Developing Tissue Culture and Genetic Transformation Techniques for Almond (*Prunus dulcis* Mill.)

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ABSTRACT

The almond (*Prunus dulcis* Mill.) is one of the most economically important nut crops and is grown in many countries with a Mediterranean-type climate. This study aimed to develop a range of tissue culture techniques that could be utilised in the improvement of this species, with the primary focus being the development of a genetic transformation system. The two key requirements for such a system, an *in vitro* shoot regeneration procedure and methodology for foreign DNA introduction, formed the basis of this research.

To develop a shoot regeneration system for almond, leaf explants from micropropagated shoot cultures of the cultivars Nonpareil and Ne Plus Ultra were incubated on Almehdi and Parfitt basal medium (AP) with various plant growth regulators and concentrations. Three auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA) in combination with two cytokinins, 6-benzyladenine (BA) and thidiazuron (TDZ), were tested at different concentrations, as was the presence and absence of casein hydrolysate (CH) on adventitious shoot regeneration. Response to the plant growth regulators varied with genotype. Of the three auxins tested, both NAA and IBA induced adventitious shoots from Ne Plus Ultra explants, whereas only IBA was effective for Nonpareil. With the cytokinins, shoot development from Ne Plus Ultra occurred in the presence of either BA or TDZ, whereas for Nonpareil, only TDZ was effective unless CH was incorporated into the basal medium. The inclusion of CH improved callus morphology, and increased regeneration frequencies for both cultivars. Maximum regeneration frequencies for Ne Plus Ultra (44.4%) and Nonpareil (5.5%) were

achieved on AP basal medium supplemented with CH, IBA (9.8 μM), and TDZ at 22.7 and 6.8 μM respectively.

As an alternative approach to shoot regeneration, the amenability of immature seed cotyledons to undergo morphogenesis was investigated. Open-pollinated fruits were collected from orchard-grown trees of four almond cultivars (Ne Plus Ultra, Nonpareil, Carmel, and Parkinson), with explants subjected to a range of tissue culture conditions, including the effects of TDZ, IBA, and the presence or absence of light for the first 7 days of culture. Shoot regeneration rates were highest for cotyledons cultured for 8 weeks on Murashige and Skoog (MS) basal medium containing 10.0 μM TDZ, followed by 4 weeks on basal medium without plant growth regulators. Regeneration levels were further improved if cotyledons were maintained in darkness for the first 7 days. The inclusion of 0.5 μM IBA in media significantly reduced the development of adventitious shoots. Regeneration rates for Ne Plus Ultra, Nonpareil, Carmel, and Parkinson under optimum conditions were 80.0%, 73.3%, 100.0%, and 86.7%, respectively.

The ability of Ne Plus Ultra and Nonpareil to root under tissue culture conditions was also tested, with microshoots subjected to a range of rooting treatments. To determine the optimum auxin for root formation, various concentrations of IBA and NAA were compared over a range of incubation periods. In addition, the effects of shoot base shading, phloroglucinol (PG), and basal salt composition were examined. The best response for both cultivars occurred following shoot insertion for 12 h into agar containing 1.0 mM IBA, followed by 2 weeks in basal medium without growth regulators supplemented with 100.0 μM PG. Extended shading of the shoot base and the inclusion of PG in media did not significantly improve rooting ability. Whilst MS basal medium with

half strength salts was suitable for rooting Ne Plus Ultra shoots, full strength AP medium produced the best results for Nonpareil. Under these conditions, 60.0% of microshoots developed roots.

The final aspect of this study investigated the effect of a range of factors influencing the transfer of an intron-containing β-glucuronidase (GUS) gene to leaf explants of Ne Plus Ultra and Nonpareil using the *Agrobacterium*-mediated transformation approach. Gene transfer was evaluated by counting the number of GUS-zones (foci) 4 days after cocultivation, as well as the number of GUS-active calli that developed on leaf explants 6 weeks post-cocultivation. *A. tumefaciens* strain EHA105 was more effective than LBA4404, and cocultivation over a 4-day period was the optimum for both almond genotypes. The presence of acetosyringone in the bacterial, plant preculture, and cocultivation medium had a varied effect on gene transfer efficiency, with its influence dependent on genotype and bacterial strain. Using an optimised procedure, 1500 leaf explants of both genotypes were transformed. While no transgenic plants were recovered, PCR and Southern blot analysis confirmed the presence of the transgene and its stable integration in callus. The implications of this technology for the improvement of almond are discussed.

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ABBREVIATIONS

Acetosyringone	AS
Almehdi and Parfitt medium	AP
Analysis of variance	ANOVA
Base pairs	bp
6-Benzyladenine	BA
5-Bromo-4-chloro-3-indolyl-β-D-glucuronide	X-gluc
Casein hydrolysate	CH
Colony forming units per millilitre	cfu mL^{-1}
2,4-Dichlorophenoxyacetic acid	2,4-D
Disodium ethylenediaminetetraacetic acid	Na_2EDTA
Ethylenediaminetetraacetic acid	EDTA
β-glucuronidase	GUS
Indole-3-acetic acid	IAA
Indole-3-butyric acid	IBA
Kilobases	Kb
Least significant difference	LSD
Messenger RNA	mRNA
Micromoles per square metre per second	µmol m^{-2} sec^{-1}
Murashige and Skoog Medium	MS
α-Naphthaleneacetic acid	NAA
Neomycin phosphotransferase II	*npt*II
Phloroglucinol	PG

Polymerase Chain Reaction	PCR
Quoirin and Lepoivre Medium	QL
Self-incompatibility	SI
Sodium dodecylsulphate	SDS
Thidiazuron	TDZ
Units per microlitre	U μL^{-1}
Watt	W

1. INTRODUCTION

1.1 ALMOND - HISTORY AND DEVELOPMENT

1.1.1 Botanical classification

Family: Rosaceae

Genus: *Prunus*

Subgenus: Amygdalus

Species: *dulcis* (Miller) D. A. Webb

Linnaeus first classified the cultivated almond in 1753 (Kester et al., 1990), assigning the botanical name *Amygdalus communis* (L.). Since then there has been a number of views on name priority, with some botanists preferring the genus *Prunus*, and others assigning it to the *Prunus* subgenus *Amygdalus* (Kester et al., 1990). In 1967, Webb proposed that the

cultivated sweet almond be assigned the taxonomic name *Prunus dulcis* (Miller) D.A. Webb, and whilst this classification has been widely adopted, a number of synonyms are still cited in the literature, including *Prunus amygdalus* Batsch and *Prunus communis* (L). The almond is closely related to several stone fruit (Kester and Asay, 1975) that are also included in the botanical family Rosaceae, including peach and nectarine (*Prunus* subgenus *Amygdalus*), apricot and plum (*Prunus* subgenus *Prunophora*), and cherry (*Prunus* subgenus *Cerasus*).

1.1.2 Origin and domestication

Almonds have been grown for many centuries, and it is reported that domestication may have started during the 3rd millennium BC (Spiegel-Roy, 1986). Cultivation of this tree crop has expanded from Central Asia, believed to be the primary site of origin, to countries bordering the Mediterranean Sea, North and South America, South Africa, and Australia by either seed or scions (Socias i Company and Felipe, 1992). Many wild almond species can still be found in Central Asia (Socias i Company and Felipe, 1992), and it is from these species that the cultivated almond is believed to have evolved. Although the exact origin is not known, two theories have been proposed in the literature (Kester et al., 1990; Socias i Company and Felipe, 1992). The first suggests that wild populations of *Prunus communis* (L.) gave rise to cultivated almond types. The natural morphology of this species with its large tree size and high proportion of sweet kernel genotypes suggests that it has a greater capability for rapid domestication compared with other *Prunus* species which tend to be small, thorny, and produce small, bitter, hard-shelled nuts. Furthermore, the variations in tree, kernel, and shell types within wild populations of *Prunus communis* (L.) and those of

present day cultivated forms, strongly suggests direct selection and domestication (Kester et al., 1990). An alternative theory hypothesises that the cultivated almond is a result of hybridisations between a number of wild species (*Prunus fenzliana*, *P. bucharica*, *P. triloba*, and *P. webbii*) which have the ability to freely intercross (Socias i Company and Felipe, 1992). Although this theory may explain some of the almond populations found along the border of the Mediterranean Sea, it remains unknown if these hybridisations directly gave rise to the cultivated forms grown today.

Almonds have been grown in South Australia since 1836, when they were introduced to Kangaroo Island as seed from the sailing vessel 'The Duke of York', five months prior to the State's Proclamation (Quinn, 1941). The introduction of almond to mainland Australia soon followed, with records showing that the Spanish cultivar, Jordan, was being grown in gardens in North Adelaide as early as 1842 (Quinn, 1941). This hard shell cultivar is believed to be one of two sources that provided germplasm for Australia's current local cultivars (Woolley et al., 2000), with its offspring producing cultivars including Chellaston, Brandis, and Johnson's Prolific. The second source was introduced much later from California and includes cultivars such as Nonpareil (synonym: Californian Papershell), IXL, and Ne Plus Ultra (Kester et al., 1990).

1.1.3 Cultivated almond

The cultivated almond is diploid with a chromosome number $2n = 2\times = 16$ (Kester et al., 1994). It is a medium sized deciduous tree and is grown in regions with cool wet winters and hot dry summers that are typical of the Mediterranean area. It has an upright bearing

nature and is usually grafted to a selected rootstock. Branches are glabrous, and usually pale green to reddish-brown in colouration. Separate leaf and flower buds occur, the former being pointed in shape, and the latter rounded, with flowering preceding leaf sprouting (Rugini, 1986). Leaves are long and ovate with a serrate margin and an acute apex (Woodroof, 1979). Flowers are hermaphroditic with a single pistil containing two ovules, both of which can develop into seed. The ovary is seated within a floral cup that is surrounded by 20-40 stamens and 5 white to pink petals (Kester, 1981). Most commercial cultivars are self-incompatible and require cross-pollination with polliniser cultivars during bloom to ensure nut set (Micke and Kester, 1978). Pollination is entomophilous and is performed by bees. The fruit consists of an egg-shaped drupe. The pericarp (hull) is generally thin (5-15 mm in length) and dry and dehisces at maturity to expose the endocarp (shell) which encloses one or two kernels (Rugini, 1986). Cultivars are classified on the basis of shell consistency as stone shell, hard shell, semihard shell, soft shell, or paper shell. Kernels vary in size, and weigh between 0.5 and 2.0 g (Woodroof, 1979).

1.1.4 Australian almond industry

Commercial production of almonds in Australia began in South Australia, with early plantings close to Adelaide and on the Central Adelaide Plains. Orchard development soon spread south to the Southern Vales and eventually north to the Northern Adelaide Plains by the mid-1960s (Anonymous, 1996). Most of the trees on the Central Adelaide Plains have been removed as a result of urban encroachment, and those in the Southern Districts have declined in recent times. However, over the last 20 years, many large commercial almond orchards have been established along the Murray Valley, between Swan Reach in

Table 1.1: World Production by Country of Almonds in 1997 (in shell).

Country	Production (metric tonnes)	Contribution to World Production (%)
USA	515,000	35.8
Spain	362,000	25.2
Italy	101,000	7.0
Iran	76,000	5.3
Pakistan	49,000	3.4
Greece	43,000	3.0
Australia	9,000	0.6
Other	283,000	19.7

(Source: Anonymous, 1997a)

Table 1.2: Summary of Australian Almond Production and Tree Numbers between 1986 and 1996.

Year	Production - Kernel (metric tonnes)	Number of Almond Trees
1986	2,432	749,799
1987	2,959	823,976
1988	2,859	917,875
1989	3,257	976,789
1990	3,490	959,804
1991	3,958	971,150
1992	4,090	1,000,000
1993	4,990	1,090,000
1994	5,020	1,120,000
1995	5,020	1,110,000
1996	5,890	1,150,000

(Source: Anonymous, 1997b)

South Australia and Swan Hill in Victoria, using production technology acquired from California (Scholefield, 1995).

The United States of America dominates the global production of almonds, with California being the main production region. In 1997, the USA accounted for 35.8% of world production (Table 1.1). Other major producers include Spain, Italy, Iran, Pakistan and Greece. Australia's crop totals less than 1% of world production (Anonymous, 1997a).

The industry in Australia has shown steady increases in production over the past 30 years (Table 1.2). In 1960, national production was 395 tonnes (kernel), with the majority produced from orchards in South Australia (Anonymous, 1996). In comparison, production in 1997 reached 7,000 tonnes (kernel), with large commercial orchards in the Murray Valley of Victoria yielding 59% of the national crop that was valued in excess of $40 million (Anonymous, 1998). Future predictions by the Australian Almond Growers Association estimate that by 2005, as young non-bearing trees reach maturity, Australia will produce a crop of 10,500 tonnes (kernel) with an approximate value of $55 million (Pocock, 1999).

The average kernel yield per tree in Australia is 5.5 kg (Anonymous, 1996), with the more productive areas in the Murray Valley achieving higher levels of up to 10.7 kg per tree. This figure compares favourably to production in the USA, where average tree productivity is 5.9 kg (Anonymous, 1989).

Currently, Australia is not self-sufficient in supply of almonds to the domestic market. During 1996-97, almond products with a value of $16.2 million, comprising 40 tonnes of kernel and 2,700 tonnes of in shell nuts, were imported, with the USA being the major supplier (Anonymous, 1998). Recently, Australian producers have begun exporting their product, and during 1996-97 nearly 1,200 tonnes in shell were exported to countries including the United Arab Emirates, Japan, France and the United Kingdom (Anonymous, 1998). In the future, the industry aims to increase supply to the domestic market and the level of exports to niche markets overseas.

1.1.5 Almond breeding in Australia

In 1997, the Australian Almond Growers Association initiated the first breeding program to be conducted in Australia aimed at producing improved almond cultivars specifically for Australian climatic and environmental conditions, and for Australian industry and consumer requirements (Sedgley, 1999). The primary objective is the development of self-fertile Nonpareil (synonym: Californian Papershell) type cultivars, or at the very least, superior pollinisers. The program is based at the Waite Campus of Adelaide University, and is using a multi-facetted approach, combining traditional hybridisation methods with the latest biotechniques, including genetic transformation.

1.2 PLANT TISSUE CULTURE

Plant tissue culture is the general term for the cultivation of plant parts such as cells, tissues, or organs, under aseptic conditions in synthetic medium *in vitro* (Donnelly and

Vidaver, 1988). The different types of plant tissue culture are categorised according to the source of the explant, and include meristem, embryo, root, anther/pollen, callus, cell suspension, and protoplast culture (Taji et al., 1992). These procedures have become powerful scientific tools and have formed the basis for a number of modern molecular approaches, including genetic transformation, that have been developed for crop improvement (Litz and Gray, 1992).

Tissue and cell culture techniques have been applied to many plant species including a number of perennial fruit crops. Whilst the most common application for tissue culture has been the mass production of clones through micropropagation, *in vitro* techniques have also been applied for generating virus-free plants, producing new genotypes through somaclonal variation, assisting breeding programs by complementing/shortening screening and selection procedures, and for the conservation of germplasm (Kester et al., 1986; Taji et al., 1992; Withers, 1992; Channuntapipat et al., 2000). However, of interest to this study is the ability to efficiently regenerate plantlets from cell and tissue cultures - a prerequisite for plant transformation research.

1.2.1 Using tissue culture to regenerate plants

There are two recognised pathways by which plants can be regenerated under tissue culture conditions - organogenesis and somatic embryogenesis (Christianson, 1987). The two pathways are reflective of different developmental events, and are proposed to be mutually exclusive, with plant cells committed to only one pathway (Ammirato, 1985).

Somatic embryos (somatic embryogenesis) can be distinguished from adventitious shoot meristems (organogenesis) on the basis of different morphological characteristics. Somatic embryos have a single cell origin, a bipolar structure with both shoot and root meristems, and a closed vascular system. In contrast, adventitious meristems have a multicellular origin, a unipolar structure with either a shoot or root meristem, and vascular tissue continuous with the underlying explant. In addition, adventitious meristems usually develop at the periphery of explants, with leaf development directly associated with the parental explant (Haccius, 1978; Litz and Gray, 1992). In order for morphogenesis to occur, an initial inductive event is required to determine the fate of the plant cells. That is, the cells acquire competence to regenerate, and can be triggered to express either an embryogenic or an organogenic potential. Morphogenesis can be either direct (ie. somatic embryogenesis from nucellus tissue) or indirect (ie. meristematic shoots from callus tissue), with the choice of explant and plant growth regulators affecting which morphogenic pathway is entered (George, 1993). Although the regeneration of plants from cell and tissue cultures has been reported in many plant species, woody perennials including fruit trees are widely regarded as recalcitrant to this methodology as they have proved difficult to regenerate under tissue culture conditions, particularly from adult explants (Laimer et al., 1988).

1.2.2 Application of tissue culture to almond and other members of the family Rosaceae

Early work on the tissue culture of almond was performed by Dale Kester at the University of California (Kester 1970; Kester et al., 1977; Tabachnik and Kester, 1977), with a number of other researchers contributing in more recent times (Mehra and Mehra, 1974;

Hisajima, 1982; Rugini and Verma, 1983; Bimal and Jha, 1985; Antonelli, 1991; Bouza et al., 1992; Scott et al., 1992; Caboni and Damiano, 1994; Dorion et al., 1994; Miguel et al., 1996). This previous research has primarily focused upon developing micropropagation protocols for almond cultivars and rootstocks, with axillary buds or 'flushed' (emerging) shoot tips being used as explants in most cases. A range of basal culture media have been trialed, including Bourgin and Nitsch (1967), Knops (Tabachnik and Kester, 1977), Murashige and Skoog (1962), Quoirin and Lepoivre (1977), and White's medium (Zdruikowskaya-Rikhter, 1980). Similarly, different plant growth regulators including the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), and the cytokinins, 6-benzyladenine (BA), kinetin, and zeatin, have been tested at various concentrations and in different combinations. Despite these efforts, difficulties in shoot multiplication and root induction are still experienced, with certain genotypes proving more recalcitrant *in vitro* than others. This has lead to the general conclusion that the tissue culture response in almond is strongly influenced by genotypic differences.

For almond, there are only a few reports where the *in vitro* regeneration of plants has been studied. Mehra and Mehra (1974) describe sporadic plantlet regeneration from callus initiated from seedling explants including leaf, stem, root, hypocotyl, and cotyledon that were exposed to a diverse range of basal media and plant growth regulator regimes. In another study, Hisajima (1982) reports that almond seeds can be induced to form multiple shoots when cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of BA. Similarly, Bimal and Jha (1985) report shoot bud differentiation from endosperm explants excised from mature almond seed cultured on the same basal medium (MS) supplemented with the auxins 2,4-D or NAA. In another study, Antonelli

(1991) describes the induction of proembryonal masses from seed cotyledons of the European cultivar Tuono, but was unable to induce morphogenesis when using MS nutrients supplemented with BA and NAA at various concentrations. More recently, Miguel et al. (1996) report shoot regeneration from juvenile and adult explants of the Portuguese cultivar Boa Casta when cultured on MS or Quoirin and Lepoivre (QL) nutrients supplemented with various levels of the cytokinin, thidiazuron (TDZ), in combination with the auxins, IBA, IAA or 2,4-D.

There have been no reports of shoot regeneration from the almond cultivars grown extensively in Australia and the USA. Therefore, the amenability of genotypes, including the premier cultivar Nonpareil (synonym: Californian Papershell), to regenerate under tissue culture conditions need to be investigated before genetic transformation research can be commenced with the almond cultivars grown in these countries.

The *de novo* regeneration of plants from tissue and cell cultures via either organogenesis or embryogenesis has been investigated in a number of other members of the Rosaceae, including apple, apricot, cherry, peach, pear, and plum (Table1.3). Cultures have been initiated from various explant sources including anthers, cotyledon, leaf, nucellus, petiole, root, stem, and zygotic embryos. In the case of organogenesis, callus induction and shoot development usually occur on the same media supplemented with both an auxin and a cytokinin (Dufour, 1990; Welander, 1988), with a high cytokinin to auxin ratio favouring caulogenesis (Hammerschlag et al., 1985). BA has been the most frequently used cytokinin for shoot induction from perennial fruit tree species, with the auxin NAA preferred for its synergistic effect on shoot induction (Litz and Gray, 1992). For somatic

Table 1.3: Regeneration from explants of temperate fruit tree species belonging to the Rosaceae family under tissue culture conditions.

Crop	Explant	Regeneration		PGRs		Reference
		Type	%	Cytokinin	Auxin & other PGRs	
Apple	L, SI	S	10-30	BA	IBA, 2,4-D	James et al., 1984
	L, SI	S	50-85	BA	NAA	Welander, 1988
	L	S	50-82	BA, TDZ	NAA	Fasolo et al., 1989
	L	S	100	BA	NAA	Predieri et al., 1989
	L	S	11-100	TDZ	NAA	Korban et al., 1992
	E	SE	30	BA	NAA	Paul et al., 1994
	L	S	49	BA	NAA	Pawlicki and Welander, 1994
	M	SE	NS	TDZ	-	Höfer et al., 1999
	P	S	33	TDZ	IAA, ABA	Saito and Suzuki, 1999
Apricot	SC	S	100	BA	2,4-D	Lane and Cossio, 1986
	SC	S	NS	BA	2,4-D	Pieterse, 1989
	E	S	80	TDZ	IBA	Goffreda et al., 1995
Cherry	SI	S	22	BA	NAA	James et al., 1984
	SC	S	70	BA	-	Lane and Cossio, 1986
	SC	S	64	TDZ	IBA	Mante et al., 1989
	L	S	9-47	TDZ	NAA	Hammatt and Grant, 1998
	R	SE	75	BA	2,4-D, GA_3	Mandegaran et al., 1999
Peach	E	S	NS	BA	NAA	Hammerschlag et al., 1985
	SC	S	33-70	TDZ	IBA	Mante et al., 1989
	SC	SE	NS	BA, KIN	2,4-D NAA	Bhansali et al., 1990
	E	SE	10	BA	IBA	Scorza et al., 1990
	SC	S	6-60	TDZ	IBA	Pooler and Scorza, 1995
Pear	SC	SE	80	BA	NAA	Mehra and Jaidka, 1985
	L	S	NS	TDZ	NAA	Chevreau et al., 1989
	L	S	100	TDZ	NAA	Chevreau et al., 1997
	L	S	23	TDZ	GA_3	Lane et al., 1998
	L	S	4-23	BA	NAA	Caboni et al., 1999
Plum	SC	S	87	TDZ	IBA	Mante et al., 1989

% Regeneration = percentage of explants giving rise to shoot buds or somatic embryos. Where more than one value is presented, two or more genotypes were studied.

