

**THE DEVELOPMENT OF**  
***WASABIA JAPONICA* (MIQ.) MATSUMURA**  
***IN VITRO***

**Cao Dinh Hung**

**Thesis submitted for degree of Master of Science**

Department of Environmental Sciences,

Faculty of Science,

University of Technology, Sydney

**MSc**

**2007**

## **CERTIFICATE OF AUTHORSHIP/ORIGINALITY**

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student

---

*January 2007*

## **ACKNOWLEDGEMENTS**

I would like to thank my principal supervisor, Dr Krystyna Johnson for her guidance and friendship throughout my whole project, and my co-supervisors, Emeritus Professor Ron Wills and Dr Debbie Shohet (School of Applied Sciences, University of Newcastle), for their guidance and advice during the last two semesters of my candidature. I am really grateful to them for their valuable expertise and support.

I wish to thank Gemma Armstrong for her direct help, advice and encouragement throughout my candidature. I also thank Tony Ye, Christine Wojak and Melinda Ellith for their support during my project research in the Biology Annex.

A special thank you is given to Professor Margaret Burchett for her comments on my presentation and editorial assistance on my thesis, and to the staff at the ANSTO and Royal North Shore Hospital for their assistance with ionising-irradiation treatments.

I thankfully acknowledge Narelle Richardson and Sue Fenech for assisting with the laboratory work at Dunbar Building, and Dr Fraser Torpy for help with the statistical analysis of the experimental results.

Countless thanks are given to the Gore Hill campus staff at the Department of Environmental Sciences, Faculty of Science, University of Technology, Sydney, who have helped me during my two-year candidature.

Sincere thanks to my colleagues Hoang Van Hung and Pham Thi Thu Nga, and Vietnamese families residing in Australia, for their friendship, encouragement and invaluable support throughout my two academic years at UTS.

This thesis would not have been possible without financial assistance from an AusAID scholarship and a grant from the Department of Environmental Sciences.

Finally, this project may not have been accomplished without the assistance and emotional support from my institute, my family and my friends in Vietnam.

## TABLE OF CONTENTS

Acknowledgements.....	i
Table of Contents.....	ii
List of Figures.....	vi
List of Tables.....	ix
List of Appendices.....	x
Abbreviations.....	xii
Abstract.....	xiv
Publication and poster.....	xvii
<b>CHAPTER 1 – INTRODUCTION.....</b>	<b>1</b>
1.1 Background and aims.....	1
1.2 Wasabi crop.....	2
1.2.1 Taxonomy, distribution and morphology.....	2
1.2.2 Bioactive compounds in wasabi.....	4
1.2.3 Wasabi cultivation.....	4
1.2.3.1 Propagation.....	4
1.2.3.2 Varieties.....	6
1.2.3.3 Production.....	7
1.2.3.4 Yield.....	8
1.2.3.5 Diseases and pests.....	9
1.2.3.6 Storage.....	9
1.2.4 Markets and uses.....	10
1.2.5 Recent studies on wasabi.....	13
1.3 Plant tissue culture.....	16
1.4 Plant mutagenesis.....	17
1.5 Experimental objectives.....	18
<b>CHAPTER 2 – <i>IN VITRO</i> PROPAGATION STUDIES.....</b>	<b>20</b>
2.1 Introduction.....	20
2.1.1 Rapid clonal propagation.....	20
2.1.2 Liquid culture for mass propagation.....	23
2.1.3 Callus culture for the production of bioactive compounds.....	25
2.2 Materials and methods.....	26
2.2.1 Clonal propagation.....	26
2.2.1.1 Sterilisation of explants for culture initiation.....	26
2.2.1.2 Experimental design for shoot proliferation, root induction and plantlet acclimatisation.....	26
2.2.1.3 Culture conditions.....	30
2.2.1.4 Growth measurement.....	30
2.2.1.5 Data analysis.....	31
2.2.2 Callus culture.....	31

