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by Dr. Nigel Urwin

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Foreword

The project described here was undertaken to produce improved germplasm for the Australia's small but growing lavender industry. Currently growers use long established varieties bred outside Australia or varieties not generally accessible to new farmers and little attention has been paid to breeding new varieties with improved yield, suitable for the Australian environment. The outcomes of this project are 'polyploid' varieties of *Lavandula angustifolia* (lavender) and *Lavandula* x *intermedia* (lavandin) primarily for use as essential oil producing plants. These plants should be higher yielding and produce equivalent or better quality oils than currently grown varieties. It is hoped that these will be adopted by the national and international lavender industries. This project was funded by RIRDC and Larkman Nurseries.

This report, an addition to RIRDC's diverse range of over 1800 research publications, forms part of our Essential Oils and Plant Extracts R&D Program, which aims to support the growth of a profitable and sustainable essential oils and natural plant extracts industry in Australia.

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

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Executive Summary

What the report is about
The report describes the development of improved varieties of lavender (*L. angustifolia*) and lavandin (*L. x intermedia*) specifically for commercial oil production. Using conventional propagation and micro-propagation techniques together with treatment of plant tissue with the mitotic spindle inhibitor colchicine, chromosome numbers in these plants were increased. This report describes the preliminary morphological, oil quality and quantity analysis from some of the plants produced. Australia produces less than 1% of the world's oil and imports approximately 90% of lavender oil consumed. Climatically there is huge potential for Australia to become a key world oil producer and it is hoped provision of this new germplasm to Australian lavender growers will improve the economics of oil production and encourage broad-acre production of high quality lavender and lavandin oils.

Target audience
Primarily this project was undertaken to provide superior germplasm to the Australian Lavender Industry so that our industry may have a competitive edge and leading technology worldwide. The report should be provided to organisations like The Australian Lavender Growers Association (TALGA). The techniques used for plant improvement in this project are equally applicable to many species with commercial potential and particularly those for which little breeding has so far been undertaken including Australian wild flowers and bush foods. The report would be of use to stakeholders in those industries.

Background
Lavender (*L. angustifolia*) is grown for its essential oil, which is used in the perfume industry. Lavandin (*L.* x *intermedia*) varieties are sterile hybrids of the species *L. angustifolia* and *L. latifolia*. These are also grown for their oil, which is of lower quality but used in various toiletries. Lavender is also grown for cut and dried flowers and several of the 39 species within the genus are grown as ornamentals. The Australian lavender industry produces less than 1% of total oil produced worldwide. However, recently there has been increased interest in lavender as an alternative crop, given the increased demand for essential oils. Little effort has been put into breeding improved cultivars of lavender for commercial oil production. Polyploidy induction (producing plants with more than two sets of chromosomes) is commonly used in agricultural and horticultural crops for plant improvement. Polyploids are generally bigger plants with increased vigour relative to their diploid counterparts.

Objectives
L. angustifolia cultivars which produce the highest grade oils are relatively small plants with smaller flowers in comparison to *L.* x *intermedia* cultivars. *L.* x *intermedia* cultivars produce 3-5 times more oil than *L. angustifolia* cultivars. Both types of lavender would benefit from increased size and vigour and so the objectives of this project were as follows:

1. Produce polyploid *L. angustifolia* varieties with improved oil yield and vigour.
2. Produce polyploid *L.* x *intermedia* cultivars with improved oil yield and vigour.
3. Produce double haploid varieties of *L. angustifolia.* Double haploid plants are completely homozygous allowing propagation of lavenders by seed.

Method and Results
The first objective was to produce polyploid *L. angustifolia* varieties in the hope that oil yield from these would be improved over traditional ones. By germinating seed in the presence of a mitotic spindle poison colchicine we produced two varieties - C6/24 and C3/2 - which were tetraploids (four sets of chromosomes). We demonstrated these tetraploids had approximately 100 chromosomes by direct counts in root tip cells and flow cytometry of nuclei, whereas common varieties have 50 and are diploid. Both tetraploids had larger flowers than diploids and preliminary GC and olfactory analysis of oil distilled from C6/24 demonstrated it was of perfume quality and produced at greater yield per mass of flower material than oils from diploid varieties. Both tetraploids were fertile and produced seed

however, seedlings produced from these were themselves infertile and had reduced genome sizes, estimated by flow cytometry.

Another approach we took towards producing polyploid *L. angustifolia* and *L.* x *intermedia* was to produce polyploid derivatives of common varieties already grown for oil rather than selecting plants produced from seed. To do this we developed tissue culture techniques for shoot culture of *L. angustifolia* varieties 'Bee' and 'Swampy' and *L.* x *intermedia* varieties 'Grosso' and 'Seal'. Following brief treatments of shoot cultures with colchicine to induce polyploidy we regenerated survivors and acclimatised the plantlets to ambient conditions. None of the surviving plants had obviously larger flowering spikes however four polyploids of Bee were obtained and verified as such by flow cytometry. These plants had smaller stature but larger flowers than diploid varieties, but because of the smaller stature would be unsuitable for oil production. To further determine if polyploidy had been induced we examined seed production in the *L.* x *intermedia* survivors and found several which produced seed indicating that conversion to tetraploidy had occurred. *L.* x *intermedia* varieties such as Grosso and Seal are normally sterile probably because of an inability of chromosomes to pair during meiosis. Induction of tetraploidy was anticipated to restore fertility as it can do in other plant genera. *L.* x *intermedia* plants were grown from some of these seeds and the ploidy level of these F_1 generation plants was assessed by chromosome counting and flow cytometry. Polyploids were indeed generated however the ploidy level of all of these was intermediate between diploid and tetraploid. This was similar to the F_1 generation of the tetraploid *L. angustifolia* C6/24 and C3/2 with most plants being approximately triploid. Similarly, these F_1 *L.* x *intermedia* plants produced no seeds and were therefore infertile.

Characteristics of these F_1 polyploid *L.* x *intermedia* plants are that they are much larger and more vigorous than the parents Grosso and Seal and they have larger flowering spikes with longer thicker peduncles. These should be excellent plants for oil production. If they are resultant from crosses of the tetraploid *L.* x *intermedia* plants and diploid *L. angustifolia* then it is likely that their oil quality will be more like *L. angustifolia* and therefore much better than typical Lavandin oils. Oil yield and quality is be assessed.

The third objective of this project was to culture *L. angustifolia* microspores or whole anthers to generate haploid plants. Once produced treatment with colchicine was intended to produce doubled haploids. These plants should be pure breeding (completely homozygous) and could be grown from seed producing a cost saving to farmers in propagation of plants. During this project considerable time and effort was spent on attempting to culture microspores and whole anthers and despite using plant material at different developmental stages, using several different media and hormone combinations we were unsuccessful in obtaining any haploids or in seeing any development of microspores or anthers in culture at all. Since haploid plants could not be produced this objective abandoned.

Overall two of the three objectives of this project have been achieved and we have produced polyploid varieties of *L. angustifolia* and *L.* x *intermedia*.

Implications for relevant stakeholders

Having produced polyploid lavender and lavandin varieties it will be necessary to evaluate these in the field in terms of oil yield and quality. The results of this project were presented to TALGA at their annual conference in Goulburn in May this year. Larkman Nurseries (industry partner) in this project will assist in obtaining Plant Breeders Rights PBR in Australia and internationally and will produce and market the plants. Further evaluation requires that the effect of environment on these plants be assessed since all previous results have been from plants grown in Wagga Wagga. These plants may perform better or worse in different environments.

Recommendations

This report should be made available to TALGA. Field trials are a long term objective and a minimum of three to four years would be required for plants to reach maturity. It is recommended that these should be carried out in conjunction with growers through an industry organisation such as The Australian Lavender Growers Association (TALGA). It is proposed that several candidate varieties are selected from the list below and replicate trials are set up in different locations around Australia (Tasmania, NSW, SA, WA, VIC) with plants being given as similar maintenance regimes as possible. Samples can then be collected and evaluated for oil yield and quality. Several growers are willing to help with these both in providing land and also in maintaining and sampling the plants. In the interim period it is also recommended that Charles Sturt University and RIRDC apply for Plant Breeders Rights (PBR) on selected varieties below.

Table A. Summary of new varieties resulting

Species/ hybrid, experiment reference number (CSU collection number)	**Ploidy Estimate**	**Characteristics**	**Application**
L. angustifolia C7/103 (CSU70)*	Diploid	Compact small flowered	Garden /culinary
L. angustifolia C6/24K (CSU95)*	Diploid	Large plant, vigorous growth	Oil production/ Garden
L. angustifolia C6/24 (CSU72) *	Tetraploid	Large flowers, large leaves good oil quality (GC and olfactory)	Oil production/ Garden
L. angustifolia C3/2 (CSU71)*	Tetraploid	Large flowers, large leaves	Oil production/ Garden
L. angustifolia C3/2/2 (CSU97)*	Approximately triploid	Large flowers, large leaves good oil quality (GC and olfactory)	Oil production/ Garden
L. angustifolia C6/24/8 (CSU92)	Approximately triploid	Large flowers, large leaves	Oil production/ Garden
L. x intermedia G7 (CSU148)	Approximately triploid	Derivative of Grosso, Large plant, large flowers, extremely vigorous growth	Oil production/ Garden
L.. x intermedia G9 (CSU150)*	Approximately triploid	Derivative of Grosso, Large plant, large flowers extremely vigorous growth.	Oil production/ Garden
L. x intermedia S2 (CSU138)*	Approximately triploid	Derivative of Seal, large plant, large flowers extremely vigorous growth.	Oil production/ Garden
L. x intermedia S3 (CSU139)	Approximately triploid	Derivative of Seal, large plant, large flowers extremely vigorous growth.	Oil production/ Garden

* = those varieties for which PBR should be sought first.

Introduction

Taxonomy and Uses of Lavenders

Lavenders belong to the Lamiaceae, a family which contains square stemmed plants such as thyme (*Thymus* spp.) and mint (*Mentha* spp.). Lavenders are contained within the genus *Lavandula* which contains 39 species, mostly of Mediterranean origin, the taxonomy of which was recently reviewed by Upson and Andrews (2004). Lavenders have been grown since Roman times for cut flowers, dried flowers and more recently their essential oils which are extracted from the flowers, traditionally by steam distillation. Within the genus there are now eight sections*: Lavandula, Stoechas*, *Dentatae, Pterostoechas, Chaetostachys, Hasikenses, Sabaudia* and *Subnudae.* Section *Lavandula* contains the majority of species grown commercially for oil and flowers. Other sections contain mainly garden ornamentals.

Lavender Oils

There are two main species and hybrids thereof which are grown commercially for their oil. These are *L. angustifolia* Miller (common name: Lavender), *Lavandula latifolia* Medicus (common name: Spike lavender*)* and hybrids of the two termed *Lavandula* x *intermedia* (common name: Lavandin) (Tucker, 1981). The oils are used mainly in the fragrance and perfume industry. Commercially, the most valuable of lavender oils is extracted from *L. angustifolia.* A lower grade oil (lavandin oil) is obtained from the hybrids and a third oil is produced is from *L. latifolia,* 'spike oil'.

