

THE ROLE OF LETTUCENIN A IN LETTUCE (*Lactuca sativa*) AGAINST
PATHOGEN *Xanthomonas campestris* pv. *oryzae*

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AGAINST PATHOGEN Xanthomonas campestris pv. oryzae.

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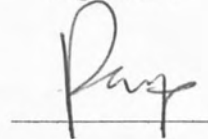
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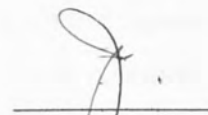
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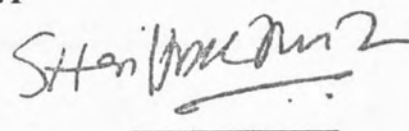
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ABSTRACT

Lettucenin A is the major phytoalexin produced by lettuce after elicited by biotic or abiotic elicitors. The production of lettukenin A in leaves can be induced by 5% CuSO₄ (w/v) and 1% AgNO₃ (w/v). A clear inhibition zone where the fungus *Aspergillus niger* failed to grow on TLC bioassay after the chromatogram was developed in hexane: ethyl acetate (1:1, v/v) at R_f 0.45 was observed. Lettukenin A was detected at a retention time of approximately 5.3 min when analyzed with HPLC in isocratic solvent system with water: acetonitrile (60:40, v/v). *In vitro* antibacterial study with *Xanthomonas campestris* pv. *oryzae* showed that this pathogen had different sensitivity to all tested concentrations of lettukenin A. The bacterium was more sensitive to higher concentration of lettukenin A (333, 533 and 667 µg ml⁻¹) than in lower concentration such as 67 µg ml⁻¹. Thus, the relationship between the bacterial growth rate and lettukenin A concentration was negatively correlated. However, the bacterial growth rate was continuing to increase after two hours of incubation time. Hence, it is suggested that *X. campestris* pv. *oryzae* might had the ability to detoxify the lettukenin A. It is also suggested that the success or failure of *X. campestris* pv. *oryzae* to invade lettuce depends on a balance between accumulation and degradation of lettukenin A at the invading site of lettuce. In summary, lettukenin A may play an important role in the resistance of lettuce against microbial infection.



ABSTRAK

Letusenin A merupakan fitoalexin utama dari sayur “lettuce” atau salad selepas dirangsang dengan perangsang biotik dan abiotik. Penghasilan letusenin A dapat dirangsangkan dengan penggunaan bahan kimia seperti 5% CuSO₄ (w/v) dan 1% AgNO₃ (w/v). Satu kawasan kerintangan yang jelas di mana kegagalan kulat *Aspergillus niger* bertumbuh dapat diperhatikan pada ujian biologi TLC pada R_f 0.45 setelah kromatogram itu dicelupkan dalam hexane: ethyl acetate (1:1, v/v). Letusenin A telah dikesan pada masa penahanan minit ke-5.3 apabila dianalisis dengan HPLC dalam sistem pelarut isokratik, iaitu air: acetonitrile ialah 60:40 (v/v). Ujian *in vitro* antibakteria letusenin A terhadap *Xanthomonas campestris* pv. *oryzae* telah menunjukkan bahawa patogen ini mempunyai tahap kepekaan yang berbeza terhadap semua kepekatan letusenin A yang diuji. Bakteria ini lebih sensitif dalam kepekatan letusenin A yang lebih tinggi (333, 533 dan 677 µg ml⁻¹) berbanding dengan kepekatan letusenin A yang rendah, iaitu 67 µg ml⁻¹. Oleh yang demikian, kadar pertumbuhan bakteria adalah berkorelasi secara negatif dengan kepekatan letusenin A. Namun demikian, kadar pertumbuhan bakteria meningkat semula dua jam selepas inkubasi. Oleh itu, *X. campestris* pv. *oryzae* dicadangkan mempunyai kebolehan untuk mendegradasikan letusenin A. Terdapat juga cadangan bahawa kejayaan atau kegagalan bakteria dalam serangan ke atas sayur salad bergantung kepada keseimbangan antara penghasilan dan degradasi letusenin A pada kawasan serangan salad. Sebagai kesimpulan, letusenin A mungkin memainkan satu peranan yang penting dalam memberikan rintangan sayur salad ke atas serangan mikroorganisma.