2.2.2.1 Selection of explants and culture conditions for callus induction .....	31
2.2.2.2 Selection of PGRs for callus proliferation.....	32
2.3 Results.....	34
2.3.1 Clonal propagation.....	34
2.3.1.1 <i>In vitro</i> culture initiation.....	34
2.3.1.2 Shoot multiplication.....	34
2.3.1.3 <i>In vitro</i> root formation.....	44
2.3.1.4 <i>In vivo</i> acclimatisation of plantlets.....	48
2.3.2 Callus culture.....	52
2.3.2.1 Callus initiation.....	52
2.3.2.2 Callus proliferation.....	55
2.4 Discussion.....	58
2.4.1 Clonal propagation.....	58
2.4.1.1 Choice of culture basal media.....	58
2.4.1.2 Choice of PGRs for shoot multiplication.....	59
2.4.1.3 Choice of PGRs for root formation.....	60
2.4.1.4 Choice of culture conditions (gelled vs. liquid media) for efficient shoot proliferation.....	61
2.4.1.5 Choice of root origins and substrates for acclimatisation of plantlets.....	63
2.4.1.6 Problems in tissue culture of <i>W. japonica</i> .....	64
2.4.2 Callus culture.....	66
2.5 Conclusions.....	69
<b>CHAPTER 3 – STUDIES IN MUTAGENESIS.....</b>	<b>70</b>
3.1 Introduction.....	70
3.1.1 Chemical mutations.....	70
3.1.2 Physical mutations.....	74
3.2 Materials and methods.....	78
3.2.1 Plant materials.....	78
3.2.2 Treatment methods.....	78
3.2.2.1 Chemical mutagens.....	78
3.2.2.1.1 Colchicine.....	78
3.2.2.1.2 Oryzalin.....	78
3.2.2.2 Physical mutagens.....	79
3.2.2.2.1 X-irradiation.....	79
3.2.2.2.2 Gamma irradiation.....	80
3.2.3 Culture conditions.....	80
3.2.4 Growth measurement.....	81
3.2.5 Data analysis.....	81
3.3 Results.....	81
3.3.1 Chemical mutagen treatments.....	81
3.3.1.1 Selection of optimal treatments based on time exposure and concentrations of mutagens.....	81
3.3.1.2. Effect of chemical mutations on <i>in vitro</i> growth and morphological variations.....	85
3.3.1.2.1 Explant weight.....	85
3.3.1.2.2 Shoot multiplication.....	86
3.3.1.2.3 Shoot height.....	87

3.3.1.2.4 Morphological variations of shoots and leaves.....	89
3.3.1.3 Survival of explants <i>in vitro</i> and <i>in vivo</i> .....	91
3.3.2. Physical mutagen treatments.....	92
3.3.2.1 Survival of explants <i>in vitro</i> and morphological variations.....	92
3.3.2.2 Effect of physical mutation on <i>in vitro</i> growth.....	96
3.3.2.2.1 Explant weight.....	96
3.3.2.2.2 Shoot multiplication.....	98
3.3.2.2.3 Shoot height.....	99
3.3.2.3 Survival of explants <i>in vivo</i> .....	100
3.4 Discussion.....	101
3.4.1 Mutation induction with polyploidy-inducing agents.....	101
3.4.2 Mutation induction with ionising-irradiations.....	104
3.5 Conclusions.....	106
<b>CHAPTER 4 – ANALYSIS OF BIOACTIVE COMPOUNDS –</b>	
<b>ALLYL ISOTHIOCYANATE.....</b>	<b>108</b>
4.1 Introduction .....	108
4.2 Materials and methods.....	111
4.2.1 Collection and preparation of <i>W. japonica</i> samples.....	111
4.2.1.1 Calli.....	111
4.2.1.2 <i>In vitro</i> mutant explants.....	111
4.2.1.3 <i>In vitro</i> non-mutant explants.....	112
4.2.1.4 <i>In vivo</i> non-mutant plants.....	112
4.2.2 Analytical methods.....	113
4.2.2.1 Extraction procedures.....	113
4.2.2.2 Gas chromatography.....	115
4.2.2.3 Mass spectrometry.....	115
4.2.2.4 Quantitative analysis of AITC.....	115
4.2.2.5 Qualitative analysis of AITC.....	116
4.2.2.6 Experimental design.....	117
4.2.2.7 Data analysis.....	119
4.3 Results.....	119
4.3.1 AITC content as affected by callus cultures.....	119
4.3.2 AITC content as affected by mutagens.....	120
4.3.3 Investigation of AITC and plant yields from plant organs.....	123
4.3.4 Necessary conditions for AITC yield optimisation.....	127
4.3.4.1 Effect of particle size.....	128
4.3.4.2 Effect of drying temperature.....	129
4.3.4.3 Effect of storage temperature.....	129
4.3.4.4 Effect of shaking conditions.....	131
4.3.4.5 Effect of solvent.....	132
4.4 Discussion.....	133
4.4.1 AITC in calli.....	133
4.4.2 AITC in mutant lines as affected by antimetabolic agents.....	134
4.4.3 AITC in mutant lines as affected by ionising-irradiations.....	135
4.4.4 AITC in <i>in vitro</i> - and <i>in vivo</i> -grown plants .....	136
4.4.5 Optimisation of AITC extraction conditions.....	138
4.5 Conclusions.....	141

