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DROUGHT TOLERANCE SCREENING IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

Khamsee Saensee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Biology

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DROUGHT TOLERANCE SCREENING IN SUNFLOWER

(*HELIANTHUS ANNUUS* L.)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สภาวะแล้งเป็นปัจจัยสำคัญหนึ่งที่จำกัดการเจริญเติบโตและผลผลิตของทานตะวันในเขตแห้งแล้งและค่อนข้างแห้งแล้ง ดังนั้นการพัฒนาพันธุ์ที่ทนต่อสภาวะแล้งจึงจำเป็นต่อการรักษาระดับผลผลิตไม่ให้ต่ำลงเมื่อพืชขาดน้ำและการเพิ่มพื้นที่การเกษตรไปยังบริเวณที่มักให้ผลผลิตต่ำกว่าปกติ วัตถุประสงค์ของงานวิจัยนี้ เพื่อประเมินความทนแล้งของทานตะวันจำนวนหลายจีโนไทป์ในระยะเริ่มงอก และระยะต้นกล้าภายใต้สภาพแวดล้อมที่ควบคุม ทั้งวิธีเพาะในจานแก้ว (Petri dish bioassay) และการปลูกแบบไร้ดิน (hydroponic culture) โดยใช้โพลีเอทิลีน ไกลคอล (PEG-6000) เป็นสารลดการแพร่ผ่านของน้ำ (osmoticum) การทดลองโดยวิธีเพาะในจานแก้ว ใช้ทานตะวัน 8 จีโนไทป์ ได้แก่ สุรนารี 471 สุรนารี 473 สุรนารี 475 แปซิฟิค 77 5A 6A 9A และ 10A นำมาทดลองภายใต้สภาพแวดล้อมที่ควบคุมโดยกำหนดสภาวะแล้งไว้ 3 ระดับ คือ 0% PEG (control) 10% PEG (-0.6 MPa) และ 20% PEG (-1.2 MPa) ผลการทดลองนี้พบว่า ลักษณะการเจริญเติบโตและสรีรวิทยาที่ศึกษาลดลงอย่างมีนัยสำคัญในทุกจีโนไทป์ ค่าดัชนีการงอก (GSI) ลดลงภายใต้สภาวะแล้งและไม่พบการงอกในทานตะวันสายพันธุ์ 5A 6A และ 10A ที่ระดับความเข้มข้น 20% PEG หลังจากการงอก 14 วัน พบว่าค่าดัชนีของความสูง (PHSI) ค่าดัชนีของความยาวราก (RLSI) ค่าดัชนีของน้ำหนักแห้ง (DMSI) และค่าดัชนีของน้ำในต้นกล้า (RWCSI) ลดลงอย่างมีนัยสำคัญ ($p < 0.05$) ตามความเข้มข้นของ PEG ที่สูงขึ้น ผลการคัดเลือกทานตะวันทนแล้งด้วยวิธี clustering และ ranking สามารถแบ่งทานตะวันได้ดังนี้ สุรนารี 473 สุรนารี 471 สุรนารี 475 และแปซิฟิค 77 เป็นกลุ่มทนแล้ง ในขณะที่จีโนไทป์ 5A 6A 9A และ 10A อยู่ในกลุ่มที่ไวต่อสภาวะแล้ง

การศึกษาในระบบไร้ดิน โดยการเพาะเลี้ยงต้นกล้าทานตะวันอายุ 14 วัน จำนวน 11 จีโนไทป์ ได้แก่ สุรนารี 471 สุรนารี 473 สุรนารี 475 แปซิฟิค 77 5A 6A 9A 10A เชียงใหม่ 1 HA429 และ HA430 ในสภาวะขาดน้ำ (10% PEG-6000) เป็นระยะเวลา 7 วัน ผลการทดลองพบว่าค่าดัชนีความสูง (PHSI) ค่าดัชนีความยาวของราก (RLSI) ค่าดัชนีน้ำหนักแห้ง (DMSI) ค่าดัชนีปริมาณน้ำสัมพันธ์ (RWCSI) ค่าดัชนีการคายน้ำ (SCSI) และค่าดัชนีจำนวนใบ (LNSI) ลดลงอย่างมีนัยสำคัญ ($p < 0.05$) ในสภาวะแล้ง ในทางตรงกันข้าม ลักษณะทางชีวเคมี ได้แก่ ปริมาณโพรลีน ปริมาณมาโลดิออลดิไฮด์ (MDA) ไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) และเอนไซม์ต้านอนุมูลอิสระ เช่น ซูเพอร์ออกไซด์ดิสมิวเทส (SOD) คะทะเลส (CAT) และแอสคอร์เบตเปอร์ออกซิเดส (APX)

มีค่าเพิ่มขึ้นในทานตะวันทุกจีโนไทป์เมื่ออยู่ภายใต้สภาวะแล้ง เมื่อทำการคัดเลือกทานตะวันทนแล้ง ด้วยวิธี clustering และ ranking โดยใช้ลักษณะสัณฐานวิทยา และสรีรวิทยาสามารถจำแนกทานตะวันออกเป็น 2 กลุ่ม คือ กลุ่มทนแล้ง ได้แก่ สุรนารี 473 สุรนารี 471 สุรนารี 475 และแปซิฟิค 77 และกลุ่มที่ไวต่อสภาวะแล้งได้แก่ 5A 6A 9A 10A เชียงใหม่ 1 HA429 และ HA430 ซึ่งผลการจัดกลุ่มที่ได้คล้ายกับผลการทดลองก่อนหน้านี้ เมื่อพิจารณาทุกดัชนีเป็นที่ประจักษ์ว่าทานตะวันพันธุ์สังเคราะห์ ได้แก่ สุรนารี 471 สุรนารี 473 สุรนารี 475 สามารถทนแล้งได้ดีกว่าพันธุ์ลูกผสมที่มีจำหน่ายในท้องตลาด (แปซิฟิค 77) และทุกจีโนไทป์ที่ทดลอง ดังนั้นจึงควรเป็นพันธุ์แนะนำสำหรับการเพาะปลูกในสภาพที่มีความแห้งแล้งและในการปรับปรุงพันธุ์ทานตะวันในอนาคต

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

KHAMSEE SAENSEE : DROUGHT TOLERANCE SCREENING IN SUNFLOWER (*HELIANTHUS ANNUUS* L.). THESIS ADVISOR : ASST. PROF. NOODUAN MUANGSAN, Ph.D. 132 PP.

ANTIOXIDANT ENZYME/ DROUGHT STRESS/ DROUGHT TOLERANCE/ HYDROPONIC CULTURE/ SUNFLOWER

Drought is one of the most significant factors restricting sunflower growth and productivity in arid and semi-arid regions. Development of drought-tolerant varieties is therefore required for maintaining yields under water deficit conditions and for the extension of agriculture to sub-optimal cropping areas. The objective of this work was to evaluate sunflower genotypes for drought tolerance at germination and seedling growth stages under laboratory conditions either in Petri dish bioassays or in hydroponic conditions using polyethylene glycol (PEG-6000) as an osmoticum. In Petri dish bioassays, eight sunflower genotypes S471, S473, S475, Pacific 77, 5A, 6A, 9A, and 10A were tested under controlled environments at three stress levels 0% PEG (control), 10% PEG (-0.6 MPa) and 20% (-1.2 MPa). Results of this experiment showed significant reduction in most studied traits among sunflower genotypes. Germination stress index (GSI) decreased under drought stress and no germination was observed in sunflower lines 5A, 6A and 10A at 20% PEG concentration. At 14-days after sowing (DAS), plant height stress index (PHSI), root length stress index (RLSI), dry matter stress index (DMSI) and relative water content stress index (RWCSI) were reduced significantly ($p < 0.05$) with increment of drought stress levels. Screening of drought tolerant genotypes using clustering and ranking methods

discriminated genotypes: S473, S471, S475 and Pacific 77 as the drought tolerant genotypes, while 5A, 6A, 9A and 10 A as the drought sensitive genotypes.

In hydroponic conditions, 14 day-old seedlings of 11 sunflower genotypes were subjected to drought stress (10% PEG-6000) and non-stress treatment (control) for 7 days. Results showed that the PHSI, RLSI, DMSI, RWCSI, stomatal conductance index (SCSI) and leaf number stress index (LNSI) were reduced under drought stress. In contrast, biochemical characters including proline, malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and antioxidant enzyme activities, i.e. superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) increased in all genotypes under drought stress. Similar to former experiments, screening drought tolerant genotypes using clustering and ranking methods based on morpho-physiological parameters discriminated genotypes: S473, S471, S475 and Pacific 77 as the drought tolerant genotypes, while 5A, 6A, 9A, 10A, CM1, HA429 and HA430 as the drought sensitive. In consideration to all indices, synthetic varieties: S473, S471 and S475 obviously showed greater drought tolerance than selected commercial variety (Pacific 77) and other tested genotypes, and therefore are recommended for planting and drought improvement program in the future.

School of Biology

Academic Year 2012

Student's Signature_____

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Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

%	=	percentage
APX	=	ascorbate peroxidase
APXSI	=	ascorbate peroxide stress index
CAT	=	catalase
CATSI	=	catalase stress index
^{o}C	=	degree Celsius
cm	=	centimeter
DW	=	dry weight
DAS	=	days after sowing
DWi	=	dry weight before
DWf	=	dry weight after
DMSI	=	dry matter stress index
FW	=	fresh weight
GSI	=	germination stress index
g	=	gram
H_2O_2	=	hydrogen peroxide
H_2O_2SI	=	hydrogen peroxide stress index
MDA	=	malodialdehyde
MDASI	=	malodialdehyde stress index
mL	=	milliliter
mg	=	milligram

LIST OF ABBREVIATIONS (Continued)

mM	=	millimolar
MPa	=	megapascal
nm	=	nanometer
O_2^1	=	singlet oxygen
O_2^-	=	superoxide anion
$OH^\bullet$	=	hydrogyl radical
PHSI	=	plant height stress index
PI	=	promptness index
PISS	=	promptness index of stressed seed
PICS	=	promptness index of control seed
PROSI	=	proline stress index
R	=	reproductive stage
RGRSI	=	relative growth rate stress index
ROSs	=	reactive oxygen species
rpm	=	revolutions per minute
RLSI	=	root length stress index
RWCSI	=	relative water content stress index
SM	=	saturated mass
SOD	=	superoxide dismutase
SODSI	=	superoxide dismutase stress index
TW	=	turgid weight
V	=	vegetative emergence

LIST OF ABBREVIATIONS (Continued)

VE	=	vegetative stage
W	=	watt
w/v	=	weight by volume
μL	=	micro liter

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Sunflower (*Helianthus annuus* L.) is one of the world's largest oilseed crops (Paniego *et al*., 2002). It is an annual plant native of Northern America. There are two types of sunflower: confectionary and oilseed types. The first type has striped hull, large seed, and an oil content of 200-300 g/kg. The second type has black seed coat, smaller seed, and an oil content of 400-500 g/kg (Tang *et al*., 2006). The kernel is composed of 67% oil, 21% protein, 3% fiber, 6% N-free extract and 3% ash. Typical sunflower oil is composed of 66-72% linoleic acid, 12% saturated acid (palmitic acid and stearic acid), 16-20% oleic acid, and less than 1% alpha-linoleic acid. Sunflower oil also contains antioxidants such as alpha-tocopherol and ascorbic acid, indicating that the oil also functions as free radical scavenger (Grompone, 2005).

Today, high amount of sunflower seeds is needed for consumers worldwide. In 2008-2009, seed production was about 33.3 million tons and accounted around 8.5% of the total oilseed production of the world (Vanaja *et al*., 2011). England consumes sunflower oil about 45% of demand. Sunflower oil is considered to be a premium cooking oil and is valued for its light taste, frying performance and health benefits because of its high content of monounsaturated and polyunsaturated fat. In industry, the oil is also used to make soap, candles, varnishes, paint, lubricant, and beauty moisturizer because of its vitamin E content that prevents drying of skin. Moreover,

the oil is used as biodiesel fuel, and sunflower meal for livestock. The dried stem makes an excellent fuel and paper.

Sunflower is well grown in central, northern and northeastern provinces of Thailand such as Lop Buri, Saraburi, Nakhon Sawan, Phetchabun, Uthai Thani, Uttaradit, Ratchaburi, Kanchanaburi, Kam Phaeng Phet, Chiang Rai, Phayao, Sa Kaeo and Nakhon Ratchasima. However, Lop Buri and Saraburi are the most famous sites. In Thailand, sunflower seeds are needed for oil extraction more than 100,000 tons per year, but farmers can produce the seeds only 20% of demand (Boomrung, 2009). The reasons for this are expensive price of seeds, seed rot disease, lack of seeds, and environmental stress factors such as drought and plant diseases.

Drought is one of the most important factors limiting the survival and growth of plants. Generally, drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation. When the plants lose water and hence turgor pressure, cells are flaccid. The cells in this condition wilt. Further water loss causes plants to shrink and die.

Drought limits crop yields. The responses of sunflower due to water stress vary greatly depending on varieties. Water deficit at any stages of sunflower development has a negative impact on sunflower yield, but severity of the drought effect depends on the sunflower development stages. Moreover, water deficit affects greatly seed quality at the flowering stage. Seed sowing generally is considered as the first critical and most sensitive stage in the life cycle of plants. Water deficit not only affects seed germination, but also increases mean germination time in crop plants (Willenborb *et al*., 2004). Schutz and Milberg (1997) has reported that germination is regulated by the amount of moisture in the medium. Mild water deficits can cause a reduction of plant turgor, or losses of 1-15% of plant water content, resulting in large changes in

growth and metabolism but rarely cause plant death unless these conditions persist for long period of time.

This study aimed to screen for drought tolerance of sunflower genotypes subjected to drought stress created with polyethylene glycol (PEG-6000). Morphological, physiological and biochemical responses due to drought stress were investigated at germination and seedling growth stages. A better understanding of the morphological, physiological, and biochemical basis of changes in drought stress tolerance could be used to select or create new genotypes of sunflower having a better productivity under drought stress conditions.

1.2 Research objectives

1.2.1 To investigate morphological and physiological responses among sunflower genotypes subjected to drought stress at germination and establishment of seedling growth stage in Petri dish bioassays.

1.2.2 To investigate morphological, physiological and biochemical responses among sunflower genotypes subjected to drought stress at seedling growth stage in hydroponic culture.

1.2.3 To evaluate sunflower genotypes for drought tolerance in laboratory conditions.

1.3 Research hypothesis

Drought stress is one of abiotic stresses, affecting plant growth and yield. Responses of plants to drought stress vary depending on genotypes and environmental conditions. Extreme drought is found in many agricultural areas. Plants exhibit responses to drought stress at molecular, cellular, and whole part levels. The research hypothesis was that drought tolerance genotypes could be selected at germination and seedling growth stages by the laboratory screening methods based on morphological, physiological, and/or biochemical characteristics.

1.4 Scope and limitation of the study

In this research study, up to 11 sunflower genotypes were used and tested against drought stress at germination and seedling growth stages under laboratory conditions either in Petri dish bioassays or in hydroponic conditions. Polyethylene glycol-6000 (PEG-6000) was used as a drought stimulator. Morphological, physiological and biochemical parameters were used to evaluate the genotypic response to induced drought stress and to determine for drought tolerance.

1.5 Expected results

The expected results from this study were:

1.5.1 Increase our understandings about sunflower responses to drought stress.

1.5.2 Morphological, physiological and/or biochemical parameters could be used to identify tolerance genotypes of sunflower to drought stress.

1.5.3 Better understanding of drought tolerance mechanisms that may be useful for sunflower genetic improvement program.

CHAPTER II

LITERATURE REVIEW

2.1 Morphology and general characteristics of sunflower

Sunflower (*Helianthus annuus* L.) is an annual plant, belonging to family Asteraceae (Compositae), and is one of the world's most important oilseed crops (Mukhtar, 2009). The genus *Helianthus* comprises about 68 species (Grompone, 2005), which includes the annual and the polyploidy perennial. Nonetheless, only annual species are crossed with the cultivated sunflowers. Sunflower is the most important source of edible oil after soybean, rapeseed and peanut, with a worldwide seed production of 33.3 million tons destined almost exclusively to oil extraction, providing 8.5% of total world volume (Vanaja *et al*., 2011).

Characteristics of sunflowers are tall shrubs, with the height between 1.5-2.5 m at flowering stage. There is a single stalk topped by a large flower. Leaves are simple, large, dark green and roughly heart shaped. Arrangement of leaves in seedling stage is opposite and in young plant is alternate. The stems are typically unbranched. The flower head (capitulum) typically has a maximum diameter of 15-30 cm.

The flower head is composed of numerous florets crowded together. The outer florets are the sterile ray florets and can be yellow, red, orange, or other colors. The heads are radiate and the ray flowers are pistillate. They are usually large and yellow, orange to reddish (Figure 2.1). Sunflowers have a type of phototropic response called heliotropism; the young flower head follows the sun. The movements become

response and when plants are rotated 180 degrees, the old response pattern is still followed for a few days. The achene (fruit) of sunflower consists of a seed, often called the kernel, and adhering pericarp, usually called the hull (Moghadasi, 2011).

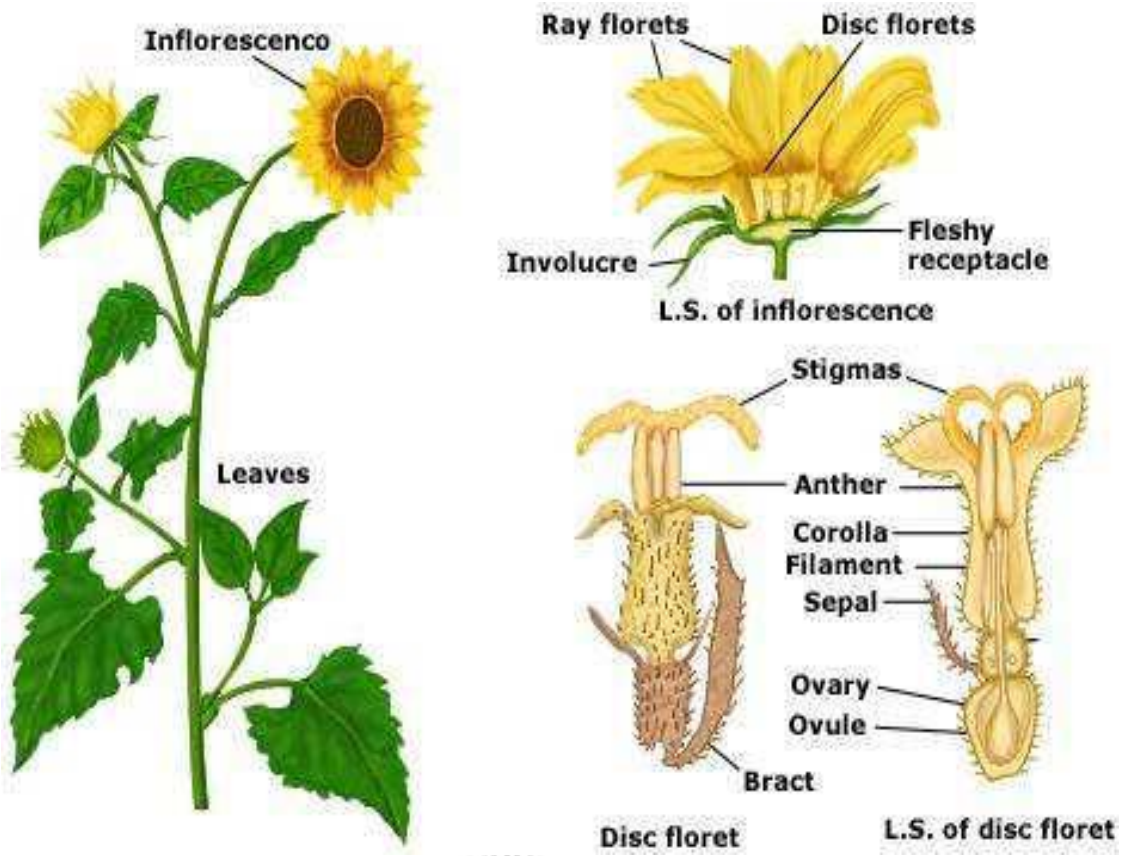

Figure 2.1 Sunflower plant. Source; Tutorvista, 2010.

2.2 Sunflower varieties

There are 3 types of sunflower varieties: Open-pollinated oil, hybrid and synthetic types (Boomrung, 2009).

2.2.1 Open-pollinated oil sunflower varieties are the traditional varieties which have been grown and selected for their desirable traits for millennia. They grow well without high inputs because they have been selected under organic condition. These varieties are hardier, have better flavor and more flexible than hybrid varieties.

2.2.2 Hybrid sunflower varieties are the first generation offspring of two distant and distinct parental lines of the same species. Seeds taken from a hybrid may either be sterile and expressing the desired traits of the parent. Hybrid seed is highly responsive seed. The seeds require more fertilizer, herbicides, pesticides, and water compared with all varieties. Some examples of this sunflower of type are Pacific 77, Pacific 44 and Hysun 33.

2.2.3 Synthetic sunflower varieties are developed by intercrossing a number of genotypes of known superior combining ability or genotypes that are known to give superior hybrid performance. When crossed in all combinations, synthetic varieties are known for their hybrid vigor and for their ability to produce usable seeds for succeeding season such as Suranaree 473, Suranaree 471, Suranaree 475 and Chiang Mai 1.

2.3 Nutritional value of sunflower seeds

Besides saturated fatty acids, sunflower seed is composed of unsaturated fatty acid (linoleic acid and oleic acid), tryptophan (amino acid), fiber and vitamin E as an antioxidant that may protect against heart disease by getting rid of harmful molecules called free radicals that can cause arteriosclerosis. It also contains other vitamins such as thiamine (vitamin B1) and pantothenic (vitamin B5), and minerals such as copper, manganese, potassium, magnesium, iron, phosphorus, selenium, calcium, and zinc. In addition, sunflower seed has low level of cholesterol (Table 2.1).

Sunflower oil contains more unsaturated fatty acid, and linoleic acid compared to other kinds of crop oil. It is found that sunflower seed oil has 83% of unsaturated fatty acid, which is less than safflower oil (87%) and corn oil (84%) (Table 2.2).

Partly dehulled sunflower meal has been substituted successfully for soybean meal in isonitrogenous diets for ruminant animals, as well as for swine and poultry feeding. Sunflower meal is higher in fiber, has a lower energy value and is lower in lysine but higher in methionine than soybean meal. Protein percentage of sunflower meal ranges from 28% for non-dehulled seeds to 42% for completely dehulled seeds (Grompone, 2005).

Table 2.1 Nutrient composition of sunflower kernel.

	Nutrient Composition per 28.35 grams
Calories	160 Joule
Calories from fat	129 Joule
Total fat	14 g
Saturated fat	2 g
Polyunsaturated fat	9 g
Monounsaturated fat	3 g
Cholesterol	0 mg
Sodium	< 2 mg
Total carbohydrate	5 g
Dietary fiber	4 g
Sugars	1 g
Protein	6 g
Vitamin A	< 3 IU
Vitamin C	< 0.2 mg
Calcium	33 mg
Iron	1.9 mg
Vitamin E	11.34 mg
Thiamine	0.65 mg
Riboflavin	0.07 mg
Niacin	1.3 mg
Folate	64.5 mcg
Magnesium	104 mg
Zinc	1.4 mg
Copper	0.4 mg

Source; National Sunflower Association, 2011.

Table 2.2 Fatty acid percentage in oil crop plants.

Items	Unsaturated fatty acid	Saturated fatty acid	Linoleic acid
Safflower oil	87	8	72
Corn oil	84	10	53
Sunflower seed oil	83	12	63
Soybean oil	80	15	52
Rice oil	80	16	37
Sesame oil	80	14	42
Cotton seed oil	71	25	50
Peanut oil	76	18	29
Palm oil	49	45	8

Source; Department of Agriculture, 2009.

2.4 Seeds and germination of sunflower

The sunflower seed is a fruit containing a single seed. The seed coat consists of the ovary wall and testa fused together. In all respects, this acts in the same way as the testa of the other seeds. The seeds are the primary ingredient in all sunflower seed products. They are generally a quarter inch long and one eighth of an inch wide. They have a black seed coat with dark or grey stripes. The coat, or hull, surrounds a small kernel, which is composed of protein and lipid.

The sunflower's germination differs from broad bean and pea because, once the radicle has emerged (Figure 2.2), it is the hypocotyl which elongates. This has the effect of either dragging the cotyledons from the fruit wall or carrying the entire fruit above soil. Sunflower germination is restricted at water potentials between -1.1 to -1.5 MPa, and is completely inhibited below -1.0 MPa (Somers *et al*., 1983). Priming of seeds by soaking in water or various solutions followed by drying before sowing also can improve germination (Smok *et al*., 1992). As the seedlings emerge from the soil,

they are already beginning to make chlorophyll and turn green. The cotyledons and apical hook unfold as the plant emerges into the light.

Figure 2.2 Sunflower seedlings.

2.5 Stages of sunflower development

The total time required for a sunflower to develop in all stages depends on the genetic background of the plant and the environmental growing condition (Schneiter and Miller, 1981). The same system can be used for classifying either a single head or branched sunflower. In stages R7 through R9, healthy, disease free heads are used to determine plant development (Table 2.3).

The development stages and associated crop management inputs of sunflower are shown in Figure 2.3. The first stage is germination and establishment of seedling, the second stage is leaf development, the third stage is flower bud stage, and the fourth is seed developmental stage.

Table 2.3 Growth stages of sunflower.

Stages	Description
VE	Seedling has emerged and the first leaf beyond the cotyledons is less than 4 cm long.
V (number) (i.e. V-1, V-2, V-3, etc.)	These are determined by counting the number of true leaves at least 4 cm in length beginning as V-1, V-2, V-3, V-4, etc. If senescence of the lower leaves has occurred count leaf scars (excluding those where the cotyledons were attached) to determine the proper stage.
R-1	The terminal bud forms a miniature floral head rather than a cluster of leaves. When viewed from directly above the immature bracts form a many-pointed star-like appearance.
R-2	The immature bud elongates 0.5 to 2.0 cm above the nearest leaf attached to the stem. Disregard leaves attached directly to the back of the bud.
R-3	The immature bud elongates more than 2.0 cm above the nearest leaf.
R-4	The inflorescence begins to open. When viewed from directly above immature ray flowers are visible.
R-5 (decimal) (i.e. R-5.1, R-5.2, R-5.3, R-5.4,R-5.5 through R-5.9, etc.)	This stage is the beginning of flowering. The stage can be divided into sub stages dependent upon the percent of the head area (disk flowers) that has completed or is in flowering. Ex. R-5.3 (30%), R-5.8 (80%) etc.
R-6	Flowering is complete and the ray flowers are wilting.
R-7	The back of the head has started to turn a pale yellow color.
R-8	The back of the head is yellow but the bracts remain green.
R-9	The bracts become yellow and brown. This stage is regarded as physiological maturity.

Source; Schneiter and Miller, 1981.

Remark; VE: Vegetative emergence, V: Vegetative stages, R: Reproductive stages.

