BIOACTIVITY OF THE ROOT EXTRACT FROM *Semiaquilegia adoxoides* (DC.) MAKINO AGAINST TOBACCO MOSAIC VIRUS (TMV)

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ฤทธิ์ของสารสกัดจากราก *Semiaquiegia adoxoides* ต่อเชื้อ
tobacco mosaic virus (TMV)

นายหลี่ หมิง

วิทยานิพนธ์นี้สำหรับการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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หลี่ หมิง : ฤทธิ์ของสารสกัดจากราก *Semiaquiegia adoxoides* ต่อเชื้อ tobacco mosaic virus (TMV) (BIOACTIVITY OF THE ROOT EXTRACT FROM *Semiaquiegia adoxoides* (DC.) MAKINO AGAINST TOBACCO MOSAIC VIRUS (TMV) อาจารย์ที่ปรึกษา : อาจารย์ ดร.โสภณ วงศ์แก้ว, 194 หน้า.

การศึกษามีวัตถุประสงค์เพื่อพัฒนาวิธีการสกัดและแยกองค์ประกอบทางเคมีจากรากของ *Semiaquiegia adoxoides* ระบุชนิดและศึกษาฤทธิ์ของสารสกัดต่อเชื้อ tobacco mosaic virus (TMV) และพัฒนาสูตรสารควบคุมสมุนไวรัสจากสารสกัด นำรากของ *S. adoxoides* มาสกัดด้วยวิธี Systematic และ nonsystematic โดยใช้ปิโตรเลียมอีเธอร์ (PE) คลอโรฟอร์ม (C) เอทิลอะซิเตต (EA) อะซิโตน (A) บิวทานอล (B) เอธานอล (E) เมธานอล (M) และน้ำเป็นตัวทำละลาย จากนั้นนำสารสกัดที่ได้ไปวิเคราะห์เชิงคุณภาพและทดสอบฤทธิ์ในการยับยั้งเชื้อ TMV โดยใช้ half leaf technique กับยาสูบใบเล็ก (*Nicotiana glutinosa*) ทั้งในสภาพใบตัด (in vitro) และสภาพพืชทั้งต้น (in vivo) เปรียบเทียบกับฤทธิ์ของสาร motoxydine hydrochloride copper acetate (Virus A) ซึ่งใช้ควบคุมโรคไวรัสที่ผลิตเป็นการค้า ผลการทดลองพบว่า วิธี systematic ให้ผลผลิตสารสกัด 38.76% ขณะที่วิธี nonsystematic ให้ผลผลิต 35.15% สารสกัดที่ได้จากการสกัดครั้งแรกด้วยเอธานอลในวิธี nonsystematic ประกอบไปด้วย แซคาไรด์ กรดอินทรีย์ แอนธราคิวิโนน ฟีนอล น้ำมันระเหย และอาจมีส่วนประกอบของอัลคาลอยด์ ฟลาโวนอย คูมาริน กรดอะมิโน แทนนิน สเตรอยด์ ไตรเตอร์พีน แต่ไม่พบโปตีนเปบไทด์ หรือลิปิด สารสกัด PE ประกอบด้วย อัคคาลอยด์ ขณะที่สารสกัด EA และ C ประกอบด้วยฟลาโวน ผลการวิเคราะห์สารสกัด PE ด้วยวิธี gas chromatography – mass spectrometry (GC-MS) พบสาร 31 ชนิด ซึ่งสามารถระบุชนิดได้ 12 ชนิดในระดับโครงสร้าง โดยส่วนประกอบที่เป็นน้ำมันระเหยมีส่วนผสมของ β-sitosterol 38.26% กรดลิโนเลอิค 18.73% กรดโอเลอิค 15.68% กรดปาล์มมิติล 13.5% และกรดสเตียริค 2.44% ผลการทดลองในสภาพใบตัดพบว่า สารสกัด PE A และ E สามารถทำให้ TMV เสื่อมสภาพได้สูงสุดเมื่อเทียบกับสารสกัดชนิดที่เหลือ คือ 68.09% 63.28% และ 60.59% ตามลำดับ และสารสกัด PE ยังออกฤทธิ์ป้องกัน (protective) ได้สูงสุดคือ 62.26% ในทั้งสองกรณีการออกฤทธิ์ของสารสกัดสูงกว่าการออกฤทธิ์ของสารควบคุม Virus A การทดลองในสภาพทั้งต้นพบว่า ฤทธิ์ในการควบคุม TMV ของสารสกัด C EA และ PE เท่ากับ 42.04% 41.83% และ 33.14% ตามลำดับ ขณะที่ฤทธิ์ในการรักษาของสารสกัด EA PE และ C เท่ากับ 72.36% 50.26% และ 47.01% ตามลำดับ ในทั้งสองกรณีการออกฤทธิ์ของสารสกัดสูงกว่าของ Virus A ผลการทดสอบด้วยวิธี direct antigen coating indirect ELISA พบว่า การเพิ่มปริมาณของเชื้อ TMV ซึ่งประเมินจากค่า A 492 ช้าลงเมื่อยาสูบได้รับสารสกัด PE C EA B และ Virus A เมื่อเทียบกับยาสูบที่ไม่ได้รับสาร (ตำรับควบคุม) ค่า A 492 ที่วัดได้จากยาสูบที่ได้รับสารสกัด

มีค่าสูงสุดหลังจากได้รับ TMV 5 วัน ขณะที่ยาสูบให้ค่าสูงสุดใน 3 วัน ค่า A 492 ที่วัดได้จากยาสูบที่ได้รับสารสกัด C มีค่าต่ำสุด ตามด้วยสารสกัด PE EA และ Virus A ในการทดสอบฤทธิ์ของการคุ้มครอง สำหรับฤทธิ์ในการรักษา ค่า A 492 เริ่มลดลงใน 7 วัน และหลังจากยาสูบได้รับสารสกัดโดยสารสกัด EA ให้ค่าต่ำสุดคือ 0.128 ตามด้วย Virus A PE และ C ตามลำดับ ระยะเวลาในการป้องกันของสารละลาย PE C EA B และ Virus A เท่ากับ 18 13 13 8 และ 8 วัน ตามลำดับ การแยกสารด้วยวิธี column chromatograph จากสารสกัด C และ EA ซึ่งออกฤทธิ์ในการยับยั้งเชื้อ TMV ได้สูงสุดได้ ส่วน B 4-3 เมื่อล้าง (elute) คอลัมน์ด้วยคลอโรฟอร์ม-เมธานอล สัดส่วน 83 : 17 (v : v) และ C 5-5 เมื่อใช้คลอโรฟอร์ม-เมธานอล สัดส่วน 75 : 25 (v : v) ส่วน B 4-3 มีฤทธิ์ป้องกัน และรักษาการติดเชื้อ TMV 52.54% และ 48.69% ตามลำดับ ขณะที่ส่วน C 5-5 มีฤทธิ์ยับยั้ง TMV 42.98% และ 62.45% ตามลำดับ การศึกษาการเปลี่ยนแปลงของเอนไซม์ในยาสูบที่ปลูกเชื้อ TMV พบว่า เมื่อยาสูบได้รับสารสกัดก่อนได้รับเชื้อไวรัส เอนไซม์คาตาเลส (CAT) เปอร์ออกซิเดส (POD) และซุปเปอร์ออกไซด์ดิสมิวเตส (SOD) มีกิจกรรมลดลงเมื่อเทียบกับยาสูบที่ไม่ได้รับสารสกัด เวลาที่กิจกรรมของเอนไซม์ทั้งสามชนิดมีค่าสูงสุด ช้าลงกว่าเวลาของกิจกรรมในยาสูบที่ไม่ได้รับสารสกัด 2 วันซึ่งสอดคล้องกับผลการวัดการเพิ่มปริมาณของ TMV โดยวิธี ELISA กิจกรรมของเอนไซม์ในยาสูบลดลงมากที่สุดเมื่อได้รับสารสกัด PE Virus A และ EA ตามลำดับ เมื่อยาสูบได้รับเชื้อ TMV ก่อนได้รับสารสกัด กิจกรรมของเอนไซม์เปลี่ยนแปลงในลักษณะเดียวกันกับการได้รับเชื้อ TMV หลังได้รับสารสกัด แต่ระดับที่ลดน้อยกว่าโดยยาสูบที่ได้รับ Virus A กิจกรรมลดลงมากที่สุด ตามด้วยสารสกัด EA PE และ C ตามลำดับ ผลการศึกษาการยับยั้ง TMV ในสภาพใบตัด พบว่า กรดสเตียริค และ β-sitosterol ที่แยกได้จากสารสกัด PE เข้มข้น 10 มก./มล. สามารถทำให้เชื้อ TMV เสื่อมสภาพ 56.86% และ 50.73% ตามลำดับ กรดสเตียริคสามารถป้องกันการเข้าทำลายจากเชื้อ TMV ได้ 57.91% ขณะที่กรดโอเลอิคมีผลในการรักษา 37.10% ซึ่งสูงกว่าฤทธิ์ของ Virus A ในทั้งสองกรณี ผลของการศึกษาในสภาพทั้งต้นเหมือนกันกับการศึกษาในสภาพใบตัด แต่ต่างกันที่ระดับของความสามารถในการยับยั้ง ระยะเวลาที่สัมผัสเชื้อ และความเข้มข้นของสารที่มีผลต่อความสามารถในการยับยั้งเชื้อ TMV โดยที่การเพิ่มระยะเวลาและความเข้มข้นช่วยให้ความสามารถในการยับยั้งเชื้อสูงขึ้น การประเมินสูตร emulsion ในน้ำ (EW) ได้คัดเลือกสูตร TK#2 ซึ่งมีส่วนผสม C 5-5 20% dimethyl sulfoxide 15% OX-7513 emulsifier 10% carboxymethyl cellulose sodium 3% propylene glycol antifreeze 5% และน้ำ 47% มาใช้ทดสอบประสิทธิภาพควบคุมโรคจากเชื้อ TMV ในยาสูบ ทั้งในสภาพเรือนทดลองและสภาพไร่นา สูตรดังกล่าวสามารถทำให้เชื้อ TMV เสื่อมสภาพ ป้องกันการติดเชื้อ และรักษาอาการจากเชื้อ TMV ได้ 68.64% 62.05% และ 64.28% ตามลำดับ เมื่อทดสอบกับยาสูบ *N. glutinosa* การทดสอบกับ *N. tabacum* พันธุ์ K326 ในสภาพไร่นาที่ระดับเจือจาง 100 และ 200 เท่า พบว่าสามารถลดการเกิดโรคจาก

TMV ได้ 67.86% และ 65.16% เมื่อเทียบกับแปลงเปรียบเทียบ การวิเคราะห์ค่าสหสัมพันธ์ระหว่าง logarithm concentration กับ infection index ได้ค่าสมการ regression y = 1.0642x+1.9218 (r = 0.9908*) และ ค่า ED 50 เท่ากับ 724.44 มก./ล.

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา________________________

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา__________________

LI MING : BIOACTIVITY OF THE ROOT EXTRACT FROM *Semiaquilegia adoxoides* (DC.) MAKINO AGAINST TOBACCO MOSAIC VIRUS (TMV). THESIS ADVISOR : SOPONE WONGKAEW Ph.D., 194 PP.

Semiaquilegia adoxoides / ROOT EXTRACTS / ANTI-TMV BIOACTIVITY

The study was conducted to develop methods for extracting and separating chemical components from *Semiaquilegia adoxoides* (DC.) Makino root, to identify and study bioactivity of the extracted compounds against tobacco mosaic virus (TMV), and to develop an antiviral formulation from the extract. Roots of *S. adoxoides* were extracted by systematic and non-systematic methods using petroleum ether (PE), chloroform (C), ethyl acetate (EA), acetone (A), butanol (B), ethanol (E), methanol (M) and water (W) as solvents. Subsequently, the resulting extracts were qualitatively analysed and tested for bioactivity on TMV both in vitro and in vivo using a half leaf technique having *Nicotiana glutinosa* as a test plant comparing with that of moroxydine hydrochloride copper acetate (Virus A), a commercial antiviral agent. Results of the experiments showed that the systematic extraction method yielded 38.76% of total extracts while the nonsystematic methods yielded 35.15%. The crude ethanol extract of the nonsystematic method contained saccharide, organic acids, anthraqurinone, phenols, volatile oils and may contain alkaloids, flavonoids, coumarin, amino acids, tannin, steroids, triterpenes but not proteins, peptides, or lipids. The PE extract contained alkaloids while the EA and C extracts contained flavones. Results of the gas chromatography-mass spectrometry (GC-MS) analysis revealed 31 different substances in the PE extract in which 12 substances were identified at a structural level. The volatile oils in the extract composed of 38.26% β-sitosterol, 18.73% linoleic acid, 15.68% oleic acid, 13.51% palmitic acid and 2.44%

stearic acid. The in vitro bioactivity test using detached leaves showed that among the seven extracts, PE, A and E extracts had higher inactivation activity than that of the rest. The inactivation percentages were 68.09%, 63.28%, and 60.59% respectively. The PE extract also had the highest inhibition rate of 62.26% for protective effects. In both cases, the root extracts had significantly higher efficacy than that of the Virus A control. In the in vivo pot experiment, protection rates of C, EA and PE extracts were 42.04%, 41.83% and 37.14% respectively. EA, PE and C extracts also had high curing effects of 72.36%, 50.26%, and 47.01% respectively, which was significantly higher than that of Virus A. Results of the direct antigen coating (DAC) indirect ELISA showed that after treatment with the PE, C, EA, B extracts, and Virus A, TMV replication measured as A_{492} was delayed compared to that of the control. The maximum A_{492} values of the extract treated tobacco were reached in 5 days while that of the control in 3 days. A_{492} of C extract treated tobacco was the lowest followed by that of the PE, EA extracts and virus A in the protective experiment. For curing activities, the A_{492} started to decline, 7 days after the extract treatment whereas EA extract had the lowest A_{492} value of 0.128 followed by Virus A, PE, and C extracts. The protective effect lasted 18, 13, 13, 8, and 8 days when tobacco was treated with PE, C, EA, B extracts and Virus A respectively. The column chromatograph of the C and EA extracts which showed the highest anti-TMV activity yielded B4-3 fraction when eluted with chloroform-methanol at 83 : 17 (V : V) ratio and C5-5 fraction when eluted with chloroform-methanol at 75 : 25 (V : V) ratio. With TMV, the B4-3 fraction had protective and curing effects of 52.54% and 48.69% while these of the C5-5 fraction were 42.98% and 62.45% respectively. Results of monitoring enzymatic changes in TMV infected leaf tissue indicated that when the extracts were applied

prior to TMV inoculation, catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities were reduced compared to those of the control non-treated tobacco. The peak activities of the three enzymes were delayed for 2 days corresponding to the result of ELISA. Enzyme activities in the treated tobacco were reduced in the order of PE extract > Virus A > EA extract. When TMV was inoculated prior to the extract treatment, changes in enzyme activities were similar to that of the protective experiment except the decrease was not as high and the order of reduction was Virus A > EA > PE > C extracts. Results of anti-TMV assay in vitro of substances separated from the PE extract indicated that stearic acid and β-sitosterol at 10 mg/ml could inactivate 56.86% and 50.73% of TMV, respectively. The protective inhibition rate of stearic acid on TMV was 57.91% while the curing inhibition rate of oleic acid was 37.10%. Their control effects were significantly higher than that of Virus A. Results of the in vivo experiment were similar to these of the in vitro, but with different levels of inhibition. Both exposure time and concentration of the inhibitors had effects on an inhibition rate which increased when the two factors increased. After a series of emulsion in water (EW), formulations had been evaluated, the TK#2 containing 20% of C5-5 fraction, 15% of dimethyl sulfoxide solvent, 10% of OX-7513 emulsifier, 3% of carboxymethyl cellulose sodium thickener, 5% of ethylene glycol antifreezer and 47% of water was selected for controlling TMV in tobacco under both greenhouse and field conditions. On *N. glutinosa*, the TK#2 had inactivation, protection and curing percentages of 68.64%, 62.05% and 64.28% respectively. On *N. tabacum* K326 under field condition, TK#2 diluted 100X and 200X could reduce TMV infection 67.86% and 65.16% compared to that of the control plot. The correlation analysis between logarithm concentration

and infection index showed that regression equation was Y=1.0642X+1.9218 ($r = 0.9908^*$) and ED50 was 724.44 mg/l.

School of Crop Production Technology Student's Signature______________________

Academic Year 2008 Advisor's Signature______________________

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LIST OF ABBREVIATIONS

A260	=	Value of absorbance at 260nm
A492	=	Value of absorbance at 492nm
AOX	=	Alterative oxidase
AVF	=	Antiviral factor
BBMV	=	Broad bean mottle virus
CAT	=	Catalase
CMC	=	Carboxymethyl cellulose sodium
CMV	=	Cucumber mosaic virus
CP	=	Capsid protein
CPMV	=	Cowpea mosaic virus
DAC	=	direct antigen coating
DHT	=	2,4-dioxohexahydro-1,3,5-triune
DMSO	=	Dimethyl sulfoxide
EC	=	Emulsion concentrate
ELISA	=	Enzyme-linked immunosorbent assay
EW	=	Emulsion in water
fg	=	femtogram
GA	=	Giberellic acid
GPX	=	Cellular Glutathione Peroxidase

LIST OF ABBREVIATIONS (Continued)

GR	=	Glutathione Reductase
IVR	=	Inhbitor of virus replication
ME	=	Microemulsion
OD	=	Optical Density
PAP	=	Phytolacca antiviral protein
POD	=	Peroxidase
PPO	=	Polyphenol oxidase
PR-P	=	Pathogenesis-related proteins
PVX	=	Potato virus X
PVY	=	Potato virus Y
ROS	=	Reactive oxygen species
SOD	=	Superoxide Dismutase
TBSV	=	Tomato bushy stunt virus
TLC	=	Thin layer chromatogram
TMV	=	Tobacco mosaic virus
TRV	=	Tobacco rattle virus
TYMV	=	Turnip yellow mosaic virus
SC	=	Suspension concentrate
SE	=	Suspension emulsions
SG	=	Soluble granules

LIST OF ABBREVIATIONS (Continued)

WDG	=	Water dispersible granules
WP	=	Wettable powder

CHAPTER I

INTRODUCTION

1. 1 Background of the selected topic

1.1.1 Chemical pesticides pollution on environment

Nowadays, pesticide pollution has become detrimental to human beings. It has proven that the long-term and massive application of synthetic pesticides resulted in the disasters to human survival environment (Zhang and Zhang, 2001). As a consequence, the ecological equilibrium has been out of balance, the ecological environment worsened, and the resistance of harmful life-form to pesticides increased. The application of pesticides kill natural enemy of pests, leading to the frequent occurrence of the rampant phenomenon of harmful life-form. At present, application of chemicals is main method for tobacco diseases control in China. It is highly effective and plays the important role when diseases and pests are rampant. The problems of “three R”, namely residue, resistance and resurgence caused by the application of chemical pesticides are more and more serious with the increased frequency of application of chemicals. According to the statistics of the Environmental Plan Bureau of the United Nations, there are 2 million people who have the acute poisoning, and approximately 40,000 people are killed every year. In the recent 20 years, the occurrence rate of cancer, liver sickness, and heart angiopathy disease of the human is increasing. It cannot be denied that there is inseparable relationship between these diseases and the long-term use of the chemical pesticides, which caused simultaneously the serious pollution of environment.

1.1.2 Organic farming and green pesticides

(1) Economical loss caused by plant viruses

Plant diseases caused by virus is difficult to prevent and control. It is estimated that the economical loss caused by plant viruses is up to 15 billion dollars every year all over the world. Tobacco viral diseases are one main plant diseases in the tobacco planting industry. The economical loss caused by tobacco mosaic virus (TMV) were more than 100 million dollars every year worldwide (Wu, 1995). In China, the tobacco industry has been also threatened seriously. There has been a decrease in both the output and the quality of tobacco leaves. In recent years, plant viral diseases have become more and more severe due to the increase of global temperature, the warmer winter, the aggravation of arid phenomenon, and the expansion of the counter-season vegetable planting area.

(2) Organic farming

Organic farming is a form of agriculture which avoids or largely excludes the use of synthetic fertilizers and pesticides, plant growth regulators, and livestock feed additives. As far as possible organic farmers rely on crop rotation, crop residues, animal manures and mechanical cultivation to maintain soil productivity and tilth, to supply plant nutrients, and to control weeds, insects and other pests (Kuepper and Gegner, 2004). According to the international organic farming organisation IFOAM, the role of organic agriculture, whether in farming, processing, distribution, or consumption, is to sustain and enhance the health of ecosystems and organisms from the smallest in the soil to human beings. Organic farming excludes the use of synthetic inputs, such as synthetic fertilizers, pesticides, herbicides and genetically modified organisms (GMOs). In addition to the exclusion of synthetic agrichemicals,

these include protection of the soil, promotion of biodiversity and outdoor grazing for livestock and poultry. Within this framework, individual farmers develop their own organic production systems, determined by factors such as climate, market conditions, and local agricultural regulations. Organic farming standards do not allow the use of synthetic pesticides, but they do allow the use of certain so-called natural pesticides, such as those derived from plants. The most common organic pesticides, accepted for restricted use by most organic standards, include Bt, pyrethrum, and rotenone. Some organic pesticides, such as rotenone, have high toxicity to fish and aquatic creatures with some toxicity to mammals including humans.

(3) Green pesticides

In the 21st century, the concept of pesticide has a profound change and does not stress certainly “killings”, but rather “regulation of the pests”. It is thought that in the 21st century, pesticides should be called “biorational pesticide” or “environment acceptable pesticide”(or “environment friendly pesticide”) (Li et al., 1993; Xue et al.,1993; Wu et al., 1995; Li et al., 2000). Therefore, in the process of searching for a safe replacer of the chemically synthetic pesticides, plant source pesticides have become alternative because plant source pesticide has the advantage of reducing harmful particle quantity entering into the environment, being safe to human and livestock, decomposing quickly in nature, being not inferior to the chemically synthetic pesticides in the toxicity aspect, and being not easy for the pathogen and pest to have resistance.

It is the best effective way to research and develop new pesticides by seeking bioactive substance from plants. In China, plant resources are very rich, and the plants that may be used as pesticides are also very many. The book, named “the

Chinese poisonous plant", listed more than 1300 poisonous plants species, many of them have the function of killing pests and pathogens. This has established the foundation for the development of plant source pesticides.

1.2 Research objectives of this study

(1) To develop extraction and separation methods for chemical components from *S. adoxoides* root.

(2) To identify the chemical components of the *S. adoxoides* extract having bioactivity against TMV.

(3) To study the bioactivity and mode of action of the extracted compounds against TMV.

(4) To develop a formulation of active antiviral compounds from *S. adoxoides* root extract.

1.3 Significance and design ideas of this study

1.3.1 Significance of this study

Plant virus diseases caused tremendous losses to agricultural production. However, so far, there are still not good control methods for plant virus disease. Tobacco, an economic crop in China, is easily infected by viruses. According to domestic and foreign reports, there are more than 40 viruses isolated from tobacco plants in the field. TMV disease is one of the important disease in tobacco production, which is widespread over the tobacco-growing areas in China. Annual incidence percentage varies from 5.40% to 8.32%, and exceeds 90% in the areas of tobacco seriously infected with TMV. TMV not only caused the loss of tobacco production, but also caused a serious decline in the quality of tobacco leaves. The tobacco leaves,

infected by TMV, its average loss is 4.78% - 58.76% with the disease-class increase. If the tobacco is infected by TMV at growing forepart, its loss of output and quality is over 90%.

Tobacco is an important crop for economic development in many provinces of China. TMV disease has been a problem that plagued tobacco production. Until now, there are no efficient anti-TMV agents at home and abroad. Many virus inhibitors can only reduce the symptom severity to certain degree. Botanical pesticides come from plants. It is safe to the crop and environmentally friendly. It has become a focus in the green agriculture. Therefore, screening anti-viral material from the plant is one of the important ways of developing anti-virus inhibitor. Domestic and foreign scholars have done a lot of work and achieved certain achievements (Chen, 2003).

Thus this study linked the development tendency and goal of modern pesticide research and development, for controlling TMV disease in tobacco. Active components of *S. doxoides* root were developed into a series of emulsion in water (EW) preparations of environment compatibility through using formulation processing of pesticide. This study not only would provide a scientific basis and clues for development and application of safe and efficient antivirus inhibitors, bionic synthesis and structure modification establish a theoretical background for biologically reasonable design and chemical decorating synthesis leading to the development of new specific botanical pesticides of safety, but also fully demonstrate the broad prospects of direct application of control TMV in the production. Thus, it has important economics significance for development and utilization of plant resources.

1.3.2 The design routes of this study

In this study, TMV, causing TMV disease of crops in agricultural production was used as the study object. It is main aim that the anti-TMV bioactivity of *S. adoxoides* extracts and separation of active components of *S. adoxoides* were studied through using the solvent extraction and biological activity tracking method. Finally, research and development of anti-TMV inhibitors of control TMV disease were carried out by modern formulation processing technique. The extraction methods of active components of *S. adoxoides*, the root powders of *S. adoxoides* were extracted with two extraction procedures.

(1) Systematic extraction : *S. adoxoides* root powders were extracted systematically with petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and water.

(2) Non-systematic extraction : After *S. adoxoides* root powders were extracted with 95% ethanol. The resulting ethanol crude extracts was then extracted with petroleum ether, chloroform, ethyl acetate, butanol, and water, respectively. Schematic figure of the technical routes was presented in Fig 1.1

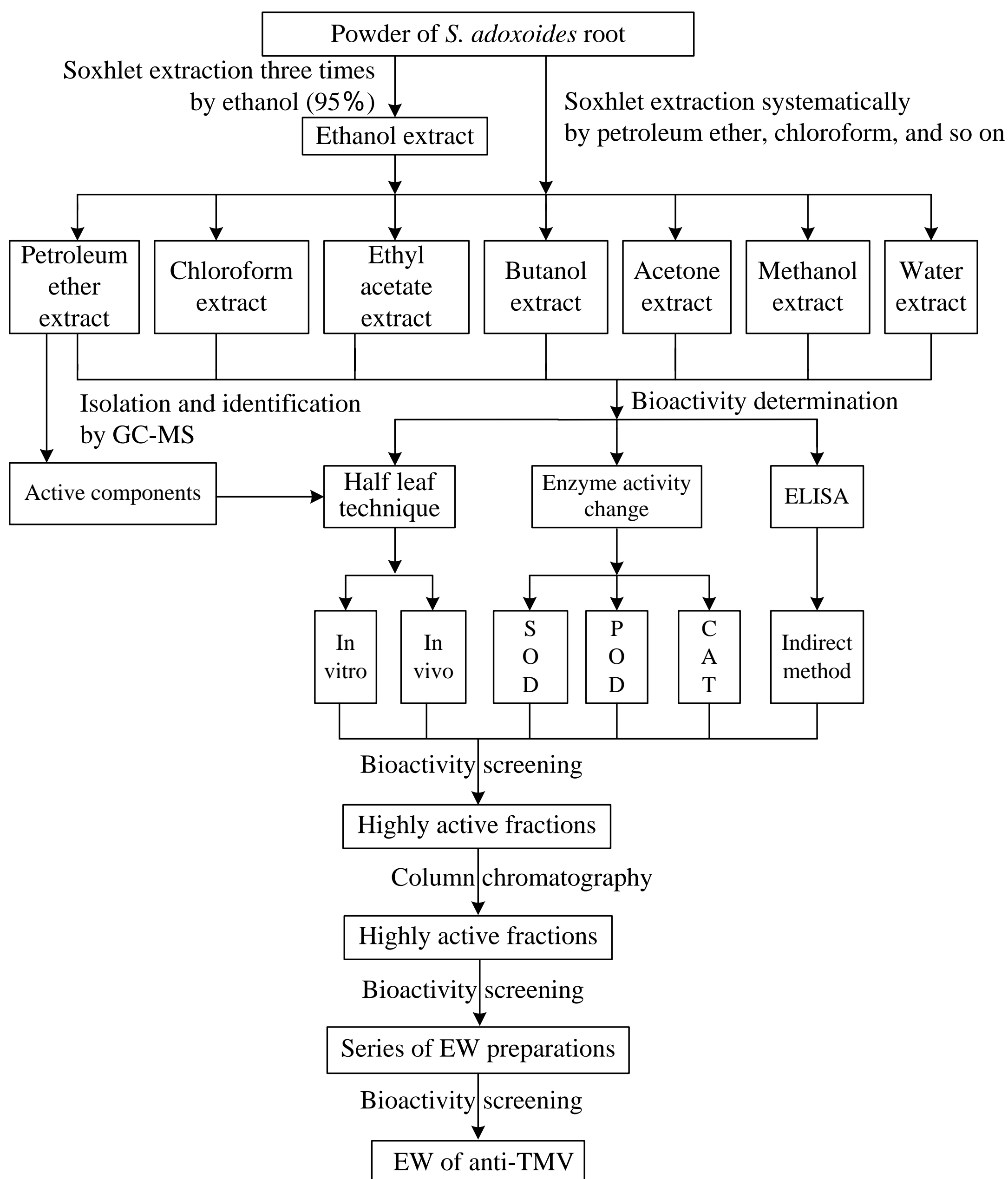

Figure 1.1 Schematic figure of the experimental routes.

1.4 Scope and limitation of the study

1.4.1 *S. adoxoides* in Guiyang area will be used as representative sample for the study.

1.4.2 The method of half-leaf local lesion assay and ELISA will be used to determine bioactivity of *S. adoxoides* extract.

1.4.3 The common TMV from Wuhan Virus Institute of China will be used as representative virus for the study.

1.4.4 Method of tracing bioactivity will be used to extract and separate chemical components from *S. adoxoides*.

1.4.5 Chemical components of the *S. adoxoides* extracts will be measured for bioactivity against TMV.

1.4.6 Only 1 to 3 chemical extracts of *S. adoxoides* that have showed high antiviral bioactivity will be further identified at a structural level.

1.4.7 Only 1 to 3 of such chemicals will be studied for mode of action against TMV.

1.4.8 Only the compound that showed the highest antiviral activities will be used to develop new plant source antiviral agent.

1.4.9 The new plant source antiviral agent will be tested for effectiveness against TMV in the field.

1.5 Expected results of the study

1.5.1 Gain suitable extraction method，technique of tracing and separating the chemical components of *S. adoxoides* roots.

1.5.2 Knowing the bioactivity of chemical components of *S. adoxoides* roots

extract against TMV.

1.5.3 Understanding the mode of action of chemical components of *S. adoxoides* roots extract against TMV.

1.5.4 Attaining the possibility of obtaining chemical components from *S. adoxoides* roots that could be developed into a commercial antiviral agent of TMV

CHAPTER II

REVIEW OF LITERATURES

2.1 Research on tobacco mosaic virus (TMV)

2.1.1 General characteristics

Tobacco mosaic virus (TMV) (Xu, 1997) is a typical member of tobacco mosaic virus (*Tobamovirus*). Mayer recorded the symptoms of TMV in 1886. Ivannovski confirmed that TMV had the filterable and infectious activity in 1892. The research history of TMV represents the history of the development of phytovirology in some degree. The virion is a long and straightly rod-shaped cylinder, 300×18nm in size. Transmission in nature relys on contacts between plants or seed to spread, reflecting its strong resistance to the external environment. The virion molecular weight is 39.4×10^6. The sedimentation coefficient is $S_{20,w}$ = 194 s and the buoyant density is 1.325 g/cm^3 in cesium chloride. The electrophoretic mobility is - 0.83 cm^2/s•V and A_{260}/A_{280} is 1.19. Its nucleic acid is ssRNA with the length of 6.4 kb and molecular quantity of 2×10^6. The molecular weight of protein coat is about 17×10^3 - 18×10^3. The virion is very stable having the thermal inactivation point of 90 ^{O}C/10 min, dilution end point of 1×10^{-6}. The isolated viruses remain infective after several decades.

2.1.2 Economic importance and distribution of TMV

TMV disease is widely distributed world wide. It is the most important tobacco diseases. Three hundred and ten kinds of plants of 30 families can be infected

by TMV. First symptom appears as vein clearing on the new leaves after being infected by TMV. Subsequently, the color around the vein turns light green and semitransparent if direct under the light. The symptoms expand from the leaf base to the leaf apex, and gradually spread to the entire leaf. Such symptoms are often found thus it is called as “common mosaic disease”. The plants with light TMV disease have clear vein in whole leaf or only at leaf tip without the yellow veins. In most cases, the green leaf area alternated with yellow, and shape of the leaf does not change, but in heavy diseased cases the leaf margin always rolls towards the back and the shape is malformed. The flower or fruit are also out of shape. If infected at seedling stage, the tobacco plant will become and remain stunted. Desease caused by TMV directly affects both the quantity and quality of tobacco yield. The disease reduces proportion of the high quality of tobacco leaves, make color of the roasted leaves uneven, contents of nicotine and carbohydrates declined, content of total nitrogen increased and the aroma deteriorated.

In China mosaic disease of tobacco is endemic nationwide. The average incidence is 20% to 30%, but can rise up to 60% to 80%. Thus there is a serious impact on the tobacco quality and quantity which limit the development of the tobacco industry. In recently years, the occurrence and damages from TMV have been increased especially in tobacco area of the northeast, Huanghuai, southeast and southwest of China (Lei, 2004).