<b>CHAPTER 5 – SIGNIFICANCE OF FINDINGS AND FUTURE DIRECTIONS.....</b>	<b>143</b>
5.1 Significance of findings.....	143
5.2 Future research needs and directions.....	145
<b>REFERENCES.....</b>	<b>148</b>
<b>APPENDICES.....</b>	<b>178</b>

## LIST OF FIGURES

Figure 1.1: A four-month-old <i>W. japonica</i> plant.....	3
Figure 2.1: Plant materials used for tissue culture, induced mutation and AITC content assessment.....	30
Figure 2.2: Plant materials used for callus culture.....	32
Figure 2.3: Effects of basal media on shoot survival, shoot height and explant weight of <i>W. japonica</i> after 1 month in culture.....	35
Figure 2.4: Vigorous shoots obtained on MS media.....	38
Figure 2.5: Low-quality shoots produced on MS media containing BA and TDZ at high concentrations.....	38
Figure 2.6: Effects of medium types ( $\frac{1}{4}$ MS, $\frac{1}{2}$ MS and MS) and medium conditions (gelled and liquid) on shoot number, shoot height and explant weight of <i>W. japonica</i> after 4 weeks in culture.....	39
Figure 2.7: Shoot proliferation of <i>W. japonica</i> after 4 weeks in culture on (a) $\frac{1}{4}$ MS, (b) $\frac{1}{2}$ MS and (c) MS media, under (A) liquid and (B) gelled culture conditions.....	39
Figure 2.8: Effects of liquid culture media ( $\frac{1}{4}$ MS, $\frac{1}{2}$ MS and MS) on shoot number, shoot height and explant weight of <i>W. japonica</i> after 6 weeks in culture..	40
Figure 2.9: Shoot proliferation of <i>W. japonica</i> after 6 weeks in culture on (a) $\frac{1}{4}$ MS, (b) $\frac{1}{2}$ MS and (c) MS liquid media.....	41
Figure 2.10: Effects of initial shoots derived from MS gelled, MS liquid, and $\frac{1}{2}$ MS liquid media on shoot number, shoot height and explant weight of <i>W. japonica</i> after 4 weeks in culture on MS gelled medium.....	42
Figure 2.11: Shoot proliferation of <i>W. japonica</i> after 4 weeks in culture on MS gelled medium containing 5 $\mu$ M BA, with initial shoots derived from 3 origins: (a) MS gelled, (b) MS liquid, and (c) $\frac{1}{2}$ MS liquid media.....	42
Figure 2.12: Effects of combinations between BA and auxins (IBA, IAA and NAA) on shoot number, shoot height and explant weight of <i>W. japonica</i> after 4 weeks in culture .....	43
Figure 2.13: Effects of combinations between BA and other cytokinins (TDZ, kinetin and zeatin) on shoot number, shoot height and explant weight of <i>W. japonica</i> after 4 weeks in culture .....	44
Figure 2.14: <i>W. japonica</i> rooted on $\frac{1}{2}$ MS medium containing IBA at (a) 1.0 $\mu$ M, (b) 5.0 $\mu$ M and (c) 10.0 $\mu$ M.....	45
Figure 2.15: Effects of $\frac{1}{2}$ MS gelled and liquid culture media on the frequency of shoot forming roots, root number and root length of <i>W. japonica</i> after 3 weeks in culture.....	47
Figure 2.16: <i>W. japonica</i> rooted on $\frac{1}{2}$ MS medium containing 5.0 $\mu$ M IBA in (a) gelled and (b) liquid culture conditions, and (c) 5.0 $\mu$ M BA plus 2.0 $\mu$ M NAA in liquid culture conditions.....	48
Figure 2.17: Effects of types of roots derived from $\frac{1}{2}$ MS gelled media with IBA at 5 $\mu$ M or 10 $\mu$ M, and from $\frac{1}{2}$ MS liquid media with 5 $\mu$ M IBA or 5 $\mu$ M BA plus 2 $\mu$ M NAA on the survival rate, total leaf number, and height of <i>W. japonica</i> plantlets grown in the greenhouse for 1 month.....	49
Figure 2.18: Growth of rooted explants for 4 weeks in greenhouse conditions, with initial roots derived from 4 origins: $\frac{1}{2}$ MS gelled media with IBA at (a) 5 $\mu$ M or (b) 10 $\mu$ M, and $\frac{1}{2}$ MS liquid media with (c) 5 $\mu$ M IBA or (d) 5 $\mu$ M BA plus 2 $\mu$ M NAA.....	50

