# ผลของคาร์บอนแบล็กและอนุภาคนาโนโลหะออกไซด์ต่อการเปลี่ยนแปลงทางสรีรวิทยาและระดับสารเมแทบอไลต์ของต้นยาสูบภายใต้สภาวะการเพาะเลี้ยงเนื้อเยื่อและการแช่น้ำ

# EFFECTS OF CARBON BLACK AND METAL OXIDE NANOPARTICLES ON PHYSIOLOGICAL AND METABOLIC CHANGES OF TOBACCO UNDER TISSUE CULTURE AND IMMERSING WATER CONDITIONS

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COLLEGE OF NANOTECHNOLOGY

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## บทคัดย่อ

ในปัจจุบันอนุภาคนาโนชนิดต่างๆ ได้ถูกนำมาประยุกต์ใช้ในผลผลิตอุตสาหกรรม และขั้นตอนทางการเกษตร เนื่องด้วยอนุภาคนาโนเป็นวัสดุมีพื้นที่ผิวสัมผัสมากและมีคุณสมบัติเฉพาะ ทั่วโลกมีการใช้อนุภาคนาโนในปริมาณหลายล้านตัน เพราะเหตุนี้การตระหนักถึงการปนเปื้อนของอนุภาคนาโนลงสู่สิ่งแวดล้อมจึงเพิ่มมากขึ้น ส่งผลให้ความกังวลถึงผลกระทบของอนุภาคนาโนต่อสิ่งมีชีวิตเพิ่มขึ้นตามไปด้วย แม้ว่าจะมีการศึกษาถึงผลของอนุภาคนาโนต่อพืชโดยนักวิจัยจำนวนมาก แต่ผลของอนุภาคนาโนที่มีต่อพืชยังคงเป็นที่ถกเถียงในเรื่องความเป็นพิษ ในงานวิจัยนี้จะทำการศึกษาผลของวัสดุคาร์บอน และ อนุภาคนาโนโลหะออกไซด์ต่อการเปลี่ยนแปลงทางกายภาพของพืชดัดแปรพันธุกรรมภายใต้สภาวะการเพาะเลี้ยงเนื้อเยื่อเปรียบเทียบกับตัวอย่างพืชสายพันธุ์ธรรมชาติ จากนั้นทำการวิเคราะห์การเปลี่ยนแปลงทางกายภาพ ซึ่งเกี่ยวพันกันกับการเปลี่ยนแปลงวิถีสังเคราะห์สารเมแทบอไลต์ของพืชที่เกิดจากผลของอนุภาคนาโนที่มีต่างชนิดและปริมาณกัน เมื่อต้นยาสูบเจริญในอาหารเพาะเลี้ยงเนื้อเยื่อที่มีการเติมน้ำตาลซูโครส 4%, คาร์บอนแบล็ก 25 มิลลิกรัมต่อลิตร, อนุภาคนาโนซิงค์ออกไซด์ที่ 10 มิลลิกรัมต่อลิตร และ อนุภาคนาโนไททาเนียมไดออกไซด์ที่ 20 มิลลิกรัมต่อลิตร ส่งผลต่อการเปลี่ยนแปลงทางสรีรวิทยา โดยมีการเพิ่มขึ้นของรงควัตถุที่ใช้ในกระบวนการสังเคราะห์ด้วยแสงและ มีการลดลงของความเสียหายของเยื่อหุ้มเซลล์ ภายใต้สภาวะดังกล่าว ต้นยาสูบมีการสะสมสารคาร์โบไฮเดรตและ สารกลุ่มฟลาโวนอยด์ เพิ่มมากขึ้น เมื่อพืชอยู่ภายใต้สภาวะการเพาะเลี้ยงเนื้อเยิ่มที่มีการเติมทั้งวัสดุคาร์บอน และ อนุภาคนาโนโลหะออกไซด์ที่ความเข้มข้นที่

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เหมาะสมร่วมกัน พบว่ามีการเปลี่ยนแปลงทางสรีรวิทยา และ การสังเคราะห์สารเมแทบอไลต์ในทางที่ดีขึ้น ในสภาวะการแช่น้ำตัวอย่างพืชที่ตัดถูกแช่ลงในสารละลายอนุภาคนาโนโลหะออกไซด์มีการเพิ่มขึ้นของรงควัตถุ และลดลงของเอทธิลีนเมื่อเปรียบเทียบตัวอย่างพืชที่ถูกตัดแช่น้ำ

**คำสำคัญ:** สารเมแทบอไลต์**,** โลหะออกไซด์, อนุภาคนาโน, พืช, สรีรวิทยา, ความเป็นพิษ

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|---|---|
| **Thesis Title** | Effects of carbon black and metal oxide nanoparticles on physiological and metabolic changes of tobacco under tissue culture and immersing water conditions |
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| **Degree** | Doctor of philosophy |
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| **Academic Year** | 2019 |
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## ABSTRACT

Nowadays, various kind of nanoparticles (NPs) are being applied in various industrial products and agricultural processes because they have high surface area with unique characteristics. Several million tons of NPs are used worldwide, consequently, the contamination of NPs in the environment is highly increasing awareness. An influence of NPs on living organisms is also being concerned. Although the effects of NPs on plants have been studied by various researchers, the debating issue of toxicity should be clarified. In this thesis, carbon-based materials and metal oxide NPs were added in planting medium under tissue culture condition in order to study their effects on the physiological changes of transgenic plant and were compared to wild type plant. Analysis of plant physiological quality under specific quality and quantity of NPs were performed. When tobaccos were under 4% sucrose, 25 mg. $L^{-1}$ carbon black, 10 mg. $L^{-1}$ ZnO NPs and 20 mg. $L^{-1}$ $TiO_2$ NPs containing medium condition. These plants showed an increase of photosynthetic pigments and a decrease of membrane injury. In these treatments, tobaccos also enhanced the accumulations of total soluble carbohydrate and flavonoids. The result of combination effect of carbon-based material and metal oxide NPs containing medium at appropriate concentration showed better physiological and metabolic changes. Moreover, after plants were cut and

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immersed in NPs solution, samples showed higher of photosynthetic pigment and lower of ethylene content when they were compared to the samples that were immersed in water.

**Keyword:** Metabolites, Metal oxide, Nanoparticles, Plant, Physiology, Toxicity

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Wilailack Chayaprasert

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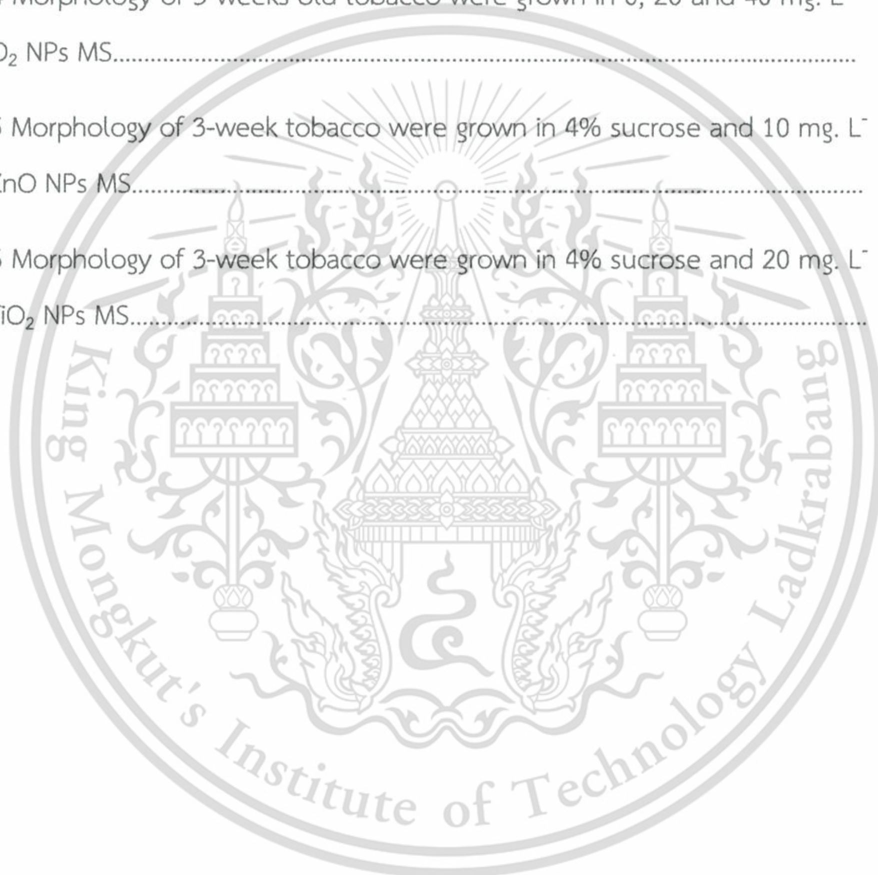


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# CHAPTER 1

# INTRODUCTION

## 1.1 Rationale

Nanomaterials have been shown marvelous applications applied in many kinds of technology including material engineering, cosmetics, medicine, and agriculture. An improvement of nanomaterial applications trends to increase every single year. The high surface area of nanoparticles induces a high rate of reaction. Different types of nanoparticles (metallic nanoparticles, carbon-based nanoparticles, metal-oxide nanoparticles) has been reported their effects on the plant developments (seed germination, root elongation, fresh and dry weight) and plant metabolite biosynthesis (carbohydrate, protein, and secondary metabolites) (Siddiqui *et al.,* 2015). However, the uses of nanomaterials are now concern about the risks and the negative effect on the environment especially, effects on a plant in our ecosystem (Lee *et al.,* 2012). The toxicity and the positive effects of nanomaterials in the plant has also presented. The effects of type and concentration of nanomaterials on the plant are important to be find out. Different results also depend on the condition of the study. The previous studies have been reported on various conditions of plant which were tested with nanomaterials. These condition were immersing in nanoparticles solution, spraying nanoparticles solution, mixing nanoparticles in soil and hydroponic system conditions (Song *et al.,* 2013; Ghanati and Bakhtiarian, 2014; Samadi e*t al.,* 2014; Narendhran *et al.,* 2016; Vecerova *et al.,* 2016). These conditions are limited in controlling the concentration of NPs that plant cloud absorbs to the cell.

The effects of nanoparticles on plant physiology and metabolites have been interested in many researchers. The functions of secondary metabolites in plant involve with many parts in plant systems, in addition, many secondary metabolites from the plant were used in human health promotion and disease cure treatments. The effects of nanoparticle on the synthesis of the secondary metabolites has been

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reported that nanoparticles both induce and reduce the accumulation of plant secondary metabolites.

The objectives of this study are aimed to evaluate the effects of type and concentration of nanoparticles on plant physiology and biomaterial synthesis in the tissue culture system. In this study, we focus on carbon-based materials and metal-oxide nanoparticles due to the carbon is the main structure of all living organism while metal-oxide nanoparticles like ZnO nanoparticles and $TiO_2$ nanoparticles are photocatalyst materials. Different plant lines are used in the experiments in order to investigate the response on medium containing various concentration of nanoparticles. Furthermore, this study aims to expose the effects of nanoparticles on cutting plant.

## 1.2 Objectives

1. To study the effects of carbon black materials or metal nanoparticles containing media on physiological and metabolic changes of tobacco under tissue culture condition

    1.1 To study the effects of carbon black materials in medium on plant

    1.2 To study the effects of metal oxide nanoparticles in medium on plant

    1.3 To study the combination effects of metal oxide nanoparticles and extra sucrose in medium on plant

2. To study on the applications of nanoparticles on the protection of cutting plant that immersed water

    2.1 To determine the effects of nanoparticles on photosynthetic pigment changes of the cutting plant

    2.2 To study the effect of nanoparticles on ethylene accumulation in cutting plant

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## 1.3 Scopes

To study the effects of type and concentration of nanoparticles on plant under tissue culture condition. Plant treated with nanoparticles were analyzed for physiological and metabolic (total soluble carbohydrate and flavonoids) changes in tissue of tobacco wild-type and transgenics overexpressing the flavonoid regulatory gene. The effects on cell membrane stability of tobacco under nanoparticle conditions were also be performed. Moreover, the concentration of each nanoparticles were studied an effect on the senescence of cutting plant.

## 1.4 Expected Results

1. The effects of carbon black material in medium on plant physiology and the accumulation of plant biomaterials

2. The effects of metal oxide nanoparticles in medium on plant physiology and the accumulation of plant biomaterials

3. The combination effects of metal oxide nanoparticles and extra sucrose in medium on plant physiology and the accumulation of plant biomaterials

4. The effects of the nanoparticles on the cutting plants that were immersed in water

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# CHAPTER 2

# LITERATURE REVIEWS

## 2.1 Nanoparticles

Nanoparticles (NPs) are world new material that become multiplicate uses in every day of our life. Year over year increase of the world supply of nanomaterial has been continued. 10-100 nm in range of nanoparticle makes a specific property that different from bulk materials, such as high surface area, size-dependent qualities and optical properties. Nanoparticles are classified into many types depending on their structure, characteristics and advantages (Bhatia, 2016). These are some examples of NPs and their applications.

### 2.1.1 Carbon- based materials

Carbon-based nanoparticles have become of interest in the scientific community due to their unique combinations of physical and chemical properties (Cha *et al.*, 2013). The wide range of superior properties in carbon nanostructures (thermal and electrical conductivity, high mechanical strength, and optical properties) and the simplicity of their structures have played an important role in the current rapid expansion of fundamental studies of carbon-based nanoparticles and their applicational use in nanotechnology. Because of their unique property, carbon-based nanoparticles have been used in a wide range of fields for example, electronics, energy, biomedicine, bio-sensing, biomolecular and agricultural industry.

### 2.1.2 Metal oxide nanoparticles

Metal oxide nanoparticles play an important role in diverse areas. It exhibits different physiochemical properties (surface, optical, thermal, and electrical properties) that are different when compared to their native bulk compounds (Rastogi *et al.*, 2017). Metal oxide nanoparticles present larger surface-to-volume ratio which increase the reactive surface atoms. The size-dependent property of metal oxide nanoparticles is the cause of using metal oxide NPs in various applications for the recent decade (Figure

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2.1) with this reason, the metal oxide nanoparticles productivity are recently rising every year (Stankic *et al.*, 2016).

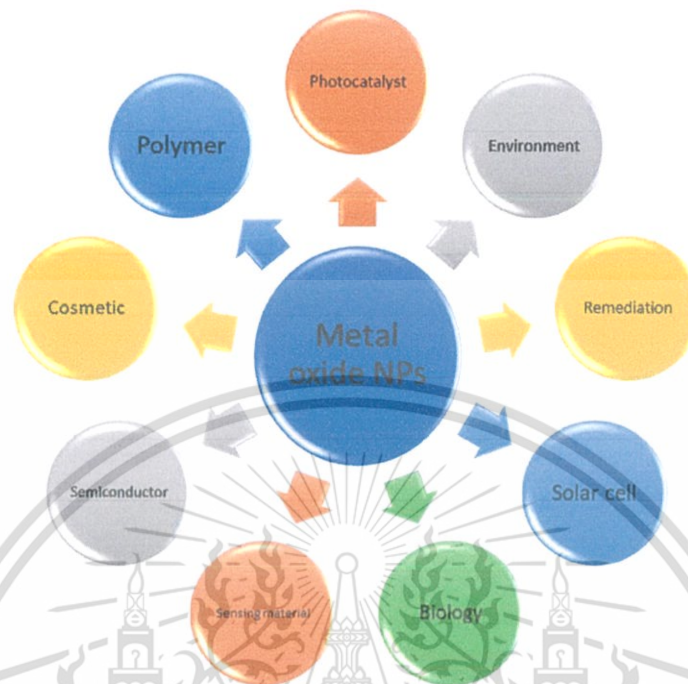


**Figure 2.1** General applications of nanoparticles metal oxide nanoparticles

## 2.2 Plant physiology

Plant physiology is the study of all process in plant including germination, growth, development, maturation, reproduction, senescent and death (Bidwell, 1979). All plant physiological process is controlled by its genetic information of each spices together with effect from environment. The sample of environmental factors are drought, temperature, lack of nutrient, chemical compound, radiation on so on (Hale and Orcutt, 1987). Plants have different basis lift and behavior from animal thereby they have different physiological process. Under changing stress environment, animals that are automobile organism can immigrate to new habitat but plants that are non-mobile organism can growth through its environment, but slowly to a limited extent. Under environmental stress, plant changes physiological process (Hale and Orcutt, 1987) and responds to predictable and unpredictable environmental effects (Bidwell, 1979). In conclusion, the basis of plant is ability to complete in survive in its

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environment and this depend on its physiological evolution to the environment. The evolutions of plant metabolite and physiology are also marked as an importance topic to study because it provides an essential link between the biochemical and ecological process (Wilkins, 1984). Moreover, the knowledges of plant physiological and metabolic changes have been interested to solve problems in agriculture technology (Bidwell, 1979).

### 2.2.1 Physiological changes

In generally, the study topic in plant physiological change are how plant covert light energy to their food, how plant obtain nutrient to use in their metabolic process, how plants control growth and development and how plant response to their environment (Hopkins, 1995). To narrow down the scope of plant physiological change, basic uniformity of cell structure needs to be expanded because the cell structure is direct relative with cell function and obviously physiological change (Mohr and Schopfer, 1995).

The cells are the simplest organisms are capable of all activity and bioreaction of life (Bidwell, 1979). The basic fact of living organisms composes of individual cell combined. Plant cells are eukaryotic cells that have various components knows as cell organelles. Organelles perform various tasks and function to sustain itself. These organelles include: The cell wall, cell membrane, nucleus and other cellular organelles. To carry the photosynthetic reaction, plant cells contain photosynthetic plastid in double membrane organelles. The most prominent are chloroplasts (Figure 2.2). In autotroph, photosynthetic pigments are one of importance metabolites because they absorb light that is used in photosynthesis. There are several types of photosynthetic pigments. Chlorophylls and carotenoids are the most important pigment in the plant. In plants, chlorophyll a and chlorophyll b are the main photosynthetic pigments. Chlorophyll molecules absorb blue and red wavelengths while carotenoids are another key group of pigments that absorb violet and blue-green light. The distribution of photosynthetic pigment type in autotroph is different and

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shown in Table 2.1. The structure of chlorophyll is a tetrapyrrole ring with Mg ion and 20-carbon side chain called a phytol unit (Figure 2.3).

The biological membranes are one of the most important part of cell structure. In eukaryotic cell, membranes are main composed lipid and protein with small amount of carbohydrate. The most abundant of lipid cell membrane are amphipathic of phospholipid (Figure 2.4). Environment changes may trig point the plant physiological change. The stable of lipid membrane can be troubled by reactive oxygen species (ROS) that are formed as a natural byproduct of the normal metabolism and environmental stress. If ROS levels increase dramatically, this may result in significant damage to cell structures by lipid peroxidation (Figure 2.5) that is chain reaction cause ROS to steal electrons from the lipids in cell membranes encourage cell damage.

