สำนักหอสมุดกลาง พระจอมเกล้าลาดกระบัง

MECHANISMS OF INHIBITION OF *TAGETES ERECTA* L. LEAF EXTRACT ON SEED GERMINATION OF *AMARANTHUS* SPP.

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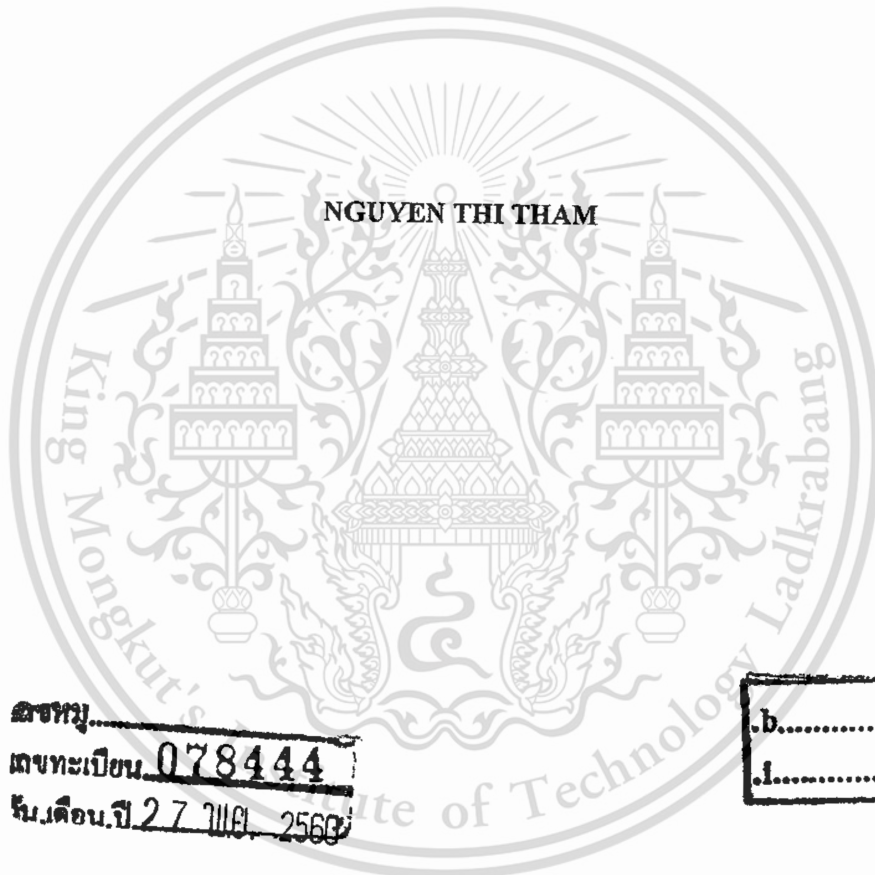

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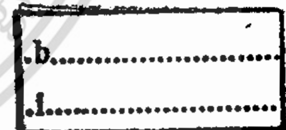

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A THESIS SUBMITTED IN PARTIAL FULFILMENT
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หัวข้อวิทยานิพนธ์	กลไกยับยั้งการงอกของสารสกัดจากใบดาวเรือง (*Tagetes erecta* L.) ต่อผักโขม (*Amaranthus* spp.)
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บทคัดย่อ

สารสกัดหยาบจากดาวเรือง เตรียมโดยสกัดใบดาวเรืองด้วยเอทานอล 75 % ในน้ำ ระเหยเอทานอลและน้ำออกจนแห้ง ได้สารสกัดหยาบเหนียวข้นละลายสารสกัดหยาบด้วยน้ำกลั่นและปรับ pH ของสารละลายด้วย 6 N HCl ให้ได้ pH 3 เพื่อให้เกิดปฏิกิริยาไฮโดรไลซีส สกัดด้วยเอทิลอะซิเตท 3 ครั้ง นำสารสกัดส่วนเอทิลอะซิเตทมาดักจับน้ำด้วย anhydrous $MgSO_4$ จากนั้นนำมาระเหยเอทิลอะซิเตทออกจะได้เป็นส่วนสกัดหยาบชั้นไฮโดรไลซ์ (Hy fraction) นำส่วนสารสกัดหยาบชั้นไฮโดรไลซ์ มาผสมกับ adjuvant ในอัตราส่วน 30:70 จะได้เป็นสารละลายเข้มข้น (SC) 30 % ของสารออกฤทธิ์ จากนั้นนำสารละลายเข้มข้น มาทดสอบการงอก การเจริญเติบโต การดูดน้ำ กิจกรรมเอนไซม์อะไมเลส และการเสื่อมสภาพของดีเอ็นเอ ของผักโขม 3 พันธุ์ ที่ระดับความเข้มข้น 250 – 8000 ppm ผลการทดลองพบว่าสารละลายเข้มข้นจากใบดาวเรือง ที่ระดับความเข้มข้น 1000 ppm สามารถยับยั้งการงอกและการเจริญเติบโตของผักโขมหนามและผักโขมไร้หนามได้อย่างสมบูรณ์ ที่ระดับความเข้มข้น 2500 ppm ขึ้นไป สามารถยับยั้งการงอกและการเจริญเติบโตของผักโขมสวนได้โดยสมบูรณ์ จากนั้นได้ทำการศึกษาผลของสารละลายเข้มข้นจากดาวเรือง ต่อการดูดน้ำและกิจกรรมของเอนไซม์ อะไมเลสของเมล็ดผักโขมสวน ผักโขมหนามและผักโขมไร้หนาม พบว่าสารธรรมชาติจากใบดาวเรืองในรูปของสารละลายเข้มข้นมีผลในการลดการดูดน้ำของเมล็ดและกิจกรรมเอนไซม์อะไมเลสของ ผักโขม 3 พันธุ์เมื่อเปรียบเทียบกับการควบคุม จากผลการศึกษาสารละลายเข้มข้นจากดาวเรือง ในการศึกษาการชักนำการงอกของเมล็ดโดยสารจิบเบอเรลลินจากภายนอกพบว่าการงอก การดูดน้ำและกิจกรรมเอนไซม์อะไมเลส ของผักโขมสวน ผักโขมหนามและผักโขมไร้หนามไม่มีความแตกต่างกันทางสถิติ ดังนั้นผลที่ได้แสดงให้เห็นว่า กลไกการยับยั้งการงอกของสารละลายเข้มข้นจากดาวเรืองไม่เกี่ยวข้องกับสารจิบเบอเรลลิน นอกจากนี้เพื่อให้เข้าใจกลไกการมีปฏิสัมพันธ์ในระดับโมเลกุลของผลการยับยั้งของสารละลาย

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เข้มข้นจากดาวเรือง ต่อการเสื่อมสภาพของดีเอ็นเอในเซลล์พืช ผลการศึกษา ดีเอ็นเอที่สกัดจากผักโขม 3 พันธุ์ ที่ได้รับการทดสอบด้วยสารละลายเข้มข้นจากดาวเรืองที่ความเข้มข้นของ 100 - 1000 ppm พบว่าผักโขมทั้ง 3 พันธุ์มีการเสื่อมสภาพของดีเอ็นเอ ดังนั้นการใช้สารสกัดจากใบดาวเรืองมีศักยภาพในการเป็นสารธรรมชาติกำจัดวัชพืช

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Thesis	Mechanisms of Inhibition of *Tagetes erecta* L. Leaf Extract on Seed Germination of *Amaranthus* spp.
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Abstract

Marigold leaf (*Tagetes erecta* L.) was extracted with 75% ethanol in water and evaporated ethanol to obtain crude ethanol extract. The crude ethanol fraction was diluted with distilled water, resulting in aqueous solution which was hydrolized by acidified to pH 3 with 6 N HCl. The filtrate was extracted with ethyl acetate three times. The ethyl acetate solutions were combined, dried over anhydrous $MgSO_4$, and then evaporated to obtain the ethyl acetate soluble hydrolyzed fraction (Hy fraction). The Hy fraction was mixed with adjuvant at the ratio of 30: 70 to give of 30% active ingredient (a.i.) in soluble concentrate formulation (SC). The SC was bioassayed on germination, seedling growth, seed imbibition, α-amylase activities and DNA degradation against 3 species of amaranth at the concentrations of 250 - 8000 ppm. The results showed that natural herbicide from *T. erecta* leaf in SC formulation at 1000 ppm inhibited germination and seedling growth completely of *Amaranthus spinosus* L. and *Amaranthus gracilis* Desf. At the higher concentration, 2500 ppm completely inhibited germination and seedling growth of *Amaranthus tricolor* L. Then, further studies were extended to explore the effects of the SC formulation from *T. erecta* leaf on seed imbibition and α-amylase activities of *A. tricolor*, *A. spinosus* and *A. gracilis* seeds. The results indicated that *T. erecta* leaf extract in SC formulation decreased in seed imbibition and α-amylase activities of 3 above species of Amaranth seeds in comparison with controls. By these results, *T. erecta* leaf extract in SC formulation had

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comparison with controls. In this study, exogenous GA_3 was also used for induction of seed germination through seed imbibition and α-amylase activity induction however it had non-significant effects against *T. erecta* leaf extract in SC formulation on *A. tricolor*, *A. spinosus* and *A. gracilis*. Thus, the present results support the hypothesis that inhibition mechanism of *T. erecta* leaf extract and exogenous GA_3 on 3 *Amaranthus* species seed germination are dependent. Besides, in order to understand molecular interactions mechanisms of the inhibition effects of crude leaf extract from *T. erecta* leaf extract in SC formulation on 3 species, effects of *T. erecta* leaf extract on the DNA degradation in plant cells were carried out. The results indicated that DNA extracted from 3 *Amaranthus* species treated with leaf extract in SC formulation from *T. erecta* by concentration of 100 - 1000 ppm according to each species showed genomic DNA degradation. Hence the use of *T. erecta* leaf extract as a potential natural herbicide for weed control might be possible.

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CHAPTER 1

INTRODUCTION

1.1 The problem and its background

Marigold (*Tagetes erecta* L.) is in *Tagetes* genus belonging to the family *Asteraceae;* comprises about 56 species distributed around the world. It is demonstrated with the high allelopathic activity in the bioassay was fractionated by simple partitioning procedure which separated the component into gross chemicals classes. Almost studies show that allelopathic activity may inhibit the seeds germination by inhibiting the induction of α-amylase activity (Kato-Noguchi and Macias, 2005; Meksawat and Pornprom, 2010; Laosinwattana et al., 2012) whereas GAs play crucial roles in aspects of plant growth and development, including seed germination, stem elongation, leaf expansion, trichome development, and flower and fruit development. Seed germination begins with water uptake by seeds and terminates with the initial elongation of the embryonic axis (Bewley, 1997). Imbibition and α-amylase activity is consistently linked with seed germination process (Chong et al., 2002). GAs is currently accepted that safeners may act either as bio-regulators influencing the amounts of a given herbicide that reaches its target site in an active form or as antagonists of herbicides common target site whereas crude marigold leaf extract in soluble concentrate formulation (SC) is studied as a natural herbicide because of its high allelochemical. However, its mechanism remains unclearly how exogenous GAs concentrations are modulated during seed germination. In some cases, herbicides may affect the main crop adversely by interfering with its essential biochemical processes such as respiration, photosynthesis, protein metabolism and hydrolytic enzyme activity (Murphy et al., 1999). However, the deeply understanding on its actions via molecular mechanisms such as gene expression, DNA degradation have not been studied yet.

This present study was carried out to evaluate the effects on allelopathic potential of *T. erecta* leaf extract in different concentrations; to understand physiological and molecular interactions mechanisms of the allelopathic effects of crude leaf extract from *T. erecta* on seed germination during *Amaranthus* spp. (*Amaranthus spinosus* L., *Amaranthus tricolor* L. and *Amaranthus gracillis* Desf.) germination. Finally, this information on *T. erecta* extracted application could be applied for customer safety as one of the potential natural herbicides.

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1.2 The purpose of the research thesis

To investigate the allelopathic effects of crude leaf extract from *Tagetes erecta* L. and exogenous GA_3 on seed germination, seed imbibition, α-amylase activity induction and DNA degradation of *Amaranthus spinosus* L., *Amaranthus tricolor* L. and *Amaranthus gracilis* Desf.

1.3 The scope of the research thesis

1.3.1 Determine extract concentration of *T. erecta* inhibit effectively on seed germination, seed imbibition and α-amylase activity induction.

1.3.2 Determine combination of exogenous GA_3 concentration and leaf extract concentration of *T. erecta* that against decreased α-amylase activity induction of *A. spinosus*, *A. tricolor* and *A. gracilis*.