Abbreviations: E, embryos: L, leaf: M, microspores: NS, not stated: P, protoplasts: R, roots: S, shoots: SC, seed cotyledons: SE, somatic embryos: SI, stem internodes.

Plant Growth Regulators (PGRs): ABA, abscisic acid: BA, 6-benzyladenine: 2,4-D, 2,4-dichlorophenoxyacetic acid: GA_3, gibberellic acid: IAA, indole-3-acetic acid: IBA, indole-3-butyric acid: KIN, kinetin: NAA, α-naphthaleneacetic acid: TDZ, thidiazuron.

embryogenesis, embryogenic cultures can be induced by culturing explants on an auxin-based medium containing 2,4-D, NAA, or IAA (Bhansali et al., 1990; Mehra and Jaidka, 1985). Although cytokinins are often incorporated, they are not usually critical for embryo initiation. Incorporation of a cytokinin during the latter stages of somatic embryo development is however necessary, as cytokinins have been demonstrated to play a regulatory role in the differentiation of embryonic organs (Litz and Gray, 1992). Regeneration of embryoids into functional organs (cotyledons, shoots, and root apices) is achieved by lowering or omitting auxins from the culture medium. A summary of plant regeneration research with woody fruit species belonging to the family Rosaceae (other than almond) is provided in Table 1.3.

1.3 GENETIC TRANSFORMATION

For many years the only approach available for the introduction of new traits or characteristics into plant species was via traditional breeding methods. These techniques, which usually rely upon sexual hybridisation followed by intensive selection procedures for individual plants expressing desirable phenotypes, are extremely lengthy processes. However, advances in the fields of molecular biology and genetic engineering have facilitated the introduction of foreign DNA into plant cells. This technique, known as genetic transformation, has been one of the major advances in plant biotechnology, and is predicted to provide an alternative method for crop development, to improve productivity and quality in a range of plant species (Gasser and Fraley, 1989). Genetic transformation has the potential not only to accelerate the plant breeding process, but also to facilitate the

introduction of novel genes irrespective of taxonomic barriers, and in doing so, broaden the gene pool available for crop improvement (Potrykus, 1991).

A number of different procedures have been developed to facilitate the genetic transformation of plant cells. These range from exploitation of the natural gene transfer system of *Agrobacterium*, to chemical treatment of protoplasts by polyethylene glycol (PEG), and the physical procedures of DNA introduction including electroporation of protoplasts and tissues, microinjection, and DNA-coated microprojectile bombardment (Potrykus, 1991). In this study, the focus will be on the *Agrobacterium*-mediated approach, as this procedure has proved most successful in transformation studies with woody plant species (Oliveira et al., 1996).

1.3.1 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* are naturally occurring plant pathogens that cause the diseases crown gall and hairy root respectively. Their mechanism of pathogenicity is a resident gene transfer system that allows the delivery of a section of DNA from the bacterium to the plant cell. Advances in molecular biology have permitted the exploitation of this system, allowing replacement of the naturally occurring genes responsible for plant disease, with alternative ones of agronomic interest. This approach, known as *Agrobacterium*-mediated transformation, has been found to provide an efficient transformation system for many gymnosperms, dicotyledonous angiosperms, and more recently some monocots (Day and Lichtenstein, 1992). The fundamentals of agroinfection will be explained with specific reference to *A. tumefaciens.*

In 1907, Smith and Townsend established that the plant disease crown gall was caused by a bacterium which they called *Bacterium tumefaciens*, later changed by Conn to *Agrobacterium tumefaciens* (Kerr, 1969a). Early efforts in understanding the mechanisms of crown gall were driven by extensive economic losses to several plant species and its widespread nature in many countries (Bernaerts and De Ley, 1963). Further research identified numerous strains and biotypes of *Agrobacterium* (Kerr and Panagopoulos, 1977), and showed that the ability to induce tumours in plants could be transmitted between bacterial strains by DNA transfer (Klein and Klein, 1953; Kerr 1969b, 1971; Kerr et al., 1977). In 1975, Allen Kerr, at Adelaide University (Kerr, 1975), proposed a model detailing the relationship between *Agrobacterium* pathogenicity and tumour induction in plants. Kerr (1975) suggested that DNA was transferred from pathogenic bacteria to plant cells by conjugation. DNA transfer was believed to be associated with two transfer factors, and correlated to genes for the metabolism of the guanidine compounds, octopine and nopaline, as well as sensitivity to bacteriocin 84, all of which were located on a circular chromosome (Kerr, 1975). Whilst it has since been shown that the oncogenic properties of *A. tumefaciens* are actually encoded on a large plasmid (Genetello et al., 1977), the model proposed by Kerr (1975) helped to elucidate the relationship between *Agrobacterium* species and plant disease, and in so doing, allowed this natural gene transfer system to be modified and used for plant genetic engineering.

The large plasmid found in oncogenic *A. tumefaciens* strains has since been termed the Tumour-inducing (Ti) plasmid, and occurs as a single copy, 150 - 250 Kb in size (Day and Lichtenstein, 1992). A segment of this plasmid, designated transfer-DNA (T-DNA), is transmitted by conjugation into individual plant cells, usually within wounded tissue. For

attachment of *Agrobacterium* to plant cells, the chromosomally encoded genes *chvA* and *chvB* are required (Day and Lichtenstein, 1992). The T-DNA segment of the Ti plasmid penetrates the plant cell nucleus, and integrates randomly within the plant genome, where it segregates stably according to Mendelian genetics (Fisk and Dandekar, 1993). Transcriptional activation of the T-DNA insert within the plant genome results in the synthesis of specific phytohormones and opines which alter cell metabolism, causing uncontrolled proliferation of infected cells and the subsequent development of tumorous galls (Schuerman and Dandekar, 1991). The T-DNA is flanked by left and right border sequences (25-bp direct repeat) which define the region to be transferred to infected cells. Transmission of T-DNA is controlled by virulence (*vir*) genes, with the *vir* regulon in the Ti plasmid being characterised into several complementation groups (including *vir* A, B, C, D, E, G) based upon mutational and DNA sequence analysis (Dandekar, 1992). Each *vir* group has a specific effect on the virulence level of the bacterium, conferring the ability and efficiency of T-DNA transfer. Gene products encoded by the *vir* region are not transferred during infection (Gasser and Fraley, 1989). The transfer (*tra*) operon, also encoded by the Ti plasmid, provides the genes for conjugal DNA transfer and is activated by opine synthesis (Day and Lichtenstein, 1992). The genes responsible for tumour induction (*onc* genes) can be removed from the Ti plasmid and replaced with virtually any gene of interest. Infection with such 'disarmed' strains allows the regeneration of morphologically normal plants that contain the foreign gene(s) stably integrated within the plant genome (Dandekar, 1992).

There are essentially two types of recombinant Ti vector plasmids that have been used for foreign gene transfer with *Agrobacterium* (Gheysen et al., 1998). The first type, '*cis*' or co-integrating vectors, contains both the T-DNA and *vir* regulon on the same plasmid. The

second type referred to as 'binary' vectors contain the T-DNA and *vir* regulon on two separate, but compatible plasmids within the same bacterium. Binary vectors are generally easier to manipulate and can replicate in both *Escherichia coli* and *Agrobacterium*. The frequency of transformation in a plant system is dependent on the type of Ti plasmid vector chosen (Schuerman and Dandekar, 1991). In addition, application of the *Agrobacterium*-mediated approach is dependent on the susceptibility of the target species to agroinfection. Extensive screening of dicotyledonous plants for tumour infection has defined a host range of about 50%, whereas only a few monocotyledonous species are susceptible. Almonds were found to be susceptible to infection by *A. tumefaciens* (De Cleene and De Ley, 1976).

1.3.2 Transformation of almond and other members of the family Rosaceae

The ultimate goal of transformation is to produce improved cultivars through the incorporation of agronomically important genes into existing cultivars. Desirable traits for fruit trees include resistance or tolerance to biological or non-biological stresses, regulation of fruit ripening, improved fruit quality and yield, reduced juvenility, and alteration of tree form and architecture (Dandekar, 1992; Oliveira et al., 1998). Although it is preferable for the trait of interest to be encoded by a single gene, in practise many of the above mentioned traits are multigenic, further adding to the complexity of crop improvement by genetic engineering (Dandekar, 1992).

Most fruit tree transformation studies to date have focused on the optimisation of gene transfer using simple reporter and marker gene systems. The genes most frequently used have been the scorable *uidA* reporter gene that encodes the enzyme β-glucuronidase (GUS)

Table 1.4: Transformation of Rosaceae species with agronomically important traits for the purpose of genetic improvement.

Crop	Gene	Gene Function	Reference
Apple	Attacin E	resistance to *Erwinia amylovora* (Fireblight)	Norelli et al., 1994
	Acetolactate synthase	resistance to herbicide Glean™	Yao et al., 1995
	*Rol*A	induces dwarfism	Holefors et al., 1998
	*Rol*B	promotes rooting	Welander et al., 1998
Apricot	Plum pox virus coat protein	plum pox virus resistance	Laimer et al., 1992
Peach	Cytokinin biosynthesis (*ipt*)	modifies growth habit	Hammerschlag and Smigocki, 1998
Pear	*Rol*C	induces dwarfism	Bell et al., 1999
Plum	Papaya ringspot virus coat protein	papaya ringspot virus resistance	Scorza et al., 1994, 1995

and the selectable *npt*II marker gene that confers resistance to the antibiotic kanamycin. Transgenic plants expressing both these genes have been regenerated from apple (James et al., 1989; Yao et al., 1995; Liu et al., 1998; Sriskandarajah and Goodwin, 1998; Bolar et al., 1999), cherry (Brasileiro et al., 1991), and pear (Mourgues et al., 1996). There have also been reports of woody fruit species being transformed with genes other than those used in marker/reporter systems. This research is summarised in Table 1.4.

For almond, there are two reports where *Agrobacterium*-mediated transformation has been investigated. Archilletti et al. (1995) describes the transfer of the *uidA* and *npt*II genes to leaf pieces of the European cultivars, Supernova and MN51. While integration of the transgenes was confirmed by Southern blot analysis, no transgenic plants were generated. More recently, Miguel and Oliveira (1999) described the regeneration of genetically modified plants expressing the same transgenes from seed-derived leaf explants of the Portuguese cultivar, Boa Casta. There have been no published reports describing *Agrobacterium*-mediated transformation of cultivars grown in Australia and the USA.

1.4 AIMS AND OBJECTIVES

The primary objective of this project was to develop tissue culture and genetic transformation techniques for almond cultivars grown commercially in Australia and the USA.

1.4.1 Specific objectives

- To develop a tissue culture based regeneration system for almond from adult (mature) explants.
- To determine the amenability of almond to regenerate from juvenile (immature) explants under tissue culture conditions.
- To establish protocols for rooting almonds *in vitro*.
- To evaluate the potential of the *Agrobacterium*-mediated approach in the genetic transformation of almond, and optimise some of the parameters involved in this complex process.

2. IN VITRO REGENERATION - ADULT TISSUE

2.1 INTRODUCTION

Conventional breeding of woody fruit species is a slow and difficult process due to high levels of heterozygosity and long generation cycles (Sriskandarajah et al., 1994). For this reason, it is important to develop gene transfer methods for fruit crops to accelerate the breeding process and broaden germplasm sources available for crop improvement. One of the prerequisites for plant transformation is a method for efficiently regenerating plants *in vitro* (De Bondt et al., 1994). However, fruit trees are amongst the most recalcitrant for *in vitro* culture, and regeneration of adventitious shoots from adult explants has proven difficult (Miguel et al., 1996; Singh and Sansavini, 1998). For the Rosaceae, whilst protocols have been established for regenerating adventitious shoots from leaf tissue of *Malus* (Pawlicki and Welander, 1994; Sriskandarajah et al., 1994) and *Pyrus* (Chevreau et al., 1989; Lane et al., 1998; Caboni et al., 1999), reports within the *Prunus* genus have

been limited to apricot (Escalettes and Dosba, 1993), cherry (Hammatt and Grant, 1998), cherry rootstock (James et al., 1984), and plum rootstock (Escalettes and Dosba, 1993).

Almond is a highly heterozygous species (Miguel et al., 1996) and most common commercial cultivars are self-incompatible. Therefore, to maintain clonal purity, seed-derived material is generally not used for propagation. Thus, in considering the application of genetic transformation to almond, it is important to attempt to develop protocols to efficiently regenerate plants from tissues taken from recognised cultivars.

While the morphogenetic capacity of almond has previously been studied, most reports of plantlet regeneration involve juvenile seed material including cotyledons, endosperm, and hypocotyl tissue (Mehra and Mehra, 1974; Hisajima, 1982; Bimal and Jha, 1985; Antonelli, 1991; Miguel et al., 1996). In the literature there is only one report of regeneration from adult almond tissue, with adventitious buds regenerated from leaf explants of the Portuguese cultivar Boa Casta (Miguel et al., 1996). There have been no published reports of adventitious regeneration from cultivars grown extensively in Australia and the USA.

The objective of this study was to determine the conditions required to facilitate the regeneration of shoots from leaf explants derived from *in vitro* cultures of the almond cultivars Nonpareil (synonym: Californian Papershell) and Ne Plus Ultra, which are grown commercially throughout Australia and the USA.

2.2 MATERIALS & METHODS

2.2.1 Explant sterilisation

Actively growing healthy shoots of Ne Plus Ultra and Nonpareil were collected from field-grown trees at the Waite Campus of Adelaide University, South Australia. After removing leaves, the shoots were dissected into nodal segments 20-30 mm in length and rinsed under running water for 2 h. The segments were sterilised by submersion in filtered 7% (w/v) calcium hypochlorite solution containing 0.05% (v/v) Tween 20 for 20 min with gentle agitation, then rinsed four times with sterile distilled water.

2.2.2 Micropropagation

To initiate *in vitro* shoot cultures, individual sterile nodal segments were cultured in 40 mL polycarbonate tubes containing 10 mL of QL medium (Quoirin and Lepoivre, 1977 - Appendix 1) without plant growth regulators (Figure 2.1). After 3 weeks, emerging shoots (Figure 2.2) were excised and transferred to MS medium (Murashige and Skoog, 1962 - Appendix 1) supplemented with 4.4 µM 6-benzyladenine (BA) and 0.05 µM indole-3-butyric acid (IBA) for Ne Plus Ultra, and AP medium (Almehdi and Parfitt, 1986 - Appendix 1) supplemented with 3.1 µM BA and 0.05 µM IBA for Nonpareil. Different media regimes were selected for the two genotypes, as preliminary experiments indicated MS basal medium was most suitable for *in vitro* manipulation of Ne Plus Ultra, and AP basal medium for Nonpareil (Ainsley, unpublished data). Shoots were maintained in 250 mL polycarbonate pots with vented lids containing 50 mL of culture media, and subcultured every 4 weeks.

Figure 2.1. Initiation of almond shoot cultures (cv. Ne Plus Ultra) from sterile nodal segments in QL medium without plant growth regulators.

Figure 2.2. Emerging shoots (cv. Ne Plus Ultra) from nodal segments after 21 days culture in QL medium without plant growth regulators.

2.2.3 Media preparation and culture conditions

All media contained 3.0% (w/v) sucrose, were solidified with 0.7% (w/v) agar (SIGMA), and adjusted to pH 5.7 prior to autoclaving (120°C for 20 min). Plant growth regulators were added to basal media prior to the adjustment of pH and before sterilisation. Cultures were maintained at 25 ± 1°C with a 16 h photoperiod (40 μmol m^{-2} s^{-1}) provided by Osram 36 W cool white fluorescent tubes.

2.2.4 Experiment 1: Effect of auxins on regeneration from almond leaf explants

The uppermost expanded leaves from 4-week-old micropropagated shoots of Ne Plus Ultra and Nonpareil were excised, dissected transversely across the midrib into 5 mm^2 sections, and placed abaxial side down in deep-sided petri dishes (100 × 20 mm - Greiner Labortechnik) containing 25 mL of regeneration medium. The regeneration medium contained AP basal nutrients, and either thidiazuron (TDZ) or BA (9.1 μM and 22.2 μM respectively), in combination with either 2,4-dichlorophenoxyacetic acid (2,4-D: 0.0, 0.5, 4.5, 9.0, 22.6 μM), α-naphthaleneacetic acid (NAA: 0.0, 0.5, 5.4, 10.7, 26.9 μM), or IBA (0.0, 0.5, 4.9, 9.8, 24.6 μM). Explants were maintained in the dark for 3 weeks, then exposed to light for a further 5 weeks (as previously described), with a passage time of 4 weeks. (Passage time refers to the period between subculturing the explants to fresh medium). Regeneration frequencies (the number of explants that developed adventitious shoots) and the number of shoots per regenerating explant were recorded after 8 weeks.

2.2.5 Experiment 2: Effect of cytokinins and casein hydrolysate (CH) on regeneration from almond leaf explants

Leaf explants were as described for experiment 1. The culture medium consisted of AP basal nutrients and contained a fixed amount of IBA (9.8 μM) with varying amounts of either BA (0.0, 4.4, 11.1, 22.2, 33.3, 44.4 μM) or TDZ (0.0, 2.3, 4.5, 6.8, 9.1, 22.7 μM). The effect of CH on regeneration was also determined by supplementing the above growth regulator regimes with CH at fixed levels of either 0.0% or 0.1% (w/v). Explants were maintained in the dark for 3 weeks, then exposed to light for 5 weeks (as previously described), with a passage time of 4 weeks. Results (as described for experiment 1) were recorded after 8 weeks.

2.2.6 Experimental design and statistical analysis

A completely randomised design with three replicates per treatment, each containing 12 leaf sections, was used for both experiments. Percentages of regenerating explants were determined and subjected to ANOVA using a factorial design. Percentages were arcsine transformed before analysis. The number of shoots per regenerating explant was determined and presented as a mean value with a standard error. Data were analysed using PlotIT version 3.2 (Scientific Programming Enterprises, USA).

2.3 RESULTS

2.3.1 Experiment 1: Effect of auxins on regeneration from almond leaf explants

The results of the effect of different auxins on the regeneration of leaf explants are shown in Table 2.1. Callus development was first observed after 7-10 days, forming at cut

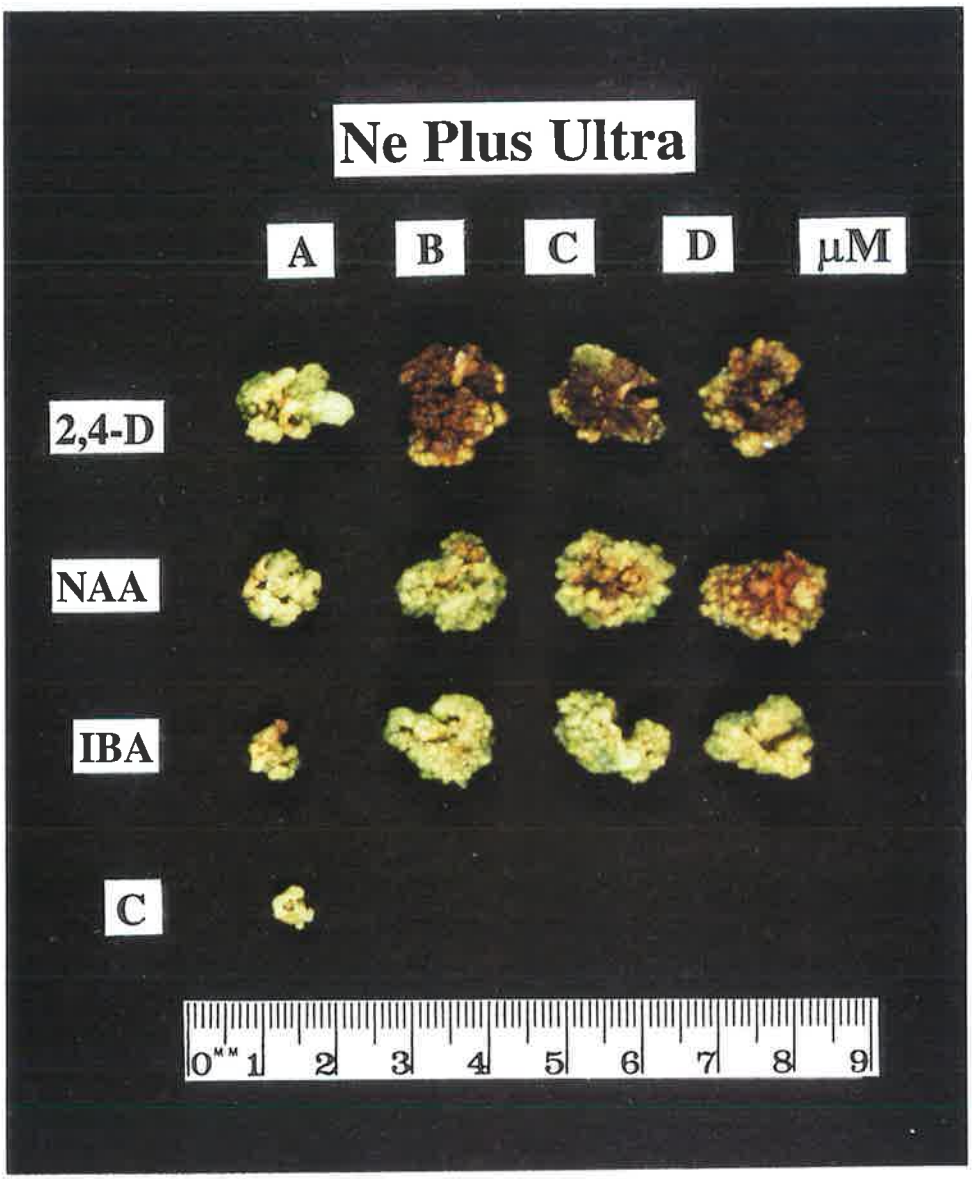

Figure 2.3. Morphology of leaf derived callus (cv. Ne Plus Ultra) after 8 weeks culture on AP medium supplemented with 22.2 µM BAP and different auxins at various concentrations.