Figure 2.19: Acclimatised nursery <i>W. japonica</i> plants at (a) one week of age in a humid environment, and (b) two months of age in the greenhouse....	51
Figure 2.20: Calli induced on the cut surfaces of explants.....	55
Figure 2.21: Responses of calli after 6 weeks in culture on MS medium supplemented with various PGRs.....	58
Figure 3.1: Experimental set up for X-irradiation treatment .....	79
Figure 3.2: Effects of a 2-day treatment on the survival rates of <i>W. japonica</i> shoot tips exposed with oryzalin and colchicine after 1 month in culture on MS shoot proliferation medium.....	82
Figure 3.3: Effects of a 4-day treatment on the survival rates of <i>W. japonica</i> shoot tips exposed with oryzalin and colchicine after 1 month in culture on MS shoot proliferation medium.....	83
Figure 3.4: Effects of an 8-day treatment on the survival rates of <i>W. japonica</i> shoot tips exposed with oryzalin and colchicine after 1 month in culture on MS shoot proliferation medium.....	84
Figure 3.5: Four-week-old <i>W. japonica</i> shoots following an 8-day treatment with colchicine at (a) 0 $\mu\text{M}$ , (b) 25 $\mu\text{M}$ , (c) 75 $\mu\text{M}$ and (d) 150 $\mu\text{M}$ .....	85
Figure 3.6: Four-week-old <i>W. japonica</i> shoots following an 8-day treatment with oryzalin at (a) 0 $\mu\text{M}$ , (b) 5 $\mu\text{M}$ , (c) 15 $\mu\text{M}$ and (d) 30 $\mu\text{M}$ .....	85
Figure 3.7: Effects of chemical mutagens and concentrations on the explant weight of <i>W. japonica</i> after 1 month, 2 months and 3 months in culture on MS medium.....	86
Figure 3.8: Effects of chemical mutagens and concentrations on shoot number of <i>W. japonica</i> after 1 month, 2 months and 3 months in culture on MS medium.....	87
Figure 3.9: Effects of chemical mutagens and concentrations on shoot height of <i>W. japonica</i> after 1 month, 2 months and 3 months in culture on MS medium.....	88
Figure 3.10: Deformed and fragile leaves of <i>W. japonica</i> produced by mutagen treatments at high doses after 2 and 3 months in culture.....	90
Figure 3.11: Healthy shoots of <i>W. japonica</i> with abundant callus-like rhizomes at the shoot bases obtained by mutagen treatments at low doses after 3 months in culture.....	90
Figure 3.12: Unhealthy shoots of <i>W. japonica</i> with some necrosis formed by mutagen treatments at high doses after 3 months in culture.....	90
Figure 3.13: Effects of the radiation treatments by gamma rays and X-rays at doses of 0, 10, 20, 40 and 80 Gy on the survival rates of <i>W. japonica</i> shoot tips at (a) 1, (b) 2 and (c) 3 months in culture on MS shoot proliferation medium.....	96
Figure 3.14: <i>W. japonica</i> shoots exposed to irradiations at high doses after 4 weeks in culture exhibited necrosis.....	96
Figure 3.15: Effects of physical mutagens and doses on explant weights of <i>W. japonica</i> after 1 month, 2 months and 3 months in culture on MS medium.....	98
Figure 3.16: Effects of physical mutagens and doses on shoot numbers of <i>W. japonica</i> after 1 month, 2 months and 3 months in culture on MS medium.....	99
Figure 3.17: Effects of physical mutagens and doses on shoot heights of <i>W. japonica</i> after 1 month, 2 months and 3 months in culture on MS medium.....	100