1.3.3 Determine the effects of *T. erecta* leaf extract on the DNA degradation in plant cells.

1.3.4 Understand the physiological and molecular mechanisms that related to the inhibition and the enhancement of seed germination of *A. spinosus*, *A. tricolor* and *A. gracilis* after controlling by crude extract from *T. erecta* and exogenous GA_3.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of marigold

Mexican marigold (*Tagetes erecta* L.) is in *Tagetes* genus belonging to the family *Asteraceae;* comprises about 56 species distributed around the world. It was almost studied as an antibiotic and antioxidant plant. Owino (1992) studied effects of marigold leaf extract and captafol on fungal parasitism of root knot nematode egg-kenyan isolates. Olabiyi and Oyedunmade (2007) studied on marigold as interplant with cowpea for the control of nematode pests (*Meloidogyne spp., Pratylenchus spp.*, and *Helicotylenchus* spp.*)*. Besides Dasgupta et al. (2012) studied on the antibacterial effect of marigold leaf extract at room temperature against 10 gram positive and 6 gram negative. The maximum antibacterial effect of Mexican Marigold leaf extract among micro- organism was obtained for *Acinetobacter* and *Propioni* bacterium acne. The results suggest that species of Mexican marigold can be useful in developing drugs for diseases like dermatitis, acne, skin races and also can be developed as antiseptic. Moreover antioxidant activity of marigold essential oil was researched by Martha Perez Gutierrez et al. (2006). The essential oil from flowers of marigold was evaluated for antioxidant activity in vitro using diphenyl-1-picrylhydrazyl (DPPH), thiocyanate, β-carotene bleaching, free radical scavenging activity and oxidation of deoxyribose assay. The GC-MS analysis of the oil has resulted in the identification of 18 components; β-caryophyllene, limonene, methyleugenol, (E)-ocimene, piperetone, piperitenone and terpinolene were the main components. It is demonstrated with the high allelopathic activity in the bioassay.

2.2 Seed development, structure and general mechanisms of germination

To elucidate the mechanisms of seed germination, it is essential to analyze the structure of seeds common to many different species and also to recognize characteristics of seeds, such as the chemical and physical properties of the testa, which vary considerably among species. The properties of the testa have been analyzed in greater detail in the next section. The two major components of seed, the embryo and the endosperm and their interaction are presented here. Seed is generally defined as a mature ovule. In angiosperm seeds, the embryo and the endosperm start to differentiate after double fertilization in the ovule. A pollen sperm nucleus penetrates into the

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ovule through one of the synergic cells and fuses with an egg cell nucleus forming a diploid embryo (Higashiyama et al., 2003). The embryo develops into globular, heart; torpedo and walking-stick stage sand eventually exhibits a mature morphology (Bewley, 1997). The triploid endosperm, derived from the fusion between another pollen sperm nucleus and two central nuclei, also differentiates during embryogenesis as does the testa, which is derived from maternal integuments. Analyzing the genetic origin of seed tissues is important for interpreting and applying results from molecular and genetic studies. Investigations using Arabidopsis mutants indicated that the development of the testa and endosperm can occur independently of embryogenesis (Ohad et al., 1996; Chaudhury et al., 1997; Kiyosue et al., 1999). However, it is also possible that there are significant interactions and communication between these tissues during seed development (Nonogaki et al., 2006). Before considering dormancy, which imposes a block to the completion of germination, it is appropriate first to consider the processes that comprise germination. Germination commences with the dry seed-imbibition and is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it imbibition and the resumption of metabolism. The imbibition by a mature dry seed is triphasic with a rapid initial uptake followed by a plateau phase. A further increase in water uptake occurs only after germination is completed, as the embryonic axes elongate because dormancy seeds do not complete germination. The influx of water into the cells of dry seeds during phase I results in temporary structural perturbations, particularly to membranes, which lead to an immediate and rapid leakage of solutes and low molecular weight metabolites into the surrounding imbibition solution. This is symptomatic of a transition of the membrane phospholipid components from the gel phase achieved during maturation drying to the normal, hydrated liquid-crystalline state (Crowe et al., 1992). Within a short time of rehydration, the membranes return to their more stable configuration, at which time solute leakage is curtailed. How repair to desiccation and rehydration induced damage to membranes and organelles is achieved is unknown. However, during the imbibition of cotton seeds, the amount of N-acetylphosphatidyl ethanol amine, a phospholipid with membrane-stabilizing properties, increases, as does that of the corresponding synthase. These molecules may be involved in maintaining or enhancing membrane integrity (Sandoval et al., 1995). Upon imbibition, the quiescent dry seed rapidly resumes metabolic activity. The structures and enzymes necessary for this initial resumption of metabolic activity are generally assumed to be present within the dry seed, having survived, at least partially intact, the desiccation phase that terminates seed maturation. Reintroduction of water during imbibition is

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sufficient for metabolic activities to resume, with turn over or replacement of components occurring over several hours as full metabolic status is achieved. One of the first changes upon imbibition is the resumption of respiratory activity, which can be detected within minutes. After a steep initial increase in oxygen consumption, the rate declines until the radicle penetrates the surrounding (Bewley, 1997).

About pattern of seed germination was studied by Miller and McDonald (2006). Prior to germination, seeds are in a "maintenance" phase that is often characterized as dormancy being imposed by ABA, metabolic blocks or some other agent hindering the transition to germination. At some point, the seed becomes sensitive to the presence of "trigger" agents. A "trigger" agent such as light or temperature alterations shift the balance of inhibitors to favor promoters such as gibberellins. A "trigger" agent can be defined as a factor that elicits germination but whose continued presence is not required throughout germination. In contrast, a "germination" agent must be present throughout the germination process. An example is gibberellic acid. The major sequence of events leading to germination is imbibition, enzyme activation, initiation of embryo growth, rupture of the seed coat and emergence of the seedling. The early stages of imbibition or water uptake into a dry seed represent a crucial period for seed germination. It is the first key event that moves the seed from a dry, quiescent, dormant organism to the resumption of embryo growth. Thus, any consideration of seed germination physiology and its resultant impact on stand establishment should focus initially on water uptake. The extent to which water imbibition occurs is dependent on three factors: (1) composition of the seed, (2) seed coat permeability, and (3) water availability.

2.3 Related researches about effect of allelopathy on bioassays

Many authors illustrated some allelochemicals may inhibit the germination of bioassays by inhibited imbibition and α-amylase activity in bioassay seeds (itchgrass (*Rottboellia cochinchinensis*), *Suregada multiflorum*, *Aglaia odorata* granules, *Melia Azedarach* L., *Echinochloa crus-galli* and *Phaseolus lathyroides* L.) during germination such as: Kato-Noguchi and Macias (2005); Meksawat and Pornprom (2010); Laosinwattana et al. (2010). However *T. erecta* has not studied about weed control through inhibit the weed germination yet. It still remains unclearly how are modulated during seed germination by its inhibition whereas GAs plays crucial roles in aspects of plant growth and development, including seed germination, stem

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elongation, leaf expansion, trichome development, and flower and fruit development (Olszewski et al., 2002).

For exogenous GA_3 studies, exogenous GA_3, relationship of GA_3 induced germination and amylase and GAs gene expression were mentioned by many findings and author groups. Mambouh and Nemat (1998) investigated efficacy of exogenous GA_3 and herbicide safener and protection. The research investigated contents of gibberellic acid (GA_3), glutathione (γ-L-glutamyl-L-cysteinyl glycine, GSH) and protein, activities of α-amylase (EC 3.2.1.1) and glutathione S-transferase (GST, EC 2.5.1.18) as well as metolachlor residues in shoots of 6-d old maize seedlings during an 8-d period following treatment with metolachlor, either alone or combined with GA_3, naphthalic anhydride (NA) or flurazole (FL). Externally applied GA_3 relieved this effect while NA and FL relatively raised the enzyme activity but still remained below control levels. Significant increases of GSH content were induced by metolachlor or its combinations; the magnitude of the increase was more pronounced with FL. GST activity was significantly enhanced by metolachlor; the effect was not influenced by GA_3, being augmented by the presence of NA and multiplied by FL. The results indicate that GA_3 compensates the loss of the endogenous GA_3 content and of α-amylase activity while FL, and to some extent NA, stimulated the detoxification rate of metolachlor by enhancing GSH content and GST activity.

The α-amylase induction in endosperm during rice seed germination is caused by gibberellin synthesized in epithelium was studied by Kaneko et al. (2002).

BiaLecka and Kepczynski (2010) studied on effect of ethephon and gibberellins A_3 on *Amaranthus caudatus* seed germination and α- and β-amylase activity under salinity stress. This study assessed the effects of different doses of ethephon and gibberellin A_3 on germination and α- and β-amylase activity in *A. caudatus* seeds exposed to different levels of salt stress. Kepczynski, and Sznigir (2013) investigated about response of *Amaranthus retroflexus* L. seeds to gibberellic acid, ethylene and abscisic acid depending on duration of stratrification and burial. The results indicated that *A. retroflexus* seed dormancy can be released either by stratification or by autumn–winter burial. The response to GA_3 and ethylene increased with increasing time of stratification. The presence of GA_3 and ethephon during stratification may stimulate germination at 35°C. Thus, both GA_3 and ethylene can partially substitute the requirement for stratification or autumn–winter burial. Both hormones may also stimulate germination of secondary dormant seeds, exhumed in September. Endogenous GAn, ethylene and ABA may be involved in the control of dormancy state and germination of *A. retroflexus*. It is possible that releasing dormancy by stratification or

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partial burial is associated with changes in ABA/ GA and ethylene balance and/or sensitivity to these hormones.

2.4 DNA degradation in plant cell review

However *T. erecta* has not studied about weed control through inhibit the weed germination yet. It still remains unclearly how molecular mechanism is during seed germination by its inhibition (Olszewski et al., 2002).

DNA degradation is a process by which DNA breaks down into smaller fragments. As DNA molecules randomly break down into smaller fragments, the STR regions of the DNA molecule can be fractured. If the STRs do not stay intact, amplification of these regions will not be successful. Degradation is more likely to occur at a large STR locus before occurring in a smaller STR locus. Generally, degradation can be easily identified because the peak heights exhibit a downward slope across the electropherogram. The process of degradation can reduce the height of some alleles, making them too low to be distinguished from background noise in the data. In severely degraded DNA samples, no results will be obtained. Two or more biological samples that make up a mixture may show different levels of degradation, which can complicate the interpretation of these samples (NFSTC Science, 2007).

Transgenic procedure is a reason of DNA degradation in plant cell that inhibit the germination. MariaTeresa et al. (2003) studied on degradation and transformability of DNA from transgenic leaves. This result indicated that while most of the DNA will be degraded inside plant cells, sufficient DNA persists to be released into the soil. The fate of transplastomic (chloroplast genome contains the transgene) tobacco plant DNA in planta was studied when the plant leaves were subjected to decay conditions simulating those encountered naturally, including grinding, incubation with cellulase or enzymes produced by *Erwinia chrysanthemi,* and attack by the plant pathogen *Ralstonia solanacearum*. Direct visualization of DNA on agarose gels, gene extraction yield (the number of amplifiable aadA sequences in extracted plant DNA), and the frequency that recipient bacteria can be transformed by plant DNA were used to evaluate the quality and quantity of plant DNA and the transgene. These measurements were used to monitor the physical and biological degradation of DNA inside decaying plant tissues.

Although various nucleases are known to function in nuclear DNA degradation in animal apoptosis, it is unclear what hydrolase is involved in nuclear degradation in plants. During Programmed Cell Death of Tracheary Elements Tracheary elements (TEs) have a unique cell

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death program in which the rapid collapse of the vacuole triggers the beginning of nuclear degradation. Jun Ito and Hiroo Fukuda (2002) determined ZEN1 is a key enzyme in the degradation of nuclear DNA. In this study, they demonstrated that an S1-type nuclease, Zinnia endonuclease 1 (ZEN1), functions directly in nuclear DNA degradation during programmed cell death (PCD) of TEs. In-gel DNase assay demonstrated the presence of a 24-kD Ca^{2+}/Mg^{2+}-dependent nuclease and a 40-kD $Zn2^{+}$-dependent nuclease as well as ZEN1 in 60-h-cultured cells that included differentiating TEs. Such cell extracts possessed the ability to degrade the nuclear DNA isolated from *Zinnia elegans* cells in the presence of Zn^{2+}, and its activity was suppressed by an anti-ZEN1 antibody, indicating that ZEN1 is a central DNase responsible for nuclear DNA degradation. The introduction of the antisense ZEN1 gene into Zinnia cells cultured for 40 h specifically suppressed the degradation of nuclear DNA in TEs undergoing PCD but did not affect vacuole collapse. Therefore based on these results, a common mechanism between animal and plant PCD is discussed.

Besides regulation of gene expression by a biologist first requires accurate identification of the target allelochemicals, to determine enzymes and the genes encoding them.