2.1.3 Current situation of TMV Control

In order to effectively control virus, domestic and international researchers are always seeking the ways to prevent plant viruses. At present, there has not been a good single measure to control tobacco virus diseases. The guidelines of

controlling tobacco virus disease are “focusing on prevention, controlling in time”. The current mainly control measures in production (Zhang, 2001) are as follows.

(1) Cultural control

The core of cultural control is health care cultivation. A large number of field practices have proved that the plants which grow well have a stronger resistance to various pests and diseases including virus diseases. Except for appropriate conditions of water, fertilizer, temperature and light, agricultural control should focus on cleaning up the residue of crops, eliminating weak sprouts, using clean water, and removing weeds, and disinfection of seedbed. Crops are normally infected with the virus at seedling stage but because of a long latent period after being infected, it is hardly noticed at the seedling stage. Thus, good handling of the seedbed can effectively reduce the incidence and the subsequence spread by vectors. The weeds are the host of many plant viruses, which are easy to become the source of infection. It should be cleared out in time.

(2) Application of plant anti-virus agent

So far, the highly effective agents of plant anti-virus have not been found. Most inhibiting substance, only applied with the virus mixed, to display its role in inhibiting infection. The results have little or no role in practical virus control (Misaghi, 1982; White, 1983). U.S. first used milk to control tobacco mosaic virus. It was reported milk proteins can inhibit the proliferation of TMV, and stop movement of the virus between cells, to avoid causing serious damage.

China has developed several virus inhibitors, such as Zhi-BING-LING, the Virus-BIKE, Golden-LEAF-PO, ningnanmycin, JUN-DU-QING, Ginkgo-LEAF extracts, and so on, these virus inhibitors have varying degrees of anti-virus effect on

tobacco control experiments. The average control effect is about 40%. Judging from the current plant disease prevention and treatment of the actual situation, no resistant varieties, or genetically modified tobacco is available. There are still some problems in addition, the cultivation of current level and scientific and technological level of farmers in China are not all high. The farmer found the symptom only when they realize it must be controled. However, for some preventive measures, they think that it is not needed to do. Therefore, the development of anti-plant virus agent has the great practical significance and potential for marketting. Since drugs are successfully applied to combat diseases of human and animals. It is not reasonable why plant virus diseases can not be treated and controlled (Qiu, 1984).

(3) Application of genetic engineering resistant plants

Viruses are intercellular pathogens closely integrated within the host's cellular machinery, and chemicals which affect the virus are also harmful to the plant. Therefore, the best way to overcome virus diseases is to breed resistant plants. Traditional breeding procedures allow movement of resistance sources only between closely related species and geneticists lack sufficient sources for their breeding programs. The use of DNA technology to manipulate and move resistance sources against plant pests in general, and viruses in particular, has been widely advocated. This would be in addition to current approaches of inserting viral genes (coat protein, satellite RNA) to obtain resistant plants.

For isolation of natural resistance genes the following approaches can be considered : via the gene product, insertional mutation with a transposon or substraction hybridization of mRNA's.

As the local lesion response depends on one single dominant gene it is

thought that it might be suitable for isolation and transformation of plants. Results have shown that a host-coded protein, termed "inhibitor of virus replication" (IVR), is induced by virus infection, and inhibits virus replication. IVR has been isolated from protoplast incubation medium and the intercellular spaces of resistant tobacco leaves inoculated with TMV. This protein effectively inhibits the replication of a spectrum of viruses. This protein had been characterized and prepared poly- and monoclonal antisera to IVR has been prepared. IVR is different from PR-proteins.

Immunoprecipitation of translation products of Poly (A) + RNA from induced resistant tissue yielded a specific product of about 20 K. This Poly (A) + RNA was used for obtaining cDNA for construction of a library. A clone that reacted positive with IVR antisera produced a protein that inhibited replication of virus (Gera, A., Aly, R., Teverovsky, E. and Loebenstein, G. 1994).

2.2 Detection method of plant viruses

Plant virus diseases are important in the agricultural production, it seriously influences on output and quality of the crop products. At present, there are no antivirus agents that can give a good treatment effect on plant virus diseases. Thus, it becomes very important to make a correct diagnosis and inspect early for the virus diseases. With nearly 100 years development of the plant virus studies, the detection methods and means are also constantly developed and improved. The commonly used methods are infection assay, serological assay, electron microscopy and molecular biological assay.

2.2.1 Infection assay

Determination of the infection of a virus is to inoculate the virus to a test plant.

Its sensitivity is relatively high in all of quantitative methods. For the design of a new quantitative method, if infection capability is not determined, it will be unable to judge the virus viability. The measuring method for infection capability of a virus includes local lesion assay, amylum-iodine spot assay, and infection titer assay.

(1) Local lesion assay

In 1929, Holmes found that TMV can cause the particular necrosis spots on the inoculated leaves of *Nicotiana glutinosa*. In a certain range of the virus concentration, the number of the generated spots is directly proportional to the strengths of the virus. This discovery becomes the basis of quantitative determination of infection of the virus (Tian, 1987). The entire mechanical transmission viruses may be determined by local lesion assay, but in reality only a few viruses would have the particular necrosis spots developed on a tested host. The number of spots that a test sample generates not only depends on the virus c concentration but also be affected by the host plant species, environment, inoculation technique, and agents or inhibitor in the plant sap.

(2) Amylum-iodine spot assay

This method is used when the virus has no local lesion hosts. Holmes (1931) found that there were distinct yellow patches formed on the tobacco leaves inoculated with TMV, but it could not be counted. If the leaves were heated to 80 ^{O}C with 95% ethanol and then dyed with the mixture of I_2 and KI (10g I_2, 30g KI, 1500ml H_2O), the amylum-iodine blue color reaction will appear at the infection point. If the leaves were picked in the afternoon and left overnight, and then dyed them with iodine, the color around the infection point was lighter than at the infection point. If the plants were put in the darkness for hours before being picked and then dyed them

with iodine, the result would be opposite. This is because the virus infection can not only reduce the formation of carbohydrates from photosynthesis but also inhibit their transport out of the infection point. The intensity of amylum-iodine is greatly affected by the environment. Thus it is less reliable than local lesion away. But under standardized conditions, the quantitative determination of the infection capability is also possible.

(3) Infection titer assay

Infectious titer methods can also be utilized when other methods can not be applied. That is the test samples can be diluted by buffer. Each dilution is inoculated to at least three systemic host plants. The last dilution that gives rise to infection is the infection titer. The shortcoming of this approach is the requirement for a large number of test plants.

2.2.2 Serological assay

At present, serological assay has high specificity, and is a common detection method for plant viruses. The method was developed in 1960s by using specific antibody. The methods include precipitation test, latex agglutination, ELISA, IEM, RIA and tissue blotting.

(1) Precipitation test

Soluble antigen is mixed with the corresponding antibody, when the ratio is appropriate the antibody will form a complex with virus antigen and precipitate appear. Since the sediments is mainly composed of antibody globulin, in order to ensure that there are sufficient antibodies, when test is being conducted, the antigen, not the antibodies should be diluted.

(2) Agglutination

A specific antiserum is added into the microbial cell suspension. When there is a certain concentration of electrolytes in the mixtures the microbial cells will agglutinate. Because virus particles are soluble antigens, it is necessary to adsorb the virus or the antibody to a particle surface such as bentonite, latex bead, carbon, and red blood cells. Widely methods that use this principle include rapid immuno-filter paper assay (RIPA) and immunogold-label assay (Jin, 2005)

(3) Enzyme-linked immunosorbent assay (ELISA)

The combination of the catalytic role of enzyme and the immune response that occurs in the enzyme marker and antigen-antibody complex not only maintains the specificity of antigen-antibody response, but also improves the sensitivity. At the same time, it is also a non-homogeneous analysis process. Namely, the response of each step followed by washing process, thereby it gets rid of the non-reactive substances interference. Since 1976 enzyme-linked immunosorbent assay (ELISA) has been used in plant virus detection and formed a variety of testing methods (Hou, 2000). Some methods have high sensitivity which can be measured up to 1-10 ng/ml concentration, are very specific and easy to operate. They have been widely used for the diagnosis and identification of viruses (Sheng, 1978; Ma, 1991). ELISA was first used for animal virus detection and has been used for the plant virus detection for the past 20 or 30 years. It was widely used as a virus quantitative method in pathology, immunology research and production practice (Ma, 2003).

2.2.3 Electron microscopy

The electron microscope has made important contributions in the field of biology, cytology and virology. In 1940, Kausche Melcher observed tobacco

mosaic virus particles with the electron microscope for the first time. The establishing of electron microscopy has played a tremendous role in promoting the development of virology. Electron microscope technique is the most direct and most accurate means of detecting the virus. It can directly observe morphology of the virus, its presence or absence. Although techniques have entered the molecular era, electro microscope still has a role that can not be replaced.

In plant virus diagnosis, the most common methods are negative staining and ultrathin sectioning. It can help to identify the virus types and understand the dynamic process of virus infection and replication.

2.2.4 Nucleic acid assay

Nucleic acid assay is most sensitive, highly specific, fast and simple. It can be used to detect a large number of samples (Hou, 2000). At present, the commonly used molecular biology assay includes the nucleic acid hybridization technology, the dsRNA electrophoretic techniques, and polymerase chain reaction technology.

2.3 Changes of enzyme activity of plant infected by TMV

2.3.1 Main enzyme activities of TMV infected plants

In a certain extent, plants can withstand the impact of external stimulation by the self-immune defense system. Virus infection can induce the reactive oxygen to accumulate (Orozco-Cardenas et.al.1999; Lamb, 1997). This is one of the early plant disease-resistant reactions (Zhao, 2002). These reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide (O^-_2), and oxygen free radicals (•OH) (Doke, 1994; Foyer, 1997; Dat. 1998). Reactive oxygen species play

an important role in plant disease resistance, and can lead to lipid peroxidation and loss of membrane permeability, thus lead to a series of physiological and biochemical changes and metabolic disorder that are harmful to plants. Under normal circumstances, there is an eliminator to remove reactive oxygen species and prevent the poisoning of the protection system and keep reactive oxygen species at a state of dynamic equilibrium. When the organisms, in the formation of reactive oxygen species engender faster than the removal and break the balance, the cell active oxygen are rapidly accumulated and the cells are stressed by oxygen. The reactive oxygen species are more active than the normal oxygen, they can react with proteins, nucleic acids and lipids and make them degraded and inactive. DNA strand breaks and lipid peroxidation, finally lead to cell structure and function damage.

After evolution, organisms form a complete and complex enzyme and non-antioxidant enzyme protection system to remove reactive oxygen species (Du, 2001). In the active oxygen removal system, the enzyme system (including SOD, CAT, POD, and APX) is more important to the removal of reactive oxygen species. Under the condition of SOD, reactive oxygen species was degraded into H_2O_2 and O_2. Then H_2O_2 was decomposed into non-toxic H_2O and O_2 by CAT, POD, and APX.

(1) Superoxide dismutase

Superoxide dismutase (SOD) is a very important enzyme in plant virus defending system. It can make superoxide radical ($•O_2^-$) rapidly disproportionated into hydrogen peroxide (H_2O_2) and oxygen molecule (O_2), to avoid $•O_2$- damaging the cell. While H_2O_2 can also induce cell necrosis (Levine, 1996; Draper, 1997), thereby inhibit expansion of the pathogen. If the $•O_2^-$ can not be removed in time, the presence of Fe_2^+ and certain physiological conditions, $•O_2^-$ can promote the Fenton reaction,

which will transformed H_2O_2 into •OH. H_2O_2 can form hydroxyl radical •OH through the Haber-Weiss reaction. •OH is the most toxic radical to cells (Cadenas, 1999). Therefore, SOD is one of the main members of the defense system in the cells. It could eliminate oxygen free radicals (O^-_2) and has a protective effect to cells. Many studies have proved that the SOD activity is relative to disease-resistance of plant.

(2) Catalase

Catalase (CAT) has been studied considerably in plant disease-resistance phenomena. Its function is to eliminate reactive oxygen species (ROS) accumulation caused by pathogen infection (mainly H_2O_2), and degrade H_2O_2 to become non-toxic H_2O and O_2. Since plant pathologist believes that CAT relative to ROS metabolism may have an important role in plant and pathogen mutual relations, many researchers have studied a variety of diseases system. The overall trend is that the CAT activity decreased after tobacco had been infected by viruses. But in various combinations, the conclusion is not entirely consistent.

After be being infected by virus, the conformation of CAT changed and the CAT activity is inhibited. As a result, the level of H_2O_2 was enhanced. Except for killing pathogens directly, H_2O_2 could activate the expression of protein genes of disease course, which was good for the response of disease-resistance. On the contrary, the excess increase of H_2O_2 in the plant would induce once more the increase of CAT activity due to its inherent resistance (Chen, 1991).

Ron Millter et al. (1999) found that in the transgenic tobacco, reduction of CAT expression quantity would enhance content of H_2O_2 in the tobacco. The resistance of tobacco markedly increased. It may cause cell putrescence induced by H_2O_2. This was another proof that CAT activity was closely related to plant

disease-resistance. It is indispensable to further study the relationship of CAT activity and plant disease-resistance. This is because that quantitative change of CAT enzyme provides a basis in screening of anti-virus inhibitors.

(3) Peroxidase

The activity of peroxidase (POD) and isozyme bands change in plants are closely related to plant disease resistance. POD is not only an important endogenesis eliminator of ROS in cells, but it takes part in the process of polymerization of the lignin that is relative to the synthesis of plant protection and the oxidation of the phenolic, and these are related to disease-resistance of the plant.

POD activity positively relates to plant disease-resistance had no universality. For POD mechanisms, Xue, (1992) thought that main role of POD was to take part in depositing of lignin in the early years. Kozlowska (2001) also pointed out that POD activity change influenced on subsequent lignification. In recent years, Wang et al. (2001) suggested that except for the above roles, POD activity played an important role in defense in the elimination of oxygen free radicals. POD activity can be induced by ROS, which was consistent to conclusion of Hige et al. (2001). Albert et al. (1997) and Weijia et al. (1999) found that the oxidation of nitrite (NO_2-) enhanced content of nitrogen oxide (NO^+), which stimulated plant-mediated resistance of the hosts. The NO^+ and SA was also important resistance pathway (Daniei et al., 2000). Yin (2005) found that the VFB can stimulate POD activity increase and enhanced disease-resistance of the host plant.

Antioxidant protection enzymes also include glutathione reductase (GR) (James et al., 1998; Bellaire, 2000), glutathione peroxidase (GPX) (Olliver et al., 2000), alterative oxidase (AOX) (Denis et al., 2003; Hannah L. Parsons et al., 1999),

deoxy ascorbic acid oxidase (DHAR), and some coenzymes. The removal of ROS involved in a series of cell metabolism and enzymatic reaction process. When a single enzyme activity is raised, the increase of the antioxidant capacity will not be affected significantly. The balance of the various antioxidant enzyme activities may be important to the plant virus. ROS only can be decomposed into water and oxygen molecules through the synergy. Produce and removals of ROS in the cells always keep a dynamic equilibrium.

ROS relative enzyme activity after or before the tobacco is treated with pesticides will help us to screen out anti-viral active substances. This will provide theoretical basis for further development of antiviral agents. If we can establish a certain correlation between these physiological and biochemical changes with the pesticide, we can quantitatively detect the changes of activity of SOD, POD, and CAT. Then the changes of activity can be used as physiological and biochemical indicators of identification pesticide that have inhibiting effect on TMV.

2.3.2 Relationship between course of diseases and enzyme activity

Under normal conditions, the ROS metabolism is in dynamic equilibrium of low levels due to protection enzymes such as SOD, POD, and CAT cleaning up the ROS. But after the pathogen infects an organ, ROS begins bursting (Han, 2002). When the plant was infected by pathogen, the function of SOD is to eliminate $O\text{-}_2$. POD and CAT will remove H_2O_2 and O_2 that are produced in the process of SOD disproportionation ($2O\text{-}_2 + 2H \rightarrow H_2O_2 + O_2$) and Haber Weiss reaction ($O_2 + H_2O_2 \rightarrow \cdot OH + O_2$) in order to avoid harm to the cells. Therefore, POD and SOD are also important endogenesis eliminator of ROS in cells. If activities of POD and SOD increase, the CAT activity will decrease (Wang, 1992). The reason

may be the resistance reaction such as increase of permeability of cell membrane, change of membrane structure and the necrosis and irritability of cells (Zhu et al., 1994).

Research results, carried out by Sean et al. (2002), showed that the CAT activity decreased after the host was infected by virus. Chen et al. (2002) also found that after the host was infected by TMV, CAT activity in the disease-resistant cultivar was lower than that of the disease-susceptible cultivar after treatment of pesticides. Studying the TMV interaction system, Kong et al. (2002) found that CAT activity always increased after inoculation. After the early phase of the compatible interaction, the CAT activity was higher than that of the incompatible interaction, but at the later stage, the CAT activity of the compatible interaction was lower than that of the incompatible interaction. Wu et al. (1999) found that after disease-susceptible tobacco cultivar being inoculated with PVY, the CAT activity initially had slight increase, and subsequently was lower than that of the tobacco treated with pesticide. Zhang et al. (2003) found that after disease-susceptible plant inoculated with virus and treated with pesticides, the CAT activity initially increased, and subsequently decreased. However, after plant immune to virus, was inoculated with virus before treatment with inhibitors, the CAT activity had invisible change. By far, there is no consistent conclusion about the change and mechanism of CAT activity after the tobacco infected by virus. But it is believed generally that the change rate of increase or decrease of the disease-resistant plants is lower than that of disease-susceptible plants. This may be result of the disease-resistant plants rapidly receiving the signal of virus as soon as possible and transmitting it quickly and start the defensive reaction in time.

Zimmermann et al. (2002) pointed out that in the initial stages of virus infection, the decrease of the activity of CAT isozyme 2 led to the accumulation of H_2O_2. And the increase of content of H_2O_2 activated the expression of CAT isozyme 3. Finally, CAT activity increased. This phenomenon can be explained by the salicylic acid-binding protein1 (SABP). When the host plants were infected by virus, the salicylic acid content increased, and combined SABP 1 (namely CAT), which caused changes of conformation and inhibited CAT activity. Finally, the level of H_2O_2 was enhanced, which can activate the proteins gene expression related to course of diseases, except for killing pathogens directly. Thus, it was in favor of disease-resistant respond for plants. On the contrary, due to inherent resistance in the plants, the high level of H_2O_2 would induce the increasing in the activity of CAT (Chen, 1991).

POD activity in the plant and the change of isozyme band is closely correlated with the plant disease-resistant. Many study results showed that after tobacco was infected by the virus, POD activity of disease-resistant or disease-susceptible tobacco always increased. But the different diseases, there was a different difference in change POD activity of disease-susceptible plants. Mao et al. (2002) found that there were changes in POD activity of disease-susceptible plants. Study results of Kong et al. (2001) showed that in the tobacco-TMV interaction, POD activity of disease-resistant or disease-susceptible tobacco always increased. POD activity change of the incompatible interaction was bigger than that of the compatible interaction. Li (1999) found that after tobacco being infected by CMV, POD activity of disease-resistant or disease-susceptible tobacco always rised. POD activity of different disease classs was opposite to the external symptoms of tobacco plants.

Experimental results of Sean et al. (2002) after tobacco being infected by virus, POD activity rapidly increased.

It can be seen that, changes of POD activity of different combinations and different disease-resistant or disease-susceptible plants are complex and can not be generalized.

In recent years, the change of SOD activity is always a subjest in plant pathology research. However, there is inconsistent conclusion in different interaction systems. Experiment results, done by Gupta et al. (1993) proved that the excess expression of the SOD activity in the transgenic tobacco could improve the tolerance of the plants. Therefore, it can be presumed that the quantity of SOD in the tobacco infected by virus could be the biological and chemical indicators of disease-resistant determination.

2.4 Present situation and prospect of antivirus agent

2.4.1 History and achievements of antivirus agent

(1) History and achievements of antivirus agent world wide

Plant virus diseases are one of important diseases in agriculture production. All economic crops are infected by at least two or three viruses (An et al., 1994). It is estimated that about 1000 virus species, naturally infect plants with important economic values. There are more than 400 viruses reported in China. The economical loss caused by plant viral disease is up to 15 billion dollars every year all over the world. Among the loss, more than billion dollars is caused by tobacco mosaic virus (TMV) (Wu et al., 1995). By being an obligate parasite, virus needs energy and enzyme system of the host cells. Therefore, it is more difficult to control plant virus

diseases and to develop antivirus agents of high selectivity (Jiang, 1995). The control of plant virus diseases is more difficult than that of other plant diseases.

In the early 19th century, Allard (1914) found that juice of *Phytolacca Americana* L. would inhibit the infection of TMV. Duggar and Armstrong (1925) found a kind of Anti virus substance from *P. americana*. Studies done by many researchers found that many plant juices contain substances inhibiting to virus (Ye et al., 1988; Zhu et al., 1989; Yang et al., 1991; Qian, 1984; Qiu, 1984). Since then, a series of researches on inhibiting agent of plant virus had been carried out (Jiang, 1992). After the1980s, with development of molecular biology, people hoped to use transgenic bioengineering technique to solve the problem of plant virus diseases. In addition, people also used reference of the technique and animal virus inhibitor to control plant viruses. But the research of anti virus agent only started at the 1950s. Scientists of USA, Germany, Japan, Israel, Netherlands and other countries have identified a large number of substances having certain antivirus activities. According to their molecular size, these substances can be divided into small molecule and large molecule substances. According to the sources, they can be categorized as synthetic chemicals and naturally active substances. The synthetic chemicals are often synthesized from naturally active substances through transformation of the individual chemical bond or gene. Researchers firstly studied the naturally active substances of anti-virus in the nature. Subsequently they screened the natural compounds of anti-virus from the higher plants, algae, bacteria, fungi and even insects. Plants in their studies included purple jasmine (Kubo et al, 1990; Habuka et al, 1991; Jorge et al, 1999), *Diranthus chinensis* (Frotschl et al, 1990; Liu Hok, 1994； Sadasivam et al, 1991), *Bougainvillea spectabilis* (Balasaraswathi et al, 1998), *Yucca filamemtosa*

(Guo, 1992), *Phytolacca americana* (Barbieri et al, 1982; Kung et al, 1990; Guo, 1992), protein in *Spinacia oleracea* (Barbieri et al, 1982) , fatty acids in rape oil (Lei, 1984), alkaloid in asphodel (Adallah et al, 1993), glucosidase in *Bougainvillea glabra* (Bolognesi et al, 1999), naphthoquinone and cumarin in *Sinojohnstonia chekiangensis* (Liu,1997), extract oils in *Ocimum santum* (Bioshop, 1995; Shukla et al, 1989), terpenes in Salvia (Tada et al, 1994), flavonoids in orange (Benavente et al, 1997), and glycoprotein in *Asarum caudigerum* (Awashi et al, 1985). But so far, only a few natural compounds are applied and the limited compounds become merchandised of effective anti-virus agents, including phytolacca antiviral protein (PAP) separated from *Phytolacca americana* and Lentemin powder. In addition, skim milk in USA, extracts of *Melia azedaeach* leaf in India, and alginic oil formulation in Israel have been developed as the commercial products. The plants and microbes that have been studied as the sources of anti-virus are listed in Table 2.1 and Table 2.2.

Table 2.1 Plants that have been reported as the sources of anti-virus*

Sources of plant extracts	**Effective component**	**Control target**
Gymnogongrus flabelliformis	Protein	TMV
Seed of *Brassica napus*	Fatty acids	TMV,TuMV
Allium cepa	Extract of Root	TMV, PVX
Dolichos purpureus	Extract of Pod	TMV
Rheum palmatum	Alkaloids	CMV
Callistephus chinensis	Essential oils	PVX
leaves of *Spinacea oleracea*	Nucleotide Acid -	TMV
Isatis tinctoria	Alkaloids, fatty acids	TMV.CMV
Bougainvillea spectabilis	Protein	TMV
Lily plants	Protein	TMV,CMV
Glycine max (L.) Merr	Lecithin	TMV
Beta vulgarisL.var.iuteaDC	Sulfonyl-Betaine	PVX
Corm of *Fritillaria imperialis*	Prolease	TMV,PVX
Bougainbillea brasiliensis	Protein	TMV,CMV
Lithospermu erythrorhizon	Naphthoquinone	TMV

* Abstracted from the Progress of Chemical controlling of Virus Diseases of Vegetable and Plant viruses. Microbiology Professional Committee of the Plant Virus of China 1998

Apart from plants listed in Tab.2.1, there are many plants that have also anti-virus substances such as *Dianthus caryophyllus*, *D. barbatus*, *D. plumarius*, *Chenopodium amaranticolor*, *C. quinoa*, *Tribulus terrestris*, *Datura stramonium*, *Solanum torvam*, *Acacia arabica*, *Agrostemma githag, Vitex cannabifoli*, and *Aloe vera*. Generally speaking, most extracts of plant have only an inactivation effect to viruses. But the research is very few on the substances of inducing resistance and curing action. (Baranwal et al, 1992 and 1997; Hou et al., 1998).

Table 2.2 Microbes that have been reported to have anti-virus activity*

Microbe	Active ingredient	Control target
Aspergillus oryzae	Polysaccharides	TMV
Bacillus subtilis	Zymosis filtrate	TMV
Penicillium of Jia Wei Sike	Incubation filtrate	TMV，PVX
Rhizoctonia Solani	Incubation filtrate	TMV，PVX
Actinomycin D	Incubation fluid	TuMV, CMV
Lentinus edodes	β-1,3glucan	TMV, CMV
Coprinus comatus	Polysaccharide	TMV
Schizophyllum commune	Incubation fluid	TMV
Bacilli	Filtrate	PVX
Basidiomycotina	Incubation fluid	TMV
Pseudomonas diminuta	Incubation fluid	TMV
Phytophthora megasperma	Polysaccharide	TMV

* Abstracted from the Progress of Chemical Controlling of Virus Diseases of Vegetables and Plant Viruses. Microbiology Professional Committee of the Plant Virus of China. 1998

Apart from the above mentioned natural substances with anti-viral activity, many chemical substances also have certain anti-viral activity. These are (1) Metal salts such as $ZnSO_4$, $CaCl_2$. (2) Plant hormones such as 2, 4-D, gibberellin, kinetin, and cytokines. (3) Vitamins such as VB2. (4) Pyrimidine and purine such as azoguanin*e* and thiouraci*l* and (5) Amino acid such as leucine.

(2) Situation of research and development of anti-virus agent in China

In China, until now, the research and development of antivirus agent

has only a history of not more than 40 years, but the progress is rapid. Qiu et al (1963) studied curing and protection functions of actinomycete metabolin on a rape viral disease. In 1984, Qiu et al found that fatty acid in rapeseed had inhibitory action to both TMV and CMV; and the extracted liquid of India pokeberry had high inactivation effect on many plant viruses. Verma and Awastihi (1980) also found that the extracted liquid of *Pseuderanthemam bicolor* had a treatment function to many kinds of plant viral disease. In 1984, Lei et al. extracted the NS-83 agent from a vegetable oil, which not only had induced resistance and tolerance to viral diseases, but also could change the agronomic characters of the crops, and can increase the yield (Lei et al., 1984). In 1997, Liu et al., screened 600 of plant species and confected the extracts the Indian pokeberry, liquorice and forsythia into a multiple agent MH11-4, which had stronger inactivation effect on TMV and CMV in vitro and could inhibit then multiplication in tobacco. The mixture appeared to have resistance inducing effect on tobacco. On the base of the antivirus mechanism, the preventing and controlling experiments to tobacco mosaic virus in the room and the field were carried out again. However, these inhibitors still do not meet the request of agricultural production.

In general, the research and development of anti-virus agent is still at an initial stage in China. These anti-virus agents are mixed with the hormones. There are few pesticides that can directly be used as anti-virus agents. The research and development of the natural anti-virus agent are also few.

2.4.2 Prospects of antivirus agent

(1) Characteristics of antiviral agent

The antivirus agents that have been developed, only a few are effective

when applied in practice. Their control effect is about 30% to 60% in the field (Jiang, 1995; Yao, 2002). The plants are injuried by some inhibitors (such as ribavirin). Therefore, it is imperative to develop anti-virus agent with high efficiency, low toxicity, and compatibility. Compared to other pesticides, an ideal antiviral agent must have the following characteristics : (1) having reliable and stable activity of anti-viral, (2) have good absorption into the plant. (3) cause no injury to plants, (4) is still effective at least for a week in the plant (5) is safe to human, livestock and the environment. (6) production cost is not too high. Under the condition of this request, the research and development of anti-virus agent will focus on the following aspects in the future : (1) to develop anti-virus agent to control virus vectors, especially aphid, (2) to develop protective and curing agents for virus disease, (3) to develop an anti-virus agent which can prevent the transmission of viruses. Concretely, anti-virus agent should have the capability of inducing host plant response to produce the partial necrosis reaction. So it can prevent subsequent infection by inducing plant produce anti-viral factor (Antiviral factor, AVF). Many studies have showed that there are some anti-virus factors (AVF) in plants. Some of the AVF are preexisting but some are induced after infection. French and Dutch researcher found that after the hypersensitive tobacco (*Nicotiana tabacum*-Xanthi-nc or SamsunNN) was inoculated with TMV, around the local lesions on the leaves will produce four kinds of proteins in which healthy plants do not have. These protein produced, were the products of the host genes and not the virus. These proteins are called pathogenesis related protein (PR-P). So far, PR-protein was found in 16 species of plants, 11 species reported form tobacco (Xu, 1989). Virus, viroid, epiphyte, bacteria and chemicals such as phenylformic acid, aspirin, salicylic acid, ethylene, cytokinin and ABA (Fraser, 1985)

can induce plant to proudce PR-protein. China's NS-83 can induce plant to produce PR-protein, too. In addition, the anti-virus agents can also inhibit the movement of virus between cells. The researches show that many plants which have highly resistance to virus also have inducer (White, 1983). When Jiang et al. (1995) induced disease-related proteins in tobacco and sugar beet with DHT, NS-83, DA-DHT, E30, they found that all the agents could induce the tobacco and sugar beet to produce PR-proteins, but the type and content of PR-proteins were different. Compared with single agent, the type and content of PR-protein which was induced by a variety of agents are much alike.

Based on the needs of agricultural production, some researchers focus on the development of natural substances in order to develop a good anti-virus agent. Other researchers focus on the new anti-virus agent by synthesization, such as "8300" in Germany (Schuster, 1991). Others focus on the development of the corresponding synergistic agent.

(2) Effect of plant extracts on plant viruses

Vincent et al. (1986) reduced the damage of broad bean mottle virus (BBMV) by dressing with barley seeds with Berberine. Lin (1987) selected anti-tomato mosaic virus (TMV-T) in 90 species of plants to find that the extracts of *Chenopodium serotiuum* and *Hosta plantaginea* had therapeutic action on TMV-T. The extracts of *Rheum officinalle*, *Forsythia suspensa*, and *Isatis tinctoria* separated from the herbal medicine had a stable curing effect to mosaic virus of chili which caused by CMV (Zhu, 1989). Wang et al; (1989) also reported that *Isatis tinctoria* can inhibit the proliferation and infection of TMV. Sadasivam (1991) determined inhibition effect of extracts of *Boerhaavia diffusa* to TSWV on groundnut and chili,

CpMV on cowpea and TMV on tobacco. The results showed that suppressed time of all the extracts could last more than 20 days to TSWV or 45 days to CpMV and TMV.Qin et al. (1997) reported the inactivation effect of extracts of *Yew hada* on CMV and inhibition to primary infection before or after inoculation. Liu (1997) mixture MH11-4 which had a better virus control effect was screened from 600 species of plants. Experiments of many years showed that the control effect of MH11-4 was superior to that of ST, NS-83, and virus A. Li et al. (1997) reported that propionyl alkannin obtained from *Lithospermi* can combine with TMV and make TMV inactivate. Xing (1998) reported that the water extracts of rhubarb had a therapeutic action on TMV causing a tomato mosaic virus. Hou et al. (1998) selected highly effective anti-virus agent from *Lithospermi* and China rose by using the changes of chlorophyll content and the characteristics of fluorescence spectrum after tobacco infected with TMV. Hou et al. (2000) reported that *Belamcanda chinensis* extract could inhibit the proliferation of TMV by using simultaneously infected system of HIV/cell. Yao et al. (2002) and Qu et al.(2002) extracted indolizidines alkaloid from *Cynanchum komarovii* which have better biological activity on TMV, PVY and TuMV. Fu (2002) separated anti-TMV protein Zb which can strongly inhibit local lesion formation on *N. glutisona* from *Flammulina velutipes*. They found that TMV particles would disintegrate when anti-TMV protein Zb and TMV were mixed by using electron microscope, but mechanism of these proteins is not clear.

