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Olive Oil
Yield, Quality and Cultivar Identification

A report for the Rural Industries Research and Development Corporation

by Kevin Robards and Rod Mailer

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Foreword

Olives were introduced to the Colony of New South Wales on 28th December 1800 by George Suttor, a London market gardener. Production never progressed beyond the cottage industry phase. Demand for olive products has increased dramatically in recent times due to higher incomes and interest in a healthy lifestyle. However, the demand is currently met by imports.

Siting of the Wagga Wagga Agricultural Institute (WWAI) and Charles Sturt University (CSU) with an Olive Grove on CSU land was an integral feature of this project. Both agencies have been actively involved with the olive industry in the provision of technical advice to growers and use of the Olive Grove as a training facility. For example, during 1996 a workshop was organised under the auspices of the Australian Olive Association (AOA) at CSU. The workshop included sessions on horticulture, sensory evaluation and production of olives and olive oil. The lack of appreciation of oil quality and flavour was identified as a key issue confronting the Australian olive industry.

Although olive trees have been harvested in Australia for long periods, little is known in regard to quality factors such as fatty acid profiles and oil contents. This type of information is considered vital in oilseed crops such as canola and sunflower where programs have been established to alter fatty acid profiles to obtain the most nutritionally desirable proportions. To achieve this, canola and high oleic sunflower have mimicked the traditional profiles of olive oil. Measurement of oil quality is essential to quality control and to cultivar selection in breeding programs.

There is currently much confusion in the identification of olive cultivars. The need to identify cultivars is considered a major requirement due to the longevity of the crop and the need to improve efficiency in growing and extraction of olive oil. It is crucial that cultivars be identified using techniques such as DNA fingerprinting. The Wagga Wagga grove has cultural and economic importance as a source of genetic material. Cultivars which show superior quality and agronomic suitability can be selected and propagated for Australian conditions.

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Executive Summary

The Wagga Wagga Olive Grove
The Wagga Wagga Olive Grove (WWOG), situated on the campus of Charles Sturt University, provides a unique opportunity to study a large range of olive cultivars from established trees. The grove is Australia's largest gene pool of mature trees representing many of the cultivars currently being grown by Australian industry. This collection is supported by a similarly aged grove at Yanco consisting of four additional cultivars. This study was important to industry as many of Australia's orchards contain only young trees that lack information on the quality parameters presented in this report. Importantly, many of the trees growing in commercial orchards have been developed from cuttings taken from the WWOG.

The grove contains several trees identified by cultivar on a map developed by the Wagga Wagga Agricultural College at the time of planting in 1895. Since the map was drawn, several trees have been removed. Over the years, many of the trees, through neglect, have been overgrown by feral trees originating from seeds dropped by birds, or by suckers growing from the root stock of the original tree. The first objective of this project therefore was to verify the identity of each tree before carrying out studies on the quality parameters.

DNA identification
DNA identification has been used at the Wagga Wagga Agricultural Institute for several years, particularly in the canola-breeding program for the registration of new cultivars and discrimination from existing cultivars. The Institute has therefore developed some expertise in the practical application of the technique. A major factor in RAPD analysis (randomly amplified polymorphic DNA) is the selection of suitable polymorphic primers which can highlight differences between closely related cultivars. This was particularly important in the Wagga grove where tree identities were vague and cuttings have been used to generate many of the current olive orchards around Australia.

Variation from the given name: The WWOG consists of 93 trees composed of 54 cultivars. Many cultivars have only one representative whereas others have several. The first significant finding was that many of the trees did not agree with the map identification. In particular, missing trees on one row had altered all tree numbers in that row. DNA allowed us to rename those trees to the correct cultivar. Some trees did not match any of the available DNA patterns and are considered to be either feral trees or grown from rootstock of the original planting.

Variation within a cultivar: Where there was more than one tree of a particular cultivar, there were often variations within the DNA pattern. Most obvious was the Manzanilla group. Using cluster analysis for all of the trees, all of the Manzanilla trees clustered within a group but all showed at least some variation from each other. Phenotypic expression agreed with the DNA patterns with fruit pit shape showing a range of shapes. Only three of the Manzanillas grouped closely with the common *Manzanilla de Sevilla* type with the others showing various levels of similarity. This raised some concern that there may be variability within individual trees. In fact, some isolated trees did have more than two cultivars within the tree although analysis of branches within several trees showed them to be uniform.

Pera Bore (three trees from Pera Bore) also showed close similarity for DNA fingerprints although the fruit shape of one tree was very dissimilar to the other two. This illustrates the limitations of DNA testing as the RAPD patterns only show similarity for a limited part of the DNA and this does not necessarily reflect characteristics such as oil content or yield.

Identification within the WWOG: Cultivar identification in the WWOG was based on comparison of DNA from reliable sources where possible. Some DNA was obtained from Cordoba, Spain. Other comparisons were made with samples from Olives Australia who had obtained DNA verification in overseas laboratories. In many instances, where several trees of an individual cultivar were present

within the orchard and DNA patterns for those trees were closely related, the identity was accepted as that shown on the original map. Many single trees of unique cultivars exist within the orchard which could not be verified and have been named as shown on the map. Some of these were named after people or places (Dr Fiiaschi, HAC - Hawkesbury Agricultural College, Pera Bore) and are likely to be unique to this collection.

As a result of the study, many of the trees have been identified and their relatedness illustrated in cluster analysis. A selection of cultivars, where identities had been verified by international comparisons, or other means have been used as standards for commercial testing and these standards are illustrated on a dendrogram within the report. A few trees could not be identified and have been labelled as unknown. All of the trees at the WWOG and several from Yanco have been analysed by cluster analysis and dendrograms have been constructed to illustrate genetic similarities.

