QUANTIFICATION OF RISHITIN IN TOMATO LEAVES AND STEMS AFTER ELICITED BY BIOTIC AND ABIOTIC ELICITORS

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DECLARATION

I declare that this writing is my own work except for citations and summaries where the sources of every each have been duly acknowledged.

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ABSTRACT

Levels of rishitin, a stress metabolite of tomato plant (*Lycopersicon esculentum* Mill.), were monitored in the leaves and stems of nine weeks old plant which were elicited with 1% silver nitrate and *Xanthomonas campestris* pv. *oryzae*. Extraction using 95% ethanol and 100% chloroform were followed by isolation via thin layer chromatography method. Quantification of rishitin in each plant part after each treatment was carried out using spectrophotometer. The experiment on leaves and stems of tomato plants treated with silver nitrate and *Xanthomonas campestris* pv. *oryzae.* produced an insignificant result for all possible combinations of elicitors and plant parts elicited except for the elicitation on tomato leaves. Two independent samples Mann-Whitney test illustrated that both plant parts and both elicitors are independent and not influenced by each other. Thus, study regarding the relationship between different plant parts and their impact on rishitin production under different treatments is crucial in order to improve plant defense system in the near future.

KUANTIFIKASI RISHITIN DALAM TOMATO SETELAH DIRANGSANG DENGAN PERANGSANG BIOTIK DAN ABIOTIK

ABSTRAK

Kuantiti rishitin, iaitu sejenis metabolit tekanan dalam pokok tomato (*Lycopersicon esculentum* Mill.), dikaji pada bahagian daun dan batang tumbuhan yang berumur sembilan minggu. Pokok tomato diberi rawatan 1% argentum nitrat dan *Xanthomonas campestris* pv. *oryzae*. Pengekstrakan menggunakan 95% etanol dan 100% kloroform diikuti dengan pengasingan melalui kaedah kromatografi lapisan nipis. Kuantifikasi rishitin pada setiap bahagian pokok setelah setiap rawatan dicapai menggunakan spektrofotometer. Eksperimen ke atas daun dan batang tomato yang dirawat dengan argentun nitrat dan *Xanthomonas campestris* pv. *oryzae.* menghasilkan keputusan yang tidak signifikan bagi kesemua gabungan yang mungkin pada jenis perangsang dan bahagian tumbuhan dirawat kecuali rangsangan pada daun tomato. Ujian dua sampel bebas Mann-Whitney menunjukkan bahawa kedua-dua bahagian tumbuhan dan perangsang adalah saling tidak bergantungan dan tidak mempengaruhi satu sama lain. Oleh yang demikian, kajian berkenaan hubungan antara bahagian tumbuhan yang berlainan serta impak ke atas penghasilan rishitin pada rawatan yang berbeza amat penting untuk meningkatkan sistem pertahan tumbuhan pada masa depan.

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g	gram
μ	micro
°C	degree Celsius
%	percentage
cm	centimeter
mL	milliliter
nm	nanometer
λ	wavelength
<	less than
>	greater than

CHAPTER 1

INTRODUCTION

1.1 Background

Plant is the largest and most important group of autotrophic life-forms on earth either in cultivated or wild form (Andrews & Tommerup, 1995). Thousands of diseases can affect these plants while each kind of crop plant can be affected by more than hundred plant diseases. Plant disease is actually a series of invisible and visible responses of plant cells and tissues to a pathogenic organism or environmental factor (Agrios, 2005).

Most plants exhibit natural resistance to microbial attack, called non-host resistance due to either the inability of parasite to recognize and infect a plant or the ability of a plant to activate its defense mechanism. However, a few specialized pathogens have evolved to parasitize a plant host and establish basic compatibility. Thus, they damage and weaken the plant with toxins, inhibit host defense mechanism, escape recognition and avoid induction of host defense mechanism. Consequently, the host plant will show severe disease symptoms especially when agriculturally and economically important crop plants are affected (Andrews & Tommerup, 1995).

In plant-pathogen interactions, plant defense reactions comprise signals generation, structural barriers, hypersensitive cell death and formation of pathogen growth inhibitors (Andrews & Tommerup, 1995). Disease resistance in plants often takes the form of a hypersensitive reaction (HR), in which the pathogen remains confined to necrotic lesions near the site of infection (Van Loon, 1997). Phytoalexins are one of the inhibitors synthesized besides pathogenesis-related (PR) proteins, 1,3-β glucanase, chitinase, thaumatine-like proteins, thionins, proteinase and polygalactorunase (Andrews & Tommerup, 1995).

