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

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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

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ABBREVIATIONS

µl	:	Microlitre
µM	:	Micro molar
µmol $m^{-2}s^{-1}$	:	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_5	:	Gamborg's medium (Gamborg *et al*., 1968)
BA	:	N^6-benzyladenine
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribonucleic acid
DW	:	Dry weight
FID	:	Flame ionisation detector
FW	:	Fresh weight
GA_3	:	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_6	:	Chu (1978) medium

NAA	:	α-naphthalene acetic acid
NaOCl	:	Sodium hypochlorite
Oryzalin	:	3,5-dinitro-N^4,N^4-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 µM. Combinations among growth regulators enhanced shoot proliferation. In order to establish a simple and efficient *in vitro* propagation protocol, ½MS liquid culture medium with 5 µM BA was used for effective shoot multiplication of *W. japonica* with reduced labour and cost. The best rooting occurred in shoots cultured on gelled ½MS basal medium supplemented with 10 µM 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 µM affecting explant growth. Conversely, a low concentration of colchicine at 25 µM 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 he 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 pharmacautical uses of this plant.

PUBLICATIONS FROM THIS PROJECT

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CHAPTER 1

INTRODUCTION

1.1 Background and aims

In recent years, the Vietnamese Government has assigned a high priority to scientific research on biotechnology. The application of biotechnology in agriculture and forestry plays a primarily important role in the national economic development strategies. One of the key research activities in agricultural biotechnology has concentrated on the *in vitro* conservation and propagation of superior germplasms derived from crops of high commercial and economical value. New regeneration techniques for the rapid multiplication of some agricultural crops such as rice (*Oryza sativa* L.), onion (*Allium fistulosum* L.), tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea*) (Viện Sinh học Nhiệt Đới, 1998), and valuable ornamental plants such as the orchid (*Cymbidium* spp.), lily (*Lilium longiflorum* L.) and carnation (*Dianthus caryophyllus*) (Phân viện Sinh học tại Đà Lạt, 1995) have so far been established. Wasabi (*Wasabia japonica* (Miq.) Matsumura), a medicinal plant newly imported to Vietnam from Japan, is currently sought after for micropropagation to satisfy the increasing domestic demands on the planting material, resulting from the rapid expansion of wasabi production areas, and for subsequent large-scale cultivation to increase the export volume. Together with the propagation of wasabi, its improvement has also been acknowledged as a major strategy designed for achieving novel varieties of high quality to supply the food and drug-processing industries. The alteration of genetic composition of plants by non-transgenic methods such as mutagenesis and breeding appears to be an excellent idea for producing new cultivars capable of competing with any potential cultivars imported from foreign countries in both crop appearance and quality.

The aims of this project were to investigate the development of appropriate plant tissue culture techniques for wasabi, and to improve the quality of wasabi varieties, if possible by mutation breeding, and hence assist the advancement of the commercial cultivation of wasabi. In particular, the project's aims have focused on the use of plant growth

regulators for the *in vitro* propagation of the current wasabi species via shoot tip multiplication and the production of calli, and on the use of chemical and physical mutagens for the improvement of bioactive compounds in the plant materials produced.

Below is a description of *W. japonica*, the bioactive compounds that provide its piquancy and flavours, and the development and applications of this species. An overview of the development in plant tissue culture and mutation breeding is presented, followed by the experimental objectives of the project.0

1.2 Wasabi crop

1.2.1 Taxonomy, distribution and morphology

Wasabi, *Wasabia japonica* (Miq.) Matsumura [syn. *Eutrema wasabi* (Sieb.) Maxim.], originates from Japan and is a member of the Brassicaceae, previously known as Cruciferae or mustard family (Ohwi, 1984; Chadwick *et al.*, 1993). In nature, it grows alongside wet mountain streams and is widely distributed from the northernmost island of Sakhalin to the southernmost island of Kyushu, Japan.

The genus *Wasabia* includes two species, *Wasabia tenuis* and *Wasabia japonica*, which are mainly distinguished by their flowers, leaf size, rhizome size and colour. Because of their wide distribution in east Asia, and their differences in ecology, cultural practices and naming history, this crop has a number of recognised varieties, and hence many aliases, including: for *Wasabia tenuis*, a wild species, variously called 'yuri wasabi', *Nasturtium tenue, Cardamine tenuis, C. bracteate, W. bracteate, Eutrema bracteate, E. hederaefolia and W. hederaefolia*; and *Wasabia japonica*, a wholly cultivated species, also known as 'swamp wasabi', 'sawa wasabi', *Cochlearia wasabi, Eutrema wasabi, Alliaria wasabi, W. wasabi, W. pungens, Lunaria japonica, E. japonica, E. okinosimensi* (Ohwi, 1984; Chadwick *et al.*, 1993). *Wasabia japonica* is now widening its distribution to Taiwan, New Zealand (Douglas and Follett, 1992; Sultana *et al.*, 2003b), USA, Australia (Barber and Buntain, 1985; Sparrow, 2004), China, Vietnam and other countries.

Ohwi (1984) describes two species of wasabi in detail. They are glabrous perennial herbs with radical, undulate-toothed, heart-shaped leaves, long petioles and leafy stems. Their flowers are bracteate with white colour, ascending sepals, clawed, obovate petals and simple stigmas. The siliques are relatively short and linear-oblong. Seeds are few

and large. The two species *W. japonica* and *W. tenuis* are distinguished primarily on the size of their rhizomes, style, petals and leaves. *W. japonica* has a stout rhizome 1–2 cm across, flowers with styles of about 2 mm long, petals of 8–9 mm in length and leaves up to 15 cm across (Figure 1.1). *W. tenuis* has a short and narrow rhizome 1–2 mm across, flowers with styles of 0.5–0.7 mm in length, petals 5–7 mm long and leaves of about 5 cm across.

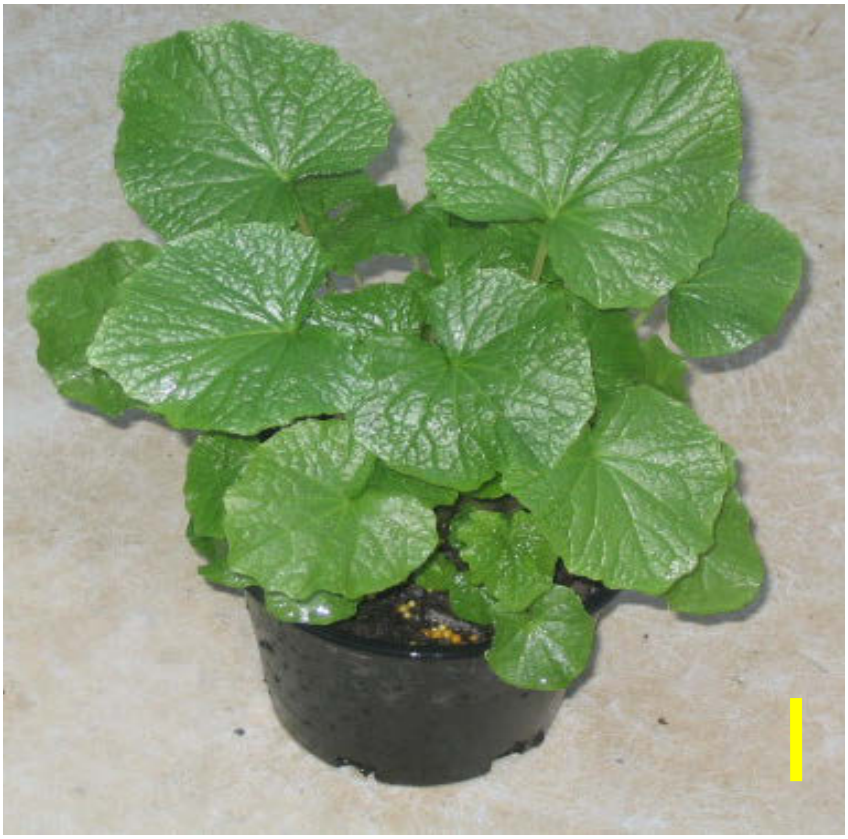


Figure 1.1: A four-month-old *W. japonica* plant (bar = 5 cm).

Siliques and seeds appear from early to mid summer (in Japan), about two months after the wasabi plant initially starts flowering. Young siliques are soft and of green colour, while old siliques are hard and black. As the quantity of flowers increases rapidly in mid-spring, the quantity of viable seeds also rises with a peak in late spring. In the weeks before and after this period, seeds are formed in a small number, particularly under low temperatures. The viability of seeds is dependent on the environmental temperatures in the period of flowering (Adachi, 1987).

Leaf development occurs depending on the changes of temperature or season. In months with extreme temperature, leaf growth becomes slower than that in moderate months. Approximately two to three new leaves appear per month during summer and winter,

whereas from mid spring to early autumn, about five to six new leaves are formed in young plants or 62 total leaves in mature plants 15 months old (Adachi, 1987). The wasabi plant starts flowering in late winter when the peduncles may be formed, and ends in early summer in Japan. In late spring, the peduncles elongate rapidly and the flowering reaches a peak.

1.2.2 Bioactive compounds in *W. japonica*

Isothiocyanates (ITCs) are major bioactive compounds responsible for the distinctive flavour of wasabi (Sultana *et al.*, 2003c). Under the mechanic injuries caused by crushing, grating, cutting or chewing in preparing food, Brassicaceous plant tissues produce ITCs, a group of volatile sulphur compounds non-existent in intact cells. Nevertheless, a group of glucosides called glucosinolates (GSLs) are localised in the intact cells, together with the hydrolytic enzyme myrosinase, but they are not in direct contact with each other (Delaquis and Mazza, 1995). Some authors claim that myrosinase exists in specialised myrosin cells (Hoglund *et al.*, 1991; Thangstad *et al.*, 1991; Hoglund *et al.*, 1992) and are thus separated from GSLs. GSLs have also been reported to be stored in cell vacuoles, together with ascorbic acid (Grob and Matile, 1979). The activity of myrosinase has been found to be inhibited by a large amount of ascorbic acid, whereas a small amount of ascorbic acid activates this enzyme (Bones and Rossiter, 1996). When hydrolysed, GSLs produce glucose and an unstable aglucone, with the latter forming ITCs by a rearrangement under neutral or alkaline pH conditions. Aglucone is, however, autolysed under pH condition as weakly acidic (pH between 3 and 6), or even with the participation of an Fe^{2+} or of an endogenous nitrile factor, to produce nitriles and elemental sulphur instead of ITCs and sulphate. The chemical reactions to form ITCs are discussed in Chapter 4.

1.2.3 Wasabi cultivation

1.2.3.1 Propagation. Planting stocks required for wasabi cultivation can be divided into three major groups: seeds, vegetative propagules and *in vitro* propagated plantlets.

Wasabi produces seeds in the spring (Chadwick, 1990). Seedlings are produced from seeds by farmers to replace harvested crops. The biggest obstacle of seedling propagation is the relatively low germination and viability of seeds (Palmer, 1990). Seed loses its viability rapidly when dried out. Soaking seeds in GA_3 is reported to enhance seedling growth for leaves in greenhouses, but not for rhizome production in

the fields (Haruki, 1978). Annually collected in mid summer in Japan and subsequently stored under moist conditions, seeds are sown in late winter in boxes filled with rice husks in black-cloth-covered houses. After about two weeks, seeds begin germination. The use of N-P-K fertiliser can be proportionated as 100-60-100 kg/ha (Follett, 1986). Between early and mid autumn, selected seedlings are lifted for planting. For field production, seedlings are required to have four to five leaves, with a minimum rhizome size of about 3 cm in height (Suzuki, 1968).

Wasabi seed displays a deep dormancy. It has less dormancy when grown under a cool temperature of about 17^{o}C. Adachi (1987) argues that a faster germination of wasabi seeds can be observed when they are matured under cool weather in early summer; whereas those maturing under hotter or colder weather show a slower germination. Many studies show that the wasabi seed dormancy can be artificially broken at an optimal temperature of 5^{o}C, or when the seed is soaked in 100 ppm GA_3 for five days (Palmer, 1990). The germination of wasabi seeds can be up to 100% at 15^{o}C after a four-month storage at 5^{o}C. Some plant growth regulators can enhance the germination of wasabi seeds, such as GA_3 at 500 ppm or kinetin at 25 ppm without any effects on the vigour of seeds (Palmer, 1990; Potts and Lumpkin, 1997).

Vegetative propagation is suitable for the regeneration of young plants from a healthy, disease-free parent plant. It is commonly cultivated for a maximum of three production cycles to prevent the spread of diseases. Wasabi is susceptible to a wide range of microbial pathogens, such as *Phoma* spp., *Fusarium* spp. and *Corynebacterium* spp. (Sparrow, 2004); therefore, for vegetative propagation, a young, healthy disease-free mother stock is most suitable. Unlike seeds, offshoots are more easily regenerated into whole wasabi plants. A two-year-old plant may have a total number of 30 offshoots. At harvesting, the offshoots developed from the main stem base are separated for replanting directly to furrows at any time of the year except winter. Although the harvest of wasabi is likely to be performed all year round, the major crop is harvested between late spring and early autumn (Follett, 1986). Whole rhizomes are able to produce young plantlets (Adachi, 1987). Rhizomes can be cut into small parts with five pieces being common, containing several buds. The pieces are sterilised before being placed into a mixture of sand and organic matter in a greenhouse. After about eight weeks, plantlets appear under a cool and humid condition, available for field

production. Additionally, wasabi roots can also be used for vegetative propagation (Adachi, 1987). Carefully removed from a mother plant, together with the attachment of a piece of rhizomal epidermis, roots produce plantlets when grown in cool and humid conditions under a plastic shade. Roots are advised to be soaked in GA_3 solution for about three hours for budding induction before transplanting (Suzuki, 1968).

Tissue culture propagation has also been used for the regeneration of disease-free wasabi plantlets, however, only a few studies on tissue culture have been reported. Plantlets have been grown in the greenhouses of the New Bio Research Company located in the Japanese mountains in Hotaka City, for the production of wasabi leaf and rhizome within three months and 12 months, respectively (Chadwick, 1990). A technique using shoot tips of wasabi for micropropagation was developed by Hosokawa *et al.* (1999). Micropropagation has been used as a protocol for keeping the integrity of wasabi cultivars and regenerating disease-free plants (Follett, 1986; Hosoki *et al.*, 1986).

1.2.3.2 Varieties. Numerous wasabi varieties grown in Japan and Taiwan have been described in Japanese reports. It was cited in the literature of Hodge (1974) that three cultivars were recognised by Japanese farmers; the cultivar with green petioles is widely cultivated, while the cultivar with reddish petioles is cultivated in Abegun, and the cultivar with poor quality is sometimes cultivated. However, Chadwick (1990) mentions 19 Japanese wasabi cultivars, with ‘Daruma’ considered the most popular one (Archer, 2001) as its environmental temperature range for cultivation appears to be wider, and five Taiwanese wasabi cultivars with the ‘Local Cultivar’ regarded as the most widely cultivated, but less resistant to disease (Chadwick *et al.*, 1993).

Japanese wasabi cultivars are commonly propagated from offshoots, and require specific cultivation and climate conditions. A disadvantage in this vegetative traditional propagation method is that diseases can be transferred to new plants from a preceding offshoot. Therefore, after every three propagation generations, offshoots are replaced by seedlings for breaking a disease cycle and rejuvenating the crop (Chadwick, 1990; Chadwick *et al.*, 1993). Four Taiwanese wasabi cultivars have been identified from an originally unknown cultivar introduced from Japan. These cultivars are highly affected by a wide range of disease-causing pathogens. Moreover, they have small rhizomes and

low quality as a result of being cultivated in soil beds, in comparison with the wasabi grown in waterbeds in Japan (Chadwick, 1990).

1.2.3.3 Production. There are two commonly used methods of growing wasabi, soil cultivation and water cultivation. Depending on the commercial purposes, one of these two cultivation methods is used. In Japan, for the harvest of leaf and petiole products, wasabi plants are grown in a soil environment; while the wasabi plants grown in flooded fields are used for harvesting rhizomes. Wasabi plants cultivated under flooded systems produce enlarged rhizomes of high quality and of higher marketable prices. Autumn and spring are considered the best seasons for wasabi cultivation, as the temperatures are moderate. Wasabi plants are particularly sensitive to the cultivation environment (Chadwick, 1990).

Soil-grown wasabi reaches marketable size by about three years after planting. Shade structures must be constructed for obtaining the cooling by air flow. Soil is enriched with compost like rapeseed cake and liquid manure, or with phosphate fertiliser before planting. Nitrogen is not necessarily supplied when the young seedlings are first grown in the soil. However, the application of a small amount of nitrogen over the growing season is needed. For stem development, additional fertilisers are required. A study on the effects of fertilisation on the AITC contents in plant parts of wasabi grown in New Zealand showed that ammonium sulphate increased the AITC amount in rhizomes and petioles, while manure increased the AITC amount in leaves (Sultana *et al.*, 2002). On the same cultivation plot, two crops are continuously harvested after three years. The wasabi crop ideally requires an ideal air temperature ranging from 8^{o}C to 18^{o}C, with the best pH range for soil from 6 to 7. It is usually grown under the bases of such trees as plum, mulberry, persimmon and cedar in Japan, but in shade houses in New Zealand.

Water-grown wasabi often needs beds constructed by gravel up to 20–30 cm high and paralleled with the water flow. Plants must be grown deeply into the bed sides so that roots and stems can reach the wet area of the gravel. Wasabi is dramatically affected by various factors such as water temperature, moisture, nutrition, aeration, pH level, dissolved oxygen level and water quality (Hodge, 1974; Douglas and Follett, 1992). Stagnant and less-oxygenated water supply inhibits the growth of wasabi plants. Aquatic-planted wasabi requires clean, highly oxygenated water with constant flow all year round. An optimal air temperature of between 12^{o}C and 15^{o}C is required for

wasabi growth (Douglas and Follett, 1992). Its growth is inhibited or even stopped at an air temperature below 5^{o}C. Headwaters are monthly supplied with slowly released fertilisers of N:P:K (12:12:12), and even with sulphur sprays for flavour enhancement.

1.2.3.4 Yield. According to Chadwick *et al.* (1993), a majority of the land areas used for wasabi production in Japan is located in mountainous regions. Due to the pollution primarily caused by fertilisers, and the land degradation caused by the construction of roads or dams, wasabi production areas are becoming increasingly narrower. Another factor that limits the production of wasabi to approximately 880 ha in Japan and 400 ha in Taiwan is the strict environmental parameters needed for its growth. In a report from a Forest Information Division of Japanese Government, the total production of both soil and water grown wasabi stems in Japan during the year 1986 was shown in Table 1.1.

Table 1.1: Production of *W. japonica* in Japan in 1986 (Chadwick *et al.*, 1993).

Locations	Wasabi production (ton per hectare per year)	
	Stems	Petioles
Iwate	19.9	30.7
Tokyo	26.0	120
Yamanashi	14.9	164
Nagano	73.0	1259
Gifu	20.5	13.9
Shizuoka	351	0.0
Tottori	5.4	76.5
Shimane	132	192
Yamaguchi	65.3	62.0
Oita	6.0	96.4
Other 42 prefectures	1450	2077

The current total production by fresh weight in Japan is approximately 5,000 tonnes per year (Barber and Buntain, 1985). Recently, a wasabi crop has been cultivated in

Tasmania, Australia with the purpose of supplying wasabi products to the Southeast Asian region during the off-season (Barber and Buntain, 1985; Sparrow, 2004).

1.2.3.5 Diseases and pests. There are a large number of viruses, bacteria and fungi that can damage the wasabi crop. Common diseases of wasabi include black rot (Lo *et al.*, 2002), leaf spots, darkening or yellowing of leaves and petioles, black vein, drooping and stem mould (Barber and Buntain, 1985). Chadwick *et al.* (1993) describe thoroughly three viral strains called tobacco mosaic virus, turnip mosaic virus and cucumber mosaic virus, two bacterial genera, which are *Erwinia* and *Corynebacterium*, and six fungal species belonging to the classes of Ascomycetes, Omycetes, Plasmodiophoromycetes and Basidiomycetes, that cause serious damage to the wasabi crop. The three viral strains decreased the growth rate of wasabi by 50% in New Zealand (Follett, 1986). The bacterial genus *Erwinia* causes soft rot of wasabi rhizomes (Goto and Matsumoto, 1986; Douglas and Follett, 1992). In Japan, wasabi is found to be susceptible to two fungal genera of the class Oomycetes; *Phoma* (Takuda and Hirosawa, 1975; Soga, 1982; Adachi, 1987) and *Sclerotina* (Adachi, 1987). *Phoma wasabie* is a new species carrying the most dangerous features of all fungi, damaging both flooded and upland grown wasabi. Lo *et al.* (2002) combined the use of fungus-free plantlets and polyethylene cover for a successful control of black rot caused by *Phoma wasabiae*. In addition, numerous types of pests attack all plant parts of wasabi, from flowers, leaves, stems to petioles and roots, causing a total plant loss or a reduction in market value (Chadwick *et al.*, 1993; Douglas, 1993). Common insects documented as attacking wasabi include aphids, thrips, crane flies and caddisfly. Aphids are considered one of the most destructive insects of wasabi, for functioning as vectors that cause a viral disease by transferring cucumber mosaic viruses to the wounds of the plant (Chadwick *et al.*, 1993). In total, more than 40 insects visiting Japanese wasabi are documented (Follett, 1986), and over 44 insect species listed as visiting wasabi flowers (Togashi, 1981).

1.2.3.6 Storage. Amongst the fresh parts of a wasabi plant, the rhizome has been used for storage in freezers. The content of ITCs in fresh wasabi rhizomes changes during storage at different temperatures and over different periods. It has been reported that the individual and total contents of *iso*-propyl ITC, *sec*-butyl ITC, AITC, 4-pentenyl ITC and 5-hexenyl ITC were unchanged during the storage of rhizomes at -10, -20 and

-80^{o}C for 8 weeks, and lost when stored at just below -4^{o}C for 6 weeks. The yield of 3-butenyl ITC was, however, reduced significantly after storage at -10, -20 and -80^{o}C for only three weeks (Sultana *et al.*, 2003d). A study on the effects of pre-storage treatment with liquid nitrogen on the yield of ITCs by the same authors suggests that it is unnecessary to utilise this fast initial freezing treatment technique.

Another method for the storage of wasabi comes in the form of powder. The wasabi powder was obtained from drying raw materials. Three drying methods of raw wasabi material, freeze-drying, hot-drying and sun-drying, were investigated by Taiwanese researchers (Tseng *et al.*, 1986) to examine the yields of AITC in wasabi powders. Results showed that the AITC content in ground wasabi products was highest after drying with hot air, followed by freeze-drying, and lowest with sun-drying. The brightness of the powder was lost with the sun-dried method after prolonged storage. The authors suggest that the 45^{o}C hot-air-drying method is best for applications in processing because the powder is able to maintain its colour and odour almost intact. Kojima and Nakano (1980), in a study on the change of ITCs in wasabi powder during storage at 5^{o}C and 30^{o}C in sealed vessels, using headspace GC, point out that the yields of AITC, *sec*-butyl ITC and 3-butenyl ITC were reduced significantly. The only stable pungent component in wasabi hydrolysates during storage at these temperatures was *iso*-propyl ITC. Nevertheless, the contents of all pungent components were shown to be fairly stable at -15^{o}C (Kojima and Nakano, 1980). These results are consistent with studies by Kojima and colleagues on stored wasabi powder derived from roots dried at below 55^{o}C, in which the yields of all ITCs decreased after a four-week storage period (Kojima *et al.*, 1982), or were slightly reduced, compared to fresh root materials (Kojima *et al.*, 1985).

1.2.4 Markets and uses

Markets. An agriculture market exists for the raw materials of wasabi such as rhizomes, leaves and petioles, while food markets exist for processed products from rhizomes, and the pharmaceutical market concentrates on products processed from bioactive compounds. Wasabi rhizome, known as wasabi stem in some literatures, is more consumable than the petiole and leaf (Barber and Buntain, 1985; Sparrow, 2004). The market demand and market value of wasabi rhizomes have been significantly increased in Japan as wasabi production areas have decreased over decades. The wasabi

consumption market has also been widening to many countries, as Japanese dishes have become popularly used outside Japan. Wasabi production in Washington can be domestically sold and exported to Asian countries (Miles and Chadwick, 1996). Wasabi produced in New Zealand is accepted by the Japanese market (Douglas, 2001). Wasabi grown in Tasmania is also acceptable to some markets in Japan, Korea and Taiwan (Barber and Buntain, 1985). Fresh wasabi rhizome is of great demand with an increasingly high price (Sparrow, 2004). Before the 1970s, Japanese and Taiwanese wasabi rhizomes of top quality were accepted at a price of $15/kg. By 1984, it increased to about $50/kg. In 1991, its wholesale price was over $75/kg in the Japanese market (Chadwick *et al.*, 1993). The high-priced wasabi has encouraged the development of methods of artificial production and the expansion of production regions into New Zealand, Korea, Brazil, Thailand and Israel.

In addition to fresh rhizome attracting agriculture markets, processed products such as pastes, pickles, purees and powder attract a principal food market (Barber and Buntain, 1985; Sparrow, 2004). A wasabi product group to assist quality standards and marketing issues has existed under the New Zealand Horticulture Export Authority (Douglas, 2001) since wasabi production was successfully commercialised.

***Uses*.** Some of the popular applications of wasabi are mentioned below.

Food and beverages. Wasabi's benefits have been recognised from as early as the tenth century in Japan, since it was recorded in a medical encyclopaedia. It is traditionally used as one of the staple culinary ingredients in Japanese dishes (Hodge, 1974). Wasabi is popularly served with raw sliced fish (sashimi) for poisoning prevention, or used with other Japanese dishes, such as sushi, chazuke rice soup, natto and buckwheat noodles (soba), as the flavour is enhanced. Fresh rhizomes can be served immediately after grating. Grated wasabi is often mixed with noodles or topped on tofu (Chadwick *et al.*, 1993). Wasabi paste commonly processed from the petioles, roots and stems, is also popularly used as seasoning in Japanese sushi bars and in North American restaurants (Archer, 2001). The leaves of wasabi can enhance odour for some kinds of foods such as sake brine, soy sauce, white rice, barbecue dips, steak, meats, salad dressing, clear soup, cheese, ice cream and crackers (Chadwick *et al.*, 1993; Archer, 2001).

In addition to the traditional uses as primary condiments in Japanese cuisine, wasabi can be used as beverages. Wasabi wine is a novel liqueur of high alcohol content that is

displayed in many of the Japanese specialty stores (Archer, 2001). Wasabi cocktail, produced from a type of quintessential distilled spirit '*shochu'* of Japan, is a special drink known as Wasabi West or Wasabi martini.

Isothiocyanates (ITCs), particularly allyl isothiocyanates (AITC), are the main pungent chemical components of wasabi that provide a specific flavour to foods and beverages; they also serve as an antidote to fish poisoning.

Medical applications. Apart from the important role of wasabi in flavouring foods and in preventing food poisoning from the raw fish by antibacterial properties, its natural, general, antibiotic properties have recently been discovered to provide health benefits. Numerous bioactive compounds contained in all parts of a wasabi plant, particularly in the rhizome, can be used for treatment of many diseases as outlined below.

- *Cancer*. As do its cabbage cousins, wasabi contains cancer-fighting isothiocyanates that help prevent colon cancer by suppressing the multiplication of human stomach cancer cells, or killing them (Fuke *et al.*, 1994; Shin and Lee, 1998; Yano *et al.*, 2000; Watanabe *et al.*, 2003; Uto *et al.*, 2005a). The substances in wasabi can also reduce the risk of hormone-related cancers such as breast and prostate cancer by eliminating the excess hormones. It is recommended by the American National Cancer Institute and the American Cancer Society that the weekly use of some servings of wasabi may dramatically decrease the risk of all types of human cancer (Archer, 2001).

- *Blood clots and cerebral thrombosis*. The ITCs are effective in inhibiting platelet aggregation as an anticoagulant agent (Kumagai *et al.*, 1994). The anticoagulant properties are necessary for the avoidance of blood clotting during surgeries, and can be applied in the treatment of old people.

- *Asthma*. Several secondary metabolites contained in wasabi have antiasthmatic applications (Dorsch *et al.*, 1985). Depree *et al.* (1999) affirm that the ω-methylthioalkyl ITCs work as agents against not only asthma but also coughs, colds, sinusitis and even anaphylaxis.

- *Heart attack*. The prevention of heart attacks is primarily based on the ability of blood-clotting inhibition by the ω-methylthioalkyl ITCs found in wasabi. The ITCs have

a faster effect on heart attack treatment than commonly prescribed aspirin (Depree *et al.*, 1999).

- *Diarrhoea*. Nakayama *et al.* (1998) identify that other compounds found in wasabi are likely to have an inhibitory effect on diarrhoea. The experiment, using solvent extracts of wasabi stems with the removal of any lipid soluble materials, shows that ITCs seem to be the indirect anti-diarrhoeal agents.

Other applications. Modern researches show new applications from wasabi compounds. ITCs are found to be capable of supplying wasabi's 'heat' (extreme piquancy). Fouling-resistant marine paint and natural wood preservatives can be produced from extracts of wasabi. Wood preservatives based on wasabi were used to replace toxic chemicals (poisonous arsenates) by the Japanese National Wasabi Association many years ago (Archer, 2001).

A research project conducted in Canada has indicated that some antifungal metabolites are also contained in wasabi (Soledade *et al.*, 1998). The blackleg fungus *Leptosphaeria maculans*, which destroys such important oilseed crops as canola and rapeseed, can be eliminated by the metabolites. Thus, wasabi can potentially be applied in the development of the natural organic fungicide industry.

1.2.5 Recent studies on wasabi

One of the recent researches conducted on *W. japonica* has focused on the germplasm preservations of seeds and meristems. Wasabi seed is commonly stored under moist conditions, at temperatures ranging from 0^{o}C to 5^{o}C, for a short-term period to maintain its germination. Seeds can show an increase in storage life at a temperature of minus 20^{o}C in mechanical refrigeration, but their viability and genetic stability appear to be lost. For long-term storage of wasabi seeds, cryopreservation is an effective method, enabling the inhibition of metabolism in the germplasm (Potts and Lumpkin, 1997). Recently, research on the cryopreservation of the 'Daruma' variety (*Wasabia japonica*) has been conducted (Potts and Lumpkin, 1997), which showed that wasabi seeds could be cryopreserved in liquid nitrogen at -196^{o}C without any negative effects on germination. Seed viability demonstrated a dependence on desiccation speed, being damaged when slowly dried, but not damaged when rapidly dried.

The wasabi meristem can be stored by the use of a cryopreservation technique (Matsumoto *et al.*, 1994; Matsumoto *et al.*, 1995). The apical meristems of the *W. japonica* cv. 'Shimane No. 3' have been successfully cryopreserved for the maintenance of wasabi germplasm (Matsumoto *et al.*, 1994). Excised at 1 mm in length, meristems were cultured on an MS medium supplemented with 30 g L^{-1} sucrose before being loaded with glycerol and sucrose for 20 minutes at 25^{o}C. A hyperhydricity solution of high concentration was employed for dehydrating the meristems for 10 minutes at the same temperature before the use of liquid nitrogen. The cryopreserved meristems showed a high rate (80–90%) of subsequent shoot regeneration. Another technique of cryopreservation using alginate-coated meristems resulted in a higher rate (95%) of shoot regeneration and an earlier recovery growth of wasabi shoots (Matsumoto *et al.*, 1995). More recently, Matsumoto and Nako (1999) have shown that the success of storage of wasabi meristems at a low temperature of -5^{o}C for two years is affected by dimethyl sulphoxide; the recovery of shoots still proved to be normal.

Other major researches have concentrated on medical applications of bioactive compounds, such as flavonoids and phytoalexins. The identification of new flavonoids and investigation of ITC productivity were carried out successfully (Yu *et al.*, 2001; Sultana *et al.*, 2003a; Murata *et al.*, 2004; Hosoya *et al.*, 2005). Hosoya and collaborators (2005) identified and clarified the structures of five flavone glycosides in fresh leaves of wasabi. These novel flavonoids are isovitexin derivatives that have a *trans*-sinapoyl group at C7. Sultana and his associates (2003a) investigated the yield of ITCs in water-grown wasabi plants of both types, flowering and non-flowering. The measurements and comparisons of ITCs stored in the leaves, petioles, rhizomes and roots of plants were conducted using GC-MS. The authors found that, in comparison with non-flowering plants, flowering plants contained a significantly greater total ITC amount in roots and rhizomes. The authors implied that differences in ITC contents would result in changes in flavour. A suggestion concerning the benefits of flowering plants was made for the crop harvest and product processing. Yu and co-workers (2001), on the other hand, observed the formation of ITC *in situ*. These authors pointed out that ITC could be released from the disruption of cells by local pressure. It was revealed that the secretion resulted from the conversion of sinigrin containing sulphur compounds into ITCs and sulphate. A project carried out by Murata and colleagues (2004) on the examination of 6-methylsulphinylhexyl ITC (6-MSITC) amounts in

various cultivars of wasabi and processed products revealed that rhizomes contained the highest 6-MSITC content, followed by roots, petioles and leaves, in that order. The contents of 6-MSITC in product types were different and rhizome-dependent. It was suggested by the authors that the choice of the rhizome part, or processed products with a higher rhizome percentage, assisted in the daily diet more effectively than other plant parts.

Phytoalexins, the constitutive secondary metabolites in wasabi, were examined for their antifungal properties. Pedras and Sorensen (1998) isolated and successfully synthesised the first antifungal phytoalexin in wasabi, named methyl-1-methoxyindole-3-carboxylate, for the resistance of crucifers to *Phoma lingam* and *P. wasabiae* causing blackleg disease. Pedras, in collaboration with colleagues (1999), extracted another two novel phytoalexins from wasabi leaf tissues, known as wasalexin A and B, which are also resistant to the blackleg fungi (*P. lingam* and *P. wasabiae*). The chemical structures of the three isolated phytoalexins were identified to contain indole rings and sulphur atoms (Pedras and Sorensen, 1998; Pedras *et al.*, 1999).

In other studies, the antimicrobial activities of ITCs were revealed (Ono *et al.*, 1998; Morimitsu *et al.*, 2000; Yano *et al.*, 2000; Watanabe *et al.*, 2003; Shin *et al.*, 2004). Morimitsu *et al.* (2000) identified the human antiplatelet and anticarcinogen activities of 6-MSITC isolated from wasabi. The initial assays conducted on rats and mice *in vivo* showed that 6-MSITC exhibited glutathione S-transferase induction and platelet aggregation inhibition activities. The anticancer properties of 6-MSITC were confirmed by Watanabe *et al.* (2003) when the ethanol extract of wasabi containing 6-MSITC was assayed to demonstrate the necrosis of human U937 cells. 6-MSITC and its homologues contained in wasabi rhizomes were reported by Ono and associates to exert antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (Ono *et al.*, 1998). Most recently, 6-MSITC was found to have excellent anti-inflammatory activities, depending upon the length of the methyl chain (Uto *et al.*, 2005a; Uto *et al.*, 2005b), and to reduce type-2 diabetes in mice by suppressing oxidative stress (Fukuchi *et al.*, 2004). The 6-methylthiohexyl ITC isolated from wasabi was reported by Yano *et al.* (2000) to have an inhibitory effect on lung tumourigenesis in mice under the pretreatment with a carcinogen agent, 4-methylnitrosamino-1-3-pyridyl-1-butanone. The findings of Shin *et al.* (2004) demonstrated that AITC contained in all wasabi plant parts (leaf, petiole,

rhizome and root) exhibited bactericidal properties to eradicate the growth of *Helicobacter pylori* strain NCTC 11637, YS 27 and YS 50. According to the authors, the leaves of the wasabi displayed higher bactericidal activities than roots. It was suggested that, in addition to AITC, other compounds in wasabi might contribute to the eradication of *Helicobacter pylori*.

1.3 Plant tissue culture

The history of plant cell, tissue and organ culture dates back to the year 1838, when Schleiden put forward the idea that a single plant cell possesses enough genetic information necessary for the regeneration of the whole plant, known as totipotency (Taji and Williams, 1996). In 1902 when plant physiology was little known, Gottlieb Haberlandt, in experimenting with a nutrient medium of inorganic salts in an attempt to induce embryos from leaf mesophyll culture, tried to investigate this idea, although unsuccessfully (Steward, 1968; Baker, 1992; Taji and Williams, 1996). It was not until the 1930s that a breakthrough in tissue culture development was made, with the discovery of plant growth regulators necessary for cell division and proliferation in a culture medium. A natural auxin, IAA, and vitamins were first discovered to be needed for the growth of meristematic tissues, the induction of calli from the cambium of carrot root and the formation of tumour tissues of tobacco (Caponetti *et al.*, 2000). Other auxins discovered by Went and colleagues advanced some successes in tissue cultures (Taji and Williams, 1996). Further progress in plant tissue culture was made during 1939–1945 when Johannes Van Overbeek added coconut milk to culture media for regenerating seedlings from heart-shape staged embryos. Rapid progress was achieved after 1950 for the success of plant tissue culture, with the initial investigations of Skoog and associates on the effects of plant growth regulators on the induction of tobacco callus. This led to the discovery of cytokinins, consisting of kinetin and adenine, for the micropropagation of horticultural and agronomic plant varieties of economic values in the 1960s (Trigiano and Gray, 2000). Following further technological advances, such as laminar flow hoods and HEPA filters, which supported sterile culture techniques in the late 1960s, studies on callus and cell suspension cultures contributed to medicinal plant industries.

Preliminary studies on the micropropagation of orchids by Morel (1965) and the development of a new medium rich in mineral salts, initially used in the rapid growth

and bioassays with tobacco callus, by Murashige and Skoog (1962), have stimulated the applications of tissue cultures of various ornamental plant species for supplying planting stocks of high quality with low cost. Until today, this increasingly developed technology has played a major role in plant propagation and improvement. Also in the 1960s, successes in plant recovery were made by doubling haploid cells to generate a homozygous breeding line where in all the recessive genes are expressed, as observed in *Datura* by Guha and Maheshwari (1966), and in *Nicotiana* by Bourgin and Nitsch (1967). These findings facilitated plant breeders and breeding programmes as well (Caponetti *et al.*, 2000). The isolation of protoplasts by removing plant cell walls and the successful cultures of the protoplasts in the 1960s had a major positive impact on somatic cultures and somatic hybridisation for overcoming the sexual incompatibility in plants. This also opened new opportunities for research on membrane transport. The regeneration of transgenic plants was initially reported thanks to the isolation of plant protoplasts, an ideal material source used in plant genetic transformation by the indirect uptake of DNA into protoplasts (Taji and Williams, 1996; Trigiano and Gray, 2000).

A further major stimulus in plant tissue culture may be attributed to the findings of restriction endonuclease enzymes at the beginning of the 1970s. This discovery has provided the background for the manipulations of the plant genome, allowing for the modification and insertion of alien genes into plant DNA strands. Genetically modified (GM) crops were produced and rapidly established due to this technologically developmental watershed, which attracted commercial interest from a great number of companies worldwide during the 1970s and 1980s (Caponetti *et al.*, 2000). The achievements of recombinant DNA technology and GM plant production via the use of plant tissue culture technology and the association of molecular biological techniques have improved the quality of food crops needed for human lives. A detailed review of *in vitro* tissue culture in terms of clonal propagation, liquid culture and callus culture is presented in Chapter 2.

1.4 Plant mutagenesis

The domestication and artificial selection of crops has occurred since the beginning of mankind's history, when common edible plants such as wheat, maize and bean were selected from the natural environment for the valuable traits (Mayo, 1980). Since the discovery of Mendel's research in the early 1900s, the introduction of genetics has laid

the scientific foundation for plant breeding, improving crop development through the understanding of hybridisation, segregation and recombination aiming at desirable traits for a certain purpose (Micke and Donini, 1993). Plant breeding nowadays focuses on characteristics such as disease resistance, drought resistance, heat tolerance or nutritional value (Gottschalk and Wolff, 1983). Many breeding methods available for plant breeders to create genetic variations of better attributes include selection breeding, combination breeding, hybrid breeding, haploid breeding, mutation breeding and synthesis breeding (Kuckuck *et al.*, 1991).

In 1901, natural mutations in some plant species were first discovered and recognised by Korschinsky (van Harten, 1998). Since the beginning of the 1940s, artificial mutation induction, by means of radiation, has been introduced to modern plant breeding (Gottschalk and Wolff, 1983), with the aim of evaluating the biological influences of radiation on the alterations of living organisms. Mutation breeding occupied a significant position in the successful development of new plant varieties, until biotechnology started to be applied to plant breeding programmes during the period from 1985 to 1995. There are two primary types of mutagens, namely physical mutagens that are generally better for tissue penetration, and chemical mutagens that produce a greater gene mutation rate. The combination of the two types of mutagens has been found to enhance mutation induction in several plant species such as rice (*Oryza sativa* L.) (Montalvan and Ando, 1998), pea (*Lathyrus sativus*) (Kumar and Dubey, 1998) and urdbean (*Vigna muno*) (Singh *et al.*, 1999). A more detailed review of chemical and physical mutagenesis is presented in Chapter 3.

1.5 Experimental objectives

Experimentally, the project was designed to establish for *W. japonica*:

- A suitable micropropagation technique for shoot-derived plantlets, using gelled and liquid media under the influences of various PGRs;
- Suitable conditions for the *in vitro* production of calli, as a basis for future induction of somatic embryogenesis and other biotechnological applications;
- Optimal concentrations of colchicine and oryzalin for chemical mutation induction by comparing the effectiveness of these two chemicals;

- Optimal radiation doses of X-rays and gamma rays for physical mutation induction by comparing the effectiveness of both these radiation sources;
- The effects of mutagenic treatments on subsequent plantlets in terms of altered levels and composition of essential oils;
- Appropriate protocols for the estimation of AITC yield from wasabi tissues.

The experimental work presented here is divided into three major sections relating to *in vitro* propagation, investigations of mutagenesis and assessments of AITC content in mutant and non-mutant plant materials.

A report on the rapid multiplication of *W. japonica* is presented in Chapter 2. This chapter outlines the best PGRs to be used for shoot proliferation and root formation. It compares liquid culture with gelled medium culture to draw a conclusion about the efficiency of culture systems. Callus culture is also described, with the aim of investigating the content of AITC, where possible. The most efficient medium is then applied as a standard micropropagation protocol.

An account of the induced mutation experiments is presented in Chapter 3. This chapter describes the applications of oryzalin and colchicine as chemical mutagens, and ionising irradiations as physical mutagens, for inducing mutagenesis in *W. japonica*. The assessments of these mutated plants for any morphological differences to the non-mutated donor plant were followed.

In Chapter 4, assessments for differences in AITC content are made for calli, and for mutant lines, *in vitro* and *in vivo* plant organs. Chapter 5 summarises the significance of the project along with a discussion of the implication of the findings for the future commercial cultivation of this plant.

CHAPTER 2

IN VITRO PROPAGATION STUDIES

2.1 Introduction

2.1.1 Rapid clonal propagation

The objective of the first study in this project was to establish a suitable method for the rapid multiplication of *W. japonica*. Clonal propagation is the production of large amounts of genetically uniform plants *in vitro* (Hulse, 1992; George, 1993). It is an efficient method and especially useful for providing young planting materials in the place of seedlings when seed production is difficult or impossible. Buds, shoots, meristems, bulb scales and node cuttings can be utilised as explants for *in vitro* culture for rapid clonal propagation.

Most of the studies in plant tissue culture describe four key steps of micropropagation, namely, culture establishment, multiplication, root induction and acclimatisation. Some researchers also consider the preparative procedures of stock plants to be an initially important step in any micropropagation programmes (Debergh and Read, 1990; George, 1993; Taji *et al.*, 2001). A general description of five steps or stages is summarised below.

Stage 0 (explant preparation). This stage, commencing prior to micropropagation, involves the selection of mother plants free from any diseases and pre-treatment by growing these stock plants under hygienic conditions for reducing contamination levels. Measures to control bacteria and viruses are essential requirements. Systemic viral diseases are regarded as serious problems in phytosanitation that require an integrated controlling procedure (Thresh and Cooter, 2005). Stage 0 has an influence on both the phytosanitation and survival rate of explants in stage 1. Explants of *Theobroma cacao*, for instance, only survived after the mother plants were grown in the greenhouse (Senawi, 1985). In that study it was recommended that stock plants, particularly tropical and subtropical ornamental crops, should be grown in greenhouses at 25^{o}C, with 75% humidity. It has also been suggested that plants be watered through capillarity or

directly to the pots instead of by means of overhead irrigation (Debergh and Read, 1990). The chemical pre-treatment of mother plants also affects explants in stage 1. Success rates in cultures at stage 1 were found to be increased by injecting a solution of 500 mg L^{-1} BA into the stem of mother plants of *Magnolia soulangeana*, or by placing stock branches of *Castanea* and *Aesculus* in a forcing solution (Read and Yang, 1985). Stage 0 is, thus, essential for the quality of explants in stage 1 and beyond.

Stage 1 (culture initiation). This stage entails the establishment of an aseptic culture of explants of choice, which are usually apical buds or axillary buds for most of the *in vitro* propagation work, from stock plants selected in stage 0. Explants are surface-sterilised to eliminate microbial contaminations before being placed on a nutrient medium. The choice of chemicals and length of exposure for surface sterilisation are dependent upon the source of experimental material. Nodal segments of *Azadirachta indica* were soaked in a 0.15% solution of mercuric chloride for 13 minutes in a laminar air-flow cabinet (Chaturvedi *et al.*, 2004). Shoot tips from seedlings of passion fruits (*Passiflora edulis* Sims.) were successfully sterilised with a 5% solution of sodium hypochlorite for 10 minutes before being established on an MS basal medium (Isutsa, 2004). An MS basal medium, that usually contains some kind of growth regulators, is the most popular for the initiation of an aseptic culture. Piatczak *et al.* (2005) demonstrated that shoot tips of *Centaurium erythraea* were initiated on an MS medium supplemented with 1 mg L^{-1} BA and 0.1 mg L^{-1} IAA after surface sterilisation. In studies on the formation of *Gladiolus* corms derived from dormant corms, Sen and Sen (1995) pointed out that lateral buds of cultivar 'Green Bay' were placed on an MS basal medium fortified with 1 mg L^{-1} BA for initiation, while Dantu and Bhojwani (1995) reported that axillary buds of cultivar 'Friendship' could be established on an MS basal medium alone. The success of stage 1 is also determined by explant origin and size, developmental stage and physiological age of an explant, and donor plant age (Pierik, 1987; Debergh and Zimmerman, 1990). It was shown in a study conducted by Medero and Enriquez (1987) that buds excised from softwood stems were more responsive than those excised from hardwood stems. The findings of Salehi and Khosh-Khui (1997) revealed that the optimal rates for shoot growth and proliferation of miniature roses (*Rosa chinensis* cv. 'Minima') were observable in explants of 9–10 mm length and 3–3.5 mm diameter. Thimmappaiah *et al.* (2002), while working with cashew, noticed that juvenile explants, whose tissues contained higher concentrations of IAA preserved

by auxin protectors stimulating *in vitro* growth (Mato *et al.*, 1994), excised from young donor plants performed better in establishment and proliferation than those derived from adult plants. Reducing the blackening of the medium, resulting from oxidation of polyphenols released from the cut surface of explants, contributes to the success of this stage. This problem could be circumvented by various techniques found in rose micropropagation; for example, by supplementing such substances as polyvinyl pyrrolidone, citric acid, ascorbic acid to the medium, and subculturing frequently (Rout *et al.*, 1999), incubating cultures in the darkness for one to two days right after inoculation to prevent the induction of polyphenol exudation in the light (Pittet and Moncousin, 1981), and adding activated charcoal into the medium and incubating for three days before transfer of cultures to the fresh medium (Curir *et al.*, 1986).

Stage 2 (multiplication). This stage requires the culture to be capable of supplying intact shoots for subsequent propagation. Shoot formation, subculture number, explant type, propagation system and cultural environment are of most interest to the propagator. The adventitious shoot regeneration is most desired in multiplication for most plant cultivars because it can result in numerous propagules in cultures. Adventitious shoots can be abundantly produced from an axillary bud after an unlimited number of subcultures on the medium of the same composition. However, adventitious shoot regeneration still has some limitations. Heterogenous plants with reduced-size fruits as final products were found in *Fragaria* after many cycles of subcultures (Debergh and Read, 1990). Undesirable traits such as off-types, useless mutations and variations also occur quite often from the multiplication method via adventitious shoot formation. Off-types may also result from supplements of an inappropriate cytokinin or appropriate cytokinins at concentrations that are too high to the culture medium. The frequency of off-type and true-to-type plants is required for evaluation to assist in increasing the trueness-to-type in cultures. Hyperhydricity, a physiological problem associated with excess water uptake and often caused by excessive levels of cytokinins, is undesirable. Hyperhydric explants proved to have a shortened survival and poor performance in acclimatisation (Barlass and Hutchison, 1996). Shoot proliferation derived from apices on hormone-containing media commonly has a higher frequency of hyperhydricity than that from axillary buds until the third subculture. Propagation techniques, if not appropriately selected, cause unwanted consequences, such as genetic variability and reduced propagation ratio, for performance in subsequent stages.

Uniform conditions, particularly of gas-exchange rates, should be notified, because these are environmental factors affecting *in vitro* plants enormously in this stage.

Stage 3 (root induction). The propagation scheme in this stage aims at the production of either cuttings or plantlets before their transfer to the natural environment. Plantlets are treated to gain self-supporting growth in soil without becoming stunted or dormant. Shoot elongation may be attempted before rooting is commenced. The efficiency of treatments is determined by both the quantity, such as shoot number, shoot length, shoot weight, rooted shoot number, root number, root length, and quality of the regenerated materials.

Stage 4 (acclimatisation). This is the last stage necessary for tissue-cultured plants to become acclimatised to the external environment prior to transfer to the fields. Although many tissue culturists mention this step only briefly, others still regard it as very important, because micropropagated plants can become moribund under greenhouse conditions for two reasons (George, 1993). Firstly, water in plantlets is rapidly lost because they fail to possess much epicuticular wax on their leaves to protect themselves against *in vivo* conditions of higher temperatures and light intensity, and lower humidity. Leaf stomata in some plant species are incompletely closed under reduced humidity. Secondly, plantlets are incapable of sufficient photosynthesis for autotrophic nutrition for at least several initial days in the external conditions, due to the fact that *in vitro* plantlets are dependent on the culture source of carbon derived from sucrose and carbohydrates. To overcome these difficulties, some authors have suggested that the maintenance of high humidity with mist, and of low light intensity by shading for the first week after transplantation to soil, is required (Pospíšilová *et al.*, 1999).

2.1.2 Liquid culture for mass propagation

Although a liquid medium has long been used for cell suspension cultures in plant tissue culture for bioactive compound production (Dodds and Roberts, 1982; Kevers *et al.*, 2005), it is also an ideal culture medium for somatic embryogenesis (Hvoslef-Eide and Preil, 2005) and for shoot regeneration in many plant species (Batista *et al.*, 2000; Varshney *et al.*, 2001; Akutsu and Sato, 2002; Grigoriadou *et al.*, 2005; Pati *et al.*, 2006). A liquid medium employed in plant tissue culture has advantages over a gelled medium. In liquid cultures, cost is reduced because gelling agents are not utilised in the medium, and more micropropagules are usually multiplied by further addition of fresh

medium without any subculture cycles. Liquid cultures also allow the use of scaled-up automated systems such as bioreactors and robotics, to raise the production and decrease labour costs in commercial micropropagation (Herman, 1995). Plant organs are, at the same time, better provided with available oxygen supply by agitation, and are in direct contact with the medium nutrient components in liquid shake cultures (Dodds and Roberts, 1982).

The problems commonly encountered when employing a liquid medium for micropropagation are the hyperhydricity of micropropagules resulting from the submersion and/or contact of tissues to the liquid, and high contamination rates due to handling, such as during nutrient input and flask sealing. Hyperhydricity in liquid cultures can be eliminated by adding growth retardants such as paclobutrazol and ancymidol, and osmotic agents, to the medium (Dodds and Roberts, 1982; Aitken-Christie *et al.*, 1995).

A considerable number of results have been gained on the micropropagation of plant cultivars via liquid cultures. Batista and co-workers (2000) explanted petiole segments of hops (*Humulus lupulus*) directly to an MS medium fortified with a combination of IAA and kinetin to have produced numerous adventitious shoots within two–three months. Piatczak and colleagues (2005) developed an efficient method for the mass propagation of *Centaurium erythraea* from shoot tips cultured on a liquid MS medium containing IAA and BA. By this method, the authors showed that the number of microshoots per explant obtained in liquid medium after four weeks in culture was three times higher than that produced in agar-solidified medium. Kadota and Niimi (2004) improved the micropropagation of *Dioscorea japonica* using the Linsmaier and Skoog (1965) medium containing BA. The authors proved that a liquid medium was superior to a gelled medium, and that stationary liquid culture was more efficient than shaken liquid culture, in relation to nodal number and fresh weight. A new container named 'RITA' (Récipient à Immersion Temporaire Automatique) has been invented to partially facilitate the *in vitro* liquid cultures as a temporary immersion system. With the support of this latest new development, improved results have been achieved on the micropropagation of several tropical crops such as coffee, banana, rubber tree and potato (VITROPIC, 2005).

2.1.3 Callus culture for the production of bioactive compounds

Of great interest to micropropagators is the application of plant tissue culture techniques to the production of valuable natural compounds originally derived from plants, such as essential oils, flavours, pigments and pharmaceuticals (Razdan, 2002; Brunakova *et al.*, 2005). In most cases, the first stage in plant tissue culture development is the formation of a callus. A callus is a dedifferentiated tumour tissue of almost unorganised cells that can be used to help solve research problems in organogenesis, embryogenesis and biochemistry (Caponetti, 1999), and can also be used to produce secondary metabolites (Doran, 1996). A callus can be initiated from various organs of many plant species (Caponetti, 1999), in which biosynthesised products are variable (Appendix 2.1). Factors affecting the formation of calli for *in vitro* phytochemical production consist of starting material, mineral nutrition, PGRs, and such physical elements as light and temperature (Pierik, 1987; Caponetti, 1999). A factor that is optimal for callus induction in one plant species or genotype may inhibit callus initiation and growth in another closely related species.

Callus culture plays an important role as a principal step in cell suspension cultures for maximising the yield of chemical substances. As a callus proliferates on the surface of a solidified medium at a relatively slow speed, cell suspension culture is usually established in a liquid culture medium after the callus is induced. To increase metabolites, fungal and bacterial elicitors are mostly introduced (for plant–microbe interaction), and the two-stage system of culture of suspended cells on the maintenance and production media is usually required (Pierik, 1987; Ngo and Shargool, 1994).

The purposes of the investigation here were to compare the effectiveness of PGRs and culture medium types for developing a protocol for the rapid multiplication of *W. japonica*, and to investigate the influences of 2,4-D concentrations and plant organs under light- and dark-culture conditions on callus cultures with the future aim of producing callus lines of high AITC content (see Chapter 4). On the basis of the literature review presented above, a set of methods was adopted for this first series of rapid propagation trials.

2.2 Materials and methods

2.2.1 Clonal propagation

2.2.1.1 Sterilisation of explants for culture initiation. Dormant buds of mature rhizomes (Figure 4.2c), collected from healthy plants grown at Dalat (ll°56'N, l08°25'E) in Southern Central Vietnam, were washed thoroughly under running tap water for 40 minutes, soaked in a solution of Viso® detergent for five minutes, rinsed five times with distilled water and then with 70% ethanol (v/v) for one minute. The dormant buds were subsequently rinsed twice with distilled water and disinfected with a 1% (w/v) aqueous solution of NaOCl for 10 minutes. After five rinses in sterile distilled water, explants consisting of dormant buds and a small portion of the rhizomes were initiated on a half-strength MS medium containing 30 g L^{-1} sucrose, 100 g L^{-1} myo-inositol, 0.1 g L^{-1} thiamin-HCl, 0.5 g L^{-1} pyridoxine-HCl, 0.5 g L^{-1} nicotinic acid, 8 g L^{-1} agar, 1.0 μM BA, 2.0 μM gibberellic acid (GA_3) and 50 mg L^{-1} streptomycin (Chemitek Co., Ltd.). Eleven integrated experiments were conducted in this segment of the project, as described in section 2.2.1.2 below.

2.2.1.2 Experimental design for shoot proliferation, root induction and plantlet acclimatisation.

Experiment 1: Effects of basal media on W. japonica shoot growth

Four-week-old *in vitro* multiple-shoots obtained from the initiation culture medium as described above were used in this study (Figure 2.1a). Multiple shoots were separated using a scalpel blade into individual shoots of the same length (Figure 2.1b). Seven media were selected for testing the survival and tolerance of the *in vitro* environment and its influences on the shoot growth:

- Full-strength MS (Murashige and Skoog, 1962);
- Half-strength MS (served as control in this assay);
- N_6 (Chu, 1978);
- B_5 (Gamborg *et al.*, 1968);
- WPM (Lloyd and McCown, 1980);
- N&N (Nitsch and Nitsch, 1969);
- KC (Knudson, 1925).

These culture media contained various components consisting of both rich and poor nutrients. Explants were placed on the basal media without supplementing any PGRs, for a period of four weeks.

Experiment 2: Effects of BA, kinetin, TDZ and zeatin on production of multiple shoots of W. japonica

Single shoots 15–20 mm in length (Figure 2.1b), separated from the clump of shoots as described in Experiment 1, were cultured on the full-strength MS medium supplemented with 7 g L^{-1} gelcarin®, 3% sucrose (w/v) and five different concentrations (0.5, 1.0, 5.0, 10.0 and 50.0 μM) BA, TDZ, kinetin or zeatin (Austratec Pty Ltd.) for four weeks for identifying optimal concentrations for each cytokinin for the shoot growth.

Shoot tips proliferated on the full-strength MS medium supplemented with 5 μM BA in this Experiment were used as initial explant sources for Experiments 3, 5, 6, 7, 8 and 9.

Experiment 3: Effects of gelled and liquid media on the production of multiple shoots of W. japonica

Explants obtained from Experiment 2 were used in this study as follows:

(a) For gelled cultures: Individual shoot tips were cultured on ¼MS, ½MS and MS media containing 7 g L^{-1} gelcarin®, 3% sucrose (w/v) and 5.0 μM BA for four weeks.

(b) For liquid cultures: Half-shoot bases (shoot tips with leaves and petioles removed) (Figure 2.1c) were cultured on ¼MS, ½MS and MS media containing 3% sucrose (w/v) and 5.0 μM BA for four weeks. Liquid medium cultures were shaken at a constant speed of 80 rpm.

Growth was compared between treatments with gelled and liquid medium cultures. The shoot number per explant, shoot length and fresh weight were measured at the end of the fourth week.

Experiment 4: Effects of continuous liquid cultures on further production of multiple shoots

Explants obtained from section (b) of the Experiment 3 were used in this study. Shoot clusters growing in Erlenmeyer flasks containing three liquid media – quarter-strength

MS, half-strength MS and full-strength MS, at the end of the fourth week, were multiplied for another two weeks by adding an equal amount of each fresh stock liquid medium containing 3% sucrose (w/v) and 5.0 μM BA to the same flasks. All cultures were shaken at 80 rpm.