The quality of lavender oils and hence market price is determined by chemical composition. Lavender oil is comprised of over 100 components, mainly terpenoid compounds. The two major constituents of *L. angustifolia* oils are linalool and linalyl acetate typically comprising 35% (w/v) each of the total oil composition. Camphor is a minor component, which has a large effect on determining oil quality. The greater the amount of camphor the lower the quality. The highest quality lavender oil has about 0.5-1% camphor whereas lavandin oil has about 5-10% making it unsuitable for many applications including incorporation into foods. Other minor components also significantly affect oil quality. Oil is generally extracted by steam distillation of flower heads although other methods such as solvent extraction and extraction in liquid CO_2 are used, (McGimpsey and Porter, 1999)

An example of a variety of *L. angustifolia* grown commercially for oil is Maillette (this variety may consist of several different varieties in reality). The most common *L.* x *intermedia* variety grown worldwide for oil is Grosso. A conservative estimate of approximately 50% of *L.* x *intermedia* oil produced worldwide is obtained from Grosso.

Lavender Propagation and Breeding

L angustifolia can be grown from seed but generally is not, due to variability in flowering time and oil quality. *L.* x *intermedia* varieties are sterile as are many interspecific hybrid plant varieties, probably as a result of problems with chromosome pairing during meiosis. Therefore the majority of lavenders are clonally propagated by cuttings. Oil producing plants are generally planted in raised beds in spring. Flowers are removed for oil extraction and plants are pruned once a year. Oil producing plants are generally replaced every 10-15 years. In addition to their commercial use lavenders are also popular garden plants and with over 100 varieties of *L. angustifolia* and *L x intermedia* available. Varieties of sections *Stoechas* and *Dentata* are increasingly popular. In all, well over three hundred varieties are grown around the world.

Worldwide very little breeding of lavenders has been undertaken to improve oil quality and yield. Some *L.* x *intermedia* varieties have been produced by allowing *L. angustifolia* and *L. latifolia* plants to flower in close proximity, collecting seed and selecting plants of intermediate phenotype. Some *L.* x *intermedia* varieties have been selected from the wild. Some attention to disease resistance has been paid in selecting plants for example the *L.* x *intermedia* variety 'Grosso' was selected as resistant to the disease 'Yellow Decline', caused by a Mycoplasma.

Natural and Induced Polyploidy

Traditional breeding programs to improve specific traits in crop species can take 10 years or more however a number of techniques can improve plant size, vigour and yield more rapidly. These include manipulation of chromosome number (ploidy). Unlike most animal species, which are diploid, plants can have multiple sets of chromosomes and are termed polyploid. Polyploidy is common and has played a major role in the evolution of flowering plants (Masterson, 1994; Otto and Whitton, 2000). Approximately 50 % of known plant species are polyploid including many crop species (e.g. potato, wheat, strawberry and marigold). Natural polyploidy is present within the Lamiaceae, for example within *Thymus* (Lopez-Pujol *et al.*, 2004), *Glechoma* (Widen and Widen, 2000) and *Lavandula* (Upson and Andrews 2004). In general polyploid plants are larger, more vigorous and have larger fruit and flowers. Modern wheat varieties are hexaploid, having six sets of chromosomes and strawberry varieties are octoploid with eight sets. Whilst polyploidy occurs in nature it can also be induced by compounds which inhibit mitotic spindle formation during cell division. Colchicine is one such compound and it is isolated from the corm or seeds of *Colchicum autumnale* (Liliaceae). Others include herbicidal compounds like trifluralin and oryzalin. Resulting induced polyploid plants also often have larger leaves, flowers, fruits and seed (Hancock, 1997; Hartwell *et al.*, 2004).

In considering induction of polyploidy to improve lavenders, chromosome number and genome size estimates should be considered to determine if further increase in these is feasible. Chromosome counts in *L. angustifolia* and *L. latifolia* vary considerably in the literature with 2n (diploid) estimates ranging between 36 and 75, (reviewed by Upson and Andrews 2004). The consensus is around 2n = 50 for both species and hybrids thereof. Chromosome number estimates suggest that both species are ancient polyploids (Upson and Andrews, 2004). The chromosome size in species of *Lavandula* is relatively small so although the genome size is unknown it is likely to be small. Therefore *Lavandula* species should tolerate further polyploidy to at least tetraploid, with approximately 100 chromosomes.

Further advantages of polyploidy induction in lavenders.

Pure breeding *L. angustifolia* varieties (Double haploids)

In addition to chemically induced polyploidy potentially resulting in larger plants with larger leaves, flowers and consequently increased oil yield, polyploidy induction may have other benefits. One problem with *L. angustifolia* varieties is that they must be propagated vegetatively to produce a uniform crop as anecdotally plants grown from seed are phenotypically variable. Producing varieties of lavenders which could be grown from seed with plants having consistent oil quality and identical flowering times would represent a significant improvement in reducing set up costs to farmers. One way to produce these without extensive crossing is to produce 'double haploids'. Usually microspores (pollen at the single nucleus stage) or whole anthers are cultured and whole plants can be produced, by *in vitro* embryogenesis, with haploid genomes. These haploid plants are infertile but brief treatment of floral meristems with colchicine or similar compounds, to double chromosome number, can result in viable seed production. Resultant diploid (actually double haploid) seedlings would be essentially homozygous at all loci and produce identical plants from seed themselves provided they are not self-incompatible.

Fertile *L.* x *intermedia* varieties

In the case of induction of polyploidy in *L.* x *intermedia* varieties, chromosome doubling is likely to result in restoration of fertility since chromosome pairing during prophase 1 of meiosis can then occur between identical duplicated chromosomes. Spontaneous polyploidy in hybrids of other plant species has occurred in nature resulting in evolution of new species and an example is the Loganberry, a fertile hybrid of wild blackberry and raspberry. Fertile *L.* x *intermedia* varieties as well as possibly being larger could then be grown from seed and crossbred with themselves or with additional species like *L. angustifolia.* The progeny of a tetraploid *L.* x *intermedia* and a diploid *L. angustifolia* should be sterile triploids which are high yielding, exhibit high vigour and produce higher quality oils closer to those of *L. angustifolia* since the relative contribution of genomes in these hybrids will be two to one for the *L. angustifolia* and *L. latifolia* genomes respectively.

Benefits to the National and International Lavender Industry

The majority of lavender is grown in Europe with the major producers being France, Bulgaria and Russia. Other lavender producing countries include Spain, China, United States, Japan, United Kingdom, Croatia, Australia and New Zealand. World production of *L. angustifolia* and *L.* x *intermedia* oils is estimated to be about 200 and 1000 tonnes per year respectively with Australia being a small player producing about 2 tonnes of high quality *L. angustifolia* oil per year. The oil yield for *L. angustifolia* is approximately 50-70kg ha^{-1} and approximately 3 to 5-fold greater for *L x intermedia* oils. Prices at the farm gate for bulk oil are around $A100 per kg for *L. angustifolia* oil and $A25 per kg for *L x intermedia* although prices vary widely (as much as 4-fold). Australia is a small producer with the largest farm in Tasmania. There are many small producers in Victoria, South Australia, Western Australia and New South Wales, tending to sell their oils/ flowers on or off farm and value adding. The main industry body is The Australian Lavender Growers Association (TALGA). The industry in general suffers from lack of information available to prospective farmers on agronomy, appropriate varieties producing high yields of high quality oils, use of herbicides as few are registered for use in Australia and high cost for plants. Expected benefits from this project would be higher yielding varieties. Polyploid *L. angustifolia* varieties might be expected to produce 2 to 5-fold more oil per hectare (similar to *L.* x *intermedia* varieties). Moreover double haploid lines of *L. angustifolia* would reduce the average cost of plants from 80 cents to approximately 20 cents per plant and enable increased production of seedlings on farms, reducing initial cost in lavender establishment. Larger and higher yielding *L.* x *intermedia* varieties would similarly improve production and profitability. Sales of seed and plants under licence at home and abroad would benefit the Australian nursery industry but is dependent on sound marketing and ensuring plant breeders rights are obtained. This benefit is difficult to quantify but considering if a single ornamental lavender (1 million plants) were sold in the U.S. under licence, with a 10 cent royalty per plant, the yield would be >$A250,000 in income to Australia alone. If these plants were adopted for oil production worldwide then the benefit would far exceed this estimate.

Objectives

As stated in the original grant application were as follows:

This project aimed to breed improved varieties of *Lavandula angustifolia* (lavender) and *Lavandula* x *intermedia* (lavandin) both of which are grown commercially for their essential oil, dried and cut flowers. In general, plant tissue culture techniques and the use of mitotic spindle inhibitors will be used to produce polyploid varieties and genetically uniform (double haploid) seed lines for commercially lavender farmers to adopt and use.

The specific outcomes of the proposed research are as follows:

1. Polyploid *L. angustifolia* and *L.* x *intermedia* varieties, which should be improved with respect to oil yield and vigour. Two *L. angustifolia* varieties 'Bee' and 'Swampy' which produce a high quality oil will be used as starting material and polyploid lines should produce similar oils at higher yield. Two *L.* x *intermedia* varieties which have been used for commercial oil production, for many years are 'Grosso' and 'Seal'. These will be used as starting material for generation of polyploid lines. In addition a number (>20) polyploids lines will be generated from *L. angustifolia* seed so that wider range of polyploid varieties with differing oil qualities and flower colours will be produced.

2. Double haploid *L .angustifolia* and fertile *L.* x *intermedia* varieties which can be propagated by seed, which are homozygous, thus removing the need for traditional and expensive vegetative propagation of plants. It is the intention to reduce the cost of plant propagation and start up costs with these lines and encourage growth in the lavender industry.

3. A new hybrid of lavender generated by doubling the chromosome number of infertile hybrids (*L.* x *intermedia*) to produce fertile derivatives, increasing the range of germplasm available for future breeding. Essentially this would represent a new species.

Methodology

Germination of *L. angustifolia* seed, colchicine treatments and vegetative propagation

L. angustifolia seeds were sown in Petri-dishes (0.2 g per dish ~200 seeds) on filter paper. The filters were wetted with 5 ml of 0.5 mg mL^{-1} gibberellic acid (GA_3) containing various concentrations of colchicine (Table 1). The potassium salt of GA_3 was used as it is soluble in water and all concentrations used. Three dishes were sown per colchicine concentration. Petri-dishes were sealed with Parafilm and incubated at 22^{o}C under cool white fluorescent lights, with a 12 hours light / 12 hours dark cycle for 7 days to germinate.

After 7 days, Petri-dishes were transferred to a glasshouse and acclimatised to natural lighting in shade for 2 days. Seedlings and seed which had not germinated were transferred to 10 cm diameter pots containing seed raising mix. Pots were covered with transparent plastic film and sub-irrigated by standing in a shallow tray of water for 1-2 weeks. Plants were then transferred to misting beds and then to ambient conditions. Surviving plants were transferred to potting mix in individual pots and were watered and fertilised as required. The experiment was repeated twice with two different batches of seed. Two different seed lots were used in these experiments. These were *L. angustifolia* 'Vera' obtained from Gippsland Seed, Silvan, Australia and *L. angustifolia* 'True Lavender' obtained from High Sun Express Seeds, Ormiston, Australia.

To propagate varieties or sports, shoots with at least three nodes were cut and the lower node and stem was dipped in Clonex® rooting hormone gel (Growth technology, Australia) which contained 3 mg mL^{-1} indole-3-butyric acid (IBA).Cuttings were placed in seed raising mix on heated misting beds and transferred to standard potting mix when roots were established.

