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Condiment paprika breeding and hybrid seed production

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Foreword

An increased demand for natural spices and colourants has expanded the demand for high quality condiment paprika worldwide. Hungary has produced the high quality condiment paprika required in the past, but the exports from that country have declined significantly over the last 15 years. By the use of the Hungarian cultivars and other genetic material in a breeding program for Australia, it is hoped to establish a viable condiment paprika industry in Australia.

To achieve these aims RIRDC has this program, which is a two year continuation of a previous project (*US-98A: Condiment Paprika: Breeding, Processing and Commercialisation (Stage 1)*). The breeding aims were to produce cultivars with high initial pigment and dry matter content suitable for direct seeding and mechanical harvesting. There is also an aim of developing a hybrid system, so that hybrid seed can be produced at low cost to gain economic access to the resultant heterosis. In this project ASAS Pty Ltd was in cooperation with industrial/commercial partners, The University of Sydney, Plant Breeding Institute-Cobbitty and the Hungarian Condiment Paprika Research Development Ltd.

This report concentrates on the plant-breeding program that has the major aim to develop cultivars suitable for mechanised production particularly to machine harvesting. Three lines are reported here that have Part 1 Plant Breeders Rights applications.

This project was funded from RIRDC core funds which are provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 1600 research publications. It forms part of our New Plant Products R&D sub-program which aims to improve existing products and develop new ones.

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Peter O'Brien
Managing Director
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Professor P. W. Bosland, Dr C. Shifriss, Dr N. Somogyi and Dr. Jae-Hyung Yoo are thanked for providing plant material.

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Executive Summary

What this report is about

This report is a two-year continuation of the RIRDC project *US-98A: Condiment Paprika: Breeding, Processing and Commercialisation (Stage 1).* Condiment paprika, which is the dried and ground flesh of seed of speciality types of *Capsicum* species, is a globally traded commodity. During the project, breeding lines were advanced, especially by single seed descent; selections were made; new germplasm with specific traits were introduced into the breeding program; work started on the use of male sterility for hybrid seed production; and one line was approved for Plant Breeders Rights (PBR).

Who is the report targeted at?

The report is intended to inform current and potential growers and other breeders of paprika of our progress towards the goal of providing them with superior material that will potentially enable the development of a paprika industry in Australia.

Objectives

The objectives of the project were as follows:

1. To breed PBR cultivars suitable for mechanical harvesting
    - these lines should have a high proportion of the total yield in the first harvest
    - in addition they should have a high dry matter content, and high pigment content
2. Develop the single seed descent method for paprika, for rapid generation advancement
3. Investigate the use of male sterility as a method of hybrid seed production.

Methods Used

The breeding program initially used standard plant breeding technology: crossing, segregation of subsequent generations from the hybrids produced, combined with selection. The selection employed was mainly visual observations of the plant type in the field, with selection for upright plants with pendulous (hanging & swinging freely) paprika-type fruits, where the majority of fruits ripened at the same time. These characteristics are aimed at good machine-harvestability. In addition, interspecific crosses with wild *Capsicum* species were undertaken to attempt to transfer two other traits of potential benefit for machine harvesting, high detachability, and the snap-off trait. High detachability refers to the easy removal of the fruit from the green fleshy calyx, while the snap-off trait relates to a fruit 'stem' that breaks easily.

A rapid breeding technique, single seed descent, was also tried. This is where single seeds from populations are grown each generation in very small containers in the glasshouse, so that only a few fruits are formed quickly. This technique allows three generations to be grown per year: two in the glasshouse and one in the field.

An important non-visual characteristic for selection of paprika is the pigment concentration in the fruit. This is measured by an Australian Spice Trade Association (ASTA) determination. The breeding program developed a methodology to measure this at the Plant Breeder's Institute-Cobbitty (PBI-Cobbitty).

An associated PhD student supported by RIRDC researched aspects of male sterility. Male sterile mutants were induced by gamma rays and chemical treatment. Male sterile genes were also sourced from other researchers. These were evaluated for fertility and for the presence of genes that restore the fertility of sterile lines. One gene selected was a good candidate for hybrid production. DNA molecular marker technology was used to search for markers closely linked to this male sterility gene.

Such markers might enable more efficient selection for sterility for the production of the female parents in any hybrid seed production system.

Results

Breeding

Continuation of the conventional breeding material enabled three lines to be selected with good paprika characteristics (high dry matter percentage and extractable pigment content), and of these, one gained Plant Breeders Rights (PBR) protection.

The single seed descent (SSD) method worked well. It enabled interspecific cross material to progress quickly to field trial; where the promising material with paprika-like quality and also with the two extra traits of detachability and snap-off were identified.

Other results

The male sterile work identified a number of male sterility genes of potential use. Particularly, male sterile 3 gene (ms3), had the characteristics needed, being uniformly male sterile with a high frequency of lines that can ‘restore’ the fertility.

The DNA marker work was successful in that a single marker was identified that was closely linked to the ms3 gene, but the presence of the marker was associated with the fertile type of the gene.

The Implications for Stakeholders

Improved cultivars and germplasm arising from this project are available for evaluation by stakeholders. The new interspecific material should be further advanced for the production of advanced paprika cultivars for possible commercialisation. The hybrid system developed by the PhD student has the possibility of allowing a commercial hybrid seed production operation to be set up in Australia, following further development. This would not have to be restricted to paprika, but could be applied to all *Capsicum* species crops.

Recommendations

1. The new cultivars, once protected by PBR, should be promoted to the industry.

2. A commercial partner should be sought to undertake the bulking up and commercialisation of the new PBR-protected cultivars.