A lot of effort has been done to explore the nature of allelopathic interactions. Some allelochemicals such as: phenoxy, benzoic acid, picolinic acid readily absorbed by foliage, less so by roots, extensively translocated, interfere with DNA, RNA and protein synthesis, results in uncontrolled cell division and elongation (Murphy et al., 1999) suggested that the stronger ability of PI312777 to suppress target weeds could be attributed to the stronger activation of the genes that function in de novo synthesis of allelochemicals. Rice accession PI312777 exhibited high allelopathic potential to suppress the growth of accompanying weeds, especially when the culture solution had low N content. Additionally, subtractive hybridization suppression was used to construct a forward cDNA library of PI312777 to investigate gene expression profiles under low N treatment. A total of 35 positive clones from the cDNA library were sequenced and annotated. According to the function category, 24 genes were classified into five groups related to primary metabolism, phenolic allelochemical synthesis, plant growth/cell cycle regulation, stress response/signal transduction, and protein synthesis/degradation.

Allelopathy is a quantitative trait. A genetic analysis of quantitative trait loci (QTL) is a promising approach to identify genes underlying this trait. Only a few crops are under genetic screening for its allelopathic properties including: rice, wheat, barley and oat. The first QTL map associated with allelopathic properties was developed in rice. A segregating population derived

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from a cross of two cultivars varying with allelopathic potential against barnyardgrass. The map contained 140 DNA markers with four main-effects QTL located on chromosome. Proteomic studies on allelopathy of rice against barnyardgrass confirmed the crucial role of three enzymes: phenylalanine ammonia-lyse (PAL), thioredoxin and 3-hydroxy-3-methilglutaril coenzyme. A reductase 3 (HMGR) is highly involved in phenols biosynthesis. Such a genetic approach may allow the location of the gene in the genome and better understanding of its function in plant allelopathy and create the chance of applying marker assisted selection (MAS) (Jensen et al., 2002; Belz, 2007).

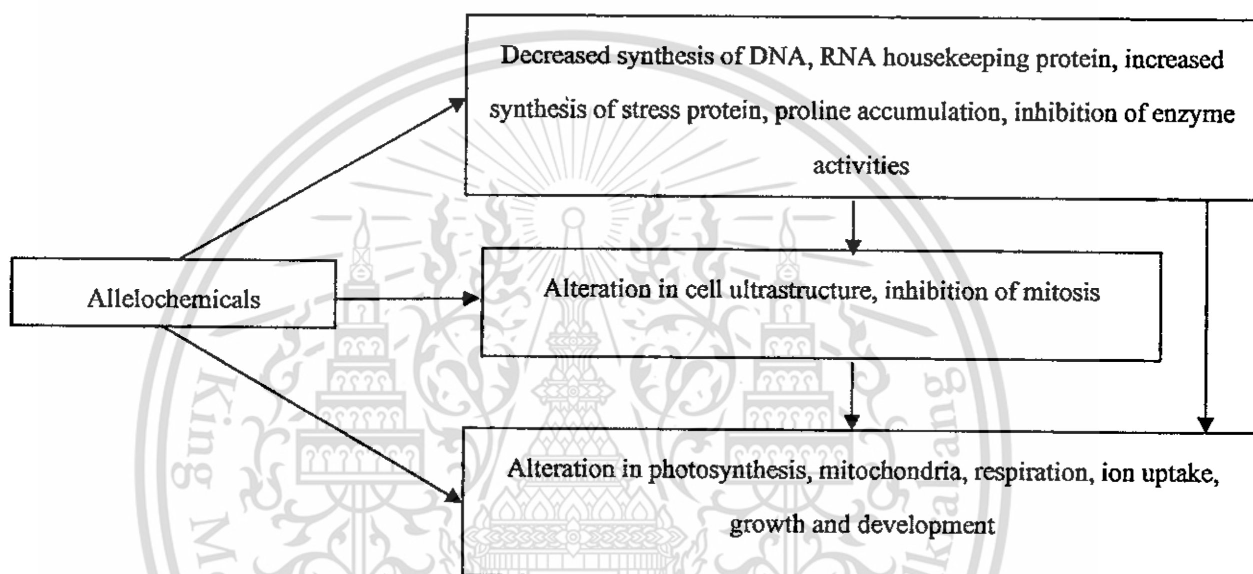

Figure 2.1 Multi site action of allelochemicals (Gniazdowska and Bogatek, 2005)

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CHAPTER 3

RESEARCH METHODOLOGY

3.1 Chemicals and instruments

3.1.1 Reagent solution preparation for α-amylase assay

1. Calcium chloride 0.1 M ($CaCl_2$ 0.1 M)

Dissolve 5.55 g of $CaCl_2$ and bring to 500 ml by distilled water

2. Acetate buffer solution (pH: 5.5)

Dissolve 150 g of sodium acetate by distilled water. Then add 15 ml acetic acid, adjust the pH to 5.5 and bring to 1000 ml by distilled water

3. Soluble starch 1% in acetate buffer solution at pH 5.5

Dissolve 1 g of soluble starch and dilute to a final volume of 100 ml by acetate buffer

4. Dinitrosalicylic acid reagent solution (DNS reagent solution)

Dissolve 1 g of 3,5 dinitrosallicylic acid in 40 ml 1 M NaOH. Then add slowly 30 g sodium potassium tartrate and dilute to a final volume of 100 ml by distilled water.

3.1.2 Reagent solution preparation for DNA extraction

1. Tris HCl 1 M (PH: 8.0)

Dissolve 60.57 g of Tris (hydroxyl methyl) aminomethane or Tris base MW and bring to 400 ml by distilled water. Then add 21 ml concentrated HCl dilute to a final volume of 500 ml, PH = 8 and autoclave.

2. EDTA 0.5 M (Ethylenediaminetetraacetic acid)

Dissolve 93.06 g of EDTA and bring to 300 ml sterile water. Then add 100 g of NaOH and adjust PH to 8 (NaOH 5 N). Dilute to a final volume of 100 ml by distilled water and autoclave

3. NaOH 5 N (Sodium hydroxyl)

Dissolve 20 g NaOH in 80 ml H_2O. Then bring to 100 ml distilled water and autoclave

4. HCl 1 N (Hydroxyl chloride)

Dissolve 8.62 ml concentrated HCl by 91.38 ml distilled water

5. NaCl 5 M (Sodium chloride)

Dissolve 29.25 g of NaCl by 80 ml H_2O. Then bring to 100 ml by distilled water and autoclave

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6. TE buffer (Tris EDTA buffer)

Dissolve 5 ml of Tris HCl 1 M (PH 8.0); 1 ml EDTA 0.5 M and bring to 500 by distilled water and autoclave

7. Ethidilium bromide 10 mg/ml

Dissolve 1 g of ethidilium bromide and bring to 100 ml sterile water

8. TAE 50X stock solution (Tris acetate EDTA)

Dissolve 60.5 g of Tris base by distilled water. Then add 14.3 ml of acetic acid and 25 ml EDTA 0.5 M. Adjust the pH to 8. Dilute to a final volume of 250 ml by distilled water and autoclave

9. CTAB buffer (Cetyltrimethyl ammonium bromide buffer)

Dissolve 11.688 g of NaCl 2 M. Add 5 ml of EDTA 25 mM and 10 ml of Tris HCl 100 mM PH 8.0. Then bring to 100 ml by sterile water and autoclave. Add 2 g of CTAB and then add 2 β-mercaptoethanol with propotion: 1 ml CTAB solution: 10 μl 2 β – mercaptoethanol

3.1.3 Other chemicals

Ethanol 99%, ethanol 70%, isopropanol, ethyl acetate, Phenol: Chloroform: isoamylalcohol = 25: 24: 1

3.1.4 Apparatus

1. Petri dish (9 and 15 cm in diameter)
2. Eppendorf 1.5 ml and kinds of tip
3. Beaker
4. Cylinder
5. Test tube
6. Curvet
7. Spatula
8. Micropipette
9. Stirring rod
10. Germination paper
11. Filter paper Whatman no. 1
12. Parafilm
13. Sterile pestle and mortar

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3.1.5 Instruments

1. Hot air oven machine
2. Magnetic stirrer
3. Rotary evaporator
4. Growth chamber
5. Autoclave machine
6. pH meter
7. Vortex machine
8. Centrifuged machine
9. Water bath
10. Gel DocTM 2000
11. UV transilluminator

3.2 Test plant preparation

The healthy seeds of *A. spinosus, A. tricolor* and *A. gracilis* were manually collected from experimental field at King Mongkut's Institute of Technology Ladkrabang and farmer fields in the Ladkrabang district, Bangkok, Thailand. Their seeds were removed from panicle by lightly shaking in collection bags to release seeds. Seeds of these species were examined and seeds with damaged seed coats were discarded.

3.3 Crude marigold leaf extracts preparation

Marigold plant was grown at the experimental field at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The mature and healthy leaves of *T. erecta* were collected at 50 days after planting, then cleaned from soil immediately with running tap water, dried-up in a hot-air oven at 45°C for 5 days and ground into powder (100 mesh) in an electrical blender. According to method of Laosinwattana (2010), the crude extracts were prepared from *T. erecta* leaf powder by extraction with 75% ethanol in water for 48 hours at room temperature and repeatedly extracted 3 times, followed by filtration through three layers of cheese cloth to remove debris.

After filtration using Whatman No. I filter paper, the filtrates was combined and evaporated in the rotary evaporator at 45°C, leaving a sticky residue (crude ethanol fraction). This

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residue was then diluted with 500 mL of distilled water and stirred vigorously on a magnetic stirrer at 45°C for 20 min, resulting in an aqueous solution which was acidified to pH 3 by 6N HCl. The filtrate was extracted with ethyl acetate three times. The ethyl acetate solutions was combined, dried over $MgSO_4$ and then evaporated to obtain the ethyl acetate soluble hydrolyzed fraction (Hy fraction) and the remains of the aqueous phase was discarded (Figure 3.1). The Hy fraction was mixed with adjuvant at the ratio of 30 : 70 to give of 30% active ingredient (a.i.) in soluble concentrate formulation (SC). The inhibitory activities from each fraction were prepared by dissolved crude of each fraction to contain different concentrations.

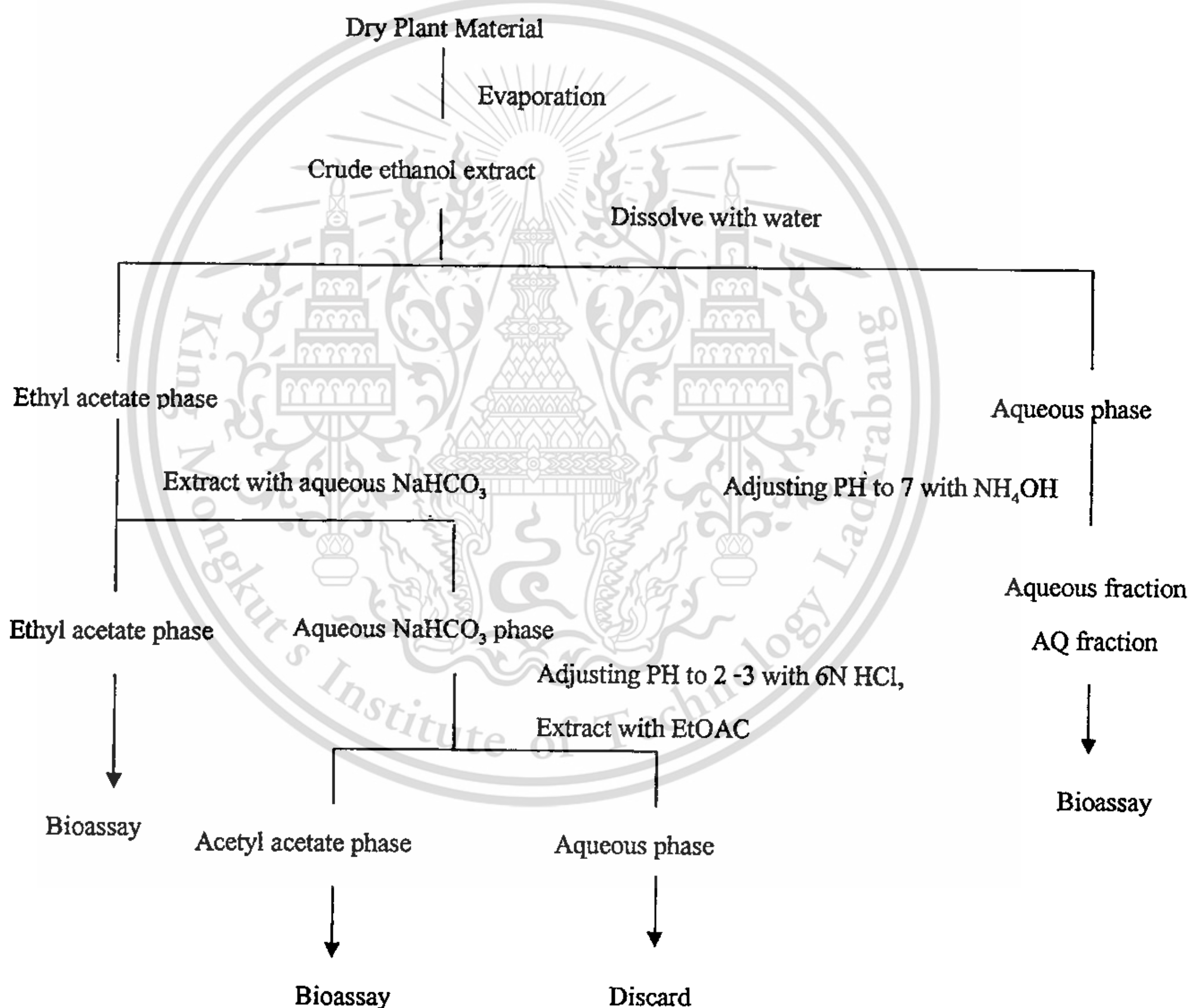

Figure 3.1 Flow chart for extraction and partially separation of active compounds from marigold dried leaves