Figure 4.1: Conversion of glucosinolates to isothiocyanates.....	108
Figure 4.2: Types of <i>W. japonica</i> materials used for extraction.....	113
Figure 4.3: Procedures for extraction of AITC from <i>W. japonica</i> .....	114
Figure 4.4: A typical regression equation for AITC standard.....	116
Figure 4.5: Mass spectra of AITC.....	116
Figure 4.6: Effects of explant parts (leaf and petiole segments) and culture conditions (light and darkness) on the accumulation of AITC.....	120
Figure 4.7: Typical gas chromatogram of AITC extracted from 3-month-old <i>in vitro</i> explants of <i>W. japonica</i> treated with mutagens.....	121
Figure 4.8: Effects of chemical mutagen treatments on AITC content of <i>W. japonica</i> after 3 months in culture on MS medium.....	121
Figure 4.9: Effects of physical mutagen treatments on AITC content of <i>W. japonica</i> after 3 months in culture on MS medium.....	122
Figure 4.10: Effects of <i>in vitro</i> culture cycle (1, 2 and 3 months) and <i>in vivo</i> growth cycle (3, 6, 9 and 12 months) on the fresh weight proportions of plant parts.....	123
Figure 4.11: Typical gas chromatogram of essential oils extracted from greenhouse-grown <i>W. japonica</i> .....	127
Figure 4.12: Effects of particle sizes of the <i>W. japonica</i> petiole samples ground to powder on the content of AITC.....	128
Figure 4.13: Relationship between the drying temperature and mean content of AITC in <i>W. japonica</i> petioles.....	129
Figure 4.14: Relationship between the storage time and mean content of AITC in <i>W. japonica</i> leaves stored at rt, 4°C, -20°C and -80°C.....	130
Figure 4.15: Effects of (a) shaking temperatures, (b) shaking time lengths and (c) shaking speeds on the yield of AITC during extraction.....	132
Figure 4.16: Effects of organic solvents on the yield of AITC during extraction.....	133

## LIST OF TABLES

Table 1.1: Production of <i>W. japonica</i> in Japan in 1986.....	8
Table 2.1: Effects of growth regulators on <i>in vitro</i> shoot induction of <i>W. japonica</i> after 4 weeks in gelled culture media.....	37
Table 2.2: Effects of growth regulators on <i>in vitro</i> root formation of <i>W. japonica</i> after 3 weeks of culture.....	46
Table 2.3: Rooted explants derived from ½MS medium containing 10 µM IBA after one-month acclimatisation in the greenhouse as affected by vermiculite, peat-moss, perlite and their combinations.....	52
Table 2.4: Callus induction after 6 weeks in culture under light conditions as affected by 2,4-D concentrations and explant sources.....	53
Table 2.5: Callus induction after 6 weeks in culture in dark conditions as affected by 2,4-D concentrations and explant sources.....	54
Table 2.6: Callus growth after 6 weeks in culture under light conditions as affected by PGR combinations.....	57
Table 3.1: Survival rates of <i>W. japonica</i> shoot tips treated with oryzalin and colchicine after 3 months in culture <i>in vitro</i> and 1-month growth <i>in vivo</i> ..	92
Table 3.2: Survival rates of <i>W. japonica</i> shoot tips treated with gamma rays and X-rays after a one-month growth in the greenhouse.....	101
Table 4.1: Essential oils isolated from <i>W. japonica</i> grown in the upland fields.....	110
Table 4.2: Some essential oils isolated from <i>W. japonica</i> grown in the flooded fields.....	111
Table 4.3: Fresh weight of <i>in vitro</i> and <i>in vivo</i> plant parts as affected by length of growth.....	124
Table 4.4: AITC content distributed in the explant parts at 1, 2 and 3 months in culture <i>in vitro</i> .....	125
Table 4.5: AITC content distributed in the plant parts at 3, 6, 9 and 12 months of growth in the greenhouse.....	126