China is a country that has rich wild plants that can provide unique conditions for scientists who study on natural active substances. Jian et al.(1999) reported that the ethanol extracts of *Astertatarlcus*, *Elsholtzia stauntonii, Rubia cordifolia*, *Saussurea amara*, *Orostachys erubescens*,and *Sophora japonica* gathered

at suburb of Beijing significantly inhibited TMV, and the activity of ethanol extracts of *Sophora japonica* was similar to that of the control Virus A. Although the ethanol extracts of *Astertatarlcus*, *Rubia cordifolia*, and *Saussurea amara* could significantly inhibit the proliferation of TMV, the effect was not as good as that of Virus A. The ethanol extracts of *Elsholtzia stauntonii* only significantly inhibited the proliferation of RMV. On the contrary, the ethanol extract of *Saussurea amara* only inhibited the infection of TMV. Therefore, except for the ethanol extracts of *Astertatarlcus* and *Rubia cordifolia*, others have the activity of anti- TMV at different stages.

2.5 Extraction and separation methods of components of Chinese herbal medicine

According to dissolved properties of Chinese herbal medicine components in the solvent, effective components could be extracted only with suitable solvents. In the extraction process, active ingredients should be avoided to be left out and impurities should be avoided to be dissolved as much as possible. With the development of science and technology, extraction and separation techniques have progressed, except for common extraction methods such as water extraction, solvent extraction (solvent extraction, including dipping extraction, circumfluent extraction, and sequent extraction), acid alkaline organic solvent extraction, water steam distillation, enzyme hydrolysis or inhibiting extraction, chemical processing, and distillation. The new extraction and purification techniques, includ supercritical fluid extraction technique, strengthen dipping technique, new adsorption clarification technique; ultracentrifugation and membrane separation technique have been applied and developed. In addition, the size of raw materials, the extraction time, extraction

temperature, equipment conditions and other factors can also affect the efficiency of extraction, which must be taken into account (Yao et al., 2002; Xu, 1993)

2.5.1 Solvent extraction

The solubility in solvent of herbal medicine ingredients is related to the nature of solvents. Solvent can be divided into hydrophilic and lipophilic. The principle of selecting appropriate solvents is that the hydrophilic or lipophilicity of herbal medicine resembles the nature of solvent. Dipping extraction, circumfluent extraction, and sequent extraction are often used to extract active ingredients of the herbs. The crucial key of the choice of solvent is polarity of the molecules. The different chemical compositions of herbal medicine have different polarity. If the effective component is needed to be extracted maximally from plants, the principle of "the polarity similar to dissolution" must be followed. The stronger the polarity is, the stronger hydrophilic is, but the weaker lipophilicity is. Vice versa the weaker the polarity is, the weaker hydrophilic is, but the stronger the lipophilicity is. The common extraction solvent of the strong lipophilicity can be divided into the following three methods.

Water : Water is a strong polar solvent. The hydrophilic components in Chinese herbal medicine, such as salts, glucide, multi-carbohydrate, tannin, amino acids, organic acid salts and base (parts alkaloid), can be dissolved by water. In order to increase the solubility of certain components, acid + water or base + water are often used as a solvent of extraction.

Hydrophilic organic solvents : These are organic solvents that can dissolve in water such as ethanol and methanol. Ethanol is the most commonly used. The dissolution of ethanol has the stronger penetration capability to Chinese herbal

medicine. The lipophilic components are difficult to dissolve in water, but have higher solubility in ethanol.

Lipophilic organic solvents : These are organic solvents that can not dissolve in water such as petroleum ether, benzene, chloroform, ethyl ether, and ethyl acetate. These solvents have strong selectivity, but are more flammable, toxic, expensive, and need special handling. Their capability of permeation into the plant tissue is weak and it takes them a long time to extract chemical components.

2.5.2 Other methods of extraction

(1) Water steam distillation extraction

The steam distillation can be applied to extract components of Chinese herbal medicine because water steam distillation destroys the components. The boiling point of these components is higher than 100 ^{O}C, therefor the components do not have the immiscibility with water and the certain vapor pressure at 100 ^{O}C. When being heated with water, The vapor pressure of components and the vapor pressure of water sums up one atmospheric pressure, the liquid start to boil and volatility substances, including volatile oil in herbal medicine, alkaloids-ephedrine, and arecoline, are brought out by water vapor.

(2) Sublimation extraction

The solid substances being heated boil away directly and concrete curdle solid compounds when being cooled. Some ingredients in Chinese herbal medicine have the nature of sublimation. Thus we can directly extract them with the method of distillation. Free hydroxyl anthraquinones, coumarin and organic acid compounds also have the nature of sublimation.

(3) Physical extraction of strengthening

After a certain physical field such as microwave or ultrasound is transfered into the traditional solvent extraction, the extracts are concentrated, which thus results in decreasing extraction time and decreasing degradation degree of active substances. Ultrasonic treatment, a physical field enhancing extraction technology, can make internal micro-gas nuclear of material shock, grow and rupture by ultrasonic cavitation action. In the extraction of natural products, microwave has characteristic of rupturing internal organization of plants in a very short time by rapid heating in the entrails of the plant. Thus microwave field can enhance extraction process efficiently so that extraction yield can be increased significantly as well as the decrease of extraction time and solvent quantity.

2.6 Research review of pesticide formulations

2.6.1 Actuality of pesticide formulations

Pesticide formulations processing is the procedure in which appropriate auxiliary agents are added to the technical products, and the mixtures form a product of certain shape, components and specifications for easy use (Liu, 1998). Under the pressure of environmental protection and the difficulties of new pesticide synthesis, new pesticide formulations have caught more and more people's attention. Some traditional pesticide formulations such as powder, wettable powder (WP) and emulsion concentrate (EC) are gradually being replaced by the new environment-friendly pesticide formulations such as suspension concentrate (SC), emulsion in water (EW), suspension emulsions (SE), microemulsions (ME), dry flowable（DF), soluble granules (SG), and capsule suspensions (CS). Some mixed

preparations of the new formulations are also constantly researched and developed, although it takes a long time for new users to accept these new formulations.

Nowadays, the stringent requirements was put forward for the safety of pesticides, it makes some enterprises not to afford outlay and time for the new pesticide research and development. Thus, people make their effort to research and develop formulations to reduce or avoid the adverse effects of pesticides and extend the pesticide life. Looking at the trends of domestic and international, pesticide formulations are developing in the direction of the water-based, granular, slow-release, multi-purpose and effortless. Accordingly, some security, economic, and effortless of the new formulations are emerging. From domestic and international trends of pesticide formulations, it can be seen that pesticide formulations are expanding to water-based, granular, slow-release, multi-purpose direction (Ling, 1999). At present, the main direction of preparation processing is to create the agents with the following functions such as reducing toxicity, enhancing safety, reducing pollution, lightening the crop injury, more secure for users, ease of use, saving labor, energy saving and reducing prices, enhancing utilization rate, and having development direction of water-borne.

2.6.2 Water-based formulations

Water is one of the three elements of mankind's survival, and it is the most abundant resources and the most inexpensive material in the world. Water-based formulations are a pesticide formulation processed from a medium or diluter. Such formulations have characteristics such as low pesticide injury, low toxicity, easily diluted, inflammable, inexplosive, easy use, being good for environmental protection, being easy measures and so on. Water-based formulations mainly include SC, EW, SE,

ME, water dispersible granules (WDG). New solid formulations mainly include SG and WDG. EW, SC, SG and WG are main formulations developed actively formulations (Leng et al., 2003; Zhong, 2003). A water-based formulation of pesticides is a liquid pesticide formulations processed from water as the medium. It includes water formulation SC, EW, SE, and ME.

2.6.3 Study progress of EW

Emulsion oil in water (EW) is an O/W emulsion formed from the tiny droplets of hydrophobic liquid or solid pesticides by entering energy and the appropriate emulsifier under the condition of solvents indiscerptible in water. Micro-emulsion (ME) is a transparent or milky liquid formed when the active ingredients, emulsifier, dispersant agent, antifreeze, stability agents, solvents and other auxiliary agents evenly disperse in the water. The differences between EW and ME are different grain diameters of active ingredients. The grain diameter of former is 0.1- 50 μm, the grain diameter of later is 0.01-0.1μm. The appearance of ME is transparent or nearly transparent. The appearance of EW is milky white. Organic solvents (such as toluene and xylene etc.) of EC are restricted due to the environment pollution. At overseas, there is merchandise EW such as 60 % butachlor, 10% fenvalerate, 5% efficient fenvalerate, 25% fenitrothion and so on. The amount of emulsifier of EW is less than one of EC and micro-emulsion. Thus, water emulsion preparation costs generally lower than the EC and micro-emulsion. In China, as a formulation of the important green water-based, EW grows very fast, and commercialization of EW also increases. According to the data of Chinese Ministry of Agriculture that EW registration began from 1993. Before 2000, there are only 30 kinds EW. But from 2000 to 2005, 78 kinds were registered. After 2006, 215 EWs

were registered. Between January-March of 2007, 85 EWs were registered. Up to April 20 of 2007, there are 214 EWs registered in China. They are about 1% of number of registered pesticides. Although the pesticide EW has a good development, the varieties of pesticide EW registered only occupy 1.2% of pesticide formulations registered in China. Its market share is also very limited. In China, EC is a main pesticide formulation, which accounting for about 45 %. The annual organic solvents (xylene) used in EC processing of is around 200,000 tons, resulting in massive waste of resources and tremendous pressure to environment. Thus speeding up the pesticide water emulsion instead of the EC has become one of the urgent tasks for science and technology workers.

2.7 Research summarization of *Semiaquilegia adoxoides*

2.7.1 Classification, identification and resource distribution of *S. adoxoides*

Semiaquilegia adoxoides (DC.) Makino is called thousand mouse excrements in Guizhou and Sichuan province. It has been mistakenly classified as *Aquilegia adoxoides* (DC.) Ohwi. It belongs to Ranunculaceae family. *S. adoxoides* mainly distributes in the Chang River basin or subtropical regions in China, and mainly distributes in districts of Guizhou, Hunan, Hubei, Sichuan, Jiangsu, Anhui, south of Shaanxi, Zhejiang, Jiangxi, and north of Guangxi. *S. adoxoides* likes to grow on low-woodland, bushes, sides of channel and road, shade of valley of 100-1050 m altitude.

2.7.2 Biology and ecology of *S. adoxoides*

(1) Biology of *S. adoxoides*

Biological characteristics of *S. adoxoides* (Fig 2.1) are as follows (Li Ming, 2003) : The height is 10-40 cm. The stems are slim and have sparsely short soft hairs. Leaves are trifoliate with long-petiole and have small wedge leaves of 3 deep clefts of infrequent coarse teeth with purple underside. The stem leaves are smaller. In late summer, stems leaves wither. The proboscis white flower is small, single-born on axillary or stem top. Each flower has 5 petal-shaped sepals and 5 spoon-shaped petals, with 8-14 stamens and 3-5 heart like leaves. The base of the flowers is cystic shaped. The seeds are black. Florescences last 3-4 months and the fruit begin to mature at summer. The slightly curving roots are anomalously and shortly columnar, or cambiform or massive. Length and diameter of the roots are 1~5 cm and 0.5~1 cm, respectively. The surfaces of the roots are dull to cinereous, and have the anomalous wrinkle and fibrous root or fibrous root marks. The top of the root having stem leaf residues are coated with several tan sheath shape lamellas. The root texture nature is soft, easy to be broken off. The xylem was yellow white or yellow brown, and slightly radiated. The cross section has multi-row cells, containing brown substances. The cortex is narrow with broad base. Cambium is annular and the xylem radials have cells of 20 rows. The trachea is radiatedly arranged (Fig 2.1).

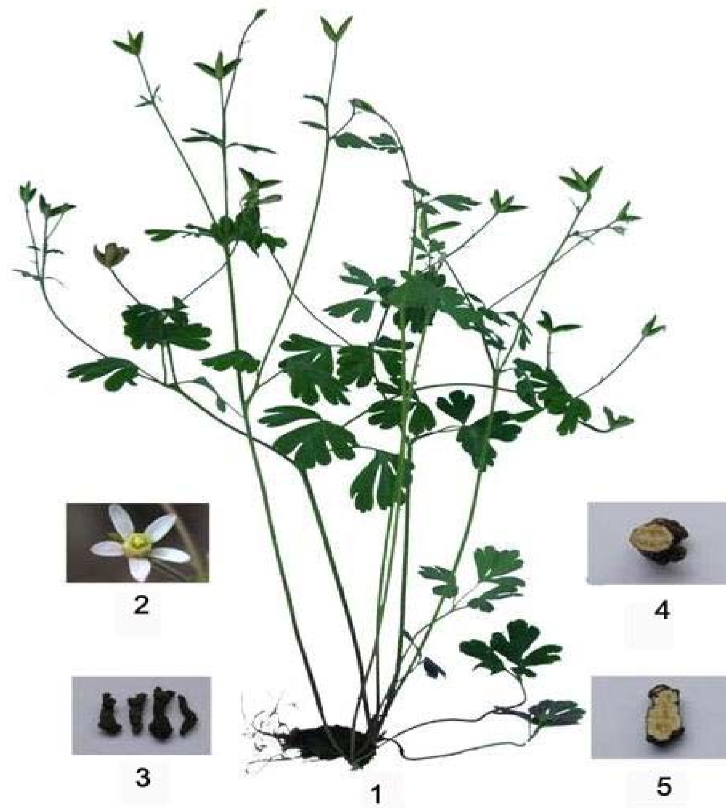

Figure 2.1 *Semiaquilegia adoxoides* (DC.) Makino

1. Stems and leaves.
2. Flowers.
3. Storage roots.
4. Cross section of the root.
5. Longitudinal section of the root.

(2) Ecological characteristics of *S. adoxoides*

S. adoxoides often grows in thin forest of mountainous regions, roadside and tomb. *S. adoxoides* likes to grow in the soil of moderate or high fertility level. Tuber and adventitious root of *S. adoxoides* distribute in the surface soil. Essential species found in *S. adoxoides* community are *Erigeron annuus* Pers., *Kqlimeris indica* L., *Commelina communis* L., *Achyranthes bidentata* Bl., *Senecio*

vulgaris L., *Sophora japonica* L., *Ligustrum lucidum* Ait., *Pinus massoniana* Lamb., *Melia azedarach* L. and *Viburnum utile* Hemsl (Li Ming, 2003).

(3) Anatomical structure of *S. adoxoides*

1) Root structure : Both adventitious roots and lateral roots have the primary structures. The periderm and the cork layer developed from the cork cambium are 7-9 cell layer containing brown substances. The cortex is narrow. The boundary of the phelloderm cell and the secondary cortex cell is not distinct, approximately has 4 cell layer of anomalous shape. The secondary bundles are 10-15 bunches. The sieve tube group is distinctive and the cambium is annular. The xylem radial has width of 20 cell layer and the trachea diameter approximately 40 μ m. The secondary structure exists in the pith (Li, 2003).

2) Stem structure : Stem cross section is circular and made up of the following several parts from external surface : l) the epidermis is composed of cells with cuticle wall and stomata. 2) cortex with multi-layered collenchymas and developed cortex parenchyma. 3) fibrovascular column composed of bundle, cumbiur and cumbiur radial. The different size vascular bundle of 6-7cells can be seen on stem cross section. The stem has a developed pith, the diameter is 1/3 of stem (Li, 2003).

3) Leaf structure : Leaf is ventro-back, has the cross-section structure of the common dicotyledon plants including three parts of the epidermis, the mesophyll and the leaf vein. l) The epidermis : both the top and bottom epidermis is composed of a cell. The stomatal apparatus distributes on the bottom epidermis, which is composed encircledly of 2 kidney-shaped guard-cells. The bottom epidermis has the superficial fair, which is composed of the single cell. 2) The mesophyll is composed encircledly of the palisade tissue and the spongy tissue. The palisade tissue

is composed of the l-2 layer column-shaped cell, whose arrangement is slightly dense and neat, there are the many chloroplasts in the cell. The spongy tissue is composed of 2-4 cells, whose the shape is anomalous and the arrangement is loose, there are few chloroplasts in the cell. 3) Leaf vein : main vein and side vein stands out undersurface. In the centre of both the main vein and the side vein, there are 4-6 bundles dispersing in the parenchyma. There is the distribution collenchyma in the bottom epidermis of the main nerve. The thin vein is not obvious (Li, 2003).

2.7.3 Chemical components and bioactivity of *S. adoxoides* extract

(1) Chemical components of *S. adoxoides* extract

S. adoxoides extract has been found to contain alkaloids, lactone, cumarin, phenolic compounds, and organic acids by using modern technology. In the study done by Zou (2004), chromatography with silica column and recrystallization were employed for isolation and purification of the compounds from *S. adoxoides* extract. The structures of the constituents were elucidated by spectral analysis. Seven compounds were isolated and identified as aurantiamide acetate (I), β-sitosterol (II), palmitic acid (III), dau-costerol (IV), cirsiumaldehyde (V), griffonilide (VI) and lithospermoside (VII). Compound I, III, V and Ⅴ were-isolated from the plants for the first time. In addition, the compounds I and V were isolated from Ranunculaceae for the fist time. 13C-NMR data of compounds I were reported in detail for the fist time. Liu (1999) also studied the chemical composition of *S. adoxoides* and two flavonoid glucosides (I and II) were isolated from the aerial part of *S. adoxoides* by column chromatography with silica gel. The structure of I, named as semiaquilinoside was established as acacetin (1-2)-O-α-L-rhamnosyl-osides by spectral methods and X-ray analysis.

(2) Biological activity of *S. adoxoides* extract

The tuberous root and the seed of *S. adoxoides* can be supplied for medicinal purposes. It has the function of disinfection and diuretic effect. There are many reports that *S. adoxoides* had been used as Chinese medicine to treat diseases such as the swollen lymphnodes, acute mastitis, swollen furuncle and blown breast carbuncle (Zhou, 1996; Cheng, 2002; Zhan et al., 2003). Active components of *S.adoxoide* have been studied by many medicine departments. Although there have been numerous records of *S.adoxoide* used as Chinese medicine, only little information related to the development of pesticide formulation has been reported. According to Chinese indigenous pesticide document, alkaloids from *S.adoxoide* have contact toxicity action to aphids, red spiders and rice borers. Inhibitors developed from exracts of *S.adoxoide* had an antifeeding effect on diamondback moth (*Plutella xylostella)* and cabbageworm (*Pieris rapae*), and a certain stomach toxicity action to *Pieris rapae* (Luo et al., 2004). But there has been no report available about the preventing and controlling effect of *S. adoxoides* extract on plant virus diseases. Li et al. (2004) studied bioactivity of chemical component of ethanol extracts of *S. adoxoides*. The results showed that the ethanol extracts had good contact poisoning function, remarkable antifeeding function and growth inhibitory action on *Pieris rapae* (Wu and Li, 2005, 2006).

CHAPTER III

EXTRACTION AND SEPARATION OF CRUDE EXTRACTS OF *S. adoxoides* ROOTS

3.1 Introduction

With the development of science and technology, there has been the new progress on the techniques of extracting and separating active components from herbal plants. Except for some common extraction methods of solvent extraction, acid or base organic solvent extraction, vapour distillation and sublimation, the new techniques of extraction and purification, such as supercritical fluid extraction, strengthening lixiviation extraction, ultracentrifugation and membrane separation and so on, have also been applied and developed. In addition, the pulverization degree of the raw material, the time and temperature of extraction, and equipments which influence the efficiency of extraction, must be taken into account (Yao et al., 2002; Xu, 1993).

In this study, the active components of *S. adoxoides* roots were extracted with two extraction procedures. First, the root powder directly extracted by petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and water using **systematic extraction.** Second, root powders of *S. adoxoides* were extracted first with 95% ethanol following by extraction with petroleum ether, chloroform, ethyl acetate, butanol, and water (**non-systematic extraction**). The total extraction rates of extracts of *S. adoxoides* were then calculated.

3.2 Materials and methods

All reagents used in this thesis were of analytical grade.

3.2.1 Collection and preparation of *S. adoxoides* plants

S. adoxoides plants used in this study were collected at south Campus, Guizhou University. After removal of the stems, leaves and roots, the roots were rinsed with clean water, air-dried in the shade and roasted in a oven for 12 hours at 45 ^{O}C. Subsequently roots of *S. adoxoides* were ground and passed through a sieve of 0.42mm mesh. The root powder was then sealed in water-repellent bags before use.

3.2.2 Extraction of active component of *S. adoxoides* root powder

(1) Systematic extraction method

Five hundred grams powder of *S. adoxoides* was transferred to a conical-bottom flask in a water-bath boiler, and extracted circularly with petroleum ether (30-60 ^{O}C). The colorless extract was filtered and the filtrates were combined. The filtrates were concentrated on a rotary evaporator to dryness. Subsequently the residual filter cake was futher extracted with chloroform, ethyl acetate, acetone, ethanol, methanol, and water, respectively. The final extract was filtered and the filtrates were concentrated with the same processes as those described for the extraction with petroleum ether. All extracts of different solvents were dried in an electric vacuum oven at 50 ^{O}C to obtain dry samples (Fig 3.1).

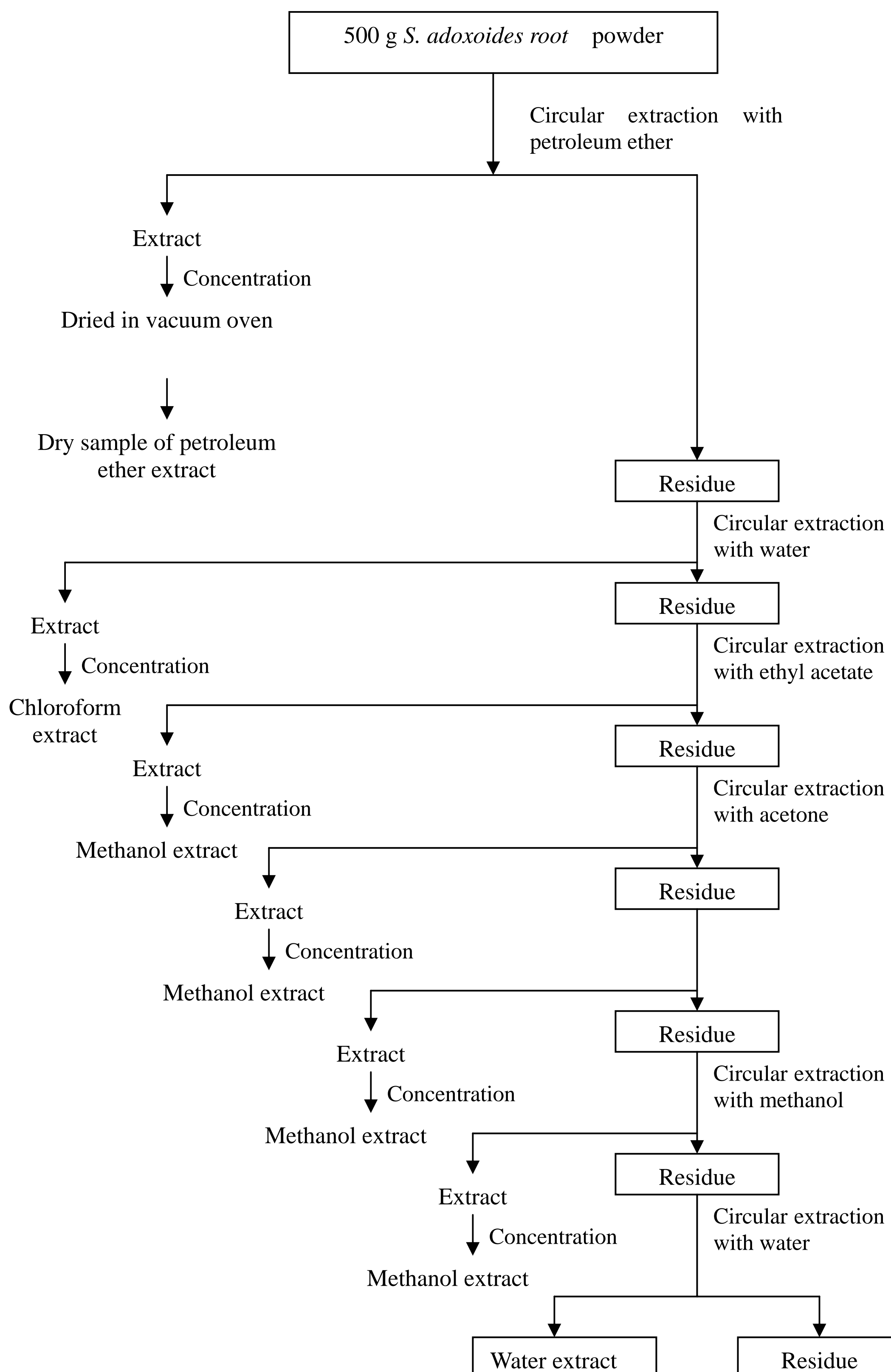

Figure 3.1 Diagram of extraction steps of *S. adoxoides* root powder by different solvents (Systematic extraction method)

(2) Non-systematic extraction method

Four hundred grams root powder were transferred to a conical-bottom flask and soaked for 72 hours in 95% ethanol. The mixtures was extracted three times at 70 °C. Subsequently the extract was filtered and the filtrate was concentrated to near dryness. The ethanol extract was futher extracted by petroleum ether, chloroform, ethyl acetate, butanol, and water respectively. Finally, petroleum ether, chloroform, ethyl acetate, butanol, and water extracts were also obtained (Fig 3.2). All extracts were stored in refrigerator at 4°C until use. The total extraction rates of petroleum ether, chloroform, ethyl acetate, butanol, and water were also calculated.

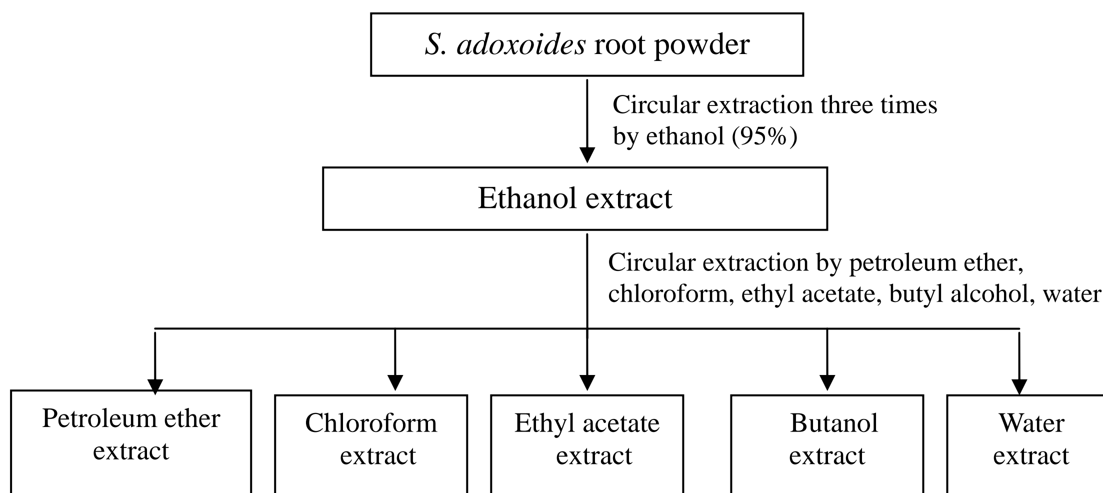

Figure 3.2 Schematic figure of extraction ethanol crude extract of *S. adoxoides* by different solvents (Non-systematic extraction method)

3.2.3 Qualitative test of extracts of *S. adoxoides* root powder

The extracts of 0.01 g each was dissolved in 10 ml water. The solution of 2 ml was transferred into a test tube and their color changes were tested with phosphor -molybdic acid, oil of vitriol, hydroboration sodium, iodizate bismuth potassium, magnesium/concentrate hydrochloric acid, and sodium hydroxide, respectively.

3.2.4 Calculate of extraction rate of *S. adoxoides* root powder

Extraction percentage of extracts with various solvent was determined according to the following formula :

$$\text{Extraction percentage } \mathbf{(\%)} = \frac{\text{total amount of the initial extract (g)}}{\text{total amount of } \textit{S.adoxoides} \text{ root powder (g)}} \times 100$$

3.3 Results

3.3.1 Extraction rate of active component of *S. adoxoides* root powder

(1) Systematic extraction

Crude extract weights and extraction rate of 500g root powder of *S. adoxoides* were shown in Table 3.1. Among the 7 solvents used, water extraction yielded the largest amount of crude extract of 90.85 g, while chloroform gave the smallest only 1.68g. The total crude extract obtained after completion of the systematic extraction from 7 solvents was 193.8 g accounting for 38.76% of the root powder weight.

Table 3.1 Extraction rate of different polar solvent extract of *S. adoxoides* root powder (Systematic extraction)

Solvent	Quantity of crude extract (g)	Extraction rate (%)
Petroleum ether	2.08	0.42
Chloroform	1.68	0.34
Ethyl acetate	5.70	1.14
Acetone	4.53	0.91
Ethanol	29.44	5.89
Methanol	59.54	11.91
Water	90.85	18.17
Total extract	**193.8**	**38.76**

(2) Non-systematic extraction

Crude extract weights and extraction rate of 400 g root powder of *S. adoxoides* were shown in Table 3.2. Among the 5 solvents used, water extraction yielded the largest amount of crude extract of 110.03 g, while ethyl acetate gave the smallest only 2.62 g. The total crude extract obtained after completion of the systematic extraction from 5 solvents was 140.56 g accounting for 35.15% of the root powder weight.

Table 3.2 Extraction rate of ethanol crude extracts of *S. adoxoides* root by different solvents (Non-systematic extraction)

Extraction solvent	Quantity of crude extract (g)	Extraction rate (%)
Petroleum ether	15.20	3.80
Chloroform	3.10	0.78
Ethyl acetate	2.62	0.66
Butanol	9.61	2.40
Water	110.03	27.51
Total extract	**140.56**	**35.15**

3.3.2 Qualitative analysis of extracts of *S. adoxoides* root

Results of the qualitative analysis of the extracts are shown in table 3.3. The colours given are those that indicator the presence of active substance reacting to the indicators. It can be seen that the petroleum ether extract contained alkaloids, whereas the chloroform extract and ethyl acetate extract contained flavones. It is uncertain those of the butanol extract and water extract contained depending on limited test.

Table 3.3 Test results of alkaloids and flavones in *S. adoxoides* root extracts of different reagents

Extracts	Reagents						Test results
	Phospho-molybdic acid	oil of vitriol	Hydroboration sodium	Iodizate bismith potassium	Magnesium powder /conc. hydrochloric acid	Sodium hydroxide	
Petroleum	Brown yellow	Maroon	No	Nacarat	Brown yellow	Brown	Alkaloids
Chloroform	Brown green	Red	Nacarat	Salmon pink	No	Buff	Flavones
Ethyl	Sepia brown	Nacarat	No	Salmon pink	No	Brown	Flavones
Butanol	No	Black	Brown yellow	Saffron	Yellow	Brown	Uncertain
Water	No	Black	Red	Red	Buff	Brown	Uncertain

3.4 Discussion and conclusions

In order to compare the efficacy of extraction methods for active component from roots of *S. adoxoides*, two extraction procedures were tested. It appears that the systematic extraction method gave a better extraction rate of 38.76% while the non-systematic extraction method gave a slightly lower rate of 35.15%, percentage of extraction by subsequent solvents were also different depending on the method used. In the systematic extraction procedure, extraction percentages of petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and water were 0.42%, 0.34%, 1.14%, 0.91%, 5.89%, 11.91% and 18.17%, respectively, but with the non-systematic extraction procedure, extraction percentages of petroleum ether, chloroform, ethyl acetate, butanol and water were 3.80%, 0.78%, 0.66%, 2.40% and 27.51%, respectively. Thus, it is indispensable to select proper extraction solvents and procedures depending on purposes of the study.

Ethanol is a hydrophilic organic solvent and the most commonly used in extraction of active components of Chinese herbal medicine due to its stronger penetration capability. Lipophilic components in herbal medicine are difficult to dissolve in water, but have a better solubility in ethanol. Petroleum ether, chloroform and ethyl acetate are lipophilic organic solvents. These solvents can not dissolve in water. Hydrophilicity of ethanol, ethyl acetate, chloroform and petroleum ether decreases in the order of ethanol > ethyl acetate> chloroform > petroleum ether. In the non-systematic extraction method of this study, Roots powder of *S. adoxoides* was extracted first with 95% ethanol following by petroleum ether and chloroform. Thus their extraction percentage was higher than those of the systematic extraction method. However, extraction percentage of ethyl acetate did not follow dissolving principle.

This is a problem need to be researched. Water is a strong polar solvent. The hydrophilic components in Chinese herbal medicine, such as salts, glucide, complexed-carbohydrate, tannin, amino acids, organic salts and base, can be dissolved by water. In this study, water extraction percentage was the highest.

The results from qualitative analysis of extracts of *S. adoxoides* showed that petroleum ether extract contained alkaloids, while chloroform extract and ethyl acetate extract contained flavones, but it is uncertain those of the butanol extract and water extract contained depending on the limited test.

CHAPTER IV

ISOLATION AND IDENTIFICATION OF CHEMICAL COMPONENTS OF EXTRACTS OF *S. adoxoides* ROOTS

4.1 Introduction

As a traditional Chinese medicine, *S. adoxoides* was embodied in "Chinese pharmacopoeia" of different editions. However, application of active components of *S. adoxoides* in agriculture has not been reported. Scientific research workers found that *S. adoxoides* contains alkaloids, lactone, coumarin, phenolic components, and organic acids etc by adopting modern analytical techniques. Liu (1999) isolated two flavonoid glucosides from the aerial part of ***S. adoxoides*** by repeated column chromatography with the silica gel. The structure of I，named as semiaquilinoside was established as acacetin (1"→2")-O-α-L-rhamnosyl-6-C-β-D-glucopyra-noside by spectral methods and X-ray analysis. Zou（2004）also isolated seven compounds from ***S. adoxoides*** through repeated chromatography with silica column and recrystallization. There is still little information available on chemical components of *S. adoxoides* roots. As a result, in this study, preliminary experiment of chemical components of *S. adoxoides* root was conducted.