2,4-D: A = 0.5 µM, B = 4.5 µM, C = 9.0 µM, D = 22.6 µM.
NAA: A = 0.5 µM, B = 5.4 µM, C = 10.7 µM, D = 26.9 µM.
IBA: A = 0.5 µM, B = 4.9 µM, C = 9.8 µM, D = 24.6 µM.
C: no auxin.

surfaces and along the midrib of leaf sections. The type and concentration of auxin in the regeneration medium influenced the morphology of the developing callus (Figure 2.3). At concentrations below 5.0 μM, auxins induced callus with a soft watery morphology, compared to higher concentrations (≥ 5.0 μM) which promoted the formation of nodular callus (Figure 2.4). It was the nodular callus type that was most conducive to the development of adventitious shoots. The incidence of browning was more prevalent for nodular callus in the presence of 2,4-D and NAA. No browning was observed at any of the IBA levels tested. At this stage, it was still possible to determine the location on the leaf from where the explant was derived. Explant sections derived from distal parts of leaves exhibited poor callus proliferation and no adventitious bud formation.

Regeneration was evident by day 21, with the formation of adventitious buds and young shoots from nodular calli on the abaxial side of the leaf surfaces. At this stage, shoots were etiolated, but chlorophyll synthesis was evident within a few days after transfer to light (Figure 2.5). Furthermore, exposure to light accelerated shoot differentiation and the development of adventitious buds into multiple leafy shoots. By day 42, fully developed shoots (Figure 2.6) could be excised from some explants for transfer to micropropagation media for multiplication and maintenance *in vitro* (Figure 2.7), using the conditions as described in Section 2.2.2 of this Chapter.

Adventitious shoot regeneration was significantly affected by the different plant growth regulator combinations and concentrations (Table 2.2), and was up to 16.6% higher in the presence of IBA than for NAA (Table 2.1). No adventitious shoots occurred in the presence of the 2,4-D concentrations tested. The two cultivars responded differently to IBA and NAA. Levels of IBA between 4.9 and 24.6 μM and NAA between 5.4 and 10.7

Figure 2.4. Nodular type callus conducive to adventitious bud formation and shoot differentiation (Bar = 5 mm).

Figure 2.5. Adventitious shoot development from a leaf explant (cv. Ne Plus Ultra) 25 days after culture initiation on regeneration medium (Bar = 5mm).

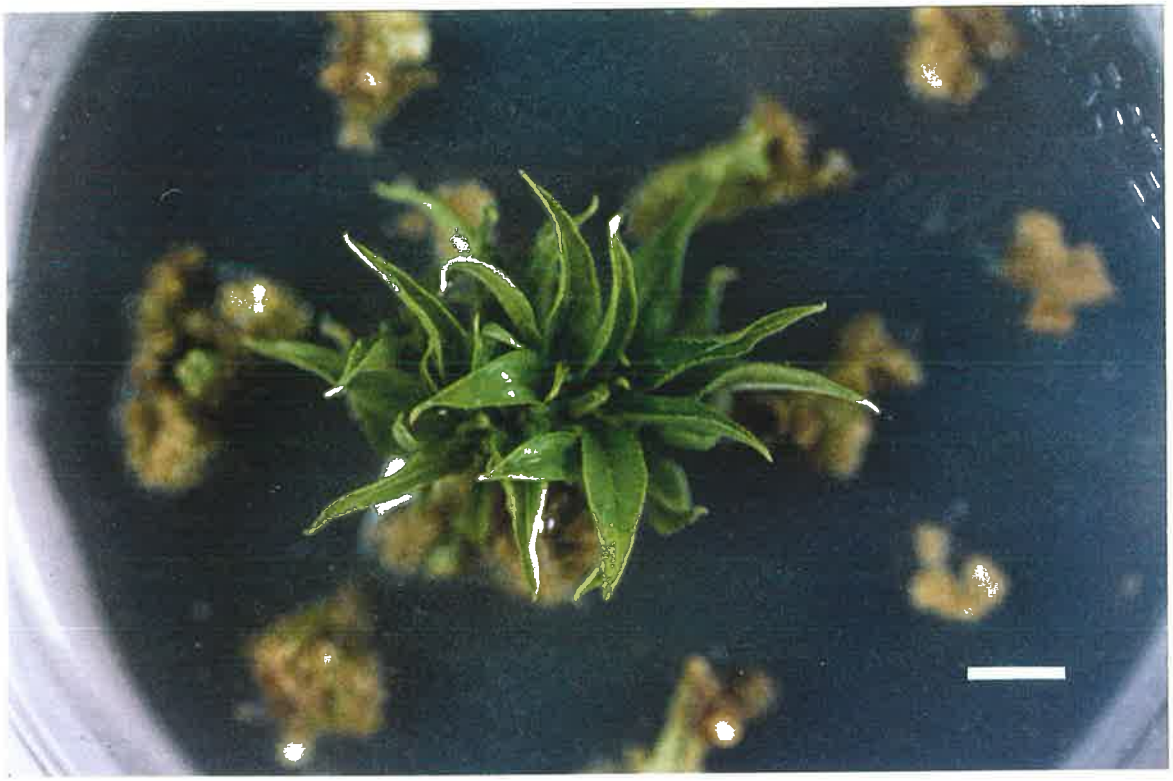

Figure 2.6. Fully developed adventitious shoot from leaf explant (cv. Ne Plus Ultra) 42 days after culture initiation on regeneration medium (Bar = 5mm).

Figure 2.7. Micropropagation of Ne Plus Ultra shoots regenerated from leaf explants.

Table 2.1: Effect of auxins on the induction of adventitious shoots from almond leaf explants (values are the means of three replicates).

Cultivar	Plant growth regulators (μM)					Regeneration frequency† (%)	Shoots per leaf section‡	Cultivar	Plant growth regulators (μM)					Regeneration frequency† (%)	Shoots per leaf section‡
	BA	TDZ	2,4-D	NAA	IBA				BA	TDZ	2,4-D	NAA	IBA		
Ne Plus Ultra	22.2	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0	Nonpareil	22.2	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
	22.2	0.0	0.5	0.0	0.0	0.0	0.0 ± 0.0		22.2	0.0	0.5	0.0	0.0	0.0	0.0 ± 0.0
	22.2	0.0	4.5	0.0	0.0	0.0	0.0 ± 0.0		22.2	0.0	4.5	0.0	0.0	0.0	0.0 ± 0.0
	22.2	0.0	9.0	0.0	0.0	0.0	0.0 ± 0.0		22.2	0.0	9.0	0.0	0.0	0.0	0.0 ± 0.0
	22.2	0.0	22.6	0.0	0.0	0.0	0.0 ± 0.0		22.2	0.0	22.6	0.0	0.0	0.0	0.0 ± 0.0
	22.2	0.0	0.0	0.5	0.0	0.0	0.0 ± 0.0		22.2	0.0	0.0	0.5	0.0	0.0	0.0 ± 0.0
	22.2	0.0	0.0	5.4	0.0	2.8	5.0 ± 0.0		22.2	0.0	0.0	5.4	0.0	0.0	0.0 ± 0.0
	22.2	0.0	0.0	10.7	0.0	0.0	0.0 ± 0.0		22.2	0.0	0.0	10.7	0.0	0.0	0.0 ± 0.0
	22.2	0.0	0.0	26.9	0.0	0.0	0.0 ± 0.0		22.2	0.0	0.0	26.9	0.0	0.0	0.0 ± 0.0
	22.2	0.0	0.0	0.0	0.5	0.0	0.0 ± 0.0		22.2	0.0	0.0	0.0	0.5	0.0	0.0 ± 0.0
	22.2	0.0	0.0	0.0	4.9	5.5	1.0 ± 0.0		22.2	0.0	0.0	0.0	4.9	0.0	0.0 ± 0.0
	22.2	0.0	0.0	0.0	9.8	16.6	2.5 ± 0.2		22.2	0.0	0.0	0.0	9.8	0.0	0.0 ± 0.0
	22.2	0.0	0.0	0.0	24.6	13.9	2.0 ± 0.4		22.2	0.0	0.0	0.0	24.6	0.0	0.0 ± 0.0
	0.0	9.1	0.0	0.0	0.0	2.8	5.0 ± 0.0		0.0	9.1	0.0	0.0	0.0	0.0	0.0 ± 0.0
	0.0	9.1	0.5	0.0	0.0	0.0	0.0 ± 0.0		0.0	9.1	0.5	0.0	0.0	0.0	0.0 ± 0.0
	0.0	9.1	4.5	0.0	0.0	0.0	0.0 ± 0.0		0.0	9.1	4.5	0.0	0.0	0.0	0.0 ± 0.0
	0.0	9.1	9.0	0.0	0.0	0.0	0.0 ± 0.0		0.0	9.1	9.0	0.0	0.0	0.0	0.0 ± 0.0
	0.0	9.1	22.6	0.0	0.0	0.0	0.0 ± 0.0		0.0	9.1	22.6	0.0	0.0	0.0	0.0 ± 0.0
	0.0	9.1	0.0	0.5	0.0	0.0	0.0 ± 0.0		0.0	9.1	0.0	0.5	0.0	0.0	0.0 ± 0.0
	0.0	9.1	0.0	5.4	0.0	8.3	1.7 ± 0.7		0.0	9.1	0.0	5.4	0.0	0.0	0.0 ± 0.0
	0.0	9.1	0.0	10.7	0.0	8.3	4.3 ± 2.4		0.0	9.1	0.0	10.7	0.0	0.0	0.0 ± 0.0
	0.0	9.1	0.0	26.9	0.0	0.0	0.0 ± 0.0		0.0	9.1	0.0	26.9	0.0	0.0	0.0 ± 0.0
	0.0	9.1	0.0	0.0	0.5	0.0	0.0 ± 0.0		0.0	9.1	0.0	0.0	0.5	2.8	8.0 ± 0.0
	0.0	9.1	0.0	0.0	4.9	5.5	1.5 ± 0.5		0.0	9.1	0.0	0.0	4.9	0.0	0.0 ± 0.0
	0.0	9.1	0.0	0.0	9.8	19.4	4.3 ± 1.1		0.0	9.1	0.0	0.0	9.8	5.5	9.5 ± 0.5
	0.0	9.1	0.0	0.0	24.6	11.1	3.5 ± 1.0		0.0	9.1	0.0	0.0	24.6	0.0	0.0 ± 0.0

† Defined as the mean number of leaf sections that produced adventitious shoots.

‡ Defined as the number of adventitious shoots per regenerating leaf section. Mean value ± standard error.

Table 2.2: Analysis of variance summary for interaction effects between genotype, cytokinin, auxin, and auxin concentration on regeneration frequency from almond leaf explants.

	Regen frequency† (%)		
Source of variation	df	MS	F
Genotype	1	992.2	33.6***
Cytokinin	1	139.0	4.7*
Genotype × Cytokinin	1	32.5	1.1
Auxin	2	561.9	19.0***
Genotype × Auxin	2	320.9	10.9***
Cytokinin × Auxin	2	18.2	0.6
Genotype × Cytokinin × Auxin	2	45.8	1.5
Auxin concentration	4	154.3	5.2***
Genotype × Auxin conc	4	134.6	4.6**
Cytokinin × Auxin conc	4	25.9	0.9
Genotype × Cytokinin × Auxin conc	4	19.8	0.7
Auxin × Auxin conc	8	124.8	4.2***
Genotype × Auxin × Auxin conc	8	101.3	3.4**
Cytokinin × Auxin × Auxin conc	8	11.3	0.4
Genotype × Cytokinin × Auxin × Auxin conc	8	12.7	0.4
Error	120	29.5	
Total	179		

† Data subjected to arcsine transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

Table 2.3: Effect of cytokinins and casein hydrolysate (CH) on the induction of adventitious shoots from almond leaf explants (values are the means of three replicates).

Cultivar	Plant Growth Regulators (μM)			CH (% w/v)	Regeneration frequency† (%)	Shoots per leaf section‡	Cultivar	Plant Growth Regulators (μM)			CH (% w/v)	Regeneration frequency† (%)	Shoots per leaf section‡
	IBA	BA	TDZ					IBA	BA	TDZ			
Ne Plus Ultra	9.8	0.0	0.0	0.0	0.0	0.0 ± 0.0	Nonpareil	9.8	0.0	0.0	0.0	0.0	0.0 ± 0.0
	9.8	4.4	0.0	0.0	0.0	0.0 ± 0.0		9.8	4.4	0.0	0.0	0.0	0.0 ± 0.0
	9.8	11.1	0.0	0.0	2.8	1.0 ± 0.0		9.8	11.1	0.0	0.0	0.0	0.0 ± 0.0
	9.8	22.2	0.0	0.0	19.4	4.4 ± 1.0		9.8	22.2	0.0	0.0	0.0	0.0 ± 0.0
	9.8	33.3	0.0	0.0	8.3	3.0 ± 1.0		9.8	33.3	0.0	0.0	0.0	0.0 ± 0.0
	9.8	44.4	0.0	0.0	5.5	4.5 ± 3.5		9.8	44.4	0.0	0.0	0.0	0.0 ± 0.0
	9.8	0.0	2.3	0.0	0.0	0.0 ± 0.0		9.8	0.0	2.3	0.0	0.0	0.0 ± 0.0
	9.8	0.0	4.5	0.0	8.3	1.3 ± 0.3		9.8	0.0	4.5	0.0	0.0	0.0 ± 0.0
	9.8	0.0	6.8	0.0	8.3	1.7 ± 0.7		9.8	0.0	6.8	0.0	0.0	0.0 ± 0.0
	9.8	0.0	9.1	0.0	16.6	2.2 ± 0.4		9.8	0.0	9.1	0.0	0.0	0.0 ± 0.0
	9.8	0.0	22.7	0.0	8.3	2.5 ± 0.5		9.8	0.0	22.7	0.0	0.0	0.0 ± 0.0
	9.8	0.0	0.0	0.1	0.0	0.0 ± 0.0		9.8	0.0	0.0	0.1	0.0	0.0 ± 0.0
	9.8	4.4	0.0	0.1	0.0	0.0 ± 0.0		9.8	4.4	0.0	0.1	0.0	0.0 ± 0.0
	9.8	11.1	0.0	0.1	11.1	1.5 ± 0.3		9.8	11.1	0.0	0.1	5.5	1.0 ± 0.0
	9.8	22.2	0.0	0.1	16.6	5.3 ± 0.8		9.8	22.2	0.0	0.1	0.0	0.0 ± 0.0
	9.8	33.3	0.0	0.1	8.3	4.0 ± 1.5		9.8	33.3	0.0	0.1	0.0	0.0 ± 0.0
	9.8	44.4	0.0	0.1	8.3	5.7 ± 0.7		9.8	44.4	0.0	0.1	0.0	0.0 ± 0.0
	9.8	0.0	2.3	0.1	16.6	2.7 ± 1.3		9.8	0.0	2.3	0.1	0.0	0.0 ± 0.0
	9.8	0.0	4.5	0.1	11.1	1.5 ± 0.5		9.8	0.0	4.5	0.1	5.5	2.0 ± 1.0
	9.8	0.0	6.8	0.1	38.9	2.2 ± 0.6		9.8	0.0	6.8	0.1	5.5	4.0 ± 3.0
	9.8	0.0	9.1	0.1	19.4	1.2 ± 0.2		9.8	0.0	9.1	0.1	5.5	4.0 ± 0.0
	9.8	0.0	22.7	0.1	44.4	3.4 ± 0.5		9.8	0.0	22.7	0.1	2.8	4.0 ± 0.0

† Defined as the mean number of leaf sections that produced adventitious shoots.

‡ Defined as the number of adventitious shoots per regenerating leaf section. Mean value ± standard error.

Table 2.4: Analysis of variance summary for interaction effects between genotype, casein hydrolysate (CH), cytokinin, and cytokinin concentration on regeneration frequency from almond leaf explants.

	Regen frequency† (%)		
Source of variation	df	MS	F
Genotype	1	6146.7	83.0***
CH	1	988.9	13.4***
Genotype × CH	1	319.5	4.3*
Cytokinin	1	1383.7	18.7***
Genotype × Cytokinin	1	219.6	2.9
CH × Cytokinin	1	440.1	5.9*
Genotype × CH × Cytokinin	1	54.8	0.7
Cytokinin concentration	4	346.8	4.7*
Genotype × Cytokinin conc	4	282.2	3.8
CH × Cytokinin conc	4	31.9	0.4
Genotype × CH × Cytokinin conc	4	46.9	0.6
Cytokinin × Cytokinin conc	4	33.5	0.5
Genotype × Cytokinin × Cytokinin conc	4	98.6	1.3
CH × Cytokinin × Cytokinin conc	4	130.4	1.8
Genotype × CH × Cytokinin × Cytokinin conc	4	143.8	1.9
Error	80	74.0	
Total	119		

† Data subjected to arcsine transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

μM promoted adventitious bud formation for Ne Plus Ultra, whereas for Nonpareil only IBA between 0.5 and 9.8 μM was effective, but only with TDZ at 9.1 μM (Table 2.1). Maximum regeneration frequencies for both Ne Plus Ultra (19.4%) and Nonpareil (5.5%) occurred for IBA at 9.8 μM (Table 2.1). The number of shoots per regenerating explant ranged from 1.0 to 5.0 for Ne Plus Ultra and 8.0 to 9.5 for Nonpareil (Table 2.1). A summary of the interaction effects between the different variables is provided in Table 2.2.

2.3.2 Experiment 2: Effect of cytokinins and CH on almond leaf explant regeneration

The results for the effect of different cytokinins and CH on the regeneration of leaf explants are shown in Table 2.3. The choice of cytokinin, its concentration, the presence of CH in the regeneration medium and interactions between these variables had significant effects on shoot morphogenesis (Table 2.4). In the absence of CH, shoot differentiation from explants of Ne Plus Ultra was observed on media containing IBA (9.8 μM) and either BA (11.1-44.4 μM) or TDZ (4.5-22.7 μM), with regeneration frequencies ranging between 2.8 and 19.4% (Table 2.3). However, high levels of BA (44.4 μM) appeared to have an inhibitory effect on callus induction, with the number of leaf explants forming callus reduced by up to 47.2% (Table 2.3). When CH (0.1% w/v) was incorporated into the basal medium, both the nodular morphology of leaf callus and the frequency of shoot regeneration were improved (Table 2.3). For Ne Plus Ultra, CH improved shoot organogenesis by up to 36.1% (Table 2.3). Whilst Nonpareil was less regenerative under the tested conditions, leaf explants developed adventitious buds when cultured on regeneration media supplemented with casein hydrolysate, IBA (9.8 μM) and either BA (11.1 μM) or TDZ (4.5-22.7 μM), at frequencies ranging between 2.8 and 5.5% (Table

2.3). Although TDZ generally yielded higher levels of adventitious regeneration, aberrations in the morphology of regenerated shoots including shoot hyperhydricity, fasciation and impeded elongation were observed at levels ≥ 9.1 μM. The number of shoots per regenerating explant ranged from 1.0 to 5.7 depending on the genotype (Table 2.3). A summary of the interaction effects between the different treatment variables is provided in Table 2.4.

Maximum regeneration frequencies were achieved on AP basal nutrients supplemented with casein hydrolysate, IBA (9.8 μM), and TDZ at 22.7 μM for Ne Plus Ultra or 6.8 μM for Nonpareil (Table 2.3).

2.4 DISCUSSION

Of the three auxins tested, IBA at 9.8 μM was found to be the most conducive to the formation of adventitious shoots. Although IBA is commonly used to induce roots in woody fruit species, its use to promote adventitious regeneration is less frequent, with NAA being preferred for its synergistic effect on shoot production (Litz and Gray, 1992). In a previous study with a Portuguese almond cultivar (Miguel et al., 1996) where adventitious shoots were induced with IAA, IBA and 2,4-D, it was reported that auxin levels above 2.5 μM either reduced or inhibited regeneration. Similar effects have also been observed for apple (Fasolo et al., 1989), apple rootstock (James et al., 1984), and cherry rootstock (James et al., 1984). However, findings from this current study suggest that the cultivars Ne Plus Ultra and Nonpareil can tolerate higher auxin levels, thus

indicating that the requirement of a high cytokinin to auxin ratio for caulogenesis is genotype-specific in almond.

The use of TDZ in woody plant tissue culture, due to its high cytokinin-like activity, has been widely reported (Huetteman and Preece, 1993; Lu, 1993; Murthy et al., 1998). In this study, TDZ was successfully used to induce adventitious shoots from two almond cultivars, confirming an earlier report by Miguel et al. (1996). Miguel et al. (1996) also reported that BA significantly reduced regeneration in the cultivar Boa Casta. In this current study, a similar trend was observed, with BA reducing adventitious shoot formation in both Ne Plus Ultra and Nonpareil. Although BA has previously been used for *in vitro* regeneration from leaf explants in other *Prunus* species (Antonelli and Druart, 1990), it appears that for almond, other cytokinins are more suitable. Whilst TDZ was the preferred cytokinin in this study, aberrations in the morphology of regenerated shoots were observed at levels $\geq$ 9.1 µM. The occurrence of shoot hyperhydricity, fasciation and reduced elongation have been associated with TDZ (Huetteman and Preece, 1993) and described in almond (Miguel et al., 1996), apple (van Nieuwkerk et al., 1986; Pawlicki and Welander, 1994), pear (Caboni et al., 1999), and rhododendron (Preece and Imel, 1991). In the current study, these problems were overcome by transferring regenerating explants (day 14-21) to a medium with either a lower level of TDZ (2.3-4.5 µM) or replacing TDZ with BA (22.2 µM).

CH is a milk protein product composed of amino acids and other substances that can be incorporated in basal media to provide plant cells with a source of organic nitrogen, calcium, phosphate and vitamins (George, 1993). Whilst CH has previously been incorporated in media used to differentiate shoots from leaf callus of almond seedling

tissues (Mehra and Mehra, 1974), the results reported in this study are the first with adult almond explants. For other woody plants, the effect of CH on adventitious regeneration from adult explants varies from inhibitory on apple and cherry rootstocks (James et al., 1984) to beneficial on apple scions (Hammerschlag et al., 1997) and blueberry (Cao et al., 1998). The ability of CH to enhance morphogenesis by inducing the development of highly regenerative nodular callus from almond leaf explants may be in part due to its amino acid component supplementing the nitrogen component of AP basal nutrients (which is low compared to those based on MS nutrients), providing cells with a readily used source of nitrogen (George, 1993).