Experiment 5: Effects of gelled medium derived-shoots and liquid mediun derived-shoots on further proliferation of multiple shoots of W. japonica

Single vigorous shoots, 15–20 mm in length, obtained from three previous culture media – full-strength MS gelled medium containing 5 μM BA (see Experiment 2), full-strength MS liquid medium containing 5 μM BA (see Experiment 3), and half-strength MS liquid medium containing 5 μM BA (see also Experiment 3), were placed on a full-strength MS gelled media supplemented with 7 g L^{-1} gelcarin, 3% sucrose and 5 μM BA for four weeks for identifying the optimal culture medium for shoot growth.

Experiment 6: Effect of combination between 5 μM BA and 1 or 2 μM NAA, IBA or IAA on further proliferation of multiple shoots of W. japonica

Explants obtained from Experiment 2 were used in this study. Single shoots 15–20 mm in length were cultured on a full-strength MS medium supplemented with 7 g L^{-1} gelcarin, 3% sucrose (w/v) and 5.0 μM BA in combination with 1 or 2 μM NAA, IBA or IAA (Austratec Pty Ltd.) for four weeks for identifying the optimal combinations for shoot growth.

Experiment 7: Effect of combination between 5 μM BA and 2 or 5 μM TDZ, kinetin or zeatin on further proliferation of multiple shoots of W. japonica

Single shoots 15–20 mm in length, obtained from Experiment 2, were cultured on a full-strength MS medium supplemented with 7 g L^{-1} gelcarin, 3% sucrose (w/v) and 5.0 μM BA in combination with 2 or 5 μM TDZ, kinetin or zeatin for four weeks for identifying the optimal combinations for shoot growth.

Experiment 8: Effects of NAA, IBA and IAA on W. japonica root formation

Single shoots derived from Experiment 2 were cultured on a ½MS medium supplemented with 7 g L^{-1} gelcarin®, 2% sucrose (w/v) and five different concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 μM) of IBA, IAA or NAA for three weeks for identifying the optimal concentrations of each auxin for root development.

Experiment 9: Effects of IBA, NAA, BA in gelled and liquid culture media on W. japonica root formation

In a more extended set of trials, single shoot tips and shoot bases derived from Experiment 2 were cultured on two types of media, ½MS gelled medium and ½MS liquid culture medium, for identifying the optimal culture condition for root formation. For the ½MS gelled medium, 5.0 µM IBA were added. For the ½MS liquid culture medium, either 5.0 µM IBA alone or a combination with 5.0 µM BA and 2.0 µM NAA were added. All cultures were incubated for three weeks.

Experiment 10. Effects of root origins on the acclimatisation of W. japonica plantlets in the greenhouse

Four types of roots, obtained from the ½MS gelled medium supplemented with IBA at 5 µM or 10 µM (see Experiment 8) (Figure 2.1d), and from the ½MS liquid shake medium with 5 µM IBA or 5 µM BA plus 2 µM NAA (see Experiment 9), were used to compare their plant growth in the greenhouse conditions. Plantlets were removed from the jars or flasks using forceps and then washed under running water to remove all the gelcarin. Washed plantlets were immediately potted in 70 mm x 50 mm plastic pots containing a selective mixture of vermiculite, perlite and peat-moss at a ratio of 1:1:1 (v/v/v) and placed on a mist bench.

Experiment 11. Effects of substrates on the acclimatisation of W. japonica plantlets in the greenhouse

Three main types of nursery substrates (vermiculite, peat-moss and perlite) and their combinations were used for *in vitro* plantlets to acclimatise in the greenhouse. After removing gelcarin, plantlets derived from the ½MS medium supplemented with 10 µM IBA (see Experiment 8) (Figure 2.1d) were potted in 70 mm x 50 mm plastic pots containing media comprised of either vermiculite, perlite or peat-moss alone, or mixtures of vermiculite : perlite (1:1); vermiculite : peat-moss (1:1); perlite : peat-moss (1:1); or vermiculite : perlite : peat-moss (1:1:1).

Figure 2.1: Plant materials used for tissue culture, induced mutation and AITC content assessment ((a) a 4-week-old multiple-shoot, (b) single shoots, (c) single shoots with bases separated away from leaves and petioles, and (d) a rooted shoot or plantlet)) (bar = 0.5 cm).

2.2.1.3 Culture conditions

In all experiments for shoot initiation, shoot multiplication and root induction, 50 mL of medium were dispensed into 250-mL jars for the gelled culture media, or 10 mL liquid medium in 100-mL Erlenmeyer flasks for the liquid culture media. Media adjusted to pH 5.8 were autoclaved at 121^{o}C at 105 kPa for 15 minutes. Explants were incubated at 24 ± 1^{o}C with a 12-hour photoperiod and irradiance of 50 µmol $m^{-2}s^{-1}$ emitted from cool white fluorescent lights. In experiments for *in vivo* plantlet acclimatisation, plantlets were placed on a mist bench or in a plastic tray covered with a clear plastic lid (Figure 2.19) under the shade for two weeks to obtain a humid environment, and then moved to the greenhouse at 25–27^{o}C, 80–85% relative humidity. They were automatically watered every day under natural light.

2.2.1.4 Growth measurement

Morphological and agronomic traits of the explants exposed to different type of media, PGRs and greenhouse substrates were assessed for their survival rate, weight, height and quantity. The survival percentage of explants was calculated by subtracting the necrotic explants from the total, and then dividing by the total. The fresh weight of explants was measured using an electronic balance (Mettler, Model AE260 Delta range) at an accuracy rate of 10 ng. The height of shoots and length of roots were measured using a millimetre ruler. The number of shoots was calculated by counting every single-leaf shoot.

2.2.1.5 Data analysis

Each treatment was represented by 25 replicates of each type of explants. The experiment was repeated three times and conducted as a completely randomised design. All data were recorded four weeks after culture for shoot growth and shoot multiplication, three weeks for root formation, four weeks and six weeks for liquid shake culture, and four weeks for acclimatisation. Statistical analyses of data were performed using a general linear model and one-way ANOVA. When ANOVA demonstrated significant treatment effects, individual mean separation was carried out using Tukey's Simultaneous Tests at 5% level of probability ($p \leq 0.05$). Means with/without standard errors (SE) were presented. The SE of means were calculated by the descriptive statistics test. Data were statistically analysed using a Minitab computer package (Minitab 14, State College, PA, 2003).

2.2.2 Callus culture

2.2.2.1 Selection of explant and culture condition for callus induction

Plant materials. Leaves and petioles isolated from four-week-old *in vitro* shoots multiplied on a full-strength MS proliferation medium supplemented with 5 μM BA (see Experiment 2 and Figure 2.2) were selected for callus induction.

Excising techniques. Leaves were symmetrically divided into (a) two parts along major veins using a scalpel blade. Petioles were cut either into three parts: (b) leaf-close segment (proximal portion), (c) middle segment (medial portion) and (d) base-close segment (distal portion) (Figure 2.2A), or into 1–2 mm segments (Figure 2.2B).

Experimental design. Excised explants were separately placed on the surface of MS media containing 2,4-D at concentrations of 0, 0.1, 0.5, 1 and 5 μM. Media were contained in transparent plastic Petri dishes (80 mm diameter) sealed with parafilm. Leaf explants were placed in both positions with either abaxial or adaxial leaf surfaces, before labelling. Petiole explants were placed horizontally on the surface of an MS medium containing the same range of 2,4-D concentrations.

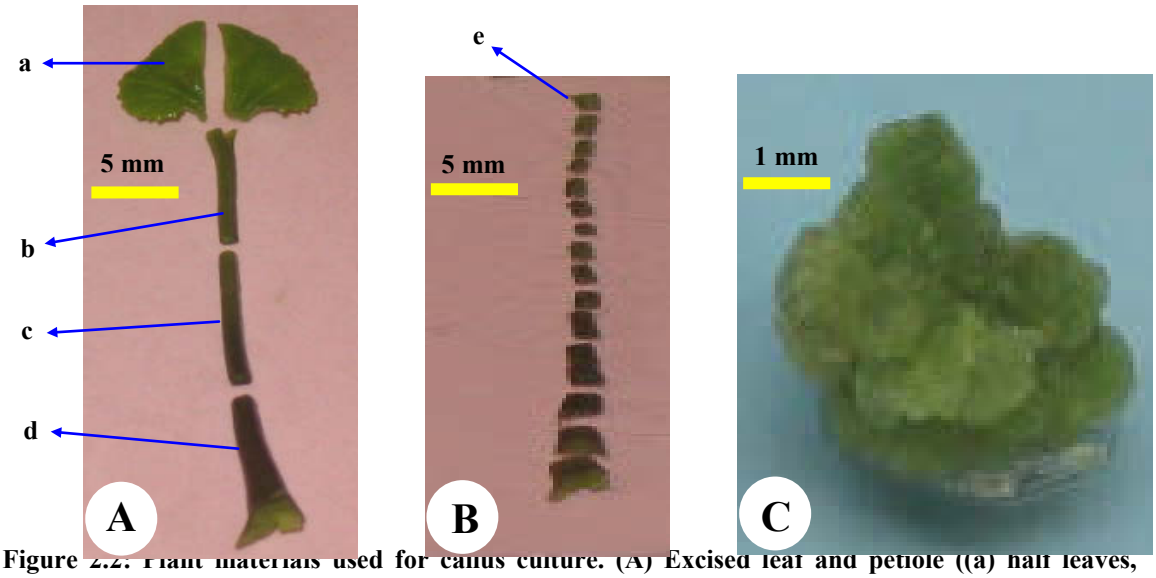


Figure 2.2: Plant materials used for callus culture. (A) Excised leaf and petiole ((a) half leaves, (b) leaf-close petiole segment, (c) middle petiole segment, (d) base-close petiole segment)); (B) petiole excised into pieces; and (C) petiole pieces-derived callus.

Culture conditions. Leaf and petiole explants were incubated in both dark and light conditions for six weeks. In dark conditions, cultures were placed in a totally dark closed-door incubator (Memmert GmbH, BM 500, Germany) set at 24 ± 1°C. In light conditions, cultures were kept in a growth chamber at 24 ± 1°C with a 12-hour photoperiod and irradiance of 50 µmol $m^{-2}s^{-1}$.

Evaluations. After six weeks in culture, the percentage of callusing and callus quality were recorded. The percentage of callusing was calculated by dividing the number of calli by the total number of explants. The quality of the calli was determined on the basis of friability under a fluorescence microscope (Olympus, Japan). Calli of high friability were marked as "+++", while those with low friability were marked as "+". Calli that were neither compact nor highly friable were marked as "++", and unviable calli were marked as "-". Each treatment was represented by 25 replicates of each type of explant. The experiment was randomly arranged and conducted three times. Statistical analyses of data were carried out as appropriate, using the methods described in section 2.2.1.5 above.

2.2.2.2 Selection of PGRs for callus proliferation

Plant materials. Six-week-old calli (Figure 2.2C) induced from petioles that had been divided into pieces of 2 mm (Figure 2.2B) and cultured on the MS medium

supplemented with 0.5 μM 2,4-D in the light conditions were used for further callus studies.

Experimental design. Explants were placed on the surface of MS media contained in transparent plastic Petri dishes (80 mm diameter). The culture media were MS supplemented with a range of PGRs, as listed below.

a) No regulators (Control)
b) 0.5 μM 2,4-D
c) 0.5 μM 2,4-D + 0.5 μM BA
d) 0.5 μM 2,4-D + 0.5 μM kinetin
e) 0.5 μM 2,4-D + 0.5 μM TDZ
f) 0.5 μM 2,4-D + 0.5 μM zeatin
g) 5 μM BA + 2 μM NAA
h) 5 μM BA + 2 μM IBA
i) 5 μM BA + 2 μM IAA
j) 1 μM NAA + 10 μM kinetin

Culture conditions. See section 2.2.2.1.

Evaluations. The growth of calli was observed every week. After six weeks in culture in the light condition, the callus quality, fresh weight and type of response were recorded. The quality of the calli was evaluated in terms of friability (see section 2.2.2.1). The biomass of the calli was measured on a fresh weight basis using a balance (see section 2.2.1.4). The type of callus response was determined by morphological differentiation, such as rooting and callusing. Each treatment was represented by 25 replicates of explants. The experiment was randomly arranged and repeated three times. All data were recorded six weeks after culture. Representative callus cultures were submitted to dark conditions for morphological observations. Statistical analyses of data were carried out as appropriate, according to the methods described in section 2.2.1.5 above.

2.3 Results

2.3.1 Clonal propagation

2.3.1.1 *In vitro* culture initiation

Cultures of *W. japonica* were successfully initiated, however, a high percentage (45–50%) of contamination was observed. The sterilisation procedures appeared to show satisfactory results (data not shown). All initiated cultures exhibited healthy shoots when subcultured onto shoot proliferation media. Results obtained from the eleven integrated Experiments are presented below.

2.3.1.2 Shoot multiplication. Results of *W. japonica* shoot growth and multiplication are presented in Experiments 1–7.

Experiment 1: Effects of basal media on W. japonica shoot growth

The effects of seven basal media types on the survival and growth of *in vitro* shoots, after four weeks in culture, are shown in Figure 2.3 below.

It can be seen that shoot tips of *W. japonica* survived on a wide range of media types, from the nutrient-enriched medium (MS) to the medium poor in mineral and organic substances (KC) (see Appendix 2.2). Necrosis of shoot tips was not detected in any of the seven basal media after four weeks of culture. Survival rates in all basal media was maximal, that is, 100%, with no significant differences recorded ($p= 0.102$).

A great tolerance of types of culture media by the shoot tips was also observed in the weights and heights achieved. However, there were significant differences in the response of shoots in terms of weight ($p= 0.004$). It was found that shoot weights in WPM and KC media were not significantly different, but they were both lower than those in ½MS, MS, B5, N6 and N&N (Figure 2.3 and Appendix 2.3). In the height response, shoot tips in WPM and KC were significantly different from each other, and were also significantly lower than those in other media. In other words, both shoot weight and shoot height responded positively in the five media: ½MS, MS, B5, N6 and N&N, and there were no significant differences in the growth parameters among these media (Figure 2.3 and Appendix 2.3).

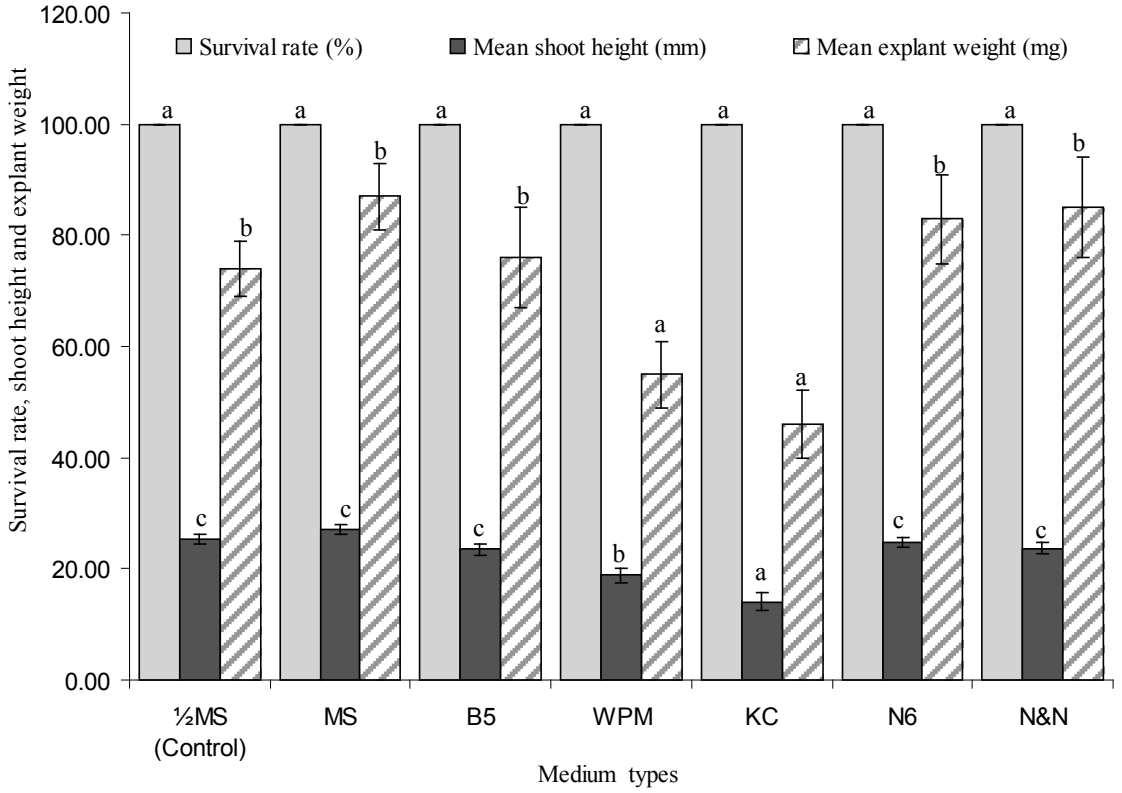


Figure 2.3: Effects of basal media on shoot survival, shoot height and explant weight of *W. japonica* after 1 month in culture. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 single shoots. Vertical lines denote standard errors.

After four weeks in culture, the stimulation of shoot multiplication was detectable only in the KC medium, and at a low multiplication rate (data not shown). New leaves produced in this medium had light green and white colours that looked deficient in some types of chlorophylls. Other media did not respond to new shoot formation.

In summary, apart from the WPM and KC media, the optimum shoot growth was obtained equally from the other five media. MS and ½MS were media rich in minerals and organic substances, and are the most popular in use in plant cell and tissue culture. Therefore, MS was selected for shoot proliferation, and ½MS for root induction in our later Experiments.

Experiment 2: Effects of BA, kinetin, TDZ and zeatin on production of multiple shoots of W. japonica

Various responses were observed in terms of shoot proliferation from shoot tips in a full-strength MS gelled medium supplemented with different concentrations of cytokinins after four weeks in culture. Data are summarised in Table 2.1.

Optimal shoot numbers were obtained with 0.5–10.0 μM BA, 0.5–10.0 μM TDZ, 5.0–50.0 μM kinetin, and 10.0 μM zeatin (p= 0.000) (Figure 2.4 and Table 2.1). Kinetin (10.0 μM) and BA (5.0 μM) gave rise to the highest shoot numbers (10.9±0.8 shoots/explant and 10.4±0.6 shoots/explant, respectively). BA (1.0–10.0 μM) and zeatin (10.0 μM) gave rise to the highest explant fresh weights (p= 0.023). Explant fresh weights ranged from 230.6±12.8 mg (at 0.5 μM kinetin) to 348.2±16.9 mg (at 5.0 μM zeatin) for the remaining concentrations and PGRs, and all of these explant fresh weights were significantly greater than the control (145.0±9.5 mg) (p= 0.012). A significant reduction in explant fresh weights was observed with BA and zeatin from the 10 μM treatments to the 50 μM treatments, while kinetin or TDZ showed no significant difference in explant fresh weights between the 10 μM and 50 μM treatments.

In contrast, average shoot heights obtained from the control (33.4±1.4 mm) and 0.5 μM zeatin (33.0±1.3 mm) were significantly higher than those from other PGRs (p= 0.006) (Table 2.1). Shoot abnormality was observable in the MS medium supplemented with either 50 μM BA or 50 μM TDZ (Figure 2.5); whereas, the shoot appearance was still stable on the MS medium containing kinetin and zeatin at the high concentrations.

BA at 5 μM gave rise to high shoot weight and shoot number per explant. Therefore, MS plus 5 μM BA was selected as the shoot multiplication medium for any of the subsequent shoot proliferation Experiments.

Table 2.1: Effects of growth regulators on *in vitro* shoot induction of *W. japonica* after 4 weeks in gelled culture media.

Growth regulators (μM)	Mean number of shoots per explant[a]	Mean height of shoots (mm)	Mean weight of explants (mg)
Control (0.0)[b]	1.0±0.0a[c]	33.4±1.4d	145.0±9.5a
BA 0.5	8.1±0.8c	22.6±0.5c	296.0±16.2bc
1	9.6±0.9c	20.6±0.9bc	368.5±20.8d
5	10.4±0.6d	15.4±0.7ab	437.6±17.3d
10	7.9±0.6c	14.8±0.5ab	386.4±24.8d
50	4.3±0.3b	13.0±0.6ab	257.8±9.6b
TDZ 0.5	7.2±0.4c	20.4±0.3bc	262.2±16.8b
1	8.7±0.6c	17.7±0.9b	336.0±24.3c
5	8.3±0.6c	11.1±0.6ab	276.3±11.3b
10	7.8±0.3c	9.9±0.7a	270.4±9.4b
50	5.8±0.3b	9.0±0.9a	259.0±8.0b
Kinetin 0.5	5.2±0.3b	20.1±1.2bc	230.6±12.8b
1	5.2±0.4b	18.7±1.1b	236.8±13.1b
5	9.7±0.8c	13.3±1.1ab	287.6±15.1bc
10	10.9±0.8d	12.1±1.2ab	347.9±22.1c
50	8.0±0.3c	7.0±0.6a	289.2±10.1bc
Zeatin 0.5	1.1±0.1a	33.0±1.3d	249.6±6.3b
1	2.4±0.1ab	26.6±1.1c	261.6±12.4b
5	4.7±0.3b	20.8±0.4bc	348.2±16.9c
10	6.7±0.3c	19.8±0.4bc	374.0±22.0d
50	4.7±0.3b	19.0±0.6b	328.3±29.5c

[a]Values are means ± standard errors of three independent experiments consisting of a total of 25 explants.
[b]Control = Full-strength MS medium free of growth regulators.
[c]Means within a column followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

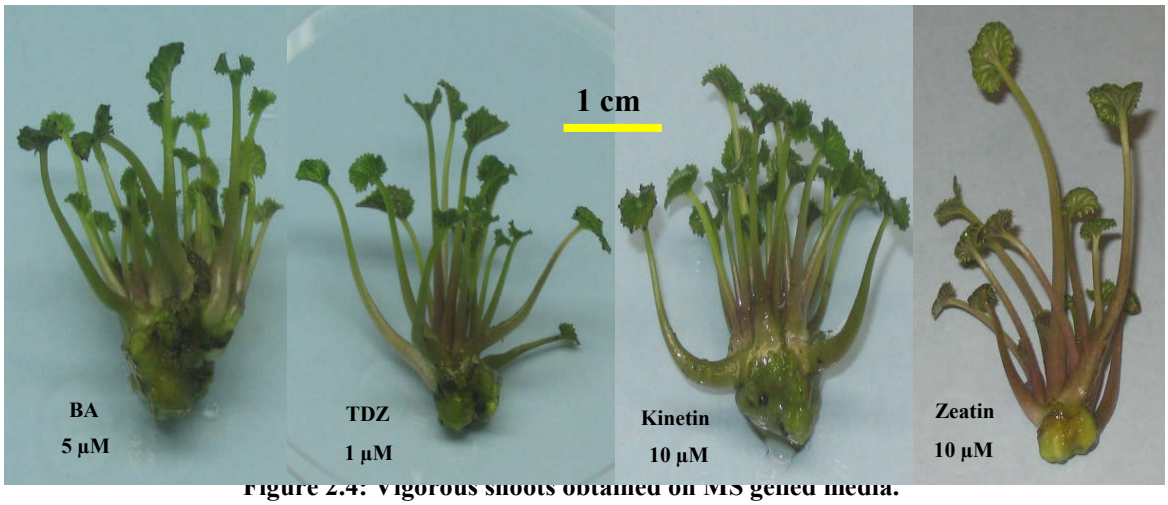


Figure 2.4: Vigorous shoots obtained on MS gelled media.

Abnormal shoot Retarded shoot

Figure 2.5: Low-quality shoots produced on MS gelled media containing BA and TDZ at high concentrations.

Experiment 3: Effects of gelled and liquid media on the production of multiple shoots of W. japonica

After four weeks in culture, significant differences in shoot number, shoot length and explant fresh weight were observed between the liquid and gelled medium treatments (Figures 2.6, 2.7 and Appendix 2.4).

Shoot numbers were significantly greater ($p= 0.001$) in half- and full-strength MS liquid media than in gelled media or the quarter-strength MS liquid medium (half-strength MS; 15.0±0.7 shoots/explant and full-strength MS; 15.3±0.9 shoots/explant).

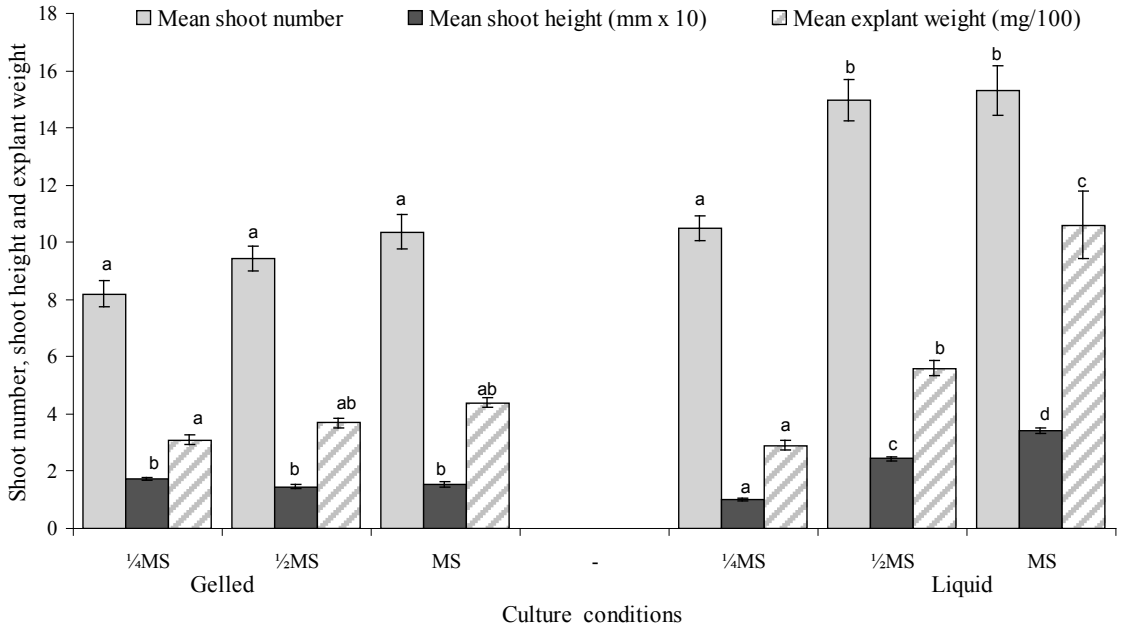


Figure 2.6: Effects of medium types (¼MS, ½MS and MS) and medium conditions (gelled and liquid) on shoot number, shoot height and explant weight of *W. japonica* after 4 weeks in culture. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

Figure 2.7: Shoot proliferation of *W. japonica* after 4 weeks in culture on (a) ¼MS, (b) ½MS and (c) MS media, under (A) liquid and (B) gelled culture conditions (bar = 1 cm).

The greatest shoot lengths and explant weights were also achieved on the full-strength MS liquid medium (34.2±1.0 mm and 1055±77 mg, respectively). There was no significant difference in the number of shoots, length of shoots and fresh weight of explants among treatments for shoots grown on gelled media (p= 0.096). Similarly, no significant difference in shoot number and explant fresh weight was found between the quarter-strength MS gelled medium and quarter-strength MS liquid medium (p= 0.062). The half-strength MS liquid medium produced statistically similar results to the

full-strength MS liquid medium in terms of shoot number (p= 0.091). The full-strength MS liquid medium was the most effective for shoot proliferation of *W. japonica*.

Experiment 4: Effects of continuous liquid cultures on further production of multiple shoots

After six weeks' culture on quarter-, half- and full-strength MS liquid media, there was a significant difference in the shoot formation of *W. japonica*. The half-strength MS liquid medium produced more shoots than the full-strength MS liquid medium (p= 0.015). The average shoot length and height on the half-strength MS liquid medium were significantly lower than on the full-strength MS liquid medium, confirming previous results (Figures 2.8, 2.9 and Appendix 2.5). A small amount of hyperhydricity was observed in shoots cultured on the full-strength MS liquid medium, whereas no hyperhydricity symptom was displayed in shoots cultured on the quarter- and half-strength MS liquid media.

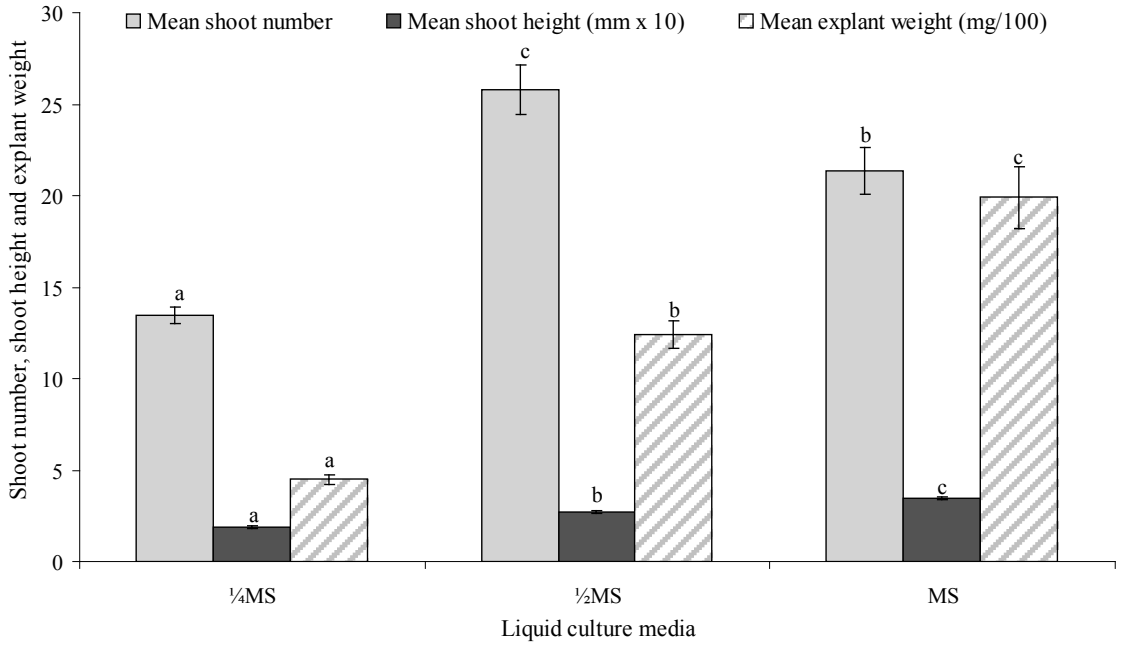


Figure 2.8: Effects of liquid shake culture media (¼MS, ½MS and MS) on shoot number, shoot height and explant weight of *W. japonica* after 6 weeks in culture. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

Figure 2.9: Shoot proliferation of *W. japonica* after 6 weeks in culture on (a) ¼MS, (b) ½MS and (c) MS liquid media (bar = 1 cm).

Experiment 5: Effects of gelled medium derived-shoots and liquid mediun derived-shoots on the further proliferation of multiple shoots of W. japonica

The further multiplication of *in vitro*-regenerated shoots derived from various origins on the MS shoot proliferation medium had an influence on shoot number and height (Figure 2.10).

Shoots originating from liquid shake culture media produced higher shoot number and height than shoots multiplied from the same gelled medium. The maximum shoot multiplication rate was obtained in shoots derived from the ½MS liquid medium (12.91±0.79 shoots/explant), and the maximum shoot height rate was attained in shoots derived from both MS and ½MS liquid media (Figure 2.10 and Appendix 2.6).

The explant weight stabilised when shoots derived from all three origins were subcultured on the gelled medium (Figure 2.10). It was observed that the uniform height occurred in shoots derived from the ½MS liquid medium, followed by the MS liquid medium. Solidified medium-derived shoots mostly comprised abundant calli, while shoots originating from the liquid culture media induced few calli (Figure 2.11). The decline in callus induction caused the explant weight reduction in the liquid culture medium-derived shoots and resulted in the non-significant difference ($p= 0.062$) in shoot weight (Figure 2.11 and Appendix 2.6).

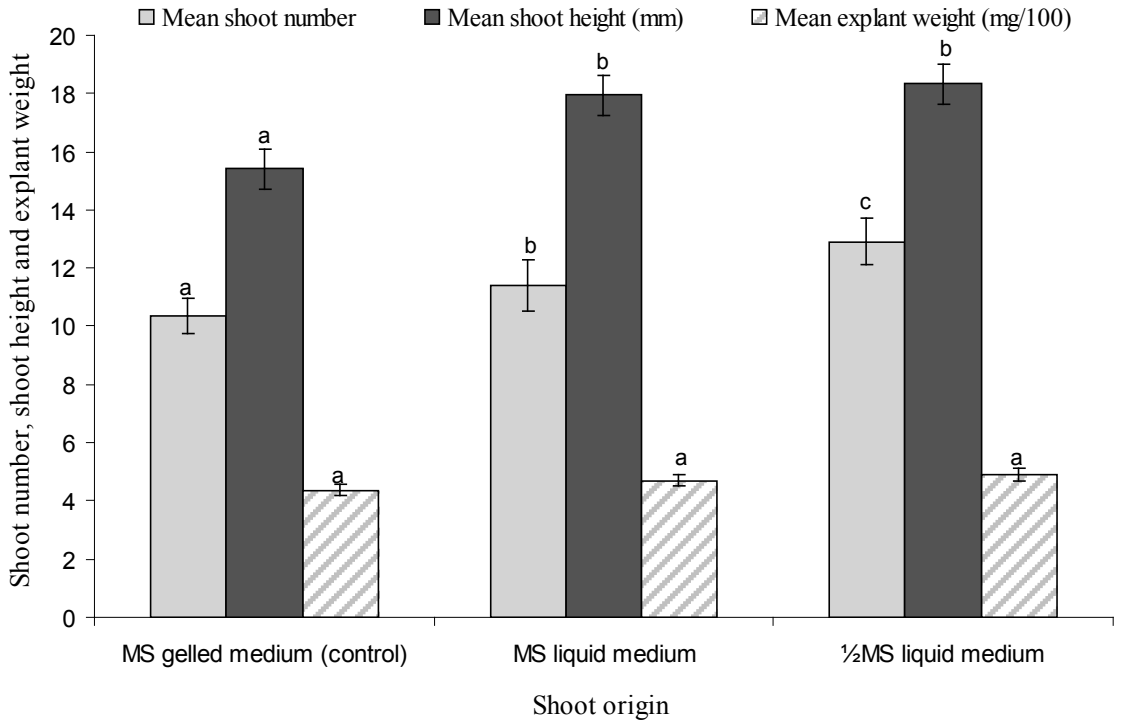


Figure 2.10: Effects of initial shoots derived from MS gelled, MS liquid, and ½MS liquid media on shoot number, shoot height and explant weight of *W. japonica* after 4 weeks in culture on MS gelled medium. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

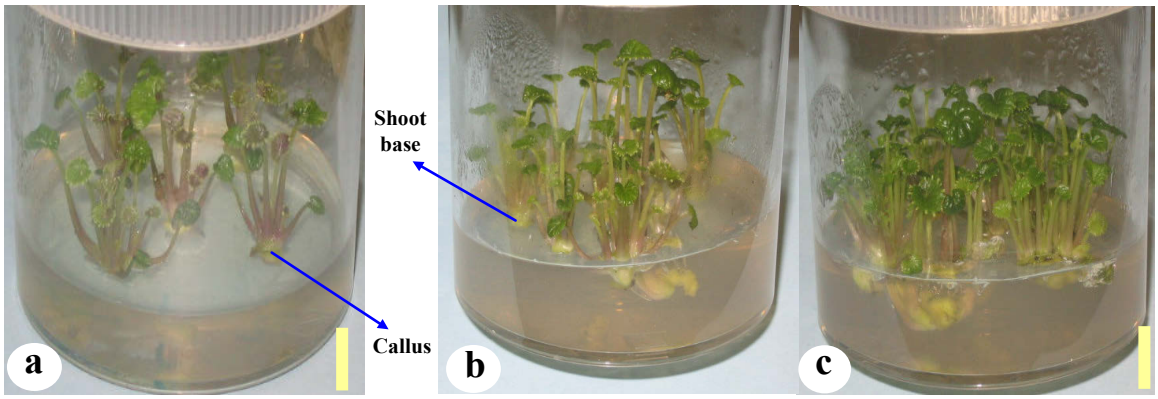


Figure 2.11: Shoot proliferation of *W. japonica* after 4 weeks in culture on MS gelled medium containing 5 µM BA, with initial shoots derived from 3 origins: (a) MS gelled, (b) MS liquid, and (c) ½MS liquid media (bars = 1 cm).

Experiment 6: Effect of combination of 5 µM BA and 1 or 2 µM NAA, IBA or IAA on further proliferation of multiple shoots of W. japonica

The incorporation of IAA, IBA and NAA at 1 or 2 µM into the MS shoot multiplication medium supplemented with 5 µM BA showed significant effects on shoot number, shoot height and explant weight (Figure 2.12 and Appendix 2.7). The only combination

between 5 μM BA and 1 μM IAA gave rise to a high shoot number compared to the control (p= 0.032). No significant difference was found in shoot height between combinations and the control (p= 0.066). The exposure of shoots to auxins and BA resulted in the highest shoot height in the combination of 5 μM BA and 2 μM IBA. The addition of 1 or 2 μM IAA to the medium significantly increased explant fresh weight (ranging from 660±52 to 716±48 mg/explant) compared to the control (438±17 mg/explant) and other combinations. In comparison with the control, the combinations of these auxins at levels of 1–2 μM and BA at 5 μM showed no significant reduction in shoot growth parameters.

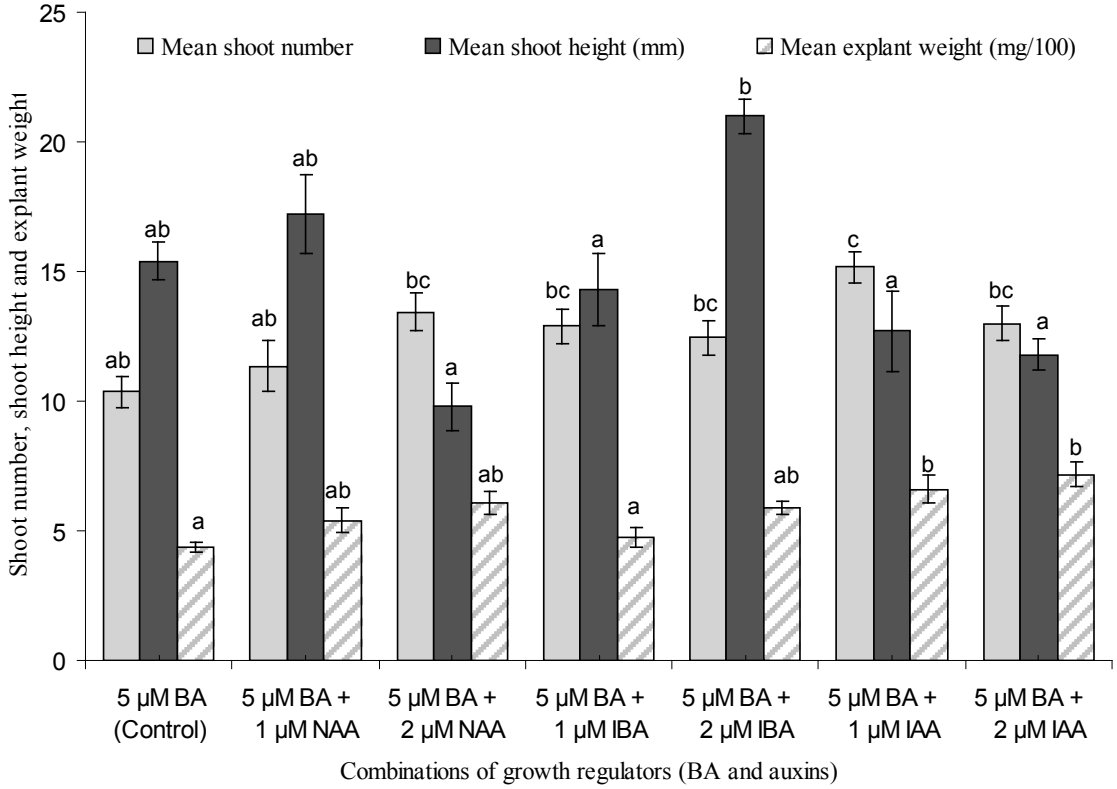


Figure 2.12: Effects of combinations between BA and auxins (IBA, IAA and NAA) on shoot number, shoot height and explant weight of *W. japonica* after 4 weeks in culture. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

Experiment 7: Effect of combination between 5 μM BA and 2 or 5 μM TDZ, kinetin or zeatin on further proliferation of multiple shoots of W. japonica

Unlike the combination between BA and auxins where no decline in shoot growth was identified, the combination between 5 μM BA and cytokinins at 2 or 5 μM was found to

exhibit fluctuations in explant weight in comparison with the control (Figure 2.13 and Appendix 2.8).

The lowest explant weight was detected when 2 μM kinetin were incorporated into the medium (p= 0.007). In contrast, a supply of 5 μM TDZ to the medium increased the average explant weight to 657±42 mg.

Shoot number increased in a number of combinations, compared to the control. TDZ and zeatin at 2 or 5 μM induced higher rates of shoot formation. The best shoot number was obtained with 5 μM zeatin, followed with 2 μM TDZ. The only type of cytokinin that showed no significant difference in shoot multiplication rate compared to the control was kinetin at 2 and 5 μM (Figure 2.13).

Apart from the similarity in shoot height between the control and combination of 5 μM BA plus 5 μM kinetin, other combinations with BA resulted in a significant reduction in this parameter, compared to the control (p= 0.020).

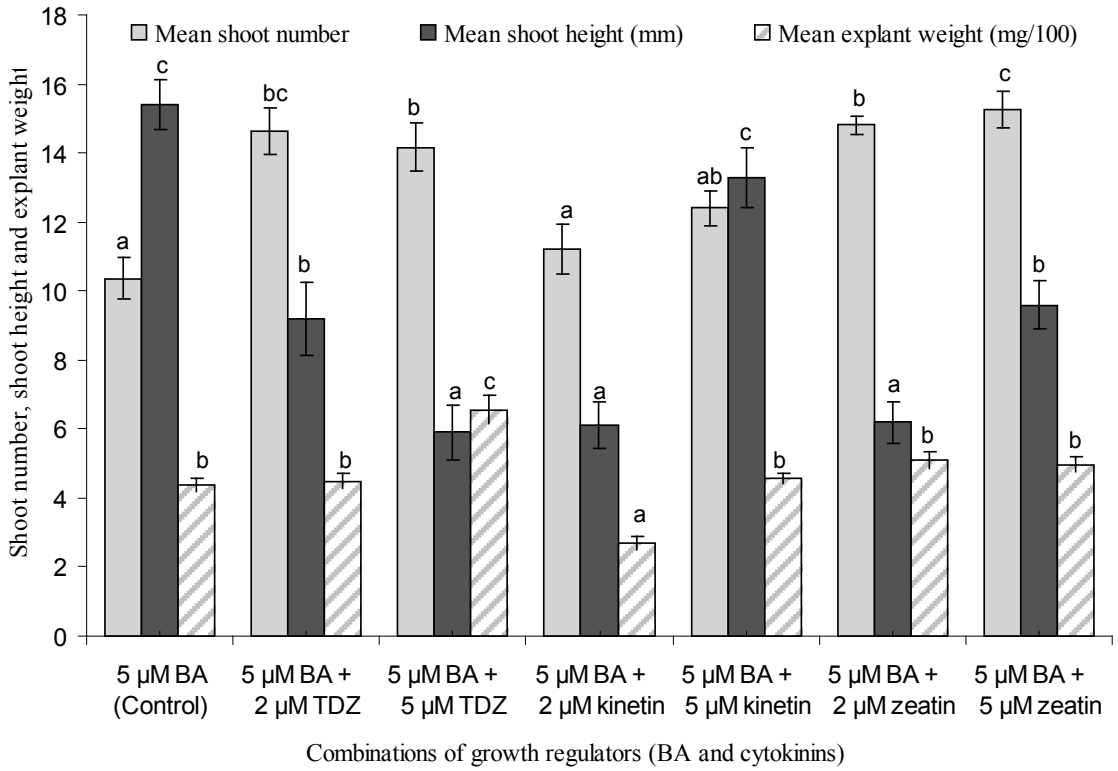


Figure 2.13: Effects of combinations between BA and other cytokinins (TDZ, kinetin and zeatin) on shoot number, shoot height and explant weight of *W. japonica* after 4 weeks in culture. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

2.3.1.3 ***In vitro*** **root formation**. Results of the Experiments 8 and 9 are presented below.

Experiment 8: Effects of NAA, IBA and IAA on W. japonica root formation

Rooting occurred in both the absence and presence of NAA, IBA and IAA (Table 2.2).

NAA, IBA or IAA (5.0–10 µM) caused microshoots to induce roots sooner than those at 0.1–1.0 µM. Roots were induced with 5.0–10.0 µM IBA or IAA after one week of culture, and with all three auxins after two weeks of culture. The percentage of root-forming shoots was high at all concentrations of PGRs, except with high levels (5.0–10.0 µM) of NAA. Nevertheless, root number was highest on IBA at 0.5 µM with 3.5±0.3 roots/shoot, and was inhibited on NAA at 10.0 µM with only 0.9±0.02 roots/shoot. Root length was greatest on IBA at 0.1–1.0 µM, while NAA strongly inhibited root elongation at all concentrations (all roots were shorter than 3 mm). Thick, strong roots were formed on 5–10 µM IBA or IAA. Roots obtained from the 10.0 µM IBA treatment connected directly to the basal stems, whereas roots induced with IBA at lower concentrations, or with other auxins, linked with stem via the callus (Figure 2.14).

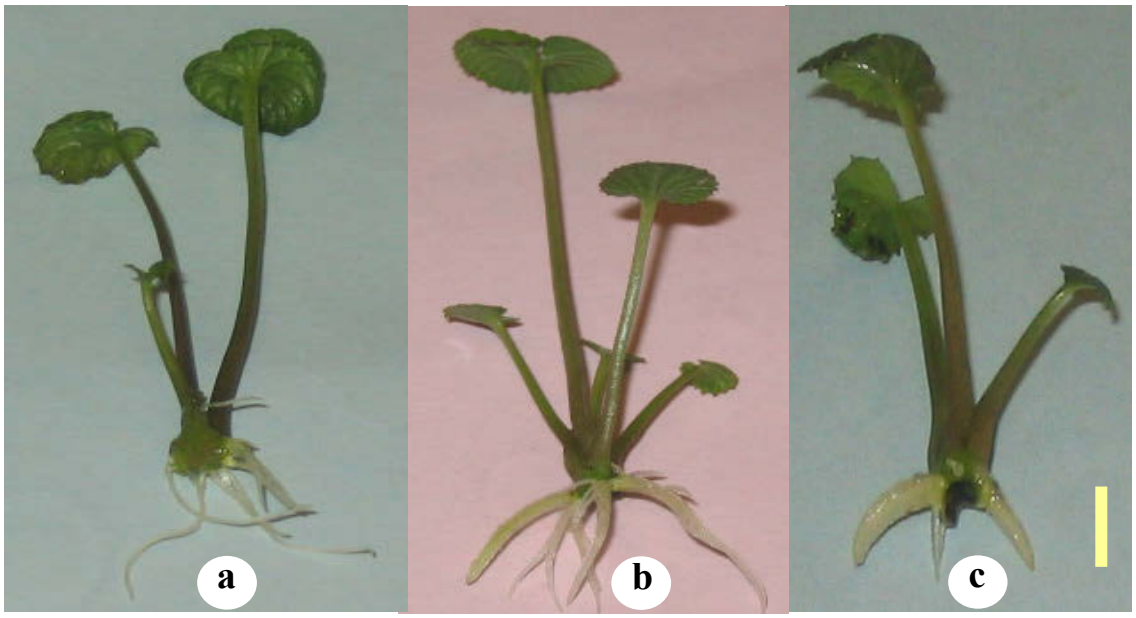


Figure 2.14: ***W. japonica*** **rooted on ½MS medium containing IBA at (a) 1.0 µM, (b) 5.0 µM and (c) 10.0 µM (bar = 1 cm), showing large-sized roots inducible in high IBA concentration, and different attachment of roots to shoots.**

Table 2.2: Effects of growth regulators on *in vitro* root formation of *W. japonica* after 3 weeks of culture.

Growth regulators (μM)	Frequency of shoots forming roots (%)[a]	Mean number of roots per shoot	Mean length of roots (mm)
Control (0.0)[b]	88.0±5.8bc[c]	1.9±0.02b	11.5±0.6b
NAA 0.1	72.0±6.2b	2.4±0.1b	2.0±0.3a
0.5	60.0±6.5b	2.3±0.03b	1.6±0.1a
1.0	60.0±6.5b	1.8±0.03b	1.5±0.1a
5.0	44.0±7.1a	1.2±0.04b	1.2±0.1a
10	40.0±7.0a	0.9±0.02a	1.2±0.1a
IBA 0.1	88.0±5.8bc	2.5±0.2b	20.2±0.9c
0.5	92.0±5.5c	3.5±0.3c	23.2±0.9c
1.0	92.0±5.5c	3.0±0.3b	24.2±1.2c
5.0	100±0.0c	2.4±0.2b	17.0±0.9b
10	96.0±4.0c	2.2±0.3b	9.1±0.7b
IAA 0.1	88.0±6.6bc	2.0±0.2b	14.7±0.9b
0.5	80.0±6.2bc	2.1±0.2b	13.7±0.8b
1.0	80.0±6.2bc	2.7±0.3b	13.0±0.8b
5.0	72.0±6.2b	2.1±0.3b	11.9±0.7b
10	72.0±6.2b	1.6±0.2b	11.0±0.6b

[a]Values are means ± standard errors of three independent experiments consisting of a total of 25 explants.
[b]Control = Half-strength MS medium free of growth regulators.
[c]Means within a column followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

Experiment 9: Effects of IBA, NAA, BA in gelled and liquid culture media on W. japonica root formation

The type of culture medium influenced root induction. It was found that liquid culture media promoted root formation in terms of quantity and length more than the solidified

medium did. In liquid culture media, the addition of different types and concentrations of auxin showed differences in root number, but not in root length. The observation on the frequency of root-forming shoots demonstrated no differences in both medium types (Figure 2.15 and Appendix 2.9).

The liquid culture medium containing 5 µM IBA was more favourable to root number rise than the gelled medium containing 5 µM IBA, but less favourable than the liquid medium supplemented with 5 µM BA and 2 µM NAA. The highest shoot length was gained in both liquid medium types in comparison with the gelled medium (p= 0.009). Roots induced in the liquid medium supplemented with 5 µM IBA reduced the growth, whereas roots induced in the liquid medium fortified with 5 µM BA plus 2 µM NAA exhibited volume growth with both primary and secondary root occurrence (Figure 2.16).

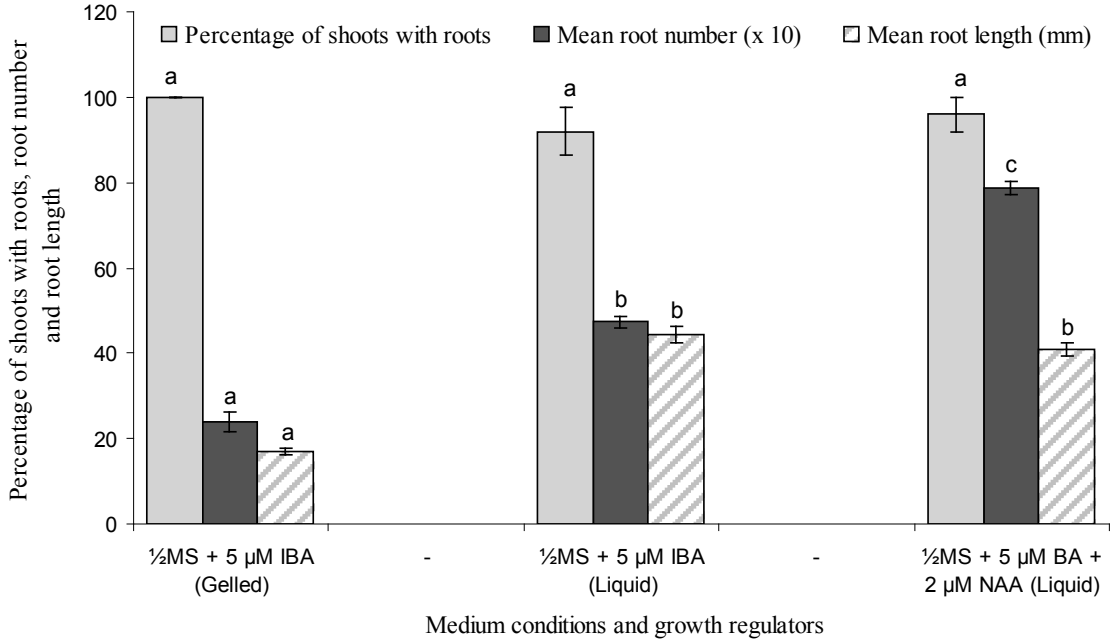


Figure 2.15: Effects of ½MS gelled and liquid culture media on the frequency of shoots forming roots, root number and root length of *W. japonica* after 3 weeks in culture. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

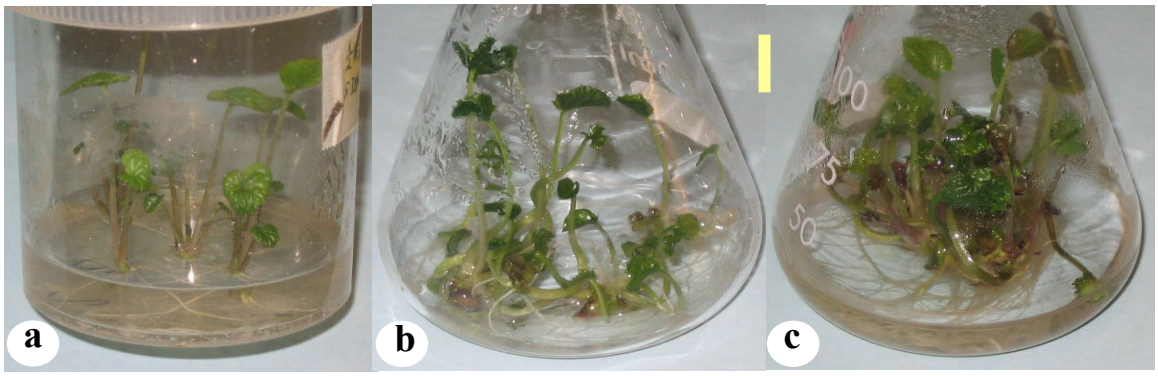


Figure 2.16: ***W. japonica*** **rooted on ½MS medium containing 5.0 µM IBA in (a) gelled and (b) liquid culture conditions, and (c) 5.0 µM BA plus 2.0 µM NAA in liquid culture conditions (bar = 1 cm).**

2.3.1.4 ***In vivo*** **acclimatisation of plantlets.** Experiments 10 and 11 are presented below.

Experiment 10. Effects of root origins on the acclimatisation of W. japonica plantlets in the greenhouse

The survival rate of plantlets after transplantation to the greenhouse was much influenced by the types of their roots. Roots derived from liquid and solidified media demonstrated plant establishment differences in the greenhouse with regard to the survival rate, shoot length and leaf quantity (Figure 2.17).

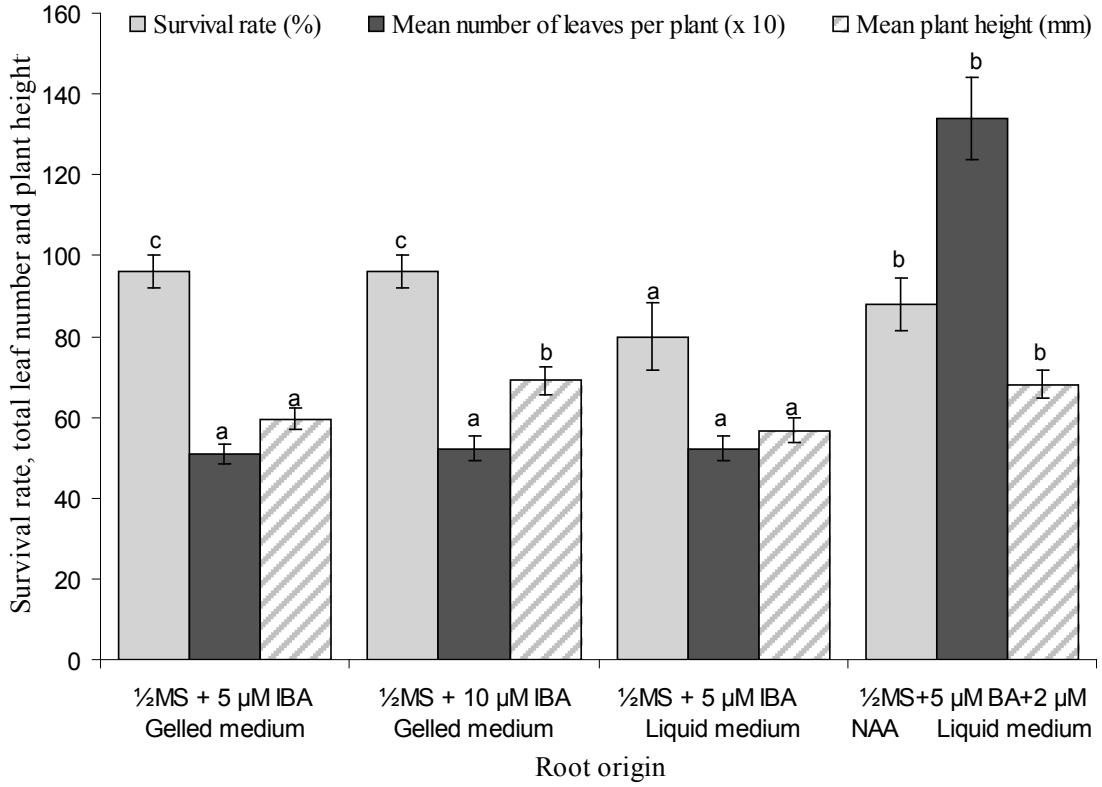


Figure 2.17: Effects of types of roots derived from ½MS gelled media with IBA at 5 µM or 10 µM, and from ½MS liquid media with 5 µM IBA or 5 µM BA plus 2 µM NAA on the survival rate, total leaf number, and height of *W. japonica* plantlets grown in the greenhouse for 1 month. The same letters in each bar type indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 25 replicates. Vertical lines denote standard errors.

A significant drop in the survival rate of plantlets established in the greenhouse was detectable when their roots were originated from liquid culture media, compared to gelled media (p= 0.001) (Appendix 2.10). Of the two liquid media, the liquid shake medium with 5 µM IBA resulted in the lowest survival rate (80±8%) of *in vitro* regenerated plantlets. There was no significant difference in the success rate of plantlets with roots originating from solidified medium containing IBA at 5–10 µM, all accounting for the highest rate of 96±4% survival.