4.2 Material and methods

4.2.1 Preparation of reagents

The following solvents-all were of analytical reagent grade.

(1) Ninhydrin reagent : 0.2 g ninhydrin was dissolved in 100 ml ethanol

(2) $FeC1_3$ reagent : water or ethanol solution of 5% $FeC1_3$

(3) Bromocresol green reagent : 0.1% bromocresol green in ethanol

(4) Picric acid reagent : 1 g picric acid was dissolved in 100 ml water

(5) $A1C1_3$ reagent : methanol solution of 2%$AlC1_3$

(6) Silicon tungstenic acid reagents : 5 g silicon tungstenic acid was dissolved in 100 ml water, pH 2

(7) Tannic acid reagent : 1g tannic acid and 1 ml ethanol were dissolved in 10 ml water

(8) Phosphomolybdate acid reagent : fresh ethanol solution of 5% phosphomolybdate acid

(9) Biuret reagents: solution A : 1%copper sulfate solution

Solution B : 40%NaOH solution

Mixed solution of solution A and B of the same volume

(10) Gelatin reagent : 10 g NaCl and 1 g gelatin were dissolved in 100 ml water

(11) Bismuth potassium iodide reagents :

Solution A : 0.85 g bismuth nitrate oxide dissolved in 10 ml acetic acid and diluted in 40 ml water

Solution B : 8 g bismuth oxide was dissolved in 20 ml water

Mixed solution of solution A and B of the same volume

(12) $Mg(Ac)_2$ reagent : methanol solution of 1% $Mg(Ac)_2$

(13) Hydroxybutyric acid iron reagents :

Solution A : fresh methanol solution of 1 N hydroxylamine hydrochloride

Solution B : 1.1 N potassium hydrochloride methanol solutions

Solution C : 1% solution of ferric of chloride dissolved in 1% hydrochloric acid when being used, solution A, B, C were dropped in the sample in order

4.2.2 Preparation of the test solutions

The root powder of *S. adoxoides* of 100 g was transferred to a conical-bottom flask in a water-bath boiler, and extracted circularly with ethanol of 95%. The colorless extract was filtered and the filtrates were combined. The filtrates were concentrated in a rotary evaporator to 100 ml. And ethanol crude extracts were then obtained.

(1) Preparation of petroleum ether extract (PEE) : 10 ml ethanol extract was extracted fully with 30 ml petroleum ether (60-90 ^{O}C). The petroleum ether extract was filtered and the filtrates were concentrated in a rotary evaporator to 10 ml.

(2) Preparation of chloroform extract (CE) : After extraction with petroleum ether, the lower part was extracted fully with 40 ml chloroform, the chloroform extract was filtered and filtrates were concentrated in a rotary evaporator to 10 ml.

(3) Preparation of ethyl acetate extract (EAE) : After extraction with chloroform, the upper and middle parts were extracted adequately with 50 ml ethyl acetate. The ethyl acetate extract was filtered and the filtrates were concentrated in a rotary evaporator to 10 ml.

(4) Preparation of butanol extract (BE) : After extraction with ethyl acetate, the middle and lower parts were extracted adequately with 50 ml butanol. The butanol extract was filtered and filtrates were concentrated in a rotary evaporator to 10 ml.

(5) Preparation of water extract (WE) : After extraction with butanol, the middle and lower parts were extracted adequately with 10 ml water. The extract was used as the solution WE.

4.2.3 Qualitative tests of chemical components of *S. adoxoides* root extracts

After a 2 ml aliquot of the solution PEE, CE, EAE, BE and WE was transferred to the 10 ml test tube, ninhydrin reagent, $FeC1_3$ reagent, bromocresol green reagent, picric acid reagent were added into the tube, respectively. Then hydrophobic, polar, and hydrophilic substances of the solution were tested through reaction characteristics of compounds.

4.2.4 Isolation and identification of chemical components from roots of *S. adoxoides*

(1) Extraction of chemical components from roots of *S. adoxoides*

One hundred grams of powder of roots of *S. adoxoides* were weighed into a flask of the Soxhlet apparatus. All solvents such as n-hexane, petroleum ether (30-60 ^{O}C), and ether used in this study were of analytical reagents grade. The extraction with petroleum ether was done for 12 h. After dehydration with anhydrous sodium sulphate, the extracts were collected in a 250 ml round-bottom flask, concentrated in a rotary evaporator to near dryness (Beijing Medical College (BMC). 1981).The resulting residues were then dissolved with n-hexane. After n-hexane was

evaporated, a yellow oily substance was obtained.

(2) Isolation and identification of the chemical components of *S. adoxoides*

1) Instruments

HP6890/HP5973 gas chromatography-mass spectrometry (GC-MS) (Hewlett-Packard Company, USA) with HP-5 silica capillary column (30 m× 0.32 mm ×0.25 μm) (Hewlett-Packard Company, USA) was used as the instruments.

2) Gas chromatographic conditions

Helium (99.999% in purity) of 1 ml/min was used as a carrier gas. Split (40 : 1) injection of 1 μl was carried out at 250 ^{O}C. The oven temperature was programmed as follows: initial temperature 60 ^{O}C, held for 1 min, then increased at rate of 10 ^{O}C /min to 170 ^{O}C, held for 1 min, and finally followed by 5 ^{O}C /min to 230 ^{O}C, and held for 18 min. Initial in the pressure column was 51.98 kPa.

3) Mass spectrometry conditions

The mass spectrometer was operated in an electron impact ionization mode. The operating conditions were as follows: ionization energy 70 eV; ion source temperature 230 ^{O}C; filament emission current 34.6μA; electron multiplier voltage 1859V; quality range 10～550u; and solvent was delayed for 4 min.

4.3 Results

4.3.1 Qualitative analysis of chemical components of *S. adoxoides* root extracts

(1) Hydrophobic substances of the petroleum ether extract (PEE)

The tested results were listed in Table 4.1. Hydrophobic substances of

the petroleum ether extract (PEE) considering the reaction that took place when the test regents reacted with the extract it could be concluded that the PEE contained volatile oils, steroids, and triterpenes, but not greases.

Table 4.1 Test results of hydrophobic substances of the petroleum ether extract (PEE) of ***S. adoxoides*** roots

Components	**Methods**	**Result***
Volatile oils	Quick volatilization of test solution	+
Greases	Quick volatilization of test solution	-
Steroids	5% solution of phosphorus molybdenic acid and ethanol	+
Triterpenes	Acetic acid - oil of vitriol reaction	+

* "+" represents positive reaction, with evident reaction. "-" represents negative reaction, with no evident reaction.

(2) Polar substances of the chloroform extract (CE)

Polar substances of the chloroform extract (CE) considering the reaction that took place when the test regents reacted with the extract it could be concluded that the CE contained alkaloids, organic acids, flavonoids, and anthraquinone, but not coumarin. The tested results were listed in Table 4.2.

Table 4.2 Test results of polar substances of the chloroform extract (CE) of ***S. adoxoides*** roots

Components	Methods	Result*
Alkaloids	Silicon tungstenic acid test	+
	Tannic acid test	+
	Picric acid test	-
	Bismuth potassium iodide test	+
Organic acid	pH paper test	+
	Bromocresol green test	-
Flavone	$AlC1_3$ test	+
	$FeCl_3$ test	+
	Hydrochloride-magnesium powder reaction	-
	$Mg(Ac)_2$ test	+
Anthraquinone	NaOH test	+
Coumarin	Hydroxybutyric acid iron test	-
Terpene	5% solution of phosphorus molybdenic acid and ethanol	-

* "+" represents positive reaction, with evident reaction. "-" represents negative reaction, with no evident reaction.

(3) Polar substances of the ethyl acetate (EAE)

The tested results were presented in Table 4.3. Polar substances of the ethyl acetate extract (EAE) considering the reaction that took place when the test regents reacted with the extract it could be concluded that the EAE contained alkaloids, organic acids, flavonoids, anthraquinone, and coumarin, but not terpenes.

Table 4.3 Test result of polar substances the ethyl acetate extract (EAE) of ***S. adoxoides*** roots

Component	Method	Result*
Alkaloids	Silicon tungstenic acid test	+
	Tannic acid test	+
	Picric acid test	-
	Bismuth potassium iodide test	+
Organic acid	pH paper test	+
	Bromocresol green test	-
Flavone	$AlC1_3$ test	+
	$FeCl_3$ test	+
	Hydrochloride-magnesium powder reaction	-
	$Mg(Ac)_2$ test	+
Anthraquinone	NaOH test	+
Coumarin	Hydroxybutyric acid iron test	-
Terpene	Fresh ethanol solution of 5%phosphorus molybdenic acid	-

* "+" represents positive reaction, with evident reaction. "-" represents negative reaction, with no evident reaction.

(4) Hydrophilic substances of the butanol extract (BE)

The tested results were presented in Table 4.4. Hydrophlic substances of the butanol extract (BE) considering the reaction that took place when the test regents reacted with the extract it could be concluded that the BE contained saccharide, organic acids, phenols, tannic acids, and amino acids, but not proteins.

Table 4.4 Test result of hydrophilic substances of the butanol extract (BE) of ***S. adoxoides*** roots

Component	Method	Result*
Saccharide	Molish reaction	+
Organic acids	pH paper test	+
	Bromocresol green test	+
Phenols	FeCl3 test	+
Tannic acids	Glutin test	-
	FeCl3test	+
Amino acids	Ninhydrin test	+
Proteins	Biuret method	-

* "+" represents positive reaction, with evident reaction. "-" represents negative reaction, with no evident reaction.

(5) Water-soluble substances of the water extract (WE)

The tested results were presented in Table 4.5. Water-soluble substances of the water extract (WE) considering the reaction that took place when the test regents reacted with the extract it could be concluded that the WE contained carbohydrate and organic acids, but not amino acids and peptide nor protein.

Table 4.5 Test result of water-soluble of the water extract (WE) of ***S. adoxoides*** roots

Component	Method	Result*
Amino acids	Ninhydrin test	-
Proteins, peptide	Biuret method	-
Saccharide	Molish reaction	+
Organic acids	pH test paper test	+
	Bromocresol green test	+

* "+" represents positive reaction, with evident reaction. "-" represents negative reaction, with no evident reaction.

4.3.2 Isolation and identification of chemical components

The results from analysis of the GC-MS total flow diagram indicate that there were 31 isolated substances of the volatile oil from roots of *S. adoxoides*. The chemical structures of 12 substances were confirmed by the method of searching NIST98 MS Gallery using G1701BA chemical workstation and consulting the relative literatures (Cong Pu-zhu,, 1987; Su Yan-fang et al., 2006; Zou Jian-hua and Jun Jun-han, 2004). Total ion flow diagram was shown in Fig. 4.1.

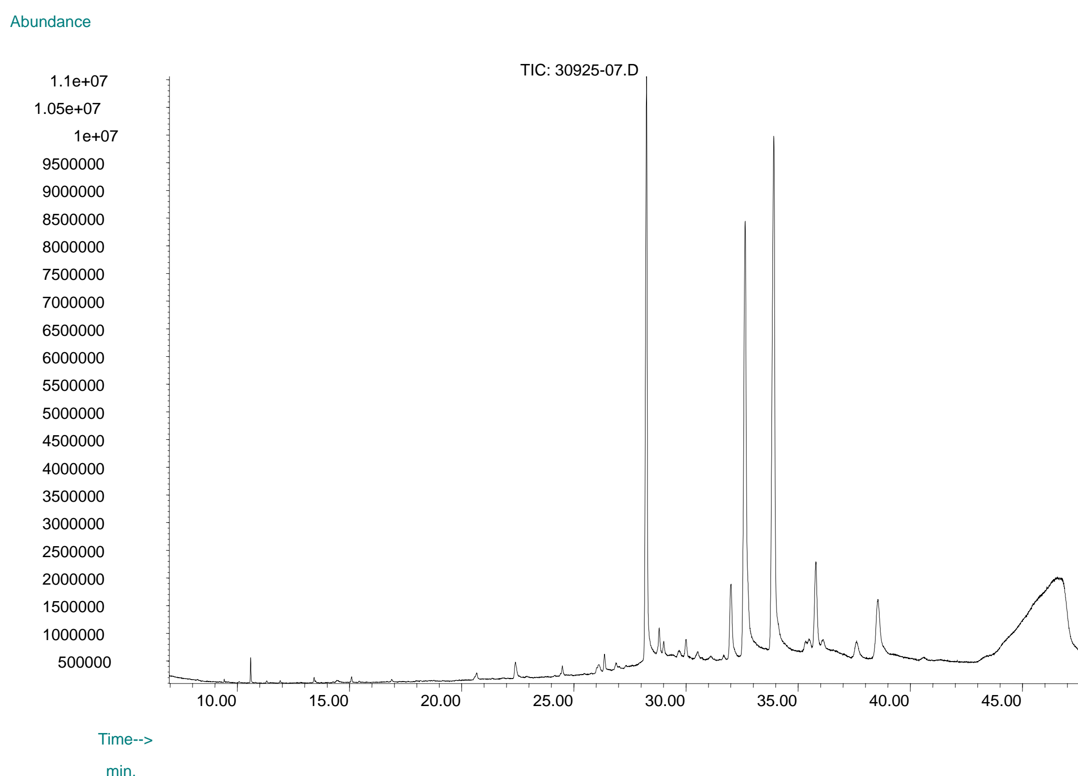

Figure 4.1 GC-MS total ion flow diagram of volatile oil in root of *S. adoxoides*

4.3.3 Content of chemical components of the volatile oil of *S. adoxoides* root

Content of each component of the volatile oil from roots of *S.*

adoxoides was confirmed by the method of peak area normalization method. The results of the analysis were shown in Table 4.5. It can be seen that content of the components detected accounted for 90.55% of the volatile oil in roots of *S. adoxoides.* They were mainly fatty acid compounds, including β-sitosterol (38.26%), linoleic acid (18.73%), oleic acid (15.68%) and palmitic acid (13.51%) (Cong, 1987; Chemistry and Chemical Industry Dictionary Editorial Board (CCIDEB, 2003).

Table 4.6 Chemical component of volatile oil from roots of *S. adoxoides*

No.	RT/min	Compound	Molecular formula	Relative mole
1	10.06	Hexanoic	$C_6H_{12}O_2$	116
2	13.43	Ocfanoic	$C_8H_{16}O_2$	144
3	15.10	Nonanoic	$C_9H_{18}O_2$	158
4	24.49	Myrisfic	$C_{14}H_{28}O_2$	228
5	26.37	Pentadecylic	$C_{15}H_{30}O_2$	242
6	28.25	Palmitic	$C_{16}H_{32}O_2$	256
7	30.01	Margaric	$C_{17}H_{34}O_2$	270
8	32.01	Stearic acid	$C_{18}H_{36}O_2$	284
9	32.64	Oleic acid	$C_{18}H_{34}O_2$	282
10	33.92	Linoleic	$C_{18}H_{32}O_2$	280
11	37.61	Arachidic	$C_{20}H_{40}O_2$	312
12	46.56	β-sitosterol	$C_{29}H_{50}O$	414

4.4 Discussion and conclusions

The preliminary experiment results of chemical components of crude extracts of *S. adoxoides* indicate that *S. adoxoides* root contain saccharide, organic acids, anthraquinone, phenols, volatile oils, and may contain alkaloids, flavonoids, coumarin, amino acids, tannin, steroids, triterpenes, and not contain proteins, peptides and lipids.

In this study, the results of chemical components of crude extracts of *S. adoxoides* was consistent with the research results of other literatures reporting that there were alkaloids, lactone, coumarin, phenols and organic acids, and in extracts of *S. adoxoides*.

High-grade fatty acid contained in roots of *S. adoxoides* is an important industrial raw material. Its main component β-sitosterol has functions of antiinflammatory, antibacterial growth and antiulcer, lowering cholesterol, cooling, and antitumor (Information Center Station of Traditional Chinese and Herbal Drugs of State Pharmaceutical Administration (ICSTCHDSPA), 1986). Components of volatile oil in roots of *S. adoxoides* also can be used to manufacture cosmetics such as pilatory perfume, shampoo, nutrition cream and so on. In addition, it can promote growth and increase productivity of animals (Yan and Ji, 2000). The experimental results in the present study were supposed to have certain guiding significance for development and use of *S. adoxoides* resources.

According to previous reports, components contained in roots of *S. adoxoides* also have insecticidal and fungicidal functions (Li et al., 1997; Luo et al., 2004; Wu and Li, 2005; Wu and Li, 2006). Currently, roots of *S. adoxoides* are one of the traditional Chinese medicines used to treat mumps, which is caused by Rubulavirus. (CUPEB, 1959; JNMC, 1975). Whether or not it could be used to control virus diseases in plants are yet to be determined. The chemical components found in this study will be further tested for bioactivity on tobacco mosaic virus (TMV). Research in direction of chemical components of other extracts by GC-MS is underway in our laboratory.

CHAPTER V

BIOASSAY OF ANTI-TMV ACTIVITY OF *S. adoxoides* EXTRACTS BY HALF LEAF METHOD

5.1 Introduction

There are many ways to determine anti-TMV activity. In this study, half leaf technique was used to determine anti-TMV activity on heart leaf tobacco (*Nicotiana glutinosa* L.). This method has advantages of appearance rapidness of a local lesion, symptoms reaction stability, easy obtaining experimental data and rapid screening of bioactive substances. These will provide the theoretic reference value for extraction and separation of extracts of *S. adoxoides* root and show good prospects for research and development of the plant source anti-virus agent.

5.2 Materials and methods

5.2.1 Host Plants and test virus

(1) Host Plants

1) Local lesion host : Heart leaf tobacco (*Nicotiana glutinosa* L.), wild species, an assay host for TMV. Its seed was purchased from Tobacco Institute of Qingzhou, Tobacco Co. LTD of China.

2) Systemic host : a general tobacco variety K326 (*N. tabacum* L.) also purchased from Tobacco Institute of Qingzhou is a systemic host of tobacco mosaic virus (TMV).

(2) Test virus

TMV common strain : obtained from Wuhan Virus Institute of Chinese Academy of Sciences, propagated and preserved in the ordinary tobacco K326 plant and kept in insect-proof greenhouse.

(3) Control pesticide

20% Virus A WP of 0.1g was diluted to 100 times (10.0 mg/ml), 20% Virus A (moroxydine hydrochloride copper acetate) WP was purchased from Heilongjiang • Qiqihar Siyou Chemical Industry Co.LTD, Heilongjiang, China.

5.2.2 Preparation of extracts of *S. adoxoides*

The appropriate amount of organic solvents and surfactants was added into petroleum ether, chloroform, ethyl acetate, acetone, and ethanol, methanol and water extract of 0.1 g. The resulting mixture were fully dissolved, mixed, and diluted to 10 ml with distilled water (10.0 mg/ml). The organic solvent and the surfactants were used as blank control. Control pesticide : virus A of 0.1 g was diluted to 100 times (10.0 mg/ml).

5.2.3 Purification and preparation of TMV

(1) Preparation of buffer

Phosphate buffer (0.01 mol/l, pH 7.2) : potassium dihydrogen phosphate (KH_2PO_4) of 0.2 g and sodium dihydrogen phosphate (Na_2HPO_4) of 2.9 g were dissolved in water of 1000ml.

Phosphate buffer (0.2 mol/l, pH7.0) : sodium dihydrogen phosphate (Na2HPO4) of 1.732 g and potassium dihydrogen phosphate (KH_2PO_4) of 1.06 g were dissolved in water of 1000 ml.

(2) Purification of TMV

Purification of TMV was performed according to the methods described by Gooding and Hebert (1967) and Chen and Feng (2001). It was slightly modified.

After 20 days of inoculation, One hundred gram fresh leaf blades of tobacco K326 infected with TMV were cut off and the veins were removed. The leaf blades were homogenized with 0.2 M phosphate buffer solution pH 7.0 containing 1% mercaptoethanol in a blender. Crude suspension of TMV was added 8% N-butanol, stirred for 15 min, centrifuged for 20 min at 4 ^{O}C (6000 rpm). Then 4% sodium chloride and 4.0% polyethylene glycol were added in supernatant and stirred for 1-1.5h, centrifuged for 20min at 4 ^{O}C (8000 rpm). The deposit was added 0.01 M phosphate buffer pH 7.2 and suspended again, stirred for 1h, centrifuged for 20 min at 4 ^{O}C (8000 rpm), supernatant were added 4% sodium chloride and 4.0% polyethylene glycol, and stirred for 1-1.5h, centrifuged for 20 min at 4 ^{O}C (8000 rpm). Finally, the deposit was suspended with 0.01 M phosphate buffer and suspended again, stirred for 1h, then centrifuged for 5 min at 4 ^{O}C (8000 rpm), the purified TMV supernatant was gained (Fig 5.1).

(3) Calculation of concentration of TMV

Concentration of TMV preparation is calculated from the extinction coefficient (E) 0.1% 1 cm 260 nm of 3.1. Formulation is as follow :

The virus concentration (mg/ml) $= A_{260} \times \text{dilution} / E^{0.1\%}{}_{1cm}$ 260nm (Tian et.al., 1987).

In the expression, E is extinction coefficient. Namely, when wavelength is 260 nm, absorption value of the suspension whose concentration is

0.1% (1 mg/ml) and light-path is 1 cm. For quantification of the unknown virus, the pure virus is first dried and weighed and diluted into the suspension whose concentration is known, then its optical density value is determined at 260 nm. Finally the value is converted to $E^{0.1\%}$ 1 cm. The width of measuring cuvette is 1 cm, if the width of measuring cup is 0.5 cm; the actual optical density value is 2 times of the obtained value.

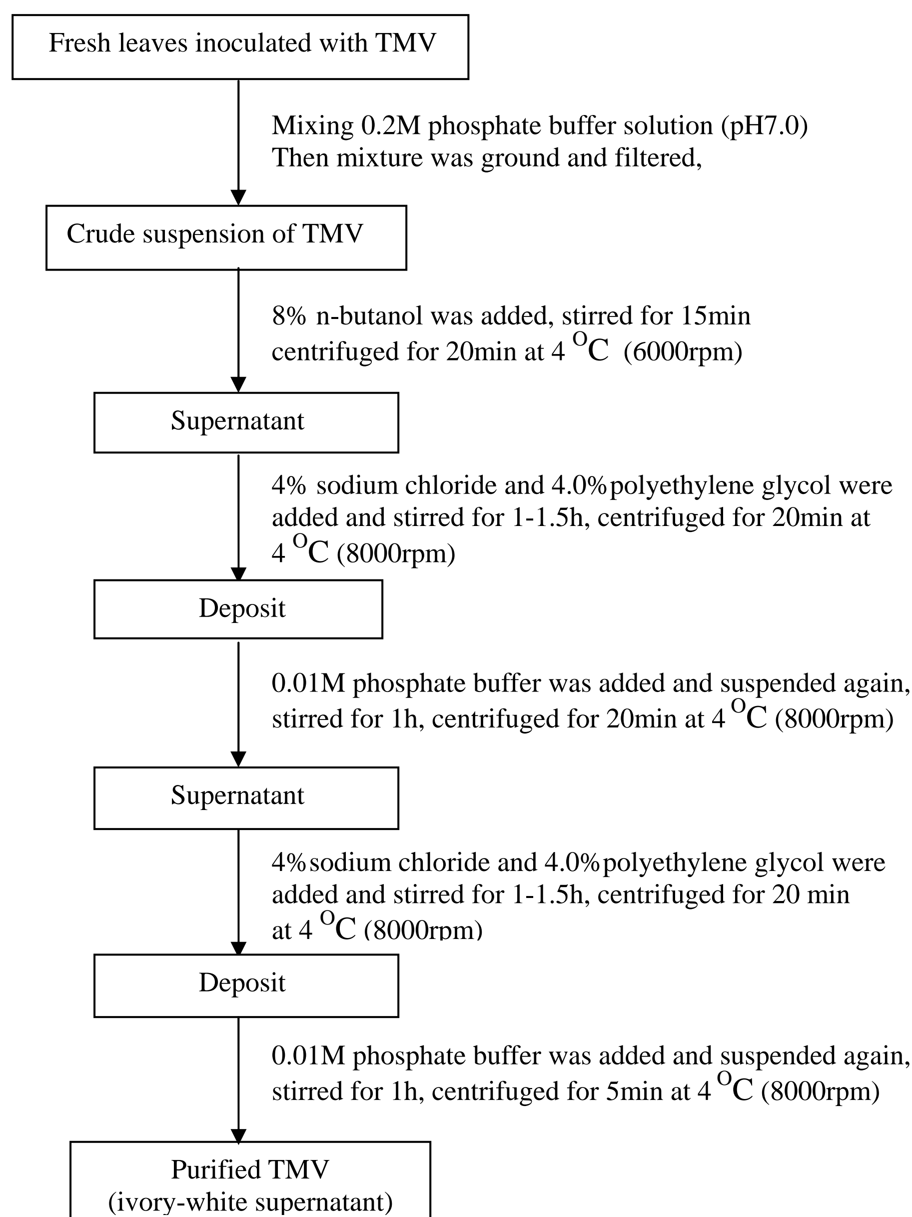

Figure 5. 1 The drawing of purification of TMV

(4) Choice of the optimal concentration of TMV

The heart leaf tobacco *N. glutinosa* was used as test material to determine biological activity of against infection of TMV. The purified TMV suspension was diluted to 100, 200, 500, 1000 and 1500 times with 0.01 M phosphate buffer pH 7.0. After silicon carbide of 400 meshs being added, the diluted suspensions were mechanically inoculated on the leaf of *N. glutinosa*, five leaves per suspension. The experiment was done in 3 replications. The inoculated tobacco plants were incubated for 2-3 days at 25 ^{O}C until lesion appeared on the leaves. The concentration that gave 15-60 lesions/leaf was considered an optimal concentration.

5.2.4 Anti-TMV activity of *S. adoxoides* extracts on *N. glutinosa*

Methods of Bioassay have referred to Fukami and Uesugi Kangyan, 1981.

A symmetrical and green leaf was split into two halves of left and right from the midrib with scissors. Then two half leaves were kept in the wetting absorbent paper in the porcelaneous dishes before use. The treatment of the bioassay was used as following :

1) Dilution of crude extracts of *S. adoxoides* : The appropriate amount of organic solvents and surfactants was added into petroleum ether, chloroform, ethyl acetate, acetone, and ethanol, methanol extract of 0.1 g, the resulting mixture were fully dissolved, mixed, and diluted to 10 ml with distilled water (10.0 mg/ml).

2) Control pesticide : 20% virus A WP of 0.1 g was diluted to 100times (10.0 mg/ml).

3) Blank control : The same quantity of the organic solvent and the

surface active agent were dissolved in water of 10 ml.

(1) In vitro inactivation activity

Leaves of *N. glutinosa* were split into two halves of left and right from the midrib with scissors and kept in wet absorbent paper in porcelaneous dishes before use. The extract solutions of *S. adoxoides*, the blank control, and 20% Virus A were mixed with the TMV suspension of 10.19 µg/ml, and left standing for 30 min. subsequently, the mixture was inoculated on the half-leaf using silicon carbide as an abrasive. The extract solution of *S. adoxoides* and 20% Virus A containing TMV were inoculated on the left half, and the mixture of blank control and TMV suspension was inoculated on the right half. The experiment was done in a complete randomized design (CRD) on 3 replicates of 5 half leaves.

(2) In vitro protection activity

The left and right half leaf were dipped for 1min in treatment and control solutions. After the excess solution was absorbed with absorbent paper and left for 2h, the TMV suspension of 10.19 µg/ml was inoculated on the leaves. The experiment was done as described in inactivation activity.

(3) In vitro curing activity

The TMV suspension of 10.19 µg/ml was inoculated on leaves of *N. glutinosa*. After 2 h, the leaf was split into two halves from the midrib with sterilized scissors. Extracts of *S. adoxoides* and 20% Virus A were treated on the left half leaf. The blank control was treated on the right half leaf. The experiment was done as described in inactivation activity.

The inoculated leaves were kept in the same dishes at 25 ^{O}C. Water was added regularly to keep the leaves fresh.

5.2.5 Bioassay of anti-TMV activity of extracts of *S. adoxoides* in the pot trial

(1) In vivo protection activity

Petroleum ether, chloroform, ethyl acetate, butanol, water extract and virus A of 10.0 mg/ml was transferred gently onto the left half leaf of the symmetry of *N. glutinosa* tobacco with brush pen. The blank control was also transferred onto the right half leaf of the same leaf. After treatment of 2h, TMV was inoculated on the leaf, respectively.

(2) In vivo curing activity

TMV was inoculated gently on the leaf of the symmetry local lesion tobacco *N. glutinosa* with brush pen. After inoculation of 2h, petroleum ether, chloroform, ethyl acetate, butanol, water extract and virus A of 10.0mg/ml was transferred gently onto the left half leaf, and blank control was also transferred onto the right half leaf of the same leaf with brush pen, respectively.

All above each treatment had three individual plants whose three leaves were treated. Then three replicates were prepared for each treatment.

5.2.6 Calculation of inhibiting rate

When numbers and size of local lesion kept stable and was clear, numbers of local lesions were record in time and inhibiting rate was then calculated according to the following formula :

$$\text{Inhibiting rate\%} = \frac{\text{average lesion number of right half leaf} - \text{average lesion number of left half leaf}}{\text{average lesion number of right half leaf}} \times 100$$

In the formula, average local lesion number was the average value of three replicates.

5.3 Results

5.3.1 TMV purification and determination of optimal concentration for bioassay

After completing the purification cycle, partially purified TMV preparation was obtained. The virus preparation was kept as suspension in 0.01 M phosphate buffer pH 7.0 at the concentration of 10.19 mg/ml.

After being diluted to 100, 200, 500, 1000 and 1500 times with inoculating buffer, the 1000 times gave the optimum number of local lesions of 48.6/leaf when inoculated onto *N.glutinosa*. At 500 times and lower dilutions, the local lesions were too numerous. While 1500 times, there were local lesions 14.4 /leaf only. As a result the 1000 times dilution having 10.19 μg/ml TMV concentration was used for bioassay (Table 5.1).

Table 5.1 Numbers of necrotic spot in *N.glutinosa* inoculated by different concentrations of TMV

Dilution time	100	200	500	1000	1500
Leaf number	5	5	5	5	5
Average number of local lesions	Local lesions merged into film	Local lesions merged into film in some sort	103.2 Much number	48.6 Optimum number	14.4 Fewer number

5.3.2 In vitro bioactivity of extracts of *S. adoxoides* root to TMV

(1) In vitro inactivation activity

Extracts from seven solvents appeared to have different inactivation

activity ranging from 34.12% to 68.09% (Table 5.2). Among them, the petroleum ether extract gave the highest inactivation activity while the water extract gave the lowest. Compared to the activity of 20% Virus A WP, the commercial pesticide, the activities of petroleum ether extract, acetone extract and ethanol extract were significantly higher. The three extracts had inactivation percentages of 68.09, 63.28 and 60.59, respectively, while Virus A had only 51.16.

Table 5.2 In vitro inactivation activity of different extracts of *S. adoxoides* root after being inoculated with 10.19ug/ml TMV mixed with 0.01g/ml extract

Extract treatments	Average local lesions of treatment	Average local lesions of control [1]	Inactivation rate [2] (%)
Petroleum ether	97	304	68.09 a
Chloroform	167	257	35.02 e
Ethyl acetate	186	311	40.19 e
Acetone	123	335	63.28 ab
Ethanol	80	203	60.59 bc
Methanol	108	234	53.85cd
Water	195	296	34.12 e
virus A	148	303	51.16 d

Note : 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

(2) In vitro protection activity

Extracts from seven solvents appeared to have different protection activity ranging from 17.48% to 65.26% (Table 5.3). Among them, the petroleum ether extract gave the highest protection activity while the water extract gave the lowest. Compared to the activity of pesticide control Virus A, the activity of petroleum ether extract was significantly higher. The activities of acetone extract and

chloroform extract were 45.35% and 39.29%, which were not significantly different from that of the pesticide control.

Table 5.3 In vitro protection activity of 0.01g/ml different extracts of *S. adoxoides* root before being inoculated with 10.19ug/ml TMV

Extract treatment	Average local lesions of treatment[1]	Average local lesions of control[1]	Protection rate[2] (%)
Petroleum ether	99	285	65.26 a
Chloroform	187	308	39.29 b
Ethyl acetate	192	283	32.16 c
Acetone	147	269	45.35 b
Ethanol	243	337	27.89 cd
Methanol	187	240	22.08 de
Water	203	246	17.48 e
Virus A	171	311	45.02 b

Note : 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(3) In vitro curing activity

Extracts from seven solvents appeared to have different curing activity ranging from 9.01%-34.24% (Table 5.4). Among them, the acetone extract gave the highest curing activity while the water extract gave the lowest. Compared to the activity of pesticide control Virus A, the activity of acetone, petroleum ether, ethanol and ethyl acetate were 34.24%, 21.03%, 20.28% and 19.93%, respectively, which were significantly higher. The activities of chloroform, methanol and water extract were lower than that of other’s, which were not significantly different from that of the pesticide control Virus A.