In addition to the effects of tissue culture conditions, genotype was shown to strongly influence regeneration (Tables 2.2 and 2.4), with Nonpareil appearing more recalcitrant to *in vitro* morphogenesis. This difference may have originated from the different micropropagation conditions used for the two genotypes, with Ne Plus Ultra shoot cultures being maintained on MS basal medium as compared to Nonpareil shoot cultures which were maintained on AP basal medium with a lower level of BA. After 4 weeks under these conditions, shoot cultures may be at different physiological stages in terms of their growth, or may have developed different morphogenic competencies, and variations of this nature would most likely influence the morphogenetic response of the cultured explants. Furthermore, the transfer of Ne Plus Ultra leaf sections to AP basal nutrients for regeneration could be considered as stress conditions due to the reduction in solute concentrations in the medium, which in turn may influence the sensitivity of the explants to the culture environment.

In the future it would be beneficial to repeat this research with a wider range of almond genotypes under identical conditions. This would provide information as to whether the genotype effects observed in this study are limited to Nonpareil, or are widely spread throughout the species.

This study was in part initiated to develop efficient regeneration protocols as a prerequisite to transformation studies with almond. Although an adequate level of regeneration was observed for Ne Plus Ultra, an increase in the frequency for Nonpareil would enhance the chance of successfully transforming and recovering transgenic plants, and requires further consideration in future studies.

3. IN VITRO REGENERATION - JUVENILE TISSUE

3.1 INTRODUCTION

One of the prerequisites for successful transformation is the ability to efficiently regenerate plants under tissue culture conditions. In the previous chapter, the regeneration capacity of adult explants from two almond cultivars was investigated. Results indicated that whereas the cultivar Nonpareil was relatively recalcitrant to the tissue culture conditions tested, up to 44.4% of explants from Ne Plus Ultra could be induced to develop adventitious shoots. However, an improvement in the level of regeneration is required to maximise the chance of successfully recovering transgenic plants. These results concur with other studies on almond where the regeneration of plants from adult tissues has been the limiting step in the transformation process (Archilletti et al., 1995; Miguel and Oliveira, 1999). In other *Prunus* species, including apricot (Lane and Cossio, 1986; Pieterse, 1989; Goffreda et al.,

1995), cherry (Lane and Cossio, 1986), ornamental cherry (Hokanson and Pooler, 2000), sour cherry (Mante et al., 1989; Tang et al., 2000), peach (Hammerschlag et al., 1985; Mante et al., 1989; Schneider et al., 1992), peach rootstock (Pooler and Scorza, 1995), and plum (Mante et al., 1989) where similar problems have been experienced, regeneration efficiency was improved by using highly morphogenic juvenile explants including cotyledons and immature embryos. While this approach would not maintain clonal integrity in almond, a system for regenerating juvenile almond explants would be a useful tool for producing somaclonal variants with resistance/tolerance to biotic and/or abiotic stresses, and provide a method for recovering transgenic plants. Although juvenile explants are not the preferred tissue type for generating variation using these methods, the recalcitrant nature of adult and somatic almond tissues to regenerate under *in vitro* conditions limits the use of alternative approaches. Hence the objective of this study was to assess the regeneration capacity of juvenile almond explants.

3.2 MATERIALS & METHODS

Open-pollinated fruits from the cultivars Ne Plus Ultra, Nonpareil (synonym: Californian Papershell), Carmel, and Parkinson, were collected 100-115 days after full bloom from orchard-grown trees at the Waite Campus, Adelaide University, South Australia. After removing hulls and shells, seeds were imbibed overnight in deionised water, then surface sterilised by immersion in 1.0% (w/v) sodium hypochlorite solution with 0.01% (v/v) Tween 20 for 20 min, followed by three rinses in sterile distilled water. Seed coats were removed, and the two cotyledons separated. Embryonic axes, and tissue immediately surrounding the embryonic axis, were excised with a scalpel and discarded. Cotyledons

were dissected transversely, and the proximal half placed abaxial side down in deep-sided petri dishes (100 × 20 mm - Greiner Labortechnik) containing 25 mL of medium.

MS basal medium (Murashige and Skoog, 1962 - Appendix 1) was supplemented with thidiazuron (TDZ: 0.0, 0.1, 1.0, 10.0, or 20.0 μM) in combination with indole-3-butyric acid (IBA: 0.0 or 0.5 μM). Media contained 3.0% (w/v) sucrose, were solidified with 0.7% (w/v) agar (SIGMA), and adjusted to pH 5.7 prior to autoclaving (120°C for 20 min). Plant growth regulators were added to the basal medium prior to adjustment of pH and sterilisation. Explants were either cultured in the dark for 7 days before being exposed to light, or were transferred directly to light without an initial dark period. Unless otherwise described, cultures were maintained at 25 ± 1°C with a 16 h photoperiod (40 μmol m^{-2} s^{-1}) provided by Osram 36 W cool white fluorescent tubes. Cotyledons were subcultured to fresh media every 4 weeks, and after 8 weeks in culture, transferred to MS medium without plant growth regulators. Shoot production was recorded 12 weeks after cotyledons were introduced into culture.

A completely randomised design with three replicates, each containing 5 cotyledons, was used for experiments. Results were analysed as one data set. Percentages of cotyledons with regenerating shoots were determined and subjected to ANOVA using a factorial design. Percentages were arcsine transformed before analysis. The number of shoots per regenerating cotyledon was determined and presented as a mean value with a standard error. Data were analysed using PlotIT version 3.2 (Scientific Programming Enterprises, USA).

3.3 RESULTS

Regenerating cotyledons were characterised by swelling of the cotyledon at the proximal end after 7 to 14 days in culture, as compared to cotyledons that did not undergo morphogenesis that remained unchanged. This was followed by the development of nodular callus (Figure 3.1), which spread distally from the point of excision from the embryonic axis. Callus development and production was greatest at the highest concentrations of TDZ (10.0 - 20.0 μM).

Regeneration was first evident after 28 days, with multiple clusters of white or green coloured adventitious buds forming on the abaxial surface of cotyledons at the proximal end (Figure 3.2). Cotyledons with little callus developed only a few adventitious buds, whereas those with larger callus masses formed more adventitious buds and subsequently more shoots. Interspersed between the adventitious buds were early embryo (proembryo) structures (Figure 3.3). These, however, did not develop beyond the heart-shape stage. New adventitious buds continued to emerge until explants were transferred to basal medium without plant growth regulators. The absence of hormones stimulated the development of adventitious buds into multiple leafy shoots (Figure 3.4). Also at this stage, a few shoots (4-5) became dominant, and the development of other young buds was inhibited. The excision of developing shoots from cotyledon tissue reduced shoot dominance and hastened elongation of the remaining shoots.

Incorporation of the cytokinin, TDZ, in the regeneration media stimulated a morphogenic response from cotyledon tissue, and varying its concentration had a significant affect on the frequency of regeneration (Table 3.2). Levels between 0.1 and 20.0 μM promoted

Figure 3.1. Nodular callus development on immature almond cotyledon 21 days after culture initiation of MS medium containing 10.0 μM TDZ (Bar = 0.5 mm).

Figure 3.2. Adventitious bud development on immature almond cotyledons 48 days after culture initiation on MS medium containing 10.0 μM TDZ (Bar = 3.5 mm).

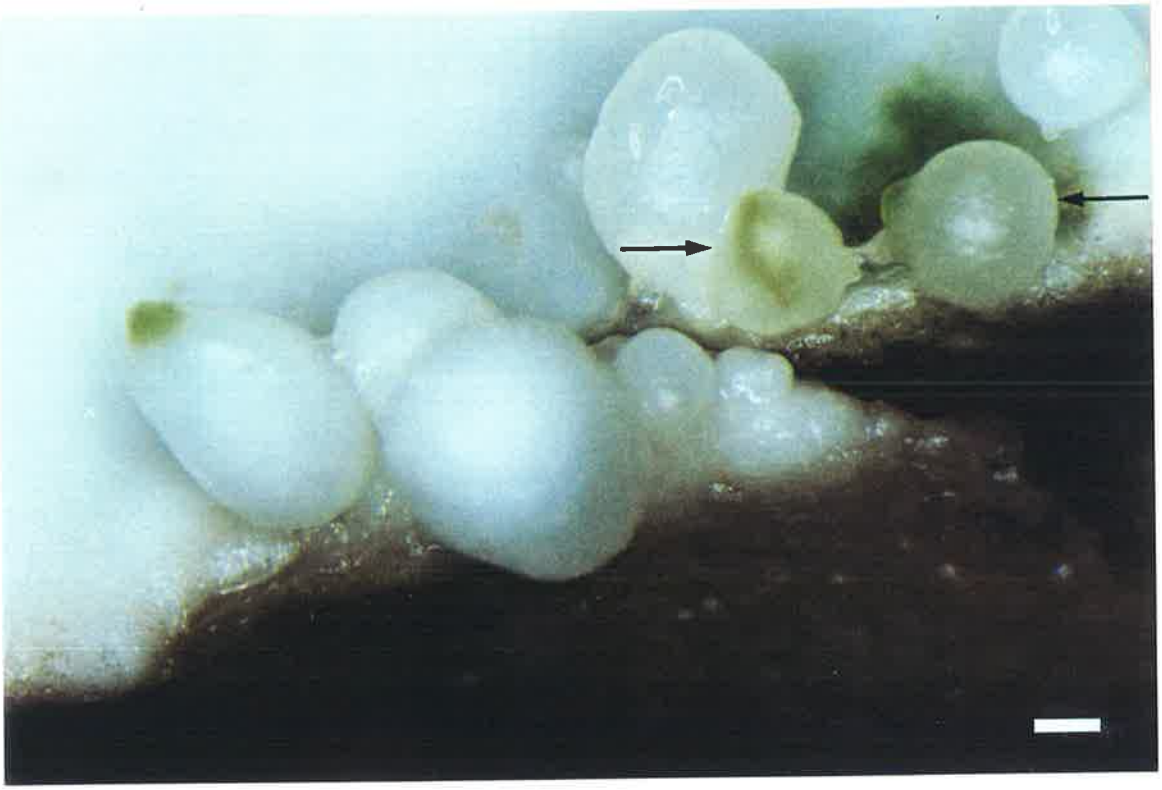

Figure 3.3. Proembryo structures that developed on immature almond cotyledons 28 days after culture initiation on MS medium containing 10.0 μM TDZ (Bar = 0.5 mm).

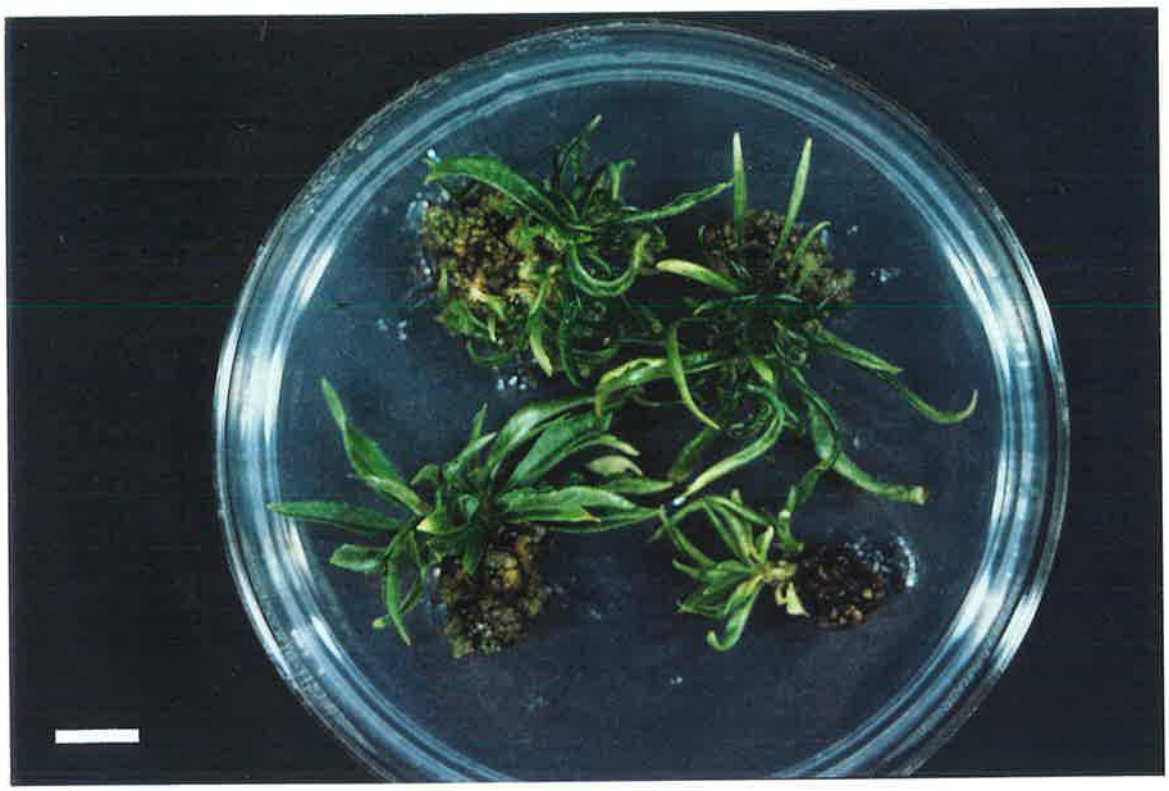

Figure 3.4. Multiple shoot production from adventitious buds on immature almond cotyledons after being transferred to MS medium without plant growth regulators (Bar = 10 mm).

Table 3.1: Effect of TDZ, IBA, and dark treatment on adventitious shoot development from immature cotyledons of four almond cultivars after 12 weeks culture on MS medium (values are the means of 3 replicates).

Cultivar	TDZ (μM)	IBA (μM)	Dark treatment (days)	Regeneration* (%)	Number of shoots†	Cultivar	TDZ (μM)	IBA (μM)	Dark treatment (days)	Regeneration* (%)	Number of shoots†
Ne Plus Ultra	0.0	0.0	0	0.0	0.0 ± 0.0	Nonpareil	0.0	0.0	0	0.0	0.0 ± 0.0
	0.0	0.5	0	0.0	0.0 ± 0.0		0.0	0.5	0	0.0	0.0 ± 0.0
	0.1	0.0	0	20.0	1.0 ± 0.0		0.1	0.0	0	0.0	0.0 ± 0.0
	0.1	0.5	0	0.0	0.0 ± 0.0		0.1	0.5	0	0.0	0.0 ± 0.0
	1.0	0.0	0	46.7	7.3 ± 2.8		1.0	0.0	0	46.7	2.5 ± 0.5
	1.0	0.5	0	0.0	0.0 ± 0.0		1.0	0.5	0	0.0	0.0 ± 0.0
	10.0	0.0	0	73.3	11.7 ± 3.3		10.0	0.0	0	66.7	10.7 ± 2.1
	10.0	0.5	0	20.0	1.0 ± 0.0		10.0	0.5	0	0.0	0.0 ± 0.0
	20.0	0.0	0	80.0	11.8 ± 3.3		20.0	0.0	0	73.3	12.6 ± 2.3
	20.0	0.5	0	33.3	2.5 ± 0.5		20.0	0.5	0	13.3	2.7 ± 0.3
	0.0	0.0	7	0.0	0.0 ± 0.0		0.0	0.0	7	0.0	0.0 ± 0.0
	0.0	0.5	7	0.0	0.0 ± 0.0		0.0	0.5	7	0.0	0.0 ± 0.0
	0.1	0.0	7	40.0	1.7 ± 0.4		0.1	0.0	7	0.0	0.0 ± 0.0
	0.1	0.5	7	0.0	0.0 ± 0.0		0.1	0.5	7	0.0	0.0 ± 0.0
	1.0	0.0	7	66.7	4.0 ± 1.4		1.0	0.0	7	80.0	3.2 ± 0.9
	1.0	0.5	7	0.0	0.0 ± 0.0		1.0	0.5	7	6.7	1.0 ± 0.0
	10.0	0.0	7	80.0	11.4 ± 2.5		10.0	0.0	7	73.3	12.3 ± 2.5
	10.0	0.5	7	33.3	5.8 ± 1.6		10.0	0.5	7	0.0	0.0 ± 0.0
	20.0	0.0	7	93.3	16.0 ± 2.9		20.0	0.0	7	80.0	9.3 ± 3.2
	20.0	0.5	7	53.3	4.0 ± 0.8		20.0	0.5	7	20.0	2.7 ± 1.2

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Table 3.1: continued.

Cultivar	TDZ (µM)	IBA (µM)	Dark treatment (days)	Regeneration* (%)	Number of shoots†	Cultivar	TDZ (µM)	IBA (µM)	Dark treatment (days)	Regeneration* (%)	Number of shoots†
Carmel	0.0	0.0	0	0.0	0.0 ± 0.0	Parkinson	0.0	0.0	0	0.0	0.0 ± 0.0
	0.0	0.5	0	0.0	0.0 ± 0.0		0.0	0.5	0	0.0	0.0 ± 0.0
	0.1	0.0	0	26.7	5.8 ± 1.5		0.1	0.0	0	26.7	2.3 ± 0.8
	0.1	0.5	0	0.0	0.0 ± 0.0		0.1	0.5	0	0.0	0.0 ± 0.0
	1.0	0.0	0	73.3	8.7 ± 3.1		1.0	0.0	0	66.7	3.4 ± 0.9
	1.0	0.5	0	0.0	0.0 ± 0.0		1.0	0.5	0	0.0	0.0 ± 0.0
	10.0	0.0	0	100.0	10.5 ± 1.5		10.0	0.0	0	73.3	15.1 ± 3.2
	10.0	0.5	0	13.3	2.5 ± 0.5		10.0	0.5	0	0.0	0.0 ± 0.0
	20.0	0.0	0	86.7	10.6 ± 2.3		20.0	0.0	0	93.3	13.5 ± 3.1
	20.0	0.5	0	13.3	2.0 ± 0.0		20.0	0.5	0	13.3	2.5 ± 0.5
	0.0	0.0	7	0.0	0.0 ± 0.0		0.0	0.0	7	0.0	0.0 ± 0.0
	0.0	0.5	7	0.0	0.0 ± 0.0		0.0	0.5	7	0.0	0.0 ± 0.0
	0.1	0.0	7	33.3	3.2 ± 1.4		0.1	0.0	7	40.0	2.3 ± 1.2
	0.1	0.5	7	0.0	0.0 ± 0.0		0.1	0.5	7	0.0	0.0 ± 0.0
	1.0	0.0	7	66.7	6.2 ± 1.7		1.0	0.0	7	60.0	5.0 ± 1.4
	1.0	0.5	7	6.7	2.0 ± 0.0		1.0	0.5	7	0.0	0.0 ± 0.0
	10.0	0.0	7	100.0	11.5 ± 2.2		10.0	0.0	7	86.7	14.2 ± 3.5
	10.0	0.5	7	26.7	1.0 ± 0.0		10.0	0.5	7	13.3	4.5 ± 2.5
	20.0	0.0	7	93.3	12.3 ± 2.3		20.0	0.0	7	86.7	20.0 ± 3.2
	20.0	0.5	7	13.3	2.5 ± 1.5		20.0	0.5	7	13.3	2.5 ± 1.5

* Defined as the number of cotyledons that produced adventitious shoots.

† Defined as the number of adventitious shoots per regenerating cotyledon. Mean values ± standard error.

Table 3.2: Analysis of variance summary for interaction effects between genotype, dark treatment, TDZ concentration and IBA presence on regeneration frequency in immature almond cotyledons.

	Regen frequency† (%)		
Source of variation	df	MS	F
Genotype	3	1596.4	9.4***
Dark treatment	1	1634.8	9.6**
Genotype × Dark treatment	3	51.6	0.3
TDZ concentration	4	18835.0	111.3***
Genotype × TDZ conc	12	408.4	2.4**
Dark treatment × TDZ conc	4	154.9	0.9
Genotype × Dark treatment × TDZ conc	12	210.4	1.2
IBA	1	75042.4	443.6***
Genotype × IBA	3	876.6	5.1**
Dark treatment × IBA	1	72.9	0.4
Genotype × Dark treatment × IBA	3	80.3	0.5
TDZ conc × IBA	4	6502.1	38.4***
Genotype × TDZ conc × IBA	12	324.0	1.9*
Dark treatment × TDZ conc × IBA	4	122.6	0.7
Genotype × Dark treatment × TDZ conc × IBA	12	130.1	0.8
Error	160	169.2	
Total	239		

† Data subjected to arcsine transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

adventitious bud formation in all cultivars except for Nonpareil, which responded only to concentrations ≥ 1.0 μM (Table 3.1). Differences in regeneration response to TDZ concentration were more evident at levels up to 10.0 μM, especially for Ne Plus Ultra, Carmel, and Parkinson, with higher concentrations in the 0.0 - 10.0 μM range producing higher regeneration frequencies and shoot numbers (Table 3.1). Although the highest level of TDZ (20.0 μM) generally produced the highest number of adventitious buds, shoot elongation was inhibited, and the incidence of shoot hyperhydricity was increased compared to cotyledons cultured on media containing lower concentrations of TDZ (data not shown). Hence, the most suitable TDZ level for all cultivars was 10.0 μM, and at this concentration, regeneration frequencies ranged from 66.7 to 100.0%, and the number of shoots per cotyledon from 10.7 to 15.1 (Table 3.1). Rooting of recovered shoots was not attempted in this study.

Dark treatment of cotyledons for 7 days had a significant affect on regeneration frequency (Table 3.2). This treatment generally increased the number of adventitious buds that formed, improving regeneration levels by up to 33.3% as compared to results from cotyledons treated with a continuous light regime (Table 3.1). Under the experimental conditions imposed of the first 7 days in the dark, 10.0 μM TDZ, and the absence of IBA, regeneration rates for Ne Plus Ultra, Nonpareil, Carmel, and Parkinson were 80.0%, 73.3%, 100.0%, and 86.7% respectively.

The inclusion of 0.5 μM IBA in regeneration media significantly affected the regeneration capacity of all cultivars (Table 3.2). Regeneration frequencies were significantly lower (33.0 - 87.0%), as were the number of shoots per cotyledon, when compared to explants cultured under identical conditions in the absence of IBA (Table 3.1). IBA also reduced

the range of TDZ concentrations that induced shoot morphogenesis, with only the higher levels (10.0 - 20.0 μM) being effective (Table 3.1). Furthermore, its inclusion in regeneration media promoted occasional root development directly from cotyledon tissue, particularly when combined with low TDZ levels (≤ 1.0 μM). Roots were stubby (> 1.0 mm thick), and the number per cotyledon ranged between 1.0 and 2.0 (data not shown). A summary of interaction effects between the treatment variables is provided in Table 3.2.