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LIST OF SYMBOLS AND ABBREVIATION

°C	degree of Celsius
kPa	kilo Pascal
>	more than
<	less than
%	percentage
pv.	pathovar
λ_{\max}	maximum absorbance
μg	microgram
g	gram
ml	milliliter
nm	nanometer
μm	micrometer
mm	millimeter
cm	centimeter
w/v	weight/volume
v/v	volume/volume
rpm	round per minute
<i>p</i>	significant value
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
HR	hypersensitive resistance
UV	ultraviolet
CuCl_2	copper(II) chloride
CuSO_4	copper(II) sulfate
AgNO_3	silver nitrate
PDA	Potato Dextrose Agar
NA	Nutrient Agar
R_f	retention factor
R_t	retention time
SD	standard deviation
OD	optical density



CHAPTER 1

INTRODUCTION

1.1 Background

Throughout their life cycles, plants may be challenged by different of environmental conditions or numerous of pathogens such as viruses, bacteria, mollicutes, fungi, nematodes, insects and others. In lettuce, it is normally attacked by *Bremia lactucae*, which is a fungus that causes downy mildew of this plant. Lettuce is also infected by *Botrytis* sp. The symptom of this disease is the formation of whitish-gray mold on the leaves. Besides that, lettuce can be attacked by lettuce mosaic virus, which cause yellowing and necrosis on leaves. Lettuce infectious yellow virus or LIYV, one type of crinivirus can infect lettuce as well and cause the leaves become yellow and stunted. Most of the viruses that cause disease in lettuce are transmitted by insects such as whiteflies and aphids (Agrios, 2005). In addition, bacteria *Xanthomonas campestris* pv. *vitiens* cause bacterial spots of lettuce (Barak *et al.*, 2002).

In Sabah and most of the plantation areas in Malaysia, chemical pesticides are used to control the diseases of lettuce and other crops (Jipanin *et al.*, 2001). These pesticides are toxic compounds and are also known as fungicides, bactericides, nematocides, herbicides and insecticides depending of the kind of pests they against. The public assumed that pesticides were only toxic to pests at which they were aimed. Therefore, large quantity of



pesticides was applied liberally on fields, farm, vegetables and fruits. Even more powerful and toxic pesticides were produced annually to control the pests at lower concentration in a higher speed (Agrios, 2005; Jipanin *et al.*, 2001).

Public concern about the safety of chemical pesticides began at 1960s. Researchers and scientists found that one of the disadvantages arises from the frequently usage of pesticides is high residue contain in these vegetables or crop products. This is because low biodegradability and persistence in the environment of the pesticides. Bird, fish, insect and other animals' deaths because of these pesticides are being accumulated and concentrated through the food chain, and finally cause pollution problems which contaminant the soil, water and air. In addition, some of the species in insects had developed genetic resistance to these chemicals and could no longer be controlled with them. Pesticides can be hazardous to human health as well, in direct or indirect ways. As the results, pesticides containing mercury, chlorinated hydrocarbons such as DDT, aldrin, endrin, lindane, chlordane and carcinogenic compounds were banned (Dahama, 2003; Gossett, 2003; Young, 1987). Recently, the desirability of using fewer and safer pesticides and the demand of consumers for fruits and vegetables which are free of chemicals are increasing. So, there is a greater need to reduce the usage of chemical pesticides by screening alternative strategies or technologies in order to improve plant disease resistance and control the pathogens.

