International Journal of Agricultural Technology 2016 Vol. 12(6): 1005-1015
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Study on DNA Degradation by Natural Herbicide from *Tagetes Erecta* L. During *Amaranthus* Spp. Seed Germination

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Nguyen Thi Tham, Chamroon Laosinwattana, MontineeTeerarak and Siriporn Sripinyowanich. (2016). Study on DNA degradation by natural herbicide from *Tagetes erecta* L. during *Amaranthus* spp. seed germination. International Journal of Agricultural Technology 12(6): 1005-1015.

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). In order to investigate the effects of SC formulation concentrations from *T. erecta* that is lower than concentrations that completely inhibited seed germination and seedling growth of *Amaranthus tricolor* L. (2500 ppm), *Amaranthus gracilis* Desf. and *Amaranthus spinosus* L. (1000 ppm) and obtain samples (leaf and stem) for DNA extraction, a dose of concentration application from 100 to 1000 ppm of *T. erecta* leaf extract in SC formulation was bioassayed on germination, seedling growth on 3 species of amaranth. The results showed 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. 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 different genomic DNA degradation. Genomic DNA extracted from *A. spinosus* showed the most sensitively degraded, followed by *A. gracilis* and *A. tricolor*. Hence the use of *T. erecta* leaf extract as a potential natural herbicide for weed control might be possible.

Keywords: DNA degradation, *Amaranthus* spp., marigold, seedling growth.

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Introduction

DNA degradation is a process by which DNA breaks down into smaller fragments. 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 (Ito, J. and Hiroo, F. 2002).

Marigold (*Tagetes erecta* L.) is in *Tagetes* genus belonging to the family *Asteraceae*; it is demonstrated with the high allelopathic potential. Almost studies showed that allelopathic activity may inhibit the seeds germination by inhibiting the induction of α-amylase activity (Meksawat, S. and Pornprom, T. (2010), Laosinwattana C *et al*.). Crude marigold leaf extract in soluble concentrate formulation (SC) was studied as a natural herbicide because of its high allelochemical. 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, T. R. 1999). However, its molecular mechanism remains unclearly during seed germination, especially in DNA degradation.

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

Materials and methods

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.

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℃ 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.

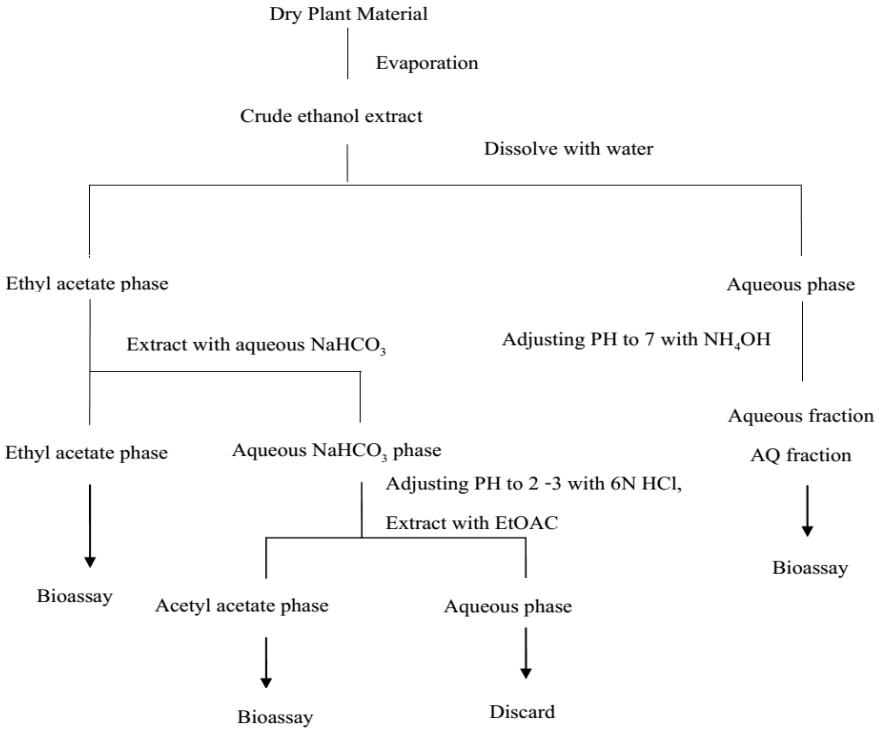

Fig. 1. Flow chart for extraction and partially separation of active compounds from marigold dried leaves

After filtration using Whatman No. I filter paper, the filtrates was combined and evaporated in the rotary evaporator at 45℃, leaving a sticky residue (crude ethanol fraction). This residue was then diluted with 500 mL of distilled water and stirred vigorously on a magnetic stirrer at 45^{o}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 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.