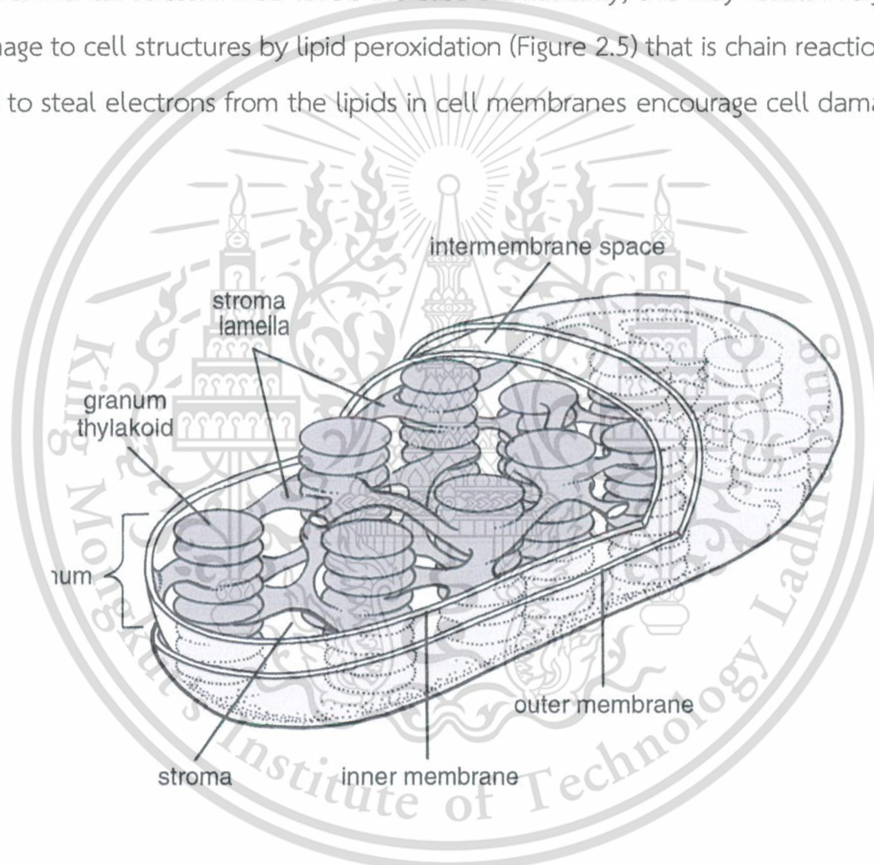


**Figure 2.2** The structure of chloroplast, (Evert, 2006).

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**Table 2.1** Distribution of photosynthetic pigments in different autotroph organisms (David, 2001; Anthony *et al.*, 2003; Grimm *et al.*, 2006).

| Pigment | Organisms |
|---|---|
| Chlorophyll A | Higher plant, Green algae, Red algae, Diatom, Blue-green algae |
| Chlorophyll B | Higher plant, Green algae |
| Chlorophyll C | Brown algae, Diatom |
| Chlorophyll D | Red algae |
| Carotenoid | Higher plant, Green algae, Red algae, Brown algae, Diatom, Bacteria |
| Phycobilin | Red algae, Blue-green algae |

**Figure 2.3** The structure of chlorophyll, National Center for Biotechnology Information. PubChem Database. Chlorophylls, CID=6449992, https://pubchem.ncbi.nlm.nih.gov/compound/6449992 (accessed on May 13, 2019)

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**Figure 2.4** The structure of phospholipid bilayer (Evert, 2006)

Unsaturated fatty acid

(lipid cell membrane)

$\cdot OH$

$H_2O$

Carbon centered radical

$O_2$

Peroxy radical

**Figure 2.5** Lipid peroxidation reaction

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## 2.3 Plant metabolites

Plant synthesize many biomolecule compounds through a metabolic pathway that converts one molecule to another with enzyme-mediated biochemical reaction same as another living organism, the metabolites are produced for both essential function and specific function. Plant metabolites are divided into two groups. First are primary metabolites that are essential for plant growth, development and reproduction, so it can be found in all plant. The other group are secondary metabolites, these metabolites are not essential for main plant growth and development but have a specific function that plant uses for surviving in its environment. Secondary metabolites are mediate the interaction of the plant with other organism and other metabolites. The difference between primary and secondary metabolites are presented in Table 2.2.

**Table 2.2** The difference between plant primary and secondary metabolites

| Primary metabolites | Secondary metabolites |
|---|---|
| Perform physiological function | Perform plant protection |
| Involve in growth, energy production, development, and reproduction | Involve in responding signal and environment correlation |
| Synthesize during the growth phase | Synthesize nearing stationary phase |
| Large quantity | Small quantity |
| Same in all plant | Unique due to plant species |

### 2.3.1 Primary metabolite in plants

The primary metabolites are used for energy and tissue construction. Primary metabolites have a key role in the survival of the plant, playing an active function in the photosynthesis, respiration, growth, development, and reproduction. In general, the sample of primary metabolites are carbohydrate, protein, lipid, nucleic acid, some

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vitamin, and cofactor. The samples of plant primary metabolites are lists bellow (Buchanan *et al.*, 2000; Caroline *et al.*, 2010).

**2.3.1.1 Carbohydrate**

Another importance primary metabolites are carbohydrate molecules, from basic monosaccharide to complex polysaccharide is an importance metabolite which can be categorized into two main groups. Structural polysaccharides are used to support plants and storage polysaccharides are used to store energy for later use by the plant. Plant perform photosynthetic system to produce sugar and create sugar-phosphate precursors that acts as precursors in several numbers of metabolite biosynthesis in the plant (Gleason and Chollet, 2012). Cellulose is a polymer of glucose (about 8,000-15,000 glucose units). The substrate for forming cellulose is Uridine diphosphate glucose (UDP-glucose). The cellulose structure is ß-1,4-linked glucose, the monomer is cellobiose (Figure 2.6). Cellulose form cell wall that is provided for supporting plant tissue and defending against pathogens and herbivores.

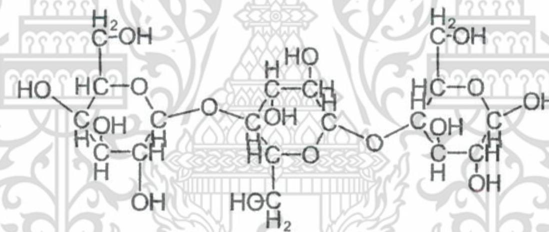

**Figure 2.6** The structure of cellulose

Starch is an importance store energy store for the plant. Plant starch is used to synthesize ATP during catabolism of glucose. Two type polysaccharides (amylose and amylopectin) compose plant starch (Figure 2.7). Amylose is a linear polymer of glucose with alpha-1,4-glycosidic. Amylopectin is also a linear polymer of glucose with alpha-1,4-glycosidic but addition with alpha-1,6-glycosidic branches occurring about every 20 monomers.

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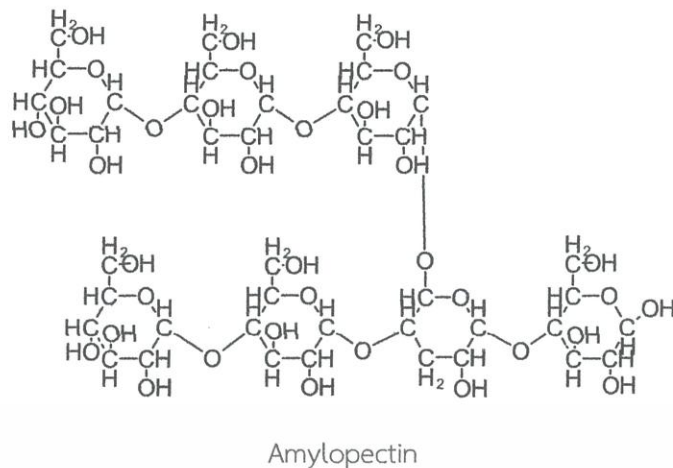
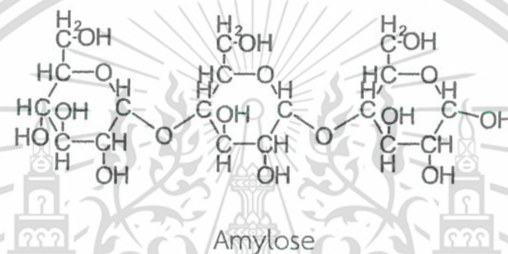


Amylopectin



Amylose

**Figure 2.7** Plant starch (amylopectin and amylose)

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**2.3.1.2 Protein**

Primary metabolites and many cofactors contain a nitrogen atom in molecules such as amino acid and nucleic acids, NADP and biotin (Gleason and Chollet, 2012). Plant needs to uptake nitrate ($NO^{3-}$), nitrite ($NO^{2-}$) and ammonia cation ($NH_4^+$) by active transport process and synthesizes many proteins including common protein (enzymes, structural proteins such as tubulin) and variety of unique proteins (protein storage, intercellular signal, and defense). The structure of amino acids that commonly found in proteins is shown in Table 2.3.

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**Table 2.3** structure of natural 20-amino acids

| Amino acid | Structure of the R group | Abbreviation | R class |
|---|---|---|---|
| Glycine | $H-CH(NH_2)-C(=O)-OH$ | Gly, G | Hydrogen, non-polar |
| Alanine | $H_3C-CH(NH_2)-C(=O)-OH$ | Ala, A | Alkyl, non-polar |
| Valine | $(H_3C)(CH_3)CH-CH(NH_2)-C(=O)-OH$ | Val, V | Alkyl, non-polar |
| Leucine | $(CH_3)_2CH-CH_2-CH(NH_2)-C(=O)OH$ | Leu, L | Alkyl, non-polar |
| Isoleucine | $H_3C-CH_2-CH(CH_3)-CH(NH_2)-C(=O)OH$ | Ile, I | Alkyl, non-polar |
| Phenylalanine | $C_6H_5-CH_2-CH(NH_2)-C(=O)OH$ | Phe, F | Aromatic, non-polar |
| Proline | $H_2C-CH_2-CH(COOH)-NH-CH_2$ (ring) | Pro, P | Cyclic, non-polar |
| Serine | $HO-CH_2-CH(NH_2)-C(=O)-OH$ | Ser, S | Alcohol, polar |
| Threonine | $H_3C-CH(OH)-CH(NH_2)-C(=O)-OH$ | Thr, T | Alcohol, polar |
| Tyrosine | $HO-C_6H_4-CH_2-CH(NH_2)-C(=O)-OH$ | Tyr, Y | Phenol, polar |

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| | | | |
|---|---|---|---|
| Cysteine | $HS-CH_2-CH(NH_2)-COOH$ | Cys, C | Thiol, polar |
| Methionine | $H_3C-S-CH_2-CH_2-CH(NH_2)-COOH$ | Met, M | Thioether, non-polar |
| Lysine | $H_2N-CH_2-CH_2-CH_2-CH_2-CH(NH_2)-COOH$ | Lys, K | Amine, polar, basic |
| Arginine | $H_2N(HN=)C-NH-CH_2-CH_2-CH_2-CH(NH_2)-COOH$ | Arg, R | Guanidine, polar, basic |
| Histidine | $C_3H_3N_2-CH_2-CH(NH_2)-COOH$ | His,H | Imidazole, non-polar |
| Tryptophan | $C_8H_6N-CH_2-CH(NH_2)-COOH$ | Trp, W | Aromatic, non-polar |
| Aspartic acid | $HO-CO-CH_2-CH(NH_2)-COOH$ | Asp, D | Carboxylic acid, polar |
| Glutamic acid | $HO-CO-CH_2-CH_2-CH(NH_2)-COOH$ | Glu, E | Amide, polar |
| Asparagine | $H_2N-CO-CH_2-CH(NH_2)-COOH$ | Asn, N | Amide, polar |
| Glutamine | $H_2N-CO-CH_2-CH_2-CH(NH_2)-COOH$ | Gln, Q | Amide, polar |

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#### 2.3.1.3 Lipids

Eukaryotic cell synthesizes unusual lipids that are important in maintaining cell membrane. In the plant, the lipids are also synthesized for being a fluidity in the thylakoids and can function in the defense system (Gleason and Chollet, 2012). There have over 300 different type of fatty acid in the plant. The biosynthesis of fatty acid is made through the acetate-malonate pathway. Lipid can be group into neutral lipids, phospholipids, glycolipids, terpenoids and waxes (Irwin, 1982).

#### 2.3.1.4 Nucleic acid

Two types of chemically similar nucleic acids, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Nucleic acids are molecules that contain genetic information that determines traits and makes protein synthesis possible. The small unit of Nucleic acids is the nucleotide that is a structure of three main molecules that are ribose sugar, phosphate and nitrogenous base (Figure 2.8). There are four basic types of nucleotide, adenine (A), guanine (G), cytosine (C), and thymine (T) in DNA while in RNA, uracil (U) is instead of thymine (T). The main role of nucleic acids is to store information that is used to make proteins. During these process DNA transfer to RNA for ultimate protein biosynthesis (Irwin, 1982). These processes call transcription and translation. Triplets of nucleotide in DNA are code for amino acid call codon. During transcription that occur in nucleus, the DNA code by mRNA then mRNA immigrate to cytoplasm. The translation process starts the codon code to amino acid sequence to synthesis protein.

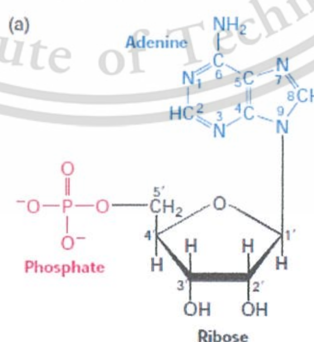


**Figure 2.8** Structure of Nucleic acids (Lodish *et al.*, 2000)

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### 2.3.2 Secondary metabolite in plants

Plants produce secondary metabolite not for growth but for existence in its environment, unlike primary metabolite that is an organic compound which involves in plant growth and organ development. In general, the accumulation of secondary metabolite in plants are low quantities but not always (Wink, 1999). The productivity of secondary metabolite may be widespread or restricted to plant species. Secondary metabolites in the plant can be divided into three groups from their precursor and metabolic pathway (Figure 2.9). They are terpenes, nitrogen-containing compound and phenolics.

**Figure 2.9** The major biosynthetic pathways of secondary metabolites (Rosa *et al.*, 2009)

#### 2.3.2.1 Terpenes

Terpenes also are known as terpenoids, some terpenes are classified in primary metabolites because they mainly involve with plant growth and development for example gibberellins hormone. however, the plant produces terpenoids to plant defending molecules. The majority of terpenoids are toxic to many herbivorous, insects

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and mammals. The pathway of terpenoid biosynthesis in the plant is shown in Figure 2.10.

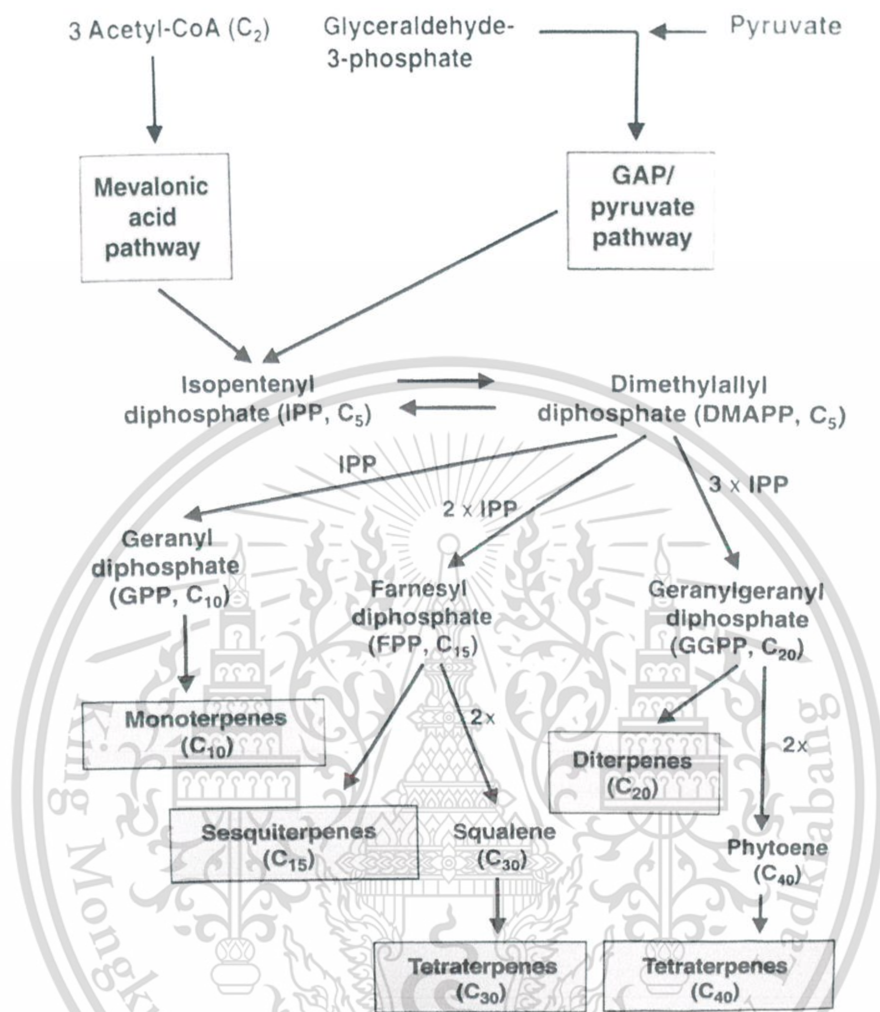


**Figure 2.10** Overview of terpenoid biosynthesis in plants (Wink, 2010)

### 2.3.2.2 Nitrogen-containing compounds

Nitrogen-containing compounds are the secondary metabolite that has nitrogen as a part of the structure. They are synthesized from amino acid and other precursors (Wink, 2010) (Figure 2.11). The most well know are alkaloids and cyanogenic glucosides because their medical properties, on the other hand, most of the alkaloids and cyanogenic glucosides have a toxicity effect on vertebrate animals.

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Figure 2.11 Overview of biosynthetic pathways of major groups of alkaloids (Wink., 2010)

#### 2.3.2.3 Flavonoids

One of the large groups of secondary metabolites is flavonoids. Flavonoids are constructed from phenylpropanoid precursor in the shikimate pathway. The mechanism of flavonoid has been studied for a long time by using analysis of structural and regulatory gene and protein crystallization technique, Overview of flavonoid biosynthesis pathway are displayed in Figure 2.12. There are several branches in the flavonoid pathway. From studying the function of flavonoid regulatory gene. There has three main types of flavonoid regulatory gene. There are the R2R3 MYB, the basic helix–loop–helix (bHLH) and the WD repeat (WD40) protein (Andersen and Markham, 2006).

The regulatory gene of flavonoid biosynthesis may be regulated in response to the environmental signal. According to many reports, environmental stress such as UV, drought, cold temperature, metal oxide and an organic compound such as sugar, hormone, pest have effect to increase rate of flavonoid regulatory gene transcription and accelerate flavonoid biosynthesis (Andersen and Markham, 2006). Flavonoid functions in plant, The main flavonoid function in the plant for red and blue

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flavonoids (Anthocyanins and 3-deoxyanthocyanins) is remarked as defense molecule, UV protection, antioxidant activity, reduce oxidative stress while yellow and colorless flavonoid (Flavones, Flavonols, and Flavanones) are labeled as stress protection, reproduction and development signal molecules (Wink, 1999). Under stress condition, plant enhances accumulation of flavonoid content to promote a defensive system for surviving.