3. Further work should be undertaken on the potential of hybrid seed production.

1. Introduction

This project is a two year extension of the RIRDC project *US-98A: Condiment Paprika: Breeding, Processing and Commercialisation (Stage 1),* which started breeding paprika cultivars for Australian production. That project identified sources of germplasm material for possible new cultivar development. Information and materials from *US-98A* have carried on into this project. The earlier project also identified that machine harvestability would be a key component required of paprika cultivars for Australia. This is because Australian labour costs would not allow Australian production to compete with low-labour cost countries in Africa, the Indian sub-continent, or Asia if hand harvesting was required.

The breeding and research program was therefore devised for providing machine harvest ready germplasm and cultivars.

Competition also means that high productivity is required. One way to develop high yielding lines is to use hybrid vigour. Consequently, hybrid production was investigated by researching male sterile paprikas and possible hybrid seed production.

2. The breeding program

2. 1 Crossing nursery

The standard crossing nursery was devised to attempt to introduce traits considered important for machine harvesting of paprika cultivars in the field. The traits relate to how the fruit will come away from the plant in the field when ripe. Certain wild *Capsicum* species have the trait of 'detachability' (the easy removal of the fruit from the calyx), and 'snap off' (the easy breakage of the pedicel between the calyx and the stem).

Initially, *Capsicum chacoense* was used as a source of the detachability trait. Interspecific crosses were made with Hungarian, New Mexican, and American paprika cultivars. The F1 plants that resulted were male sterile. In addition, the fruits of these were hybrids were very soft when ripe; too soft to be useful for machine harvesting. As a result, the two species *C. eximium* and *C. pubescens* were introduced as new sources of the detachability gene into paprika, or to allow introduction. These species were crossed with *C. baccatum* to create an intermediate form which could be crossed with paprika cultivars, which have the snap off pedicel genes. The three species are described below.

Capsicum baccatum
In South America, *C. baccatum* is the most commonly grown species, where it is called 'aji'. Three botanical varieties are recognized: *C. baccatum* var. *baccatum*, *C. baccatum* var. *pendulum*, and *C. baccatum* var. *microcarpum*. *C. baccatum* flowers have yellow, brown, or dark green spots on the corolla. Fruits vary in pungency from non-pungent to very hot. They embody unique aromatics and flavours that can be overpowering to some people. This chilli is used to make marinated fish. Aji Amarillo is the most common *C. baccatum* in Peru. In the United States it is sometimes called 'Yellow Pepper'. The pods are 10-15 cm long and deep orange in colour when ripe. This pod type has been known in Peru since ancient Inca times, where it is represented in drawings and pottery.

Capsicum pubescens
C. pubescens forms a distinct genetic lineage. This *Capsicum*, first described by Ruiz and Pavon (1971), did not receive wide attention from taxonomists until recently. Morphologically, it is unlike any other domesticated *Capsicum*, having large purple or white flowers infused with purple and fruits with brown/black seeds. Genetically it belongs to a tightly-knit group of wild taxa including *C. eximium* (Bolivia and Northern Argentina), *C. cardenasii* (Bolivia), and *C. tovarii* (Peru). *C. pubescens* is still primarily cultivated in South America. This species remains virtually unknown to the rest of the world. Two of the major difficulties in transferring this species to other regions include its growth requirements for a cool, frost-free environment and long growing season, and the fleshy nature of the fruit that leads to rapid deterioration and spoilage. Common names include 'rocoto' or 'locoto' in South America. We introduced it as a lure for bees to attend *Capsicum* flowers for hybrid production.

Capsicum chacoense
C. chacoense is distinct from any other species. Tall, slender plants branch out only after 15-25 nodes, but from then on many branches appear. The flowers are erect and white, and the anthers are distinctly yellow and produce a profuse amount of nectar. Fruits are small and when fully matured are red and soft and easily detachable from the calyx (Somos 1985). The fruit detachability is the characteristic which is being utilised to develop a cultivar that lends itself easily to mechanical harvesting. Somos (1985) indicated that the detachability and softness are caused by a pleiotrophic effect of one gene. Some other authors, such as Greenleaf (1986), suspected that the easy separation of mature fruit from the calyx and the soft flesh are caused by two separate genes. Daskalov and Poulos (1994) listed the detachability as an incomplete dominant gene which can be easily modified by genes that control the fruit form, calyx, and placenta, and allocated the symbol of Ps. The gene that is causing soft flesh is distinct from Ps, is a dominant gene, and the allocated symbol is S. When we are talking about detachability, one has to know that there are three types of detachability. One, where the calyx easily

separates from the plant, but fruits are soft. The second is when a line carries the detachable gene, and the fruit firmness is acceptable. The third type is semi detachable, which allows the separation of fruit from the plant with a little force; however the fruit stays attached to the plant even at the fully ripened stage, conserving a nice firm fruit.

2.2.1 Interspecific material produced

Problems often occur during interspecific hybridisation, which prevent the easy recovery of hybrids. In the material worked on here incompatibility was a problem, but this was overcome using the embryo rescue technique to rescue hybrid embryos before they died, and therefore produce F1 interspecific hybrids.

During the 2002-2003 season the crossing nursery was placed in the field. There was significant bird damage to some crosses, but 30 crosses out of the 138 attempted were successful. In cooperation with a Spanish paprika breeder a further 20 crosses were completed during the 2002-2003 season.

To avoid any crosspollinations bird problems during the 2003-2004 season, the crossing nursery was placed in a tunnel house.