Gelled medium-derived roots showed the similarity in the total leaf number of plants. The dramatic increase in leaf quantity was only detected in plants with roots induced in the liquid medium containing a combination of 5 µM BA and 2 µM NAA. From this medium, a total number of leaves per plantlet could increase to 13.4±1.0 (Appendix 2.10), resulting from the previous production of a cluster of *in vitro*-rooted shoots (Figure 2.18). This multiple plantlet was separated into individual plants after a four-month growth under greenhouse conditions (data not shown). Roots derived from

this medium also gave rise to taller plants, compared to those derived from gelled or liquid media supplemented with 5 µM IBA, but showed no difference to those derived from gelled medium containing 10 µM IBA.

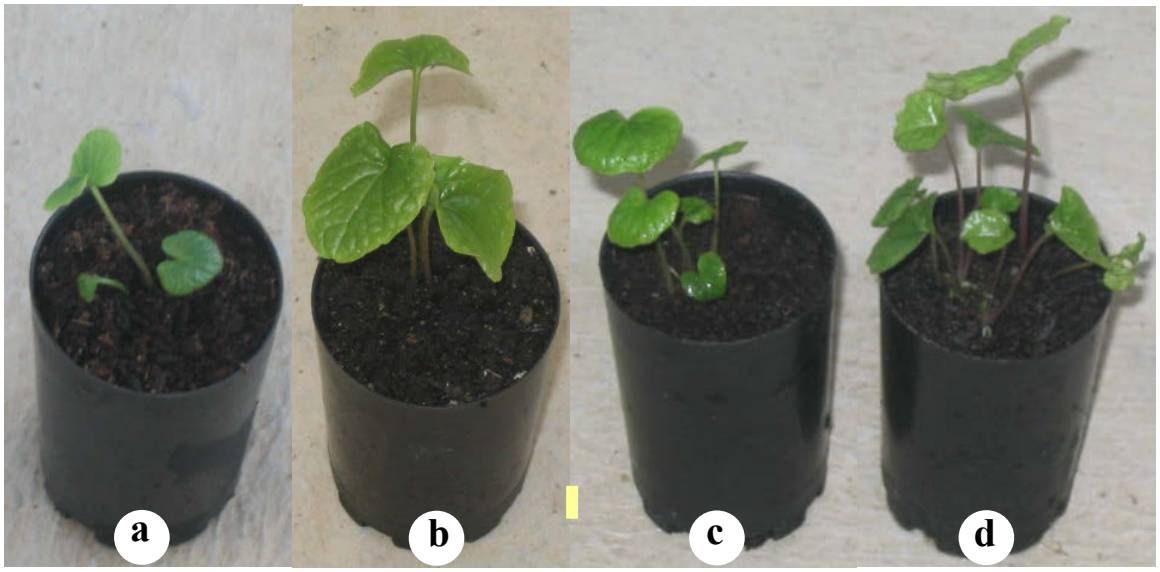


Figure 2.18: Growth of rooted explants for 4 weeks in greenhouse conditions, with initial roots derived from 4 origins: ½MS gelled media with IBA at (a) 5 µM or (b) 10 µM, and ½MS liquid media with (c) 5 µM IBA or (d) 5 µM BA plus 2 µM NAA (bar = 1 cm).

In comparing the frequency of survival and the growth of individual plantlets in the greenhouse, plants with roots originating from the solidified medium supplemented with 10 µM IBA were the best. This type of plant was chosen for the greenhouse establishment of *in vitro* regenerated plantlets in the subsequent trials.

Experiment 11. Effects of substrates on the acclimatisation of W. japonica plantlets in the greenhouse

Micropropagated plants with vigorous roots obtained from the ½MS medium supplemented with 10 µM IBA were potted on the selected mixture of perlite: vermiculite: peatmoss (1:1:1) for one month's growth in plastic trays. Plants grew very well in the greenhouse with a high rate of survival, fluctuating from 96% to 100%. After five weeks of acclimatisation, uniform new leaves were formed and the leaf area commenced a relatively rapid expansion (Figure 2.19).

Figure 2.19: Acclimatised nursery *W. japonica* plants at (a) one week of age in a humid environment, and (b) two months of age in the greenhouse, showing uniform growth and phenotypic similarity to parental stock (bars = 5 cm).

Prior to the selection of mixture, an experiment was conducted on substrate suitability. The results showed that the acclimatisation of plantlets in the greenhouse affected the frequency of survival slightly, and the plant height and leaf quantity moderately when popular substrates were utilised (Table 2.3).

Vermiculite alone or a mixture of vermiculite, peat moss and perlite at a ratio of 1:1:1 significantly increased the survival rate to 96%, compared to other substrates (p= 0.003). The average number of leaves was promoted in all substrate mixtures containing vermiculite. The mixtures with vermiculite were also favourable to plant height growth, the combination of vermiculite, peat moss and perlite being the best (p= 0.000) for plant height (59.57±2.73 mm). Inhibitory effects on growth indexes (survival rate, total leaf and plant height) were found only with peat moss, in which the pH value was lower than the requirement for this plant species.

Taking into account all growth parameters and health conditions (Table 2.3 and Figure 2.19), a mixture comprising vermiculite, peatmoss and perlite (1:1:1) was used for the acclimatisation of any *in vitro* wasabi plantlets in later assays.

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.

Substrates & their combinations	Survival rate (%)[a]	Average total number of leaves per plant	Average plant height (mm)
Vermiculite	96±4b[b]	4.3±0.2bc	48.5±1.7bc
Perlite	88±7a	4.2±0.2b	46.1±2.2b
Peat moss	88±7a	3.4±0.2a	42.8±1.0a
Vermiculite: perlite (1:1)	92±6a	5.1±0.2d	54.4±2.6c
Vermiculite: peatmoss (1:1)	92±6a	4.9±0.3cd	51.2±2.7c
Perlite: peatmoss (1:1)	88±6a	4.5±0.2bc	46.3±1.2a
Vermiculite: perlite: peatmoss (1:1:1)	96±4b	5.1±0.3d	59.6±2.7d
P value	*0.003*	*0.001*	*0.000*

[a]Values are means ± standard errors of three independent experiments consisting of a total of 25 explants.
[b]Means within a column followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

2.3.2 Callus culture

2.3.2.1 Callus initiation

The effect of 2,4-D concentrations and explant types (Figure 2.2) including excised leaves (a), proximal portion (b), medial portion (c), distal portion (d) and 1–2 mm excised portion (e) of petioles on the frequency of callus formation and callus quality after six weeks in culture are shown in Table 2.4 below.

The induction of calli, from cut ends of leaves and petiole segments, started to occur in the second week in the culture on the surface of an MS medium containing 2,4-D at concentrations ranging from 0.1 to 5 µM under light conditions. Neither the leaf cut ends nor the petiole segments could initiate calli on the MS medium free of 2,4-D

(control). Necrosis of petiole segments and swelling of leaves were observed on the control (Table 2.4 and Figure 2.20).

The frequency of callusing showed a wide range of variation among treatments with differences in levels of 2,4-D and explant types (Table 2.4 and Figure 2.20). Petiole segments (d) and (e) displayed the highest callusing rates overall (92–96%), with 1 µM and 0.5 µM 2,4-D supplements, respectively, to the medium, compared with segments (b) and (c), and excised leaves. At 0.1 µM and 5 µM of 2,4-D, percentages of callusing were reduced and almost inhibited in leaf-cut surfaces (20–28%) (p= 0.001).

Table 2.4: Callus induction after 6 weeks in culture under light conditions as affected by 2,4-D concentrations and explant sources.

MS media plus 2,4-D (µM)	Callusing (%)					Callus quality				
	Leaf[a] (a)	Petiole (b)	Petiole (c)	Petiole (d)	Petiole (e)	Leaf (a)	Petiole (b)	Petiole (c)	Petiole (d)	Petiole (e)
0.0	0.0a[b]	0.0a	0.0a	0.0a	0.0a	-	-	-	-	-
0.1	28ab	72bc	72bc	80cd	84cd	+	+	++	++	++
0.5	32b	76bc	80cd	88cd	92d	+	+	++	+++	+++
1.0	36b	84cd	88cd	92d	96d	+	++	++	+++	+++
5.0	20a	52b	52b	56b	60bc	+	+	+	+	++

[a]Values are means of three independent experiments consisting of a total of 25 explants.
[b]Means followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.
Note: +++: Good quality; ++: Average quality; + : Poor quality; and -: Necrosis

In the presence of light, the calli showed a deep green colour with different levels of friability. No friable callus was observed in leaves and in petiole segments with 5 µM 2,4-D supplement. Calli were friable on all petiole segments with 2,4-D at 0.5–1 µM, and best on petiole segments (d) and (e). 2,4-D at 0.1 µM and 5 µM failed to promote callus friability in all the explants.

Table 2.5: Callus induction after 6 weeks in culture in dark conditions as affected by 2,4-D concentrations and explant sources.

MS media plus 2,4-D (μM)	Callusing (%)					Callus quality (friability level)				
	Leaf[a] (a)	Petiole (b)	Petiole (c)	Petiole (d)	Petiole (e)	Leaf (a)	Petiole (b)	Petiole (c)	Petiole (d)	Petiole (e)
0.0	0.0a[b]	0.0a	0.0a	0.0a	0.0a	-	-	-	-	-
0.1	48b	80cd	84cd	96de	88cd	+	+	++	+++	+++
0.5	76bc	88cd	96de	100e	100e	++	++	+++	+++	+++
1.0	56b	84cd	88cd	88cd	84cd	+	+	+	++	+++
5.0	20a	52b	56b	60b	60b	+	+	+	+	++

[a]Values are means of three independent experiments consisting of a total of 25 explants.
[b]Means followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.
Note: +++: Good quality; ++: Average quality; + : Poor quality; and -: Necrosis

The explants swelling on the 2,4-D-containing medium incubated in total darkness produced more abundant and friable calli than those cultured in the light, after six weeks in culture. Calli induced in the dark displayed a yellow-white colour in petiole segments and light yellow-greenish in leaves (Figure 2.20 and Table 2.5).

All (d) and (e) petiole segments when cultured on the 0.5 μM 2,4-D-containing medium in darkness formed calli (100%). The petiole segments displayed higher percentages of callusing than the leaves with 2,4-D supplements at 0.1–1 μM. A high level of 2,4-D (5 μM) reduced the survival frequency to 52–60% in the petiole segments, which showed no differences from the percentages of callusing in the leaves on the medium with 0.1–1 μM 2,4-D. At 5 μM 2,4-D, leaves showed minimum callusing frequency (20%). No callus induction occurred on the control from all the explants, with only observable swelling of leaves and necrosis of petioles.

Calli emerged precociously on both types of explants after the first week in culture in darkness. The growth of calli was rapid in all petiole segments, particularly in segment (e) (Figure 2.20). In leaf-cut ends, calli grew more slowly but lasted longer (until the

ninth culture week). Callus formation was best on the medium with 0.5 µM 2,4-D, where the highest friability could be observed in segments (c), (d) and (e) (Table 2.5). Callus quality was worst with 5 µM 2,4-D in all explants, and with 0.1 and 1 µM 2,4-D in segments (a) and (b). Segment (e) was, however, best for callus friability and growth with 0.1–1.0 µM 2,4-D supplements (Table 2.5).

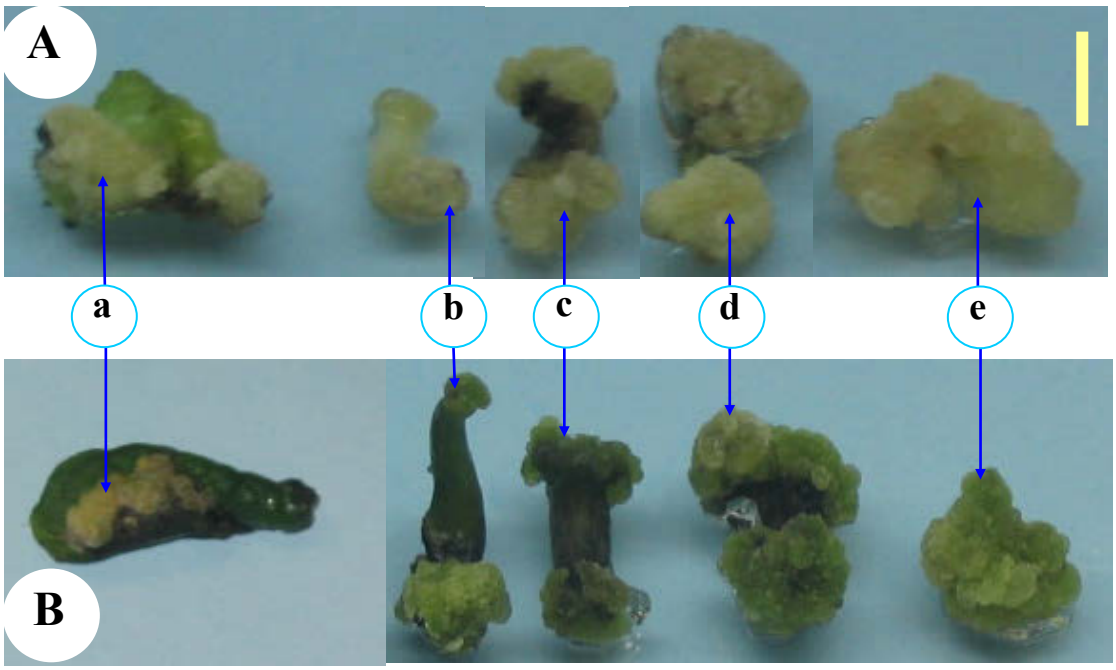


Figure 2.20: Calli induced on the cut surfaces of explants ((a) half-leaf, (b) leaf-close petiole segment, (c) middle petiole segment, (d) base-close petiole segment, and (e) 1–2 mm petiole segments)) on MS medium containing 0.5 µM 2,4-D after 6 weeks in culture under (A) dark and (B) light conditions (bar = 2 mm).

2.3.2.2 Callus proliferation

Callus response was different when petiole-derived calli were cultured on the MS medium supplemented with various PGR combinations at different ratios (Table 2.6 and Figure 2.21). The petiole segments failed to yield further calli on the hormone-free medium for another six weeks in the light (see control). IAA and IBA, when combined with 5 µM BA, induced explants to form roots (Table 2.6h, i and Figure 2.21). A similar response was found on the combination between 1 µM NAA and 10 µM kinetin (Table 2.6j). Calli incubated on medium with 2,4-D alone or in combination with other cytokinins formed callus (Table 2.6b–f). NAA in combination with 5 µM BA induced a yield of further calli with no roots (Table 2.6g). Root growth was much faster on media with supplements of a combination of NAA and kinetin than other PGR combinations, with long primary and secondary roots.

Biomass increased in all types of callus explants from corresponding initial calli, except for the control. The lowest increase of callus biomass was observed in the medium with 0.5 μM 2,4-D and 0.5 μM zeatin, followed by the medium containing 0.5 μM 2,4 D alone or in combination with 0.5 μM BA (p= 0.041) (Table 2.6). In combinations between 0.5 μM 2,4-D and other four cytokinins, the medium with the supplement of 0.5 μM 2,4-D, plus either 0.5 μM TDZ or 0.5 μM kinetin, gave rise to the highest biomass weight (Table 2.6d, e). In media with combinations between 0.5 μM BA and the other three auxins (IAA, IBA and NAA), callus weight was non-significantly different (p= 0.061) (Table 2.6g–i). The medium fortified with 1 μM NAA plus 10 μM kinetin increased biomass weight more slowly than that found in the medium with 5 μM BA plus 2 μM NAA or in the medium with 0.5 μM 2,4-D plus 0.5 μM kinetin (Table 2.6), and reduced to below its initial callus weight value when roots developed thoroughly (data not shown).

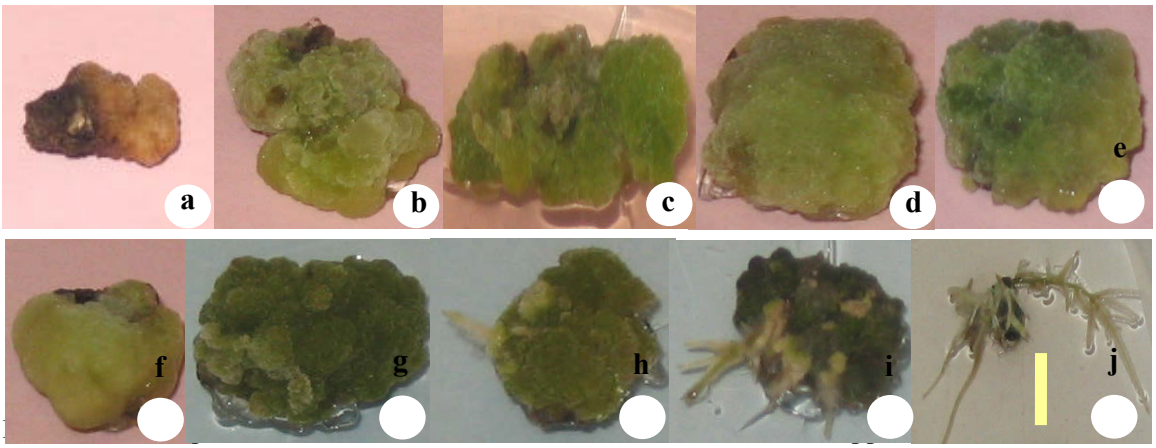
The friable and rapid-growing callus was achieved on the medium with 0.5 μM 2,4-D plus 0.5 μM BA or 0.5 μM TDZ after six weeks in culture (Table 2.6 and Figure 2.21c, e). This similarity was also found on the medium supplemented with 0.5 μM BA plus 2 μM NAA (Figure 2.21g). In media with rooting responses, dark green calli with low friability level were formed (Figure h–j). Compact and little callus aggregates of slow growth occurred on the medium containing 0.5 μM 2,4-D plus 0.5 μM zeatin (Figure 2.21f). Larger sized compact callus aggregates of relatively rapid growth were achieved on the medium containing 0.5 μM 2,4-D alone or in combination with 0.5 μM kinetin (Figures 2.21b, d). The callus turned browned, darkened and subsequently became moribund on the medium with no growth regulators (Figure 2.21a).

Most of the live calli subjected to the dark conditions for further proliferation were viable and homogenously friable (data not shown), however, fast growing callus aggregates were formed on the medium containing 0.5 μM 2,4-D plus 0.5 μM kinetin (Figure 2.21d). These callus aggregates were compared to those obtained in the light conditions for AITC accumulation (see Chapter 4).

Table 2.6: Callus growth after 6 weeks in culture under light conditions as affected by PGR combinations.

PGR Components	Callus FW (mg)[a]	Callus quality	Type of response
a) No regulators (control)[b]	-	-	None
b) 0.5 µM 2,4-D	172 ± 9b[c]	++	Callusing
c) 0.5 µM 2,4-D + 0.5 µM BA	210 ± 9bc	+++	Callusing
d) 0.5 µM 2,4-D + 0.5 µM kinetin	290 ± 10d	++	Callusing
e) 0.5 µM 2,4-D + 0.5 µM TDZ	318 ± 11de	+++	Callusing
f) 0.5 µM 2,4-D + 0.5 µM zeatin	120 ± 8a	+	Callusing
g) 5 µM BA + 2 µM NAA	337 ± 12e	+++	Callusing
h) 5 µM BA + 2 µM IBA	312 ± 10de	+	Rooting
i) 5 µM BA + 2 µM IAA	280 ± 10d	+	Rooting
j) 1 µM NAA + 10 µM kinetin	243 ± 10cd	+	Rooting

[a]Values are means ± standard errors of three independent experiments consisting of a total of 25 explants.
[b]Control = MS medium free of growth regulators.
[c]Means followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.
Note: +++: Good quality; ++: Average quality; + : Poor quality; and -: Necrosis



PGRs ((a) no regulator, (b) 0.5 µM 2,4-D, (c) 0.5 µM 2,4-D + 0.5 µM BA, (d) 0.5 µM 2,4-D + 0.5 µM kinetin, (e) 0.5 µM 2,4-D + 0.5 µM TDZ, (f) 0.5 µM 2,4-D + 0.5 µM zeatin, (g) 5 µM BA + 2 µM NAA, (h) 5 µM BA + 2 µM IBA, (i) 5 µM BA + 2 µM IAA, and (j) 1 µM NAA + 10 µM kinetin)) (bar = 5 mm).

2.4 Discussion

2.4.1 Clonal propagation

2.4.1.1 Choice of culture basal media. The choice of various basal media was made for optimal shoot tip establishment and subsequent proliferation. The different media affected the height and weight, but not the frequency of wasabi explants in the initiation stage. All the seven media used in the initiation culture stage are free of PGRs, hence, the influence of explant tolerance accounts for the medium compositions. The significant reduction in explant height and weight, which was found only in the two media WPM and KC, may be due to the use of calcium nitrate salts at high levels (556 mg L^{-1} and 1000 mg L^{-1}, respectively) in replacement with potassium nitrate salts (see Appendix 2.2). Other media including ½MS, MS, B5, N6 and N&N exhibited a more tolerant growth of wasabi explants. In plant tissue culture, KC, N&N and N6 are media commonly used for orchid, anther and cereal cultures, respectively (Gamborg and Phillips, 1996). MS and ½MS basal media are most widely used in tissue culture for the establishment of numerous explants such as *Cucumis hystrix* (Compton *et al.*, 2001) and *Lavandula viridis* (Dias *et al.*, 2002), while WPM and Gamborg's B5 are typical for initiating woody plant tissues as observed in *Arbutus unedo* (Mereti *et al.*, 2002). Wasabi shoot tips proved to be more widely tolerant to the cultural medium compositions because all of these basal media favoured a maximum increase in the survival rate and morphological appearance of explants.

2.4.1.2 Choice of PGRs for shoot multiplication. The use of plant growth regulators in tissue culture media of *Wasabia japonica* is required for rapid multiplication and rooting. The use of various cytokinins had different influences on the shoot multiplication rate and health of wasabi explants. Although the effectiveness of BA, TDZ and zeatin has previously been compared in the clonal propagation of *W. japonica* (Hosokawa *et al.*, 1999), the importance of kinetin in the stimulation of shoot proliferation in many plant species cannot be ignored (Paek and Hahn, 2000; Borthakur and Singh, 2002; Soniya and Das, 2002; Stajner *et al.*, 2002; Arikat *et al.*, 2004). In this study, of the four cytokinins tested, both 5.0 μM BA and 10.0 μM kinetin proved to be the best for shoot multiplication (Table 2.1). However, comparing both shoot number and explant weight, 5.0 μM BA was the most effective for shoot proliferation of *W. japonica*. Hosokawa and colleagues (1999) also found for a cultivar of *W. japonica* that 5.0 μM BA maximised the number of both shoot tips and petioles, confirming BA as an optimal cytokinin for shoot multiplication of *W. japonica*.

The combinations between auxins and cytokinins at shoot multiplication stage affect shoot organogenesis in various plants under a tissue culture system. Shoot buds derived from leaf midribs of *Beta palonga* produced more shoots on the MS medium supplemented with an association of 1 mg L^{-1} IAA and 1 mg L^{-1} BA than with either 1 mg L^{-1} IAA or 1 mg L^{-1} BA alone (Mitra and Mukherjee, 2001). The effects of BA and NAA on the frequency of shoot proliferation were also found on lateral buds of *Achillea filipendulina* cv. 'Parker' incubated on an MS medium supplemented with a combination of 1 mg L^{-1} IAA and 2 mg L^{-1} BA, indicating a higher frequency than with 1 mg L^{-1} IAA plus 4 mg L^{-1} BA (Evenor and Reuveni, 2004). Our study on the interactions between BA and NAA showed no different responses on shoot number, shoot height and explant weight, whereas differences were observed with combinations between BA and other PGRs (Figures 2.12 and 2.13). A study on the micropropagation of *Ceropegia candelabrum* by Beena and colleagues (2003) indicated that combinations of 8.87 μM BA with 2.46 μM IBA or 2.85 μM IAA enhanced shoot multiplication rate (7.8 or 4.6 shoots per explant, respectively), and was reduced with a 2.69 μM NAA addition (3.1 shoots per explant), compared to 8.87 μM BA alone (3.81 shoots per explant) when axillary buds were cultured on an MS medium. However, the percentage response was not different between BA alone and BA in combinations with IBA and

IAA (Beena *et al.*, 2003). *In vitro* propagation of *W. japonica* showed a higher frequency of shoot proliferation rates and explant weight in the medium supplemented with 5 μM BA plus 1 μM IAA, and no different responses were found in the MS medium with 5 μM BA plus 1–2 μM IBA. The enhancement of shoot proliferation rate is detected on the association between different cytokinins themselves (Grigoriadou *et al.*, 2002; Babu *et al.*, 2003; Herath *et al.*, 2004). BA at 1 μM in combinations with zeatin at 5–20 μM promoted the shoot proliferation rate and microshoot number of Greek olive explants placed on WPM medium to 6.8 and 1.85, respectively, compared to the control of 1 μM BA (4.3 and 1.03, respectively) (Grigoriadou *et al.*, 2002). Results obtained by Herath and co-workers (2004) in establishing shoot proliferation of *Hibiscus cannabinus* cv. 'Tainung 2' indicated that a negative effect on microshoot numbers was observed on an MS medium with 8.8 μM BA alone, while a positive effect on shoot regeneration percentage was found with 8.8 μM BA plus 9.2 μM kinetin, and vice versa. Babu and colleagues (2003), in a study with nodal segments of camphor tree (*Cinnamonum camphora*) cultured on WPM medium, showed that BA and kinetin in combinations positively affected shoot proliferation efficiency, compared to either kinetin or BA alone, or kinetin in combinations with zeatin. Microshoot numbers and shoot elongation of *W. japonica* were, however, enhanced with 5 μM BA plus 2–5 μM zeatin, whereas shoot elongation and explant weight were reduced with 5 μM BA plus 2 μM kinetin. The efficiency of shoot regeneration (%) of wasabi was highest in all the treatments with combinations of PGRs (data not shown). The mechanism of auxin–cytokinin integration (auxin to kinetin ratio) to obtain optimal shoot regeneration in wasabi micropropagation requires further investigations, however, it is obviously true in many plants that a higher level of cytokinin than auxin induced further microshoot regeneration responses, while an equal level of both facilitated callus induction.

2.4.1.3 Choice of PGRs for root formation. The induction of roots on a hormone-free medium has been reported for various plants, such as *Arbutus unedo* (Mereti *et al.*, 2002), *Isoplexis canariensis* and *I. chalcantha* (Bermúdez *et al.*, 2002). Although microshoots of *W. japonica* could also be rooted on a hormone-free medium (Hosokawa *et al.*, 1999), the use of three auxins, namely IBA, IAA and NAA, in our study resulted in refining an optimal auxin and its concentrations for root formation. Media supplemented with auxins at high concentrations caused *W. japonica* root formation

earlier than those without auxins or with auxins at low concentrations. IBA and IAA initiated greater production of roots for *W. japonica* than NAA. For *in vitro* propagation of *Arbutus unedo*, 10.0 μM IBA or IAA showed the efficiency for root induction; however, successful acclimatisation was observed with IAA due to the formation of branched roots (Mereti *et al.*, 2002). We found that vigorous root growth of *W. japonica* was achieved only on a high concentration of IBA (10.0 μM or greater), where roots were short, thick and directly attached to the stems without intermediate calli (Figure 2.14). An observation was made that these plants appeared to grow faster than those from other treatments. Utilising IBA at a high level (20.7 μM) induced optimal roots for *Olea europaea* (Santos *et al.*, 2003). Wasabi roots induced in a medium free of growth regulators, or with IAA and IBA at low concentrations were long and thin, and the callus forming between roots and stems might inhibit nutrient absorption (Figure 2.14). Rai and Misra (2005) also reported that IBA ranging from 0.57 μM to 5.71 μM induced long, thin roots of *Carissa carandas in vitro* and subsequently limited the growth of the plantlets in the greenhouse. When comparing three auxins (IAA, IBA and NAA), IBA was most effective for root formation and acclimatisation of *W. japonica* in the greenhouse.

Culture conditions also affected the root formation capacity of micropropagated wasabi shoots. A liquid medium stimulated the increase in root number and root length more rapidly than did a gelled medium. This was apparently observed when an equal level of 5 μM IBA was added to both the media. The greater quantity and faster elongation of roots in a liquid medium may have resulted from aeration in an aqueous solution under a shaking condition (Hvoslef-Eide and Preil, 2005). However, the frequency of shoots with roots was significantly lower in the liquid medium than in the gelled medium. Moreover, roots of plantlets cultured in liquid condition were thinner and weaker, which could reduce the survival rate of plantlets in the *in vivo* phase. Hence, they are undesirable in a tissue culture system.

2.4.1.4 Choice of culture conditions (gelled vs. liquid media) for efficient shoot proliferation. Liquid shake cultures have proven extremely successful in plant tissue culture (Tewary and Oka, 1999; Han *et al.*, 2004; Kadota and Niimi, 2004; Piatczak *et al.*, 2005). Thus, the purpose of establishing a liquid culture system for *W. japonica* was to save labour and expense by eliminating or reducing gelling agents in large-scale *in*

vitro propagation, which would consequently reduce the cost of micropropagated plantlets. Furthermore, the selection of different types of liquid media for shoot development aimed at optimising the proliferation of shoots in culture. The liquid medium displayed greater multiplication rates and fresh weight increases for *W. japonica* shoots than did the gelled medium. Shoot proliferation was also improved by almost three times in the liquid medium, compared to the gelled medium in *Dioscorea japonica* (Kadota and Niimi, 2004) and *Centaurium erythraea* (Piatczak *et al.*, 2005). The positive effects of liquid culture are possibly because the shoots are totally submerged in the liquid medium, facilitating the uptake of nutrients and PGRs over the whole shoot surface, otherwise limited in gelled media by the gelcarin matrix. In the case of *W. japonica* shoots, this was perhaps further promoted by the better supply of oxygen and sucrose under the rotary culture (Hvoslef-Eide and Preil, 2005). All shoots initiated in liquid culture media generated uniform and vigorous shoots when subcultured onto gelled media for further shoot formation, which may have been due to the extra amount of sucrose accumulated in liquid medium-cultured shoots. Shoots grown in all liquid MS media fortified with 5.0 µM BA continued to proliferate from four weeks to six weeks, but appeared to multiply more slowly in full-strength MS medium than in half-strength MS medium, with no rooting observed at week 6. It was found that shoots of *Morus indica* grown in full-strength MS liquid medium supplemented with 0.5–2.0 mg L^{-1} N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) proliferated within four weeks, and induced rooting when the culture period was lengthened from week 4 to week 6 (Tewary and Oka, 1999). Shoot proliferation and development were, hence, affected by the level of MS nutrient salts and activity of cytokinins. However, the fresh weight of *W. japonica* explants failed to show any slow increases on the full-strength MS liquid medium within six weeks, which may be partially accounted for by the explants with excess water uptake. Culture in a liquid medium has been known to cause a hyperhydricity phenomenon in some species (Paek *et al.*, 2001). However, hyperhydricity symptoms were little observed among *W. japonica* shoots. Those hyperhydric individuals transferred onto a gelled medium demonstrated a reduction in hyperhydricity symptoms (data not shown). In field cultivation, wasabi grown under water, particularly in clean and oxygenated water, supplied a higher concentration of essential oils than wasabi grown in soil (Chadwick, 1990; Depree *et al.*, 1999). From this viewpoint, liquid cultures might improve the

quality of wasabi in comparison to a gelled medium. However, confirmation of this hypothesis depends on future research conducted on the chemical component analysis of wasabi. Liquid culture could at least provide wasabi material for other applications. We conclude that regeneration of wasabi shoots in liquid culture was more useful than the gelcarin-based culture method.

The regeneration capacity of wasabi shoot tip explants by the two methods and the successful transfer of rooted plantlets to soil after acclimatising to greenhouse conditions reveal that *in vitro* propagation should be a useful protocol in the conservation and mass propagation of this medicinal species of high commercial value.

2.4.1.5 Choice of root origins and substrates for acclimatisation of plantlets. Micropropagated plants of *W. japonica* transferred to the greenhouse directly from culture flasks behaved differently in their survival and growth, depending on the types of *in vitro* root. Plantlets with more vigorous roots derived from the gelled medium containing 10 μM IBA proved to be greater in height, but neither in survival nor total leaf number, as compared to those with 5 μM IBA. Plantlets rooted on liquid culture media showed a reduction in their survival, while plant height and leaf number were affected by the addition of PGRs. The liquid medium with 5 μM BA plus 2 μM NAA proved to be most effective for plant growth *in vivo*, as it increased both height and leaf number of plantlets significantly, compared to either the gelled medium or liquid medium with 5 μM IBA (Figure 2.17). The significant increase in leaf number during the acclimatisation phase was accounted for by the supplement of BA to the medium, which had stimulated an increase in the shoot number of plantlets during the *in vitro* phase (Figure 3.18d).

After *in vivo* transfer of the 10 μM IBA containing gelled medium-derived plantlets, substrates had slight effects on the acclimatisation of wasabi plants. Mixtures in the presence of vermiculite appeared to be more successfully acclimatised than others free of vermiculite. The mixture consisting of vermiculite, perlite and peat-moss (1:1:1 v/v/v) proved to be the best substrate for the survival, leaf development and plant height (Table 2.3). Unlike *W. japonica*, other plants were successfully hardened to survive *in vivo* conditions in a mixture of peat-moss and perlite (Pruski *et al.*, 2005), or in a peat: sand mixture (Jang *et al.*, 2003; Martinussen *et al.*, 2004). Wasabi plantlets were also well rooted in a sterilised sand substrate maintained in a mist chamber at 85% humidity

(data not shown). Grown in substrates as vermiculite, perlite and peatmoss individually or in combinations, wasabi plantlets showed a uniform growth without any morphological defects, exhibited normal development and were subsequently well established into the soil. However, a drawback encountered in the UTS greenhouse was the relatively high temperature (20–25^{o}C) during the day time which limited the further development of wasabi plants, because the optimum growth temperature for this species ranges between 8^{o}C and 18^{o}C (Chadwick, 1990). Hence, for further studies on wasabi in the greenhouse, temperature and shading need to be fully controlled.

2.4.1.6 Problems in tissue culture of W. japonica. Physiological and genetical malformation problems such as recalcitrance, hyperhydricity and somaclonal variation are normally associated with the tissue culture of plants *in vitro* (Cassells and Curry, 2001). The hyperhydricity in *in vitro* culture of wasabi shoots was slightly influenced by cytokinin types and their concentrations. BA induced less hyperhydric shoots of *W. japonica* on the MS medium at 10 μM, and commenced to form more hyperhydric explants at 50 μM. TDZ at 50 μM behaved similarly to BA at the same level. Kinetin at 50 μM triggered hyperhydricity after an incubation period greater than four weeks. However, zeatin induced no hyperhydric shoots at all concentrations. In the cultured explants of *Pyrus pyrifolia*, however, TDZ was found to induce greater hyperhydricity than BA and kinetin (Kadota and Niimi, 2003). Retarded and abnormal shoots of wasabi were also observed with BA, TDZ and kinetin, but not with zeatin, at high concentrations (Figure 2.5). High levels of some cytokinins were also found to increase the occurrence of hyperhydricity in explants of *Ceratopetalum gummiferum* (Armstrong and Johnson, 2001) and *Eustoma grandiflorum* (Paek and Hahn, 2000).

Hyperhydricity is a metabolic activity disorder caused by a combination of chemical and physical factors resulting from the *in vitro* environment (Pâques and Boxus, 1987). The occurrence of this abnormal developmental phenomenon is primarily responsible for an excess of cytokinins triggering rapid cell division in shoot tips (Kataeva *et al.*, 1991), high relative humidity (Kataeva *et al.*, 1991; Gribble, 1999), dark conditions with low temperature (Williams and Taji, 1987) and the formation of ice by the storage of *in vitro* explants at low temperature (Debergh *et al.*, 1992). Callus-derived shoot clusters of *Lavandula vera* mostly exhibited hyperhydricity when grown in the close culture system (Tsuro *et al.*, 2000). Non-hyperhydric plantlets of *Gypsophila paniculata*

cultured in a liquid medium displayed hyperhydric growth by high water availability restricting transpiration (Gribble, 1999). Wasabi explants grown in the liquid culture media, however, still exhibited less hyperhydricity. This is partly due to the use of relatively low concentrations of cytokinins. Hyperhydric microplants displayed glassy appearance, translucent and brittle leaves and hypertrophic stems (Williams and Taji, 1991; Armstrong and Johnson, 2001). They faced difficulties in long-term shoot production and *in vivo* acclimatisation (Pâques and Boxus, 1987; Barlass and Hutchison, 1996; Paek and Hahn, 2000), and are thus undesirable in plant micropropagation. In order to reduce hyperhydricity in plant tissue culture, various satisfactory solutions have been suggested, such as the addition of appropriate gelling agents (Williams and Taji, 1991; Curtis and Shetty, 1996; Armstrong and Johnson, 2001), the inoculation of polysaccharide-producing soil bacteria (Ueno and Shetty, 1998), the supplementing of polysaccharide extracts to the culture media (Shetty *et al.*, 1996; Whitehouse *et al.*, 2002), and the use of silver nitrate (Mayor *et al.*, 2003). It is recommended that an interaction between many factors influencing hyperhydricity should be simultaneously examined to avoid this undesirable phenomenon.

Pathogens were relatively serious problems encountered in *in vitro* cultures of wasabi. At the initiation stage, wasabi cultures were contaminated with bacteria at a high rate. In a study using shoot apices for propagation, Hosoki *et al.* (1986) showed that the loss rate of shoot accounted for 30 to 40% due to contaminants surviving inside wasabi stems' wrinkles. In our research dealing with dormant buds, initial explants exhibited a greater than 40% loss due to bacterial and fungal contaminations. After successive subculture cycles for two years, wasabi leaves became yellow, with some small black dots. The proximal portion of petiole was withered, while the medial and distal portions remained healthy. The yellowing started from the two outermost leaves and spread to the inner ones prior to the outermost leaves becoming dry. By removing the leaves and petioles, leaving only shoot bases on the culture medium, the survival rate of wasabi explants was not reduced. Microorganisms associating with wasabi explants appeared to be latent bacteria present in dormant buds, which may derive from the establishment stage under an incomplete aseptic culture, and then persist in the liber tissues. The association of microorganisms with wasabi shoots could have caused the loss of sample materials and the risk of false results. Re-initiation of wasabi cultures after several cycles of subculture is, thus, required to gain new healthy samples. Screening

vegetatively micropropagated plants for contaminating microorganisms by culture indexing on fungal and bacteriological media has been a proposed strategy (Raju and Trolinger, 1986; Cassells, 1991; Kane, 2000). Bacterial screening of tissue cultures would be a necessary tool for eliminating a systemic pathogen from wasabi shoots.

Toxicity is another drawback occurring in wasabi culture. High concentrations (50 μM and above) of BA, TDZ and kinetin caused toxicity to the explants which are, hence, undesirable in micropropagation. Similarly high levels of zeatin, however, did not induce toxicity. Conversely, 0.5 μM zeatin exhibited as being too low to have any significant effects on the multiplication rate and height of shoots. Low levels of all the cytokinins tested failed to induce any toxicity symptoms.

Nevertheless, wasabi calli were initially produced with very low levels of cytokinins. The formation of calli was progressing at the shoot bases with individual cytokinins, but appeared to be reduced with combined concentrations between cytokinins and auxins. Our observations showed that concentrations of BA initiated callus growth faster than concentrations of kinetin. Callus growth was the least evident in the medium with zeatin. While under the influence of TDZ, calli were the most profuse (Figure 2.4). It was assumed that the stronger cytokinin activity probably caused the callus induction in more abundance. Callus development increased explant weight, but did not affect the shoot growth of wasabi. The only increase in explant weight with concentrations of 5 μM BA and 1–2 μM IAA was also due to the production of a small amount of calli. However, calli did not develop in liquid shake cultures in different media (Figure 2.7). The significant increase in explant weights of liquid cultures in MS and ½MS media compared to ¼MS medium was, therefore, responsible for the increase in shoot height and shoot number, and partially for the hyperhydricity.

2.4.2 Callus culture

The use of 2,4-D in tissue culture medium of *W. japonica* is necessary for callus induction, although other PGRs can be effective. The use of different concentrations of 2,4-D affected the friability of callus from leaf and petiole explants differently in both types of culture condition, darkness and light. A low dose of 2,4-D (0.5–1.0 μM) appeared to be more suitable for callus induction of *W. japonica* (Tables 2.4 and 2.5). 2,4-D is a very popular auxin used for callus initiation in many plants, such as

Rudbeckia hirta (Luczkiewicz *et al.*, 2002), *Hypericum perforatum* (Pasqua *et al.*, 2003), *Genista tinctoria* (Luczkiewicz and Glod, 2003) and *Linum narbonense* (Vasilev and Ionkova, 2005). A report by Hosokawa and collaborators revealed that NAA and IAA are able to induce calli in wasabi (Hosokawa *et al.*, 1999). Eun and colleagues stated that NAA exhibits more effectiveness than IAA in somatic embryo formation from wasabi calli derived from cotyledon tissue (Eun *et al.*, 1996). Our experiments pointed out that NAA and IAA at high levels (25–50 μM) initiate calli from leaves and petioles after an inoculation period of six days, faster than those at lower levels (5–10 μM), and a combination between auxin and cytokinin produces calli in some culture stages (data not shown). The consistency of callus emergence on medium without 2,4-D supplement is also identified in other species (Oncina *et al.*, 2000; Toker *et al.*, 2003; Gyorgy *et al.*, 2004). The organs of *Trigonella foenum-graecum* were placed on a B_5 medium with 3 μM NAA and 500 mg L^{-1} of malt extract for callus formation (Oncina *et al.*, 2000). Seedling leaves of *Rhodiola rosea* and explants of *Ecballium elaterium* were cultured on an MS medium with BA and NAA in combinations for callus initiation and development (Toker *et al.*, 2003; Gyorgy *et al.*, 2004). The majority of explants used for callus induction are from leaves of the seedlings. Callus cultures of *Nasturtium montanum* (*Cruciferae*) (Songsak and Lockwood, 2004) and *Ammi majus* (Staniszewska *et al.*, 2003) were, for instance, established from seedlings. However, wasabi explants grown in sterile conditions can be utilised for callus initiation. Both leaves and petioles of *W. japonica* at one month of age could initiate calli on MS medium with 2,4-D supplement. In petiole fragments 1 cm in length, the distal portion is more effective on callus proliferation than the proximal and medial portions (Tables 2.4, 2.5 and Figure 2.20). Division of these petiole fragments into segments at 1–2 mm long enhanced the callus emergence (Tables 2.4 and 2.5). Petioles in all cases led to a faster and more thorough formation of callus than did leaves. Explant age and light conditions have influences on callus cultures. Matkowski reported that calli initiated and proliferated rapidly in young stems and leaves of *Pueraria lobata* (Wild.) Ohwi, while its adult leaves and petioles exhibited a slow callus growth when explants were cultured on various tested media (Matkowski, 2004). A photoperiod of 16-hours light/8-hours darkness was optimal for callus induction of *Hypericum perforatum* cv. 'Topas' and *Ecballium elaterium* (Pasqua *et al.*, 2003; Toker *et al.*, 2003), whereas a continuous light was required for callus cultures of *Nasturtium*

montanum, *Cleome chelidonii*, *Rudbeckia hirta* L., *Genista* spp., *Linum* spp., and *Trigonella* sp. (Oncina *et al.*, 2000; Luczkiewicz *et al.*, 2002; Luczkiewicz and Glod, 2003; Songsak and Lockwood, 2004; Vasilev and Ionkova, 2005). In our studies, explants of *W. japonica* cultured under either a photoperiod of 12-hours light/12-hours darkness or in complete darkness resulted in a callus initiation. However, under the darkness, calli from petioles were initiated earlier than in the light, and the petioles promoted a completed callus formation (Figure 2.20).

The growth stage of calli may require other types of PGR and culture media. A work on medium types inclusive of MS and B_5 by Oncina *et al*. demonstrated that both media were recommended for the callus initiation (callogenesis) stage of *Trigonella foenum-graecum*, while B_5 was unsuitable for the growing stage, as its callus growth percentage was much lower (3%, compared to 98% in the first stage) (Oncina *et al.*, 2000). The authors also pointed out that an MS medium was more effective for the callus growth of leaf and stem than all other media trialled. An MS basal medium was appropriate for the callus growth of *W. japonica* leaves and petioles, despite the fact that only 2-mm petiole segments were used in this stage as a result of full callogenesis in the first stage (Table 2.6). Callus cultures of wasabi grew best with the addition of 5 μM BA plus 2 μM NAA to the MS basal medium. Therefore, 2,4-D was unnecessary for the biomass accumulation of wasabi calli. Cotyledons, leaves and stems of *Rhodiola sachalinensis* were positively affected by IBA, IAA and NAA alone or in combinations with BA, and negatively affected by 2,4-D, for the second stage of calli (Wu *et al.*, 2003). The callus growth of *Pueraria lobata* was, however, satisfactory with a 2,4-D addition, and beneficial with the presence of both 2,4-D and either BA or NAA in the MS medium (Matkowski, 2004). In wasabi explants, 2,4-D was only essential for callus growth performance when it was combined with kinetin, TDZ or zeatin, because the presence of 2,4-D alone or together with BA in the medium showed a slower growth rate at this stage, although 2,4-D was an essential component in the initiation stage. In contrast, *Genista* species required an identical set of the two phytohormones at the respectively equal concentrations (22.6 μM 2,4-D and 23.2 μM kinetin) for both stages of callus induction and growth performance (Luczkiewicz and Glod, 2003). Unlike explants of *Rhodiola sachalinensis* (Wu *et al.*, 2003), wasabi petiole segments showed an inappropriate effect on callus growth with the addition of IBA and IAA to the basal medium containing 2,4-D, while callus biomass was well accumulated in the presence

of 2,4-D and other cytokinins, and could be maintained until the third generation of callus under light conditions. Thus, the callogenesis of *W. japonica* is induced with 2,4-D supplement, and the subsequent stage requires the presence of both types of phytohormones.

2.5 Conclusions

In summary, it was found that *W. japonica* can be rapidly multiplied *in vitro*. Starting explants could survive on a range of seven basal media (half-strength MS, full-strength MS, B5, WPM, KC, N6 and N&N). Among four cytokinins (BA, TDZ, kinetin and zeatin), zeatin proved to be less suitable for shoot proliferation than BA, TDZ and kinetin. An MS medium supplemented with 5 μM BA, 3% sucrose and solidified with 0.7% gelcarin was the most effective for shoot multiplication. Shoot proliferation could be improved with the addition of 1 μM IAA or 5 μM TDZ into the MS medium containing 5 μM BA. Half- and full-strength MS liquid culture media produced greater shoot numbers, shoot height and explant weight than did gelled media. Rooting was successfully achieved *in vitro* with half-strength MS gelled medium supplemented with 5–10 μM IBA, giving the highest frequency. Problems of contamination, hyperhydricity and toxicity caused by high levels of cytokinins that were encountered in the *in vitro* cultures remain to be resolved. Plantlets were successfully acclimatised on a mixture of vermiculite, perlite and peat-moss (1:1:1) under greenhouse conditions.

The studies on callus culture show that *W. japonica* calli can be initiated and further proliferated on MS media supplemented with 2,4-D from leaves and petioles *in vitro*. Petioles produced calli faster and more effectively than did leaves. An excised-petiole size at 2 mm was the most effective for callus induction on the media with the additions of 0.5 μM 2,4-D in the dark, and 1.0 μM 2,4-D in the light. The proliferation of calli was achieved on the media with 0.5 μM 2,4-D plus either 0.5 μM BA or 0.5 μM TDZ.

CHAPTER 3

STUDIES IN MUTAGENESIS

3.1 Introduction

Having developed a successful method for the *in vitro* propagation of *W. japonica* (reported in Chapter 2), the aim of this component of the project was to investigate the utility and feasibility of applying mutagenesis for crop improvement in *W. japonica*. There are many plant breeding methods that can be utilised to change many different characteristics of plants such as colour, size, resistance to disease and the quality of secondary metabolites. These methods include combination breeding, hybrid breeding, haploid breeding or mutation breeding (Kuckuck *et al.*, 1991). Natural mutations in plants were first observed and reported by Korschinsky in 1901 (van Harten, 1998). The history of induced mutations only dates back to the beginning of the twentieth century. Today, mutation breeding with radiation and chemical mutagens has been successfully utilised for the development of new varieties with specific attributes of growth, yield, disease-resistance and the production of specific chemical contents.

3.1.1 Chemical mutations

Mutagenic chemical compounds are numerous and show different activities. The most popularly applied mutagens include ethyl methane sulphonate (EMS), diethyl sulphate (DES), ethylene imine (EI), propane sultone, N-methyl-N-nitroso urethane (MNU) and several of their related components (Gottschalk and Wolff, 1983). A strong chemical for mutagenesis is sodium azide (NaN_3) (Sander and Muehlbauer, 1977). So far, the utilisation of chemical mutagens in plant breeding has been limited to some popular agents, such as EMS and MNU. EMS was effectively applied in the mutagenesis of *Ipomoea purpurea* (Bhate, 2001) and *Beta vulgaris* (Hohmann *et al.*, 2005). EMS was successfully used to change the erucic acid content in *Brassica carinata* (Barro *et al.*, 2001). MNU was reported to induce mutation on chickpeas (*Cicer arietinum*) at a higher efficiency than gamma rays (Kharkwal, 1998).

Commonly used mutagens that have a function in the disruption of cell division to form polyploid levels in plants include colchicine and oryzalin. These antimitotic mutagens are mainly used for inducing chromosome doubling. In addition to the fact that the utilisation of both these antimitotic agents in plant breeding is simple and highly effective in many plant cultivars, the elimination of some problems in physiology and genetics is also feasible (Tosca *et al.*, 1995).

Mutations caused by oryzalin. Oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) is a herbicide, in products such as trifluralin (α,α,α-trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine) and pronamide (*N*-(1,1-dimethylpropynyl) 3,5-dichlorobenzamide) (Bartels and Hilton, 1973). Like various dinitroaniline compounds, oryzalin was found to have a much stronger activity than colchicine when binding to the plant tubules, and consequently depolymerising microtubules at lower doses. It was also able to induce the disappearance of both cortical and spindle tubules of root cells (Bartels and Hilton, 1973; Morejohn *et al.*, 1987; Bouvier *et al.*, 1994). Oryzalin is successfully applied at much lower doses than colchicine (Geoffriau *et al.*, 1997; Thao *et al.*, 2003b; de Carvalho *et al.*, 2005). Successes were achieved at low concentrations of oryzalin treatments on kiwifruit *Actinidia deliciosa* (Chalak and Legave, 1996), on *Lilium* and *Nerine* for tetraploidy induction (van Tuyl *et al.*, 1992), and on apples for haploidy induction (Bouvier *et al.*, 1994). However, oryzalin showed a less effective capacity in chromosome doubling than did colchicine (Sanford, 1983; Tosca *et al.*, 1995). It also causes no risk to human health and less toxicity to plant materials. Oryzalin displayed weaker toxicity than colchicine when applied to haploid plantlets of gerbera (*Gerbera jamesonii*) (Tosca *et al.*, 1995) and triploid kiwifruit (Chalak and Legave, 1996). However, studies on the effects of oryzalin on the morphology and physiology of oryzalin-treated plants have been less intensively conducted than studies on the effects of colchicine.

Mutations caused by colchicine. Colchicine is one of the most popular chromosome-doubling and hazardous chemicals applied in plant breeding, recognised in the 1930s (Sanford, 1983; Tosca *et al.*, 1995). Many researches focusing on the effects of colchicine on the meiotic mechanism, morphogenesis and physiology of plant cells have significantly contributed to basic plant science (Eleftheriou, 1993; Hassan and Jones, 1994). The paring of chromosomes in prophase and metaphase was obviously affected

by colchicine, and had an influence on the appearance of univalent chromosomes in *Crepis capillaris* (Abberton and Callow, 1996), and the reduction of chiasma frequency observed in *Lolium multiflorum* (Hassan and Jones, 1994). It was considered that due to unsuccessful chiasma formation identified in *Rhoeo discolor*, the chromosomes failed to pair, or separated right after being paired, leading to an increase in univalent and chain formation but a decrease in ring formation (Verma *et al.*, 1992). In the spermatogenesis of treated pollen grains of *Allium ursinum*, colchicine was responsible for the extension of the leptonene stage in meiosis (the first stage of prophase) and for the pairing of homologous chromosomes in the zygotene (the second stage of prophase), resulting in the chiasma reduction of pollen (Loidl, 1988).

It was found that colchicine affected the chromosomal association in various plant species. In *Phlox drummondii*, colchicine caused an increase in the formation of quadrivalent, bivalent and univalent chromosomes at metaphase (Verma *et al.*, 1993). In tomatoes, colchicine increased the number of bivalent chromosomes while decreasing the formation of multivalent chromosomes (Zeerak, 1991). In *Sorghum versicolor*, colchicine caused an increase in the pairing of quadrivalent, trivalent, bivalent and univalent chromosomes in prophase and metaphase (Sun *et al.*, 1994). The partial desynapsis in the meiosis of *Lathyrus odoratus* and *L. pratensis* was attributed to the treatment of colchicine (Khawaja and Ellis, 1987). In addition to the chromosomal effects, colchicine also influences the growth and development of plants. Colchicine was found to reduce seed germination in radishes (Scialabba *et al.*, 1995) and *Cyclamen perscium* (Takamura and Miyajima, 1996). Following seed treatment with colchicine, the delay in gemination and decrease in the survival of seedlings quantity were reported on *Trichosanthes anguina* (Datta, 1992) and *Crepis capillaris* (Abberton and Callow, 1996), while the increase in the maturation rate of seedlings was observed in *Lolium multiflorum* (Hassan and Jones, 1994). In some hybrids of *Rosa* treated with colchicine, there were changes in the growth and survival rate of plants (Ma *et al.*, 1997). The suppression of cell division was found in Japanese persimmon (*Diospyros kaki*) when treating protoplasts with colchicine (Tamura *et al.*, 1996). In *Lolium multiflorum*, the effects of colchicine treatment reduced the frequency of chiasmas and increased the univalent chromosomes not only in parent seedlings but also in the next untreated offspring (Hassan and Jones, 1994). In tomatoes, morphological alterations were passed from colchicine-treated parents to the next generation (Zeerak, 1991).

The effectiveness of both oryzalin and colchicine depends upon species, treatment doses and the length of exposure of plant samples to the agents (Bartels and Hilton, 1973; Gottschalk and Wolff, 1983; Tosca *et al.*, 1995). Further examples of the successful applications of oryzalin and colchicine in some plant species are shown in Appendices 3.1 and 3.2.

Other popular chemical mutagens for chromosome doubling induction include trifluralin, amiprophosmethyl (APM), nitrous oxide and caffeine. APM and trifluralin are dinitroaniline herbicides of the greatest appeal to plant breeders, which have been used for inducing chromosome doubling of *Brassica napus* (Zhao and Simmonds, 1995; Hansen and Andersen, 1996), *Nicotiana plumbaginifolia* (Verhoeven *et al.*, 1990) and *Beta vulgaris* (Hansen *et al.*, 1998). Nitrous oxide was found to be successful in the haploid doubling induction of *Triticum aestivum* (Hansen *et al.*, 1988), whereas caffeine successfully induced chromosome doubling in common wheat plants (Thomas *et al.*, 1997).

The performance of chromosome doubling is frequently observed not only in haploid plants for forming homozygous lines, as reported in food crops such as wheat (Redha *et al.*, 1998) and maize (Saisingtong *et al.*, 1996), but also in polyploids that are mostly desirable to plant breeding because of the expression of changes of various characteristics such as size and colour in comparison with their parents. Tetraploids have been successfully induced in bananas (Hamill *et al.*, 1992) and grapes to increase fruit size (Stebbins, 1956). In *sorghum versicolor*, tetraploids exhibited greater plant height than diploids, where 20% of autotetraploids was necrotic due to physiological problems (Sun *et al.*, 1994). In tomatoes, however, tetraploids showed larger stomata, larger flowers, darker leaves, longer fruiting and broader tolerance to low temperatures, but smaller fruit size than diploids (Zeerak, 1991). The negative effects of plant polyploids are commonly found in plant vigour reduction, pollen sterility, genetic imbalances and physiological disturbances. A high number of chromosomes reduced the growth rates in polyploids of Japanese persimmon *Diospyros kaki* (Tamura *et al.*, 1996) and blueberries *Vaccinium* sp. (Goldy and Lyrene, 1984). The yield of *Camellia sinensis* (Wachira and Ng'etich, 1999) was reduced with increasing ploidy levels, which were partly attributed to photosynthetic capacity reduction. A reduction in pollen viability that may lead to a decreased seed set has been studied in the polyploids of

colchicine-induced cyclamens (Takamura and Miyajima, 1996), in hybrids of *Sesamum* (Biswas and Bose, 1998), and also in mutants of *Chrysanthemum morifolium* (Datta and Datta, 1998). A decrease in seed set despite the high fertility of pollen was responsible for the failure of conjugation between pollen tube and ovarian cavity in the mutants of *Nicotiana tabacum* (Chung *et al.*, 1996), or for the inequality of chromosome distribution in mutants of *Sorghum versicolor* (Sun *et al.*, 1994).

3.1.2 Physical mutations

Physical mutagens consist of ionising radiation such as X-ray, gamma rays, UV rays, neutrons, particles and protons (Gottschalk and Wolff, 1983; Taji *et al.*, 2002). The most popularly used irradiations in plant mutagenesis are gamma rays and X-rays. X-rays are produced by electrical electrons in high vacuum, with a wavelength ranging from 0.001 to 10 nm, allowing the radiation to penetrate into plant tissues variably to a depth of between several millimetres (by long wavelengths) and several centimetres (by short wavelengths). Gamma rays emitted from radioisotopes can penetrate more deeply into plant tissues for a few centimetres, as they have shorter wavelengths than X-rays.

In induced mutations by physical mutagens, the key factors affecting plant materials include radiation dose, temperature, recurrence and pre- and post-treatments (Gottschalk and Wolff, 1983; Ahloowalia and Maluszynski, 2001). Radiation doses are considered to be high, medium or low if the energy amount being exposed to plant materials ranges above 10 kGy, between 1 kGy and 10 kGy, or below 1 kGy, respectively. The sterilisation of food products requires high doses of irradiation, whereas low doses are usually used to induce mutations in seed-propagated plants, optimally between 60 and 700 Gy for seed materials of rice, wheat, maize and beans (Ahloowalia and Maluszynski, 2001). For tissue-cultured plant materials, irradiation doses are used at much lower levels, typically from 2 to 5 Gy in callus cultures, and from 15 to 20 Gy in stem cuttings (Ahloowalia and Maluszynski, 2001). Irradiation dose levels have different effects on the mutagenic efficiency of plant species and plant materials. For example, low doses of radiation were found to have stimulatory influences on the germination of carrot seeds (Mensah and Eruotor, 1993) and peroxidase activity of sweet potato (Lage and Esquibel, 1997). An ionising radiation dose higher than 25 Gy caused a loss in the regenerative capacity of date palm callus cultures, while a dose of 10 Gy was lethal for callus cultures of sweet potato. Similarly, the lethal dose was

60 Gy and the optimal dose was from 5 to 10 Gy for sugarcane callus cultures, and the optimal dose for the survival of *in vitro* cultured potatoes was 20 Gy (IAEA, 1997). The application of gamma and X-irradiations in some plant species is described in Appendices 3.3 and 3.4.