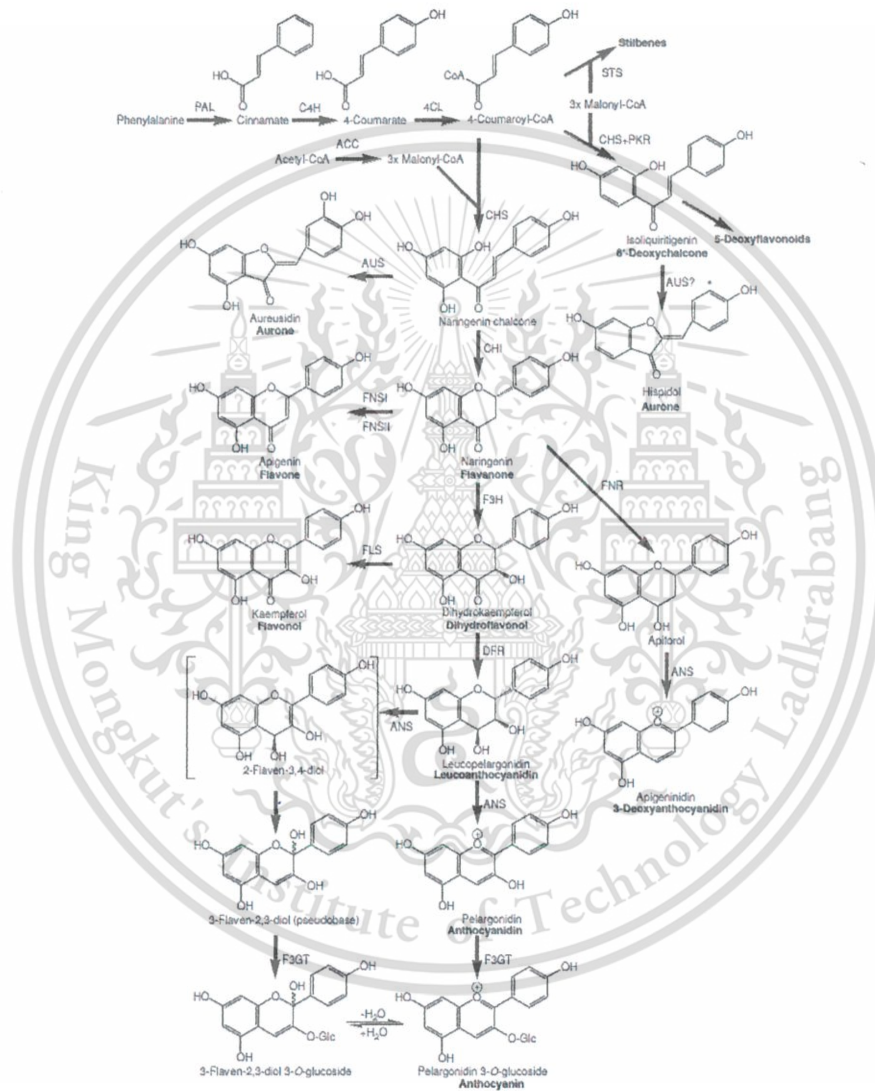


**Figure 2.12** General phenylpropanoid and flavonoid biosynthetic pathways (Andersen and Markham, 2006)

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## 2.4 Tissue culture technique

Plant tissue culture technique is called second green revolution which can improve both quantity and quality of crop yield in agricultural technology and also have been used broadly in plant biology experimental researches because this technique give many advantages such as shorten the time taken for plant growth, reducing the area requirement, improvement large scale of plant metabolites, increasing in screening plant cell and plant cell improvement for genetic transformation (Dodds and Roberts, 1985; Collin and Edwards, 1998). The origin of tissue culture technique is from the theory of totipotent of Swan, 1839. Totipotent stem cells have the potential to create an entire organism. Several researchers had been performed experiment on plant tissue isolate and differentiation lead the tissue culture technique successful. Nowadays, tissue culture technique is a part of the study of plant physiological change, plant metabolites, plant genetic engineering and so on. This *in vitro* technique assists controlling of experimental condition. To perform tissue culture technique, parts of plant such as root, stem, leaf, flower and shoot were isolated and sterilized. These plant tissues were grown in media as broth and agar. Plant tissues were differentiated into new plant under *in vitro* condition (Figure 2.13).

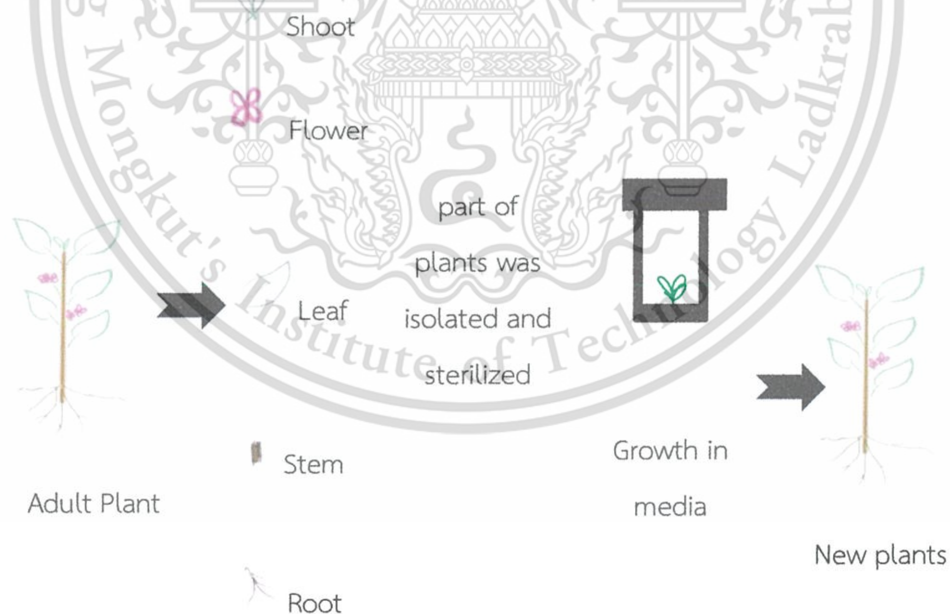


**Figure 2.13** Brief of plant tissue culture technique

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## 2.5 Post-harvest technology

In the study of plant physiological and metabolic change, senescence and aging in plants is one of the most interesting because understanding of senescence mechanism can be applied to extend plant life after being harvested. At first, post-harvest technology is developed to prolong life of agricultural products due to increasing of agricultural product demanding. After post-harvest living biological system will be deteriorated (Wills *et al.*, 1998). The rate of deteriorated is affiliate with various factor depending on plant species and environment after harvest. Extending of post-harvest life of plant still require more knowledge from scientific researches. To study and improvement of post-harvest technology, senescence of plant needs to be measured for progress in understanding plant aging (Noodén and Leopold, 1988). These can be measured change of morphology or metabolite and also senescence hormone like ethylene after plants were harvested. The techniques of post-harvest that are used to handling shelf life nowadays are harvesting in either matured green stage, precooling after harvest, cleaning or disinfecting and using chemical to suppress the action of ethylene (Arah *et al.*, 2016).

## 2.6 Effects of nanomaterials on plant

Nanoparticles can induce both plant development and synthesis of substances in different plant variety. Therefore, they are required in the agricultural process, because it's low-cost and has the ability to enhance agricultural productivity. NPs in culture medium have a potentiality to activate modifications of plant physiology (Figure 2.14) (Aslani *et al.,* 2014). NPs are enhanced their use in agricultural process; thus they are concerned about their side effects. According to the results of many research, effects of NPs on plant depend on species, stage of the growth, methodology of experiment, type of NPs and concentration of NPs. When plant interaction with NPs, it can uptake and accumulate in a plant cell. These activate changing gene expressing (Burklew *et al.,* 2012; Kaveh *et al.*, 2013; Syu *et al.,* 2014) and biosynthesis that effect on plant growth and development including of plant biosynthesis (both primary and secondary

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metabolites) (Ghanati and Bakhtiarian, 2014; Vecerova *et al.,* 2016). Overall NPs influence plant physiological and metabolic changes.

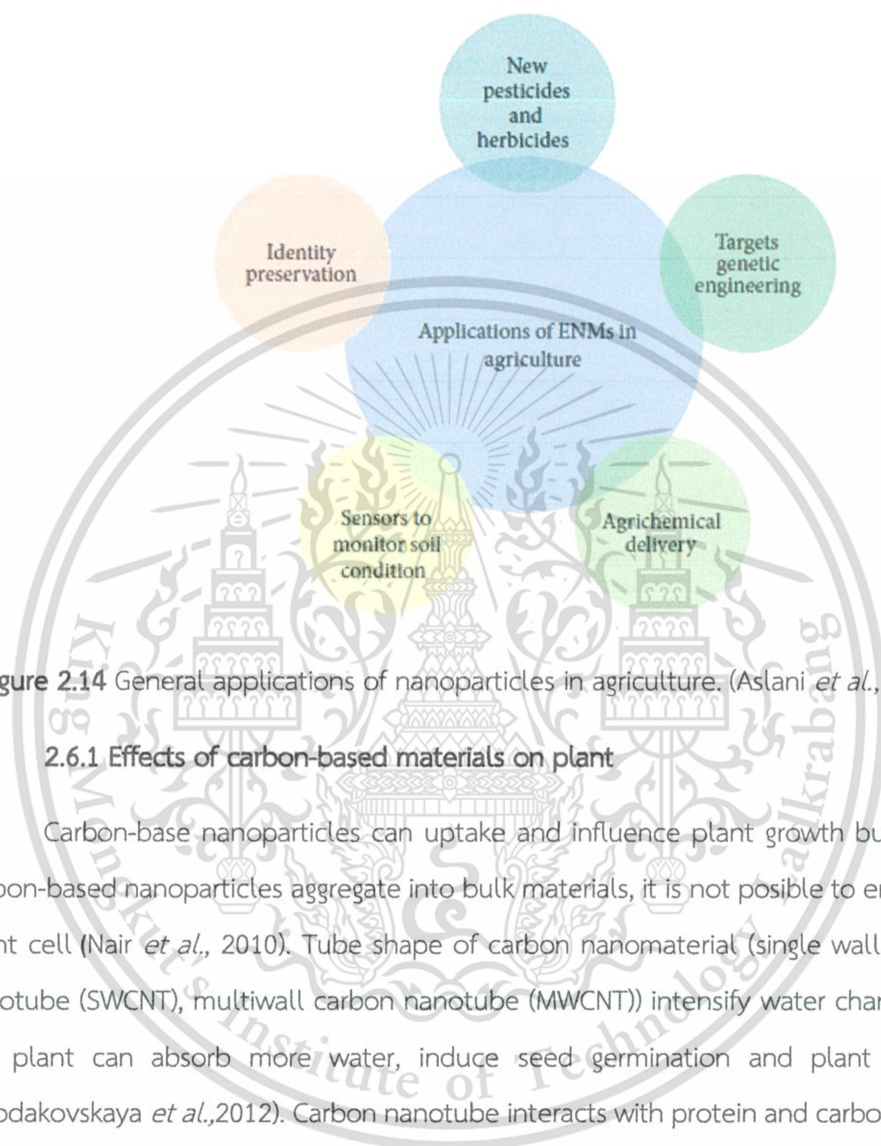


**Figure 2.14** General applications of nanoparticles in agriculture. (Aslani *et al.*, 2014)

**2.6.1 Effects of carbon-based materials on plant**

Carbon-base nanoparticles can uptake and influence plant growth but when carbon-based nanoparticles aggregate into bulk materials, it is not posible to enter the plant cell (Nair *et al.*, 2010). Tube shape of carbon nanomaterial (single wall carbon nanotube (SWCNT), multiwall carbon nanotube (MWCNT)) intensify water channel, so the plant can absorb more water, induce seed germination and plant growth (Khodakovskaya *et al.,*2012). Carbon nanotube interacts with protein and carbohydrate (Hazarika and Maji, 2014). Therefore, it may disturb cellular metabolism. The cytotoxicity of carbon-based nanoparticles on plant cell also been found (Jia *et al.*, 2005). High concentration of carbon nanomaterials effects reducing root hair, biomass, producing plant growth inhibitor and activating program cell death in Arabidopsis (Shen *et al.*, 2010; Yuan *et al.,* 2011).

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### 2.6.2 Effects of metal oxide nanomaterials on plant

Metal oxide nanoparticles have an effect on plant growth and development. Several studies have been shown that metal oxide nanoparticles can uptake and effect to plant biosynthesis system owe to it act as co-factor for many enzymes in plants. On the other hand, a high concentration of metal oxide nanoparticle treatment leads to phytotoxicity (Ma *et al.,* 2010; Yang *et al.*, 2015). Overall, each plant has a different effect depending on plant species, stage of growth, experiment condition, and type of nanomaterials. The most studies presented that several kinds of metal oxide nanomaterials effected on the plant are $Al_2O_3$, $CeO_2$, $Cu_2O$, $Fe_3O_4$, $TiO_2$ and ZnO nanoparticles (Aslani *et al.,* 2014).

#### 2.6.2.1 Effects of Zinc oxide nanomaterials on plant

Zinc is very important for all living organism including plants. It is a co-factor for various enzymes. In the plant, Zinc is used for chlorophyll synthesis, fertilization, pollen function, and germination. These are why ZnO nanoparticles (NPs) have been used in plant technology. Effect of ZnO NPs on the plant is very interesting because it effects on organisms in ecosystem. Many studies found that ZnO NPs have a different effect on plants when compare with the bulk material (Prasad *et al.*, 2012; Bandyopadhyay *et al.*, 2015; Kouhi *et al.*,2015; Narendhran *et al.*, 2016). However, the result of the effect of ZnO NPs are the same as showed in other nanomaterials that is when plants are treated with high concentration on ZnO NPs, the toxicity effect happened (Ma *et al.*, 2010; Mukherjee *et al.*, 2014; Bandyopadhyay *et al.,* 2015; Yang *et al.*, 2015;). The effect of ZnO NPs on the plant is being decided by plant species, conditions and the main factor is the amount of ZnO NPs that plant uptake to their cell.

#### 2.6.2.2 Effects of titanium dioxide nanomaterials on plant

$TiO_2$ nanoparticles have a photo-catalyzed characteristic. It can find in various products for example paint, dyes, plastic, drugs and cosmetics. $TiO_2$ NPs are

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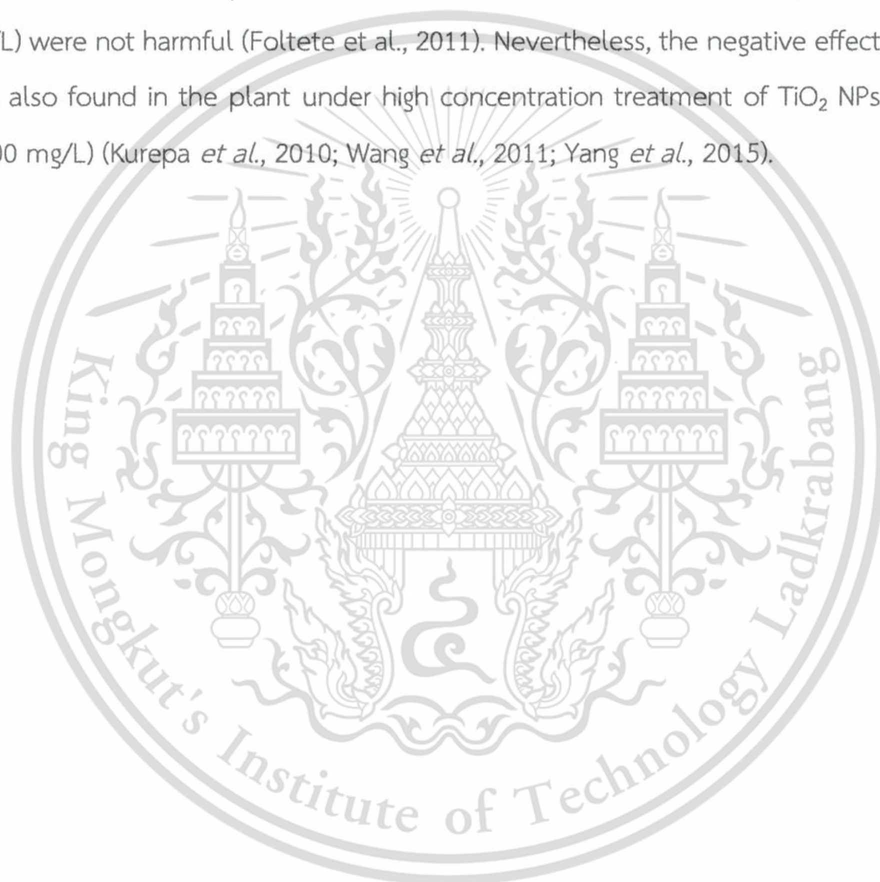
studied in changing of plant physiology. $TiO_2$ NPs could induce seed germination and reduce germination time because it helps the adsorbed water of seed (Feizi *et al.*, 2012; Mahmoodzadeh *et al.*, 2013; Mahmoodzadeh *et al.*, 2014). Several studies found that $TiO_2$ NPs activate nutrient absorption in plant and enhance plant dry weight, chlorophyll activity and enzyme activity (Fashui *et al.*, 2005; Gao *et al.*, 2008; Mingyu *et al.,* 2009; Pocic *et al.*, 2016). Moreover, $TiO_2$ NPs showed an ability to breakdown of organic substance and to reduce reactive oxygen species by activating plant defend system (Zheng *et al.*, 2005; Yang *et al.*, 2006). According to the study of the effect of $TiO_2$ NPs in the model plant (*Vicia faba*), the low concentration of $TiO_2$ NPs (5, 25, 50 mg/L) were not harmful (Foltete et al., 2011). Nevertheless, the negative effect of $TiO_2$ NPs also found in the plant under high concentration treatment of $TiO_2$ NPs (2,000-4,000 mg/L) (Kurepa *et al.*, 2010; Wang *et al.*, 2011; Yang *et al.*, 2015).

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# CHAPTER 3

# RESEARCH METHODOLOGY

## 3.1 Plant materials

Tobacco (*Nicotiana tabacum*) provided from DNA technology laboratory, college of nanotechnology, KMIT'L

## 3.2 Chemical substances

1. Chemical for tissue culture medium: Murashige and Skoog (MS) medium (Murashige and Skoog, 1962)
2. Carbon-based materials: Sucrose, Carbon black (the average size of 52 nm, surface area 30 $m^2/g$)
3. Nanoparticles: Zinc oxide (the average size of 30.4 nm ±3.1 nm) and Titanium dioxide (the average size of 24.0 ±1.9 nm)
4. Chemical for photosynthetic analysis: Acetone (Fisher Scientific®, USA)
5. Chemical for lipid peroxidation analysis: Trichloroacetic acid and Thiobarbituric acid (Himedia®, India)
6. Chemical for total soluble carbohydrate analysis: Methanol (Fisher Scientific®, USA), Sulfuric acid (Himedia®, India), Phenol (Fisher Scientific®, USA)
7. Chemical for flavonoid analysis: Methanol (Fisher Scientific®, USA), Hydrochloric acid (EMSURE®, USA), Chloroform (Fisher Scientific®, USA)

## 3.3 Equipment

1. Autoclave (TOMY SX-500; TOMY®, Japan)
2. Balance (AG204; METTLER TOLEDO®, Switzerland)
3. Benchtop UV transilluminator with the digimage system (AG204; Major Science®, Taiwan)
4. Centrifuge (SPECTRAFUGE 16M; Labnet®, USA)

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5. Electro conductivity Meter (CON900A MTAST®, USA)
6. Gel electrophoresis (MJ105, Major Science®, Taiwan)
7. Glassware and plasticware (Erlenmeyer flask, cylinders, separating funnel, pipettes, test tube, test tube with cap and test tube with screw cap)
8. High-speed refrigerated microcentrifuge (MX-305, TOMY ®, Germany)
9. Incubator (IB11-E; Lab Companion ®, Korea)
10. Incubator shaker (Innova 2000; SCIENCETIFIC ®, Thailand)
11. Laminar air flow (BHA48; FASTER®, Netherland)
12. Master cycler (Eppendorf ®, Thailand)
13. Microwave oven (LG ®, Thailand)
14. Power supply (MP-300V)
15. pH meter
16. Spectrophotometer (SPEKOL 1500; Analitik jena®, Germany)
17. Water Bath (DI N40050; Memmert®, Germany)

## 3.4 Methods

### 3.4.1 Study the effects of carbon materials or metal nanoparticles containing media on physiological and metabolic changes of the plant under tissue culture condition

Shoot of tobaccos were grown on Murashige and Skoog (MS) under 25±2 °C, 16-h light/ 8-h dark for 3 weeks. All sample were analyzed their morphology and the content of plant biomaterials.