Lettuce or salad continues to increase in popularity and has bright future in Malaysia. It is grown for local market and export such as to Singapore. In Sabah, the total plantation area of lettuce increased from 39.1 hectares in 2002 to 49.0 hectares in 2003 (Department of Agriculture Sabah, 2003a, 2003b). For Malaysian and people in other countries, lettuce is grown for its leaves, which are eaten raw as salad or cooked as vegetable (Grubben & Sukprakarn, 1994). However, this species is always infected with bacterial spots caused by *Xanthomonas campestris* pv. *vitians* (Barak *et al.*, 2002). Plants attacked by this pathogen



have the symptoms of spots on leaves and thus will decrease its commercial value in market. Farmers may use chemicals or other cultural practices to control this disease, thus increasing the expenses. These are economic and financial losses for both farmers and agricultural department (Jipanin *et al.*, 2001).

The first step in any infection is the recognition of host by pathogen or in the opposite way – recognition of pathogen by host. Early recognition between host and pathogen can be explained by gene-for-gene concept. There are 4 possible gene combinations: a) host resistance gene recognizes pathogen elicitor molecules and triggers defense reactions; b) pathogen lacks elicitor and thus, host resistance gene can't recognize this elicitor and no defense reaction is activated; c) host lacks receptors to recognize pathogen elicitor and defense reaction is not activated; and d) host lacks the resistance gene while pathogen carries virulent gene thus defense mechanism is not activated (Agrios, 2005).

Phytoalexins are produced in plants after stimulation of various types of phytopathogenic microorganisms, chemical, mechanical injury or stress. Resistance occurs when their concentrations are sufficient to inhibit the growth of fungi, bacteria or other pathogens (Agrios, 2005; Cruickshank, 1963; Ebel, 1986; Hammerschmidt, 1999; Kuć, 1972, 1995; Mansfield, 2000; Nicholson & Hammerschmidt, 1992; VanEtten *et al.*, 1989). Although phytoalexins have been studied in numerous years, but little is known with its direct effect to plant resistance. So, it is hoped that we can have much better view of it after the study in this field. Beside that, the level of phytoalexin accumulation after infection will be a good criterion to study plant-bacteria interaction.

There is a positive linear relationship between the amount or time of phytoalexin produced and the degree of disease resistance. This relationship is also known as

quantitative relationship. In other words, accumulation of phytoalexin can be correlated with the degree of pathogen restriction. When there is a higher rate or quantity of phytoalexin accumulated, the smaller lesion size and so for the number of the bacterial cells is found in the host plant (Hammerschmidt, 1999). Therefore, it is important to determine whether the interaction between the concentration of lettucenin A and *Xanthomonas campestris* pv. *oryzae* follows the same pattern of expression.

In this study, abiotic elicitors – CuSO₄ and AgNO₃ are used for elicitation. Thin Layer Chromatography or TLC is used to recover lettucenin A from plant tissue extracts. The samples are then subjected to High Performance Liquid Chromatography or HPLC for quantification. Different concentrations of lettucenin A are tested on *Xanthomonas campestris* pv. *oryzae* to verify the antimicrobial effect of different concentration of lettucenin A to *X. campestris* pv. *oryzae*. The antifungal activities of lettucenin A have been assessed in terms of the bacteria growth compared with that in control without lettucenin A.

1.2 Research objectives

Objectives of the study are:

- i) To carry out a preliminary TLC bioassay with lettucenin A.
- ii) *In vitro* study the role of lettucenin A in different concentrations against pathogen *Xanthomonas campestris* pv. *oryzae*.



1.3 Hypothesis

- i) Null Hypothesis, H_0 : High concentration of lettucein A is unable to inhibit the growth of pathogen *Xanthomonas campestris* pv. *oryzae*.
- ii) Alternative Hypothesis, H_1 : High concentration of lettucein A is able to inhibit the growth of pathogen *Xanthomonas campestris* pv. *oryzae*.