Mutation breeding by radiation commonly provides new and improved traits for plant breeding. It is particularly of benefit to species of natural high variability problems, such as found in taro with vegetative reproduction (Malamug *et al.*, 1994), where traditional breeding is impossible, such as seen in *Bougainvillea* producing no flowers (Datta, 1991), and of time-consuming sexual reproduction cycles, as observed in *Gladiolus*, enabling the production of flowers only after several seasons (Misra, 1998). More popularly, as seed can be treated by irradiation in a dry condition, and subsequently handled for sowing with a method similar to that used with untreated seeds, numerous plant varieties of legumes, maize, barley, peas and cereals have been successfully produced by physical mutation. The method has been aimed at producing the alteration of seed storage substances such as proteins, lipids and carbohydrates (Gottschalk and Wolff, 1983).

In cut-flower industries, flower colour and size are the most desirable characteristics that can be improved by irradiation. Applying X-rays, the flower colours of *Achimenes* cv. 'Paul Arnold' (Broertjes, 1972) and *Chrysanthemum morifolium* were altered more rapidly than by traditional breeding methods (Datta, 1991). Other important plant species that have achieved variations in flower colour by means of irradiation breeding include *Rosa* spp. (Datta, 1991), *Dianthus* 'Mystere' (Cassells *et al.*, 1993), *Dendranthema grandiflora, Gerbera jamesonii* (Jerzy and Zalewska, 1996) and *Gladiolus* (Misra, 1998). Altered flower shape by irradiation was found in *Portulaca* (Datta, 1991).

Irradiation can induce mutations to beneficially alter the morphology and habit of plants. Leaf mutants were found both in ornamental plants such as *Lantana depesas* (Datta, 1991), *Dianthus* 'Mystere' (Cassells *et al.*, 1993) and *Gerbera jamesonii* (Jerzy and Zalewska, 1996), and food crops such as mulberries (Meesilpa and Phadvibulya, 1991) and taro (Malamug *et al.*, 1994). Changes in the size and height of plants were observed in rice (Singh *et al.*, 1998) and taro (Malamug *et al.*, 1994). Alteration in habit was identified in *Achimenes* (Broertjes, 1972), *Rosa* spp. (Walther and Sauer, 1986) and

Dendranthema grandiflora (Jerzy and Zalewska, 1996), while a low-temperature tolerant mutant was achieved in *Chrysanthemum morifolium* (de Jong *et al.*, 1991).

Pest- and disease-resistant mutants have also been achieved by irradiation. Resistance to tuber moth was found in potato mutants (Saour *et al.*, 1999). A serious disease of a common bean caused by bacterial blight was prevented after irradiating the bean cultivar *Phaseolus vulgaris* 'UNECA' with gamma rays (Mohamed *et al.*, 1995). A similar blight found on cultivars of Indian rice (*Oryza sativa* L.), which is known to be the second most serious disease in Southeast Asia caused by *Xanthomonas oryzae*, was prevented by gamma irradiation (Agrawal *et al.*, 2005). Disease resistance was also improved by the irradiations of grapefruit and *Dianthus* 'Mystere' (Meesilpa and Phadvibulya, 1991).

Irradiation at low doses increases yield and chemical components in many crops. The growth of mung bean (*Vigna radiata)* (Singh and Sharma, 1993) and potatoes (Saour *et al.*, 1999) was increased after gamma irradiation at low doses, which resulted in the high yields. It was identified that low doses of irradiation also increased the amount of vitamin C and nitrogen in potato leaves (Saour *et al.*, 1999), and the protein amount in chickpeas (*Cicer arietinum*) by 18.8% compared to their control (Kharkwal, 1998), and the oleic acid content in soybeans (Rahman *et al.*, 1994).

Additionally, somatic hybridisation has been obtained by the radiation induction of protoplasts. The irradiated protoplast (the donor) is fused to the non-irradiated protoplast (the recipient) to improve fertility. Some plant species to which the method has been successfully applied include *Nicotiana plumbaginifolia, Atropa belladonna* (Gleba *et al.*, 1988), *Brassica oleracea*, *Brassica campestris* (Yamashita *et al.*, 1989), *Festuca arundinacea* and *Lolium multiflorum* (Spangenberg *et al.*, 1994).

Irradiations have positively contributed to the improvement of desirable attributes in the morphology, habit and yield of secondary metabolites in a large number of plant species. Numerous improved varieties have been officially commercially released, such as *Malus domestica* cv. 'Golden Haidegg', *Musa acuminata* cv. 'Novaria', *Prunus cerasus* cv. 'Plodorodnaya Michurina' and *Prunus avium* cv. 'Compact Stella 35B11' (Predieri, 2001). However, in parallel with the benefits gained by physical mutations, problems such as undesired forms are also observed in many plant varieties. With chromosome mutations, not only the structural changes of chromosome occur, but also

the newly rearranged chromosomes may not exist after irradiation (Gottschalk and Wolff, 1983; Kuckuck *et al.*, 1991). In another case, the cells become moribund because the injured DNA cannot be normally replicated (Ahnström, 1989). Consequently, the plant may die because of the excessive cell death.

A high dosage of irradiation can also result in a decrease of growth, germination ability and disorder in the morphology and physiology of plant growth, or even cause the death of the plant (Kuckuck *et al.*, 1991). For instance, a 3 kGy dose of gamma rays stopped the growth of French beans after a one-week treatment due to the damage of DNA causing a disorder in photosynthesis and stomatal regulation (Saakov *et al.*, 1992). The survival rate was decreased with high-dose irradiation on seedlings of Lima beans (Mensah and Eruotor, 1993), in tissue-cultured explants of *Alpinia purpurata* (Fereol *et al.*, 1996), and in *in vitro* leaves of apples (James *et al.*, 1988) and *Rosa hybrida* (Ibrahim *et al.*, 1998). With these high doses, the treatments were considered unsuccessful. For the optimisation of mutation frequency, the level of radiation and treatment duration are expected to be initially determined. A recommendation was that a lower irradiation dose causing less than 20–50% (LD_{20}–LD_{50}) of total plant death is regarded as significant in any plant breeding programmes (Kuckuck *et al.*, 1991).

Another problem associated with the irradiation involves the low efficiency of mutation. The cause derives from the capacity of the damage restoration of DNA by itself before the expression of mutation (Micke and Donini, 1993). In point mutations, dominant alleles tend to be changed into recessive ones for the most part (Kuckuck *et al.*, 1991), resulting in recessive mutations. The frequency of observed mutation is, therefore, commonly low. An observation following irradiation revealed that only 2.5% of mutants was discernible on mung beans in the M_2 generation (Singh and Sharma, 1993), and one mutant out of more than nine thousand soybean plants was visible in producing higher amounts of oleic acid (Rahman *et al.*, 1994). Low efficiency can also be caused by the speed of cell division. The healthy or less damaged cells dominate mutated cells because the former divide more rapidly. Such an example was observed on the mutation of *Alpinia purpurata* plantlets (Fereol *et al.*, 1996).

The purpose of these studies were to compare the effectiveness of two chemical mutagens (oryzalin and colchicine) and to investigate the irradiation capacity of two physical mutagens, X-rays and gamma rays, for inducing mutagenesis in *in vitro* shoot

tips of *W. japonica*, with the future aim of producing mutant lines of improved AITC content (see Chapter 4).

3.2 Materials and methods

3.2.1 Plant materials

Mother stock material of *W. japonica* was grown in the MS medium described in Chapter 2. Stock plants were subcultured onto fresh media every four weeks. The experiments with both chemical and physical mutagens were carried out using the actively growing multiple shoots obtained from the MS medium containing 5 μM BA (Figure 2.1b). Single shoot tips were excised from these four-week-old multiple-shoots using a forceps. For treatments with the physical mutagens, cultures were contained in Petri dishes or test tubes kept in an esky container, which was incubated prior to transport to and from ionising-radiation sources to suppress stress.

3.2.2 Treatment methods

3.2.2.1 Chemical mutagens

3.2.2.1.1 Colchicine (C-9754, Sigma) was dissolved in 1% (v/v) DMSO (Dimethyl sulphoxide, Sigma) and added to water in appropriate levels. Colchicine solutions were then added to the MS basal medium before autoclaving. Single shoot tips, 10 mm in length, aseptically excised from *in vitro* multiple shoots, were treated with 0, 25, 75 and 150 μM colchicine by placing them vertically onto the gelled MS basal media containing 0, 25, 75 and 150 μM colchicine, respectively. For each concentration, a total of fifty shoot tips were placed in 10 round plastic containers, each with five explants, and kept in the plant growth room for two, four and eight days. After treatment, shoot tips were removed from the media, carefully washed four times with sterile distilled water, briefly dried on sterile filter papers, and then immediately grown in shoot proliferation medium (the MS medium containing 5 μM BA as described in Chapter 2, section 2.2.1.2) free of colchicine for monthly assessments.

3.2.2.1.2 Oryzalin (DowElanco, Analytical Standard) was dissolved in 1% (v/v) DMSO, filter-sterilised, and then added to the MS basal media before autoclaving. Individual shoot tips 10 mm in length were exposed to oryzalin at 0, 5.0, 15 and 30 μM by placing vertically onto the gelled MS basal media supplemented with 0, 5.0, 15 and 30 μM oryzalin, respectively. For each concentration, fifty shoot tips were cultured in

10 round plastic jars, each with five explants, and incubated in the growth chamber for two, four and eight days. After incubation, the explants were removed from the media, washed four times with sterile distilled water, briefly dried on sterile filter papers, and then immediately transferred onto shoot proliferation fresh MS medium (as described for colchicine treatment above), free of oryzalin. Explants were observed monthly and assessed throughout three propagation cycles for a total of three months.

3.2.2.2 Physical mutagens

3.2.2.2.1 X-irradiation. Individual shoots were horizontally placed on plastic Petri dishes (80 mm diameter) with 10 shoot tips per dish, and five dishes per experimental batch. Sterile distilled water was then added to each dish to submerge the shoots for more uniform movements of X-rays when irradiated. Only one Petri dish was placed in the middle of a 30 cm x 40 cm Perspex box for each treatment (Figure 3.1).

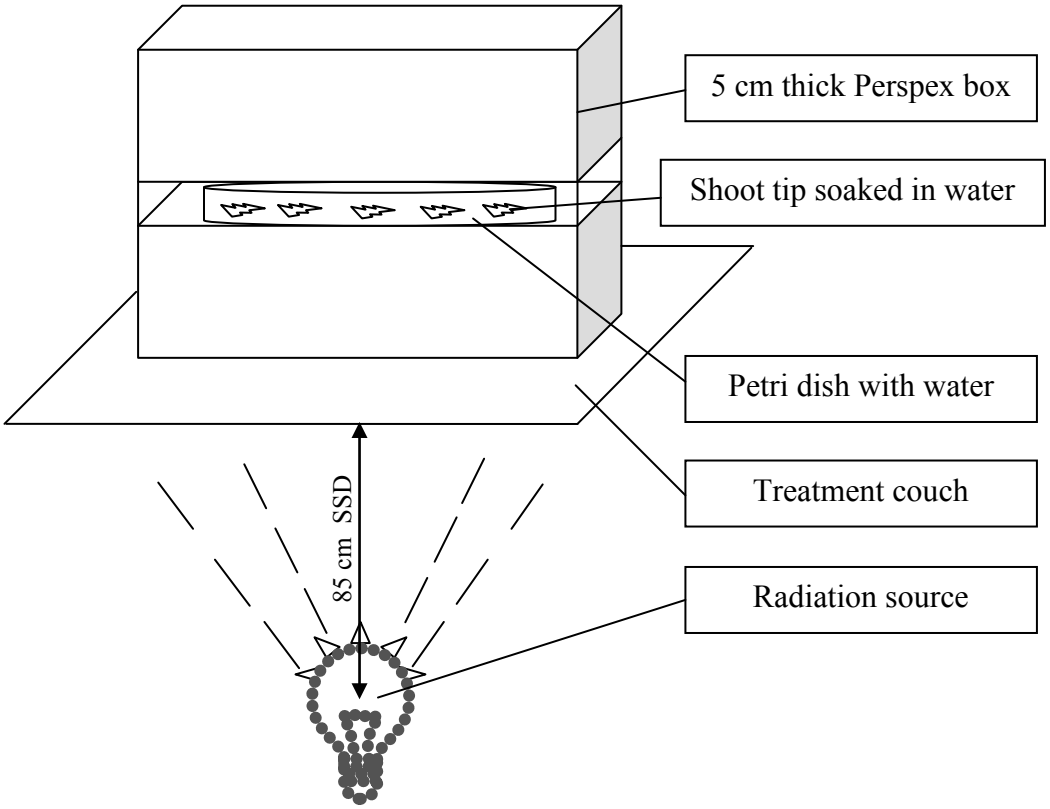


Figure 3.1: Experimental set up for X-irradiation treatment.

X-irradiation was performed at the Radiotherapy Unit, Royal North Shore Hospital of Sydney. The dose rate applied was 820 monitor units/minute at a depth of 85 cm (85 SSD, 180^{o} gantry, ColliRNT 0.0^{o}) from the radiation source to the treatment couch.

Radiation was administered in a field at 20 cm x 20 cm, and aimed at 1.3 cm from the bottom of the Perspex box.

The cadcheck was used to obtain the total monitor units (MU) per treatment as follows:

0 Gy = 0 MU
10 Gy = 700 MU
20 Gy = 1400 MU
40 Gy = 2800 MU
80 Gy = 5600 MU

Within one hour after treatment by radiation, all shoot tips were rinsed twice with sterile distilled water and transferred onto the fresh culture media. All cultures were distributed at random in the plant growth chamber for growth observations and measurements.

3.2.2.2.2 Gamma irradiation. The gamma rays were obtained from a cobalt 60 source with a measured dose rate of 6.65 Gray/minute, at the Australian Nuclear Science and Technology Organisation (ANSTO) in Lucas Heights, NSW.

Twenty-five test tubes per experimental lot, each tube containing one shoot tip, were evenly arrayed in a round cardboard cylindrical container. For radiation emission measurement, Fricke dosimeters were sited throughout the array to monitor the dose received by each batch of tubes. A larger metal cylindrical container covering the cardboard was placed in a radiation emission facility containing the cobalt source for a time calculated to gain the required dose. Five doses obtained from gamma irradiation were 0, 10, 20, 40 and 80 Gray.

Immediately after gamma radiation exposure, all shoot tips were rinsed three times with sterile distilled water and transferred onto the fresh culture media. All cultures were incubated in the plant growth room for growth assessments.

3.2.3 Culture conditions

In all treatments with mutagens, 50 mL of MS gelled medium were dispensed into 250-mL jars, or 10 mL medium into 50-mL test tubes. Media adjusted to pH 5.8 were autoclaved at 121^{o}C at 105 kPa for 15 minutes. Cultures were incubated in the plant growth room at 24 ± 1^{o}C with a 12-hour photoperiod and irradiance of 50 μmol $m^{-2}s^{-1}$ emitted from cool white fluorescent lights for periods of one, two and three months.

3.2.4 Growth measurements

In all treatments with mutagens, the survival rate, height, weight and number of shoot per explant were recorded, and shoot proliferation fresh media replaced at four-week intervals of post-treatment growth. Parameters of explant health and condition were measured as described in section 2.2.1.4 of Chapter 2, except for the shoot number counted on the basis of twin-leaf shoot. Each shoot tip was cultured for three continuously vegetative cycles (three months) in the shoot proliferation MS medium, and its progeny was severally marked at each subculture. After the third cycle of culture, each multiple shoot was subdivided into single shoots, and subcultured on MS fresh media. The aim of this subculture was to reduce or eliminate chimeras in cultures. Every three single shoot tips were isolated from each multiple shoot and separately numbered. One single shoot was cultured on a fresh shoot multiplication medium. The other two were placed on a rooting medium supplemented with 10 μM IBA for three weeks, and later transplanted into a mixture of vermiculite, perlite and peat-moss (1:1:1) in the greenhouse conditions, as described in section 2.2.1.3 of Chapter 2, for further observations on morphological alterations and mutants during plant development.

3.2.5. Data analysis

Statistical analyses of *in vitro* culture data were carried out as appropriate, using the methods described in section 2.2.1.5 of Chapter 2.

3.3 Results

3.3.1 Chemical mutagen treatments

Actively growing shoot explants at four weeks of age, which were derived from the previous gelled MS shoot multiplication medium and later cultured on the MS medium containing chemical mutagens, showed a wide range of variations on the survival, morphology and synthesised AITC content.

3.3.1.1. Selection of optimal treatments based on time exposure and concentrations of mutagens

In treatments of shoot explants with colchicine and oryzalin for two, four and eight days, it was found that treatment time length and concentration of chemical mutagens affected shoot explant survival percentage after four weeks in culture (Figures 3.2, 3.3 and 3.4).

The survival ratios of shoot tips forming new shoots after a two-day exposure with chemical mutagens ranged from 84% (with 30 μM oryzalin) to 100% (with 25 μM colchicine) (Figure 3.2). In concentrations of oryzalin (0, 5, 15 and 30 μM) and colchicine (0, 25, 75 and 150 μM) assayed, shoot mortality was only observed in the medium with 15 μM and 30 μM oryzalin supplements at a rate of 8% and 12%, respectively. Shoot mortality present on the medium with these high doses of oryzalin was initially observed in the third culture week. One hundred per cent of shoot explants was viable with the colchicine supplement at 25 μM and in the untreated control, but 98% of shoots produced more shoots on the medium supplemented with colchicine at high concentrations (75 μM and 150 μM). The percentage of shoots that failed to produce new shoots was higher on the medium with 30 μM oryzalin (4%) than with the 5–15 μM oryzalin supplement (2%).

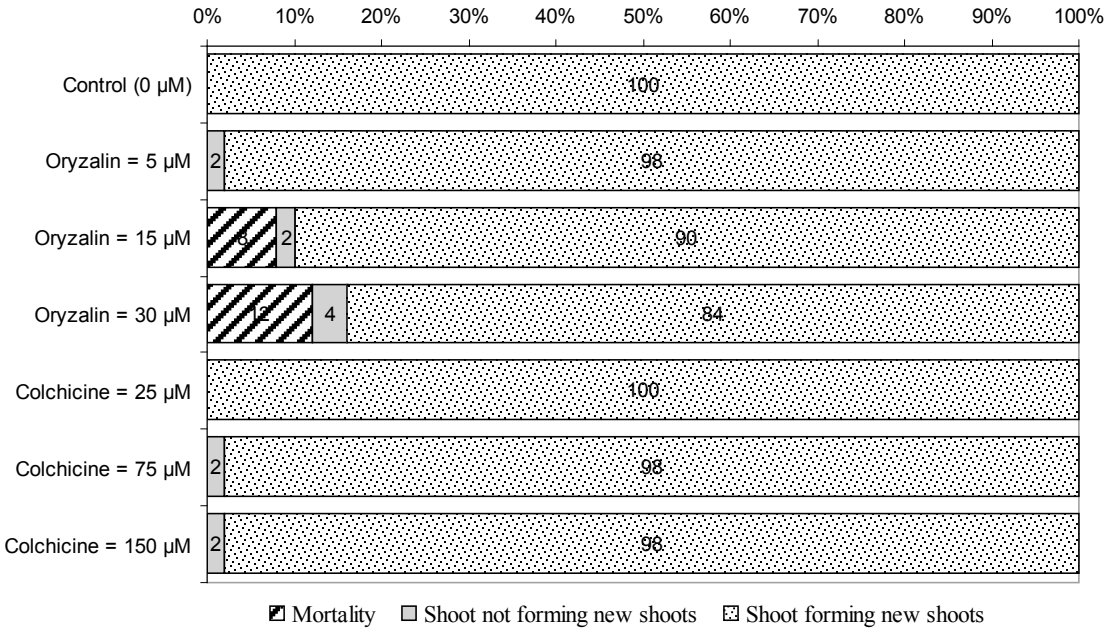


Figure 3.2: Effects of a 2-day treatment on the survival rates of *W. japonica* shoot tips exposed with oryzalin (at 0, 5, 15 and 30 μM) and colchicine (at 0, 25, 75 and 150 μM) after 1 month in culture on MS shoot proliferation medium. Values are the means of 50 replicates.

In a four-day treatment with chemical mutagens at the same concentration ranges as above, the mortality rate and percentage of shoots inhibiting new shoot formation increased slightly (Figure 3.3). None of the concentrations of oryzalin or colchicine causing a 50% reduction of shoot growth in the first four-week vegetative cycle after treatment (LD_{50}) was detectable. The percentage of shoot-regenerating explants

decreased more slowly in the increasing concentrations of colchicine (100, 98, 94 and 90%) than in the increasing concentrations of oryzalin (100, 96, 82 and 74%).

Mortality commenced to occur in explants treated with colchicine at a high dose (75 μM) and with oryzalin at a low dose (5 μM). The highest mortality rate was recorded on shoot explants exposed to 30 μM oryzalin (18%). A low inhibition rate to produce new shoots appeared on treated explants with 25 μM colchicine (2%). More optimal doses for survival were obtained with colchicine treatment than with oryzalin treatment.

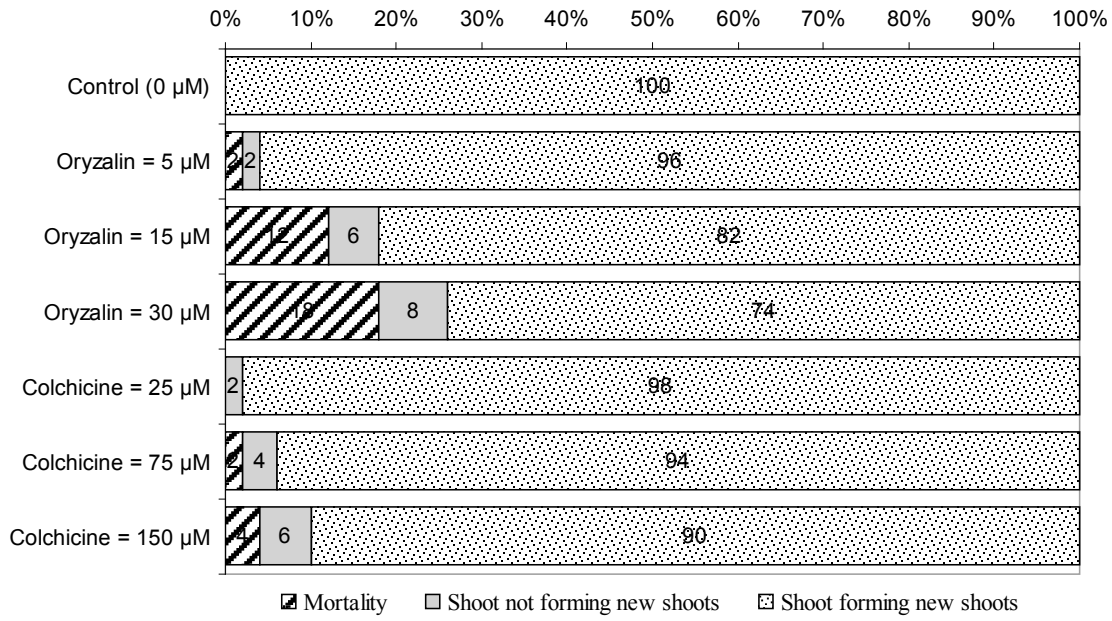


Figure 3.3: Effects of a 4-day treatment on the survival rates of *W. japonica* shoot tips exposed with oryzalin (at 0, 5, 15 and 30 μM) and colchicine (at 0, 25, 75 and 150 μM) after 1 month in culture on MS shoot proliferation medium. Values are the means of 50 replicates.

The least effective treatment involved the use of chemical mutagens at the two above-mentioned ranges of concentrations for eight days (Figures 3.4, 3.5 and 3.6). The number of shoot-regenerating explants reduced rapidly within treatments with oryzalin and colchicine at all concentrations after the four-week culture. The lethal concentration for 50% of the regenerating shoots occurred in treatments with both chemical mutagens. LD_{50} rate (50% mortality) was close to 150 μM of colchicine and lay between 15 μM and 30 μM of oryzalin. Low doses of colchicine (25 μM) and oryzalin (5 μM) almost maintained the high shoot survival rates at 92% and 86%, respectively. The rate of regenerating shoots obtained with 15 μM oryzalin decreased strikingly in an eight-day treatment, compared to a four-day treatment.

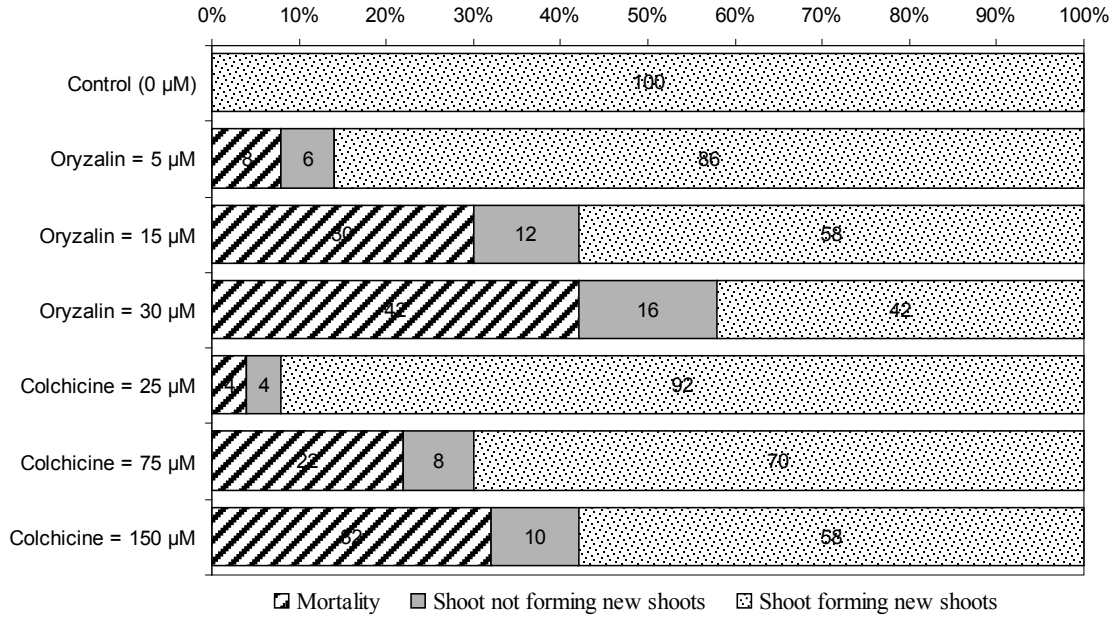


Figure 3.4: Effects of an 8-day treatment on the survival rates of *W. japonica* shoot tips exposed with oryzalin (at 0, 5, 15 and 30 µM) and colchicine (at 0, 25, 75 and 150 µM) after 1 month in culture on MS shoot proliferation medium. Values are the means of 50 replicates.

High inhibition rates of new shoot production occurred in all treated explants, particularly on shoots treated with 30 µM oryzalin (16%). Unregenerating shoots accounted for 10% in 150 µM colchicine, resulting in a survival rate of 58%, equal to the value of regenerating shoots found in 15 µM oryzalin. The highest inhibitory effect on regenerating shoots was exhibited by the 30 µM of oryzalin that led to a survival rate of 42%.

Shoot necrosis occurred at the first week in culture after treatment with a mortality rate increasing at the fourth culture week on all doses of oryzalin and colchicine. The lowest mortality rate could only be observed on explants treated with colchicine at 25 µM (4%), and the highest mortality rate observed with 30 µM oryzalin (42%). At high doses of colchicine (75–150 µM), this rate increased dramatically in an eight-day treatment (22–32%), compared to a four-day treatment (2–4%).

Apparent chimerism was observed in explants treated with high doses of mutagens (15–30 µM oryzalin and 75–150 µM colchicine). The undesirable chimera appeared on both shoots and outer leaves and increased over the culture period. At week 4 in the culture, apparent chimeras were also detected in explants with a low dose of oryzalin (5 µM).

Depending on the LD_{50} values that were not close to any concentrations appearing lethal, and unregenerating explants in a four-day treatment with both chemical mutagens, we selected shoot materials under the four-day treatment period for the morphological assessments during the next two months in culture.

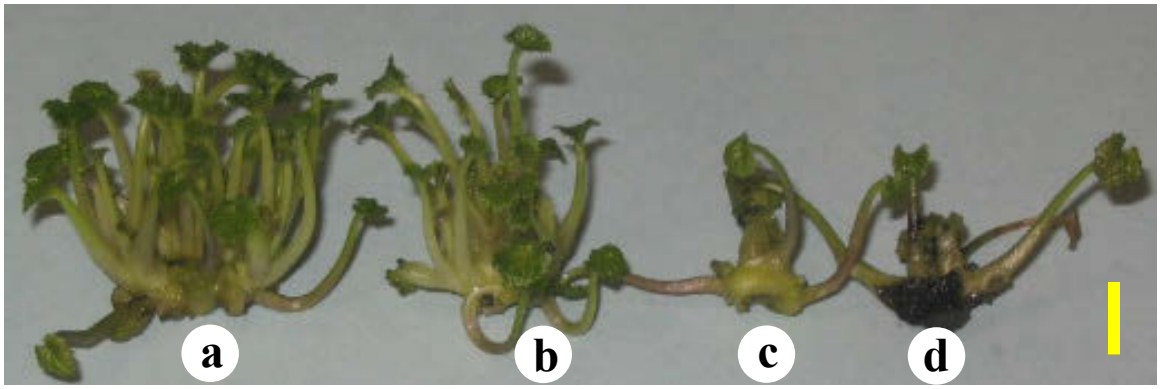


Figure 3.5: Four-week-old *W. japonica* shoots following an 8-day treatment with colchicine at (a) 0 µM, (b) 25 µM, (c) 75 µM and (d) 150 µM (bar = 1 cm).

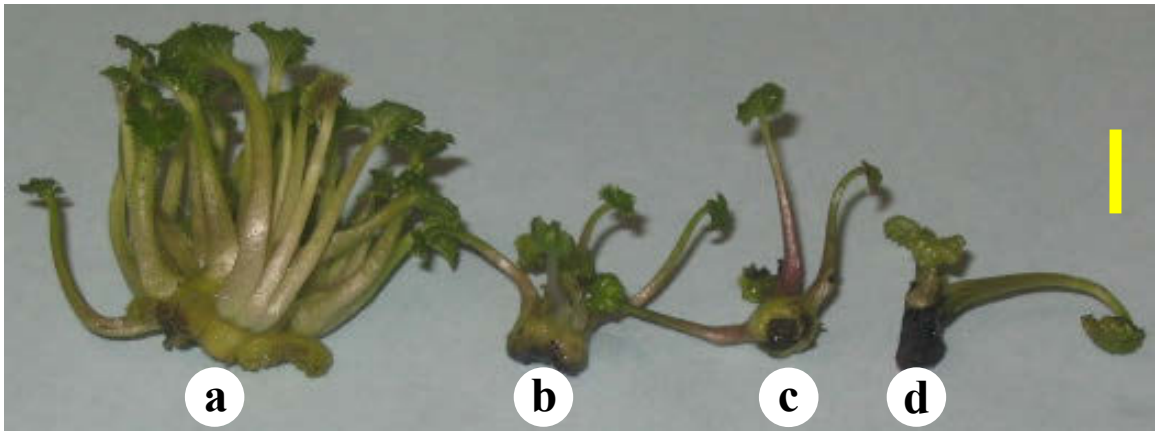


Figure 3.6: Four-week-old *W. japonica* shoots following an 8-day treatment with oryzalin at (a) 0 µM, (b) 5 µM, (c) 15 µM and (d) 30 µM (bar = 1 cm).

3.3.1.2. Effect of chemical mutations on in vitro growth and morphological variations

3.3.1.2.1 Explant weight

Explant weight was affected by both oryzalin and colchicine after a four-day treatment (Figure 3.7 and Appendix 3.5). After one month in culture, the weights of explants treated with 15–30 µM oryzalin were non-significantly different from each other, but significantly smaller than untreated explant weight (p= 0.041). All treated explants with colchicine at all concentrations were, however, not different from the untreated control

in terms of explant weight after the same length of culture (p= 0.064). There was no different response regarding explant weight between the control and 5 μM oryzalin treatment.

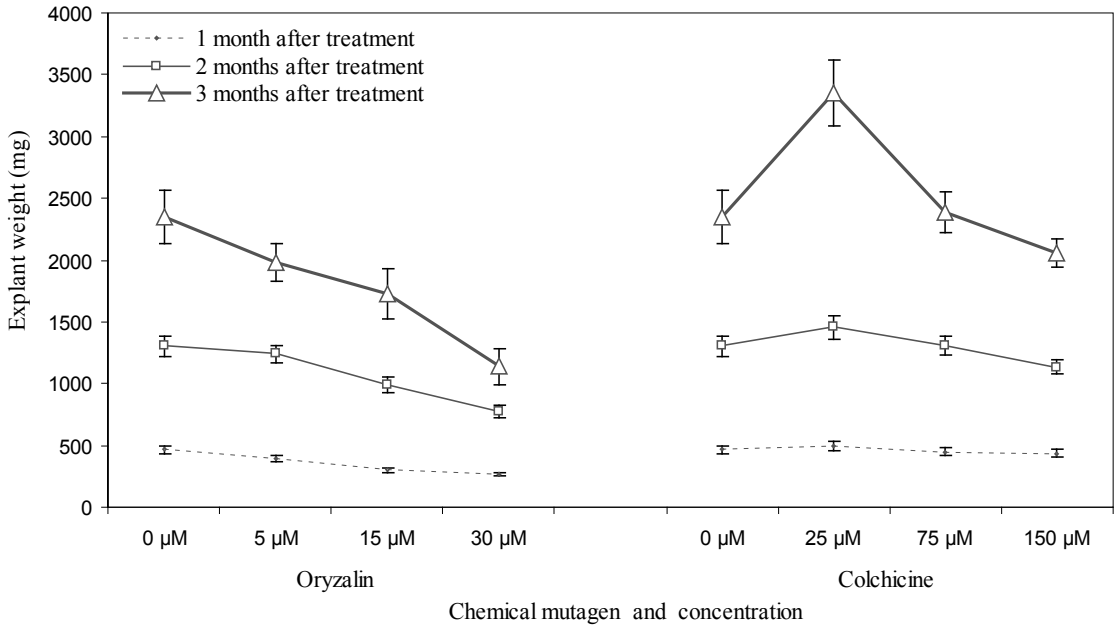


Figure 3.7: Effects of chemical mutagens and concentrations on the explant weight of *W. japonica* after 1 month, 2 months and 3 months in culture on MS medium. Values are the means of 50 single shoots. Vertical lines represent standard errors.

After two months in culture, significant differences in the explant weight of all treated plants were found only in a high dose of oryzalin (30 μM) compared with untreated control plants. After three months in culture, this significant effect was exhibited in both oryzalin at a high dose (30 μM) and colchicine at a low dose (25 μM). A high dose of oryzalin decreased explant weight to 48% (p= 0.034), while a low dose of colchicine increased the weight of the explant by 42%, compared with that of the control (p= 0.000).

The explant weight increased monthly in culture among doses of mutagens, significantly in all doses of colchicine and low doses of oryzalin (data not shown).

3.3.1.2.2 Shoot multiplication

Oryzalin and colchicine affected the number of shoots per explant after 30, 60 and 90 days in culture (Figure 3.8 and Appendix 3.5). During the first 30 days, all treated explants produced significantly smaller numbers of new shoots than did untreated explants (p= 0.012). The shoot numbers obtained with 150 μM colchicine and

5 μM oryzalin were significantly different from each other, but both were not different from those with 75 μM colchicine. Oryzalin at 15 and 30 μM showed no differences, and had the highest inhibitory effect on shoot numbers. The lowest inhibition on the multiplication of shoots was in the treatment with 25 μM colchicine.

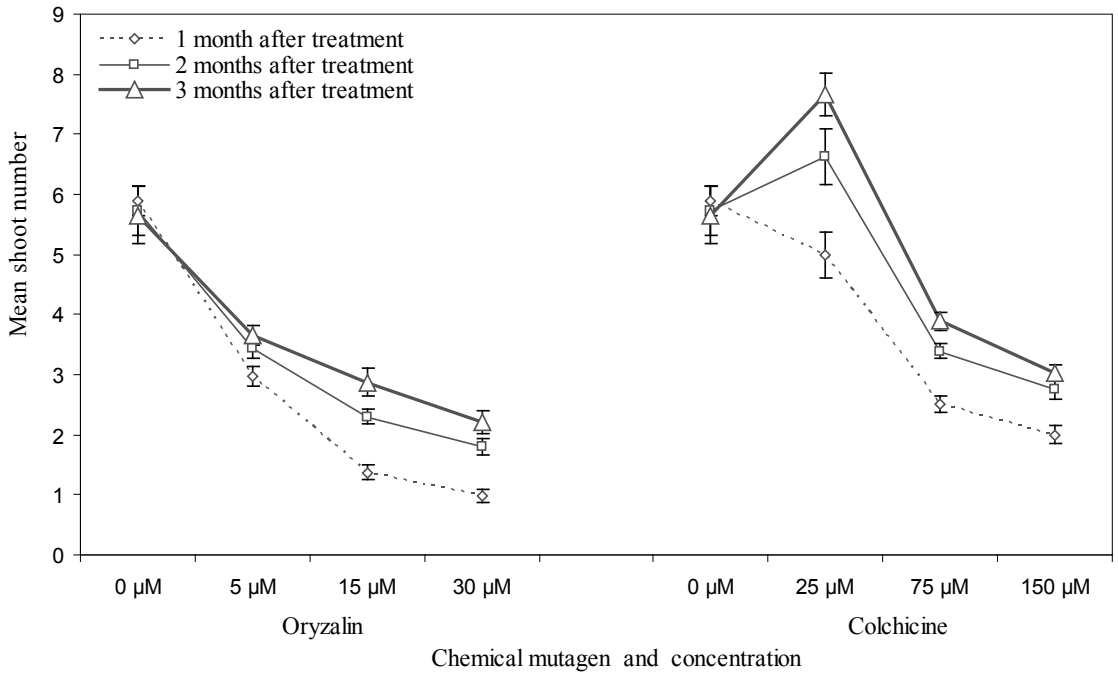


Figure 3.8: Effects of chemical mutagens and concentrations on shoot number of *W. japonica* after 1 month, 2 months and 3 months in culture on MS medium. Values are the means of 50 single shoots. Vertical lines represent standard errors.

Following 60 days in culture, colchicine at 25 μM increased shoot numbers to the same shoot multiplication rate as found in the untreated control. Shoot numbers were reduced in treated plants by other doses of mutagens and were not significantly different from each other. After 90 days in culture, colchicine at 25 μM accelerated the regeneration of new shoots by 36% of the control. Colchicine and oryzalin at other concentrations maintained the reduction and showed no different effects in shoot multiplication.

3.3.1.2.3 Shoot height

The effects of oryzalin and colchicine on shoot height under a four-day treatment were recorded (Figure 3.9 and Appendix 3.5). Shoot elongation fluctuated during the culture period of 90 days. By the thirtieth day, there was a significant reduction in shoot elongation (p= 0.000). Oryzalin at doses ranging from 15–30 μM and colchicine from 75–150 μM showed shorter shoots than control explants, and these were not

significantly different from each other. Elongation of shoots was found not to be different among the control, 5 μM oryzalin and 25 μM colchicine treatments.

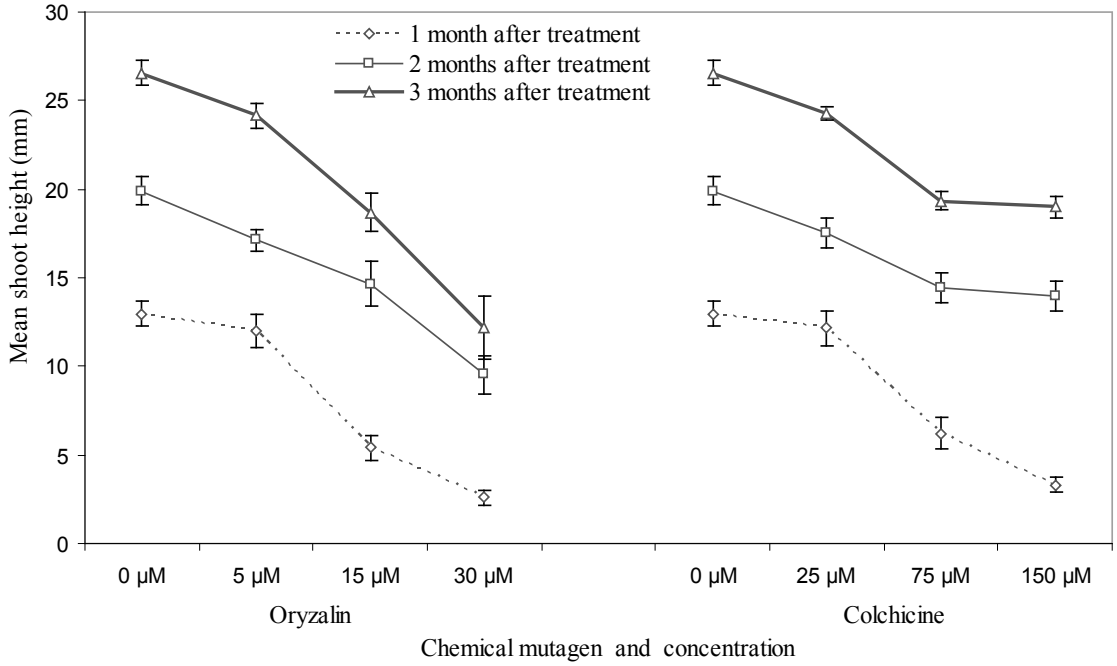


Figure 3.9: Effects of chemical mutagens and concentrations on shoot height of *W. japonica* after 1 month, 2 months and 3 months in culture on MS medium. Values are the means of 50 single shoots. Vertical lines represent standard errors.

By the sixtieth day, shoot height varied between concentrations and treatments. An increase in shoot elongation was experienced with 15 μM oryzalin and 75–150 μM colchicine, where there were no differences compared to that in untreated plants. Explants with 30 μM oryzalin treatment still maintained a reduction in shoot height ($p= 0.049$). Oryzalin at 5 μM and colchicine at 25 μM retained a balanced rate in shoot elongation, compared to the control.

By the ninetieth day, shoot elongation was significantly reduced in both mutagens at high doses, and dramatically decreased in high doses of oryzalin ($p= 0.003$). A reduction in shoot length was repeated in 15–30 μM oryzalin and 75–150 μM colchicine. However, a dose at 30 μM of oryzalin inhibited shoot length more effectively than a 15 μM dose. The maintenance in shoot elongation as the untreated control was detected in 5 μM oryzalin and 25 μM colchicine.

3.3.1.2.4 Morphological variations of shoots and leaves

Phenotypic variations occurred among the regenerated explant materials treated with high doses of mutagens (30 μM oryzalin and 150 μM colchicine) on the second week in culture. The two outermost leaves of explants with 15–30 μM oryzalin or 75–150 μM colchicine were fragile and phenotypically different from the control leaves (Figure 3.10b, c).

In the exposures with colchicine, browning of leaves and shoots occurred on day 35 for the dose of 150 μM, and day 60 for the dose of 75 μM. Shoot blackening was experienced only in 150 μM colchicine on day 60. In the treatments with oryzalin, however, browning and blackening of leaves and petioles occurred earlier. On day 30 in culture, both 15 μM and 30 μM of oryzalin caused browned shoots, and shoots turned black on day 40. Leaf deformity was also detected in high doses of both colchicine and oryzalin on day 60. Leaves were cleaved, with a gradually narrowed surface area (Figure 3.10b, c). Healthy shoots were observed in treatments with oryzalin at 5 μM and colchicine at 25 μM until day 90. These low doses of oryzalin and colchicine also induced callus-like rhizomes of larger size than those in untreated plants (Figure 3.11a, b). Unhealthy shoots were, however, observed only in treatment with 30 μM oryzalin after 60 days, and in treatments with both 30 μM oryzalin and 150 μM colchicine after 90 days in culture. Callus-like rhizomes still occurred on the bases of unhealthy shoots on day 90 with larger sizes found in 30 μM oryzalin than in 150 μM colchicine treatments (Figure 3.12a, b).

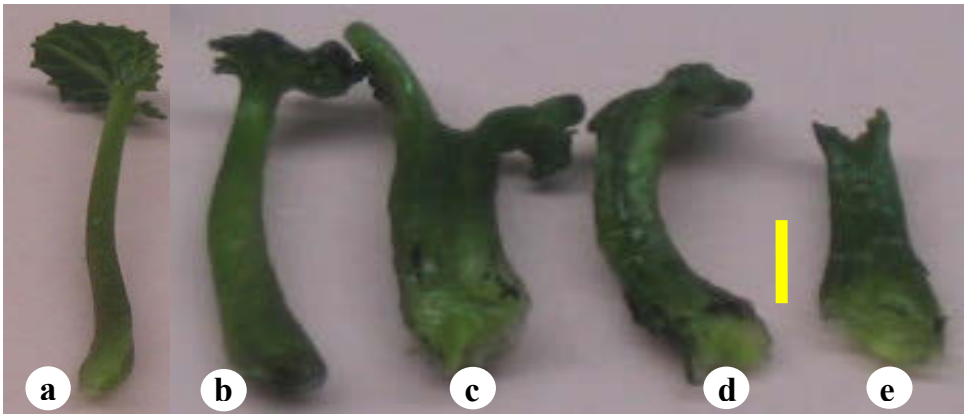


Figure 3.10: Deformed and fragile leaves of *W. japonica* produced by mutagen treatments at high doses after 2 and 3 months in culture ((a) control, (b) 75 & 150 µM colchicine, (c) 15 & 30 µM oryzalin, (d) 40 Gy of gamma rays and (e) 40 Gy of X-rays)) (bar = 0.5 cm).

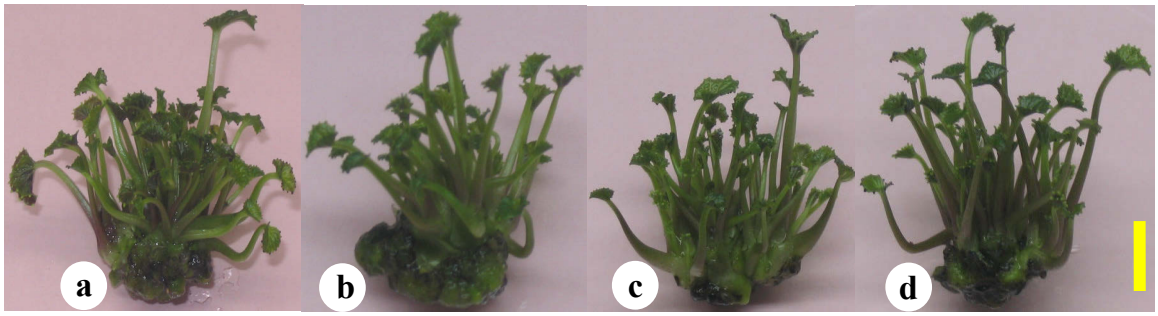


Figure 3.11: Healthy shoots of *W. japonica* with abundant callus-like rhizomes at the shoot bases obtained by mutagen treatments at low doses after 3 months in culture ((a) 25 µM colchicine, (b) 5 µM oryzalin, (c) 10 Gy of gamma rays and (d) 10 Gy of X-rays)) (bar = 1cm).

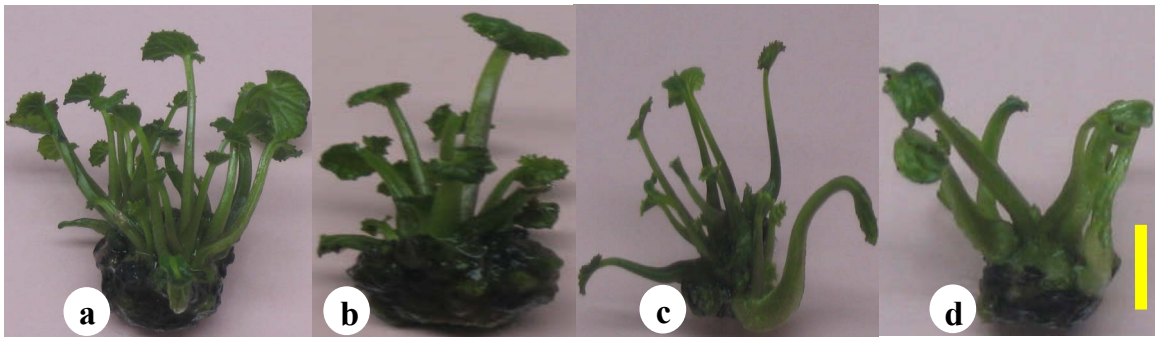


Figure 3.12: Unhealthy shoots of *W. japonica* with some necrosis formed by mutagen treatments at high doses after 3 months in culture ((a) 150 µM colchicine, (b) 30 µM oryzalin, (c) 40 Gy of gamma rays and (d) 40 Gy of X-rays)) (bar = 1 cm).

3.3.1.3 Survival of explants in vitro *and* in vivo

Explant survival *in vitro* and *in vivo* was also affected by chemical mutagen treatment (Table 3.1). After three months of culture *in vitro*, the survival rates of shoot tips treated with the highest concentrations of oryzalin and colchicine decreased to 70% and 88%, respectively, while the control maintained the highest survival rate, at 100% (p= 0.001). A slight and equal reduction (90%) in explant survival was found from either 5 µM oryzalin or 75 µM colchicine, but not significantly different from the control. At a low dose (25 µM) of colchicine, the survival rate was reduced by only 4% after three months in culture, which showed no difference from the untreated plants. Explant viability decreased to 74% in 15 µM oryzalin; however, it exhibited no different response from the lowest concentrations of mutagens.

The survival rate of explant at the one-month growth stage in the greenhouse conditions was slightly greater than that at three months *in vitro*. The lowest reduction in explant survival to 80–82% was observed from oryzalin at both doses of 15 µM and 30 µM (p= 0.000). There was no significant difference in the survival (98%) between a high dose of colchicine and the control. Low doses of oryzalin and colchicine still maintained the highest explant survival rate (100%), as with the control. The mortality of explants grown *in vivo* appeared on the third week after transplantation. On the fourth week of *in vivo* growth, no further necrosis of explants occurred.

After two months of further development of plants *in vivo*, morphological observations appeared to show that there were variations in leaf parameters in several treated plants when compared to the control plants (data not shown). A difference in leaf shape was observable in 5 µM oryzalin, while treatment with 150 µM colchicine varied the leaf size. Four out of 50 plants exhibited oval-shaped leaves, and two out of 49 plants produced heart-shaped leaves in smaller sizes. All untreated plants remained with normal heart-shaped leaves. Leaf-altered characteristics were not detected in any other treatments. There were no other changes observed in all treatments after a two-month growth of plants.

Table 3.1: Survival rates of *W. japonica* shoot tips treated with oryzalin and colchicine after 3 months in culture *in vitro* and 1-month growth *in vivo*.

Chemical mutagens and concentrations	Survival rate (%)[a]	
	3 months *in vitro*(1)	1 month *in vivo*(2)
Control[b]	100±0c[c]	100±0b
Oryzalin = 5 µM	90±4bc	100±0b
Oryzalin = 15 µM	74±6c	82±6a
Oryzalin = 30 µM	70±7a	80±6a
Colchicine = 25 µM	96±3c	100±0b
Colchicine = 75 µM	90±4bc	98±2b
Colchicine = 150 µM	88±5b	98±2b
P value	0.001	0.000

[a]Values are means ± standard errors of three independent experiments consisting of a total of 50 explants.
[b]Control denotes both MS medium free of growth regulators for (1) *in vitro* cultures and (2) untreated plantlets grown in the greenhouse.
[c]Means within a column followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

3.3.2. Physical mutagen treatments

3.3.2.1 Survival of explants in vitro and morphological variations

Irradiations with doses at 0, 10, 20, 40 and 80 Gy had effects on the survival of explants after one, two and three months in subsequent culture (Figure 3.13). It was found as a general trend in the treatment with irradiation that when the irradiated dose and culture length increased, the number of regenerating explants was reduced, and the mortality and unregenerating explants increased.

In the first 30 days after exposure, explants treated with 10 Gy exhibited the highest survival rate as found in untreated control plants (100%) (Figure 3.13a). This consistency was also identified in the exposure with gamma irradiation at 20 Gy. X-irradiation at 20 Gy, however, induced necrotic and unregenerating shoots at a rate of 4% and 8%, respectively. The application of 40 and 80 Gy of gamma rays did not induce mortality, but inhibited the number of regenerating shoots at 8% and 16%, respectively. The 40 Gy and 80 Gy treatments with X-irradiation induced both mortality (8% and 40%) and unregenerating (12% and 16%) shoot numbers. It was evident that the rates of regenerating in shoots were reduced more quickly with X-irradiations than with gamma irradiations, and ranged from 44% to 100% in X-ray treatments and 84–100% in gamma-ray treatments. The dose closest to LD_{50} was 80 Gy from X-irradiation.

The health of explants was stable in low dose treatments, and gradually degraded by treatments with increasing doses, within 30 days. Shoot necrosis was detectable in treated plants during two weeks of exposure. The 80 Gy doses from two irradiation sources produced increasingly necrotic leaves and shoots after four weeks in culture (Figure 3.14). Deformity in the leaves occurred with irradiations at 80 Gy, but not with irradiations at lower doses.

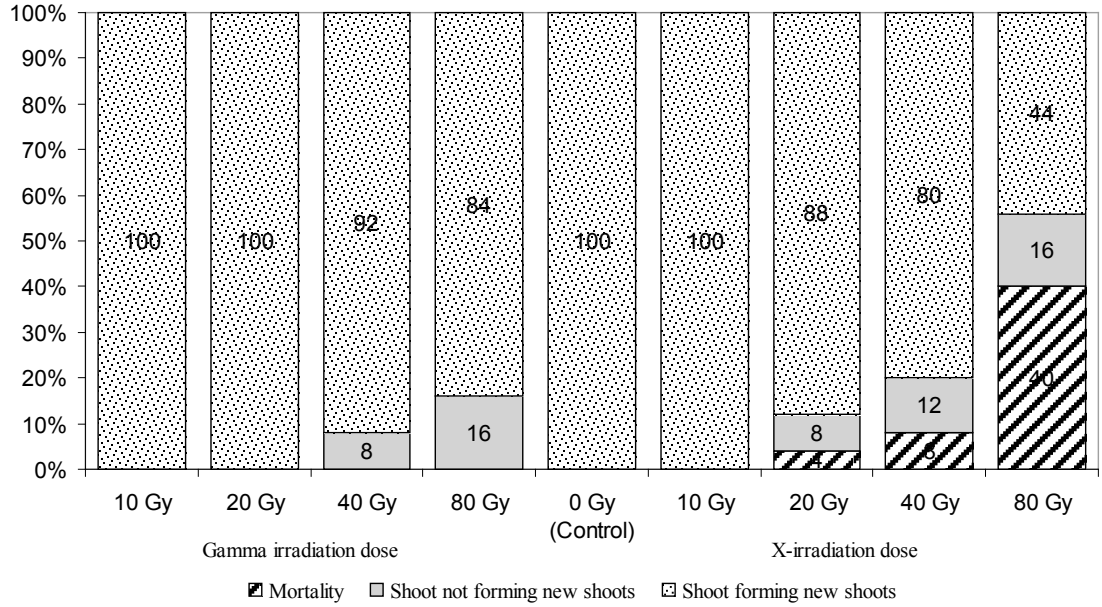
By the sixtieth day, the increase in mortality rate of explants reduced survival to 96%, 88% and 72% with X-rays, and 100%, 96% and 88% with gamma rays, of 10, 20 and 40 Gy, respectively (Figure 3.13b). The highest dose (80 Gy) from two types of irradiation led to a complete death or absence of regeneration in the explants. The 10 Gy treatment with gamma irradiation maintained the highest rate of survival (100%). There was an occurrence of unregenerating explants irradiated with 10 Gy of X-radiation and 20 Gy of gamma radiation, at a frequency of 4%. Irradiation of the explants with 40 Gy resulted in the presence of the unregenerating shoots at an increased rate in gamma radiation (from 8% to 12%), and at a reduced rate in X-radiation (from 12% to 8%). A similar result was evident with the 20 Gy of X-rays, where the absence of regeneration in new shoots occurred at a reduced rate from 8% to 4%. Nevertheless, the mortality rate increased from 4% to 8% with this dose of X-irradiation from 30 days to 60 days in culture. A dramatic increase in the mortality of explants was observed with 40 Gy of X-ray at a rate from 12% to 20% after a further one month in culture. Necrosis

of shoots was not detected in the treatments with 20 and 40 Gy of gamma irradiations, while it was detectable with X-irradiation at the same doses.

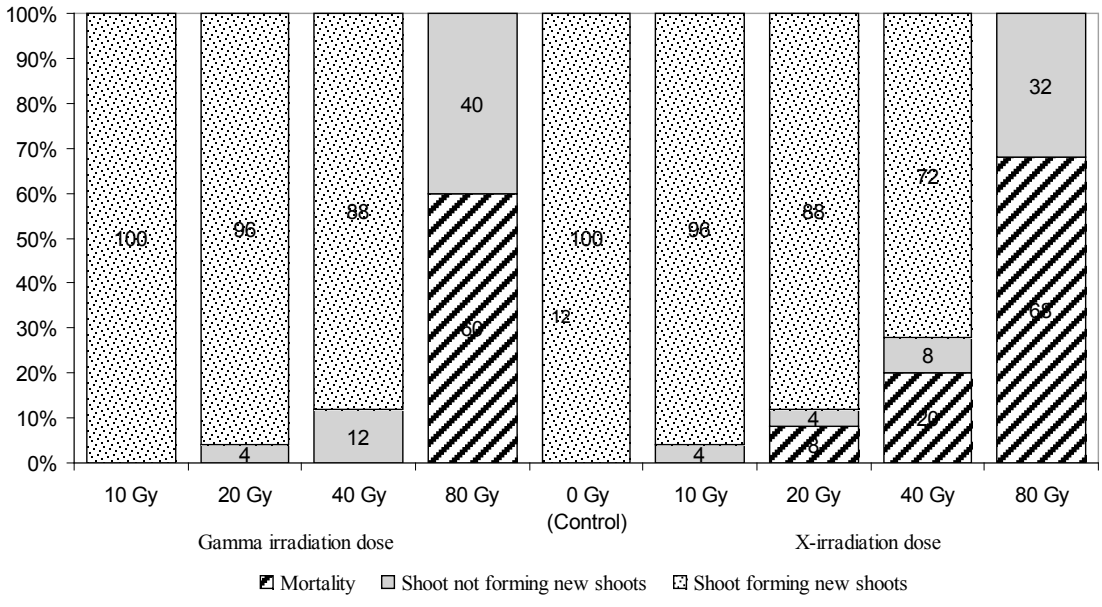
Explant health under the 20 and 40 Gy doses of both irradiations was improved by the end of the second month. However, browning of shoots under the 80 Gy treatments continued to occur until all the explants were totally dead. Deformity in the leaves started to appear under the 40 Gy treatments (Figure 3.10d, e). Leaves were fragile and much smaller than in the control, with short and swelling petioles of a bigger size. The majority of explants with the 40 Gy dose formed outermost leaves with areas as narrowed as petioles. Deformed leaves were not detectable with the 10 Gy of both irradiations.

