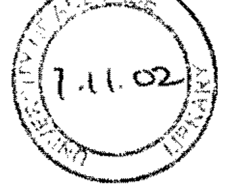

ALMOND IMPROVEMENT VIA MICROPROPAGATION, CRYOPRESERVATION, AND *S*-ALLELE IDENTIFICATION

Chockpisit Channuntapipat

B.Sc. (Botany), M.Sc. (Agriculture)

Submitted in fulfilment of the requirement for the degree of

Doctor of Philosophy

Department of Horticulture, Viticulture and Oenology

Waite Agricultural Research Institute

Adelaide University

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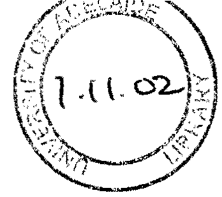
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ABSTRACT

The Australian almond improvement program was initiated in 1997 to develop improved cultivars that are adapted to local conditions and consumer demands. The program combines molecular techniques along with the traditional approach of controlled hybridisation with mass selection. This research project was carried out to assist the Australian almond improvement program in the areas of micropropagation, cryopreservation, and rapid identification of self-incompatibility genotypes of almond.

Micropropagation was accomplished successfully for two commercially important almond cultivars ('Nonpareil' 15-1 and 'Ne Plus Ultra') and an almond/peach hybrid rootstock by culturing shoot tips, about 0.7 cm long with 3 – 5 leaves, on appropriate shoot multiplication media. For 'Nonpareil' 15-1, AP medium with 0.049 μM IBA, 3 μ M BAP, 0.058 M sucrose, and 0.7% agar at pH 5.7 was effective. MS medium with 0.049 μM IBA, 5 μM BAP, 0.088 M sucrose, and 0.7% agar at pH 5.7 was suitable for 'Ne Plus Ultra'. For the almond/peach hybrid rootstock, MS medium supplemented with 10 μM BAP, 0.088 M sucrose, and 0.7% agar provided the best shoot proliferation. Shoots of the rootstock, about two cm long, readily produced roots after one week in the dark and two weeks in the light on half strength MS medium supplemented with 2.4 μM IBA, 0.088 M sucrose and 0.7% agar at pH 5.7, with 88.0% rooting efficiency. The two almond cultivars did not readily produce roots, but, at about 1.5 cm long, were micrografted successfully onto the rootstock. These micrografted plantlets were acclimatised and transferred to potting mix with 92% survival.

Shoot tips of the two almond cultivars and the almond/peach hybrid rootstock were cryopreserved successfully using a one-step vitrification technique. Three-week-old *in vitro* cultures were cold-hardened at 4°C on multiplication media (Murashige and Skoog for 'Ne Plus Ultra' and the hybrid rootstock; Almehdi and Parfitt for 'Nonpareil' 15-1) for three weeks. Shoot tips, 2 – 2.5 mm long, were excised and precultured for

one day at 4°C on the same basal medium, without plant growth regulators, supplemented with 0.7 M sucrose. After the preculture, the shoot tips were incubated in vitrification solution at 25°C for 45 min for the almond cultivars and 60 min for the almond/peach hybrid rootstock, and then stored under liquid nitrogen (LN) for up to 24 months. After rapid thawing at 30°C, the shoot tips were washed with the appropriate liquid basal medium containing 1.0 M sucrose and then cultured on the same basal medium, solidified with agar, but excluding NH_4NO_3 or $(NH_4)_2SO_4$. Shoot regeneration was usually observed within 2 – 3 weeks. Survival of shoots after thawing varied from 56-80% for 'Ne Plus Ultra', 35 – 53% for 'Nonpareil' 15-1, and 62 – 82% for the almond/peach hybrid rootstock. Non-vitrified shoots that were stored on basal medium at 3.5 – 5°C showed good survival up to six months, but thereafter survival decreased rapidly. Cryopreservation has considerable potential for long-term storage of almond germplasm, but future research should be aimed at improving the regeneration of 'Nonpareil' 15-1, the most important commercial cultivar grown in Australia.

The genetic stability of almond DNA to both *in vitro* culture and the cryopreservation process was evaluated by comparing the fingerprints of the DNA from the original orchard trees, from the *in vitro* cultures before and after cryopreservation for up to 24 months, and from plants regenerated from *in vitro* cultures. The fingerprints were prepared by initially digesting the DNA with two isoschizomer pairs of restriction enzymes, one of each pair being 'methylation sensitive' and the other 'methylation insensitive', followed by amplification of the digested products using randomly amplified polymorphic DNA (RAPD) with six different 10-mer primers. Changes in methylation were found between the original orchard trees and *in vitro* cultures, and there was also the possibility that some structural changes may have occurred. However, no methylation or structural changes could be attributed to the cryopreservation procedure. Plants regenerated from the *in vitro* cultures before and after cryopreservation should be monitored carefully in the future for changes in morphology compared to the original trees.

Partial genomic and cDNA sequences of the self-incompatibility alleles *S1*, *S2*, *S7*, *S8*, *S9*, *S10*, *S23*, and *Sf* were obtained from *Prunus dulcis* cvs 'Anxaneta' (*S2S9*),

'Cristomorto' (*S1S2*), 'Ferragnes' (*S1S3*), 'Gabaix' (*S5S10*), 'Ne Plus Ultra' (*S1S7*), 'Nonpareil' 15-1 (*S7S8*), 'Primorskiy' (*S5S9*), 'Ramilette' (*S6S23*), and IRTA Selection 12-2 (*SfSf*). Total DNA was extracted from leaves, and cDNA was prepared from total RNA extracted from styles. The partial cDNA sequences of the *S1* allele from 'Ferragnes', and the *S7* and *S8* alleles from 'Nonpareil' 15-1 matched those reported in the literature for the alleles *Sb*, *Sc*, and *Sd* respectively. The sequences of the *S1*, *S2*, *S7*, *S8*, *S9*, *S10*, *S23*, and *Sf* alleles found in genomic DNA contained introns of 562, 253, 1,530, 2,208, 1,343, 710, 494, and 662 bp respectively, and partial exons of 510, 537, 489, 498, 486, 495, 489, and 543 bp respectively. In addition, one allele of the Australian cultivars, 'Johnston's Prolific' and 'Pierce', was identified and found to have the same sequence as *S23* in 'Ramilette', suggesting that this cultivar may have been an early introduction to Australia from Spain. The exon/intron splice junction sites of all alleles followed the GT/AG consensus sequence rule, and the sequences were found to be highly conserved.

Both the length and the sequence of each intron was unique, and a technique of identifying the *S*-alleles of almond was developed based on primers that targetted the intron sequences. The use of these primers has increased the speed, precision, and efficiency with which the incompatilibity genotypes of almond cultivars can be detected, compared to other published techniques. The primers confirmed the *S*-allele specificities for 26 out of 30 cultivars for which published information is available, and are currently in use in the Australian almond improvement program to identify incompatibility groups in the breeding progeny. Future work should be directed towards obtaining the sequences of the introns for the remaining known *S*-alleles, *S3* to *S6*, and *S11* to *S22*.