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**3.4.1.1 The effects of carbon materials in medium on plant**

- Plants were cultured on medium containing 3% and 4% Sucrose or 0, 25 and 100 mg. $L^{-1}$ carbon black (CB)

**3.4.1.2 The effects of metal oxide nanoparticles in medium on plant**

- Plants were cultured on medium containing 0, 10 and 20 mg. $L^{-1}$ ZnO NPs

- Plants were cultured on medium containing 0, 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs

**3.4.1.3 The combination effects metal oxide nanoparticles and extra sucrose in medium on plant**

- Plants were cultured on 4% sucrose medium containing with 10 mg. $L^{-1}$ ZnO NPs or 20 mg. $L^{-1}$ $TiO_2$ NPs

Analysis of plant physiological changes

- Photosynthetic pigments

Chlorophyll A, chlorophyll B, and carotenoids were extracted with 1.5 ml acetone (Reiss, 1994) from 50 mg of the sample that were ground to a powder with liquid nitrogen. After keeping at 4 °C for 48 hr., all samples were quantitated photosynthetic pigment with a spectrophotometer at absorbance 470, 644 and 662 nm. Chlorophyll A, Chlorophyll B and Carotenoid content of each samples were calculated using equations below.

$$\text{Chlorophyll A } (\mu g.\ gFW^{-1}) = 9.784(A_{662}) - 0.99(A_{644})$$

$$\text{Chlorophyll B } (\mu g.\ gFW^{-1}) = 21.42(A_{644}) - 4.56(A_{662})$$

$$\text{Carotenoid } (\mu g.\ gFW^{-1}) = \frac{(1,000(A_{470}) - 1.9(ChA) - 63.14(ChB)}{214}$$

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- Percentage on cell membrane injury

Ion leakage method was used to detect cell membrane injury (Bajji *et al*, 2002). All samples were weighted 0.3 g and cut to same small pieces. 10 ml of deionized water was added to the sample then the sample were placed at room temperature for 20 min. and measured the initial electrical conductivity content ($EC_i$). The sample was continued to incubate at room temperature for 4 hr. and the final electrical conductivity content ($EC_f$) of the sample were measured. All sample were boiled for 2 min then the total electrical conductivity content ($EC_T$) of sample were measured. Percentage of cell membrane injuring were estimated by the equation.

$$\% \text{ cell membrane injury} = [\frac{EC_f - EC_i}{EC_T - EC_i}] \times 100$$

- Lipid peroxidation

For measurement of cell membrane injury, the lipid peroxidation reaction on cell membrane was detected by the amount of MDA derived from lipid peroxidation reaction (Hodges, 1999). Leaves of tobacco were ground then 1.5 ml of 1% trichloroacetic acid (TCA) were added and incubated for 1 hr. with 250 rpm agitating at room temperature. The sample were centrifuged at 10,000 rpm. The supernatant of the sample was divided into two tubes in volume 500 µl per each. One group of samples was added 500 µl 20% TCA and the other group was added 500 µl mixed solution of 20% TCA and 0.5% thiobarbituric acid (TBA). All sample were boiled for 30 min. and measured an absorbance. TCA solution was measured at 532 and 600 nm. TCA and TBA solution at 440, 532 and 600 nm. All absorbance value was calculated malondialdehyde content using the formula below.

$$\text{MDA (nmol/ml)} = [\frac{(A - B)}{157{,}000}] \times 10^{6}$$

$$A = [(A_{532\,(2nd)} - A_{600(2nd)}] - [A_{532(1st)} - A_{600(1st)}],\ B = [A_{440(2nd)} - A_{600(2nd)}] \times 0.0571$$

*1st = first group that were added 20% TCA

*2nd = second group that were added mixed solution (20%TCA and 0.5% TBA)

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- Total soluble carbohydrate

Total soluble carbohydrate content was measured by the phenol-sulfuric method (Shout *et al.,* 2003) by ground 0.5 g of leaf sample with liquid nitrogen then added 2 ml of 80% ethanol. After incubating at 80 °C for 15 min. All sample were agitated 250 rpm at room temperature for 1 hr. and kept at 4 °C for 24 hr. then centrifuged at 10,000 rpm for 10 min to separate 400 µl supernatant. Samples were mixed with 400 µl deionized water and chloroform. The solutions were diluted ten times with deionized water. 500 µl of the solution was added 500 µl of 5 % phenol and 2.5 ml of sulfuric acid, placed at room temperature for 10 min then the sample was measured an absorbance value at wavelength 490 nm. to estimate amount of total soluble carbohydrate by total soluble carbohydrate standard curve.

- Flavonoid

0.5 g of the sample was ground to a powder with liquid nitrogen then extracted with 2 ml of acid-methanol solvent (Harborne, 1998). After agitation at 250 rpm at room temperature for 2 hr., the extraction was added 1 ml chloroform then centrifuged at 10,000 rpm at 4°C for 10 min. The upper part of the supernatant was used for detection the content of flavonoid in unit Abs. $gFW^{-1}$ by spectrophotometry (*p*-Coumaric acid = 305 nm., Naringenin = 330 nm., Apigenin = 336 nm., Kaempferol = 368 nm., Pelargonidin = 520 nm.).

**3.4.2 Study the application of nanoparticles on cutting plants that were immersed in water**

Tobacco leaves were cut and immersed in 30 ml of water without or with nanoparticles Solutions (10 mg. $L^{-1}$ ZnO NPs, 20 mg. $L^{-1}$ $TiO_2$ NPs). All sample were covered with lids and placed in the control condition. After 3 days of incubation, all sample were analyzed in comparing to the control.

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**3.4.2.1 Analysis of photosynthetic pigments**

Three main photosynthetic pigments (Chlorophyll A, Chlorophyll B and Carotenoid) were detected by the same methods that were mentioned above.

**3.4.2.2 Ethylene**

After 3 days of incubation, water without or with nanoparticle solution which were immersed by plant, were used for detecting the concentration of soluble ethylene by the reaction of decolonization of $KMnO_4$. The solution was mixed to 0.16 µM $KMnO_4$ with 1% sulfuric acid for 10 min (Chayaprasert and Sompornpailin, 2019). The solutions were measured at an absorbance value of the remaining $KMnO_4$ (525 nm) and converted to the percentage of ethylene content.

**3.4.3 Statistical analysis**

The experiments were performed in a completely randomized design (CRD) at least 3 duplications. Using one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) for Statistical analysis. The differences among means value significant at a P value ≤ 0.05 were determined by LSD test.

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# CHAPTER 4

# RESULTS

## 4.1 The effects of carbon materials or metal nanoparticles containing media on physiological and metabolic changes of plant under tissue culture condition

### 4.1.1 The effects of carbon materials in medium on plant

The plant culture medium (MS) that contain 3% and 4% of sucrose have been studied the effects on plant physiology. The tobaccos grown in 4% sucrose MS show higher growth rate than the same line grown in 3% MS (Figure 4.1). However, morphologies of the same tobacco line in both conditions were similar. Tobacco grown in 4% sucrose MS contained photosynthetic pigments higher than Tobacco grown in 3% sucrose MS did. Under 4% sucrose in MS condition, the main photosynthetic pigment (Chlorophyll A) was increased 74.99% in WT and 34.40% in *TT8* that were higher than the content of Chlorophyll A in the same tobacco lines that were grown in 3% sucrose.

Sub-photosynthetic pigment (Chlorophyll B and Carotenoid) content was also be increased when tobaccos were grown in 4% sucrose MS. Chlorophyll B content was be increased about 10.58% and 41.40% in WT and *TT8,* respectively. Carotenoid content was be increased about 37.53% and 38.58% in WT and *TT8,* respectively (Table 4.1). The levels of cell membrane injury of tobaccos grown in 4% sucrose MS were reduced in comparing with the same line of tobacco in 3% sucrose MS (Table 4.2). WT and *TT8* plant showed the reducing percentage of cell membrane injury about 40.33% and 25.83%, respectively.

The primary metabolite (total soluble carbohydrate) content of tobaccos grown in 4% sucrose are higher than that of content in 3% sucrose MS at significant level (Table 4.3). WT in 4% sucrose MS, total soluble carbohydrate was increased 24.47%, while total soluble carbohydrate of *TT8* in 4% sucrose MS was increased 29.01%. The transgenic tobaccos accumulated higher level of total soluble

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carbohydrate than WT did in both 3% and 4% sucrose MS. A biosynthesis of secondary metabolite (flavonoids) also showed higher in tobaccos that were grown in 4% sucrose MS treatment. (Table 4.4) *p*-Coumaric acid were increased 41.56% and 74.17% in WT and *TT8* transgenic, respectively. Naringenin and Apigenin accumulated higher when tobaccos were grown in 4% sucrose MS. The content of Naringenin in WT and *TT8* tobacco accumulated 58.11% and 67% higher than the content in tobacco that were grown in 3% sucrose MS. WT and *TT8* tobacco increased Apigenin content 35.83% and 69.32% when were grown in 4% sucrose MS. For Kaempferol and Pelargonidin accumulation, the highest level of these flavonoid subgroup found in *TT8* transgenic tobaccos that were grown in 4% sucrose MS condition while the another treatment was not different at a significant level.

**Figure 4.1** Morphology of 3-weeks old WT and *TT8*-transgenic tobacco were grown in 3% and 4% sucrose MS

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**Table 4.1** Effects of sucrose in culture medium on plant photosynthetic pigments

| Pigments ($\mu g.\ gFW^{-1}$) | | 3% sucrose MS | 4% sucrose MS |
|---|---|---|---|
| Chlorophyll A | WT | $23.74 \pm 1.31^{c}$ | $41.54 \pm 0.27^{a}$ |
| | *TT8* | $30.52 \pm 0.76^{bc}$ | $41.01 \pm 1.12^{a}$ |
| Chlorophyll B | WT | $9.53 \pm 1.94^{c}$ | $10.54 \pm 1.07^{bc}$ |
| | *TT8* | $10.82 \pm 2.56^{bc}$ | $15.30 \pm 3.04^{a}$ |
| Carotenoid | WT | $9.59 \pm 0.56^{b}$ | $13.20 \pm 0.27^{a}$ |
| | *TT8* | $9.09 \pm 1.28^{b}$ | $12.59 \pm 3.08^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.2** Effects of sucrose in culture medium on plant cell membrane injury

| | | 3% sucrose MS | 4% sucrose MS |
|---|---|---|---|
| Percentage on cell membrane injury (%) | WT | $28.14 \pm 0.41^{a}$ | $16.79 \pm 0.38^{c}$ |
| | *TT8* | $24.55 \pm 6.24^{ab}$ | $18.21 \pm 4.00^{c}$ |

The different letters represented a significant at $p \leq 0.05$

**Table 4.3** Effects of sucrose in culture medium on total soluble carbohydrate

| Metabolite ($\mu g.\ ml^{-1}$) | | 3% sucrose MS | 4% sucrose MS |
|---|---|---|---|
| Total soluble carbohydrate | WT | $21.63 \pm 1.44^{d}$ | $26.92 \pm 1.13^{c}$ |
| | *TT8* | $46.67 \pm 2.39^{b}$ | $60.21 \pm 2.82^{a}$ |

The different letters represented a significant at $p \leq 0.05$

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**Table 4.4** Effects of sucrose in culture medium on the accumulations of plant secondary metabolites

| Metabolites (Abs. $gFW^{-1}$) | | 3% sucrose MS | 4% sucrose MS |
|---|---|---|---|
| *p*-Coumaric acid | WT | $9.25 \pm 5.3^{c}$ | $13.09 \pm 0.63^{b}$ |
| | *TT8* | $19.46 \pm 7.47^{b}$ | $33.88 \pm 14.47^{a}$ |
| Naringenin | WT | $9.74 \pm 2.51^{c}$ | $15.40 \pm 5.77^{b}$ |
| | *TT8* | $21.70 \pm 5.74^{b}$ | $36.24 \pm 13.68^{a}$ |
| Apigenin | WT | $9.70 \pm 4.10^{b}$ | $13.23 \pm 4.81^{b}$ |
| | *TT8* | $18.22 \pm 4.96^{b}$ | $30.85 \pm 12.30^{a}$ |
| Kaempferol | WT | $1.96 \pm 0.33^{bc}$ | $2.74 \pm 0.25^{b}$ |
| | *TT8* | $2.19 \pm 0.34^{b}$ | $3.42 \pm 0.75^{a}$ |
| Pelargonidin | WT | $0.072 \pm 0.026^{b}$ | $0.076 \pm 0.21^{b}$ |
| | *TT8* | $0.083 \pm 0.016^{b}$ | $0.122 \pm 0.19^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

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Another carbon-based material that was used in experiments, was carbon black. In this experiment, plant physiology of tobacco under carbon black condition were different from those of non-carbon black condition. Root of tobacco in carbon black treatment were higher quantity and thicker than root in non-carbon black MS, moreover when tobacco was grown in carbon black MS, the plant was increased plant

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height (Figure 4.2). The content of photosynthesis pigments was induced when tobaccos were grown in carbon black MS (Table 4.5). WT enhanced chlorophyll A about 19% in 25 and 100 mg. $L^{-1}$ carbon black MS. *TT8*-transgenic enhanced about 18% in 25 mg. $L^{-1}$ carbon black MS and 31.39% in 100 mg. $L^{-1}$ carbon black MS. Media with carbon black induced chlorophyll B content in both WT and *TT8*-transgenic. Under carbon black condition, WT contained chlorophyll B about 100% while *TT8*-transgenic contained chlorophyll B only about 20-40% higher than non-carbon black condition. Carotenoid content was reduced when tobaccos were grown in carbon black condition.

To clarification more effect of carbon black on physiological of tobacco percentage of cell membrane injury and MDA content were measured. For the percentage of cell membrane injury in both WT and *TT8*-transgenic reduced when tobaccos were grown in carbon black MS. The percentages of cell membrane injury in 25 mg. $L^{-1}$ and 100 mg. $L^{-1}$ carbon black reduced about 12% in WT tobacco and 7-15% in *TT8*-transgenic tobacco (Table 4.6). The MDA content of WT and *TT8*-transgenic in carbon black MS were less than tobacco in non-carbon black MS. WT plant reduces MDA content about 20% in 25 mg. $L^{-1}$ carbon black MS and about 40% in 100 mg. $L^{-1}$ carbon black MS. MDA content in *TT8*-transgenic that were grown in carbon black MS also reduce but not significant (Table 4.6). In carbon black treatment, plant accumulated higher total soluble carbohydrate about 9.00 and 14.80 % for WT and 11.37 and 19.56 % for *TT8*-transgenic when they were grown in 25 mg. $L^{-1}$ and 100 mg. $L^{-1}$ carbon black MS, respectively (Table 4.7). In comparison with the same treatment, *TT8*-transgenic accumulated total soluble carbohydrate higher than WT 64.91%, 68.48% and 71.89% in 0, 25 and 100 mg. $L^{-1}$ carbon black, respectively. Carbon black effected on inducing the flavonoid accumulation (Table 4.8). WT contained precursor and early flavonoid substrate higher about 10-20% when were grown in carbon black MS. Late flavonoid substrate accumulated in WT increased about 100%. *TT8*-transgenic increased early flavonoid content about 30-60% and late flavonoid substrate accumulated higher about 100%.

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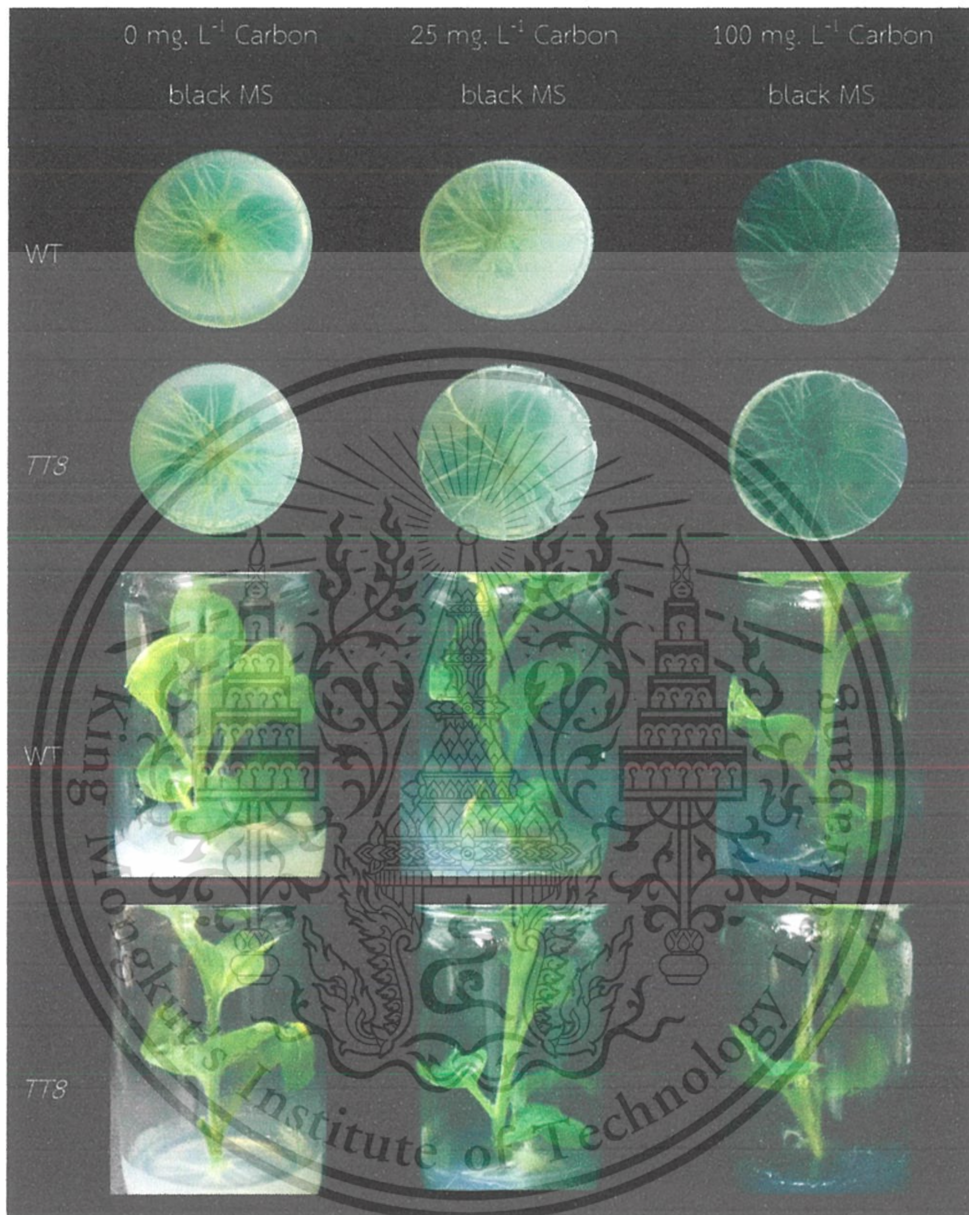