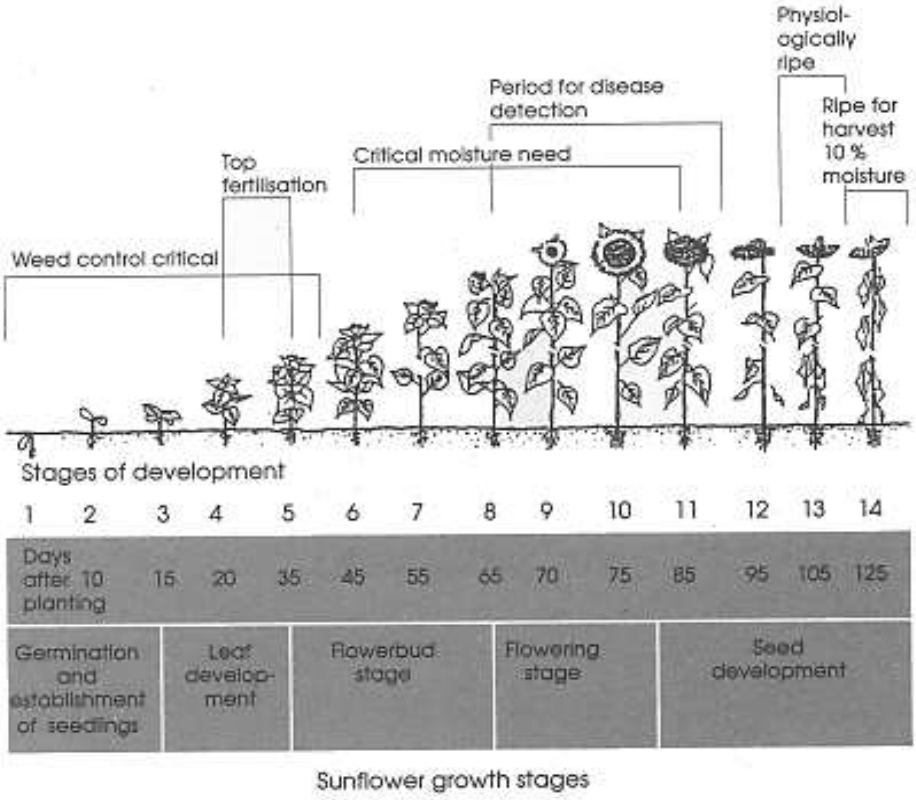

Figure 2.3 A schematic representation of sunflower developmental stages.

Source; Agriculture Environmental Affairs and Rulal Department, 2005.

2.6 Sunflower cultivation in Thailand

In Thailand, sunflower cultivation started in 1988, about 1,200 ha, and produced 670 tons of seed yield. Later this crop increased about 20 folds to 21,000 ha in 1994 with the seed production of 8,548 tons. Sunflower is not a new crop in Thailand as it has been grown for birdseed and confectionery for more than three decades (Laosuwan, 1997).

Planting time of sunflower before harvesting is about 90-100 days. It is more tolerance to drought stress when compared to other crop plants. It needs water about 300-400 m^3 per rai. The cultivation places in Thailand are Lop Buri province (55%), Saraburi province (17%), Nakhon Sawan province (14%), and Phetchabun province

(13%) (Boomrung, 2009).

Thailand sunflower breeding program started in 1973 by importing the seeds of 18 varieties from abroad and first cultivation was taken in Maha Sarakham and Chiang Mai provinces. However, output level was not satisfactory (Laosuwan, 1997). The farmers wanted hybrid cultivars because they could self-pollinate and give high yield, but the disadvantage is an expensive seed price, which makes sunflower farming not worthwhile at that time.

Still, Thailand has to import sunflower seeds to meet the demand in the country Figure 2.4 shows the quantity and value of imported sunflower seeds. For the production of seed in the country, although the prices of the seeds and seed oil have increased, the production capacity tends to stay the same (Table 2.4).

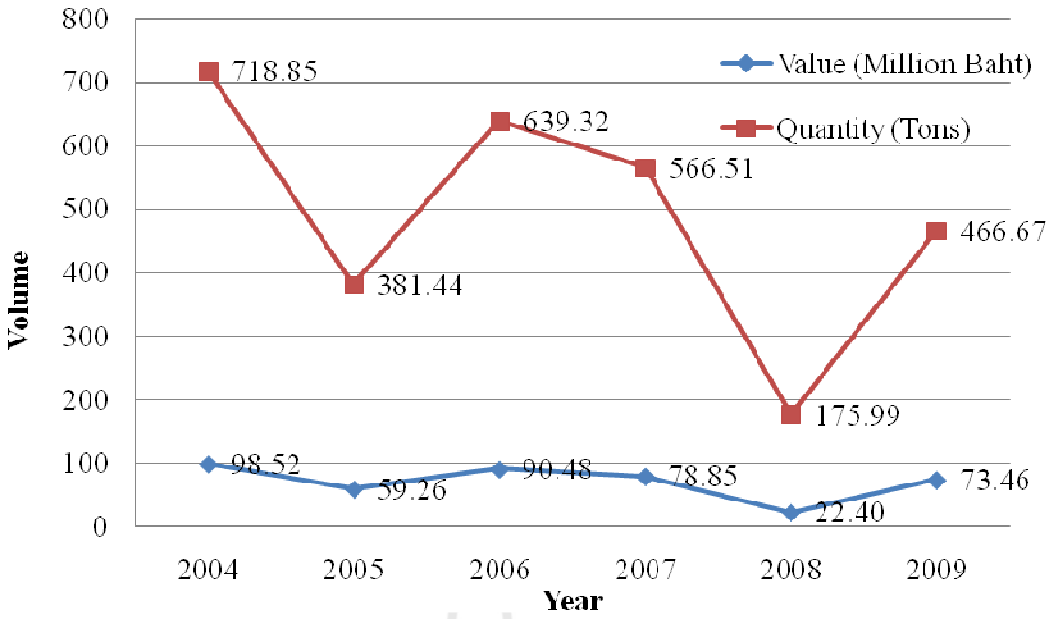

Figure 2.4 Quantity and value of the imported sunflower seeds between 2004 and 2009. Source; Office of Agricultural Economic, 2009.

Table 2.4 Seed price of sunflower for 2005-2010.

Productivity quantity	Years					
	2004-2005	2005-2006	2006-2007	2007-2008	2008-2009	2009-2010
Output quantity: 1000xTons	22,089	33,176	24,229	22,999	22,565	22,316
Seed price at marketing (baht/kg)	11.28	12.12	8.72	11.38	18.64	18.49
Seed price at the extraction factory	11.52	11.38	10.61	12.28	19.07	18.79
Price of pure oilseed at Bangkok (baht/litter)	45.30	45.13	43.75	45.01	67.87	83.13

Source; Department of Internal Trade, 2010.

2.7 Drought stress

Drought is a period or condition of unusually dry weather within a geographic area where rainfall is normally present. During a drought period there is a lack of precipitation. However, its characteristics vary significantly from one region to another. Drought produces a large number of impacts that affect the social, environmental, and economical standards of living. Its effects spread far beyond the physical effects of drought itself. Some direct impacts of drought are reduced crop, rangeland, and forest productivity, reduced water levels, increased fire hazard, increased livestock and wildlife death rates, and damage to wildlife and fish habitat. A reduction in crop productivity usually results in less income for farmers, increased prices of food, unemployment, and migration.

Plants are immobile, which means they cannot select their own environment; thus, they have evolved a wide range of unique and sophisticated survival strategies to cope with drought stress. Under drought stress, plant hormones such as ethylene (ET), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) are endogenously produced. Low-molecular-weight molecules primarily regulate protective responses against drought stress through synergistic and antagonistic action. In addition, plants produce reactive oxygen species (ROS) as a key link to drought stress signaling (Apel and Hirt, 2004).

Drought stress induces a signal transduction resulting in accumulation of proteins protecting the plants from drought stress (Muoma *et al*., 2010) as shown in Figure 2.5. To achieve the understanding of this series of coordinated molecular reactions, studies of genomics, proteomics and transcriptomics must work in a concerted effort to provide information which leads to the solution to drought stress.

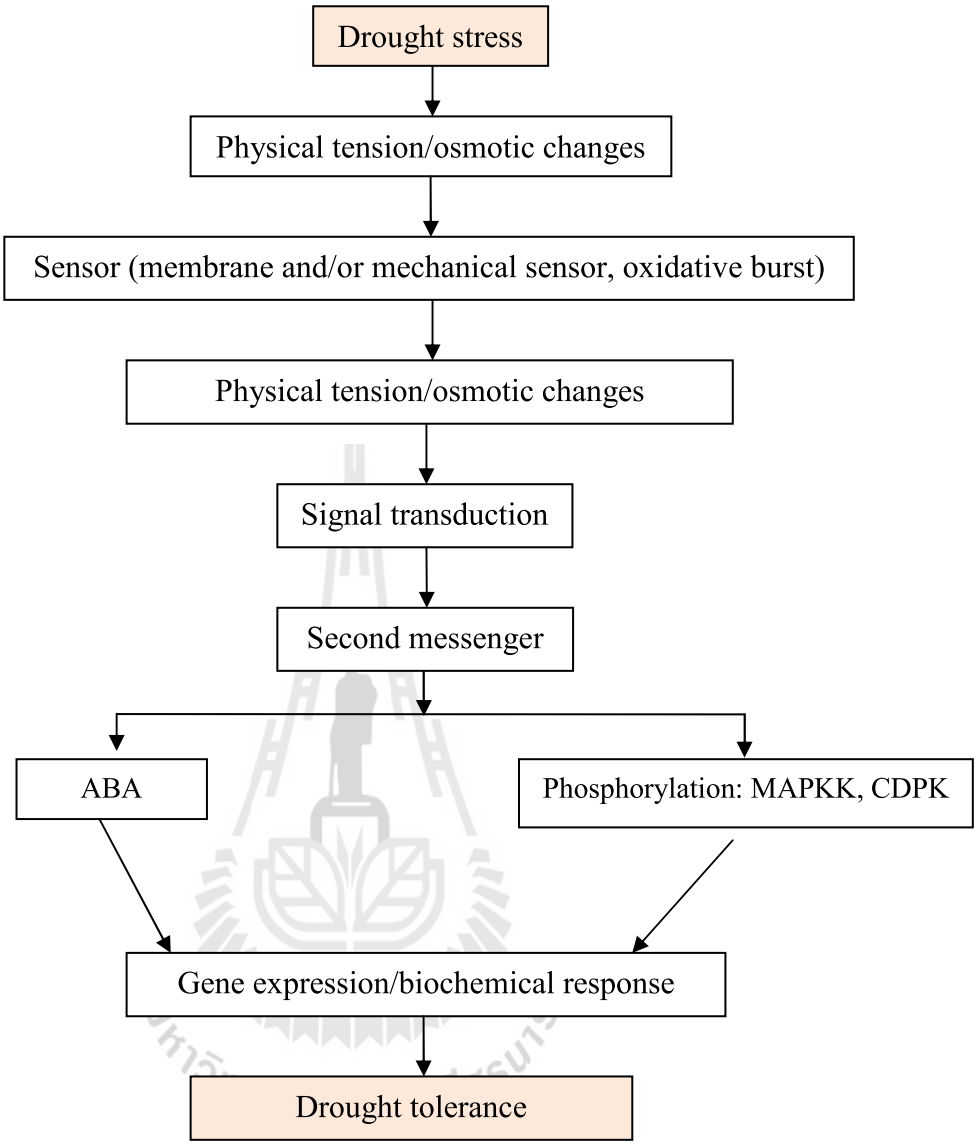

Figure 2.5 Drought stress and signal transduction leading to drought stress tolerance in plants. Source; Muoma *et al*., 2010.

2.8 Effects of drought stress on plants

Drought stress has a marked adverse effect on morphological, physiological, biochemical and molecular changes.

2.8.1 Morphological and physiological changes

Plants are exposed to a variety of biotic or abiotic stresses, such as disease, drought, salt and freezing that influence their development, growth and productivity. Water is vital for plant growth, development and productivity. Permanent or temporary water deficit limits the growth and distribution of natural and artificial vegetation and the performance of cultivated crop plants more than any other environmental factors (Kamel and Loser, 1995).

2.8.1.1 Plant growth

Common effects of drought stress on sunflower morphology include the decrease in plant height, leaf area, head diameter, yield per plant, dry weight, and plant biomass weight per hectare due to water deficit. However, some sunflower varieties showed better tolerance to stress than others (Tahir *et al*., 2002; Khalilvand *et al*., 2007). Moreover, the early root and hypocotyl growth and root branching pattern in sunflower seedling hybrids were significantly influenced by water deficit (Seiler, 2007). The stem length was decreased under drought conditions in soybean, citrus and potato (Specht *et al*., 2001). The reduction in plant height was associated with a decline in the cell enlargement and more leaf senescence in *Abelmoschus esculentus* under water stress (Bhatt and Rao, 2005).

Effects of drought stress imposed at germination stage in laboratory have been reported. The germination stress index (GSI), plant height stress index (PHSI),

root length stress index (RLSI), and dry matter stress index (DMSI) were used to evaluate phenotypic response to stress in sunflower. RLSI was observed in all sunflowers classified as drought tolerance. GSI, PHSI and DMSI decreased in drought sensitive varieties (Ahmad *et al*., 2009).

2.8.1.2 Leaf number

Drought stress affects morphological characters of plants. It induces plant adaption to reduce the loss of water vapor, by inhibiting the differentiation of leaf buds; leading to reduction in leaf number. It has been reported that reduced plant height, leaf weight, leaf area and leaf number at flowering stage were observed in sunflower (Hussain *et al.*, 1994; Taheri-Asghari *et al*., 2009).

2.8.1.3 Stomatal conductance

Drought stress affects the capability of leaf water absorption and leaf anatomy of plants. Chartzoulakis *et al*. (2002) showed that leaf anatomy was changed in water stress, which could have accounted for the decreased stomatal conductance. The plant response is complex because it reflects over space and time the integration of stress effects and responses at all underlying levels of organization (Blum, 1996). Sdoodee and Kaewkong (2006) reported that stomatal conductance of neck orange (*Citrus reticulata* Blanco) leaves decreased during the progress of water stress. Therefore, water status is an important factor to determine the progress of water stress in neck orange. Sdoodee and Somjun (2008) reported that stomatal conductance in the drought treatment was significantly lower than that of the control along the experimental period; stomatal conductance therefore was a sensitive indicator of water stress in neck orange.

2.8.1.4 Relative water content

Relative water content (RWC) is an important characteristic that influences plant water relations. RWC relates to water uptake by the roots as well as water loss by transpiration. A decrease in the RWC in response to drought stress has been noted in wide variety of plants. When plants are subjected to drought, leaves exhibit large reductions in RWC and water potential. RWC was also affected by the interaction of severity, duration of the drought event and species (Nayyar and Gupta, 2006; Yang and Miao, 2010).

2.8.1.5 Photosynthesis

Plants use water as a configuration message in the light synthetic process and water is a payout electron called photolysis in redox reaction (Dietz, 2003). In addition, the water also serves to maintain a balance in life such as balances of temperature and osmoregulation in the body of the plants. When plant loses water, it adapts by closing its stomata. Stoma is the center of control of water and gases exchange such as CO_2, transpiration, and photosynthesis (Jones, 1998). In dry season, crops reduce water loss, starting from plants with yellow called chlorosis, chlorophyll loses function. Photosynthesis is reduced as well as growth is inhibited. The plant has a reduced growth rate in both the vegetative and reproductive stages.

2.8.2 Biochemical changes

Water stress develops when land plants absorb less water from the environment through their roots than is transpired from their leaves. The relative water content, water potential and turgor of cells are decreased and the concentrations of ions and other solutes in the cells are increased, thereby decreasing the osmotic potential. Stomatal pores in the leaf surface progressively close decreasing the conductance to water vapor and thus slowing transpiration and the rate at which water deficit develops. Also, photosynthetic assimilation of CO_2 decreases, often concomitant with, and frequently ascribed to, decreasing conductance to CO_2. However, decreased of water is also considered to be caused by inhibition of the photosynthesis in Calvin cycle. Water loss from plant tissues under drought conditions results in growth inhibition and in a number of other metabolic changes. These include abscisic acid (ABA) accumulation (MacRobbie, 1991), the decreased photosynthesis (Hsiao, 1973) and solute accumulation (Hoffmann, 2010).

The maintenance of total water potential during water deficit can be achieved by osmotic adjustment. A reduction in cellular water potential below the external water potential, resulting from a decrease in osmotic potential, allows water to move into the cell. The osmotic potential inside the cell is lowered by the accumulation of osmolytes in cytoplasm.

Osmolytes are organic compounds affecting osmosis of cells. They are soluble in the solution within a cell, or in mixing in extracellular fluid. Osmolytes play a role in maintaining cell volume and fluid balance. For example, when the cell swells due to external osmotic pressure, membrane pores open and allow efflux of osmolytes, which carry water with them, restoring normal cell volume. Sugars, glycerol, amino acid (proline, glycine butaine, dehydrin), sugar alcohol (manitol) and other low

molecular weight metabolites are classified as compatible solutes for the propose of maintaining cell water status at favorable level (Morgan, 1984). Like proline, a low molecular weight compound is also accumulated in plants and has some role in protecting harmful effect of water stress (Stewart and Lee, 1974). Proline may act as a regulation molecule to activate multiple responses. In young leaf of sunflower under water stress, proline accumulation was found in tolerance genotypes more than sensitive genotypes (Cechin *et al*., 2006).

2.8.3 Cellular and molecular changes

At the cellular level, drought stress causes shrinkage of cells, cell membrane injury, and production of free radicals that cause damage to the cellular apparatus (Kang and Zhang, 1997).

Cellular response to water deficit varies depending on the degree of water limitation, the duration of the stress, plant species and developmental stage of the plant/cell (Mullet and Whitsitt, 1996). Water stress poses unique challenges to plant cells dependent on hydrostatic skeleton and cell wall for growth and development (Figure 2.6) (Moore *et al*., 2008). The cell wall is a composite structure consisting of cellulose-hemicelluloses frame-work embedded within a matrix of pectin and proteins, which allows it to respond to stress imposed upon it from many directions (Carpita and Gibeaut, 1993). The cell wall responds to turgor pressure by inutility elastic and plastic, which result in reduced cell growth (Brett and Waldron, 1996).

Molecular responses to water deficit have identified multiple changes in gene expression (Figure 2.7). Genes expressed during stresses are anticipated to promote cellular tolerance of dehydration through protective functions in the cytoplasm, alteration of cellular water potential to promote water uptake, control of ion

accumulation and further regulation of gene expression (Parry, 1993).

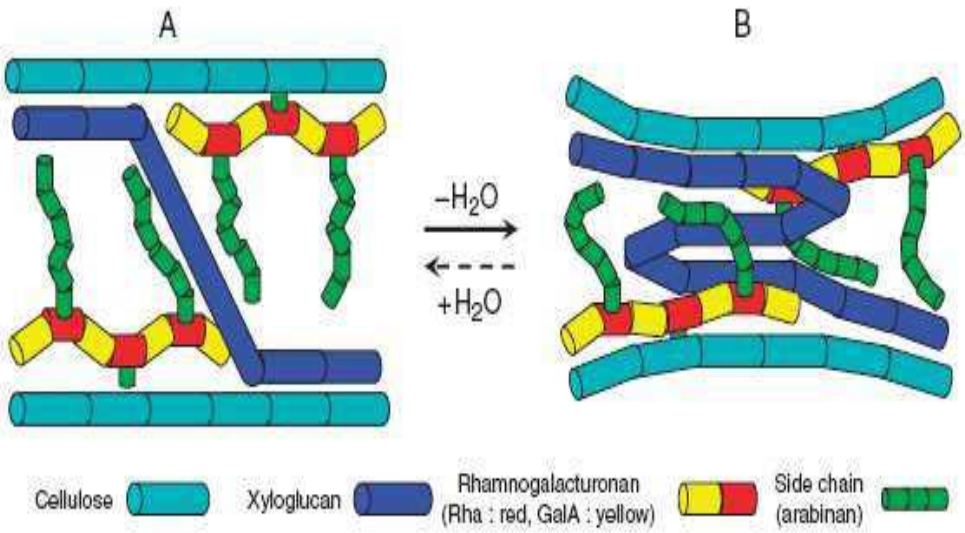

Figure 2.6 A model representing the effect of water loss on plant cell wall structure. (A) Hydrated plant cell wall and (B) dehydrated cell wall.

Source; Moore *et al*., 2008.

There are many genes related to drought tolerance such as *SunTIP*, *ERF2*, *ERF5*, *HaDhn1*, *HaDhn2*, *Sdi5* (sunflower drought induced), *Gdi15*, *Hahb-4*, *HAS1* or *HAS1.1*. Their transcript levels under drought stress are abundant and they have important roles in the drought response. Genes *Sdi5* and *HaRPS28* showed the highest expression in fully expanded leaves and they were linked to the production of certain ACC oxidase antioxidant or dehydrins while *HAS1* and *HAS1.1* had more expression in root when compared with leaves. *ERF2* and *ERF5* relate to ethylene synthesis (Ouvrard, 1996).

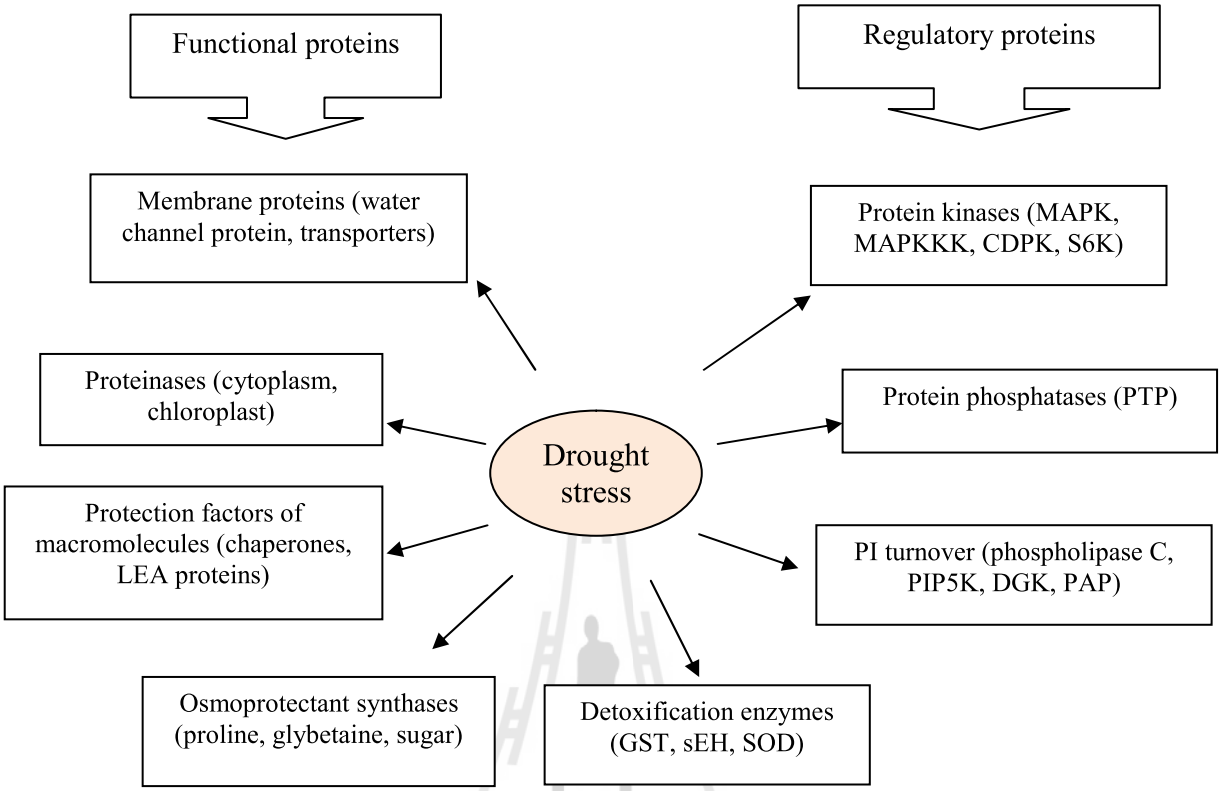

Figure 2.7 Drought stress-inducible genes and their possible functions in stress tolerance and response. Source; Muoma *et al*., 2010.

2.8.4 Osmotic stress

Plants are composed of water 80-90% of biomass. Water in the cell is called intracellular fluid. The function of fluid is to control turgor pressure in cell level. Water is involved in metabolic process and acts to maintain osmotic pressure in the cell. Plants lose water from transpiration but the water is replaced with water absorption at root.

Water loss in plant results in plasma membrane broken called plasmolysis. For osmotic adjustment mechanism, the plant cells turnover proteins and synthesize osmolytes such as proline, chorine and glycine betaine. The accumulation of

osmolytes during stress is well documented. Recent studies have demonstrated that the manipulation of genes involved in the biosynthesis of low-molecular-weight metabolites, such as proline, have improved plant tolerance to drought and salinity in a number of crops (Molinari *et al*., 2004; Zhu *et al*., 2005).

2.8.5 Oxidative stress

Drought induces the generation of reactive oxygen species (ROSs) causing lipid peroxidation, which leads to membrane injury, protein degradation, enzyme inactivation and the disruption of DNA strands (Becana *et al*., 1998). ROSs can act as second messengers involved in stress signal transduction pathway (Foyer and Noctor, 2005), but excessive ROSs production under water deficit can disturb plant cell metabolism. Plants protect cells and subcellular systems from the cytotoxic effects of these active oxygen radicals through both non-enzymatic and enzymatic antioxidant systems such as carotenoids, ascorbic acid, α-tocopherol, peroxidase and catalase (Munné-Bosch and Alegre, 2000). Reactive oxygen intermediates (ROIs) are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two or three electrons to O_2 to form a superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($OH^\bullet$), respectively. In contrast to atmospheric oxygen, ROIs are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of the cell.

2.8.5.1 Lipid peroxidation and hydrogen peroxide

The end product of lipid peroxidation, the MDA content reflects the degree of the peroxidation of membrane lipids (Taulavuori *et al*., 2001). H_2O_2 is a non-reactive oxygen species (ROSs) like singlet superoxide (O_2^1). Because of its

relative stability, H_2O_2 has received particular attention as a signal molecule involved in the regulation of specific biological processes such as plant-pathogen interactions. H_2O_2 is generated by a two-electron reduction of O_2 (Foyer and Noctor, 2000). It damages the membrane lipids, and induces protein denaturation and DNA mutation (Foyer and Halliwell, 1976). The MDA and H_2O_2 contents significantly increased with drought stress progressed in the two poplar species sunflower (Bailly *et al*., 1996), but MDA and H_2O_2 possessed negative effects on drought tolerance.

2.8.5.2 Scavenging of ROSs in cells

ROS-scavenging is cellular balance between prooxidants and antioxidants in plant tissues during stress (Arora *et al*., 2002). ROI-scavenging enzymes of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Figure 2.8). ROSs are considered toxic by-products of aerobic metabolism, which are disposed of using antioxidants. The major ROI-scavenging enzymes pathways of plants include SOD, found in almost all cellular compartments, the ascorbate-glutathione cycle is found in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes, glutathione peroxidase and CAT in peroxisomes. The reduction of oxygen form superoxide, hydrogen peroxide and hydroxyl radicals are the principle mechanism of oxygen activation in most biological systems. However, in photosynthetic plants, the formation of singlet oxygen is important. Activated oxygen is often formed as a component of metabolism to enable "complex" chemical reactions, such as the polymerization of lignin, but in other instances activated oxygen is formed by the electron transport systems, as a result of perturbations in metabolism caused by chemical or environmental stress (Mittler, 2002).

2.9 Plant adaptation to water deficit

Water deficit stress is known as drought stress, which reduces agricultural productivity mainly with disrupting the osmotic equilibrium and membrane structure of cell. Water deficit can affect plants in different ways. In order to maintain RWC within the limit, plants must regulate water loss and uptake via physiological adjustment. Water stress induces various biochemical and physiological responses in plants. Plants respond and adapt to water stress in order to survive by the following ways (Mitra, 2001).