Interspecific combinations being used involve *C. chacoense*, *C. chinense*, *C. baccatum* and various other *C. annum* cultivars, which were 'top-crossed' by accessions and between each other. From 69 successful crosses out of 450, 60 were interspecific ones. Table 1 lists the material produced. Interspecific parents listed in the pedigrees were made in 2002-2003.

Table 1. **Successful interspecific and top-crosses made in 2003-2004**

CO4	Pedigree	No. of seed
1	Sunired/Bambino	7
2	Sunired/Papri King	25
3	Sunired/Papri King	8
4	Sunired//Aji Amarilo bac./Cap.eximium	3
5	Sunired//Aji amariloC.bac./C.eximium	23
6	Sunired/Aji Amarilo C.bacatum	12
7	Aji Amarilo C.bac/C.eximium//Papri mild	1
8	Aji Amarilo Bac/Cap.eximium//Papri Mild	1
9	Aji Amarilo Cap.bac/Papri Mild	4
10	Aji Amarilo Cap.bac/Papri Mild	3
11	Aji Amarilo C.bac/C.eximium//Spanish Spice	4
12	Aji Amarilo C.bac/C.eximium//Aji amarilo C.bac./Papri Queen	3
13	Aji Amarilo C.bac/C.eximium//Aji amarilo C.bac./Papri Queen	3
14	Aji Amarilo C.bac/C.eximium//Aji Amarilo Bac.	1
15	Aji Amarilo C.bac/C.eximium//Aji Amarilo Bac.	2
16	Aji Amarilo C.bac/C.eximium//Co.NFD.3b	1
17	Aji Amarilo C.bac/C.eximium//Papri King	2
18	Aji Amarilo bac./eximium//Cap. bac.L. Aji/Papri Queen	1
19	Aji Amarilo bac./eximium//Cap. bac.L. Aji/Papri Queen	2
20	Aji Amarilo bac./eximium//Cap. bac.L. Aji/Papri Queen	3
21	Chacoense/Aji Amarilo bacatum	10
22	Chacoense/Aji Amarilo bacatum	11
23	Chacoense/Aji Amarilo bacatum	13
24	Chacoense/Aji Amarilo bacatum	10
25	Co.57-13.3.b/Aji Amarilo C.bacatum	8
26	Co.57-13.4.b/Aji Amarilo C.bacatum	10
27	Co.57-13.4b/Cap.bacatum Pendulum	2
28	Cap.Chinense Jacq./Papri Mild	8
29	Papri Mild//Aji Amarilo C.bac/C.eximium	5
30	Papri Mild/C.bac.Pend.	10
31	Papri Mild/C.bac.Pend.	7
32	Cap.bac. Pend./Kalocscai 801	5
33	Cap.bac. Pend./Papri King	2
34	Cap.bac. Pend./Papri King	4
35	Cap.bac. Pend./Papri King	3
36	Cap.bac.Pend./Papri Mild	5
37	Cap.bac.Pend./Papri Mild	2
38	Cap. bac.Pend./Papri Mild	2
39	Cap. bac.Pend./Co.57-13.4.b	3
40	Cap. bac.Pend./Co.57-13.4.b	3
41	Cap. bac.Pend./Co.57-13.3.b	1
42	Cap.eximium/Aji Amarilo bac.//Papri King	1
43	Cap.eximium/Aji Amarilo bac.//Papri King	2
44	Cap.eximium/Aji Amarilo bac.//Papri King	1
45	Cap.eximium/Aji Amarilo bac//Szegedi 80	1
46	Cap.eximium/Aji amarilo bac.//Co.57-13.4.b	2
47	Cap.eximium/Aji Amarilo bac//Papri Mild	2
48	Cap.eximium/Aji Amarilo bac.//Cap. bac.L. Aji/Papri Queen	4
49	Cap.eximium/Aji Amarilo bacatum	3
50	Cap.eximium/Aji Amarilo bacatum	3
51	Cap.eximium/Aji Amarilo bacatum	8
52	Cap.Eximium/Cap. Bac.Pend	6
53	C.eximium/Cap. bac.Pend	10
54	Cap.eximium/Cap. bac.Pend	8
55	Cap.eximium//Cap.bacatum L.Aji/Papri Queen	7
56	Cap.eximium//Cap.bacatum L.Aji/Papri Queen	8
57	Cap.eximium//Cap.bacatum L.Aji/Papri Queen	3
58	Cap.eximium//Cap.bacatum L.Aji/Papri Queen	12
59	Cap.bac.L.Aji/Papri Queen//Cerise Sweet	4
60	Cap.bac.L.Aji/Papri Queen//Cerise Sweet	3
61	Cap.bac.L.Aji/Papri Queen//C.eximium	5
62	Cap.bac.L.Aji/Papri Queen//C.eximium	2
63	Cap. bac.L. Aji/Papri Queen//Cap.eximium/Aji amarilo bac.	3
64	Conquistador/Cap.bacatum Pend.	6
65	Papri King/AjiAmarilo C.Bac	2
66	Super shepherd/Papri king	20
67	Bambino/Sunired	3
68	Cap.Chinense Jacq./Papri Mild	8
69	Spanish spice//Aji Amarilo Bac./C.eximium	6

Note: The *Capsicum eximium* received from USDA and mentioned above was subsequently found to be *C. frutescens.*

2.2 Selection of cultivars

Initial material for the selection of cultivars came from the material flowing into this project from project US-89A. Cultivar trials were undertaken in both the 2002-2003 and 2003-2004 growing seasons.

2.2.1 Cultivar Trial 2002-2003

The purpose of this trial was to select potential cultivars and strains for further testing and PBR purposes. This trial included comparative varieties from the U.S.A. (2), central Europe (8) and six of our selections.

