

# CONTROLLING BITTER-SEED CONTAMINATION IN THE NSW *LUPINUS ALBUS* INDUSTRY

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## ABSTRACT

**The Australian *Lupinus albus* L. industry relies on being able to supply 100% low-alkaloid seed for animal feed and for human snack food. In 2000 it was discovered that some seed lots of commercial, sweet (low-alkaloid) cultivars in New South Wales were contaminated with bitter seeds (high alkaloid), mainly at low levels < 2% but up to a maximum of 13%. *Albus* lupin is partially outcrossing (~8%), and bitter plants have higher fitness, so that without corrective action, the contamination was projected to increase over time. An industry-wide plan was developed to help growers detect and eliminate contaminated seed lots. The main detection method used was a UV lamp which causes bitter seeds to fluoresce so that they can be easily counted and seed lots with contamination discarded. The aim was to reduce commercial contamination until new, 100% sweet cultivars were produced in order to replace the old contaminated stock. More recently, an industry wide education campaign has been conducted to ensure that new, high-yielding and disease-resistant cultivars are not contaminated by admixture or pollen movement from older contaminated cultivars.**

## KEYWORDS

alkaloid content, white lupin, outcrossing

## INTRODUCTION

The Australian *Lupinus albus* L. industry relies on being able to supply 100% low-alkaloid seed for animal feed and for human snack food. The Food Standard for alkaloid content in lupins is 0.02% (w/w) (ANZFA, 2008). The Australian Receival Standard regarding bitter seed contamination in *albus* lupins (NACMA, 2008) is four bitter seeds per 400 g, equivalent to 1 seed in 286 (assuming an average *albus* seed size of 0.35 g) or 0.35% w/w. In 2000 it was discovered that some NSW seed lots of commercial, sweet (low-alkaloid) cultivars in New South Wales were contaminated up to 13% with bitter seeds (high alkaloid), well above all acceptable levels. In addition, two cultivars released in 1998 (Magna and Minibean) were shown to be contaminated and were withdrawn from commerce, and a third potential new release (75B09-2-3) was also contaminated at a low level (1 in 12,000 seeds) and was abandoned.

This paper describes the response of industry to the detection of bitter seed contamination in commercial seed lots. This included a survey of commercial seed lots to determine how widespread the problem was, a modelling exercise to describe a number of scenarios moving forward, and the development of an industry plan to protect new releases of 100% sweet, low-alkaloid cultivars from future contamination.

## MATERIALS AND METHODS

### SURVEY OF COMMERCIAL SEED LOTS

Seed samples for testing were received from all lupin-growing areas of NSW.

*Albus* growers were asked to voluntarily send in 2 kg samples (~5,700 seeds) from all seed lots intended for sowing (Luckett, 2007a). Testing was free and confidential, and a detailed result certificate was produced for the grower (and/or their agronomy advisor).

### TESTING METHODS

Available testing methods for alkaloid content in lupins include: Dragendorff reagent (usually impregnated onto filter paper and used to test petiole sap), iodine test of cut seed or seed leachate, UV lamp of whole seed, or wet chemistry of ground seed samples. Each method has its strengths and weaknesses. Wet chemistry was routinely used in Australian breeding programs prior to 2000 but was not designed to detect low-frequency seed contamination. We found Dragendorff testing to be too insensitive and subjective. In 2000 we settled on UV testing at 365 nm wavelength, plus iodine testing for ambiguous cases. Seed lots were examined in the dark then weighed and counted for seed size, and any suspect (putative bitter) fluorescing seeds at low frequency were tested by iodine. For the iodine test, single seeds were soaked overnight at room temperature in 2.5 mL of distilled water. Next day, 1 drop of saturated iodine solution (2.8 g KI plus 2 g I dissolved in 100 mL) was added to the leachate. A brown precipitate was immediately formed with bitter seeds, while the colour remained yellow for sweet seeds.

Bitter seeds fluoresce under UV light of wavelength 365 nm, and a single seed can easily be detected among thousands of sweets (although it is tedious). However, UV doesn't detect heterozygous zygotes (caused by outcrossing) inside a sweet maternal seed coat. Consequently, UV always underestimates the actual bitter plant frequency using seeds from plants grown in the field. UV can be used successfully to eliminate bitterness if all plants are grown in containment (i.e. pollinators are excluded and there is no outcrossing) and the testing is conducted for a minimum of two consecutive generations. This is the approach used in Australian breeding programs where testing is essential because bitter germplasm is often used as a common source of other important genes (e.g. disease resistance).

UV testing is followed up by iodine testing of individual seeds in two circumstances: 1) where the seed fluorescence is marginal; or 2) where a commercial seed lot is contaminated at a very low frequency and we want to be absolutely sure that the suspect seeds are bitter (i.e. to minimise any false positive reporting). In practice, all suspect seeds from our UV testing have turned out to also be positive high-alkaloid with iodine.