Following 90 days in culture, the growth of explants was affected by both low and high doses of radiations. There was a reduction in the regenerating explant numbers in all of the radiation doses. Necrosis of explants commenced under the 40 Gy of gamma rays from unregenerating shoots, at a mortality rate of 4% (Figure 3.13c). The presence of explants unable to produce shoots was evident under the 10 Gy treatment with gamma irradiation at 4%. Most of the explants that regenerated at a dose of 20 Gy on day 30 were unable to survive on day 90. An average of 4% and 8% of mortality rates was found under the 20 Gy dose of gamma ray and X-ray, respectively, from regenerating explants. The percentage of explants that inhibited further shoot formation was found to increase under the 20 Gy treatment by 4% and to stabilise under the 40 Gy of X-ray at 8%. Increasing the quantity of unregenerating explants by 4% was also identified under the 10 Gy of X-ray. Irradiation with 80 Gy of both gamma ray and X-ray led to a reduction in the unregenerating explants (40–24% and 32–16%, respectively) as a result of an increase in lethal explants. The absence of regenerating explants continued to be maintained in this highest dose. Leaf abnormality was more detectable under the 40 Gy of X-radiation than in gamma radiation (Figure 3.10d, e).

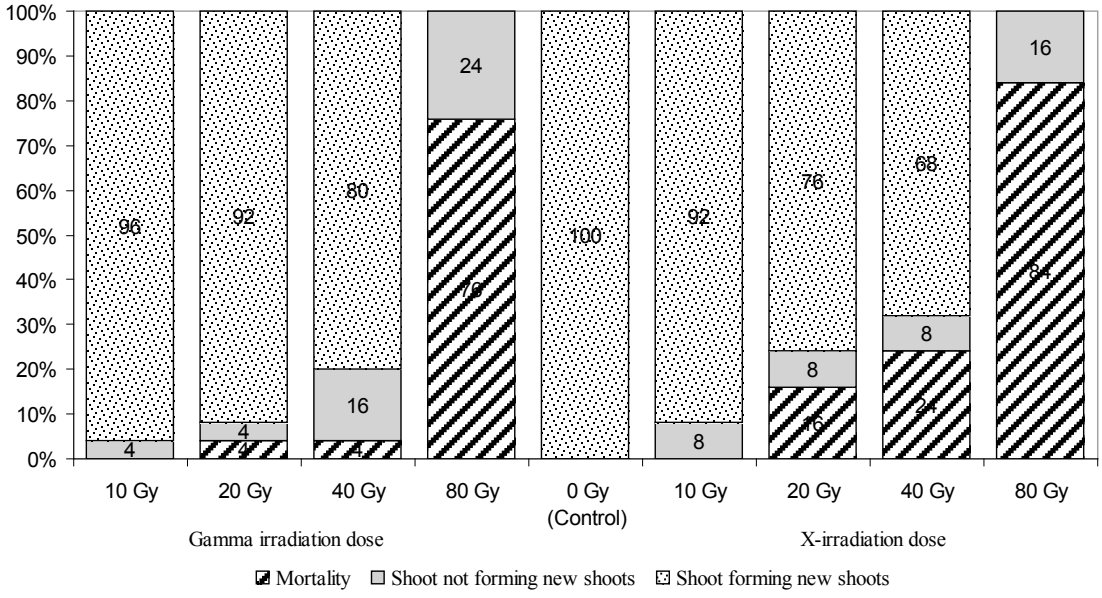
As the dose of 80 Gy caused a high mortality rate from day 60 in culture (60–68%), it was excluded from the following assays on the growth and development of explants. Based on radiosensitivity, the dosage range from 0 Gy to 40 Gy was, therefore, selected to assess the morphological variations of explants throughout a culture period of three months.



(a) Mortality rates of explants treated with irradiations after 1 month in culture.



(b) Mortality rates of explants treated with irradiations after 2 months in culture.



(c) Mortality rates of explants treated with irradiations after 3 months in culture.

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 *W. japonica* shoot tips at (a) 1, (b) 2 and (c) 3 months in culture on MS shoot proliferation medium. Values are the means of 25 replicates.

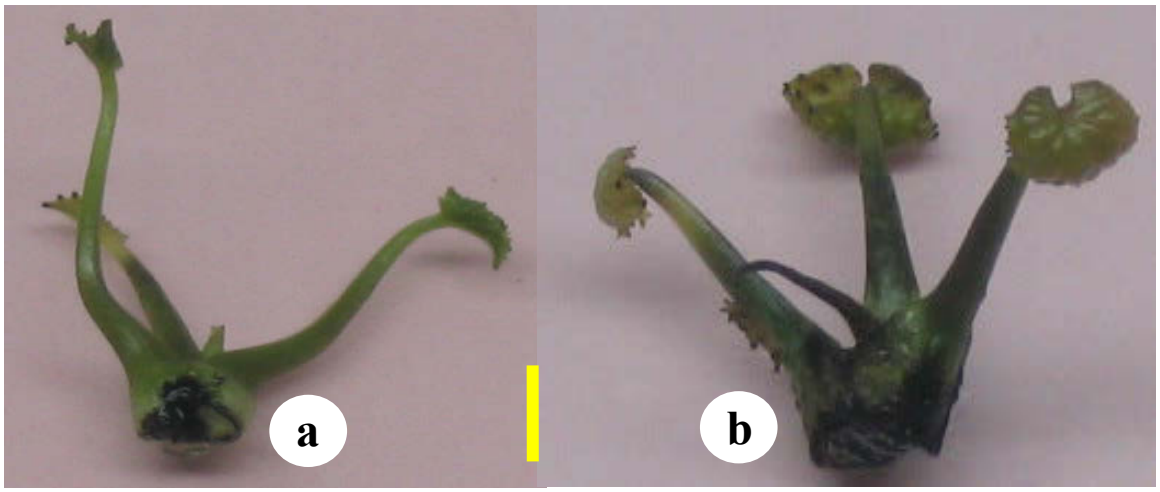


Figure 3.14: *W. japonica* shoots exposed to irradiations at high doses ((a) gamma rays at 80 Gy and (b) X-rays at 80 Gy)) after 4 weeks in culture exhibited necrosis (bar = 0.5 cm).

3.3.2.2 Effect of physical mutation on in vitro growth

3.3.2.2.1 Explant weight

Analysis of variance showed a significant effect of irradiation on explant weight from the application of 0, 10, 20 and 40 Gy of gamma ray and X-ray following 30, 60 and 90 days in culture (Figure 3.15 and Appendix 3.6).

In the first month after treatment, the difference in explant weight was observable only between a 40 Gy treatment and other treatments (p= 0.006). Irradiations of the explants with 40 Gy resulted in a drop in explant weight to 67.2% and 62.6% over the untreated explants for X-ray and gamma ray, respectively. Between the 40 Gy of gamma ray and 40 Gy of X-ray, the explant weight revealed no significant effects. There were also no significant differences in the weight of shoots among 0, 10 and 20 Gy treatments. The only dose that assisted the explant weight to resemble all other doses was the 20 Gy of X-ray.

In the second month after treatment, the effects of irradiation on explant weight, however, varied dramatically, and were significantly different among irradiation doses. Significant difference was shown between the control and 20 Gy treatments with X-irradiations (p= 0.033). The 20 Gy also induced a higher explant weight than the 40 Gy of X-ray. Similarly, shoots treated with the 20 Gy of gamma ray showed a significant increase in explant weight compared to the 40 Gy. However, the 20 Gy of gamma ray showed no significant difference in explant weight from its control. In both irradiations at a dose of 10 Gy, the explant weight was similar to that of the controls.

The rapid growth of callus-like rhizomes in treatments at low doses continued to increase the explant weight and differ the irradiation doses in the third month (p= 0.005). The weight of shoots was improved with the 20 Gy of X-ray but was not significantly different from the control. The 40 Gy of X-ray maintained a lower level in the rates of explant weight at 39% and 33% over the 20 Gy and control treatments, respectively. Explants under the 40 Gy of gamma ray produced a significantly lower weight accounting for 53% of the size of its control, but showed no difference with the 20 Gy treatment. Explant weights induced with the 10 Gy of X-ray, and 10 Gy and 20 Gy of gamma rays demonstrated no significant differences from the controls. Between the same dose levels of 10, 20 and 40 Gy, the reduction of explant weights was observed with X-rays compared to gamma rays on day 60, but this was undetected on day 90.

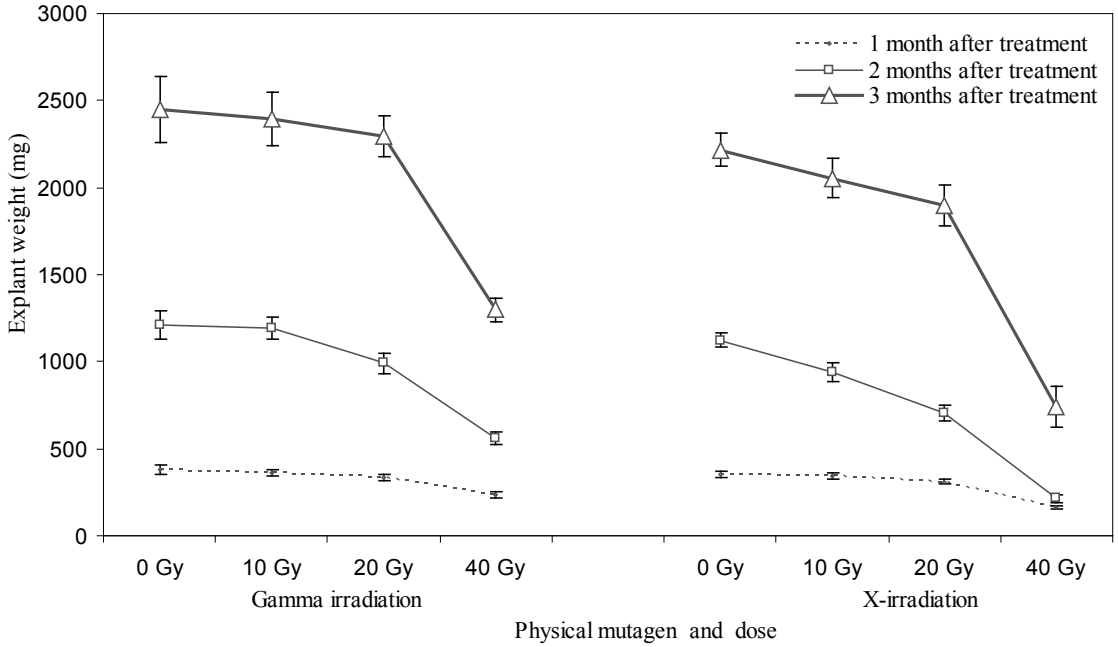


Figure 3.15: Effects of physical mutagens and doses on the explant weights of *W. japonica* after 1 month, 2 months and 3 months in culture on MS medium. Values are the means of 25 single shoots. Vertical lines represent standard errors.

3.3.2.2.2 Shoot multiplication

There were no differences in responses in shoot proliferation between the same levels of treatments with X-rays and gamma rays within three months in culture (Figure 3.16 and Appendix 3.6). However, among different dose levels, the effects of irradiations on shoot multiplication rate occurred strongly after one month and slightly more after two to three months in culture.

By the thirtieth day after treatment, explants under the 20 Gy and 40 Gy exhibited a significantly lower multiplication rate accounting for 80% and 53% for gamma rays, and 80% and 37% for X-rays, respectively, of the number of shoots of the controls ($p= 0.000$). Of these two treatments, the 40 Gy had significantly higher inhibitory effects in multiplication rate than did the 20 Gy. Reduction in shoot production was not significantly detectable between the 10 Gy and control treatments. There was also no difference in shoot number between the 20 Gy and 10 Gy treatments with irradiation.

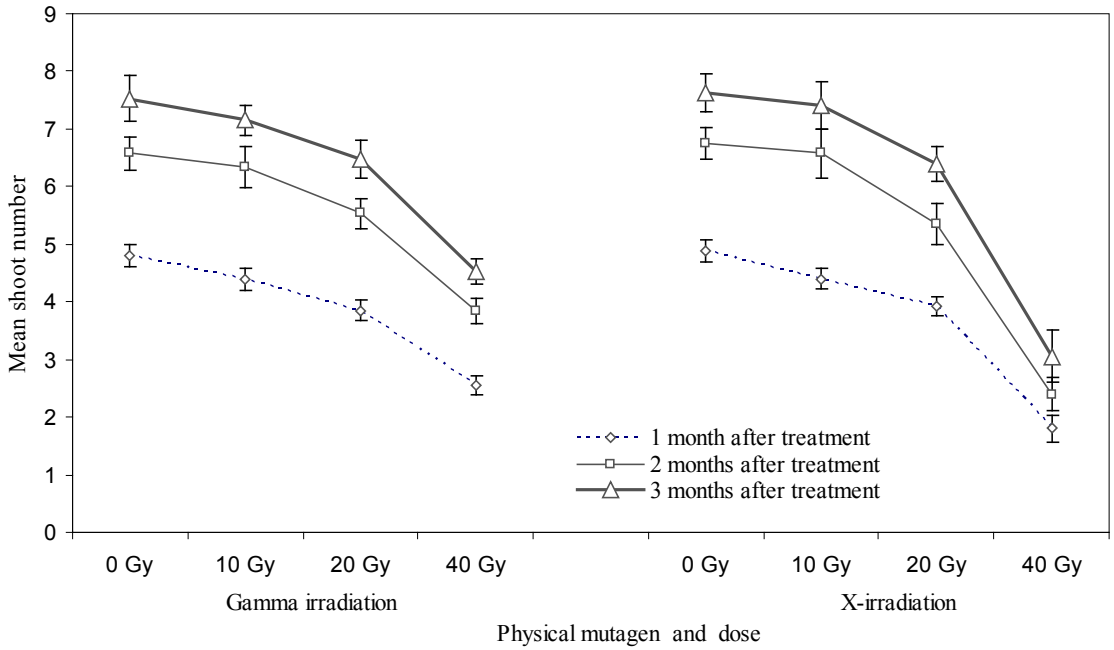


Figure 3.16: Effects of physical mutagens and doses on shoot numbers of *W. japonica* after 1 month, 2 months and 3 months in culture on MS medium. Values are the means of 25 single shoots. Vertical lines represent standard errors.

Following 60 and 90 days in culture, shoot numbers significantly fewer than the control were observable only with the 40 Gy treatments. Irradiations with 10 Gy and 20 Gy of gamma rays and X-rays produced shoot numbers non-significantly different from each other and from the controls.

Less callus-like rhizomes were produced with irradiations than with colchicine or oryzalin. Healthy shoots were observable with 10 Gy treatments, but not with 20 Gy treatments. Unhealthy shoots were detectable under the 40 Gy of irradiations. Shoots with deformed leaves were found less with 40 Gy of gamma ray than with 40 Gy of X-ray (Figures 3.11c, d and 3.12c, d). Unlike the deep green leaves and thick petioles observed in explants treated with chemical mutagens, light green leaves and normal-sized petioles mostly arose from shoots exposed to irradiation.

3.3.2.2.3 Shoot height

The effects of irradiation on shoot height occurred slightly after one month and more obviously after two to three months in culture (Figure 3.17 and Appendix 3.6). In the first 30 days after treatment, the 40 Gy of X-ray, and 20 Gy and 40 Gy of gamma rays produced significantly shorter shoots than did the control ($p = 0.044$). Differences in shoot length among these three treatments were not found. Treatments with 10–20 Gy

of X-rays and 10 Gy of gamma ray were identified to produce no differences in shoot length from the controls.

By the sixtieth day, treatments by X-rays at both 20 Gy and 40 Gy produced significantly shorter shoots than did the control (p= 0.049). Lower doses of X-irradiation reduced shoot length non-significantly compared to the control. Similarly to X-ray treatments, shoot length was significantly reduced to 70% under the 40 Gy, and revealed no differences in the 10 Gy and 20 Gy of gamma rays, compared to the control.

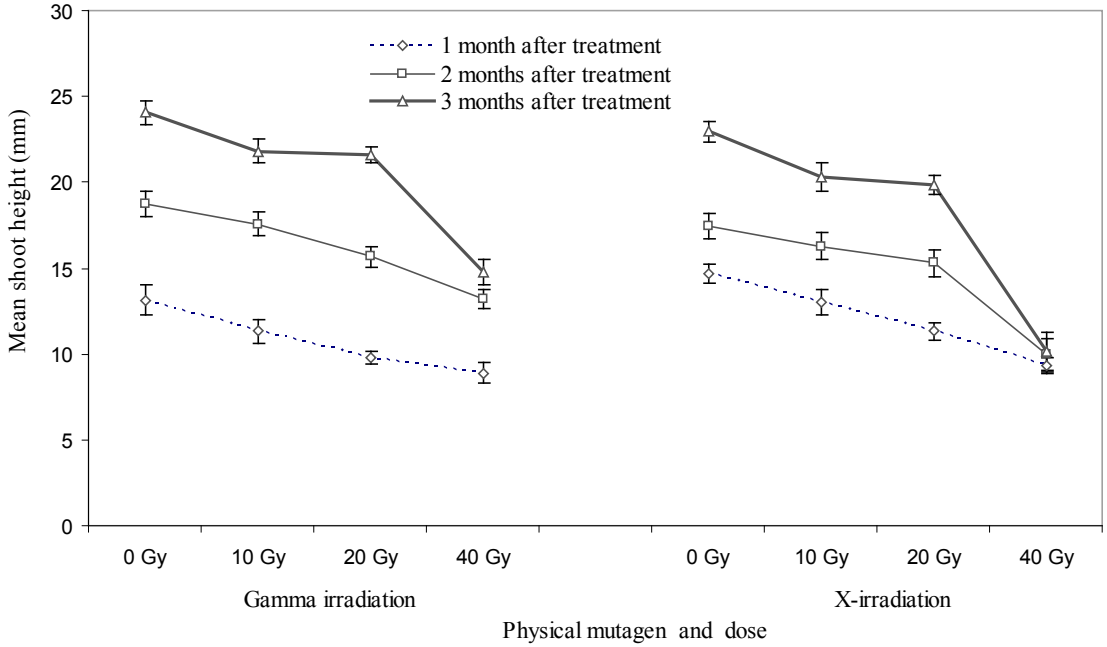


Figure 3.17: Effects of physical mutagens and doses on shoot heights of *W. japonica* after 1 month, 2 months and 3 months in culture on MS medium. Values are the means of 25 single shoots. Vertical lines represent standard errors.

Following 90 days in culture, the 40 Gy treatments of X-ray and gamma ray were significantly different to the controls, with shoot lengths 44% and 61% shorter than the controls (p= 0.000). Shoots were also significantly 69% shorter with 40 Gy of X-ray than with 40 Gy of gamma ray. Shoot length was still unaffected by treatments of 10 Gy and 20 Gy of both types of radiation. The 40 Gy of X-ray obviously exhibited no significant difference in shoot length between 30, 60 and 90 days in culture.

3.3.2.3 Survival of explants in vivo

It was shown in Table 3.2 that irradiation with gamma rays and X-rays led to an effect on the survival of rooted shoots following a four-week transplantation. There was no significant difference between the *in vivo* survival rates of the plantlets in the 10 Gy and

20 Gy gamma irradiation treatments, compared to the control (p= 0.102) (Table 3.2). Nevertheless, there was a drop in the survival rate of the plantlets in the 40 Gy gamma irradiation treatment, with 90% of plantlets surviving, compared to 100% in the control treatment.

Likewise, plantlets under the 40 Gy X-irradiation treatment showed a significantly poorer survival rate, which occupied only 70%, compared to 100% under the control treatment (p= 0.044) (Table 3.2). Plantlets under the 20 Gy X-irradiation treatment exhibited a significantly greater survival rate than plantlets treated with 40 Gy of X-ray, but demonstrated a significantly poorer survival rate than the control. The 10 Gy of X-ray and gamma ray showed no difference in the *in vivo* survival rate of plantlets. Similarly, no difference was detectable between plantlets treated with 20 Gy of X-ray and 40 Gy of gamma ray; however, plantlets in the former treatment had a significantly poorer survival rate than plantlets treated with 20 Gy of gamma ray.

Table 3.2: Survival rates of *W. japonica* shoot tips treated with gamma rays and X-rays after a one-month growth in the greenhouse.

Radiation dose	Survival rate (%)[a]	
	Gamma irradiation	X-irradiation
Control[b]	100±0c[c]	100±0c
10 Gy	98±2c	96±3c
20 Gy	96±3c	90±4b
40 Gy	90±4b	70±7a

[a]Values are means ± standard errors of three independent experiments consisting of a total of 50 explants.
[b]Control = Untreated plantlets grown in the greenhouse.
[c]Means followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

3.4 Discussion

3.4.1 Mutation induction with polyploidy-inducing agents

This is the first report on the performance of *in vitro*-grown *W. japonica* after a mutagenic treatment. Treatments of *W. japonica* shoot-tips with polyploidy-inducing agents (colchicine and oryzalin) affected explant growth, multiplication and survival. The influence on explant survival was worse with higher concentrations and longer

treatment durations (Figures 3.2, 3.3 and 3.4). Oryzalin appeared to have a more adverse influence than colchicine. As little as 5 μM of oryzalin influenced the regenerative capacity of shoot tips, after a treatment length of even two days. The regenerable capacity of explants treated with colchicine at 25 μM was unaffected until day 4 in treatment and after four weeks in culture. The higher toxicity of oryzalin compared to colchicine was also found in the chromosome-doubling induction of onion clones when treated with a lower dose of oryzalin (50 μM) than of colchicine (2.5 mM) for providing an optimal number of double-haploid plants (Geoffriau *et al.*, 1997). This is similar to the findings by de Carvalho *et al.* (2005) in annatto (*Bixa orellana*), Timofeeva *et al.* (2000) in winter wheat and van Tuyl *et al.* (1992) in *Lilium* and *Nerine*. However, a work in the breeding of *Musa acuminate* by *in vitro* polyploidisation indicated a contradictory effect on the toxicity of these two agents, wherein colchicine proved to be more phytotoxic than oryzalin (van Duren *et al.*, 1996). It seems that the toxic difference in these two antimitotic agents is species dependent.

In the treatments on a range of different plant species with chromosome-doubling agents, a detrimental effect was visible on the *in vitro* growth stages of explant (Wan *et al.*, 1991; Takamura and Miyajima, 1996; van Duren *et al.*, 1996; Chauvin *et al.*, 2003). The beneficial effects, if any, commenced in the *in vivo* developmental stages or field-grown stages of plantlets. In the currently studied species of *W. japonica*, however, the positive influence on the *in vitro* proliferation was observable in the treatment with 25 μM colchicine. This level of colchicine significantly increased the explant growth after three months in culture, and shoot multiplication after one month in culture (Appendix 3.5). Lower levels of colchicine may also have increases in the *in vitro* growth of *W. japonica* shoots. However, experiments with the lower-treatment concentrations were not conducted in our project. The effects of colchicine at concentrations lower than 25 μM on the growth of *W. japonica* apparently need further investigations because these levels can be used to stimulate microshoot production of wasabi *in vitro* with little fear of its producing genetic variations. However, chromosome counts were beyond the scope of this study. The phenomenon of wasabi plant growth enhancement by low-dose treatments with colchicine needs to be further investigated in a range of plant species for a reasonable and adequate explanation of the action mechanism of colchicine in the plant. It is known that, biochemically, the toxicity of colchicine and oryzalin is responsible for the cell-division suppression. Oryzalin was

attributed to the inhibition of microtubule formation, triggering a disruption in mitosis in explant meristematic cells (Strachan and Hess, 1983; Morejohn *et al.*, 1987), while colchicine was attributed to the interference with the meiotic prophase (Loidl, 1988).

Dimethyl sulphoxide (DMSO) represents one of the solvents that has been used with chemical mutagen treatments for improving the uptake of these agents into the explants, due to a greater permeability of plant cells (Omar *et al.*, 1989; van Duren *et al.*, 1996; Timofeeva *et al.*, 2000). The application of DMSO with oryzalin at 5 μM and colchicine at 25 μM or 75 μM in the gelled culture medium appeared to be useful for the *in vitro* mutation induction of wasabi explants. Using oryzalin at 15–30 μM and colchicine at a high concentration (150 μM) with 1% DMSO resulted in a relatively high mortality rate of wasabi shoot tips. It has been reported in the polyploid induction of banana plantlets *in vitro* that a 2% solution of DMSO stabilised a high rate of autotetraploids in the treatment with 0.5% colchicine, whereas DMSO reduced the survival of explants exposed to higher levels of colchicine (Hamill *et al.*, 1992). It may be implied that DMSO in combination with high doses of colchicine is a trigger for increasing the lethality rate in wasabi explants. A 1% solution of DMSO is, thus, identified as an optimal level for dissolving chromosome-doubling agents, which are effective for wasabi plantlet growth, when used in low concentrations. In addition to strengthening the penetration of colchicine into the plant cells, DMSO is regarded as a plant cryopreservant causing damages to chromosomes, as found in the induced mutation of *Solanum* spp. (Chauvin *et al.*, 2003) and *Manihot esculenta* (Awoleye *et al.*, 1994), particularly in combination with oryzalin. Hence, the effects of DMSO on wasabi shoot tips need to be further studied.

Greenhouse selection has been attempted for the continuous two months after transplantation. Several plants displayed interesting leaf traits. Out of 150 plants treated with oryzalin and 149 plants treated with colchicine, four plants (2.67%) with oval-shaped leaves were obtained from the 5 μM oryzalin treatment, and two plants (1.34%) with smaller sized leaves in normal heart shapes were detected from the 150 μM colchicine treatment. The mutation rate, as found in the majority of plants, is very low (Broertjes, 1972; Singh and Sharma, 1993), possibly owing to the recessive mutations. The changes in the leaf shape of wasabi *in vivo* are not in complete relation to the changes in the AITC contents *in vitro*, since the treatment with 5 μM oryzalin

failed to alter the AITC level while inducing a leaf morphology alteration (Figure 4.8). Perhaps these plants of altered leaf shape are not polyploids. This is in agreement with the fact that polyploids typically display larger plant parts, such as fruits, leaves and flower petals, than the diploids (Takamura and Miyajima, 1996; Shao *et al.*, 2003). Somaclonal variation may be attributed to this case in wasabi, because it was identified in a great number of species (Buiatti, 1989). Observation and analysis of these leaf-altered wasabi plants, however, need to be carried out for identifying their responses and ploidy levels. Furthermore, plant variations need to be assessed through many subsequent generations for ensuring that mutants produced are stable.

3.4.2 Mutation induction with ionising-irradiations

The treatment of *W. japonica* shoot tips with radiation (gamma rays and X-rays) *in vitro* affected explant growth, multiplication and survival. X-irradiation seemed to have a greater influence on the growth of explant than gamma radiation from the second month after treatment. Reduction in weight was observed from 20 Gy onwards in X-irradiation treatments, but only from 40 Gy gamma treatment, from the second subcultures. Likewise, reduced shoot lengths were found from the 20 Gy X-ray treatment, but only from the 40 Gy gamma treatment. Shoot multiplication was, however, not different between the two types of radiations during an incubation period of three months after treatment. The significant effects of multiplication in the treatments with these two rays were detected from 20 Gy on the thirtieth day, but were visible only from 40 Gy on the sixtieth and ninetieth days. The survival rates were lower in X-irradiation treatments than gamma treatments during 90 days in culture, and were reduced with higher doses applied.

The greater detrimental effects of X-rays compared to gamma rays may be partially attributed to the fact that the X-irradiation dose rate (820 monitor units/minute equivalent to 11.71 Gy/minute) was higher than the gamma radiation dose rate (6.65 Gy/minute). The influence of X-rays on the wasabi plant tissue might have been more detrimental if the explants had been irradiated without the addition of water. In *Prunus avium* (sweet cherry), the frequency of mutations under exposure to the air environment was markedly higher than in explants exposed to water (Saamin and Thompson, 1998). The interference of water that contributes to the reduction of X-ray capacity in plant mutations was also observed in seeds where the radiosensitivity by

X-rays was higher with lower water content (IAEA, 1977). In contrast, data in the literature (Ahnström, 1989) showed that OH^- radicals can be separated from water molecules by the ionising irradiations, including X-irradiation and gamma radiation (Halliwell and Gutteridge, 1989), and lead to the changes in DNA of plant cells by reacting with H^+ atoms derived from the DNA structure, which consequently stimulate the mutation rate increase. The contradictory impact of water on the mutations by irradiation needs to be considered in more detail from other reports.

The reduction in the regenerative capacity of irradiated cultures has been widely reported for various crops for which radiation dosages are different when they begin to affect the regenerative capacity. In the treatment of *Rosa hybrida* leaf explants by X-rays, it has been found that the regenerating rate decreased between 47% and 0% when doses increased from 25 Gy to 100 Gy, respectively (Ibrahim *et al.*, 1998). A decreasing rate of regeneration was also observed in *Dianthus* with a 20 Gy dose (Cassells *et al.*, 1993) and in apples with a 30 Gy dose (James *et al.*, 1988) from X-irradiations. Similarly, treatments of explants *in vitro* with gamma radiation have been shown to reduce the multiplication rate of *Alpinina purpurata* irradiated at a 15 Gy dose onwards (Fereol *et al.*, 1996), to decrease the production of microtubers of *Solanum tuberosum* at a dose from 10 Gy (Al-Safadi *et al.*, 2000) and to decline to 50% in the survival rate of lotus (*Nelumbo nucifera* Gaertn.) when irradiated at 2 krad (Arunyanart and Soontronyatara, 2002).

The detrimental effects on wasabi explant growth tended to be reduced over a subculture length of three months after treatments by both gamma rays and X-rays. In the X-ray treatments, the 20 Gy dose significantly decreased the explant weight in the second subculture, but not in the third subculture, compared to the non-treated control. Similar responses for shoot multiplication in the X-irradiation experiments were observable between the first subculture and second subculture. However, there was a balance in the reduction in the effect on shoot height over time by X-ray treatments. Similarly for gamma irradiation treatments, the rates in shoot multiplication and shoot height were significantly reduced from the unirradiated control in the 20 Gy treatment in the first subculture, but not in the second and third subcultures. A detrimental influence was observed on the explant weight that failed to increase when treated with gamma rays, but remained stable, over three successive subcultures. Similar behaviour

in the decline in effects over time of X-rays were observed in the *in vitro* explant of *Dianthus* (Cassells *et al.*, 1993) showing a reduction in secondary nodal production from primary nodes over successive subcultures, but not in the third subculture, and of gamma rays as detected in tissue-cultured plants of *Alpinia purpurata* (Fereol *et al.*, 1996).

The formation of chimeral mutants, one of the primary problems in mutagenesis, has occurred in a variety of plant species exposed to ionising radiations (Broertjes, 1966; de Loose, 1979; Parliman and Stushnoff, 1979; Roest *et al.*, 1981; Cassells *et al.*, 1993; Mandal *et al.*, 2000a). Chimerism was less observed in all experiments with gamma rays and X-rays irradiating wasabi explants *in vitro*. It is possible that the chimeric problem in mutagenesis is reduced by irradiating *in vitro* shoots as detected in *Pyrus communis* (Predieri and Zimmerman, 2001), in comparison with the irradiation of *in vivo*-grown shoots and seeds. The findings by other authors (Broertjes, 1972; Miedema, 1973; Roest *et al.*, 1981) demonstrate that the chimerism can be eliminated by the use of adventitious-bud techniques, because apices in buds derive from single cells. The low frequency of apparent chimeras by means of irradiating wasabi shoots may be due to the induction of the majority of non-chimeric solid mutants as observed in *Begonia* x *hiemalis* (Roest *et al.*, 1981), and partly due to the progressive reduction by dissolving chimeras during the three propagation cycles of subcultures as observed in *Dianthus* (Cassells *et al.*, 1993). The low rate of chimerism occurring in wasabi explants treated with gamma rays and X-rays is advantageous for the selection and micropropagation of promising mutants. This advantage could be perhaps explained on the concept of a monocellular apex; that is, the shoot apices on the wasabi shoot tips arise from single epidermal cells, which are similar to the formation of adventitious buds from leaf-derived callus.

3.5 Conclusions

Mutations can be achieved in *W. japonica* by treating shoot tip explants with either colchicine, oryzalin, X-rays or gamma rays. Oryzalin proved to be more phytotoxic to this species than did colchicine. Similarly, X-rays appeared to cause a higher mortality rate to explants than did gamma rays. Growth responses of plants were negatively affected by ionising irradiations and chemical mutagens at high doses. Several plants appeared to exhibit alterations on leaf shape and size after chemical treatments. Further

observations and assessments are needed of these *in vitro* and *in vivo* plants for several growing seasons to ensure that these mutations are stable.

CHAPTER 4

ANALYSIS OF BIOACTIVE COMPOUNDS – ALLYL ISOTHIOCYANATE

4.1 Introduction

Having experimentally produced *in vitro* stocks of *W. japonica*, both with and without the application of mutagenic techniques (Chapters 2 and 3), the next step required was to investigate AITC levels in the tissues produced. Analyses were therefore made of AITC concentrations in calli, and in shoot-derived plantlets, in both untreated (control) and experimental batches which had been subjected to chemical or physical mutagens.

A variety of isothiocyanates (ITCs) derived from glucosinolates (GSLs) provide a variety of different pungent tastes and the distinctive odour of *Wasabia japonica* (Depree *et al.*, 1999). The reactions required to form ITCs can be illustrated as below:

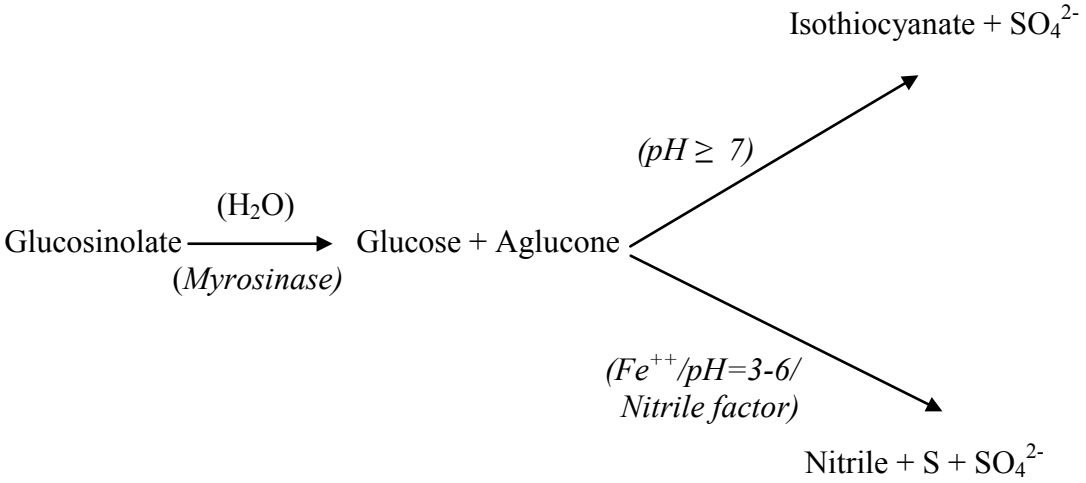


Figure 4.1: Conversion of glucosinolates to isothiocyanates.

The biosynthesis of GSLs (β-thioglucoside-*N*-hydroxysulphates) originates from amino acids. The amino acids synthesised into GSLs follow a common pathway. Some GSLs have a direct origin from protein amino acids; for example, alanine, valine and tyrosine contain methyl-, isopropyl-, and *p*-hydroxybenzyl side chains, respectively, but most

require several modifications in structure, that possibly occur prior to the formation of side chains (Fenwick *et al.*, 1983).

The major pungent component of *W. japonica* is allyl isothiocyanate (AITC), also known as 2-propenyl ITC. AITC has an antimicrobial activity (Isshiki *et al.*, 1992). The activity of AITC against some of the most commonly occurring food-associated micro-organisms, and its minimum inhibitory concentrations, are presented in Appendix 4.1.

Ina *et al.* (1989) discovered three important compounds in wasabi, namely, 6-methylthiohexyl ITC, 7-methythioheptyl ITC and 8-methylthiooctyl ITC, which give fresh odour, sweetish odour and weakly pungent odour, respectively. Fenwick *et al.* (1983) found several glucosinolates present in wasabi: methyl, isopropyl, 2-propenyl, 3-butenyl, 4-pentenyl, 5-hexenyl, 6-heptenyl, 3-methylpetenyl, 1-metylpropyl and 2-phenylethyl (by side chain). The principal ITCs extracted from *W. japonica* include allyl, n-butyl, 3-butenyl, 4-pentenyl, 5-hexenyl, 2-phenylethyl, 5-methylthiopentyl, 6-methylthiohexyl, 7-methylthioheptyl, 8-methylthiooctyl, 5-methylsulphinylpentyl, 6-methylsulphinylhexyl, and 7-methylsulphinylheptyl types (Depree *et al.*, 1999). Kumagai *et al.* (1994) compared the contents of volatile compounds in leaves, petioles, rhizomes and roots of soil-grown wasabi, as shown in Table 4.1.

Sultana *et al.* (2003a) analysed seven major ITCs from four main parts of the 18-month-old wasabi plants grown in flooded fields in New Zealand (Table 4.2). The results show that the distribution of ITCs differs in the various plant parts. The total ITC content in rhizomes was higher than that in other plant parts, of which, the AITC content was the highest. It was further found that the total ITC level in the epidermis and vascular tissue of rhizomes in flowering wasabi was higher than that in non-flowering wasabi at the same age, and that outer tissues of the rhizome contained a higher content of ITC than inner tissues (Sultana *et al.*, 2003a).

Individual ITCs have different flavours (Masuda *et al.*, 1996) (Table 4.2). The aroma of wasabi paste can be gradually lost after processing, due to the volatility of ITCs. A method of helping to maintain the odorants of wasabi is to store it in dried form. Kojima and Nakano (1980) found that some wasabi powders maintained their pungency when stored at a temperature of -15^{o}C in an airtight container. However, 50% of the pungency was lost when stored at room temperature for about a month, but it may be restored by adding L-ascorbic acid to the material (Kojima *et al.*, 1982).

Table 4.1: Essential oils isolated from wasabi grown in the upland fields (Kumagai *et al.*, 1994).

Number	Substituents	Extracted oil (mg kg^{-1} fresh weight)			
		Leaf	Petiole	Rhizome	Root
1	1-penten-3-ol	6.3	-	0.1	-
2	3-butenonitrile	23.5	31.1	8.7	43.9
3	*iso*-propyl ITC	3.3	-	-	-
4	*trans*-2-hexenal	149.4	-	-	-
5	*sec*-butyl ITC	8.1	19.4	6	43.9
6	*cis*-2-penten-1-ol	3.1	2.7	0.2	0.3
7	*iso*-butyl ITC	10.5	0.9	0.3	1.1
8	1-hexanol	0.3	0.1	1.4	0.9
9	Allyl ITC	550.7	795.7	832.6	793.9
10	Unknown	146	-	-	-
11	*trans*-2-hexen-1-ol	5.1	0.4	5.4	0.9
12	3-butenyl ITC	42.9	84.2	69.6	44.4
13	4-pentenyl ITC	4.5	12.7	36.5	7.3
14	5-hexenyl ITC	-	1.8	9.4	1.8
15	6-heptenyl ITC	-	1.4	0.6	9.5
16	Unknown	26.5	-	-	0.8
17	4-methylthiobutanonitrile	0.9	0.1	-	0.4
18	3-methylthiopropyl ITC	3.9	3.6	1.3	4.1
19	6-methylthiohexanonitrile	1.4	1.1	0.1	1.7
20	4-methylthiobutyl ITC	-	-	0.1	1.5
21	7-methylthioheptanonitrile	8.3	4.7	0.2	7.9
22	5-methylthiopentyl ITC	0.8	2.1	5.8	3.6
23	6-methylthiohexyl ITC	4.5	16.6	19.8	24.9
24	7-methylthioheptyl ITC	1.6	21	1.8	7.3

Table 4.2: Some essential oils isolated from wasabi grown in the flooded fields (Sultana *et al.*, 2003a) with the odorants described (Masuda *et al.*, 1996).

Ordinal Number	Substituents	Extracted oil (mg kg^{-1} fresh weight)				Flavours
		Leaf	Petiole	Rhizome	Root	
1	Allyl ITC	1090	484	4270	2071	Strong pungent
2	3-butenyl ITC	29.9	11.7	111.5	54.1	Green, pungent
3	4-pentenyl ITC	41.5	17.9	107.1	50.0	Green, pungent
4	5-hexenyl ITC	5.4	8.6	20.2	9.1	Pungent, fatty
5	*iso*-propyl ITC	5.6	3.3	17.0	8.1	Chemical
6	*sec*-butyl ITC	12.2	6.3	36.9	16.9	Chemical
7	*iso*-butyl ITC	3.1	1.2	11.1	5.5	Sweet, chemical

In this project AITC levels were investigated in calli, and in shoot-derived plantlets, both in untreated (control) and in experimental batches which had been subjected to chemical or physical mutagens (see Chapters 2 and 3)**.**

4.2 Materials and methods

4.2.1 Collection and preparation of *W. japonica* samples

4.2.1.1 Calli. Fast-growing calli, obtained from either excised leaves or 1–2 mm petiole segments at six weeks in culture on MS medium containing 0.5 μM 2,4-D in both light and dark conditions (see Chapter 2), were transferred onto an MS fresh medium containing 0.5 μM 2,4-D plus 0.5 μM kinetin for a further eight weeks, with one subculture at a four-week interval. The callus cultures were maintained at 24 ± 1°C, under two consistent light regimes (in the light and in total darkness) as previously described in section 2.2.2.1. At eight weeks, four types of calli, namely light-cultured leaf callus, dark-cultured leaf callus, light-cultured petiole callus and dark-cultured petiole callus, were harvested. These calli were removed from the culture's plastic jars using a forceps, washed under cold water to remove all gelcarin, and then briefly dried with sterile tissue paper. Samples were then separated into individual callus types for analysis and comparison of AITC contents between calli.

4.2.1.2 In vitro mutant explants. Multiple shoots grown on MS media containing 5 μM BA, under *in vitro* culture conditions as described in section 2.2.1.3, were collected at three months after treatments with chemical and physical mutagens. Fifty,

50, 25 and 25 multiple shoots derived from shoot-tips treated with colchicine, oryzalin, X-rays and gamma rays, respectively, were thoroughly washed in cold water to remove the carryover gelling agent (gelcarin). Whole fresh multiple shoots consisting of leaves, petioles and shoot bases of each type of mutants were grouped together and used for comparing their AITC content with those in other mutant types. Samples were rapidly dried with tissue paper before analysis.

4.2.1.3 In vitro non-mutant explants. Multiple shoots grown on MS media containing 5 µM BA, under *in vitro* culture conditions as described in section 2.2.1.3, were collected at one, two and three months. One hundred multiple shoots at each age size were removed from 20 round plastic jars with a forceps, and then carefully washed under running cold faucet water for a complete removal of gelcarin. They were then dried rapidly with tissue paper. Fresh multiple shoots were either grouped together as a whole for comparing their AITC contents with the mutant explants, or separated into leaves, petioles and shoot bases using sharp scissors for comparing their AITC contents in each part. Samples were then dried rapidly with tissue paper before analysis.

4.2.1.4 In vivo non-mutant plants. *W. japonica* plants derived from *in vitro*-raised plantlets were freshly harvested in late winter from the UTS greenhouse located at Gore Hill, Sydney, Australia. Plants were grown in 15 cm x 20 cm (diameter x height) round plastic pots containing potting mixture (Hortico™, Australia) with the fertiliser Osmocote® (N:P:K=8:1:4) for three, six, nine and 12 months. They were shaded with one outermost layer of glass, one layer of white net and two innermost layers of green net, automatically watered every day, maintained in the greenhouse at 15–25^{o}C and 80–85% relative humidity and under natural light prior to collection. Thirty, 20, 10 and eight freshly collected plants at three, six, nine and 12 months of age, respectively, were carefully washed in cold water. Four plant parts (leaves, petioles, rhizomes and roots) of each age size were separated into same-size classes for: width (for leaves), length (for petioles) and weight (for rhizomes and roots), using a sharp knife and scissors (Figure 4.2a, b, c, d). Samples were then allowed to dry rapidly with tissue papers before analysis of AITC in each plant part.

For experiments on the necessary conditions for AITC yield optimisation, 16 nine-month-old plants were collected from the above cultivation location and conditions, for each experiment. Fresh leaves and petioles were separated using sharp scissors, and then

prepared as above. Fresh leaves were used for experiments on solvent, storage temperature and shaking effects, while fresh petioles were used for experiments on the effects of drying temperatures and particle sizes of the sample.

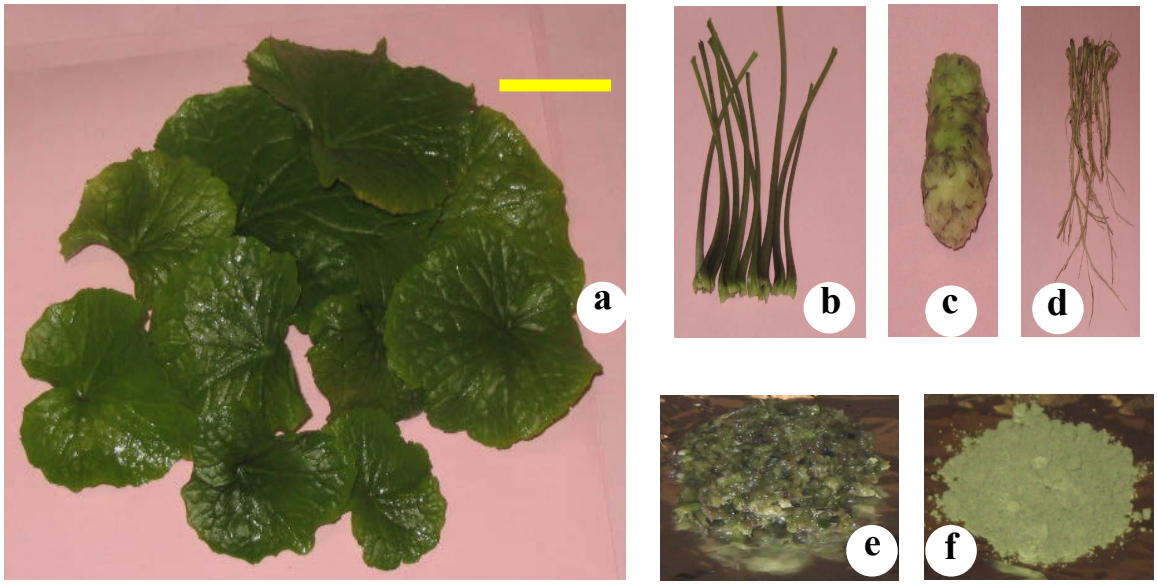


Figure 4.2: Types of *W. japonica* materials used for extraction ((a) leaves, (b) petioles, (c) rhizome, (d) roots, (e) finely-ground fresh rhizome, and (f) ground dried petioles, obtained from a nine-month-old greenhouse plant)) (bar = 5 cm).

4.2.2 Analytical methods

4.2.2.1 Extraction procedures

Fresh samples were cut into small pieces, finely crushed using pestle and mortar, and then homogenised (Figure 4.2e). Dried samples were cut with a sharp knife, pulverised to powder with an electric ball mill (07302, GmbH, Fritsch) and then homogenised (Figure 4.2f). The extraction procedure followed the methods described by Sultana *et al.* (2003a) and Songsak and Lockwood (2004), with slight modifications. In brief, 2 grams of sample from each plant part were randomly selected and weighed into a 15-mL polypropylene plastic centrifuge tube (Polypro, Birmingham). Amounts of 3.5 mL of chilled reverse osmosis (RO) water and 2.5 mL of CH_2Cl_2 (UN1593, Merck KGaA, Darmstadt) were added. The tube was continuously agitated for one hour at room temperature by a shaker at 200 rpm. The mixture was then separated by a centrifuge (Z230 Chermle, Labnet, NJ) at 4300 rpm for five minutes at 20^{o}C, into three layers – organic solution, aqueous solution and residue (Sultana *et al.*, 2003a). The dichloromethane layer was exhaustedly withdrawn with a Pasteur pipette and placed in

a plastic vial, then concentrated by evaporating the solvent under a stream of nitrogen to a required volume. The dichloromethane extract was stored at -20^{o}C for subsequent analysis. The method is summarised in Figure 4.3.

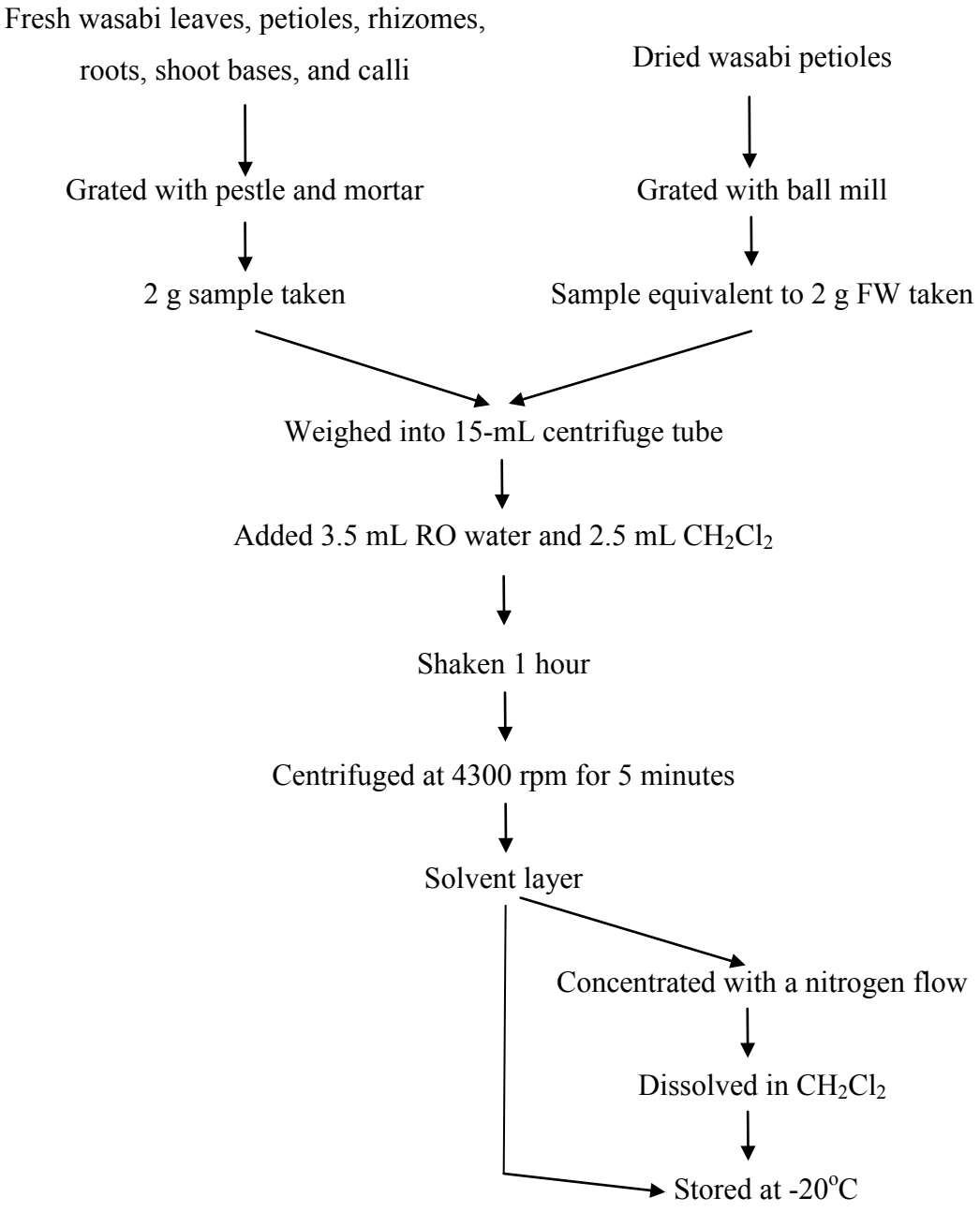


Figure 4.3: Procedures for extraction of AITC from *W. japonica*.

4.2.2.2 Gas chromatography

Analysis of AITC was performed on a gas chromatograph (Varian Star, 3400CX, Redwood) equipped with an Econo-Cap EC-WAX capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) (Alltech, Deerfield, IL), a FID detector and a Varian 8035 automatic sampler. The flow rate for the He carrier gas was 2.3 $mLmin^{-1}$ with an inlet pressure of 85 kPa. The temperature of the inlet was 160^{o}C, and that of the detector was 250^{o}C. A 2-μl sample of extract solution was injected. The temperature program for separation was 50^{o}C to 100^{o}C at a rate of 5^{o}C/min, then 100^{o}C to 200^{o}C at a rate of 10^{o}C/min, and finally held isothermally at 200^{o}C for 2 minutes (Sultana *et al.*, 2003a).

4.2.2.3 Mass spectrometry

GC-MS analysis was conducted on a chromatograph (Varian Star, 3400CX, Redwood) with a split ratio of 30:1, coupled to a mass spectrometer detector (Varian, Saturn). Helium was the carrier gas, at 2 $mLmin^{-1}$. Ionisation energy and accelerating voltage were 70 eV and 4000 V, respectively (Sultana *et al.*, 2003a). The ion source temperature was 250^{o}C and the scan mass spectra ranged from 49 to 629 nm (School of Applied Sciences, University of Newcastle, Ourimbah).

4.2.2.4 Quantitative analysis of AITC

Quantification of AITC was carried out on the basis of peak area and retention time data from an AITC authentic standard (Pestanal, Sigma-Aldrich, Laborchemikalien, St. Louis, MO). An external AITC standard was used and injected separately from the wasabi essential oils. Two injections from each of three different concentrations of AITC standard were conducted to establish a regression line for the peak area against AITC concentration. AITC in the essential oils was calculated from the regression equation of AITC standard. A typical AITC standard regression line is shown in Figure 4.4.

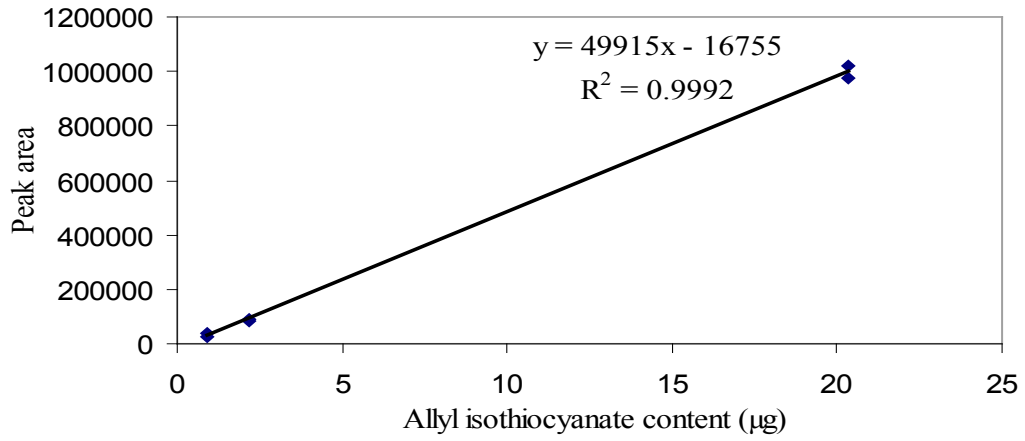


Figure 4.4: A typical regression equation for AITC standard.

4.2.2.5 Qualitative analysis of AITC

Identification of volatile components was determined on the basis of mass spectra and chemical evidence. Mass spectra of ITCs were compared with mass spectroscopic data of Kjaer *et al.* (1963), Gilbert and Nursten (1972), Slater (1993) and Kumagai *et al.* (1994). ITCs were confirmed using base peaks as molecular weights, and *m/e* fragments. AITC was identified on the basis of a base peak of M^+99 (Mw = 99), and two fragments of M72 (*m/z* 72) and M41 (*m/z* 41) corresponding to molecular weight portions of ions separated from the AITC molecule at the two mono-linkage positions. Figure 4.5 demonstrates the identity of AITC.

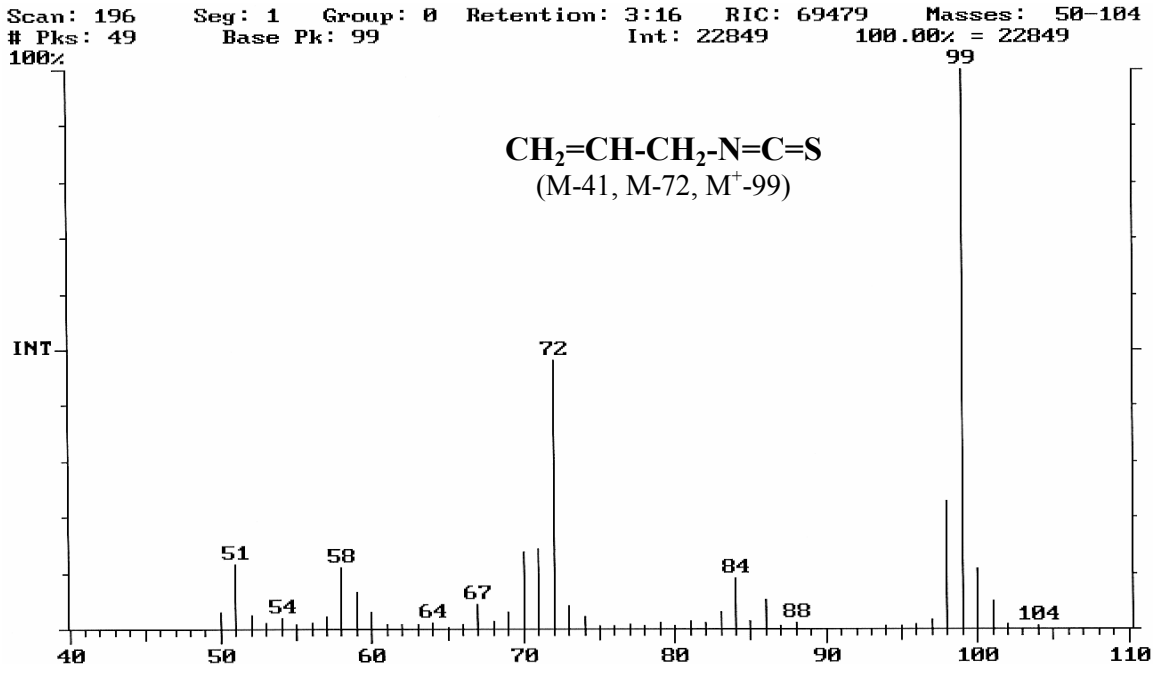


Figure 4.5: Mass spectra of AITC.

4.2.2.6 Experimental design

Experiment 1: Effects of callus origins and light regimes on the synthesis of AITC in calli

A design of 2 x 2 factorial was performed to compare the two callus types (leaf callus and petiole callus) and two culture conditions (dark and light).

Experiment 2: Effects of oryzalin and colchicine on the synthesis of AITC in plantlets

Mutant explants obtained from four concentrations of oryzalin (0, 5, 15 and 30 μM) and four concentrations of colchicine (0, 25, 75 and 150 μM) were compared to each other. Treatments at 0 μM were used as controls (non-mutant explants).