Water stress also results in stomatal closure, reduced transpiration rates, a decrease in the water potential of plant tissues, a decrease in photosynthesis, growth inhibition, accumulation of abscisic acid (ABA), proline, mannitol, sorbitol, formation of radical scavenging compounds (ascorbate, glutathione, α-tocopherol etc.), and synthesis of new proteins.

1. Drought escape is the ability of plants to live until life cycle, before it affects drought stress, such as shorting of its life cycle or delaying time of blossom.

2. Drought avoidance is the ability of plants to lifeline by reducing the loss of water in when experiencing with drought condition such as the roll of leaf, thick leaf blade, stomatal closure and the root system and to fathom the depth of field density.

3. Drought tolerance is ability of plants to grow when experiencing drought condition such as reduced water potential to adjust the level of solute in cells by osmotic adjustment, cell wall elasticity, reduced in cell size for protection water critical period in a cell. Plants adapt gene expression then transfer to daughter generation called temporary acclimation.

4. Drought recovery is ability of plants to revive after stress. After experiencing the dry state, which may cause any part of plants withered plants can grow the new branch and recover once it is watered again.

5. Drought resistance is a complex trait, expression of which depends on action and interaction of different morphological, physiological and biochemical characters.

2.10 Antioxidant enzymes in drought tolerance mechanism

Antioxidants are substances that inhibit oxidative damage to target bio-molecules such as lipids and proteins. There are two types of antioxidants:

1. Enzymatic antioxidant type includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and ascorbate peroxidase (APX).

2. Non-enzymatic antioxidant type includes ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, flavonoid etc.

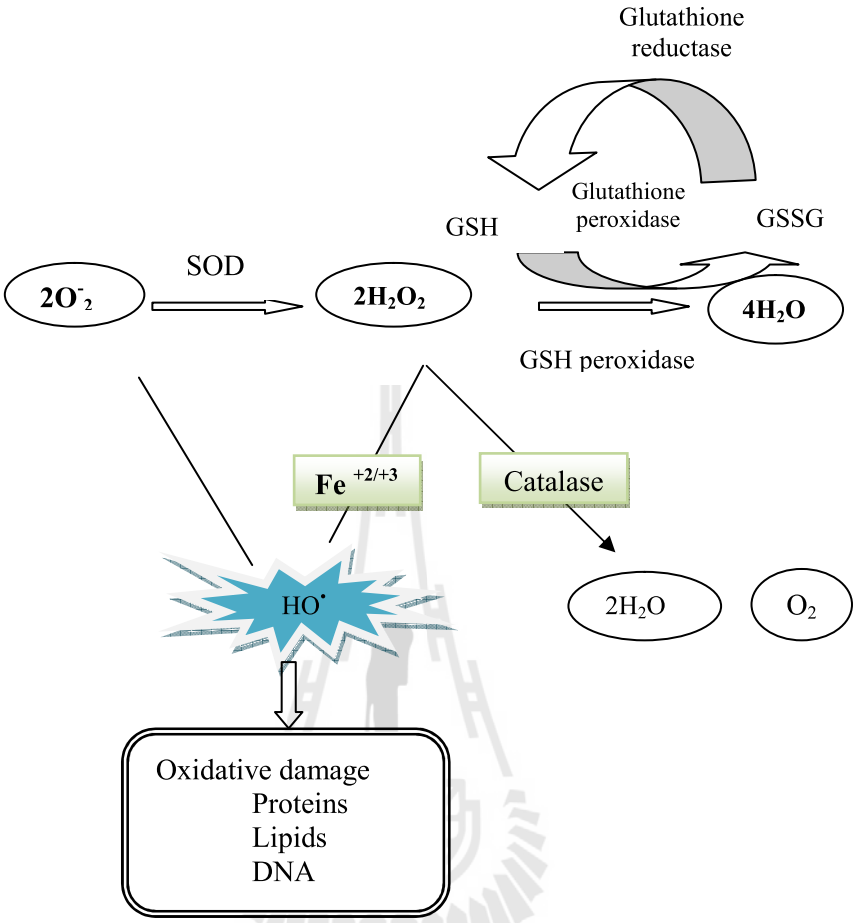

Figure 2.8 Relationship between the production of prooxidants and antioxidants in cells leading to a strengthened production of free radicals like activated (O_2) and reactive oxygen species which could lead to serious cellular damage.

Source; modified from Wakamatsu *et al*., 2008.

2.10.1 Superoxide dismutase (SOD; EC 1.15.1.1)

Superoxide dismutase (SOD) is an enzyme which acts as a catalyst in the process of dismutation of superoxide anion (O_2^-) into oxygen and hydrogen peroxide (Bannister *et al*., 1987). It is therefore a critical antioxidant defense which is present in nearly all cells which are exposed to oxygen.

There are three distinct types of SOD classified on the basis of the metal. Mn-SOD is localized in mitochondria, Cu-Zn SOD is in the cytosol and chloroplast, and Fe-SOD is in chloroplast (Bannister *et al*., 1987). Oxygen, the most essential element for aerobic life, is relatively stable. Because the electrons of oxygen (O_2) are directional with a parallel spin state, its divalent reduction is kinetically limited by the relatively slow electron spin inversion process. When O_2 is involved in a reaction, it has to be activated, permitting spin inversion of one electron at a time. This univalent pathway requires the generation of intermediates. The first reduced intermediate is the superoxide anion (O_2^-). O_2 activation may occur in different compartments of the cell (Grene, 2002), including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol. Because the measurement of O_2^- is technically difficult, evidence for increased O_2^- within the cell comes from changes in the concentrations of antioxidant molecules and changes in the specific activities of enzymes, such as superoxide dismutase, peroxidases, and enzymes of the Asada-Halliwell pathway (Foyer and Halliwell, 1976; Quartacci and Navari-Izzo, 1992).

2.10.2 Catalase (CAT; EC 1.11.1.6)

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide (H_2O_2) into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes by oxidases involved in ß-oxidation of fatty acids, the photorespiration and purine catabolism. Catalase is one of the first enzymes to be isolated in a highly purified state. Multiple forms of catalase have been described in many plants. These forms have been cloned from maize (Redibaugh *et al*., 1988). Maize has three isoforms termed cat-1, cat-2 and cat-3, which are on separate chromosomes and are differentially expressed and independently regulated (Scandalias, 1990).

2.10.3 Ascorbate peroxidase (APX; EC 1.11.1.11)

Ascorbate peroxidase (APX) is an important enzyme scavenging and protecting cells against the toxic effect of H_2O_2 in higher plants. It is one of the important enzymes in ascorbate-glutathione cycle that APX converts of H_2O_2 to H_2O and O_2 using ascorbate as the specific electron. APX isozymes are distributed in four distinct cellular compartments: thylakoid membrane-bound ascorbate peroxidase (tAPX), mitochondrial membrane-bound APX (mitAPX), stromal ascorbate peroxidase (sAPX) and cytosolic ascorbate peroxidase (cAPX) (Kawakami *et al*., 2002).

2.11 Hydroponic culture

Hydroponic culture (also called aquaculture) is a method of cultivating plants by growing them in soilless, etc., through which water containing dissolved inorganic nutrient salts is pumped (Hoagland and Arnon, 1950). The water culture method for growing plants without soil debunking the exaggerated claims made about hydroponics. A growing medium function is to aerate and support the root system of the plant and to channel the water and nutrients. Different growing mediums work well different types of hydroponic systems. It is a light, airy type of growing medium that allows plenty of oxygen to penetrate the plant's root system. The principles that apply to soil fertilizers also apply to hydroponic nutrient solution (Figure 2.10). A hydroponic nutrient solution contains all the elements that the plants normally would get from the soil. The nutrient solutions are the slightly more expensive and the easiest to use. They dissolve quickly and completely into the reservoir and often have an added pH buffer. Plants can grow hydroponically within a pH range of 5.8- 6.8

which are considered optimal.

The using polyethylene glycol (PEG) induced water deficit in hydroponic culture has been widely reported. Polyethylene glycol is a polymer produced in a range of molecular weights. In 1961 a paper published in 'Science' (Lagerwerff *et al*., 1961) indicated that PEG can be used to modify the osmotic potential of nutrient solution culture and thus induce plant water deficit in a relatively controlled manner, appropriate to experimental protocols. It is assumed that PEG of large molecular weight does not penetrate the plants and thus is an ideal osmoticum for use in hydroponic root medium.

Figure 2.9 Hydroponic culture.

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CHAPTER III

MATERIALS AND MATHODS

3.1 Experiment I: Effects of drought stress on sunflower responses in Petri dish bioassays

3.1.1 Plant materials

Sunflower seeds of 4 lines, 3 synthetic and 1 hybrid varieties were used in this experiment (Table 3.1).

Table 3.1 Sunflower genotypes and their origin for the experimental study in Petri dish bioassay.

Name	Sunflower genotypes	Origin
5A	Inbred line	SUT
6A	Inbred line	SUT
9A	Inbred line	SUT
10A	Inbred line	SUT
Suranaree 471 (S471)	Synthetic	SUT
Suranaree 473 (S473)	Synthetic	SUT
Suranaree 475 (S475)	Synthetic	SUT
Pacific 77	Hybrid	Pacific Seeds (Thai) Ltd.

SUT = Suranaree University of Technology.

3.1.2 Plant growth and water stress treatment

The experiment was carried out in the Center for Scientific and Technological Equipment (F2 building). Eight sunflower genotypes: 5A, 6A, 9A, 10A, S471, S473, S475 and Pacific 77 (medium tolerance) were tested against drought stress at germination and establishment of seedling growth stage (1-14 days after sowing, DAS) under laboratory conditions (25±3 °C) in Petri dish bioassays. Polyethylene glycol with a molecular weight of 6000 (PEG-6000) was used as a drought stimulator and two water stress levels of -0.6 MPa and -1.2 MPa were developed by dissolving 10 and 20 g of PEG per 100 mL distilled water, respectively. Apart from the stress treatments, one control treatment with no stress was applied. The seeds of each sunflower genotype were split pericarp before sterilization. The seeds were soaked with 70% ethanol for two times (two minutes per time) and were surface sterilized with 10% sodium hypochlorite solution for 10 minutes, and finally rinsed four times with distilled water. Ten seeds of each sunflower genotype were placed in each Petri dish containing 2-layer of Whatman filter papers. The experiment was laid out in a completely randomized design (CRD) with three replications. Five milliliters of designated treatment solution were applied in each Petri dish. Numbers of germinated seeds were counted daily and data were recorded for 14 days. A seed was considered for germination when both plumule and radicle have emerged to 5 mm. Fourteen-day-old seedlings was subjected to analysis of morphological and physiological characteristics.

3.1.3 Determination of growth and physiological characteristics

3.1.3.1 Germination stress index (GSI)

After 10 days, the numbers of germinated seeds were recorded and the Promptness index (PI) and GSI were calculated using the formula proposed by Bouslama and Schapaugh (1984).

Promptness index (PI)

$$= nd_2\ (1.0) + nd_4\ (0.8) + nd_6\ (0.6) + nd_8\ (0.4) + nd_{10}\ (0.2)$$

When; nd_2, nd_4, nd_6, nd_8, nd_{10} represent the percentage of germinated seeds at 2, 4, 6, 8 and 10 days after sowing, respectively.

Germination stress index (GSI) = (PISS / PICS) ×100

When; PISS is the promptness index of stressed seed

PICS is the promptness index of control seed

3.1.3.2 Plant height stress index (PHSI)

PHSI = (Plant height of stressed plant / Plant height of controlled plant) ×100

3.1.3.3 Root length stress index (RLSI)

RLSI = (Root length stressed plant / Root length of controlled plant) ×100

3.1.3.4 Dry matter stress index (DMSI)

DMSI = (Dry matter of stressed plant / Dry matter of controlled plant) ×100

3.1.3.5 Relative water content stress index (RWCSI)

RWC was estimated according to Turner (1981) and was evaluated from the equation given below. Seedlings of each treatment were cleaned and weighted in fresh weight, then floated for 24 hours in distilled water at room temperature. Dry weight was the weight estimated after drying the seedlings at 70 °C for 4 hours.

RWCSI = (RWC of stressed plants / RWC of control plants) ×100

$$RWC = [(FW - DW) / (TW - DW)] \times 100$$

When; FW is the fresh weight of the seedlings, TW is the weight at full turgid, measured after floating the seedlings for 24 hours, DW is the weight estimated after drying.

3.2 Experiment II: Effects of drought stress on sunflower responses in hydroponic culture

3.2.1 Plant materials

Sunflower seeds of 4 lines, 4 synthetic and 3 hybrid varieties were used in this experiment (Table 3.2).

Table 3.2 Sunflower genotypes and their origin for the experimental study in hydroponic culture.

Name	Sunflower genotypes	Origin
5A	Inbred line	SUT
6A	Inbred line	SUT
9A	Inbred line	SUT
10A	Inbred line	SUT
Suranaree 471 (S471)	Synthetic	SUT
Suranaree 473 (S473)	Synthetic	SUT
Suranaree 475 (S475)	Synthetic	SUT
Pacific 77	Hybrid	Pacific Seeds (Thai) Ltd.
HA429	Hybrid	USDA
HA430	Hybrid	USDA
Chiang Mai 1 (CM1)	Synthetic	Department of Agriculture

SUT = Suranaree University of Technology.

3.2.2 Plant preparation and drought treatment

The experiment II was carried out at the Center for Scientific and Technological Equipment (F2 building) and Suranaree University farm. Eleven sunflower genotypes: 5A, 6A, 9A, 10A, S471, S473, S475, Chiang Mai 1 (CM1), HA429, HA430 and Pacific 77 (medium tolerance) were used for drought tolerance screening. Before planting in hydroponic system, seeds were surface steriled with 10% sodium hypochlorite for 10 minutes, and then washed with distilled water three times and finally soaked in 1 mM gibberellic acid (GA3) for 1 hour. Later, seeds were germinated in tray containing perlite until a pair of true leaves emerged. Then, these seedlings were transferred to hydroponic culture with Hoagland nutrient solution (APPENDIX A), which was renewed once a day after measured electrical conductivity (EC). The solution was oxygenated with air diffuser to prevent root asphyxia. At the beginning of the 14 DAS, PEG-6000 was progressively added to the nutrient solution. Two water stress levels of control (no PEG) and -0.6 MPa (10% PEG) were developed using PEG-6000 as an osmoticum. Stonatal conductance was measured on the third pair of leaves from top of sunflower plants every two day after added 10% PEG concentration. At 21 days after sowing (DAS), seedlings were subjected to morphological, physiological, biochemical, and antioxidant enzyme activity analyses.

3.2.3 Measurements of morphological and physiological characteristics

3.2.3.1 Relative growth rate stress index (RGRSI)

Relative growth rate (RGR) was done as followed by the method of Dionisio-Sese and Tobita (1998) and expressed as g^{-1} day.

$$RGR = \frac{\ln DW_f - \ln DW_i}{T_i - t_f}$$

When; DW_i and DW_f are the dry weight at the beginning (T_i) and end (t_f) of sampling period and In is the natural logarithm.

RGRSI = (RGR of stressed plant / RGR of controlled plant) ×100

3.2.3.2 PHSI, RLSI and DMSI were analyzed as previously described in **3.1.2**

3.2.3.3 Relative water content stress index (RWCSI)

Leaf relative water content was estimated by recording the saturated mass (SM) of 0.5 g fresh leaf samples (the third pair of leaves) by keeping in water for 4 hours, followed by drying in hot air oven till constant dry mass (DM) was achieved (Wheatherley, 1950).

RWCSI = (RWC of stressed plant / RWC of controlled plant) x 100

3.2.3.4 Stomatal conductance stress index (SCSI)

Simultaneous measurements of stomatal conductance were performed on midday at 2 days interval during the experimental period. Young fully expanding leaves (the third pair of leaves) from the outer part of canopy were used for the measurement of stomatal conductance using AP4 porometer (Delta-T Devices, UK), and measurements were made in six replications on each plant.

SCSI = (Stomatal conductance of stressed plant / Stomatal conductance of controlled plant) x 100

3.2.3.5 Leaf number stress index (LNSI)

Leaf number was estimated by recording the young leaves. Numbers of leaves were counted at 14 DAS. A leaf was counted when a leaf has reached 4 mm in length.

LNSI = (Leaf number of stressed plant / Leaf number of controlled plant) x 100

3.2.4 Measurement of proline content

Proline content was determined following the method of Bates *et al*. (1973). Leaf samples served for proline determination were harvested at the same time from the third-pair of leaves from top. A 0.1 g of frozen powder was mixed with 5.0 mL aliquot of 3% (w/v) sulfosalicylic acid in glass tubes covered with the top and boiled in a water bath at 100 °C for 30 minutes. The mixtures were centrifuged at 2,000 rpm for 5 minutes at 25 °C. Then, a 200 µl aliquot of the extract was mixed with 400 µl distilled water and 2.0 mL of the reagent mixture (30 mL glacial acetic acid, 20 mL distilled water and 0.5 g of ninhydrin), and boiled at 100 °C for 1 hour. After cooling the mixture, 6.0 mL of toluene were added. The chromophore containing toluene was separated and the absorbance was read at 520 nm, using toluene as a blank. Proline concentration was calculated using L-proline as the standard curve.

3.2.5 Measurement of lipid peroxidation

Lipid peroxidation was estimated according to the method of Dhinds and Matowe (1981). Ground frozen tissues (0.2 g) were transferred to a 1.5 mL eppendorf tube and homogenized following addition of 1 mL of TCA-TBA-HCl reagent, 15% (w/v) trichloroacetic acid (TCA), 0.25 M HCl, 0.37% (w/v) 2-thiobarbituric acid (TBA) and 0.01% butylated hydroxytoluene. After homogenization, samples were

vortexed for a few seconds then incubated at 90 °C for 30 minutes in a hot block, then chilled in ice and centrifuged at 12,000 rpm for 10 minutes. Absorbance was measured at 535 nm and 600 nm.

3.2.6 Measurement of hydrogen peroxide level

H_2O_2 levels were measured colorimetrically as described by Mukherjee and Choudhuri (1983). Leaf samples (0.5 g) were homogenized in ice cool of 5 mL 0.1% (w/v) TCA. Each homogenate was centrifuged at 6,000 rpm for 15 minutes and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM K-phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The intensity of the yellow color of the supernatant was measured at 410 nm (extinction coefficient = 0.28 μmol cm^{-1}). The blank consisted of 0.1 % TCA instead of the leaf extract.

3.2.7 Antioxidant enzyme activities

3.2.7.1 Extraction of enzymes

For SOD enzyme extraction, leaf samples (0.5 g) were homogenized in ice cool of 0.75 mL extraction buffer (0.1 M Na-phosphate buffer (pH 7.5), containing 0.5 mM ethylene diaminetetraacetic acid (EDTA)) with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 12,000 rpm for 15 minutes, 4 ^{o}C. The supernatant was used for enzyme activity assay.

For CAT enzyme extraction, leaf samples (1.0 g) were homogenized in ice cool of 10 mL extraction buffer (0.1 M K-phosphate buffer (pH 7.5), containing 0.5 mM ethylene diaminetetraacetic acid (EDTA)) with pre-chilled pestle and mortar.

Each homogenate was transferred to centrifuge tubes and was centrifuged at 12,000 rpm for 15 minutes, 4 ^{o}C. The supernatant was used for enzyme activity assay.

For APX enzyme extraction, the leaf samples (0.5 g) were homogenized in ice-cold 10 mL of 0.1 M K-phosphate buffer (pH 7.5) containing 0.5 mM EDTA, 2 mM ascorbate (AsA) and 5% polyvinyl pyrrolidone (PVP) with pre-chilled pest and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 12,000 rpm for 15 minutes, 4 ^{o}C. The supernatant was used for enzyme activity assay.

3.2.7.2 Superoxide dismutase assay

SOD activity was measured according to the method of Stewart and Bewley (1980). One unit of SOD activity is the amount of enzyme activity that causes 50% inhibition of the initial rate of the reaction in the absence of enzyme. About 3 mL of reaction mixture, containing 0.1 mL of 1.5 M sodium bicarbonate, 0.2 mL of 200 mM methionine, 0.1 mL of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 mL of 3 mM EDTA, 1.5 mL of 100 mM potassium phosphate buffer (pH 7.5), 0.95 mL distilled water and 0.05 mL of enzyme extraction, taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 mL of 60 µM riboflavin and placed the tube below a light source of two 18 W florescent lamps for 15 minutes. The reaction was stopped by switching off the light and covered the tubes with black cloth for 20 minutes. Tubes without enzyme developed maximal color. Non-irradiated complete reaction mixtures which did not develop color served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity is taken as the quantity of enzyme which reduced the absorbance reading of sample to 50% in comparison with tubes lacking enzymes.

3.2.7.3 Catalase assay

CAT activity was assayed by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm (Aebi, 1983). One unit of enzyme activity was equal to 1 μmol of H_2O_2 decomposed per minute. Then reaction mixtures contained 1.5 mL of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mL of 75 mM H_2O_2, 0.05 mL enzyme extract and distilled water were added to make up the volume to 3 mL. The reaction started by adding H_2O_2 and decrease in absorbance was recorded at 240 nm for 1 minute. Enzyme activity was computed by calculating among of H_2O_2 decomposed.

3.2.7.4 Ascorbate peroxidase assay

APX activity was assayed according to Asada (1992). The reaction was initiated by the addition of H_2O_2. The H_2O_2 dependent oxidation of ascorbate was followed monitoring the decrease in absorbance at 290 nm due to the oxidation of APX in the first 30 seconds from the start of the reaction (extinction coefficient = 2.8 mM^{-1} cm^{-1}). One unit of APX is the amount of enzyme that oxidizes 1 μmol of ascorbate per minute at room temperature. Two mL reaction mixtures contained 25 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM AsA, 1 mM H_2O_2 and 50 μl of the enzyme sample. No change in absorption was found in the absence of AsA in the test medium.

3.3 Statistical analysis

Statistical evaluation of all parameters was analyzed as Factorial experiment with five replications using SPSS 17.0 for Windows (SPSS 2008, Inc., Chicago, IL). All data were analyzed by the analysis of variance (ANOVA). Significant difference at $p<0.05$ among genotypes and treatments were determined by Duncan's multiple range test (DMRT). Principal component analysis (PCA) and clustering analysis were used to determine the relationship among genotypes and indices based on morphological, physiological and biochemical parameters. PCA and cluster analysis were conducted with R program, while the ranking method (Farshadfar *et al*., 2012) was measured to determine the most desirable drought tolerant genotypes according to all indices.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Effects of drought stress on sunflower responses in Petri dish bioassays

This study was conducted to understand the plant tolerance based on morphological and physiological characters in response to drought stress at germination and establishment of seedling growth stage. Osmotic solution, PEG-6000, was used to induce water stress, as most of the researchers (Lagerwerff *et al*., 1961; Zgallai *et al.*, 2005).

Sunflower plants exposed to PEG-induced drought stress from the germination stage through to seedling growth stage (1-14 DAS) showed a significant reduction in germination percentage and plant growth parameters in all sunflower genotypes. The results of combined analyses of variances indicated the significant differences among genotypes for dry matter stress index (DMSI) and significant genotype × PEG concentration interaction for all traits with the exception of root length stress index (RLSI) (Tables 4.1). In addition, analyses of variances indicated the significant differences among PEG concentrations for PHSI and DMSI (Table 4.1).

Drought significantly influenced the PHSI and DMSI indices (Table 4.1) and caused a decrease in all indices (Table 4.2). The mean PHSI and GSI values reduced from 34.75% to 9.01% (3.9 folds) and 51.58% to 22.53% (2.3 folds) under drought stress conditions at 10% PEG and 20% PEG, respectively (Table 4.2).

4.1.1 Germination stress index (GSI)

Figure 4.1 describes the effect of PEG concentrations on GSI in eight sunflower genotypes at 1-10 DAS. Seed germination decreased as osmotic potential became more negative. Inhibition of seed germination was greatest under the lowest osmotic potential, -1.2 MPa (20% PEG). The GSI at 10 DAS ranged from 15.07% to 83.64% for the -0.6 MPa (10% PEG) compared to 0 to 51.81% at -1.2 MPa indicating more pronounced differences among genotypes at the lower osmotic potentials. At 10% PEG, the maximum GSI value was recorded in sunflower var. S473 (83.64%) which line 5A was the lowest (15.07%). At 20% PEG, the minimum value 51.81% was recorded in sunflower var. S473 while no germination was observed in lines 5A, 6A and 10A (Figure 4.1). These results were similar to those of Ahmad *et al*. (2009) and Mohammed *et al*. (2002). Kaya *et al*. (2006) reported that germination delayed in higher PEG concentrations, but no seed germination was observed at -1.2MPa of PEG treatments. Bouslama and Schapaugh, (1984) was recorded the increased in osmotic stress with the decreased in germination rate on soybeans.

Table 4.1 Analysis of variances for morphological and physiological stress indices of sunflower plants exposed to different PEG-6000 concentrations at germination and establishment of seedling growth stage.

S.V.O	d.f.	Mean square				
		GSI	PHSI	RLSI	DMSI	RWCSI
Genotypes (G)	7	2865.084**	1432.037**	1486.147**	3042.599**	2701.219**
PEG Concentration (C)	1	10137.434**	7951.343**	8456.175**	12745.435**	20755.074**
G X C	7	133.045	280.303	624.279**	1258.052	367.207**
Error	32	148.675	54.627	118.723	703.090	8.929
C.V. (%)		27.631	21.480	23.981	28.742	30.080

** =Significant at 0.01 probability level.

GSI: germination stress index, PHSI: plant height stress index, RLSI: root length stress index, DMSI: dry matter stress index, and RWCSI: relative water content stress index.

Table 4.2 The values are mean of morphological and physiological stress indices of sunflower plants under water stressed germination and establishment of seedling growth stage.

Genotypes	GSI		PHSI		RLSI		DMSI		RWCSI	
	10%PEG	20%PEG	10%PEG	20%PEG	10%PEG	20%PEG	10%PEG	20%PEG	10%PEG	20%PEG
S471	79.95ab	41.49a	54.76a	23.52a	59.10ab	36.87a	73.81ab	64.23a	79.95b	58.63b
S473	83.64a	51.81a	55.62a	33.18a	66.07a	46.7a	78.64a	70.59a	91.90a	67.29a
S475	60.90bc	35.72ab	43.72a	12.01b	63.13ab	59.13a	69.36ab	40.91b	76.21b	44.62c
Pacific77	52.71c	35.82ab	56.39a	3.37bc	58.00ab	55.60a	57.82abc	36.39b	71.14c	37.06d
5A	15.07e	0c^{ng}	9.62b	0c^{ng}	47.23b	0b^{ng}	53.33bc	0c^{ng}	60.63e	0e^{ng}
6A	40.69cd	0c^{ng}	21.22b	0c^{ng}	54.10ab	0b^{ng}	45.04c	0c^{ng}	47.44g	0e^{ng}
9A	49.97cd	15.38bc	25.31b	0c^{ng}	54.10ab	38.63a	56.89abc	9.78c	64.84d	5.58e
10A	29.72de	0c^{ng}	11.36b	0c^{ng}	47.57b	0b^{ng}	22.73d	0c^{ng}	53.79f	0e^{ng}
Mean	51.58	22.53	34.75	9.01	56.16	29.62	57.20	27.74	68.24	26.65

Means followed by the same letters are not significantly different at p <0.05 level of probability using DMRT; ng: no germination.