**Figure 4.2** Morphology of 3-weeks old tobacco were grown in 0, 25 and 100 mg. $L^{-1}$ carbon black MS.

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**Table 4.5** Effects of carbon black in culture medium on plant photosynthetic pigments

| Pigments (µg. gFW$^{-1}$) | | 0 mg. L$^{-1}$ carbon black MS | 25 mg. L$^{-1}$ carbon black MS | 100 mg. L$^{-1}$ carbon black MS |
|---|---|---|---|---|
| Chlorophyll A | WT | 30.73±0.31$^{b}$ | 36.85±0.50$^{a}$ | 36.51±0.07$^{a}$ |
| | *TT8* | 28.41±3.18$^{b}$ | 33.65±2.23$^{ab}$ | 37.32±3.00$^{a}$ |
| Chlorophyll B | WT | 4.233±0.42$^{b}$ | 11.57±0.14$^{a}$ | 11.96±0.33$^{a}$ |
| | *TT8* | 9.88±1.92$^{a}$ | 11.70±2.02$^{a}$ | 13.77±3.20$^{a}$ |
| Carotenoid | WT | 15.17±0.26$^{b}$ | 14.84±0.30$^{a}$ | 15.23±0.26$^{a}$ |
| | TT8 | 9.03±2.61$^{b}$ | 11.60±2.43$^{ab}$ | 11.61±2.14$^{ab}$ |

The different letters in each row of same material represented a significant at p ≤0.05

**Table 4.6** Effects of carbon black in culture medium on cell membrane

| | | 0 mg. L$^{-1}$ carbon black MS | 25 mg. L$^{-1}$ carbon black MS | 100 mg. L$^{-1}$ carbon black MS |
|---|---|---|---|---|
| Percentage on cell membrane injury (%) | WT | 58.14±0.54$^{a}$ | 51.16±0.60$^{b}$ | 50.63±0.59$^{b}$ |
| | *TT8* | 51.14±2.12$^{b}$ | 47.69±1.81$^{b}$ | 43.51±2.09$^{c}$ |
| MDA (nmol/ml) | WT | 0.79±0.03$^{a}$ | 0.49±0.01$^{b}$ | 0.46±0.01$^{bc}$ |
| | *TT8* | 0.42±0.12$^{bc}$ | 0.35±0.08$^{bc}$ | 0.30±0.06$^{c}$ |

The different letters in each row of same material represented a significant at p ≤0.05

**Table 4.7** Effects of carbon black in culture medium on total soluble carbohydrate

| Metabolite (µg. ml$^{-1}$) | | 0 mg. L$^{-1}$ carbon black MS | 25 mg. L$^{-1}$ carbon black MS | 100 mg. L$^{-1}$ carbon black MS |
|---|---|---|---|---|
| Total soluble carbohydrate | WT | 40.25±2.22$^{c}$ | 43.88±1.00$^{c}$ | 46.17±3.25$^{bc}$ |
| | *TT8* | 66.38±16.91$^{abc}$ | 73.92±14.41$^{ab}$ | 79.36±11.75$^{a}$ |

The different letters in each row of same material represented a significant at p ≤0.05

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**Table 4.8** Effects of carbon black in culture medium on plant secondary metabolites

| Metabolites (Abs. gFW$^{-1}$) | | 0 mg. L$^{-1}$ carbon black MS | 25 mg. L$^{-1}$ carbon black MS | 100 mg. L$^{-1}$ carbon black MS |
|---|---|---|---|---|
| *p*-Coumaric acid | WT | 12.35±0.14$^{c}$ | 13.49±0.31$^{c}$ | 15.29±0.13$^{bc}$ |
| | *TT8* | 20.24±2.67$^{b}$ | 26.90±5.11$^{a}$ | 29.58±4.09$^{a}$ |
| Naringenin | WT | 12.56±0.09$^{d}$ | 13.75±0.36$^{d}$ | 16.68±0.10c$^{d}$ |
| | *TT8* | 22.90±5.32$^{abc}$ | 28.19±5.40$^{ab}$ | 32.35±4.40$^{a}$ |
| Apigenin | WT | 14.64±0.06$^{c}$ | 15.15±0.07$^{c}$ | 14.99±0.58$^{c}$ |
| | *TT8* | 19.43±3.90$^{bc}$ | 25.49±6.34$^{ab}$ | 30.92±4.39$^{a}$ |
| Kaempferol | WT | 2.43±0.08$^{c}$ | 2.49±0.11$^{c}$ | 2.70±0.06$^{bc}$ |
| | *TT8* | 2.56±0.08$^{c}$ | 2.94±0.31$^{b}$ | 3.32±0.24$^{a}$ |
| Pelargonidin | WT | 0.059±0.001$^{d}$ | 0.114±0.005$^{c}$ | 0.201±0.003$^{a}$ |
| | *TT8* | 0.077±0.035$^{d}$ | 0.150±0.024$^{b}$ | 0.235±0.010$^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

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### 4.1.2 The effects of metal oxide nanoparticles in medium on plant

#### 4.1.2.1 ZnO nanoparticles

Under ZnO NPs condition, the morphologies of tobacco (WT, *TT8,* and *PAP1* transgenics) that were grown in MS medium with or without ZnO NPs as control are shown in Figure 4.3. Three-weeks tobacco WT and transgenics in ZnO NPs condition had a higher height than the same line in non ZnO NPs MS. Chlorophyll A in WT increased 22.38% and only 4.84% in 10 and 20 mg. $L^{-1}$ ZnO NPs MS, respectively. For transgenic tobacco, *TT8* were enhanced the accumulation of chlorophyll A 41.44% and 25.47% in 10 and 20 mg. $L^{-1}$ ZnO NPs MS, respectively. Chlorophyll A content in *PAP1* transgenic were promoted about 22.56% and 9.26% in 10 and 20 mg. $L^{-1}$ ZnO NPs MS, respectively. The content of chlorophyll B in all lines of samples were also increased in ZnO NPs MS conditions (Table 4.9). Chlorophyll B content in WT increased about 22.41% and 8.96%, in *TT8* transgenic increased about 46.68% and 18.13%, in *PAP1* transgenic increased about 22.14% and 1.93% in 10 and 20 mg. $L^{-1}$ ZnO NPs MS from 0 mg. $L^{-1}$ ZnO NPs MS (Table 4.9). Plants that were grown under ZnO NPs MS, showed higher accumulation of carotenoid content than those tobaccos in non ZnO NPs MS. The highest carotenoid contents were found in 10 mg. $L^{-1}$ ZnO NPs MS but insignificantly different from the content in 20 mg. $L^{-1}$ ZnO NPs MS condition (Table 4.9). The cell membrane injury in 10 mg. $L^{-1}$ ZnO NPs MS condition were reduced from control treatment 19.43%, 13.79% and 10.47% in WT, *TT8,* and *PAP1* transgenic, respectively. The MDA content of tobaccos in 10 mg. $L^{-1}$ ZnO NPs MS are different between plant lines, WT contained 34.25% MDA, *TT8*-transgenic contained 19.64% and *PAP1*-transgenic contained 24.56% MDA which were lower than the MDA content of each line in 0 mg. $L^{-1}$ ZnO NPs MS (Table 4.10).

WT tobacco accumulated total soluble carbohydrate higher about 41.68% in 10 mg. $L^{-1}$ ZnO NPs MS and 58.06% in 20 mg. $L^{-1}$ ZnO NPs MS from WT tobacco in MS

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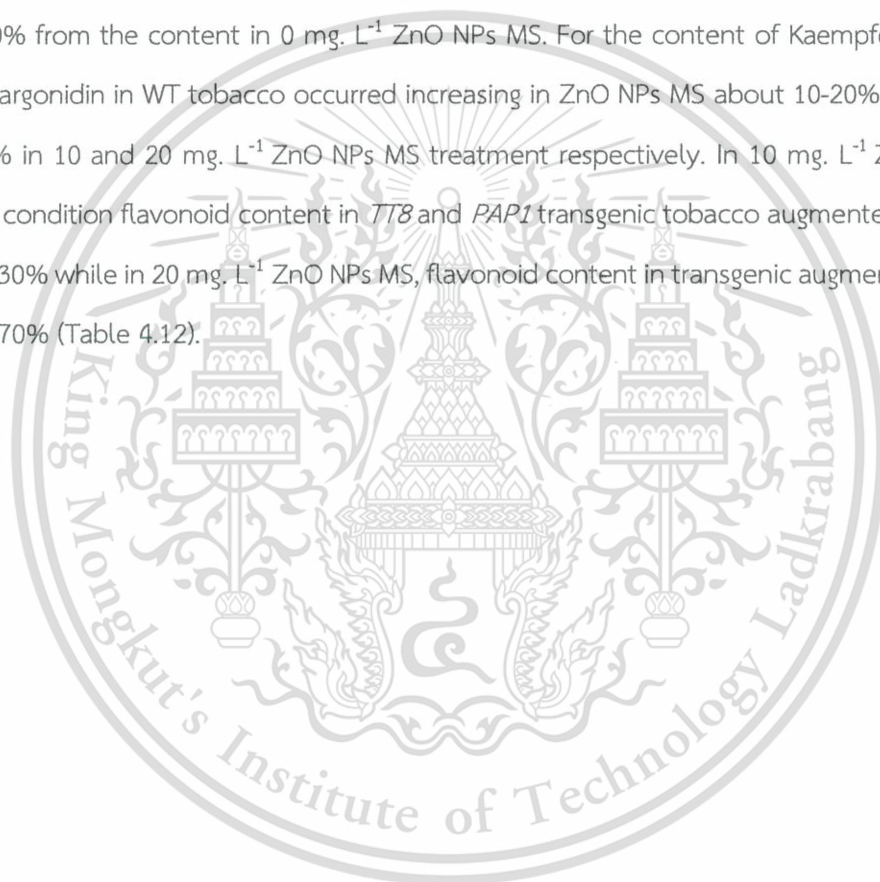
without ZnO NPs. *TT8*-transgenic increased total soluble carbohydrate content about 17.11% and 29.17% when were grown in 10 and 20 mg. $L^{-1}$ ZnO NPs MS, respectively. The total soluble carbohydrate content in *PAP1*-transgenic also increased when were grown in ZnO NPs MS 5.03% and 13.63% in 10 and 20 mg. $L^{-1}$ ZnO NPs MS, respectively (Table 4.11)

WT contained *p*-Coumaric acid, Naringenin and Apigenin content higher about 12-25% when were grown under 10 mg. $L^{-1}$ ZnO NPs MS. When WT were grown in 20 mg. $L^{-1}$ ZnO NPs MS, there three flavonoid sub-group content increased more than 100% from the content in 0 mg. $L^{-1}$ ZnO NPs MS. For the content of Kaempferol and Pelargonidin in WT tobacco occurred increasing in ZnO NPs MS about 10-20% and 50-70% in 10 and 20 mg. $L^{-1}$ ZnO NPs MS treatment respectively. In 10 mg. $L^{-1}$ ZnO NPs MS condition flavonoid content in *TT8* and *PAP1* transgenic tobacco augmented about 10-30% while in 20 mg. $L^{-1}$ ZnO NPs MS, flavonoid content in transgenic augment about 40-70% (Table 4.12).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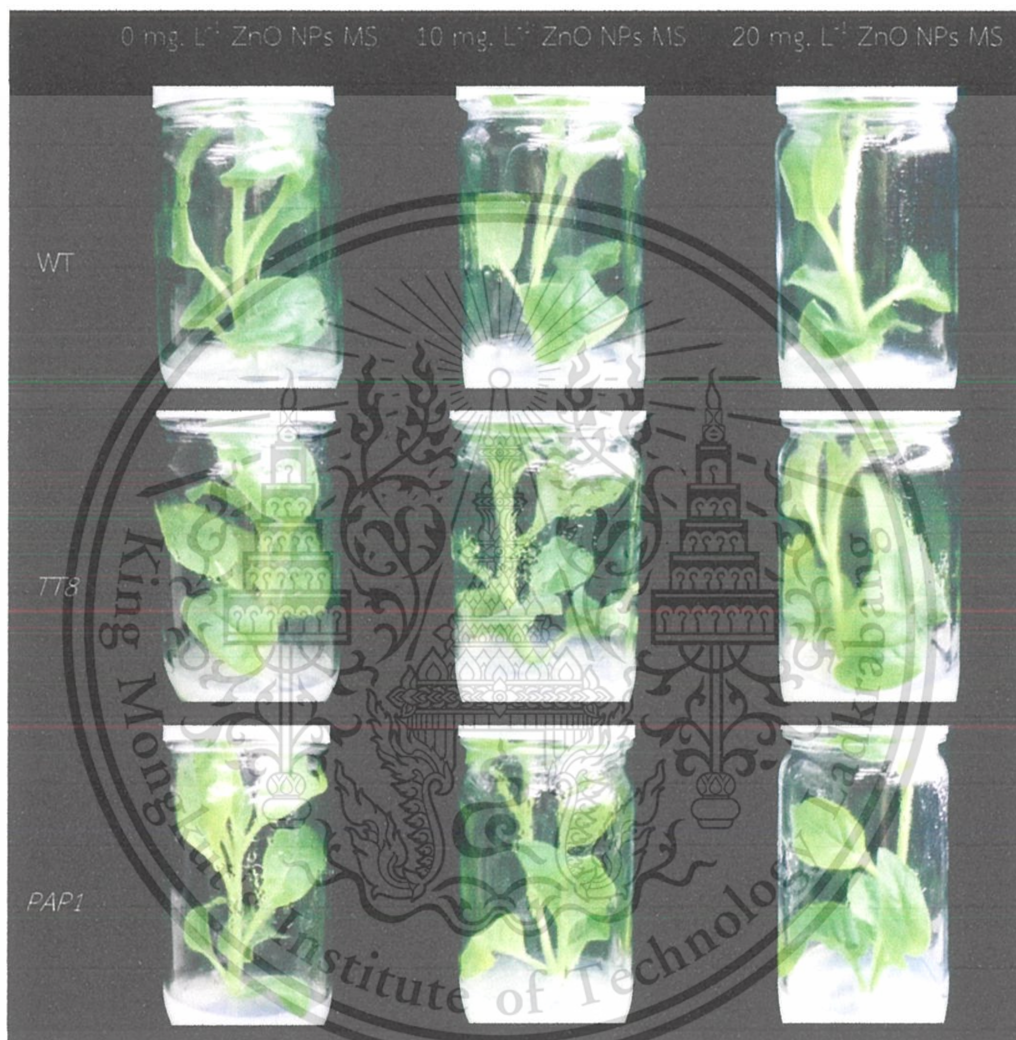


**Figure 4.3** Morphology of 3-weeks old tobacco were grown in 0, 10 and 20 mg. $L^{-1}$ ZnO NPs MS.

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**Table 4.9** Effects of ZnO NPs in culture medium on photosynthetic pigments

| Pigments ($\mu g.\ gFW^{-1}$) | | 0 mg. $L^{-1}$ ZnO NPs MS | 10 mg. $L^{-1}$ ZnO NPs MS | 20 mg. $L^{-1}$ ZnO NPs MS |
|---|---|---|---|---|
| Chlorophyll A | WT | $17.96\pm0.07^{d}$ | $21.98\pm0.70^{cd}$ | $18.83\pm0.53^{d}$ |
| | *TT8* | $27.44\pm0.76^{bc}$ | $38.81\pm0.80^{a}$ | $34.43\pm1.09^{ab}$ |
| | *PAP1* | $29.04\pm0.23^{bc}$ | $35.59\pm0.94^{ab}$ | $31.73\pm0.27^{ab}$ |
| Chlorophyll B | WT | $6.27\pm0.26^{e}$ | $7.67\pm0.21^{de}$ | $6.83\pm0.18^{e}$ |
| | *TT8* | $10.13\pm0.76^{cd}$ | $14.86\pm0.78^{a}$ | $11.99\pm0.59^{abc}$ |
| | *PAP1* | $11.09\pm0.50^{bc}$ | $13.77\pm0.59^{ab}$ | $11.31\pm0.63^{bc}$ |
| Carotenoid | WT | $5.87\pm0.03^{cd}$ | $5.94\pm0.07^{cd}$ | $5.38\pm0.06^{d}$ |
| | *TT8* | $8.00\pm0.24^{bc}$ | $11.09\pm0.32^{a}$ | $10.05\pm0.29^{ab}$ |
| | *PAP1* | $8.71\pm0.34^{ab}$ | $10.61\pm0.37^{a}$ | $10.28\pm0.38^{ab}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.10** Effects of ZnO NPs in culture medium on cell membrane

| | | 0 mg. $L^{-1}$ ZnO NPs MS | 10 mg. $L^{-1}$ ZnO NPs MS | 20 mg. $L^{-1}$ ZnO NPs MS |
|---|---|---|---|---|
| Percentage on cell membrane injury (%) | WT | $33.91\pm0.70^{a}$ | $19.43\pm0.21^{bcd}$ | $23.96\pm0.15^{b}$ |
| | *TT8* | $22.98\pm0.74^{b}$ | $13.79\pm0.34^{de}$ | $16.95\pm0.70^{cd}$ |
| | *PAP1* | $20.76\pm0.53^{bc}$ | $10.47\pm0.28^{e}$ | $16.35\pm0.22^{cd}$ |
| MDA ($nmol.ml^{-1}$) | WT | $0.73\pm0.01^{a}$ | $0.48\pm0.02^{c}$ | $0.62\pm0.01^{b}$ |
| | *TT8* | $0.56\pm0.02^{d}$ | $0.45\pm0.03^{d}$ | $0.51\pm0.01^{d}$ |
| | *PAP1* | $0.57\pm0.01^{d}$ | $0.43\pm0.02^{d}$ | $0.51\pm0.02^{d}$ |