Experiment 3: Effects of ionising-radiation on the synthesis of AITC in plantlets

Mutant explants obtained from four doses (0, 10, 20 and 40 Gy) of both gamma rays and X-rays were compared to each other. Treatments at 0 Gy were used as controls (non-mutant explants).

Experiment 4: Investigation of fresh weight percentages and AITC yields from in vitro- and in vivo-grown plants

For *in vitro* plants, a design of 3 x 3 factorial was carried out to compare the three plant parts (leaf, petiole and base) and three plant age sizes (one, two and three months). For *in vivo* plants, a design of 4 x 4 factorial was conducted to compare the four plant parts (leaf, petiole, rhizome and root) and four plant age sizes (three, six, nine and 12 months). The fresh weight percentage of each plant part was calculated by dividing the total fresh weight of the whole plant, and then multiplying by 100. The fresh weight of plant parts was measured using an electronic balance (Mettler, Model AE260 Delta range) at an accuracy rate of 10 ng.

Experiment 5: Effects of sample particle sizes on the AITC extraction

Six sieves (BS 410, Endecotts, London) with apertures at 1000, 500, 250, 210, 106 and 63 μm were selected and arranged into a pile in an order that the uppermost sieve has larger apertures than the next sieve down, and so on in order, so that the lowest of the six sieves has the smallest apertures. Petioles dried at 30^{o}C and pulverised with an

electrical ball mill as described above were sieved until all particles remained on a sieve.

Experiment 6: Effects of drying and storage temperatures on the loss of AITC

- Drying temperature. Four ovens (Labec, Sydney) were set at 30, 40, 50 and 60^{o}C. Petioles were dried at all the four temperatures until constant weights were reached before being pulverised to powder for extraction.

- Storage temperature. Three freezers were set at 4, -20 and -80^{o}C. Fresh leaf samples were stored in all three freezers and collected for extraction on days 2, 7, 14 and 28. For the defrosting and refreezing Experiments, samples stored for two weeks were transferred to room temperature for one day, and then placed back in these freezers until day 28 for extraction.

Fresh petiole and leaf samples, following extraction with dichloromethane at 20^{o}C at 200 rpm for one hour, were used as controls for drying and storage temperature Experiments, respectively.

In the two Experiments 7 and 8 below, the extraction procedures were slightly different, according to the individual treatments as specified, from those described in section 4.2.2.1 above.

Experiment 7: Effects of shaking conditions on AITC extraction

The whole Experiment was divided into three treatments as detailed below.

- Shaking speed: A shaker was set at 100, 200, 300 and 400 rpm. Extraction was performed with dichloromethane for one hour at 20^{o}C at all four shaking speeds.

- Shaking time: Shaking was for 15, 30, 60, 120 and 180 minutes. Extraction was carried out with dichloromethane at 20^{o}C at 200 rpm for all four shaking times.

- Shaking temperature: Extraction was carried out with dichloromethane at 200 rpm for one hour at 35^{o}C, room temperature (rt), and 0^{o}C (rt with ice). For rt with ice, centrifuge tubes containing ground leaf samples were placed in the middle of a 10 cm-thick ice layer during shaking.

Fresh leaf samples following extraction with dichloromethane at 20^{o}C at 200 rpm for one hour were used as controls for these three treatments.

Experiment 8: Effects of solvents on the AITC extraction

Five selected solvents were dichloromethane (Merck KgaA, Darmstadt), chloroform (UN 1888, BDH, Clayton South), n-hexan (HiperSol Grade, BDH, London), diethyl ether (Analytical Grade, Univar, Seven Hills) and petroleum ether (Sigma-Aldrich, Castle Hill). AITC in fresh leaf samples was extracted with each type of solvent for one hour at 20^{o}C, using a shaker set at 200 rpm.

4.2.2.7 Data analysis

For each treatment, data were presented from the means of six replicates. The experiment was conducted twice and performed as a randomised block design. Statistical analyses of data were carried out using a general linear model, and one-way and two-way ANOVAs. When ANOVAs demonstrated significant treatment effects, individual mean separation was performed using Tukey's Simultaneous Tests at a 5% level of probability ($p \leq 0.05$). Means with/without standard errors (SE) were presented. The SE of means were calculated by the descriptive statistics test. Data were statistically analysed using a Minitab computer package (Minitab 14, State College, PA, 2003). For GC analyses, a Star Chromatography Workstation software program (Version 4.5) was employed to determine calibration curves.

4.3 Results

4.3.1 AITC content as affected by callus cultures

Fast-growing and friable homogenous callus, obtained from leaves and petioles in both dark and light conditions after three subcultures with a total of 14 weeks, exhibited differences in AITC accumulation by GC-MS (Figure 4.6).

The amount of AITC varied between origin-homogenous calli incubated in different light regimes. In darkness, there was no significant difference in the AITC content between calli of different origins ($p= 0.000$). The AITC content in both leaf and petiole calli cultured in the light was significantly higher than when cultured in the dark. In the light, the AITC content in leaf-derived calli was significantly higher than that in petiole-derived calli.

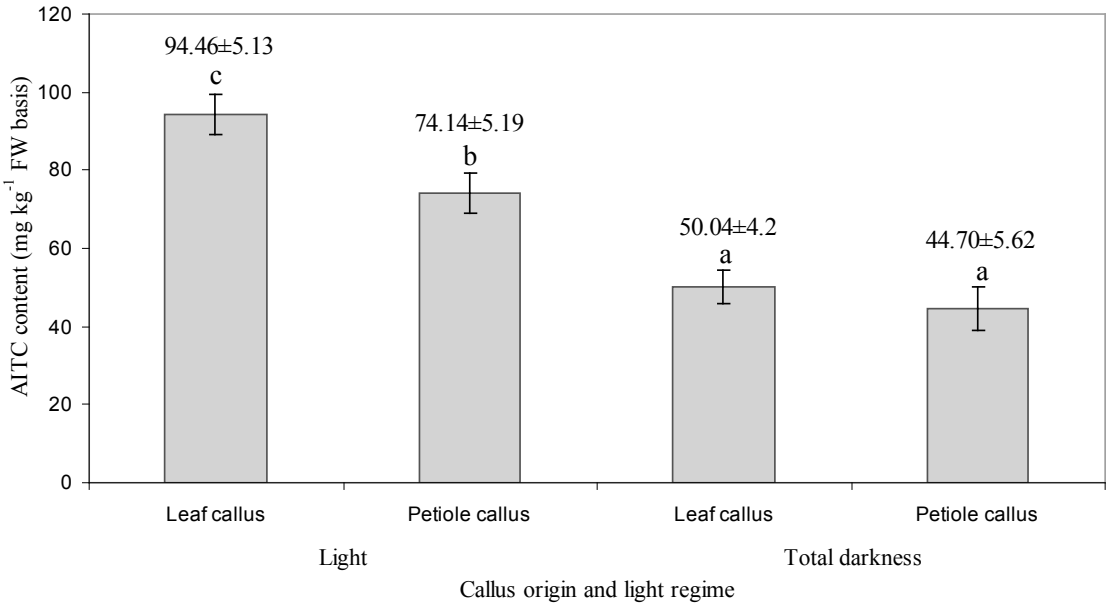


Figure 4.6: Effects of explant parts (leaf and petiole segments) and culture conditions (light and darkness) on the accumulation of AITC. The same letters in the graph indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Values are the means of 6 samples. Vertical lines denote standard errors.

4.3.2 AITC content as affected by mutagens

The accumulation of AITC was affected by oryzalin and colchicine treatments after 90 days of shoot growth *in vitro*.

Figure 4.8 and Appendix 4.2 show that an increased amount of AITC was achieved by the treatment with 15 µM oryzalin (p= 0.000), but oryzalin at doses higher or lower than 15 µM showed no significant differences in AITC accumulation from untreated plants. However, treatments with colchicine at all concentrations increased the amount of AITC significantly. There were no differences in the AITC levels accumulated by the treatments with 25 µM and 75 µM colchicine. Colchicine at 150 µM stimulated the synthesis of AITC to the highest level at 151±11 mg kg^{-1} FW basis, increasing by about 100% compared to the control.

Figure 4.7 shows that the GC-MS chromatograms of dichloromethane extracts of chemical mutagen-induced explants *in vitro* displayed further peaks (with retention times at 7.0, 10.1 and 11.2). These peaks were not found in the extracts of untreated control explants by the chromatograms. Extracts of three-month-old explants from either 5 µM oryzalin or 25 µM colchicine treatments showed all three peaks, while extracts of explants from either 15 µM oryzalin or 75 µM colchicine treatments showed only one of these three peaks. Areas of the three peaks were greater in 5 µM oryzalin

and 25 µM colchicine treatments than in 15 µM oryzalin and 75 µM colchicine treatments, however, all of these peak areas were much smaller than the peak area of AITC (data not shown).

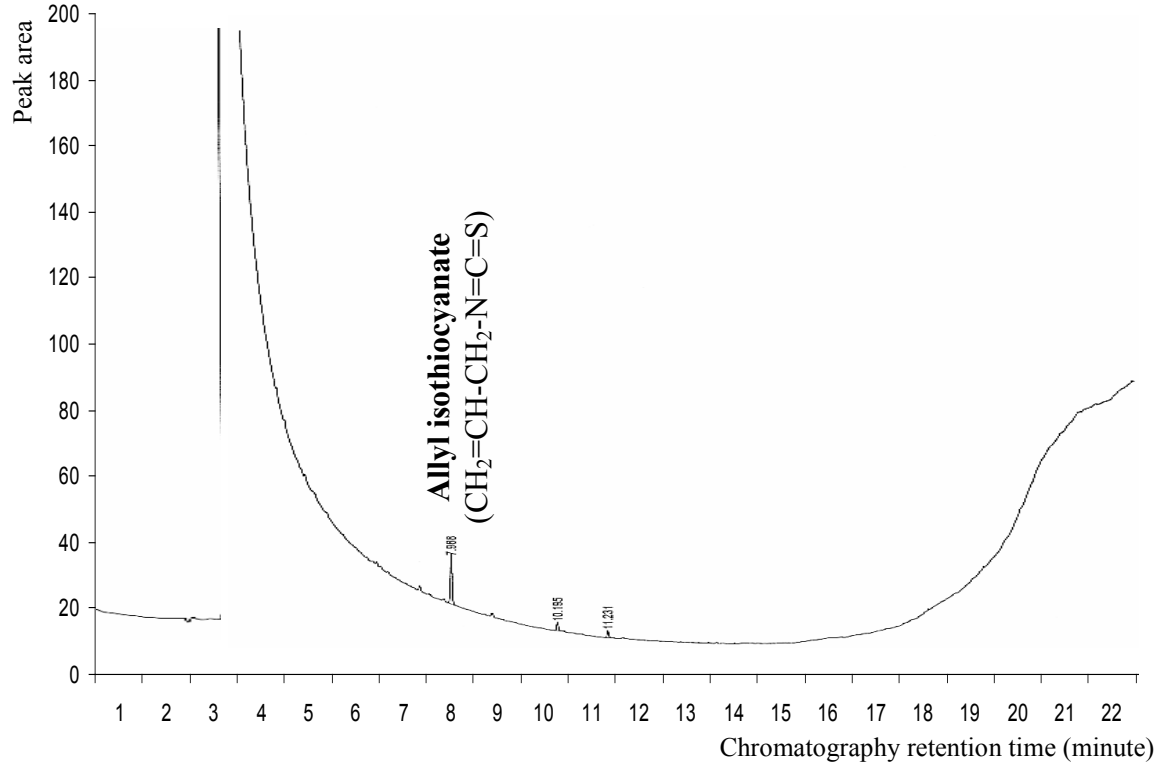


Figure 4.7: Typical gas chromatogram of AITC extracted from 3-month-old *in vitro* explants of *W. japonica* treated with mutagens.

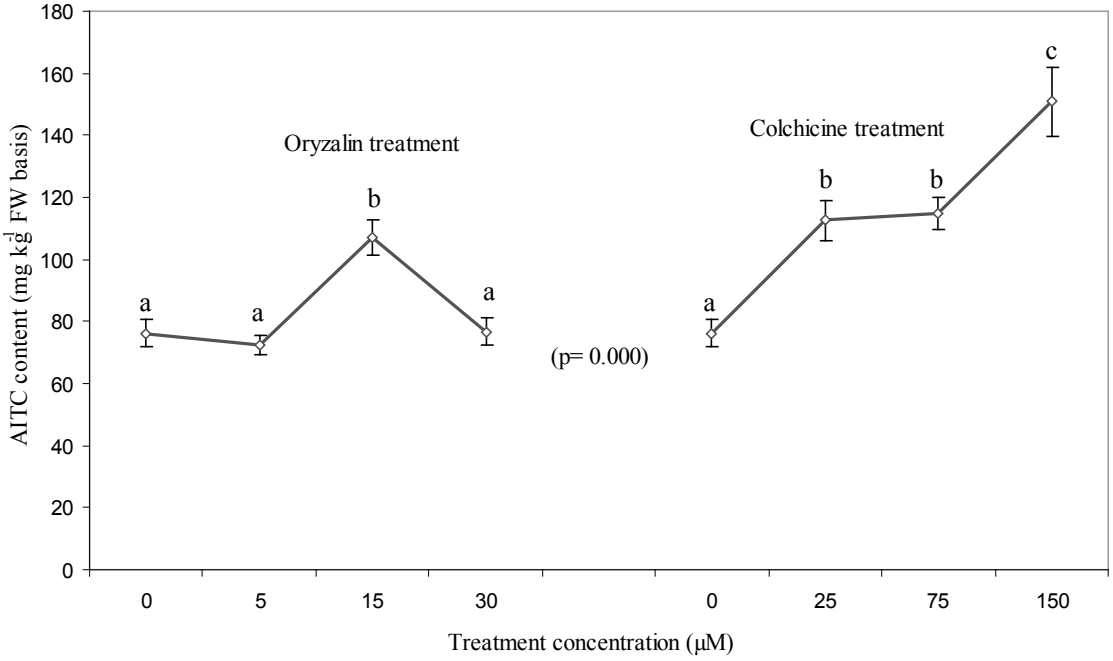


Figure 4.8: Effects of chemical mutagen treatments on AITC content of *W. japonica* after 3 months in culture on MS medium. Values are the means of 6 samples extracted from 50 whole explants. Vertical lines represent standard errors.

The data in Figure 4.9 and Appendix 4.3 show that the effects of irradiation on AITC content were evident following 90 days in culture *in vitro*, with all treatments producing explants with significantly lower levels of AITC than the control (p= 0.003).

Within gamma radiation doses, the drop in the accumulation of AITC content was significantly different (p= 0.001). The lowest reduction was found with the highest dose at 40 Gy, followed by the 20 Gy and then the 10 Gy, to rates at 61%, 72% and 80%, respectively, of the AITC amounts of the control. Likewise, the AITC content was reduced to 70%, 62% and 46% of the control when the shoot tips were irradiated with 10, 20 and 40 Gy of X-rays, respectively. Similar to gamma treatments, the effect of AITC accumulation between doses of X-rays showed significant differences (p= 0.002).

Comparison of X-rays and gamma rays showed that the effect of equal dose levels on AITC amount was also significantly different (p= 0.000). The 10, 20 and 40 Gy of X-rays induced a reduction of AITC content to 85%, 84% and 72% of the equalled doses of gamma rays, respectively.

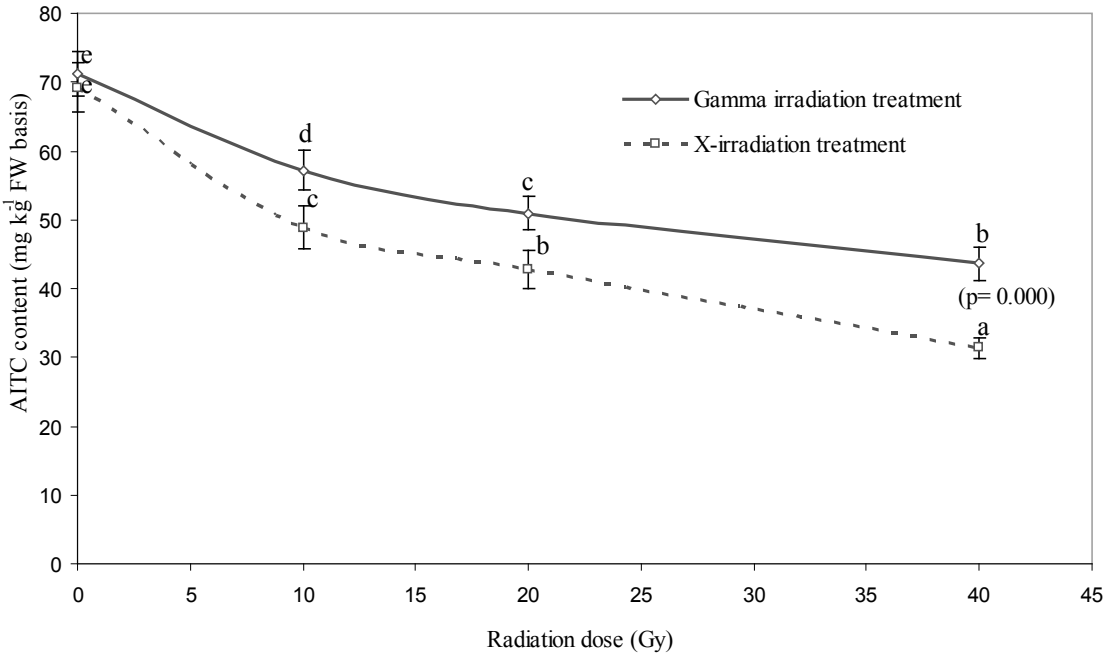


Figure 4.9: Effects of physical mutagen treatments on AITC content of *W. japonica* after 3 months in culture on MS medium. Values are the means of 6 samples extracted from 25 whole explants. Vertical lines represent standard errors.

4.3.3 Investigation of AITC and plant yields from plant organs

The data in Figure 4.10 and Table 4.3 show that wasabi explants cultured *in vitro* exhibited significant increases in the fresh weight of leaves, petioles and bases at 30-day intervals. However, the percentage of fresh weight petiole to the total explant weight was significantly reduced from 56% to 40% between one and three months in culture (p= 0.037). In contrast, the fresh weight percentages of leaf and base increased significantly within this growth period, but showed no difference between one month and two months.

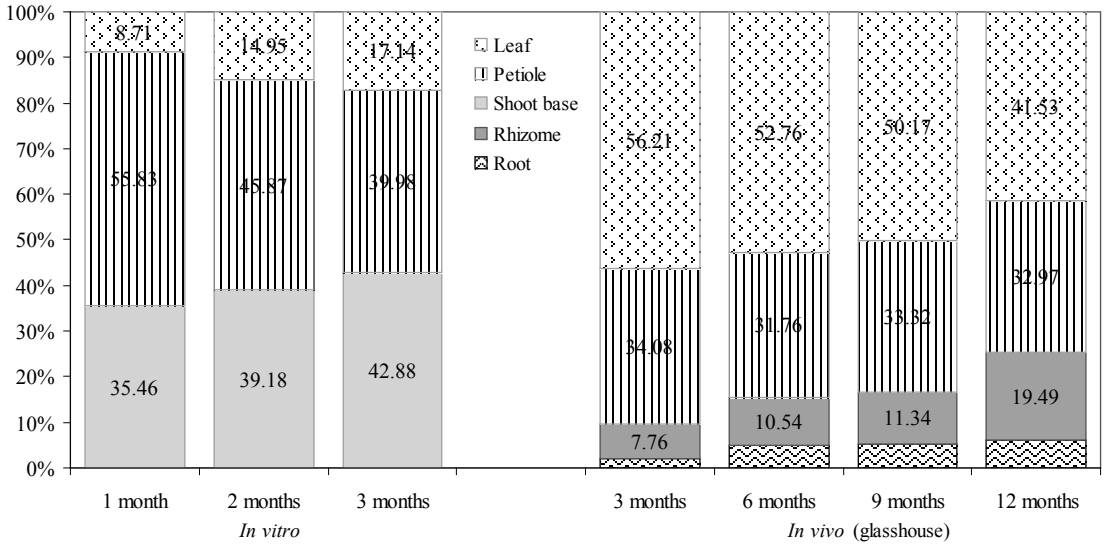


Figure 4.10: Effects of *in vitro* culture cycle (1, 2 and 3 months) and *in vivo* growth cycle (3, 6, 9 and 12 months) on the fresh weight proportions of plant parts. Values are the means of 25 replicates.

In wasabi plants raised in the greenhouse, there was also a significant increase in the fresh weight of leaves, petioles, rhizomes and roots during a development period from three to nine months. Nevertheless, between month 9 and month 12, harvested plants showed that weights of leaves, petioles and roots stabilised, while the rhizome continued to increase its fresh weight significantly. However, the weight proportions of plant parts altered differently compared to those obtained from *in vitro* explants. There was no difference in the percentage of petiole fresh weight to the total plant weight over a period of 12 months (p= 0.150). A significant drop in the fresh weight percentage was observable in leaves between three months and nine months but not between three months and six months. Another significant decrease in the leaves' fresh weight percentage was detected from nine months to 12 months (p= 0.014). The fresh weight percentages of rhizomes and roots showed no different responses at months 6 and 9,

however, a significant reduction in these percentages was found between month 3 and month 12, or even between month 3 and month 6.

Table 4.3: Fresh weight of *in vitro* and *in vivo* plant parts as affected by length of growth.

Growth cycles	Fresh weight distribution in a plant/explant (mg)				
	Leaf[a]	Petiole	Shoot base	Rhizome	Root
In vitro					
1 month	40.6±3.8a[b]	260±23a	165±17a	None	None
2 months	195±17b	598±18b	511±23b	None	None
3 months	403±20c	940±15c	1008±41c	None	None
P value	*0.000*	*0.000*	*0.000*	-	-
In vivo					
3 months	3549±207a	2152±246a	None	490±34a	123±9a
6 months	12436±921b	7487±184b	None	2485±183b	1165±81b
9 months	24053±776c	15975±1502c	None	5437±165c	2479±181c
12 months	19992±1887c	15872±897c	None	9382±334d	2893±262c
P value	*0.001*	*0.002*	-	*0.000*	*0.001*

[a]Values are means ± standard errors of three independent experiments consisting of a total of 50 explants.
[b]Means within a column followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

The distribution of AITC content in the explants grown *in vitro* is shown in Table 4.4.

Table 4.4: AITC content distributed in the explant parts at 1, 2 and 3 months in culture *in vitro*.

Explant part	AITC content (mg kg^{-1} FW basis) after *in vitro* culture		
	1 month[a]	2 months	3 months
Leaf	38.7±1.7a[b]	55.0±4.5a	67.9±5.1a
Petiole	52.4±3.0b	55.1±3.6a	60.2±5.0a
Shoot base	71.1±2.2c	79.7±5.4b	89.6±3.6b
P value	*0.000*	*0.002*	*0.001*

[a]Values are means ± standard errors of three independent experiments consisting of a total of 6 samples.
[b]Means within a column followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

AITC content differed (p= 0.000–0.002) among the three *in vitro* explant parts of wasabi following 30, 60 and 90 days in culture. In the first 30 days, the level of AITC was reduced in the following order: shoot base > petiole > leaf. However, from day 60 to day 90, the AITC level increased faster in leaves than in petioles, resulting in no significant difference between leaves' and petioles' AITC amounts. The highest AITC level was measured in the shoot base at 71.1±2.2, 79.7±5.4 and 89.7±3.6 mg kg^{-1} fresh weight basis after one, two and three months, respectively.

The distribution of AITC content in the plants grown *in vivo* (in the greenhouse) is shown in Table 4.5. The AITC level in the four plant parts after three, six, nine and 12 months of plant growth in the greenhouse showed significant differences. During the 12 months, the concentration of AITC in *W. japonica* leaves was significantly higher than those in petioles while rhizomes contained the greatest concentration of AITC. Generally, the order of AITC content was: rhizome > leaf > petiole. At three months, the AITC content ranged from 236±23 to 845±17 mg kg^{-1} among the four plant parts. Of these, roots and leaves showed no significant differences in AITC content, thus at this age, AITC was: rhizome > root = leaf > petiole. From month 9 to month 12, roots contained a significantly greater level of AITC than leaves, which led to the order of AITC content being: rhizome > root > leaf > petiole.

Table 4.5: AITC content distributed in the plant parts at 3, 6, 9 and 12 months of growth in the greenhouse.

Plant part	AITC content (mg kg^{-1} FW basis) after *in vivo* culture			
	3 months[a]	6 months	9 months	12 months
Leaf	425±25b[b]	499±24bc	619±16acd	652±32a
Petiole	236±23f	277±10fg	361±23eg	440±26ce
Rhizome	845±17i	943±17i	1129±86j	1508±73hj
Root	415±18be	520±12bde	804±22i	1144±52k

[a]Values are means ± standard errors of three independent experiments consisting of a total of 6 samples.
[b]Means followed by the same letters are not significantly different at the 5% level by Tukey's Simultaneous Tests.

Comparison of the AITC yields in each plant part between different growth lengths, however, showed only slight differences. The only significantly different response in the content of AITC synthesised in leaves was between three-month-old and nine-month-old plants, and between six-month-old and 12-month-old plants. Petioles showed a similar effect of age on the AITC level as that found in leaves. However, the yield of AITC synthesised in petioles was substantially lower than in leaves, corresponding to 0.56, 0.57, 0.58 and 0.67 times for plants of three, six, nine and 12 months, respectively. From Table 4.5, it can be calculated that the leaves contributed 17–22% of the total ITC yield of the plant. In rhizomes and roots, however, AITC levels were significantly lower in the six-month-old plants than in the nine-month-old plants. No significant differences in AITC content were found between rhizomes of nine- and 12-month-old plants. Both roots and rhizomes contained significant higher levels of AITC in plants of 12 months of age than those in the plants of three and six months of age. The distribution of AITC in rhizomes was, therefore, summarised as: three months = six months < nine months = 12 months; whereas, the summary of AITC distribution in root followed another order: three months = six months < nine months < 12 months.

Apart from AITC, further four volatile compounds were identified by GC-MS from greenhouse-grown wasabi extracts (Figure 4.11). Their concentrations were much lower than the AITC content in the same plant parts, according to the peak areas recorded

(data not shown). All of these four compounds were present in leaves, petioles, roots or rhizomes of the 12-month-old plants; however, they were not all identified in every part of the three- or six-month-old plants.

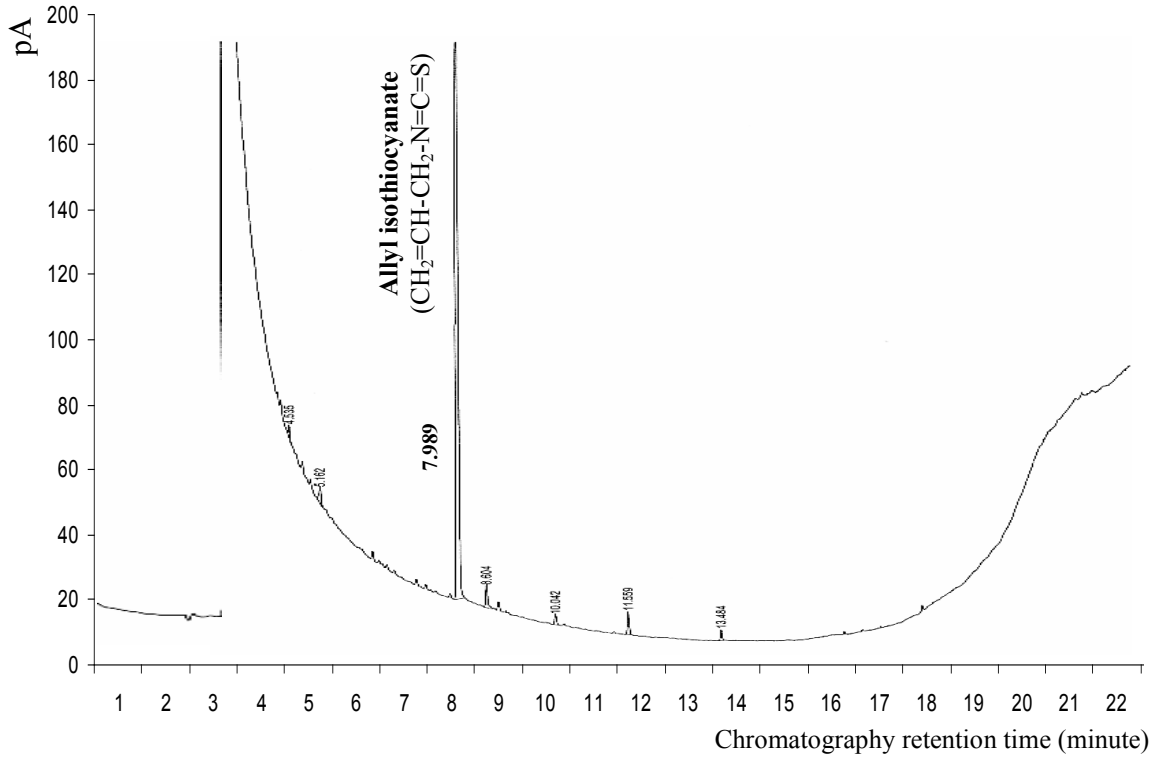


Figure 4.11. Typical gas chromatogram of essential oils extracted from greenhouse-grown *W. japonica*.

These compounds were identified from their mass spectra (Appendix 4.4) to be:

1) 2-Methylthioethyl ITC Mw = 133 Mf: $CH_3S(CH_2)_2$-N=C=S

2) 5-Methylthiopentyl ITC Mw = 175 Mf: $CH_3S(CH_2)_5$-N=C=S

3) 6-Methylthiohexyl ITC Mw = 189 Mf: $CH_3S(CH_2)_6$-N=C=S

4) *cis*-2-Penten-1-ol Mw = 86 Mf: $CH_3CH_2CH=CHCH_2$-OH,

or 1-Penten-3-ol Mw = 86 Mf: $CH_2=CHCH(OH)CH_2CH_3$

4.3.4 Necessary conditions for AITC yield optimisation

It was clear from the results of the analyses obtained above that the experimental conditions require further refinement for the optimisation of methodology.

4.3.4.1 Effect of particle size

The effects of particle size from dried petioles on the mean yield of AITC are presented in Figure 4.12. No significantly different response was found between any of the mean levels of AITC for the particle sizes ranging from 500 to 1000 µm (p= 0.021) but there were significant increases in the AITC yields from the sizes between 210 and 250 µm (p= 0.000). The AITC content obtained with a size of 210 µm was 409±24 mg kg^{-1} FW basis, which was 1.3 times higher than the mean yield obtained from the 1000 µm size. Significant declines in the AITC level were observed from the particle sizes in a range from 106 µm to less than 63 µm. At a particle size of 106 µm, the AITC content was 3334±19 mg kg^{-1}, accounting for 81% of the 210 µm size, and showed no significant difference from the mean level of the 500 µm size. Particles at 63 µm, however, produced a significantly smaller amount of AITC (222±15 mg kg^{-1}), a reduction of 54% to the mean level from the 210 µm size, as compared to all the particle sizes larger than 210 µm. The highest loss of mean level was detectable in the size at less than 63 µm, where the AITC content was only 95.9±6.9 mg kg^{-1}, equivalent to 23%, the mean level of the 210 µm size.

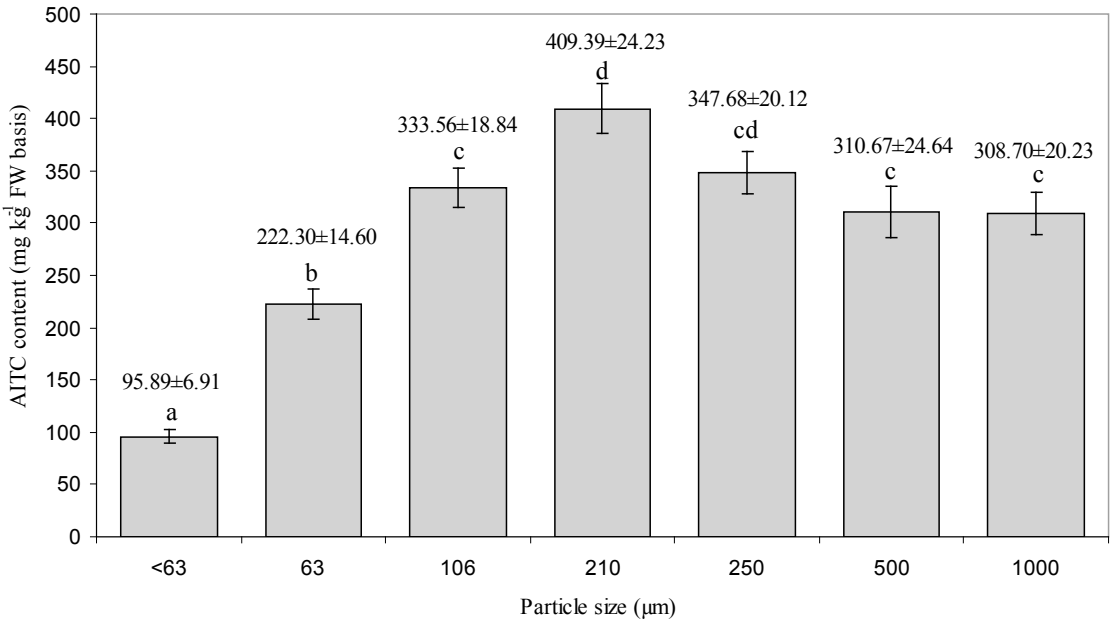


Figure 4.12: Effects of particle sizes of the *W. japonica* petiole samples ground to powder on the content of AITC. The same letters above the bars indicate that values are not significantly different. Values are the means of 6 replicates (n=6). Vertical lines represent standard errors.

4.3.4.2 Effect of drying temperature

The effects of drying temperature on the loss of AITC are shown in Figure 4.13. A significant (p= 0.001) linear relationship exists between drying temperatures and the mean content of AITC for *W. japonica* petioles, indicating that AITC yield decreased as the temperature increased. The best drying was at 30°C, where the reduction in AITC level was not significantly different compared to the fresh petiole (control).

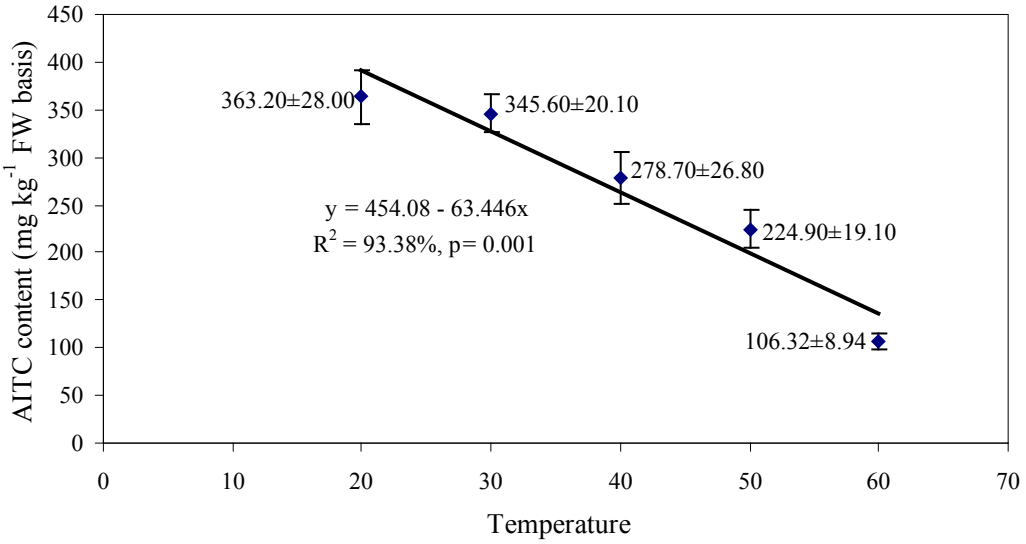


Figure 4.13: Relationship between the drying temperature and content of AITC in *W. japonica* petioles ((20°C = control temperature (rt for freshly extracted samples); 30°C–60°C = drying temperatures; error bars = mean AITC content ± SE (n=6)).

4.3.4.3 Effect of storage temperature

Trends for AITC yield during storage of fresh leaves of *W. japonica* at room temperature (rt), 4°C, -20°C and -80°C for a period of four weeks are shown in Figure 4.14.

The yield of AITC decreased significantly (p= 0.001–0.041) as the storage time increased at each temperature. At -80°C, the mean yield of AITC showed no significant difference from the control (no storage) during two weeks of storage; however, it was significantly reduced (by 31%) after four weeks. At -20°C, AITC was unaffected by storage time until the end of the second week, when 19% of AITC yield was lost, as compared to the control. A four-week storage at -20°C showed a significant AITC loss compared to a two-week period. At 4°C, AITC was significantly lost by 36% after two days compared to the control, and dramatically reduced (by 74%) after four weeks of storage. The AITC loss occurred rapidly in leaves stored at rt, from 619±39 mg kg^{-1} to

300±19 mg kg^{-1} and 146±12 mg kg^{-1} after two and seven days, respectively. After two weeks of storage at rt, AITC was unable to be detected.

Regression equations with different slopes showed that AITC loss increased as the temperature increased. Within two days of storage, a significant decline in AITC was found at 4°C compared to -20°C (p= 0.021). However, there were no significant loss of AITC between -20°C and -80°C (p= 0.062). Similarly, no significant difference in AITC yields was found between rt and 4°C. After seven days of storage, the loss of AITC was higher in storage at 4°C than at -20°C, but lower than at rt. Mean AITC yields obtained at -20°C and -80°C after this period of storage showed no significant difference. After two weeks of storage, mean AITC yields decreased to 595±30, 499±23 and 291±18 mg kg^{-1} at -80°C, -20°C and 4°C, respectively. After four weeks of storage, AITC yields obtained at 4°C and -20°C were not significantly different from each other, but both were significantly lower than the yield gained at -80°C.

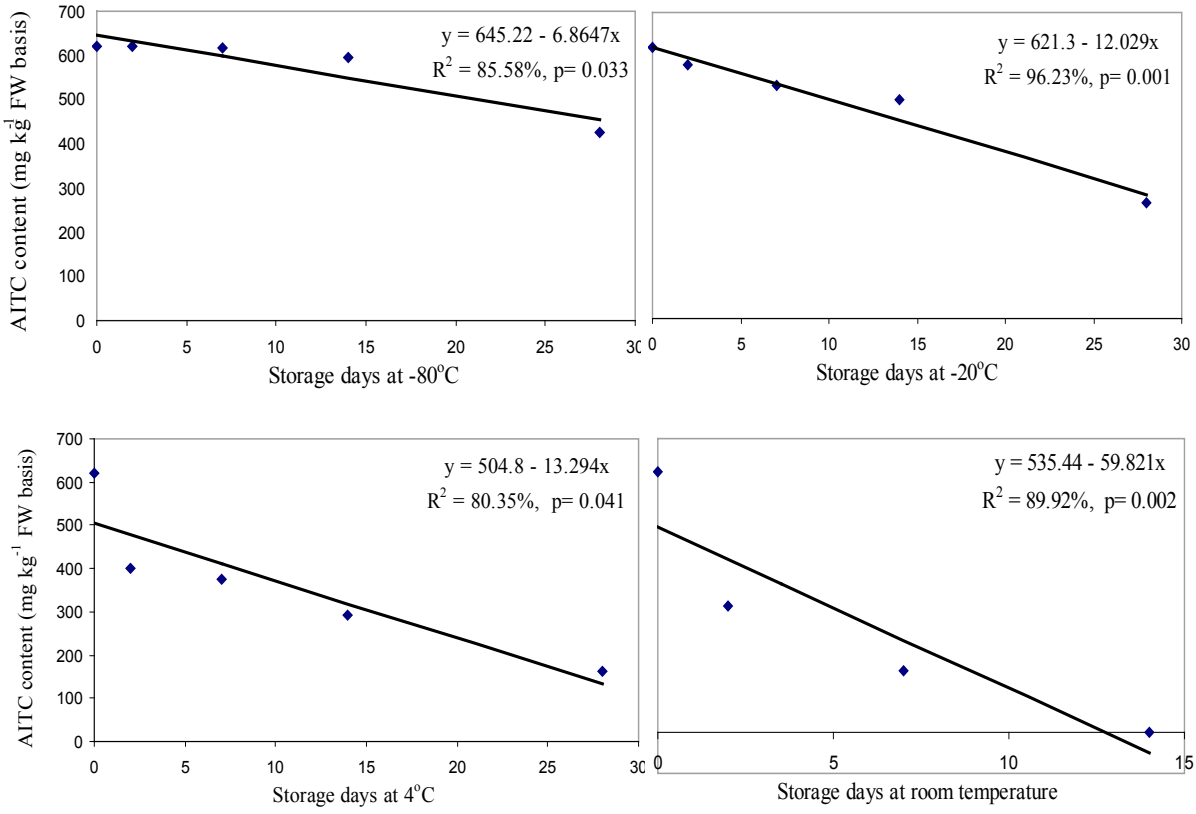


Figure 4.14: Relationship between the storage time and mean content of AITC in *W. japonica* leaves stored at rt, 4°C, -20°C and -80°C.

Defrosting and then refreezing the fresh leaves at -80°C, -20°C and 4°C resulted in drastic losses of AITC by 68%, 74% and 81%, respectively, compared to the control.

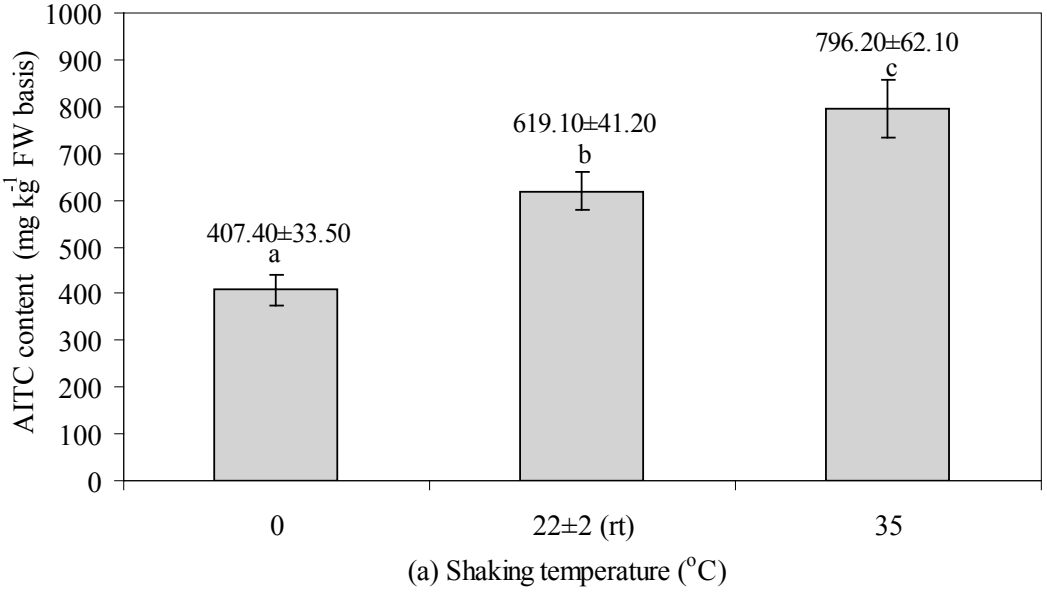
There was no significant difference in the reduction of AITC between these three temperatures. After four weeks at rt, defrosting and then refreezing caused an absence of AITC in the fresh leaves (data not shown).

4.3.4.4 Effect of shaking conditions

The effects of shaking temperature, shaking time length and shaking speed on the yield of AITC from fresh leaves are shown in Figure 4.15. The level of AITC was significantly reduced with decreasing the temperature from 35^{o}C to 0^{o}C (with ice) (p= 0.009) (Figure 4.15a). Shaking at 35^{o}C resulted in an AITC content of 796±62 mg kg^{-1}. A significant decline in the AITC level of 22% was obtained when fresh leaves were shaken at room temperature and a 49% decline at 0^{o}C.

Increasing the shaking time significantly increased the yield of AITC (Figure 4.15b). There was a significant gradual increase in the yield of AITC to two hours, from 400±40 mg kg^{-1} to 691±53 mg kg^{-1} (p= 0.002); however, a minor change in the level of this component was found after two hours (p= 0.062).

The shaking speed showed a significant increase in the yield of AITC between 100 rpm and 200 rpm, from 337.4±28.3 to 619±33 mg kg^{-1} FW basis (p= 0.000). However, it stabilised at a speed of 300 rpm and yielded at about 650±23 mg kg^{-1} (Figure 4.15c).



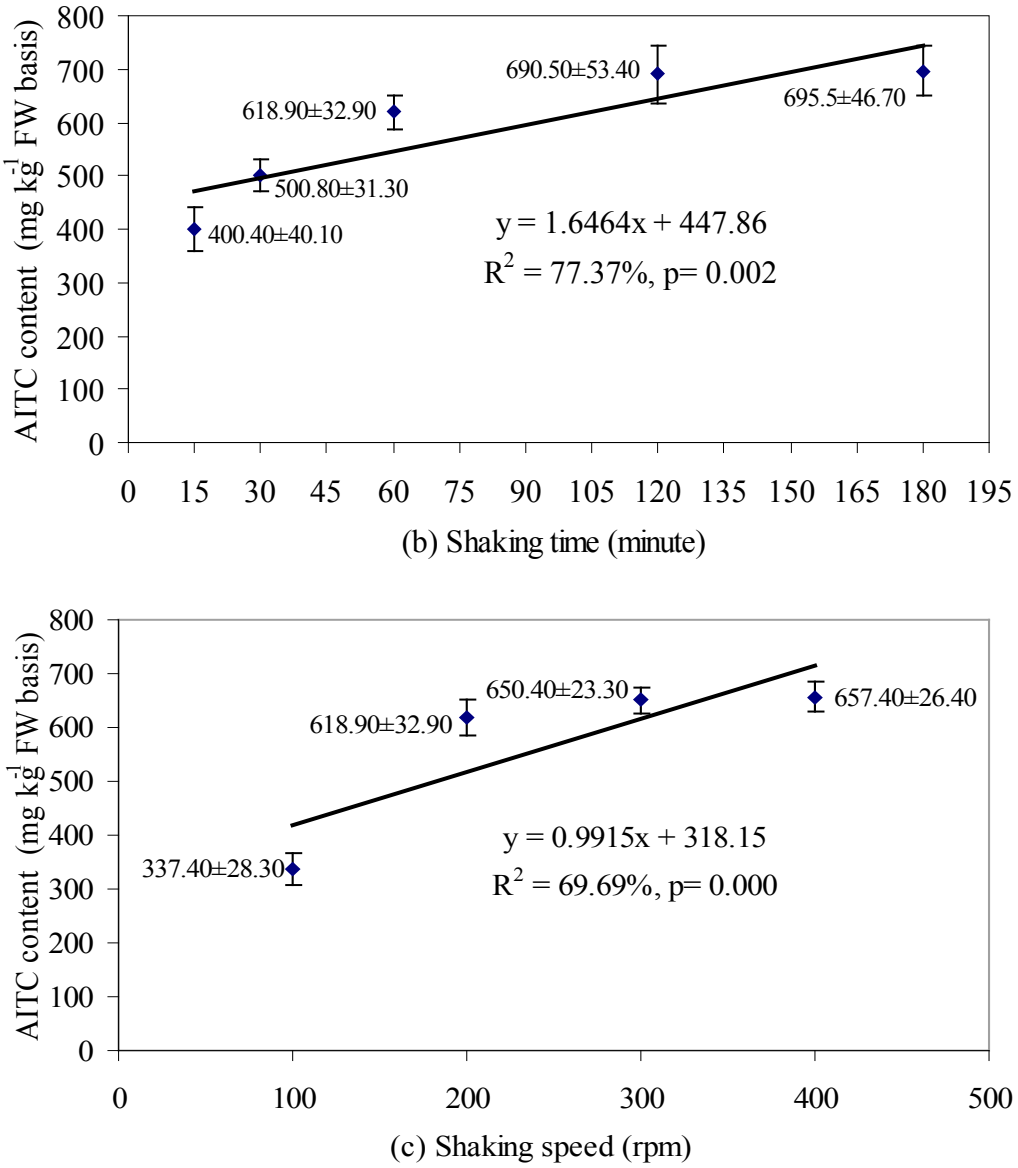


Figure 4.15: Effects of (a) shaking temperature, (b) shaking time and (c) shaking speed on the yield of AITC during extraction. The same letters above the bars indicate that values are not significantly different. Error bars = mean AITC content ± SE (n=6).

4.3.4.5 Effect of solvent

Investigation into the effects of five organic solvents on the yield of AITC showed that organic solvents significantly affected the yields (Figure 4.16).

The highest AITC yield (619±56 mg kg^{-1} FW basis) was obtained when wasabi samples were extracted with dichloromethane (p= 0.005). There was no significant difference in the yield of AITC with diethyl ether and chloroform solutions but the yield was 32–43% lower than from dichloromethane. The lowest AITC level was obtained with n-hexane

(152±13 mg kg^{-1}), which accounted for only 24% of that from dichloromethane; however, it showed no significant difference from petroleum ether.

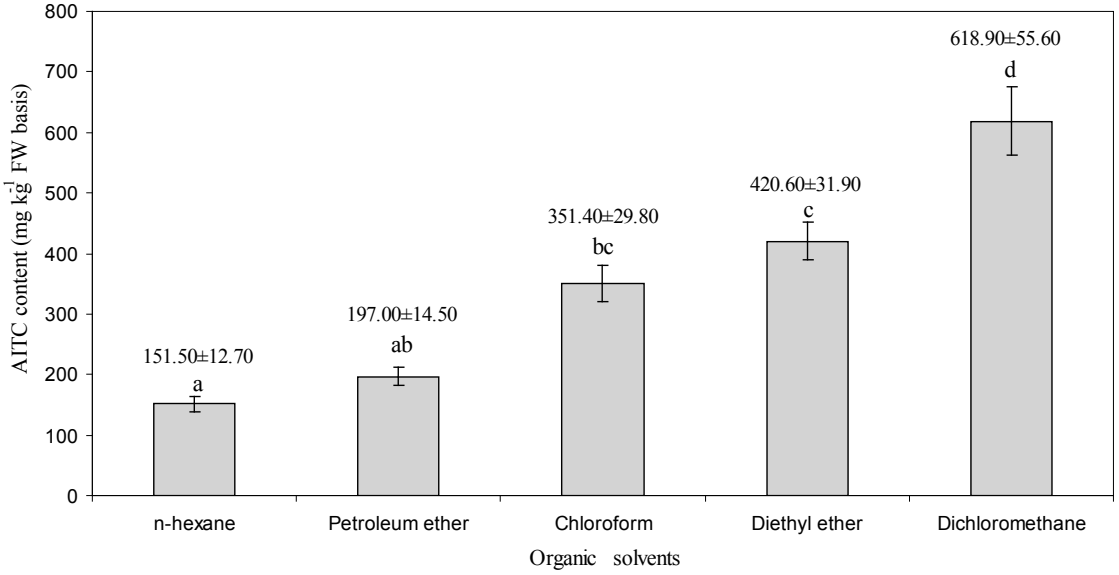


Figure 4.16: Effects of organic solvents on the yield of AITC during extraction. The same letters above the bars indicate that values are not significantly different. Values are the means of 6 replicates (n=6). Vertical lines represent standard errors.

4.4 Discussion

4.4.1 AITC in calli

Calli of wasabi were found capable of synthesising AITC with explant source and light conditions having an active impact on the synthesis of AITC in wasabi calli derived from both leaf and petiole segments, with leaf calli producing more AITC than petiole calli in the light. The difference in the capacity to produce ITCs between organs of *in vivo* wasabi plants, where leaves contain a larger content of AITC than petioles (Sultana *et al.*, 2003a), or between organs of *in vivo* explants cultured *in vitro*, is perhaps retained in the *in vitro* callus explants. The dissimilarity in the distribution of secondary metabolites in calli has also been found in callus cultures of *Pueraria lobata*, in which *in vitro* root callus synthesised more isoflavonoid compounds than leaf callus and stem callus (Matkowski, 2004), due partially to a higher level of isoflavones contained in the *in vivo* roots. Explanations for these differences are possibly based on the metabolism of different organs or cells. It is implied that specific tissues and cells of different origins possess a particularly biosynthetic potential. Therefore, the callus culture of *W. japonica* is capable of laying an initial background for further research on the metabolic regulation of ITCs in the development of plants. Parallel effects on the reaction of calli

to the light have been observed in the callus cultures of *Genista tinctoria* and *Rudbeckia hirta* (Luczkiewicz *et al.*, 2002; Luczkiewicz and Glod, 2003). The increased production of pulchelin E from root tissues of *R. hirta* indicated that light is a crucial factor for the synthesis of this compound, as it fails to be accumulated in darkness. Genistein esters in calli of *G. tinctoria* are regulated by the light regime in a different way. The dry weight proportions between genistin malonate and genistin acetate are reversed in calli cultured in the changes of light regime. The influence of light on the production of AITC in wasabi calli may be associated with increased enzyme activity. In addition, under *in vivo* conditions, there is a higher rate of volatilisation of AITC from leaves and petioles than from rhizomes and roots. This is in agreement with the findings of Sultana *et al.* (2003a, b) that ITCs are accumulated at relatively higher amounts in the *in vivo* plant organs without light access, such as rhizomes and roots grown under soil, than in organs with light access, like leaves and petioles. However, it is also possible that a metabolic pathway to synthesise AITC occurs in another tissue of the wasabi plant with the light factor, and there is a translocation process to transfer this compound to the storage and perennating tissues located in roots and rhizomes.

4.4.2 AITC in mutant lines as affected by polyploid-inducing agents

Mutation breeding is utilised to improve desirable agronomic characteristics in plants. In this study, colchicine and oryzalin were found to enhance the synthesis of AITC in *W. japonica* cultures, although it could be considered that colchicine was more efficient than oryzalin for AITC synthesis. A modification in erucic acid level by chemical mutagenesis has been observed from the double haploid lines of a Brassicaceous plant (*Brassica carinata*) with the level reduced by half in three lines, and increased in six other lines (Barro *et al.*, 2001). However, in the wasabi mutagenesis experiments, no reduction in AITC content was detected from both chromosome-doubling agents. Moreover, some peak areas fluctuated and other minor peaks occurred by GC-MS analysis, showing variations in other bioactive compounds (data not shown). The increase in AITC levels from treated explants suggests that a few mutant lines may have been established, and this possibility will be investigated in later studies. The changed condition might have been via an influence on the biosynthesis of ITCs or on the degradation of glucosinolate at different levels. To confirm this hypothesis, progenies

need to be established into soils in the greenhouses for more reliable evaluations, as plants would grow in conditions closer to the actual field environment.

4.4.3 AITC in mutant lines as affected by ionising-irradiations

No previous work has been reported on the *in vitro* mutagenesis of *W. japonica* by irradiation. The majority of studies on the mutation breeding have focused on the development of new cultivars with altered appearance traits such as flower colour, flower shape and fruit size (Gottschalk and Wolff, 1983). In this study, gamma rays and X-rays inhibited AITC accumulation in wasabi shoot tips in the dosage range at 10–40 Gy. X-rays appeared to have a greater effect on the reduction in AITC accumulation.

It is not possible to decide whether the effects on AITC decline by irradiation were responsible for the mutants at the *in vitro* stage, because it may be argued that these AITC-reduced variations occurred due to physical mutagens altering the physiological and biochemical processes (Berezina and Kaushanskii, 1989). Effects of radiation actions on the plant physiology that were not attributed to genetic changes have been observed on the *in vitro* growth of potatoes (Al-Safadi *et al.*, 2000) and grapevines (Charbaji and Nabulsi, 1999) by the use of low gamma doses. The reduced content of AITC in wasabi explants caused by gamma rays and X-rays may be explained by a degradation in biosynthesis of a certain amino acid that constitutes into GSLs, by the negative influence on the myrosinase activity that degrades GSLs into ITCs, or by the inhibition in medium mineral nutrient absorption. The effects of ionising radiation on enzyme activity, which result from the production of free radicals, such as H^+ and OH^-, and the subsequent formation of H_2O_2, by the light exposure from the radiation source (Dalton *et al.*, 1991), have been found in sweet potatoes (Lage and Esquibel, 1997), in which peroxidase was greater in leaves of explants treated with gamma rays.

If the reduction in AITC levels in wasabi materials did not originate from changes in physiological or biochemical processes, the gene instability by irradiation resulting in mutants could be the cause. However, to confirm the occurrence of a wasabi mutant line, there need to be careful field assessments of the progenies grown in soil for several generations over the following harvesting periods to achieve stability in AITC contents. From all of the treatments, irradiated-wasabi plantlets grown in the UTS greenhouse for a period of two months demonstrated no responses to altered morphological variations.

Studies on the differences in AITC contents in irradiated-wasabi plants grown in the greenhouse are progressing.

4.4.4 AITC in in vitro- and in vivo-grown plants

In vitro. The distribution of AITC in the three parts of microshoots was slightly changed over a three-month period of subculture. The accumulation of AITC was greater in leaves than in petioles and bases. Leaves possessed less AITC than petioles in the first subculture. Shoot bases accumulated a higher AITC yield than leaves and petioles in all three subcultures. The rapid increase of AITC in leaves may be explained on the basis of developing photosynthesis and tissue function. Leaf surfaces are narrow after a one-month growth of *in vitro* wasabi shoots, and become larger after two- and three-month growths when shoot multiplication is reduced. Thus, one-month-old leaves have a lower capacity in photosynthesis of secondary metabolites than the two- and three-month-old leaves. This explanation is well correlated to the higher accumulation of AITC in petioles than in leaves in the first 30 days in culture. It may also be that *in vitro* leaves contain a lower number of metabolically active tissues than petioles and bases, particularly at the one-month period, because leaves are less mature.

The maturity of the tissue in relation to changes in AITC levels was confirmed by Sultana *et al.* (2003b). Indeed, the metabolic activity of mature plant organs performs a large number of functions in plants, such as storage and photosynthesis (Taiz and Zeiger, 1991). Storage function assists in maintaining GSLs, precursors of AITC, in Brassicaceous plants, as GSLs are reported to be stored in vacuoles (Grob and Matile, 1979; Delaquis and Mazza, 1995).

The investigation of AITC in wasabi microshoots suggests that the changes in AITC yield are related to the age of plant parts. In addition, the possession of the highest level of AITC in bases, compared to leaves and petioles of *in vitro* shoots, is consistent with the accumulation of ITCs and AITC in the rhizomes of field-grown wasabi plants (Sultana *et al.*, 2003b). It can be expected, under field conditions, that there would be higher rates of volatilisation of AITCs from the leafy shoots than from the rhizomes. It is interesting, however, that the same trend was found here in *in vitro* material.

In vivo. In the current work, distribution of AITC in the four parts of greenhouse-grown plants increased differently over a period of 12 months. The leaves and petioles of the young plants possessed significantly lower concentrations of AITC than older plants.