Olive quality characteristics

Olive quality parameters have been studied for all of the cultivars over three years to develop an understanding of cultivar and seasonal variation. The Yanco site was included in addition to the WWOG to determine site affects. The major parameters of interest to growers include oil yield and quality factors that are used to grade olive oil as extra virgin or other. Other factors that contribute to oil characteristics were studied including phenol content, moisture and fatty acid profiles.

Oil content and fatty acids were analysed at four harvest dates during maturation. Additionally samples were taken from selected trees on a fortnightly basis to study the development of these components. The oil content, as expected, increased in a sigmoidal fashion, with a slow and continuous increase toward the end of maturity. Comparisons between all of the cultivars are made within the report. The point at which the rapid increase in oil levels out provides a good indicator for determining optimum time of harvest.

More unexpected was the development of fatty acid profiles in which linolenic acid, a minor fatty acid in olives, was as high as 30% at early fruit development. After 10 weeks there was a rapid decline in linolenic and increase in the desirable oleic acid. These findings are also valuable in determining acceptable times of harvest to ensure fatty acids are within IOOC limits for extra virgin olive oil.

Oleic acid is a monounsaturated fatty acid and considered to be nutritionally beneficial. The report compares cultivars and indicates that although some oils have relatively low levels, others are more acceptable. Additionally, palmitic acid, which is a saturated fat and considered undesirable, shows considerable variation between the cultivars.

The range of phenolic compounds was measured in three cultivars and oleuropein found to be the dominant phenol. This component decreased with fruit maturity whereas other phenols increased. The range and proportions of the phenols were significantly different for each of the cultivars and indicates that actual phenol components are more important than the total polyphenol content generally used to evaluate olive oils.

The WWOG has several deficiencies in terms of scientific research as many of the trees have been neglected and are in poor health. Due to road development, erosion and lack of pruning and irrigation, the trees are difficult to compare for yield or oil content. However the orchard continues to be a valuable genetic resource for the olive industry. It has provided an opportunity to compare fatty acid profiles of a wide range of cultivars that can help in future selection of nutritionally superior olive oil. The DNA extract from these trees has also provided valuable cultivar fingerprints for many cultivars not readily available elsewhere. Renewed interest and ongoing improvements to the orchard will provide a valuable gene pool for many years in the future.

1. Introduction

The current interest in olive production and processing across Australia has been stimulated by an increase in the value of imported olive products into Australia from $30 million in 1988 to $110 million in 1997. Rising imports and domestic consumption have encouraged new plantings of olive trees in Australia (McEvoy & Gomez, 1999). Nevertheless, olive production (and consumption) remains highly concentrated in the Mediterranean region where it generates regional employment and is an important source of income in rural areas, thereby reducing migration to urban areas. It also provides ecological benefits by contributing to soil retention and reducing erosion. As stated often in the past and lately by Fausto Luchetti: "The olive tree, in particular, goes beyond the bounds of the purely economic and encompasses and embodies the other aspects mentioned. Consolidating and defending its survival equates with consolidating and defending our own cultural identity, what in English is so neatly summed up as our way of life."

World production and consumption of olive oil and table olives reached record levels in 1996-97 but production exceeded consumption. The ensuing surplus makes it necessary for the sector to heighten its competitiveness (de los Angeles Blanco Sandia and Alvarez Martinez, 1997). Although consumption of olive products in most olive producing-countries has increased very slowly over the last decade, and in some cases even decreased, consumption in new markets such as the United States, Canada, Australia and particularly Japan has risen much faster (see Tables 1 and 2).

Table 1 : Trend in Consumption of Olive Oils, 1990-91 to 1997-98 (tonnes x 1000)

Country	1990-91	1991-92	1992-93	1993-94	1994-95	1995-96	1996-97 (prov.)	1997-98 (est.)
Italy	540.0	630.0	640.0	600.0	600.0	600.0	620.0	620.0
Spain	394.1	418.7	421.4	421.0	409.2	331.9	470.0	470.0
Greece	204.0	203.0	197.0	196.0	197.0	177.0	180.0	200.0
USA	88.0	79.0	104.0	111.5	115.5	101.0	134.5	150.0
Portugal	27.0	45.0	49.9	59.0	58.0	58.4	61.5	62.0
Japan	4.0	4.5	5.0	6.5	8.5	16.5	25.0	35.0
Canada	10.0	10.0	13.0	12.0	15.0	14.0	20.0	23.5
Australia	13.5	12.5	16.0	16.5	19.0	15.5	18.0	23.0

Source: IOOC.

Australian producers must be aware of this background and recognise that the olive crop extends beyond the economic to having significant social implications. Moreover, olive oil, and table olives to a lesser extent, are traded as a commodity in world markets, and are therefore highly dependent on world production and consumption levels. The Australian market for olive products is expanding as per capita consumption increases but domestic production will increase substantially as large numbers of new plantings reach bearing age. The magnitude of future production is important to the viability of the local industry.

