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*Original Article*

# Effects of biocides on chlorophyll contents of detached basil leaves

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**Abstract**

Herbicides and insecticides have been widely and intensively used in agricultural areas worldwide to enhance crop yield. However, many biocides cause serious environmental problems. In addition, the biocides may also have some effects on the treated agricultural crops. To study effects of biocides on chlorophyll content in detached basil leaves, 2,4-D dimethylamine salt (2,4 D-Amine), paraquat, carbosulfan, and azadirachtin, were chosen as representatives of biocide. After applying the chemicals to detached basil leaves overnight in darkness, chlorophyll contents were determined. Only treatment with 2,4 D-Amine resulted in reduction of chlorophyll contents significantly compared to treatment with deionized (DI) water. In the case of paraquat and carbosulfan, chlorophyll contents were not significantly changed, while slightly higher chlorophyll contents, compared to DI water, after the treatment with azadirachtin, were observed. The results indicated that 2,4 D-Amine shows an ability to accelerate chlorophyll degradation, but azadirachtin helps to retard chlorophyll degradation, when each biocide is used at the concentration recommended by the manufacturer.

**Keywords:** chlorophyll, biocide, agriculture, detached leave

## 1. Introduction

Leaf is one essential part of plants, since it plays several important roles such as food production (photosynthesis), food storage, water transport, gas exchanging (respiration), and protection of vegetative and floral buds. In addition to the plants themselves, leaves are also necessary to other living organisms, because the photosynthesis process generates oxygen which is released to an environment. Plant leaves are also a major source of food for human and other animals. In green leaf plants, quality of leaves can be simply determined by chlorophyll content in the leaves, because chlorophyll is responsible for the characteristic green colour of several vegetables and also fruit. However, this green pigment can degrade to other forms of molecule with colour changes such as phaeophytin and phaeophorbide, olive-brown compounds, and the degradation products can be further metabolized to colourless compounds (Heaton and Marangoni, 1996; Eckhardt *et al.*, 2004). There are several factors affecting chlorophyll loss such as normal aging, lack of light, temperature changes, nutrient deficiency, and viral infection etc (Wann, 1930).

The loss of chlorophyll in response to aging resulting in natural senescence is an inevitable process in plant development. Although senescence is a genetically controlled program, environmental or external factors are able to hasten or repress senescence (Lichtenthaler, 1987; Holden, 1972; Gan and Amasino, 1997). The external factors induce changes in gene expression (Weaver *et al.*, 1998; Chen *et al.*, 2011), which initiates senescence. Leaf senescence has been studied in several aspects including physiology, biochemistry, and molecular studies, using naturally (aging) or/and artificially (environmental stresses, such organ detachment, exposure to darkness, drought, and exposure to acid etc.) induced leaf senescence approaches. These studies have shown that the processes of natural and artificial senescence have some regulatory mechanisms in common, but are not totally identical (Weaver *et al.*, 1998; Canetti *et al.*, 2002;

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Becker and Apel, 1993). Since chlorophyll degradation is widely used to represent quality loss of green plant products such as vegetable crops, manipulation of senescence is very important for agricultural applications. Leaf or plant organ detachment is a condition that can accelerate the process of senescence. Therefore, postharvest storage is a critical process for quality control of crop yield in certain crops, because the yield can be lost during postharvest storage. Detachment of leaves from plant resources causes water and nutrient deficiency. In addition, lack of light or darkness condition during storage causes photosynthetic inhibition. Thus, conditions in postharvest storage can absolutely affect the crop yield. The external factors can influence senescence either singly or in combination with different levels of effect.

As some chemical treatments can also affect chlorophyll contents in detached leaves (Holden, 1972), chemical contamination of postharvest crops may also influence the degradation process of chlorophyll. Although attached leaves can recover their chlorophyll contents, the contaminating chemicals could affect chlorophyll contents after the leaves are detached from plant. Since biocides have been intensively used in most agricultural areas, contamination with biocides and their metabolites generally occurs. This study presents the effects of several biocides, both herbicides and insecticides, on chlorophyll contents after applying the biocides to detached basil leaves in darkness condition. Only chlorophyll a and b were monitored in this work, because they are the most commonly found components of green leaf plants. The selected biocides are representative of different groups of biocide: 2,4 D-Amine is salt form of acidic biocide, paraquat is basic biocide, carbosulfan is nonionic biocide, and azadirachtin is a natural extract biocide. Results obtained from the study not only contribute to our knowledge about the effect of chemicals on chlorophyll loss, but may also provide information to be aware of when choosing a herbicide or insecticide for agricultural applications.