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3.4 Methodology

3.4.1 Effects of different concentrations of *T. erecta*, exogenous GA_3 and the combination of *T. erecta* and exogenous GA_3 on seed germination, seed imbibition and α-amylase activity induction of *A. tricolor*, *A. spinosus* and *A. gracilis.*

Experiment 1. Effects of different concentrations of *T. erecta* on seed germination, seed imbibition and α-amylase activity induction

The crude solution from *T. erecta* was diluted with distilled water to 250 ppm, 500 ppm, 750 ppm, 1000 ppm, 2000 ppm, 4000 ppm, 8000 ppm. 5 ml of each concentration was added to each petri dish (9 cm in diameter) containing 2 layers of germination paper and then 20 healthy seeds of test plant were placed on the germination paper as per treatment.

Control: Distilled water

Treatment 2: 250 ppm

Treatment 3: 500 ppm

Treatment 4: 750 ppm

Treatment 5: 1000 ppm

Treatment 6: 2000 ppm

Treatment 7: 4000 ppm

Treatment 8: 8000 ppm

After the completion of experiment 1, the best result was used for next treatment and its sign was Concentration*

Experiment 2. Effects of the combination of *T. erecta* and exogenous GA_3 on seed germination, seed imbibition and α-amylase activity induction

The combination of *T. erecta* and exogenous GA_3 concentration was carried out about Concentration* + different concentrations of exogenous GA_3.

Exogenous GA_3 (Gibberellic acid) (Eastman organic chemicals Rochestor, N.Y, USA.) stock solution was prepared and stored at 5°C. Then stock solution was diluted with distilled water to contain different concentrations (0.1 ppm, 0.2 ppm, 0.4 ppm and 0.8 ppm).

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5 ml of above concentration combination (Concentration* + different concentrations of exogenous GA_3) was added to each petri dish (9 cm in diameter) containing 2 layers of germination paper.

Treatment 1: Concentration* + 0.1 ppm GA_3

Treatment 2: Concentration* + 0.2 ppm GA_3

Treatment 3: Concentration* + 0.4 ppm GA_3

Treatment 4: Concentration* + 0.8 ppm GA_3

The control was only received distilled water. Each treatment had 3 - 4 replications in a completely randomized design (CRD).

All petri dishes were placed at a growth chamber with condition (white 840 Climacell 707, Munich, Germany) at 25-32°C, 12h dark/ light photoperiod light intensity of 100 μmol $m^{-2}s^{-1}$, and relative humidity of 80%. After 7 days, germination % (%) (SG), shoot length (SL) and root length (RL) were observed and recorded in all treatments.

The inhibition percentage relative to control was calculated from following equation:

SG, SL or RL (% of control) = (Sample extracts/ Control) x 100 (1)

Seed imbibition and assay of α-amylase activity induction:

Measurement of seed imbibition was performed using method of Turk and Tawaha (2003). Four replicates of 100 healthy seeds of *A.spinosus*, *A. tricolor* and *A. gracilic* were weighed and recorded as original seed weight (W_1). These seeds were separately germinated in 5 ml of each treatment, distilled water as control (according to above treatments). Seed weights were recorded as final seed weight (W_2) for each concentration and exposure time. Seed imbibition percentage was calculated from following equation:

$$\text{Seed imbibition (\%)} = [(W_2 - W_1)/ W_1] \times 100 \quad (2)$$

Measurement of activity of α-amylase was performed by following the method of Bernfield (1955) and Sadasivam and Manickam (1996). After measuring water uptake, seeds (100 seeds for one determination) were homogenized with 4 mL ice-cold solution of 0.1 M $CaCl_2$ and centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was used as enzyme extract. The α - amylase was then assayed by measuring rate of generation of reducing sugars from soluble starch. The reaction medium (2 ml) contained 1 ml of 1% soluble starch in acetate buffer solution at pH 5.5. The assay medium was incubated for 15 min at 37°C. The reaction was terminated by

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addition of 1 ml DNS reagent (40 mM 3,5 dinitrosalicylic acid, 0.4 N NaOH and 1M K-Na tartrate), and immediately heated in a boiling water bath for 5 min. The mixture was cooled under running tap water. The intensity of colour was measured as absorption at 560 nm in a spectronic GENESYS 20 spectrophotometer (Thermo Electron Corporation, USA). A standard graph was prepared using maltose, and the amount of α-amylase present in sample was calculated from standard curve and expressed as μmol maltose/min/g (FW).

The concentration of α- amylase activity was calculated from following equation:

$$X = (Y + 0.005) / 0.0026 \quad (3)$$

X = Concentration of α- amylase activity

Y = Spectotrophometer measurement

The amount of α-amylase activity (μmol/min/g (FW) was calculated from following equation:

$$\frac{X \times V}{T \times g\,(Fw) \times M\,(maltose) \times 0.25} \quad (4)$$

X = Concentration of α- amylase activity

V = Total volume

T = Incubation time

g (Fw) = Weight of seed

M (maltose) = Molecular weight of maltose

3.4.2 Effects of natural herbicide from *Tagetes erecta* L. on DNA degradation of *Amaranthus* spp.

Experiment 3. Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. spinosus* and *A. tricolor* and *A. gracilis* for DNA extraction

The SC formulation from *T. erecta* was diluted with distilled water to100 ppm to 1000 ppm according to each species. Five ml of each concentration was added to each petri dish (9 cm in diameter) containing 2 layers of germination paper and then 20 healthy seeds of test plant were placed as per treatment. The control was only received distilled water. Each treatment had 4 replications in a completely randomized design (CRD):

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For *A. tricolor,* the SC formulation from *T. erecta* was diluted with distilled water to 250, 500, 750 and 1000 ppm

Control: Distilled water

Treatment 2: 250 ppm

Treatment 3: 500 ppm

Treatment 4: 750 ppm

Treatment 5: 1000 ppm

For *A. gracilis* and *A. spinosus*, the SC from *T. erecta* was diluted with distilled water to 100, 200, 300and 400 ppm.

Control: Distilled water

Treatment 2: 100 ppm

Treatment 3: 200 ppm

Treatment 4: 300 ppm

Treatment 5: 400 ppm

Then all petri dishes were placed in a growth chamber with condition (cool white 840 Climacell 707, Munich, Germany) at 25 - 32°C, 12h dark/ light photoperiod light intensity of 100 µmol $m^{-2}s^{-1}$, and relative humidity of 80%. After 7 days, germination percentage (%) (SG), shoot length (SL) and root length (RL) were observed and recorded in all treatments.

The inhibition percentage relative to control was calculated from following equation:

G, SL or RL (% of control) = 100 – [(sample extracts/control) x 100] (5)

Experiment 4. Effect of different concentrations of SC formulation from *T. erecta* on DNA degradation in plant cells

The SC formulation from *T. erecta* was diluted with distilled water to 250, 500, 750, 1000 ppm for *A. tricolor* while 100, 200, 300 and 400 ppm for *A. spinosus* and *A. gracilis*. Ten ml of each concentration was added to each petri dish (15 cm in diameter) containing 2 layers of germination paper and then 100 healthy seeds of *Amaranthus* spp. were placed as per treatment. The control was only received distilled water. Each treatment had 3 replications in a completely randomized design (CRD):

All petri dishes were sealed with parafilm and placed in a growth chamber.

After 12 days, Amaranths leaves and stems were collected and thencovered by alumium foil and kept in -80°C refrigerator for 3 days before DNA extraction.

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Method of extraction of genomic DNA from plant tissue (CTAB method):

A hundred mg leaf and stem of *Amanranthus* spp. for each determination was ground in liquid nitrogen with prechilled mortar and pestle. A 500 µl hot CTAB solution (60°C) was immediately added to the homogenized solution. The flour sample was then transferred into 1.5-mL DNase-free microcentrifuge tube (eppendorf). After mixing the content with vigorous vortex mixing, flour sample was incubated in water bath at 60°C for 15 minutes. Samples were then centrifuged immediately at 12,000 rpm for 10 min at 4°C. The upper aqueous phase was carefully transferred to a new 1.5-mL eppendorf. A 700 µL phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added. The samples was mixed well by inversion and placed on ice for 10 min and then centrifuged at 12,000 × g for 10 min at 4°C. The upper aqueous phase will be transferred to a new 1.5-mL eppendorf. To the precipitated DNA, 500 µL isopropanol will be added. The sample were placed in -80°C refrigerator for 1 hour and centrifuged to obtain DNA for 12,000 rpm for 15 min at 4°C. Then the supernatant was discarded, and the DNA pellets were washed carefully with 400 µL 70% ethanol at RT and dissolved by 30 µl TE buffer and kept in 4°C refrigerator.

Method of quality assessment of extracted DNA:

Spectrophotometer was used to determine the purity of the extracted DNA. Nucleic acids absorbed light at 260nm and the amount of light absorbed was used to calculate the purity and amount of DNA. By using a spectrophotometer that emits a light at 260nm that passed through the sample, the concentration of DNA in the sample was determined. The more light was absorbed the more nucleic acid is present in the sample. Interference by contaminants was calculated by using a ratio. Since proteins absorbed at 280nm, the ratio of absorbance A of a sample at 260nm and the absorbance A at 280nm was used to estimate the purity of the DNA sample.

Two µl of the DNA sample was mixed with 498 µl pure HPLC-grade water to make a 1/ 250 dilution with a total volume of 500 µl in a 1.5 ml eppendorf tube. In order to concentrate the fluid in the bottom of the tube, the tube was mixed well and centrifuged for a few seconds. Sixty µl of the dilution was pipetted in to the bottom of a plastic disposable cuvette. Sixty µl pure water were placed a cuvette containing the blank in position in the spectrophotometer. The clear sides were placed in line with the direction of the light ray. The photometer and programme were switched on to measure double stranded DNA. The blank was measured by pushing the button marked "Blank" and made sure the result reads 0. The cuvette containing the diluted sample was

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placed in position in the photometer and pressed the "Sample" button to measure. The instrument showed its readings for absorbance at 230, 260, 280 and 320 nm as well as the ratio of the absorbance A260/ A280 nm and A260/ A230 nm. It also showed the concentration of DNA in the sample in ng/µl.

The DNA degradation was accessed by electrophoresis in highly percentage of agarose gel (approximately 2% w/v) containing 5 mg/ml ethidium bromide. Then, the degradation of plant genomic DNA was determined by Gel Doc™ 2000 and UV transilluminator.

3.5 Data analysis/ Statistical analysis

Each treatment consists of four replications in completely randomized design (CRD). Analysis of variance was calculated for all data and comparisons between treatments will be made at probability level $p \leq 0.05$ using Tukey's test.

3.6 Duration and location of thesis

My thesis was carried out during 1.5 years (6/ 2013 – 12/ 2014) in laboratory and greenhouse experiments, Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Effects of different concentrations of *T. erecta*, exogenous GA_3 and the combination of *T. erecta* and exogenous GA_3 on seed germination, seed imbibition and α-amylase activity induction of *A. tricolor.*

4.1.1 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. tricolor.*

The results showed that *T. erecta* leaf extract in soluble concentrate formulation had significant allelopathic effects against *A. tricolor* (Table 4.1). At 1000 ppm dose, germination of seed of *A. tricolor* was inhibited by 17.5%. Its shoot and root length inhibition were 32.83 and 31.52%, respectively. By increasing the dose of application at 2500, 4000 and 8000 ppm, the inhibition magnitude was complete. In general, there are no significant differences in the inhibitory effect between on root length and shoot length. These results indicate that *T. erecta* leaf extract contains some inhibitory principles upon inhibited germination and seedling growth. However, the nature of inhibitory principles contained in *T. erecta* leaf crude extract is unknown. Thus, further studies were carried out to evaluate and understand interaction mechanisms of allelopathic potential about inhibition on seed imbibition and α-amylase activities of *A. tricolor* seeds.