Similarly, roots and rhizomes accumulated significantly higher contents of AITC in older plants. The suggestion is that the optimal harvest time is in either the ninth month or twelfth month, when AITC contents are highest. Since the level of AITC in leaves and petioles remained relatively constant after cultivation for nine to 12 months, it shows that the physiological yellowing has not yet occurred in the one-year-old plant aerial parts. The plant aerial organs at the senescence stage result in the loss of GSLs, precursors of AITC, as identified in the senescent leaves of *Arabidopsis thaliana* (Brown *et al.*, 2003). This has also been confirmed by the findings of Verkerk *et al.* (2001), that GSLs were strongly autolysed by the endogenous myrosinase in senescent florets of broccoli (*Brassica olerucea*) due to much cell damage. A further investigation is thus necessary to be carried out to identify the time when AITC concentration starts to decline. In the one-year period of wasabi growth, considering the fresh weight proportions of plant parts, the nine-month-old leaves occupied a significantly higher weight percentage than the twelve-month-old leaves; whereas petioles remained unchanged in their fresh weight percentage between nine and 12 months of age. Therefore, it is best to harvest the leaves of greenhouse-raised wasabi plants at the age of nine months.

There is a consistency in the increasing synthesis of AITC over developmental stages between *in vitro* and *in vivo* plant organs, and this is also in agreement with the findings of Sultana *et al.* (2003b) on the yield of ITCs in wasabi rhizomes. However, the proportions of AITC distributed among the leaves and petioles of *in vitro* and *in vivo* plants are not totally consistent. The leaves of greenhouse-grown plants accumulated a greater AITC level than petioles from the third month *in vivo*, and maintained this higher value through 12 months of growth, while leaves of *in vitro*-grown shoots failed to possess a higher AITC level than did the petioles. It is suggested that metabolite biosynthesis starts to occur strongly in wasabi leaves only at the post-*in vitro* stage, which may be due to the growth stimulation by natural light. The rhizomes yielded still higher AITC level, followed by roots, than leaves and petioles. This could be explained on the basis of the locations for biosynthesis and storage of these products, which have been discussed earlier. That is, it may be that AITC is synthesised in the mature leaves of wasabi, and then transported to storage tissues localised in roots and rhizomes. An investigation into the mechanism of GSL transport has demonstrated that benzylglucosinolate synthesised in leaves of *Tropaeolum majus* was transported to other

tissues (Lykkesfeldt and Moller, 1993). Also, GSLs that accumulated in the seeds of *Brassica napus* have physicochemical properties to allow the mobility of phloems (Brudenell *et al.*, 1999). It is also argued that GSLs synthesised in mature leaves of *Brassica nigra* are transported to key sinks by the phloem, resulting in a higher level of GSLs in younger leaves (Merritt, 1996). The findings by Du and Halkier (1998), however, show that GSLs are synthesised in the seeds of *Sinapis alba* owing to the co-localisation of all the enzymes necessary for GSL synthesis.

Whatever the debates, the highest amount of AITC is accumulated in the rhizomes of wasabi grown in soil and water under field conditions (Sultana *et al.*, 2000, 2002, 2003a, 2003b), or in soil under greenhouse conditions. Although further investigations on translocation need to be conducted, these results support the concept that the age and organs of wasabi plants are involved in AITC rate variations, comparable with the data on *Arabidopsis thaliana* (Brown *et al.*, 2003). The difference in AITC accumulated in greenhouse-grown wasabi plant parts, which follows the order: rhizomes > roots > leaves > petioles, is similar to the report by Sultana *et al.* (2003a) on the flowering and non-flowering tissues of the hydroponic-grown wasabi, while difference in the total fresh weight (of the greenhouse-grown wasabi plant parts) is: leaves > petioles > rhizomes > roots.

4.4.5 Optimisation of AITC extraction conditions

Temperature is one of the most important factors affecting the stability of ITC content in wasabi materials (Kojima and Nakano, 1980; Ina *et al.*, 1981; Kojima *et al.*, 1982, 1985; Yong-Gang *et al.*, 1994; Ohta *et al.*, 1995; Sultana *et al.*, 2003d). The present research on the effects of drying temperature on wasabi petioles shows that increasing the temperature increases the loss of AITC but the effect was not severe until 40^{o}C. An observation by Yong-Gang *et al.* (1994) on heating cramble cake, a by-product derived from a Brassicaceous vegetable known as *Crambe abyssinica*, at 100–110^{o}C for 60–80 minutes showed a 60% loss of ITC. The apparent loss of AITC concentration from raw petioles to high-temperature-heated materials may account for the changes in the chemical structure of ITCs, or the evaporation of the GSLs as reported by Yong-Gang *et al.* (1994). To maintain petiole quality, it is suggested that a drying temperature be kept below 50^{o}C. This is supported by the findings of Tseng *et al.* (1986), that composition in wasabi products was best maintained by drying at 45^{o}C.

In the present experiments dealing with the effects of storage temperatures on the AITC yield of fresh wasabi leaves, substantial changes were recorded. While the AITC content in fresh rhizomes remained unchanged for a storage length of eight weeks at -20^{o}C and -80^{o}C (Sultana *et al.*, 2003d), the AITC yield in fresh leaves stored at these temperatures was significantly reduced by the fourth week. The opposite to fresh leaves was further shown from the results of Kojima and Nakano (1980), in that the contents of ITCs in the hydrolysates of wasabi powder were unaffected during storage at -15^{o}C. In this respect, it can be argued that fresh leaves demonstrate a faster alteration in composition than do other materials in cold storage. The decline in AITC concentration in fresh leaves occurred more rapidly (after only two days of storage) with temperatures at 4^{o}C (cool temperature) and approximately 20^{o}C (room temperature), and the AITC was totally lost during a two-week storage at room temperature. The latter is partly in agreement with the observations of Rodrigues and Rosa (1999) on the storage of fresh broccoli inflorescences at different temperatures (room temperature, cool temperature and cold temperature), in that the contents of total and individual GSLs were most significantly reduced at room temperature. It was reported by Kojima and Nakano (1980), who conducted a study on the storage of wasabi powder, that an increase of water content in wasabi during storage triggered a decrease in the level of volatile compounds. However, the loss of AITC on storage at 4^{o}C accounted for the instability of AITC as concluded by Sultana *et al.* (2003d), after showing no correlation between the moisture content and the loss of ITCs at cool temperature. The same may be true for the reduction of AITC during storage at room temperature. A storage temperature higher than 20^{o}C may cause an AITC loss at a higher rate, as indicated in the report by Kojima *et al.* (1982) on the wasabi root powder stored at 30^{o}C in a sealed vessel. A considerable loss that occurred when the leaves were defrosted and subsequently refrozen could result from the fact that GSLs were hydrolysed by the plant enzyme myrosinase into AITC which was then removed away from leaf tissues by its highly volatile nature. The statement on the formation and removal of the breakdown products was supported by the observation of Sultana *et al.* (2003d) on rhizomes defrosted and then frozen at -15^{o}C.

Little is known about the effects of cool and cold temperatures on the AITC content alteration in wasabi leaves harvested from a greenhouse. The target of storing fresh wasabi leaves at different temperatures aims to help consumers and food processors to

minimise the risk of AITC loss during storage. This is particularly important in Vietnam where the storage conditions for the preservation of post-harvest agricultural products are not fully controlled.

The study on the shaking methods for efficient extraction indicated that temperature had a significant influence on the stability of the AITC content. In a range between 0^oC and 37^oC, increasing the temperature decreased the degradation of AITC in the aqueous solution (Ina *et al.*, 1981; Depree and Savage, 1996). The sensitivity of degradation to temperature depends upon the reaction between water molecules and OH^- ions of AITC molecule (Ohta *et al.*, 1995). Degradation was stable at below -5^oC (Ina *et al.*, 1981; Depree and Savage, 1996). It has also been observed in the current experiments with shaking time and speed that the decomposition of AITC stopped completely at 60–120 minutes and 200 rpm, respectively.

Investigations reported on the effects of particle size of dried petioles, after grinding, on the stability of AITC content are scarce. The results in this study showed that particle size distribution was important for classifying dried wasabi materials into groups before extraction, as each size group may contain a different level of AITC. Particle size at 210 μM appeared to be optimal for maximising AITC yield from wasabi petioles. The high yield of AITC could be extracted from particles at smaller size because they have larger total surface areas for the contact with aqueous solution. However, at a size of 63 μM, particles contained a reduced amount of AITC. The AITC level continued to decline significantly at a size below 63 μM, and may be completely lost in particles at an extremely small size. This was possibly because the yields of AITC produced from particle sizes larger than 63 μM were affected by the penetration and extraction of dichloromethane, while the yields gained from particle sizes equal to or smaller than 63 μM were affected by the volatilisation of AITC from dichloromethane.

In the research on the effects of organic solvents on the extraction efficiency, dichloromethane proved to be most effective for extraction, which could harvest 619±56 mg of AITC from 2 grams of fresh leaves. Hexane, however, appeared to be least effective because it only harvested 152±13 mg of AITC. The stability of AITC in hexane was studied by Ina *et al.* (1981), who stated that the decomposition of AITC occurred gradually in the solution of water and methanol, while stabilised in hexane, acetone and ethyl acetate. This statement was confirmed by Etoh *et al.* (1994). Boiling

70–90% methanol was popularly used for GSL extraction of Brassicaceous plants before using high-performance liquid chromatography (Rodrigues and Rosa, 1999; Verkerk *et al.*, 2001; Brown *et al.*, 2003) for performing the GSL analysis. However, dichloromethane was widely used in reports by Sultana *et al.* (2003b, 2003c, 2003d) for individual ITC extraction prior to using gas chromatography–mass spectroscopy. Hence, the correlation between methanol and dichloromethane for optimal extraction may need to be compared.

4.5 Conclusions

The studies show that *W. japonica* can synthesise AITC from consecutive callus cultures derived from leaves and petioles under *in vitro* culture conditions. Accumulation of AITC on the MS medium supplemented with 0.5 μM 2,4-D for the first six weeks, and then with 0.5 μM 2,4-D plus 0.5 μM kinetin for another eight weeks, was greater under the light conditions than under dark conditions from both types of explants (leaf callus and petiole callus), with higher yields obtained from leaf calli.

Mutant plants in terms of AITC yield alteration were produced *in vitro*. All mutated plants under the ionising irradiation treatments showed lower AITC contents than the non-mutated plants, whereas none of the mutants induced by chemical mutagens exhibited lower AITC contents than the control, with the higher AITC yields produced by colchicine treatments.

AITC content was investigated from *in vitro* explants monthly and *in vivo* plants every three months. Rhizomes contain the most AITC compared to leaves, petioles and roots of the plants grown in the greenhouse at three, six, nine and 12 months. Similarly to *in vitro* explants, the shoot bases account for the highest level of AITC in comparison with leaves and petioles. A different response was recorded with the AITC content rate between leaves and petioles. This rate was lower in the *in vitro* explants at one month, but higher in the *in vitro* explants at two and three months, and *in vivo* plants within 12 months.

Further investigations into the effects of storage temperatures on the loss of AITC yield showed that the leaves of *W. japonica* were affected at two days, two weeks and four weeks after storage at 4^{o}C, -20^{o}C and -80^{o}C, respectively. In contrast, shaking the

ground leaves for more than 60 minutes and 200 rpm at 35^{o}C, using dichloromethane as a solvent for extraction, drying the petioles below 40^{o}C and then grinding at the size of 210 μm before extracting, resulted in the greatest AITC yields.

CHAPTER 5

SIGNIFICANCE OF FINDINGS AND FUTURE DIRECTIONS

This report has described a series of investigations into protocols for the *in vitro* propagation of *W. japonica* using liquid and gelled media, callus induction, root induction, acclimatisation, induced mutations by chemical and physical mutagens, the accumulation of AITC in callus cultures and in plants, and optimisation of the AITC extraction conditions of *W. japonica*.

5.1 Significance of findings

Transplant material plays a primary role in cultivation. Cultivation of *W. japonica* to supply the Japanese market has recently spread to several countries such as Taiwan, New Zealand (Douglas and Follett, 1992; Sultana *et al.*, 2003b) and Australia (Barber and Buntain, 1985; Sparrow, 2004). Transplant material is thus growing in importance. However, *W. japonica* seeds exhibit a deep dormancy, resulting in a low rate of germination (Chadwick, 1990; Palmer, 1990); while offsets separated from an elite mother plant are highly contaminated with various diseases such as leaf spots, chronic wilt and petiole veins after several production cycles (Chadwick *et al.*, 1993; Sparrow, 2004). Micropropagated plantlets can partially overcome these problems for large-scale cultivation. In accordance with the use of liquid cultures in the shoot multiplication stage of the *W. japonica* propagation cycle, the price set for commercial plantlets can be reduced due to simple handling and low labour costs (Hvoslef-Eide and Preil, 2005); otherwise, plantlets are expensive (Hosoki *et al.*, 1986). The use of a liquid medium for *in vitro* culture is also an ideal subject for future studies on the production of numerous plantlets via bioreactors and the synthesis of bioactive compounds via cell suspension cultures.

Wasabia japonica has already proven to be one of the important Japanese native crops due to its bioactive compounds used for the applications in food and pharmaceutical

products. The major market interest lies in the value of the isothiocyanates contained in the rhizomes of *W. japonica*. The market appeal for *W. japonica* will remain unchanged if the Japanese food and pharmaceutical industries continually rely on the current cultivars of this crop for the majority of its population. Cultivars with desirably altered characteristics should be developed to satisfy the market's demand and sustain the current high prices for *W. japonica*. Novel cultivars can be obtained by a range of plant breeding techniques. The foundation studies described in Chapters 3 and 4 indicate that several preliminary results on the alterations of AITC content in *W. japonica* can be achieved with mutation breeding at the *in vitro* phase. Indeed, treatments with both forms of chemical and physical mutagens were shown to produce AITC variants. Colchicine and oryzalin treatments resulted in several promising varieties in terms of improved AITC content and altered leaf shape and size. Ionising irradiation treatments produced varieties with reduced AITC contents; however, these varieties may have other desirable characteristics such as pathogen resistance and rhizome size enlargement.

The aim of initiating callus cultures derived from *in vitro* leaves and petioles in this project was to prepare useful materials for future studies, as calli play an important role in various agricultural, biotechnological and biochemical applications. In plant propagation, calli are largely established as a precursor for somatic embryogenesis and plantlet regeneration. Somatic embryos of *W. japonica* were reported to be differentiated via calli derived from cotyledon tissues, and subsequently produced multiple shoots (Eun *et al.*, 1996). Direct somatic embryogenesis from embryogenic calli derived from cotyledons has been initiated in such species as *Cephaelis ipecacuanha* A. Richard (Rout *et al.*, 2000), *Cajanus cajan* L. Millsp (Singh *et al.*, 2003) and *Dianthus* spp. (Pareek and Kothari, 2003). By histological observations, the characteristic embryogenic calli developed into somatic embryos through globular, torpedo and heart-shaped stages, followed by embryo maturation and germination to form plantlets. In *W. japonica*, it has been reported that the induction of embryogenic calli was positive for cotyledon explants and negative for hypocotyl and radicle tissues (Eun *et al.*, 1996). Furthermore, the induction of organogenesis from calli of shoot-tip, leaf, petiole, stem or corm origins has been widely reported (Al-Juboory *et al.*, 1998; Tsuro *et al.*, 2000; Roy and Banerjee, 2003; Thao *et al.*, 2003a). Direct regeneration of adventitious shoots from calli was achieved, and plantlet regeneration followed. In

addition to the primary application of callus cultures in plant breeding, another aim in establishing wasabi calli was to provide initial explants for the later use in the production of bioactive metabolites by cell suspension cultures. The accumulation of phytoalexins (Pedras and Sorensen, 1998), wasalexins A and B (Pedras *et al.*, 1999) from *W. japonica* and glucosinolates from cell suspension cultures of a Brassicaceous plant (*Nasturtium montanum*) (Songsak and Lockwood, 2004) has displayed the importance of calli in some culture stages. Like the supplements of L-cysteine, L-methionine and L-tryptophan in a range at 2, 50, 100 and 200 ppm to the culture media for enhancing the degradation products of GSLs in *Nasturtium montanum* tissues, the exposure of callus and suspended cells to elicitors, such as jasmonic acid and its derivatives at 50 μM, for a dramatic stimulation in producing secondary metabolites has been achieved in such plants as *Ammi majus* L. (Staniszewska *et al.*, 2003), *Prunus cerasus* L. (Blando *et al.*, 2005), *Trigonella foenum-graecum* L. (Oncina *et al.*, 2000) and *Vitis vinifera* (Nagamori *et al.*, 2001). Therefore, the cell suspension culture of wasabi calli for the improvement in the synthesis of glucosilonates appears to be feasible.

5.2 Future research needs and directions

In plant breeding by mutagenesis, a continuous assessment of altered characteristics over several production cycles is required before the end products can be released for commercialisation. Therefore, monitoring of stable traits, that is, the improved AITC contents, in mutant varieties of *W. japonica* should be continued for several years because each production cycle of *W. japonica* lasts for approximately 18 months. Studies based on somatic embryogenesis in this species are also needed. The contents of other important ITCs, which are described in section 4.3.3 (Chapter 4), including 2-Methylthioethyl ITC, 5-Methylthiopentyl ITC, 6-Methylthiohexyl ITC and *cis*-2-Penten-1-ol, need to be urgently assessed in both mutated and non-mutated plants, because these ITCs are present in the currently studied plant. There is a considerable need for further identification of new bioactive compounds in *W. japonica* mutants, because ionising irradiation has been reported to change the chemical components in many plants (Rahman *et al.*, 1994; Saour *et al.*, 1999). Indeed, Chapter 4 appeared to show several new unknown compounds because the three minor peaks with three retention times, namely 7.0, 10.1 and 11.2, were displayed in the extracts of chemical

mutagen-induced explants, and were not exhibited in the untreated explants. An individual component of ITCs has a specific aroma. For example, 3-butenyl ITC has a pungent odour while *iso*-butyl ITC has a chemical-like flavour (Sultana *et al*., 2003). It was also reported that a minor change in the ITC components could significantly alter the overall flavour profile of *W. japonica* (Gilbert and Nursten, 1972). Components with retention times at 7.0, 10.1 and 11.2 would, therefore, be likely to have differences in the flavour of *W. japonica*. Some of the flavour ITC components have effective chemopreventive roles with pharmacological properties (Depree *et al*., 1999). Thus, whether the nutritional and condimental values of the components with the three above-mentioned retention times are useful or not, investigations on these components would be necessarily carried out in a near future. However, to fully identify these components, the total costs are relatively expensive. Regarding a purchase of one millilitre of an ITC standard chemical component such as AITC, it costs approximately AUD 100. Individual and total concentrations of ITCs are measured using many methods such as GC-MS (Kumagai *et al.*, 1994; Sultana *et al.*, 2002, 2003b, 2003c, 2003d), UV spectrometry (Sultana *et al.*, 2000) and head-space gas chromatography (Kojima and Nakano, 1980; Kojima *et al.*, 1982). ITC concentrations can also be reversely calculated from the total glucosinolate content which is measured by high-performance liquid chromatography (HPLC) (Rodrigues and Rosa, 1999; Verkerk *et al.*, 2001). Unknown bioactive compounds can be identified using spectroscopic data and chemical evidence (Hosoya *et al.*, 2005).

Other plant traits such as leaf number, rhizome size, plant height and disease resistance need to be ascertained before any commercialisation can occur. *W. japonica* is susceptible to a large number of diseases (Chadwick *et al.*, 1993; Sparrow, 2004). Eliminating diseases in wasabi plants by irradiation with gamma rays appears to be an excellent idea for future studies, as gamma-ionising irradiation could be applied to induce mutative plants resistant to diseases (Mohamed *et al.*, 1995; Agrawal *et al.*, 2005). In continuing our current work, selection of a mutant wasabi line free of disease becomes an essential step in the near future.

Currently, the plant-breeding process is simplified and accelerated with molecular studies for providing improved methods for identification, isolation and selection of desired mutants (Maluszynski *et al.*, 1995). There are many techniques employed to

determine desirable mutants. Selection of mutated materials in the field is popularly performed following the plant-to-progeny growing method. The ploidy level can be, however, determined by flow cytometry. This technique is more advantageous than traditional methods such as chromosome staining and counting, because it requires only a small amount of tissue and can be performed early in the plant development, or prior to the visible genetic changes, resulting in the reduction of time for plant selection. Flow cytometry has been successfully applied for determining the ploidy level in various species such as *Alocasia* spp., *Manihot esculenta* and *Diospyros kaki* (Awoleye *et al.*, 1994; Tamura *et al.*, 1996; Thao *et al.*, 2003b), and also for determining polysomaty in diploid and tetraploid *Portulaca grandiflora* (Mishiba and Mii, 2000).

Hydroponics is a simple system of growing plants without soil (Romer, 2002). As *W. japonica* proliferated rapidly in liquid cultures (see Chapter 2) and its rhizomes produced a greater level of ITCs in water cultivation, compared to soil cultivation (Sultana *et al.*, 2003b), it is suggested that *W. japonica* be investigated into growing in hydroponic systems for enhancing the growth and quality of this plant.

In conclusion, the studies described in my thesis are an exciting step forward in the growth of the *W. japonica* industry. Improved propagation technology, and induced mutation by the use of physical and chemical mutagens have appeared to be successful on this crop at the *in vitro* stage, and so can assist in future growth and development of the industry.

REFERENCES

Abberton M T, Callow R S. (1996). Nucleotypic influences on chromosome-specific chiasma variation in *Crepis capillaris*. I. Responses to early colchicine treatment and chromosome doubling. *Genome*; **39**: 1078–1085.

Adachi S. (1987). *Wasabi saibai.* Shizuoka Prefecture Agricultural Experiment Station: Shizuoka, Japan.

Adaniya S, Shirai D. (2001). *In vitro* induction of tetraploid ginger (*Zingiber officinale* Roscoe) and its pollen fertility and germinability. *Scientia Horticulturae*; **88**: 277–287.

Agrawal P K, Sidu G S, Gosal S S. (2005). Induction of bacterial blight resistance in elite Indian rice (*Oryza sativa* L.) cultivars using gamma irradiation and ethyl methane sulfonate. In: Shu Q, Lagoda P J L (Eds), *Mutation Breeding Newsletter and Reviews*, IAEA: Wien; pp. 17–18.

Ahloowalia B S, Maluszynski M. (2001). Induced mutations – A new paradigm in plant breeding. *Euphytica*; **118**: 167–173.

Ahnström G. (1989). Mechanism of mutation induction. *Vortrage für Pflanzenzüchtung*; **16**: 153–160.

Aitken-Christie J, Kozai T, Takayama S. (1995). Automation in plant tissue culture-general introduction and overview. In: Aitken-Christie J, Kozai T, Smith M A L (Eds), *Automation and Environmental Control in Plant Tissue Culture*, Kluwer: Dordrecht; pp. 1–18.

Akutsu M, Sato H. (2002). Induction of proembryos in liquid culture increases the efficiency of plant regeneration from *Alstroemeria* calli. *Plant Science*; **163**: 475–479.

Al-Juboory K H, Skirvin R M, Williams D J. (1998). Callus induction and adventitious shoot regeneration of gardenia (*Gardenia jasminoides* Ellis) leaf explants. *Scientia Horticulturae*; **72**: 171–178.

Al-Safadi B, Ayyoubi Z, Jawdat D. (2000). The effect of gamma irradiation on potato microtuber production *in vitro*. *Plant Cell, Tissue and Organ Culture*; **61**: 183–187.

Archer S J. (2001). *Wasabia japonica*. Urban agriculture notes. Canada's office of urban agriculture: Canada.

Arikat N A, Jawad F M, Karam N S, Shibli R A. (2004). Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill.). *Scientia Horticulturae*; **100**: 193–202.

Armstrong G, Johnson K. (2001). Micropropagation of *Ceratopetalum gummiferum*, an important Australian cut flower crop. *In Vitro Cellular & Developmental Biology – Plant*; **37**: 173–177.

Arunyanart S, Soontronyatara S. (2002). Mutation induction by gamma and X-ray irradiation in tissue cultured lotus. *Plant Cell, Tissue and Organ Culture*; **70**: 119–122.

Awoleye F, van Duren M, Dolezel J, Novak F J. (1994). Nuclear DNA content and *in vitro* induced somatic polyploidization cassava (*Manihot esculenta* Crantz) breeding. *Euphytica*; **76**: 195–202.

Babu K N, Sajina A, Minoo D, John C Z, Mini P M, Tushar K V. (2003). Micropropagation of camphor tree (*Cinnamonum camphora*). *Plant Cell, Tissue and Organ Culture*; **74**: 179–183.

Baker F W G (Ed.). (1992). *Rapid Propagation of Fast-growing Woody Species*. C.A.B. International for CASAFA: London, England.

Barber M, Buntain M. (1985). Wasabi. In: Keith H (Ed.), *The New Rural Industries, a Hand-book for Farmers and Investors*; RIRDC Publication: Canberra; pp. 219–224.

Barlass M, Hutchison J. (1996). Commercial micropropagation of Australian native plants. In: Taji A, Williams R (Eds), *Tissue Culture of Australian Plants*, University of New England Press: Armidale; pp. 180–203.

Barro F, Escobar J F, Vega M D L, Martin A. (2001). Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. *Plant Breeding*; **120**: 262–264.

Bartels P G, Hilton J L. (1973). Comparison of trifluralin, oryzalin, pronamide, propham, and colchicine treatments on microtubules. *Pesticide Biochemistry and Physiology*; **3**: 462–472.

Batista D, Ascensao L, Sousa M J, Pais M S. (2000). Adventitious shoot mass production of hop (*Humulus lupulus* L.) var. 'Eroica' in liquid medium from organogenesis nodule cultures. *Plant Science*; **151**: 47–57.

Beena M R, Martin K P, Kirti P B, Hariharan M. (2003). Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum*. *Plant Cell, Tissue and Organ Culture*; **72**: 285–289.

Berezina N M, Kaushanskii D A. (1989). *Presowing Irradiation of Plant Seeds*. Oxonian Press: New Delhi.

Bermúdez P P, Seitz H U, Gavidia I. (2002). A protocol for rapid micropropagation of endangered *Isoplexis*. *In Vitro Cellular & Developmental Biology – Plant*; **38**: 178–182.

Bhagwat B, Duncan E J. (1998). Mutation breeding of Highgate (*Musa acuminata*, AAA) for tolerance to *Fusarium oxysporum* f.sp. *cubense* using gamma irradiation. *Euphytica*; **101**: 143–150.

Bhate R H. (2001). Chemically induced floral morphological mutations in two cultivars of *Ipomoea purpurea* (L.) Roth. *Scientia Horticulturae*; **88**: 133–145.

Biswas A K, Bose K. (1998). Induced allotetraploidy in sesame. *Indian Journal of Genetics and Plant Breeding*; **58**: 137–141.

Blando F, Scardino A P, de Bellis L, Nicoletti I, Giovinazzo G. (2005). Characterization of *in vitro* anthocyanin-producing sour cherry (*Prunus cerasus* L.) callus cultures. *Food Research International*; **38**: 937–942.

Bones A M, Rossiter T J. (1996). The myrosinase-glucosinolate system, its organization and biochemistry. *Physiologia Plantarum*; **97**: 194–208.

Borthakur M, Singh R S. (2002). Direct plantlet regeneration from male inflorescences of medicinal yam (*Dioscorea floribunda* Mart. & Gal.). *In Vitro Cellular & Developmental Biology – Plant*; **38**: 183–185.

Bourgin J P, Nitsch J P. (1967). Obtention de *Nicotiana* haploïdes à partir d'étamines cultivées *in vitro*. *Annales de Physiologie Végétale*; **9**: 377–382.

Bouvier L, Fillon F R, Lespinasse Y. (1994). Oryzalin as an efficient agent for chromosome doubling of haploid apple shoot *in vitro*. *Plant Breeding*; **113**: 343–346.

Broertjes C. (1966). Mutation breeding of chrysanthemums. *Euphytica*; **15**: 156–162.

Broertjes C. (1972). Mutation breeding of *Achimenes*. *Euphytica*; **21**: 48–62.

Brown P D, Tokuhisa J G, Reichelt M, Gershenzon J. (2003). Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry*; **62**: 471–481.

Brudenell A J P, Griffiths H, Rossiter J T, Baker D A. (1999). The phloem mobility of glucosinolates. *Journal of Experimental Botany*; **50**: 745–756.

Brunakova K, Babincova Z, Cellarova E. (2005). Production of taxanes in callus and suspension cultures of *Taxus baccata* L. In: Hvoslef-Eide A K, Preil W (Eds), *Liquid Culture Systems for In Vitro Plant Propagation*, Springer: Dordrecht; pp. 567–574.

Buiatti M. (1989). Use of cell and tissue cultures for mutation breeding. *Vortrage für Pflanzenzüchtung*; **16**: 179–200.

Caponetti J D. (1999). Nutrition of callus cultures. In: Trigiano R N, Gray D J (Eds), *Plant Tissue Culture Concepts and Laboratory Exercises*, 2nd ed., CRC Press: Boca Raton; pp. 39–44.

Caponetti J D, Gray D J, Trigiano R N. (2000). History of plant tissue and cell culture. In: Trigiano R N, Gray D J (Eds), *Plant Tissue Culture Concepts and Laboratory Exercises*, 2nd ed., CRC Press: Boca Raton; pp. 11–17.

Cassells A C. (1991). Problems in tissue culture: Culture contamination. In: Debergh P C, Read P E (Eds), *Micropropagation*, Kluwer Academic Publishers: Dordrecht, The Netherlands; pp. 31–44.

Cassells A C, Curry R F. (2001). Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: Implications for micropropagations and genetic engineers. *Plant Cell, Tissue and Organ Culture*; **64**: 145–157.

Cassells A C, Walsh C, Periappuram C. (1993). Diplonic selection as a positive factor in determining the fitness of mutants of *Dianthus* 'Mystère' derived from X-irradiation of nodes in *in vitro* culture. *Euphytica*; **70**: 167–174.

Chadwick C I. (1990). Wasabi, *Wasabia japonica* (Miq.) Matsum., a semi-aquatic crop from Japan, Washington State University, Dissertation, 121 pp.

Chadwick C I, Lumpkin T A, Elberson L R. (1993). The botany, uses and production of *Wasabia japonica* (Miq) (*Cruciferae*) Matsum. *Economic Botany*; **47**: 113–135.

Chalak L, Legave J M. (1996). Oryzalin combined with adventitious regeneration for an efficient chromosome doubling of trihaploid kiwifruit. *Plant Cell Reports*; **16**: 97–100.

Charbaji T, Nabulsi I. (1999). Effect of low doses of gamma irradiation on *in vitro* growth of grapevine. *Plant Cell, Tissue and Organ Culture*; **57**: 129–132.

Chaturvedi R, Razdan M K, Bhojwani S S. (2004). *In vitro* clonal propagation of an adult tree of neem (*Azadirachta indica* A. Juss.) by forced axillary branching. *Plant Science*; **66**: 501–506.

Chauvin J E, Souchet C, Dantec J P, Ellisseche D. (2003). Chromosome doubling of 2x *Solanum* species by oryzalin: Method development and comparison with spontaneous chromosome doubling *in vitro*. *Plant Cell, Tissue and Organ Culture*; **73**: 65–73.

Chen Q F, Wang C L, Lu Y M, Shen M, Afza R, Duren M V, Brunner H. (2001). Anther culture in connection with induced mutations for rice improvement. *Euphytica*; **120**: 401–408.

Chu C C. (1978). The N6 medium and its applications to anther culture of cereal crops, *Proceedings of Symposium on Plant Tissue Culture*, Science Press: Beijing, China; pp. 43–50.

Chung C S, Yoshida K T, Takeda G. (1996). Reproductive characteristics of amphidiploids derived from an interspecific hybrid between *Nicotiana trigonophylla* Dun. and *N. tabacum* L. *Breeding Science*; **46**: 29–33.

Compton M E, Pierson B L, Staub J E. (2001). Micropropagation for recovery of *Cucumis hystrix*. *Plant Cell, Tissue and Organ Culture*; **64**: 63–67.

Curir P, Damiano C, Cosmi T. (1986). *In vitro* propagation of some rose cultivars. *Acta Horticulturae*; **189**: 221–224.

Curtis O F, Shetty K. (1996). Growth medium effects on vitrification, total phenolics, chlorophyll, and water content of *in vitro* propagated oregano clones. *Acta Horticulturae*; **426**: 489–504.

Dalton D A, Post C J, Langeberg L. (1991). Effects of ambient oxygen and of fixed nitrogen on concentrations of glutathione, ascorbate, and associated enzymes in soybean root nodules. *Plant Physiology*; **96**: 812–818.

Dantu P K, Bhojwani S S. (1995). *In vitro* corm formation and field evaluation of corm-derived plants of *Gladiolus*. *Scientia Horticulturae*; **61**: 115–129.

Das A, Gosal S S, Sidhu J S, Dhaliwal H S. (2000). Induction of mutations for heat tolerance in potato by using *in vitro* culture and radiation. *Euphytica*; **114**: 205–209.

Datta K, Datta S K. (1998). Palynological interpretation of gamma rays and colchicine induced mutations in chrysanthemum cultivars. *Israel Journal of Plant Sciences*; **46**: 199–207.

Datta S K. (1991). Role of mutation breeding in floriculture. *Plant Mutation Breeding for Crop Improvement. Volume 1 – Proceedings of a Symposium*, Vienna: IAEA: Vienna; pp. 273–281.

Datta S K. (1992). Assessment of single and combined X-ray and colchicine treatment on *Trichosanthes anguina* L. *Journal of Nuclear Agriculture Biology*; **21**: 293–298.

Datta S K, Chakrabarty D, Mandal A K A. (2001). Gamma ray-induced genetic manipulations in flower colour and shape in *Dendranthema grandiflorum* and their management through tissue culture. *Plant Breeding*; **120**: 91–92.

de Carvalho J F R P, de Carvalho C R, Otoni W C. (2005). *In vitro* induction of polyploidy in annatto *(Bixa orellana)*. *Plant Cell, Tissue and Organ Culture*; **80**: 69–75.

de Jong J, Huitema J B M, Preil W. (1991). Use of *in vitro* techniques for the selection of stress tolerant mutants of *Chrysanthemum morifolium*. *Plant Mutation Breeding for Crop Improvement. Volume 2 – Proceedings of a Symposium*, Vienna: IAEA: Vienna; pp. 149–155.

de Loose R. (1979). Radiation induced chimeric rearrangement in flower structure of *Rhododendron simsii* Planch. (*Azalea indica* L.). Use of recurrent irradiation. *Euphytica*; **28**: 105–113.

Debergh P, Aitken-Christie J, Cohen D, Grout B, von Arnold S, Zimmerman R, Ziv M. (1992). Reconsideration of the term 'vitrification' as used in micropropagation. *Plant Cell, Tissue and Organ Culture*; **30**: 135–140.

Debergh P, Zimmerman R H. (1990). *Micropropagation: Technology and Application*. Kluwer Academic: Dordrecht.

Debergh P C, Read P E. (1990). Micropropagation. In: Debergh P C, Zimmerman R H (Eds), *Micropropagation: Technology and Application*, Kluwer Academic: Dordrecht.

Delaquis P J, Mazza G. (1995). Antimicrobial properties of isothiocyanates in food preservation. *Food Technology*; **49**: 73–84.

Depree J A, Howard T M, Savage G P. (1999). Flavour and pharmaceutical properties of the volatile sulphur compounds of wasabi (*Wasabia japonica*). *Food Research International*; **31**: 329–337.

Depree J A, Savage G P. (1996). Storage properties of a wasabi-flavoured mayonnaise. *Proceedings of the Nutrition Society of New Zealand*; pp. 142–150.

Dias M C, Almeida R, Romano A. (2002). Rapid clonal multiplication of *Lavandula viridis* L'Her through *in vitro* axillary shoot proliferation. *Plant Cell, Tissue and Organ Culture*; **68**: 99–102.

Dodds J H, Roberts L W. (1982). *Experiments in Plant Tissue Culture*. Cambridge University Press: Cambridge.

Doorenbos J, Karper J J. (1975). X-ray induced mutations in *Begonia* x *hiemalis*. *Euphytica*; **24**: 13–19.

Doran P M. (1996). Cell culture technology for secondary metabolite production with reference to Australian plants. In: Taji A M, Williams R R (Eds), *Tissue Culture of Australian Plants*, University of New England: Armidale; pp. 240–283.

Dorsch W, Adam O, Weber J, Ziegeltrum T. (1985). Antiasthmatic effects of onion extracts – Detection of benzyl, and other isothiocyanates (mustard oil) as antiasthmatic compounds of plant origin. *European Journal of Pharmacology*; **107**: 17–24.

Douglas J. (2001). *Wasabi or Japanese Horseradish – Wasabia japonica*. New Zealand Institute for Crop and Food Research.

Douglas J A. (1993). New crop development in New Zealand. In: Janick J, Simon J E (Eds), *New Crops*: Wiley, New York.

Douglas J A, Follett J M. (1992). Initial Research on the Production of Water-grown Wasabi in the Waikato, *Twenty-second annual conference*, Agronomy Society of New Zealand: Lincoln University Canterbury, New Zealand; pp. 57–60.

Du L, Halkier B A. (1998). Biosynthesis of glucosinolates in the developing silique walls and seeds of *Sinapis alba*. *Phytochemistry*; **48**: 1145–1150.

Eleftheriou E P. (1993). Differentiation of abnormal sieve elements in roots of wheat (*Triticum aestivum* L.) affected by colchicine. *New Phytologist*; **125**: 813–827.

Etoh H, Jian-Sheng W, Yagi A, Kawano T, Kishima I, Ina K. (1994). Stabilization of isothiocyanates in wasabi and horseradish by high pressure treatment and addition of proteins. *Journal of the Japanese Society of Food and Technology*; **41**: 531–535.

Eun J S, Ko J E, Kim Y S. (1996). Propagation by means of somatic embryogenesis from immature embryo of *Wasabia japonica*. *Korean Journal of Breeding*; **28**: 21–28.

Evenor D, Reuveni M. (2004). Micropropagation of *Achillea filipendulina* cv. 'Parker'. *Plant Cell, Tissue and Organ Culture*; **79**: 91–93.

Fedoreyev S A, Pokushalova T V, Veselova M V, Glebko L I, Kulesh N I, Muzarok T I, Seletskaya L D, Bulgakov V P, Zhuravlev Y N. (2000). Isoflavonoid production by callus cultures of *Maackia amurensis*. *Fitoterapia*; **71**: 365–372.

Fenwick G R, Heaney R K, Mullin W J. (1983). Glucosinolates and their breakdown products in food and food plants. *CRC Critical Reviews in Food Science and Nutrition*; **18**: 123–201.

Fereol L, Louis S, Luce L. (1996). Effects of gamma radiation on *in vitro* plantlets of *Alpinia purpurata*. *Journal of Horticultural Science*; **71**: 243–247.

Follett J M. (1986). *Wasabi. Production of Four Traditional Japanese Vegetables in Japan*. MAFTECH Ruakura Agricultural Centre: Hamilton, New Zealand.

Fuke Y, Ohishi Y, Iwashita K, Ono H, Shinohara K. (1994). Growth suppression of MKN-28 human stomach cancer cells by wasabi (*Eutrema wasabi* Matxim.). *Journal of the Japanese Society for Food Science & Technology*; **41**: 709–711.

Fukuchi Y, Kato Y, Okunishi I, Matsutani Y, Osawa T, Naito M. (2004). 6-methylsulfinylhexyl isothiocyanate, an antioxidant derived from *Wasabia japonica*

Matsum., ameliorates diabetic nephropathy in type-2 diabetic mice. *Food Science and Technology Research*; **10**: 290–295.

Gamborg O L, Miller R A, Ojima K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*; **50**: 151–158.

Gamborg O L, Phillips G C (Eds). (1996). *Plant Cell, Tissue and Organ Culture*. Narosa: Springer-Verlag Berlin Geidelberg.

Geoffriau E, Kahane R, Bellamy C, Rancillac M. (1997). Ploidy stability and *in vitro* chromosome doubling in gynogenic clones of onion (*Allium cepa* L.). *Plant Science*; **122**: 201–208.

George E F. (1993). *Plant Propagation by Tissue Culture* (2nd ed.). Exegetics: Edington, England.

Gilbert L, Nursten H E. (1972). Volatile constituents of horseradish roots. *Journal of Science and Food Agriculture*; **23**: 527–539.

Gladstones J S. (1958). Induction of mutation in the West Australian blue lupin (*Lupinus Digitatus* Forsk.) by X-irradiation. *Australian Journal of Agricultural Research*; **9**: 473–482.

Gleba Y Y, Hinnisdaels S, Sidorov V A, Kaleda V A, Parokonny A S, Boryshuk N V, Cherep N N, Negrutiu I, Jacobs M. (1988). Intergeneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Atropa belladonna* obtained by gamma fusion. *Theoretical and Applied Genetics*; **75**: 760–766.

Goldy R G, Lyrene P M. (1984). *In vitro* colchicine treatment of 4x blueberries, *Vaccinium* sp. *Journal of American Society of Horticultural Science*; **109**: 336–338.

Goto M, Matsumoto K. (1986). Causal agents associated with the internal black rot syndrome of Japanese horseradish (*Eutrema wasabi* Maxim.). *Annals of the Phytopathological Society of Japan*; **52**: 59–68.

Gottschalk W, Wolff G. (1983). *Induced Mutations in Plant Breeding*. Springer-Verlag: Berlin; New York.

Green A G, Marshall D R. (1984). Isolation of induced mutants in linseed (*Linum usitatissimum*) having reduced linolenic acid content. *Euphytica*; **33**: 321–328.

Gribble K. (1999). The influence of relative humidity on vitrification, growth and morphology of *Gypsophila paniculata* L. *Plant Growth Regulation*; **27**: 179–188.

Grigoriadou K, Vasilakakis M, Eleftheriou E P. (2002). *In vitro* propagation of the Greek olive cultivar '*Chondrolia Chalkidikis*'. *Plant Cell, Tissue and Organ Culture*; **71**: 47–54.

Grigoriadou K, Vasilakakis M, Tzoulis T, Eleftheriou E P. (2005). Experimental use of a novel temporary immersion system for liquid culture of olive microshoots. In: Hvoslef-Eide A K, Preil W (Eds), *Liquid Culture Systems for In Vitro Plant Propagation*, Springer: Dordrecht; pp. 263–274.

Grob K, Matile P H. (1979). Vacuolar location of glucosinolates in horseradish root cells. *Plant Science Letter*; **14**: 327–335.

Guha S, Maheshwari S C. (1966). Cell division and differentiation in the pollen grains of *Datura in vitro*. *Nature*; **212**: 97–98.

Gyorgy Z, Tolonen A, Pakonen M, Neubauer P, Hohtola A. (2004). Enhancing the production of cinnamyl glycosides in compact callus aggregate cultures of *Rhodiola rosea* by biotransformation of cinnamyl alcohol. *Plant Science*; **166**: 229–236.

Halliwell B, Gutteridge J M. (1989). *Free Radicals in Biology and Medicine*. Oxford University Press: New York.

Hamill S D, Smith M K, Dodd W A. (1992). *In vitro* induction of banana autotetraploids by colchicine treatment of micropropagated diploids. *Australian Journal of Botany*; **40**: 887–896.

Han B H, Yu H J, Yae B W, Peak K Y. (2004). *In vitro* micropropagation of *Lilium longiflorum* 'Georgia' by shoot formation as influenced by addition of liquid medium. *Scientia Horticulturae*; **103**: 39–49.

Hansen A L, Gertz A, Joersbo M, Andersen S B. (1998). Antimicrotubule herbicides for *in vitro* chromosome doubling in *Beta vulgaris* L. ovule culture. *Euphytica*; **101**: 231–237.

Hansen F L, Andersen S B, Due I K, Olesen A. (1988). Nitrous oxide as a possible alternative agent for chromosome doubling of wheat haploids. *Plant Science*; **54**: 219–222.

Hansen N J P, Andersen S B. (1996). *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin and AMP in *Brassica napus* microspore culture. *Euphytica*; **88**: 159–164.

Haruki K. (1978). *Leaf Cultivation of Wasabi in Greenhouses*. Mitsubishi & Monsanto P.R. Publications: Japan.

Hassan L, Jones R N. (1994). Long-range effects of colchicine sensitivity on meiosis in *Loium multiflorum* L. (Italian ryegrass). *Heredity*; **73**: 65–71.

Herath S P, Suzuki T, Hattori K. (2004). Multiple shoot regeneration from young shoots of kenaf (*Hibiscus cannabinus*). *Plant Cell, Tissue and Organ Culture*; **77**: 49–53.

Herman E B. (1995). *Regeneration and Micropropagation: Techniques, Systems and Media 1991–1995*. Agritech Consultants: Shrub Oak (N.Y.).

Hodge W H. (1974). Wasabi – Native condiment plant of Japan. *Economic Botany*; **28**: 118–129.

Hoglund A S, Lenman M, Falk A, Rask L. (1991). Distribution of myrosinase in rapeseed tissues. *Plant Physiology*; **95**: 213–221.

Hoglund A S, Lenman M, Rask L. (1992). Myrosinase is localized to the interior of myrosin grains and is not associated to the surrounding tonoplast membrane. *Plant Science*; **85**: 165–170.

Hohmann U, Jacobs G, Jung C. (2005). An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breeding*; **124**: 317–321.

Hosokawa K, Oikawa Y, Yamamura S. (1999). Clonal propagation of *Wasabia japonica* by shoot tip culture. *Planta Medica*; **65**: 676.

Hosoki K, Tunoda T, Hamada M, Seo M. (1986). Wasabi tissue culture and multiplication. *Agriculture and Horticulture*; **61**: 85–86.

Hosoya T, Yun Y S, Kunugi A. (2005). Five novel flavonoids from *Wasabia japonica*. *Tetrahedron*; **61**: 7037–7044.

Hughes J, Tregova A, Tomsett A B, Jones M G, Cosstick R, Collin H A. (2005). Synthesis of the flavour precursor, allin, in garlic tissue cultures. *Phytochemistry*; **66**: 187–194.

Hulse J H. (1992). Plant cell and tissue culture: Progress and prospects. In: Baker F W G (Ed.), *Rapid Propagation of Fast-growing Woody Species*, C.A.B. International for CASAFA: London, England.

Hvoslef-Eide A K, Preil W. (2005). *Liquid Culture Systems for In Vitro Plant Propagation*. Springer: Dordrecht, Netherlands; Norwell, MA, U.S.A.

IAEA. (1977). *Manual on Mutation Breeding*, 2nd ed., Technical report series No. 119: Vienna: IAEA.

IAEA. (1997). Report 2nd FAO/IAEA research co-ordination meeting on *in vitro* techniques for selection of radiation induced mutations adapted to adverse environmental conditions, International Atomic Energy Agency: Vienna.

Ibrahim R, Mondelaers W, Debergh P C. (1998). Effects of X-irradiation on adventitious bud regeneration from *in vitro* leaf explants of *Rosa hybrida*. *Plant Cell, Tissue and Organ Culture*; **54**: 37–44.

Ina K, Ina H, Ueda M, Yagi A, Kishima I. (1989). ω-methylthioalkyl isothiocyanates in wasabi. *Agricultural and Biological Chemistry*; **53**: 537–538.

Ina K, Nobukuni M, Sano A, Kishima I. (1981). Stability of allyl isothiocyanate (Studies on the volatile components of wasabi and horseradish, Part III). *Nippon Shokuhin Kogyo Gakkaishi*; **28**: 627–631.

Ishikawa T, Takayama T, Ishizaka H. (1999). Amphidiploids between *Alstroemeria ligtu* L. hybrid and *A. pelegrina* L. var. 'Rosea' induced through colchicine treatment and their reproductive characteristics. *Scientia Horticulturae*; **80**: 235–246.

Isshiki K, Tokuoka K, Mori R, Chiba S. (1992). Preliminary examination of allyl isothiocyanate vapor for food preservation. *Bioscience Biotechnology and Biochemistry*; **56**: 1476–1477.

Isutsa D K. (2004). Rapid micropropagation of passion fruit (*Passiflora edulis* Sims.) varieties. *Scientia Horticulturae*; **99**: 395–400.

James D J, Passey A J, Rugini E. (1988). Factors affecting high frequency plant regeneration from apple leaf tissue cultures *in vitro*. *Journal of Plant Physiology*; **132**: 148–154.

Jang G W, Kim K S, Park R D. (2003). Micropropagation of venus fly trap by shoot culture. *Plant Cell, Tissue and Organ Culture*; **72**: 95–98.

Jerzy M, Zalewska M. (1996). Polish cultivars of *Dendranthema grandiflora* Tzvelev and *Gerbera jamesonii* Bolus bred *in vitro* by induced mutations. *Mutation Breeding Newsletter*; **42**: 19.

Joseph R, Yeoh H H, Loh C S. (2004). Induced mutations in cassava using somatic embryos and the identification of mutant plants with altered starch yield and composition. *Plant Cell Reports*; **23**: 91–98.

Kadota M, Niimi Y. (2003). Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in *in vitro* pear cultivar shoots. *Plant Cell, Tissue and Organ Culture*; **72**: 261–265.

Kadota M, Niimi Y. (2004). Improvement of micropropagation of Japanese yam using liquid and gelled medium culture. *Scientia Horticulturae*; **102**: 461–466.

Kane M E. (2000). Culture indexing for bacterial and fungal contaminants. In: Trigiano R N, Gray D J (Eds), *Plant Tissue Culture Concepts and Laboratory Exercises*, 2nd ed., CRC Press: Boca Raton.

Kataeva N V, Alexandrova I G, Butenko R G, Dragavtceva E V. (1991). Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured *in vitro*. *Plant Cell, Tissue and Organ Culture*; **27**: 149–154.

Kevers C, Bare G, Gaspar T, Thonart P, Dommes J. (2005). Optimisation of *Panax ginseng* liquid cell cultures for biomass accumulation and ginsenoside production. In: Hvoslef-Eide A K, Preil W (Eds), *Liquid Culture Systems for In Vitro Plant Propagation*, Springer: Dordrecht; pp. 547–555.

Kharkwal M C. (1998). Induced mutations for improvement of protein in chickpeas (*Cicer arietinum L.*). *Indian Journal of Genetics*; **58**: 61–68.

Khawaja H I T, Ellis J R. (1987). Colchicine-induced desynaptic mutations in *Lathyrus odoratus* and *L. pratensis*. *Genome*; **29**: 859–866.

Kjaer A, Ohashi M, Wilson J M, Djerassi C. (1963). Mass spectra of isothiocyanates. *Acta Chemica Scandinavica*; **17**: 2143–2154.

Knudson L. (1925). Physical study of the symbiotic germination of orchid seeds. *Botanical Gazette*; **79**: 345–379.

Kojima M, Hamada H, Toshimitsu N. (1985). Changes in isothiocyanates of *Wasabia japonica* roots by drying. *Nippon Shokuhin Kogyo Gakkaishi*; **32**: 886–891.

Kojima M, Hamada H, Yamashita M. (1982). Studies on evaluation of quality of Japanese horseradish (wasabi) powder by gas chromatography. XI. Studies on the stability of dried wasabi powder. *Journal of the Japanese Society for Food Science and Technology*; **29**: 232–237.

Kojima M, Nakano Y. (1980). Studies on evaluation of quality of Japanese horseradish (wasabi) powder by gas chromatography. X. Studies of changes in pungent components during storage of horseradish, mustard and wasabi powder. *Journal of the Japanese Society for Food Science and Technology*; **27**: 86–88.

Kuckuck H, Kobabe G, Wenzel G. (1991). *Fundamentals of Plant Breeding*. Springer-Verlag: Berlin; New York.

Kuksova V B, Piven N M, Gleba Y Y. (1997). Somaclonal variation and *in vitro* induced mutagenesis in grapevine. *Plant Cell, Tissue and Organ Culture*; **49**: 17–27.

Kumagai H, Kashima N, Seki T, Sakurai H, Ishii K, Ariga T. (1994). Analysis of volatile components in essential oil of upland wasabi and their inhibitory effects on platelet aggregation. *Bioscience Biotechnology and Biochemistry*; **58**: 2131–2135.

Kumar S, Dubey D K. (1998). Influence of separate and simultaneous applications of gamma rays, DES and EMS on meiosis in khesari (*Lathryus sativus* L.). *Journal of Genetics and Breeding*; **52**: 295–300.

Lage C L S, Esquibel M A. (1997). Growth stimulation produced by methylene blue treatment in sweet potato. *Plant Cell, Tissue and Organ Culture*; **48**: 77–81.

Linsmaier E M, Skoog F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum*; **18**: 100–127.

Lloyd G, McCown B. (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Combined Proceedings of the International Plant Propagation Society*; **30**: 421–427.

Lo C T, Wang K M, Hu M F, Wang C H. (2002). Integrated control of black rot disease of wasabi, caused by *Phoma wasabiae*. *Botanical Bulletin of Academia Sinica*; **43**: 219–225.

Loidl J. (1988). The effect of colchicine on synaptonemal complex formation in *Allium ursinum*. *Experimental Cell Research*; **178**: 93–97.

Luczkiewicz M, Glod D. (2003). Callus cultures of *Genista* plants – *In vitro* material producing high amounts of isoflavones of phytoestrogenic activity. *Plant Science*; **165**: 1101–1108.

Luczkiewicz M, Zarate R, Migas W, Migas P, Verpoorte R. (2002). Production of pulchelin E in hairy roots, callus and suspension cultures of *Rubeckia hirta* L. *Plant Science*; **163**: 91–100.

Lykkesfeldt J, Moller B L. (1993). Synthesis of benzylglucosinolate in *Tropaeolum majus* L.: Isothiocyanates as potent enzyme inhibitors. *Plant Physiology*; **102**: 609–613.

Ma Y, Byrne H H, Chen J. (1997). Amphidiploid induction from diploid rose interspecific hybrids. *HortScience*; **32**: 292–295.

Malamug J J F, Yazawa S, Asahira T. (1994). Morphological variants induced from shoot tips of taro (*Colocasia esculanta* (L.) Schott) treated with gamma radiation. *Scientia Horticulturae*; **58**: 105–113.

Maluszynski M, Ahloowalia B S, Sigurbjörnsson B. (1995). Application of *in vivo* and *in vitro* mutation techniques for crop improvement. *Euphytica*; **85**: 303–315.

Mandal A K A, Chakrabarty D, Datta S K. (2000a). Application of *in vitro* techniques in mutation breeding of chrysanthemum. *Plant Cell, Tissue and Organ Culture*; **60**: 33–38.

Mandal A K A, Chakrabarty D, Datta S K. (2000b). *In vitro* isolation of solid novel flower colour mutants from induced chimeric ray florets of chrysanthemum. *Euphytica*; **114**: 9–12.

Martinussen I, Nilsen G, Svenson L, Junttila O, Rapp K. (2004). *In vitro* propagation of cloudberry (*Rubus chammaemorus*). *Plant Cell, Tissue and Organ Culture*; **78**: 43–49.

Masuda H, Harada Y, Tanaka K, Nakajima M, Tabeta H. (1996). Characteristic odorants of wasabi (*Wasabia japonica* Matsum.), Japanese horseradish, in comparison

with those of horseradish (*Armoracia rusticana*). *Biotechnology for Improved Foods and Flavors*; **637**: 67–78.

Matkowski A. (2004). *In vitro* isoflavonoid production in callus from different organs of *Pueraria lobata* (Wild.) Ohwi. *Journal of Plant Physiology*; **161**: 343–346.

Mato M C, Mendez J, Vazquez A. (1994). Polyphenolic auxin protectors in buds of juvenile and adult chestnut. *Plant Physiology*; **91**: 23–26.

Matsumoto T, Nako Y. (1999). Effect of dimethylsulfoxide on *in vitro* storage of wasabi meristems at low temperature. *Plant Biotechnology*; **16**: 243–245.

Matsumoto T, Sakai A, Takahashi C, Yamada K. (1995). Cryopreservation of *in vitro* grown apical meristems of wasabi (*Wasabia japonica*) by encapsulation-vitrification method. *Cryo-Letters*; **16**: 189–196.

Matsumoto T, Sakai A, Yamada K. (1994). Cryopreservation of *in vitro* grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. *Plant Cell Reports*; **13**: 442–446.

Mayo O. (1980). *The Theory of Plant Breeding*. Clarendon Press: Oxford.

Mayor M L, Nestares G, Zorzoli R, Picardi L A. (2003). Reduction of hyperhydricity in sunflower tissue culture. *Plant Cell, Tissue and Organ Culture*; **72**: 99–103.

Medero S, Enriquez M J. (1987). *In vitro* propagation of 'Golden Times' roses – Factors affecting shoot tips and axillary bud growth and morphogenesis. *Acta Horticulturae*; **212**: 619–624.

Meesilpa P, Phadvibulya V. (1991). Induced mutation in mulberry using gamma radiation. *Plant Mutation Breeding for Crop Improvement. Volume 1 – Proceedings of a Symposium*, Vienna: IAEA: Vienna; pp. 513–517.

Mensah J K, Eruotor P G. (1993). Genetic variation in agronomic characteristics of lima beans induced by seed irradiations. *Tropical Agriculture*; **70**: 342–344.

Mereti M, Grigoriadou K, Nanos G D. (2002). Micropropagation of the strawberry tree, *Arbutus unedo* L. *Scientia Horticulturae*; **93**: 143–148.

Merritt S Z. (1996). Within-plant variation in concentrations of amino acids, sugar, and sinigrin in phloem sap of black mustard, *Brassica nigra* (L.) Koch (*Cruciferae*). *Journal of Chemical Ecology*; **22**: 1133–1145.

Micke A, Donini B. (1993). Induced mutations. In: Hayward M O, Bosemark N O, Romogosa I (Eds), *Plant Breeding Principles and Prospects – Series 1*, Chapman and Hall: London.

Miedema P. (1973). The use of adventitious buds to prevent chimerism in mutation breeding of potato. *Euphytica*; **22**: 209–218.

Miles C A, Chadwick C I. (1996). Wasabi article. *PNW Sustainable Agriculture Newsletter*; **8**: 1–3.

Mishiba K I, Mii M. (2000). Polysomaty analysis in diploid and tetraploid *Portulaca grandiflora*. *Plant Science*; **156**: 213–219.

Misra R L. (1998). Radiation induced variability in *Gladioli*. *Indian Journal of Genetics*; **58**: 237–239.

Mitra S K, Mukherjee K K. (2001). Direct organogenesis in Indian spinach. *Plant Cell, Tissue and Organ Culture*; **67**: 191–194.

Mohamed M F, Coyne D P, Read P E. (1995). A radiation-induced mutant with resistance to common bacterial blight disease in common beans. *HortScience*; **30**: 577–578.

Montalvan R, Ando A. (1998). Effect of gamma-radiation and sodium azide on quantitative characteristics in rice (*Oryza sativa* L.). *Genetics and Molecular Biology*; **21**: 81–85.

Morejohn L C, Bureau T E, Mole-Bajer J, Bajer A S, Fosket D E. (1987). Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. *Planta*; **172**: 252–264.

Morel G. (1965). Clonal propagation of orchids by meristem culture. *Cymbidium Society News*; **20**: 3–11.

Morimitsu Y, Hayashi K, Nakagawa Y, Fujii H, Horio F, Uchida K, Osawa T. (2000). Antiplatelet and anticancer isothiocyanates in Japanese domestic horseradish, wasabi. *Mechanisms of Ageing and Development*; **116**: 125–134.