## 2. Materials and Methods

### 2.1 Plant material and treatments

Germinated Thai basil (*Ocinum sanctum)* was purchased and raised in growth pots in a natural environment. Analyses were performed after the harvested leaves were treated with biocide solutions of 2,4 D-Amine, active ingredient 72%, 1,1-dimethyl-4,4-bipyridinium ion, active ingredient 27.6% (Paraquat), Carbosulfan, active ingredient 20%, and 100% neem's extract (Azadirachtin) individually. Distilled water was used as a control experiment.

2,4 D-Amine manufactured by Millennium Farm Ltd, Paraquat manufactured by Syngenta Crop Protection Ltd, Carbosulfan manufactured by Sharp Formulator Ltd, and Azadirachtin manufactured by DR Agriculture Ltd were purchased from commercial sources in Bangkok, Thailand.

### 2.2 Chlorophyll content determination

For determining chlorophyll contents of Thai basil detached leaves after application of different biocides, 2.5 g leaf was homogenized with 15 mL of pure acetone. 2.0 g of anhydrous sodium sulfate ($Na_2SO_4$) was then added to the sample. Chlorophyll extract was filtered through filter paper. The extracts from each sample were made to a known volume, and the chlorophyll extracts were measured using spectrophotometer at the wavelengths 644.8 and 661.6 nm. Chlorophyll a and b contents were determined according to equations 1.1 and 1.2 (Lichtenthaler, 1987).

$$C_a\ (\mu g/mL) = 11.24A_{661.6} - 2.04A_{644.8} \qquad \text{(Eq 1.1)}$$

$$C_b\ (\mu g/mL) = 20.13A_{644.8} - 4.19A_{661.6} \qquad \text{(Eq 1.2)}$$

where $C_a$ is a concentration of chlorophyll a (μg/mL),
$C_b$ is a concentration of chlorophyll b (μg/mL), and
A is absorbance at given wavelengths

## 3. Results

### 3.1 Effects of 2,4 D-Amine on the chlorophyll a and b in chlorophyll extracts

To monitor effects of herbicide, 2,4 D-Amine on chlorophyll degradation, different amounts of the herbicide were added to the chlorophyll extracts. Contents of chlorophyll a and b were determined immediately after herbicide treatment using a spectrophotometer. Chlorophyll a and b contents in the extracts after adding the herbicide at different amounts are presented in Table 1.

The recommended concentrations of 2,4 D-Amine for applying to plants are in the range of approximately 1.5-3.0 g/L. In this study, the final concentrations of 2,4 D-Amine added to chlorophyll extracts were as follows, 2.16, 6.48, and 10.80 g/L. The results of chlorophyll content measurement show that both $C_a$ and $C_b$ in the extracts were decreased comparing to the control, approximately 80% of $C_a$ and 50% of $C_b$ remaining in the extracts. There was no significant change on chlorophyll content from adding different 2,4 D-Amine concentrations, although higher concentrations of herbicide were added to the extracts. However, percent decreases in $C_b$ were greater than those in $C_a$. This indicated that $C_b$ is more sensitive to 2,4 D-Amine than $C_a$. Structures of $C_a$ and $C_b$ are very similar with only one functional group different on the molecules. $C_a$ contains a methyl group, while $C_b$ bears a formyl group on the sidechain of the porphyrin ring. Therefore, $C_b$ could possibly be converted to $C_a$ by reduction of 7-formyl into 7-methyl group when $C_b$ is exposed to environmental stresses resulting in higher levels of $C_a$ as found in this case. There are some works reporting about $C_b$-to-$C_a$ conversion (Houimli *et al.*, 2010; Fang *et al.*, 1998; Scheumann *et al.*, 1998).

Table 1. Chlorophyll a and b contents of Thai basil extracts in the presence of various concentrations of 2,4 D-Amine.

| Concentration of 2, 4 D-Amine (g/L) | $C_a$ (μg/g) | Relative % of $C_a$ | $C_b$ (μg/g) | Relative % of $C_b$ |
|---|---|---|---|---|
| Control* | 263.0±0.6 | 100.0 | 94.7±0.8 | 100.0 |
| 2.16 g/L | 215.2±0.8 | 81.8 | 47.7±0.9 | 50.4 |
| 6.48 g/L | 214.7±1.2 | 81.6 | 54.1±0.7 | 57.1 |
| 10.80 g/L | 211.2±0.9 | 80.3 | 47.8±0.9 | 50.5 |

*Control is distilled water added to chlorophyll extract instead of 2,4 D-Amine.