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Table 4.1 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. tricolor.*

Concentration (ppm)	Inhibition (% of control)		
	Seed germination	Shoot length	Root length
1000	17.5b[1]	32.83b	31.52b
2500	100a	100a	100a
4000	100a	100a	100a
8000	100a	100a	100a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.1.2 Effects of different concentrations of *T. erecta* on inhibition of seed imbibition and α-amylase activity induction of *A. tricolor*

Data that further showed the differences in the percentage of seed imbibition between control and concentration application of natural herbicide from *T. erecta* leaf extract in soluble concentrate formulation at different imbibition periods presented in Table 4.2. The percentages of imbibition in the control seeds exhibited a marked increase by prolonging the imbibition periods and had the highest seed imbibition at all the imbibition time whereas the percentage of imbibition in treated seeds increased slightly by prolonging the imbibition period. After imbibiton time of 12 hours, the percentage of seed imbibition inhibited remarkably according to the increasing the dose of application during the whole experiment.

Many findings indicate that between the imbibition and α-amylase activities induction had closed relation (Kato-Noguchi and Macias, 2005; Bewley, 1997; Teerarak and Laosinwattana, 2012). Thus, further studies were carried out to evaluate about inhibition on α-amylase activities of *A. tricolor* seeds. The activities of α-amylase of *A. tricolor* seeds were also investigated and the results were shown in Table 4.3. Under the same extract concentration, α-amylase activity increased by prolonging the imbibition period, especially in control. For all treatment conditions, no significant differences in α-amylase activity after 6 hours imbibition time were observed. After imbibition time of 12 and 24 hours, an increased concentration of natural herbicide from crude marigold leaf extract in soluble concentrate formulation inhibited induction α-amylase activity.

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Table 4.2 Effects of different concentrations of *T. erecta* on inhibition of seed imbibition of *A. tricolor.*

Concentration (ppm)	Seed imbibitions (%)		
	6 hours	12 hours	24 hours
Distilled water	28.14a[1]	39.42a	66.51a
1000	27.75a	35.92b	40.97b
2000	27.97a	33.84bc	38.9b
4000	26.34a	36.18c	37.74b
8000	23.9a	32.96d	36.35b

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

Table 4.3 Effects of different concentrations of *T. erecta* on α-amylase activity induction of *A. tricolor.*

Concentration	α-Amylase activity (μmol maltose $min^{-1}g^{-1}$FW)		
	6 hours	12 hours	24 hours
Distilled water	5.85a[1]	10.91a	14.32a
1000 ppm	5.91a	9.2ab	12.61a
2000 ppm	6.21a	8.07abc	12.45ab
4000 ppm	6.23a	7.71bc	8.71bc
8000 ppm	5.57a	7.38c	6.26c

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.1.3 Effects of *T. erecta* and exogenous GA_3 on inhibition of seed germination of *A. tricolor*

As shown in Table 4.4, seed inhibition on *A. tricolor* (% of control) after treated with combination of *T. erecta* leaf extract considered as natural herbicide and exogenous GA_3 was slightly decreased throughout the entire experiment with respect to untreated exogenous GA_3 (2500 ppm *T. erecta* leaf extract). This decrease was most pronounced with applied 0.01 ppm GA_3 and the inhibition of seed germination, shoot length and root length reached about 57.50;

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82.11 and 97.29%, respectively. However by increasing the dose of applied GA_3 at 0.1 ppm, the inhibition increased remarkably and inhibition of shoot length and root length at all treatment was still more than 90%. Hence this result suggests that these applied GA_3 had non-significant to prevent and relieve the herbicide effect on *A. tricolor.*

Table 4.4 Effects of *T. erecta* and exogenious GA_3 on inhibition of seed germination of *A. tricolor.*

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Root length
1000 ppm crude extract	12.50d[1]	36.01b	71.44bc
1000 ppm crude extract + 0.005 ppm GA_3	18.75d	44.50b	79.26b
1000 ppm crude extract + 0.01 ppm GA_3	11.25d	25.46b	63.33c
1000 ppm crude extract + 0.1 ppm GA_3	8.75d	44.04b	74.65bc
2500 ppm crude extract	100a	100a	100a
2500 ppm crude extract + 0.005 ppm GA_3	71.25bc	97.71a	97.80a
2500 ppm crude extract + 0.01 ppm GA_3	57.50c	82.11a	97.29a
2500 ppm crude extract + 0.1 ppm GA_3	83.75ab	93.81a	98.40a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test ($p=0.05$)

4.1.4 Effects of *T. erecta* and exogenous GA_3 on seed imbibtion and α-amylase activity induction of *A. tricolor*

In this research, exogenous GA_3 was used for induction of seed germination by α-amylase activity after complete inhibition because of applied 2500 ppm *T. erecta* leaf extract. The results in Table 4.5 showed that exogenous GA_3 had non-significant effects against *T. erecta* leaf extract in soluble concentrate formulation on *A. tricolor* entire experimental period (after 6 an 12 hours imbibition) by both of seed imbibition and α-amylase activity induction. After 6 hours imbibition, seed imbibition and α-Amylase activity were 24 – 26% and 5 – 6 μmol maltose $min^{-1}g^{-1}FW$ at all of treatments, respectively. After 12 hours imbibition, seed imbibition and α-Amylase activity were about 32 - 36% and 9 - 11 μmol maltose $min^{-1}g^{-1}FW$, respectively. Therefore, this result suggests that exogenous GA_3 had non-significant effects against *T. erecta* leaf extract in soluble concentrate formulation on *A. tricolor.*

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Table 4.5 Effects of *T. erecta* and exogenous GA_3 on seed imbibtion and α-amylase activity induction of *A. tricolor* (after 6 and 12 hours imbibition).

Concentration	Seed imbibitions (%)		α-Amylase activity (μmol maltose $min^{-1}g^{-1}$FW)	
	6 hours	12 hours	6 hours	12 hours
Distilled water	24.70a[1]	36.56a	5.84a	11.48a
2500 ppm Crude extract	24.99a	32.28a	5.45a	9.42b
2500 ppm crude extract + 0.005 ppm GA_3	25.67a	32.36a	6.14a	9.16b
2500 ppm crude extract + 0.01 ppm GA_3	26.03a	34.06a	6.23a	9.53b
2500 ppm crude extract + 0.1 ppm GA_3	24.38a	33.31a	5.69a	8.58b

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.2 Effects of different concentrations of *T. erecta*, exogenous GA_3 and the combination of *T. erecta* and exogenous GA_3 on seed germination, seed imbibition and α-amylase activity induction of *A. spinosus*

4.2.1 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. spinosus*

The Table and Figure below showed the inhibition of seed germination and seedling growth of *A. spinosus* by *T. erecta* leaf extract in soluble concentrate formulation. At 1000 – 8000 ppm doses, seed germination of *A. spinosus* was inhibited completely whereas there was not inhibition of shoot length and root length at control. Hence *T. erecta* leaf extract contains some inhibitory principles upon inhibited germination and seedling growth. Thus, further studies were carried out to evaluate and understand interaction mechanisms of allelopathic potential about inhibition on seed imbibition and α-amylase activities of *A. spinosus* seeds.

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Table 4.6 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. spinosus*.

Concentration (ppm)	Inhibition (% of control)		
	Seed germination	Shoot length	Root length
Distilled water	12.50b[1]	0.00b	0.00b
1000	100.00a	100.00a	100.00a
2000	100.00a	100.00a	100.00a
4000	100.00a	100.00a	100.00a
8000	100.00a	100.00a	100.00a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

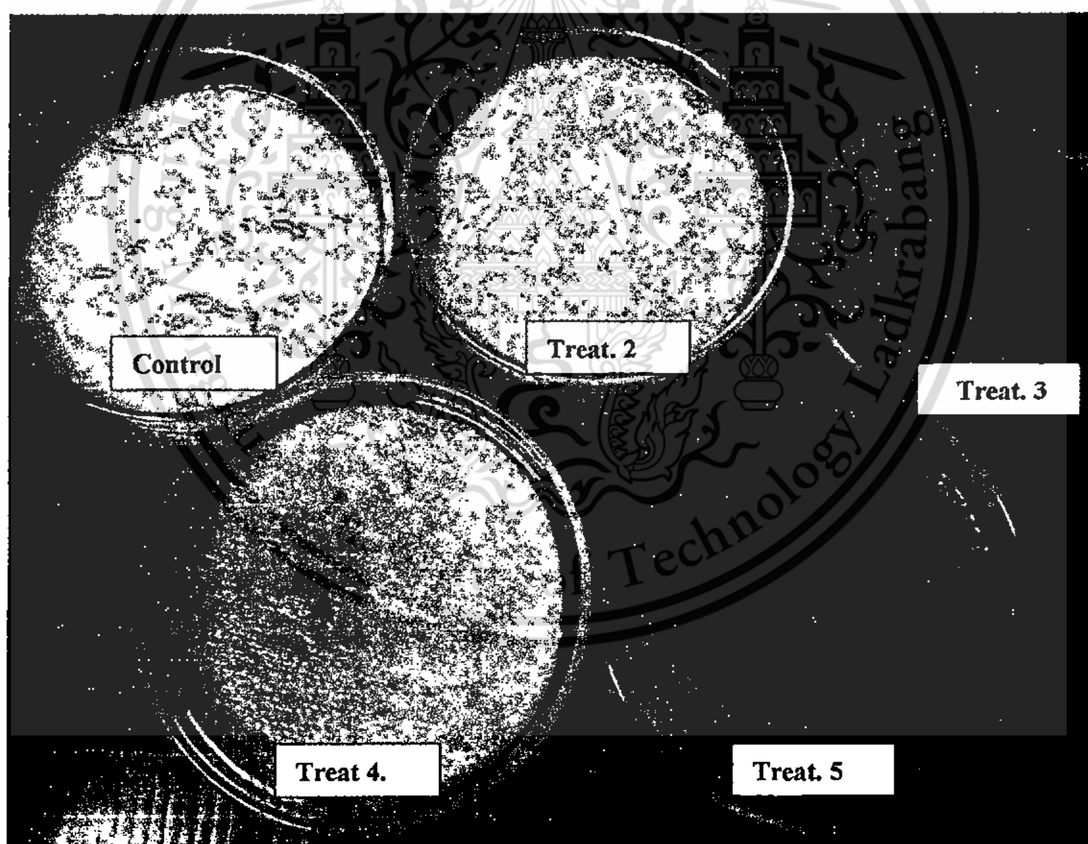

Figure 4.1 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. spinosus*

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4.2.2 Effects of different concentrations of *T. erecta* on seed imbibition and α-amylase activity induction of *A. spinosus*

Data below showed the differences in the percentage of seed imbibition between control and concentration application of *T. erecta* leaf extract in soluble concentrate formulation at different imbibition periods. The percentages of imbibition in the control seeds markedly increased by prolonging the imbibition periods and had the highest seed imbibition at all the imbibition time whereas the percentage of imbibition in treated seeds increased slightly by prolonging the imbibition period. After imbibiton time of 12, 24 and 36 hours, the percentage of seed imbibition inhibited significantly upon the increasing the dose of application during the entire experiment. After imbibiton time of 12, 24 and 36 hours, seed imbibition inhibited from 8.14, 14.38 and 105.64% in comparison with the control.

The activities of α-amylase of *A. spinosus* seeds were also investigated and the results were shown below within the same concentration of *T. erecta* leaf extract, α-amylase activity increased by prolonging the imbibition period, especially in control. After imbibition time of 12, 24 and 36 hours, increased concentration of *T. erecta* leaf extract in soluble concentrate formulation inhibited remarkably induction α-amylase activities were 9.4, 22.8 and 41.46 μmol maltose $min^{-1}g^{-1}$FW, respectively. By these results, the mechanisms of allelopathic potential of *T. erecta* leaf extract inhibited seed imbibition and α-amylase activities at high concentration on *A. spinosus*.

Table 4.7 Effects of different concentrations of *T. erecta* on seed imbibition of *A. spinosus*.