Table 5.4 In vitro curing activity of 0.01g/ml different extracts of *S. adoxoides* root after being inoculated with 10.19ug/ml TMV

Extract treatment	Average local lesions of treatment	Average local lesions of control [1]	Curing rate [2] (%)
Petroleum ether	169	214	21.03 b
chloroform	225	258	12.79 cd
Ethyl acetate	241	301	19.93 b
Acetone	169	257	34.24 a
Ethanol	173	217	20.28 b
Methanol	236	274	13.87 c
Water	212	233	9.01 d
Virus A	259	292	11.30 cd

Note : 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

5.3.3 In vivo anti-TMV activity of extracts of *S. adoxoides* root in the pot

(1) In vivo protection activity

Extracts from five solvents appeared to have different protection activity ranging from 23.98% to 42.04% (Table 5.5). Among them, the Chloroform extract gave the highest protection activity while the water extract gave the lowest, compare to the protection activity of 20% Virus A WP, the commercial pesticide, the activities of chloroform extract and ethyl acetate extract were significantly higher. There two extracts had protection percentages of 42.04 and 41.83 respectively while Virus A had only 31.28.

Table 5.5 In vivo protection activity of 0.01g/ml different extracts of *S. adoxoides* root before being inoculated with 10.19ug/ml TMV

Extract treatment	Average local lesions of treatment	Average local lesions of control [1]	Protection rate [2] (%)
Petroleum ether	73	117	37.14 ab
Chloroform	68	119	42.04 a
Ethyl acetate	71	122	41.83 a
Butanol	75	108	30.47 bc
Water	81	107	23.98 bc
Virus A	75	110	31.28 bc

Note : 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) In vivo curing activity

Results of in vivo curing activity of extracts of *S. adoxoides* showed that after inoculation of 2 h, extracts of *S. adoxoides* had good curing activity to TMV under condition of the pot experiment, with inhibition ranging from 20.86%-72.36%. Ethyl acetate extract had the best treatment effect. Its inhibition rate was up to 72.36%, which was significantly higher than pesticide of control Virus A. Petroleum ether and chloroform extract also had high treatment effect, with treating rate of 50.26 % and 47.01%, respectively, however, there was no significant difference to be found, compared to pesticide of control (Table 5.6)

Table 5.6 In vivo curing activity of 0.01g/ml different extracts of *S. adoxoides* root after being inoculated with 10.19ug/ml TMV

Extract treatment	Average local lesions of treatment [1]	Average local lesions of control [1]	Curing rate [2] (%)
Petroleum ether	64	129	50.26 b
Chloroform	63	120	47.01c
Ethyl acetate	34	123	72.36 a
Butanol	79	102	25.57 d
Water	88	112	20.86 d
virus A	47	108	56.61 b

Note : 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT.

5.4 Discussion and conclusions

The roots of *S. adoxoides* have been used as Chinese herbal medicine for years; however, anti-virus activity of their extracts of *S. adoxoides* has never been reported. In this study, it was the first time that the inactivation, protection and curing activities on TMV of *S. adoxoides* extracts were found in vitro. Among the seven extracts, petroleum ether, acetone and ethanol extracts had higher inactivation activity than that of the rest. Their inhibiting rates to TMV were 68.09%, 63.28% and 60.59%, respectively.

The petroleum ether extract still showed the best performance when tested for protection activity in which it gave 65.26% inhibiting rate. With the same test, acetone extract seem to loose its efficacy slightly but still second rank while the ethanol extract did not perform that well. Considering the similarity in term of the chance of active chemicals in the extract contacting with the virus particles in

inactivation and protection experiments, it is expected that the performance of each extract should be the same regardless of the test. The activities of both petroleum ether and acetone extracts do support the assumption. The decrease in activities of acetone and ethanol extracts in the protective activity tests could result from the poorer stability of the different active chemicals in extracts compared to that obtained by petroleum ether extraction.

Extracts from many higher plants contain substances which inhibit infection by viruses. For example, sap from species of *Phytolacca* is one of the most potent inhibitors, which isolated from the sap of *P.esculenta* and be found that it had the composition expected for a glycoprotein. Under appropriate conditions it precipitated TMV as paracrystalline threads, and also precipitated tomato bushy stunt virus. Phenolic substance may react with the inactive protein in various ways. Many rosaceous species are commonly infected with a range of viruses and yet the extracts of these plants are usually potent virus inhibitors. Water extracts from macerated leaves, stems, or roots of strawberry plants contain sufficient tannins to precipitate all the plant proteins in extract. If this precipitate is removed the supernatant fluid still contains enough tannin to precipitate added TMV and make it noninfectious for *Nicotiana glutinosa* (Chandniwala. 2002).

A different performance was observed when the extracts were tested for their curing activity. With their test, whether or not the active chemicals are effective depending on both their action on the virus and on the plant. The three extracts still performed considerable well but the petroleum ether extract has lost its first rank to the acetone extract. It is interesting to note that all the extracts as well as the Virus A seem to have much lower curing activity when compared to that of the inactivation

and protection activities. This indicates that the active chemicals in the extract must be in contact with the virus particles in order to express their full capacity in inhibiting the virus.

The results of the pot experiment showed that protective inhibiting rate of chloroform, ethyl acetate and petroleum ether extract of *S. adoxoides* to TMV was 42.04%, 41.83% and 37.14 %, respectively. Ethyl acetate, petroleum ether and chloroform extract also had high curing effect, with treating rate of 72.36%, 50.26 % and 47.01%, respectively. Ethyl acetate extract had the highest inhibition rate of 72.36%, which was significantly higher than pesticide of control Virus A.

CHAPTER VI

APPLICATION OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SCREENING ANTI-TMV ACTIVITY OF EXTRACTS OF *S. adoxoides* ROOT

6.1 Introduction

In order to prevent and control tobacco mosaic virus disease, it is needed to detect state of tobacco mosaic virus in time. Although biological assay would serve this purpose, they often time-consuming, lack of precision, and difficult to achieve early diagnosis. An alternative one is serological assay. In the rencent 10 years, serological assay has been applied in many domains and has got rapid progress. Serological assay can detect minimum viral antigen in plants. Thus it can be used for early diagnosis of tobacco mosaic virus disease both in plants and seeds.

Enzyme linked immunosorbent assay (ELISA) is an assay that combine enzyme labeled compound and the immune reaction of antigen-antibody complex. It can keep both the sensitivity of catalytic reaction and the specificity of the antigen-antibody reaction. Thus the sensitivity is greatly improved. In addition, it is a non-homogeneous analysis process. Namely, the reaction of each step is followed by a washing process. As a result, the non-reactive substances interference can be excluded. ELISA is being widely applied in diagnosis and determination of virus due to its high sensitivity (concentration of 1-10 mg/ml can be detected), specificity and Easy operation (Sheng, 1978; Ma et al., 1981). This study was further conducted

based on the previous section of preparatory screening of anti-TMV activity of extracts of ***S. adoxoides*** roots using an indirect ELISA. The first antibody was the specific anti-serum corresponding with virus needed to be detected. The second antibody was enzyme labeled antibody specific to the first antibody.

6.2 Materials and methods

6.2.1 Test reagents and buffer preparation

Main regents and buffer preparation are in appendix.

6.2.2 Experimental methods

(1) In vivo protective activity of root extracts of *S. adoxoides*

In *vivo* protective activity of root extracts of *S. adoxoides* was divided into following two sub-projects.

a. The tobacco K326 (*Nicotiana tabacum*) having the same growing trend was chosen as host plant. The *S. adoxoides* root extract of 10.0 mg/ml each, Virus A, and check solution were spread on the top 3-6 leaves with brush. After 1 day, TMV suspension of 13.0 μg/ml concentration was inoculated on the leaf. At the end of 1, 3, 5, 7 and 9 days after inoculation, sampling and ELISA assay were performed, respectively. Each treatment was done in 3 replications (3 plants) of 3-leaves/plant using complete randomized design (CRD) design.

b. The *S. adoxoides* root extract of 10.0 mg/ml each, Virus A, check solution were spread on the top K326 (*Nicotiana tabacum*) 3-6 leaves with brush. After 1, 3, 5, 10 and 15 days, TMV suspension of 13.0 μg/ml concentration was inoculated on the leaf. At the end of 3 days after inoculation, sampling and ELISA assay were performed, respectively. Each treatment was done in 3 replications (3

plants) of 3-leaves/plant using CRD design.

(2) In vivo curing activity of root extracts of *S. adoxoides*

The tobacco K326 (*Nicotiana tabacum*) having the same growing trend was chosen as host plant. TMV suspension of 13.0 µg/ml concentration was inoculated on the top 3-6 leaves. After 1-day inoculation, the *S. adoxoides* root extract of 10.0 mg/ml each, Virus A, and check solution were spread on the top 3-6 leaves with brush. At the end of 1, 3, 5, 7, 9-day after inoculation, sampling and ELISA assay were performed, respectively. Each treatment was done in 3 replications (3 plants) of 3-leaves/plant using CRD design.

6.2.3 Preparation of samples and ELISA assay

At the specified time 0.05 g of tissue was collected from the inoculated leaves having one plant represented one replication. Subsequently the tissue was homogenated in 1 ml of carbonate coating buffer filtered through cheesecloth and centrifuged. The resulting supernatant was then assayed for TMV by Direct antigen coating indirect (DAC-indirect) ELISA as followed.

One hundred microlitre of the samples was put into each well of a microtiter plate and incubated in a moist chamber one night at 4 ^{O}C. After decanting, the wells were washed 3 times with PBS-T, 2 minutes each time. The 100µl of 1 : 1000 TMV antiserum diluted in conjugate buffer was put in the wells and incubated at 37 ^{O}C for one hr. The wells were washed again with PBS-T followed by adding 1 : 10000 horse radish peroxidase conjugated antirabbit IgG antiserum in conjugated buffer 100µl in each well. The plate was incubated again for 1hr at 37 ^{O}C. After being washed with PBS-T, OPD and H_2O_2 in substrate buffer was put in the wells, 100 µl each and incubated at 37 ^{O}C for 15min. in the dark. Stop and acidified the mixture

with 20 µl of 1N HCl. Leave standing for 1min. then read the reaction in ELISA plate reader at 492 nm.

6.3 Results

6.3.1 In *vivo* protective activity of extracts of *S. adoxoides*

(1) Absorbance of TMV inoculated tobacco collected at different time after being treated with the extract

It can be seen from Fig 6.1 that with the increase of time, A_{492} value increased. After 7days, the A_{492} values of TMV were slightly decreased. After being treated with extracts of petroleum ether, chloroform, ethyl acetate and water. A_{492} value of TMV reached the maximum value at 5 days while that of positive control, butanol and Virus A reached maximum at 3 days. The results suggested that the replication of TMV was delayed after tobacco was treated with PE, C, EA and water extract. For all treatments, when A_{492} values of TMV reached the maximum which ranged from 0.073 to 0.305. A_{492} value of TMV was the lowest (0.073) for chloroform extract, which indicated that chloroform extract had the best protective effect. The protective effect of petroleum ether extract, ethyl acetate extract and water decreased in the order.

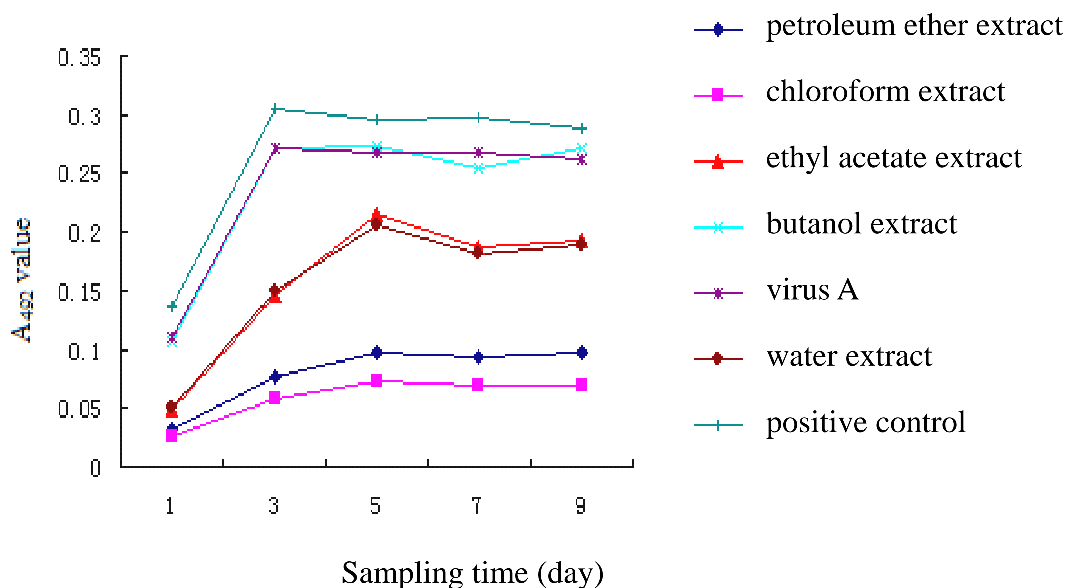

Figure 6.1 A_{492} of TMV inoculated tobacco collected at different time after being treated with *S. adoxoides* root extract (Protection activity)

(2) Absorbance of TMV inoculated tobacco at different time after interval treatment with root extract

The *S. adoxoides* extract of 10.0 mg/ml, virus A, CK solution were spread on leaf with brush after 1, 3, 5, 10 and 15 days, TMV was inoculated on the leaf. At the end of 3-day inoculation, sampling and determination were performed, respectively.

Content of TMV was the highest, with the extension of inoculation after treatment. Infection of TMV presented the increase trend, which mean that inhibiting effect of the extracts to TMV was negatively correlated with A_{492} value of TMV (Fig 6.2). At 1day after treatment with chloroform, petroleum ether, ethyl acetate, butanol extracts, and virus A. The corresponding A_{492} value of TMV was 0.053, 0.093, 0.150, 0.267, 0.272 and 0.146, respectively. After comparison, it can be found that the protective effect of chloroform extract and petroleum ether extract were

the best and that of ethyl acetate extract, butanol extract, and Virus A decreased in the order.

Inoculation of TMV was then carried out after a certain period of treatment with inhibitors, the inhibiting ability of inhibitors to TMV was weakened. Inhibiting ability of extracts of *S. adoxoide* to TMV was negatively correlated with the A_{492} value of TMV. A_{492} value of TMV was 0.296, 0.298, 0.294, 0.300, 0.301 and 0.298 for chloroform extract, petroleum ether extract, ethyl acetate extract, butanol extract, and virus A, respectively. Ethyl acetate, chloroform, petroleum ether and butanol extracts, and Virus A had the lasting of protective action of 18, 13, 13, 8 and 8 days, respectively.

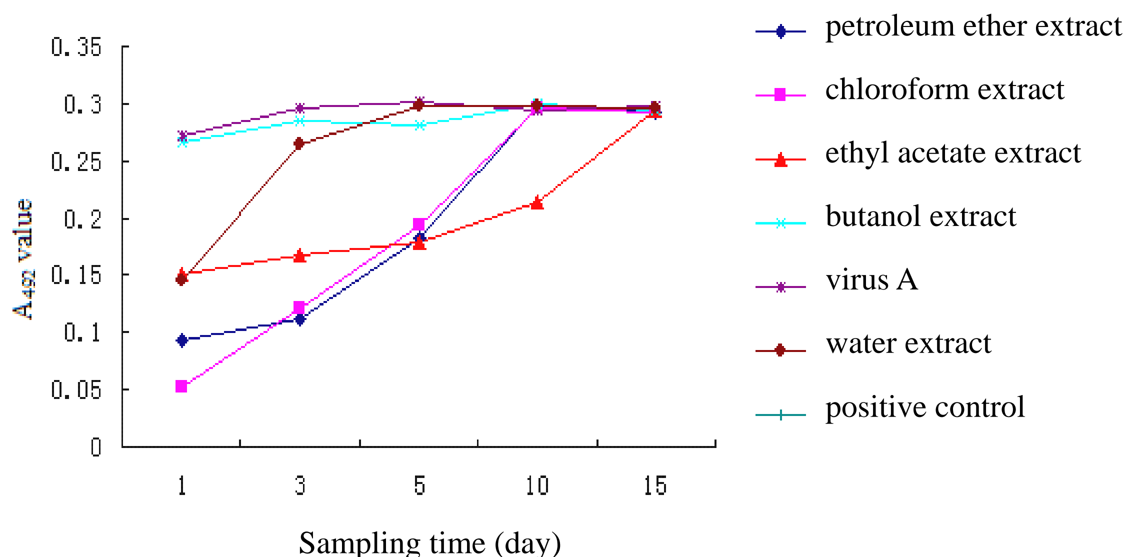

Figure 6.2 A_{492} of TMV inoculated tobacco sampled 3 days after TMV inoculation and treated with extracts at different time (Protection activity)

6.3.2 In *vivo* curing activity of extracts of *S. adoxoides*

One day after TMV was inoculated on leaf, petroleum ether extract, chloroform extract, ethyl acetate extract, butanol extract, water extract and Virus A were applied at 1, 3, 5, 7 and 9 days after the inoculation. Determination of A_{492} value of TMV inoculation tobacco was carried out respectively. Results were shown in Figure 6.3. From 1 to 7 days, the A_{492} value of TMV increased. After 7 days, A_{492} value of TMV exhibited decline trend. For positive control, A_{492} value of TMV was the highest (0.308) at 3days. When A_{492} values of TMV was at their maximum for all treatments, the values varied from 0.128 to 0.308. Ethyl acetate extract, with the lowest A_{492} value of TMV (0.128), had the best curing effect to TMV, which showed that ethyl acetate extract inhibited the infection or replication of TMV. The curing effect of virus A, petroleum ether extract, and chloroform extract declined in the order.

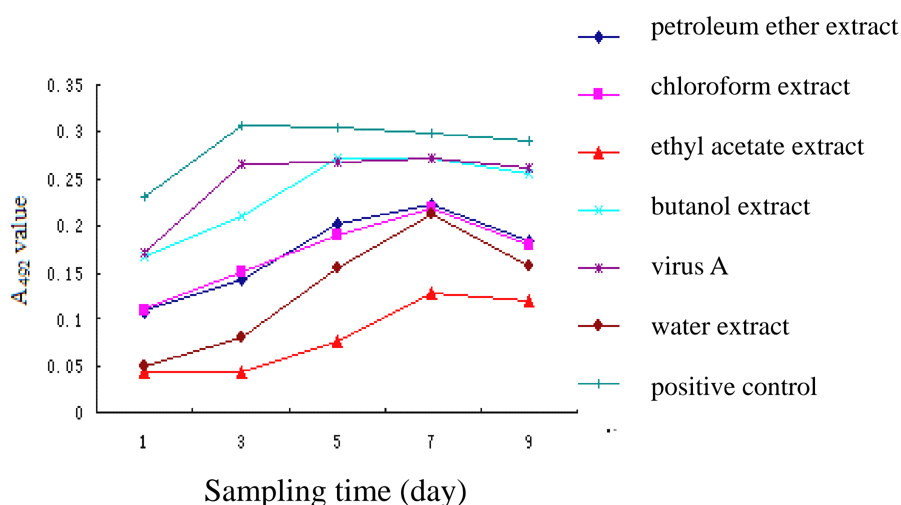

Figure 6.3 A_{492} of tobacco 1 day after being inoculated with TMV and extracts being applied, the samples were collected at 1, 3, 5, 7 and 9 days after extract treatment (Curing activity)

6.4 Discussion and conclusions

ELISA can detect minimum virus rapidly, sensitively and it also has the characteristics of ease operation. After treatment with extracts of *S. adoxoides*, infection quantity of the virus, in the host plant would change. Thus, action effect of extracts of *S. adoxoides* can be indirectly reflected through determination of the A_{492}. The in vivo results of extracts of *S. adoxoides* showed that the protective effect of chloroform extract, petroleum ether extract, ethyl acetate extract and water extract declined in the order. However, the lasting time of ethyl acetate extract, chloroform extract, petroleum ether extract, butanol extract, and virus A decreased in the order. During determination of in vivo curing action of the extracts, it was found that the curing effect of chloroform extract petroleum ether extract, virus A, and ethyl acetate extract increased in the order.

At present, some successes are achieved on research of anti-virus active substances. Advantages of the half leaf technique which is a conventional detection method are its simplility, and accuracy of selecting material, and the virus can be detected not only qualitatively, but also quantitatively. Half leaf method has great limitations however. Firstly, needs larger amount of the substance tested, which has certain difficulty for research of the natural products. Secondly, the plant growth could be restricted by growing season. Wu (2000) believed that ELISA has the advantage of speed, high sensitivity, being free from growing season restrictions of the plant growth. But its shortcoming is its accuracy. A relatively rapid and accurate anti-virus detection method can be established, if ELISA can be combined with the half leaf technique.

As a detection method of plant virus, ELISA has a certain degree of complementary role to the half leaf technique. The results presented here show that both the half leaf technique and ELISA had basically the same results. ELISA has advantages of rapidness, simpleness, and high sensitivity. In the past 10 years, there are rapid developments on polymerase chain reaction technique. If polymerase chain reaction technique combines with ELISA, it is expected to rapidly and accurately be used for selecting high-activity drugs.

Determination of anti-TMV activity and screening of active substances is mainly related to inoculation concentration, tobacco variety, test temperature and seedling age. Since the crude extracts contain a variety of substances, and some substances are very small, therefore, the small anti-virus part may contain with high anti-virus activity. In vitro inactivation, protection and curing action of drug can be used as an important indicator for screening anti-virus substances. However, because there are large differences between inside and outside plant tissue, the substance with in vitro inactivation may not always have the same effect to virus inside the plant. In this study, despite of certain differences between results of in vitro and in vivo treatment, the trend was consistent.

CHAPTER VII

SEPARATION OF ANTI-TMV ACTIVE INGREDIENTS OF EXTRACTS of *S. adoxoides* ROOTS

7.1 Introduction

In the previous study, the crude ethanol extracts of ***S. adoxoides*** were futher extracted by petroleum ether, chloroform, ethyl acetate, butanol and water, respectively. Determination of bioactivity of the extracts was also conducted. The results showed that chloroform, ethyl acetate extracts had the best anti-TMV activity. In this study, separatation of anti-TMV active ingredients of chloroform and ethyl acetate extracts would be carried out by thin layer chromatography (TLC) and column chromatography techniques. Screening of the active components of root extracts of ***S. adoxoides*** was performed by bioactivity tracking method using *Nicotiana glutinosa* as TMV indicator.

7.2 Materials and methods

7.2.1 Extraction of anti-TMV active ingredients of *S. adoxoides* root extracts

S. adoxoides root powder of 200g was transferred to a conical-bottom flask soaked for 24 h in 95% ethanol at room temperature and extracted for 3 hr three times in a water-bath boiler at 60 ^{O}C. The resulting extract was concentrated to near dryness, the ethanol crude extract was futher extracted by petroleum ether, chloroform, ethyl acetate, butanol, and water, respectively. Subsequently, activated carbon was

added into the final extracts of each solvent. The mixtures were stirred on electric mixer at 40-50 ^{O}C in order to decolor the extracts. Only chloroform extract (labeled as B) and ethyl acetate extract (labeled as C) were used in this study depending to the previous results.

7.2.2 Thin layer chromatography (TLC) of root extracts of *S. adoxoides*

Silica GF254 and water were ground into a mortar. Water solution of 0.5~0.7% carboxymethyl cellulose sodium (CMC)) was added into the resulting mixture. The mixture was stirred uniformly to remove bubbles. Then it was put on glass chromatography plates, baked at 110 ^{O}C for 30 min and stored in a blast drying oven before use. Subsequently 5 μl of the extracts having 0.5-2 mg/ml was spot into the plate. Each spot was 1-2 cm apart. The spot plate was put in a sealed chamber containing solvent of chloroform and methanol (20 : 1) as expanding solvent. After the sample had moved 10-15 cm away from the starting point the plates were taken out to allow solvents volatilizing. The active ingredients were detected by optical colored location, steam colored location, and reagent colored location, respectively.

7.2.3 Column chromatography of anti-TMV active parts of *S. adoxoides* root extracts

Silica gel of 200~300 meshes was placed in a beaker after which the solvents were added, the mixture was stirred uniformly. In this study, the proportion of diameter and length of the chromatography column was 1 : 10. The uniform silica gel was transferred into the chromatography column by wet packing method. The samples were added into the column and eluted gradiently with 100 ml petroleum ether-ethyl acetate (98 : 2, V : V), 100ml petroleum ether-ethyl acetate (95 : 5, V : V), 100ml petroleum ether-ethyl acetate (90 : 10, V : V), 100ml petroleum ether-ethyl

acetate (80 : 20, V : V), 100ml petroleum ether-ethyl acetate (70 : 30, V : V), and 100ml petroleum ether-ethyl acetate (50 : 50, V : V), respectively. In order to avoid delay of samples, a small amount of acetic acid added into the eluants. For the second chromatography column, the proportion of the eluants would be adjusted according to the results of the TLC Preliminary experiment

7.2.4 Separation procedure of anti-TMV active parts of *S. adoxoides* root extracts

(1) Separation procedure of chloroform extract (B) of *S. adoxoides* root

After extract B was eluted gradiently with petroleum ether-ethyl acetate and the resulting fractions were assayed for anti-TMV bioactivity, the results showed that both B3 and extract B4 fractions had high anti-TMV activity. B3 and B4 were futher eluted with the chloroform-methanol of different ratio (Figure 7.1) being.

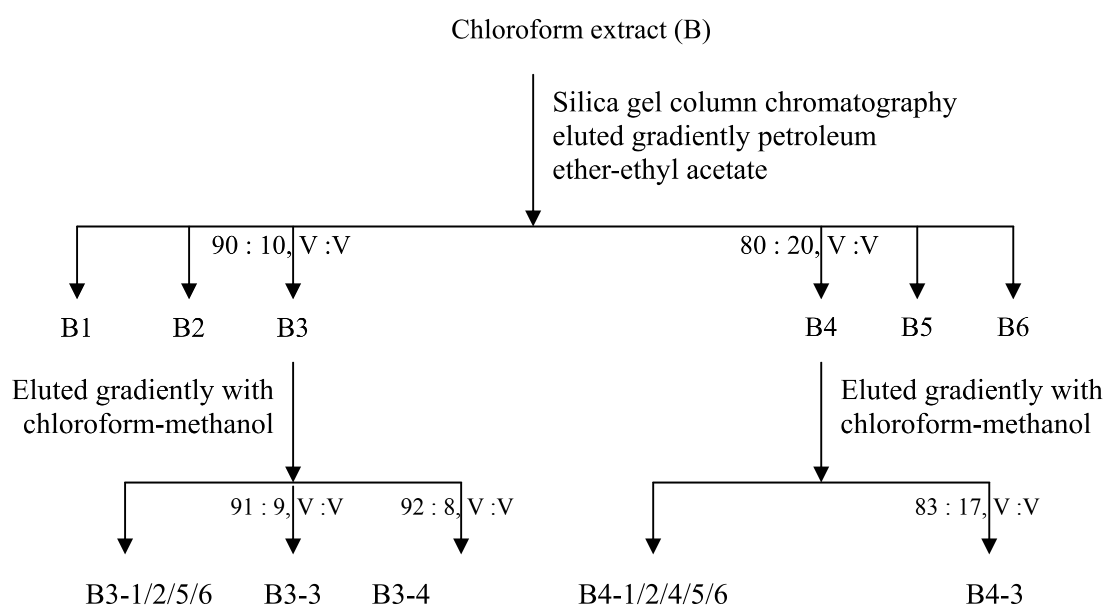

Figure 7.1 Flowchart of separation procedure of chloroform extract (B) of ***S. adoxoides*** roots

(2) Separation procedure of ethyl acetate extract (C) of *S. adoxoides* root

After extract C being eluted gradiently with chloroform-methanol and the wanting fractions were assayed for anti-TMV bioactivity, the results showed that both C4 and extract C5 fractions had high anti-TMV activity. C4 and C5 were futher eluted with the chloroform-methanol of different ratio (Figure 7.2) being. Among these extracts, extract C5-5 was dehydrated with anhydrous sodium soleplates, filtrated, and allowed to solvents volatilizing. Then there were the buff deposits in extract C5-5. After re-crystallization, the buff deposits became the buff powders.

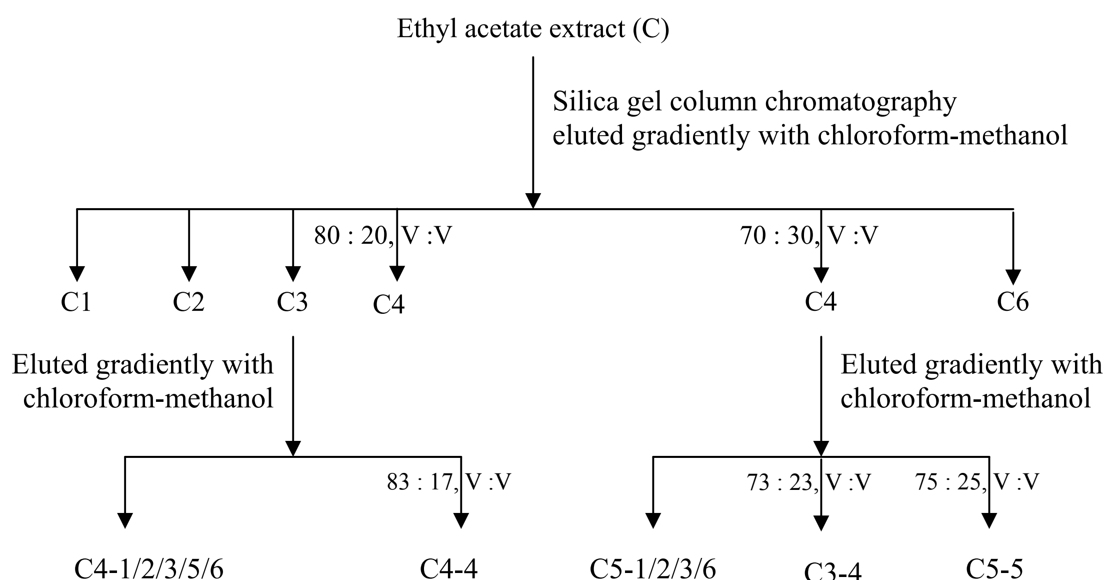

Figure7.2 Flowchart of separation procedure of ethyl acetate extract (C) of ***S. adoxoides*** roots

7.2.5 Anti-TMV activity of active fractions of *S. adoxoides* root extracts in the pot experiment

(1) In vivo protection activity

In vivo protection activity of f of B1, B2, B3, B4, B5, B6, B3-1/2/ 3/4/5/6, B4-1/ 2/3/4/5/6, C1, C2, C3, C4, C5, C6, C3-1/2/3/4/5/6 and C4-1/2/3/4/5/6 were tested with the same procedure as those described in **5.2.5 (1)**.

(2) In vivo curing activity

In vivo curing activity of fractions of B1, B2, B3, B4, B5, B6, B3-1/2/ 3/4/5/6, B4-1/2/3/4/5/6, C1, C2, C3, C4, C5, C6, C3-1/2/3/4/5/6 and C4-1/2/3/4/5/6 were tested with the same procedure as those described in **5.2.5 (2)**.

7.3 Results

7.3.1 In vivo anti-TMV bioactivity of the B1-B6 of *S. adoxoides* root extracts

(1) Protective activity of the chloroform fractions B1-B6

From the anti-TMV bioactivity assay, B4 showed the highest inhibition percentage of 49.22, which was significantly different from that of the other fractions. Protection effect of B3, B2, B5, B1 and B1 decreased in the order, with inhibition percentage of 43.33 %, 35.74 %, 32.28%, 28.63 % and 25.57%, respectively (Table 7.1).

Table 7.1 In vivo protective activity of chloroform fractions B1-B6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
B1	19.67	27.56	28.63 d
B2	17.78	27.67	35.74 c
B3	16.56	29.22	43.33 b
B4	14.56	28.67	49.22 a
B5	19.11	28.22	32.28 c
B6	22.33	30.00	25.57 d

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) Curing activity of the chloroform fractions B1-B6

From the anti-TMV bioactivity assay, B3 showed the highest inhibition percentage of 48.87, which was significantly different from that of the other fractions. Inhibition percentage of B3, B4, B1, B2, B5 and B6 was 48.87%, 41.63%, 37.36%, 36.52%, 35.59% and 26.14%, respectively. There were significantly differences between inhibition percentages of B4, B1, B2, B5, B4 and B3 (Table 7.2).

Table 7.2 In vivo curing activity of chloroform fractions B1-B6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
B1	17.89	28.56	37.36 bc
B2	18.55	29.22	36.52 c
B3	15.11	29.55	48.87 a
B4	16.67	28.56	41.63 b
B5	19.11	29.67	35.59 c
B6	21.67	29.34	26.14 d

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

7.3.2 In vivo anti-TMV bioactivity of the B3-1-6 of *S. adoxoides* root extracts

(1) Protective activity of chloroform fractions B3-1-6

From the anti-TMV bioactivity assay, B3-3 and B3-4 showed the highest inhibition percentage of 49.61 and 48.42, which was significantly different from that of the other fractions. Protective effect of B3-3, B3-4, B3-2, B3-5, B3-6 and B3-1 declined in the order, with inhibition percentage 49.61%, 48.42%, 39.78%, 38.50%, 33.19% and 31.53%, respectively. There were significantly differences between inhibition percentages of B3-4, B3-2, B3-5, B3-6, B3-1and B3-3 (Table 7.3).