Table 2 : Per Capita Consumption of Table Olives, 1993-1997 (kg)					
Country	1993	1994	1995	1996 (prov.)	1997 (est.)
Syria	5.56	5.42	5.07	5.49	5.02
Spain	3.04	2.98	2.93	2.52	2.77
Greece	2.03	2.88	2.87	1.91	2.38
Italy	2.27	1.98	2.19	1.87	2.27
Turkey	1.51	2.25	2.12	2.46	2.12
Morocco	1.43	1.31	1.09	1.07	1.05
USA	0.61	0.62	0.67	0.59	0.60
Australia	0.51	0.57	0.53	0.55	0.60
Canada	0.47	0.55	0.49	0.57	0.57
Japan	0	0	0.01	0.02	0.02

Future domestic production of olive oil (the major commodity) will clearly depend on the levels of yield per tree and oil content. The available data are widely divergent with a minimum yield per tree of 12 kg based on the average for Mediterranean countries (Weiying, Mingquan and Ning 1998), and a maximum yield per tree of 70 kg, based on values provided by local nurseries (Olives Australia 1997). Olive oil content has been quoted at 20% by domestic nurseries supplying olive trees (Olives Australia 1997) but was 13.5% from a survey of olive producers in South Australia and Victoria (McEvoy et al. 1998). This project addresses both aspects of production.

The success of the Australian industry will require significant penetration of the domestic market (McEvoy and Gomez, 1999) which is dominated by imports (mainly from Spain and Italy). This represents a challenge for Australian producers who will need to develop production, processing and marketing strategies to deliver the desired outcome. The report by McEvoy and Gomez has identified a target group and "consumers meeting the 'target profile' have a strict notion of quality". Moreover, quality and health aspects are important considerations in influencing consumers to purchase olive oil.

As stated by McEvoy and Gomez: "The qualitative research found that consumers who buy olive oil because of its health benefits later become accustomed to its flavour. This is of relevance to the Australian industry, as it implies that it is possible to educate consumers into developing a taste for good quality oil. The qualitative research also indicated that although health benefits were perceived as important by consumers, they could not explain why this was the case. However, the fact that consumers perceive olive oil as 'healthy' provides the base for a promotional campaign emphasising such benefits and possibly explaining the reasons for them".

The requirements of a target population for a product are important when considering quality and the ability to deliver a quality product. Also there is no future in marketing olive products at a cost which is unacceptable to the consumer. From the perspective of the grower/processor, the oil content of the fruit represents a major quality consideration. On the other hand, the fatty acid profile and phenolic content are important in relation to nutritional quality. Apart from its use as an edible oil, olive oil can be used as an ingredient in cosmetic products or used for medicinal purposes. *Producers must be encouraged to examine new and novel uses.*

Given the anticipated level of local production, producers also need to consider potential export markets, especially in the Asia - Pacific region where Japan represents the most promising market. Japan has traditionally imported low volumes of olive oil but these have grown at a rapidly increasing rate. A number of factors have contributed to the increasing use of olive oil in Japan but an important factor affecting consumption is the health aspects of olive oil. Japanese consumers have become increasingly health conscious with growing concerns about health and food safety (USDA 1994). The role played by the IOOC in promoting the health benefits of olive oil in Japan should not be underestimated. A recent survey found that olive oil users like to cook, are more careful about

their health and are prepared to pay extra for quality cooking ingredients (McEvoy and Gomez, 1999).

The potential exists therefore to increase local cultivation and production for both domestic and export markets although the olive oil industry faces some key issues that could hinder this development. As stated by Boskou (1996): "Olive oil has a unique position among edible oils because of its delicate flavor, stability and health benefits." However, oil flavours are lost due to handling methods and oxidation occurs with primitive equipment and off-flavours develop due to a lack of application of appropriate technology (see, for example, Beer, 1996/97).

Characterisation of olive oil can be an extremely difficult task because of the variation in composition caused by extraction methods, and environmental and storage conditions and can be further complicated by the presence of adulterants. The quality of olive oil is a result of both chemical and sensorial properties, and therefore, is ultimately determined by the consumer. Olive oil is assessed through analytical means designed to quantify certain chemical components deemed essential to oil quality. Presently, the analytical methods employed for qualitative purposes are time consuming and complex. Current research is therefore aimed at providing rapid analyses that preclude the need for extensive sample preparation, and are suited to online control.

This project examined a number of aspects of olive quality but financial constraints have not allowed an examination of the full range of parameters but rather those selected as most critical early indicators in discussions between the Chief Investigators and Michael Burr.

2. Objectives

This project, which was developed in consultation with the olive industry, addresses the following:

- To develop family trees (relatedness) for selected olive cultivars by developing DNA identification procedures and producing a database of DNA fingerprints to meet Objective 2. This data is necessary to ensure that future projects identify cultivars correctly and comparisons of data are on the same cultivars.
- To develop a database on Australian olives which may be used to indicate best time to harvest the olives, the variability of individual cultivars between sites and the variability in quality between select cultivars by measuring quality parameters such as oil content, fatty acids, polyphenols, etc. based on IOOC standards.

3. Methodology

3.1 Olive Trees

Trees from the CSU grove were selected because of its historical and cultural significance and their use in propagation. There are currently 93 trees in this collection. A second grove at Yanco was also examined which contains several trees of each of a limited number of well characterised cultivars. This grove comprises 6 blocks of 35 trees with four cultivars, namely, Hardy's Mammoth, Corregiola, Sevillano and Verdale.

RIRDC provided funds for evaluation of olive cultivars growing in the olive grove on CSU campus and the NSW Agriculture grove at Yanco. The funds provided were used to employ a Technical Assistant to harvest and chemically analyse fruit from the large number of trees, incorporating a wide range of cultivars.

In addition to this work, NSW Agriculture has carried out extensive DNA analysis on the trees to confirm the cultivar identities and investigate genetic differences. NSW Agriculture provided the funds for this analysis.

3.2 DNA Testing of Olive Cultivars.

Leaves were harvested from each of 93 trees within the Wagga Wagga and 35 trees at Yanco orchard during December 1998. At this time, the trees had young, light green leaves that have been shown to yield the best quality DNA. Additionally, DNA was obtained from various sources including Olives Australia P/L and the olive gene bank in Cordoba, Spain. Some samples were obtained from Waite Institute but these had not been verified and represented different cultivars to those at Wagga Wagga.