GSI: germination stress index, PHSI: plant height stress index, RLSI: root length stress index, DMSI: dry matter stress index, and RWCSI: relative water content stress index.

The GSI was used to account differences in the rate of germination due to osmotic stress (Bouslama and Schapaugh, 1984). High values of GSI indicated a high rate of germination. The rate of germination indicated by GSI was inversely related to moisture stress. The highest GSI average over treatments was 67.73% for sunflower var. S473 and the lowest 7.54% for line 5A. According to Ahmad *et al*. (2009), drought stress has an inhibitory effect on sunflower seed germination. Increasing drought stress levels caused delay in seedling emergence as a result of reducing cell division and plant growth metabolism. Somers *et al*. (1982) reported that at higher PEG concentrations seedling emergence of four sunflower cultivars was reduced at -11 and -15 bars and completely inhibited at -21 bars. The results of these previous studies are similar with results of our present study.

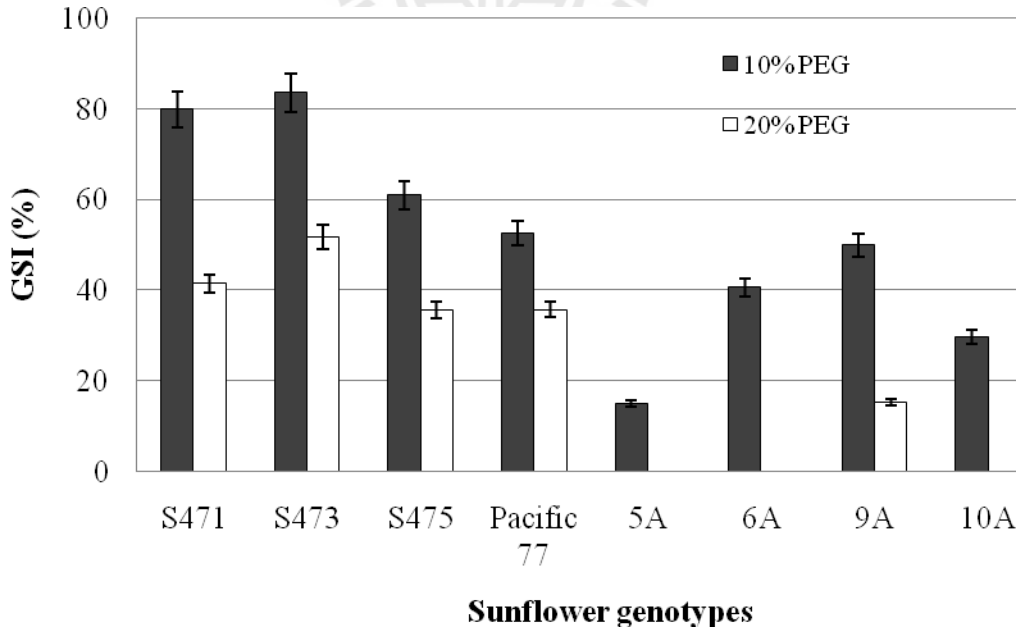


Figure 4.1 Germination stress index (GSI) of sunflower plants in response to 10% and 20% PEG concentrations. The values shown are means ± SE.

4.1.2 Plant height stress index (PHSI)

Analysis of variance on PHSI in sunflower plants was significantly influenced by PEG concentrations and the interaction of genotypes × PEG concentrations was not significant as presented in Table 4.1.

The PHSI was decreased in both 10% and 20% PEG concentrations in all genotypes. At 10% PEG level, Pacific 77 had the maximum PHSI values (56.39%), followed by S473 and S471 and 5A had the lowest. At 20% PEG, S473 maintained the highest PHSI (33.18%) while the PHSI was not recorded in 5A, 6A, 9A and 10A at this water stress level (Figure 4.2). The highest PHSI average over treatments was 44.40% for sunflower var. S473 and the lowest 4.81% for line 5A. Water stress on sunflower has been reported to reduce plant height, root length and number of stomata (Pirjol-Sovulescu *et al.*, 1974). The results of PHSI under drought stress are agreement with the findings of Ashraf and Waheed (1993) and Ahmad *et al*. (2009) that the PHSI showed significant differences among sunflower genotypes and was decreased with the increase in PEG concentrations.

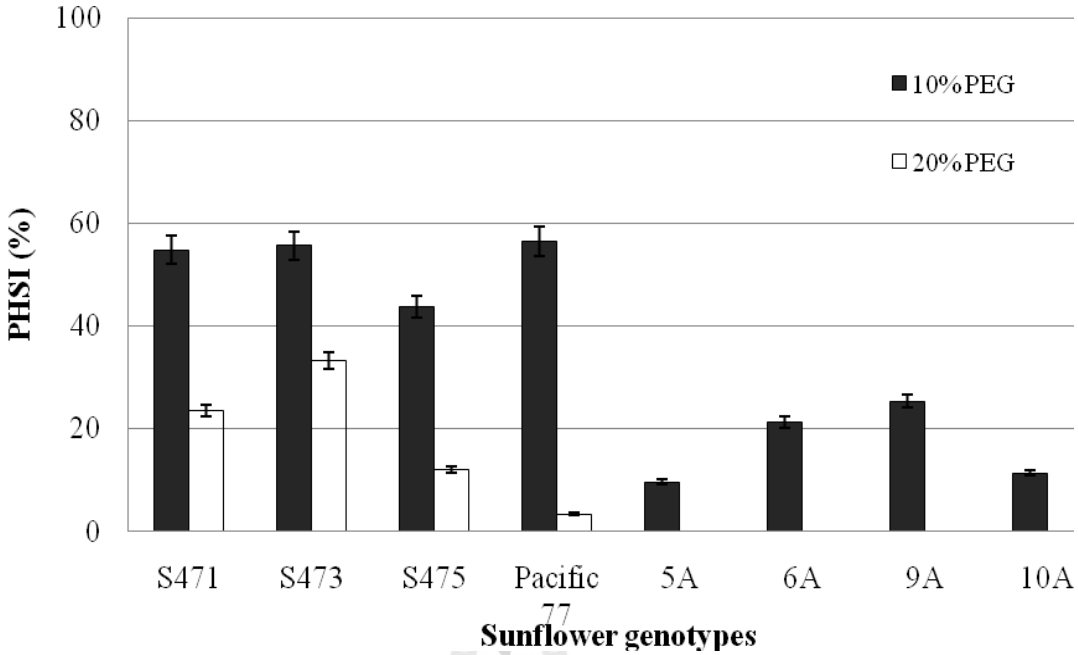


Figure 4.2 Plant height stress index (PHSI) of sunflower plants in response to 10% and 20% PEG concentrations. The values shown are means ± SE.

4.1.3 Root length stress index (RLSI)

Analysis of variance for the RLSI data in different sunflower genotypes was not significantly influenced by genotypes, PEG concentrations and the interaction of genotypes × PEG concentration as shown in Table 4.1.

Figure 4.3 describes the effect of 10 % and 20% PEG concentrations on the RLSI in sunflower seedlings. At 10% PEG, the highest RLSI value was recorded in sunflower var. S473 (66.07%) while the minimum value of 47.23% was recorded in line 5A. At 20% PEG level, the highest RLSI value was recorded in S475 (59.54%), while sunflower inbred lines showed minimum RLSI values (Figure 4.3). It is interesting to note that sunflower line 9A had the highest RLSI value among inbred lines at 20% PEG. The finding that root length decreased in increasing of drought stress levels is agreement with the finding of Ben-Rouina *et al.* (2006). Nejad (2011)

reported that major parameters in drought conditions such as root length, number, weight and root volume, decreased in mild water stress (50% of the amount of irrigation treatments). Root length increased in conditions of severe water stress (25% of the amount of irrigation treatments), but reduced root length compared to the control treatments.

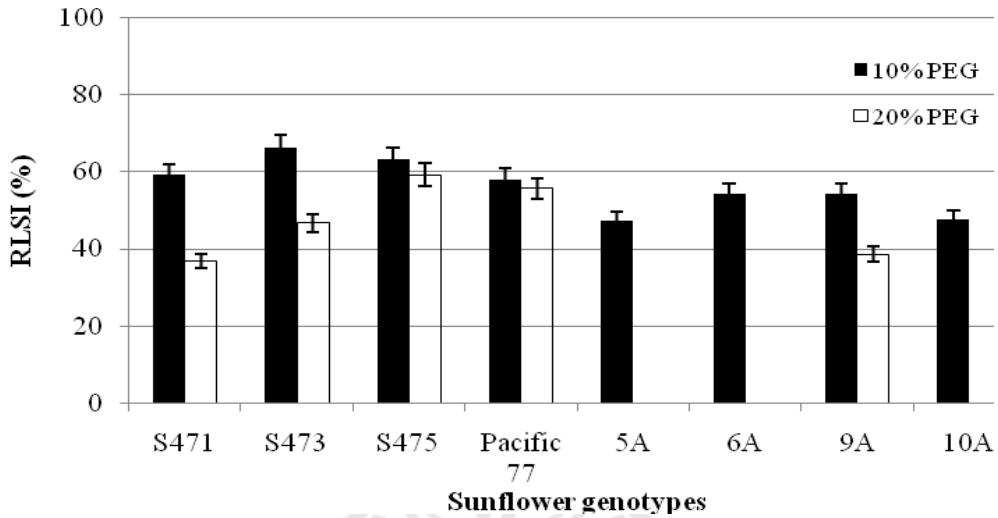


Figure 4.3 Root length stress index (RLSI) of sunflower plants in response to 10% and 20% PEG concentrations. The values shown are means ± SE.

4.1.4 Dry matter stress index (DMSI)

Analysis of variance for the data DMSI in different sunflower genotypes revealed significant among genotypes, PEG concentrations, and the interaction of genotypes x PEG concentrations as shown in Table 4.1.

The DMSI was decreased under drought stress in all genotypes (Figure 4.4). At 10% PEG level, the maximum DMSI value was observed in sunflower var. S473 (78.64%) and the minimum value 22.73% was in line 10A. The DMSI values were declined when exposed to 20% PEG concentration and sunflower var. S473 had the

highest DMSI value (70.59%), followed by var. S471 (64.23%). Moreover, among four inbred lines genotype 9A had the highest DMSI value, 56.89% and 9.78% at 10% and 20% PEG concentrations, respectively. Reduced biomass under drought stress was observed in several plant species including sunflower (Tahir and Mehid, 2001; Ahamad *et al.,* 2009) and soybean (Specht *et al*., 2001).

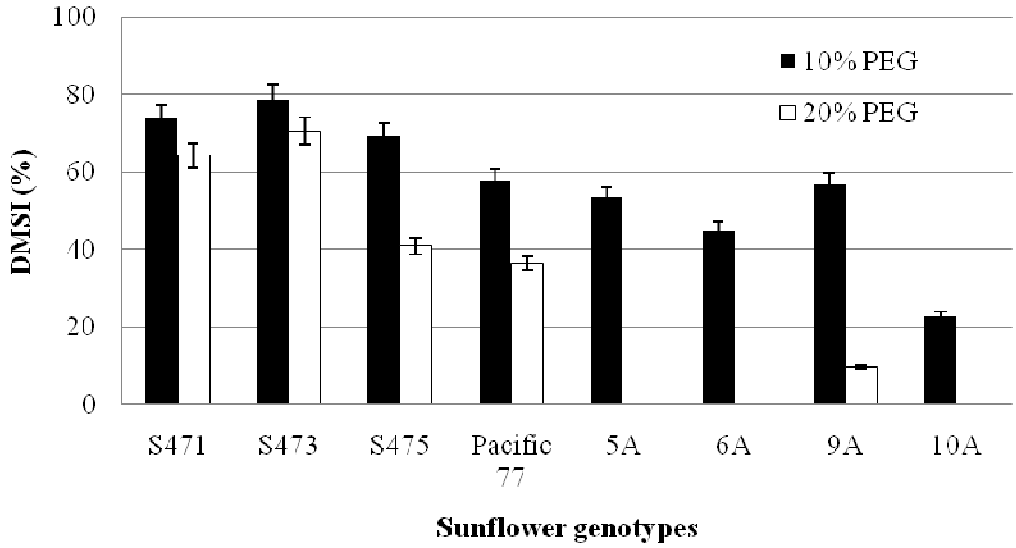


Figure 4.4 Dry matter stress index (DMSI) of sunflower plants treated with 10% and 20% PEG concentrations. The values shown are means ± SE.

4.1.5 Relative water content stress index (RWCSI)

Figure 4.5 describes the effect of drought stress levels on RWCSI in sunflower seedlings. Exposure of plants to drought led to noticeable decreases in relative water content. The minimum RWCSI value was in sunflower line 6A at 10% PEG level, which the maximum was in genotype S437 (91.90%). At 20% PEG level the maximum RWCSI value was in sunflower var. S473 (67.29%). Genotype 9A had the highest RWCSI values at both PEG concentrations among inbred lines. Some genotypes of sunflower, e.g. S473 maintained the RWCSI extremely well at both

drought stress levels. These results were agreement with those of Siddique *et al.* (2000) who found that drought stress induced decreasing in relative water content in wheat. In additional the higher leaf water potential and relative water content as well as lower leaf temperature were associated with a higher photosynthetic rate. Generally, when cells are subjected to slow dehydration, compatible solutes are accumulated in the cells resulting in maintenance of cell water content in spite of the reduction in tissue water potential (Rhodes and Samaras, 1994).

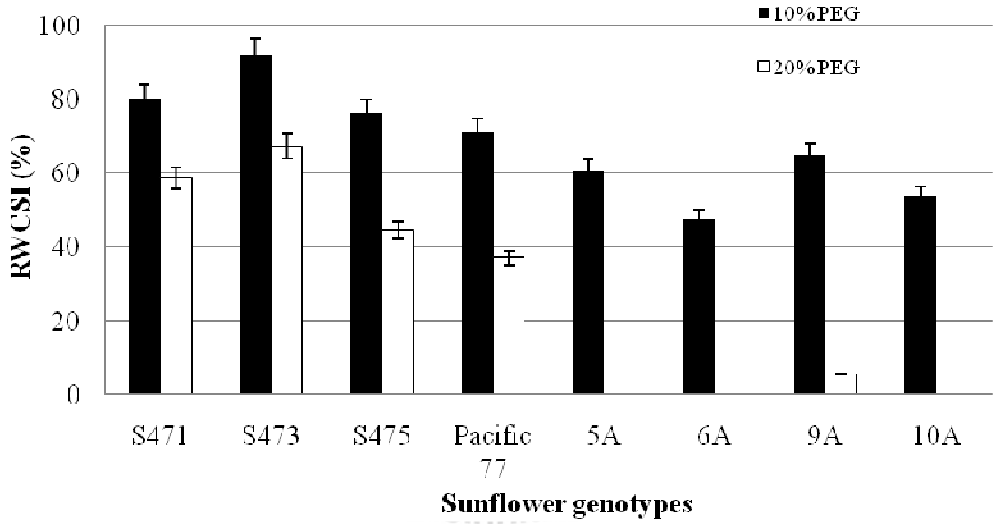


Figure 4.5 Relative water content stress index (RWCSI) of sunflower plants in response to 10% and 20% PEG concentrations. The values shown are means ± SE.

4.2 Effects of drought stress on sunflower responses in hydroponic culture

To study the response of sunflower plants to drought stress, controlled water deficit (drought) was developed based on the hydroponic culture at seedling growth stage (14-21 DAS). Plants were grown under well-watered conditions, and drought stress was applied by adding 10% PEG-6000 at 14 DAS. Seven days after drought initiation, growth, physiological and biochemical parameters as well as antioxidant enzyme activities were measured.

4.2.1 Plant growth and physiological parameters

The mean values showed that PHSI, RLSI, DMSI, RWCSI, relative growth rate stress index (RGRSI), stomatal conductance stress index (SCSI) and leaf number stress index (LNSI) of all sunflower genotypes were significantly decreased under water stress condition (Table 4.3).

Table 4.3 The values mean of morphological, physiological and biochemical stress indices of sunflower plants under drought stress condition at seedling growth stage.

Genotypes	PHSI	RLSI	DMSI	SDMSI	RDMSI	RWCSI	RGRSI	SCSI	LNSI
S471	74.86b	59.99bcd	75.06ab	79.15ab	63.78a	73.35b	67.38a	70.042b	66.25ab
S473	83.88a	71.49ab	80.77a	85.29a	71.56a	80.65a	77.99a	86.00ab	72.71ab
S475	73.64b	62.86bc	65.46abc	67.42abc	61.23a	71.99b	70.79a	91.77a	66.55ab
Pacific 77	71.91bc	82.72a	64.57abc	64.40abc	73.02a	70.85b	69.63a	79.16ab	54.48b
5A	64.38cd	55.17bcde	41.35d	38.47d	46.19a	68.50b	57.90a	90.45a	60.40ab
6A	57.39d	42.93de	54.67bcd	58.33bcd	48.38a	57.54c	57.18a	75.67ab	57.77ab
9A	67.81bc	41.03de	60.03abcd	61.16bcd	60.26a	69.38b	67.49a	81.55ab	75.51ab
10A	59.05d	37.48e	46.35cd	48.22cd	43.68a	61.64c	61.47a	81.07ab	58.38ab
CM1	70.86bc	53.35bcde	56.51bcd	54.97bcd	62.93a	69.72b	56.33a	81.99ab	69.81ab
HA430	68.58bc	50.20cde	51.36cd	55.09cd	40.63a	68.57b	58.39a	78.81ab	63.22ab
HA429	69.25bc	46.18cde	48.29cd	46.01cd	57.60a	68.82b	62.17a	68.48b	82.42a
Mean	69.24	54.86	58.58	59.86	57.21	69.18	64.25	80.45	66.13

Means with the same letter superscript is not significantly different at 5% level by DMRT.

PHSI: plant height stress index, RLSI: root length stress index, DMSI: dry matter stress index, SDMSI: stem dry matter stress index, RLSI: root length stress index, RWCSI: relative water content stress index, RGRSI: relative growth rate stress index, SCSI: stomatal conductance stress index, and LNSI: leaf number stress index.

4.2.1.1 Plant height stress index (PHSI)

Drought stress caused a reduction in plant height in term of PHSI of all sunflower genotypes in the hydroponic system (Table 4.3). The maximum PHSI value was recorded in sunflower var. S473 (83.88%) while the minimum value was in line 6A (57.39%). Decreases in plant height were recorded in sunflower seedlings (Vasilui *et al.,* 1971; Ahmad *et al*., 2009) and triticale seedlings (Atak *et al*., 2006).

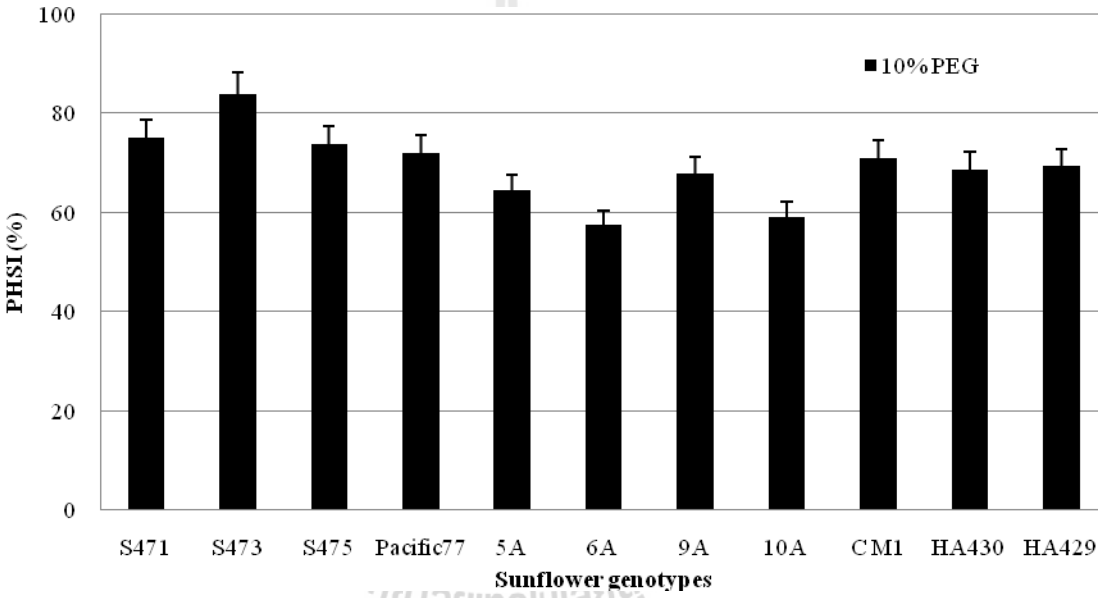


Figure 4.6 Effect of drought stress on plant height stress index (PHSI) in sunflower plants. The bars indicate SE (n=5).

4.2.1.2 Root length stress index (RLSI)

Figure 4.7 shows the effect of drought stress on root length in sunflower seedlings. When the plants were treated with 10% PEG-6000 for 7 days, the root length was decreased compared with the control. The maximum RLSI value was recorded in sunflower var. Pacific 77 (82.72%), while the minimum value was found in line 10A (37.48%). Root length is an important trait against drought stress in plant varieties; in general, variety with longer root growth has resistant ability for drought (Leishman and Westoby, 1994). The result in this study was similar to the finding of Ahmad *et al.* (2009) in that the RLSI of sunflower plants were lower under water stress and were different among genotypes. Root characteristics such as root length density, root thickness, and rooting depth and distribution have been established as constituting factors of drought resistance. In this experiment, drought stress was also effective in reducing root density as shown in Figure 4.8.

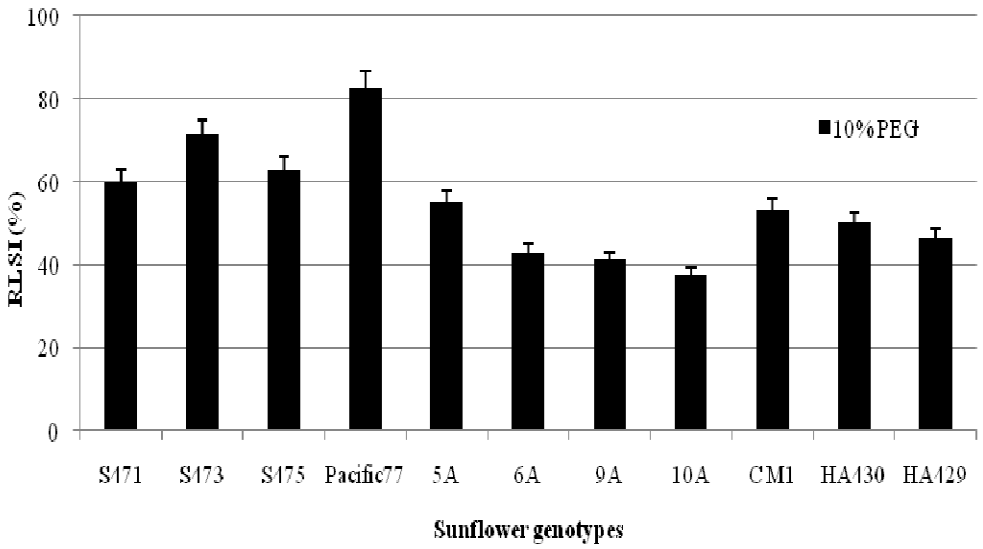


Figure 4.7 Effect of drought stress on root length stress index (RLSI) in sunflower plants. The bars indicate SE (n=5).

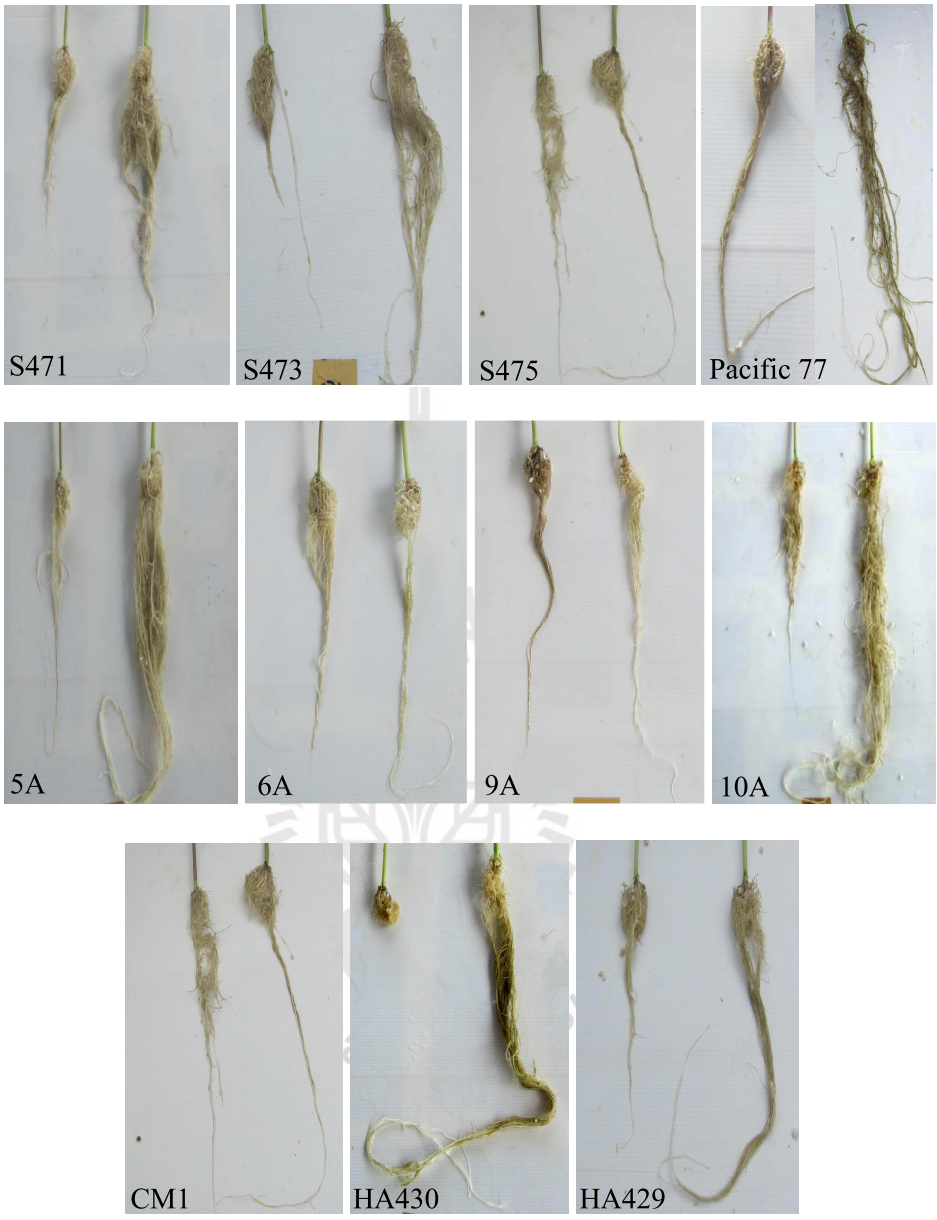
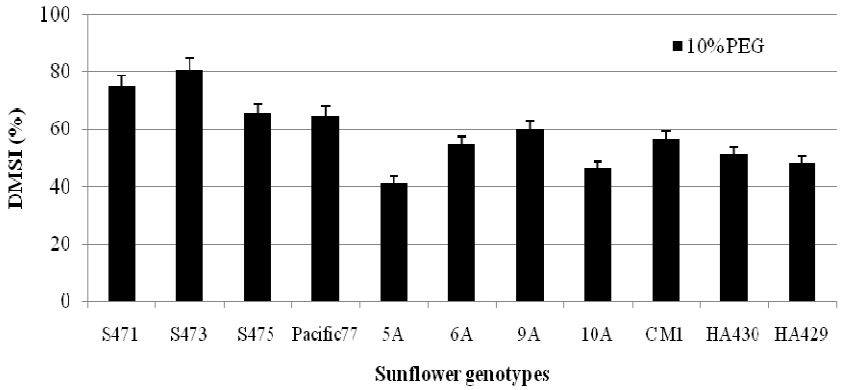


Figure 4.8 Sunflower root length and density under drought stress (left) and control treatment (right).