Phytoalexins are compounds which ward off pathogens and produced by plants following infection. Phytoalexins from potato (rishitin), *Arabidopsis* (camalexin) and pea plant (pisatin) have been extensively studied before. However, in this experiment, rishitin from tomato plant will be studied instead (Woolhouse, 1979). Plant tissue culture was also used to study the production of phytoalexins by plants cells. Since the phytoalexins are secondary products, their production stimulus provides a system for examining their regulation in plant tissue cultures. Synthesis of phytoalexins in treated cells is quite remarkable. If the regulation of secondary pathway is understood, therefore it should be possible to increase the amount of commercially interesting compounds formed from similar secondary pathways in other species (Collin, 1987).

There are two types of tomato diseases; parasitic and non-parasitic. Parasitic diseases are the common ones caused by living organisms like bacteria, fungi and viruses. Whereas non-parasitic diseases are caused by unfavourable environmental conditions pertaining moisture, temperature and mineral elements (Gould, 1983).

Some of the more common diseases of tomatoes are fusarium wilt, early blight, anthracnose, fruit rot, gray leaf spot and late blight which are all caused by fungi. However, there are a number of bacterial diseases of tomatoes that also need strong considerations such as bacterial spot, bacterial wilt and bacterial canker (Gould, 1983).

The major obstacle in this research is the lack of literature concerning phytoalexins in tomato plant. Most of the previous researches were done on rishitin in potatoes since the compound is among the major phytoalexins synthesized in potatoes. Since there has not been much research done on rishitin elicitation in tomato, therefore it is high time to carry out this research thus contribute to the world of agriculture.

1.2 Objective

The objective of this study is to quantify rishitin accumulation in tomato leaves and stems after elicited by abiotic ($AgNO_3$) and biotic (*Xanthomonas campestris* pv. *oryzae*) elicitors.

CHAPTER 2

LITERATURE REVIEW

2.1 Tomato

2.1.1 Background / Origins

The tropical family of Solanaceae which is also known as the nightshade family consists of 75 genera and 2000 species. Lycopersicon is amongst the important vegetable genera besides Solanum and Capsicum (Rubatzky & Yamaguchi, 1997). Botanically, the tomato (*Lycopersicum esculentum* Mill.) is classified as a fruit. However, it is generally regarded as a vegetable since the Supreme Court of USA has pronounced tomato as one in 1893. This is because the tomatoes are used with other vegetables for composite dishes and condiments. (Goose & Binsted, 1973).

The tomato originated from Mexico, Central, South America and the Galapagos Islands (Goose & Binsted, 1973; Jones, 1999). The Vera Cruz and Puebla of Mexico are centers of domestication. Then it was cultivated in the narrow, dry tropical, coastal areas of Peru, Ecuador, Bolivia and northern Chile. Consequently, it spread to European countries like England, Spain, France, central Europe and tropical America (Goose & Binsted, 1973; Rubatzky & Yamaguchi, 1997).

It is believed that the name "tomato" comes from the Nahuatl language of Mexico. In France, it is called "pomme d'amour" or love apple (Rubatzky & Yamaguchi, 1997). In 1554, Italy reported the tomato's name as Moor's Apple, Mala Aurea, or Pomi d'oro which means golden apple due to its colour then, bright yellow (Goose & Binsted, 1973; Jones, 1999). From 16th to early 20th century the tomato was used for decoration and even believed to be a poisonous Solanaceous species like belladonna and mandrake (Goose & Binsted, 1973; Rubatzky & Yamaguchi, 1997).

In the meantime, the tomato's colour changed to bright red though there is still a variety with a yellowish tinge. The tomato was started to be used as edible food around 1750 in the U.S.A., whereby Thomas Jefferson (1781) being amongst those who grew it. Whereas Sir Edward Sabine (b.1788); an astronomer and geodentist, issued the first instructions for tomato cultivation in England (Goose & Binsted, 1973). George Washington Carver grew and introduced tomato into the diet among the poor in Alabama (Jones, 1999).