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**Table 4.11** Effects of ZnO NPs in culture medium on total soluble carbohydrate

| Metabolite ($\mu g. ml^{-1}$) | | 0 mg. $L^{-1}$ ZnO NPs MS | 10 mg. $L^{-1}$ ZnO NPs MS | 20 mg. $L^{-1}$ ZnO NPs MS |
|---|---|---|---|---|
| Total soluble carbohydrate | WT | $41.88 \pm 0.18^{e}$ | $59.33 \pm 0.14^{e}$ | $66.19 \pm 0.75^{e}$ |
| | *TT8* | $60.17 \pm 0.95^{d}$ | $70.47 \pm 0.51^{b}$ | $77.73 \pm 0.93^{a}$ |
| | *PAP1* | $63.74 \pm 0.88^{cd}$ | $66.95 \pm 0.97^{bc}$ | $72.43 \pm 0.91^{ab}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.12** Effects of ZnO NPs in culture medium on plant secondary metabolites

| Metabolites (Abs. $gFW^{-1}$) | | 0 mg. $L^{-1}$ ZnO NPs MS | 10 mg. $L^{-1}$ ZnO NPs MS | 20 mg. $L^{-1}$ ZnO NPs MS |
|---|---|---|---|---|
| *p*-Coumaric acid | WT | $5.57 \pm 0.17^{c}$ | $6.76 \pm 0.16^{bc}$ | $12.46 \pm 0.22^{abc}$ |
| | *TT8* | $18.45 \pm 0.48^{abc}$ | $20.64 \pm 0.59^{a}$ | $25.73 \pm 0.32^{a}$ |
| | *PAP1* | $13.17 \pm 0.16^{abc}$ | $16.13 \pm 0.28^{ab}$ | $18.23 \pm 0.29^{a}$ |
| Naringenin | WT | $7.23 \pm 0.67^{c}$ | $9.21 \pm 0.15^{c}$ | 19.54±0.43ab |
| | *TT8* | $16.82 \pm 0.40^{b}$ | $21.43 \pm 0.57^{ab}$ | $23.88 \pm 0.25^{a}$ |
| | *PAP1* | $14.62 \pm 0.52^{b}$ | $18.03 \pm 0.26^{ab}$ | $21.23 \pm 0.30^{a}$ |
| Apigenin | WT | $6.10 \pm 0.08^{e}$ | $6.78 \pm 0.03^{e}$ | $13.74 \pm 0.12^{d}$ |
| | *TT8* | $15.47 \pm 0.29^{cd}$ | $22.82 \pm 0.34^{b}$ | $26.92 \pm 0.13^{a}$ |
| | *PAP1* | $13.39 \pm 0.32^{d}$ | $18.86 \pm 0.45^{c}$ | $22.97 \pm 0.27^{b}$ |
| Kaempferol | WT | $0.70 \pm 0.01^{g}$ | $1.09 \pm 0.02^{fg}$ | $1.72 \pm 0.01^{ef}$ |
| | *TT8* | $2.48 \pm 0.05^{cde}$ | $3.19 \pm 0.03^{bc}$ | $4.03 \pm 0.12^{a}$ |
| | *PAP1* | $2.11 \pm 0.02^{de}$ | $2.48 \pm 0.04^{cd}$ | $3.12 \pm 0.04^{ab}$ |
| Pelargonidin | WT | $0.034 \pm 0.001^{e}$ | $0.042 \pm 0.001^{e}$ | $0.050 \pm 0.002^{de}$ |
| | *TT8* | $0.073 \pm 0.002^{cd}$ | $0.102 \pm 0.002^{ab}$ | $0.125 \pm 0.002^{a}$ |
| | *PAP1* | $0.073 \pm 0.002^{cd}$ | $0.087 \pm 0.002^{bc}$ | $0.102 \pm 0.001^{bc}$ |

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**4.1.2.2 $TiO_2$ nanoparticles**

The chlorophyll A level increased when all line tobaccos were grown in 20 mg. $L^{-1}$ $TiO_2$ NPs MS (Table 4.13). For WT increased chlorophyll A content 4.85%. *TT8* tobacco increased 9.19% and *PAP1* increased 8.43%. When tobaccos were grown in 40 mg. $L^{-1}$ $TiO_2$ NPs MS, the chlorophyll A content dropped 26.59% in WT and 4.46% in *TT8* tobacco. Only *PAP1* contained chlorophyll A content, 0.50%, higher than *PAP1* in non-$TiO_2$ NPs MS. The result of chlorophyll B was the same as chlorophyll A result. WT and *TT8* transgenic had chlorophyll B level 14.07% and 11.32% which were higher than the same line in non $TiO_2$ NPs MS but decreased 17.96% and 12.58% in WT and *TT8* transgenic from the content in non $TiO_2$ NPs MS when they were grown in 40 mg. $L^{-1}$ $TiO_2$ NPs MS. For *PAP1* tobacco increased 32.70% of chlorophyll B content when were grown in 20 mg. $L^{-1}$ $TiO_2$ NPs MS and increased only 11.44% when were grown in 40 mg. $L^{-1}$ $TiO_2$ NPs MS. Carotenoid content in WT in 20 mg. $L^{-1}$ $TiO_2$ nanoparticle MS was 22.78% higher than carotenoid content of WT in MS without $TiO_2$ NPs. Transgenic tobaccos accumulated the higher level of carotenoid when they were grown in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS, 29.74% and 20.72% for *TT8*, 25.05% and 9.22% for *PAP1*. The result of cell membrane stability when tobaccos were grown in 0, 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS shown in Table 4.14. WT were reduced the percentage of cell membrane injury in 20 mg. $L^{-1}$ $TiO_2$ NPs MS 17.70%, but the percentage of cell membrane injury of WT were induced 13.84% in 40 mg. $L^{-1}$ $TiO_2$ NPs MS. The percentage of cell membrane injury of *TT8* transgenic tobaccos were reduced in both $TiO_2$ NPs MS treatment 53.28% and 4.36% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS, respectively. *PAP1* transgenics in 20 mg. $L^{-1}$ $TiO_2$ NPs MS had percentage of cell membrane injury 22.86% lower than in 0 mg. $L^{-1}$ $TiO_2$ NPs MS but the percentage of cell membrane injury in *PAP1* increased 9.74% in 40 mg. $L^{-1}$ $TiO_2$ NPs MS. The MDA content of all tobacco was reduced when they were grown in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS. WT decreased

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43.46% and 35.42%, *TT8* decreased 24.10% and 4.58% and *PAP1* decreased 43.71% and 15.05% from MDA content in the same line of tobacco in MS without $TiO_2$ NPs.

Total soluble carbohydrate in WT increased 32.41% and 57.62% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS, respectively. Transgenic tobaccos also accumulated higher total soluble carbohydrate when were in $TiO_2$ NPs MS than in non $TiO_2$ NPs MS. *TT8* increased 18.23% and 32.12% while *PAP1* increased 15.9% and 28.67% when transgenics were grown in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS, respectively (Table 4.15). The flavonoid contents of tobacco that was grown in 0, 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs treatment is shown in Table 4.16. Flavonoid content in transgenic tobaccos were accumulated higher than WT. Tobacco in $TiO_2$ NPs MS treatment showed increasing of flavonoid accumulation. the *p*-Coumaric acid in WT accrued 14.21% and 156.72% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS treatment while *TT8* and *PAP1* transgenic accrued only 12-13% and 32-33% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS treatment. Naringenin and apigenin content in WT increased about 20-30% and 50-60% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS treatment, respectively. for transgenics increased about 18-20% and 20-50% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS, respectively. Kaempferol subgroup in WT contained 9.30% and 76.27% in 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs treatment. *TT8* and *PAP1* accumulated kaempferol higher about 20-50% in $TiO_2$ NPs treatment. The content of pelargonidin in WT increased 20-40% in 20 mg. $L^{-1}$ $TiO_2$ NPs treatment and 30-70% in 40 mg. $L^{-1}$ $TiO_2$ NPs treatment. The increasing rate of pelargonidin in transgenics when they were grown in $TiO_2$ NPs treatment were lower than WT. Transgenic tobacco accumulated pelargonidin about 14-30% higher in 20 mg. $L^{-1}$ $TiO_2$ NPs treatment and about 20-60% higher in 40mg. $L^{-1}$ $TiO_2$ NPs treatment.

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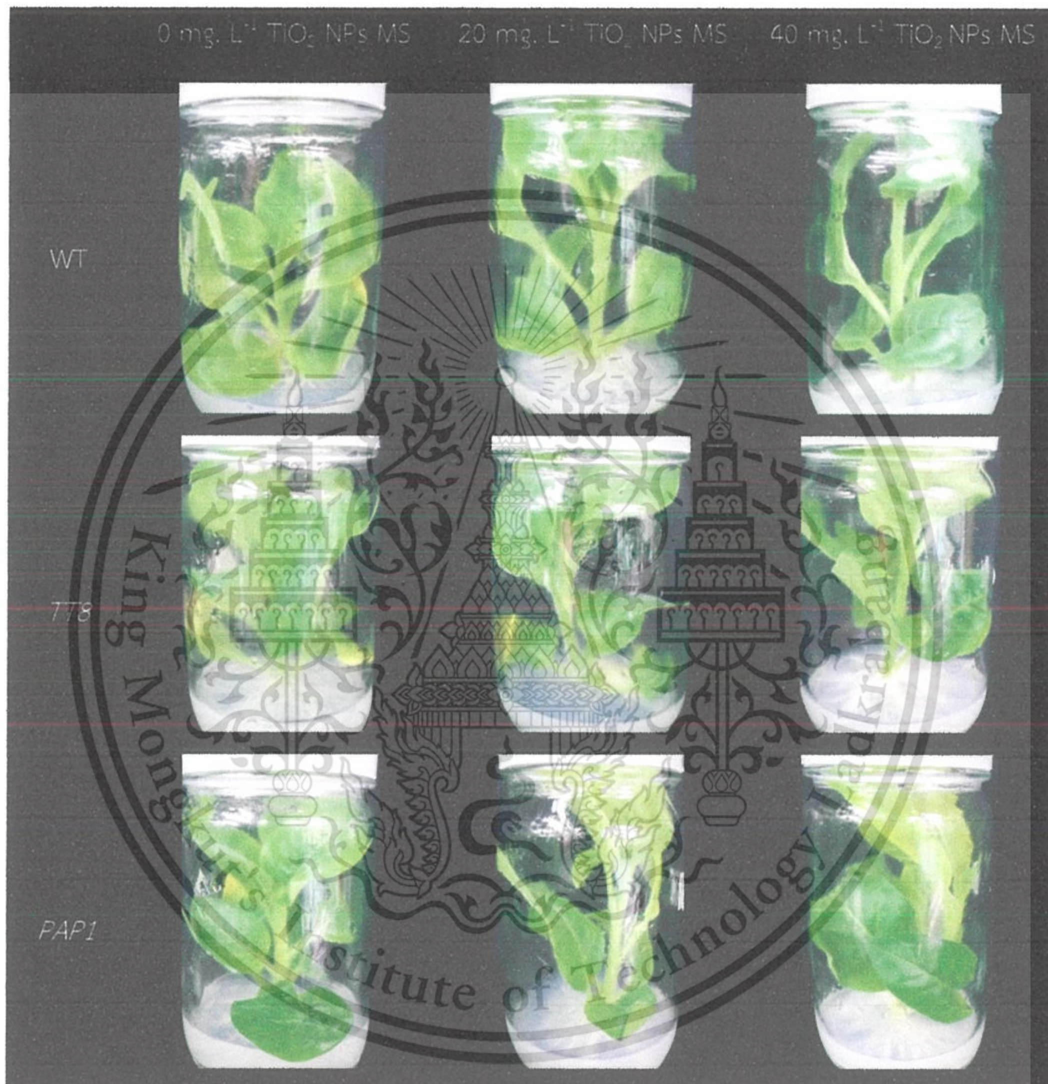


**Figure 4.4** Morphology of 3-weeks old tobacco were grown in 0, 20 and 40 mg. $L^{-1}$ $TiO_2$ NPs MS.

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**Table 4.13** Effects of $TiO_2$ NPs in culture medium on photosynthetic pigments

| Pigment s ($\mu g.\ gFW^{-1}$) | | 0 mg. $L^{-1}$ $TiO_2$ NPs MS | 20 mg. $L^{-1}$ $TiO_2$ NPs MS | 40 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|---|
| Chlorophyll A | WT | $28.22\pm0.39^{b}$ | $29.59\pm0.311^{ab}$ | $20.72\pm0.86^{b}$ |
| | *TT8* | $30.26\pm1.91^{ab}$ | $33.04\pm4.03^{a}$ | $28.91\pm4.83^{ab}$ |
| | *PAP1* | $28.60\pm3.62^{ab}$ | $31.01\pm3.31^{a}$ | $28.74\pm4.21^{ab}$ |
| Chlorophyll B | WT | $9.07\pm0.51^{a}$ | $10.35\pm0.74^{a}$ | $7.44\pm0.24^{a}$ |
| | *TT8* | $10.39\pm3.61^{a}$ | $11.56\pm3.00^{a}$ | $9.08\pm2.11^{a}$ |
| | *PAP1* | $9.36\pm1.51^{a}$ | $12.43\pm1.74^{a}$ | $10.44\pm1.34^{a}$ |
| Carotenoid | WT | $7.29\pm0.57^{d}$ | $8.95\pm0.53^{bcd}$ | $7.08\pm0.66^{d}$ |
| | *TT8* | $8.34\pm1.50^{bcd}$ | $10.82\pm1.52^{a}$ | $10.07\pm1.28^{ab}$ |
| | *PAP1* | $7.52\pm1.42^{cd}$ | $9.40\pm0.95^{abc}$ | $8.21\pm1.28^{bcd}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.14** Effects of $TiO_2$ NPs in culture medium on cell membrane

| | | 0 mg. $L^{-1}$ $TiO_2$ NPs MS | 20 mg. $L^{-1}$ $TiO_2$ NPs MS | 40 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|---|
| Percentage on cell membrane injury (%) | WT | $50.33\pm1.23^{ab}$ | $41.42\pm0.33^{bc}$ | $57.29\pm0.80^{a}$ |
| | *TT8* | $33.56\pm13.90^{cd}$ | $15.68\pm10.63^{e}$ | $32.10\pm16.81^{cd}$ |
| | *PAP1* | $20.30\pm6.60^{de}$ | $15.66\pm16.81^{e}$ | $22.28\pm10.01^{de}$ |
| MDA ($nmol.\ ml^{-1}$) | WT | $0.56\pm0.01^{a}$ | $0.32\pm0.01^{bc}$ | $0.36\pm0.01^{b}$ |
| | *TT8* | $0.30\pm0.09^{bc}$ | $0.23\pm0.05^{cd}$ | $0.29\pm0.09^{bcd}$ |
| | *PAP1* | $0.35\pm0.09^{b}$ | $0.20\pm0.04^{d}$ | $0.30\pm0.04^{bc}$ |

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**Table 4.15** Effects of $TiO_2$ NPs in culture medium on total soluble carbohydrate

| Metabolite ($\mu g.\ ml^{-1}$) | | 0 mg. $L^{-1}$ $TiO_2$ NPs MS | 20 mg. $L^{-1}$ $TiO_2$ NPs MS | 40 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|---|
| Total soluble carbohydrate | WT | $30.08\pm1.63^{d}$ | $39.83\pm0.71^{d}$ | $47.42\pm1.39^{d}$ |
| | *TT8* | $43.83\pm2.87^{ab}$ | $51.83\pm5.16^{ab}$ | $57.92\pm6.79^{a}$ |
| | *PAP1* | $41.64\pm6.56^{c}$ | $48.28\pm5.15^{bc}$ | $53.58\pm5.79^{ab}$ |

The different letters in each row of same material represented a significant at $p \le 0.05$

**Table 4.16** Effects of $TiO_2$ NPs in culture medium on plant secondary metabolites

| Metabolite (Abs/g.FW) | | 0 mg. $L^{-1}$ $TiO_2$ NPs MS | 20 mg. $L^{-1}$ $TiO_2$ NPs MS | 40 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|---|
| *p*-Coumaric acid | WT | $5.45\pm0.09^{f}$ | $6.22\pm0.06^{f}$ | $14.04\pm0.07^{e}$ |
| | *TT8* | $19.87\pm2.93^{bc}$ | $22.60\pm2.47^{b}$ | $26.27\pm3.00^{a}$ |
| | *PAP1* | $15.97\pm3.74^{de}$ | $17.92\pm2.61^{de}$ | $21.36\pm1.44^{cd}$ |
| Naringenin | WT | $6.13\pm0.05^{d}$ | $7.73\pm0.19^{d}$ | $9.56\pm0.07^{d}$ |
| | *TT8* | $18.73\pm5.28a^{bc}$ | $21.75\pm5.00^{ab}$ | $24.13\pm5.03^{a}$ |
| | *PAP1* | $15.49\pm2.79^{c}$ | $16.59\pm2.78^{bc}$ | $19.57\pm1.78^{abc}$ |
| Apigenin | WT | $6.07\pm0.08^{e}$ | $6.31\pm0.07^{e}$ | $7.58\pm0.16^{e}$ |
| | *TT8* | $19.09\pm4.21^{bc}$ | $22.20\pm4.70^{ab}$ | $24.24\pm4.71^{a}$ |
| | *PAP1* | $12.64\pm1.39^{d}$ | $15.30\pm3.22^{cd}$ | $17.66\pm2.57^{bc}$ |
| Kaempferol | WT | $1.51\pm0.03^{e}$ | $1.65\pm0.04^{de}$ | $2.67\pm0.07^{bc}$ |
| | *TT8* | $2.45\pm0.37^{bcd}$ | $3.19\pm0.74^{ab}$ | $3.77\pm1.03^{a}$ |
| | *PAP1* | $1.59\pm0.44^{de}$ | $2.04\pm0.45^{cde}$ | $2.42\pm0.55^{bcd}$ |
| Pelargonidin | WT | $0.042\pm0.001^{e}$ | $0.054\pm0.001^{cd}$ | $0.058\pm0.001^{c}$ |
| | *TT8* | $0.055\pm0.005^{cd}$ | $0.070\pm0.007^{b}$ | $0.084\pm0.007^{a}$ |
| | *PAP1* | $0.049\pm0.005^{de}$ | $0.055\pm0.005^{cd}$ | $0.062\pm0.007^{c}$ |

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### 4.1.3 the combination effects of metal oxide nanoparticles and extra sucrose in medium on plant

#### 4.1.3.1 Sucrose and ZnO nanoparticles

After tobacco was treated under 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS for 3-weeks, the sample in treatment shown better morphology than in the control condition. There were higher and had darker green on leaves in compare with same line tobacco that was grown in normal MS (Figure 4.5). Photosynthetic pigment content was also increased (Table 4.17). In WT under 4% sucrose and 10 mg. $L^{-1}$ ZnO MS treatment contained chlorophyll A, chlorophyll B and carotenoid 8.57%, 12.32% and 14.55 % higher than WT in normal MS. Both transgenics accumulated photosynthetic pigment higher than WT. Chlorophyll A content in transgenics were higher than WT about 9-13%, chlorophyll B content in transgenics were higher than WT about 110-130%, carotenoid content in transgenics were higher than WT about 1.2-10% in normal MS. In 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS transgenic synthesized chlorophyll A, chlorophyll B and carotenoid about 13-16%, 20-40% and 10-40% higher than WT. Transgenic tobaccos increased accumulation of chlorophyll A about 10%, chlorophyll B about 25%, carotenoid about 30% when were growth in 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS than in normal MS. Plant cell injury after were treated with 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS that were measured by the amount of MDA shown in Table 4.18. WT reduced MDA content 49.95% under 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS. Transgenics reduced MDA content 36.12% and 50.81% for *TT8* and *PAP1* respectively. In the same treatment, *TT8* and *PAP1* contained lower MDA than WT did about 20-30% and 10-20% in 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS than the same line in normal MS.