Colchicine treatment of whole plants and cutting material

Colchicine at various concentrations in 50% glycerol was applied to the terminal nodes of whole plants grown in pots in the glasshouse after removal of flowers. For cuttings, at least three nodes were removed from plants. The lower pair of leaves was removed and cuttings were submerged in colchicine solutions of various concentrations for different times. Cuttings were rinsed with distilled water and the end of the stem including the lower node stripped of leaves was dipped in Clonex® rooting hormone gel. Cuttings were planted in seed raising mix and placed on heated misting beds until roots formed.

Shoot culture initiation and maintenance

All lavender varieties were cultured on Murashige and Skoog (MS) medium containing minimal organics and vitamins, 2% sucrose and 0.9% agar. The hormones benzylamino purine (BAP) and naphthalene acetic acid (NAA) were added to the medium after autoclave sterilisation. The stock hormones were filter sterilised and added to sterile media prior to pouring plates. Tissue was taken from glasshouse grown plants and surface sterilised by a brief rinse with 70% ethanol followed by 1.25% w/v sodium hypochlorite, 0.005% Tween 20 for 20 min with vigorous agitation. Tissue was rinsed in four changes of sterile distilled water. Nodes were excised with 5 mm of stem tissue either side and most leaf tissue was removed. Nodes were semi-submerged in the agar and cultures were incubated at 25^{o}C under cool white fluorescent lights on a 16 hr light, 8 hr dark cycle. Every six to eight weeks nodes were excised from new shoots and sub-cultured on the same media.

Colchicine treatment of in vitro grown shoots

Nodes were placed on fresh medium 3 days prior to treatment and then placed on fresh medium after treatment. Treatments consisted of submerging nodes in filter sterilised 0.1% colchicine, 2% dimethylsulphoxide (DMSO) solution for various times, (DMSO alone in control treatments) followed by rinsing three times in sterile distilled water.

Root induction and transfer to ambient conditions

Cultured shoots and shoots surviving colchicine treatments were excised and placed in half strength MS medium, 1% sucrose and 0.9% agar without hormones. After root formation plantlets were acclimatised to glasshouse conditions in their Petri-dishes, firstly in shade and then reducing humidity by slowly removing the lids. Plantlets were washed and placed in moist seed raising mix. Pots were covered with plastic film and sub-irrigated for several weeks prior to finally removing the film. Plants were then watered and fertilised as required.

Chromosome number determination

Chromosome counts were conducted on seedling root tips and also root tips of newly struck cuttings. Root tips (~1mm) were excised into iced water and incubated for 24 hrs to increase the number of metaphase cells. Root tips were then fixed in ethanol-acetic acid (3:1 v/v) overnight. Fixed root tips were placed in 1-2 drops of aceto-orcein stain (Sharma and Sharma, 1999) acidified with one tenth volume of 1M HCl on a microscope slide. The root tips were heated briefly and squashed beneath a coverslip. Chromosomes were counted in metaphase spreads from at least five different plants or seedlings for each variety assessed.

Flow cytometry

For relative genome size determination nuclei were isolated and stained with 4'6-diamidino-2-phenylindole (DAPI) using a CyStain® UV precise P kit obtained from Partec, (Munster, Germany). Nuclei from leaf tissue were isolated and stained according to the manufacturer's instructions. Samples were analysed using a Partec PA-I flow cytometer (Partec, Munster, Germany). For absolute genome size determination (C-value), propidium iodide (PI) was used as the DNA stain. Nuclei were released from 0.5 cm^2 of leaf tissue by chopping with a razor blade for 30 s in 2 mL of modified Galbraith's nuclei isolation buffer (Galbraith *et al.*, 1983) containing 45 mM magnesium chloride, 30 mM trisodium citrate, 20 mM 3-morpholinopropanesulfonic acid (pH 7.3), 0.1% triton X-100, 50 µg mL-1 PI and 100 µg mL-1 ribonuclease A. Nuclei were passed through a 30 µm nylon filter and were kept on ice for 10 mins prior to analysis using a Quanta 488 flow cytometer (Beckman Coulter, Sydney, Australia). For C- value determination nuclei were isolated from *L. angustifolia* C7/103, *Petroselinum crispum* (parsley) and *Lycopersicon esculentum* 'Grosse Lisse' (tomato). Five samples of leaf were analysed for each species and samples were analysed alternately. C-values were determined from the mean peak fluorescence of samples and references according to Dolezel and Bartos (2005). The experiment was repeated twice using different plants on different days. For each set of samples the 1C value for lavender was calculated using the 1C values for tomato and parsley listed in Bennett and Leitch (2005).

Plant measurements

Flower, leaf and seed material was obtained from lavender varieties in the collection at Charles Sturt University at Wagga Wagga. Seed weight was determined by weighing batches of 10 seeds (Ten seed weight) with 10 replicates for each variety. Twenty measurements were made of peduncle width, bud weight (single flower bud), spike length, and number of whorls per spike for each variety at time of flowering. The number of flowers per spike for twelve spikes was measured for each variety. Weight of at least ten individual flowering spikes were also determined for some varieties.

Scanning electron microscopy

Leaves or unopened flower buds were attached to flat brass stubs, quick-frozen at approx. -170^{o}C in a cryotransfer unit (Oxford CT1500, Oxford Instruments, Sydney, Australia) attached to a JEOL 6400 scanning electron microscope (JEOL Australasia Limited, Sydney), gold-coated then observed at 15 kV on the cold stage (-150 to -180^{o}C) of the SEM (Craig and Beaton, 1996). For measurements, photomicrographs were taken at x150 magnification and stomatal aperture (guard cell length) and oil gland diameters (the widest point for each oil gland) were measured. Forty eight oil glands and forty stomata were measured for each variety. The experiment was repeated twice.

Distillation

Oils were obtained from *L. angustifolia* varieties by steam distillation using a 50 L capacity stainless steel still and condenser. Water (4 L) was placed in the bottom of the still, the still and condenser were assembled and the still was heated using a gas burner until distillate emerged. The lid and condenser were removed and up to 1 kg of flower heads was placed in a mesh basket above the level of the water. The still was assembled again and distillate collected until little or no further oil emerged. The time taken for distillation was approximately 20 min and 600-900 ml of distillate was collected in this time. Oil was collected in separating funnel. Oils were immediately stored in dark glass vials with minimal head space to prevent oxidation. Oils were stored at 8^{o}C prior to analysis.

Gas Chromatography of Essential Oils

Gas chromatography (GC) was outsourced and performed by Dr. Ian Southwell, NSW Department of Primary Industry, Wollongbar Agricultural Institute, Wollongbar.

Results

Production of polyploid *L. angustifolia* from seed

Effect of colchicine and gibberellic acid on *L. angustifolia* germination and early seedling growth

One approach we used to induce polyploidy in *L. angustifolia* was to germinate seed in the presence of colchicine at various concentrations on filter paper in Petri-dishes for 1 week. Survival of seedlings in such an experiment is shown in Table 1. Many batches of *L. angustifolia* seed had poor germination rates or required long times to germinate in water. Gibberellic acid (GA_3) has been used to break dormancy in seed (Taylorson and Hendricks, 1977; Bewley, 1997) and we found 0.5 mg mL^{-1} to be optimum for lavender and most seed which germinated did so within 14 days of imbibition. Colchicine had little effect on germination of seedlings, however, seedling survival was affected at all concentrations used with no seedlings surviving 55 days post imbibition on 1g L^{-1} and approximately 50% surviving at day 55 on 3.6 mg L^{-1}, relative to those germinating on GA_3 alone (Table 1). Seedlings were visibly affected by high concentrations of colchicine, being shorter and having broader stems than controls (Figure 1.). Surviving seedlings were grown to flowering and examined for the presence of larger flowers on some sports relative to other flowers on the same plant. Two plants surviving 125 mg L^{-1} and 15.6 mg L^{-1} colchicine had sports with visibly larger flowers. These were propagated as putative polyploids and gave rise to two independent varieties C3/2 (CSU71) and C6/24 (CSU72) respectively. Another plant which survived 7.8 mg L^{-1} and which did not have larger flowers was propagated and was designated C7/103 (CSU70). All plants including the three derived varieties were grown under field conditions in the lavender collection at Charles Sturt University under conditions which allowed open pollination. Appendix 1 contains a list of varieties (both common and resulting from this project) in the collection and throughout this report they are referred to by there original designation and CSU collection number eg C7/103 (CSU70) as above.

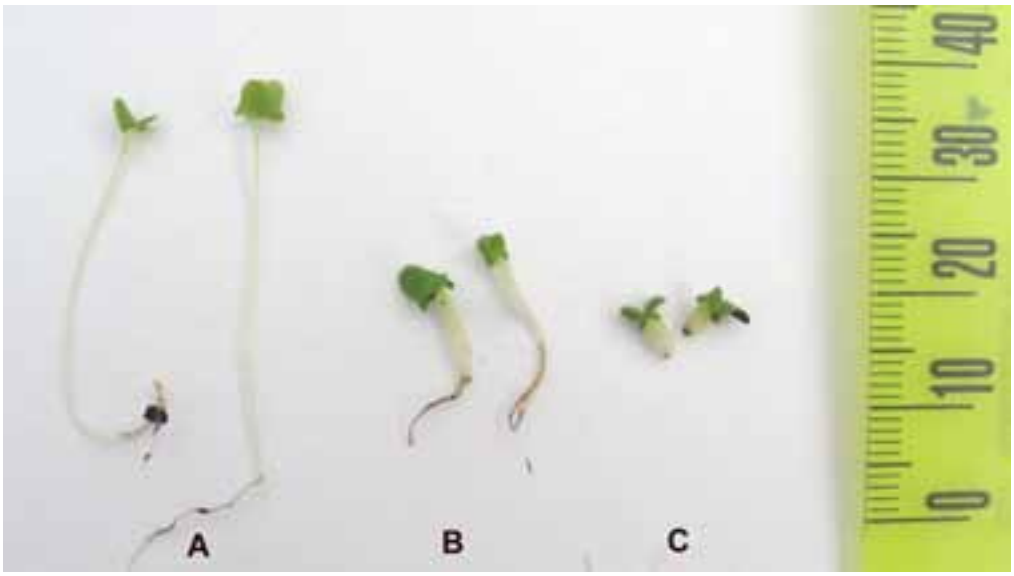

Figure 1. Effect of colchicine on early seedling growth. Seedings were photographed 1 week after transfer to potting mix. Seedling were germinated in the presence of colchicine at A) 0 g/L B) 0.0625 g/L and C) 1g/L.

Table 1. Effects of colchicine and gibberellic acid on germination of *L. angustifolia* seed. Data shown are the mean number of surviving seedlings at the number of days after sowing with +/- 2 x standard error (2SE) in brackets (n=3).