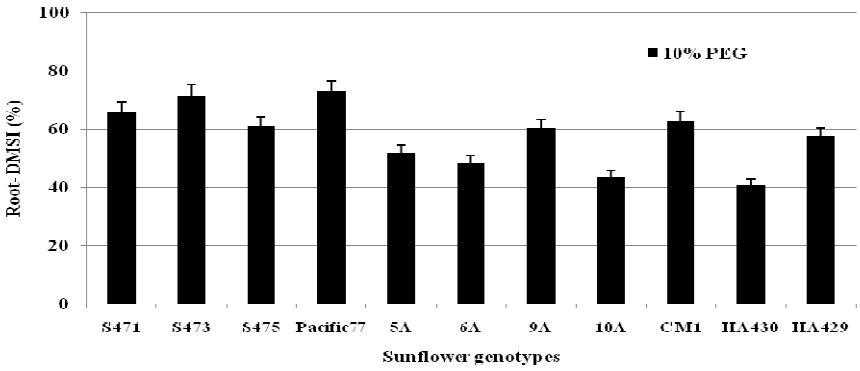
4.2.1.3 Dry matters stress index (DMSI)

Drought stress significantly decreased dry weight of sunflower plants, i.e. 58.58% for the DMSI average. The DMSI maximum and minimum values were found in sunflower var. S473 (80.77%) and line 5A (41.35%), respectively (Figure 4.9). Both shoot and root dry weight were affected and decreased in the range of 38.47% to 85.29%, and 40.63% to 73.02 %, respectively. The DMSI data in this study were similarly recorded in rice (Khan *et al.,* 2000) and chickpea (Mahdi and Al-Mutawa, 2003).

A



B



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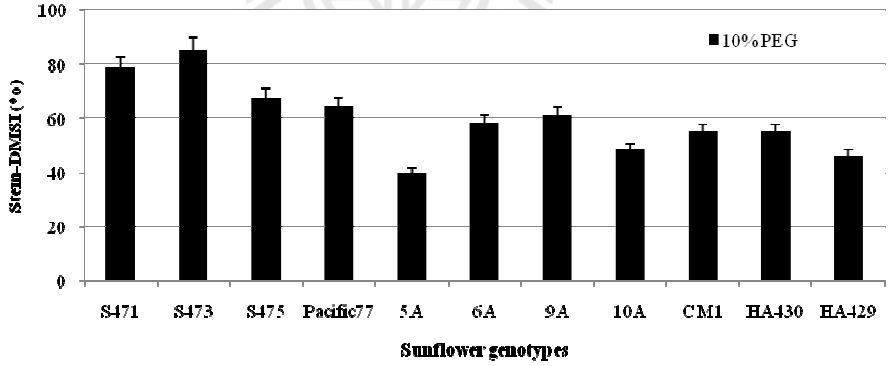

Figure 4.9 Effect of drought stress on dry matter stress index (DMSI, A), root dry matter stress index (RDMSI, B), and shoot dry matter stress index (SDMSI, C) in sunflower plants. The bars indicate SE (n=5).

4.2.1.4 Relative water content stress index (RWCSI)

Figure 4.10 describes effect of drought stress on leaf relative water content of sunflowers. At 10% PEG, the maximum value was in sunflower var. S473 (80.65%) while the minimum value was in line 6A (57.54%). Kumar *et al.* (2011) reported water deficit induced by PEG-6000 affected decreasing of RWC in Pigeon pea.

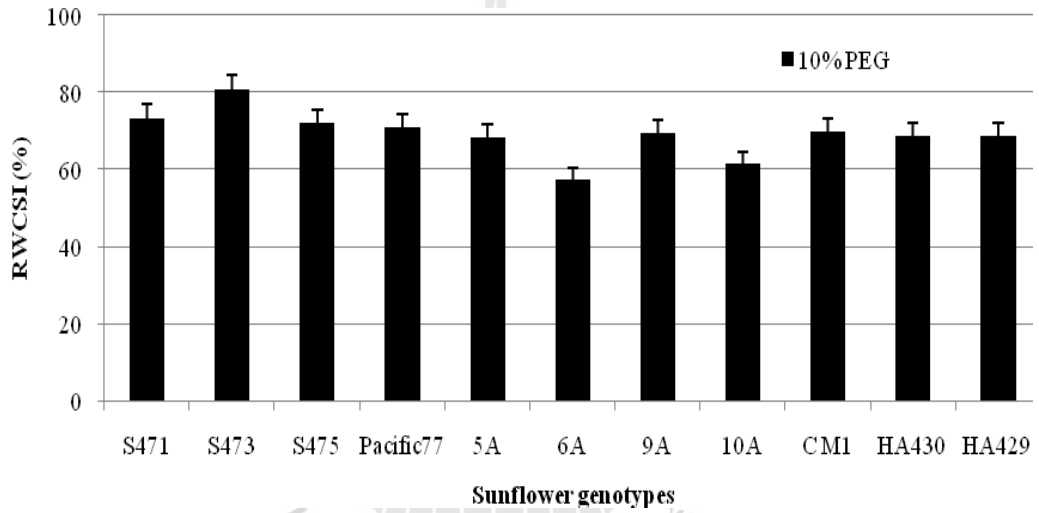

Figure 4.10 Effect of drought stress on relative water content stress index (RWCSI) in sunflower plants. The bars indicate SE (n=5).

4.2.1.5 Relative growth rate stress index (RGRSI)

Data on effect of drought stress on relative growth rate stress index (RGRSI) are presented in Table 4.3.

Figure 4.11 describes the effect of drought stress on RGRSI of sunflower seedlings. The data of relative growth rate were different among genotypes. At 10% PEG, the maximum RGRSI value was recorded in sunflower var. S473 (77.99%), which the minimum RGRSI value was found in var. CM1 (56.33%).

Drought stress reduced productivity of yield and plant growth. Thus, an understanding of drought stress and water use in relation to plant growth is of importance for sustainable agriculture.

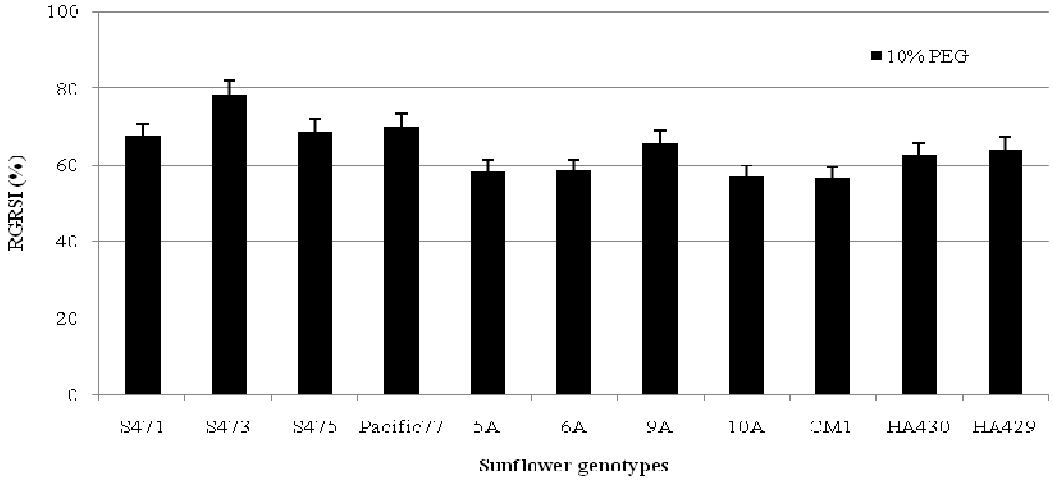

Figure 4.11 Effect of drought stress on relative growth rate stress index (RGRSI) in sunflower plants. The bars indicate SE (n=5).

4.2.1.6 Stomatal conductance stress index (SCSI)

Figure 4.12 shows the effect of drought stress on stomatal conductance in sunflower seedlings applied with 10% PEG-6000. Under drought stress, the highest SCSI value was recorded in sunflower var. S475 (88.12%), while the lowest was in genotype var. HA429 (68.48%). These results suggest that drought stress affected the loss of turgor pressure in the plant cells and photosynthesis metabolism in chloroplast. The changes in leaf physiology such as reduction in relative water content, stomatal conductance and stomatal closure have been observed in sunflower plant (Benilloch-Gonzalez *et a*l., 2010).

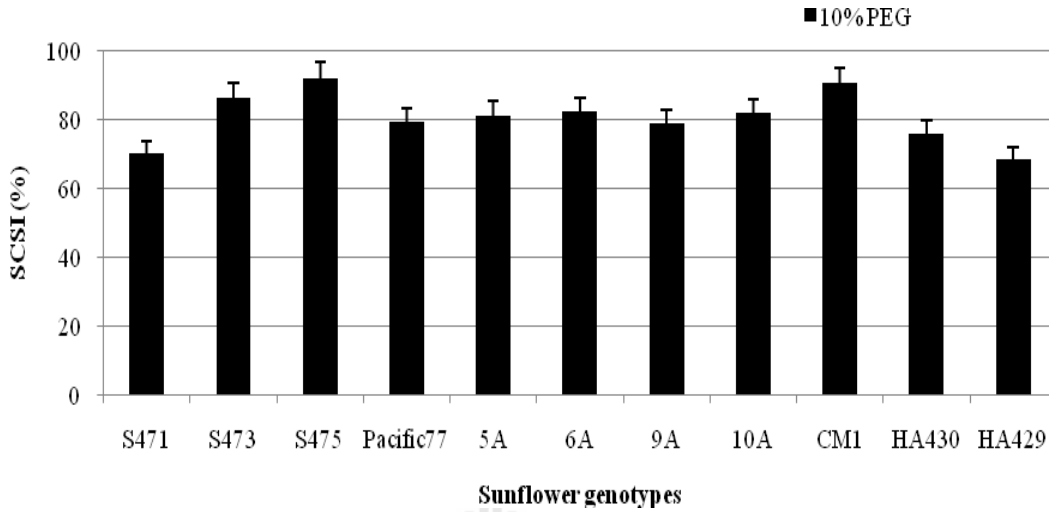

Figure 4.12 Effect of drought stress on stomatal conductance stress index (SCSI) in sunflower plants. The bars indicate SE (n=5).

4.2.1.7 Leaf number stress index (LNSI)

The first visual symptom of drought stress was leaf rolling, and severity of leaf rolling as well as leaf drying increased with duration of drought stress. This led to decreasing of leaf number in plants of all sunflower genotypes in the range of 54.48% to 82.42% as shown in Table 4.3 and Figure 4.13. The maximum LNSI value was recorded in genotype HA429 (82.42 %), while the minimum was recorded in sunflower var. Pacific 77 (54.48%). Furthermore, it was observed that stressed plants had smaller leaves compared with the control, and the leaves became pale green to yellowish-green that might due to inhibited chloroplast and chlorophyll synthesis (Figure 4.14).

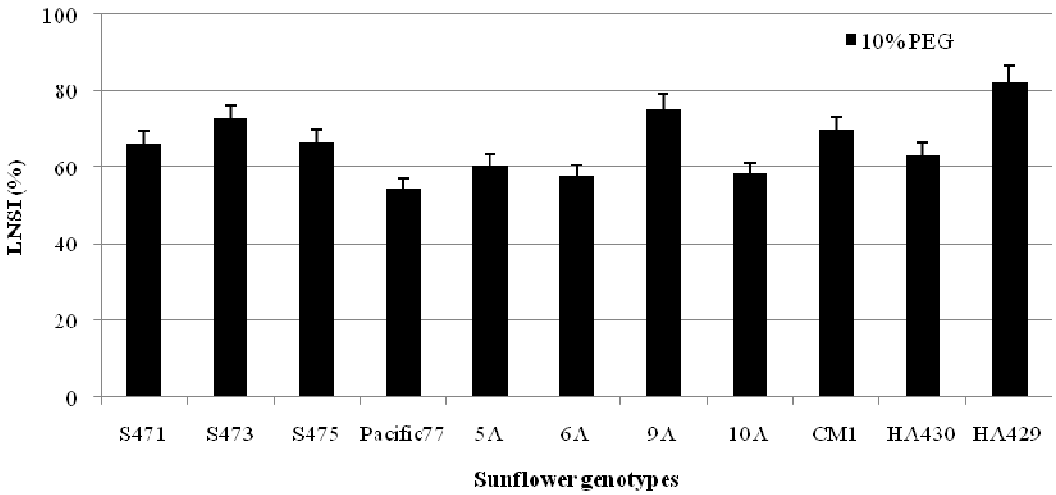

Figure 4.13 Effect of drought stress on leaf number stress index (LNSI) in sunflower plants. The bars indicate SE (n=5).

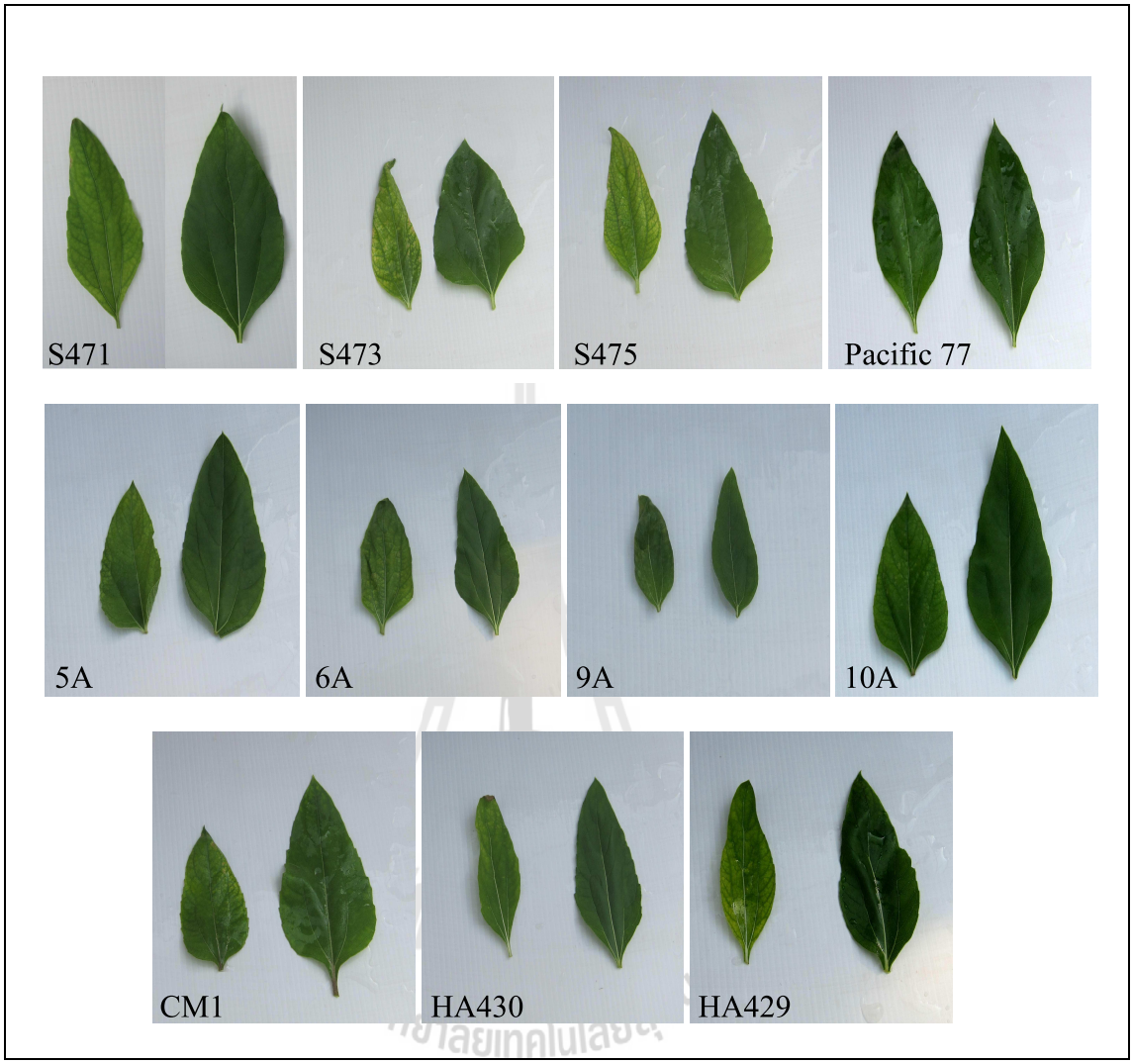

Figure 4.14 Morphology of the third pair of leaves from top of sunflower plants under drought stress (left) and control treatment (right).

4.2.2 Effects of progressive drought stress on proline content, malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) and antioxidant enzyme activities

Understanding plant responses to drought stress is essential, and there is a need to know possible biochemical mechanisms and drought tolerance for the improvement of crops.

4.2.2.1 Proline content

Data on effect of drought stress on accumulated proline content are presented in Table 4.4. Under drought stress, plants accumulated proline at higher levels and the maximum PROSI value was found in sunflower var. S473 (269.52%), while the minimum value was in 10A (141.53%). Accumulation of proline is a widespread plant response to environmental stresses, including drought and salt stress. Plants under drought stress may survive by, among other mechanisms, maintaining cell turgor and reducing evaporative water loss by accumulating compatible solutes (Yancey *et al.*, 1982). In this study, the increase in accumulation of free proline in plant leaves was similar to those of Stewart and Lee (1974) and Cechin *et al.* (2006). Slama *et al.* (2007) reported that proline is regarded as a source of energy, carbon and nitrogen for recovering tissues under water deficit.

Table 4.4 Mean comparisons of sunflower plants on biochemical stress indices under drought stress.

Genotypes	PROSI	H_2O_2SI	MDASI	SODSI	CATSI	APXSI
S471	201.19bc	281.89ab	162.19ab	170.24acb	188.23a	149.13a
S473	269.52a	352.79a	193.85a	219.84a	223.23a	214.68a
S475	244.75ab	268.76ab	160.96ab	199.32ab	195.28a	300.91a
Pacific77	189.85bc	176.72b	159.95ab	138.58bc	138.30a	158.12a
5A	160.96c	266.41ab	145.48b	140.54bc	135.31a	178.47a
6A	177.69c	185.63ab	157.38ab	169.90abc	162.36a	146.66a
9A	191.09bc	198.41ab	132.18b	126.79c	141.94a	148.64a
10A	141.53c	241.06ab	147.73b	123.30c	178.68a	136.98a
CM1	184.60bc	244.63ab	142.79b	140.68bc	127.43a	147.45a
HA430	172.05c	177.39b	164.70ab	174.10abc	174.88a	213.89a
HA429	167.97c	214.90b	160.15ab	170.41abc	153.68a	146.09a
Mean	191.02	237.14	157.03	161.25	165.39	176.46

Means with the same letter(s) in each column are not significantly different ($p < 0.05$).

PROSI: proline stress index, MDASI: malondialdehyde stress index, H_2O_2SI: hydrogen peroxide stress index, SODSI: superoxide dismutase stress index, CATSI: catalase stress index, and APXSI: ascorbate peroxidase stress index.

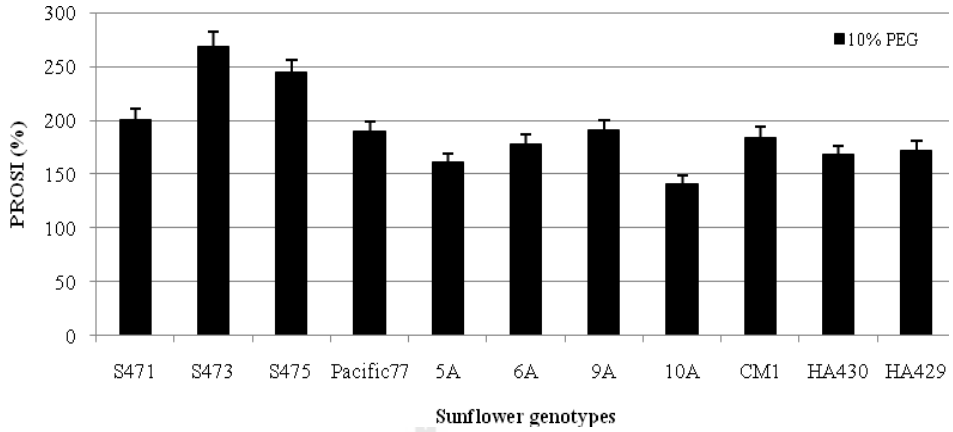

Figure 4.15 Effect of drought stress on proline stress index (PROSI) in eleven sunflower genotypes. The bars indicate SE.

4.2.2.2 Oxidative stress: malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

One of the biochemical changes occurring when plants are subjected to stress condition is the accumulation of reactive oxygen species (ROS) which are inevitable by products of normal cells. Plants have evolved several mechanisms that allow perceiving the stresses and rapidly regulating their physiology and metabolism to cope them. Acclimation of plants to drought is considered to promote antioxidants defense systems to face the increased levels of reactive oxygen species, which in turn, cause membrane damage by lipid peroxidation and indicated by MDA content, which is one main parameter for evaluating membrane oxidation extent and are toxic for the cells (Shao *et al*., 2005).

The occurrence of oxidative stress induced by drought stress was monitored by determining oxidative stress index such as H_2O_2, and by analyzing membrane damage through measurement of MDA levels. After drought treatment,

H_2O_2 and MDA were increased in all genotypes relative to control. The sunflower plants growing in 10% PEG-induced stress media significantly accumulated high MDA content as expressed in term of MDASI in the range of 114.85% - 246.63% over control. Enhanced MDA content indicated lipid peroxidation in response to PEG-induced oxidative stress. Under drought stress, the highest MDASI value was recorded in sunflower var. S473 (193.85%), while the lowest was found in sunflower line 9A (132.18%) (Table 4.4 and Figure 4.16). These observations are in conformity with an earlier observation of Zhang and Kirkram (1996) that reported MDA accumulation in sunflower and sorghum seedlings in response to drought while PEG increased lipid peroxidation in sunflower, but had no effect in sorghum. Furthermore, Bailly *et al*. (1996) reported that sunflower seed deterioration during accelerated aging was closely related to lipid peroxidation.

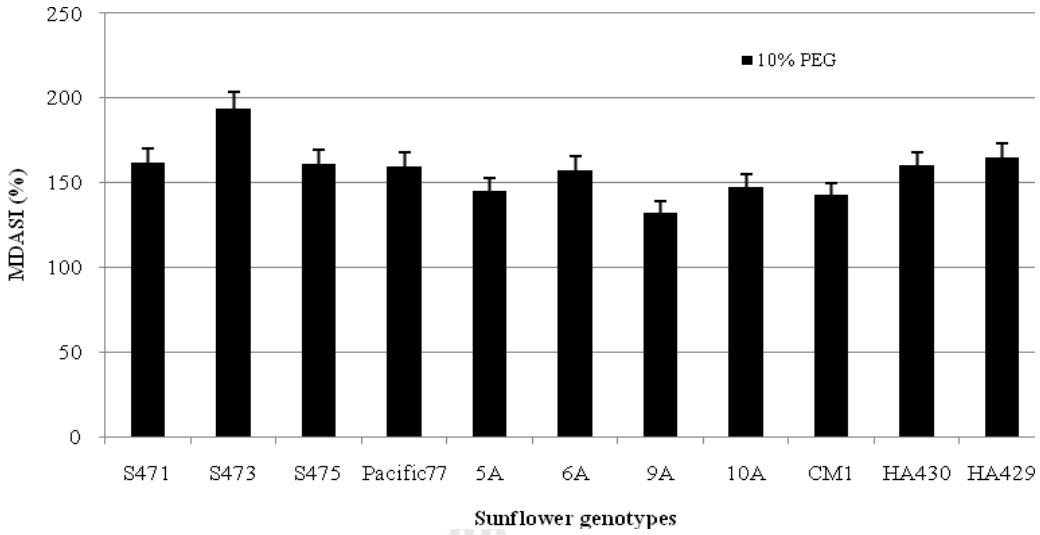

Figure 4.16 Effect of drought stress on accumulation of malondialdehyde stress index (MDASI) in sunflower plants. The bars indicate SE.

The plants growing in PEG-contaminated media significantly enhanced H_2O_2 content as expressed in term of H_2O_2SI (124.62% - 411.73 %) over control. The maximum H_2O_2SI value was recorded in sunflower var. S473 (352.79%), while the minimum was found in var. Pacific 77 (176.72%) (Figure 4.17). Such a result is in agreement with earlier studies by Lunar *et al*. (2005) who reported that induced H_2O_2 accumulation correlated with decreases in soil water content in wheat.

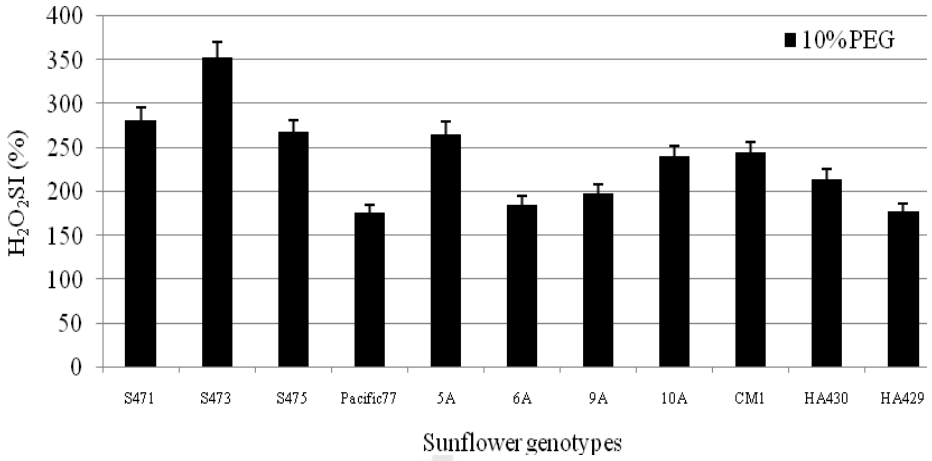

Figure 4.17 Effect of drought stress on hydrogen peroxide stress index (H_2O_2SI) in sunflower plants. The bars indicate SE.

4.2.2.3 Antioxidant enzymes: Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX)

The experimental findings showed that there was a difference among antioxidant enzyme activities (SOD, CAT and APX) in control and drought conditions in hydroponic culture as shown in Table 4.4. Higher activities of all three antioxidant enzymes were observed in response to PEG-induced drought stress. SOD is a family of metalloenzymes that catalyze the dismutation of O_2^- to hydrogen peroxide and oxygen gases. It has four major of SODs (MnSOD, FeSOD, NiSOD and Cu/ZnSOD), and plays an important role in protection. SOD responds to a variety of environmental stresses and plants acquire tolerance to them by increasing the SOD activity, as well as the activity of H_2O_2 detoxifying enzymes (Foyer and Halliwell, 1976). As shown in Figure 4.18, SOD activity index in leaves of sunflower plants increased in the range of (123.30 - 219.84%). The highest SODSI was detected in sunflower genotype S473 (219.84%), while the lowest was in genotype 10A

(123.30%). Sod activity in sorghum and sunflower were decreased by drought (Zhang and Kirkram, 1996).