Phenotypic observations were made for PBR purposes (see section 'The PBR Part1 lines').

Yield and dry matter content were recorded and statistically analysed (Table 3). Ripe and unripened fruits were harvested separately to estimate the product harvested at the first harvest.

Whilst the two cultivars from the U.S.A., 'Papri Queen' and 'Conquistador', produced the highest total yield, one of our selections (Co57-13.3) produced the highest yield of ripe fruit. This line would therefore have the highest yield under mechanised production. Three of our lines, named 'Sunired', 'Earlysuni', and 'Cerise Sweet', were selected for inclusion in a second cultivar trial for the 2003-2004 seasons and as candidates for commercialisation.

'Cerise Sweet' and 'Sunired' were planted as buffers and then harvested for processing and production of milled paprika.

'Sunired' produced 89% of its total yield in the first harvest. 'Earlysuni' was early maturing with high dry matter and pigment content. 'Cerise Sweet' gave the highest pigment production per unit area.

Six to eight plants per cultivar were selfed for pure seed production.

Table 2. **Cultivar trial yield and dry matter content 2002-2003**

Cultivar	**Plot yield**			**1st Harvest**		**Yield**	**Dry matt**	**1/2 prod**	**ASTA**	**Pigment**	
	Ripe	Unripe	Total	t/ha	%	t/ha	%	t/ha		g/kg	kg/ha
Szegedi 80	5	2.17	7.17	12.5	70.4	17.93	13.57	1.7	336	8.38	13.77
Kalocsai 801	5.81	1.05	6.86	14.5	84.9	17.15	11.76	1.7	198	4.95	8.56
Co.801.2, Sunired	8.51	1.03	9.54	21.3	89.2	23.97	12.58	2.66	221	5.53	14.44
Co.801.8	7.33	1.23	8.56	18.3	84.4	21.39	11.69	2.18	206	5.15	11.14
Mauthner	5.58	1.94	7.52	14	69.4	18.79	14.47	2.02	311	7.53	15.25
Cerise Sweet	6.89	1.91	8.8	18	78.7	21.99	16.25	2.92	271	6.85	20
Szegedi 20	6.71	1.48	8.18	16.8	81.5	20.45	14.31	2.43	317	7.9	19.11
Co.20.8, Earlysuni	6.1	2.85	8.94	15.3	67.7	22.37	15.24	2.29	264	6.05	14.13
Szegedi 57-13	6.22	1.69	7.91	15.6	78.2	19.78	14.21	2.18	232	5.8	12.39
Co.57-13.3	6.67	2.66	9.33	16.7	71.4	23.32	14.21	2.38	266	6.65	15.49
Co.57-13.4	5.65	2.17	7.82	14.2	70.5	19.55	13.61	1.92	233	5.83	11.58
Mihalyteleki	5.56	1.27	6.84	13.9	81	17.09	13.29	1.84	304	7.63	13.91
Bibor	6.99	1.1	10.9	17.5	86.4	20.22	14.61	2.57	238	5.95	15.18
Conquistador	2.74	11.2 **	13.3 **	6.88	19.2**	34.86 **	8.28 **	0.75	194	4.88	3.63
Papri Queen	10.3	6.18 **	16.43 **	25.7	61.9	41.07 **	13.2	3.39	252	6.3	20.52
LSD 0.01	3.26	1.4	4.88	8.15	15.8	9.27	3.28	1.16	89	2.18	7.76
SD	2.23	2.62	3.11	2.22	17.3	7.79	23.21	0.8	59.1	1.45	5.56

2.2.2 Cultivar Trial 2003-2004

This trial had the purpose to further test the chosen candidates from the 2002-2003 cultivar trial. The trial included three of our candidates submitted for PBR registration ('Sunired', 'Earlysuni', and 'Cerise Sweet'), as well as some other selections from the program and other Hungarian cultivars as controls.

There was a very heavy virus infection of this trial. Consequently, no yield data was collected, but the epidemic allowed scoring of virus resistance of the lines to be undertaken (Table 2). There were no significant differences between cultivars for the mean number of infected plants.

Table 3. **Paprika cultivar trial 2003-2004**

Accession #	Cultivar	Plants /plot	Mean number of infected plants/plot
39	Szegedi 80	24	4.75
40	Mauthner	24	4.5
238	Cerise Sweet(2002)	24	4.5
238	Cerise Sweet(2003)	24	5.25
185	Kalocsai 801	24	4.5
229	Sunired(2001)	24	6
229	Sunired(2003)	24	5.25
228	Co.801.8	24	7
191	Szegedi 57-13	24	5.25
233	Co.57-13.4	24	5.75
235	Co.57-13.3	24	4.5
188	Szegedi 20	24	7
227	Earlysuni(2001)	24	7.25
227	Earlysuni(2003)	24	6.5

2.2.3 Semi-commercial Trial

A semi-commercial trial was conducted of 'Cerise Sweet'. This was undertaken to increase seed of 'Cerise Sweet', which was significantly the best line for dry matter production per unit area, and has the highest pigment content.

Figure 1. Field seed production of Cerise Sweet

A local seedling supplier, John Vella of Berwick Speedy Seedlings Pty Ltd, was very interested in trialling paprika *Capsicums* for his growers. John Vella has a major client base in the eastern states of Australia and was looking at paprika *Capsicums* as an alternative crop for his tomato growers. The tomato crop has similar agronomy requirements and John Vella was targeting tomato producers that grow tomatoes for processing.