2.1.2 Taxonomy

Two cultivated *Lycopersicon* species are red, smooth and self-pollinated which belong to the subgenus *Eulycopersicon*. Whereas the wild species are green, pubescent and cross-pollinated. They belong to the *Eriopersicon* subgenus. Useful characteristics of the wild species are transferred to improve the cultivated species (Rubatzky & Yamaguchi, 1997).

At first, the tomato was placed in the genus *Solanum* along with potato and identified as *Solanum lycopersicon*. Then, this designation was changed to *Lycopersicon esculentum*. *Lycopersicon* means "wolf peach" and *esculentum* means "edible" in Greek words (Jones, 1999).

2.2 Plant Defense Mechanism

2.2.1 General Introduction

Plants have evolved a large variety of sophisticated defence mechanisms to resist the colonization by microbial pathogens and parasites. These can be divided into three major categories (Kombrink & Schmelzer, 2001):

i) immediate, early defence responses of the directly invaded plant cells, starting with signal recognition and transduction and frequently leading to rapid cell death called hypersensitive response (HR)

ii) local gene activation in the close vicinity of infection sites, resulting in the *de novo* synthesis of numerous secondary products, including phytoalexins, in the reinforcement of structural barriers, such as the cell wall, or in indirect inhibition of the pathogen

iii) systemic activation of genes encoding pathogenesis-related (PR) proteins, including chitinases and 1,3-ß-glucanases, which are directly or indirectly inhibitory towards pathogens and have been associated with the phenomenon of systemic acquired resistance (SAR).

Putative defense compounds or systems for disease resistance in plants (Kuć, 2001):

i) Passive and/or wound responses:

Waxes, cutin, phenolic glycosides, phenols, quinones, steroid glycoalkaloids, suberin, terpenoids and proteins (thionins)

ii) Increases after infection:

Phytoalexins, reactive oxygen species/free radicals, calcium, silicon/silicates, polyphenoloxidases, peroxidases, phenolic cross-linked cell wall polymers, hydroxyproline and glycine-rich glycoproteins, thionins, antimicrobial proteins and peptides, chitinases, β-1,3-glucanases, ribonucleases, proteases, callose, lignin, lipoxygenases and phospholipases

2.2.2 Hypersensitive Response

The hypersensitive response (HR) was first identified in 1915 and since then has been observed as a general feature in numerous plant-pathogen interactions (Kombrink & Schmelzer, 2001). The HR's resistance is expressed only as a result of the specific recognition between plant and pathogen (Van Loon, 1997). It is defined as a rapid, localized necrosis of cells at the infection site and it occurs in resistant plants in response to pathogenic viruses, bacteria, fungi or nematodes. It has been suggested that the HR is a form of programmed cell death (PCD) in plants (Kombrink & Schmelzer, 2001).

2.2.3 Induced Resistance

a. Nature of Induced Resistance

Resistance is the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor. Induced resistance is the phenomenon that a plant, once appropriately stimulated, exhibits an enhanced resistance upon 'challenge' inoculation with a pathogen (Van Loon, 1997).

Induction of disease resistance in plants by necrotizing pathogens is a general phenomenon, and that the induced resistance is non-specific with respect to both the inducing and the challenging pathogen. Thus, a primary infection of cucumber with the fungus *Colletotrichum lagenarium*or with tobacco necrosis virus (TNV) led to enhanced resistance against fungi, bacteria and viruses causing various foliar and root diseases. In all cases symptom expression due to the challenging pathogen was substantially reduced, sometimes to the extent that infection was hardly apparent. These observations indicate that induced resistance constitutes a mechanism through which the level of general resistance to pathogens is increased. That this enhanced resistance depends on extant mechanisms is illustrated by examples showing their increased expression upon challenge inoculation. (Van Loon, 1997).

b. Terminology

The term 'induced resistance' has been used synonymously with 'acquired resistance', 'acquired immunity' and 'immunization'. The term immunization is misleading because plants neither possess a circulatory system, nor immune surveillance; the mechanisms must be entirely different. Induced resistance is nonspecific. Induced disease resistance has been adopted as a general term and defined as 'the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (inducing agents) (Van Loon, 1997).

