

## TABLE OF CONTENTS

	<b>Page</b>
TABLE OF CONTENTS	i
LIST OF TABLES	iii
LIST OF FIGURES	iv
LIST OF ABBREVIATIONS	x
INTRODUCTION	1
LITERATURE REVIEWS	3
<i>Plumbago indica</i> L.	3
Plumbagin	6
The Application of Immunological Methods in Plant	
Secondary Metabolite Studies	8
Production of Secondary Metabolites Using Plant Tissue Culture	12
Nutrient and Environmental Factors Influencing the	
Accumulation of Secondary Metabolites	12
Elicitor Treatment as Strategy to Improve Production of	
Secondary Metabolites	14
Hairy Root Culture	21
<i>In vitro</i> Production of Plumbagin	22
MATERIALS AND METHODS	25
Materials	25
Methods	26
Quantitative Analysis of Plumbagin in Various Plant Parts	
and <i>in vitro</i> Cultures of <i>P. indica</i> L.	26

**TABLE OF CONTENTS (cont'd)**

	<b>Page</b>
Immunolocalization of Plumbagin in <i>Plumbago indica</i> L.	27
Establishment of Hairy Root, Callus and Cell Suspension Cultures	31
Study of Hairy Root, Callus and Cell Suspension Cultures and Plumbagin Production	32
Effects of Biotic and Abiotic Elicitation of Plumbagin in Hairy Root and Cell Suspension Cultures	34
<b>RESULTS AND DISCUSSION</b>	<b>41</b>
Quantitative Analysis of Plumbagin in Various Plant Parts and <i>in vitro</i> Cultures of <i>P. indica</i> L.	41
Immunolocalization of Plumbagin in <i>P. indica</i> L.	44
Establishment of Hairy Root, Callus and Cell Suspension Cultures and Growth Study	55
The Production of Plumbagin in Various Types of Culture	65
Effects of Biotic and Abiotic Elicitation of Plumbagin in Hairy Root and Cell Suspension Cultures	75
<b>CONCLUSION</b>	<b>108</b>
<b>LITERATURE CITED</b>	<b>110</b>
<b>APPENDICES</b>	<b>130</b>

**LIST OF TABLES**

<b>Table</b>		<b>Page</b>
1	Chemical Constituents of <i>P. indica</i> L.	4
2	Examples of increased production of phytochemicals, using biotic and abiotic elicitors, in plant cells and tissue culture system of several plant species	19
3	<i>In vitro</i> studies of <i>Plumbago</i> spp.	23
4	Studies on <i>in vitro</i> production of plumbagin	24
5	Concentrations of elicitors used in the study of the effect of concentration in hairy root cultures	37
6	Concentrations of elicitors used in the study of the effect of concentration in cell suspension cultures	39
7	Comparison of various elicitors for plumbagin stimulation	107
<b>Appendix Table</b>		
1	Composition of plant tissue culture media	131
2	Composition of Czapek's Dox medium	132

## LIST OF FIGURES

<b>Figure</b>		<b>Page</b>
1	<i>Plumbago indica</i> L. (Plumbaginaceae)	5
2	The chemical structure of plumbagin	6
3	The biosynthetic pathway of plumbagin	8
4	Schematic diagram of signal transduction events involved in the stimulation of anthraquinone synthesis by chitosan.	17
5	Calibration curves of standard plumbagin for HPLC analysis	42
6	The HPLC chromatograms of plumbagin standard plumbagin in callus and plumbagin in hairy root	42
7	Plumbagin content in various parts of <i>in vivo</i> and <i>in vitro</i> <i>Plumbago indica</i> L.	43
8	The color change during the conjugation procedure.	46
9	Analysis of plumbagin-protein conjugates using SDS-PAGE	47
10	MALDI TOF mass spectra of samples from plumbagin-carrier protein conjugates	47
11	Antibody titers of the rabbit antisera raised against plumbagin-BSA conjugate determined by ELISA.	49
12	Immunolocalization of plumbagin in cross sections of <i>Plumbago indica</i> L. plant parts (collected in November) using fluorescein isothiocyanate (FITC) labeled secondary antibody.	52
13	Immunolocalization of plumbagin in cross sections of 3-year-old <i>Plumbago indica</i> L. roots using fluorescein isothiocyanate labeled secondary antibody.	53