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). For *A. tricolor*, the SC formulation from *T. erecta* was diluted with distilled water to 250, 500, 750 and 1000 ppm. For *A. gracilis* and *A. spinosus*, the SC from T. erecta was diluted with distilled water to 100, 200, 300and 400 ppm. Then all petri dishes were placed in a growth chamber with condition (cool white 840 Climacell 707, Munich, Germany) at 25 - 32oC, 12h dark/ light photoperiod light intensity of 100 µmol m-2s-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]

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^{o}C refrigerator for 3 days before DNA extraction.

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^{o}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^{o}C for 15 minutes. Samples were then centrifuged immediately at 12,000 rpm for 10 min at 4 ℃. 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 ℃. The upper aqueous phase will be transferred to a new 1.5-mL eppendorf. To the precipitated DNA, 500 µL isopropanol was added. The sample were placed in -80^{o}C refrigerator for 1 hour and centrifuged to obtain DNA for 12,000 rpm for 15 min at 4^{o}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^{o}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

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 DocTM 2000 and UV transilluminator.

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.

Results

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.

Table 1. 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)

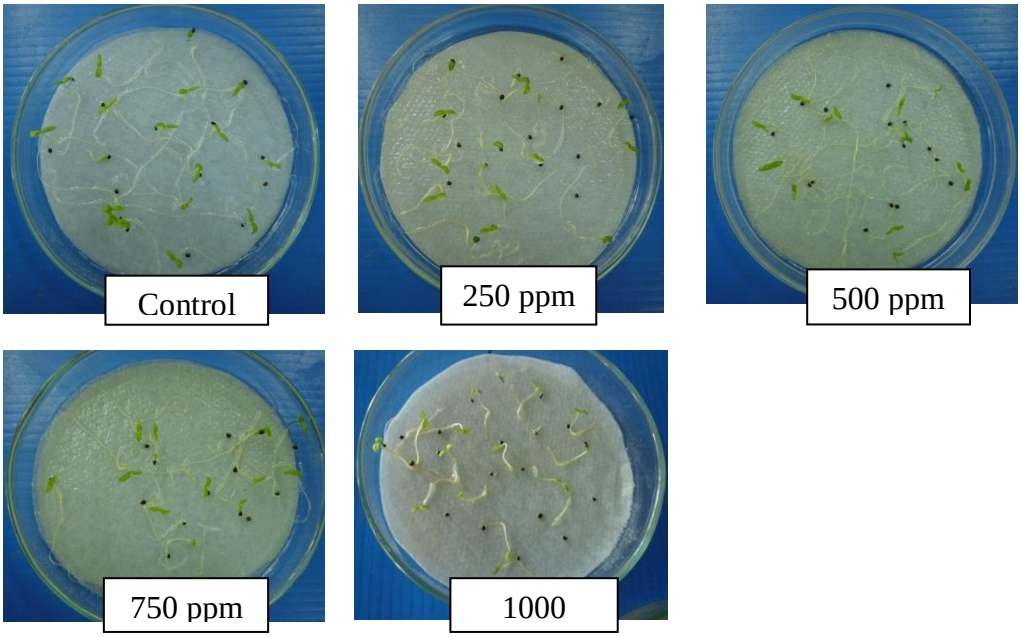

Fig. 2. Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. tricolor*

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

Table 2. 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)

The table 2 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 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.

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

Table and Figure 3 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.

Table 3: 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)