## LIST OF APPENDICES

Appendix 2.1: Some examples of <i>in vitro</i> production of valuable secondary metabolites in callus cultures from medicinal plant organs.....	178
Appendix 2.2: Nutritional composition of plant tissue culture media used (mg L <sup>-1</sup> )...	179
Appendix 2.3: Statistics on the differences in the survival rate, shoot height and explant weight of <i>W. japonica</i> among the seven basal media.....	180
Appendix 2.4: Statistics on the differences in the shoot number, shoot height and explant weight of <i>W. japonica</i> among 3 medium types (¼MS, ½MS and MS) in two culture conditions (gelled and liquid).....	180
Appendix 2.5: Statistics on the differences in the shoot number, shoot height and explant weight of <i>W. japonica</i> among 3 medium types (¼MS, ½MS and MS) under liquid culture for 6 weeks.....	180
Appendix 2.6: Statistics on the differences in the shoot number, shoot height and explant weight of <i>W. japonica</i> with initial shoots derived from 3 origins (MS gelled, MS liquid, and ½MS liquid media), after 4 weeks in culture on MS gelled medium with 5 µM BA .....	181
Appendix 2.7: Statistics on the differences in the shoot number, shoot height and explant weight of <i>W. japonica</i> among combinations between BA and auxins after 4 weeks in culture.....	181
Appendix 2.8: Statistics on the differences in the shoot number, shoot height and explant weight of <i>W. japonica</i> among combinations between BA and other cytokinins after 4 weeks in culture.....	181
Appendix 2.9: Statistics on the differences in the percentage of shoots forming roots, root number and root length of <i>W. japonica</i> between gelled and liquid culture conditions after 3 weeks in culture.....	182
Appendix 2.10: Statistics on the differences in the survival rate, total leaf number and height of <i>in vitro</i> plantlets of <i>W. japonica</i> , with initial roots derived from 4 origins after 4-weeks' growth in the greenhouse.....	182
Appendix 3.1: Some common plant varieties that were successfully treated with colchicine.....	183
Appendix 3.2: Some common plant varieties that were successfully treated with oryzalin.....	184
Appendix 3.3: Some common plant varieties that were successfully treated with gamma rays.....	185
Appendix 3.4: Some common plant varieties that were successfully treated with X-rays.....	186
Appendix 3.5: Statistics on the differences in the health and conditions of <i>W. japonica</i> explants treated with chemical mutagens at different concentrations after 1, 2 and 3 months in culture on MS shoot proliferation medium.....	187
Appendix 3.6: Statistics on the differences in the health and conditions of <i>W. japonica</i> explants treated with physical mutagens at different doses after 1, 2 and 3 months in culture on MS shoot proliferation medium.....	188
Appendix 4.1: Minimum level of AITC against organisms.....	189
Appendix 4.2: Statistics on the differences in the AITC content of <i>W. japonica</i> explants exposed with chemical mutagens at different concentrations after 3 months in culture on MS shoot proliferation medium.....	189

Appendix 4.3: Statistics on the differences in the AITC content of <i>W. japonica</i> explants exposed with physical mutagens at different doses after 3 months in culture on MS shoot proliferation medium.....	190
Appendix 4.4: Possible mass spectra of the four compounds extracted from greenhouse-grown <i>W. japonica</i> .....	191

## ABBREVIATIONS

μl	:	Microlitre
μM	:	Micro molar
μmol m <sup>-2</sup> s <sup>-1</sup>	:	Micromoles per square meter per second
¼MS	:	Quarter-strength MS (Murashige and Skoog, 1962)
½MS	:	Half-strength MS
2,4-D	:	2,4-dichlorophenoxyacetic acid
AITC	:	Allyl isothiocyanate
ANOVA	:	Analysis of variance
B <sub>5</sub>	:	Gamborg's medium (Gamborg <i>et al.</i> , 1968)
BA	:	N <sup>6</sup> -benzyladenine
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribonucleic acid
DW	:	Dry weight
FID	:	Flame ionisation detector
FW	:	Fresh weight
GA <sub>3</sub>	:	Gibberellic acid
GC	:	Gas chromatography
GC-MS	:	Gas chromatography–Mass spectrometry
GSLs	:	Glucosinolates
Gy	:	Gray
He	:	Helium
HEPA	:	High-Efficiency Particulate Air
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3- butyric acid
ITCs	:	Isothiocyanates
KC	:	Knudson (1925)
Mf	:	Molecular formula
mM	:	Mili molar per litre
MS	:	Full-strength MS
MU	:	Monitor unit
Mw	:	Molecular weight
N&N	:	Nitsch & Nitsch
N <sub>6</sub>	:	Chu (1978) medium

NAA	:	$\alpha$ -naphthalene acetic acid
NaOCl	:	Sodium hypochlorite
Oryzalin	:	3,5-dinitro-N <sup>4</sup> ,N <sup>4</sup> -dipropylsulphanilamide
pA	:	Peak area
PGRs	:	Plant growth regulators
ppm	:	Part per million
rpm	:	Round per minute
rt	:	Room temperature
TDZ	:	Thidiazuron
UTS	:	University of Technology, Sydney
v/v	:	Volume per volume
w/v	:	Weight per volume
WPM	:	Woody plant medium (Lloyd and McCown, 1980)
Zeatin	:	6-(4-hydroxy-3-methyl-2-butenylamino)purine