3.4 DISCUSSION

The current study reports the development of a high-frequency shoot regeneration system from immature seed explants of almond. Cotyledons were excised from the seeds of four almond cultivars, and tested for regeneration potential under a range of tissue culture conditions. Following previously reported procedures (Mante et al., 1989; Pooler and Scorza, 1995; Tang et al., 2000), explants were taken from the proximal ends of the cotyledons and the embryonic axes discarded.

Culturing explants in the dark has been demonstrated to improve adventitious bud development (Predieri and Fasolo Fabbri Malavasi, 1989; Famiani et al., 1994), with a period of 7 days sufficient for shoot organogenesis to occur (Korban et al., 1992). In this study a similar light regime was tested, and under these conditions, cotyledons cultured on MS medium supplemented with 10.0 μM TDZ resulted in regeneration levels of at least 73.3% for all four cultivars. These levels are substantially better than those reported for adult tissues (6.0 - 44.0%) under similar conditions (Miguel et al., 1996; Ainsley et al.,

2000). The genotype-specific variability previously experienced with explants derived from adult almond tissue (Ainsley et al., 2000) was reduced by using juvenile explants.

Although regeneration from immature almond cotyledons has been reported, the frequency of shoot morphogenesis has been low. Mehra and Mehra (1974) describe sporadic shoot regeneration (below 10.0%) from cotyledons subjected to various combinations of basal nutrients and plant growth regulators. A more recent study (Antonelli, 1991) with cotyledons of the European cultivar, Tuono, cultured on MS medium with various concentrations of BA and NAA, did not achieve shoot morphogenesis. The high levels of adventitious regeneration described in this study are most likely attributable to the inclusion of TDZ in the regeneration medium. The potent cytokinin activity of this plant growth regulator, especially for woody plant tissue culture, has been well documented (Huetteman and Preece, 1993; Lu 1993; Murthy et al., 1998). The current findings also concur with those on other *Prunus* species where TDZ has been effective in promoting regeneration from cotyledon tissue (Mante et al., 1989; Goffreda et al., 1995; Pooler and Scorza, 1995) and increases the potential use of TDZ for tissue culture practices with almond.

In the present study, the inclusion of IBA (0.5 μM) in the regeneration media significantly reduced the frequency of adventitious regeneration. These findings contrast with previous reports of regeneration in other *Prunus* species where IBA was required for shoot regeneration from cotyledons (Mante et al 1989; Pooler and Scorza, 1995), and in some instances, promoted organogenesis in the absence of other plant growth regulators (Goffreda et al., 1995). However, the concentrations of IBA detailed in these reports were higher (1.25 - 5.0 μM) than the level tested in this study, and hence, additional research is

required to determine the optimum level for almond cotyledons. The finding in Chapter 2, where regeneration of adult leaf explants was improved with increased levels of IBA provides further justification for this.

The use of matured cotyledons to allow year-round experimentation has been demonstrated in ornamental cherry (Hokanson and Pooler, 2000) and peach rootstock (Pooler and Scorza, 1995). This would also prove useful with almond. However, preliminary experiments with mature almond cotyledons resulted in severe fungal and bacterial contamination following the initiation of cultures, preventing the collection of any useful data (results not shown). Further experiments are therefore required to overcome exogenous/endogenous contaminants and determine the regeneration potential of mature almond cotyledons.

The findings in this study are significant for future tissue culture and genetic transformation research with almond. The described regeneration system provides an opportunity to generate somaclonal variants (Hammerschlag, 1992), and recover genetically modified plants. It is acknowledged that plantlets regenerated from zygotic embryo tissue will be genetically different from the parental cultivar, and that the phenotype would be an unknown variable. Therefore, either extensive field-testing would need to be conducted, or the regenerated material introduced into a breeding program, before its release, as a new cultivar would be possible. Whilst this regeneration system may not be the preferred approach for generating variation in the gene pool (either via tissue culture induced mutations or genetic engineering), it is an acceptable system for species such as almond where regeneration from adult and somatic explants is either limited or not possible. Hence, until regeneration from these tissues is improved/possible,

the approach described in this study affords the opportunity to introduce variation and genes outside the scope of conventional improvement methods into almond.

4. IN VITRO ROOT FORMATION

4.1 INTRODUCTION

The induction of roots *in vitro* is an important step in plant micropropagation and genetic transformation protocols, but has often proved difficult, particularly when rooting shoots of mature woody plants (George, 1996). Most reports of adventitious root induction from woody species have involved treatments with exogenous auxins (George, 1996) such as indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA). In addition, other factors including basal salt composition, phenolic compounds, explant physiology, photoperiod, light intensity, and light quality have also been shown to affect adventitious rooting (Damiano et al., 1991; Rugini et al., 1993; De Klerk et al., 1999).

Although rooting of several *Prunus* species has been reported, including apricot (Marino et al., 1993; Perez-Tornero et al., 2000), cherry (Hammatt and Grant, 1997), peach (Hammerschlag et al., 1987; Fouad et al., 1995), and plum (Aier and Sharma, 1990), *in*

vitro rooting of almond has proven difficult (Kester and Gradziel, 1996). Reports describing rooting of adult almond explants are limited, and have primarily focused on cultivars grown in Europe (Rugini and Verma, 1983; Caboni and Damiano, 1994; Caboni et al., 1997). Results from these previous studies demonstrated that, for almond, the conditions required for the induction of roots under tissue culture conditions are genotype-specific. Of the cultivars grown in Australia and the USA, there has been one previous report with the cultivar Nonpareil (Tabachnik and Kester, 1977). In that study Nonpareil was recalcitrant to the conditions tested, and only limited rooting was reported.

This study aimed to develop an improved rooting protocol for almond by examining the effects of auxin concentration and exposure time, shoot base shading, basal salt composition, and the presence of the phenolic compound phloroglucinol on adventitious root formation, using the cultivars Nonpareil and Ne Plus Ultra that are grown commercially throughout Australia and the USA.

4.2 MATERIALS & METHODS

4.2.1 Explant sterilisation and micropropagation

Actively growing shoots of the cultivars Ne Plus Ultra and Nonpareil (synonym: Californian Papershell) were sterilised, and *in vitro* cultures initiated and maintained as described in Chapter 2, Sections 2.1 and 2.2, respectively.

4.2.2 Rooting pretreatment

4-week-old micropropagated shoot cultures were transferred to 250 mL tissue culture pots containing a basal medium of MS (Murashige and Skoog, 1962 - Appendix 1) for Ne Plus Ultra or AP (Almehdi and Parfitt, 1986 - Appendix 1) for Nonpareil, without plant growth regulators. Shoots were maintained at 4°C with low light intensity (5 μmol m^{-2} s^{-1}) for 4 weeks.

4.2.3 Experiment 1: Effect of chronic auxin treatment and shoot base shading on *in vitro* rooting of Ne Plus Ultra

Pretreated elongated shoots were harvested and wounded by splitting their base vertically through the pith, approximately 2 mm up the stem. Rooting media consisted of ½-strength MS salts supplemented with either IBA or NAA at 0.0, 2.5, 5.0 or 10.0 μM. MS-based salts were used for all experiments with Ne Plus Ultra as previous experiments have shown them to be superior for *in vitro* manipulations with this almond genotype (Ainsley, unpublished data). The effect of shoot base shading on root initiation by these treatments was examined with or without black food dye (0.2% v/v: CORELLA, Australian Food Ingredient Suppliers). For rooting experiments, 125 mL polycarbonate tubes containing 20 mL of medium were used. Treated shoots were kept in the dark for 3 days, then exposed to light. After 2 weeks, shoots were transferred to ½-MS nutrients without plant growth regulators or food dye. Rooting frequency, the number of roots on rooted explants, and root lengths were recorded after 4 weeks.

4.2.4 Experiment 2: Effect of acute auxin treatment and shoot base shading on *in vitro* rooting of Ne Plus Ultra

Explants were pretreated as described for experiment 1. Shoot stems were dipped for 1 min in 5.0 mM solutions of either IBA or NAA to a depth of approximately 5 mm, or inserted for 12 h into water-agar (0.6% w/v) containing either 1.0 mM IBA or 1.0 mM NAA, then transferred to media containing ½-MS nutrients without growth regulators, ± black food dye (0.2% v/v). Shoots were kept in the dark for 3 days, then exposed to the light. After 2 weeks, shoots were transferred to ½-MS medium without food dye. Results (as described for experiment 1) were recorded after 4 weeks.

4.2.5 Experiment 3: Effect of phloroglucinol (PG) on *in vitro* rooting of Ne Plus Ultra

Explants were pretreated as described for experiment 1. Shoots were inserted for 12 h into water-agar (0.6% w/v) containing either 1.0 mM IBA or 1.0 mM NAA, then transferred to media comprising ½-MS salts with PG at 0.0, 1.0, 10.0 or 100.0 µM. Shoots were kept in the dark for 3 days, then exposed to light. After 2 weeks, shoots were transferred to ½-MS medium without PG. Results (as described for experiment 1) were recorded after 4 weeks.

4.2.6 Experiment 4: Effect of acute IBA treatment, basal salt composition and PG on *in vitro* rooting of Nonpareil

Explants were pretreated as described for experiment 1. The choice of parameters for this experiment was based upon results from experiments 1-3. Shoots were inserted for 12 h into water-agar (0.6% w/v) containing either 0.5 or 1.0 mM IBA, then transferred to AP_1 (½-strength AP salts), AP_2 (½-strength nitrogen: 1250 mg L^{-1} KNO_3 and 135 mg L^{-1}

$(NH_4)_2SO_4$, plus other components as per Almehdi and Parfitt, 1986) or AP_3 (unmodified) media ± 100.0 µM PG. For experiments with Nonpareil, MS basal nutrients were not used, as previous experiments showed that AP basal salts were superior for *in vitro* manipulations with this almond genotype (Ainsley, unpublished data). Shoots were kept in the dark for 3 days, then exposed to light. After 2 weeks, shoots were transferred to the same basal medium, but without PG. Results (as described for experiment 1) were recorded after 4 weeks.

4.2.7 Media preparation and culture conditions

All media contained 3.0% (w/v) sucrose, 0.7% (w/v) agar (SIGMA), and were adjusted to pH 5.7 prior to autoclaving (120°C for 20 min). Plant growth regulators and rooting co-factors were added to basal media prior to adjustment of pH and sterilisation. Unless otherwise described, cultures were maintained at 25 ± 1°C with a 16 h photoperiod (40 µmol m^{-2} s^{-1}) provided by Osram 36 W cool white fluorescent tubes.

4.2.8 Acclimatisation of rooted plantlets

Plantlets with roots between 5.0 and 10.0 mm in length were randomly selected for establishing in the glasshouse. Explants were removed from culture, and the roots gently washed in distilled water to remove any residual medium. Shoots were planted into 100 mm plastic pots containing Nu-Erth premium potting mix (Nu-Erth Horticultural Supplies, South Australia) and covered with 500 mL clear polycarbonate culture pots to maintain high humidity. Over 4 weeks, relative humidity was slowly decreased by gradually removing polycarbonate pots. Plantlets were acclimatised in a glasshouse at 25 ± 2°C under natural daylight.

4.2.9 Experimental design and statistical analysis

Four experiments were conducted over a 12-month period, with the data from each experiment being analysed separately. Treatments were replicated 10 times, with each replicate comprising 1 explant. Rooting frequencies (before conversion into percentages) were statistically analysed by the χ^2 test in contingency tables according to De Fossard (1976). Root number and root length were statistically analysed by ANOVA using a factorial design. Root numbers were transformed into square roots prior to analysis, whereas for root length data analysis was performed on the original values. The number of roots and root lengths were also presented as mean values with a standard error. Data were analysed using PlotIT version 3.2 (Scientific Programming Enterprises, USA).

4.3 RESULTS

4.3.1 Experiment 1: Effect of chronic auxin treatment and shoot base shading on *in vitro* rooting of Ne Plus Ultra

Continuous exposure of shoots to IBA or NAA for a 2-week period at concentrations up to 10.0 μM did not promote adventitious root development. Although some root primordia formed on stem tissue exposed to auxin concentrations ≥ 5.0 μM, primordia outgrowth was inhibited, and structures were restricted to < 0.5 mm in size. In addition, auxin concentrations ≥ 5.0 μM resulted in shoot tip necrosis, leaf abscission, and the

development of soft friable callus at the stem base. Callus development was reduced if shoot bases were subjected to shading for 2 weeks.

4.3.2 Experiment 2: Effect of acute auxin treatment and shoot base shading on *in vitro* rooting of Ne Plus Ultra

Both the quick dip and 12 h incubation techniques promoted the formation of root structures that first appeared 3-4 days after treatment, as small (< 0.5 mm), dome-shaped, cream-coloured primordia. These primordia originated from undamaged stem tissue approximately 10 mm above the base of the shoot, and were not observed on either wounded tissue or from the shoot base. Within 7 days, the primordia elongated and developed into roots. Roots were white, non-branched (Figure 4.1), and ranged in length from 8.9 - 18.3 mm (Table 4.1). The number of roots per rooted shoot ranged between 1.0 and 3.0 (Table 4.1).

There was a significant difference between the two acute auxin treatments (χ^2 test, $P \leq 0.01$, Table 4.2), with insertion of shoots into water-agar containing 1 mM auxin for 12 h yielding higher rooting frequencies compared to the quick-dip approach (Table 4.1). Shoots incubated for 12 h in water-agar containing either 1.0 mM IBA or 1.0 mM NAA that were then transferred to medium without black dye developed roots at frequencies of 50.0% and 40.0% respectively (Table 4.1). However, if black dye was incorporated into the medium, root formation was reduced by up to 40.0% (Table 4.1), and both root number and root length decreased (Table 4.1). In contrast, the quick-dip treatments were generally unsuccessful in promoting root development in Ne Plus Ultra shoots, with rooting only occurring following an IBA dip and the subsequent transfer of shoots to medium containing food dye (Table 4.1). The effect of food dye on rooting ability was significant

Figure 4.1. Adventitious root development on Ne Plus Ultra shoot 4 weeks after being cultured in agar containing 1.0 mM IBA for 12 hr (Bar = 10 mm).

Figure 4.2. Rooted Ne Plus Ultra plant 12 weeks after outplanting (Bar = 40 mm).

Table 4.1: Experiment 2 - Effect of acute auxin treatment and shoot base shading on *in vitro* root formation in Ne Plus Ultra. Shoots were either dipped into 5 mM IBA/NAA solutions or inserted for 12 h into water-agar with 1 mM IBA/NAA before transfer to basal medium ± dye.

Rooting treatment	Auxin conc. (mM)	Black food dye	Root induction[†] (%)	Number of roots[‡]	Root length[‡] (mm)
IBA dip	5.0	-	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0
IBA dip	5.0	+	10.0 [b]	1.0 ± 0.0	18.3 ± 0.0
NAA dip	5.0	-	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0
NAA dip	5.0	+	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0
IBA agar[*]	1.0	-	50.0 [b]	3.0 ± 0.6	10.8 ± 1.6
IBA agar[*]	1.0	+	10.0 [b]	1.0 ± 0.0	8.9 ± 0.0
NAA agar[*]	1.0	-	40.0 [b]	2.0 ± 0.7	9.9 ± 2.6
NAA agar[*]	1.0	+	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0

[*] 0.6% w/v SIGMA agar.

[†] Defined as the number of microshoots that produced roots. Mean values followed by different letters are significantly different as determined by the χ^2 test at the 0.05 probability level - data was converted to percentages following analysis.

[‡] Mean value ± standard error.

Table 4.2: Experiment 2 - Treatment effects on rooting frequency of Ne Plus Ultra shoots.

Treatment effect	df	Chi-Square	*P*
+/- Food dye	1	6.746	**
Auxin (IBA/NAA)	1	0.422	NS
Auxin application (1 min / 12 h)	1	10.540	**

NS = not significant; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** $P \leq 0.001$

Table 4.3: Experiment 2 - Summary of treatment interactions as determined by analysis of variance for root number and root length data.

	Root number†			Root length		
Source of variation	df	MS	F	df	MS	F
Auxin treatment	1	6.2	12.2 *	1	127.6	6.2 *
Auxin	1	0.9	1.9	1	20.8	1.0
Auxin treat × Auxin	1	0.4	0.9	1	0.2	0.01
Food dye	1	4.7	9.3 *	1	86.6	4.2 *
Auxin treat × Food dye	1	6.2	12.2 **	1	179.5	8.7 **
Auxin × Food dye	1	0.1	0.2	1	2.3	0.1
Auxin treat × Auxin × Food dye	1	0.4	0.9	1	6.6	0.3
Error	72	0.5		72	20.6	
Total	79			79		

† Data subjected to square root transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

as determined by the χ^2 test (Table 4.2), and in most instances reduced the formation of adventitious roots. No significant difference was detected between IBA and NAA for the induction of roots in experiment 2 (Table 4.2). Significant interactions between auxin treatment, food dye presence, and auxin treatment × food dye presence did however affect root number and root length (Table 4.3).

After 4 weeks, rooted shoots were outplanted to a greenhouse. At least 70.0% of the plantlets transferred survived acclimatisation procedures and developed into phenotypically normal plants (Figure 4.2). There were no differences in survival rates between the different rooting treatments (data not shown). Shoots that did not develop roots showed shoot tip necrosis, leaf yellowing and leaf abscission after 4 weeks in culture. These symptoms were more prevalent on shoots that had been treated with NAA.

4.3.3 Experiment 3: Effect of phloroglucinol (PG) on *in vitro* rooting of Ne Plus Ultra

The effect of including PG in media on rooting frequency varied depending on the auxin used for root induction (Table 4.4). When shoots were subjected to PG following 12 h in water-agar (0.6% w/v) containing 1.0 mM NAA, the number of explants that developed roots decreased significantly (Table 4.4), as did the mean root length (Table 4.4). Comparatively, changes in the rooting frequency of shoots cultured for 12 h in water-agar (0.6% w/v) containing 1.0 mM IBA were not significant (Table 4.4). No significant differences in rooting frequency were detected when comparing the different levels of PG tested on the Ne Plus Ultra microshoots (χ^2 test, $P > 0.1$, Table 4.5). Similarly, no significant interactions between PG concentration or auxin × PG concentration were detected for root number and root length data (Table 4.6). The number of roots ranged

Table 4.4: Experiment 3 - Effect of phloroglucinol (PG) on rooting of Ne Plus Ultra shoots: explants were placed in water-agar containing 1.0 mM IBA/NAA for 12 h before transfer to basal medium with PG.

Rooting treatment*	PG (µM)	Root induction† (%)	Number of roots†	Root length‡ (mm)
IBA (1 mM)	0.0	50.0 [b]	3.0 ± 0.6	10.8 ± 1.6
	1.0	40.0 [b]	2.3 ± 0.3	9.8 ± 1.9
	10.0	50.0 [b]	2.0 ± 0.6	7.4 ± 1.5
	100.0	60.0 [b]	2.5 ± 0.7	15.8 ± 2.5
NAA (1 mM)	0.0	40.0 [b]	2.0 ± 0.7	9.9 ± 2.6
	1.0	10.0 [a]	1.0 ± 0.0	8.7 ± 0.0
	10.0	10.0 [a]	2.0 ± 0.0	5.5 ± 3.1
	100.0	20.0 [a,b]	4.0 ± 2.0	5.7 ± 0.5

* 12 h culture in water-agar (0.6% w/v) containing 1.0 mM IBA or NAA.

† Defined as the number of microshoots that produced roots. Mean values followed by different letters are significantly different as determined by the χ^2 test at the 0.05 probability level - data was converted to percentages following analysis.

‡ Mean values ± standard error.

Table 4.5: Experiment 3 - Treatment effects on rooting frequency of Ne Plus Ultra shoots.

Treatment effect	df	Chi-Square	*P*
PG concentration	3	2.198	NS
Auxin (IBA/NAA)	1	11.091	***

NS = not significant; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** $P \leq 0.001$

Table 4.6: Experiment 3 - Summary of treatment interactions as determined by analysis of variance for root number and root length data.

	Root number[†]			Root length		
Source of variation	df	MS	F	df	MS	F
Auxin	1	10.9	7.8**	1	329.4	8.4**
PG concentration	3	1.6	1.2	3	59.6	1.5
Auxin × PG conc	3	0.1	0.03	3	40.2	1.0
Error	72	1.4		72	39.4	
Total	79			79		

[†] Data subjected to square root transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

from 1.0 to 4.0 (Table 4.4) and the root length from 5.5 to 15.8 mm (Table 4.4) depending on the rooting treatment.

For Ne Plus Ultra, maximum rooting was achieved by inserting shoots for 12 h into water-agar (0.6% w/v) containing 1.0 mM IBA, followed by 2 weeks in ½-MS salts supplemented with 100.0 µM PG (Table 4.4). Under these conditions, 60.0% of shoots developed multiple roots.

4.3.4 Experiment 4: Effect of acute IBA treatment, basal salt composition and PG on *in vitro* rooting of Nonpareil

The development of root primordia and elongation of root structures was as described for Ne Plus Ultra. For rooted explants, root number ranged from 1.0 - 7.0 (Table 4.7) and root length from 3.4 - 11.3 mm (Table 4.7) depending on the treatment. IBA concentration significantly affected rooting frequency (χ^2 test, $P \leq 0.05$, Table 4.8), with the number of shoots that developed adventitious roots following insertion into water-agar (0.6% w/v) containing 1.0 mM IBA up to 20.0% higher than those treated with 0.5 mM IBA (Table 4.7). Although the inclusion of 100 µM PG in media following the exposure of shoots to IBA increased rooting by up to 10.0%, its effect was not significant as determined by the χ^2 test ($P > 0.5$, Table 4.8). Of the three basal salts tested, AP_3 (full-strength AP salts) was significantly better than AP_1 (χ^2 test, $P \leq 0.001$) or AP_2 (χ^2 test, $P \leq 0.001$), and under certain conditions, induced up to 3-fold the level of rooting (Table 4.7). AP_1 and AP_2 basal salts were not significantly different (χ^2 test, $P > 0.25$) with respect to rooting frequency, with the number of Nonpareil shoots that developed roots on these media ranging from 0.0 to 20.0% (Table 4.7). Significant interactions between basal salt composition and basal

Table 4.7: Experiment 4 - Effect of acute IBA treatment, basal salt composition and phloroglucinol (PG) on *in vitro* of Nonpareil shoots: explants were placed in water-agar containing 0.5/1.0 mM IBA for 12 h then transferred to media with different levels of basal salts and PG.