Colchicine concentration (g/L)	Number of surviving seedlings Day 14	Day 42	Day 55
0 (No GA_3)	2 (0-5)	5 (4-6)	4 (3-4)
0	51 (45-58)	36 (30-42)	32 (26-37)
1	38 (9-66)	7 (1-12)	0 (0-1)
0.5	52 (40-64)	9 (6-12)	1 (0-2)
0.25	35 (8-63)	6 (1-11)	4 (0-8)
0.125	63 (52-73)	13 (8-17)	3 (1-4)
0.0625	44 (27-62)	14 (7-22)	6 (3-8)
0.03125	53 (47-59)	20 (15-24)	11 (6-17)
0.0156	49 (45-54)	26 (19-34)	13 (2-24)
0.0078	57(49-66)	33 (30-35)	22 (19-25)
0.0036	50 (38-62)	27(22-33)	17 (14-20)

Chromosome number in *L. angustifolia*

To determine the number of chromosomes in *L. angustifolia* we counted chromosomes in root tip squashes of seedlings derived from the varieties 'Bee', 'Lavenite Petite', 'Swampy', 'Egerton Blue' and one of the seed lines (Vera) from which the putative polyploids described above were derived. All varieties had 2n =50 chromosomes (Figure 2A&B). *L. angustifolia* chromosomes were small and of similar size. To assess chromosome numbers in the putative polyploids, chromosomes were counted in squashes of root tips excised from newly propagated cuttings of C3/2 and C6/24. Both these varieties had greater than 90 chromosomes per cell so were found to be tetraploid (Figure 2C&D). The exact number of chromosomes in these lines could not be determined because the chromosomes were too numerous, and the observed frequency of metaphase spreads in root tips was very low.

To support the chromosome count data we studied the relative DNA content of leaf nuclei from the putative tetraploids and controls by flow cytometry. Nuclei were stained with DAPI. G1 peaks of the putative tetraploids C3/2 and C6/24 had nuclei with approximately twice the fluorescence of nuclei from variety C7/103 which also survived colchicine treatment and was derived from the same seed batch. Therefore flow cytometry confirmed that C3/2 and C6/24 were tetraploid (Figure 3).

Twelve varieties of *L. angustifolia* from our collection were also analysed and these were as follows: 'Hidcote', 'Imperial Gem', 'Ashdown Forest', 'Bosisto', 'Rosea', 'Lavenite Petite', 'Folgate', 'Munstead', 'Princess Blue', 'Nana Atropurpurea', 'Lady', and 'Irene Doyle'. All varieties had G1 peaks with similar fluorescence to C7/103 and were therefore determined to be diploid. Analysis of twenty individual seedlings, grown from seed harvested from four other plants surviving colchicine treatment indicated three seedlings were also polyploid and all were derived from another plant 'C6/26' which survived 15.6 mg L^{-1} colchicine. These plants were designated C6/26/A, C6/26/B and C6/26/C and were also placed in the collection at CSU. Eight seedlings from seed collected from the original plant that had the sport from which C3/2 was derived were all determined to be diploid and therefore the original surviving plant was probably chimeric in respect of tissue ploidy level.

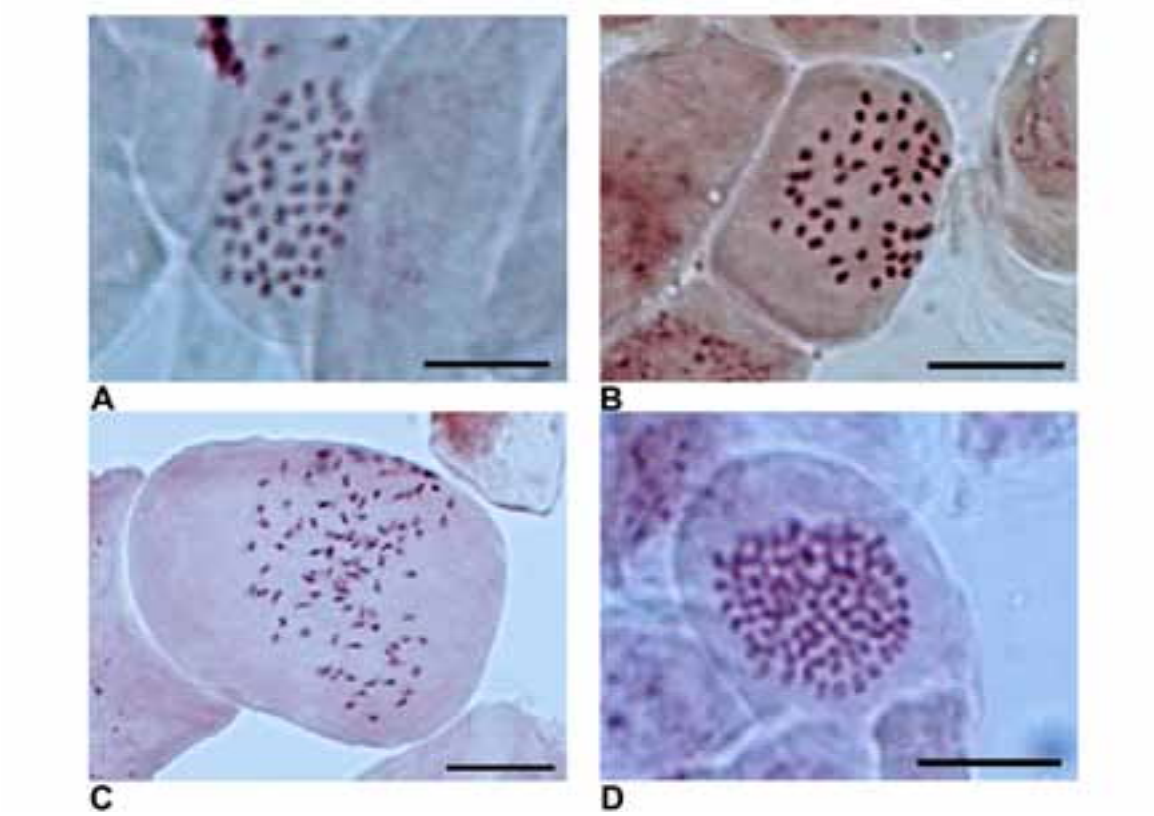

Figure 2. Chromosomes from root tip cells of diploid and tetraploid L. angustifolia. Photomicrographs are of metaphase cells from root tips of seedlings of diploid varieties Vera (A) , Egerton Blue (B), and rooted cuttings of tetraploid varieties C6/24 (C) and C3/2 (D), taken at x1000 magnification. The bar in D represents 10 µm.

Eight seedlings derived from the plant giving rise to the C6/24 sport were found to vary in their ploidy level. Two appeared to be triploid having a single G1 peak between diploid and tetraploid, suggesting they were derived from cross-pollination by nearby diploid varieties in the lavender collection. The remaining six seedlings appeared to be diploid, again indicating the original plant was mixoploid. In summary, out of approximately 100 plants from one experiment two plants were identified carrying tetraploid sports and one plant was identified which gave rise to polyploid seedlings.

Estimate of the *L. angustifolia* genome size

Whilst chromosome numbers in *L. angustifolia* varieties have been assessed previously, (reviewed by Upson and Andrews, 2004), absolute genome size or C-value has not been reported. To determine the size of the *L. angustifolia* genome we measured fluorescence of leaf nuclei stained with propidium iodide (PI), relative to the fluorescence of nuclei from reference plants of known genome size using flow cytometry. DAPI was not used for this purpose since it binds preferentially to AT base pairs and differences in GC/AT ratios of species can grossly affect genome size estimates (Dolezel *et al.*, 1992). We used a tomato variety 'Grosse Lisse' and parsley as references. The 1C values of these reference species

were reported to be 2.3 pg for parsley and 1.0 pg for tomato (Bennett and Leitch, 2005). To determine genome size we chopped leaf tissue and stained nuclei from different species separately because of interference in nuclear staining when we chopped the tissues together. We chopped and stained the test and reference tissue samples alternately and analysed by flow cytometry.

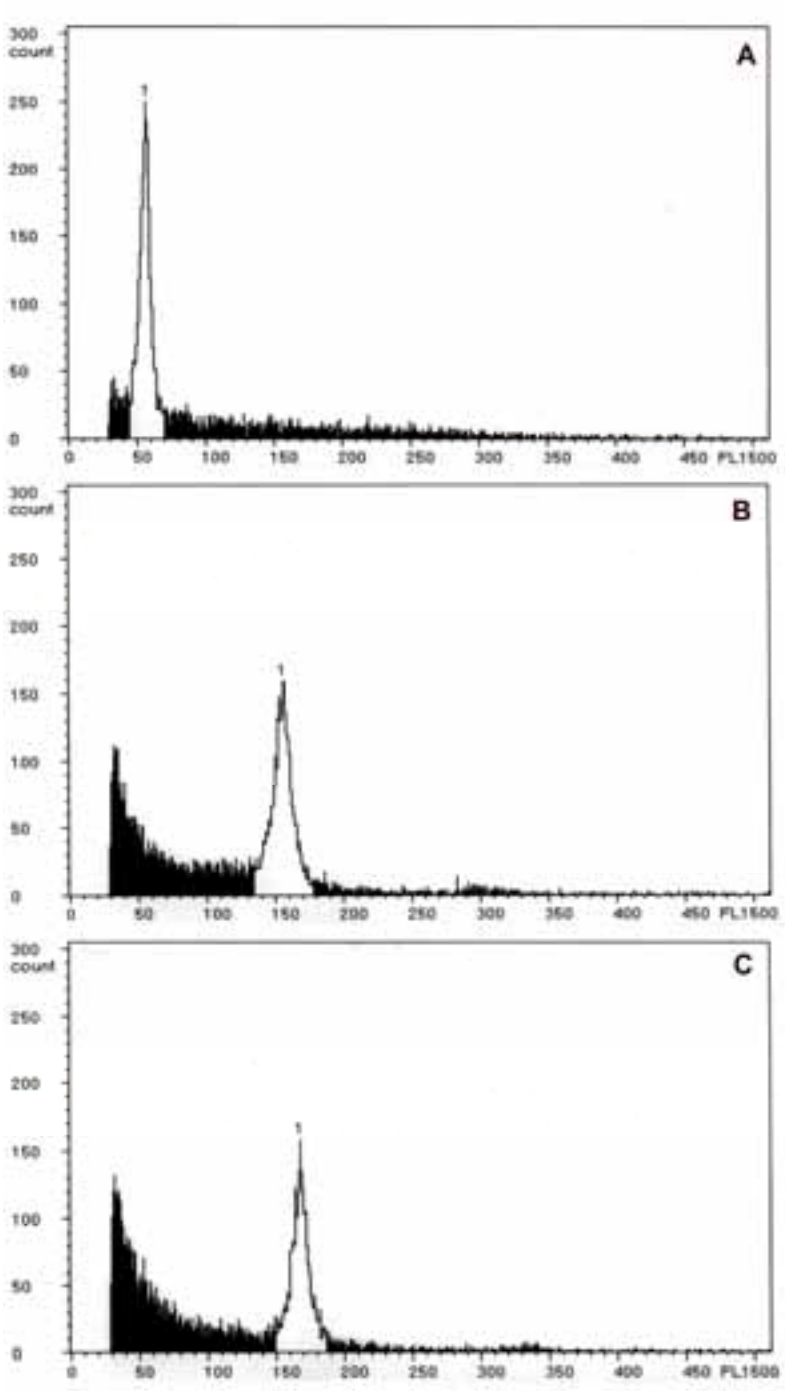

Figure 3. Flow cytometry of tetraploid and diploid L. angustifolia varieties DAPI fluorescence of nuclei (x axis) vs. number of nuclei counted (y axis) in variety C7/103, diploid control (**A**), C6/24 tetraploid (**B**) and C3/2 tetraploid (**C**).