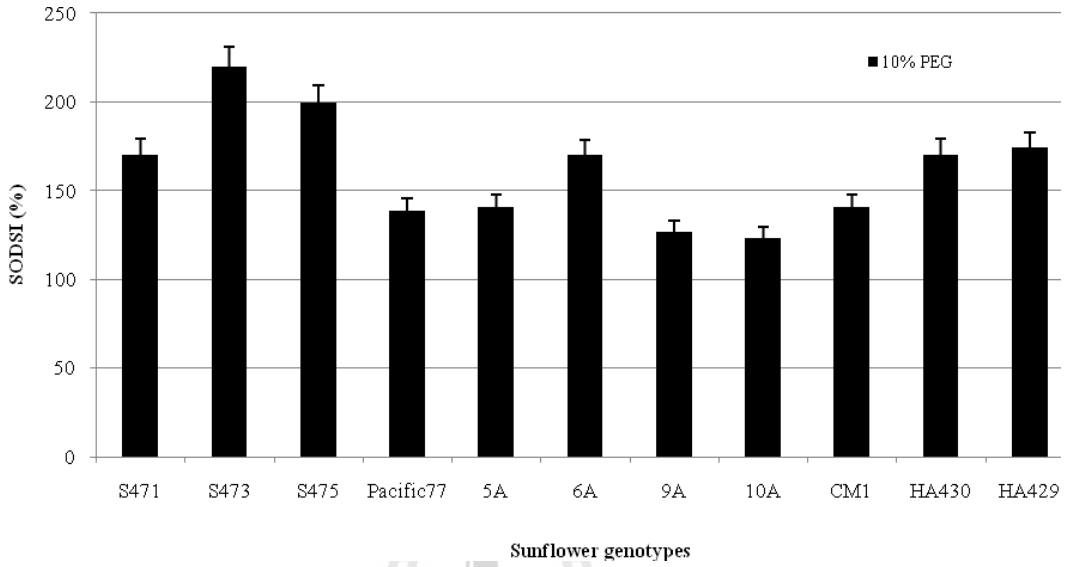

Figure 4.18 Effect of drought stress on superoxide dismutase stress index (SODSI) in sunflower plants. The bars indicate SE.

Catalase is an enzyme that scavenges hydrogen peroxide and converts it to water and molecular oxygen. In plants, catalase is found predominantly in glyoxysomes where it functions chiefly to remove H_2O_2 formed in photorespiration or in ß- oxidation of fatty acid in the glyoxysomes (Date *et al*., 2000). Increase in CAT activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism (Katsuhara *et al*., 2005). The experimental result showed that drought stress enhanced the activities of CAT and APX in sunflower plants. The CATSI was the highest in leaf tissues of sunflower var. S473 (223.23%), while the lowest was found in var. CM1 (127.43%) (Figure 4.19).

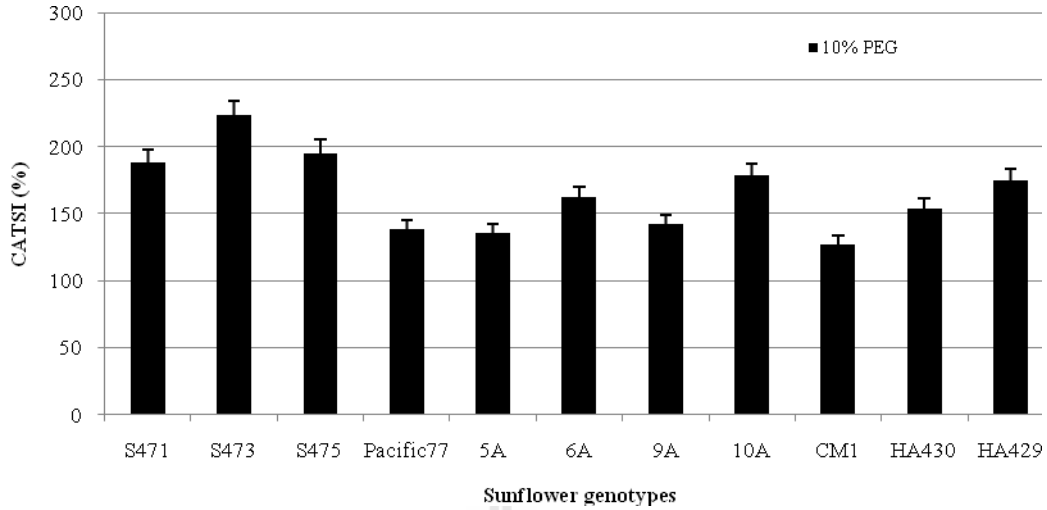

Figure 4.19 Effect of drought stress on catalase stress index (CATSI) in sunflower plants.

Figure 4.20 describes the effect of drought stress on the APXSI in sunflower seedlings that was grown in hydroponic culture. Sunflower var. S475 showed the highest APXSI (300.91%), while the lowest was indicated in line 10A (136.98%). Catalase (CAT) and ascorbate peroxidase (APX) are the major enzymatic cellular scavengers of H_2O_2 (Noctor and Foyer, 1998). The drought stress has activated unbalance of H_2O_2 in the cells to protect the cell from H_2O_2 induced oxidative stress, plants therefore synthesized APX for dismutation the H_2O_2 (Wakamatsu *et al*., 2008).

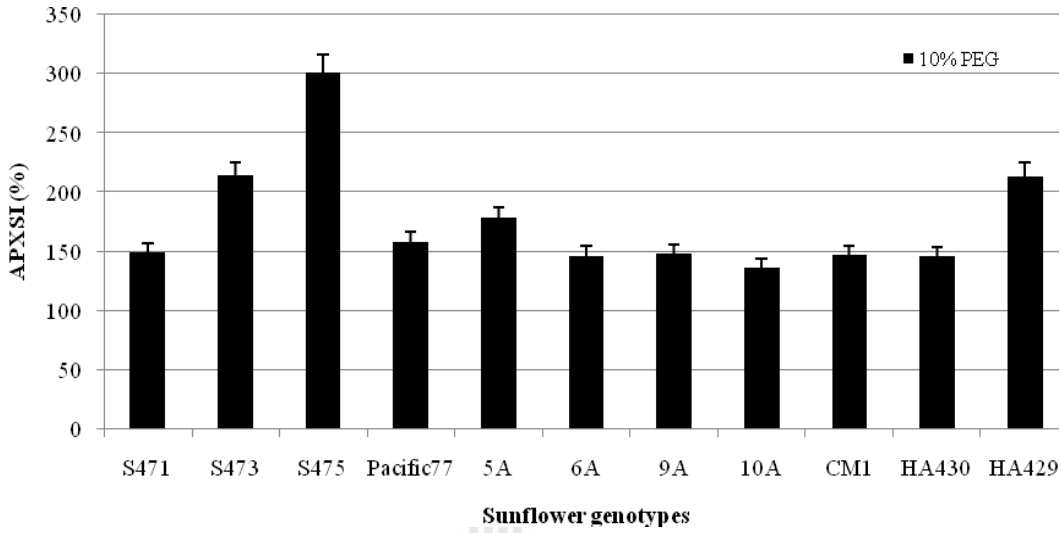

Figure 4.20 Effect of drought stress on ascorbate peroxidase stress index (APXSI) in sunflower plants. The bars indicate SE.

4.3 Evaluation of sunflower genotypes for drought tolerance based on morphological, physiological and biochemical parameters

Effects of drought stress on sunflower plants at germination and seedling growth stages were evaluated. All sunflower genotypes under stressed treatment varied dramatically in response to drought treatment in terms of morphological, physiological and biochemical parameters.

4.3.1 Determination of sunflower genotypes for drought tolerance based on principal component analysis (PCA) and clustering method

To better understand the relationships and similarities among the drought stress in sunflower genotypes, principle component analysis (PCA) based on the rank correlation matrix was used. The main advantage of using PCA over cluster analysis is that each statistics can be assigned to one group.

In experiment I, eight sunflower genotypes were determined their drought tolerance in terms of GSI, PHSI, RLSI, DMSI and RWCSI at the germination and establishment of seedling growth stage. The relationships among different indices using PCA are displayed in a biplot of Dim 1 and Dim 2 (Figure 4.21). The cosine of the angle between the vectors of two indices approximates the correlation coefficients between them, suggesting that the angles are informative enough to allow a whole picture about the interrelationships among the morpho-physiological indices. G2 indices were positively correlated (an acute angle) whereas G1 had weak correlation with G3. Obviously the PCA revealed two distinct groups of all genotypes, and the first group was considered as drought tolerant genotypes: S473, S471, S475 and

Pacific 77, while the other was considered as drought sensitive genotypes: 10A, 9A, 6A and 5A (Figure 4.22).

Similarly clustering was made to categorize drought indices into components for the understanding of the share components that contribute to major variation in the study. Cluster analysis based on morpho-physiological parameters as GSI, PHSI, RLSI, DMSI and RWCSI divided sunflower plants into two groups. The first group included genotypes from S471, S473, S475 and Pacific 77, while the second group included genotypes: 5A, 6A, 9A and 10A (Figure 4.23).

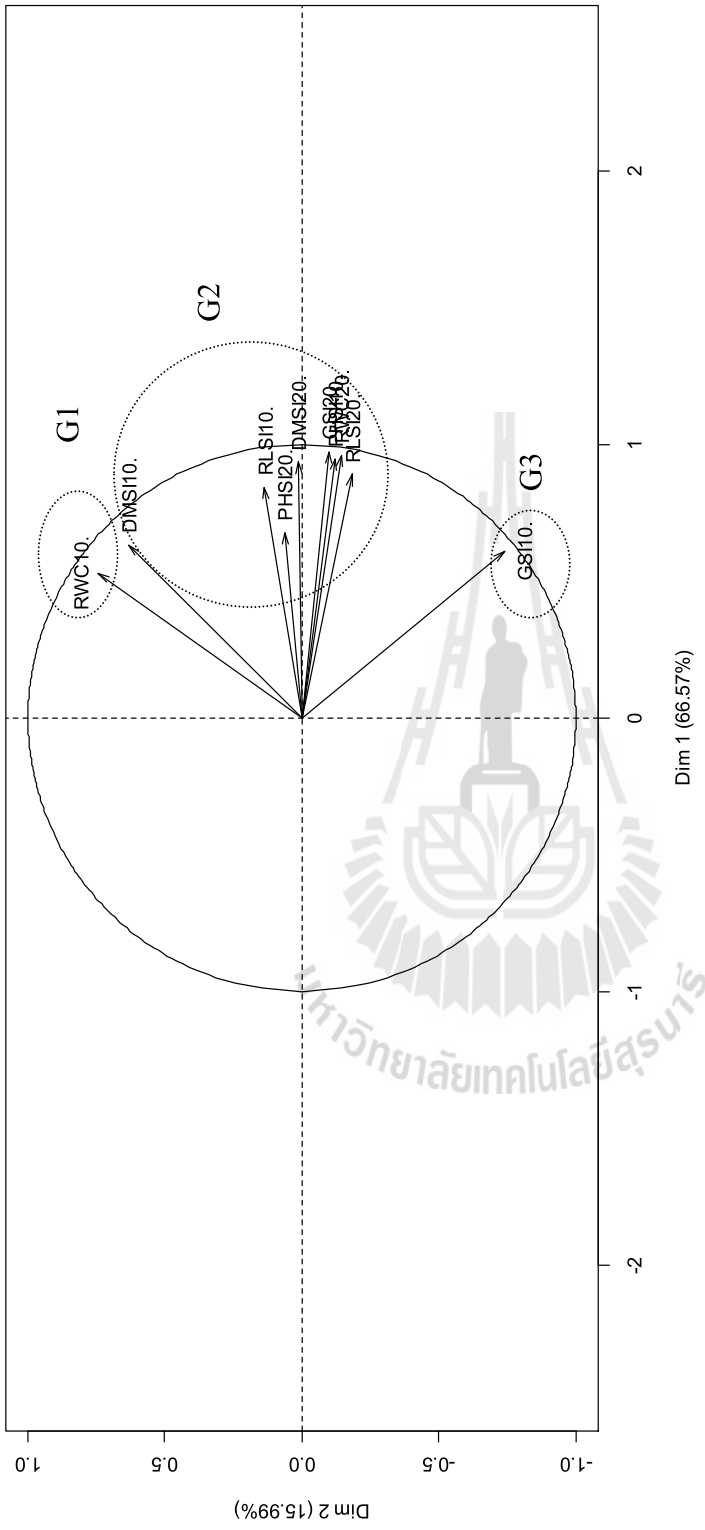

Figure 4.21 Biplot analysis of morpho-physiological indicators of drought tolerance in sunflower plants in Petri dish bioassays.

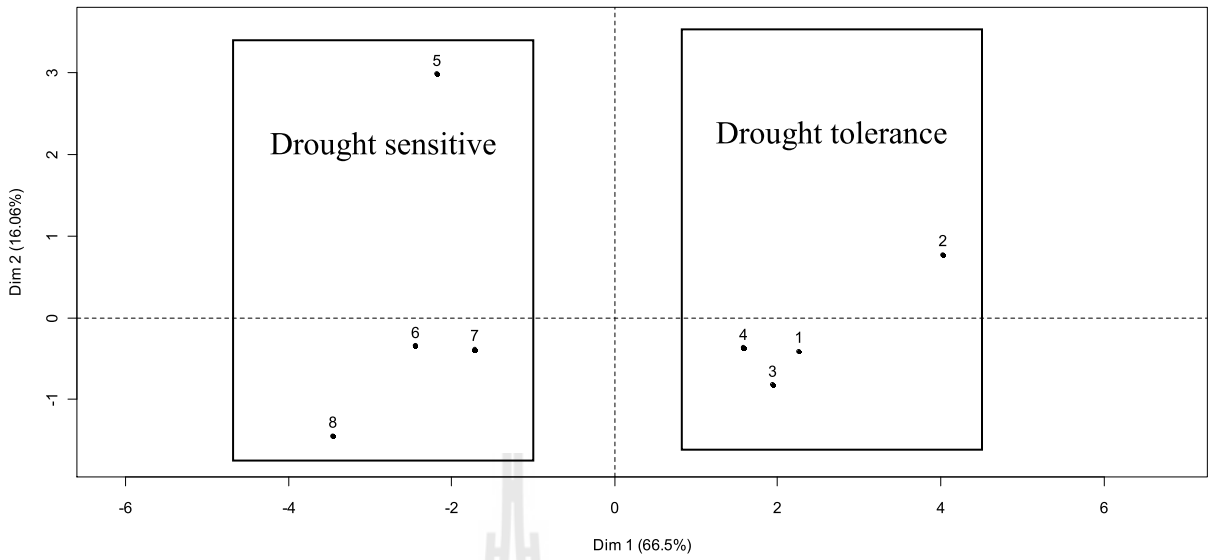

Figure 4.22 Principal component analysis (PCA) of sunflower plants at germination and establishment of seedling growth stage under water stress (number inside: 1 = S471, 2 = S473, 3 = S475, 4 = Pacific 77, 5 = 5A, 6 = 6A, 7 = 9A, 8 = 10A).

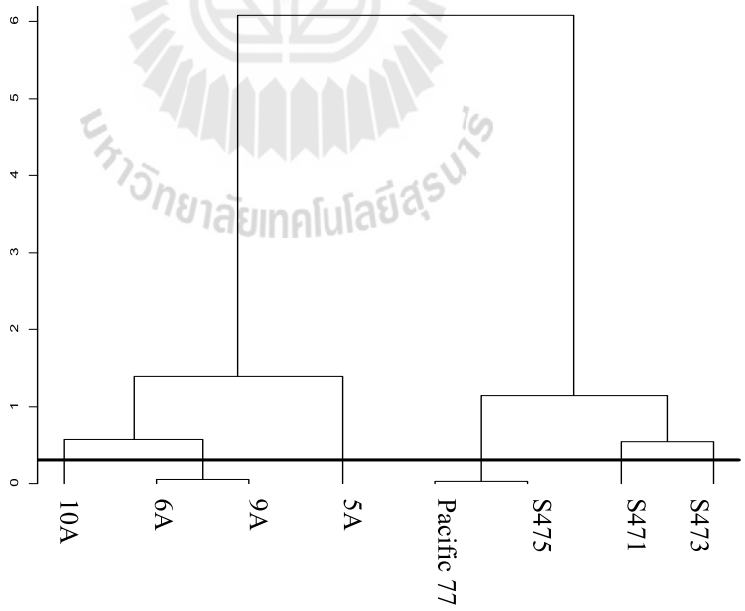

Figure 4.23 Dendrogram of cluster analysis of eight sunflower genotypes based on all morpho-physiological characters standardized to mean 0 and standard deviation = 1.

In experiment II, eleven sunflower genotypes were determined in term of PHSI, RLSI, DMSI, RWCSI, SCSI, LNSI, PROSI, MDASI, H_2O_2SI, SODSI, CATSI and APXSI of seedlings using PCA. The relationships among different indices using PCA are displayed in a biplot of Dim 1 and Dim 2 (Figure 4.24). G2 indices were positively correlated (an acute angle), the same conclusion was obtained for the G1 indices. Weak correlation (almost right angle) was observed between G1 with G2 and G2 with G3 indices. In contrast, G1 was negatively correlated with G3 indices (obtuse angle). As the result of this analysis, the PCA revealed two distinct groups of all genotypes as shown in Figure 4.23. The first group was defined as drought tolerant genotypes: S473, S471, S475 and pacific 77, while the other as sensitive genotypes: HA430, HA429, CM1, 10A, 9A, 6A and 5A. The results from experiment II are in agreement with those obtained in the first experiment.

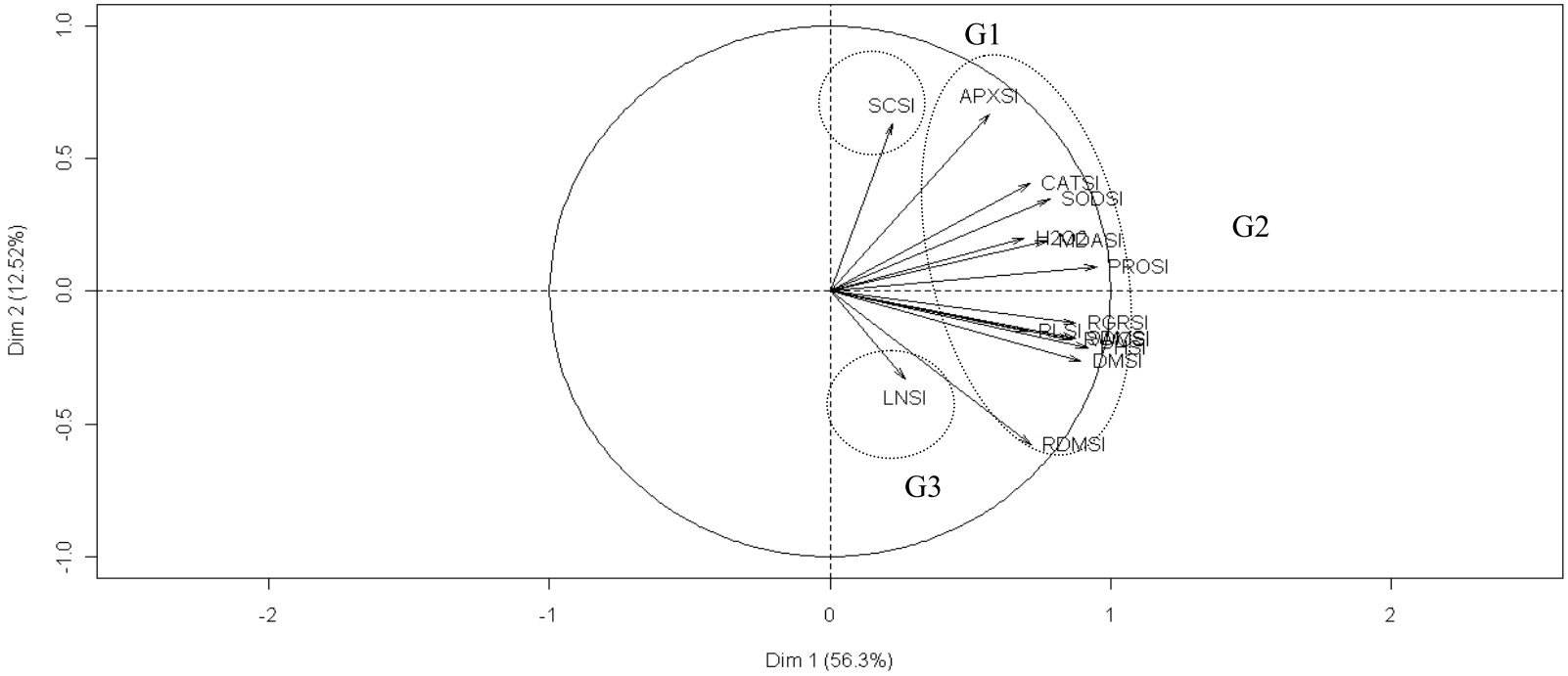

Figure 4.24 Biplot analysis of morpho-physiological and biochemical indicators of drought tolerance in sunflower plants under hydroponic culture.

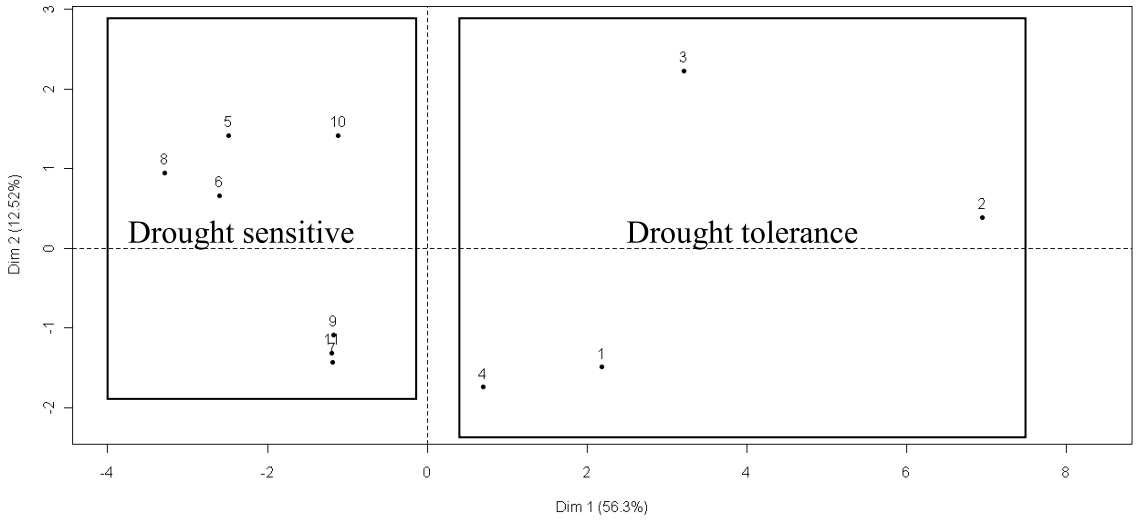

Figure 4.25 Principal component analysis (PCA) for morpho-physiological and biochemical characters of eleven sunflower genotypes under water stress (number inside: 1 = S471, 2 = S473, 3 = S475, 4 = Pacific 77, 5 = 5A, 6 = 6A, 7 = 9A, 8 = 10A, 9 = CM1, 10 = HA430 and 11 = HA429).

Figure 4.26 shows a dendrogram with cluster analysis based on all parameters. The result classified sunflower plants into two groups. The first group included genotypes from S471, S473, S475 and pacific 77, while the second group included genotypes 5A, 6A, 9A, 10A, CM1, HA429 and HA430.

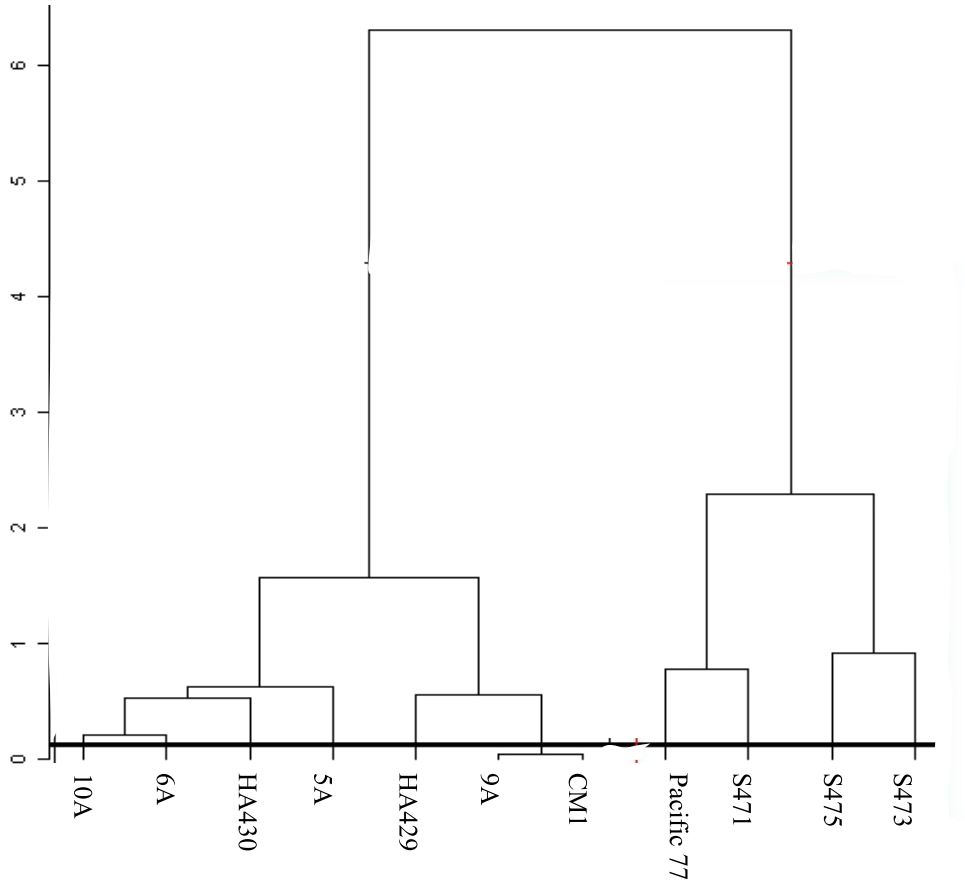

Figure 4.26 Dendrogram of cluster analysis based on morpho-physiological and biochemical characters of sunflower plants in hydroponic culture standardized to mean 0 and standard deviation = 1.

4.3.2 Determination of sunflower genotypes for drought tolerance based on ranking method

The identification of drought tolerance genotypes based on single criterion might be contradicted. The rank method was used to determine on overall judgment. All indices, mean rank and standard deviation of rank of all drought tolerance criteria were calculated, rank sum (RS) and the final rank were shown in Table 4.5, 4.6 and 4.7.

In experiment I, based on all criteria in terms of morpho-physiological indices the most desirable drought tolerant genotypes were identified and found that genotypes: S473 (RS = 1.97), followed by S471 (RS = 3.47), S475 (RS = 3.78) and Pacific 77 (RS = 4.47) were the most drought tolerant genotypes, respectively. While genotypes: 5A (RS = 7.37), 10A (RS = 7.24), 6A (RS = 6.98) and 9A (RS = 5.22) were the most sensitive to drought, respectively (Table 4.5).