## ABSTRACT

The aim of this project was to investigate the feasibility of the development of *in vitro* methods for assisting in the plant breeding of the important culinary and medicinal plant, *Wasabia japonica* Matsumura, through rapid plantlet production and improved plant quality, by the application of micro-propagation and induced mutation techniques. The synthesis and accumulation of a key valuable chemical component was also examined, through callus culture, tissue culture, and induced mutation.

*In vitro* propagation systems by means of gelled and liquid culture were developed for *W. japonica*. *In vivo* dormant buds were used as a primary source of explants, following disinfection in alcohol, NaOCl and detergent. Explants were successfully initiated in culture followed by a rapid shoot multiplication on gelled basal media containing nutrient salts of Murashige and Skoog (MS), vitamins, sucrose and growth regulators. Amongst various cytokinins used (BA, TDZ, kinetin and zeatin) BA proved to be the most effective, with the best concentration at 5  $\mu$ M. Combinations among growth regulators enhanced shoot proliferation. In order to establish a simple and efficient *in vitro* propagation protocol,  $\frac{1}{2}$ MS liquid culture medium with 5° $\mu$ M BA was used for effective shoot multiplication of *W. japonica* with reduced labour and cost. The best rooting occurred in shoots cultured on gelled  $\frac{1}{2}$ MS basal medium supplemented with 10° $\mu$ M IBA. Over ninety per cent of plantlets were successfully acclimatised to *in vivo* conditions, exhibiting normal development.

The effects of *in vitro* mutation induction by physical and chemical mutagens on wasabi shoot tips were also investigated. Gamma rays and X-rays as physical mutagens in the range of 10 to 40 Gy, reduced the allyl isothiocyanate content of *in vitro* explants, to a greater extent as doses increased. X-ionising irradiation had a more detrimental influence on survival and growth of wasabi tissues than gamma-ionising irradiation. Colchicine and oryzalin as chemical mutagens, however, did not reduce the content of this compound. Oryzalin appeared to be more phytotoxic than colchicine, with a concentration of 15  $\mu$ M affecting explant growth. Conversely, a low concentration of colchicine at 25  $\mu$ M appeared to be favourable for the growth and development *in vitro*. Leaf morphology appeared to be altered in several greenhouse-grown plants treated with chemical mutagens, exhibiting typical characteristics of polyploids. Further studies

of morphological effects and assessments of the alteration of allyl isothiocyanate need to be undertaken on *in vivo*-grown plants.

The yield of allyl isothiocyanate was investigated in calluses, *in vitro* mutated- and non-mutated explants, and *in vivo* plants. Light conditions and leaf tissues proved to be more effective than dark conditions and petiole tissues, respectively, for the accumulation of this component in callus cultures. Shoot bases accumulated higher yield than leaves and petioles in the *in vitro* explants over three months in culture. Similarly, roots and rhizomes produced higher yields than leaves and petioles of *in vivo* plants over a period of 12 months. Leaves accumulated greater amounts of allyl isothiocyanate than petioles *in vitro*, but lower amounts than petioles *in vivo*. Thus it appears that the harvest of allyl isothiocyanate in plant leaves and petioles *in vivo* could be most effectively commenced after growth for nine months.

These results lay a scientific foundation on which a more elaborated and effective system of *in vitro* propagation and improvement for *W. japonica* can be achieved. Further studies on the analysis of chemical components need to be conducted for the pharmaceutical uses of this plant.

## PUBLICATIONS FROM THIS PROJECT

Hung C D, Johnson K, Torpy F. (2006). Liquid culture for efficient micropropagation of *Wasabia japonica* (Miq.) Matsumura. *In Vitro Cellular & Developmental Biology – Plant*, **42**(6): In press.

Hung C D, Johnson K, Armstrong G, Wojak C. Liquid culture for efficient micropropagation of *Wasabia japonica* (Miq.) Matsumura. In: *Contributing to a Sustainable Future, Proceedings of the Australian Branch of the IAPTC&B, Perth, Western Australia*, The Australasian Plant Breeding Association Inc. (Conference poster no. 21).