The 'Cerise Sweet' seed was provided to Berwick Speedy Seedlings to grow seedlings for this trial. Six week old seedlings were then transplanted by a two operator machine in a trial site located in the Penrith Lakes area of the Cumberland basin. An area of 500 m^2 was bedded and sprayed with pre emergent herbicide one month before transplanting. The seedlings were transplanted three rows per bed, with forty centimetres between rows and thirty centimetres between

plants. This provided a plant density of around 83,000 plants per hectare. Overhead irrigation was used in conjunction with drip irrigation to maximise water efficiency. Additional nutrients were supplied via the drip system.

The University of Sydney assisted John Vella in the management of the crop and provided advice when necessary. The crop was harvested by hand when more than 80% of fruits were fully matured.

This fruit was layered in a closed room for one week and then seed was extracted by a method devised for our research material. This involved an electric propeller in a 60 litre drum with water. The floating flesh was removed and dried for oleoresin production and the heavier seed was extracted from the bottom of the drum, then rinsed and dried. The University of Sydney was able to process 19.5 kg of seed and 13 kg of dried skin from this trial. Samples were taken for pigment testing and found to have an average value of 328 ASTA. This is a better quality compared to Hungarian standards and suppliers from Australia.

Figure 2. Harvested fruit of Cerise Sweet

Bulk seed is being stored in the seed room and the germination was tested regularly (Table 4).

Table 4. **Germination of bulk seed in store (April 2004**)

Cultivar	**Kg of seed**	**Germination (%)**
Mauthner	1.4	76
Szegedi 80	10.5	23
Earlysuni	38	86
Sunired	20	82
Cerise Sweet	19.3	74

2.3 Single Seed Descent material

We are cooperating with the Szeged Paprika Research Station in Hungary to utilise the alternative seasons in Europe and Australia. During the 2002-2003 season, two to three F1 plants from 71 crosses were received from Hungary and were selfed for pure seed production. In the 2003-2004 season 43 crosses were received from Hungary and 9 crosses also carried out at the PBI Cobbitty were included in an F1 nursery where 2-3 plants for each combination were selfed. Many of the hybrids exhibited the targeted traits related to possible mechanical harvesting. ASTA testing was done for all individually harvested plants.

Figure 3. Harvest of F1s

Based on pigment content and other characteristics, 50 populations were selected for F2 generation for selection of suitable individuals (this was then sown in the SSD method).

Figure 4. F1 hybrid plant

The 'Single Seed Decent' (SSD) method was experimentally employed to advance the generations more quickly and economically, and the method has proved very effective. This involved growing the plants in a very small amount of potting media in seedling trays until maturity in microclimates at the PBI Cobbitty glasshouse throughout the year, to quickly advance the generations towards fixation of traits. Early generations, F2 and F3, were included in this method for the last two years. During SSD, selection was limited to the elimination of plants with erect fruit and the selection of plants with apparent high pigment content based on fruit colour. In the 2003-2004 season, F2 and F3 generations were grown in the 'off' season, which made it possible to transplant the F4 generation late in October.

Figure 5. Growth of SSD material in a microclimate environment

The F4 generation material derived from SSD was treated as a separate trial in which most of the combinations performed very well. Phenotypic observations have been recorded for the target traits. A total of 183 selected plants were selfed and harvested individually. The skin was tested for ASTA and further selections were undertaken, based on pigment content, using as a standard a constantly high pigment cultivar. The most outstanding combinations were Sud Afrikai/K57-231 (426 ASTA) and Del-Afrikai/Jaranda (400 ASTA).

Advanced Lines

A total of 441 single plants were selected and selfed in the field during the 2002-2003 season and a further 250 single plants were similarly selected for the 2003-2004 season, and then subsequently harvested. Phenotypic observations were recorded for the traits of interest for mechanical harvesting, and all the lines have been tested for snap off and detachability of the fruit from the calyx. Disease resistance has also been scored. ASTA was measured for all of them (Table 5).

Table 5. **Average of ASTA values for different combinations of single plants selected from advanced lines (2002-2003 season**)

Pedigree	Generation	Number of selected plants	ASTA
Bant/chac//Sz20*3	F3	37	190
Cent/chac//Sz20*3	F3	4	226
C98.78/Conquistador	F3	12	160
Sz80/NMSweet	F3	30	203
Bant/Chac//Sz20*2	F4	36	206
Cent/Chac//Sz20*2	F4	9	216
Cent/Chac// Mauthner	F4	8	217
Cent/Chac//Conquistador*2	F4	14	175
Papri Mild/K801	F4	5	188
C97.15/Sz80	F4	14	222
C98.51.1/Conquistador	F4	3	290
Sz20/Papri King	F4	21	250
Sz20/PM 1231	F4	5	185
SzNFD/Papri King	F4	13	223
Sz57-13/Papri Mild	F4	40	239
Sz57-13/Jaranda	F4	24	201
Papri Mild/Jaranda	F4	22	145
Papri Mild/K801	F4	6	176
Papri Mild/Conquistador	F4	9	162
HM2/Super Sheperd	F4	12	134
Kalocsai 801 2*/Jalapeno	F5	24	176
C98.15/ Szegedi 20	F5	22	266
C97.15.b21/Mauthner	F5	8	215
K57-231/Jalapeno	F6	33	126

Further selections were undertaken based on ASTA results. Phenotypic observations were recorded for the mechanical harvesting traits; particularly detachability, snap off and disease resistance. Most of the selections during the 2002-2003 season were open pollinated. The best combinations, which now carry the gene for detachability, are ones that have *C. chacoence* as one of the parents. For example, C97.15.b21/Mauthner, where C97.15.b21 is a combination of Szegedi 20/*C. chacoence*.