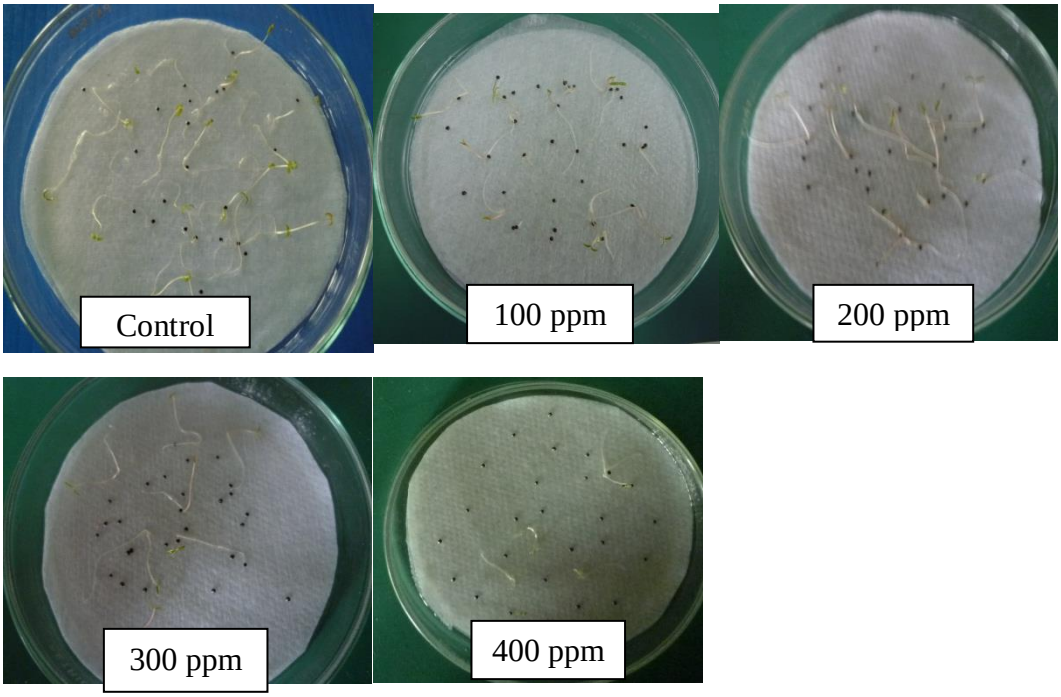

Fig. 3. Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. spinosus*

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

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*.

At higher concentration of *T. erecta* in SC formulation from 250 ppm to 1000 ppm, genomic DNA of *A. tricolor* show more degraded (genomic DNA fragments of varying sizes appear as a long smear). Fig. 4 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 (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 Fig. 5. Lane 2 (control) genomic DNA showed clear band (normalized DNA) whereas lane 3, 4, 5 showed genomic DNA degradation.

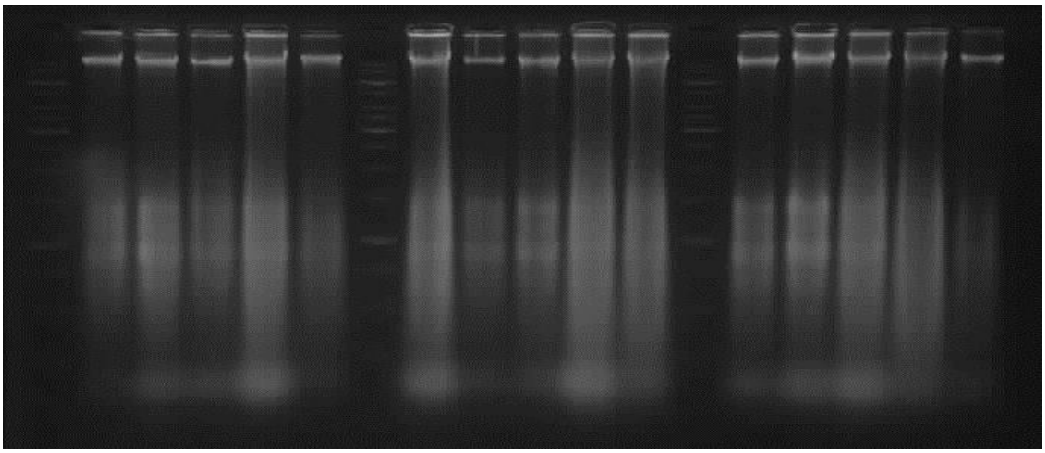

Fig. 4. Electrophoresis of DNA extracted from *A. gracilis* treated with different concentration of *T. erecta*; lane 1, 7, 13: DNA ladder 1kb, lane 2, 8, 14: control; lane 3, 9, 15: 100 ppm; lane 4, 10, 16: 200 ppm; lane 5, 11, 17: 300 ppm; lane 6, 12, 18: 400 ppm

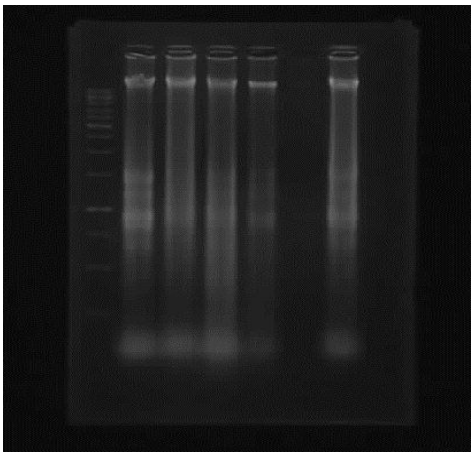

Fig. 5. Electrophoresis of DNA extracted from *A. spinosus* treated with different concentration of *T. erecta*; lane 1: DNA ladder 1kb; lane 2, 6: control; lane 3: 100 ppm; lane 4: 200 ppm; lane 5: 300 ppm

Conclusions

At the concentration is lower than the concentration of SC formulation that completely inhibited seed germination and seedling growth of *Amaranthus* spp. (less than 1000 ppm) still contains inhibition on germination and seedling growth.

DNA extracted from *Amaranthus* spp. treated with leaf extract in SC formulation from *T. erecta* by concentration of 100-1000 ppm showed different genomic DNA degradation. Genomic DNA extracted from *A. spinosus* showed the most sensitively degraded, followed by *A. gracilis* and *A. tricolor*.

Acknowledgement

This research was supported by grants from Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand.

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(Received: 11 September 2016, accepted: 31 October 2016)