### 3.2 Effects of 2,4 D-Amine, Carbosulfan, Paraquat, and Azadirachtin on the chlorophyll a and b in chlorophyll extracts in darkness

To observe an effect of biocides on chlorophyll extract in the long term, the herbicides: 2,4 D-Amine and paraquat, and insecticides: carbosulfan and azadirachtin were added to the chlorophyll extracts. The concentrations of these biocides applied to chlorophyll extracts were within the recommended range for applying to plants. The biocide-added chlorophyll extracts were then kept in the dark for 18 hours before the chlorophyll contents were determined. The amounts of chlorophyll a and b in the presence of various biocides are shown in Table 2.

As presented in Table 2, chlorophyll a and b contents in the biocide-containing extract were not significantly different from the control after being kept in the dark for several hours. Chlorophyll contents of the extract 2,4 D-Amine and other studied biocides showed similar levels as the control when the biocide-added extracts were incubated for several hours in the dark.

### 3.3 The changes of chlorophyll contents in detached leaves in a contact with various solutions

As biocide did not cause significant change in chlorophyll level in the extract comparing to the control in long term of storage, detached leaves were then studied to observe whether or not biocide effect the content of chlorophyll was manifest in the plant leaves. Fresh detached Thai basil leaves were submerged in various solution including distillated water, HCl (aq), and 2.16 g/L of 2,4 D-Amine, and kept in darkness for 18 hours. Chlorophyll was then extracted from the leaves for determining chlorophyll content.

Compared to the control treatment, as shown in Table 3, detached leaves exposed to 2.16 g/L 2,4 D-Amine, and to 1.0 and 10.0 mM HCl (aq) showed significantly lower chlorophyll contents. Thus, the results indicate that contact with these solutions results in enhanced degradation rate of chlorophyll. As it is well known that chlorophyll is a pH sensitive compound, high concentrations of HCl (aq) therefore cause the loss of the magnesium from the center of the chlorophyll molecule resulting in the molecule termed *phaeophytin*. However, 2,4 D-Amine solution has similar pH as DI water which is close to 7. Therefore, 2,4 D-Amine appears to involve a different pathway from pH dependence in chlorophyll degradation.

In addition, biocides are generally applied to plants by spraying. Therefore, to mimic reality, different solutions were applied to detached Thai basil leaves by spraying method. The treated leaves were then kept in the dark under room temperature for 18 hours prior to performing chlorophyll extraction procedure for determining chlorophyll contents in the detached leaves.

Either submerging detached leaves in the solutions or spraying the solutions to detached leaves showed similar results (Table 4), in which chlorophyll contents, both $C_a$ and $C_b$, after HCl (aq) and 2,4 D-Amine solutions were almost

Table 2. Chlorophyll a and b contents of Thai basil extracts in the presence of 2,4 D-Amine, Paraquat, Carbosulfan, and Azadirachtin, kept in darkness for 18 hours prior to chlorophyll determination.

| Herbicide | $C_a$ (μg/g) | Relative % of $C_a$ | $C_b$ (μg/g) | Relative % of $C_b$ |
|---|---|---|---|---|
| Control* | 168.5±0.8 | 100.0 | 33.6±0.5 | 100.0 |
| 2,4 D-Amine (2.16 g/L) | 169.9±1.3 | 100.8 | 34.2±0.2 | 101.8 |
| Paraquat (1.66 g/L) | 170.2±1.6 | 101.0 | 37.4±1.2 | 111.3 |
| Carbosulfan (1.20 g/L) | 171.4±0.1 | 101.8 | 35.2±0.6 | 104.9 |
| Azadirachtin (3.0% v/v) | 173.8±1.2 | 103.2 | 36.6±0.4 | 108.7 |

*Control is distilled water added to chlorophyll extract instead of biocides.