CHAPTER 2

LITERATURE REVIEW

2.1 Lettuce

2.1.1 Origin and uses

Lettuce or *Lactuca sativa* is a member in Family of Compositae or Asteraceae. Compositae is one of the largest angiosperm or flowering families. This family contains over 25,000 species including dandelion, chicory and endives (Mabberley, 1997). Linnaeus named this plant as *Lactuca*, which derived from the word 'lac', meaning 'milk' in Latin. This is because the sap of lettuce is milk-like. Our modern lettuce is believed to evolve in Mediterranean or the Middle East from the wild *Lactuca serriola*. Until now, lettuce is still popular and always cultivated as leafy vegetable and eaten raw as salad. It is grown well in temperate country, and also in subtropical and tropical lands (Grubben & Sukprakarn, 1994).

2.1.2 Descriptions and ecology

Lettuce is a very variable, glabrous annual or biennial herb. Leaves arrangement, size, shape, and colour always differ between cultivars. However, it usually forms a dense basal rosette and later with a tall, branched, flowering stem. The colour of leaves always comes with green, sometimes with red anthocyanin. Lettuce grows best at moderate day temperature at



15-20 °C and cool nights, like in the highlands. While in lowlands, it always cultivated during the coolest season. Lettuce adapts to soil with good structure, high fertility and water capacity. It prefers the sandy-loam soils with slightly alkaline and does not tolerate acid soil with pH < 6 (Grubben & Sukprakarn, 1994).

2.1.3 Types

There are 5 common types of lettuce which can be differentiated by leaf arrangement and structure. The first type is 'Butterhead' lettuce. The head contains overlapping leaves while the inner leaves are thin, oily and buttery in texture. This variety is the most popular variety in cool temperate country such as Europe. 'Crisp' lettuce, also known as Iceberg lettuce has dense, thick and crunchy leaves with prominent flabellate veins and midribs that resemble cabbage. 'Cos' lettuce has elongated leaves and forms a tall, loose and cylindrical head. While 'Bunching' lettuce has thin, broad and curled leaves in loose rosette stem. The last type of lettuce is 'Stem' lettuce. This type of lettuce has thick stem and is always used as celery stalk. Its crispy leaves are eaten as salad (Grubben & Sukprakarn, 1994; Simpson & Ogorzaly, 1995).

2.1.4 Diseases

Lettuce it is often attacked by *Bremia lactucae*, which are fungi that cause downy mildew in this species. Another fungal pathogen, *Botrytis* sp. causes disease which has the symptom of whitish-gray mold formation on the leaves. Besides that, lettuce can be attacked by lettuce mosaic virus and cause yellowing and necrosis on leaves. This virus is transmitted by aphids. Lettuce infectious yellow virus or LIYV can infect lettuce as well and cause the leaves become yellow and stunted. LIYV is one type of crinivirus and is transmitted by



whiteflies (Agrios, 2005). While bacterial pathogen *X. campestris* pv. *vitiensis* cause bacterial spots of lettuce (Barak *et al.*, 2002).

2.1.5 Prospects

Lettuce is one type of vegetables that becomes and more important recently in agriculture, both for local use and export. This is proved by estimated plantation area of salad (lettuce) in Sabah increased from year 2002 to year 2003 (Department of Agriculture, Sabah, 2003a, 2003b) and the increased for export from 1997 to 2000.

Table 2.1 Estimated plantation area of lettuce in Sabah of year 2002 and 2003 (Department of Agriculture, Sabah, 2003a, 2003b).

Area in Sabah	Year 2002 (hectares)	Year 2003 (hectares)
Tawau	2.0	2.0
West Coast	35.9	47.0
Interior Divisions	1.2	-
Total	39.1	49.0

Table 2.2 Export of lettuce in Sabah of year 1997-2000 (Jipanin *et al.*, 2001).

Year	Tone	RM
1997	126	330 000
1998	123	328 000
1999	153	398 000
2000	170	434 000



2.1.6 Classification

Below is the taxonomy or scientific classification of lettuce (Mabberley, 1997):

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Compositae (Asteraceae)

Genus: *Lactuca*

Species: *sativa*

2.2 Mechanisms of defense in plants

Every species of plants will be facing a challenge throughout their life cycles by numerous of pathogens and microorganisms such as viruses, bacteria, mollicutes, fungi and nematodes. Generally, plants defense themselves from pathogens by a combination of these 2 actions: the physical barriers that limit or inhibit the pathogens from entering and spreading through the plants and secondly, the biochemical reactions which occur in the cells and tissues, producing substances that are toxic to the pathogens or creating the conditions that will inhibit the pathogens growth (Agrios, 2005).