Table 7.3 In vivo protective activity of chloroform fractions B3-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment Average local lesions	Control Average local lesions	Inhibition rate* (%)
B3-1	19.78	28.89	31.53 c
B3-2	17.33	28.78	39.78 b
B3-3	14.33	28.44	49.61 a
B3-4	14.67	28.44	48.42 a
B3-5	17.22	28.00	38.50 b
B3-6	19.00	28.44	33.19 c

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

(2) Curing activity of the chloroform fractions B3-1-6

From the anti-TMV bioactivity assay, B3-4 showed the highest inhibition percentage of 53.27, which was significantly different from that of the other fractions. Curing effect of B3-4, B3-3, B3-2, B3-5, B3-1and B3-6 declined in the order, with inhibition percentage of 53.27%, 43.81%, 43.02%, 42.52%, 36.79% and 33.34%, respectively. There were significantly differences between inhibition percentages of B3-4, B3-2, B3-5, B3-1, B3-6 and B3-3 (Table 7.4).

Table 7.4 In vivo curing activity of chloroform fractions B3-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
B3-1	18.33	29.00	36.79 c
B3-2	16.78	29.45	43.02 b
B3-3	16.11	28.67	43.81 b
B3-4	13.45	28.78	53.27 a
B3-5	16.22	28.22	42.52 b
B3-6	19.33	29.00	33.34 d

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

7.3.3 In vivo anti-TMV bioactivity of the B4-1-6 of *S. adoxoides* root extracts

(1) Protective activity of chloroform fractions B4-1-6

From the anti-TMV bioactivity assay, B4-3 showed the highest inhibition percentage of 52.54, which was significantly different from that of the other fractions. Protection effect of B4-6, B4-1, B4-5, B4-4, B4-2 and B4-3 increased in the order, with inhibiting rate of 29.01%, 30.21%, 35.29%, 41.33%, 42.67% and 52.54%, respectively. There were significantly differences between inhibition percentages of B3-4, B4-6, B4-1, B4-5, B4-4, B4-2 and B4-3 (Table 7.5).

Table 7.5 In vivo protective activity of chloroform fractions B4-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
B4-1	19. 78	28. 33	30.21d
B4-2	16.11	28.11	42.67 b
B4-3	13.67	28.78	52.54 a
B4-4	16.89	28.78	41.33 b
B4-5	18.33	28.34	35.29 c
B4-6	20.67	29.11	29.01d

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

(2) Curing activity of of chloroform fractions B4-1-6

From the anti-TMV bioactivity assay, B4-3 showed the highest inhibition percentage of 48.69, which was significantly different from that of the other fractions. Curing effect of B4-3, B4-4, B4-2, B4-5, and B4-1and B4-6 decreased in the order, with inhibition percentages of 48.69%, 42.64%, 39.76%, 37.13%, 36.04% and 31.19%, respectively. There were significantly differences between inhibition percentages extract B3-4, extract B4-6, B4-1, B4-5, B4-4, B4-2 and B4-3 (Table 7.6).

Table 7.6 In vivo curing activity of chloroform fractions B4-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
B4-1	18.33	28.67	36.04 d
B4-2	17.67	29.33	39.76 bc
B4-3	15.00	29.22	48.69 a
B4-4	16.44	28.67	42.64 b
B4-5	18.45	29.33	37.13 cd
B4-6	20.11	29.22	31.19 e

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

7.3.4 In vivo anti-TMV bioactivity of the C1-C6 of *S. adoxoides* root extracts

(1) Protective activity of the ethyl acetate fractions C1-C6

From the anti-TMV bioactivity assay, C4 showed the highest inhibition percentage of 46.72, which was significantly different from that of the other fractions. Protection effect of C4, C5, C3, C6, C2 and C1 declined in the order, with inhibiting rate of 46.72%, 42.36%, 38.96%, 35.11%, 34.77% and 30.58%, respectively. There were significantly differences between inhibition percentages of C5, C3, C6, C2, C1 and C4 (Table 7.7).

Table 7.7 In vivo protective activity of ethyl acetate fractions C1-6 of *S. adoxoides* root to TMV

Active parts	Treatment Average local lesions	Control Average local lesions	Inhibition rate* (%)
C1	19.67	28.33	30.58 e
C2	18.33	28.11	34.77 d
C3	17.55	28.78	38.96 c
C4	15.33	28.78	46.72 a
C5	16.34	28.34	42.36 b
C6	18. 89	29. 11	35.11d

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) Curing activity of the ethyl acetate fractions C1-6

From the anti-TMV bioactivity assay, C5 showed the highest inhibition percentage of 58.72, which was significantly different from that of the other fractions. Curing effect of C6, C2, C1, C3, C4 and C5 increased in the order, with inhibiting rate of 37.64%, 37.83%, 38.71%, 45.07%, 51.94% and 58.72%, respectively (Table 6.8).

Table 7.8 In vivo curing activity of ethyl acetate fractions C1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
C1	18.11	29.55	38.71d
C2	17.89	28.78	37.83 d
C3	15.67	28.56	45.07 c
C4	13.78	28.67	51.94 b
C5	12.11	29.33	58.72 a
C6	18.22	29.22	37.64 d

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

7.3.5 In vivo anti-TMV bioactivity of the C4-1-6 of *S. adoxoides* root extracts

(1) Protective activity of the ethyl acetate fractions C4-1-6

From the anti-TMV bioactivity assay, C4-4 showed the highest inhibition percentage of 48.47, which was significantly different from that of the other fractions. Protective effect of C4-5, C4-3, C4-6, C4-1 and C4-2 decreased in the order, with inhibiting rate of 42.46%, 39.33%, 38.02%, 35.16% and 34.37%, respectively (Table 7.9).

Table 7.9 In vivo protective activity of ethyl acetate fractions C4-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
C4-1	18.44	28.44	35.16 d
C4-2	18.44	28.11	34.37 d
C4-3	17.44	28.78	39.33 c
C4-4	15.00	29.11	48.47 a
C4-5	16.56	28.78	42.46 b
C4-6	17.56	28.34	38.02 c

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) Curing activity of the ethyl acetate fractions C4-1-6

From the anti-TMV bioactivity assay (Table 7.10), C4-4 showed the highest inhibition percentage of 51.55, which was significantly different from that of the other fractions. Protective effect of C4-5, C4-3, C4-2, C4-6 and C4-1 declined in the order, with inhibition rate of 45.06%, 43.53%, 39.37%, 38.80% and 36.12%, respectively.

Table 7.10 In vivo curing activity of ethyl acetate fractions C4-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
C4-1	18.44	28.89	36.12 d
C4-2	17.44	28.78	39.37 c
C4-3	16.11	28.56	43.53 b
C4-4	13.89	28.67	51.55 a
C4-5	16.11	29.33	45.06 b
C4-6	17.89	29.22	38.80 c

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

7.3.6 In vivo anti-TMV bioactivity of the C5-1-6 of S. adoxoides root extracts

(1) Protective activity of the ethyl acetate fractions C5-1-6

From the anti-TMV bioactivity assay (Table 7.11), C5-4 and C5-5 showed the highest inhibition percentage of 45.17 and 42.98, which was significantly different from that of the other fractions. Protective effect of C5-3, C5-2, C5-6 and C5-1 decreased in the order, with inhibition rate of 39.04%, 37.14%, 37.11% and 31.78%, respectively.

Table 7.11 In vivo protective activity of ethyl acetate fractions C5-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment Average local lesions	Control Average local lesions	Inhibition rate* (%)
C5-1	19.56	28.67	31.78 c
C5-2	17.67	28.11	37.14 b
C5-3	17.67	29.00	39.04 b
C5-4	15.78	28.78	45.17 a
C5-5	16.22	28.4	42.98 a
C5-6	18.44	29.33	37.11 b

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) Curing activity of the ethyl acetate fractions C5-1-6

From the anti-TMV bioactivity assay (Table 7.12), C5-5 showed the highest inhibition percentage of 62.45, which was significantly different from that of the other fractions. Protective effect of C5-4, C5-6, C5-3, C5-2 and C5-1 declined in the order, with inhibition rate of 55.57%, 50.95%, 48.06%, 42.16% and 35.49%, respectively.

Table 7.12 In vivo curing activity of ethyl acetate fractions C5-1-6 of *S. adoxoides* root to TMV

Active parts	Treatment	Control	Inhibition rate* (%)
	Average local lesions	Average local lesions	
C5-1	18.56	28.78	35.49 e
C5-2	16.44	28.44	42.16 d
C5-3	14.78	28.44	48.06 c
C5-4	12.78	28.78	55.57 b
C5-5	10.89	29.00	62.45 a
C5-6	14.33	29.22	50.95 c

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

7.4 Discussion and conclusions

The separation and identification of effective components of chloroform fraction (B) and ethyl acetate fraction (C) of *S. adoxoides* roots were conducted by column chromatography and thin layer chromatography (TLC). Ethanol crude extracts of *S. adoxoides* were eluted gradiently with petroleum ether-ethyl acetate and the chloroform-methanol. Among the 18 components of fraction B, Only two white powders of B3-3 and B3-4 were obtained by bioactivity tracing method using the anti-TMV bioactivity assay. After detection with TLC, B3-3 and B3-4 were determined primarily to be the same substance (β- sitosterol).

From the 18 components of fraction C, Only one buff powder of C5-5 was obtained by bioactivity tracing method using the anti-TMV bioactivity assay. The melting point of C5-5 was 188 ^{O}C ~190 ^{O}C. C5-5 became red after it reacted with hydrochloric acid-Mg powder. Methanol solution of C5-5 had two main absorption

bands of 300-400 nm 240-285 nm in the ultraviolet spectrum. Thus C5-5 was determined primarily to be flavone compound. For fractions B and C, The whole separation effect was not very good. This may be relative to choice of eluants. In addition, B3-3, B3-4 and C5-5 were not identified further due to their impurities. Therefore, further research should be devoted to choose appropriate eluants and purified B3-3, B3-4 and C5-5.

Results of bioactivity by half-leaf technique showed that B4-3 separated from chloroform fraction (B) had better protective and curing effect, with inhibiting percentage of 52.54 and 48.69 to TMV, respectively. Similarly, C5-5 separated from ethyl acetate fraction (C) had better protective and curing effect, with inhibiting rate of 42.98% and 62.07%, respectively. Other components of fraction C also had good anti-TMV bioactivity. For example, C5-4 had protective and curing activity inhibiting rate of 45.17% and 55.57%, respectively. Thus, in the following study, B4-3 and C5-5 would be used as the objectives to process their formulation and determine anti-TMV bioactivity.

CHAPTER VIII

INFLUENCE OF *S. adoxoides* ROOT EXTRACTS ON ENZYME ACTIVITY OF TMV INFECTED TOBACCO

8.1 Introduction

In recent years, there are large numbers of work made on physiological and biochemical indictors of the plant before and after diseases, particularly on the reactive oxygen species (ROS) and its pathogenesis relating (PR) enzymes. The ROS has not only vital role in the plant disease-resistance, but also it could cause peroxidation of the fat membrane and lost of permeability resulting in series of physiological and biochemical changes, metabolic disorder, and injuries to the plant.

At present, tobacco mosaic virus (TMV) is one of the most serious viruses in China's tobacco production. However, there is little available information on the relationship of the ROS, the PR enzymes and anti-TMV agents. In this study, the dynamic changes of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) enzyme activity of TMV infected tobacco leaves were investigated in conditions of protection and curing.

8.2 Material and methods

8.2.1 Preparation of buffers

Buffers used for enzyme extraction were as follow.

(1) Preparation of stock solution :

Solution A : 71.64 g Na2HPO4·12H2O was dissolved in 1 litre distilled water

Solution B : 27.20 g KH_2PO_4 was dissolved in 1 litre distilled water

(2) Preparation of 0.05 M catalase (CAT) extraction buffer pH 6.8 :

Solution A	49.0 ml
Solution B	51.0 ml
Distilled water	200.0 ml

(3) Preparation of 0.05 M peroxidase (POD) extraction buffer pH 7.0 :

Solution A	61.0 ml
Solution B	39.0 ml
Distilled water	200.0 ml

(4) Preparation of 0.05 M superoxide dismutase (SOD) extraction buffer pH 7.8 :

Solution A	91.5 ml
Solution B	8.5 ml
Distilled water	200.0 ml

8.2.2 Experimental methods

(1) Protective activity assessment

The top 3-6 leaves of variety K326 (*N. tabacum*) were used as experimental material. One day after the root extracts of different solvent and Virus A were applied on the leaves, TMV was inoculated. After 1, 3, 5, 10 and 15 days, the

leaves sampling was performed and the physiological indices (CAT, POD and SOD) of the host plant were determined, respectively.

(2) Curing activity assessment

The top 3-6 leaves of variety K326 (*N. tabacum*) were used as experimental material. One day After TMV was inoculated, the root extracts of different solvent and Virus A were applied on the leaves. After 1, 3, 5, 10 and 15 days of spray, the leaves sampling was performed and the physiological indices (CAT, POD and SOD) of the host plant were determined, respectively.

8.2.3 Preparation of crude enzymes

The leaf blade surface was cleaned by wet gauze. After the big vein had been removed, and 0.05 g leaf was cut into pieces, was put into a precooling mortar, subsequently 1.0 ml 0.05 M phosphate buffer pH 7.0, a drop of 1% polyvinylpyrrolidone, and small amount of quartz sand were added into the mortar, and the mixture was ground, filtered and transferred translated into a centrifuge tube, and centrifuged for 15 min at 4000 g at 4 ^{O}C. The resulting supernatant was collected for POD enzyme activity assay.

The catalase (CAT) was extracted by 1.0 ml 0.05 M buffer phosphate pH 6.8. The superoxide dismutase (SOD) was extracted by 1.0 ml 0.05 M buffer phosphate pH 7.8.

8.2.4 Determination of enzyme activity

(1) CAT activity

After peroxide of 1ml and water of 0.9 ml were added into the 2 ml test tube, 0.1 ml crude enzyme solution was transferred into the reacting system. After reaction, the decrease A_{240} was then recorded. The absorbance was recorded every

1min for 4 min. Hydrochloride buffer was used as a control. A reduction of 0.1 A_{240} in 1 min is 1 enzyme activity unit (U).

The CAT activity was calculated as the following equation :

$$\text{CAT activity} = \frac{\Delta A_{240} \times V_T}{0.1 \times V_1 \times t \times W_F}$$

Where as :

V_T	=	total volume of the crude enzyme solution (ml);
V_1	=	volume of the crude enzyme solution used as mensurement (ml);
W_F	=	fresh weight of the sample (g);
0.1	=	0.1A_{240} reduction is 1 enzyme activity unit (U);
t	=	the interval from CAT being added to A_{240} being read (min).

(2) POD Activity

After 1ml Peroxide, 0.95 ml 1-hydroxy-2-methoxybenzene, 1 ml 0.05 M phosphate buffer pH 7.0, and 0.05 ml crude enzyme solution was added into a 3 ml test tube. After reaction, the decrease A_{470} was then recorded. The absorbance was recorded every 1 min for 4 min. Hydrochloride buffer was used as control. A reduction of 0.01 A_{470} in 1 min is 1 enzyme activity unit (U).

The POD activity was calculated as the following equation :

$$\text{POD activity} = \frac{\Delta A_{470} \times V_T}{0.01 \times V_1 \times t \times W_F}$$

Where as :

V_T = total volume of the crude enzyme solution (ml);

V_1 = volume of the crude enzyme solution used as mensurement (ml);

W_F = fresh weight of the sample (g);

0.01 = $0.01A_{470}$ reduction is 1 enzyme activity unit (U);

t = the interval from CAT being added to A470 being read (min.).

(3) SOD Activity

After 2.5 ml methionine, 0.25 ml nitroblue tetrazolium (NBT), 0.15 ml riboflavin 0.05 ml 0.05 M phosphate buffer pH = 7.8, 0.1 ml crude enzyme solution was added into the 3 ml test tube. The control test tube having no enzyme solution, after the various test tubes were irradiated by the lamp light for approximate 20 min, the determination of the enzyme activity was performed at 560 nm. The 50% enzyme quantity inhibited is 1 SOD enzyme activity unit (U).

The SOD activity was calculated as the following expression :

$$\text{SOD activity} = \frac{(A_0 - A_S) \times V_T}{0.5A_0 \times W_F \times V_1}$$

Where as :

A_0 = light absorption value of the control irradiated by light;

A_s = light absorption value of the sample;

V_t = total volume of the sample (ml);

V_1 = volume of the sample used as mensuration;

W_F = fresh weight of the sample (g).

8.3 Results

8.3.1 Enzyme activity in protective action

(1) CAT activity

The results indicated that the CAT activity seem to decrease or did not changes during the 3 days period after TMV inoculation when the tobacco was pretreated with the extracts or Virus A compared to that of the untreated (Fig 8.1). During the 5 days period, CAT activity appeared to increase in all treatments except the control which seem to decline slightly, which showing the largest disease index was postponed for 2 days. After 5 days, CAT activity in all treatments decline. Among the four extracts, tobacco treated with PEE seem to have the lowest CAT activity indicating its best protective activity corresponding to the result in chapter Ⅴ. The protective effect of CE, Virus A and EAE decreased in the order (Fig 8.1).

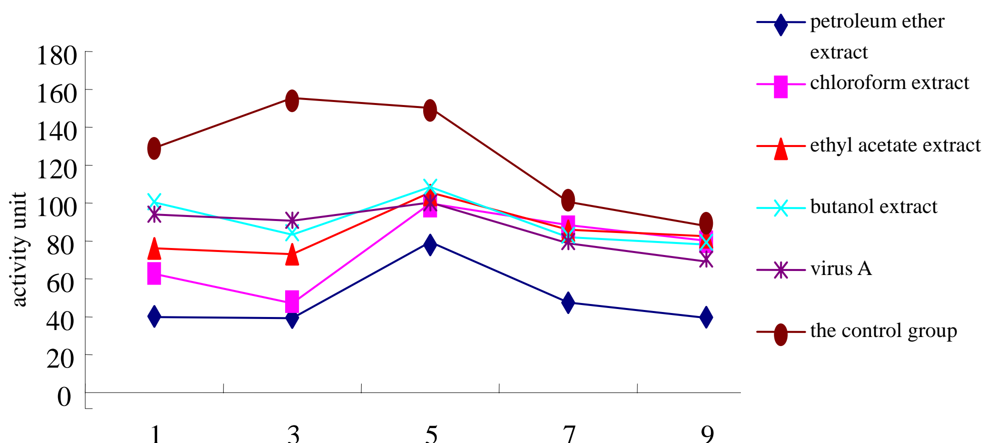

Figure 8.1 CAT activities in *N. tabacum* inoculated with TMV 1 day after being treated with root extracts of *S. adoxoides*

(2) POD activity

The results indicated that the POD activity increased period of 1 day to 3 days after TMV inoculation when the tobacco was pretreated with the extracts or virus A and control. During the 3 days period, the POD activity appeared to decrease speedy in all treatments except the control which seem to decline slightly (Fig 8.2). Among the four extracts, tobacco treated with PEE seem to have the lowest POD activity indicating its best protective activity corresponding to the result in chapter Ⅴ. The protective effect of CE, virus A and EAE declined in the order, too (Fig 8.2).

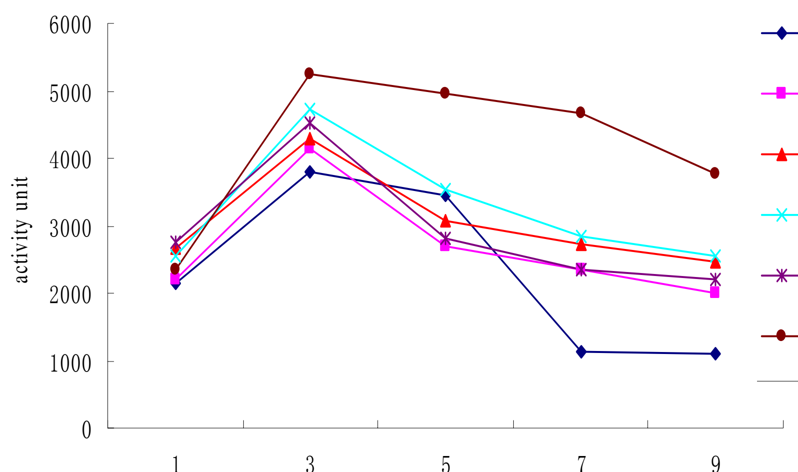

Figure 8.2 POD activities in *N. tabacum* inoculated with TMV 1 day after being treated with root extracts of *S. adoxoides*

(3) SOD activity

The results indicated that the SOD activity seem to increase slightly during the 3 days period after TMV inoculation when the tobacco was pretreated with the extracts or virus A compared to that of the untreated (Fig 8.3). After 3 days, SOD activity appeared to decrease in all treatments but which seem to decline slightly,

Among the four extracts, tobacco treated with PEE seem to have the lowest SOD activity indicating its best protective activity corresponding to the result in chapter Ⅴ. The protective effect of CE, EAE and Virus A also decreased in the order (Fig 8.3).

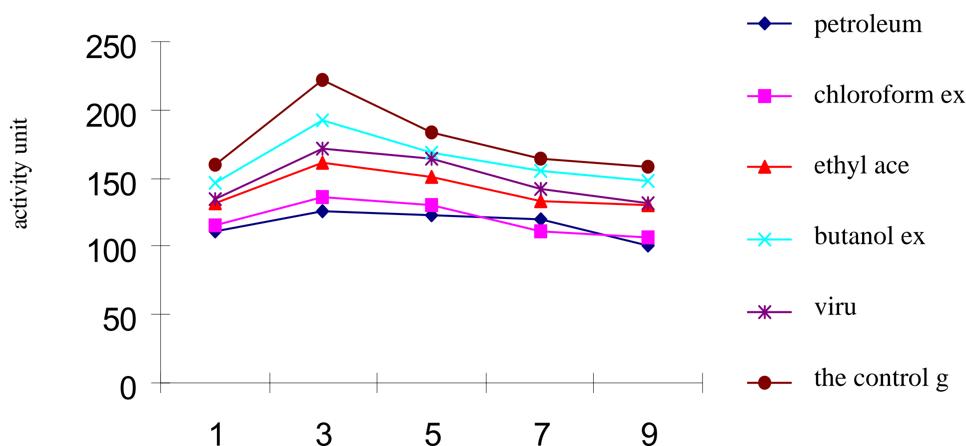

Figure 8.3 SOD activities in *N. tabacum* inoculated with TMV 1 day after being treated with root extracts of *S. adoxoides*

8.3.2 Enzyme activity in curing action

(1) CAT activity

The results indicated that CAT activity appeared to decrease during 3 days after TMV inoculation in tobacco treated with all extracts including the control and Virus A (Fig 8.4). The activity in CE, Virus A, EAE and control tobacco still declined during the 5 days period but those treated with PEE and BE seem to increase right until the 9 days period. After declining for 7 days, CAT activity in tobacco treated with CE and Virus A rised up again during the 9 days period. Among all treatments, tobacco treated with Virus A seem to have the lowest CAT activity at 9 days but considering the overall activity, tobacco treated with EAE seem to have

lowest CAT activity. The curing effect of Virus A, CE and PEE decreased in the order (Fig 8.4).

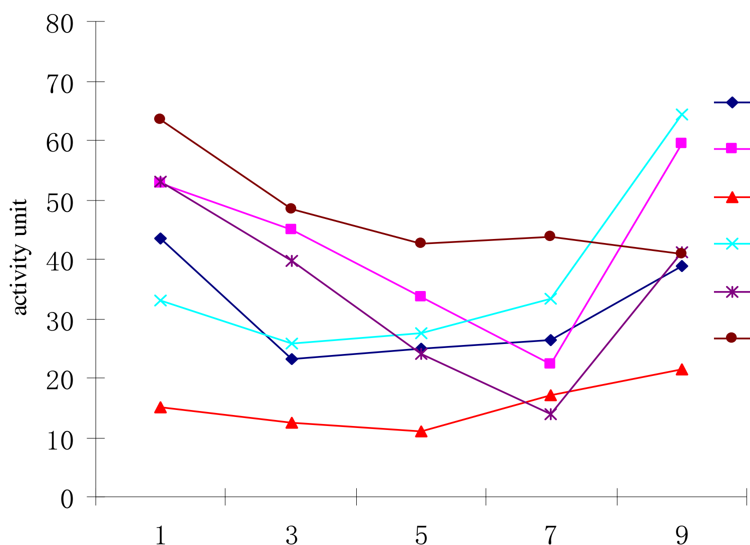

Figure 8.4 CAT activities in *N. tabacum* inoculated with TMV 1 day before being treated with root extracts of *S. adoxoides*

(2) POD activity

The results indicated that POD activity appeared to increase during 3 days after TMV inoculation in tobacco treated with all extracts including the control and Virus A (Fig 8.5). The activity in CE still increase during the 5 days period, but those treated with PEE, EAE and BE seem to decrease right until the 7 days period. After declining for 7 days, POD activity in tobacco treated in all treatments slightly rised up again during the 9 days period. Among all treatments, considering the overall activity, tobacco treated with EAE seems to have the lowest POD activity, followed by the Virus A, PEE and CE, respectively (Fig 8.5).

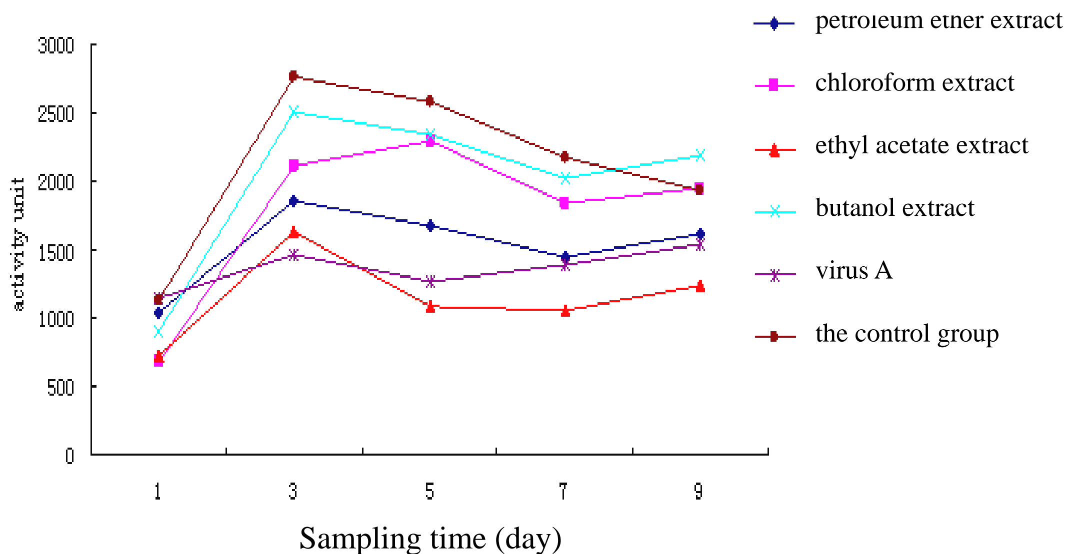

Figure 8.5 POD activities in *N. tabacum* inoculated with TMV 1 day before being treated with root extracts of *S. adoxoides*

(3) SOD activity

The results indicated that SOD activity appeared to increase during 3 days after TMV inoculation in tobacco treated with all extracts and Virus A, but the control showed to rised up quickly (Fig 8.6). The SOD activity in all treatments still declined during the 7 days period. After declining for 7 days, SOD activity in tobacco treated with all extracts including Virus A seem to rised up slightly again during the 9 days period. Among all treatments, tobacco treated with EAE seems to have the lowest SOD activity at 9 days, which means EAE had the best curing effect. The curing effect of Virus A, CE and PEE decreased in the order (Fig 8.6).

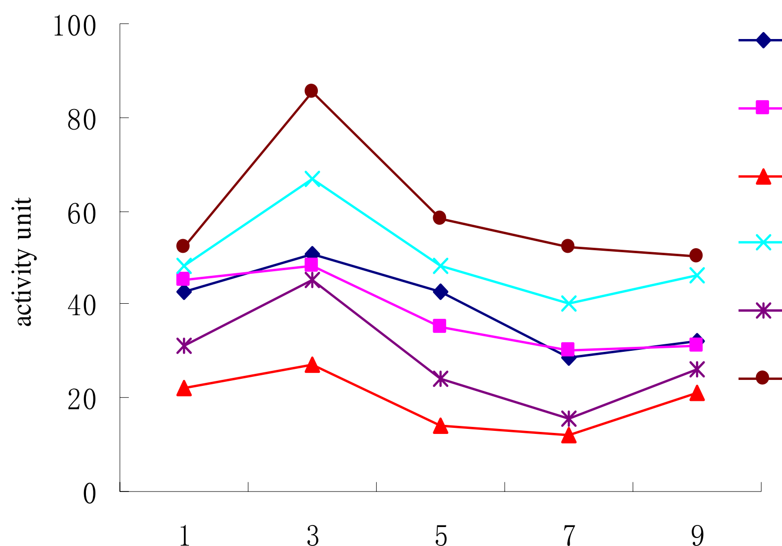

Figure 8.6 SOD activities in *N. tabacum* inoculated with TMV 1 day before being treated with root extracts of *S. adoxoides*

8.4 Discussion and conclusions

8.4.1 Changes of the CAT, POD and SOD activities as protective effect

The CAT activity decreased during the 3 days period, and subsequently showed the trend of increase. The CAT activity reached the maximum at 5 days. By contraries, for the control group, the CAT activity increased during the 3 days period, and decreased subsequently; got to the maximum at 3 days, which showing the largest disease index was postponed for 2 days. The change of the activity of POD and SOD exhibit the trend of initial rise followed by subsequent decline. After the treatment, the curve level representing CAT activity of all treated tobacco was always significantly lower than that of the control group, which showed that the disease state of host was reduced after treatment. Protective effect of PEE was the best, CE, Virus A and EAE decreased in the order.

8.4.2 Changes of the CAT, POD and SOD activities as curing effect

The CAT activity decreased in the first place, and subsequently increased. The curve level of POD and SOD activity had the change trend of initial increase followed by subsequent decrease and final increase. After the treatment, the curve level representing enzyme activity was always lower than that of the control group, which indicated that extracts of ***S. adoxoides*** had curing effect to TMV or enhanced the disease-resistant of the host. Curing effect of EAE was the best, Virus A, PEE and CE declined in the order.

This means that when the host is infected by TMV, there was ROS hurting the host plant. The SOD enzyme can remove the ROS by the protection enzyme system, and then the activity enzyme increased. Among these extracts, after treatment with ethyl acetate extract, the enzyme activity increased slowly. Probably it had better curing action, which making invasion ability decline or stopping expansion of virus. When there were the local lesions on the host, in order to keep balance of enzyme activity in the host, its enzyme activity would decrease. If the reaction of the host was allergic, its enzyme activity would increase slightly.

8.4.3 Relationship between changes of enzyme activity and bioactivity of *S. adoxoides* extracts

(1) Relationship between changes of CAT activity and bioactivity of *S. adoxoides* extracts

Results presented showed that CAT activity was up to the maximum at 5 days. Compared to control, the CAT activity was always lower after treatment with *S.adoxoide* extracts and Virus A. For control, CAT activity was got to the maximum at 3 days. This was probably caused by interaction of the above mentioned actions. After

the host plant being treated with inhibitors, immunity was enhanced and the virus infection capability was reduced. Thus, the fastigium of virus was postponed. Probably extracts of ***S. adoxoides*** had a direct effect on TMV and made infection capability of virus to decline. When the host plants were infected by TMV, enzyme activity would decrease, and content of H_2O_2 in the hosts would increase, and the infection would be reduced. After the hosts were infected by TMV, there were large numbers of ROS and the balance was broken. Thus, the normal signal could not be transmitted. When the infections were more serious, CAT produced in the plant was not enough to decompose H_2O_2 and the plant would produce a large number of CAT. When the plant tissue was necrotic, CAT activity would resume and its activity would decrease to maintain its original balance. Time of CAT activity decreased to the lowest point was at 3 days or 5 days or 7 days. This was probably because the different penetrating time of extracts of ***S. adoxoides*** made to inhibit infection of TMV.

(2) Relationship between changes of POD activity and bioactivity of *S. adoxoides* extracts

Results in Fig 8.2 indicated that POD activity is gradually decreased after rapid increase for the control group in protection. However, results in Fig 8.5 showed that POD activity rapidly increased and decreased gradually for the control group in curing. This may be results that plants startuped the protection enzyme system and POD activity increased and removed H_2O_2 after the plant infected by virus. From the Fig 8.5 it can be seen that POD activity presented the trend of initially rapid increase followed by subsequently gradually decrease.

