Speed breeding methodologies delivers two new barrel medic cultivars to farmers within 6.5 years of breeding commencing

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Abstract
Powdery mildew (PM, *Erysiphe trifolii*) is a widespread disease of barrel medics (*Medicago truncatula*) and is implicated in reduced fertility of sheep. Existing barrel medic cultivars have short spines which can get caught in wool. Some farmers wanting to produce wool with low vegetable fault grow spineless burr medic cultivars when their soil type is better suited to barrel medics. Speed breeding techniques were used to produce commercial quantities of a PM-resistant and a spineless cultivar within 6.5 years of breeding commencing. This is at least three years earlier than if traditional methods were used. The PM resistant cultivar was developed by transferring PM resistance from the strand medic cultivar Seraph into a barrel medic cultivar Paraggio background (BC_4 was achieved). The spineless cultivar was developed by transferring the spineless trait from Cyfield into cultivar Sultan-SU background (BC_6 was achieved). Lessons learnt in this work will assist future speed breeding of annual pasture legumes.

Introduction
Barrel medics are widely grown ley legume pastures in the neutral to alkaline soils in southern Australia. They provide high quality feed to livestock and benefit following cereal crops by fixing nitrogen and reducing cereal disease levels. Powdery mildew (PM) is a common disease of barrel medics and existing cultivars are susceptible (Ballard *et al*. 2012). A PM resistant strand medic cultivar Seraph has been developed (IP Australia). Several traits have been transferred between barrel and strand medics by way of an inter-specific cross followed by backcrossing to develop the cultivars Herald, Cheetah, Lynx, Sultan-SU and Jester-SU (IP Australia) and, hence, it is likely that PM resistance can be transferred from a strand medic into a barrel medic background.

Barrel medic cultivars have short spines which contaminate wool (8.3-11.2 g pod / 100g greasy belly wool) whereas spineless pods from cvs. Cyfield and Tornafield (disc medic) were never retained in fleeces (Brownlee and Denney 1985). Wild burr medics (*M. polymorpha*) have interlocking spines which cause significant wool contamination and to avoid this all cultivars are spineless. Barrel and burr medics are suited to different soils (Nichols *et al*. 2012). Farmers seeking low wool fault often sow spineless burr medic cultivars when barrel medics are better suited to their soils. We aimed to develop a spineless barrel medic cultivar to decrease vegetable fault in fleece.

Speed breeding methodologies have been developed that greatly increase the number of generations that can be grown per year (Pazos-Navarro 2017). For our two breeding objectives we used backcrossing breeding combined with speed breeding methods. We report on modifications that we made to speed breeding methods. We also saved a year by changes in the pre-commercial seed build-up phase. The new cultivars will be delivered to farmers within 6.5 years of the first cross being made which is at least three years sooner than if traditional breeding methods were used.

Methods
Plants were grown in controlled environment room set at 24°C day – 18°C night and 20 h photoperiod as per Pazos-Navarro (2017). Embryo dormancy in subclover is overcome by rescuing immature seeds into tissue culture 24 DAP (Pazos-Navarro 2017). However, we view this as laborious and time bound and prefer to harvest seed. In our medic breeding programs we have traditionally overcome embryo dormancy in annual medics by storing in an oven at 45°C for three weeks. In a pilot study we found embryo dormancy was overcome in our genotypes by placing seed in a refrigerator (~ 4 °C) for four days (Garcia *et al*. 2006) followed by incubating in the dark at 15°C (Bolingue *et al*. 2010).

Initially we made crosses using method 3 of Pathipanawat (1994) and then changed to the method of Veerappan *et al*. (2014). For our spineless cultivar, the early season cultivar Sultan-SU was chosen as our recurrent parent and has traits of tolerance to sulfonylurea (SU) herbicide residues, bluegreen aphid and spotted alfalfa aphid resistance, B tolerance and suitable hard seed levels (Peck and Howie 2012, tested as

Z2438). We used the spineless cultivar Cyfield (Oram 1990) as our donor parent. For BC_1, BC_3 and BC_6 we used spineless F_2 plants as the female plant and for the other backcrosses we used a F_1 plant of prior backcross as our pollen parent and made enough crosses to ensure at least a 99% chance of achieving at least one F_1 plant carrying the spineless trait. BC_6 was achieved and 16 F_2 spineless plants were selected.