Physical barriers can be divided into 2 groups: the preexisting defense structures and induced defense structures. Preexisting structures are first line of defense of plants against pathogens. These include waxes and cuticles on leaf surfaces, thickness and toughness of the epidermal cell walls, size, location and shapes of stomata and lenticels. Induced defense



structures are cytoplasmic reactions, formation of cork layers, abscission layers, tyloses and deposition of gums (Agrios, 2005; Maor & Shirasu, 2005).

Biochemical reactions can be divided in 2 groups as well. Preexisting chemical defenses are exudation of some fungitoxin compounds, phytoanticipins, phenolic compounds, tannins, dienes, lectins and others in leaves, young fruits or seeds which will inhibit development of pathogens. Another type is induced biochemical defenses including hypersensitive responses, production of pathogenesis-related proteins and secondary metabolites such as phenolic compounds and phytoalexins (Agrios, 2005; Grayer & Kokubun, 2001). Resistance or defense of plants against pathogens is controlled directly or indirectly by the genetic materials of the host and of the pathogen. According to this concept, there are 3 types of plant resistances.

2.2.1 Non-host resistance

This type of resistance is the most common form of plant resistance in nature and is expressed by every plant. A plant can defend itself from a pathogen because it is not a host for that pathogen. The reason is genetic makeup of that plant is different from the host plants. Thus, the interaction between plant and pathogen is unsuccessful (Agrios, 2005). Preformed or preexisting defenses are major components in this type of resistance (Heath, 2000). For example, destructive oomycete pathogen *Phytophthora infestans* do not cause disease and macroscopic symptoms in crucifer *Arabidopsis thaliana* because the interaction between them was non-host pathosystem. *A. thaliana* is not the host for *P. infestans*. However, localized hypersensitive response was observed in *A. thaliana* epidermal cells. Infections were observed in tomato after treated with *P. infestans* because tomato is the host for this pathogen (Huitema *et al.*, 2003; Kamoun, 2001). Other examples are non-host resistance of parsley and *Nicotiana* to *Phytophthora* spp. (Kamoun, 2001).

2.2.2 Race-non specific resistance

This type of resistance is also known as partial, quantitative or horizontal resistance. After attacked by certain pathogen, a plant may develop no disease, mild disease or severe disease. In other words, it is exhibited in varying degrees, from minimal to maximal each with a small contribution to the resistance. This is because the resistance depends on the specific genetic makeup of the plant itself and also the pathogen. Many minor genes are involved in protecting the plant from pathogens. Presence or formations of preexisting or induced defense structures depend on these genes. Thus, there will be different level of resistance against pathogen attacks (Agrios, 2005; Sadasivam & Thayumanavan, 2003).

2.2.3 Gene-for-gene interaction

It is also known as qualitative or horizontal resistance. In this type of resistance, defense of a host plant against its pathogens is through the presence of matching pairs of genes for disease between the host plant and the pathogens. Host resistance is specific and determined by gene-for-gene interaction between a resistance (*R*) gene in host and avirulence (*avr*) gene in pathogen. It is initiated by highly molecular recognition between these 2 genes. Recognition is accomplished by the detection of elicitors that originate from pathogen or degradation from plant cell walls. Race-specific elicitors are encoded by the pathogen *avr* gene while specific receptors are encoded by the host *R* gene. Absence of either determinant will lead to the breakdown of resistance. After recognition, signaling events become initiated and trigger some responses such as synthesis of reactive oxygen species, changes in gene transcription, hypersensitive responses and cell death, necrosis and lesions, formation of antimicrobial compounds such as phytoalexins and others. For examples, *Rpg1* gene confers resistance of barley against *Puccinia graminis* pv. *triticae*. *RRS-1R* gene of



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