We could transfer the detachability gene from *C. chacoence* to our combinations, but together with this gene in some individuals we have also gained the gene responsible for softness of fruit, which was not present in other individuals of this combination. All individuals fully detachable and not soft were selected and tested for ASTA. Most of the selections result with high ASTA content (over 200 ASTA).

Figure 6. Detachability trait exhibited by two F6 lines

A total of 51 of our selected lines from the later generations with a very high ASTA content (mean ASTA value of 310) have been included in our first year Variety and Strain Trial in future work. All have the snap off gene and 41 lines also have the detachability trait, which allows the calyx to be removed from the fruit. F6-F7 advanced lines have an average 274 ASTA, while the F5 selections mean was 266.

2.4 ASTA method

Selection for pigment content was a key characteristic in the breeding program (Table 6), so that a method for analysis of this parameter was developed. Extractable colour was measured in units of the American Spice Trade Association (ASTA) according to ASTA official analytical method 20.1 (ASTA, 1997). Four representative sub-samples of each ground sample (~100 mg) were weighed and transferred to a 100 ml volumetric flask. Acetone (100ml) was added. The flasks were shaken and kept in the dark for at least 16 hours at room temperature. Portions of each of the sample extracts were then transferred to 96 well plates, or to cuvettes of a spectrophotometer. The absorption was recorded at 460 nm with a ZEISS PM2A spectrophotometer and calibrated with an acetone blank, or with a similarly calibrated 96 well plate reader.

ASTA colour units were calculated using the following formula:

$$ASTA\ color = \frac{Absorbance_{460nm}\ of\ the\ sample\ extract \times 16.4 \times If}{Sample\ weight\ (g)}$$

In this formula, *If* is the instrument correction factor. *If* is calculated by dividing the declared absorbance of a glass reference standard by absorbance obtained at 465 nm on glass reference standard.

The ASTA method was performed at PBI Cobbitty, and also at the NATA-registered laboratory of BRI Pty Ltd, North Ryde, where the exact ASTA official analytic method 20.1 was used. This was to compare our results with an accredited laboratory. Two minor variants of our method using 96 well plates were tested, where the plates were measured with either its own perspex lid, or where the plates were sealed with cling wrap, as well as using the PM2A spectrophotometer.

Table 6. **ASTA pigment testing on samples at PBI Cobbitty and BRI**

Sample	ASTA using the lid to cover the plate	ASTA using cling wrap to cover the plate	ASTA measured using ZEISS PM2A spectrophotometer	ASTA measured in BRI
1	233	232	295	295
2	129	141	198	220
3	127	136	191	190
4	118	113	178	170
5	138	147	194	225
6	170	160	221	230
7	170	173	238	260
8	187	180	232	210
9	142	160	193	180
10	96	98	192	200
11	118	118	165	155
12	159	162	205	220
13	143	143	220	240
14	105	110	192	190
15	138	136	148	150
16	129	133	200	210

Correlation coefficients between the ASTA values obtained in BRI Australia and the other ASTA values obtained in PBI Cobbitty were calculated to find the best method we would use on our samples in the work. We had a very good correlation between the ASTA values obtained at the BRI and ASTA values obtained by us at PBI Cobbitty using the glass filter spectrophotometer (ZEISS PM2A). The 96-well methods were not reliable enough in our hands to be used for selection.

This method was therefore used to provide the ASTA data to enable selection of the breeding material for ASTA values.

3. The PBR lines

‘Cerise Sweet’ was granted Plant Breeders Rights in November 2006.

4. Hybrid breeding

This work represents the PhD thesis work of Natalia Nagy, who was supported by a RIRDC PhD scholarship. The objective of this part of the project was to develop a system for producing hybrid seed of paprika (*Capsicum annuum* var. annuum *Longum* Group) in commercial quantities at an affordable cost in Australia. The production of hybrid seed in *Capsicum* is time- and labour- intensive, and therefore expensive, but more than justified by the expressed hybrid vigour in increased yield and ASTA content. Various aspects of hybrid seed production based on male sterility were investigated.

4.1 Male sterile lines

To establish male sterile paprika lines for production of hybrid seed, male sterility was induced in paprika genotypes using gamma irradiation or EMS. Evidence from the literature indicated that male sterile (ms) mutants with agronomically acceptable characteristics in other forms of *Capsicum* could be recovered after mutagenesis. Four male sterile individuals were selected after gamma irradiation (Table 7), and two after EMS treatment. F2 segregation ratios confirmed monogenic recessive inheritance of male sterility. Tests of allelism showed that male sterility in the gamma-irradiated mutants was not controlled by genes ms3 or ms5 that were available in introduced lines of other *Capsicum* types. However, it became clear that new sources of germplasm could be obtained in agronomically adapted paprika genotypes by mutation breeding.

Table 7. **List of male sterile plants selected after gamma irradiation. Codes 2, 9, 11 and 20 were the lines selected for further work.**

Code	Generation	Fertility (visual)	Pollen colour	Pollen Staining (%)	Germ. (%)
1	M1	sterile	purple	0	0
2	M1	sterile	white	2	1
3	M1	sterile	purple	0	0
4	M1	sterile	purple	4	6
5	M1	sterile	purple	0	0
6	M2	sterile	purple	7	6
7	M1	sterile	purple	0	0
8	M2	sterile	purple	0	0
9	M1	sterile	purple	0	0
10	M1	sterile	purple	0	0
11	M1	partial fertile	purple	2	3
12	M1	sterile	purple	5	4
13	M1	sterile	purple	10	8
14	M1	sterile	purple	0	0
15	M2	sterile	purple	0	0
16	M1	sterile	purple	2	3
17	M1	sterile	purple	0	0
18	M1	sterile	purple	0	0
19	M2	sterile	white	10	10
20	M2	sterile	purple	2	0

4.2 Molecular marker tagging of a male sterility gene

Because the gene ms3 is widely used to develop hybrid *Capsicum* in other countries, an attempt was made to tag this gene to allow identification of sterile seedling plants. The amplified fragment length polymorphism (AFLP) technique identified a dominant marker co-segregating in repulsion with male sterility, using bulks of fertile and sterile plants in the cross Rapires (ms3)/Conquistador. This marker mapped 2.7 cM from Ms3. Sterility was identified by the absence of the marker, when converted to a PCR format, has the potential for acceptable identification of male sterile individuals in segregating paprika populations at an early growth stage.