Table 2. Characteristics of some diploid and tetraploid varieties of *L. angustifolia.*
Data shown are means with +/- 2SE in brackets.

L. angustifolia variety	Bud weight (mg)	Number of Whorls per Spike	Number of Flowers per spike	Peduncle thickness (mm)	Ten seed weight (mg)	Oil gland diameter (µm)	Stomatal guard cell length (µm)
Lady	4.56 (3.89-5.22)	4.20 (3.97-4.43)	27.7 (24.6-30.8)	1.14 (1.07-1.22)	4.11 (3.37-4.85)		
Lavenite Petite	7.33 (6.79-7.89)	5.65 (5.39-5.91)	38.2 (33.7-42.7)	1.55 (1.46-1.65)	5.83 (5.34-6.32)		
Hidcote	5.63 (5.06-6.19)	6.45 (6.18-6.72)	51.5 (44.0-59.0)	1.41 (1.33-1.48)	4.14 (3.78-4.50)		
Bee	6.22 (5.52-6.91)	9.90 (9.55-10.25)	77.8 (71.3-84.3)	1.72 (1.65-1.80)	6.49 (5.95-7.03)		
Imperial Gem	6.20 (5.60-6.80)	5.80 (5.52-6.08)	40.3 (36.1-44.5)	1.50 (1.42-1.57)	3.95 (3.58-4.32)		
Swampy						67.4 (66.0-68.7)	26.8 (25.9-27.7)
C7/103	6.07 (5.54-6.59)	5.75 (5.46-6.04)	48.5 (45.3-51.7)	1.50 (1.43-1.57)	3.99 (3.61-4.37)	64.1 (62.1-66.2)	27.1 (26.2-28.0)
C3/2	9.26 (8.34-10.17)	6.30 (6.04-6.56)	53.4 (49.3-57.5)	2.12 (2.01-2.23)	8.40 (7.20-9.60)	85.5 (83.9-87.2)	38.3 (37.2-39.4)
C6/24	11.64 (10.63- 12.65)	6.10 (5.81-6.39)	44.7 (40.1-49-3)	1.83 (1.73-1.92)	9.37 (8.34-10.40)	87.7 (85.3-90.1)	41.3 (40.2-42.3)

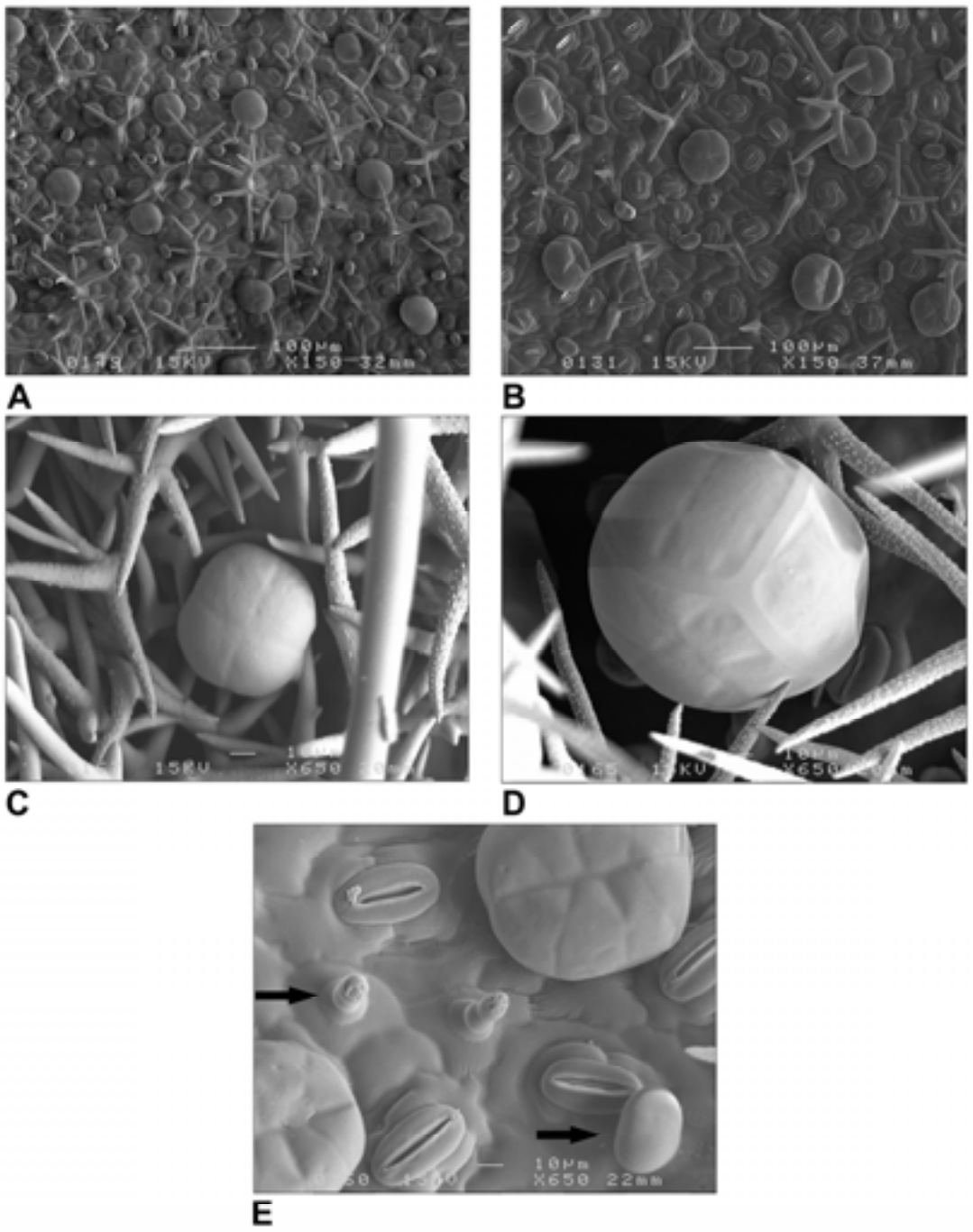

Figure 4. Scanning electron micrographs of diploid and tetraploid L. angustifolia. Images are of the lower surfaces of leaves the diploid variety C7/103 (A) and tetraploid variety C6/24 (B) at x150 magnification. Calyx surfaces of unopened flowers of C7/103 (C) and C6/24 (D) at x650 magnification. The arrows in (E) point to two types of capitate glandular trichomes on the abaxial surface of a leaf of C6/24.

Relative to tomato, C7/103 had a 1C value of 0.90 pg (+/- 0.07) and relative to parsley it was estimated to be 0.79 pg (+/-0.07). The 1C-value relative to tomato was probably the closer of the two estimates since the tomato genome size is closer to lavender being slightly greater. This preliminary estimate suggests *L. angustifolia* has a small to medium-sized genome for a plant, consistent with the observed size of chromosomes in metaphase spreads in root tips.

Characteristics of the tetraploid lavenders C3/2 and C6/24

Within approximately one hundred plants grown from seed, which were colchicine treated or controls from the same commercial batch of seed, considerable morphological variation was observed in flower colour, flowering spike size, peduncle length and growth habit. No two plants were observed to be identical. The tetraploid varieties C3/2 and C6/24 were originally selected as sports having visibly larger flowers in the hope that plants having larger flowers would have enhanced oil yield. To quantify this character the varieties C3/2, C6/24 and C7/103 were planted in our lavender collection and grown under field conditions, with irrigation. In their second flowering season flower material was collected from these and other *L. angustifolia* varieties for evaluation. The weight of individual flowers, number of whorls of flowers per spike, flower number per spike, peduncle thickness and seed weights were measured. Flower (bud) weight and seed weight were greater in the tetraploids when compared to all other *L. angustifolia* varieties assessed (Table 2). In both characteristics C6/24 was greater than C3/2 and bud weight and seed weight in C6/24 was over twice that of the variety 'Lady'. Interestingly, the number of whorls of flowers per spike did not differ between the tetraploids and other varieties measured with the exception of a variety 'Bee'. This variety had approximately a third more whorls per spike and a third more flowers per spike (Table 2). Visually the tetraploids appeared to have larger and thicker leaves and had measurably thicker peduncles (Table 2 and Figure 5).

Flowers, seeds, oil glands and stomata are all larger in *L. angustifolia* tetraploids

In other genera induced polyploids have often been screened by measuring stomatal guard cell length. We also measured stomatal guard cell length and oil gland diameter on the abaxial leaf surfaces of C3/2, C6/24, C7/103 and *L. angustifolia* 'Swampy' using SEM. Oil gland diameter and guard cell length in both tetraploids were just under one third greater than in the controls (Table 2 and Figure 4.). The term 'oil gland' used here refers to peltate glandular trichomes, commonly found on foliage and flowers of members of the Lamiaceae (Werker, 1993; Werker, 2000), which are thought to contain the bulk of essential oil (Hallahan, 2000). Peltate glandular trichomes were also present on the calyces of the tetraploid and diploid lavender flowers and were visibly larger on the tetraploids relative to controls (Figure 4). Other surface features we observed on lavender leaves and calyces were dendritic, complex, non-glandular trichomes and on leaves we observed two types of capitate glandular trichomes (Figure 4.).

Seeds were collected from C6/24 and C3/2 demonstrating that these plants were fertile. The F_1 plants resulting from these and another polyploid 'C6/26' were planted in the collection CSU 87-99 (except CSU95). None of these plants produced any seed and they are therefore infertile *L. angustifolia*. Preliminary genome sizing using flow cytometry has shown that all these F_1 plants had smaller genomes than the tetraploids and the majority are probably triploids resulting from cross fertilisation of the tetraploids by pollen from diploid plants in the collection. The phenotype of these F_1 plants was intermediate between tetraploids and diploids in terms of flower size and peduncle thickness (Figure 5). To begin evaluation of oil produced by the tetraploids oil was distilled from 400 to 900g of flower material of six *L. angustifolia* varieties including C6/24. This tetraploid produced nearly a third more oil for an equivalent weight of flowers than any of the five diploid varieties (Table 3).

Figure 5. Comparative photograph of L. angustifolia floral spikes from polyploid and diploid varieties. Left to right the varieties are C6/26/B (CSU87) polyploid, C6/24/K (CSU95) diploid, Hidcote (CSU8) diploid, Bee (CSU57) diploid, Lavenite Petite (CSU30) diploid, C7/103 (CSU70) diploid, C3/2 (CSU71) polyploid, C6/24 (CSU72) polyploid, C3/2/3 (CSU98) polyploid.

In terms of composition C6/24 had a very similar profile to other *L. angustifolia* oils and whilst none of the oils analysed fitted the ISO standard exactly most were close perhaps reflecting growing conditions in Wagga Wagga or distillation technique (Table 3.). In addition to chemical analysis a professional perfumer was contracted to evaluate these six oils and the evaluation was performed blind. The perfumer was given a sample of the six oils labelled 1-6 and asked to evaluate these and specifically asked to decide whether they were perfume quality or not. Surprisingly whilst all oils marginally failed GC analysis the oils from *L. angustifolia* Bee and C6/24 were determined to be of perfume quality, tetraploids therefore can potentially produce high quality oils similar in chemical compositions to their diploid counterparts.