Concentration (ppm)	Seed imbibitions (%)		
	12 hours	24 hours	36 hours
Distilled water	23.95a[1]	38.68a	130.42a
1000	19.59a	30.49b	33.95b
2000	23.58a	30.68b	29.74bc
4000	18.73a	24.50b	29.26bc
8000	15.81a	24.30b	24.78c

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

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Table 4.8 Effects of different concentrations of *T. erecta* on α-amylase activity induction of *A. spinosus.*

Concentration (ppm)	α-Amylase activity (μmol maltose $min^{-1} g^{-1}$ FW)		
	12 hours	24 hours	36 hours
Distilled water	17.76a[1]	26.18a	46.43a
1000	15.34ab	19.32b	20.50b
2000	12.98b	14.83b	21.07b
4000	9.85c	9.47c	14.29bc
8000	8.36c	3.38c	4.97c

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.2.3 Effects of *T. erecta* and exogenous GA_3 on inhibition of seed germination and seedling growth of *A. spinosus*

As shown in below Table and Figure, in comparison of control, *A. spinosus* seeds had non-significant on seed germination and seedling growth after applied exogenous GA_3 (from 0.1 – 0.8 ppm). Seed inhibition of *A. spinosus* (% of control) after being treated with concentration combination of *T. erecta* leaf extract considered as natural herbicide and exogenous GA_3 was slightly decreased in whole experiment in comparison with untreated exogenous GA_3 (1000 ppm *T. erecta* leaf extract). This decrease was most pronounced with applied 0.1 ppm GA_3 and the inhibition of seed germination, shoot length and root length were 68.75; 80.30 and 95.39%, respectively. However by increasing the dose of applied GA_3 at 0.2 to 0.8 ppm, the inhibition increased. In addition, shoot length and root length still had a remarkable inhibition (more than 80%). These applied GA_3 had non-significant to prevent and relieve the herbicide effect on *A. spinosus.*

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Table 4.9 Effects of *T. erecta* and exogenous GA_3 on inhibition of seed germination and seedling growth of *A. spinosus.*

Concentration	Inhibition (% of control)		
	Seed germination	Shoot length	Root length
1000 ppm Crude extract	100.00a[1]	100.00a	100.00a
0.1 ppm GA_3	2.50c	-6.06c	-3.95c
0.2 ppm GA_3	0.00c	18.18b	19.74b
0.4 ppm GA_3	2.50c	15.15b	5.26c
0.8 ppm GA_3	3.75c	9.09b	15.79b
1000 ppm crude extract + 0.1 ppm GA_3	68.75b	80.30a	95.39a
1000 ppm crude extract + 0.2 ppm GA_3	88.75ab	87.88a	100.00a
1000 ppm crude extract + 0.4 ppm GA_3	85.00ab	87.88a	100.00a
1000 ppm crude extract + 0.8 ppm GA_3	98.75a	100.00a	100.00a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.2.4. Effects of *T. erecta* and exogenous GA_3 on seed imbibition and α-Amylase activity induction of *A. spinosus*

Exogenous GA_3 was used for induction of seed germination by α-amylase activity after complete inhibition because of applied 2500 ppm *T. erecta* leaf extract in this study. The results in the Table 4.10 and showed that exogenous GA_3 had non-significant effects against *T. erecta* leaf extract in soluble concentrate formulation on *A. spinosus* entire experimental period (after 12 hours imbibition) by both of seed imbibition and α-amylase activity induction. Seed imbibition and α-amylase activity in all treatments in whole experiment were about 20 - 23% and 9 - 12 μmol maltose $min^{-1}g^{-1}FW$, respectively. Even though in comparison with control, treatments were applied with only exogenous GA_3 had a marked indifference of seed imbibition and α-amylase activity induction.

Therefore this result suggests that exogenous GA_3 had non-significant effects against *T. erecta* leaf extract in soluble concentrate formulation on *A. spinosus.*

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Table 4.10 Effects of *T. erecta* and exogenous GA_3 on seed imbibition and α-Amylase activity induction of *A. spinosus* (after 12 hours imbibition).

Concentration	Seed imbibitions (%)	α-Amylase activity (μmol maltose $min^{-1}g^{-1}FW$)
Distilled water	23.32a[1]	12.26ab
1000 ppm Crude extract	20.37a	8.78dc
0.1 ppm GA_3	24.26a	13.06a
0.2 ppm GA_3	23.20a	12.79a
0.4 ppm GA_3	23.77a	12.54a
0.8 ppm GA_3	21.06a	11.85abc
1000 ppm crude extract + 0.1 ppm GA_3	20.12a	9.16bdc
1000 ppm crude extract + 0.2 ppm GA_3	19.27a	8.98bdc
1000 ppm crude extract + 0.4 ppm GA_3	21.17a	8.54d
1000 ppm crude extract + 0.8 ppm GA_3	20.14a	8.95dc

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.3 Effects of different concentrations of *T. erecta*, exogenous GA_3 and the combination of *T. erecta* and exogenous GA_3 on seed germination, seed imbibition and α-amylase activity induction of *A. gracilis*

4.3.1 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. gracilis*

Table 4.11 and the Figure 4.2 showed that *T. erecta* leaf extract in soluble concentrate formulation had significant allelopathic effects against *A. gracilis*. At 250 ppm dose, germination of seed of *A. gracilis* was inhibited by 68.33% while its shoot and root length inhibition were 24.14 and 3.81%, respectively. By increasing the dose of application at 750 ppm, germination of seed of *A. gracilis* was inhibited by 88.33% whereas its shoot and root length inhibition were 96.55 and 95.24%, respectively. At reached concentrations of 1000 – 8000 ppm, the inhibition magnitude was complete. This suggests that *T. erecta* leaf extract contains some inhibitory principles upon inhibited germination and seedling growth. Hence, next experiments were carried

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out to evaluate and understand interaction mechanisms of allelopathic potential about inhibition on seed imbibition and α-amylase activities.

Table 4.11 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. gracilis*.

Concentration (ppm)	Inhibition (% of control)		
	Seed germination	Shoot length	Root length
250	68.33c[1]	24.14b	3.81b
500	85.83b	87.93a	88.57a
750	88.33b	96.55a	95.24a
1000	100.00a	100.00a	100.00a
2000	100.00a	100.00a	100.00a
4000	100.00a	100.00a	100.00a
8000	100.00a	100.00a	100.00a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

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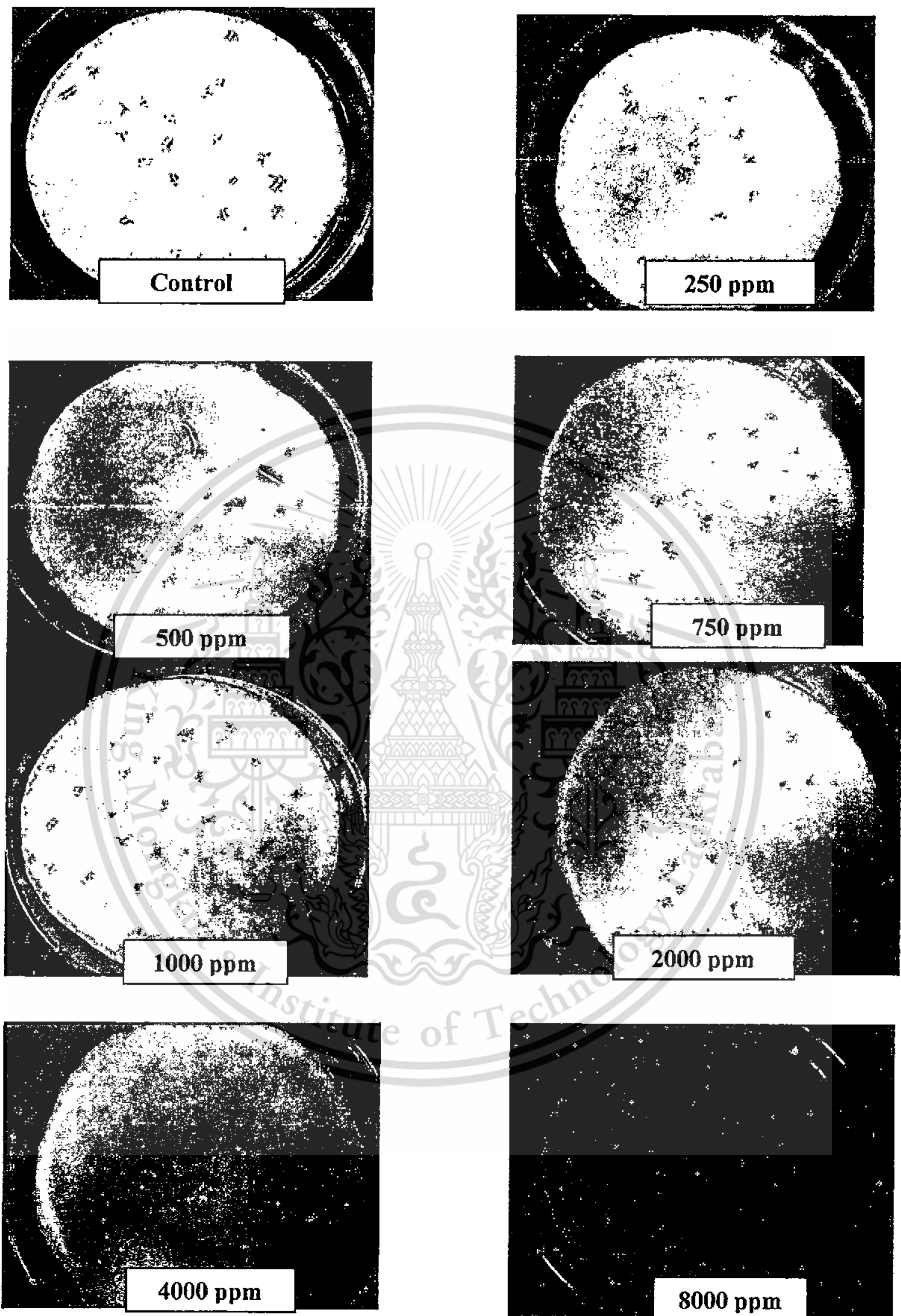

Figure 4.2 Effects of different concentrations of *T. erecta* on inhibition of seed germination and seedling growth of *A. gracilis*

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4.3.2 Effects of different concentrations of *T. erecta* on seed imbibition and α-amylase activity induction of *A. gracilis*

Table 4.12 showed the differences in the percentage of seed imbibition between control and concentration application of *T. erecta* leaf extract in soluble concentrate formulation at different imbibition periods. By prolonging the imbibition periods, the percentages of imbibition in the control seeds markedly increased and had the highest seed imbibition at all the imbibition time whereas the percentage of imbibition in treated seeds increased slightly by prolonging the imbibition period. After imbibition time of 12, 24 and 36 hours, the percentage of seed imbibition inhibited significantly according to the increasing the dose of application during the entire experiment. During above imbibition time, seed imbibition was significantly inhibited from 1.83, 10.15 and 10.01%, respectively in comparison with the control.

The activities of α-amylase of *A. gracilis* seeds were also investigated and the results were shown in Table 4.13. Within the same concentration of *T. erecta* leaf extract, α-amylase activity increased by prolonging the imbibition period, especially in control. After imbibition time of 12, 24 and 36 hours, by increased concentration of *T. erecta* leaf extract in soluble concentrate formulation reached to 1000 ppm, an inhibited induction α-amylase activities were 2.05, 10.34 and 6.39 μmol maltose $min^{-1}g^{-1}$FW, respectively in comparison with the control. By going on these results, the inhibition of seed imbibition and α-amylase activities was shown at the higher concentration of *T. erecta* leaf extract by its allelopathic potential on *A. gracilis*.

Table 4.12 Effects of different concentrations of *T. erecta* on seed imbibition of *A. gracilis*.