IBA concentration* (mM)	Basal salts	PG (μM)	Root induction† (%)	Number of roots‡	Root length‡ (mm)
0.5	AP_1	0.0	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0
	AP_1	100.0	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0
	AP_2	0.0	0.0 [a]	0.0 ± 0.0	0.0 ± 0.0
	AP_2	100.0	10.0 [b]	1.0 ± 0.0	4.7 ± 0.0
	AP_3	0.0	30.0 [b,c]	7.0 ± 3.2	8.5 ± 0.8
	AP_3	100.0	40.0 [b,c]	6.0 ± 1.8	11.3 ± 1.1
1.0	AP_1	0.0	10.0 [a]	2.0 ± 0.0	3.9 ± 0.9
	AP_1	100.0	20.0 [b,c]	4.0 ± 3.0	6.3 ± 0.9
	AP_2	0.0	20.0 [b,c]	2.0 ± 1.0	6.8 ± 2.0
	AP_2	100.0	20.0 [b,c]	3.5 ± 1.5	3.4 ± 1.2
	AP_3	0.0	50.0 [c]	4.6 ± 0.8	4.5 ± 0.5
	AP_3	100.0	60.0 [c]	6.7 ± 1.6	7.1 ± 0.6

AP_1 = ½ salts; AP_2 = ½ nitrogen; AP_3 = unmodified AP salts.

* 12 h culture in water-agar (0.6% w/v) containing auxin.

† Defined as the number of microshoots that produced roots. Mean values followed by different letters are significantly different as determined by the χ^2 test at the 0.05 probability level - data was converted to percentages following analysis.

‡ Mean values ± standard error.

Table 4.8: Experiment 4 - Treatment effects on rooting frequency of Nonpareil shoots.

Interaction	df	Chi-Square	*P*
IBA concentration	1	5.941	*
PG concentration	1	0.442	NS
Basal salt composition	2	19.540	***

NS = not significant; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** $P \leq 0.001$

Table 4.9: Experiment 4 - Summary of treatment interactions as determined by analysis of variance for root number and root length data for Nonpareil.

	Root number†			Root length		
Source of variation	df	MS	F	df	MS	F
IBA concentration	1	7.8	3.7	1	9.3	1.2
Basal salt composition (BSC)	2	11.3	5.4**	2	39.3	4.9**
IBA conc × BSC	2	0.3	0.2	2	0.4	0.1
PG	1	1.0	0.5	1	18.9	2.4
IBA conc × PG	1	0.5	0.3	1	6.5	0.8
BSC × PG	2	20.4	9.8***	2	67.9	8.6***
IBA conc × BSC × PG	2	0.1	0.1	2	1.1	0.1
Error	108	2.1		108	7.9	
Total	119			119		

† Data subjected to square root transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

salt composition × PG presence affected root number and root length (Table 4.9). Nonpareil shoots that did not develop roots within 4 weeks displayed severe shoot tip necrosis, leaf yellowing and leaf abscission.

For Nonpareil maximum rooting was achieved by inserting shoots for 12 h into water-agar (0.6% w/v) containing 1.0 mM IBA, followed by 2 weeks in full-strength AP salts supplemented with 100.0 μM PG (Table 4.7). Under these conditions, 60.0% of shoots developed multiple roots.

4.4 DISCUSSION

This study demonstrates the successful rooting of two cultivars of almond. High levels of rooting, up to 60%, were achieved by culturing shoots in agar containing an acute level of auxin for a short period of time, followed by a passage in basal medium without growth regulators.

In earlier studies with almonds (Rugini and Verma, 1983; Caboni and Damiano, 1994; Caboni et al., 1997), rooting levels of up to 75% were achieved by the application of exogenous auxins including IAA, IBA, and NAA at concentrations between 2.5 and 10.0 μM over a 2 to 4 week period. In this study, similar experiments with the genotype Ne Plus Ultra failed to induce adventitious root formation. While early stage root primordia were observed, the presence of auxin inhibited primordia outgrowth. This inhibitory action is well known, and it has been reported that, for some plant species, auxin concentration

needs to be either reduced or eliminated after a short time in order for root initiation and elongation to proceed after induction (George, 1996; De Klerk et al., 1997).

For fruit trees, it has been proposed that root induction occurs within a few hours of auxin application and that prolonged exposure may not be necessary (Collett, 1988). Therefore, a more suitable approach may be a pulse (or acute) application, whereby an auxin is applied at a high concentration over a short period of time. In this study, two pulse methods were tested. For Ne Plus Ultra, although both approaches induced rooting, insertion of shoots into water-agar containing a high concentration of auxin for 12 h yielded significantly better responses than the quick-dip approach. With Nonpareil, the quick-dip method was not tried, as shoot culture in an auxin-agar promoted satisfactory rooting. Whilst both the quick-dip (Lane and McDougald, 1982; Harbage et al., 1998) and short-term incubation methods (De Klerk et al., 1997) have successfully been applied to *Malus* species, the application of pulse treatments to induce rooting in *Prunus* has not been widely investigated. Popov et al. (1976) reported adventitious root formation in sour cherry following an 18-h incubation in 0.25 mM IBA solution, and more recently, Miguel and Oliveira (1999) reported root development on adventitious shoots derived from almond seedling explants, following an overnight incubation in Gelrite containing 1.0 mM IAA. Based on the recalcitrant nature of almond to undergo root formation, the success achieved in the present study following an acute application of auxin, suggests that this approach should be further investigated, and considered with other woody species for which rooting has proven difficult.

Of the two auxins tested, IBA was more conducive (particularly for shoots exposed to PG following auxin treatment) to the formation of adventitious roots. It should be noted that

NAA was not tested with Nonpareil, as IBA induced a satisfactory rooting response. This decision was further supported by a previous study with Nonpareil where it was shown that NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations between 0.05 and 5.0 μM were unsatisfactory for root development (Tabachnik and Kester, 1977). In previous studies with European almond cultivars, IBA has been compared to other auxins. Whilst there have been some results similar to those reported in the current study (Caboni et al., 1997), there have also been contrasting findings (Rugini and Verma, 1983; Caboni and Damiano, 1994) with IAA and NAA proving more effective. This suggests that auxin-response is genotype-specific in almond.

Although darkness during the first week of the rooting phase has been found to be essential in stimulating rooting in some woody species (Rugini et al., 1993), shoot etiolation can induce early senescence, cause shoot yellowing, and a reduction in plantlet survival (Rugini et al., 1988). While prolonged periods of darkness (10-14 days) have been used to promote root development in some European almonds (Rugini and Verma, 1983; Caboni and Damiano, 1994), preliminary experiments with Ne Plus Ultra using similar regimes caused severe leaf yellowing (data not shown). In addition, a dark treatment of 3 days was found to be sufficient to induce rooting in both the genotypes tested in the current study. It has also been reported that excluding light from the root zone by overlaying the media surface with black polycarbonate granules and painting the outside of the culture tube, improves rooting in almond (Rugini et al., 1988, 1993). In this study, a similar environment was achieved by adding black food dye to the media. Results with Ne Plus Ultra did not concur with earlier findings, as root formation was significantly reduced following this treatment. The food dye tested in this study was commercially produced, and the details of its chemical composition were not available. Therefore it is unclear

whether the response of Ne Plus Ultra was genotype related, or a phytotoxic effect associated with the composition of the black food dye. This is an area that requires further investigation in future experiments.

Phloroglucinol is a tri-hydroxyphenol that has been used to stimulate *in vitro* rooting in a number of plant species (George, 1996). It is suggested that PG enhances rooting by either influencing auxin metabolism or, alternatively, by maintaining tissue redox potential in the reduced state (Hammatt, 1994). Whilst PG has enhanced adventitious rooting in *Malus* (James and Thurbon, 1981; Zimmerman, 1984) and *Pyrus* (Wang, 1991), its effectiveness on *Prunus* species is less clear. When tested on peach, PG significantly improved root formation (Hammerschlag et al., 1987), whereas its effect on cherry ranged from promotive (Hammatt, 1993, 1994) to inhibitory (Jones and Hopgood, 1979; Poniedzialek et al., 1986), depending on genotype. In plum, there was no effect (Jones and Hopgood, 1979). This is the first test of PG with almond, and for both cultivars, PG failed to significantly improve adventitious root formation. Although this response may be genotype related, the physiological state of the shoot tissue may also be a factor. With cherry, PG was most effective before the cultivars became apparently rejuvenated as a result of continued subculture, and thus more responsive to auxin (Hammatt and Grant, 1993). This may explain the lack of tissue response in Ne Plus Ultra and Nonpareil, with shoot material used in this study derived from 2-year-old microcultures that had been regularly subcultured. As the optimum concentration in this study was 100 µM (the upper limit tested), it is necessary to trial PG at a wider range of concentrations including higher levels to determine its true effect and optimum concentration with these genotypes. Future research should also consider alternative phenolic compounds such as chlorogenic acid and

catechol that have been shown to enhance adventitious rooting in other fruit species (Hammerschlag et al., 1987).

For experiments with Nonpareil, AP basal nutrients were used. This was based upon earlier research where MS salts proved unsatisfactory for the *in vitro* manipulation of this genotype (Ainsley, unpublished data). Although AP basal medium with reduced nitrogen levels has been used for rooting peach rootstocks (Almehdi and Parfitt, 1986), the modifications tested in this study decreased root formation and induced leaf yellowing in Nonpareil. In comparison to other culture media (such as MS) that are commonly used to root woody species, the total molarity of the macronutrient ions is much lower in AP salts. Therefore, while it may be appropriate to reduce the concentration of higher molarity media during rooting (George, 1996), the results with Nonpareil suggest modifications to the strength of AP salts have the potential to limit rooting ability and shoot survival. It is most likely that an insufficient nitrogen ion concentration caused these negative effects with Nonpareil, as nitrogen was reduced in both the modified versions of AP medium tested in this study.

The protocols recommended in this study vary from those previously reported for other almond cultivars, confirming that *in vitro* rooting in almond is genotype specific. Alternatively, the differences observed in this study could be attributed to the different growing conditions of the stock plants, or subtle variations in the culture environment. These findings are significant for future micropropagation and genetic transformation research with almond (Ainsley et al., 2000, 2001).

5. AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION

5.1 INTRODUCTION

The slow and difficult process of breeding woody fruit species by conventional breeding methods makes them ideal targets for gene transfer technologies. In Chapters 1 and 2, protocols for regenerating plants from different almond tissues were developed. This study follows on from this research, investigating the next stage in the transformation process by assessing parameters that are known to effect gene transfer.

Although efficient methods for genetically modifying *Malus* (James et al., 1989; Welander et al., 1998; Bolar et al., 1999) and *Pyrus* species (Mourgues et al., 1996; Bell et al., 1999) have been developed, reports within the *Prunus* genus have been limited to apricot (Laimer da Câmara Machado et al., 1992), peach (Smigocki and Hammerschlag, 1991), and plum

(Scorza et al., 1995). For almond, although gene transfer to adult leaf explants using the *Agrobacterium*-mediated approach has generated stably transformed calli (Archilletti et al., 1995), the recovery of transgenic plants has only been achieved using juvenile explants (Miguel and Oliveira, 1999). Furthermore, there have been no published reports of gene transfer to cultivars that are the focus of the industry in Australia and the USA. Hence, this study aimed to determine the conditions most suitable for the introduction of foreign genes to the cultivars Ne Plus Ultra and Nonpareil using the *Agrobacterium*-mediated approach by examining the effects of bacterial strain, cocultivation period, and the phenolic compound acetosyringone on gene transfer.

5.2 MATERIALS & METHODS

5.2.1 Plant material

Leaf explants were taken from micropropagated shoot cultures of the cultivars Ne Plus Ultra and Nonpareil (synonym: Californian Papershell) which were initiated and maintained as previously described in Chapter 2, Sections 2.1 and 2.2, respectively.

5.2.2 Bacterial strains and growth conditions

Disarmed *Agrobacterium tumefaciens* strains, EHA105 (Hood et al., 1993) and LBA4404 (Hoekema et al., 1983), carrying the binary vector p35SGUSint (Vancanneyt et al., 1990) were used in all experiments. This dual expression vector consists of the *npt*II gene under the control of the nopaline synthase (NOS) promoter, and the *gusA*-coding region containing a plant intron linked to the cauliflower mosaic virus 35 S (*CaMV35S*) promoter

(Appendix 3). The *npt*II gene acts as a selectable marker, conferring resistance to the antibiotic kanamycin, and the *gusA* gene encodes the enzyme β-glucuronidase (GUS), acting as a scorable reporter gene. The presence of an intron in the *gusA* gene prevents GUS-expression in bacteria. Strain EHA105 was maintained at 28°C on YEB medium (0.5 % (w/v) Difco Bacto beef extract, 0.5 % (w/v) peptone, 0.5 % (w/v) sucrose, 0.1 % (w/v) yeast extract and 1.5 % (w/v) Difco Bacto agar, pH 7.2) supplemented with 85 μM kanamycin. Strain LBA4404 was maintained on the same medium additionally supplemented with 170 μM streptomycin. For cocultivation, bacteria were grown overnight (200 rpm, 28°C) in liquid YEB medium, pelleted, and resuspended to an OD_{550} of 0.3. Bacterial suspensions were then diluted 1:10 (v/v) to give a density of 2×10^8 cfu mL^{-1} in cocultivation medium comprising AP basal nutrients (Almehdi and Parfitt, 1986 - Appendix 1) supplemented with TDZ (22.7 or 6.8 μM for Ne Plus Ultra and Nonpareil respectively), 9.8 μM IBA, 3% (w/v) sucrose, casein hydrolysate (CH: 0.1% w/v), ± 20 μM acetosyringone (AS), pH 5.4.

5.2.3 Tests for kanamycin sensitivity of leaf pieces

Preliminary tests were conducted to assay the resistance threshold of untransformed tissue to kanamycin. Leaf sections, as described in Chapter 2 Section 2.4, were plated abaxial side down on cocultivation medium containing 9.0, 17.0, 25.0, 34.0, 68.0, 103.0, 137.0, or 172.0 μM kanamycin. The effect of the antibiotic on callus development and shoot regeneration was determined after 8 weeks.

5.2.4 Plant tissue culture and transformation

The uppermost, fully-expanded leaves from 4-week-old micropropagated shoot cultures of Ne Plus Ultra and Nonpareil were excised and dissected into sections as previously described in Chapter 2, Section 2.4. Leaf sections were precultured for 3 days, abaxial side down on regeneration medium comprising cocultivation medium solidified with 0.7% (w/v) agar (SIGMA). Following this, explants were suspended in the bacterial inoculum on a rotary shaker (100 rpm) at 22°C for 1 h, transferred to sterile filter paper saturated with cocultivation medium and incubated at 22°C for 2-5 days. After cocultivation, explants were washed twice by gentle agitation on a rotary shaker (100 rpm) in liquid medium without plant growth regulators containing 1 mM cefotaxime (Claforan, Hoechst) for 20 min, blotted on sterile filter paper, and transferred for 3 days to regeneration medium supplemented with plant growth regulators and 630 µM cefotaxime. Selection for putative transformants on regeneration medium was achieved in the presence of 25 µM kanamycin, which was previously determined to be the optimum concentration for the almond cultivars used (Section 5.3.1 of this Chapter). Explants were subcultured every 2 weeks. Plant growth regulators were added to basal media and the pH adjusted prior to autoclaving (120°C for 20 min). Explants were maintained under darkness for 4 weeks, then exposed to light conditions with a 16 h photoperiod (40 µmol m^{-2} s^{-1}) provided by Osram 36 W cool white fluorescent tubes, at 25 ± 1°C.

5.2.5 Histochemical β-glucuronidase (GUS) assay

Leaf explants were assayed for activity of the *gusAint* reporter gene following the histochemical staining procedure described by Jefferson et al. (1987). Explants were incubated overnight at 37°C in 100 mM sodium phosphate buffer (pH 7.0) containing 0.5

mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na_2EDTA, 0.5% (v/v) Triton X-100, and 0.5 mg L^{-1} X-Gluc (Progen) dissolved in a small amount of dimethyl sulphoxide. Following the incubation, a solution comprising 95% (v/v) ethanol: 1% (v/v) glacial acetic acid was used to remove chlorophyll and fix tissues. To assess transient *gusAint* activity, leaf explants were stained with X-Gluc solution (as described above) 4 days after cocultivation was completed. Quantification was made by counting the number of blue spots (also known as foci) on the leaf surfaces using a stereomicroscope. After a further 6 weeks, calli that had developed on leaf explants and survived antibiotic selection were assayed for GUS activity using the same procedure. The number of calli with blue zones (foci) was recorded. Control explants were also tested for GUS activity using the same methods.

5.2.6 PCR and Southern blot analysis

DNA extraction

For PCR and Southern blot hybridisation, total genomic DNA was extracted from putatively transformed callus lines and controls following the method of Mekuria et al. (1999).

PCR analysis (Polymerase Chain Reaction)

PCR was performed in a PTC-100™ Programmable Thermal Controller (MJ Research Inc). The primers used for amplification of a 366-bp fragment of the *gusAint* gene were 5′-CCCGGCAATAACATACGGCGT-3′ and 5′-CCTGTAGAAACCCCAACCCGT-3′ and those used for amplification of a 700-bp fragment of the *npt*II gene were 5′-GAGGCTATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGCGATACCGTA-3′. For each PCR, the amount of DNA used was 50 ng of the appropriate plasmid as a positive

control that was prepared following standard procedures (Sambrook et al., 1989) and 100 ng of plant genomic DNA. The reaction mixture contained 0.2 mM each of dCTP, dGTP, dATP and dTTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), and 1.5 units of Taq polymerase (Life Technologies). The PCR conditions consisted of 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C (*gusAint* primers) or 65°C (*npt*II primers) for 1 min, and 72°C for 1 min, with a final elongation step of 72°C for 5 min. PCR products were separated by electrophoresis on 1.6% (w/v) agarose gels (Seakem LE) using TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8.0 adjusted with boric acid) and stained with ethidium bromide (0.5 μg mL^{-1}). To ensure the DNA fragments obtained were not false positives produced by contaminating bacteria, an additional PCR using primers for the amplification of a bacterial kanamycin resistance gene (*npt*I) located outside the T-DNA borders was performed. The primers for this were 5′-ATCGGCTCCGTCGATACTAT-3′ and 5′-CGTTCCACATCATAGGTGGT-3′. The reaction was carried out using conditions identical to those described above, except that the annealing temperature of the primers was 56°C.

Southern blot analysis

DNA (6 μg) from putatively transformed callus lines was digested overnight in 1 × universal buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM β-mercaptoethanol, pH 8.0) at 37°C with 60 U of *Hind*III (Boehringer - 25 U μL^{-1}) to generate an internal fragment of 2.8 Kb, corresponding to the *gusAint* gene, or 30 U of *Eco*RI (Boehringer - 40 U μL^{-1}) to generate a T-DNA border fragment. Digested samples, along with equal amounts of DNA from non-transformed lines were fractionated by gel electrophoresis in 1% (w/v) agarose (Seakem LE) using TBE buffer and capillary blotted to Hybond N+ membranes (Amersham) following standard procedures (Sambrook et al.,

1989). A 366-bp fragment from the *gusAint* gene generated through a PCR reaction (as described above) was used as a probe. The DNA fragment was purified using a BRESAspin™ PCR purification kit (GeneWorks, Adelaide), labelled with α^{32}P-dCTP (GeneWorks, Adelaide - 3000 Ci mmole^{-1}) using a GIGAPRIME DNA labelling Kit (GeneWorks, Adelaide), with unincorporated radionucleotides removed using S-200 HR MicroSpin columns (Amersham). Prehybridisation and hybridisation were carried out at 65°C for 3 h and 15 h respectively, in buffer containing 5 × Denhardt's solution (1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone, 0.15% (w/v) bovine serum albumin), 7.5% (w/v) dextran sulphate, 0.5% (w/v) sodium dodecylsulphate (SDS), 0.9 M NaCl, 30 mM PIPES (pH 6.8), 7.5 mM Na_2EDTA, and 100 μg mL^{-1} salmon sperm DNA fractionated by autoclaving. Membranes were washed twice at 65°C in 2 × SSC (300 mM NaCl, 30 mM trisodium citrate), 0.1% (w/v) SDS, and once in 1 × SSC, 0.1% (w/v) SDS, then exposed to X-ray film (Fuji RX) at -80°C with an intensifying screen for 2-5 days.

5.2.7 Experimental Design and statistical analysis

A completely randomised design with ten replicates, each containing 15 leaf sections, was used for all experiments. The number of GUS-active explants (leaf and calli), the number of GUS-zones, and the number of GUS-positive calli per explant was determined and presented as a mean value with a standard error. Percentage data for the number of GUS-active explants were subjected to ANOVA using a factorial design. Percentages were arcsine transformed before analysis. Data were analysed using PlotIT version 3.2 (Scientific Programming Enterprises, USA).

5.3 RESULTS

5.3.1 Tests for kanamycin sensitivity of leaf pieces

To determine the effect of kanamycin concentration on the selection of transformed tissues, micropropagated leaf sections were cultured on regeneration medium containing various concentrations of kanamycin. At levels ≥ 68 μM, kanamycin induced severe necrosis, and explants of both cultivars died without any callus formation. At 34 μM kanamycin, some initial callus formation was observed during the first 2 weeks of culture, but by 8 weeks, explants had turned brown and there was no evidence of adventitious bud development. When explants were cultured on regeneration medium containing kanamycin at concentrations between 9 and 25 μM, nodular callus formation occurred, and a few adventitious buds could be regenerated. Based on the sensitive nature of leaf explants to kanamycin, a level of 25 μM was used for selection procedures for both almond cultivars. Whilst this level has the potential to give rise to escapes, higher concentrations compromised explant viability to the extent that even if transformation occurred, cells were unlikely to survive the selection procedure.

5.3.2 Assessment of factors affecting transient GUS activity

Genotype, bacterial strain, and cocultivation period significantly affected the level of transient GUS activity in the almond leaf explants 4 days after cocultivation. There was also a significant interaction between genotype × bacterial strain × acetosyringone, and genotype × bacterial strain × cocultivation period × acetosyringone (Table 5.2).

