in the salt-soluble protein globulins. This reveals that the UTLIEF technique is still efficiency for the hybridity test of this cross if only suitable extraction buffer is chosen.

Thailand exported approximately 105.47 tons of cucumber seeds in 2011 which more than 90% of it was hybrid varieties and was worth around 9.3 million US dollar (FAOSTAT, 2014). Hence, the effective hybridity test is extremely essential for Thailand seed industry. In this study, we have shown that the UTLIEF technique using water as the protein extract solvent is the cost effective and reliable technique for cucumber hybridity test.

CONCLUSION

For hybridity test of cucumber seed, the success of UTLIEF technique using 2-chroloethanol as the seed proteins extract solvent was reported in the past. However, this method is not applicable in Thailand. Therefore, we attempted to use water as extract solvent which is cheaper and safer to work with. The UTLIEF using water as seed protein extract solvent and the gel pH gradients of 2-11 were carried out. The results revealed that this condition was successful for verifying F1 hybrid seed and their parents eight pairs out of 10. This suggests that the water is the promising seed protein extract for carrying out UTLIEF for cucumber seed hybridity test. This application can help breeders and seed producers to verify the hybrid purity more efficiency in term of cost and safety.

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