Concentration (ppm)	Seed imbibitions (%)		
	12 hours	24 hours	36 hours
Distilled water	24.74a[1]	32.83a	33.83a
250	27.55a	28.77ab	28.00ab
500	26.49a	27.37abc	26.40ab
750	22.03a	25.94bc	25.93ab
1000	22.91a	22.68c	23.82b

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

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Table 4.13 Effects of different concentrations of *T. erecta* on α-amylase activity induction of *A. gracilis.*

Concentration (ppm)	α-Amylase activity (μmol maltose $min^{-1}g^{-1}FW$)		
	12 hours	24 hours	36 hours
Distilled water	11.95a[1]	19.56a	12.64a
250	10.06a	15.70ab	13.43a
500	9.60a	13.99ab	7.62b
750	10.08a	9.94b	7.91b
1000	9.90a	9.22b	6.25b

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

4.3.3 Effects of *T. erecta* and exogenous GA_3 on inhibition of seed germination and seedling growth of *A. gracilis*

As shown in Table 4.14, *A. gracilis* seeds after being treated with concentration combination of *T. erecta* leaf extract and exogenous GA_3, their combination still inhibited completely seed germination and seedling growth throughout the entire experiment (100% inhibition of control) whereas *A. gracilis* seed was applied with only exogenous GA_3 had non-significant promotion seedling growth in comparison of control. Hence, this result suggests that these applied exogenous GA_3 had non-significant to prevent and relieve the *T. erecta* leaf extract effect on *A. gracilis.*

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Table 4.14 Effects of *T. erecta* and exogenous GA_3 on inhibition of seed germination and seedling growth of *A. gracilis.*

Concentration	Inhibition (% of control)		
	Seed germination	Shoot length	Root length
1000 ppm crude extract	100.00a[1]	100.00a	100.00a
0.1 ppm GA_3	67.50b	10.71b	-12.50b
0.2 ppm GA_3	45.83c	-10.71b	-1.56b
0.4 ppm GA_3	72.50b	21.43b	-9.38b
0.8 ppm GA_3	56.67bc	14.29b	-12.50b
1000 ppm crude extract + 0.1 ppm GA_3	100.00a	100.00a	100.00a
1000 ppm crude extract + 0.2 ppm GA_3	100.00a	100.00a	100.00a
1000 ppm crude extract + 0.4 ppm GA_3	100.00a	100.00a	100.00a
1000 ppm crude extract + 0.8 ppm GA_3	100.00a	100.00a	100.00a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

Comparisons of different concentrations of *T. erecta* on *Amaranthus* spp. (*A. tricolor*, *A. spinosus* and *A. gracilis*)

The above results showed allelopathic potential of *T. erecta* leaf extract had significantly different inhibition on difference of amaranth species. The inhibitory effects of seed germination and seedling growth on *A. tricolor* were less than those on *A. spinosus* and *A. gracilis.* At dose of 1000 ppm *T. erecta* leaf extract in soluble concentration formulation inhibition of control was complete whereas the inhibition on *A. tricolor* was 2500 ppm *T. erecta* leaf extract.

The effects of different concentrations of *T. erecta* on inhibition of seed imbibition and α- amylase activity induction had the most significant in *A. gracilis* followed by *A. spinosus.*

In this study, allelopathic inhibition was accordance with other studies (Laosinwattana et al., 2010; Han et al., 2008; Chong et al., 2002). The findings showed the inhibitory effects of *Saururaceae* (*Houttuynia cordata* Thunb.) varied with weed indicator species evaluated; the aqueous extracts from young leaves of *M. azeradach* inhibited *E. crus-galli* seed germination; ginger aqueous extracts inhibited imbibition in seeds of chive and soybean; seed was inhibited the imbibition because of limited in specific enzymes required for metabolism of reserved food and hence exhibited poor seed germination; the α-amylase activity catalyzes endosperm starch

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hydrolysis and transformation into soluble sugars and hence its utilization for providing energy during seed germination; mechanism of aqueous extract from young leaf stage of *M. azeradach* may be associated with inhibition of activity of α-amylase. It was shown that α-amylase activity was inhibited by presence of allelochemicals. Besides Kato-Noguchi and Macias (2005) also had reported that lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds treated by 6-methoxy-2-benzoxazolinone (MBOA) reduced activity of α-amylase during seed germination.

4.4 Effects of natural herbicide from *Tagetes erecta* L. on DNA degradation of *Amaranthus* spp.

4.4.1 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. tricolor, A. gracilis* and *A. spinosus* for DNA extraction

4.4.1.1 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. tricolor* for DNA extraction

In order to investigate the effects of SC formulation from *T. erecta* under the concentration that completely inhibited seed germination and seedling growth of *A. tricolor* (2500 ppm) and obtain samples (leaf and stem) for DNA extraction, a dose of concentration application from 250 to 1000 ppm of *T. erecta* leaf extract in SC formulation was treated with *A. tricolor*. Data showed the differences in the percentage of seed inhibition (% of control). At dose of 250 to 1000 ppm *T. erecta* leaf extract in SC formulation, the inhibition (% of control) of seed germination and shoot length was less than 10% and not differ significantly by Tukey's Studentized Range Test (p=0.05) whereas the inhibition of root length was 12.37 to 53.67% on *A. tricolor* depending on the concentration application.

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Table 4.15 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. tricolor*.

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Rootlength
250	7.5a[1]	7.26a	12.37c
500	7.5a	6.85a	15.93bc
750	10a	17.34a	38.76ab
1000	7.5a	20.16a	53.63a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

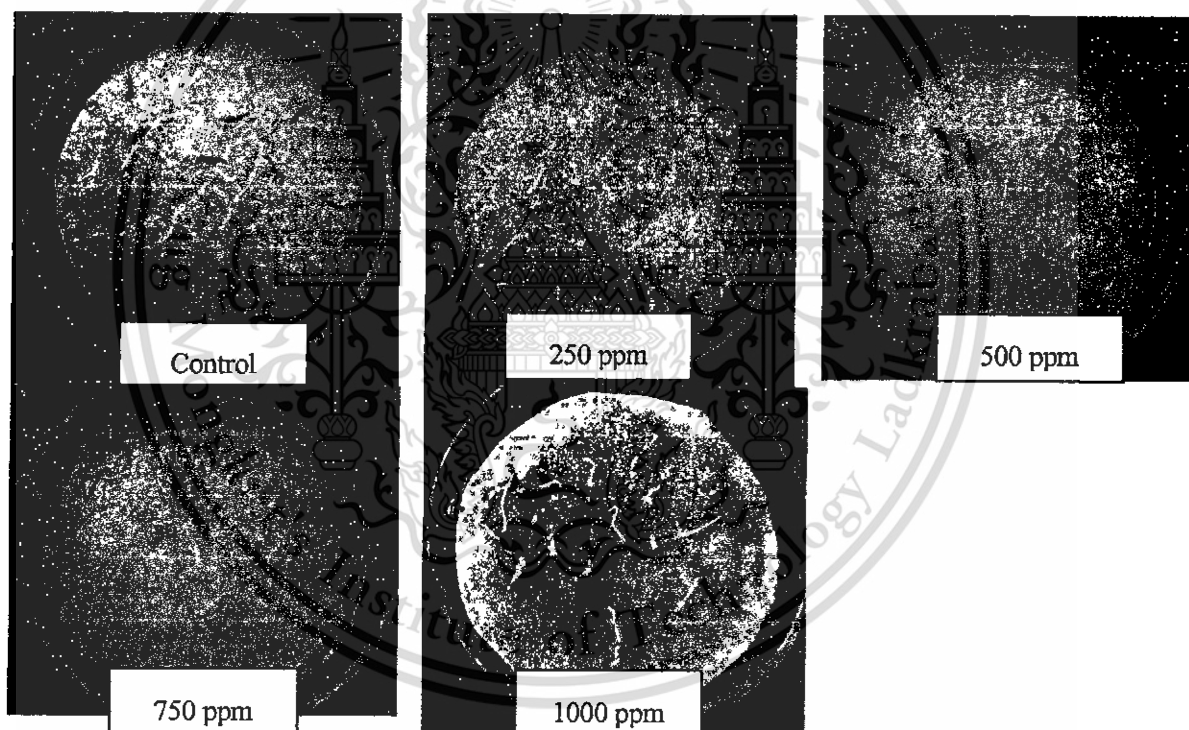

Figure 4.3 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. tricolor*

4.4.1.2 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. gracilis* for DNA extraction

Table and Figure below showed the inhibition of *T. erecta* leaf extract in SC formulation from 100 to 400 ppm on seed germination and seedling growth of *A. gracilis*. At concentration of

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100 ppm, germination of seed of *A. gracilis* was inhibited significantly by 30%. Its shoot and root length inhibition were 46.54 and 29.18%, respectively. By increasing the dose of application at 400 ppm, germination of seed of *A. gracilis* was inhibited by 78.75% whereas its shoot and root length inhibition were 92.26 and 97.77%, respectively. This suggests that at concentration less than complete inhibition concentration of *T. erecta* leaf extract (1000 ppm) still contains some inhibitory principles upon inhibited germination and seedling growth. Hence, next experiments were carried out to obtain these samples and extract genomic DNA to understand interaction mechanisms of allelopathic potential about inhibition on DNA degradation.

Table 4.16 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. gracilis*.

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Rootlength
100	30b[1]	46.54c	29.18c
200	40b	49.47bc	32.96c
300	72.5a	63.03c	65.14b
400	78.75a	92.29a	97.77a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

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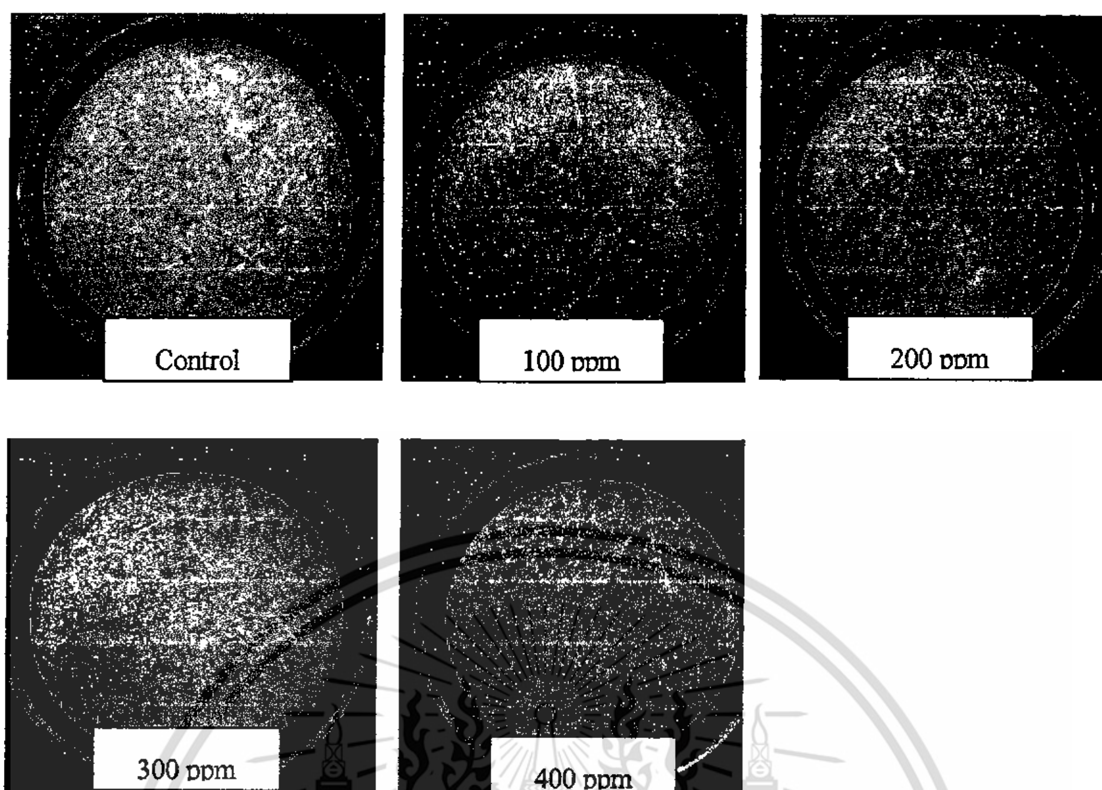

Figure 4.4 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. gracilis*

4.4.1.3 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. spinosus* for DNA extraction

Table 4.17 and Figure 4.5 showed that *A. spinosus* seeds were treated *T. erecta* leaf extract in SC formulation from 100 - 400 ppm inhibited seed germination and seedling growth according to the increasing of *T. erecta* concentration. At concentration of 100 ppm, germination of seed of *A. spinosus* was inhibited significantly by 20% while its shoot and root length inhibition were 42.29 and 26.73%, respectively. By increasing the dose of application at 400 ppm, seed germination of *A. spinosus* was inhibited by 78.75% whereas its shoot and root length inhibition were 84.31 and 98.11%, respectively. This suggests that at lower concentration of *T. erecta* leaf extract (less than 1000 ppm) still contains inhibition on germination and seedling growth. Thus, next experiment was studied to obtain these samples and extract its DNA genomic to understand molecular mechanisms of allelopathic potential on DNA degradation of *Amaranthus* spp.