Two Hardy's Mammoth and two Corregiola trees in the Yanco grove were sampled from four branches around the tree to look at any variability within a tree.

3.2.1 RAPD Amplification

Three commercial sets of 100 x 10 bp oligonucleotide primers were obtained from University of British Columbia. These were screened against olive DNA to select six primers, which produced maximum polymorphism and clear repeatable band patterns. Although the initial study showed these to give excellent results, two primers from Operon Techniologies (Alameda CA USA) were also included to allow comparison with other laboratories within Australia using Operon primers. The six primers selected for the study, based on their usefulness included UBC519, UBC521, UBC563, UBC574 and two Operon OPA5 and OPZ13. The RAPD reactions and DNA sequence amplification procedures were as described by Mailer et al. (1997).

3.2.2 Data Analysis

Electrophoresis gels were photographed using a Compuscope CCD 800 / 1600 digital camera and the images stored as TIF files. The number and size of DNA fragments within each sample were determined for qualitative assessment. Cluster analysis was used to illustrate similarity among samples using the program NT-SYS.

3.3 Oil content and composition, fruit yield

Olives were harvested from the groves in Wagga Wagga and Yanco at four stages of development during three seasons. These stages were;
(1) prior to stone hardening (6-12 January 1998 only),
(2) post stone hardening (24-27 February 1998, 24 February-1 March 1999),
(3) at colour change (6-14 April 1998, 5th-11 May 1999, 23-25 May 2000) and
(4) at full colour development (i.e. black) (15-22 June 1998, 8-14 July 1999, 7-10 August 2000).

At each stage, the fruit was analysed for oil content, fatty acid composition and flesh to pit ratio. Fruit from four trees in the Wagga Wagga grove were randomly selected for a more detailed study, harvesting the fruit weekly to follow the development of oil content, fatty acid composition and flesh to pit ratio. The fresh fruit weight was also determined by weighing 10 olives and calculating the average weight per olive. The time of harvest was measured in weeks after flowering (WAF). Flowering date was estimated when 50% of the flowers on the tree were fully open.

3.3.1 Oil Content

The seed was removed from the olive using a scalpel, and the olive flesh and seed were dried at 80°C for 24 hours in a draft forced oven prior to oil extraction. These conditions were determined as optimum for drying without damage to oil quality**.** The dry weight of the olives was recorded and they were stored in a desiccator until further analysis. The flesh was ground for approximately 30 seconds using a Sorvall Omni-mixer and oil was extracted from the powder using Butt tube (Goldfisch) extraction apparatus for 16 hours (overnight) using cellulose extraction thimbles.

3.3.2 Fatty Acids

Oil (100mg) was mixed with petroleum spirit (3mL b.p. 40-60 °C) in a small test tube. Sodium methoxide (0.5mL, 1.15% sodium in methanol solution; 0.5M) was added, and mixed for 15 seconds. The sample was allowed stand for 10 minutes and bromothymol blue (0.1 mL; 0.1% w/v in methanol) was added followed by hydrochloric acid (0.4 ml; 1 M). Sodium carbonate (0.6 mL, 1.5%) was added and the solution mixed thoroughly. Distilled water was added to bring the solvent layer to the top of the test tube and allowed to stand for 5 minutes before transferring the solvent layer to GC vials. The fatty acid profile was determined by gas chromatography using a BPX70 capillary column (30m, 0.25 μm, 0.22 μm ID) and a flame ionisation detector. The temperature program involved an initial 8 minute isothermal period at 185°C followed by a linear ramp of 10°C/minute to a final temperature of 220°C, held for 3 minutes. Injector (split mode) temperature was 240°C with a split ratio of 1:50. Detector temperature was 250°C. Data were analysed using Star Workstation Chromatography software V 4.51.

3.3.3 Flesh to Pit Ratio

The seeds from each oil extraction were cleaned of all residual flesh, weighed and flesh to pit ratio calculated.

3.3.4 Fruit Yield

Trees were covered with bird netting , while the ground underneath the tree was covered with weed mats. As fruit fell from the tree in the latter stages of the season, the fruit was collected and weighed. Fruit weight, oil content, moisture and fatty acid profile were determined. The data from theses analysis are yet to be statistically analysed.

3.4 Phenols

Olive samples were picked randomly from trees of different cultivars (Manzanillo and Cucco) in Wagga Wagga and Yanco (Hardys Mammoth) at various stages of physiological development as reflected by skin colouration. Olive samples of at least 50 grams were selected for each fruit colour. The olives were refrigerated at 4^{o}C prior to processing. The fruit was hand-pitted and freeze dried over a 2-3 day interval according to the moisture content of the fruit samples. The freeze dried olives were blended into a fine powder using a general purpose electric blender. The powdered samples were stored in screw top plastic jars that were kept in desiccators. Analysis was performed as soon as practicable, although there were no notable changes observed in freeze-dried samples stored for up to four months.