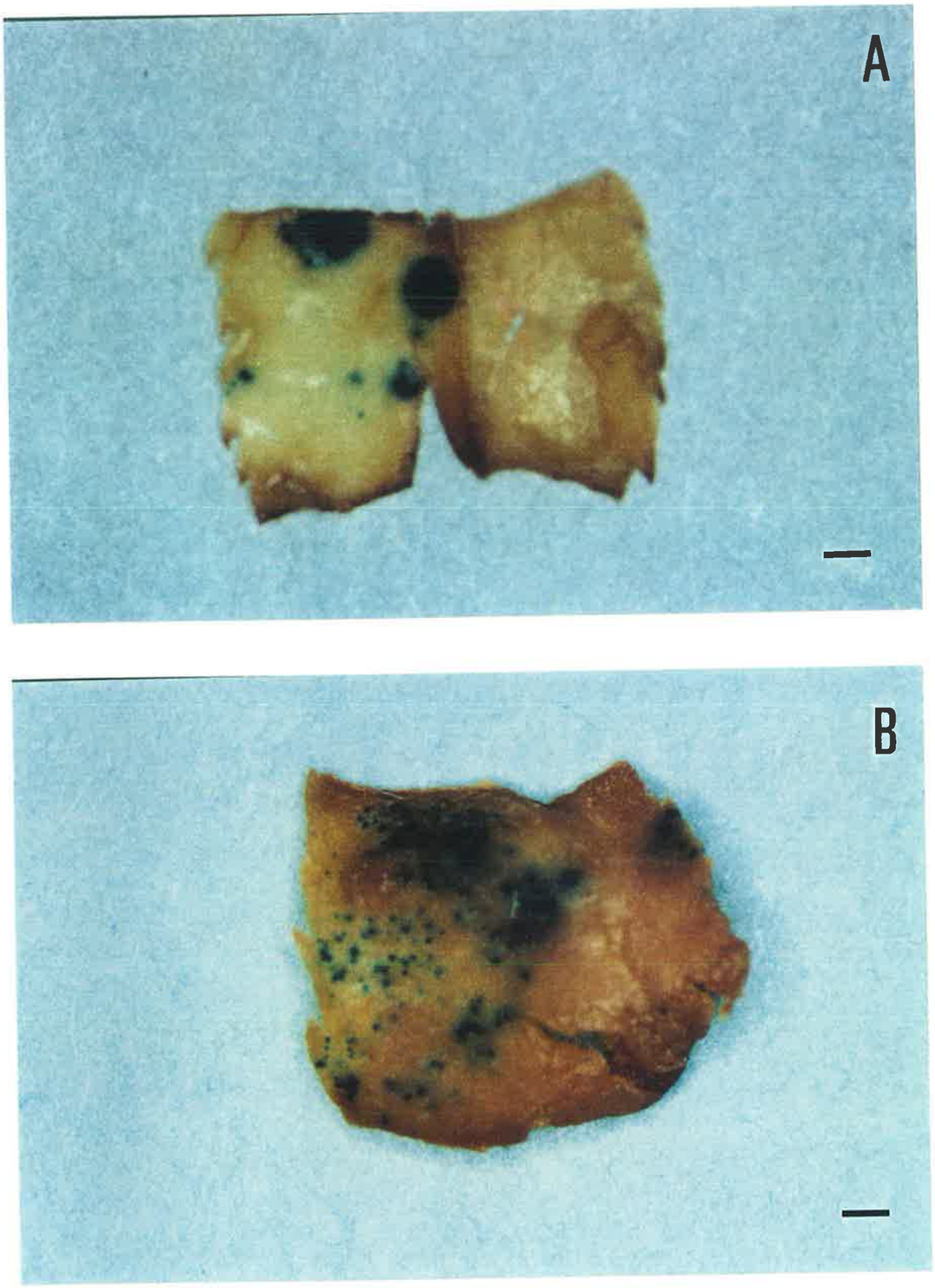


Figure 5.1. Transient GUS expression in Ne Plus Ultra leaf explants 4 days after cocultivation. A/B: examples of the different histochemical staining patterns observed following cocultivation with *A. tumefaciens* strain EHA105 (Bar = 1 mm).

Table 5.1: Effect of *Agrobacterium* strain, cocultivation period, and the presence of 20 µM acetosyringone (AS) on transient GUS activity in Ne Plus Ultra and Nonpareil leaf explants 4 days after cocultivation (values are the means of three replicates ± standard error).

Strain	Cocultivation (days)	AS	Ne Plus Ultra		Nonpareil	
			GUS activity† (%)	GUS zones‡	GUS activity† (%)	GUS zones‡
LBA4404	2	-	13.9 ± 7.3	1.4 ± 0.2	5.6 ± 5.6	4.5 ± 3.5
	2	+	13.9 ± 2.8	8.0 ± 3.2	2.8 ± 2.8	2.0 ± 0.0
	3	-	33.3 ± 8.3	4.6 ± 1.6	11.1 ± 4.9	4.1 ± 1.1
	3	+	55.6 ± 14.7	6.1 ± 1.3	5.6 ± 9.6	3.5 ± 2.5
	4	-	33.3 ± 8.3	3.8 ± 1.2	38.9 ± 10.0	8.1 ± 2.8
	4	+	50.0 ± 4.8	9.6 ± 2.8	33.3 ± 17.4	7.5 ± 2.5
	5	-	69.4 ± 5.6	4.0 ± 0.7	25.0 ± 12.7	4.1 ± 1.2
	5	+	69.4 ± 11.1	4.4 ± 0.8	33.3 ± 8.3	3.6 ± 1.1
	Average		*42.4 ± 7.9*	*5.2 ± 1.5*	*19.5 ± 8.9*	*4.7 ± 1.8*
EHA105	2	-	22.2 ± 5.6	6.4 ± 3.2	0.0 ± 0.0	0.0 ± 0.0
	2	+	16.7 ± 12.7	2.5 ± 1.0	2.8 ± 2.8	3.0 ± 0.0
	3	-	75.0 ± 12.7	4.1 ± 0.7	11.1 ± 4.9	3.0 ± 0.7
	3	+	55.6 ± 10.0	4.6 ± 1.8	36.1 ± 7.4	6.9 ± 1.1
	4	-	66.7 ± 4.8	7.8 ± 1.6	27.8 ± 10.0	7.5 ± 2.5
	4	+	61.1 ± 7.4	4.8 ± 1.0	50.0 ± 12.7	10.1 ± 2.9
	5	-	77.8 ± 7.3	3.7 ± 0.5	55.6 ± 15.5	14.5 ± 2.7
	5	+	72.2 ± 7.4	6.6 ± 2.1	66.7 ± 12.7	14.5 ± 8.2
	Average		*55.9 ± 8.5*	*5.1 ± 1.5*	*31.3 ± 8.3*	*7.4 ± 2.3*

† Defined as the number of leaf explants with GUS activity.

‡ Defined as the number of GUS zones (Foci) per explant.

Table 5.2: Analysis of variance summary for interaction effects between genotype, *Agrobacterium* strain, cocultivation period, and the presence of 20 μM acetosyringone on transient GUS activity in Ne Plus Ultra and Nonpareil leaf explants 4 days after cocultivation.

		Gus Activity† (%)	
Source of variation	df	MS	F
Genotype	1	6327.1	41.0***
Bact strain	1	1247.9	8.1**
Genotype × Bact strain	1	0.9	0.0
Cocultivation	3	6020.4	39.0***
Genotype × Cocult	3	106.7	0.7
Bact strain × Cocult	3	163.3	1.1
Genotype × Bact Strain × Cocult	3	218.3	1.4
Acetosyringone	1	0.2	0.0
Genotype × Acetosyringone	1	135.4	0.9
Bact strain × Acetosyringone	1	12.4	0.1
Genotype × Bact strain × Acetosyringone	1	2202.3	14.3***
Cocult × Acetosyringone	3	87.5	0.6
Genotype × Cocult × Acetosyringone	3	22.7	0.1
Bact strain × Cocult × Acetosyringone	3	31.7	0.2
Genotype × Bact strain × Cocult × Aceto	3	497.3	3.2*
Error	64	154.2	
Total	95		

† Data subjected to arcsine transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

Agrobacterium strain

Transient GUS activity was measured 4 days after cocultivation, and for both bacterial strains, dark blue spots (Figure 5.1) indicative of GUS presence were observed primarily in undamaged tissue closely linked with vascular bundles or surrounding leaf mesophyll. GUS activity along the cut edges of explants was minimal (< 10%).

Bacterial strain had a significant affect on transient GUS activity at the 0.01 probability level (Table 5.2). In general, cocultivation with strain EHA105 resulted in more GUS-positive explants as compared to explants cocultivated with strain LBA4404 (overall mean responses of 43.6% and 30.9% respectively - Table 5.1). However, the significance of bacterial strain varied with genotype. For Ne Plus Ultra, EHA105 produced higher levels of GUS activity than LBA4404 (overall mean responses of 55.9% and 42.4% respectively - Table 5.1), whereas for Nonpareil the corresponding mean response values for the same strains were 31.3% and 19.5% respectively. A difference in the size of GUS-zones following cocultivation with the two strains was also observed. Strain LBA4404 generated smaller more widely distributed spots compared to EHA105 which tended to generate larger zones that often coalesced, covering up to 15% of the leaf surface.

Cocultivation period

Cocultivation period had a significant effect on gene transfer efficiency (Table 5.2), with the number of GUS positive explants increasing with its prolongation (Table 5.1). For Ne Plus Ultra, the frequency of GUS active explants following 2 days cocultivation ranged from 13.9 to 22.2% compared with 69.4 to 77.8% when cocultivation was extended to 5 days (Table 5.1). Similar, although smaller increases were observed for Nonpareil (Table

5.2). It was also noted, that an extension in the cocultivation period brought about an increase in the number of GUS-positive zones. A consequence of this however was zone coalescence.

Influence of AS

Although the presence of AS in media did not have a significant affect on GUS activity (Table 5.2), under certain conditions, it did appear to have a positive influence on T-DNA gene transfer (Table 5.1). For Ne Plus Ultra explants transformed using LBA4404, AS increased the numbers of GUS-active explants by up to 22.3% and GUS-positive zones by as much as 2.5-fold following a 3-4 day cocultivation period (Table 5.1). In contrast, GUS activity in Ne Plus Ultra explants transformed with EHA105 was reduced in the presence of AS over all the cocultivation periods tested. The number of GUS-positive zones was also generally reduced (Table 5.1). For Nonpareil, GUS activity after transformation with LBA4404 was reduced in the presence of AS (up to 5.5%), as were the number of GUS zones. However, GUS activity in Nonpareil explants after transformation with EHA105 was increased by up to 25.0% in the presence of AS, with little effect on the number of GUS zones (Table 5.1).

5.3.3 Assessment of factors affecting the activity of the GUS gene in transgenic callus

The average number of GUS-positive calli that developed on leaf explants maintained on selection medium 6 weeks after cocultivation is shown in Table 5.3. For both cultivars, the number of transformed calli appearing on explants was lower than the number of explants

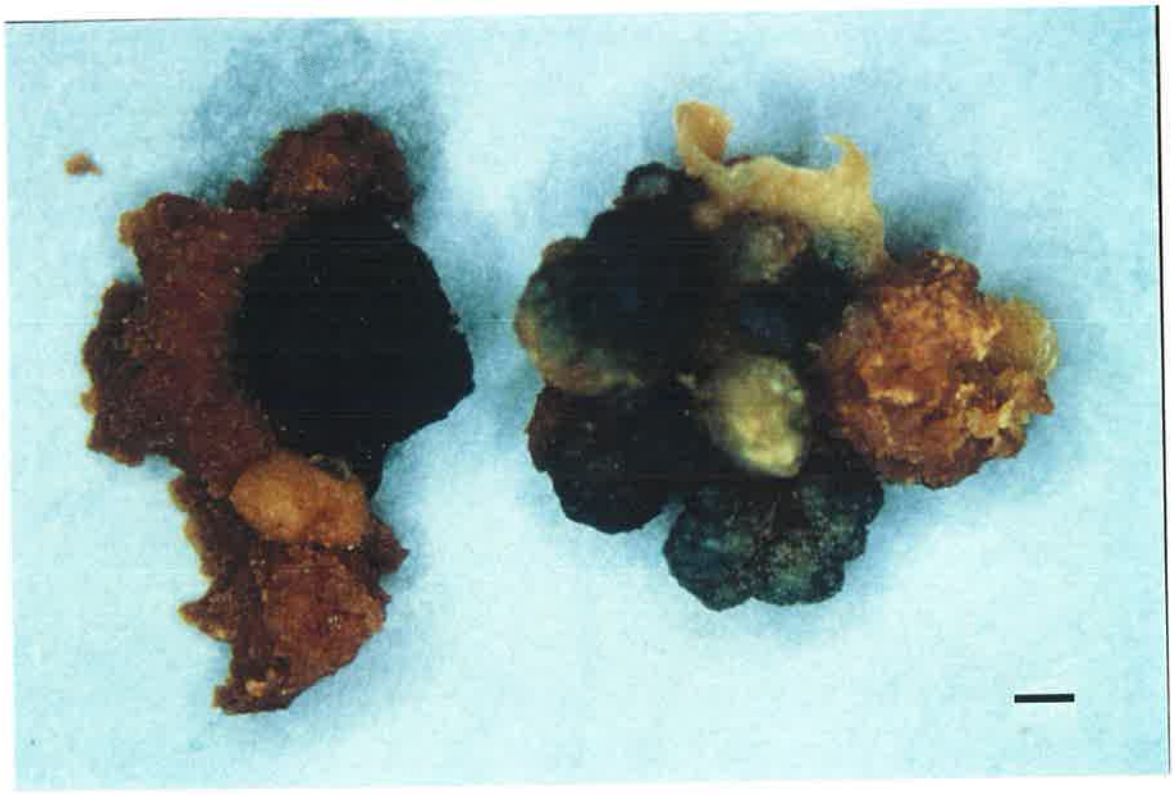


Figure 5.2. Stable GUS expression in Ne Plus Ultra leaf callus 3 months after cocultivation with *A. tumefaciens* strain EHA105 (Bar = 2 mm).

Table 5.3: Effect of *Agrobacterium* strain, cocultivation period, and the presence of acetosyringone (AS) on the activity of the GUS gene in callus originating from Ne Plus Ultra and Nonpareil leaf explants 6 weeks after cocultivation (values are the means of three replicates ± standard error).

Strain	Cocultivation (days)	AS	Ne Plus Ultra		Nonpareil	
			GUS activity† (%)	GUS calli per explant‡	GUS activity† (%)	GUS calli per explant‡
LBA4404	2	-	5.6 ± 2.8	3.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0
	2	+	5.6 ± 5.6	1.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
	3	-	8.3 ± 8.3	3.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0
	3	+	2.8 ± 2.8	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	4	-	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 2.8	1.0 ± 0.0
	4	+	8.3 ± 4.8	2.7 ± 0.9	2.8 ± 2.8	1.0 ± 0.0
	5	-	8.3 ± 0.0	2.0 ± 0.6	2.8 ± 2.8	2.0 ± 0.0
	5	+	11.1 ± 5.5	1.0 ± 0.0	2.8 ± 2.8	1.0 ± 0.0
	Average		*6.3 ± 3.8*	*1.8 ± 0.8*	*1.4 ± 1.4*	*0.6 ± 0.0*
EHA105	2	-	8.3 ± 4.8	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	2	+	8.3 ± 0.0	1.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	3	-	11.1 ± 7.4	1.5 ± 0.3	2.8 ± 2.8	2.0 ± 0.0
	3	+	8.3 ± 0.0	1.3 ± 0.3	11.1 ± 2.8	2.3 ± 1.0
	4	-	11.1 ± 2.8	2.0 ± 1.0	2.8 ± 2.8	1.0 ± 0.0
	4	+	11.1 ± 2.8	2.5 ± 1.0	11.1 ± 7.4	2.0 ± 0.7
	5	-	11.1 ± 5.6	2.3 ± 0.3	8.3 ± 4.8	1.3 ± 0.3
	5	+	8.3 ± 0.0	2.3 ± 1.3	13.8 ± 7.3	1.6 ± 0.4
	Average		*9.7 ± 2.9*	*1.8 ± 0.7*	*6.2 ± 3.5*	*1.3 ± 0.3*

† Defined as the number of leaf explants with GUS activity.

‡ Defined as the number of GUS zones per explant.

Table 5.4: Analysis of variance summary for interaction effects between genotype, *Agrobacterium* strain, cocultivation period, and acetosyringone presence on GUS activity in callus originating from Ne Plus Ultra and Nonpareil leaf explants 6 weeks after cocultivation.

		Gus activity† (%)	
Source of variation	df	MS	F
Genotype	1	1244.2	13.9***
Bact strain	1	1092.7	12.3***
Genotype × Bact strain	1	0.6	0.0
Cocultivation	3	225.4	2.5
Genotype × Cocult	3	72.7	0.8
Bact strain × Cocult	3	70.6	0.8
Genotype × Bact Strain × Cocult	3	103.3	1.1
Acetosyringone	1	138.2	1.6
Genotype × Acetosyringone	1	29.3	0.3
Bact strain × Acetosyringone	1	71.2	0.8
Genotype × Bact strain × Acetosyringone	1	76.3	0.9
Cocult × Acetosyringone	3	39.6	0.4
Genotype × Cocult × Acetosyringone	3	29.9	0.3
Bact strain × Cocult × Acetosyringone	3	32.9	0.4
Genotype × Bact strain × Cocult × Aceto	3	58.5	0.7
Error	64	88.9	
Total	95		

† Data subjected to arcsine transformation before analysis.

* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

that tested positive for transient GUS activity 4 days after cocultivation (compare Tables 5.1 and 5.3). However, high numbers of GUS spots (foci) in leaf explants shortly after cocultivation tended to lead to the development of calli with more GUS spots. The frequency of explants that developed GUS-active calli ranged from 0 to 11.1% and 0 to 13.8% for Ne Plus Ultra and Nonpareil respectively (Table 5.3). The maximum values for GUS-active calli per explant were 3.0 and 2.3 for Ne Plus Ultra and Nonpareil respectively (Table 5.3). A summary of the main effects and interactions is provided in Table 5.4. This analysis indicates that genotype and bacterial strain were the major parameters affecting the production of putatively transformed callus.

5.3.4 Production of transgenic callus

For both almond cultivars, cocultivation over a 4-day period with strain EHA105 containing the p35SGUSint plasmid induced the highest level of GUS activity. However, for Nonpareil, maximum GUS activity was only achieved in the presence of 20 μM AS. Using these conditions, 1500 explants each of Ne Plus Ultra and Nonpareil were infected with *A. tumefaciens* and subjected to kanamycin selection. While at least 15% of both Ne Plus Ultra and Nonpareil explants developed calli under selection conditions, no plants were recovered. The elimination of kanamycin from the selection medium after 4 weeks did not induce adventitious bud development.

After an additional 3 months, calli that survived selection were analysed for GUS activity by histochemical testing. All samples returned a positive result (Figure 5.2). To further confirm the presence of the *gusAint* and *npt*II genes in the callus tissue, 5 samples for each cultivar were randomly selected for PCR and Southern blot analysis.

PCR analysis of transgenic callus

The transgenic nature of the calli was confirmed by PCR analysis on DNA extracted from the putatively transformed tissue. The 366-bp and 700-bp fragments expected to be amplified by primers designed for the *gusAint* and *npt*II genes respectively were observed in all five Ne Plus Ultra calli samples (Figure 5.3: lanes 5-9) and the p35SGUSint plasmid DNA used as a positive control (Figure 5.3: lane 3). No amplification was observed for the non-transformed control (Figure 5.3: lane 4). Similar results were observed with Nonpareil (data not shown). An additional PCR, using primers designed for the bacterial *npt*I gene located outside the T-DNA borders, did not amplify a band of the expected size, indicating that bacterial contamination was not present (data not shown).