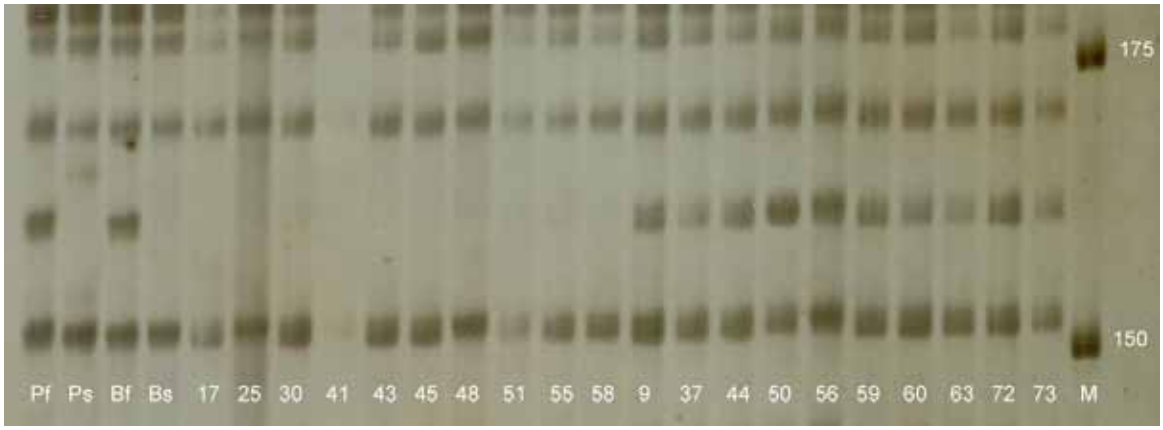

Figure 7. Amplification of the AFLP primer combination PgA/MagC gives a 160bp fragment present only in male fertiles in segregating populations of ms3. There is a segregating fragment at 160bp (between the 175 and 150bp marker on the right) that is present in the fertile parent (Pf), absent from the sterile ms3 parent (Ps), present in the bulk of 10 fertile lines (Bf, and the 10 fertile lines 9-73), and absent from the bulk of 10 sterile lines (Bs and the 10 sterile lines 10-58).

4.3 Possible vegetative production of male sterile lines

Vegetative propagation of genetic male sterile lines provides an alternative way for economic production of hybrid seed without the necessity of phenotype identification, progeny testing, or marker screening. Moreover, vegetative propagation can be cost effective in the maintenance of cms lines. Male sterile *Capsicum* lines were successfully propagated during this study as cuttings in the greenhouse. Rooting was achieved in Jiffy propagation plugs after 4-5 weeks of culturing in misting chambers. A single Ethrel application (250 ppm) was sufficient to delay flowering and to induce vegetative growth. An attempt was made to propagate ms lines *in vitro*. A standard disinfecting procedure was established and applied in the studies to identify shoot induction medium. Despite some positive results, *in vitro* propagation was at so low a frequency that it could not be used on a large-scale because of low reproducibility.

4.4 Production of hybrid seed in the field

The effects of different planting designs and propagation methods on the efficiency of hybrid seed production using genetic and cytoplasmic male sterile lines were evaluated. The level of cross-pollination of *Capsicum* in the Camden area (50km of southwest of Sydney, NSW) was determined to be 58.8% to 60.1%. No significant differences in seed yield per plant were detected among genetic and cytoplasmic male sterile lines. Popagation, whether by seed or cutting, had no significant effect on hybrid seed production. The highest hybrid seed yields were achieved in plots where a 2 female: 1 male ratio was applied.

Adequate natural cross-pollination occurs in the Camden region to produce hybrid seed utilising stable cms and fertility restoring lines. On the other hand, gms lines could be propagated as cuttings on a large scale assuring that only male sterile plants are planted into the seed production field, together with the seed propagated pollen source. As a result, the production field will show a uniform pattern ensuring the efficient usage of available land and other resources.

5. Production

Milling and sampling the harvested and dried paprika consisted of the milling of samples from the cultivar trials, milling of larger quantities, and some test-milling trials. The test milling resulted in a very highly milled paprika. The cultivar samples ranged from high ASTA values of 336, down to 194 ASTA.

During the 2002-2003 season, to increase the availability of milled product of 'Cerise Sweet' and 'Sunired', fully ripened fruits were harvested and milled.

Table 8. **Amount of milled paprika processed in the end of 2002-2003 season**

Name	Milled paprika (kg)	ASTA
Cerise sweet	5.3	221
Sunired	6.8	190
Over 200 ASTA (mixed lines)	3	220
Under 200 ASTA (mixed lines)	3	156

The test milling indicated that when a medium screen was used, the milled product was not fine enough. The product was sifted and what did not pass through a 0.3 mm screen was then milled again. The product that had a small particle size gave a higher pigment extraction. All the samples from cultivars were sent to BRI for pigment testing.