#### MODELLING BITTER FREQUENCY

As a result of the survey of commercial seed lots it appeared the wild-type, high-alkaloid, dominant allele (*Pauper*) had escaped into commerce. It was not definitively determined how the *Pauper* allele had escaped, nor where from. It was decided in the 1980s that all Australian *L. albus* cultivars would use the *pauper* low-alkaloid gene (and not *exiguus* or *nutricius*). This was to avoid complementation between different genes in different cultivars via outcrossing. Also *pauper* conditioned the lowest overall alkaloid level (typically 0.002%) with a minimal effect from the environment (Harrison and Williams, 1982; Harrison and Williams, 1983; Williams and Harrison, 1983). Australian *L. albus* breeding programs have always aimed for zero tolerance of bitter seed contamination for new cultivars. However, since *albus* lupin is partially outcrossing (~8%) (Faluyi and Williams, 1981; Green *et al.* 1980), and bitter plants have higher fitness (Cowling *et al.* 1998), there was uncertainty about the level of bitter contamination over time with or without corrective action.

A spreadsheet model was used to investigate the likely increase in bitter seed and bitter plant contamination frequency over time. Several parameters were included: the outcrossing rate, the fitness disadvantage of the sweet types over the bitter types, the initial bitter allele frequency in a contaminated seed lot, and the number of generations the seed lot was grown without corrective action. The formulae of Allard and

Workman (1963) were used in the model. The overall expected total seed alkaloid concentration (mg/kg) was calculated assuming that sweet seeds were 0.002% w/w total alkaloid (200 mg/kg), and bitter seeds were 1.0% w/w. French data (Cowling *et al.* 1998) suggest that sweet plants have a reduced fitness (seed yield) compared to bitter plants of 0.96 or 0.8.

#### INDUSTRY PLAN AND TESTING

Each year a sowing threshold for contamination was decided by industry. In 2001 the threshold was set at 0.2%, was reduced to 0.1% in 2004, and became zero in 2008. The aim was to encourage growers to replace contaminated seed but without completely halting the industry due to insufficient seed supply. There was no legal compulsion to supply samples for testing, nor to grow bitter-free crops. As a result we do not know the true level of farmer and trader co-operation and to what degree our test result recommendations were adhered to. The approach was to rely on peer pressure and the possibility of contaminated seed lots being rejected by traders if the contamination level got too high.

#### INTRALOCUS RECOMBINATION OF *PAUPER*

The sweet *albus* industry in Australia was dominated by two cultivars: Kiev-mutant (ex-Ukraine) and Ultra (ex-Germany). It was suggested that these cultivars might contain different alleles of *pauper* and that, in a hybrid, intralocus recombination (Buschges *et al.* 1997) could occur and result in a bitter allele (Rex Oram, personal communication). This hypothesis was tested by crossing Kiev-mutant with Ultra in 2000 and 2001, selfing the F<sub>1</sub>s and F<sub>2</sub>s and examining about 65,000 F<sub>3</sub> seed progeny.

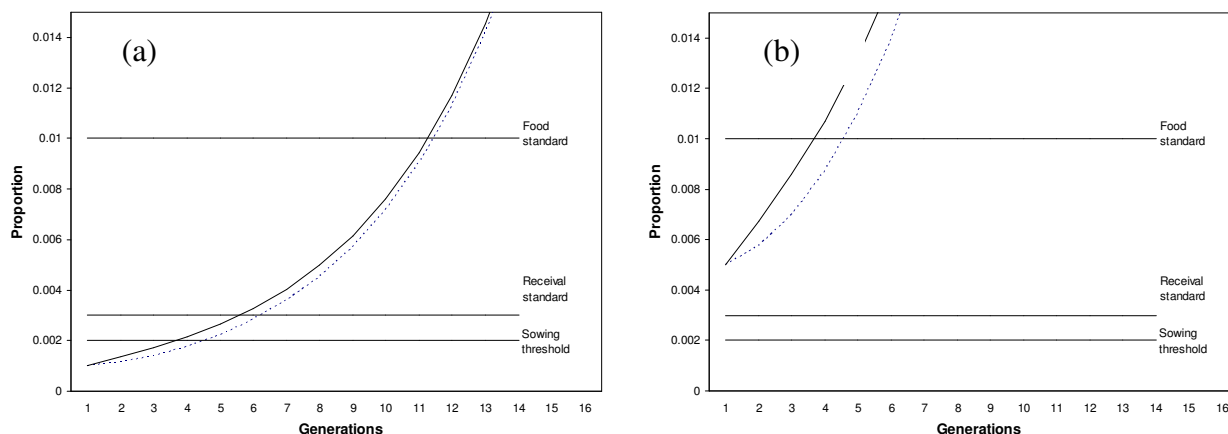
## RESULTS AND DISCUSSION

#### MODELLING OF BITTER FREQUENCY

Fig. 1 shows two examples of the expected increase in bitter seed and plant frequency over time with different starting contamination frequencies. It took about 11 generations for the Food Standard for alkaloids to be exceeded when only 0.1% of the initial seeds were bitter. When the initial value was 0.5% the Standard is exceeded in only four generations. This evidence was sufficient to convince the *albus* industry that bitterness could not be ignored and a control plan was required.