For reversed phase liquid chromatography (RPLC), powdered olive sample (1 gram) was reconstituted with sodium carbonate solution (5 mL;1 M). The solution was swirled and left to sit for 15 minutes at ambient temperature (optimized in the ranges 10 - 60 min and ambient to 60^{o}C). This mixture was then filtered using a buchner funnel apparatus fitted with Advantec 55 mm filter paper and a hand pump. The olive mass was recovered and transferred to the same conical flask to which a further 5 mL portion of carbonate solution was added. The flask was again swirled, left to stand for 15 minutes and filtered using fresh filter paper. Both filtrates were combined and transferred to a 25 mL separating funnel. The combined filtrate was washed once with hexane (5 mL) to remove lipoidal material (as verified by TLC of the washed filtrate on silica gel 60) and the aqueous phase recovered and adjusted to a pH of approximately 4 (optimized over the range of pH 1.5 to 7.3) with hydrochloric acid (4-5 mL; 3 M). The acidified extract was subjected to solid-phase extraction (SPE) using a Waters reversed phase (C18) Sep-Pak cartridge that had been conditioned with methanol (6 mL) followed by nanopure water (6 mL). Phenolic compounds were eluted using methanol + water (5 mL; 50+50 v/v). Sample extracts were collected in screw top scintillation vials and stored at 4^{o}C where necessary prior to RPLC analysis.

Alternatively, phenols have been recovered from the freeze-dried material by extraction with 50% aqueous methanol. The extract was washed with hexane and chromatographed without further purification. In either case, the eluate was diluted (1:10) with the same solvent and filtered using 0.45 μm Cameo 25AS acetate filters and Terumo plastic syringes before being injected into the RPLC system. Peak identification was based on retention time and spiking of sample extracts with authentic materials in conjunction with spectral data.

Total phenols

Total phenols were determined following reaction with Folin-Ciocalteu reagent as follows. Eluent from SPE (0.5 mL, after appropriate dilution but usually 1:10) or a phenolic standard was mixed with Folin-Ciocalteu reagent (5 mL, 1:10 diluted with nanopure water) and sodium carbonate solution (4 mL, 1 M). The mixture was heated for 15 minutes at 45^{o}C in a water bath. Absorbance of the solutions was measured at 765 nm against a reagent blank. Total phenol values are expressed as mg equivalents of phenol per gram of dry mass olive sample.

4. Results and Discussion

4.1 DNA Fingerprint

4.1.1 Yanco Grove

The Yanco trees showed good similarity within the four trees that were sampled four times around the tree. The trees were uniform within each tree and also showed good similarity within cultivars. Overall, most trees clustered within the individual cultivars, however, there were some trees which were obviously misnamed on the original map.

4.1.2 Wagga Wagga Olive Grove

Cultivar identification has focused on the Wagga site because of the problems identified in our previous report where we noted that further studies would be undertaken in the next twelve months. These trees were difficult to analyse because of the complexity of the dendrogram. Initial studies showed that only a few of the cultivars, which were identified as similar to others, clustered together. This was due to the orchard having one tree less than indicated on the map. Further investigation of the dendrogram indicated the position from where the tree had been removed. It was then possible to rename the trees from the original plan and clustering of similar cultivars was achieved.

Variation from the map
The olive grove map, produced several years ago to identify the trees, indicates that there are 94 trees in the Wagga Wagga collection including 55 cultivars. The current grove contains only 93 trees and many of these were apparently grafted to rootstock. Over the 103 years since the trees were planted, suckers from some of these rootstock appear to have developed and some trees show more than one type of fruit. It is also possible that some of the trees had been removed and existing trees may be self-sown ferals. Despite this, the diversity within the grove offers an excellent source of genetic material for future research and agriculture.

Variation within cultivars
Many of the trees could be matched and labelled with DNA from other sources. It was clear that there is considerable variability within cultivars and on only a small number of trees did DNA match completely with the six selected primers. This was also the case with trees from the Yanco grove in which there were only four cultivars with large numbers of each. Using good primers selected for their ability to detect polymorphisms, significant variation could be shown within and between cultivars.

The cultivar, which is most abundant within the Wagga Wagga grove, is Manzanillo (or Manzanilla). DNA analysis indicated that within this group of trees there was considerable variation. This is also evident in the fruit shape. Comparisons with other trees identified as Manzanillo indicate that this cultivar is very diverse and the literature lists many sub-cultivars of Manzanilla. The most common appears to be Manzanilla de sevilla but the range indicated within trees studied within Australia show that there may be other types.

Sub-cultivars of Manzanilla

Manzanilla de Carmona - Spain *Manzanilla de Tortosa - Spain*
Manzanilla dos Hermanas - Spain *Manzanilla Almodovar - Spain*
Manzanilla Italy - Italy
Manzanilla Carrasquena - Portugal

Manzanilla real Argentina - Arg	*Manzanilla*
Comune Argentina - Arg	
Hass Improved Manzanilla - USA	*Manzanilla*
Israel - Israel	

Standard DNA

For several of the cultivars in the grove at Wagga there was only one tree. As some are very unusual cultivars they could not be verified although the DNA patterns were used to relate them to other cultivars. Where there were more than two trees, or standard DNA was obtained from other sources, including cultivars outside of the Wagga collection, a set of standards were identified. The dendrogram relating these standards is illustrated below.

The list of standards and the method of verification is given in Table 3.