For our PM cultivar we chose Paraggio as our recurrent parent as it is slow to develop PM (Ballard *et al.* 2012), is widely grown, bluegreen aphid resistant (Peck and Howie 2012), B tolerant (Howie 2012), and resistant to phoma which allows it to maintain low coumestrol (a phytoestrogen) levels (Barbetti 2007). In our experience with making inter-specific crosses some genotypes cross easier and have less inter-specific problems than others. For the inter-specific cross, we also used barrel medic cv. Sephi as it has the second lowest PM (Ballard *et al*. 2012) and the male sterile line tap due to the ease of making multiple crosses. When conducting each backcross, we aimed for a minimum of 99% chance of being successful by obtaining a minimum of 7 seeds from pollen from 7 F_1 plants whose parent was PM resistant. Glasshouse powdery mildew screens were conducted in a glasshouse based on the method of Ballard *et al*. (2012). A population of powdery mildew was collected from the field in South Australia and maintained on cultivar Jester plants. As soon as we had achieved enough F_1 for each backcross we transferred male plants to a PM screen and used Paraggio as our susceptible control. We scored our breeding plants when all Paraggio plants had PM visible and again one week later. F_2 BC_4 (BC_3 Paraggio) plants were selected and progeny tested to find plants homozygous for PM resistance. F_3 plants were grown and 16 plants selected.

We increased seed from 16 lines of each cultivar target along with recurrent parents and existing cultivars as controls. Seeds were collected from eight lines with the highest DM production. In 2019 they were sown at 10 kg/ha in small plots at Penfield South Australia. In spring they were irrigated to average spring rainfall. DM was scored (0-100) in winter and spring and seed yield determined. In 2019 when lines entered field evaluation trials, we increased seed from each line targeting ~ 2kg. Pods of each line were collected but we only extracted seed of the two lines that were identified as suitable for cultivar release. 2 kg of seed was sown in 0.5 ha in 2020. Rather than keep some seed in reserve in case of a disaster we seed increased another 2 kg at different site in 2020. In 2021 we have sown 10 ha of Penfield and 5 ha of Emperor and with a predicted yield 500 kg seed/ha, 5000 kg and 2500 kg of seed is expected to be produced, enough for first commercial sales in autumn 2022.

Results

Inter-specific problems of chlorophyll deficient plants, abnormal growth and low fertility were readily noticeable in inter-specific F_1 and F_2 plants. Sephi × Seraph had less inter-specific problems than Paraggio × Seraph which in turn was less than tap × Seraph. The inter-specific problems in BC_1 Paraggio were greater than in Paraggio × (Sephi x Seraph) and we dropped the BC_1 Paraggio from the breeding program. Inter-specific crosses between barrel and strand medics have low success rates but the most difficult cross to achieve is BC_1 due to low fertility in F_2 plants of inter-specific plants. Despite multiple attempts, we achieved nil success when using method 3 of Pathipanawat *et al*. (1994). However, when we changed to the crossing method of Veerappan *et al*. (2014) we achieved success and adopted it for all future crosses. For spineless crosses and later backcrosses for PM we achieved ~ 80% success, which is higher than ~ 30% we expect with the Pathipanawat *et al*. (1994) method.

For the early season spineless cultivar, 12 generations were grown in our CER at a rate of 5.1 generations per year. For the mid-season PM cultivar, 9 generations were grown in our CER at a rate of 3.9 generations per year.