#### INTRALOCUS RECOMBINATION

No bitter seeds were detected in the F<sub>3</sub> and so intralocus recombination appears to be rare and unlikely to be the cause of bitterness in commercial seed lots. It is a possibility that the *pauper* locus exhibits relatively-high reversion rates but this has not been specifically examined.



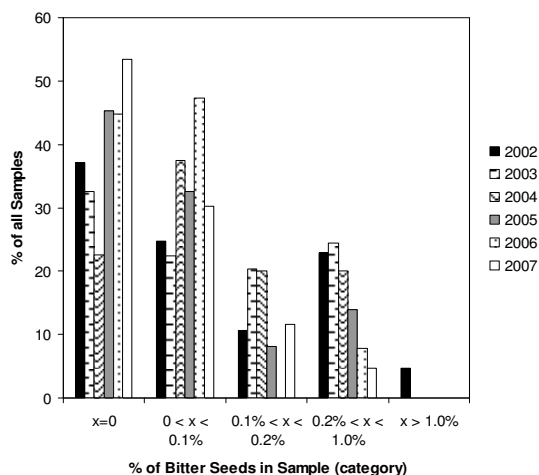
**Fig. 1.** The projected increase in fluorescent seed frequency (dotted line) and bitter plant numbers (solid line) over time from repeat growing of a bitter contaminated seed lot of *Lupinus albus*. Starting frequency of bitter seeds was (a) 1/1000, (b) 1/200. Outcrossing rate ( $t = 0.08$ ) and relative fitness of sweet plants compared to bitter ( $f = 0.8$ ) were the same in both cases. Horizontal lines represent the Food Standard, the Receiving Standard, and the recommended sowing threshold for contamination (see text).

**SURVEY RESULTS**

Seed samples for testing were received from all lupin-growing areas of NSW. The number of samples has been quite low following the initial testing year (Fig. 2) and subsequent years probably represents only a small proportion of the total growers, seed lots and planted area (despite extensive publicity on the need to test). The control plan has clearly succeeded in reducing the incidence of seed lots with very high bitter contamination rates (Fig. 2), however, some low-level contamination still remains after six years. It seems likely that this will persist until the new cultivars are adopted. The next two years are very important – as Luxor and Rosetta are adopted in NSW there is an ideal opportunity to eliminate bitterness.

**GUIDE FOR INDUSTRY**

The aim of the industry plan was to reduce (and possibly eliminate) commercial contamination until new, 100% sweet cultivars were produced to replace the old. Two 100% sweet cultivars, Luxor and Rosetta, were released from the Wagga breeding program in 2005. More recently, the aim of the work has been to educate the industry and to ensure that these new cultivars are not contaminated by admixture or pollen movement from old, contaminated cultivars. To our knowledge the contamination problem has been confined to NSW and has not affected crops in other states (e.g. Western Australia, although *albus* growing there has been halted by Anthracnose). All the lupin grain traders in NSW have been equipped and trained to conduct their own UV testing.



**Fig. 2.** Bitter seed contamination frequencies (%) in commercial *L. albus* seed samples (2002-2007), expressed as % total samples tested (2002,  $n = 170$ ; 2003,  $n = 49$ ; 2004,  $n = 40$ ; 2005,  $n = 86$ ; 2006,  $n = 38$ ; 2007,  $n = 43$ ) presented in five contamination categories.

UV testing is conducted on all seed sown in the Wagga breeding program, and all pedigree seed is kept in containment until the last possible stage of seed increase. However, low-level contamination can still occur via pollen movement from older cultivars once the pedigree seed goes into the field, unless careful procedures are adopted.

Outcrossing in *L. albus* is mainly due to foraging bees, which can be seen forcing their way into unopened flowers. Pollinator-mediated contamination of seed crops is a long-recognised problem (Bateman, 1947). Bees (and other pollinators) will have two effects 1) to move pollen between plants in the same *albus* crop thereby helping to increase the outcrossing rate and, potentially, the spread of bitterness; and 2) to move bitter pollen from one *albus* crop to another. This intercrop transfer can be up to 10 km in extreme cases (Eastham and Sweet, 2002; Glover, 2002).

A guide for industry to reduce current contamination and to prevent future contamination consisted of the following advice:

- Growers to move to the new cultivars and remove all older *albus* cultivars, even if they have received a bitter-free test result, from the farm (deliver or feed to stock).
- Growers to annually send sowing seed samples to NSW DPI for testing.
- Check that farm neighbours are not growing old cultivars within 2 km.
- Consider pooling the seed increase crop of a new cultivar with other growers.
- Grow small areas of any new *albus* cultivar in the middle of a narrow-leaf crop (agronomy is similar and no pollen contamination will occur).
- During the season carefully control any volunteer *albus* plants on the farm before flowering.
- Discourage the growing of bitter lupini bean crops in sweet *albus* areas (Luckett, 2007b).

It also became clear during this work that UPOV rules for lupin bitter seed testing (only 1 in 100) (UPOV 2004) and Australian Certified Seed Rules regarding isolation distances and testing requirements (Smith and Baxter, 2002) were insufficiently strict and needed tightening to be effective in preventing the spread of bitter seed contamination in *Lupinus albus*.

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