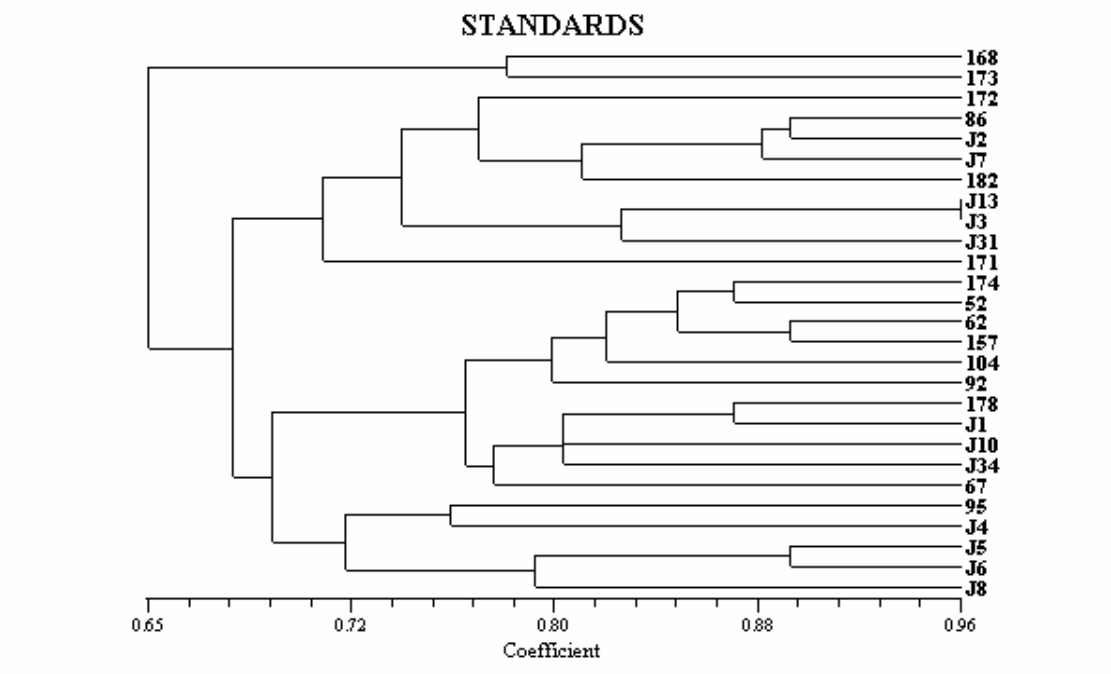


Table 3. LIST OF STANDARDS

NAME	ORIGIN	DNA #	COMMENTS
Kalamata	Olivegrove	J34	Matched in Cordoba and two other cultivars from commercial sources
Manzanilla de Sevilla	Cordoba	178	Leaves sent from Cordoba, extracted ORL
Hardys Mammoth	Olivegrove	J1	Matched with other Hardys Mammoth from Yanco
Sevillano	Olivegrove	J10	Matched in Cordoba
Hoji Blanco	Waite (Loxton)	174	Commercial sample
Large Fruited	Wagga Olive Centre	52	Two trees in grove clustering together
Oje Blanco Doncel	Wagga Olive Centre	162	Two trees in grove clustering together
Pendulina	Waite (Irymple)	157	Matched with Wagga tree
Tarascoa	Wagga Olive Centre	104	Matched with Wagga tree
Cucco	Wagga Olive Centre	92	Matched with other Tarascoa trees at Wagga
Nevadillo Blanco	Wagga Olive Centre	67	Matched with other Nevadillo Blanco at Wagga Wagga
Koroneviki	Waite (Sunraysia)	172	Commercial sample
Dr Fiiaschi	Wagga Olive Centre	86	Only tree known
Boutillon	Olivegrove	J7	Commercial sample, matched Wagga tree
Boultillan	Olivegrove	J2	Matches with Boutillon
Corregiolla	Olivegrove	182	Matched other Corregiolla trees in Wagga Wagga and Yanco
FS17	Waite (Lewis)	171	Commercial source
Frantoio	Olivegrove	J13	Matched in Cordoba
Mediterranean	Olivegrove	J3	Matched other commercial samples of Mediterranean
Barouni	Olivegrove	J31	Matched in Cordoba
Pera Bore	Wagga Olive Centre	95	Three trees in grove all match
UC13A6	Olivegrove	J4	Matched in Cordoba
Verdalian	Olivegrove	J5	Commercial sample closely related to Verdale
SA Verdale	Olivegrove	J6	Matched other commercial samples
Verdale	Olivegrove	J8	Commercial sample closely related to Verdalion and SA Verdale
Barnea	Waite (PRC)	168	Matched other Barnea from othe commercial sources
Arbiquina	Waite (Loxton)	173	Commercial variety

4.1.3 Summary

The Yanco grove can be confidently identified as containing four cultivars including Hardy's Mammoth, Corregiola, and Verdale. The fourth cultivar, of which there were only six trees labelled as Sevillano were not the same as Sevillano in the Wagga grove. Not all trees in the grove were labelled correctly.

An early map of the Wagga Wagga Grove shows 94 trees of a wide selection of cultivars. The trees are based on a collection of material from California, South Australia and Victoria. Spennemann (2000) indicates that they were derived from seeds and cuttings. The interest in olives was lost for many years after the initial sowing and the olive grove deteriorated. In relation to the original map, two trees are missing from obvious locations in the collection with a third missing from somewhere along the main roadway. The latter complicates cultivar identification. The identity of the trees in the olive grove has been verified or estimated, where evidence wasn't sufficient. Where a tree has been

verified against outside sources of DNA or confirmed against more than one tree, the cultivar has been identified as verified. If there is only one tree and no other comparison, it has been indicated as such. If the DNA does not match with trees of known identity, it has been labelled as not verified.

For 42 of the cultivars, each cultivar is represented by a single tree. There are 12 Manzanillo No 14, 13 Sevillano trees and several other cultivars represented by 2 or 3 trees.

Many of the trees show signs that more than one type of olive is present on a single tree i.e. from rootstock or feral olives growing at the base of the tree. As a result of the method of propagation, particularly from seeds that may not breed true to type, and subsequent poor management, the map identifying cultivars within the orchard appears to have several errors. Additionally, several trees have been removed from the original plan and possibly new, feral, trees have germinated within the grove. This has made DNA identification of the cultivars particularly difficult.

Despite the problems described, it has been possible to identify several of the trees. Approximately 45 trees have been described as either agreeing with the name as shown on the map, or renamed based on comparison with DNA from other trees.