### LIST OF FIGURES (cont'd)

<b>Figure</b>		<b>Page</b>
14	Immunolocalization of plumbagin in cross sections of <i>Plumbago indica</i> L. leaf using fluorescein isothiocyanate labeled secondary antibody.	54
15	The dry growth index (DGI) of hairy roots on different liquid media and different sucrose concentrations after culture at $25\pm 2^{\circ}\text{C}$ , $55\ \mu\text{mol}/\text{m}^2/\text{s}$ 16 h light, for 6 weeks.	56
16	Hairy roots of <i>Plumbago indica</i> L. cultured in different liquid media and different sucrose concentrations after culturing at $25\pm 2^{\circ}\text{C}$ , $55\ \mu\text{mol}/\text{m}^2/\text{s}$ 16 h light, for 6 weeks.	57
17	Effects of 2,4-D and cytokinin (BA and kinetin) on the growth of <i>Plumbago indica</i> L. leaf- and internode-derived callus.	59
18	Compact callus (A) induced from leaf and friable callus (B) from internode of <i>Plumbago indica</i> L. cultured in MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.2 mg/l BA.	60
19	The friable calli induced from various explants of <i>Plumbago indica</i> L. cultured in MS-B5 supplemented with 0.2 mg/l NAA, 0.2mg/l 2,4-D and 0.5 mg/l kinetin.	60
20	The growth patterns of <i>Plumbago indica</i> L. cell suspension cultures in MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l kinetin	62

### LIST OF FIGURES (cont'd)

<b>Figure</b>		<b>Page</b>
21	The DGI of cell suspension culture obtained from various explant parts in dark and light conditions.	62
22	The DGI of 21 day-old, root derived <i>Plumbago indica</i> L. cell suspension cultures grown in dark condition in MA-B5 medium supplemented with a constant concentration of NAA (0.2 mg/l) and various concentration of kinetin.	64
23	Effects of culture media and sucrose concentrations on plumbagin production of <i>Plumbago indica</i> L. hairy root culture	67
24	Effects of cytokinin (BA and kinetin) and 2,4-D on plumbagin content in leaf-and internode-derived callus of <i>Plumbago indica</i> L.	67
25	Effects of kinetin and 2,4-D concentrations on plumbagin content of <i>Plumbago indica</i> L. cell culture obtained from internode-derived callus under dark and light conditions.	72
26	Effects of kinetin and 2,4-D concentrations on plumbagin content of <i>Plumbago indica</i> L. cell culture obtained from root-derived callus under dark and light conditions.	73
27	Plumbagin released from root-derived cell cultures into the culture media (MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2-0.6 mg/l 2,4-D and 0-0.5 mg/l kinetin) after harvested cell.	74
28	Growth curve of <i>Plumbago indica</i> L. suspension cell culture and plumbagin accumulation in cells and in culture medium.	74

**LIST OF FIGURES (cont'd)**

<b>Figure</b>		<b>Page</b>
29	Effect of chitin at different concentrations on growth, the accumulation and release of plumbagin in hairy root cultures of <i>Plumbago indica</i> L. on day 8 <sup>th</sup> after elicitation.	78
30	Plumbagin contents in culture liquid medium as compared between stop and continuous shaking.	79
31	Effect of chitosan at different concentrations on growth, the accumulation and release of plumbagin in hairy root cultures of <i>Plumbago indica</i> L. on day 5 <sup>th</sup> after elicitation.	81
32	Effect of chitosan at different concentrations on growth, the accumulation and release of plumbagin in hairy root cultures of <i>Plumbago indica</i> L. in 1/2 MS liquid medium on day 2 <sup>nd</sup> after elicitation.	82
33	Effects of acetic acid at different concentrations on the accumulation and release of plumbagin in hairy root cultures of <i>Plumbago indica</i> L. in 1/2 MS liquid medium on day 5 <sup>th</sup> after elicitation.	84
34	The plumbagin was released into 1/2 MS liquid culture medium after elicited with various elicitors compared to control as indicated by the color of the medium.	85
35	Effect of precipitated yeast extract at different concentrations on growth, and the accumulation and release of plumbagin in hairy root cultures of <i>Plumbago indica</i> L. on day 8 <sup>th</sup> after elicitation	87