(3) Relationship between changes of SOD activity and bioactivity of *S. adoxoides* extracts

The injury of the plant by virus was related to the changes of SOD activity (Huang, 2005). Results in Fig 8.3 showed that SOD activity initially rised and then declined. SOD activity was always lower than that of treatment with control. This indicated that when the host was infected by TMV, ROS was product and injured the plant. And then plant startup its protection enzyme system to remove ROS. Finally, the enzyme activity increased. Among the all extracts, the increase of enzyme activity was the lowest after treatment with PEE. This was probably caused by PEE of better protective effect that made the infection capability of TMV decrease or inhibits expansion of TMV. When there were local lesions on the host, the enzyme activity would decrease in order to maintain its balance in the plant.

It can be seen from the Fig 8.6 that the SOD activity got to the maximum at 3 days, and decreased, and then increased to its second peaks at the 9 day. But for the control group, the SOD activity presented the change trend of initial increase followed by decrease. It can be speculated that the plant protect the membrane from harmfulness through enhancing the stability and activity of SOD after the plant was inoculated by virus. During the process of the rise of SOD activity, the tobacco leaves cannot be injured by TMV. But when it is beyond a certain threshold, the SOD activity started to decline and the tobacco leaves was began to be injured. The lower the enzyme activity was, the stronger the inhibiting ability of the extracts to TMV was.

CHAPTER IX

BIOACTIVITY of *S. adoxoides* ROOT ACTIVE COMPONENTS AGAINST TMV

9.1 Introduction

In past experiments, isolation and identification of petroleum ether extracts were conducted by using gas chromatography-mass spectrometry (GC-MS) technique. Results showed that there were peak-line maps of 31 substances in the extract. The chemical structures of 12 substances had been identified. The content of β-sitosterol, linoleic acid, oleic acid, palmitic acid, ocfanoic acid, pentadecylic acid, margaric acid, stearic acid and arachidic acid was 38.26%, 18.73%, 15.68%, 13.51%, 0.04%, 0.30%, 0.41%, 2.44% and 0.72%, respectively. Previous results of the antiviral activity assay of ***S. adoxoides*** extracts showed that petroleum ether extracts had the best anti-TMV bioactivity. Among the 12 substances, only β-sitosterol, oleic acid and stearic acid had higher anti-TMV bioactivity than the rest compounds of extract. So in this study β-sitosterol, oleic acid and stearic acid separated from petroleum ether extracts of ***S. adoxoides*** were used for further experiment.

9.2 Materials and methods

9.2.1 Active components

The β-sitosterol, oleic acid and stearic acid being selected as active components, which were separated and identified from *S. adoxoides* root extracts in Laboratory of Institute of Crop Protection of Guizhou University.

9.2.2 Bioassay of anti-TMV activity of active components from *S. adoxoides* root extracts in vitro

Bioassay of anti-TMV activity of the actvic components from *S. adoxoides* root extracts on *N. glutinosa* was conducted with the same processes as described in 5.2.4.

(1) In vitro inactivation activity

0.1 g β-sitosterol, stearic acid and oleic acid were dissolved in 10 ml water. Virus A solution was also diluted to 100 times. The compound solution was mixed with the same volume of TMV suspension of 10.19 μg/ml. After 30 min, the mixture was inoculated on the left half leaf using silicon carbide as an abrasive. The right half leaf was inoculated with the virus plus the equal volume of water as check.

(2) In vitro protective activity

The left and right half leaf was dipped for 1min in treatment solution and control solution as in (1), respectively. The leaves were removed from the solutions and excess solution was absorbed with absorbent paper. After 2 h, the TMV suspension of 10.19 μg/ml was inoculated on the left half leaf using silicon carbide as an abrasive. The right half leaf was inoculated with the virus plus the equal volume of water as check.

(3) In vitro curing activity

TMV suspension of 10.19 μg/ml was inoculated on the leaf of tobacco. After 2 h, the leaf was split into two halves of left and right from the midrib with sterilized scissors. The active components and Virus A solutions as in (1) were treated on the left half leaf and the blank control solution was treated on the right half leaf.

(4) Effect of inactivation time of β-sitosterol on TMV in vitro

0.1 g β-sitosterolf was dissolved in 10 ml water. The solution was mixed with the same volume of purified TMV suspension of 10.19 μg/ml. After 5, 10, 30 and 60 min. the mixture was inoculated on the left half leaf with silicon carbide. The experiment was then conducted with the same procedure as those described above.

(5) Effect on bioactivity of active components of different concentrations in vitro

The β-sitosterol, stearic acid and oleic acid were diluted 20 g/ml, 10 g/ml, 5 g/ml, 2.5 g/ml, 1.25 g/ml with water, respectively. Anti-TMV activities of the active components of different concentrations were assayed using described as above in vitro for protection or curing.

9.2.3 Bioassay of anti-TMV activity of active components from *S. adoxoides* root extracts in pot trial

Bioassay of anti-TMV activity of the actvic components from *S. adoxoides* root extracts on *N. glutinosa* was conducted with the same processes as described in 5.2.5.

(1) Inactivation in vivo

The component solutions were mixed with the same volume purified TMV suspension of 10.19 μg/ml. After 30 min., the mixture was inoculated on the left half leaf using silicon carbide as an abrasive. The right half leaf was inoculated with the virus plus the equal volume of water as check.

(2) Protective activity in vivo

The active component and Virus A solutions were transferred gently

onto the left half leaf with brush pen. The blank control was also transferred onto the right half of the same leaf. Each treatment had three individual plants. Two hours after treatment, TMV suspension of 10.19 μg/ml was inoculated on the both halves.

(3) Curing activity in vivo

The TMV suspension of 10.19 μg/ml was inoculated on tobacco leaf of *N. glutinosa*. After inoculation of 2 h, 24 h and 48 h, the active components and Virus A solutions were treated on the left half leaf and the blank control solution was treated on the right half leaf.

9.2.4 Calculation of inhibition rate

Calculation of inhibiting rate was conducted with the same processes as described in 5.2.6.

9.3 Results

9.3.1 Bioactivity of active components from *S. adoxoides* root extracts on TMV in vitro

(1) In vitro inactivation

The inhibiting effects of β-sitosterol, oleic acid and stearic acid of 10mg/ml to TMV were listed in the Table 9.1. Stearic acid had the best inactivating activity, with inhibiting percentage of 56.86%. Inhibition rate of β-sitosterol was 50.73%. There were significant differences between inactivation effect of stearic acid, β-sitosterol and the control virus A.

Components	Average local lesions of treatment	Average local lesions of control	Inhibition rate* (%)
β-sitosterol	101	205	50.73 bc
Oleic acid	90	150	40.00 c
Stearic acid	88	204	56.86 a
Virus A	148	303	51.16 ab

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) In vitro protective activity

In vitro protective action results of components of β-sitosterol, oleic acid, stearic acid separated from petroleum ether extract of *S. adoxoides* were presented in Table 9.2. It can be seen that stearic acid of 10 mg/ml had the better protective action with inhibition rate of 57.48%.There was significant difference between protective effect of stearic acid and the control virus A. The inhibition rate of oleic acid of 10 mg/ml was 49.38 %, there was not significant difference between protective effect of oleic acid and the control virus A.

Table 9.1 In vitro protective activity of active compounds of *S. adoxoides* root extracts to TMV

Components	Average local lesions of treatment	Average local lesions of control	Inhibition rate* (%)
β-sitosterol	178	232	23.27 c
Oleic acid	82	162	49.38 b
Stearic acid	182	428	57.48 a
Virus A	171	311	45.02 b

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(3) In vitro curing activity

As can be seen from Table 9.3, which three components of oleic acid, stearic acid and β-sitosterol of 10 mg/ml had good curing effect, with the inhibiting percentage of 37.4%, 28.00% and 25.32%, respectively. The curing effect was significantly higher than that of the control of virus A.

Table 9.2 In vitro curing activity of active compounds of *S. adoxoides* root extracts to TMV

Components	Average local lesions of treatment	Average local lesions of control	Inhibition rate* (%)
β-sitosterol	118	158	25.32 b
Oleic acid	97	155	37.41 a
Stearic acid	90	125	28.00 b
Virus A	259	292	11.30 c

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

9.3.2 Effect of inactivation time of β-sitosterol of *S. adoxoides* root extracts on TMV in vitro

Effect of inhibition rate of 10mg/ml β-sitosterol different inactivation time on TMV was presented in Table 9.4. It can be seen that after mixture of 5min, the inactivation rate was only 11.58%, but increased to 42.52% after 10 min. After 30 min and 60 min, the inhibition percentages were 56.38 and 61.03, respectively. This showed that the longer time TMV was mixed with β-sitosterol, the higher inhibition rate was. However, inhibition rate increased slowly after 30 min.

Table 9.3 Inhibition rate of different inactivation time of β-sitosterol of *S. adoxoides* root extracts on TMV

inactivation time (min)	Average local lesions		Inhibition rate* (%)
	Treatment	Control	
5	33.6	38.0	11.58 c
10	19.6	34.1	42.52 b
30	13.0	29.8	56.38 a
60	18.2	46.7	61.03 a

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

9.3.3 Effect on bioactivity of active components of *S. adoxoides* root extracts of different concentrations in vitro

(1) In vitro protective activity of stearic acid of different concentrations

In vitro protective action results of stearic acid of different concentrations to TMV presented in the Table 9.5 and Fig 9.1. In general, the protective effect of stearic acid increased with increase of concentration. Under the condition of the range of the low concentration (1.25 mg/ml - 5 mg/ml), the protective effect of stearic acid increased rapidly. However, under the condition of the range of the high concentration (5 mg/ml - 20 mg/ml), the protective effect of stearic acid increased slowly. There was no significant difference treatment effect of stearic acid of 5 mg/ml, 10 mg/ml and 20 mg/ml, which had inhibition percentage of 57.48%, 59.55% and 63.77%, respectively.

Table 9.4 In vitro protective action of stearic acid of *S. adoxoides* root extracts of different concentrations to TMV

Concentration (g/mL)	Average local lesions	Average local lesions	Inhibition rate*
20	125	345	63.77 a
10	182	428	59.55 a
5	108	267	57.48 a
2.5	162	233	30.47 b
1.25	156	185	15.68 c

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

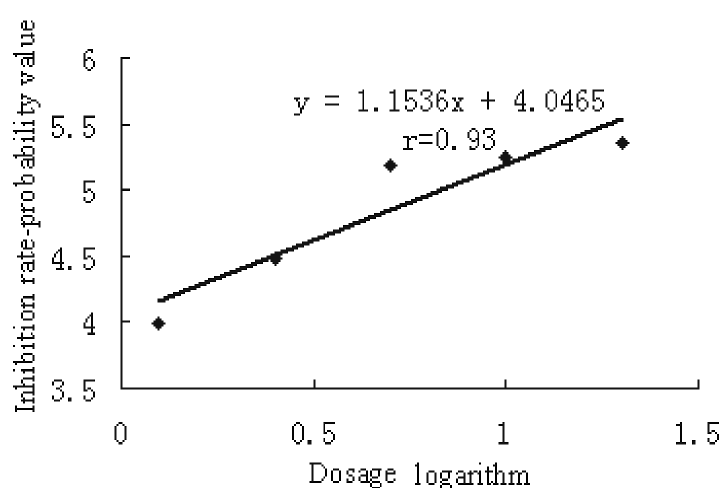

Figure 9.1 Correlation between concentrations of stearic acid and inhibition rate on *N.glutinosa* (Protective action)

(2) In vitro curing activity of stearic acid of different concentrations

The treatment effect of stearic acid increased with increase of concentration (Table 9.6 and Fig 9.2). The inhibition percentage of stearic acid of

1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml were 12.81%, 17.14%, 28.29%, 32.69% and 37.96%, respectively. There were some differences in different concentrations.

Table 9.5 In vitro curing activity of stearic acid of *S. adoxoides* root extracts of different concentrations to TMV

Concentration (g/mL)	Average local lesions	Average local lesions of control	Inhibition rate* (%)
20	134	216	37.96 a
10	140	208	32.69 ab
5	180	251	28.29 b
2.5	116	140	17.14 c
1.25	177	203	12.81 c

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

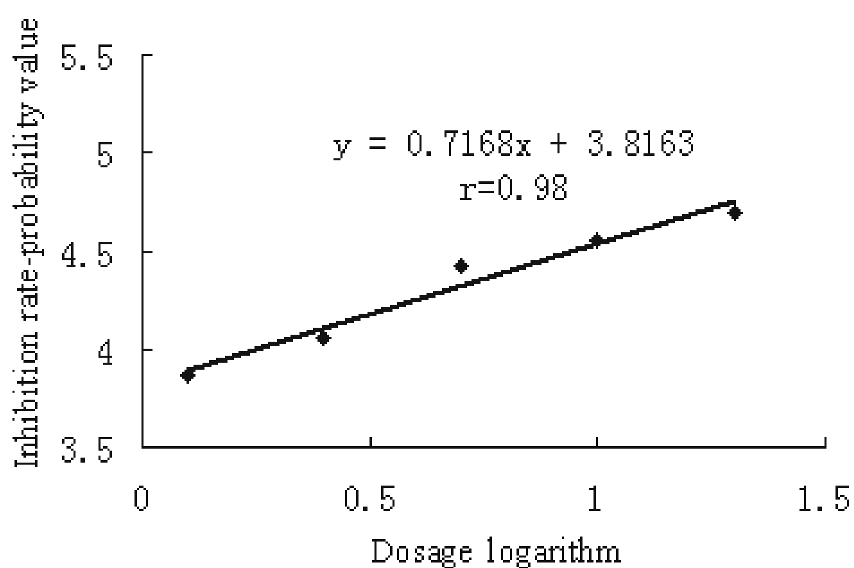

Figure 9.2 Correlation between concentrations of stearic acid and inhibition rate on *N.glutinosa* (Curing activity)

(3) In vitro curing activity of oleic acid of different concentrations

The curing effect of oleic acid also increased with increase of concentration (Table 9.7 and Fig 9.3). The inhibiting rate of oleic acid of 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml was 21.63%, 28.79%, 30.77%, 37.10% and 38.71%, respectively. There existed some differences in different concentrations of oleic acid.

Table 9.6 In vitro curing activity of oleic acid of *S. adoxoides* root extracts of different concentrations to TMV

Concentration (g/mL)	Average local lesions of treatment	Average local lesions	Inhibition rate* (%)
20	190	310	38.71 a
10	117	186	37.10 a
5	135	195	30.77 b
2.5	174	245	28.98 b
1.25	173	221	21.72 b

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

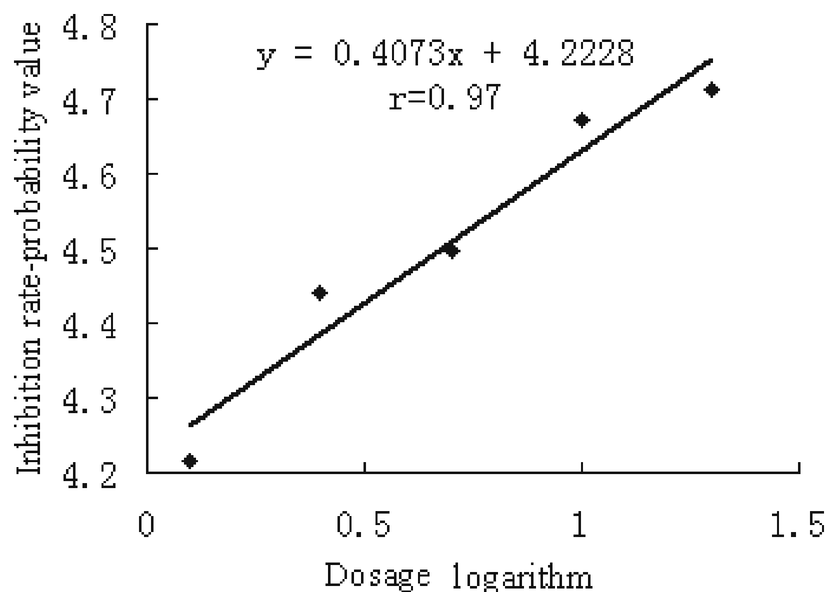

Figure 9.3 Correlation between concentrations of oleic acid and inhibition rate on *N.glutinosa* (Curing action)

(4) In vitro curing activity of β-sitosterol of different concentrations

The curing effect of β-sitosterol of different concentrations was listed in Table 9.8 and Fig 9.4. The treatment effect ofβ-sitosterol also increased with increase of concentration. The inhibiting rate of β-sitosterol of 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml was 14.71%, 18.75%, 19.78%, 25.40% and 27.08%, respectively. But, the changes of inhibition percentage of every two concentrations (for example, 10 mg/ml and 20 mg/ml) were not obvious.

Table 9.7 In vitro curing activity of β-sitosterol of *S. adoxoides* root extracts of different concentrations to TMV

Concentration (g/mL)	Average local lesions of treatment	Average local lesions of control	Inhibition rate* (%)
20	140	192	27.08 a
10	141	189	25.40 ab
5	73	91	19.78b c
2.5	193	237	18.57 c
1.25	116	136	14.71 c

* 1. Averaged from 3 replicates of 5 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

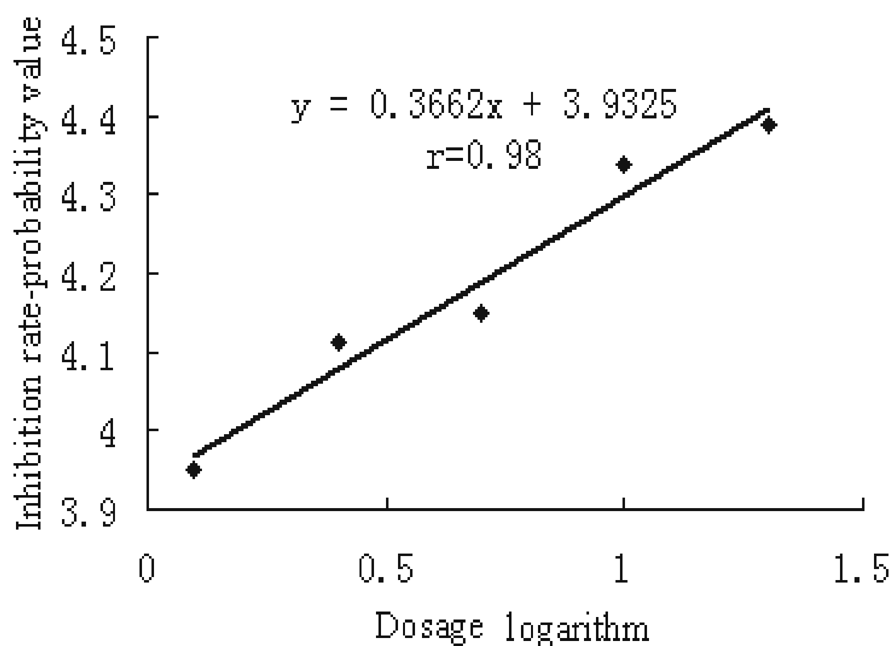

Figure 9.4 Correlation between concentrations of β-Sitosterol and inhibition rate on *N.glutinosa* (Curing action)

9.3.4 Anti-TMV activity of active components from *S. adoxoides* root extracts in pot trial

(1) In vivo inactivation

In vivo inactivation results of active compounds of *S. adoxoides* to TMV were listed in Table 9.9. Stearic acid of 0.01 g/ml had the best inactivating activity, with inhibition percentage of 60.50%. The inhibition rate of β-sitosterol of 0.01 g/ml was also up to 57.14%. There was not much significant difference from virus A. There were not significant differences between stearic acid, β-sitosterol and the control Virus A.

Table 9.8 In vivo inactivation of active compounds of *S. adoxoides* root extractsto TMV

Compounds	Ave. local lesion number of	Ave. local lesion number of control	Inhibition rate* (%)
β-sitosterol	69	161	57.14 a
Oleic acid	73	111	34.23 b
Stearic acid	79	200	60.50 a
Virus A	50	120	58.33 a

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) In vivo protective action

In vivo protective action results showed that stearic acid of 10 mg/ml had better protective effect with inhibition rate of 48.82%.There was significant difference between protective effect of stearic acid and the control Virus A. The inhibition rate of oleic acid of 10 mg/ml was 38.30%, there was not significant

difference between protective action of oleic acid and the control Virus A (Table 9.10).

Table 9.9 In vivo protective action of active compounds of *S. adoxoides* root extracts to TMV

Compounds	Ave. local lesion number of	Ave. local lesion number of control	Inhibition rate*
B-sitosterol	152	199	23.62 c
Oleic acid	145	235	38.30 b
Stearic acid	65	127	48.82 a
Virus A	180	300	40.00 b

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(3) In vivo curing activity

Through comparing data of Table 9.11, it can be found that that curing activity of oleic acid, stearic acid and β-sitosterol of 10mg/ml declined in the order when time expending. After TMV being inoculated of 2 h, 24 h and 48 h, inhibition percentages of oleic acid to TMV were 58.33%, 46.15% and 42.27%, respectively. For stearic acid, inhibition rate to TMV was 41.67%, 39.58% and 33.96%, respectively. Their curing effects were higher than that of the control of virus A. It can be seen from data of Table 9.11, for the three active components, curing effect of inoculation after 2 h was higher that of inoculation after 24 h and 48 h, which meansing that TMV disease should be control in field as soon as possible.

Table 9.10 In vivo curing activity of active compounds of *S. adoxoides* root extracts to TMV after inoculation of 2 h, 24 h and 48 h

Compounds	2 h			24 h			48 h		
	Lesions		Inhibition rate[2] (%)	Lesions		Inhibition rate[2] (%)	Lesions		Inhibition rate[2] (%)
	T[1]	C[1]		T[1]	C[1]		T[1]	C[1]	
β-sitosterol	66	87	24.14 c	72	99	27.27 c	98	133	26.32 c
Oleic acid	50	120	58.33 a	70	130	46.15 a	56	97	42.27 a
Stearic acid	42	72	41.67 b	116	192	39.58 b	35	53	33.96 b
Virus A	86	132	34.85 b	30	44	31.82 c	168	196	14.29 d

Note : 1. T is local lesions of treatment and C is local lesions of control. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

9.4 Discussion and conclusions

9.4.1 Anti-TMV action of active components from *S. adoxoides* root extracts in vitro

The biological activity of active components of petroleum ether extracts of *S. adoxoides* was further studied. The results presented here showed that active components of *S. adoxoides* to TMV had certain bioactivity. Inactivation inhibition percentages of stearic acid, oleic acid and β-sitosterol of 10mg/ml to TMV were 56.86%, 40.00% and 50.73%, respectively. Protective action percentages of stearic acid, oleic acid and β-sitosterol were 57.48%, 49.38% and 23.27%, respectively. Curing action inhibition percentages of oleic acid, stearic acid and β-sitosterol of 10mg/ml to TMV were 37.41%, 28.00% and 25.40%, respectively. In vitro results of inactivation, protection and curing activity of active components of

different concentrations to TMV showed that the inhibition rate of stearic acid, oleic acid and β-sitosterol to TMV was positively correlative with its concentration. Results showed that the longer time TMV was mixed with β-sitosterol, the higher inhibition rate was. However, inhibition rate increased slowly after 30 min.

9.4.2 Anti-TMV effect of active components from S. adoxoides root extracts in pot trial

In vivo determination results of bioactivity showed that of oleic acid, stearic acid and β-sitosterol separated from petroleum ether extracts of *S. adoxoides* had certain bioactivity to TMV. Their protective effect was higher that of curing effect. At the concentration of 0.01 g/ml, the stearic acid and β-sitosterol had a good inactivity effect, with inhibition rate of 60.50% and 57.14%, respectively. The protective activity of stearic acid and oleic acid was the best and inhibition percentage was 48.82% and 38.30%, respectively.

Oleic acid of 10mg/ml also had better curing effect, with inhibition percentage of 58.33%, 46.15% and 42.27%, respectively after inoculation of 2 h, 24 h and 48 h. For stearic acid, inhibition rate to TMV was 41.67%, 39.58% and 33.96%, respectively. Their curing effects were significantly higher than that of the control of Virus A. For the three active components, curing effect of inoculation after 2 h was higher that of inoculation after 24 h and 48 h, which meansing that TMV disease should be control in field as soon as possible. In this study, it was found that there were differences between results of in vivo and in vitro experiment, but the trends were consistent.

CHAPTER X

DEVELOPMENT AND APPLICATION OF EMULSION IN WATER OF ANTI-TMV ACTIVE COMPONENTS OF *S. adoxoides* ROOT EXTRACT

10.1 Introduction

Through research and development of formulations, the adverse effects of pesticides on the environment and human health can be reduced or avoided, and the application time of pesticides can be prolonged. From domestic and international trends of pesticide prescription, it can be seen that pesticide formulations are expanding to water-based, granular, slow-release, multi-purpose direction. Accordingly, there are emerging some new formulations of security, economic, and energy saving (Ling, 1999). At present, the main direction of preparation processing is to create the agents with the functions such as reducing toxicity, enhancing safety, reducing pollution, lightening the crop injury, more security for users, ease of use, saving labor and energy, reducing prices, and enhancing utilization rate

Water-based formulations are liquid formulations of pesticides made from water as the medium. Such formulations have characteristics such as low pesticide injury, low toxicity, easily diluted, uninflammable, inexplosive, easy use, being good for environmental protection, and being easy measures and so on. Water-based formulations mainly include : suspension concentrate (SC), emulsion in water (EW), suspension emulsions (SE), microemulsion (ME), soluble granules (SG) and water

dispersible granules (WDG). Now, SC, EW and WDG are main formulations developed actively (Leng et al., 2003; Zhong, 2003). EW, emulsion oil in water, is an O/W emulsion formed from the tiny droplets of hydrophobic liquid or solid pesticides in water by entering energy, appropriate emulsifier, and other auxiliary agents under the condition of limited solvents.

Synthetically comparaed and analysised all above results, B4-3 and C5-5 had better protective and curing effect to TMV. Thus, in this study, B4-3 and C5-5 would be used as the objectives to process their formulation of EW and determine anti-TMV bioactivity.

10.2 Material and methods

10.2.1 Test active compounds

The active compounds B4-3 (labeled as TK) and C5-5 (labeled as TK-1) were separated from chloroform extract (labeled as B) and ethyl acetate extract (labeled as C) of *S. adoxoides* root extracts and used as process formulation of EW, respectively.

10.2.2 Preparation and Processing technics of emulsion in water (EW)

As a pesticide formulation, EW should have good thermal storage stability, anti-freezing dissolving stability, and water dilution stability, therefore, formulation is more complicated. The EW formulation often contains active ingredient, limited solvent, specific emulsifier, dispersant, general emulsifier, antifreeze, defoamer, anti-microbial agent, density regulator, pH-regulator, thickener, coloring agent, and odour adjusting agent. Among these auxiliary agents, some are essential, but some are not. Screening and optimization of the various components and contents should be accurately conducted in order to obtain an excellent performance and cheap price of

water emulsion.

(1) Choice of solvent

Solvent of 5g, water of 10 ml was added into the effective components of 1g TK or 1g TK-1, respectively. The resulting mixture was mixed in an electric mixer. Then results were observed and recorded. In this study, benzene, toluene, xylene and dimethyl sulfoxide (DMSO) were tested as solvents. Selection of solvents was determined according to their solubility.

(2) Choice of emulsifier

In this study, 10 commonly used emulsifiers OX-0656, OX-0658, OX-8686, OX-8656, OX-7513, OX-708, emulsifier-0201, emulsifier-0204, emulsifier-500 and Tween-80 were tested. Each emulsifier of 2 g was added into the effective components of 1 g TK or 1 g TK-1, respectively. The resulting mixture was mixed in an electric mixer. After 10 ml water was added, the results were observed and recorded. Suitable emulsifiers were chosen, according to the physical and chemical properties of EW formulations.

(3) Choice of antifreeze

The effective components of 1g TK or 1g TK-1 was dissolved in 10 ml water, respectively. Antifreeze of 1 g was added into the mixture. The resulting mixture was mixed in an electric mixer and stored in a refrigerator at -5 ^{O}C. After 2 weeks, results were observed and recorded. In this study, 6 commonly used antifreezes : propylene glycol, glycerin, urea, ammonium sulphate and sodium chloride were tested. Suitable antifreezes were chosen according to the physical and chemical properties of EW preparation.

(4) Choice of thickener

Active components of 1 g TK or 1 g TK-1 was dissolved in water of 10 ml, respectively. Thickener of 1 g was added into the mixture. The resulting mixture was mixed in an electric mixer and stored in a refrigerator at -5 ^{O}C. After 2 weeks, results were observed and recorded. In this study, 4 commonly used thickener : polyacrylate thickener, carboxymethyl cellulose sodium (CMC), sodium alginate, and gum arabic were tested. Suitable thickeners were chosen according to the physical and chemical properties of EW preparation.

(5) Choice of other auxiliary agents

In EW processing, other auxiliary agents such as pH-regulator, the coloring agent also may be used. These auxiliary agents were selected according to the practical conditions.

(6) Processing technics of emulsion in water (EW)

Processing of EW generally had the following three processes (Fig 10.1).

First : TK or TK-1 compounds, solvents, specific emulsifiers, common emulsifiers were mixed together. The resulting mixtures were stirred in emulsification and shear mixer (DJ-11) into uniform oily liquid.

Second : water, antifreezes, antimicrobial agents, coloring agent, and defoamers were mixed together. Then mixtures were stirred in DJ-11 mixer into uniform water liquid.

Finally : Under the stiring at high speed in emulsification and shear mixer BME-100, the first oily liquid was added slowly into the second water liquid. After mixing 20 min. at high-speed in BME-100 mixer, a homogeneous and stable

water emulsions be obtained.

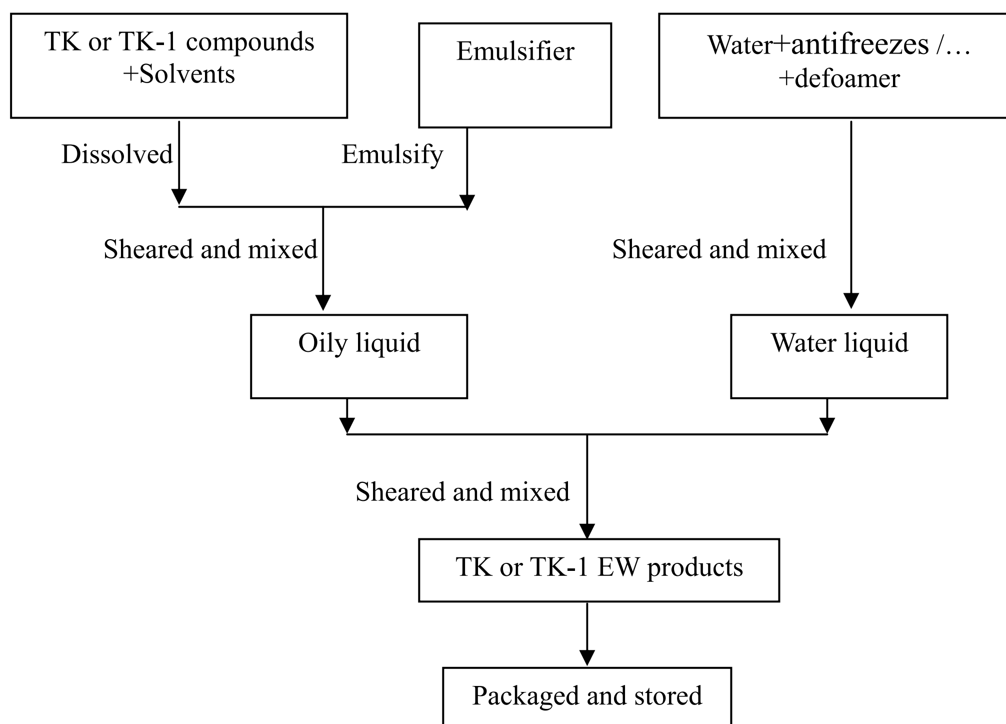

Figure 10.1 Flowchart of processing technics of emulsion in water (EW)

10.2.3 Prescriptions and quality control index of 20% TK EW formulations

(1) Contents of active components

Contents of active components of 20% TK EW were determined according to their physical and chemical properties of active components, the dissolution properties of solvents, emulsification of emulsifiers, and the stability of TK EW formulation. In principle, the higher contents of active components were, the lower costs of EW would be since EW of high contents of active components is good for reducing packaging and transport.

(2) Thermal storage stability

EW should be tested for storage stability in the reservoir, to maintain good emulsion state. It should be qualified if EW stays uniform after being rocked gently.

EW was stored in an incubator at 54 ± 2 ^{O}C for 14 days. It would be acceptable if decomposition percentage of active components was less than or equal to 5%. EW should keep the emulsion state, and oily layers should not occur in EW. EW was qualified if the emusion elicit few oily drops but disappear after being rocked gently.

(3) Stability of cold storage

Appropriate samples were sealed in glass bottles and stored in a refrigerator at 0 ^{O}C, -5 ^{O}C or -9 ^{O}C. After 1 week or 2 weeks, samples were observed. Samples were qualified when they did not delaminate or crystalize. Choice of temperature was determined according to practical conditions. In this study, -5 ^{O}C was used as the standard temperature.

(4) Determination of pH value

Determination of pH values was conducted at room temperature with pH digital indicator. The pH is very important to the stability of the EW, especially the active chemical components.