Table 3. Composition of essential oils distilled from *L. angustifolia* varieties in the CSU lavender collection. Limits on components are shown for International Standards Organisation (ISO) and Australian standard *L. angustifolia* oils are shown. Figures in bold are out of the range of the ISO standard.

Component	ISO Standard French	Standard Australian	CSU66 Egerton Blue	CSU57 Bee	CSU8 Hidcote	CSU30 Lavenite Petite	C7/103 CSU70	C6/24 CSU72
limonene	0.0- 0.5	0- 0.5	**1.1**	**1.0**	**1.2**	**0.9**	**0.7**	**0.6**
β-phellandrene	0.0- 0.5	0 - 0.5	**0.8**	**1.1**	**1.0**	0.3	0.4	0.2
cis-β-ocimene	4.0 - 10.0	3.0 - 9.0	5.1	9.0	7.3	4.0	**25.5**	**3.1**
3-octanone	0.0- 2.0	2.0 - 5.0	0.2	0.2	0.2	0.2	0.2	0.1
1,8 cineole	0 - 1.0	0 - 1.0	0.8	0.8	**1.3**	**1.2**	**1.0**	**1.1**
trans-β-ocimene	1.5 - 6.0	0.5 - 1.0	1.6	3.2	5.4	1.9	4.1	**6.1**
linalool	25 - 38	25 - 38	25.4	28.6	**18.3**	21.9	**15.6**	33.0
camphor	0.0- 0.5	0.0 - 0.5	0.5	0.5	**0.7**	**0.8**	0.4	**0.6**
terpinen-4-ol	2.0 - 6.0	1.5 - 6.0	**1.1**	3.5	3.3	4.7	4.4	**1.6**
lavandulol	>0.3	>0.3	0.7	0.6	0.5	1.0	0.3	0.7
α-terpineol	0 - 1.0	0 - 1.0	0.3	0.2	0.3	0.2	0.2	0.2
linalyl acetate	25 - 45	25 - 45	41.6	31.2	36.9	36.5	27.2	38.4
lavandulyl acetate	>2.0	>1.0	7.5	4.3	7.4	8.7	4.8	2.5
α-santalene			1.3	0.9	1.6	1.6	0.8	0.9
β- caryophyllene			1.2	4.0	2.7	2.9	2.8	2.9
trans- β-farnesene			2.0	0.7	0.7	2.7	1.9	0.6
octen-3-ol			0.5	0.3	0.7	0.7	0.0	0.3
octen-3-yl acetate			0.4	0.7	1.1	1.4	1.0	0.4
borneol			0.5	0.5	0.5	0.1	0.6	0.4
Percentage oil (v/w)			**0.50**	**0.66**	**0.46**	**0.69**	**0.53**	**0.86**
Total %			**92.6**	**91.3**	**91.1**	**91.7**	**91.9**	**93.7**

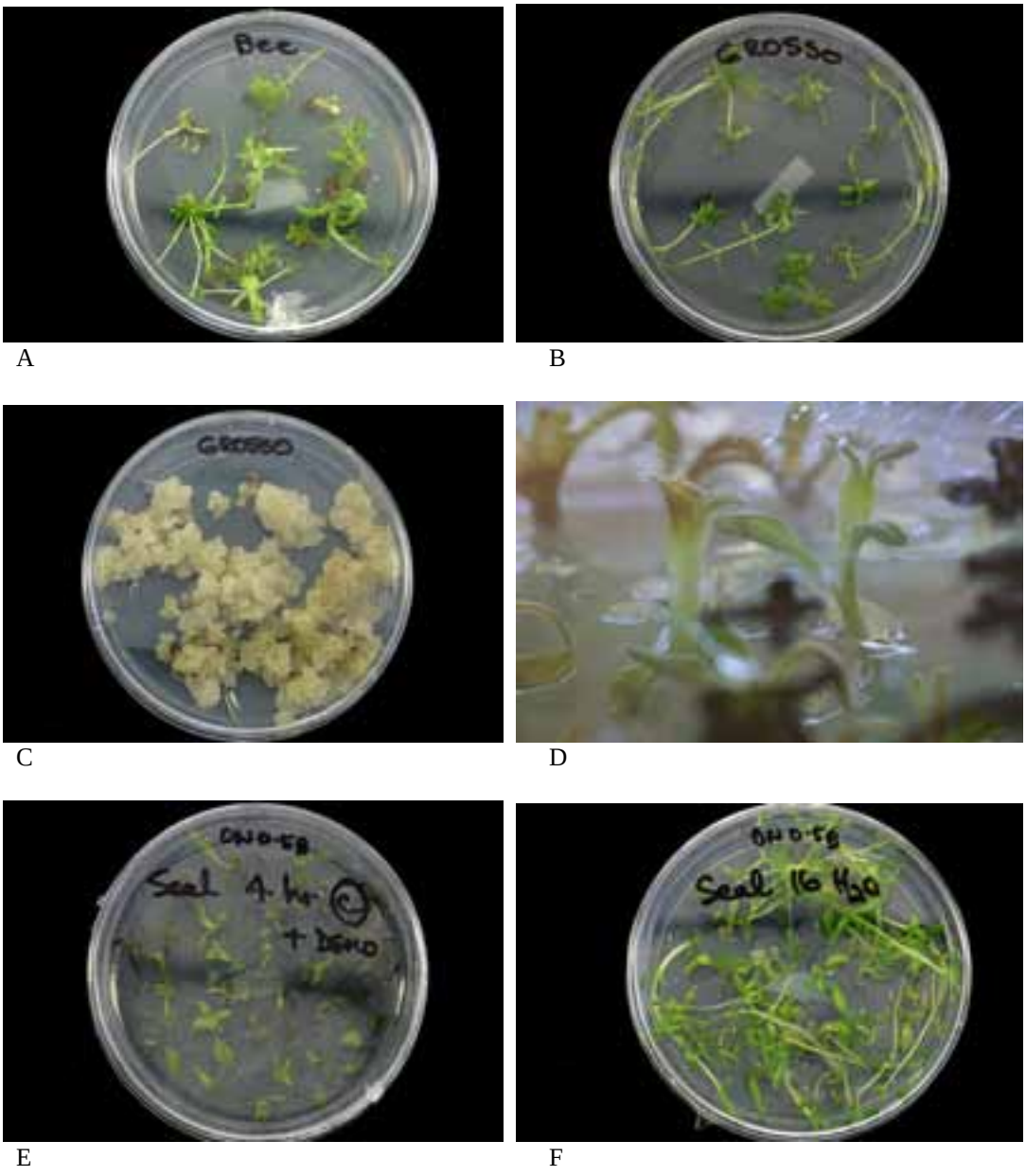

Figure 6. Tissue culture and induction of polyploidy in vitro.

Shoot cultures of *L. angustifolia* Bee (A), *L.* x *intermedia* Grosso (B), Callus culture of *L x intermedia* Grosso (C). Shoots 2 weeks after treatment with 0.1 % colchicine, (D) note the fattening of the stems at the top of the shoot . Cultures of *L x intermedia* Seal after treatment with 0.1% colchicine for 4 hours (E) or controls treated with water for 16 hours (F). Plates were photographed two weeks after treatment. Note slow growth and recovery of colchicine treated shoots.

Production of polyploid *L. angustifolia* and *L.* x *intermedia* by colchicine treatment of shoot cultures, whole plants, and cuttings.

Shoot cultures of *L. angustifolia* varieties Bee, Swampy and *L.* x *intermedia* varieties of Grosso and Seal were initiated using standard MS medium and we determined optimum concentrations of cytokinin (benzylaminopurine, BAP) and auxin (napthalene acetic acid, NAA) using factorial experiments. In addition we determined what concentrations of hormones were optimum for callus induction in the same experiments. For shoot culture initiation and maintenance we found 0.5 mg L^{-1} BAP without an auxin to be best for all the varieties. For callus culture we found 5 mg L^{-1} NAA and 1 mg L^{-1} BAP the best overall (Figure 6).

L. angustifolia Bee tetraploids

To induce polyploidy in shoots, multiplied shoots were removed from the maintenance medium and placed in a 0.1% colchicine solution for 4, 8 and 16 hrs. Plants were washed, returned to medium without colchicine to determine the number of explants surviving the treatments. The optimum time for colchicine treatment was 16 hrs and survival was approximately 10-30%. In general, after colchicine treatment, shoots in culture took several months to recover and initially grew poorly both in culture (Figure 6) and when transferred to pots. Following colchicine treatments shoots were sub-cultured at least twice and then placed on half strength MS medium with 1% sucrose without hormones to induce root formation. Roots were visible 3-4 weeks after transfer. Colchicine produced similar effects on in vitro grown shoots as it did on seedlings with stems fattening and leaves of treated explants enlarging (Figure 6). Plantlets were then acclimatised to glasshouse conditions and potted up. Plants were observed for the presence of larger flowers on treated plants relative to controls treated with water and none were observed except for four Bee plants. These plants were shorter than normal and had malformed spikes with larger flowers. Flow cytometry using DAPI to stain nuclei revealed that two of these plants had genome sizes greater than control Bee plants but smaller than the expected tetraploids. Two plants had close to double the genome size and seed was collected from one of these indicating it was fertile and tetraploid. These plants were not examined further as they would be unsuitable for oil production due to their reduced overall size and poor form.

Polyploid *L. x intermedia*

Because no physical differences in these initial plants were observed to indicate induction of polyploidy was successful we examined only the *L.* x *intermedia* plants further and examined these for production of viable seed. Production of polyploids in other hybrids often results in restoration of fertility and production of seed. We found both Grosso and Seal plants, which were either whole plants, or derived from cuttings treated with colchicine, or explants which were treated in vitro, produced seed whereas none of the untreated control plants from any experiment produced seed.

In one experiment in which shoot cultures were treated with 0.1% colchicine for 16 hr, 5 out of 21 Grosso derived plants produced seed with one plant producing 80 seeds. In the same experiment 29 out of 72 Seal derived plants produced seed with one plant producing 90 seeds. Some plants derived from cuttings treated with colchicine also produced seed but survival of cuttings treated with colchicine was less than 10%. Seeds were also recovered from whole Seal plants which had 0.1% colchicine applied to terminal nodes.