### LIST OF FIGURES (cont'd)

<b>Figure</b>		<b>Page</b>
36	Effects of fungal mycelium at different concentrations on growth, the accumulation and release of plumbagin in hairy root cultures of <i>Plumbago indica</i> L. for 7 days of elicitation	88
37	Growth and plumbagin content (mg/l) in hairy root culture harvested at different time of elicitation with 500 mg/l chitin.	90
38	Growth and plumbagin content (mg/l) in hairy root culture harvested at different time of elicitation with 400 mg/l chitosan.	90
39	The change of cell color from gray to yellow after chitin elicitation at different concentrations in <i>Plumbago indica</i> L. cell suspension culture.	92
40	Effect of chitin at different concentrations on growth, the accumulation and release of plumbagin in cell suspension cultures of <i>Plumbago indica</i> L. on 3 <sup>rd</sup> day after elicitation.	92
41	The change of cell color from gray to yellow color after chitosan elicitation at different concentrations in <i>Plumbago indica</i> L. cell suspension culture.	94
42	Effect of chitosan at different concentrations on growth, the accumulation and release of plumbagin in cell suspension cultures of <i>Plumbago indica</i> L. on 2 <sup>nd</sup> day after elicitation.	94
43	Effects of chitin and chitosan elicitors on cell death in <i>Plumbago indica</i> L. cell suspension culture.	96

**LIST OF FIGURES (cont'd)**

<b>Figure</b>		<b>Page</b>
44	Cell growth and plumbagin production in <i>Plumbago indica</i> L. cell suspension cultures after elicited yeast extract (A) and precipitated yeast extract (B).	97
45	Effect of <i>Collectotrichum capsici</i> mycelium at different concentrations on <i>Plumbago indica</i> L. cell growth and on the accumulation and release of plumbagin in cell suspension cultures on day 7 <sup>th</sup> after elicitation.	99
46	The <i>Plumbago indica</i> L. cell suspension culture cells turned to pale yellow after elicitation with <i>Collectotrichum capsici</i> mycelium at different concentrations.	99
47	Dry growth index and plumbagin content (mg/l) in cell suspension culture of <i>Plumbago indica</i> L. harvested at different time of elicitation with 200 mg/l chitosan.	100
48	Plumbagin content of <i>Plumbago indica</i> L. hairy root and cell suspension cultures multiple treated by chitosan elicitor.	102
49	Plumbagin content (mg/l) in cells and culture media of <i>Plumbago indica</i> L. hairy root (A) and cell suspension cultures (B) after treated with various elicitors.	105
50	The change of cell color after elicitation with different elicitors in <i>Plumbago indica</i> L. cell suspension cultures	106

## LIST OF ABBREVIATIONS

2,4-D	=	2,4-Dichlorophenoxy acetic acid
ANOVA	=	analysis of variance
B5	=	Gamborg (1968) medium
BA or BAP	=	<i>N</i> <sup>6</sup> -benzyladenine or <i>N</i> <sup>6</sup> -benzylaminopurine
BSA	=	bovine serum albumin
CRD	=	completely randomized design
Da	=	Dalton
DMRT	=	Duncan's Multiple Range Test
DW	=	dry weight
DGI	=	dry growth index
ELISA	=	enzyme-linked immunosorbent assay
FW	=	fresh weight
FGI	=	fresh growth index
IAA	=	indole-3-acetic acid
IBA	=	indole-3-butyric acid
kDa	=	kilodalton
LS	=	Linsmair and Skoog (1965) medium
M	=	membrane
MALDI-TOF	=	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
McC	=	McCown and Liloyd (1981) medium
MS	=	Murashige and Skoog (1962) medium
NAA	=	1-naphtalene acetic acid
OVA	=	bovineserum albumin
PBS	=	phosphate buffer saline
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
v/v	=	volume by volume
w/v	=	weight by volume