Table 3. Chlorophyll contents in Thai basil detached leaves floated in various solutions, kept in darkness for 18 hours.

| Solutions | $C_a$ (μg/g) | Relative % of $C_a$ | $C_b$ (μg/g) | Relative % of $C_b$ |
|---|---|---|---|---|
| Control* | 36.4±7.0 | 100 | 11.4±2.7 | 100 |
| 2,4 D-Amine (2.16 g/L) | 20.0±4.4 | 54.9 | 7.4±0.9 | 64.9 |
| 10.0 mM HCl (aq) | 16.1±3.4 | 44.2 | 2.8±0.7 | 24.6 |
| 1.0 mM HCl (aq) | 20.3±4.0 | 55.8 | 6.6±1.6 | 57.9 |
| 0.1 mM HCl (aq) | 40.1±4.1 | 110.2 | 10.4±0.7 | 91.2 |

*Control is detached Thai basil leaves submerged in distilled water and kept in darkness for 18 hours.

Table 4. Chlorophyll contents in Thai basil detached leaves treated with acidic and 2,4 D-Amine solutions by spraying, kept in darkness for 18 hours.

| Solutions | $C_a$ (μg/g) | Relative % of $C_a$ | $C_b$ (μg/g) | Relative % of $C_b$ |
|---|---|---|---|---|
| Control* | 101.7±3.7 | 100.0 | 23.4±1.2 | 100.0 |
| 1.0 mM HCl (aq) | 56.3±6.2 | 55.4 | 12.9±1.1 | 55.1 |
| 2,4 D-Amine (2.16 g/L) | 55.4±7.8 | 54.5 | 12.3±1.8 | 52.6 |

*Control is distilled water spraying to the detached leaves.

50% lower than the contents obtained from the control. This indicates that chlorophyll degradation pathway is easily interrupted by external factors.

### 3.4 Effects of 2,4-D concentrations on chlorophyll degradation in detached Thai basil leaves

To observe the effect of biocide concentrations on the change of chlorophyll content, detached Thai basil leaves were sprayed with different concentrations of 2,4 D-Amine.

A decrease of both $C_a$ and $C_b$ contents was observed with the chlorophyll remaining in the leaves approximately 50% compared to the control (Table 5). The results imply that 2,4 D-Amine not only causes damage to chlorophyll directly in long period of contact time, but also affects proteins or enzymes involved in the chlorophyll degradation pathway, since chlorophyll contents in the plant extracts had the same levels as the control, while the changes of chlorophyll contents can be clearly observed between the control and the detached leaves treated with 2,4 D-Amine solution.

The correlation between degradation rate of chlorophyll and concentration of 2,4 D-Amine remains unclear because of a relatively high variation of chlorophyll contents measured in the detached leaves. However, a difference in 2,4 D-Amine concentration in the range of 2.16 to 10.80 g/L does not seem to cause a great difference on chlorophyll degradation rate.

### 3.5 Effects of Paraquat, Carbosulfan, and Azadirachtin on chlorophyll degradation in detached Thai basil detached leaves

As 2,4 D-Amine treated leaves showed lower remaining chlorophyll contents in the leaves compared to the control, other biocides were then studied to observe their effects on chlorophyll contents. Another herbicide, paraquat, and two insecticides, carbosulfan and azadirachtin, were sprayed separately to detached Thai basil leaves, and the leaves were kept in the dark for 18 hours prior to chlorophyll extraction and determination. The biocides were diluted with distilled water before applying to the plants as recommended for each chemical application.

No conclusion could be drown about chlorophyll degradation in the leaves treated with carbosulfan and paraquat solutions as there was considerable variation in chlorophyll determinations in the detached leaves (Table 6). However, 3% azadirachtin provided slightly higher amounts of $C_a$ in the leaves than the control. In the case of chlorophyll b, treatments of the detached leaves with biocides and with water gave similar levels of chlorophyll content. In addition, degradation rates of $C_a$ and $C_b$ are not different when the biocides were applied to the detached leaves.

Table 5. Chlorophyll a and b contents in Thai basil detached leaves treated with 2,4 D-Amine solutions, kept in darkness for 18 hours.

| Concentration of 2, 4 D-Amine (g/L) | $C_a$ (μg/g) | Relative % of $C_a$ | $C_b$ (μg/g) | Relative % of $C_b$ |
|---|---|---|---|---|
| Control* | 133.2±2.2 | 100.0 | 30.2±2.1 | 100.0 |
| 2.16 g/L | 71.8±9.9 | 53.9 | 15.3±2.4 | 50.7 |
| 4.32 g/L | 51.1±3.0 | 38.4 | 11.3±0.1 | 37.4 |
| 10.80 g/L | 63.3±5.5 | 47.5 | 14.6±3.3 | 48.3 |

*Control is distilled water spraying to the detached leaves.