In experiment II, the ranking method based on criteria in terms of morpho-physiological indices showed that genotypes: S473 (RS= 2.53), followed by S475 (RS = 4.38), S471 (RS = 6.69) and Pacific 77 (RS = 7.42) were the most drought tolerant genotypes, respectively. While genotypes: 5A (RS = 10.87), 6A (RS = 10.80), 10A (RS = 10.73), HA429 (RS = 10.13), HA430 (RS = 9.16), CM1 (RS = 8.20) and 9A (RS = 7.96) were the most sensitive drought genotypes, respectively (Table 4.6).

Furthermore, based on all criteria in term of morphological, physiological and biochemical indices using the ranking method, it showed that genotypes: S473 (RS = 2.21), followed by S475 (RS = 4.01), S471 (RS = 5.98) and CM1 (RS = 9.01) were the most drought tolerant genotypes, respectively. While genotypes: 10A (RS = 11.07), 5A (RS = 10.56), 6A (RS = 10.10), HA429 (RS = 9.59), HA430 (RS = 9.34),

Pacific 77 (RS = 9.32) and 9A (RS = 9.10) were the most sensitive drought genotypes, respectively (Table 4.7).

Overall, the results based on morphological, physiological and biochemical parameters using the ranking method at germination and seedling growth stages under laboratory conditions were identified. In consideration to all indices, genotypes: S473, S471, S475 and Pacific 77 showed the best rank mean and low standard deviation of ranks in stress conditions, hence they were identified as the drought tolerant genotypes, while genotypes 5A, 6A, 9A, 10A, HA429, HA430 and CM1 as the drought sensitive genotypes. The ranking methods have been used for screening quantitative indicators of drought tolerance in wheat (Mohammadi *et al*., 2011) and in rye (*Secale cereale* L.) (Farshadfar *et al*., 2003).

Table 4.5 Ranks (R), ranks mean ($\overline{R}$), standard deviation of ranks (SDR) and rank sum (RS) on morpho-physiological parameters of seedling indicators of drought tolerance in Petri dish bioassays.

Genotypes	GSI				PHSI				RLSI				DMSI				RWCSI				$\overline{R}$	SDR	RS	Final rank
	10% PEG	R	20% PEG	R	10% PEG	R	20% PEG	R	10% PEG	R	20% PEG	R	10% PEG	R	20% PEG	R	10% PEG	R	20% PEG	R				
S471	79.95	2	41.49	2	54.76	3	23.52	2	59.10	3	36.87	5	73.81	2	64.23	2	79.95	2	58.63	2	2.5	0.972	3.47	2
S473	83.64	1	51.81	1	55.62	2	33.18	1	66.07	1	46.7	3	78.64	1	70.59	1	91.90	1	67.29	1	1.3	0.675	1.97	1
S475	60.90	3	35.72	4	43.72	4	12.01	3	63.13	2	59.13	1	69.36	3	40.91	3	76.21	3	44.62	3	2.9	0.876	3.78	3
Pacific 77	52.71	4	35.82	3	56.39	1	3.37	4	58.00	4	55.6	2	57.82	4	36.39	4	71.14	4	37.06	4	3.4	1.075	4.47	4
5A	15.07	8	0	6	9.62	8	0	5	47.23	7	0	6	53.33	6	0	6	60.63	6	0	6	6.4	0.966	7.37	8
6A	40.69	6	0	6	21.22	6	0	5	54.10	5	0	6	45.04	7	0	6	47.44	8	0	6	6.1	0.876	6.98	6
9A	49.97	5	15.38	5	25.31	5	0	5	54.10	5	38.63	4	56.89	5	9.78	5	64.84	5	5.58	5	4.9	0.316	5.22	5
10A	29.72	7	0	6	11.36	7	0	5	47.57	6	0	6	22.73	8	0	6	53.79	7	0	6	6.4	0.843	7.24	7

Table 4.6 Ranks (R), ranks mean ($\bar{R}$), standard deviation of ranks (SDR) and rank sum (RS) on morpho-physiological parameters of seedling indicators of drought tolerance in hydroponic culture.

Genotypes	PHSI	R	RLSI	R	DMSI	R	SDMSI	R	RDMSI	R	RWCSI	R	RGRSI	R	SCSI	R	LNSI	R	$\bar{R}$	SDR	RS	Final Rank
S471	74.86	2	59.99	4	75.06	2	79.15	2	63.78	3	73.35	2	67.38	5	70.04	10	66.25	6	4.0	2.69	6.69	3
S473	83.88	1	71.49	2	80.77	1	85.29	1	71.56	2	80.65	1	77.99	1	86.00	3	72.71	3	1.7	0.87	2.53	1
S475	73.64	3	62.86	3	65.46	3	67.42	3	61.23	5	71.99	3	70.79	2	91.77	1	66.55	5	3.1	1.27	4.38	2
Pacific 77	71.91	4	82.72	1	64.57	4	64.40	4	73.02	1	70.85	4	69.63	3	79.16	7	54.48	11	4.3	3.08	7.42	4
5A	64.38	9	55.17	5	41.35	11	38.47	11	46.19	8	68.50	9	57.90	9	90.45	2	60.40	8	8.0	2.87	10.87	11
6A	57.39	11	42.93	9	54.67	7	58.33	6	48.38	9	57.54	11	57.18	10	75.67	9	57.77	10	9.1	1.69	10.80	10
9A	67.81	8	41.03	10	60.03	5	61.16b	5	60.26	6	69.38	6	67.49	4	81.55	5	75.51	2	5.7	2.29	7.96	5
10A	59.05	10	37.48	11	46.35	10	48.22	9	43.68	10	61.64	10	61.47	7	81.07	6	58.38	9	9.1	1.62	10.73	9
CM1	70.86	5	53.35	6	56.51	6	54.97	8	62.93	4	69.72	5	56.33	11	81.99	4	69.81	4	5.9	2.32	8.20	6
HA430	68.58	7	50.20	7	51.36	8	55.09	7	40.63	11	68.57	8	58.39	8	78.81	8	63.22	7	7.9	1.27	9.16	7
HA429	69.25	6	46.18	8	48.29	9	46.01	10	57.60	7	68.82	7	62.17	6	68.48	11	82.42	1	7.2	2.91	10.13	8

Table 4.7 Ranks (R), ranks mean ($\overline{R}$), standard deviation of ranks (SDR) and rank sum (RS) on morpho-physiological and biochemical parameters of seedling indicators of drought tolerance in hydroponic culture.

Genotypes	PH SI	RL SI	DM SI	SDM SI	RDM SI	RWC SI	RGR SI	SC SI	LN SI	PRO SI	H_2O_2 SI	MDA SI	SOD SI	CAT SI	APX SI	$\overline{R}$	SDR	RS	Final Rank
S471	2	4	2	2	3	2	5	10	6	3	2	3	5	3	5	3.80	2.18	5.98	3
S473	1	2	1	1	2	1	1	3	3	1	1	1	1	1	2	1.47	0.74	2.21	1
S475	3	3	3	3	5	3	2	1	5	2	3	4	2	2	1	2.80	1.21	4.01	2
Pacific 77	4	1	4	4	1	4	3	7	11	5	11	6	9	9	10	5.93	3.39	9.32	6
5A	9	5	11	11	8	9	9	2	8	10	4	9	8	10	4	7.80	2.76	10.56	10
6A	11	9	7	6	9	11	10	9	10	7	9	7	6	6	8	8.33	1.76	10.09	9
9A	8	10	5	5	6	6	4	5	2	4	8	11	10	8	6	6.53	2.56	9.09	5
10A	10	11	10	9	10	10	7	6	9	11	6	8	11	4	11	8.87	2.20	11.07	11
CM1	5	6	6	8	4	5	11	4	4	6	5	10	7	11	7	6.60	2.41	9.01	4
HA430	7	7	8	7	11	8	8	8	7	8	10	2	3	5	3	6.80	2.54	9.34	7
HA429	6	8	9	10	7	7	6	11	1	9	7	5	4	7	9	7.07	2.52	9.59	8

CHAPTER V

CONCLUSION

Drought stress is one of the major constraints to sunflower production and yield stability especially in arid and semi-arid areas, and is getting worse as the climate change worldwide. In this thesis study, sunflower genotypes were evaluated for their drought tolerance at germination and seedling growth stages under laboratory conditions either in Petri dish bioassays or in hydroponic culture. Morphological, physiological and biochemical parameters were examined and analyzed under drought stress conditions using PEG-6000 as a drought stimulator. Summary of conclusions follow:

At germination and establishment of seeding growth stage in Petri dish bioassays, all eight sunflower genotypes, namely S471, S473, S475, Pacific 77, 5A, 6A, 9A and 10A, showed a delay in seed germination time with the increasing of PEG concentrations and germination percentage decreased significantly under higher PEG concentrations, resulting in the decrease in germination stress index (GSI). In addition, plant high stress index (PHSI), root length stress index (RLSI), dry matter stress index (DMSI) and relative water content stress index (RWCSI) were found to decrease under drought treatments, indicating that these indices are sensitive indicators to drought stress. Based on principle component analysis (PCA) of these morpho-physiological indices, the genotypes S473, S471, S475 and Pacific 77 were considered to be drought-tolerant, while 5A, 6A, 9A and 10 A were drought-sensitive.

Moreover, according to the mean rank and standard deviation of ranks of all laboratory drought tolerance criteria, the genotypes: S473 (RS= 1.97) followed by genotypes: S471 (RS= 3.47), S475 (RS= 3.78) and Pacific 77 (RS= 4.47) were identified as the most drought tolerant genotypes, respectively. While genotypes: 5A (RS= 7.37), 10A (RS= 7.24), 6A (RS= 6.98) and 9A (RS= 5.22) were the most sensitive to drought, respectively.

At seeding growth stage under hydroponic culture, eleven sunflower genotypes were included for drought tolerance screening. After 7 days of progressive drought treatment of 14 day-old seedlings using 10% PEG-6000 as an osmoticum, the PHSI, RLSI, DMSI, RWCSI, relative growth rate stress index (RGRSI), stomatal conductance index (SCSI) as well as leaf number stress index (LNSI) were found to decrease in drought stress. Based on principle component analysis (PCA) of these morpho-physiological indices, the genotypes: S473, S471, S475 and Pacific 77 were considered to be drought-tolerant, while 5A, 6A, 9A, 10A, CM1, HA430 and HA429 were drought-sensitive. According to the mean rank and standard deviation of ranks of all drought tolerance criteria, the genotypes: S473 (RS= 2.21) followed by S475 (RS= 4.01), S471 (RS= 5.98), and CM1 (RS= 9.01) were the most drought tolerant genotypes, respectively. While genotypes: 10A (RS= 11.07), 5A (RS= 10.56), 6A (RS= 10.09), HA429 (RS= 9.58), Pacific 77 (RS= 9.36), HA430 (RS= 9.34) and 9A (RS= 9.09) were the most sensitive to drought, respectively.

On the other hand, the proline content of the stressed leaves increased significantly under drought stress conditions. It was observed that there were differences in the proline levels among different genotypes. Drought tolerant genotypes such as S473, S471 and S475 accumulated more proline than sensitive genotypes such as 5A, 6A, 10A and CM1. These results suggest that an enhanced

proline level might be an important factor for stress adaptation.

When plants exposed to prolonged drought stress, oxidative damage due to the over production of reactive oxygen species (ROS) was unavoidable, as seen by increase in lipid peroxidation in term of malondialdehyde (MDA) content. In order to cope with continuous ROS production, sunflower plants have enhanced enzymatic and nonenzymatic antioxidants, which function as an extremely efficient cooperative system. According to the experimental results, activities of some ROS-scavenging enzymes, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were enhanced in all sunflower genotypes in response to drought stress. SOD is a major scavenger of O_2^- and its enzymatic action results in the formation of H_2O_2 and O_2. Higher H_2O_2 levels were also detected in all genotypes exposed to PEG-induced drought stress, indicating that O_2^- was scavenged by SOD activation. As a result of increased H_2O_2, CAT and APX, major enzymatic cellular scavengers of H_2O_2 molecule, were enhanced to remove the highly toxic H_2O_2 produced into water and molecular O_2. Therefore, drought acclimated plants showed increase in overall enzyme activities. These results suggest that increased activities of SOD, CAT and APX in leaves of stressed plants contribute to their enhanced drought tolerance. Drought tolerant genotypes such as S473, S475 and S471 had higher levels of antioxidant enzyme activities, whereas drought-sensitive genotypes: 5A, 6A, 9A and 10A had lower levels.

Overall, all sunflower genotypes in this study can be classified using the clustering method into 2 groups: S473, S471, S475 and Pacific 77 were the drought tolerance genotypes, whereas 5A, 6A, 9A, 10A, CM1, HA429 and HA430 were the drought sensitive genotypes. The variation in performance of sunflower genotypes determined by morphological, physiological and biochemical indices at germination

and seedling growth stages is a reliable and efficient procedure for screening sunflower genotypes for drought stress tolerance. Selection based on a combination of drought stress indices as well as PCA analysis may provide a more useful criterion for improving drought resistance of sunflower. It is worth to note that synthetic varieties: S471, S473 and S475 performed extremely well among all sunflower genotypes treated with drought stress, and should therefore be recommended for cultivation in dry land areas.

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APPENDICES

APPENDIX A

HYDROPONIC MEDIA

A.1 Hydroponic media (Hoagland and Arnon, 1950)

Stock solution A

$(NH_4)_2HPO_4$ (20-53-0)	1,450 g
$MgSO_4$	1,500 g
KNO_3	7,500 g
Micronutrient Solution	
$(NH_4)_2MoO_4$	1 g
H_3BO_3	30 g
$MnSO_2.4H_2O$	68 g
$ZnSO_4.7H_2O$	20 g
$CuSO_4.5H_2O$	5 g

Preparation of stock A: each chemical component was dissolved in water then poured to a tank, to make a volume 50 liters and pH 5.5-6.0 (adjusted with HNO_3 or NaOH). The solution was kept in a plastic container in the dark and stored at room temperature. Do not store at sunlight.

Stock solution B

$Ca(NO_3)_2.4H_2O$	6,700 g
Fe-EDTA	250 g

Preparation of stock B: each chemical component was dissolved in water and then poured to a tank, to make a volume 50 liter and pH 5.5-6.0 (adjusted with HNO_3 or NaOH). The solution was kept in a plastic container in the dark and stored at room temperature. Do not store in sunlight.

APPENDIX B

CHEMICAL REAGENTS

B.1 Chemical reagent solution for SOD extraction

0.1 M Sodium phosphate buffer (pH 7.5)

Disodium hydrogen phosphate (Na_2HPO_4)	17.79 g
Sodium dihydrogen phosphate (NaH_2PO_4)	15.60 g
Ethylene diamintetraacetic (EDTA)	0.19 g

Preparation: all components were weighted and then added to sterile distilled water, mixed thoroughly until dissolved and adjusted pH 7.5 and brought volume up to 1,000 mL. Solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes.

B.2 Chemical reagent solution for CAT extraction

0.1 M Potassium phosphate buffer (pH 7.5)

Dipostassium hydrogen phosphate (K_2HPO_4)	17.42 g
Postassium dihydrogen phosphate (KH_2PO_4)	13.61 g
Ethylene diamintetraacetic (EDTA)	0.15 g

Preparation: all components were weighted and then added to sterile distilled water, mixed thoroughly until dissolved and adjusted to pH 7.5 and brought volume up to 1,000 mL. Solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes.

B.3 Reaction mixture for superoxide dismutase

1.5 M Sodium carbonate

Sodium carbonate 46.5 g

Preparation: dissolved 46.5 g of sodium carbonate in distilled water; made up to a volume 250 mL. Solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes.

200 mM methionine

Methionine 1.49 g

Preparation: dissolved 1.49 g of methionine in sterile distilled water; made up to a volume 50 mL.

2.25 mM Nitrotatrazolium blue (NBT)

NBT 18 mg

Preparation: dissolved 18 mg of NBT in sterile distilled water; made up to a volume 10 mL.

3 mM EDTA

EDTA 44 mg

Preparation: dissolved 44 mg of EDTA in sterile distilled water; made up to a volume 50 mL.

100 mM phosphate buffer

K_2HPO_4 8.71 g

KH_2PO_4 6.80 g

Preparation: dissolved both components in sterile distilled water; made up to a volume 1,000 mL.

60 µM riboflavin

Riboflavin	23 mg

Preparation: dissolved 23 mg of riboflavin in sterile distilled water; made up to a volume 1,000 mL.

B.4 Reaction mixture for catalase

0.1 mM phosphate (pH 7.0)

K_2HPO_4	4.35 g
KH_2PO_4	3.40 g

Preparation: dissolved both phosphate components in sterile distilled water; made up to a volume 1,000 mL.

2.75 mM H_2O_2

Preparation: diluted 0.85 mL of H_2O_2 with distilled water, made up a volume to 100 mL.

B.5 Chemical solution for APX extraction

0.1 M Phosphate buffer (pH 7.5)

K_2HPO_4	17.42 g
KH_2PO_4	13.6 g
0.5 mM EDTA	0.15 g
Ascorbic acid	0.35 g
5% PVP	50 g

Preparation: all components were weighted and then added to sterile distilled water, mixed thoroughly until dissolved and pH 7.5 (adjusted with KOH) and

then made up a volume to 1,000 mL. The solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes.

B.6 Reaction mixture of catalase

25 mM Phosphate buffer

K_2HPO_4	4.35 g
KH_2PO_4	3.40 g

Preparation: dissolved both components in sterile distilled water 1,000 mL.

0.1 mM EDTA

EDTA	29 g

Preparation: dissolved 29 g of EDTA in sterile distilled water 1,000 mL

1 mM H_2O_2: diluted H_2O_2 with sterile distilled water.

0.25 mM L-ascorbic acid

Ascorbic acid	11 mg

Preparation: dissolved 11 mg of AsA in sterile distilled water 1,000 mL.

B.7 Chemical reagents for proline extraction

3% Sulfosalicylic acid

Sulfosalicylic acid	15 g

Preparation: dissolved 15 g of sulfosalicylic acid in sterilized distilled water then made a volume to 500 mL.

B.8 Chemical reagents for MDA extraction

15% Trichloroacetic acid (TCA)	15 g
0.37% 2-Triobarbituric acid (TBA)	0.37 g
0.01% Butylate hydroxytoluene (BHA)	0.37 g
0.25N HCl	17.5 mL

Preparation: Dissolved all components in sterilize distilled water 52.5 mL then added 0.25N HCl 17.5 mL.

B.9 Chemical reagents for hydrogen peroxide extraction

0.1% (w/v) Trichloroacetic acid (TCA)

TCA 0.25 g

Preparation: dissolved 0.25 g of trichloroacetate acid in sterilized distilled water then made a volume to 100 mL.

10 mM K-phosphate buffer (pH 7.0)

K_2HPO_4 0.435 g

KH_2PO_4 0.340 g

Preparation: dissolved K_2HPO_4 and KH_2PO_4 in sterile distilled water; made up to a volume 250 mL.

1 M Potassium iodine (KI)

KI 41.503 g

Preparation: Dissolved potassium iodine in sterilize distilled water; made up to a volume 250 mL.

APPENDIX C

CALIBRATION CURVES

C.1 Standard curve of proline

Prepare the L-proline by diluting the 120 µg/L, L-proline stock with distilled water, following below the table.

Table C.1 Preparation of L-proline stock solutions.

Proline concentration ($\mu g\ L^{-1}$)	Standard of stock 0.12 µg/mL (mL)	Distilled water (mL)
20	1.7	8.3
40	3.3	6.7
60	5.0	5.0
80	6.6	3.4
100	8.3	1.7
120	10.0	0

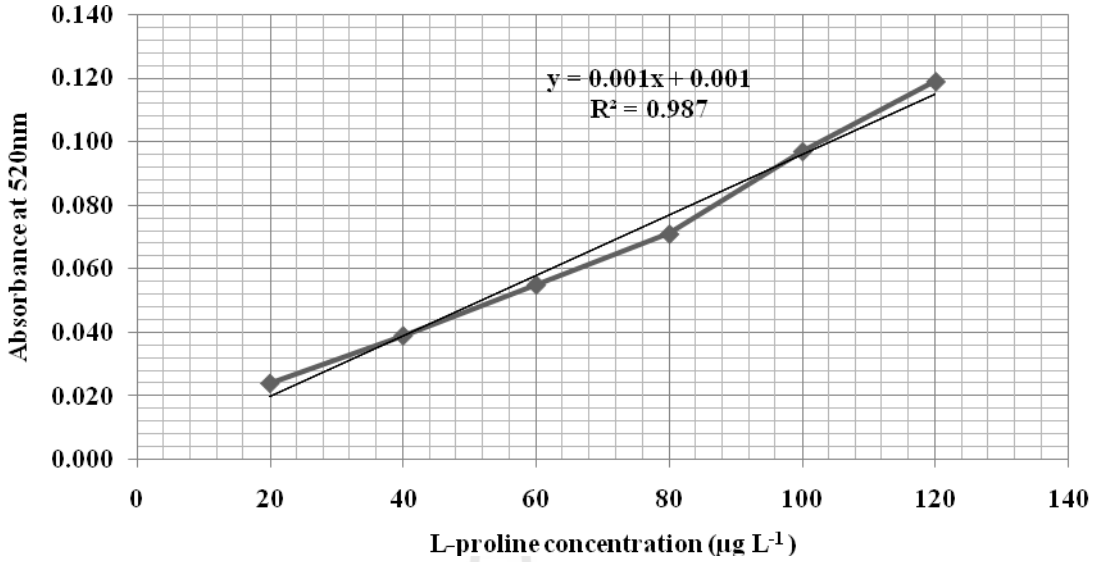

Figure C.1 Standard curve of L-proline content.

C.2 Standard curve of hydrogen peroxide

Prepare the hydrogen peroxide by diluting the 500 mmol/mL, the stock with distilled water, following below the table.

Table C.2 Preparation of hydrogen peroxide stock solutions.

H_2O_2 concentration (mmole L^{-1})	Standard of stock (mL)	Distilled water (mL)
50	1.0	9.0
100	2.0	8.0
200	4.0	6.0
300	6.0	4.0
400	8.0	2.0
500	10.0	0.0

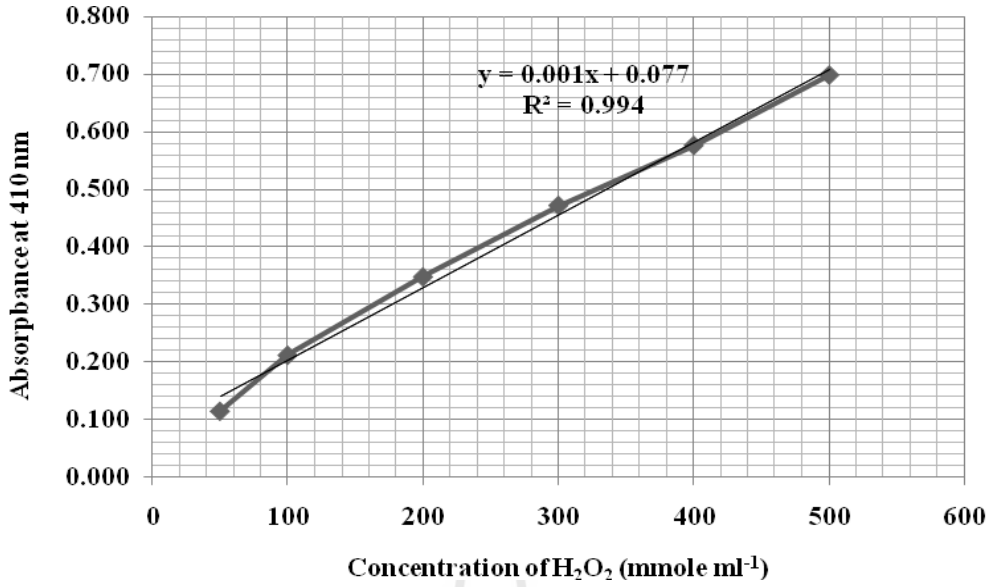

Figure C.2 Standard curve of hydrogen peroxide content.

APPENDIX D

STATISTICAL DATA

Table D.1 Analysis of variances for proline accumulation (PRO), malodialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) of sunflower genotypes exposed to 10% PEG-6000 concentration at seedling growth stage.

S.V.O	d.f.	Mean Square					
		PRO	MDA	H_2O_2	SOD	APX	CAT
Genotypes (G)	10	6062.89	1952.10	30507.51	2659.05	540.05*	409.02*
PEG Concentration (C)	1	93158.67	50987.82	12486.12	21924.86	7234.24	6143.18
G X C	10	2162.74	1092.19	33610.79	813.61*	269.99*	256.63*
Error	110	237.75	248.87	4224.21	268.88	546.5	161.98
F-Test		ns	Ns	*	ns	*	*
C.V. (%)		39.23	28.88	34.06	15.28	24.00	25.66

*Significant at 5% levels of probability, respectively. ns, not significant.

Table D.2 Analysis of variances for dry weight (DW), plant height (PH), relative water content (RWC), stomatal conductance (SC) and relative growth rate (RGR) of sunflower plants exposed to 10% PEG concentration at seedling growth stage.

		Mean Square				
S.V.O	d.f.	DW	PH	RWC	SC	RGR
Genotypes (G)	10	4.639	378.47	141.401	0.068*	0.001*
PEG Concentration(C)	1	36.911	5649.24	18316.454	3.317	0.032
G X C	10	0.584*	21.02*	61.975*	0.060*	0.00004916*
Error	88	0.602	8.49	29.978	0.191	0

* = Significant at 0.05 probability level.

Table D.3 Mean comparisons of sunflower plants on proline (PRO), malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and antioxidant enzyme activities under drought stress.

Genotypes	Mean square											
	PRO		MDA		H_2O_2		SOD		CAT		APX	
	Control	10%PEG	Control	10%PEG	Control	10%PEG	Control	10%PEG	Control	10%PEG	Control	10%PEG
S471	67.95a	133.78bc	53.25e	131.33ab	183.80bcd	482.77b	57.43ab	85.93bc	24.25a	45.43abc	46.67a	62.25a
S473	63.43a	173.13a	94.60ab	146.68a	137.67bcd	566.83a	56.90ab	110.30a	28.85a	55.22a	45.03a	68.8a
S475	57.33abc	145.95b	87.18abc	122.52bc	173.373bcd	429.87bc	46.03b	96.65ab	25.40a	52.70ab	24.58a	58.97a
Pacific77	64.38a	118.13cd	70.37cde	120.73bcd	190.530abc	322.23de	59.17ab	77.20bcd	29.97a	32.98cd	40.97a	57.32a
5A	49.00bcd	73.33e	70.28cde	101.80de	134.22bcd	255.37e	68.00a	73.78bcd	22.23a	24.62c	27.85a	43.42a
6A	31.57e	78.37e	64.95de	105.92cde	160.86bcd	297.00de	27.05c	55.20bc	23.37a	27.30d	41.75a	49.95a
9A	60.75d	109.8d	70.75cde	90.38e	261.20a	325.50de	66.82a	78.13bcd	27.58a	35.78bcd	37.68a	46.69a
10A	46.10e	62.87e	103.77a	121.68bc	149.77bcd	301.07de	64.97a	75.27bcd	19.08a	39.77abcd	54.05a	58.93a
CM1	57.42abc	100.98d	78.32cde	89.98e	110.273d	360.10ce	60.07ab	74.07bcd	25.87a	33.75cd	46.68a	57.30a
HA430	71.47a	120.95cd	73.27cde	124.70bc	120.89cd	314.18de	28.52c	50.20e	20.83a	34.37bcd	50.78a	52.40a
HA429	36.02de	72.57e	81.80bcd	125.18bc	208.48ab	315.82e	22.97c	64.72cde	17.37a	32.30cd	36.87a	59.77a
Mean	55.04	108.17	77.14	115.62	166.46	360.98	50.72	76.50	24.07	37.66	41.17	55.98

Means with the same letter superscript is not significantly different at 5% level by DMRT.