During the 2003-2004 season, from 42 kg dried paprika harvested from the cultivars, 15 kg seed was separated from the flesh mechanically using different sieves. A total of 27 kg remained as a seedless flesh. This amount was divided into two lots. One lot was milled as seedless and the other lot had 15% seed added, which is the normal condiment paprika production method. These two lots were sent to BRI Australia for milling and ASTA testing. Another ASTA testing was carried out here at PBI.

Table 9. **ASTA results of the processed quantities**

Samples	ASTA content BRI	ASTA content PBI
Paprika seedless	265	245
Paprika with seed	190	193

The milled product was tasted and compared to Hungarian standards as well as to some samples from retail suppliers here in Australia. One of the samples was imported from Israel and supplied by McCormick, and the other one was a Hungarian style supplied by Fancy Spice. We tested these samples for pigment content and it is interesting to note that our samples have much higher ASTA content and a much better taste and aroma than the retail samples.

Table 10. **ASTA results of our product and other paprika retail products**

Name	ASTA
Paprika with seed (radiated)	193
McCormick sample	96
Fancy Spice sample	73

A total pf 14 kg of paprika seedless, 4 kg of paprika with seed and 3 kg of 'Cerise Sweet' was sent for oleoresin extraction to Solvent's Australia Pty Ltd. Oleoresin for smallgoods manufacturing and as a spice is required to be extracted from high quality paprika - preferably from Hungarian or Basque types of fruits. In this case, taste and bouquet is extremely important. The price is dependent on the ASTA values that could range from 100-400 ASTA. The ASTA values are adjusted after extraction by diluting the extract with various vegetable oils. The oleoresin extraction process automatically sterilises the product.

All milled product has been sanitised by ionising radiation using a dose of 10 kGy of gamma radiation. Another ASTA testing has been done for all irradiated milled paprika.

Figure 8. Paprika produced from the parent line of Cerise Sweet line produced from project trial plots

Appendix :

1. Poster paper

Capsicum:

Cost Effective Hybrid Seed Production

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Introduction

Paprika (*Capsicum annuum* L. var. *annuum* covar. *longum*) is a crop where hybrid vigour is not exploited widely as the production of hybrid seed is costly in time and labour, and therefore expensive. To decrease the cost of the hybrid seed we aim to exploit cross pollination by native Australian bees, so that male sterile paprika plants can give high hybrid seed production.

Research Strategy

Generating male sterile lines
back crossing
mutagenesis

→ **Identification of male sterile plants**
phenotypic markers
molecular markers

→ **Vegetative propagation**
cuttings
micropropagation

→ **Hybrid seed production**
Pollination by native Australian bees (Figure 4) under field conditions

Results

Back crossing

- 7 elite cultivars carrying *ms3* gene (Figure 1),
- 1 elite cultivar with a Peterson CMS line demonstrated to be stable under our conditions,
- All BC derivates have initial ASTA ~ 160 a week after harvesting.

Figure 1: Fruits and flowers of sterile (S) and fertile (F) plants (*ms3* gene)

Mutagenesis

- From over 10,000 irradiated seeds 40 male sterile plants were identified,
- The inheritance of male sterility will be further studied.

Phenotypic markers

- Male sterile germplasm was grown in insect proof tunnel houses in field,
- 2 years observations (Table 1) confirm that reliable phenotypic markers are:
  - plant height at 50% fruit maturity,
  - fruit length/width (L/W) ratio.

Table 1: Mean of 2 years observations confirms the significant difference ($P \leq 0.05$) of sterile genotypes over fertiles

	Lines	Sterile plants*	Fertile plants
Plant height (cm)	MS3	133	98
	MS5	142	118
	MS3/Sonia/Tasso	137	106
	MS3/Sun7-13	140	107
Fruit* L/D	MS3	3.71	3.95
	MS5	3.92	3.09
	MS3/Sonia/Tasso	3.93	3.41
	MS3/Sun7-13	3.95	3.94

*Performance

Identifying molecular markers for *ms3* gene

- Using AFLP® method combined with BSA (Bulk Segregant Analysis),
- DNA extracted at 4-leaf stage,
- Polymorphism was detected among sterile and fertile bulks by 4 primer combinations (Figure 2),
- Linkage between *ms3* and the polymorphism will be studied on the population.

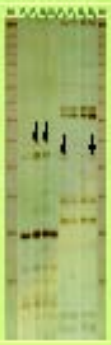

Figure 2: AFLP DNA fingerprints of sterile and fertile parents and bulks of 2 of the primer combinations which produced polymorphism

P_1 Sterile Parent
P_2 Fertile Parent
B_1 Fertile Bulk
B_2 Sterile Bulk
M Marker (1 kb ladder)

Vegetative propagation

- Male sterile plants were propagated as cuttings,
- 7 different genotypes (GMS and CMS),
- Oasis® cubes used as growing medium,
- Rooting percentage of two node cuttings between 70-85% after 5 weeks (Figure 3).

Figure 3: 1 and 5 week old cuttings in Oasis® cubes

Anticipated Outcome

- Male sterile paprika lines for commercial production of hybrid seed,
- Identification of male sterile plants at seedling stage by using molecular markers,
- Economical propagation of male sterile plants as cuttings,
- A field planting design e.g. alternating rows of male sterile and fertile plants to maximizing pollination efficiency utilizing native bees (Figure 4).

Figure 4: A stingless bee (*Trigona carbonaria*) from Queensland and NSW

Conclusion

We believe that by applying this system we can produce commercial quantities of hybrid paprika seed at a reasonable price.

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