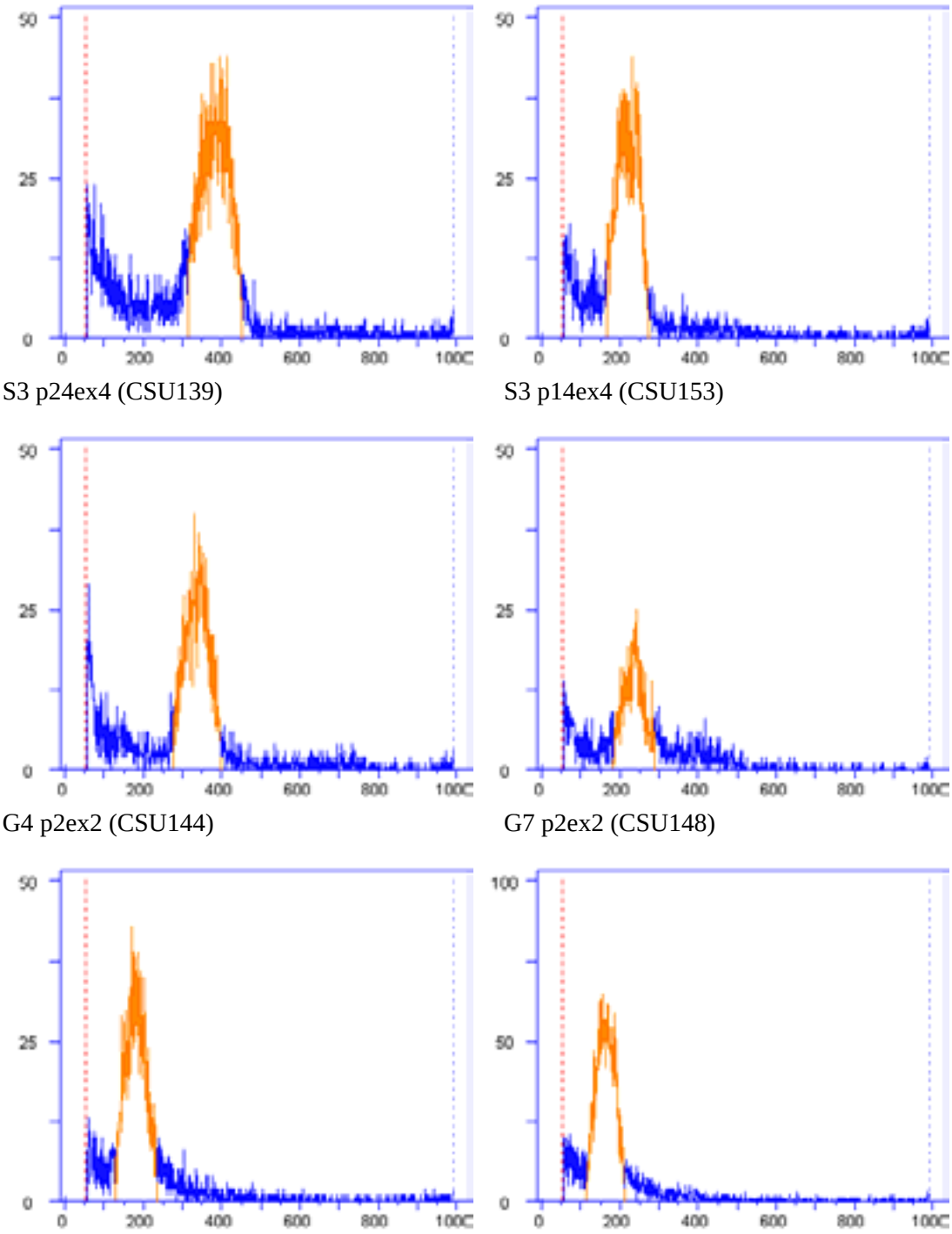

Figure 7. Flow cytometry of polyploid and diploid L. x intermedia varieties.
DAPI fluorescence of nuclei (x axis) vs. number of nuclei counted (y axis) in the varieties shown.

A

B

Figure 8. Comparative photograph of L. x intermedia floral spikes from polyploid derivatives of Grosso and Seal.

A. Left to right the varieties are G4 p2ex2 (CSU 144) polyploid, Grosso (CSU3) diploid, Abrialii (CSU22) diploid, Seal (CSU48) diploid.

B. Left to right the varieties are S3 p14ex4 (CSU153) polyploid, Grosso (CSU3) diploid, Abrialii (CSU22) diploid, Seal (CSU48) diploid.

Seeds from Grosso and Seal plants were visibly larger than those obtained from tetraploid *L. angustifolia*. In September 2004 seeds from *L.* x *intermedia* Grosso and Seal plants were germinated in Petri-dishes and plants were grown in pots until large enough to be transferred to the field. These were then planted in the CSU collection in August 2005 and they flowered in December 2005/ January 2006.

To determine the ploidy levels of these plants initially we counted chromosomes in *L. latifolia* and *L.* x *intermedia* Grosso. In both case and as in *L. angustifolia* the number was 50. We then counted chromosomes in young root tips obtained from the putative polyploid *L.* x *intermedia* plants. It was not possible to get accurate chromosome counts on any of these because they were two numerous but all had more than 50 with most having around 70-80 chromosomes, suggesting these plants were not tetraploid. Flow cytometry using DAPI to stain nuclei confirmed this finding (Figure 7) and showed all the plants had genome sizes intermediate between diploid and tetraploid. One plant, a derivative of Seal, had a genome size close to tetraploid (CSU140). These plants produced substantial flowers in their first season however oil was not distilled as we saved flowers to harvest for seed. None of these plants produced any seed and therefore the plants are infertile. Morphological variation was observed between plants derived from each variety and therefore these were genetically distinct. The plants were extremely vigorous and produced very large flowering spikes with long thick peduncles, much larger than those of either Grosso or Seal (Figure 8). Flowering spikes were weighed from four varieties, two polyploids derived from Seal and two from Grosso and these were compared to spikes taken from various common *L.* x *intermedia* and *L. angustifolia* varieties. The spike weights from the polyploid *L.* x *intermedia* varieties were approximately twice that of most control *L.*x *intermedia* varieties. The polyploid G4 p2ex2 (CSU144) derived from Grosso had a mean spike weight nearly three time that of the parent and flowers are obviously larger (Table 4).

Table 4. Average floral spike weight for some diploid and polyploid varieties of *L. angustifolia* and *L.* x *intermedia.* Data shown are the means with +\- SE in brackets and n was between 10 and 15 for all varieties. The numbers (CSU 8 etc) refer to the particular accessions within the CSU collection..

Species/ hybrid	Variety	Spike weight (g)
L. angustifolia		
	Hidcote (CSU 8)	0.49 (0.35-0.64)
	Bee	0.69 (0.50-0.87)
	Lavenite Petite	0.49 (0.36-0.63)
	C7/103	0.36 (0.25-0.47)
	C6/24	1.13 (0.77-1.49)
	C3/2	1.10 (0.78-1.41)
	C2/6/B	1.28 (0.91-1.64)
	C2/4/K	0.91 (0.66-1.16)
	C3/2/3	0.75 (0.53-0.96)
L.* x *intermedia		
	G4 p2ex2 (CSU144)	1.66 (1.20-2.12)
	G7 p2ex2 (CSU 148)	1.56 (1.11-2.01)
	S3 p24ex4 (CSU 139)	1.00 (0.70-1.30)
	S3 p14ex4 (CSU153)	1.76 (1.27-2.24)
	Abrialli	0.93 (0.66-1.19)
	Seal	0.91 (0.63-1.18)
	Hidcote Giant	0.89 (0.64-1.15)
	Impress Purple	0.83 (0.60-1.06)
	Grosso (CSU 20)	0.62 (0.44-0.80)

Discussion

L. angustifolia polyploids.

The first objective of the project was to produce polyploid *L. angustifolia* varieties in the hope that oil yield from these would be improved over traditional ones. By germinating seed in the presence of colchicine we produced two tetraploid varieties C6/24 and C3/2. These were produced by vegetative propagation of sports found on surviving seedlings. We demonstrated that these were tetraploid and had approximately 100 chromosomes whereas four common varieties (diploids) had 50 chromosomes by direct counts in root tip cells. Upson and Andrews (2004) reviewed estimates of chromosome number in *L. angustifolia* and these were between 2n=36 and 2n=54 with most being around 50. Flow cytometry of leaf nuclei confirmed tetraploidy in C6/24 and C3/2 and demonstrated 12 common varieties of *L. angustifolia* were all diploid. Of the two tetraploids, C6/24 was the most vigorous and produced the largest flowers. Preliminary GC and olfactory analysis of oil distilled from flowers demonstrated C6/24 produced perfume quality oil at greater yield per mass of flower material.

Both tetraploids were fertile and produced seed however seedlings produced from these plants were themselves infertile and had reduced genome sizes, estimated by flow cytometry. There are two main possible explanations. Firstly, *L. angustifolia* is a predominantly self-fertilising species and the genomes of the tetraploids were unstable resulting in loss of chromosomes at some point prior to seed formation. Secondly, *L. angustifolia* maybe a predominantly cross-pollinating species and C6/24 and C3/2 were fertilised by nearby diploid varieties in the collection giving rise to triploids. Triploids would be expected to be infertile. The latter explanation is favoured since if the genomes were unstable then variation might have been observed in C6/24 and C3/2 in propagated sports and this was not the case. Whilst the progeny of C6/24 and C3/2 are not tetraploid they are vigorous plants, larger than diploids and should be superior germplasm for essential oil production.

During this project it was discovered that this was not the first attempt to use colchicine to induce polyploidy in lavender. A Bulgarian group in the 1980's did some experiments and Raev *et al.* (1996) summarised their findings. They used 100 and 250mg L^{-1} colchicine to soak lavender seed prior to germination and the ploidy of resultant plants was assessed only visually by leaf and flower size and also by morphology of pollen. No estimates of chromosome number were made either in the controls or plants generated so the ploidy level of these remains unknown. None of the lines they generated has been reported elsewhere or is commercially available, however they found that pollen from polyploids was larger and had eight furrows rather than six found in common diploid varieties. Similarly, we found pollen grains from C3/2 and C6/24 had eight furrows and C7/103 had six (data not shown).

Using flow cytometry we estimated that *L. angustifolia* has a haploid genome size of 0.9 pg. This is a small to medium sized genome for a plant. This is the first estimate of genome size of any species in this genus.

We cultured shoots of two varieties of *L. angustifolia* (Bee and Swampy) in vitro and treated these with colchicine. None of the surviving plants had obviously larger flowering spikes however four polyploids of Bee were obtained and verified as such by flow cytometry. These plants had smaller stature but larger flowers (buds), but because of the smaller stature were unsuitable for oil production. No Bee or Swampy plants were identified with improved phenotypes so polyploids of these varieties were not pursued.

L. x *intermedia* polyploids

Lavandins are sterile however a single report in the literature by Vinot and Bouscary (1971) reported a fertile lavandin that spontaneously occurred in the field. Chromosome estimates in the sterile lavandin and in the fertile derivative were 50 and 100 respectively suggesting polyploidy can occur naturally at low frequency in the wild within this genus. The fertile lavandin is no longer in cultivation or in any collection.

In this project we cultured two common varieties of *L.* x *intermedia* (Grosso and Seal) and treated shoots with colchicine. Surviving plants were visually assessed for larger flowering spikes which were

not observed. To further determine if polyploidy had been induced we examined seed production from these plants and found several which produced seed indicating that conversion to tetraploidy had occurred. Plants were grown from some of these seeds and the ploidy level of this F_1 generation was assessed by chromosome counting and flow cytometry. Polyploids were generated however the ploidy level of all of these was intermediate between diploid and tetraploid. This was similar to the F_1 generation of the tetraploid *L. angustifolia* C6/24 and C3/2 with most plants being approximately triploid. In addition, phenotypic variability was also observed in these plants and the F_1 *L.* x *intermedia* plants produced no seeds. There are again two main possible explanations for this as with the *L. angustifolia*. They are that the plants self-pollinate and chromosomes are lost at some point or alternatively that they were cross-pollinated by a diploid species like *L. angustifolia* resulting in triploid progeny. It is possible that the intermediate ploidy level results from both processes and further work will be necessary to determine which of these actually occurs and understand the breeding systems operating in lavenders.