Southern blot analysis of transgenic callus

Confirmation of T-DNA integration in the calli lines was obtained by Southern blot analysis, using a fragment of the *gusAint* gene as a probe. After digestion of DNA with *Hind*III, hybridisation signals of 2.8 Kb were detected in all samples tested (Figure 5.4: lanes 8-12), corresponding with the internal T-DNA fragment of the *gusAint* cassette. An approximation of the number of inserts in each sample was made after digestion of DNA with *Eco*RI. As the T-DNA of p35SGUSint contains only one recognition site for *Eco*RI (outside of the p35SGUSint sequence), digestion of plant DNA with this enzyme should yield hybridising fragments composed of T-DNA and flanking almond sequences. Hence, the number of bands detected in this manner is expected to equal the minimum number of T-DNA inserts. The calli lines of Ne Plus Ultra had between 2 and 5 bands, and those of the Nonpareil lines between 2 and 3 bands (Figure 5.4: lanes 2-6). The different hybridisation patterns are indicative of random integration, with the variation in band intensity highlighting differences in copy number at the integration sites. The largest

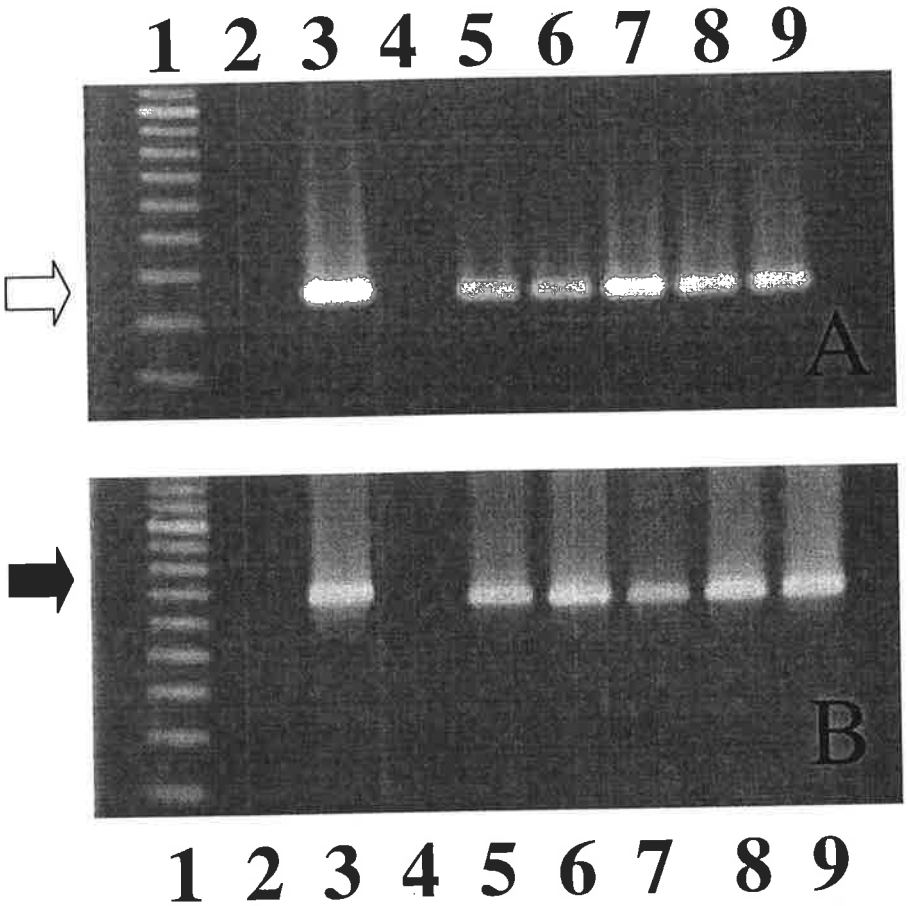


Figure 5.3. Electrophoretic analysis of PCR products from five putative transgenic callus lines of cultivar Ne Plus Ultra with primers for *gusAint* (A) and *npt*II (B) genes. White and black arrowheads point to amplification of the 366-bp and 700-bp fragments of the *gusAint* and *npt*II genes respectively. [lane1, 100-bp DNA ladder; lane 2, no DNA control; lane 3, positive control - p35SGUSint plasmid; lane 4, negative control - non-transformed callus DNA; lanes 5-9, putative transgenic callus lines]

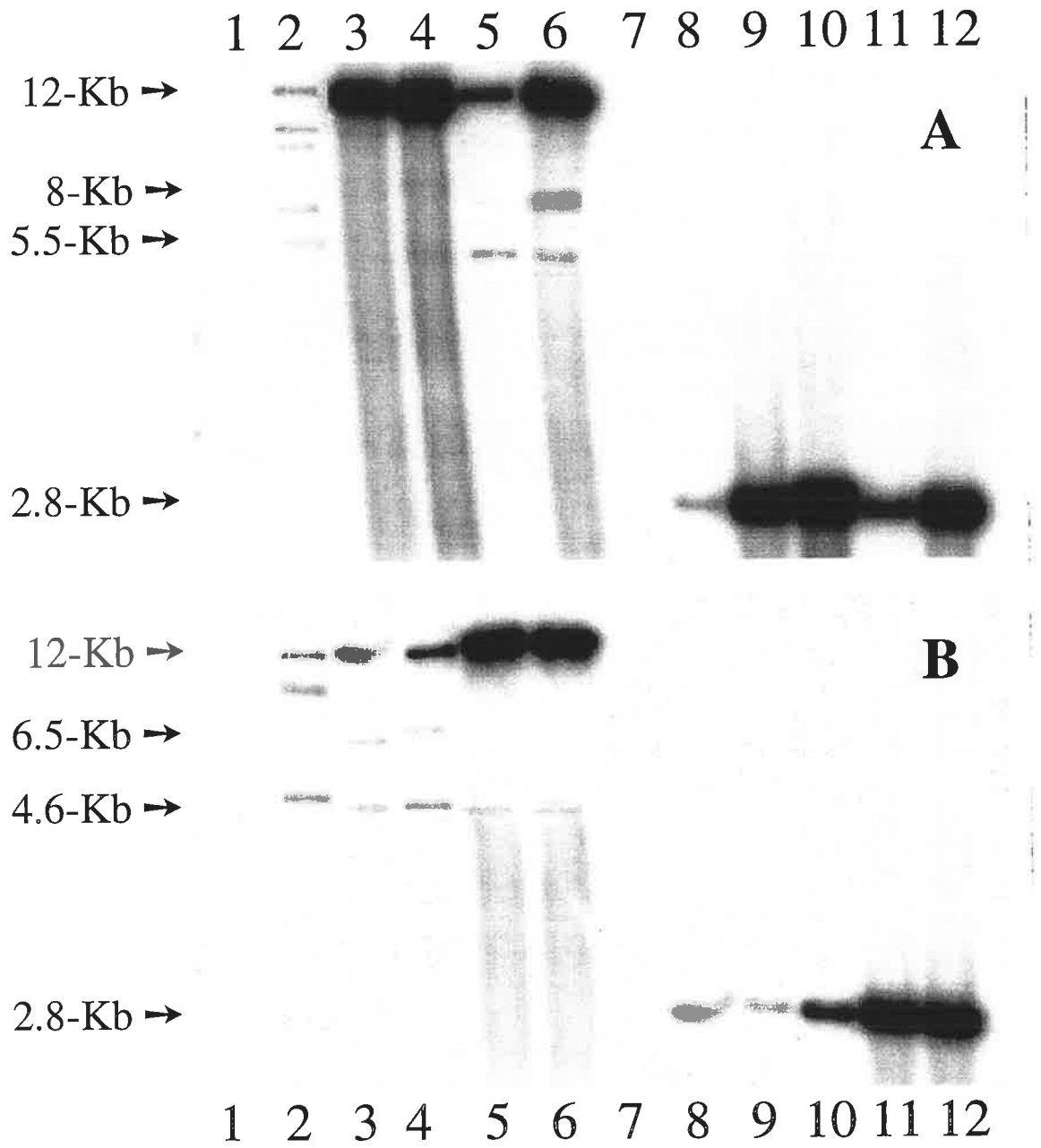


Figure 5.4. Southern blot analysis of Ne Plus Ultra (A) and Nonpareil (B) explants transformed with EHA105 (p35SGUSint). A 366-bp fragment of the *gusAint* gene was used as a probe. [lane 1, total genomic DNA from non-transformed callus (6 μg) digested with *Eco*RI; lanes 2-6, total genomic DNA from putative transgenic callus lines (6 μg) digested with *Eco*RI; lane 7, total genomic DNA from non-transformed callus (6 μg) digested with *Hind*III; lanes 8-12, total genomic DNA from putative transgenic callus lines (6 μg) digested with *Hind*III]

bands were estimated to be 12 Kb (Figure 5.4A: lanes 3,4,6 and Figure 5.4B: lanes 5,6) and most likely represent the insertion of multiple copies of the gene in tandem, as indirect or direct repeats of this nature are expected to give bands that equal combinations of the minimum restriction fragment size (refer to Appendix 3).

5.4 DISCUSSION

This current study reports the successful transfer of two reporter genes to leaf explants taken from mature plants of the almond cultivars, Ne Plus Ultra and Nonpareil.

In this study, explants were precultured on regeneration medium for 3 days prior to inoculation and cocultivation with *Agrobacterium.* This pretransformation step was introduced following reports with other *Prunus* species, where it has been shown that

ts improves transformation frequency and the recovery of transgenic
, 1991; Laimer et al., 1992; Miguel and Oliveira, 1999). Although the
s is not known, it has been proposed that during preculture, leaf tissues
gical and developmental shift to enter morphogenic competency, and
s inserted following this short period, the recipient cells have already
ation pathway (McHughen et al., 1989).

faciens strains tested in this study, cocultivation with EHA105 generally
ls of GUS activity in leaf sections compared to LBA4404. This concurs
t on the almond cultivar Boa Casta, where strain EHA105 yielded up to
active explants when compared with strain LBA4404 (Miguel and
Several other studies have also reported strain EHA105 (or strain

EHA101 from which it is derived) to be the most effective for transformation in woody fruit species (De Bondt et al., 1994; Bell et al., 1999). The effectiveness of this strain is attributed to the hypervirulence of the oncogenic parent strain A281 (Hood et al., 1993). A281 is characterised by a high level of *virG* expression, which produces the product necessary for activation of the inducible virulence genes (De Bondt et al., 1994).

Although cocultivation periods of more than 3 days have been successfully used with woody fruit species (Mourgues et al., 1996), 2-3 days cocultivation is standard for most transformation protocols, as longer periods have frequently resulted in *Agrobacterium* overgrowth (Cervera et al., 1998). Previous studies with almonds have used fixed cocultivation periods of 2 (Archilletti et al., 1995) and 3 (Miguel and Oliveira, 1999) days, and there are no reports on almond of varying the cocultivation time. In the present study, while cocultivation over 5 days provided the optimum transformation efficiency for both almond cultivars, elimination of *Agrobacterium* from leaf tissue was difficult, particularly for explants cocultivated with strain EHA105. Hence, cocultivation over 4 days was preferred. For longer periods, explants could be vacuum infiltrated with a low pH medium followed by short-term exposure to high levels of cefotaxime as described by Hammerschlag et al. (1997). On that study, increasing the acidity of the medium was effective in eliminating *A. tumefaciens* from plant cultures, and vacuum infiltration allowed explants to be treated with high levels of antibiotics for a short term without negative effects on shoot regeneration (Hammerschlag et al., 1997).

Acetosyringone is a phenolic compound produced during wounding of plant cells and in previous studies has been shown to induce transcription of the virulence genes of *A. tumefaciens* (James et al., 1993). The beneficial role of AS has been demonstrated in the

genetic transformation of woody fruit species including apple (James et al., 1993), citrange (Cervera et al., 1998), and kiwifruit (Janssen and Gardner, 1993). In a recent report with the almond genotype, Boa Casta, Miguel and Oliveira (1999) describe the stimulation of *Agrobacterium* virulence on media supplemented with 20 μM AS, but they do not detail its effect on transformation efficiency. In this study, the effect of AS on gene transfer to the cultivars Ne Plus Ultra and Nonpareil was variable, suggesting that its ability to act as a virulence enhancer is dependent on genotype and bacterial strain. These findings concur with those of Godwin et al. (1991) who reported that while the virulence of strains A281 and Ach5 (the wild strains from which EHA105 and LBA4404 were respectively derived) was enhanced by AS, its promotive effect was dependent on the plant species used.

The concentration of AS used in the present study is less than that used in some of the previous studies on plant transformation (Godwin et al., 1991; James et al., 1993; Cervera et al., 1998), and additional experiments testing a range of AS concentrations should be undertaken to determine the optimum level for almond.

In summary, these results demonstrate that *A. tumefaciens* strain EHA105 is more efficient than strain LBA4404 for transformation of the commercially important almond cultivars Nonpareil and Ne Plus Ultra. Results also suggest that cocultivation period and the presence of AS can influence the efficiency of gene transfer. This is, however, dependent on both genotype and the culture environment. The inability to recover transgenic plants in this study highlights the need to improve regeneration from adult tissue.

In the longer term, the development of a transformation system for almond will facilitate the genetic modification of this important nut species for various agronomic traits.

Potential strategies to improve regeneration and the potential implications of genetic modification technology for almond are discussed in Chapter 6.

6. SIGNIFICANCE OF RESEARCH & FUTURE DIRECTION

The objective of this study was to develop a range of *in vitro* techniques that can be applied to the commercially important almond cultivars Ne Plus Ultra and Nonpareil that are grown extensively throughout Australia and the USA. Prior to this study, there were no published reports detailing this type of research with these almond genotypes. Hence, this is the first report detailing protocols for inducing adventitious shoots from adult leaf tissue and juvenile seed cotyledons, the induction of adventitious roots under tissue culture conditions, and the transfer of reporter genes, using disarmed strains of *Agrobacterium tumefaciens*, to these almond genotypes. These techniques are prerequisites to genetic modification research, and without them, this type of technology could not be used in the future for improving almond. A summary of the protocols described in this study is provided in Appendix 4.

The inability to recover transgenic almond plants from adult explants in this study highlights the need for improving the regeneration system used during the transformation process. One possibility would be the use of shoot apices in preference to leaf sections as explants for regenerating adventitious shoots. This approach has been successfully used with apple (Caboni et al., 2000), and preliminary experiments with the almond genotypes Ne Plus Ultra and Nonpareil suggest it may be more efficient than the current (described) protocol (Ainsley, unpublished data). Alternatively, immature seed cotyledons that have proven in the current study to be highly morphogenic could be used in transformation studies. Although regenerated plants would not be true to type, they could be used as parental material in breeding programs, with their desirable traits incorporated by traditional hybridisation techniques. Consideration should also be given to the use of a different selection strategy for transformed tissue, as the selective antibiotic (kanamycin) may well be impeding the regeneration of transgenic shoots. In some apple cultivars where extreme sensitivity to kanamycin prevented antibiotic selection immediately after cocultivation, alternate periods of selection and non-selection, and the application of kanamycin only to regenerated shoots was shown to improve the recovery of transgenic plants (James et al., 1989; Yao et al., 1995). Another consideration is the method for introducing the foreign DNA. Alternative methods including biolistics (Barcelo and Lazzeri, 1998) or chloroplast engineering (Daneill, 2000) may also be suitable with almond, and along with the above-mentioned parameters, should be tested in future studies with Ne Plus Ultra and Nonpareil.

Additional research is also required to test the effectiveness of novel selection strategies. These include the phosphomannose-isomerase (Negrotto et al., 2000; Wang et al., 2000) or xylose-isomerase (Haldrup et al., 1998) systems. Both are independent of antibiotic or

herbicide resistance genes, providing transgenic cells with the ability to utilise alternative carbon sources. The use of these types of selection systems will be more readily accepted by public/consumers as compared with the current antibiotic systems that have brought about much contention and debate.

To date, most transformation studies with perennial fruit species have focused on the use of marker and reporter genes to optimise conditions for DNA transfer. The emphasis of this research is now shifting towards the introduction of desirable traits such as those conferring resistance/tolerance to biotic and/or abiotic stresses, improved yield, and the regulation of growth or fruit ripening (Schuerman and Dandekar, 1991; Scorza, 1991). Following optimisation of the protocols described in the current study, similar research can begin with almond.

For almond, one of the most promising applications for genetic transformation is the regulation of self-incompatibility (SI). Almond displays a form of homomorphic gametophytic SI, with determination governed by a single multi-allelic gene (Socias i Company and Felipe, 1988). Cultivation, therefore, requires planting of at least two cross-compatible cultivars that flower at the same time and the distribution of beehives throughout the orchard to ensure there are enough pollinating agents. At harvest, it is necessary to collect different cultivars separately due to differences in maturation and nut quality (Dicenta and Garcia, 1993). These factors have a large impact on the profitability of the crop, and hence, the production of self-compatible cultivars is a priority in almond improvement programs throughout the world.

In other species, including *Nicotiana alata*, *N. tabacum*, and *Petunia inflata*, where similar SI systems exist, experiments have shown that it is possible to overcome SI using an antisense RNA strategy to down-regulate the genes involved (Lee et al., 1994; Murfett et al., 1995; Murfett and McClure, 1998). This strategy is based on the introduction of small sequences of RNA (known as 'antisense RNA') that are complementary to the messenger RNA (mRNA) of the target gene. Expression of the endogenous gene is apparently inhibited by the formation of a duplex between its mRNA and the antisense RNA, which in turn blocks the availability of mRNA for translation (Bird and Ray, 1991). Similar experiments are currently being tested with apple (Broothaerts et al., personal communication), and in the future may provide an avenue for overcoming SI in almond.

Other possibilities for the genetic modification of almond include the introduction of virus resistance (ie. resistance to Prune Dwarf Virus or Prunus Necrotic Ringspot Virus), resistance/tolerance to biotic and abiotic stress (ie. nematode resistance, salinity tolerance), modification of nut quality, and alteration of tree architecture. In all instances, it would be necessary to identify the gene(s) that confer these modifications. Where the DNA sequence is an unknown factor, this would involve the construction and screening of genomic and/or cDNA libraries for appropriate sequences, or in instances where similar modifications have been tested in other species, gene sequences could be constructed based on previously reported gene codes. Transformation constructs for introduction into *Agrobacterium* would then be made. This would involve linking the gene sequences with a range of both constitutive and tissue specific promoters (the latter to facilitate targeting of transgene expression). Experiments would then be conducted to test the effectiveness of these constructs, determine the specificity of promoters and the effect of the introduced

gene(s) at both the genetic and phenotypic level. Underlying these experiments would be the *in vitro* techniques developed in the current study.

Whilst the application of genetic transformation technology to almond is not a short-term answer to improving this species, it provides a more rapid approach to the current breeding techniques. Genetic transformation technology also affords the opportunity to introduce genes outside the gene pool currently being used for almond improvement, irrespective of taxonomic barriers. It should be stressed however, that these techniques are not intended to replace current traditional hybridisation programs, but to complement them, and provide a multi-facetted approach in the pursuit of developing improved almond cultivars.

Appendix 1: Composition of Plant Tissue Culture Media

COMPONENT	AP	MS	QL
	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})
MACROSALTS			
NH_4NO_3	-	1650.00	400.00
$(NH_4)_2SO_4$	270.00	-	-
KNO_3	2500.00	1900.00	1800.00
$CaCl_2.2H_2O$	150.00	440.00	-
$Ca(NO_3)_2.4H_2O$	-	-	1200.00
$NaH_2PO_4.H_2O$	150.00	-	-
KH_2PO_4	-	170.00	270.00
$MgSO_4.7H_2O$	190.00	370.00	-
MICROSALTS			
$MnSO_4.H_2O$	20.00	16.90	0.76
H_3BO_3	4.50	6.20	6.20
$ZnSO_4.7H_2O$	2.00	8.60	8.60
KI	0.75	0.80	0.08
$Na_2MoO_4.2H_2O$	0.06	0.25	0.25
$CuSO_4.5H_2O$	0.05	0.025	0.025
$CoCl_2.6H_2O$	0.03	0.025	0.025
IRON SOLUTION			
Na_2EDTA	37.20	37.30	37.20
$FeSO_4.7H_2O$	28.00	27.80	27.80
VITAMINS			
myo-Inositol	25.00	100.00	100.00
Pyridoxine-HCl	2.00	0.50	0.5
Thiamine-HCl	-	0.10	0.4
Glycine	-	2.00	-
Nicotinic Acid	-	0.50	0.50

AP - (Almehdi and Parfitt, 1986)
MS - (Murashige and Skoog, 1962)
QL - (Quoirin and Lepoivre, 1977)

Appendix 2: Plant Growth Regulator & Antibiotic Conversion Table

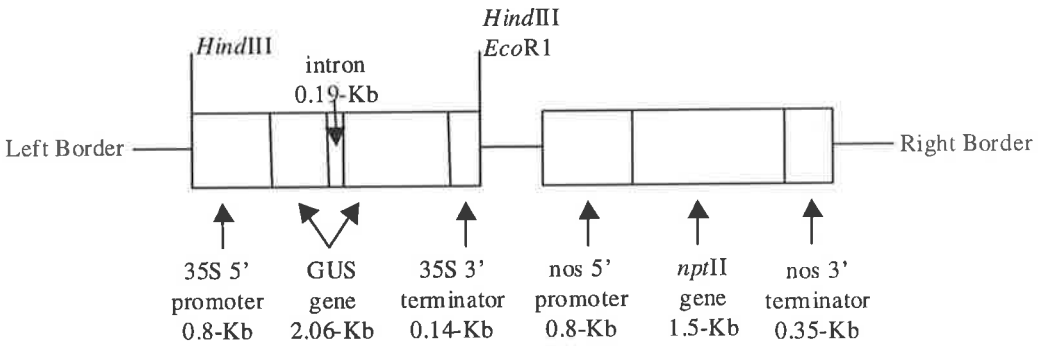
Plant Growth Regulators:

BAP		IBA		NAA		PG		TDZ		2,4-D	
µM	$mg.L^{-1}$	µM	$mg.L^{-1}$	µM	$mg.L^{-1}$	µM	$mg.L^{-1}$	µM	$mg.L^{-1}$	µM	$mg.L^{-1}$
3.1	0.7	0.05	0.01	0.5	0.1	1.0	0.13	0.1	0.02	0.5	0.1
4.4	1.0	0.5	0.1	2.5	0.5	10.0	1.3	1.0	0.2	4.5	1.0
11.1	2.5	2.5	0.5	5.4	1.0	100.0	13.0	2.3	0.5	9.0	2.0
22.2	5.0	5.0	1.0	10.7	2.0			4.5	1.0	22.6	5.0
33.3	7.5	10.0	2.0	26.9	5.0			6.8	1.5		
44.4	10.0	24.6	5.0	1000.0	186.2			9.1	2.0		
		500.0	101.6	5000.0	931.0			10.0	2.2		
		1000.0	203.2					20.0	4.4		
		5000.0	1016.0					22.7	5.0		

Antibiotics:

Cefotaxime		Kanamycin		Streptomycin	
µM	$mg.L^{-1}$	µM	$mg.L^{-1}$	µM	$mg.L^{-1}$
630.0	300.8	9.0	5.2	170.0	248.0
1000.0	477.4	17.0	9.9		
		25.0	14.6		
		34.0	19.8		
		68.0	39.6		
		85.0	49.5		
		103.0	60.0		
		137.0	79.8		
		172.0	100.2		

Appendix 3: Schematic Partial Map of p35SGUSint Plasmid



Appendix 4: Tissue Culture & Genetic Transformation Protocols for Ne Plus Ultra & Nonpareil

Summary details of the described protocols:

Adventitious shoot regeneration from adult leaf tissue:

Cultivar - Ne Plus Ultra:

AP basal medium + 9.8 μM IBA + 22.7 μM TDZ + CH (0.1% w/v).

Cultivar - Nonpareil:

AP basal medium + 9.8 μM IBA + 6.8 μM TDZ + CH (0.1% w/v).

(For both cultivars, 3 weeks in dark conditions followed by 5 weeks in the light.)

Adventitious shoot regeneration from juvenile cotyledon tissue:

Cultivars - Ne Plus Ultra, Nonpareil, Carmel and Parkinson:

MS basal medium + 10.0 μM TDZ (8 weeks).

MS basal medium containing no plant growth regulators (4 weeks).

(For all cultivars, 1 week dark followed by 11 weeks in the light.)

In vitro root formation:

Cultivar - Ne Plus Ultra:

Water-agar (0.6 % w/v) + 1.0 mM IBA (12 h).

½-strength MS salts + 100.0 μM PG (2 weeks).

½-strength MS salts containing no plant growth regulators or rooting cofactors (2 weeks).

Cultivar - Nonpareil:

As for Ne Plus Ultra but using full-strength AP basal salts.

(For both cultivars, shoots kept in dark for the first 3 days.)

Agrobacterium-mediated genetic transformation:

Cultivar - Ne Plus Ultra:

4 days cocultivation with *A. tumefaciens* strain EHA105.

Cultivar - Nonpareil:

As for Ne Plus Ultra, but using a medium supplemented with 20 μM AS.

(For both cultivars, a 3-day preculture period is necessary. Elimination of bacteria is achieved using cefotaxime (630 μM) and selection of transformed tissue on medium containing kanamycin (25 μM).)

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