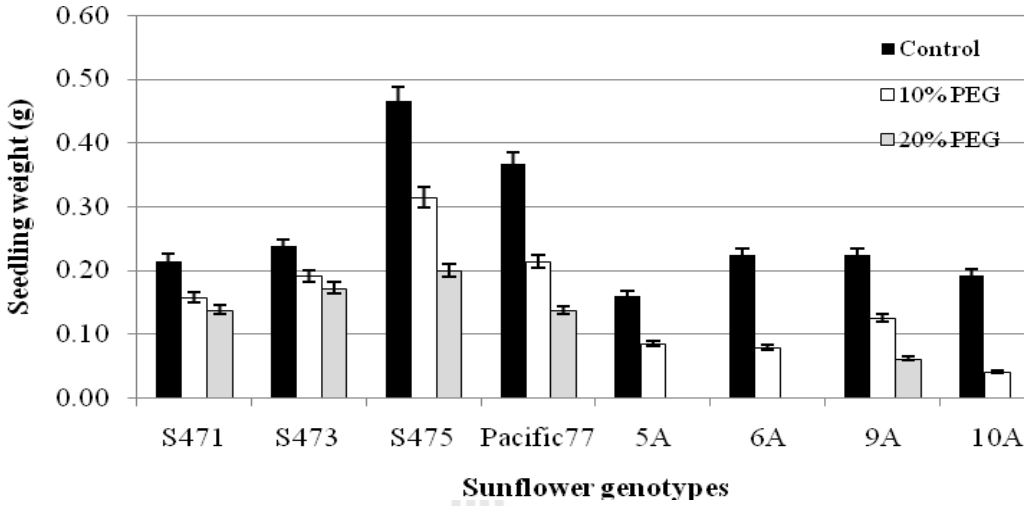

Figure D.1 Effect of drought stress on seedling weight in sunflower plants in Petri dish bioassays. The bars indicate SE.

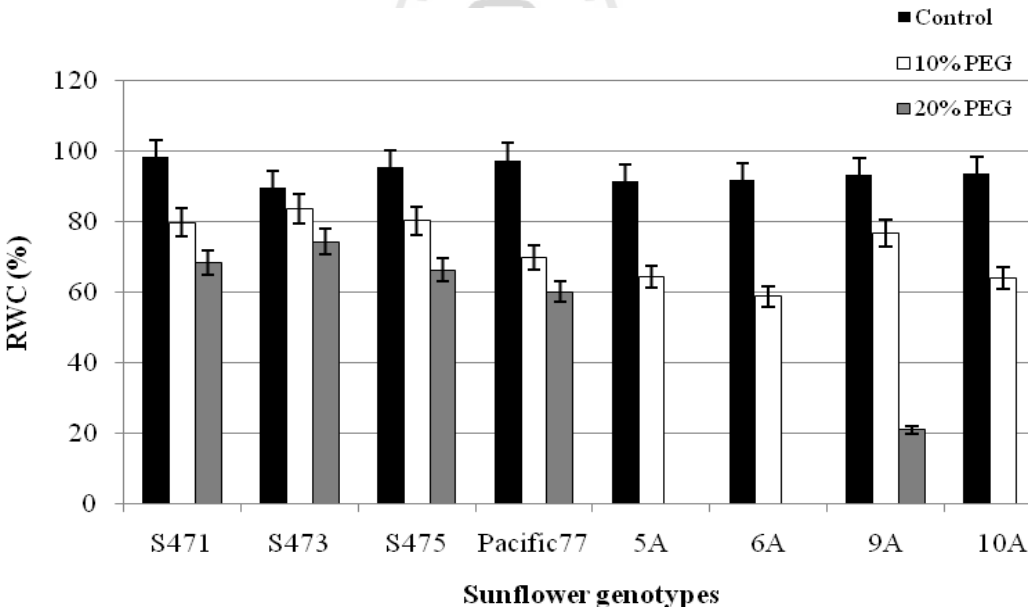

Figure D.2 Effect of drought stress on relative water content (RWC) in sunflower plants Petri dish bioassays. The bars indicate SE.

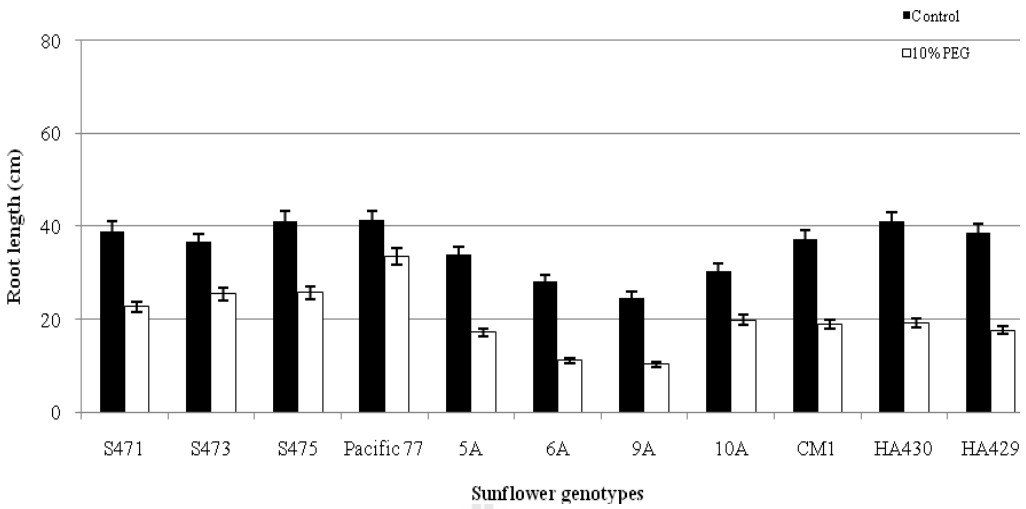

Figure D.3 Effect of drought stress on root length in sunflower plants under hydroponic culture. The bars indicate SE.

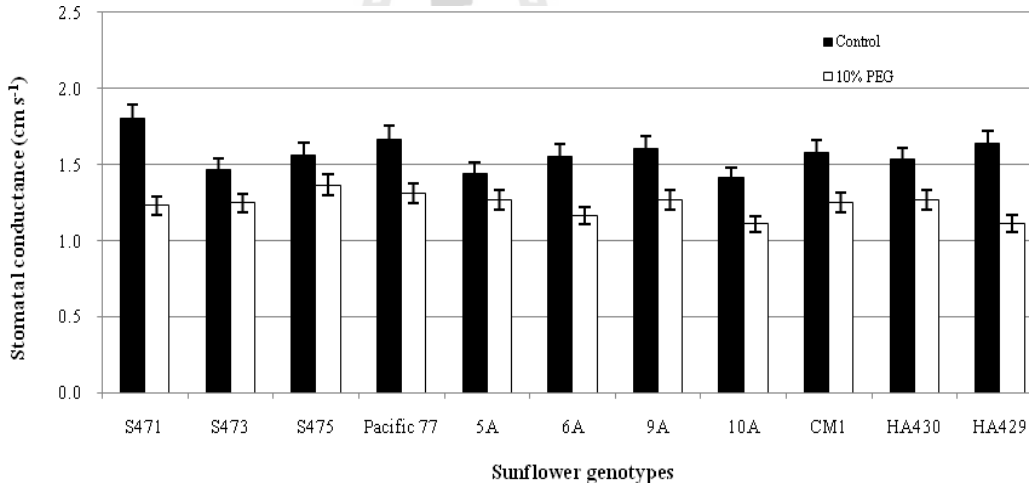

Figure D.4 Effect of drought stress on stomatal conductance in sunflower plants under hydroponic culture. The bars indicate SE.

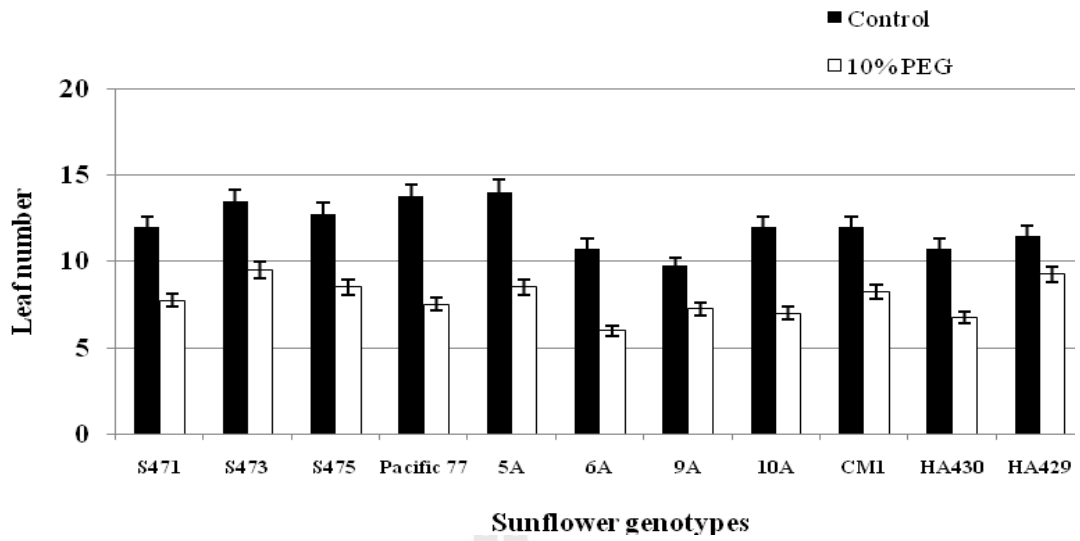

Figure D.5 Effect of drought stress on leaf number in sunflower plants under hydroponic culture. The bars indicate SE.

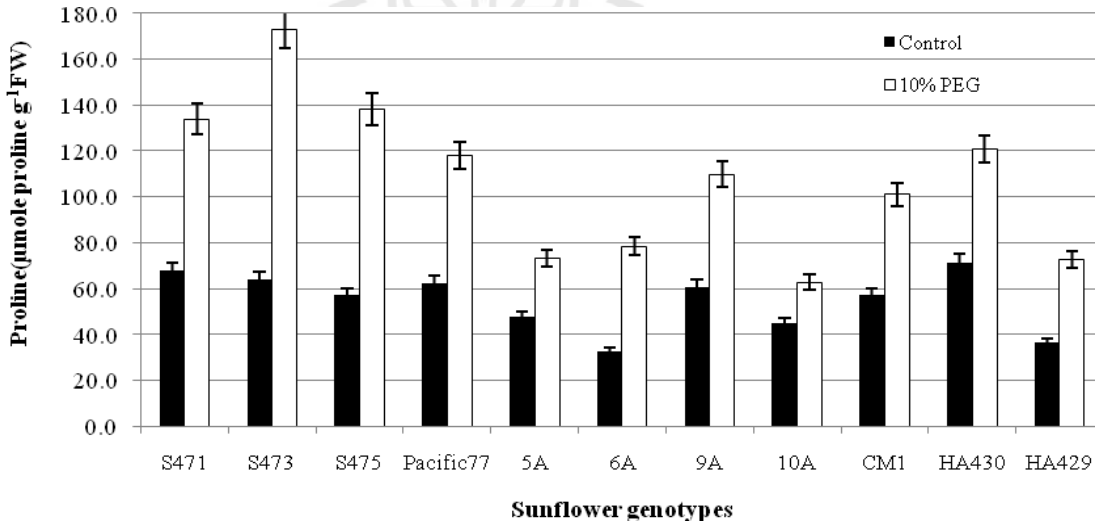

Figure D.6 Effect of drought stress on proline accumulation in sunflower plants under hydroponic culture. The bars indicate SE.

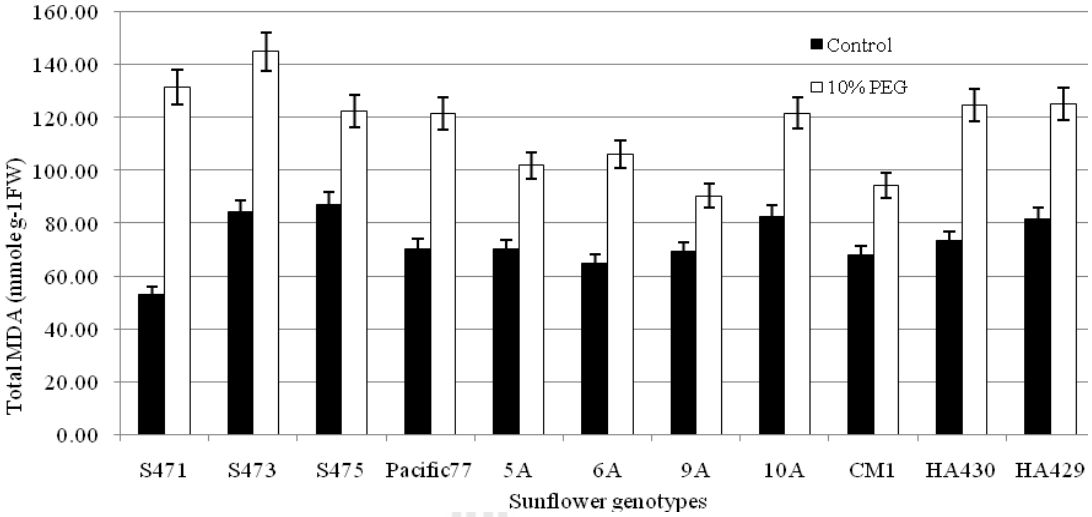

Figure D.7 Effect of drought stress on accumulation of malondialdehyde (MDA) in sunflower plants under hydroponic culture. The bars indicate SE.

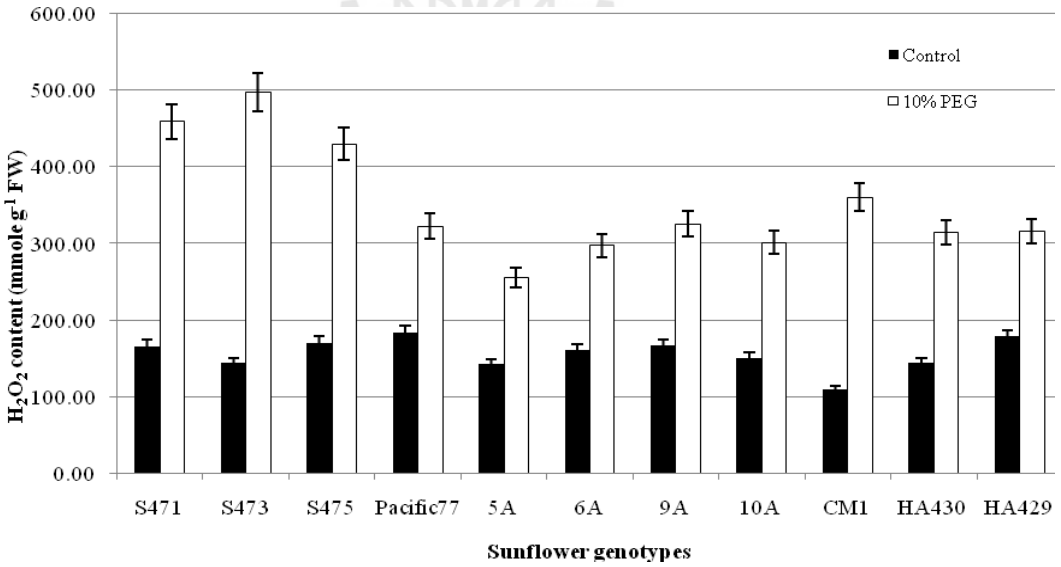

Figure D.8 Effect of drought stress on hydrogen peroxide in sunflower genotypes under hydroponic culture.

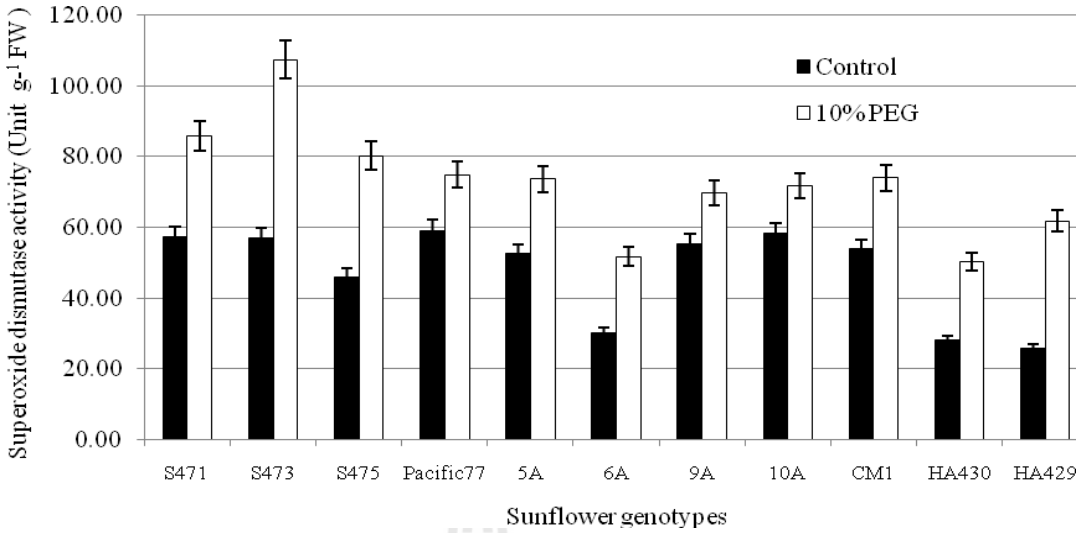

Figure D.9 Effect of drought stress on superoxide dismutase enzyme activity (SOD) in sunflower plants under hydroponic culture.

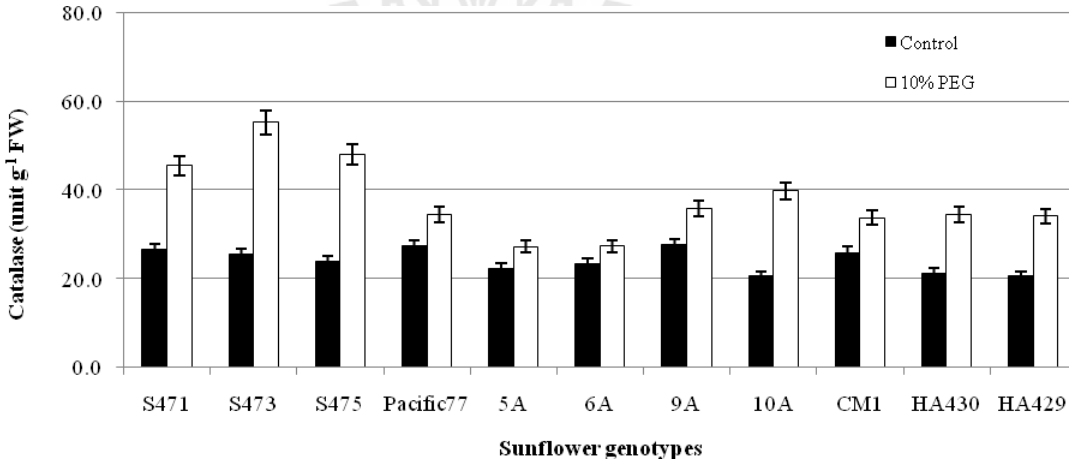

Figure D.10 Effect of drought stress on catalase enzyme activity (CAT) in sunflower plants under hydroponic culture.

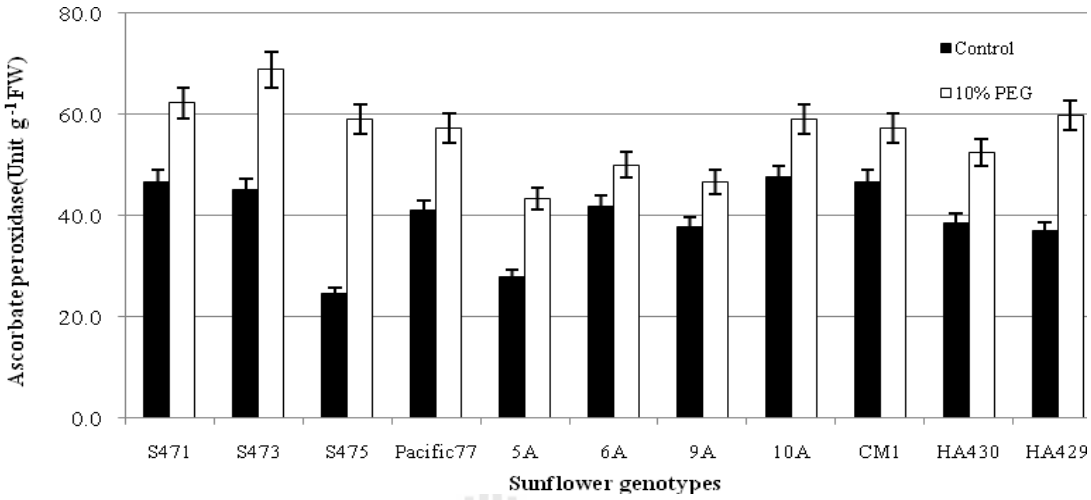

Figure D.11 Effect of drought stress on ascorbate peroxidase enzyme activity (APX) in sunflower plants under hydroponic culture.

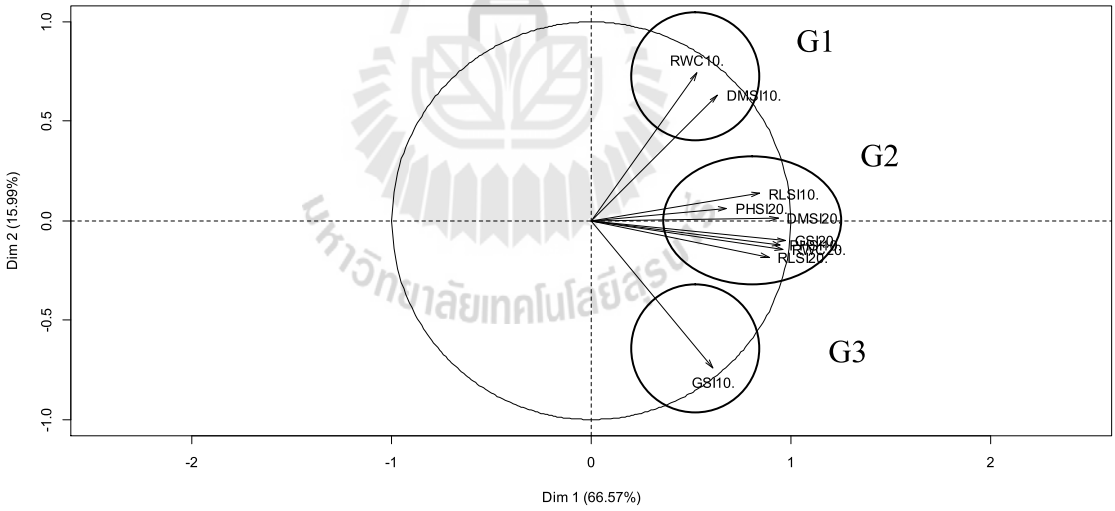

Figure D.12 Principal component analysis (PCA) based on morphological and physiological parameters of drought stress of sunflower plants in Petri dish bioassays.

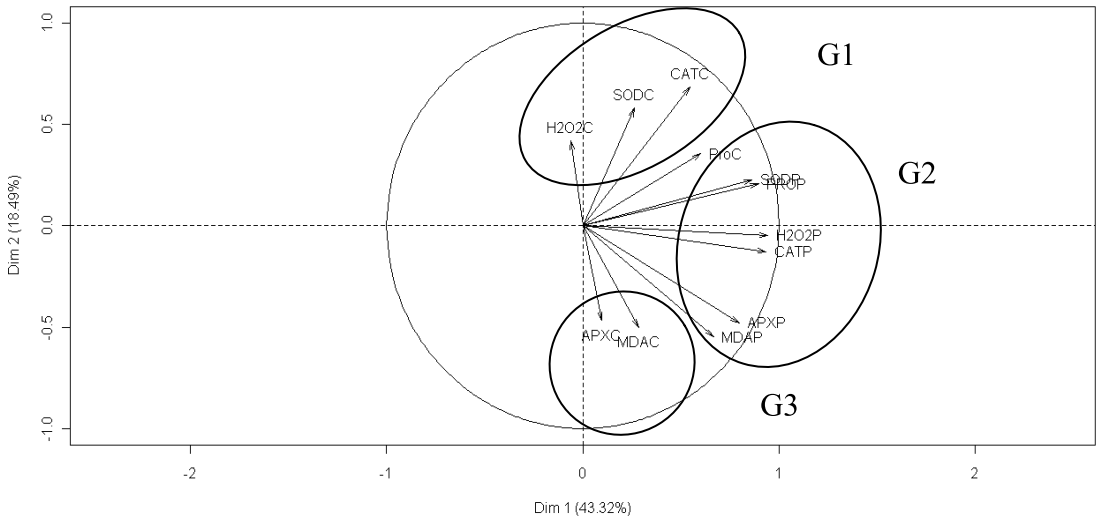

Figure D.13 Principal component analysis (PCA) based biochemical parameters of drought stress of sunflower plants in hydroponic culture.

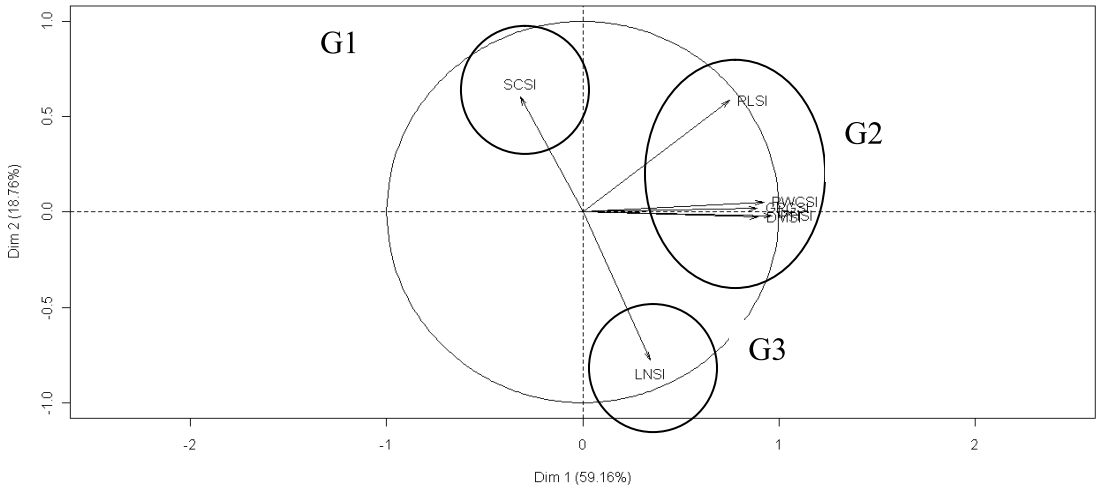

Figure D.14 Principal component analysis (PCA) based on morphological and physiological parameters of drought stress of sunflower plants in hydroponic culture.

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Awards	-
Grants and Fellowships	Suranaree University of Technology
Position and Place of Work	-