Moreover, tobacco that was grown in MS with 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs accumulated higher metabolites than tobacco in control MS (Table 4.19-4.20). Both transgenics contained higher primary and secondary metabolites than WT. The total soluble carbohydrate content of tobaccos that were growth in 4% sucrose

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and 10 mg. $L^{-1}$ ZnO NPs MS increased 78.50%, 45.07% and 32.36% in WT, *TT8,* and *PAP1* respectively. Secondary metabolites content of tobacco that was grown in MS or MS with 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS are shown in Table 4.20. *TT8* and *PAP1* transgenic accumulated higher flavonoid content than WT. In normal and 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS both transgenics showed increasing of flavonoid content than WT. All sample also increased flavonoid content when were treated under 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS. WT increased more than 100% while transgenics increased about 80-100%.

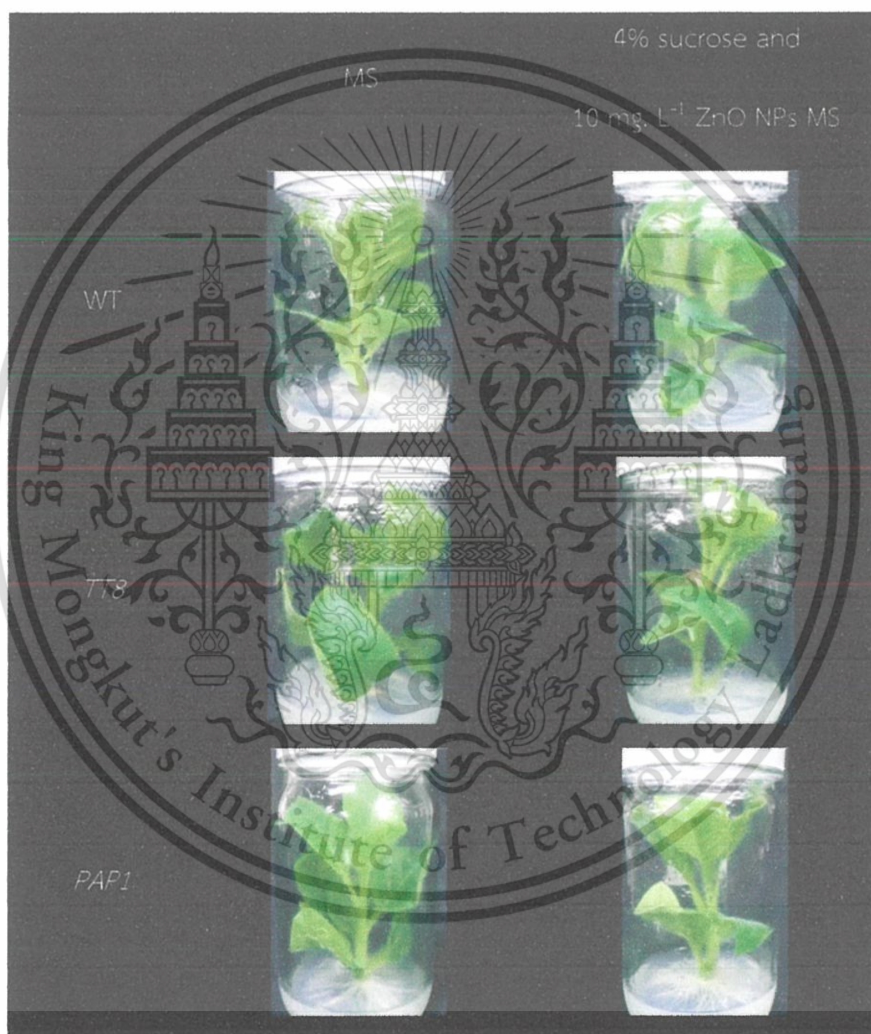


**Figure 4.5** Morphology of 3-week tobacco were grown in 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs MS

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**Table 4.17** Effects of extra sucrose and ZnO NPs in culture medium on photosynthetic pigments

| Pigments ($\mu g. gFW^{-1}$) | | MS | 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs |
|---|---|---|---|
| Chlorophyll A | WT | 30.44±0.33$^{c}$ | 33.05±0.32$^{b}$ |
| | *TT8* | 33.30±1.65$^{b}$ | 37.49±1.65$^{a}$ |
| | *PAP1* | 34.32±2.19$^{b}$ | 38.43±2.19$^{a}$ |
| Chlorophyll | WT | 9.03±1.92$^{d}$ | 19.17±0.62$^{c}$ |
| | *TT8* | 19.51±1.06$^{c}$ | 24.72±1.06$^{ab}$ |
| | *PAP1* | 21.51±1.27$^{bc}$ | 27.06±1.27$^{a}$ |
| Carotenoid | WT | 7.80±0.33$^{c}$ | 8.94±0.66$^{bc}$ |
| | *TT8* | 7.90±0.47c | 9.77±0.25$^{b}$ |
| | *PAP1* | 8.61±0.45$^{bc}$ | 12.28±0.51$^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.18** Effects of extra sucrose and ZnO NPs in culture medium on cell membrane

| | | MS | 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs |
|---|---|---|---|
| MDA (nmol. $ml^{-1}$) | WT | 1.08±0.03$^{a}$ | 0.54±0.01$^{cd}$ |
| | *TT8* | 0.70±0.03$^{bc}$ | 0.45±0.02$^{d}$ |
| | *PAP1* | 0.82±0.3$^{b}$ | 0.40±0.03$^{d}$ |

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**Table 4.19** Effects of extra sucrose and ZnO NPs in culture medium on total soluble carbohydrate

| Metabolite ($\mu g. ml^{-1}$) | | MS | 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs |
|---|---|---|---|
| Total soluble carbohydrate | WT | $43.79 \pm 2.15^{c}$ | $78.17 \pm 2.69^{a}$ |
| | *TT8* | $55.97 \pm 1.01^{c}$ | $81.19 \pm 1.45^{a}$ |
| | *PAP1* | $60.07 \pm 0.72^{b}$ | $79.52 \pm 0.72^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.20** Effects of extra sucrose and ZnO NPs in culture medium on plant secondary metabolites

| Metabolite (Abs/g.FW) | | MS | 4% sucrose and 10 mg. $L^{-1}$ ZnO NPs |
|---|---|---|---|
| *p*-Coumaric acid | WT | $4.86 \pm 0.02^{e}$ | $10.22 \pm 0.19^{de}$ |
| | *TT8* | $21.28 \pm 0.59^{c}$ | $41.27 \pm 1.03^{a}$ |
| | *PAP1* | $18.03 \pm 0.46^{cd}$ | $31.23 \pm 0.53^{b}$ |
| Naringenin | WT | $5.39 \pm 0.09^{c}$ | $11.72 \pm 0.11^{c}$ |
| | *TT8* | $29.65 \pm 1.87^{b}$ | $53.72 \pm 0.97^{a}$ |
| | *PAP1* | $26.07 \pm 1.01^{b}$ | $42.15 \pm 1.01^{a}$ |
| Apigenin | WT | $5.08 \pm 0.09^{f}$ | $11.15 \pm 0.20^{e}$ |
| | *TT8* | $23.51 \pm 1.14^{c}$ | $51.72 \pm 1.33^{a}$ |
| | *PAP1* | $17.63 \pm 1.00^{d}$ | $38.64 \pm 0.81^{b}$ |
| Kaempferol | WT | $0.86 \pm 0.09^{c}$ | $2.51 \pm 0.07^{b}$ |
| | *TT8* | $3.04 \pm 0.09^{b}$ | $5.61 \pm 0.10^{a}$ |
| | *PAP1* | $3.38 \pm 0.10^{b}$ | $6.39 \pm 0.19^{a}$ |
| Pelargonidin | WT | $0.025 \pm 0.001^{d}$ | $0.084 \pm 0.006^{b}$ |
| | *TT8* | $0.060 \pm 0.002^{c}$ | $0.118 \pm 0.003^{a}$ |
| | *PAP1* | $0.060 \pm 0.003^{bc}$ | $0.121 \pm 0.005^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

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#### 4.1.3.2 Sucrose and $TiO_2$ nanoparticles

After tobacco were treated in 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS, all samples are significantly grown faster than the same line of tobacco under normal MS (Figure 4.6). The photosynthetic pigments of tobacco samples were increased when tobaccos were grown under 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS (Table 4.21). Chlorophyll A of WT increased 12.86% and Transgenics increased about 18% from the content in normal MS. For chlorophyll B content of tobacco in 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS treatment in WT were not significantly different from normal MS but transgenic increased chlorophyll B about 20%. Carotenoid content in samples that were treated in 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS were higher than those sample in normal MS 3.51% in WT, 23.37% in *TT8* and 18.16% in *PAP1* (Table 4.21). A reaction of lipid peroxidation that show cell membrane injury was decreased in WT and transgenic tobaccos (Table 4.22) under 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS condition. WT tobacco showed the lower content of MDA 64.43%, transgenic decreased MDA content about 45-50%. The primary metabolites of tobaccos are shown in Table 4.23. Total soluble carbohydrate of all sample was increased when were grown under 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ nanoparticle MS. WT increased total soluble carbohydrate 55.11% and transgenic increased about 20%. The content of secondary metabolites are shown in Table 4.24. All sample appeared increasing all flavonoid subgroup when were treated in 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS. WT tobacco increased *p*-Coumaric acid more than 100% while transgenic increased only 30-40% but in comparison in same treatment, both transgenic contain *p*-Coumaric acid was higher than WT. Naringenin and apigenin in WT accumulated 60-90% higher, *TT8* and *PAP1* accumulated about 30% higher than the content in normal MS. Kaempferol content increased more than 100% in WT and about 30% in transgenics. The late flavonoid subgroups (Pelargonidin) increased about 60-70% when samples were grown under 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS condition.

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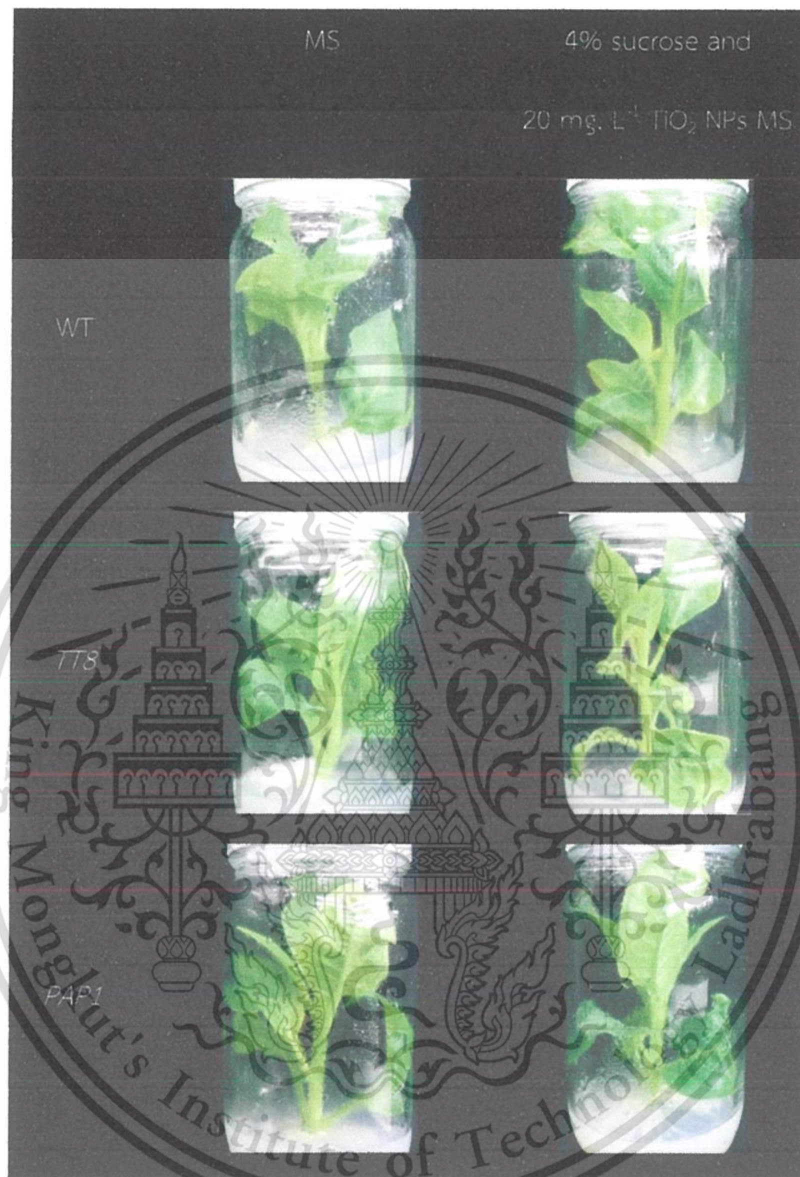


**Figure 4.6** Morphology of 3-week tobacco were grown in 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS

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**Table 4.21** Effects of extra sucrose and $TiO_2$ NPs in culture medium on photosynthetic pigments

| Pigments (µg. $gFW^{-1}$) | | MS | 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|
| Chlorophyll A | WT | $26.93\pm0.07^{c}$ | $30.39\pm0.05^{bc}$ |
| | *TT8* | $32.28\pm0.20^{bc}$ | $38.24\pm0.23^{a}$ |
| | *PAP1* | $29.76\pm0.23^{c}$ | $35.38\pm0.20^{b}$ |
| Chlorophyll B | WT | $9.46\pm0.14^{c}$ | $9.35\pm0.09^{c}$ |
| | *TT8* | $11.32\pm0.42^{abc}$ | $14.28\pm0.41^{a}$ |
| | *PAP1* | $10.31\pm0.33^{bc}$ | $13.29\pm0.38^{ab}$ |
| Carotenoid | WT | $8.38\pm0.13^{b}$ | $8.68\pm0.03^{b}$ |
| | *TT8* | $8.90\pm0.11^{b}$ | $10.98\pm0.17^{a}$ |
| | *PAP1* | $8.99\pm0.11^{b}$ | $10.62\pm0.14^{a}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.22** Effects of extra sucrose and $TiO_2$ NPs in culture medium on cell membrane

| | | MS | 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|
| MDA (nmol. $ml^{-1}$) | WT | $0.65\pm0.01^{a}$ | $0.23\pm0.01^{d}$ |
| | *TT8* | $0.40\pm0.01^{b}$ | $0.21\pm0.02^{de}$ |
| | *PAP1* | $0.32\pm0.01^{c}$ | $0.18\pm0.01^{e}$ |

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**Table 4.23** Effects of extra sucrose and $TiO_2$ NPs in culture medium on total soluble carbohydrate

| Metabolite ($\mu g.\ ml^{-1}$) | | MS | 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs MS |
|---|---|---|---|
| Total soluble carbohydrate | WT | $30.17\pm1.51^{d}$ | $46.79\pm0.80^{bc}$ |
| | *TT8* | $46.87\pm0.75^{bc}$ | $57.19\pm0.48^{a}$ |
| | *PAP1* | $43.03\pm0.37^{c}$ | $54.38\pm0.60^{ab}$ |

The different letters in each row of same material represented a significant at $p \leq 0.05$

**Table 4.24** Effects of extra sucrose and $TiO_2$ NPs in culture medium on plant secondary metabolites

| Metabolite (Abs/g.FW) | | MS | 4% sucrose and 20 mg. $L^{-1}$ $TiO_2$ NPs |
|---|---|---|---|
| *p*-Coumaric acid | WT | $5.42\pm0.38^{e}$ | $14.37\pm0.31^{d}$ |
| | *TT8* | $18.56\pm0.48^{bc}$ | $24.69\pm0.48^{a}$ |
| | *PAP1* | $15.67\pm0.36^{cd}$ | $21.95\pm0.36a^{b}$ |
| Naringenin | WT | $6.07\pm0.07^{c}$ | $9.49\pm0.16^{c}$ |
| | *TT8* | $20.09\pm0.60^{b}$ | $27.20\pm0.23^{a}$ |
| | *PAP1* | $17.10\pm0.21^{b}$ | $21.67\pm0.16^{ab}$ |
| Apigenin | WT | $5.41\pm0.20^{d}$ | $10.49\pm0.03^{c}$ |
| | *TT8* | $18.01\pm0.51^{bc}$ | $25.10\pm0.28^{a}$ |
| | *PAP1* | $15.78\pm0.39^{bc}$ | $20.66\pm0.27^{ab}$ |
| Kaempferol | WT | $0.76\pm0.03^{e}$ | $1.83\pm0.05^{cd}$ |
| | *TT8* | $2.59\pm0.76^{bc}$ | $3.75\pm0.06^{a}$ |
| | *PAP1* | $1.64\pm0.04^{d}$ | $2.73\pm0.07^{b}$ |
| Pelargonidin | WT | $0.049\pm0.001^{d}$ | $0.062\pm0.001^{cd}$ |
| | *TT8* | $0.057\pm0.002^{d}$ | $0.097\pm0.003^{b}$ |
| | *PAP1* | $0.081\pm0.002^{bc}$ | $0.158\pm0.003^{a}$ |

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## 4.2 An application of nanoparticles in post harvesting technology of cutting plant that immersed water

### 4.2.1 Effect of nanoparticles on photosynthetic pigment changes of the cutting plant

Leaves of tobacco were harvested and immersed in water or nanoparticles solution, a little senescence appearance (dry texture, sulky edge, pale color, and yellow spot) emerges, especially in WT tobacco. Chlorophyll content of all samples were shown in Table 4.25. Chlorophyll A of WT and *TT8* transgenic reduced about 25% while *PAP1* transgenic reduced about 30% in water condition. In ZnO NPs solution, chlorophyll A content of WT reduced only 3% while *TT8* and *PAP1* transgenics reduced about 10-20%. Samples in $TiO_2$ solution decreased their chlorophyll A content about 12-19%. Another photosynthetic pigment (Chlorophyll B and Carotenoid) resulted in less reducing when the sample was immersed in nanoparticles solution than water about 50%.

**Table 4.25** Effect of nanoparticles on photosynthetic pigment changes of the cutting plant

| Pigments ($\mu g.\ gFW^{-1}$) | | before immersing | water | 10 $mg.\ L^{-1}$ ZnO NPs | 20 $mg.\ L^{-1}$ $TiO_2$ NPs |
|---|---|---|---|---|---|
| Chlorophyll A | WT | $34.16\pm0.16^{ab}$ | $25.67\pm0.17^{ed}$ | $33.01\pm0.38^{ab}$ | $29.83\pm0.38^{bcd}$ |
| | *TT8* | $31.83\pm0.48^{bc}$ | $23.84\pm0.68^{e}$ | $27.96\pm0.14^{cde}$ | $27.46\pm0.68^{cde}$ |
| | *PAP1* | $37.62\pm1.67^{a}$ | $26.95\pm0.07d^{e}$ | $30.29\pm0.28^{bcd}$ | $30.29\pm0.12^{bcd}$ |
| Chlorophyll B | WT | $9.49\pm0.04^{ab}$ | $9.33\pm0.08^{ab}$ | $9.54\pm0.21^{ab}$ | $9.58\pm0.01^{ab}$ |
| | *TT8* | $12.69\pm0.76^{a}$ | $8.60\pm0.65^{b}$ | $11.07\pm0.30^{ab}$ | $10.47\pm0.70^{ab}$ |
| | *PAP1* | $12.54\pm0.78^{a}$ | $8.07\pm0.62^{b}$ | $10.27\pm0.52^{ab}$ | $9.764\pm0.19^{ab}$ |
| Carotenoid | WT | $8.21\pm0.05^{cd}$ | $4.98\pm0.02^{e}$ | $8.13\pm0.32^{cd}$ | $7.26\pm0.39^{cd}$ |
| | *TT8* | $10.01\pm0.22^{ab}$ | $6.50\pm0.48^{de}$ | $8.49\pm0.17^{bc}$ | $7.37\pm0.31^{cd}$ |
| | *PAP1* | $11.08\pm0.73^{a}$ | $7.86\pm0.19^{cd}$ | $8.27\pm0.13^{bcd}$ | $8.48\pm0.13^{bc}$ |

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#### 4.2.2 Effect of nanoparticles on ethylene accumulation in cutting plant

After samples were immersed in water or NPs solution for 3 days, ethylene content of all samples found highest in samples that were immersed in water. In ZnO NPs solution, WT reduced ethylene content 32.63% while *TT8* transgenic reduced ethylene content 11.94% and *PAP1* transgenic reduced ethylene content 40% from the content in water treatments. In $TiO_2$ NPs solution, ethylene content in WT, *TT8* and *PAP1* decreased 61.22%, 17.91% and 65.71% respectively when compare to the same line in water condition (Table 4.26).