Winter DM production, spring DM production and seed yield of existing cultivars and lines chosen for new cultivars is compared in Table 1. The newly developed PM resistant cultivar Emperor performed equal to its recurrent parent Paraggio. The newly developed spineless cultivar Penfield performed equal to its recurrent parent Sultan-SU. No PM occurred in the field trial, so we were unable to determine the yield benefit that PM resistance provides. The PM resistance of Emperor was initially established with a glasshouse test and subsequently confirmed during its seed build-up prior to field evaluation. Hardseed levels were measured at the end of April and Emperor and Penfield had levels similar to their recurrent parents. Figure 1 shows spineless pods of Penfield and spiny pods of Sultan-SU.

Table 1: Winter and Spring DM score (0-100), seed yield (kg/ha) and mean percentage maximum (% max.) of the 3 parameters for existing cultivars and new PM resistant cultivar Emperor and spineless cultivar Penfield. Superscript M indicates mid-season and E indicates early-season.

Line	Winter	Spring	Seed	% max.
Paraggio^{M}	84	83	1534	87
Emperor^{M}	82	80	1633	87
Jester^{M}	75	65	1707	79
Lynx^{M}	83	57	1240	72
Sultan-SU^{E}	83	62	2011	85
Penfield^{E}	86	72	2384	96
Parabinga^{E}	84	67	1529	81
Caliph^{E}	74	47	1757	72
LSD	*17*	*19*	*517*	

Figure 1: Spineless pods of Penfield and spiny pods of Sultan-SU

Discussion

To our knowledge this is the first time that annual pasture legume cultivars have been developed using speed breeding principles. Commercial seed will be available within 6.5 years of commencing breeding, which is at least three years sooner than if traditional methods were used. For the mid-season cultivar Emperor, we achieved 3.9 generations per year and for the early season cultivar Penfield we achieved 5.1 generations per year. This is faster than 2-2.5 generations per year achieved in the development of recent cultivars Sultan-SU and Jester-SU (which were developed by backcrossing breeding). Seed increasing ~ 2kg of all lines the year lines entered field evaluation saved a year in the precommercial seed build-up phase. Speed breeding principles used in this work is applicable to any inbreeding annual pasture species and crop species.

Adoption of a more efficient crossing method (Veerappan *et al*. 2014) was critical in maintaining speed by ensuring we obtained enough crosses with the first flush of flowers. We also needed to make more crosses than traditionally as we were unable to screen for PM resistance before making crosses. The method of Veerappan *et al*. (2014) may be applicable to other legume genera.

We modified the speed breeding method of Pazos-Navarro (2017) by harvesting seed rather than rescuing immature seed into tissue culture 24 DAP. Harvesting seed adds ~ 7 days per generation but saves labour and means you are not time bound. It allowed us to sow each generation as a batch rather than staggered. For our genotypes the method of Garci (2006) followed by 15°C in the dark (Bolingue 2010) overcame dormancy. But in subsequent work we have encountered genotypes where this method did not work. When working with a new genotype we suggest a pilot study is required to determine if embryo dormancy can be overcome in a timely fashion. We have conducted further research on breaking seed dormancy which we hope to publish soon.

Backcrossing is a conservative breeding strategy with the aim of developing new cultivars performing similar to their recurrent parent except when the new trait provides an advantage. All lines within our two cohorts performed well in the field. In 2021, the two new cultivars will be sown along with recurrent parents at multiple sites to ensure they are performing as expected. PM is increasingly being reported as a problem in annual medics and farmers believe this is causing poorer livestock production and reduced lambing percentage. More out-of-season rainfall is being experienced which is increasingly providing a green bridge for PM and is the likely reason for PM being encountered more often. We expect the PM resistant cultivar Emperor to be readily taken up by farmers. Brownlee and Denney (1985) report that spineless medic pods do not get caught in wool. Farmers with a focus on wool quality will readily take up the spineless cultivar Penfield. A modern spineless cultivar will allow breeders to use spiny accessions with high agronomic performance in their breeding programs. The spineless trait is controlled by a single recessive gene and 25% F_2 plants will be spineless and a further 50% will carry the spineless trait.

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