The term 'induced resistance' seem to imply that resistance was absent, but became present as a result of the action of an inducing agent. In fact, induced resistance is dependent on extant resistance mechanisms and, thus, resistance must be operative to begin with. Resistance to primary infection can result from the presence of preformed defensive barriers, but often depends on inducible resistance mechanisms, the infecting pathogen triggering defense responses through the release of elicitors which, in turn, lead to the expression of novel anti-pathogenic activities. Induced resistance is the additional capacity for defensive activities resulting from the primary infection, and dependent on the concomitant triggering of resistance responses. Once a plant has been stimulated in this way, it can express this enhanced defensive capacity irrespective of whether the challenging pathogen gives rise to an incompatible or to a compatible interaction (Van Loon, 1997).

2.3 Phytoalexins

2.3.1 Definition

The term phytoalexin originated from Greek whereby phyton means plant and alexin means warding off compound. In 1940, phytoalexins originally was defined as a chemical compound produced by living host cells only when these are invaded by a parasite and consequently necrosis occur. Then in 1956, it was redefined as antibiotics that are the result of interaction of two different metabolic systems, host and parasite thus inhibit the growth of microorganisms pathogenic to plant (Purkayastha, 1995).

Phytoalexins are low molecular weight antifungal and antimicrobial compounds that inhibits the development of a microbe on hypersensitive tissue formed when host plant cell come in contact with the parasite or in response to cellular injury, infection and metabolic stress (Agrios, 2005; Allaby, 2004; Kuć, 1972). They function in plant's multicomponent response mechanism for disease resistance. Their accumulation speed and magnitude is crucial to determine disease resistance or susceptibility to fungal and bacterial diseases (Kuć, 1972).

2.3.2 Characteristics

In 1963, a few basic postulates of the phytoalexins theory were formed following an experiment on potato:

i) phytoalexin inhibits the fungus growth in the hypersensitive tissue only when the parasite comes in contact with the host cells

ii) defensive reaction takes place only in living cells

iii) inhibitory substance may be regarded as the product of necrobiosis of host cells

iv) phytoalexin is nonspecific in its toxicity towards fungi

v) basic response of resistant and susceptible plants is similar but the speed of phytoalexin formation differs

vi) defense reaction is restricted to the tissue colonized by fungus and its immediate neighbourhood

vii) resistant state is acquired, not inherited

viii) speed of host reaction is determined by host cell's sensitivity (Kuć, 1990).

In situ localization and quantification proved that phytoalexins accumulates at the right time, concentration and location for effective resistance. However, there were studies showed that phytoalexins tolerant to virulent fungi will be detoxified by the latter (Hammerschmidt & Dann, 1999). Detoxification of phytoalexins by fungi may have crucial consequences for the practical application of these defense compounds and for the genetic transformation of fungi and plants. Phytoalexins accumulate in plants or cell cultures only transiently, because they are readily degraded or polymerized by extracellular peroxidases (Barz *et al.*, 1990).

The phytoalexins role can be evaluated using mutant deficient in its synthesis and elucidating biosynthetic pathways (Hammerschmidt & Dann, 1999). But some isoflavonoid compounds in legumes which classically inhibit pathogens, also serve as chemoattractants, promoters of microbial growth and inducers of nodulation genes in *Rhizobium* and *Bradyrhizobium* bacteria. These newly found biological functions of

isoflavonoids clearly complicate the traditional definitions of phytoalexins and phytoanticipins (Dakora & Phillips, 1996).

Phytoalexins are accumulated along with lignification, suberization, callose formation and synthesis of agglutinins and inhibitors of extracellular microbial hydrolases during multi-component response mechanism for disease resistance and wound repair. Phytoalexins have low specificity for induction and also for its activity (Kuć, 1984).

Disease resistance in plants includes these stages: synthesis of phytoalexins, systematically produced de novo enzymes (chitinases, beta-1,3-glucanases, proteases), which generate antimicrobial compounds and protective biopolymers (peroxidases, phenoloxidases), biopolymers which restrict the spread of pathogens (hydroxyproline rich glycoproteins, lignin, callose), and compounds that regulate the induction and/or activity of the defense compounds (elicitors of plant and microbial origin), immunity signals from immunized plants and compounds releasing immunity signals (Heil & Bostock, 2002; Kuć, 1990).

Phytoanticipins are preformed antimicrobial compounds which are found in healthy plants that may represent in-built chemical barriers to infection by potential pathogens (Osbourn, 1999)

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