Table 6. Chlorophyll contents in Thai basil detached leaves treated with various biocides, kept in darkness for 18 hours.

| Biocide | $C_a$ (μg/g) | Relative % of $C_a$ | $C_b$ (μg/g) | Relative % of $C_b$ |
|---|---|---|---|---|
| Control* | 75.5±7.9 | 100.0 | 18.0±2.2 | 100.0 |
| Paraquat (1.66 g/L) | 87.2±7.7 | 115.5 | 18.2±1.0 | 101.1 |
| Carbosulfan (1.20 g/L) | 79.0±8.2 | 104.6 | 16.9±2.0 | 93.9 |
| Azadirachtin (3% v/v) | 95.7±10.6 | 126.8 | 20.2±2.1 | 112.2 |

*Control is distilled water spraying to the detached leaves.

## 4. Discussion

Chlorophyll degradation or loss in leaves is conveniently used as an indicator for plant senescence, because the change of green colour is easily observed. However, there are several factors influencing senescence. These can either enhance or inhibit plant senescence. Herbicides and insecticides are chemicals widely used in agriculture to increase crop yields. However, some biocides can also affect growth, photosynthesis, and chlorophyll synthesis of plants.

2,4 D-Amine in the range between 2.16 to 10.80 g/L seems to increase degradation rate of chlorophyll at the early stage of contact when 2,4 D-Amine was added directly to chlorophyll extract, because $C_a$ and $C_b$ contents decreased significantly compared to the control. However, after several hours of contact, there was no obvious difference between chlorophyll content in treated and control extracts. This indicates that contact time between biocide and chlorophyll extract is a critical factor resulting in changing rate of chlorophyll degradation. 2,4 D-Amine will increase degradation rate of chlorophyll to some point and then will slow down the rate. Paraquat, carbosulfan, and azadirachtin also showed the same behavior as 2,4 D-Amine when the biocide-added chlorophyll extracts were kept in the dark for several hours.

Agricultural crops are generally contaminated with biocides. Therefore, to observe effects of biocides on the change of chlorophyll content in the crops during post-harvest storage, herbicides and insecticides were applied to detached Thai basil leaves. In the case of 2,4 D-Amine, either submerging or spraying the detached leaves with such solution resulted in the loss of $C_a$ and $C_b$ to about 50%. In addition, increasing concentrations of 2,4 D-Amine up to 5 times the recommended values caused the same level of chlorophyll loss as the recommended concentration. Interestingly, for several hours of storage, chlorophyll contents in detached leaves treated with 2,4 D-Amine had lower levels than chlorophyll content in the control, whereas there was no significantly different chlorophyll contents between biocide-added chlorophyll extract and the control. This indicates that 2,4 D-Amine may also affect other pathways of chlorophyll degradation, not only by direct action on the chlorophyll molecule.

Paraquat and carbosulfan application to detached leaves provided similar contents of chlorophyll in leaves as the control, while azadirachtin showed slightly higher amounts than the control. Therefore, azadirachtin may have a property to inhibit senescence or chlorophyll degradation in detached Thai basil leaves. Since carbosulfan and azadirachtin are pesticides, they should not result in harmful effect to plants, and the corresponding results were observed by non-decreasing chlorophyll contents compared to the control after the pesticide treatment. Chlorophyll contents in paraquat-treated leaves were also comparable to the control. This may result from paraquat, a basic herbicide, acting by inhibiting the photosynthesis process of green leaf plants. Thus, it does not cause acceleration of chlorophyll degradation. 2,4 D-Amine is a herbicide against broad-leaf weeds causing acceleration of foliar senescence, chloroplast damage, and chlorosis with subsequent disruption of the vascular system. Consequently, chlorophylls are quickly degraded. Hence, chlorophyll of basil, a dicot, could possibly be affected by 2,4 D-Amine.

This study demonstrates that some biocides have an influence on chlorophyll degradation. In growing plants, the loss of chlorophyll at small levels may not affect plant growth or plant quantity, because plants can recover chlorophyll by photosynthesis process, which cannot be occurred in post-harvest crops. Therefore, it is critical if the contaminated chemicals cause an increase of chlorophyll degradation rate. Thus, it is necessary to be aware of the chemicals applied to crops not only in terms of the effects on the environment such as contamination and toxicity, but also in terms of their effects on the crops themselves to protect and obtain good quality of agricultural crops.

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