Characteristics of these F_1 polyploid *L.* x *intermedia* plants are that they are larger and more vigorous than the parents Grosso and Seal and they have larger flowering spikes with longer peduncles. These should be excellent plants for oil production. If they are resultant from crosses of the tetraploid *L.* x *intermedia* plants and diploid *L. angustifolia* then it is likely that their oil quality will be more like *L. angustifolia* and therefore much better than typical Lavandin oils. Oil quality will be assessed in the coming season.

Conventional *L.* x *intermedia* varieties like Grosso are more vigorous than the parent species *L. angustifolia* and *L. latifolia* because of hybrid vigour. If the *L.* x *intermedia* polyploids are resultant of crosses of tetraploid Grosso and Seal plants with *L. angustifolia* then further hybrid vigour would be expected and is perhaps what we observed.

Production of doubled haploid *L. angustifolia*

One of the main objectives of this project was to culture *L. angustifolia* microspores or whole anthers to generate haploid plants. Once produced treatment with colchicine was intend to produce doubled haploids. These plants should be completely homozygous and could be grown from seed with little variation between plants. During this project considerable time and effort was spent on attempting to culture microspores and whole anthers. Despite using plant material at different developmental stages, using several different media and hormone combinations we were unsuccessful in obtaining any haploids or in seeing any development of microspores in culture at all. Since haploid plants could not be produced this objective was abandoned.

Implications

Two of the three objectives of this project have been achieved and we have produced polyploid varieties of *L. angustifolia* and *L.* x *intermedia*. The next stage will be to test yield and quality of oils produced by these plants and examine how they grow in various environments. These plants should produce higher yields of oil per hectare at similar quality or better than current varieties. Field trials are a long term objective and perhaps should be carried out in conjunction with growers through an industry organisation such as The Australian Lavender Growers Association (TALGA). There are several candidate *L. angustifolia* and *L.* x *intermedia* varieties worth assessing. These are *L. angustifolia* C6/24, C3/2, at least two of the polyploids (probable triploids) and C6/24/K (a diploid produced during this project). In addition two varieties of polyploids derived from Grosso and two from Seal should be trialled. The industry partner is also interested in propagating *L. angustifolia* C7/103 (diploid) as an ornamental variety. This amounts to a minimum of 10 varieties worth protecting, however the industry partner Larkman Nurseries cannot afford to pay for Plant Breeders Rights applications for all these so it is difficult to know how best to proceed. It is necessary to protect and disseminate the germplasm we have produced to maximise benefit on investment by RIRDC, CSU and Larkman Nurseries. One option is not to obtain PBR at all and allow all varieties to be propagated and trialled freely.

Recommendations

RIRDC might provide advice on how best to proceed with protecting, marketing and disseminating these plants through Larkman Nurseries and abroad. The way this is eventually done will affect royalties and becomes particularly important if PBR's are to be obtained abroad.

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Appendix

Charles Sturt University Lavender Collection Key

Accession Number (CSU)	Species	Variety	Description
1	L. dentata	?	White flowered
2	L. stoechas	Fairy Wings	
3	Lxintermedia	Grosso	
4	L. stoechas	Pukehou	
5	L. dentata	Monet	
6	Lxheterophylla	?	
7	L. dentata	var candicans	grey leaved
8	L. angustifolia	Hidcote	
9	L. stoechas	Lavender lace	
10	L. dentata	Linda Ligon	Variegated
11	L. latifolia		
12	L x intermedia	Old English	
13	L. angustifolia	Lady	
14	L x intermedia	Impress Purple	
15	L stoechas	Evelyn Cadzow	
16	L x lanata	Joan Head	
17	L viridis		
18	L x intermedia	Alba	White flowered
19	L. canariensis		Fern leaf
20	L x intermedia	Grosso	
21	L. stoechas	Kew Red	
22	L x intermedia	Abrialli	
23	L x intermedia	Miss Donnington	
24	L x allardii	Jurat Giant	large leaved allardii
25	L. stoechas	Willowbridge Snow	
26	L. stoechas	Avonview	
27	L. stoechas	Sugar plum	
28	L. lanata		Woolly lavender
29	L. angustifolia	Princess Blue	
30	L. angustifolia	Lavenite Petite	
31	L. angustifolia	Folgate	lot number (Muntons 1450)
32	L. buchii var buchii		
33	L. stoechas	Marshwood	
34	L. stoechas	Regal Splendour	
35	L. latifolia		Plant Number 8
36	L. angustifolia	Munstead	
37	L. stoechas subsp Luisieri	Tickled Pink	
38	L x intermedia	Yuulong	
39	L. stoechas	Plum	
40	L. angustifolia	Grey Lady	
41	L. stoechas	Ploughmans Purple	
42	L. angustifolia	?	White flowered
43	L. stoechas	Major	

44	L. angustifolia	Hidcote	
45	L. angustifolia	Royal Purple	
46	L. angustifolia	Blue Mountain	
47	L. dentata	Royal Crown	
48	L x intermedia	Seal	
49	L x allardii		Labelled Mitchum lavender
50	L. angustifolia	Twickle Purple	
51	L. angustifolia	Bosisto	
52	L. angustifolia	Nana atropurpurea	
53	L. angustifolia	Egerton Blue	
54	L. angustifolia	Irene Doyle	
55	L. angustifolia	Ashdown Forest	From Muntons UK
56	L x allardii	Crestwood	Shorter peduncle cultivar
57	L. angustifolia	Bee	
58	L. angustifolia	Pacific Blue	
59	L x allardii		
60	L. angustifolia	Rosea	
61	L. stoechas	Snowball	
62	L. angustifolia	Hidcote Pink	
63	L. stoechas	Winter Purple	
64	L. angustifolia	C9 variegated	
65	L. angustifolia	Swampy	
66	L. angustifolia	Egerton Blue	Plant Number 3
67	L x allardii		
68	L x intermedia	Grosso	CSU/TC from 1 plant
69	L x intermedia	Intermedia	Plant 1 from Muntons
70	L. angustifolia	Eunice Wilkcoxson	
71	L. angustifolia	C3/2	Tetraploid
72	L. angustifolia	C6/24	Tetraploid
73	L. viridis		
74	L. angustifolia	Maillet	
75	L. stoechas	Helmsdale	
76	L. angustifolia	Avice Hill	
77	L. stoechas	Fat head	
78	L. angustifolia	Imperial gem	
79	L x intermedia	Vera	
80	L x intermedia	Super	
81	L. stoechas	Darling Crown	
82	L. angustifolia	Trileaf	From polyploidy expt
83	L. rotundifolia		From seed (JH)
84	L. pinnata		From seed (JH)
85	L. hybrid ?	Sidonie	Front garden home
86	L. dentata	LC'02	given at conference
87	L. angustifolia	C6/26/B	Polyploid
88	L. angustifolia	C3/2/4	Polyploid
89	L. angustifolia	C6/24/2	Polyploid
90	L. angustifolia	C6/26/C	Polyploid

91	L. angustifolia	C6/26/A	Polyploid
92	L. angustifolia	C6/24/8	Polyploid
93	L. angustifolia	C3/2/1	Polyploid
94	L. angustifolia	C6/24/9	Polyploid
95	L. angustifolia	C6/24/K	Diploid
96	L. angustifolia	C3/2/6	Polyploid
97	L. angustifolia	C3/2/2	Polyploid
98	L. angustifolia	C3/2/3	Polyploid
99	L. angustifolia	C6/24/4	Polyploid
100	L. dentata (hybrid)	D2	
101	L. stoechas	Beverly	
102	L. stoechas	Somerset Mist	
103	L. stoechas	Merle	
104	L x intermedia	Bridestowe	
105	L x intermedia	Fred Boutain	
106	L. dentata	Paleface	
107	L. x intermedia	Sussex	
108	L. angustifolia	Miss Katherine	
109	L. x lanata	Richard Gray	
110	L. x intermedia	Lullingstone Castle	
111	L. angustifolia	ssp. Angustifolia	
112	L. angustifolia	Miss Muffet	
113	L. angustifolia	Little Lottie	
114	L. x intermedia	Sachet	
115	L. x intermedia	Hidcote Giant	
116	L. angustifolia	Sarah	
117	L. x intermedia	Bogong	
118	L. stoechas	Pastel Dreams	
119	L. x intermedia	Dutch Group	
120	L. angustifolia	Coconut Ice	
121	L. dentata	Ploughman's Blue	
122	L. x intermedia	Wilson's Giant	
123	L. angustifolia	Blue Scent	
124	L x allardii	Derwent Grey	
125	L. stoechas hybrid	Henry Dunant	
126	L. x intermedia	Merriwa Mist	
127	L. stoechas	Bella Purple	
128	L. stoechas	Bella Pink	
129	L. x intermedia	Warburton's Silver Edge	
130	L. stoechas	Willowbridge White	
131	L. x intermedia	Bibbenluke	
132	L. x intermedia	Chaix	
133	L. dentata	Targét	
134	L. x intermedia	Arabian Knight	
135	L. stoechas	Gurli	
136	L. dentata	Mt Lofty	
137	L x intermedia S1(p24ex4)	Seal seedling from p24 exp 4 sown 29/7/04	Polyploid
138	L x intermedia S2(p24ex4)	Seal seedling from p24 exp 4 sown 29/7/04	Polyploid

139	L x intermedia S3(p24ex4)	Seal seedling from p24 exp 4 sown 29/7/04	Polyploid
140	L x intermedia S4(p24ex4)	Seal seedling from p24 exp 4 sown 29/7/04	Polyploid
141	L x intermedia S5(p24ex4)	Seal seedling from p24 exp 4 sown 29/7/04	Polyploid
142	L x intermedia G1(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
143	L x intermedia G2(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
144	L x intermedia G4(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
145	L. stoechas	Wine red	Polyploid
146	L x intermedia G5(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
147	L x intermedia G6(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
148	L x intermedia G7(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
149	L x intermedia G8(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
150	L x intermedia G9(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
151	L x intermedia G10(p2ex2)	Grosso seedling from p2 exp 2 sown 6/8/04	Polyploid
152	L x intermedia S1(p14ex4)	Seal seedling from p14 exp 4 sown 29/7/04	Polyploid
153	L x intermedia S3(p14ex4)	Seal seedling from p14 exp 4 sown 29/7/04	Polyploid
154	L. angustifolia	Fiona English	
155	L. angustifolia	Boston Blue	

Improvement of Lavender Varieties by Manipulation of Chromosome Number

RIRDC publication number 08/200

by Dr. Nigel Urwin

Australia produces less than 1% of the world's oil and imports approximately 90% of lavender oil consumed. Climatically there is huge potential for Australia to become a key world oil producer and it is hoped provision of this new germplasm to Australian lavender growers will improve the economics of oil production and encourage broad-acre production of high quality lavender and lavandin oils.

This report describes the development of improved varieties of lavender (L. angustifolia) and lavandin (L. x intermedia) specifically for commercial oil production. Using conventional propagation and micro-propagation techniques together with treatment of plant tissue with the mitotic spindle inhibitor colchicine, chromosome numbers in these plants were increased.

This report describes the preliminary morphological, oil quality and quantity analysis from some of the plants produced.

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