It is clear that there is considerable variation within cultivars based on DNA testing. The variation within cultivars is however generally less than the variation between cultivars and it is therefore possible to discriminate between most cultivars. This is not always the case however and there is some overlap in some cultivars. There is also the problem of nomenclature in that several names exist for each cultivar. Corregiola for instance seems to be mixed up with many different names such as: (**SYNONYMS -** Pignatello, Fiorentino, Pendagliolo, Frantoiano, Gentile, Grossaio).

4.2 Flesh to Pit Ratio

There was a large variation in fresh weight between cultivars without a consistent pattern of weight increase. The ratio of flesh to pit (Table 4) shows a general increase during olive development. This can be explained by the pit reaching full development very early in the season, while the flesh continues to develop. It has been suggested that the flesh to pit ratio for olives should be at least 5:1 for olives to be acceptable as table olives rather than for oil production (Rahmani et al., 1997). Our studies show that although there is a huge range in the flesh to pit ratios for each harvest, which could be attributed to the large number of varieties which were studied, the average even at full maturity was only 5.2:1. To facilitate comparison of results, all data are normalised to a 50% moisture content.

Table 4: Flesh to pit ratios for each harvest calculated at 50% moisture

n=119	**Harvest 2**			**Harvest 3**			**Harvest 4**		
	Av	**Mi**	**Ma**	**Av**	**Mi**	**Ma**	**Av**	**Mi**	**Ma**
Flesh:Pit	2.6	1.0	5.1	4.1	2.0	6.7	5.2	1.8	8.6

Flesh to pit ratios were determined using 10 olives. This was only carried out for one season This is now considered to have been insufficient for statistically viable result and will be re-investigated.

4.3 Oil Content

Oil content (Table 5) showed a constant, rapid increase between harvest 1 and 3, followed by a plateau towards the end of the maturation period coinciding with harvest 4. The maximum and minimum oil contents at each harvest exhibited a large range.

Table 5: Change in oil content during maturation of olives harvested at Wagga Wagga and Yanco (at 50% Moisture).

N=119	Average oil content (%)	Minimum oil content (%)	Maximum oil content (%)
Harvest 1	2.54	0.56	6.97
Harvest 2	12.79	3.00	20.99
Harvest 3	19.76	9.73	25.92
Harvest 4	22.07	11.30	27.90

The accumulation of oil in the olives followed the typical sigmoidal curve. While the shape of the graph was similar for each cultivar, the final content and the rate at which it reached that point were significantly different. There is a general trend at the end of the season for the oil content to decrease, probably due to the degradation of the oil that would be occurring at this stage of the season. There is a correlation between fresh fruit weight and oil content (at 50% moisture) (data not included). The larger fruit showed a rapid increase in oil content, with the peak oil content occurring at an earlier stage of the season than the smaller, slower maturing fruit.

4.4 Fatty Acid Profile

Development of fatty acids (Table 6; Figure 1) showed very similar trends across all cultivars. Linolenic acid content was very high at the beginning of fruit development, but rapidly decreased in the first 10 weeks followed by a gradual decline to about 1 percent of the total fatty acids. This is commercially very important to growers because IOOC standards set the level of linolenic acid at below 1% of the total fatty acids for the oil to be labelled as edible olive oil. At the beginning of fruit development, the palmitic acid content was high, followed by a sudden and extreme decrease until about 10 weeks after flowering. The palmitic acid content then showed a gradual decrease until the end of fruit development. Oleic acid, quantitatively the most important fatty acid, increased dramatically in the first 10 weeks of development and then plateaued. Linoleic acid content was high at the beginning of development followed by a rapid decrease in the first 10 weeks after flowering. It then remains stable for a number of weeks. Palmitoleic acid and stearic acid contents remained constant throughout the entire season with no significant changes.

Table 6: Fatty acid content of selected trees at each harvesting stage from Wagga Wagga and Yanco.

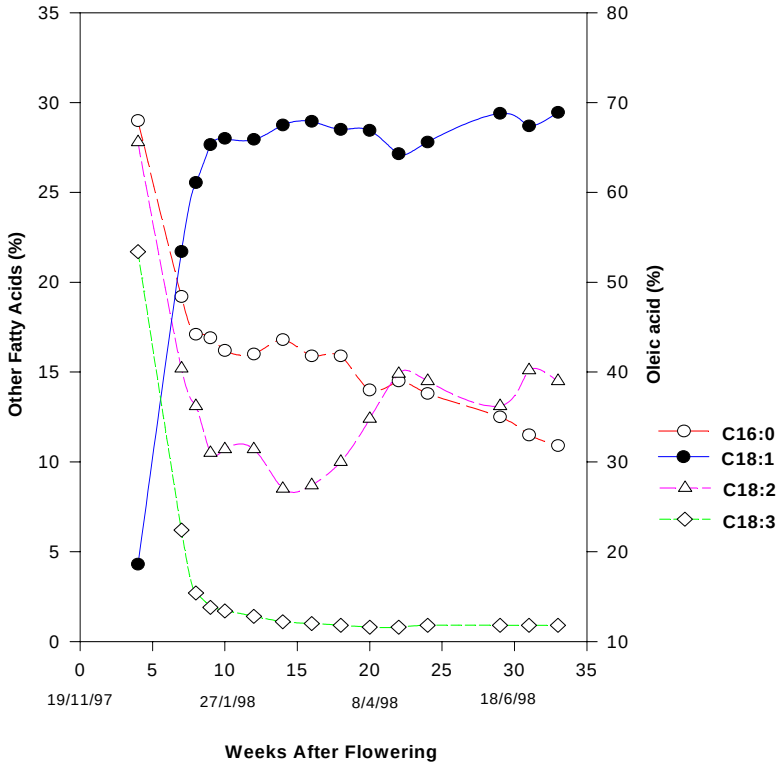
n=119	Harvest 1			Harvest 2			Harvest 3			Harvest 4		
	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max
Palmitic acid (C16:0)	17.5	14.1	21.9	16.8	13.1	24.2	15.8	10.7	20.6	13.7	7.6	17.8
Palmitoleic acid (C16:1)	0.7	0.3	1.4	0.9	0.1	3.4	1.2	0.1	3.4	1.2	0.6	3.4
Stearic acid (C18:0)	2.0	1.1	3.0	2.1	1.5	3.6	2.2	1.1	3.5	2.4	1.5	5.2
Oleic acid (C18:1)	63.7	50.3	72.4	67.7	57.5	76.0	63.4	48.8	77.2	62.8	47.8	79.1
Linoleic acid (C18:2)	10.4	4.8	17.8	9.8	2.7	18.0	15.2	2.8	26.4	17.6	4.0	32.2
Linolenic acid (C18:3)	3.5	0.5	7.9	1.2	0.7	2.4	0.9	0.5	1.6	0.9	0.6	1.4