(5) Determination of the emulsion drop fineness

Fineness of oil-water emulsion drop of emulsion was tested by laser granularity distributor (JL-9200). It is necessary that the drop size of oil-water emulsion of the sample was steady. Generally, the diameter of oil-water emulsion drop should be 0.1~50 μm. When the diameter is 5~20 μm, EW has the highest stability.

(6) Determination of viscosity

In this study, viscosity of the EW was determined by using digital viscosity meter (NDJ-5S). Thickener is very useful to help the stability of EW formulations, but high viscosity is not good to dilute and packed.

(7) Water dilution capacity

It is qualified if one drop of EW formulation was added in 100 ml water and formed uniform liquid after being rocked gently. Commercial EW having a high concentration is often diluted before used in the field. Thus, the function of EW should not be influenced by water of different quality.

(8) Determination of prescriptions of 20% TK EW formulations

Through selecting a variety of auxiliary agents, the quality control index, 7 kinds 20% TK EW various prescriptions determined having the different physical state, were appraised using described as above. The optimal prescriptions of 20% TK EW were assayed for determination of anti-TMV bioactivity.

10.2.4 Bioassay of 20% TK EW formulations for TMV in the pot trial

The 0.1 ml selected EW formulations containing 20% TK were dissolved in 10 ml water. Virus A solution was also diluted to 100 times. Bioassay of anti-TMV activity of the 20% TK EW formulations made from ***S. adoxoides*** root extracts on *N. glutinosa* was conducted with the same processes as described in 5.2.5. Calculation of inhibiting rate was conducted with the same processes as described in 5.2.6.

(1) In vivo inactivation of 20% TK series EW

The 20% TK EW formulations solutions were mixed with the same volume of 10.19 μg/ml purified TMV suspension. After 30 min, the mixture was

inoculated on the left half leaf using silicon carbide as an abrasive. The right half leaf was inoculated with the virus plus the equal volume of water as check.

(2) In vivo protective activity of 20% TK series EW

The 20% TK EW formulations solutions were transferred gently onto the left half leaf with brush pen. The blank control was also transferred onto the right half of the same leaf. Each treatment had three individual plants. Two hours after treatment, TMV suspension of 10.19 µg/ml was inoculated on the both halves.

(3) In vivo curing activity of 20% TK seriesEW

The TMV suspension of 10.19 µg/ml was inoculated on tobacco leaf of *N. glutinosa*. After inoculation of 2 h, the 20% TK EW formulations solutions and Virus A solutions were treated on the left half leaf, and the blank control solution was treated on the right half leaf.

10.2.5 Determination of 20% TK #2 EW of different concentrations for TMV in pot trial

20% TK #2 EW formulation was diluted to 50, 100, 200, 400, 1000 times with water, respectively. The TMV suspension of 10.19 µg/ml was inoculated on tobacco leaf of *N. glutinosa*. After inoculation of 2 h, the 20% TK EW formulations solutions and Virus A solutions were treated on the left half leaf, and the blank control solution was treated on the right half leaf.

10.2.6 Determination of controlling effect of 20% TK #2 EW for TMV in the field

The experiment was conducted on *Nicotiana tabacum* K326 in randomized complete block design (RCBD) having 7 treatments that were the best 20% TK #2 EW formulation diluted 50, 100, 200, 400 and 1000 times with water,

0.001 g/ml Virus A and water blank control with 4 replications. The tobacco plants were inoculated with TMV suspension of 10.19 µg/ml. In each replication 5 sampling spots within a plot of 20 m^2 were randomly allocated and 5 plants were observed for TMV symptoms 7 days after the last spraying of the solutions of 20% TK #2 EW. The sprays were done twice 7 days apart. The plants were at 8-10 leaves stage when the experiment was conducted at the farm of Guizhou University.

Disease severity was taken as 0-4 disease scores as followed : control effect of TMV was calculated.

0 = There are no symptoms on the whole plant;

1 = vein clearing on less than 1/3 of leaves are mosaic;

2 = 1/3-1/2 of leaves is mosaic, or a few leaves are abnormal, or plant height is less than 2/3 of the normal plants;

3 = 1/2-2/3 of leaves is mosaic, or plant hight is 1/3~1/2 less than of the normal plants;

4 = all leaves of the whole plant are mosaic, severely abnormaly, height of the diseased plants is less than 2/3 of the normal plants.

Control effect was calculated with the following expressions :

$$\text{Disease incidence (\%)} = \frac{\text{number of diseased plants}}{\text{number of investigated plants}} \times 100$$

$$\text{Disease index} = \frac{\sum [\text{disease score} \times \text{plant number}]}{\text{plant number investigated} \times \text{number of the last score}} \times 100$$

$$\text{Control effect (\%)} = \frac{\text{disease index of control plot - disease index of treatment plot}}{\text{disease index of control plot}} \times 100$$

10.3 Results

10.3.1 Determination of auxiliary agents of 20% TK EW formulations

(1) Determination of solvent

In the processing of pesticide formulations, some commonly used solvents such as benzene, toluene and xylene have strong volatility and flammability, and are harmful to human health and environment. Therefore the aromatic solvents such as xylene should be avoided in large-scale processing. Results in (Table 10.1) showed that TK dissolved completely in dimethyl sulfoxide (DMSO) of 5 g. TK-1 dissolved completely in both xylene and DMSO of 5 g. So in this study, DMSO was used as a solvent due to its wide dissolution range, low pollution, and less toxic to humans.

Table 10.1 Dissolved quality of TK active ingredients from *S. adoxoides* root extracts in different solvents

Solvents	Dissolution results	
	TK	TK-1
Benzene	Small amount dissolved	Reasonably dissolved
Toluene	Reasonably dissolved	Reasonably dissolved
Xylene	Reasonably dissolved	Completely dissolved
DMSO	Completely dissolved	Completely dissolved

(2) Determination of emulsifier

Emulsifier can produce a film on the oil granul surface base on space-resistance and electrostatic effect. The polar end of emulsifier is bound to water and its non-polar end is bound to oil. So an oil granul can combine and grow up, which keeps emulsion stable. It can be seen from Table 10.2 that different emulsifiers had the different emulsification effect for TK and TK-1. Among the ten emulsifiers, emulsification effect of OX-7513 was clearly transparent for TK and TK-1 and it was the best emulsifier in series of preparations of 20% TK EW. EM-500 and EM-0201 was optional emulsifier too. Two emulsifiers such as EM-500 and EM-0201 also can be used as emulsifier in series of preparations of 20% TK EW due to their transparent emulsification effect for TK and TK-1.

Table 10.2 Emulsification effect of TK active ingredients from *S. adoxoides* root extracts in different emulsifiers

Emulsifier	**Emulsification effect**	
	TK	**TK-1**
OX-0656	Turbid	Translucent
OX-0658	Turbid	Translucent
OX-8686	Turbid	Turbid
OX-8656	Stratified	Turbid
OX-7513	Clearly transparent	Clearly transparent
OX-708	Turbid	Translucent
EM-500	Translucent	Transparent
T-80	Stratified	Turbid
EM-0201	Translucent	Transparent
EM-0204	Turbid	Translucent

(3) Determination of anti-freeze

In order to ensure storage stability of EW in the low-temperature, choice of the anti-freeze was conducted. Results in Table 10.3 showed that the ethylene glycol shown excellent antifreeze effect for both TK and TK-1, thus it was the best anti-freeze in series of preparations of 20% TK EW. Propylene glycol and glycerin normally are used as antifreeze, but also can be used as emulsifier for in series of preparations of 20% TK EW due to their better effect for TK and TK-1.

Table 10.3 Anti-freeze effect of TK active ingredients from *S. adoxoides* root extracts in different antifreeze

Anti-freeze	Anti-freeze effect	
	TK	**TK-1**
Ethylene glycol	Clearly transparent	Clearly transparent
Propylene glycol	Translucent	Transparent
Glycerin	Translucent	Translucent
Urea	Stratification	Crystal
Ammonium sulphate	Crystal	Transparent
Sodium chloride	Turbid	Crystal

(4) Determination of thickener

Active component of 1 g TK or 1 g TK-1 was dissolved in water of 10 ml. Thickener of 1 g was added into the mixture. The resulting mixture was mixed in an electric mixer and stored in arefrigerator at -5 ^{O}C. After 2 weeks, results in Table 10.4 showed that the CMC of 1 g had a good physical state and good liquidity for TK and TK-1, with viscosity of 436 and 520 mPa.s. Thus, the CMC can be used as thickener in series of preparations of 20% TK EW.

Table 10.4 The viscosity or status of TK active ingredients from *S. adoxoides* root extracts in different thickener

Thickener	Viscosity (mPa.s) or status	
	TK	TK-1
Polyacrylate	Sedimentation	267
CMC	436	520
Alginate	315	Crystal
Arab pastern	Stratification	Translucent

(5) Determination of other auxiliary agents

As an anti-TMV inhibitor, the object of the EW is TMV. TMV exists in the undersurface of epidermal cells. Few auxiliary agents such as penetrating agent can enhance the permeability of TK or TK-1. Thus, it is necessary to select other auxiliary agents penetrating agent, coloring agent, and smell regulator and so on.

10.3.2 Prescriptions and quality control index of 20% TK EW formulations

(1) Determination of prescriptions of 20% TK EW formulations

Through selecting a variety of auxiliary agents, there 7 prescriptions were determined. Results of processing craft of the laboratory and various prescriptions showed the different physical states. Among 7 prescriptions, prescriptions #1, #2, #3, #6 and #7 had better characters. Prescriptions of #4 and #5 were not suitable to be studied due to stratification and turbid phenomena, respectively (Table 10.5).

Table 10.5 Prescription determination of series of 20% TK EW in15% DMSO solvent, 3% CMC thickener and 47% water

Prescription number	Active components	Emulsifier	Antifreeze	Result
1	20% TK	OX-7513 of 10%	5% ethylene glycol	Good
2	20% TK-1	OX-7513 of 10%	5% ethylene glycol	Good
3	20% TK-1	OX-7513 of 10%	5% propylene glycol	Good
4	20% TK-1	Em-0201 of 10%	5% ethylene glycol	Stratification
5	20% TK-1	Em-0201 of 10%	5% propylene glycol	Poor
6	20% TK-1	Em-500 of 10%	5% ethylene glycol	Good
7	20% TK-1	Em-500 of 10%	5% propylene glycol	Good

(2) Quality control index of 20% TK EW formulations

Results of quality control presented in Table 10.6 showed that the prescriptions of #1, #2 and #6 measured up to the standards of EW quality control index. Prescriptions of #3 and #7 did not meet the standards of EW quality control index due to stratification phenomenon in the thermal storage stability and turbid phenomenon in cold storage stability, respectively.

Table 10.6 Index of quality control of 20% TK EW formulations

Prescription number	Thermal storage stability	Cold storage stability	pH	Granule distribution	Viscosity mPa.s	Water dilution capacity	Result
1	Good	Good	7.1	Uniform	536	Good	√
2	Good	Good	6.2	Uniform	453	Good	√
3	Stratification	Good	5.2	General	317	Good	×
6	Good	Good	5.4	Uniform	384	Good	√
7	Good	Stratification	6.0	Uniform	256	Good	×

10.3.3 Anti-TMV bioactivity of 20% TK EW formulations in the pot trial

(1) In vivo inactivation of series of 20% TK EW

In vivo inactivation experiment, inhibition percentage of prescription of #1, #2 and #6 to TMV was 57.70%, 68.64% and 59.62%, respectively (Table 10.7), which aws higher than that of virus A. Prescription of #2 had the highest inhibition percentage of 68.64%, which was significant different from that of prescription of #1 and #6 and significantly different from that of virus A. Inhibition percentage of prescription of #1 and #6 was also different from that of virus A.

Table 10.7 In vivo inactivation of 20% TK EW formulations to TMV

20% TK EW	Average local lesions of treatment	Average local lesions of control	Inhibition rate* (%)
1	12.56	29.67	57.70 bc
2	9.44	30.11	68.64 a
6	11.67	28.89	59.62 b
Virus A	12.33	28.33	56.48 c

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

(2) In vivo protection action of series of 20% TK EW

In vivo protection action experiment, inhibition percentage of prescription of #1, #2 and #6 to TMV was 4.47%, 62.05%,and 54.73%, respectively (Table 10.8), which were higher than that of virus A. Inhibition percentage of prescription of #2 was significantly different from that of prescription of #1 and #6. Inhibition percentage of prescription of both #1 and #6 was not significant higher than

that of virus A.

Table 10.8 In vivo protection action of 20% TK EW formulations to TMV

20% TK EW	**Average local lesions of treatment**	**Average local lesions of control**	**Inhibition rate* (%)**
1	13.33	29.33	54.47 b
2	11.55	30.45	62.05 a
6	13.78	30.45	54.73 b
Virus A	15.11	30.67	50.72 b

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

(3) In vivo curing activity of series of 20% TK EW

In vivo curing action experiment, inhibition percentage of prescription of #1, #2 and #6 to TMV the at 59.92%,64.28% and 56.69%, respectively (Table 10.9), which were higher than that of Virus A. Inhibition percentage of prescription of #2 and was not significant higher than that of prescription of #1 and #6. There are not significant differences between inhibition percentages of prescription of #1 and, both #6 and Virus A. Inhibition percentage of prescription of #2, #1 and #6 was significantly different from that of Virus A, respectively.

Table 10.9 In vivo curing activity of 20% TK EW formulations to TMV

20% TK EW	Average local lesions of treatment	Average local lesions of control	Inhibition rate* (%)
1	11.78	29.33	59.93 a
2	10.33	29.11	64.28 a
6	12.44	28.78	56.69 ab
Virus A	14.00	29.11	51.95 b

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

10.3.4 Bioactivity of 20% TK #2 EW of different concentrations for TMV in the pot trial

In vivo anti-TMV bioactivity of TK #2 EW of different concentrations was presented in Table 10.10. The results showed that anti-TMV bioactivity of TK #2 EW increased with rise of concentrations. Inhibition percentage of TK #2 EW of 50 times, 100 times, 200 times and 500 times was 76.60, 69.26, 60.45 and 43.07, respectively. The correlation analysis between concentration logarithm and inhibition rate (probability value) showed that regression equation was $Y=1.0642X+1.9218$ ($r=0.9908^*$), ED50 was to 724.44 mg/l (Fig 10.2). Thus, TK #2 EW diluted to 200 times can be used as the field recommended dosage in the field control.

Table 10.10 In vivo anti-TMV bioactivity of TK-#2 EW of different concentrations

Times of dilution	Average local lesions of treatment	Average local lesions of control	Average inhibition rate (%)
50	6.63	28.33	76.60 a
100	9.12	29.67	69.26 b
200	11.34	28.67	60.45 c
500	16.51	29.00	43.07 d
1000	20.63	28.67	28.04 e

* 1. Averaged from 3 replicates of 9 half leaves.

2. Means followed by the same letter in the column are not statistically different at P<0.01 by DMRT

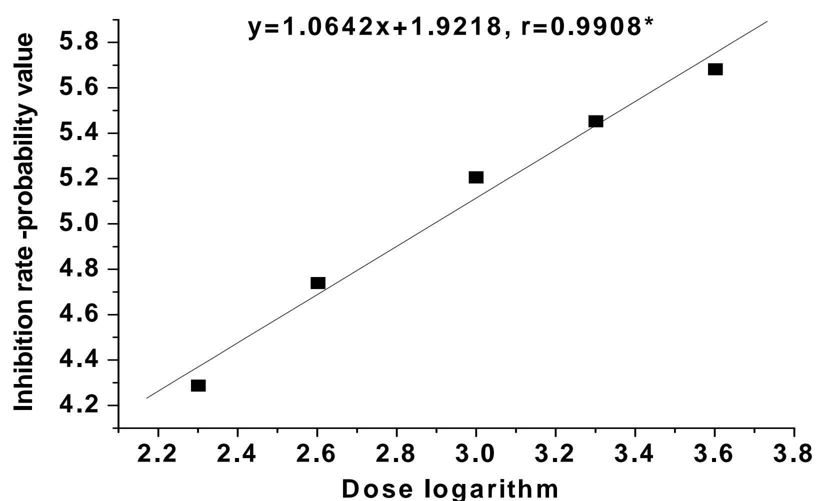

Figure 10.2 The virulence regression equation of TK #2 EW against TMV in the pot

10.3.5 Controlling effect of 20% TK #2 EW of different concentrations for TMV in the field

Field experimental results (Table 10.11) showed that inhibition percentage of TK #2 EW of 400 times, 200 times and 100 times was 46.93%, 65.16% and 67.86%, respectively. Inhibition percentages of TK #2 EW of 100 times and 200 times was significantly different from that of TK #2 EW of 400 times and virus A, respectively. However, there were not differences between inhibition percentage of TK #2 EW of 100 times and TK #2 EW of 200 times. Control effect of TK #2 EW of 400 times was significantly lower than that of virus A.

Table 10.11 Controlling effect of TK #2 EW to TMV in the field

Treatments (diluted to times)	Disease incidence (%)	Disease index	Control effect* (%)
#2 EW 400	60.00	19.27	46.93 c
#2 EW 200	42.67	12.65	65.16 a
#2 EW 100	40.00	11.67	67.86 a
Virus A 100	53.33	16.93	53.37 b
Water	73.33	36.31	0.00

* 1. Averaged from 4 replicates of 5 plants.

2. Means followed by the same letter in the column are not statistically different at $P<0.01$ by DMRT

10.4 Discussion and conclusions

10.4.1 Stability mechanism and quality control standards of EW

A water emulsion pesticide product must have a long shelf life. Single emulsifier often can not make the water emulsion system maintain its stability after a

long storage period. Other auxiliary agents such as stabilizer, common emulsifier, and thickener, decentralized agents should be added in to EW to keep the system stable. At present, there are four theories, including the volume theory (Cui et al., 1992), dual interface film theory (Walstra, 1986), directed embed theory (Xiao et al., 2003), and coalescence velocity theory (Davies, 1957). Since the limited laboratory conditions, emulsifying properties of emulsifiers were evaluated only from their appearance. In order to screen the better emulsifier, water emulsion stability is needed to be studied in-depth, and emulsifying properties of emulsifiers should be analyzed with quantitative and qualitative methods.

As a new, environmental protection and water-based formulation, EW was studied less in China. Relevant departments and research organizations do not confirm the unification standards of EW quality control. Most standards of the EW were established based on practical experience. For example, cold storage stability of water emulsion at 0 ^{O}C, -5 ^{O}C and -9 ^{O}C. There were differences in stability under cold storage under different tempretures. Thus, these provide uncertainties factors to the quality of EW.

10.4.2 Control effect of TK #2 EW to TMV in the field

In the field experiment, TK #2 EW of 100 times and 200 times, which had inhibition percentage of 67.86% and 65.16%, respectively, which was significantly different from that of Virus A. This showed that TK #2 EW of 100 times and 200 times has a better prospects and development. In the field control experiment of TMV, TK #2 EW diluted to 200 times can be used as the field recommended dosage. According to practical conditions, spray should be conducted 2 to 3 times in the planting season, every 7 days. Bioactivity of TK #2 EW toTMV on the *N.tabacum*

K326 was good, but not very ideal. The possible reasons were as follows : (1) TK #2 EW contains many compounds, of which may be passive on the host plants. (2) Application approach is not right and the application quantity of TK #2 EW is not enough to inhibit TMV infection. (3) The instability of physical and chemical properties, easy decomposition and inferior penetrating capability of TK #2 EW.

10.4.3 Extension and application of anti-TMV activity of TK #2 EW

The transmitting, invasion, DNA replication, and formation of a new virus particle of TMV are an indivisible way, as long as the appropriate means and inhibitors are found, the large area occurrence of the TMV could be controlled. In this study, there was a good result that TK #2 EW had good anti-TMV bioactivity on the tobacco K326 in the field and in pot experiment of the laboratory. However, a majority of experiments were carried out in the laboratory, which indicating that large numbers of experiments are needed in order to prove effectiveness and extend of TK #2 EW and apply it. In addition, the period of validity, toxicity and degradation dynamics are also conducted. These are the study directions need to be carried out before the application in large area.

In this study, it was made sure that extracts of *S. adoxoides* had good inactivation, protection and curing action on TMV. Anti-TMV bioactivity of active components of petroleum ether extracts of *S. adoxoides* was also carried out by using biological activity tracking method. Some components had a better inhibiting action to TMV. A series of 20% TK EW were developed through using active components of TK and TK-1 separated from extracts of *S. adoxoides*.This whould provide an important theoretical basis and practical guidance for research and development of antiviral inhibitors of plant sources.

CHAPTER XI

CONCLUSION AND RECOMMENDATION

In this study, linking the development tendency and goal of the modern pesticide research and development, taking the viral disease of the important economic crops in the agricultural production as the main objectives, the expecting goals have already been accomplished. The characteristic and the innovation of the important results of the study are as follows :

1. The extraction methods of active components of *S. adoxoides* was explored, the root powders of *S. adoxoides* were extracted with two extraction procedures. Systematic extraction method is better than non- systematic extraction method according to total percentage of *S. adoxoides* root extracts, the former has more than 38%.

2. The test results of ethanol crude extracts of *S. adoxoides* root showed that *S. adoxoides* contain saccharide, organic acids, anthraquinone, phenols, volatile oil, and may contain alkaloids, flavonoids, coumarin, amino acids, tannin, steroids, triterpene, and not contain proteins, peptides and lipids. There were 31 isolated substances of petroleum ether extract of *S. adoxoides.* The chemical structures and content of 12 substances were also identified. Content of components detected was 90.55% of total extracts from root of *S. adoxoides.*

3. The results by half leaf local lesion technique showed that it was the first time that inactivation, and protection activity of anti-TMV of extracts of *S. adoxoides* root was found in vitro and in the pot experiment, inhibition percentage of which were

up to 68.09% and 65.26%, respectively. Ethyl acetate extract, petroleum ether extract, and chloroform extract also had high curing effect, with curing inhibion rate of 72.36%, 50.26% and 47.01%, respectively, which was significantly higher than control pesticide Virus A.

4. Anti-TMV activity of extracts of *S. adoxoides* root was studied further with ELISA. The results showed that after treatment with inhibitors, infection amount of the virus that getting to the maximum value was delayed and the symptom of host plant also lightened with increase of time.

5. For determining active fractions of ethanol crude extracts of *S. adoxoides* root, including petroleum ether, chloroform, ethyl acetate, butanol and water, were assessed. Results of bioactivity by half-leaf local lesion technique showed that active fraction B4-3, eluted from B with 100mL chloroform-methanol (83 : 17,V : V), had good protective and curing effect, with inhibiting percentage of 52.54% and 48.69 % to TMV, respectively. Active fraction C5-5, separated from C with 100mL chloroform-methanol (75 : 25, V : V), had good curing and protective effect, with inhibiting rate of 62.07% and 42.98 % to TMV, respectively.

6. For assessing the physiological biochemistry changes of the host plants after treatment with extracts of *S. adoxoide* root, the variety of CAT, POD and SOD activities were studied.

7. Anti-TMV bioactivity of active compounds was further assessed resorting to three compounds of β-sitosterol, oleic acid, stearic acid separated from petroleum ether extracts of *S. adoxoides* root. Results showed that these three compounds of *S. adoxoides* to TMV had certain bioactivity.

In vitro experiment, stearic acid : inactivation and protective percentage was 56.86% and 57.91%, oleic acid curing percentage was 37.10%. Their control effect was significantly higher than that of Virus A.

In vivo : inactivation of stearic acid : 60.50%, protective of stearic acid : 48.82% Oleic acid curing activity : 58.33%, respectively. It was found that there were differences between results of in vivo and in vitro experiment, but the trends were consistent.

8. A series 20% TK EW formulations were developed. Prescription and quality control index of TK EW were determined primarily. Each quality control index of TK EW is thought to be good through using formulation processing and quality inspection. 20% TK #2 EW is the best formulation which contained active ingredients and auxiliary agents as follow : 20% TK-1, 15% DMSO solvent, 10% OX-7513 emulsifier, 5% ethylene glycol antifreeze, 3% CMC thickener, and 47% water.

9. Bioactivity of 20% TK #2 EW against TMV in tobacco was studied in the pot and field experiment, the results show that, inactivation, protection, and curing inhibition percentages on the tobacco (*N.glutinosa*) were 68.64%, 62.05%, and 64.28%, respectively in the pot experiment and 20% TK #2 EW of 100 times and 200 times on the common tobacco K326 (*N.tabacum*) had inhibition percentage of 67.86% and 65.16%, respectively in the field experiment, which was significantly higher than that of and Virus A.

10. Anti-TMV of active components from extracts of *S. adoxoides* root and their separation and purification and their influence on enzyme activity of TMV hosts were researched by using the half leaf local lesion method and ELISA technique, a series of 20% TK EW were developed primarily through using active components of

TK and TK-1 separated from extracts of *S. adoxoides*, this would establish an important theoretical basis for biologically reasonable design and chemical decorating synthesis of pesticide, and provide the practical guidance and the important clue for research and development of antiviral inhibitors of the new specifically bionic of safety, high effectiveness and better environmental compatibility. This not only can provide new approach and method of creation and development of new specifically bionic pesticides, but also the new plant source of antiviral agent which has a broad application prospect. This would provide a powerful technical support for the development and exportation of the green food or the organic food which are social, economical and ecological significance for the continuable development of world agriculture and environmental protection.

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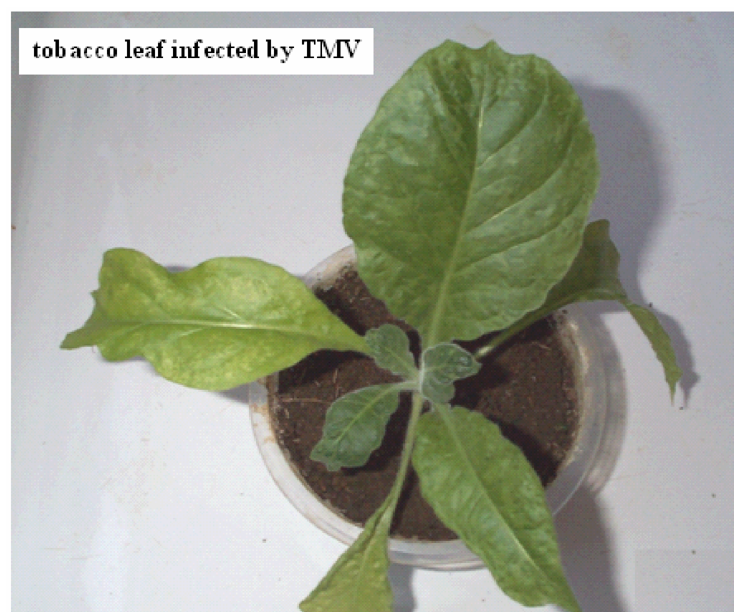
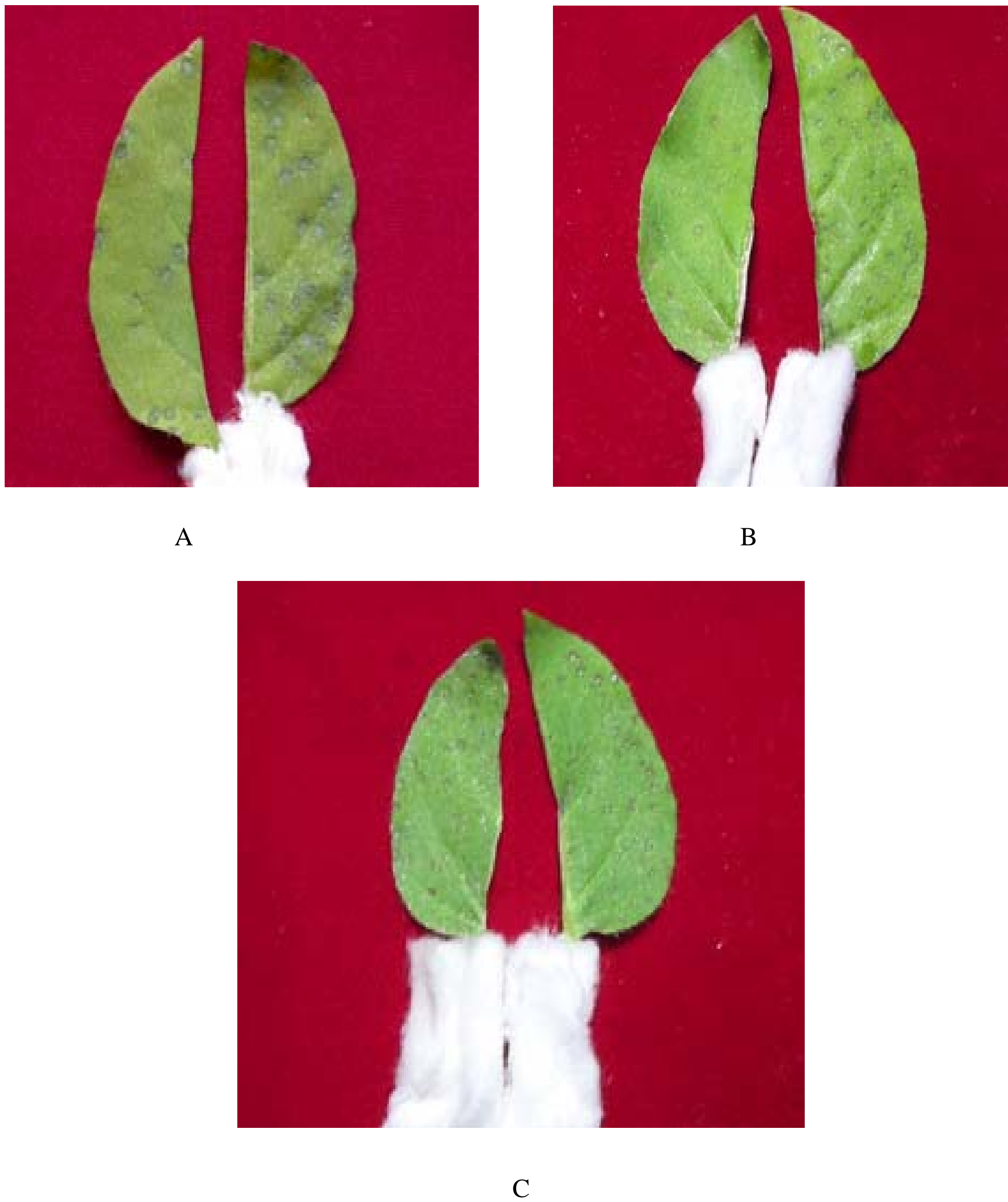
APPENDIX

I. ATTACHED FIGURES



Attached figure 1 : the symptom of *Nicotiana tabacum* with TMV inoculation



Attached figure 2 : In vitro control effect of stearic acid of 10 mg/ml to TMV on the leave of *Nicotiana glutinosa*

Note : Left half leaf : treatment; right half leaf : comparison.

A : inactivation activity;

B : protective activity;

C : curing activity

Attached figure 3 : Occurrence of TMV disease in the feild

Attached figure 4 : Occurrence of TMV disease in the feild

II. Test reagents and buffer preparationare as follows.

(1) 30% hydrogen peroxide (A.R.)

(2) Tris (hydroxymethyl)-methanamin (A.R.)

(3) TMV anti-serum

(4) Enzyme labeled antibody (sheep anti-rabbit IgG)

(5) Calf serum

(6) Coating buffer solution（0.05MCarbonate buffer solution pH9.6）:

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g
H_2O	1000 ml

(7) Washing solution（PBST : 0.01M Phosphate buffer solution pH7.4）:

NaCl	8.00 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_4 \cdot 12H_2O$	2.93 g
Tween-20	0.5 ml
H_2O	1000 ml

(8) Conjugate buffer (100mLPBST containing 2g calf serum albumin and 2g polyvinyl pyrrolidone)

(9) Substrate buffer solution（Note : 4 mg ortho-phenylene diamine and 4 µl H_2O_2 were added before use）

Citric acid	4.8 g
$Na_2HPO_4 \cdot 12H_2O$	7.163 g

H_2O	500 ml
H_2O_2	4 µl

(10) Stop reagent is 1 N HCl

BIOGRAPHY

Mr. Li Ming was born on February 19, 1959 in Guizhou province, China. He received Bachelor degree from Department of Plant Protection, Agricultural College of Guizhou University in 1982. In 1984, he attended advanced mycology courses in the Plant Protection Department of Agricultural University of China. From 1999～2001, he finished all courses and credits prescribed by the postgraduate plan comparable to MSc and attended courses on Production Technology of Agricultural Crops and Environment Safety in the International Cultivating Center of China-Israel and gained a certificate.

Since 1994, he has successively served as deputy director of Plant Protection Department, chief of Natural Pesticides Research Institute, and Vice-president of Agricultural College of Guizhou University. Now he is the chief of Evaluation Division of the Education and Academic President's Office of Guizhou University, a professor, Academic leader of Guizhou University, Pesticides Registration Field Experimental Technique Director of the Agriculture Ministry of China, Member of Chinese Plant Disease Chemical Control Committee, Member of Guizhou Crop Variety Approval Committee, and Outstanding Young Talent of Science and Technology in Guizhou Province.

He has conducted 26 research projects at national, provincial and ministerial levels, published 39 papers and co-written of 2 textbooks. He was awarded the Scientific and Technological Progress second-grade medal of Guizhou Province, second-grade trophy of Agricultural Harvest of Guizhou Province, and 2 awards of outstanding thesis from Chinese Society of Plant Pathology.