Murashige T, Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*; **15**: 473–497.

Murata M, Uno M, Nagai Y, Nakagawa K, Okunishi I. (2004). Content of 6-methylsulfinylhexyl isothiocyanate in wasabi and processed wasabi products. *Journal of the Japanese Society for Food Science and Technology – Nippon Shokuhin Kagaku Kogaku Kaishi*; **51**: 477–482.

Murray B E, Craig I L. (1962). A comparison of somatic and pollen X-irradiation of a polyploid *Medicago sativa* L. *Radiation Botany*; **1**: 209–214.

Nagamori E, Hiraoka K, Honda H, Kobayashi T. (2001). Enhancement of anthocyanin production from grape (*Vitis vinifera*) callus in a viscous additive-supplemented medium. *Biochemical Engineering Journal*; **9**: 59–65.

Nakayama H, Suzuki T, Suzuki Y. (1998). Effect of wasabi on ion secretion in guinea pig colon. *Nippon Nogeikagaku Kaishi – Journal of the Japan Society for Bioscience Biotechnology and Agrochemistry*; **72**: 499–507.

Narayan M S, Venkataraman L V. (2000). Characterisation of anthocyanins derived from carrot (*Daucus carota*) cell culture. *Food Chemistry*; **70**: 361–363.

Ngo T T, Shargool P D. (1994). *Biotechnological Applications of Plant Cultures*. CRC Press: Boca Raton.

Nitsch J P, Nitsch C. (1969). Haploid plants from pollen grains. *Science*; **163**: 111–117.

Ohta Y, Takatani K, Kawakishi S. (1995). Decomposition rate of allyl isothiocyanate in aqueous solution. *Bioscience Biotechnology and Biochemistry*; **59**: 102–103.

Ohwi J. (1984). *Flora of Japan (in English)*. Smithsonian Institution: Washington, D.C.

Omar M S, Novak F J, Brunner H. (1989). *In vitro* action of ethylmethanesulphonate on banana shoot tips. *Scientia Horticulturae*; **40**: 283–295.

Oncina R, Botia J M, Del Rio J A, Ortuno A. (2000). Bioproduction of diosgenin in callus cultures of *Trigonella foenum-graecum* L. *Food Chemistry*; **70**: 489–492.

Ono H, Tesaki S, Tanabe S, Watanabe M. (1998). 6-methylsulfinylhexyl isothiocyanate and its homologues as food-originated compounds with antibacterial activity against

Escherichia coli and *Staphylococcus aureus*. *Bioscience Biotechnology and Biochemistry*; **62**: 363–365.

Paek K Y, Hahn E J. (2000). Cytokinins, auxins and activated charcoal affect organogenesis and anatomical characteristics of shoot tip cultures of *Lisianthus* (*Eustoma grandiflorum* (Raf.) Shinn). *In Vitro Cellular & Developmental Biology – Plant*; **36**: 128–132.

Paek K Y, Hahn E J, Son S H. (2001). Application of bioreactors for large-scale micropropagation systems of plants. *In Vitro Cellular & Developmental Biology – Plant*; **37**: 149–157.

Palmer J. (1990). Germination and growth of wasabi (*Wasabia japonica* (Miq.) Matsumura). *New Zealand Journal of Crop and Horticultural Science*; **18**: 161–164.

Pâques M, Boxus P. (1987). Vitrification: Review of Literature. *Acta Horticulturae*; **212**: 155–166.

Parc G, Canaguier A, Landre P, Hocquemiller R, Chriqui D, Meyer M. (2002). Production of taxoids with biological activity by plants and callus culture from selected *Taxus* genotypes. *Phytochemistry*; **59**: 725–730.

Pareek A, Kothari S L. (2003). Direct somatic embryogenesis and plant regeneration from leaf cultures of ornamental species of *Dianthus*. *Scientia Horticulturae*; **98**: 449–459.

Parliman B J, Stushnoff C. (1979). Mutant induction through adventitious buds of *Kohleria*. *Euphytica*; **28**: 521–530.

Pasqua G, Avato P, Monacelli B, Santamaria A R, Argentieri M P. (2003). Metabolites in cell suspention cultures, calli, and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. *Plant Science*; **165**: 977–982.

Pathirana R, Wijithawarna W A, Jagoda K, Ranawaka A L. (2002). Selection of rice for iron toxicity tolerance irradiated caryopsis culture. *Plant Cell, Tissue and Organ Culture*; **70**: 83–90.

Pati P K, Rath S P, Sharma M, Sood A, Ahuja P S. (2006). *In vitro* propagation of rose – A review. *Biotechnology Advances*; **24**: 94–114.

Pedras M S C, Sorensen J L. (1998). Phytoalexin accumulation and antifungal compounds from the crucifer wasabi. *Phytochemistry*; **49**: 1959–1965.

Pedras M S C, Sorensen J L, Okanga F I, Zaharia I L. (1999). Wasalexins A and B, new phytoalexins from wasabi: Isolation, synthesis, and antifungal activity. *Bioorganic & Medicinal Chemistry Letters*; **9**: 3015–3020.

Phân viện Sinh học tại Đà Lạt. (1995). Kết quả nghiên cứu khoa học 5 năm 1991–1995: Đà Lạt; pp. 67–96.

Piatczak E, Wielanek M, Wysokinska H. (2005). Liquid culture system for shoot multiplication and secoiridoid production in micropropagated plants of *Centaurium erythraea* Rafn. *Plant Science*; **168**: 431–437.

Pierik R L M. (1987). *In Vitro Culture of Higher Plants*. Martinus Nijhoff: Dordrecht, The Netherlands.

Pittet H, Moncousin C. (1981). Multiplication nouvelle du rosier. *Rev Hortic Suisse*; **54**: 169–173.

Pospíšilová J, Tichá I, Kadlecek P, Haisel D, Plzáková. (1999). Acclimatization of micropropagated plants to *ex vitro* conditions. *Biologia Plantarum*; **42**: 481–497.

Potts S E, Lumpkin T A. (1997). Cryopreservation of *Wasabia spp*. seeds. *Cryo-Letters*; **18**: 185–190.

Predieri S. (2001). Mutation induction and tissue culture in improving fruits. *Plant Cell, Tissue and Organ Culture*; **64**: 185–210.

Predieri S, Zimmerman R H. (2001). Pear mutagenesis: *In vitro* treatment with gamma rays and field selection for productivity and fruit traits. *Euphytica*; **117**: 217–227.

Pruski K, Astatkie T, Nowak J. (2005). Tissue culture propagation of Mongolian cherry (*Prunus fruticosa*) and Nanking cherry (*Prunus tomentosa*). *Plant Cell, Tissue and Organ Culture*; **82**: 207–211.

Rahman S M, Tagaki Y, Kubota K, Miyamoto K, Kawakita T. (1994). High oleic acid mutant in soybean induced by X-ray irradiation. *Bioscience, Biotechnology and Biochemistry*; **58**: 1070–1072.

Rai R, Misra K K. (2005). Micropropagation of Karonda (*Carissa carandas*) through shoot multiplication. *Scientia Horticulturae*; **103**: 227–232.

Raju B C, Trolinger J C. (1986). Pathogen indexing in large-scale propagation of florist crops. In: Zimmerman R H, Griesbach R J, Hammerschlag F A, Lawson R H (Eds), *Tissue Culture as a Plant Production System for Horticultural Crops*, Mertinus Nijhoff Publishers: Dordrecht; pp. 135–138.

Razdan M K. (2002). *Introduction to Plant Tissue Culture* (2nd ed.). Science Publishers: Enfield, NH.

Read P E, Yang Q. (1985). Novel plant growth regulator delivery systems for *in vitro* culture of horticultural crops. *Acta Horticulturae*; **212**: 55–59.

Redha A, Attia T, Bueter B, Saisingtong S, Stamp P, Schmid J E. (1998). Improved production of doubled haploids by colchicine application to wheat (*Triticum aestivum* L.) anther culture. *Plant Cell Reports*; **17**: 974–979.

Rodrigues A S, Rosa E A S. (1999). Effect of post-harvest treatments on the level of glucosinolates in broccoli. *Journal of the Science of Food and Agriculture*; **79**: 1028–1032.

Roest S, van Berkel M A E, Bokelmann G S, Broertjes C. (1981). The use of an *in vitro* adventitious bud technique for mutation breeding of *Begonia* x *hiemalis*. *Euphytica*; **30**: 381–388.

Romer J. (2002). *Hydroponic Gardening in Australia*. R & D Aquaponics: Wetherill Park; pp. 7–13.

Rose J B, Kubba J, Tobutt K R. (2000). Induction of tetraploidy in *Buddleia globosa*. *Plant Cell, Tissue and Organ Culture*; **63**: 121–125.

Rout G R, Samantaray S, Das P. (2000). *In vitro* somatic embryogenesis from callus cultures of *Cephaelis ipecacuanha* A. Richard. *Scientia Horticulturae*; **86**: 71–79.

Rout G R, Samantaray S, Mottley J, Das P. (1999). Biotechnology of the rose: A review of recent progress. *Scientia Horticulturae*; **81**: 201–228.

Roy J, Banerjee N. (2003). Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. 'Oculatum Hk. f.' *Scientia Horticulturae*; **97**: 333–340.

Saakov V, Lang M, Schindler C, Stober F. (1992). Changes in chlorophyll florescence and photosynthetic activity of French bean leaves induced by gamma radiation. *Photosynthetica*; **27**: 369–383.

Saamin S, Thompson M M. (1998). Radiation-induced mutations from accessory buds of sweet cherry, *Prunus avium* L. cv. 'Bing'. *Theoretical and Applied Genetics*; **96**: 912–916.

Saisingtong S, Schmid J E, Stamp P, Buter B. (1996). Colchicine-mediated chromosome doubling during anther culture of maize (*Zea mays* L.). *Theoretical and Applied Genetics*; **92**: 1017–1023.

Salehi H, Khosh-Khui M. (1997). Effects of explant length and diameter on *in vitro* shoot growth and proliferation rates of miniature roses. *Journal of Horticultural Science*; **72**: 673–676.

Sander C, Muehlbauer F J. (1977). Mutagenic effects of sodium azide and gamma irradiation in *Pisum*. *Environmental and Experimental Botany*; **17**: 43–47.

Sanford J C (Ed.). (1983). *Ploidy Manipulation*. Purdue University Press: West Lafayette, Indiana.

Santos C V, Brito G, Pinto G, Fonseca H M A C. (2003). *In vitro* plantlet regeneration of *Olea europaea ssp*. *maderensis*. *Scientia Horticulturae*; **97**: 83–87.

Saour G, Makee H, Al-Oudat M. (1999). Susceptibility of potato plants grown from tubers irradiated with stimulation doses of gamma irradiation to potato tuber moth, *Phthorimaea operculella* Zeller (Lep., *Gelechiidae*). *Journal of Applied Entomology*; **123**: 159–164.

Scialabba A, Melati M R, Di L C. (1995). Colchicine-induced changes in the growth and secondary wall decomposition of cotyledonless radish seedlings. *Acta Botanica Gallica*; **142**: 773–785.

Sen J, Sen S. (1995). Two-step bud culture technique for a high frequency regeneration of *Gladiolus* corms. *Scientia Horticulturae*; **64**: 133–138.

Senawi B M T. (1985). Evaluation of the difficulties in *in vitro* propagation of *Theobroma cacao* L. and *Cocos nucifera* L., State University of Ghent (Belgium), Dissertation, 124 pp.

Shao J, Chen C, Deng X. (2003). *In vitro* induction of tetraploid in pomegranate (*Punica granatum*). *Plant Cell, Tissue and Organ Culture*; **75**: 241–246.

Shetty K, Carpenter T L, Curtis O F, Potter T L. (1996). Reduction of hyperhydricity in tissue cultures of oregano (*Origanum vulgare*) by extracellular polysaccharide isolated from *Pseudomonas* spp. *Plant Science*; **120**: 175–183.

Shin I S, Lee J M. (1998). Study on antimicrobial and antimutagenic activity of horseradish *(Wasabia japonica)* root extracts. *Bulletin of the Korean Fisheries Society*; **31**: 835–841.

Shin I S, Masuda H, Naohide K. (2004). Bactericidal activity of wasabi (*Wasabia japonica*) against *Helicobacter pylori*. *International Journal of Food Microbiology*; **94**: 255–261.

Singh N D, Sahoo L, Sarin N B, Jaiwal P K. (2003). The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Millsp). *Plant Science*; **164**: 341–347.

Singh S, Richharia A K, Joshi A K. (1998). An assessment of gamma rays induced mutants in rice (*Oryza sativa* L.). *Indian Journal of Genetics*; **58**: 455–463.

Singh V P, Sharma R. (1993). Gamma rays- and EMS-induced leaf mutants in mung bean (*Vigna radiata* (L.) Wilczeck). *Current Science*; **65**: 636–638.

Singh V P, Singh M, Pal J P. (1999). Mutagenic effects of gamma rays and EMS on frequency and spectrum of chlorophyll and macromutation in urdbean (*Vigna mungo* L. Hepper). *Indian Journal of Genetics and Plant Breeding*; **59**: 203–210.

Sinhamahapatra S P. (1986). Empirical evaluation of single plant selection strategies in X-irradiation population of jute (*Corchorus capsularis* L.). *Journal of Nuclear Agriculture Biology*; **15**: 13–17.

Slater G P. (1993). Analysis of thiocyanates and isothiocyanates by ammonia chemical ionization gas chromatography–mass spectrometry and gas chromatography – Fourier transform infrared spectroscopy. *Journal of Chromatography*; **648**: 433–443.

Soga O. (1982). Stimulative production of flaviolin by *Phoma wasabiae* causal fungi of blackening of root of wasabi, *Wasabia japonica*. *Agricultural and Biological Chemistry*; **46**: 1061–1064.

Soledade M, Pedras C, Sorensen J L. (1998). Phytoalexin accumulation and antifungal compounds from the crucifer wasabi. *Phytochemistry*; **49**: 1959–1965.

Songsak T, Lockwood G B. (2004). Production of two volatile glucosinolate hydrolysis compounds in *Nasturtium montanum* and *Cleome chelidonii* plant cell cultures. *Fitoterapia*; **75**: 296–301.

Soniya E V, Das M R. (2002). *In vitro* micropropagation of *Piper longum* – An important medicinal plant. *Plant Cell, Tissue and Organ Culture*; **70**: 325–327.

Spangenberg G, Valles M P, Wang Z Y, Montavon P, Nagel J, Potrykus I. (1994). Asymmetric somatic hybridisation between tall fescue (*Festuca arundinacea* Schreb.) and irradiated Italia ryegrass (*Lolium multiflorum* Lam.) protoplasts. *Theoretical and Applied Genetics*; **88**: 509–519.

Sparrow A. (2004). Wasabi. In: S Salvin, M Bourke, T Byrne (Eds), *The New Crop Industries Handbook*. Rural Industries Research and Development Corporation: Canberra; pp. 98–103.

Srivastava H K, Tyagi B R. (1986). Effects of seed irradiation on yield and quality of essential oil in palmarosa (*Cymbopogon martinii* Stapf.). *Euphytica*; **35**: 369–380.

Stajner N, Bohanec B, Jakse M. (2002). *In vitro* propagation of *Asparagus maritimus* – A rare Mediterranean salt-resistant species. *Plant Cell, Tissue and Organ Culture*; **70**: 269–274.

Staniszewska I, Krolicka A, Malinski E, Lojkowska E, Szafranek J. (2003). Elicitation of secondary metabolites in *in vitro* cultures of *Ammi majus* L. *Enzyme and Microbial Technology*; **33**: 565–568.

Stebbins G L. (1956). Artificial polyploidy as a tool in plant breeding. In: Stebbins G L (Ed.), *Genetics in Plant Breeding*. Brookhaven National Laboratory: New York.

Steward F C. (1968). *Growth and Organization in Plants*. Addison-Wesley Publishing: Reading, MA.

Strachan S D, Hess F D. (1983). The biochemical mechanism of action of the dinitroaniline herbicide oryzalin. *Pesticide Biochemistry and Physiology*; **20**: 141–150.

Sultana T, McNeil D L, Porter N G, Savage G P. (2003a). Investigation of isothiocyanate yield from flowering and non-flowering tissues of wasabi grown in a flooded system. *Journal of Food Composition and Analysis*; **16**: 637–646.

Sultana T, Porter N G, Savage G P, McNeil D L. (2003b). Comparision of isothiocyanate yield from wasabi rhizome tissues grown in soil or water. *Journal of Agricultural and Food Chemistry*; **51**: 3586–3591.

Sultana T, Savage G P, McNeil D L, Porter N G, Clark B. (2003c). Comparison of flavour compounds in wasabi and horseradish. *Food, Agriculture & Environment*; **1**: 117–121.

Sultana T, Savage G P, McNeil D L, Porter N G, Martin R J. (2000). Flavour components in the rhizome of soil-grown wasabi. *Proceedings of the Nutrition Society of New Zealand*; **25**: 95–106.

Sultana T, Savage G P, McNeil D L, Porter N G, Martin R J, Deo B. (2002). Effects of fertilisation on the allyl isothiocyanate profile of above-ground tissues of New Zealand-grown wasabi. *Journal of the Science of Food and Agriculture*; **82**: 1477–1482.

Sultana T, Savage G P, NcNeil D L, Porter N G. (2003d). The yield of isothiocyanates in wasabi rhizomes stored at different temperatures. *Food, Agriculture & Environment*; **1**: 39–45.

Sun Y, Cheng S Q, Liang G H. (1994). Induction of autotetraploid plants of *Sorghum versicolor*. *Cytologia (Tokyo)*; **59**: 109–114.

Suzuki M. (1968). Wasabi, Unpublished report. Kyoto University.

Taiz L, Zeiger E (Eds). (1991). *Plant and Cell Architecture in Plant Physiology*. The Benjamin/Cummings: Redwood, California.

Taji A, Kumar P K, Lakshmanan P. (2002). *In Vitro Plant Breeding*. Food Products Press: New York, London, Oxford.

Taji A M, Williams R R (Eds). (1996). *Tissue Culture of Australian Plants*. University of New England: Armidale, N.S.W.

Takamura T, Miyajima I. (1996). Colchicine induced tetraploids in yellow-flowered cyclamens and their characteristics. *Scientia Horticulturae*; **65**: 305–312.

Takuda T, Hirosawa T. (1975). Several factors for the formation of pycnidia *Phoma wasabiae* on diseased leaves of wasabi, *Wasabia japonica*. *Agricultural Research*; **50**: 53–57.

Tamura M, Tao R, Sugiura A. (1996). Production of dodecaploid plants of Japanese persimmon (*Diospyros kaki* L.) by colchicine treatment of protoplasts. *Plant Cell Reports*; **15**: 470–473.

Tewary P K, Oka S. (1999). Simplified clonal multiplication of mulberry using liquid shake culture. *Plant Cell, Tissue and Organ Culture*; **59**: 223–226.

Thangstad O P, Evjen K, Bones A. (1991). Immunogold-EM localization of myrosinase in *Brassicaceae*. *Protoplasma*; **161**: 85–93.

Thao N T P, Ozaki Y, Okubo H. (2003a). Callus induction and plantlet regeneration in ornamental *Alocasia micholitziana*. *Plant Cell, Tissue and Organ Culture*; **73**: 285–289.

Thao N T P, Ureshino K, Miyajima I, Ozaki Y, Okubo H. (2003b). Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments. *Plant Cell, Tissue and Organ Culture*; **72**: 19–25.

Thimmappaiah, Shirly R A, Sadhana P H. (2002). *In vitro* propagation of cashew from young trees. *In Vitro Cellular and Development Biology – Plant*; **38**: 152–156.

Thomas J, Qin C, Howes N. (1997). Chromosome doubling of haploids of common wheat with caffeine. *Genome*; **40**: 552–558.

Thresh J M, Cooter R J. (2005). Strategies for controlling cassava mosaic virus disease in Africa. *Plant Pathology*; **54**: 587–614.

Timofeeva O, Khokhlova L, Belyaeva N, Chulkova Y, Garaeva L. (2000). Cytoskeleton-induced alterations of the lectin activity in winter wheat under cold hardening and abscisic acid (ABA). *Cell Biology International*; **24**: 375–381.

Togashi K. (1981). Insects visiting flowers of *Wasabia japonica* (Miq.) Matsumura. *Bulletin of Ishikawa Forest Experiment Station*; **11**: 21–26.

Toker G, Memisoglu M, Toker M C, Yesilada E. (2003). Callus formation and cucurbitacin B accumulation in *Ecballium elaterium* callus cultures. *Fitoterapia*; **74**: 618–623.

Tosca A, Pandolfi R, Citterio S, Fasoli A, Sgorbati S. (1995). Determination by flow cytometry of the chromosome doubling capacity of colchicine and oryzalin gynogenetic haploids of *Gerbera*. *Plant Cell Reports*; **14**: 455–458.

Trigiano R N, Gray D J. (2000). *Plant Tissue Culture Concepts and Laboratory Exercises* (2nd ed.). CRC Press: Boca Raton.

Tseng C Y, Tsai T C, Tseng Y K. (1986). Studies on the processing of wasabi produced in Taiwan. I. Production of wasabi powder. *Food Science, China*; **13**: 60–70.

Tsuro M, Koda M, Inoue M. (2000). Efficient plant regeneration from multiple shoots formed in the leaf-derived callus of *Lavandula vera*, using the 'open culture system'. *Scientia Horticulturae*; **86**: 81–88.

Ueno K, Shetty K. (1998). Prevention of hyperhydricity in oregano shoot cultures is sustained through multiple subcultures by selected polysaccharide-producing soil bacteria without re-inoculation. *Applied Microbiology and Biotechnology*; **50**: 119–124.

Uto T, Fujii M, Hou D. (2005a). Inhibition of lipopolysaccharide-induced cyclooxygenasa-2 transcription by 6-(methylsulfinyl) hexyl isothiocyanate, a chemopreventive compound from *Wasabia japonica* (Miq.) Matsumura, in mouse macrophages. *Biochemical Pharmacology*; **70**: 1772–1784.

Uto T, Fujii M, Hou D-X. (2005b). 6-(methylsulfinyl)hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages. *Biochemical Pharmacology*; **70**: 1211–1221.

van Duren M, Morpurgo R, Dolezel J, Afza R. (1996). Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. *Euphytica*; **88**: 25–34.

van Harten A M. (1998). *Mutation Breeding: Theory and Practical Application*. Cambridge University Press: Cambridge.

van Tuyl J M, Meijer H, van Diën M P. (1992). The use of oryzalin as an alternative for colchicine in *in vitro* chromosome doubling of *Lilium* and *Nerine*, *Poster from the VIth International Symposium on Flower Bulbs*.

Varshney A, Dhawan V, Srivastava P S. (2001). A protocol for *in vitro* mass propagation of Asiatic hybrids of lily through liquid stationary culture. *In Vitro Cellular & Developmental Biology – Plant*; **36**: 383–391.

Vasilev N P, Ionkova I. (2005). Cytotoxic activity of extracts from *Linum* cell cultures. *Fitoterapia*; **76**: 50–53.

Verhoeven L A, Sree R K, Dijkhuis P. (1990). A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell suspension cultures of *Nicotiana plumbaginifolia*. *Planta*; **182**: 408–414.

Verkerk R, Dekker M, Jongen W M F. (2001). Post-harvest increase of indolyl glucosinolates in response to chopping and storage of *Brassica* vegetables. *Journal of the Science of Food and Agriculture*; **81**: 953–958.

Verma R C, Vyas P, Raina S N. (1992). The effects of colchicine on meiosis in the *Rhoeo discolor* stabile complex translocation heterozygote. *Genome*; **35**: 611–613.

Verma R C, Vyas P, Rao A N. (1993). Colchicine induced chromosomal interchange and colchitetraploidy in *Phlox drummondii*. *Indian Journal of Genetics*; **53**: 187–192.

Viện Sinh học Nhiệt Đới. (1998). Tuyển tập các công trình nghiên cứu khoa học (1993–1998), Nhà xuất bản Nông nghiệp TP.HCM; pp. 377–592.

VITROPIC. (2005). RITA, *Temporary immersion system for plant tissue culture*; pp. 1–2, Website. http://perso.wanadoo.fr/vitropic/rita/en/accueil.htm. Last Updated Date 12/12/2005. Accessed in 2005.

Wachira F N, Ng'etich W K. (1999). Dry matter production and partion in diploid, triploid and tetraploid tea. *Journal of Horticultural Science and Biotechnology*; **74**: 507–512.

Walther F, Sauer A. (1986). Analysis of radiosensitivity – A basic requirement for *in vitro* somatic mutagenesis III. Rose cultivars. *Gartenbauwissenschaft*; **51**: 40–43.

Wan Y, Duncan D R, Rayburn A L, Petolino J F, Widholm J M. (1991). The use of antimicrotubule herbicides for the production of doubled haploid plants from anther-derived maize callus. *Theoretical and Applied Genetics*; **81**: 205–211.

Wan Y, Petolino J F, Widholm J M. (1989). Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. *Theoretical and Applied Genetics*; **77**: 889–892.

Watanabe M, Ohata M, Hayakawa S, Isemura M, Kumazawa S, Nakayama T, Furugori M, Kinae N. (2003). Identification of 6-methylsulfinylhexyl isothiocyanate as an apoptosis-inducing component in wasabi. *Phytochemistry*; **62**: 733–739.

Whitehouse A B, Marks T R, Edwards G A. (2002). Control of hyperhydricity in *Eucalyptus* axillary shoot cultures grown in liquid medium. *Plant Cell, Tissue and Organ Culture*; **71**: 245–252.

Williams R R, Taji A M. (1987). Effects of temperature, darkness and gelling agent on long-term storage of *in vitro* shoot cultures of Australian woody plant species. *Plant Cell, Tissue and Organ Culture*; **11**: 151–156.

Williams R R, Taji A M. (1991). Effect of temperature, gel concentration and cytokinins on vitrification of *Olearia microdisca* Black *in vitro* shoot cultures. *Plant Cell, Tissue and Organ Culture*; **26**: 1–6.

Witjaksono, Litz R E. (2004). Effect of gamma irradiation on embryogenic avocado cultures and somatic embryo development. *Plant Cell, Tissue and Organ Culture*; **77**: 139–147.

Wu S, Zu Y, Wu M. (2003). High yield production of salidroside in the suspension culture of *Rhodiola sachalinensis*. *Journal of Biotechnology*; **106**: 33–43.

Yamashita Y, Terada R, Nishibayashi S, Shimamoto K. (1989). Asymmetric somatic hybrids of *Brassica*: Partial transfer of *B. campestris* genome into *B. oleracea* by cell fusion. *Theoretical and Applied Genetics*; **77**: 189–194.

Yano T, Yajima S, Virgona N, Yano Y, Otani S, Kumagai H, Sakurai H, Kishimoto M, Ichikawa T. (2000). The effect of 6-methylthiohexyl isothiocyanate isolated from *Wasabia japonica* (wasabi) on 4-(methylnitrosamino)-1-(3-pyridyl)-1-buatnone-induced lung tumorigenesis in mice. *Cancer Letters*; **155**: 115–120.

Yong-Gang L, Steg A, Smits B, Tamminga S. (1994). Cramble meal: Removal of glucosinolates by heating with additives and water extraction. *Animal Feed Science and Technology*; **48**: 273–287.

Yu E Y, Pickering I J, George G N, Prince R C. (2001). *In situ* observation of the generation of isothiocyanates from sinigrin in horseradish and wasabi. *Biochimica et Biophysica Acta (BBA) – General Subjects*; **1527**: 156–160.

Zambrano A Y, Demey J R, Fuchs M, Gonzalez V, Rea R, De Sousa O, Gutierrez Z. (2003). Selection of sugarcane plants resistant to SCMV. *Plant Science*; **165**: 221–225.

Zeerak N A. (1991). Induced tetraploidy in tomato (*Lycopersicon esculentum* var. 'Cerasiforme'). *Phyomorphology*; **41**: 35–41.

Zhao J, Simmonds D H. (1995). Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. *Physiologia Plantarum*; **95**: 304–309.

Zhao J P, Simmonds D H, Newcomb W. (1996). Induction of embryogenesis with colchicine instead of heat in microspore of *Brassica napus* L. cv. 'Topas'. *Planta*; **198**: 433–439.

APPENDICES

(Note: Appendices are numbered in accordance with the chapters in which each was first referred to in the text.)

Appendix 2.1: Some examples of *in vitro* production of valuable secondary metabolites in callus cultures from medicinal plant organs.

Bioactive compounds synthesised	Main properties	Plant varieties	Callus sources	Content (mg kg^{-1} or %)	References
Alliin	Flavour-enhancing	*Allium* spp. (onion, garlic, chives & leek)	Meristems (shoot apices)	0.02–2.18%	(Hughes *et al.*, 2005)
Anthocyanins (pale orange)	Food-coloured	*Daucus carota* (black carrot)	Seedlings	0.05%	(Narayan and Venkataraman, 2000)
Cucurbitacin B	Analgestic (anti-sinusitis)	*Ecballium elaterium*	Stem nodes	1.126%	(Toker *et al.*, 2003)
Cyanidin 3-glucoside	Anti-oxidant	*Prunus cerasus* L. (sour cherry)	Leaf tissues	1–45*	(Blando *et al.*, 2005)
Diosgenin	Contraceptive	*Trigonella foenum-graecum* L.	Leaves, stems & roots	100–1900*	(Oncina *et al.*, 2000)
Genistin	Anti-inflammatory	*Genista tinctorial* (*Fabaceae*)	2 mm cotyledon sections	30.16*	(Luczkiewicz and Glod, 2003)
Glucosinolates	Anti-carcinogenic	*Nasturtium montanum* Wall (*Cruciferae*)	Seedlings	8.7–18*	(Songsak and Lockwood, 2004)
Hypericins	Diuretic	*Hypericum perforatum* (*Clusiaceae*)	Leaflets	0.007–0.015%	(Pasqua *et al.*, 2003)
Isoflavonoid glycosides	Anti-alcoholic	*Pueraria lobata* (Wild). Ohwi	Root, leaf, stem segments	99.3–571.6*	(Matkowski, 2004)
Isoflavonoids	Hepato-protective	*Maackia amurensis*	Petioles, inflores-cences, apical meristems	20800**	(Fedoreyev *et al.*, 2000)
Lignans (Justicidin B)	Anti-asthmatic	*Linum narbonense & Linum leonii* (*Linaceae*)	Seedlings	1570–2220**	(Vasilev and Ionkova, 2005)
Pulchelin E	Immuno-stimulating	*Rudbeckia hirta* L. (*Asteraceae*)	Cotyledon sections	8300*	(Luczkiewicz *et al.*, 2002)
Sadidroside	Anti-anoxic	*Rhodiola sachalinensis*	Root, stem, leaf and cotyledon explants	130–5530**	(Wu *et al.*, 2003)
Taxoids	Carcinostatic	*Taxus spp.* (yew trees, *Taxaceae*)	Excised roots	0.032%	(Parc *et al.*, 2002)

((mg kg^{-1} content values are expressed as FW (*) and DW (**) bases))

Appendix 2.2: Nutritional composition of plant tissue culture media used (mg L^{-1}).

Composition	¼MS	½MS	MS	WPM	N&N	B_5	N_6	KC
Macronutrients								
NH_4NO_3	412.5	825	1650	400	720	-	-	-
$(NH_4)_2SO_4$	-	-	-	-	-	134	463	500
KNO_3	475	950	1900	-	950	2500	2830	-
$CaCl_2.2H_2O$	110	220	440	150	166	150	166	-
$Ca(NO_3)_2.4H_2O$	-	-	-	556	-	-	-	1000
$MgSO_4.7H_2O$	92.5	185	370	370	185	250	185	250
KH_2PO_4	42.5	85	170	170	68	-	400	250
$NaH_2PO_4.H_2O$	-	-	-	-	-	150	-	-
Micronutrients								
$FeSO_4.7H_2O$	27.8	27.8	27.8	27.8	27.8	-	27.8	25
$Na_2EDTA.2H_2O$	37.3	37.3	37.3	33.6	37.3	-	37.3	-
$MnSO_4.4H_2O$	22.3	22.3	22.3	22.3	25	-	3.3	7.5
$MnSO_4.H_2O$	-	-	-	-	-	10	-	-
$ZnSO_4.7H_2O$	8.6	8.6	8.6	8.6	10	2	1.5	-
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.25	0.025	0.025	0.025	-
$CoCl_2.6H_2O$	0.025	0.025	0.025	-	-	0.025	-	-
KI	0.83	0.83	0.83	0.83	-	0.75	0.8	-
H_3BO_3	6.2	6.2	6.2	6.2	10	3	1.6	-
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-
Organic supplements and vitamins								
myo-Inositol	100	100	100	100	100	100	-	100
Thiamine-HCl (Vitamin B_1)	0.1	0.1	0.1	1.0	0.5	10	1.0	0.1
Nicotinic acid	0.5	0.5	0.5	0.5	5	1.0	0.5	0.5
Pyridoxine-HCl (Vitamin B_6)	0.5	0.5	0.5	0.5	0.5	1.0	0.5	0.5
Glycine	2	2	2	2	2	-	40	2
Biotin	-	-	-	-	0.2	-	-	-
Carbon source (sucrose)	30000	30000	30000	20000	20000	20000	50000	20000
Medium pH	5.75	5.75	5.75	5.6	5.5	5.5	5.8	5.8

Appendix 2.3: Statistics on the differences in the survival rate, shoot height and explant weight of *W. japonica* among the seven basal media (graphically shown in Figure 2.3). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. Statistical analysis compares values in each column.

Basal media	Survival rate (%)	Mean height of shoots (mm)	Mean weight of fresh explants (mg)
½MS	100±0a	25.3±0.9c	74±5b
MS	100±0a	27.1±1.0c	87±6b
B5	100±0a	23.5±1.1c	76±9b
WPM	100±0a	18.8±1.2b	55±6a
KC	100±0a	14.1±1.5a	46±6a
N6	100±0a	24.7±0.9c	83±8b
N&N	100±0a	23.7±1.0c	85±9b
P value	*0.102*	*0.002*	*0.004*

Appendix 2.4: Statistics on the differences in the shoot number, shoot height and explant weight of *W. japonica* among 3 medium types (¼MS, ½MS and MS) in two culture conditions (gelled and liquid) (graphically shown in Figure 2.6). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Medium types and conditions		Mean number of shoots per explant	Mean height of shoots (mm)	Mean weight of fresh explants (mg)
Gelled	¼MS	8.2±0.5a	17.2±0.4b	315±16a
	½MS	9.4±0.4a	14.6±0.8b	371±17ab
	MS	10.4±0.6a	15.4±0.7b	438±17ab
P value		*0.118*	*0.207*	*0.071*
Liquid shake	¼MS	10.5±0.4a	10.2±0.6a	292±17a
	½MS	15.0±0.7b	24.4±0.8c	561±27b
	MS	15.3±0.9b	34.2±1.0d	1055±77c
P value		*0.003*	*0.002*	*0.001*

Appendix 2.5: Statistics on the differences in the shoot number, shoot height and explant weight of *W. japonica* among 3 medium types (¼MS, ½MS and MS) under liquid culture for 6 weeks (graphically shown in Figure 2.8). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Medium types	Mean number of shoots per explant	Mean height of shoots (mm)	Mean weight of fresh explants (mg)
¼MS	13.4±0.4a	18.6±0.6a	449±27a
½MS	25.8±1.3c	27.0±0.8b	1241±76b
MS	21.4±1.3b	34.7±0.8c	1989±168c
P value	*0.015*	*0.034*	*0.012*

Appendix 2.6: Statistics on the differences in the shoot number, shoot height and explant weight of *W. japonica* 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 (graphically shown in Figure 2.10). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Shoot origin	Mean number of shoots per explant	Mean height of shoots (mm)	Mean weight of fresh explants (mg)
Gelled MS medium (control)	10.4±0.6a	15.4±0.7a	438±17a
Liquid shake MS medium	11.4±0.9b	17.9±0.7b	470±18a
Liquid shake ½MS medium	12.9±0.8c	18.3±0.7b	491±21a
P value	*0.002*	*0.015*	*0.062*

Appendix 2.7: Statistics on the differences in the shoot number, shoot height and explant weight of *W. japonica* among combinations between BA and auxins after 4 weeks in culture (graphically shown in Figure 2.12). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Growth regulators (µM)	Mean number of shoots per explant	Mean height of shoots (mm)	Mean weight of fresh explants (mg)
BA=5 (Control)	10.4±0.6ab	15.4±0.7ab	438±17a
BA=5, NAA=1	11.4±1.0ab	17.2±1.5ab	540±48ab
BA=5, NAA=2	13.4±0.7bc	9.8±0.9a	607±46ab
BA=5, IBA=1	12.9±0.7bc	14.3±1.4a	474±37a
BA=5, IBA=2	12.4±0.7bc	21.0±0.7b	589±28ab
BA=5, IAA=1	15.2±0.6c	12.7±1.5a	660±52b
BA=5, IAA=2	13.0±0.6bc	11.8±0.6a	716±48b
P value	*0.032*	*0.000*	*0.015*

Appendix 2.8: Statistics on the differences in the shoot number, shoot height and explant weight of *W. japonica* among combinations between BA and other cytokinins after 4 weeks in culture (graphically shown in Figure 2.13). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Growth regulators (µM)	Mean number of shoots per explant	Mean height of shoots (mm)	Mean weight of fresh explants (mg)
BA=5 (Control)	10.4±0.6a	15.4±0.7c	438±17b
BA=5, TDZ=2	14.6±0.7bc	9.2±1.1b	449±22b
BA=5, TDZ=5	14.2±0.7b	5.9±0.8a	657±42c
BA=5, kinetin=2	11.2±0.7a	6.1±0.7a	271±19a
BA=5, kinetin=5	12.4±0.5ab	13.3±0.9c	457±13b
BA=5, zeatin=2	14.8±0.3b	6.2±0.6a	511±24b
BA=5, zeatin=5	15.2±0.5c	9.6±0.7b	498±20b
P value	*0.006*	*0.020*	*0.007*

Appendix 2.9: Statistics on the differences in the percentage of shoots forming roots, root number and root length of *W. japonica* between gelled and liquid culture conditions after 3 weeks in culture (graphically shown in Figure 2.15). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Medium conditions & PGRs' concentrations	Frequency of shoot forming roots (%)	Mean number of roots per shoot	Mean length of roots (mm)
½MS + 5 µM IBA (gelled) (control)	100±0a	2.4±0.2a	17.0±0.9a
½MS + 5 µM IBA (liquid)	92±6a	4.7±0.1b	44.4±1.9b
½MS+ 5 µM BA+ 2 µM NAA (liquid)	96±4a	7.9±0.2c	40.8±1.6b
P value	*0.210*	*0.004*	*0.009*

Appendix 2.10: Statistics on the differences in the survival rate, total leaf number and height of *in vitro* plantlets of *W. japonica*, with initial roots derived from 4 origins (½MS gelled media with IBA at 5 µM or 10 µM, and ½MS liquid media with 5 µM IBA or 5 µM BA plus 2 µM NAA) after 4-week growth in the greenhouse (graphically shown in Figure 2.17). Values are the means ± standard errors of 25 replicates (n=25). The same letters in each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level. The statistical analysis compares values in each column.

Root origin	Survival rate (%)	Mean number of leaves per plant	Mean height of plants (mm)
½MS + 5 µM IBA (gelled medium) (control)	96±4c	5.1±0.3a	59.6±2.7a
½MS + 10 µM IBA (gelled medium)	96±4c	5.2±0.3a	69.1±3.5b
½MS + 5 µM IBA (liquid medium)	80±8a	5.2±0.3a	56.7±3.0a
½MS+5 µM BA+2 µM NAA (liquid medium)	88±7b	13.4±1.0b	68.1±3.4b
P value	*0.001*	*0.000*	*0.012*

Appendix 3.1: Some common plant varieties that were successfully treated with colchicine.

Plant varieties	Explant	Exposure method	Doses	Exposure length	Traits improved	References
Alstremeria hybrids	Rhizomes	Dipping in colchicine-containing solution	0.5%	1–7 days	Flower size, vigorous growth	(Ishikawa *et al.*, 1999)
Buddleia globosa	*In vitro* sub-apical shoots	Soaking and shaking in colchicine-containing solution	0.01–0.1%	1–3 days	Leaf form & size, plant height	(Rose *et al.*, 2000)
Cyclamen persicum	Tuber segments	Incorporated into culture media	0.01%	4 days	Petal size, chalcone accumulation	(Takamura and Miyajima, 1996)
Oryza sativa L.	Roots of haploid plants	Immersing in colchicine-containing solution	0.001–0.02%	0.5 day	Callus induction; plant regeneration & fertility	(Chen *et al.*, 2001)
Portulaca grandiflora	Seeds	Miracloth-wrapped and transfered into liquid culture media	0.1%	2 days	Leaf shape & petal number	(Mishiba and Mii, 2000)
Punica granatum	*In vitro* shoots	Incorporated into culture media	0.001%	30 days	Root and leaf length	(Shao *et al.*, 2003)
Sorghum versicolor	Seeds	Placed on colchicine-saturated paper	0.01%	5 days	Plant height & growth length	(Sun *et al.*, 1994)
Zea mays L.	Anther-derived callus	Incorporated into liquid culture media	0.025 & 0.05%	1–3 days	Fertile frequency of inbred lines	(Wan *et al.*, 1989)
Zingiber officinale	Shoot tips	Incorporated into culture media	0.2%	4–14 days	Pollen fertility & germination rate	(Adaniya and Shirai, 2001)
Brassica napus L. cv. 'Topas'	Micro-spores	Incorporated into culture media	25–50 μM	1.5–2 days	Embryo-genesis	(Zhao *et al.*, 1996)

Appendix 3.2: Some common plant varieties that were successfully treated with oryzalin.

Plant varieties	Explant	Exposure method	Doses	Exposure length	Traits improved	References
Allium cepa L.	*In vitro* plantlets	Incorporated into culture media	10–200 μM	1 day	Ploidy stability	(Geoffriau *et al.*, 1997)
Alocasia 'Green Velvet'	Shoot tips	Incorporated into culture media	0.005–0.05%	2-6 days	Leaf shape	(Thao *et al.*, 2003b)
Bixa orellana	*In vitro* seedlings	Incorporated into culture media	5–30 μM	15 & 30 days	Polyploidisa-tion	(de Carvalho *et al.*, 2005)
Solanum spp.	*In vitro* apical shoots	Coating in oryzalin-containing agarose solution	0.0001–0.001%	1 day	Optimal tetraploid level	(Chauvin *et al.*, 2003)
Triticum aestivum L.	Roots of seedlings	Incubating in oryzalin-containing solution	15 μM	3 hours	Frost resistance (via lectin activity)	(Timofeeva *et al.*, 2000)
Musa acuminata	Shoot tips	Incorporated into liquid culture media	30 μM	7 days	Non-chimeric tetraploids	(van Duren *et al.*, 1996)
Malus hybrid	*In vitro* shoots	Immersed in oryzalin-containing solution	5–30 μM	1 day	Chromosome doubling	(Bouvier *et al.*, 1994)
Gerbera jamesonii	Shoots	Incorporated into liquid culture media	30–480 μM	2 days	Diploidisation induction & chimeral reduction	(Tosca *et al.*, 1995)

Appendix 3.3: Some common plant varieties that were successfully treated with gamma rays.

Plant varieties	Explants	Doses	Traits improved	References
Chrysanthemum morifolium cvs. 'Purnima' & 'Colchi Bahar'	Rooted cuttings	10–20 Gy	Flower colour	(Mandal *et al.*, 2000b)
Cymbopogon martinii Stapf.	Dry seeds	50–1000 Gy	Essential oil (geraniol) yield & quality	(Srivastava and Tyagi, 1986)
Dendranthema grandiflorum cv. 'Puja'	Rooted cuttings	1.5–25 Gy	Flower colour and shape	(Datta *et al.*, 2001)
Linum usitatissimum cv. 'Glenelg'	Ari-dry seeds	300–900 Gy	Linoleic acid content	(Green and Marshall, 1984)
Manihot esculenta Crantz	Somatic embryos (at globular stage)	50 Gy	Starch & amylose contents	(Joseph *et al.*, 2004)
Musa spp., AAA Group cv. 'Highgate'	Corms of *in vitro* shoots	2–50 Gy	Fungal tolerance	(Bhagwat and Duncan, 1998)
Nelumbo nucifera Gaertn.	*In vitro* plantlets	20–60 Gy	Healthy rhizome development	(Arunyanart and Soontronyatara, 2002)
Oryza sativa L.	Caryopses	100 & 150 Gy	Plant height, seed yield & Fe toxicity tolerance	(Pathirana *et al.*, 2002)
Persea Americana	Embryogenic cultures	59–209 Gy	Somatic embryo development	(Witjaksono and Litz, 2004)
Saccharum spp.	Calli	20 Gy	Viral tolerance	(Zambrano *et al.*, 2003)
Solanum tuberosum cvs. 'Kufri Jyoti' & 'Kufri Chandramukhi'	*In vitro* plantlets	20 & 40 Gy	Heat tolerance	(Das *et al.*, 2000)
Solanum tuberosum cvs. 'Spunta', 'Draga' & 'Diamant'	Nodal segments (*in vitro* shoots)	2.5–15 Gy	Microtuber number and size	(Al-Safadi *et al.*, 2000)
Vitis Vinifera cv. 'Podarok Magaracha'	Calli	5–500 Gy	Primary & embryogenic callus formation	(Kuksova *et al.*, 1997)

Appendix 3.4: Some common plant varieties that were successfully treated with X-rays.

Plant varieties	Explants	Doses	Traits improved	References
Begonia hybrid	Leaf cuttings	15–25 Gy	Growth habit; colour, shape & size of flowers and leaves	(Doorenbos and Karper, 1975)
Chrysanthemum 'Hortensien Rose'	Rooted cuttings	15 Gy	Flower colour, shape & size	(Broertjes, 1966)
Corchorus capsularis L. cv. 'JRC-212'	Dry seeds	30 & 90 Gy	Fibre yield	(Sinhamahapatra, 1986)
Glycine max (L.) Merr. var. 'Bay'	Dry seeds	214 Gy	Oleic acid content	(Rahman *et al.*, 1994)
Lupinus digitatus Forsk	Dry seeds	100–1250 Gy	Early-flowering	(Gladstones, 1958)
Medicago sativa L.	Pollen	160 Gy	Seed set	(Murray and Craig, 1962)
Nelumbo nucifera Gaertn.	*In vitro* plantlets	10–50 Gy	Shoot growth	(Arunyanart and Soontronyatara, 2002)
Rosa hybrida	*In vitro* leaflets	25–100 Gy	Adventitious bud regeneration	(Ibrahim *et al.*, 1998)
Solanum tuberosum	Tuber slices	20 Gy	Chimerism reduction	(Miedema, 1973)
Trichosanthes anguina L.	Dry seeds	180 Gy	Seed oil & punicic acid content	(Datta, 1992)

Appendix 3.5: Statistics on the differences in the health and conditions of *W. japonica* explants treated with chemical mutagens at different concentrations after 1, 2 and 3 months in culture on MS shoot proliferation medium (graphically shown in Figures 3.7, 3.8 & 3.9). Values are the means of 50 replicates (n=50). The same letters in each monthly section of each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level.

Chemical mutagen & concentration	Mean weight of fresh explants (mg)	Mean number of shoots	Mean height of shoots (mm)
1 month in culture			
Control (0 µM)	466±29b	5.9±0.2e	13.0±0.7b
Oryzalin 5 µM	394±21b	3.0±0.2c	12.0±0.9b
Oryzalin 15 µM	300±17a	1.4±0.1ab	5.4±0.7a
Oryzalin 30 µM	263±14a	0.97±0.11a	2.6±0.4a
Colchicine 25 µM	491±39b	5.0±0.4d	12.2±1.0b
Colchicine 75 µM	446±31b	2.5±0.1bc	6.2±0.9a
Colchicine 150 µM	437±28b	2.0±0.2b	3.3±0.5a
P value	*0.041*	*0.012*	*0.000*
2 months in culture			
Control (0 µM)	1304±80bc	5.7±0.4b	19.9±0.8b
Oryzalin 5 µM	1243±69bc	3.4±0.2a	17.1±0.7b
Oryzalin 15 µM	991±63b	2.3±0.1a	14.7±1.3ab
Oryzalin 30 µM	775±51a	1.8±0.1a	9.6±1.1a
Colchicine 25 µM	1455±90c	6.6±0.5b	17.6±0.8b
Colchicine 75 µM	1307±79bc	3.4±0.1a	14.4±0.9ab
Colchicine 150 µM	1136±52bc	2.8±0.2a	14.0±0.8ab
P value	*0.034*	*0.000*	*0.049*
3 months in culture			
Control (0 µM)	2350±221b	5.7±0.5b	26.6±0.7c
Oryzalin 5 µM	1986±152b	3.7±0.2a	24.2±0.7c
Oryzalin 15 µM	1728±203b	2.9±0.2a	18.7±1.1b
Oryzalin 30 µM	1138±144a	2.2±0.2a	12.2±1.8a
Colchicine 25 µM	3350±268c	7.7±0.4c	24.3±0.4c
Colchicine 75 µM	2390±165b	3.9±0.2a	19.4±0.5b
Colchicine 150 µM	2056±113b	3.0±0.1a	19.0±0.6b
P value	*0.000*	*0.006*	0.003

Appendix 3.6: Statistics on the differences in the health and conditions of *W. japonica* explants treated with physical mutagens at different doses after 1, 2 and 3 months in culture on MS shoot proliferation medium (graphically shown in Figures 3.15, 3.16 & 3.17). Values are the means of 25 replicates (n=25). The same letters in each monthly section of each column indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level.

Physical mutagen & doses	Mean weight of fresh explants (mg)	Mean number of shoots	Mean height of shoots (mm)
1 month in culture			
Gamma irradiation 0 Gy	377±25b	4.8±0.2c	13.2±0.8b
Gamma irradiation 10 Gy	361±17b	4.4±0.2bc	11.3±0.7ab
Gamma irradiation 20 Gy	337±17b	3.9±0.2b	9.8±0.4a
Gamma irradiation 40 Gy	237±17a	2.6±0.2a	8.9±0.6a
X-irradiation 0 Gy	352±19b	4.9±0.2c	14.7±0.6b
X-irradiation 10 Gy	343±15b	4.4±0.2bc	13.0±0.7ab
X-irradiation 20 Gy	309±15ab	3.9±0.2b	11.3±0.5ab
X-irradiation 40 Gy	163±9a	1.8±0.2a	9.3±0.5a
P value	*0.006*	*0.015*	*0.044*
2 months in culture			
Gamma irradiation 0 Gy	1208±81d	6.6±0.3b	18.7±0.8d
Gamma irradiation 10 Gy	1194±65d	6.3±0.4b	17.6±0.7cd
Gamma irradiation 20 Gy	991±58cd	5.5±0.3b	15.7±0.6acd
Gamma irradiation 40 Gy	561±33b	3.8±0.2a	13.2±0.6ab
X-irradiation 0 Gy	1121±41cd	6.8±0.3b	17.5±0.8cd
X-irradiation 10 Gy	938±55bc	6.6±0.4b	16.3±0.8bcd
X-irradiation 20 Gy	705±49b	5.4±0.7b	15.3±0.8ab
X-irradiation 40 Gy	212±27a	2.4±0.3a	10.0±1.0a
P value	*0.033*	*0.037*	*0.049*
3 months in culture			
Gamma irradiation 0 Gy	2451±188c	7.5±0.4b	24.1±0.7e
Gamma irradiation 10 Gy	2393±153c	7.2±0.3b	21.8±0.7de
Gamma irradiation 20 Gy	2295±114bc	6.5±0.3b	21.6±0.5ce
Gamma irradiation 40 Gy	1298±69ab	4.5±0.2a	14.7±0.7b
X-irradiation 0 Gy	2217±93bc	7.6±0.3b	23.0±0.6de
X-irradiation 10 Gy	2054±111bc	7.4±0.4b	20.3±0.8cd
X-irradiation 20 Gy	1897±117b	6.4±0.3b	19.8±0.5cd
X-irradiation 40 Gy	740±114a	3.1±0.5a	10.2±1.1a
P value	*0.005*	*0.011*	*0.000*

Appendix 4.1: Minimum level of AITC against organisms (Isshiki *et al.*, 1992).

Bacteria	Amount (ng mL^{-1})	Yeasts	Amount (ng mL^{-1})	Moulds	Amount (ng mL^{-1})
Bacillus cereus	90	*Candida albicans*	22	*Alternaria alternata*	22
Bacillus subtilis	110	*Candida tropicalis*	22	*Aspergillus niger*	37
Escherichia coli	34	*Hansemula anomala*	37	*Aspergillus flavus*	37
Pseudomonas aeruginosa	54	*Saccharomyces cerevisiae*	22	*Fusarium graminearum*	16
Salmonella enteritidis	110	*Torulaspora delbreuckii*	18	*Fusarium oxysporum*	22
Salmonella typhimurium	54	*Zygosaccharomyces rouxii*	16	*Fusarium solani*	34
Staphylococcus aureus	110	-	-	*Mucor racemosus*	62
Staphylococcus epidermidis	110	-	-	*Penicillium chrysogenum*	62
Vibrio parahaemolyticus	54	-	-	*P. citrinum*	22
-	-	-	-	*P. islandicum*	22

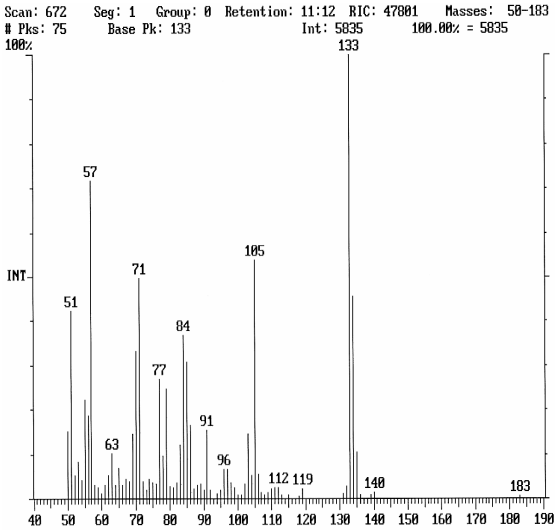
Appendix 4.2: Statistics on the differences in the AITC content of *W. japonica* explants exposed with chemical mutagens at different concentrations after 3 months in culture on MS shoot proliferation medium (graphically shown in Figure 4.8). Values are the means of 6 replicates (n=6) performed from a population of 50 explants. The same letters indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level.

Mutagen and concentration	AITC content (mg kg^{-1} FW basis)
Control (0 µM)	76.1±4.4a
Oryzalin 5 µM	72.4±3.3a
Oryzalin 15 µM	107.1±5.9b
Oryzalin 30 µM	76.7±4.5a
Colchicine 25 µM	113±7b
Colchicine 75 µM	115±5b
Colchicine 150 µM	151±11c
P value	*0.000*

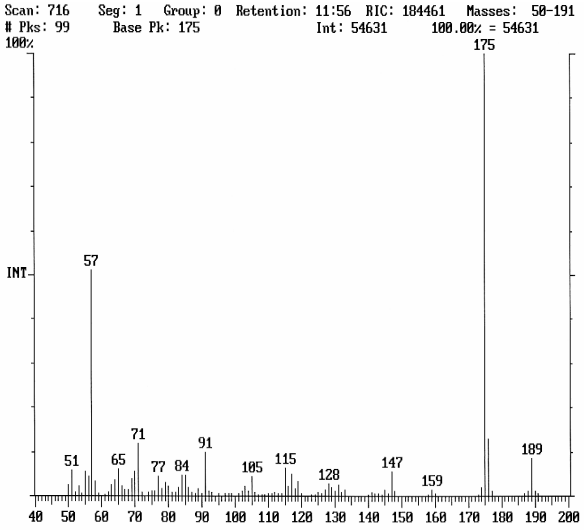
Appendix 4.3: Statistics on the differences in the AITC content of *W. japonica* explants exposed with physical mutagens at different doses after 3 months in culture on MS shoot proliferation medium (graphically shown in Figure 4.9). Values are the means of 6 replicates (n=6) performed from a population of 25 explants. The same letters indicate that values are not significantly different, according to Tukey's Simultaneous Tests, 5% level.

Treatment dose	**AITC content (mg kg^{-1} FW basis)**		*P value*
	Gamma irradiation	**X-irradiation**	
0 Gy	71.2±3.2e	69.2±3.5e	
10 Gy	57.2±2.9d	48.9±3.1c	
20 Gy	51.0±2.4c	42.8±2.8b	
40 Gy	43.6±2.5b	31.4±1.6a	
P value	*0.001*	*0.001*	*0.000*

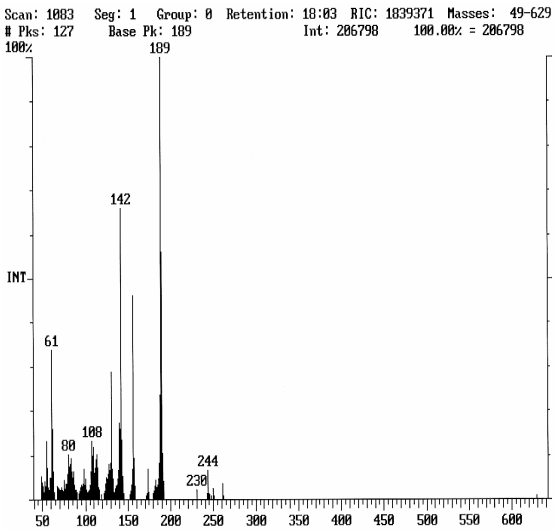
Appendix 4.4: Possible mass spectra of the four compounds extracted from greenhouse-grown *W. japonica* ((a) 2-Methylthioethyl ITC, (b) 5-Methylthiopentyl ITC, (c) 6-Methylthiohexyl ITC and (d) either 1-Penten-3-ol or *cis*-2-Penten-1-ol)).



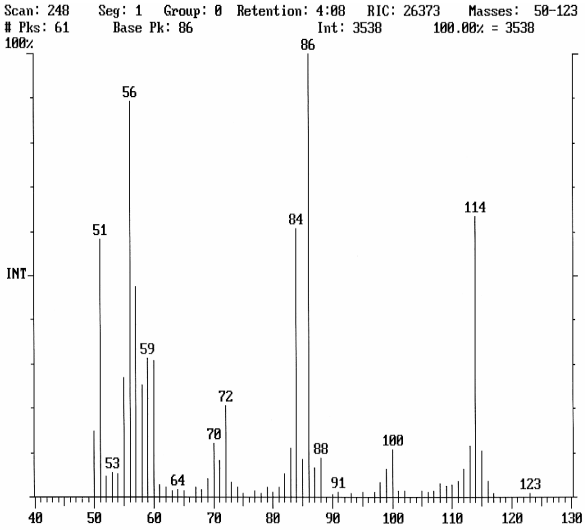
(a) CH_3-S-CH_2-CH_2-N=C= S (*m/e* 133)



(b) CH_3-S-CH_2-CH_2-CH_2-CH_2-CH_2-N=C= S (*m/e* 175)



(c) CH_3-S-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-N=C= S (*m/e* 189)



(d) CH_2=CH-CH(OH)-CH_2-CH_3 or CH_3-CH_2-CH=CH-CH_2-OH (*m/e* 86)