*Note - All results are reported as percentage of total fatty acids

Data are presented for the 1998 season. However, the same trends were observed in subsequent seasons although there is some variation in the final amount of each fatty acid according to cultivar type

A full analysis of the data for 1998 and 1999 has been completed and submitted to the Australian Journal of Experimental Agriculture and is currently under review.

Figure 1: Development of fatty acids in tree 44 from the Wagga Wagga olive grove, measured at bi-weekly intervals. The trend was typical of all trees tested.



4.5 Phenols

The results show that the phenolic profile of olive drupes is extremely complex and raise serious questions about the use of total phenols as a predictive parameter. Oleuropein is generally the dominant phenol in early season and its content decreases with fruit development. The other significant phenols (based on content) in Manzanillo and Cucco were tyrosol, verbascoside and ligstroside. Changes in content of other phenolic compounds including the flavonoids during maturation were insignificant by comparison. However, the phenolic profile of Hardys Mammoth was quite different and investigations are still continuing to identify whether this is varietal or spatially determined.

The oleuropein content of Cucco fruit at initial harvest was 22 ± 4 mg g^{-1} (all data are reported on a dry mass basis) and then showed a progressive decrease with maturation from green, through purple and black fruit. Indeed, oleuropein was not detected in Cucco fruit at black maturation on day 43. In contrast, the oleuropein content of Manzanillo showed an initial slight decrease but then increased by some 50% from 50 ± 5 mg g^{-1} to 100 ± 5 mg g^{-1} between harvests 2 and 3 (covering a period of seven days) before decreasing again. The change in oleuropein content was reflected by a corresponding change in total phenols which increased from 20 ± 2 mg g^{-1} (expressed as mg equivalents phenol per

gram dry mass) to 150 mg g^{-1} between harvests 2 and 3 followed by a rapid decline to 20 mg g^{-1} at harvest 4 with a slight monotonous decrease thereafter. The apparent discrepancy between oleuropein content and total phenols is attributed to the non-specificity of the latter measurement and also to the method of expressing results as mg equivalents of phenol. The increase in oleuropein content during the early stages of development has been attributed to a growth phase that occurs prior to that of green maturation and is characterised by an accumulation of oleuropein. A closer examination of the changes occurring during this short period is warranted and should include an examination of elenolic acid glucoside which is not present prior to green maturation. In the case of the Cucco samples, the results suggest that harvesting commenced too late to observe the growth phase with its rapid accumulation of oleuropein. The total phenol content of the Cucco fruit was constant throughout maturation at 10 ± 2 mg g^{-1}.

Manzanillo fruits exhibited a gradual decrease in verbascoside with maturation while in Cucco fruits verbascoside was not initially detected. However, in this variety, verbascoside started to accumulate after day 21, peaked and then decreased once again to an undetectable level at black maturation. No significant change in tyrosol concentration was observed in Manzanillo fruits with maturation whereas the tyrosol concentration in Cucco fruits decreased by approximately 50% by harvest at day 28 before increasing again at black maturation to be approximately equivalent to the initial concentration found in green fruit sampled on the first harvesting date.

The ligstroside content of Manzanillo olives remained relatively constant although there was a slight but significant peak at approximately 35 days. In contrast, the ligstroside content of Cucco fruits showed a large increase between harvests on days 21 and 28. This increase was accompanied by a corresponding increase in verbascoside and a decrease in tyrosol content. Our studies of the roles of these compounds in fruit development are continuing.

5. Implications and Recommendations

This research demonstrates that the gross features of fruit development are largely independent of environment and agronomic practices. The groves at Wagga Wagga and Yanco that have been used for propagation have now been characterised. Further research is necessary to determine the suitability of some of the more unusual cultivars for commercial propagation.

The industry has expanded exponentially since this research was commenced. Its future viability will depend on a commitment to quality and finding alternative uses for olive products.

5.1 Dissemination of Results

Various aspects of this work have been published as follows.
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Conference Proceedings
XIXth International Conference on Polyphenols, Groupe Polyphenols, 1-4 September 1998, Lille, France.

XXth International Conference on Polyphenols, Groupe Polyphenols, September 2000, Munich, Germany.

The chief investigators are in regular contact with members of the Australian Olive Association and representatives have attended AOA Annual General Meetings. As a result of this grant, collaboration has been developed with Professor Lavee (Israel) and Professors Montedoro and Servili (University of Perugia, Italy).

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