**Table 4.26** Effects of nanoparticles on ethylene

| Metabolite (% v/v) | | water | 10 mg. $L^{-1}$ ZnO NPs | 20 mg. $L^{-1}$ $TiO_2$ NPs |
|---|---|---|---|---|
| Ethylene | WT | 0.0147±0.0002[a] | 0.0099±0.0001[b] | 0.0057±0.0051[d] |
| | *TT8* | 0.0067±0.0007[c] | 0.0059±0.0001[d] | 0.0055±0.0016[d] |
| | *PAP1* | 0.0035±0.0001[e] | 0.0021±0.0002[f] | 0.0012±0.0008[g] |

The different letters represented a significant at $p \leq 0.05$

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# CHAPTER 5

# DISCUSSION

## 5.1 The effects of carbon materials or metal nanoparticles containing media on physiological and metabolic changes of plant under tissue culture condition

### 5.1.1 The effects of carbon materials in medium on plant

carbon-based materials can be localized and accumulated in roots, flowers, fruits, and leaves of plant (Anindita *et al.*, 2011; Larue *et al.*, 2012) because of smaller size of nanoparticles, their accumulations are higher than bulk materials, so it has more affections and interactions with plant cell. Both sugar and carbon black are a carbon source for metabolite synthesis and a signal molecule that regulates various pathway in the plant (Gloria and Daniel, 2001; Oliveira *et al.*, 2001; Paul and Pellny, 2003). Although, carbon-based materials could induce plant pigment (Barbinta-Patrascu *et al.*, 2014; Juergens *et al.*, 2015) but in fact when plant has high concentration of sugar in cell it would be inhibitor for plant pigment synthesis (Wolff and Price, 1960; Stadnichuk *et al.*, 1998). Our result showed significant differences between tobacco grown in 3% sucrose MS and 4% sucrose MS (Table 4.2). The significant differences found in plant grown under normal MS and CB treatment nevertheless no significant difference between the both concentrations of CB, in this experiment (Table 4.6). In this result, media containing carbon-based materials could effect on reducing damage of plant cell. However, multiwalled carbon nanotube at 5-50 mg/L concentrations could induce the DNA damage result in onion (Ghosh *et al.,* 2011). The negative results of carbon-based materials on plant were also found in different types of carbon nanomaterials and depended on concentration of carbon-based materials. Therefore, these materials effected phytotoxicity, increased reactive oxygen species (ROS) (Cañas *et al.,* 2008; Lin *et al.*, 2009; Begum and Fugetsu, 2012; Abdel-Aziz *et al.*, 2016) and DNA damaging (Ghosh *et al.,* 2011; Ghosh *et al.,* 2015).

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The concentration of carbon material in planting media effected on the plant physiology responding. Tobacco grown under media containing 4% sucrose or adding carbon black had higher content of total soluble carbohydrate than those under normal condition (Table 4.3 and Table 4.7). Carbohydrate contents were increased because of carbon-based materials that were added in media. The increasing carbohydrate in different types of plant (Barley, Tomato, Wheat and Maize) that were treated with carbon-based materials, have been reported (Khodakovskaya *et al.,* 2009; Khodakovskaya *et al.,* 2011; Lahiani *et al.,* 2013; Wang *et al.,* 2012). Carbon-based materials in MS media and Total soluble carbohydrate in plant cell impact the incresing of flavonoids contents (Table 4.4 and Table 4.8). This happen because carbon-based materials and carbohydrates are regulating molecule in flavonoid pathway. Moreover, various flavonoid regulatory genes contain carbohydrate signaling promotor which can be up/down regulated by carbohydrate molecules (Ohto *et a*l., 2001; Andersen and Markham, 2006; Tognetti *et al.,* 2013).

### 5.1.2 The effects of metal oxide nanoparticles in medium on plant

Zinc (Zn) is one of the essential nutrients for living organisms (Silva and Uchida, 2000; Kaur *et al.,* 2014; Coyne and Mikkelsen, 2015). It plays role as functional and structural component of more than 300 enzymes (Maret, 2013). Hence, the effect of Zn on plant development and metabolic process had been reported in Arabidopsis, Sesame, Chickpea, Peanut, Gram and Mung (Mahajan *et al.,* 2011; Prasad *et al.,* 2012; Pathania *et al.,* 2016; Narendhran *et al.,* 2016). In this work, the results of photosynthetic pigment of WT and transgenics tobaccos accumulated higher when samples under ZnO NPs MS conditions than the sample under MS without ZnO NPs (Table 4.9). The morphology of tobacco in ZnO NPs MS treatment showed a better growth rate than tobacco in control condition (Figure 4.3). ZnO NPs in MS induce total soluble carbohydrate in tobacco samples (Table 4.11). The reason is Zn that is one of the co-regulate of plant growth and developed hormones, induce plant physiological and metabolic processes. Less content of MDA and low percentage of cell membrane injury showed in samples under ZnO NPs MS conditions (Table 4.10). The content of

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flavonoids also found under ZnO NPs conditions were higher than samples under non ZnO NPs MS treatment (Table 4.12). These results occur because plant generates ROSs under metal stress (Michalak, 2006; Landa *et al.*, 2016). ROSs caused plant adjusting the rate of flavonoid biosynthesis to inhibit the potential of ROSs (Cakmak, 2000).

Under $TiO_2$ NPs in MS condition, tobacco slightly increased accumulation of photosynthetic pigments (Table 4.13). The results of increasing amount of chlorophyll in plants that were treated with $TiO_2$ NPs have been reported (peppermint, tomato and spinach) (Su *et al.*, 2009; Qi *et al.*, 2013; Samadi *et al.*, 2014). Other studies showed the effects of $TiO_2$ NPs on plant development and changing rate of metabolites (Zheng *et al.*, 2005; Gao *et al.*, 2008; Kurepa *et al.*, 2010; Wang *et al.*, 2012). Under $TiO_2$ NPs condition, tobacco had higher height than tobacco under normal MS. $TiO_2$ NPs has a beneficial effect on suppling water and organic compound. This mechanism enhances plant growth rate and development. The percentage of cell membrane injury and MDA content of sample under $TiO_2$ NPs MS were less than control conditions (Table 4.14). In 40 mg. $L^{-1}$ $TiO_2$ NPs MS, tobaccos had higher percentage of cell membrane injury and MDA content than the same line in 20 mg. $L^{-1}$ $TiO_2$ NPs MS. This provided that $TiO_2$ NPs also gave a negative effect on plant cell because $TiO_2$ NPs lead organism to produce reactive oxygen species, when $TiO_2$ NPs were radiated with ultraviolet (UV) (Khot *et al.*, 2012; Feizi *et al.*, 2013; Clément *et al.*, 2013). Other researches also indicated the phytotoxicity effect of $TiO_2$ NPs on plant cell such as reducing of germination rate, root length, shoot length, biomass and enzyme activity (Ma *et al.*, 2010; Song *et al.*, 2013; Yang *et al.*, 2015; Siddiqi and Husen, 2017). The result of this work showed that tobacco enhance flavonoids (main antioxidant molecules) when they were ground under $TiO_2$ NPs MS treatment (Table 4.16) because $TiO_2$ NPs in MS act as stress signal to stimulate an antioxidant activity of plant mechanism systems (Foltête *et al.*, 2011; Song *et al.*, 2013). Tobacco samples of this experiment showed the increasing content of total soluble carbohydrate (Table 4.15). Our result similar to the result of $TiO_2$ NPs increased amount of carbohydrate in barley have been reported (Pošćić *et al.*, 2016). These occur because $TiO_2$ lead plant

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to increase their primary metabolite synthesis (Hund-Rink and Simon, 2006; Paret *et al.*, 2013; Qi *et al.*, 2013).

**5.1.3 the combination effects of metal oxide nanoparticles and extra sucrose in medium on plant**

In this research, 4% sucrose in medium were used as basal medium for adding metal oxide nanoparticles, because sucrose are type of carbon-based material which are less toxic than carbon black (Jia *et al.*, 2005). The morphology of plant under sucrose and ZnO NPs MS treatment were not different from the plant under MS without carbon-base material and metal oxide NPs (Figure 4.5) on the other hand plant under sugar and $TiO_2$ NPs treatment shown better growth rate than plant under normal MS (Figure 4.6). Both carbon-base materials and metal oxide nanoparticles are signaling molecule that regulates mechanism of various biomaterial pathway of the plant. In this experiment, the result of cell membrane stability (Table 4.18 and Table 4.22) showed less MDA content when tobacco were grown under carbon-base material and metal oxide NPs MS than tobacco under normal MS. In summary, the effect of two type of materials promoted the defending system of plant cell. The photosynthetic pigment content of tobacco under carbon-base material and metal oxide NPs MS were higher than control condition (Table 4.17 and Table 4.21). The result of total soluble carbohydrate content of plant were induced under carbon-based and metal oxide nanoparticles condition (Table 4.19 and Table 4.23), due to the carbohydrate synthesis in plant cell are elevated in responding to oxidative condition. The accumulation of flavonoids was significantly higher when tobaccos were under media containing 4%sucrose and metal oxide NPs (Table 4.20 and Table 4.24) especially in transgenics tobaccos (*TT8* and *PAP1*). The signaling molecules are multiplied and elevated the flavonoid biosynthesis. Moreover, ZnO and $TiO_2$ NPs also effect on gene regulation and changing gene expression level and enzyme activity (Yang *et al.*, 2006; Gao *et al.*, 2008; Aslani *et al.*, 2014). The other reason is metal oxide NPs induced oxidative damage and antioxidant defense systems. Under metal oxide stress, ROS (reactive oxygen species) formation were activated (Díaz and Merino, 2006; Wojtaszek, 1997). Overuse of ROS

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signaling can cause harmful for plant cell (Mittova *et al.,* 2000). When plant is facing with a plenty of ROS signals, the defend systems were opened as a consequence of the flavonoid content of samples under carbon base material and metal oxide NPs MS were increased (Table 4.20 and Table 4.24). It may seem that metal oxide NPs have a toxicity effect of plant cell as prior researches have suggested (Hong *et al.,* 2005; Mohammadi *et al.,* 2014; Kouhi *et al.,* 2015). Notwithstanding, under optimal concentration of metal oxide NPs indicate the effect on induce plant metabolites without damaging of plant cell.

## 5.2 An application of nanoparticles in post harvesting technology of cutting plant that immersed water

### 5.2.1 Effect of nanoparticles on photosynthetic pigment changes of the cutting plant

Changing of plant physiology causes by nanoparticle especially metal oxide nanoparticles were found in various plant species (Zhao *et al.,* 2013; Rizwan *et al.,* 2017; Tripathi *et al.,* 2017). This cloud expands from size range of nanoparticle that small enough to reach and accumulate in plant cell through vascular tissue (Rizwan et al., 2017). Accumulation of nanoparticle lead to change of cellular reaction and appear different physiology from sample under non-nanoparticles condition. Although, The negative effects of NPs on plant physiological changes (deficient of biomass, belated root elongation and premature senescent) were also found but it depend on plant species, type of nanoparticles, condition of study and concentration of nanoparticles (Zhao et al., 2013; Nair and Chung, 2014). In this study, after leave sample was cut and immersed into water, 10 mg. $L^{-1}$ ZnO NPs or 20 mg. $L^{-1}$ $TiO_2$ NPs solution for 3 days the phenotype of leaves turned a slight senescence (dry texture, sulky edge, pale color, and yellow spot). Chlorophyll A, Chlorophyll B, and Carotenoid contents in leaves that were immersed into the water were reduces more than photosynthetic pigment content in leaves that were immersed into NPs solution (Table 4.25). The underlying concept of inducing photosynthetic pigment by ZnO and $TiO_2$ NPs were found in cucumber, tomato, peppermint, wheat and various plants (Priyank and Venkatachalam,

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2006; Zhao *et al.,* 2013; Samadi *et al.,* 2014; Mohammadia *et al.*, 2014; Raliya *et al.,* 2015; Amooaghaie *et al.,* 2016). This because Ti and Zn support an enzyme activity that involves with chlorophyll synthesis (Marschner, 1995; Mirshekali *et al.,* 2012, Tlustos *et al.,* 2005; Lyu *et al.,* 2017).

### 5.2.2 Effect of nanoparticles on ethylene accumulation in cutting plant

The ethylene contents in the sample under NPs solution emerged less than the ethylene content under control condition moreover the transgenic lines could be detected ethylene lass than WT in the comparison between same conditions (Table 4.26). The result showed that NPs have an efficiency to reduce the amount of ethylene from plant sample. The ZnO and $TiO_2$ are two type of metal catalyst surface for hydrogenation of ethylene besides that, these two materials have high efficiency for photocatalytic reaction (Dent and Kokes, 1962; Dent and Kokes, 1969). According to the result of other studies, ZnO and $TiO_2$ have an ability to speed up a hydrogenation reaction of ethylene but it limits in surface area of reaction (Kenji *et al.,* 1996; Panpranot *et al*., 2006; Shabaker *et al.,* 2003; Baker *et al.,* 2012). The reason of why ethylene of *PAP1* transgenic could be detected rarely under NPs solution, is ethylene and flavonoid biosynthesis are relevant. They use same regulatory factor and downregulation each other (Wang *et al.,* 2002; Buer *et al.*, 2006; Lewis *et al.,* 2011).

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# CHAPTER 6

# SUMMARY AND SUGGESTION

## 6.1 Summary

This experiment found that an appropriate concentration of each type materials for enhancing plant metabolites without damaging plant tobacco cells. When 4% sucrose were added in MS, both primary and secondary metabolites were induced. While both concentration of carbon black (25-100 mg. $L^{-1}$) in MS effected on inducing plant metabolite accumulations and producing similar in plant morphology. The concentration of metal oxide NPs used in this research were 0-20 mg. $L^{-1}$ ZnO NPs and 0-40 mg. $L^{-1}$ $TiO_2$ NPs. Under this condition, 10 mg. $L^{-1}$ ZnO NPs and 20 mg. $L^{-1}$ $TiO_2$ NPs in planting media, effected on inducing the accumulation of plant metabolites and reducing plant cell injury in compare to non-metal oxide NPs did. However, the highest accumulation of plant metabolites (of total soluble carbohydrate and flavonoids) was found when plant grown in media containing 20 mg. $L^{-1}$ ZnO NPs or 40 mg. $L^{-1}$ $TiO_2$ NPs. When 4% sucrose culture medium was added with 10 mg. $L^{-1}$ ZnO NPs or 20 mg. $L^{-1}$ $TiO_2$ NPs, the treated plants showed the better result in reducing cell injury and inducing plant biosynthesis than normal medium without nanoparticle did. The materials that were used in this experiment play role as a signaling molecules and up regulate pathway of primary and secondary metabolites of tobacco. An obvious example of this is carbon-based materials that are principal material to synthesis all plant metabolites and have an influence on physiological of plant. Metal oxide NPs act as co enzyme in various important plant biosynthesis beside that, metal oxide NPs induce oxidative reaction and trigger plant antioxidant system. For post-harvesting experiment, ZnO NPs and $TiO_2$ NPs in concentrations of 10 and 20 mg. $L^{-1}$, respectively have the ability to slow down the senescent process and maintain photosynthetic pigments of tobacco cell. Tobacco leave samples that were immersed into NPs solution could be detected ethylene content less than that were immersed into water.

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Due to this tissue this means NPs can reduce the concentration of ethylene gas that produced by the plant and retard aging process in plant cell system.

## 6.2 Suggestion

Our studies underlying the concept of the effect of NPs on plant physiological and metabolic changed of tobacco to indicate the suitable NPs for enhancing plant biomaterials. According to the amount of information on this topic is insufficient to all plant species because each plant species, stage of plants and part of plants differently respond to their environment signals. This means more studies about the effect of NPs on the other plant biomaterials of each plant stage are still required. For the use of nanoparticles in cutting plant application, the result of this study still limits in short term therefore, more researches about the application of NPs effect on cutting plants to reduce plant senescence are still required.

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# APPENDIX

## 1. Chemical for tissue culture medium: MS medium composition (Murashige and Skoog, 1962)

Macro elements

| | |
|---|---|
| $NH_4NO_3$ | 1,650.00 mg. $L^{-1}$ |
| $KNO_3$ | 1,900.00 mg. $L^{-1}$ |
| $CaCl_2 \cdot 2H_2O$ | 440.00 mg. $L^{-1}$ |
| $MgSO_4 \cdot 7H_2O$ | 370.00 mg. $L^{-1}$ |
| $KH_2PO_4$ | 170.00 mg. $L^{-1}$ |

Microelements

| | |
|---|---|
| $Na_2EDTA \cdot 2H_2O$ | 37.30 mg. $L^{-1}$ |
| $FeSO_4 \cdot 7H_2O$ | 27.80 mg. $L^{-1}$ |
| $H_3BO_3$ | 6.20 mg. $L^{-1}$ |
| $MnSO_4 \cdot 4H_2O$ | 22.30 mg. $L^{-1}$ |
| $ZnSO_4 \cdot 7H_2O$ | 8.60 mg. $L^{-1}$ |
| KI | 0.83 mg. $L^{-1}$ |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.25 mg. $L^{-1}$ |
| $CoCl_2 \cdot 6H_2O$ | 0.025 mg. $L^{-1}$ |
| $CuSO_4 \cdot 5H_2O$ | 0.025 mg. $L^{-1}$ |

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Organic compound

| | |
|---|---|
| Myo-inositol | 100.00 mg. $L^{-1}$ |
| Glycine | 2.00 mg. $L^{-1}$ |
| Nicotinic acid | 0.50 mg. $L^{-1}$ |
| Pyridoxine-HCl | 0.50 mg. $L^{-1}$ |
| Thymine-HCl | 0.01 mg. $L^{-1}$ |
| Agar | 8,000 mg. $L^{-1}$ |
| pH | 5.6-5.8 |

## 2. Chemical for photosynthetic pigment content

propan-2-one

## 3. Chemical for lipid peroxidation content

1% trichloroacetic acid (TCA)

20% trichloroacetic acid (TCA)

Mixed solution of 20% trichloroacetic acid and 0.5 % thiobarbituric acid (TBA)

## 4. Chemical for total soluble carbohydrate content

80%, $C_2H_5OH$

5% phenol, $C_6H_5OH$

$H_2SO_4$

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## 6. Chemical for flavonoid extraction

3: 2 of 1% HCl in Methanol: water

Chloroform, $CHCl_3$

## 7. Chemical for ethylene content

0.16 μM $KMnO_4$

Cycloalkene

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