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Table 4.17 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. spinosus.*

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Rootlength
100	20c[1]	42.29c	26.73c
200	35c	47.61bc	31.74c
300	53.75b	59.57b	65.37b
400	78.75a	84.31a	98.11a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

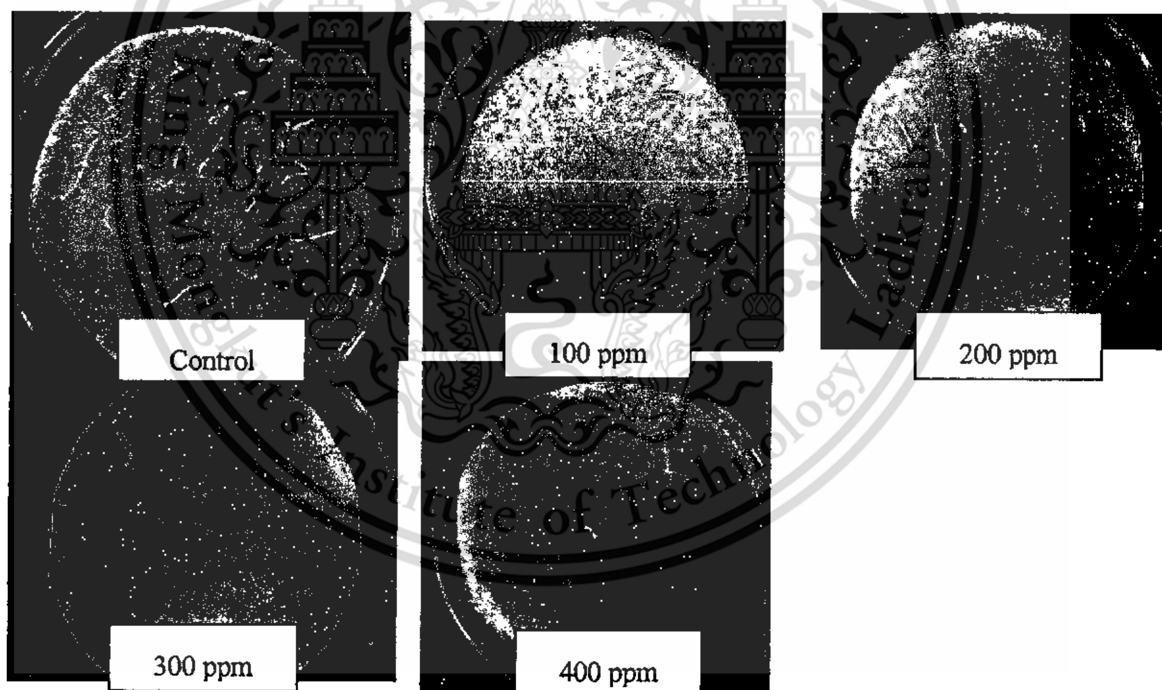

Figure 4.5 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. spinosus*

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4.4.2 Effects of different concentrations of SC formulation from *T. erecta* on DNA degradation in plant cells

4.4.2.1 Spectrophotometer results

Samples were analyzed with a spectrophotometer that measured absorbance at 230nm, 260nm, 280nm and 320nm, as well as calculating the ratios A260/ A280 and A260/A230 and the concentration of DNA in ng/µl of the samples. A260/A280 ratios were used to estimate the sample purities obtained with each of the three species of amaranth that treated with natural herbicide from *T. erecta*. In general, the ratio A260/ A280 were summarized in Tables 4 to 6 with the displays all the spectrophotometer results of all the samples between 1.6 and 2.0. Therefore, these samples can be used for electrophoresis to determine DNA degradation by natural herbicide from *T. erecta*.

Table 4.18 Spectrophotometer data of extracted DNA from *A. tricolor*

	Sample (ppm)	Concentration (ng/ µl)	A260/ A280	A260 /A230	A230	A260	A280	A340
Rep1	Control	3989.3	**1.83**	1.95	0.1625	0.3195	0.175	0.025
	250	1982.25	**1.69**	1.515	0.1045	0.1585	0.0935	0.02495
	500	5948.4	**1.64**	1.215	0.391	0.476	0.291	0.099
	750	4492.9	**1.64**	1.465	0.245	0.3595	0.22	0.0735
	1000	2080.65	**1.56**	1.24	0.1665	0.1665	0.107	0.0365
Rep 2	Control	2280.45	**1.54**	1.33	0.137	0.182	0.1185	0.029
	250	2977.6	**1.63**	1.24	0.1905	0.238	0.1455	0.0515
	500	1093.55	**1.49**	0.89	0.0985	0.0875	0.059	0.0265
	750	5319.1	**1.58**	1.47	0.284	0.4255	0.267	0.104
	1000	4638.9	**1.75**	1.675	0.221	0.371	0.212	0.0265
Rep 3	Control	2113.75	**1.73**	1.775	0.094	0.169	0.097	0.0235
	250	2248.7	**1.44**	1.3	0.141	0.1795	0.1245	0.0635
	500	2623.85	**1.62**	1.265	0.1635	0.2095	0.128	0.049
	750	3167.8	**1.63**	1.235	0.236	0.2535	0.1555	0.057
	1000	4027.05	**1.70**	1.755	0.1835	0.372	0.1795	0.052

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Table 4.19 Spectrophotometer data of extracted DNA from *A. gracilis*

	Sample (ppm)	Concentration (ng/ µl)	A260/ A280	A260/ A230	A230	A260	A280	A340
Rep1	Control	217.25	**1.82**	1.05	0.0165	0.0175	0.0415	0.0005
	100	390	**2.02**	1.83	0.0135	0.024	0.0105	0
	200	415.8	**1.78**	1.215	0.032	0.0375	0.022	0.0005
	300	705.75	**1.78**	1.565	0.029	0.042	0.023	0.0015
	400	370.3	**1.93**	1.28	0.0295	0.0295	0.0155	0.002
Rep 2	Control	644.8	**1.58**	1.18	0.028	0.0515	0.033	0.007
	100	811.55	**1.89**	1.38	0.043	0.067	0.0375	0.01
	200	727.6	**1.72**	1.515	0.049	0.0505	0.0295	0.0025
	300	702.2	**1.73**	1.26	0.0405	0.0575	0.0315	0.004
	400	827.75	**1.8**	1.46	0.04	0.0535	0.032	0.003
Rep 3	Control	430.35	**1.37**	1.17	0.045	0.033	0.027	0.0025
	100	600.25	**1.73**	1.125	0.037	0.048	0.028	0.013
	200	970.25	**1.67**	1.235	0.0475	0.0775	0.046	0.006
	300	785.45	**1.77**	1.195	0.0645	0.0625	0.0355	0.0055
	400	893.2	**1.84**	1.29	0.0575	0.0715	0.039	0.0015

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Table 4.20 Spectrophotometer data of extracted DNA from *A. spinosus*

	Sample (ppm)	Concentration (ng/ µl)	A260/ A280	A260/ A230	A230	A260	A280	A340
	Control	765.5	**1.71**	1.77	0.0345	0.0615	0.036	0.001
	100	389.9	**1.73**	0.725	0.0435	0.032	0.017	0.005
Rep1	200	245.85	**1.92**	0.85	0.0235	0.0195	0.01	0.0005
	300	1845.2	**1.21**	1.21	0.1265	0.1475	0.1235	0.103
	400	-	-	-	-	-	-	-
	Control	248.85	**1.35**	0.99	0.02	0.019	0.0145	0.0035
	100	1053.6	**1.15**	0.99	0.0855	0.0845	0.073	0.0685
Rep 2	200	349.75	**1.55**	0.95	0.0295	0.028	0.0225	0.0465
	300	248.65	**1.77**	0.845	0.0215	0.027	0.0115	0
	400	-	-	-	-	-	-	-
	Control	431.4	**1.92**	1.45	0.034	0.0325	0.018	0
	100	285.95	**1.67**	0.67	0.034	0.0315	0.0135	0.001
Rep 3	200	479.15	**1.74**	1	0.0385	0.0285	0.022	0.0015
	300	248.65	**1.77**	0.845	0.0215	0.027	0.0115	0
	400	-	-	-	-	-	-	-

4.4.2.2. Assessment of DNA degradation by electrophoresis on 2% agarose gel

Because nucleic acids are negatively charged ions at neutral or basic pH in an aqueous environment, they can be mobilized by an electric field. Gel electrophoresis is a technique used to separate molecules on the basis of size, using this charge. The nucleic acids can be separated as whole chromosomes or fragments. The nucleic acids are loaded into a slot near the negative electrode of a semisolid, porous gel matrix and pulled toward the positive electrode at the opposite end of the gel. Smaller molecules move through the pores in the gel faster than larger molecules; this difference in the rate of migration separates the fragments on the basis of size. There are molecular weight standard samples that can be run alongside the molecules to provide a

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size comparison. Nucleic acids in a gel matrix can be observed using various fluorescent or colored dyes. Distinct nucleic acid fragments appear as bands at specific distances from the top of the gel (the negative electrode end) on the basis of their size. A mixture of genomic DNA fragments of varying sizes appear as a long smear, whereas uncut genomic DNA is usually too large to run through the gel and forms a single large band at the top of the gel.

Figure 4.6 showed good quality and normalized DNA of *A. tricolor* in lane 2, 8, 14 and 20 whereas degraded genomic DNA in other lanes such as: 3, 4, 5, 6, 9, 10,11, 12, 15, 16, 17, 18, 21, 22, 23, 24. At higher concentration of *T. erecta* in SC formulation from 250 ppm to 1000 ppm, genomic DNA show more degraded (genomic DNA fragments of varying sizes appear as a long smear)

Figure 4.6 Electrophoresis of DNA extracted from *A. tricolor* treated with different concentration of *T. erecta*; lane 1, 7, 13,19: DNA ladder 1kb, lane 2, 8,14, 20: control, lane 3, 9, 15, 21: 250 ppm, lane 4, 10, 16, 22: 500 ppm, lane 5, 11, 17, 23: 750 ppm, lane 6, 12, 18, 24: 1000 ppm

Figure 4.7 showed good quality and normalized DNA of *A. gracilis* in lane 2, 8, 14 (control) whereas degraded genomic DNA of *A. gracilis* in other lanes (except marker lanes: 1, 7, 13). By increasing the dose of application from 200 - 400 ppm, genomic DNA of *A. gracilis* showed more degraded (genomic DNA fragments of small sizes appear as a longer smear). This suggests that at concentration less than complete inhibition concentration of *T. erecta* leaf extract

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(1000 ppm) DNA genomic of *A. gracilis* was broken and degraded. This is also molecular mechanisms of allelopathic potential of *T. erecta* leaf extract on DNA degradation of *Amaranthus* spp.

This was similar with DNA degradation of *A. spinosus* showed in Figure 4.8. Lane 2 (control) genomic DNA showed clear band (normalized DNA) whereas lane 3, 4, 5 showed genomic DNA degradation.

Figure 4.7 Electrophoresis of DNA extracted from *A. gracilis* treated with different concentration of *T. erecta*; lane 1, 7, 13: DNA ladder 1kb, lane 2, 8, 14: control; lane 3, 9, 15: 100 ppm; lane 4, 10, 16: 200 ppm; lane 5, 11, 17: 300 ppm; lane 6, 12, 18: 400 ppm

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Figure 4.8 Electrophoresis of DNA extracted from *A. spinosus* treated with different concentration of *T. erecta*; lane 1: DNA ladder 1kb; lane 2, 6: control; lane 3: 100 ppm; lane 4: 200 ppm; lane 5: 300 ppm

Comparison of DNA degradation of *A. tricolor*, *A. spinosus* and *A. gracilis*

The genomic DNA was degraded by being treated with concentration application of *T. erecta* in SC formulation from 100 ppm to 1000 ppm on 3 species of amaranth in comparison with control. The genomic DNA of 3 amaranth species showed different degradation according to each species. Genomic DNA extracted from *A. spinosus* showed the most sensitively degraded, following by *A. gracilis* and *A. tricolor*.

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CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

The crude extract from *T. erecta* had significant allelopathic effects against 3 species of amaranth. It completely inhibited on seed germination and seedling growth of *A. spinosus* and *A. gracilis* at the concentration of 1000 ppm whereas 2500 ppm on *A. tricolor*. The mechanisms of allelopathic potential of *T. erecta* crude extract are inhibition on seed imbibition and α-amylase activities at high concentration on amaranth.

Exogenous GA_3 was used for induction of seed germination by seed imbibition and α-amylase activity induction however it had non-significant effects against *T. erecta* leaf extract in soluble concentrate formulation on *A. tricolor, A. spinosus* and *A. gracilis*. Therefore this suggests that mechanism of *T. erecta* leaf extract on *Amaranthus* spp. through seed germination is not affected by exogenous GA_3.

DNA extracted from *Amaranthus* spp. treated with leaf extract in SC formulation from *T. erecta* by concentration of 100 - 1000 ppm showed genomic DNA degradation. Hence the use of *T. erecta* leaf extract as a potential natural herbicide for weed control might be possible.

5.2 Suggestions

5.2.1 Further studies about molecular and cytogenetic mechanisms of *T. erecta* leaf extract effects on *A. tricolor, A. spinosus* and *A. gracilis*: study on protein, DNA degradation in polyacrylamide electrophoresis, gene expression and cytotoxicity should be carried out.

5.2.2 Effects of *T. erecta* leaf extract on seed germination and seedling growth of 3 above amaranth species in green house should be carried out.

5.2.3 Effects of leaf extract of *T. erecta* on other bioassays should also be carried out.

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