

Intraspecific Hybridisation of *Boronia heterophylla* F. Muell

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Boronia heterophylla is cultivated for cut flower production. Three cultivars dominate production, 'Red', 'Cameo', and 'Moonglow'. A variety of colors and an extended flowering period are demanded by local and overseas markets. The aim of this study was to develop procedures for a *Boronia heterophylla* breeding program through intraspecific hybridization. This may yield progeny with desirable characteristics, ideally increased vigor, and a range of flower colors and flowering times. Nine pollination combinations were attempted, each self pollination and all reciprocal crosses. Seed set varied from 17 to 95%. Embryo rescue (was employed to produce hybrid plants using half strength Murashige and Skoog (MS) basal media and it was most successful (75%) 5-6 weeks after pollination. All shoots multiplied on media containing MS salts + NAA (0.1 mg/l) + BA (0.4 mg/l). All shoots transferred to medium containing half strength MS salts + NAA (4 mg/l) produced roots. Plantlets were acclimatized to sterile potting mix (in a small chamber within a glass house. This intraspecific hybridization system was very successful and plants were obtained for all pollination treatments except for selfed 'Moonglow'. Embryo rescue may provide a system for germinating other species with difficult to germinate or dormant seed.

Key words: embryo rescue, decoated seed culture, ornamental plant

INTRODUCTION

Boronia heterophylla F. Muell is an upright bush endemic to south-western of Western Australia. Abundant flowers are born along the stems in spring and plants are cultivated for the production of cut flowers. Flowers are one cm long, have four petals and are usually deep pink, but referred to as 'Red'. Two other rare color forms have been brought into cultivation, 'Cameo' which has white flowers with pink stripes along the central vascular tissue of each petal, and 'Moonglow' which has pure white flowers (Plummer 1996). A variety of colors is demanded by local and overseas markets (Plummer *et al.* 1998). 'Red' *Boronia* has a very short harvest period of 1-2 weeks in September/October and this restricts the time when product is available. 'Cameo' flowers one week earlier and 'Moonglow' one to two weeks later than 'Red'. 'Cameo' and 'Moonglow' are less vigorous than 'Red', and produce fewer, shorter stems. For harvest, and longer stems and greater productivity are desired for commercial production. Intraspecific hybridization within these varieties may yield progeny with desirable characteristics, ideally increased vigor and a range of colors and flowering times.

Intraspecific hybridization has not been attempted in *Boronia*. Weston *et al.* (1984) claims that *B. heterophylla* is self-incompatible. Controlled pollinations are therefore required to determine if seed set is possible. In *Boronia megastigma*, anthers dehisce and stigmas are receptive

when flowers begin to open but cross-pollination does not occur until flowers are fully open and it is naturally undertaken by a specific moth which pollinates flowers as it holds onto the style whilst it ovi-deposits its egg in an ovule of the flower (Bussell *et al.* 1995). This vector is required for pollination and presumably *B. heterophylla* is similar. Like many difficult to germinate Australian species, *Boronia* seed have dormancy and low viability (Bell *et al.* 1993) but these factors can be overcome in *B. megastigma* using embryo culture (Sisteberio & Plummer 1994) and a mechanism to germinate seed will be required for *B. heterophylla*.

The aim of this study was to develop procedures for a *Boronia heterophylla* breeding program through intraspecific hybridization.

MATERIALS AND METHODS

Plant Materials. Three cultivars of *Boronia heterophylla* were used, 'Red' [Royal Horticultural Society (RHS) Colour Chart no. 57B], 'Cameo' (White RHS no. 155D; pink stripes RHS no. 57B), and 'Moonglow' (White RHS no. 155D) (Anonymous 1990). Plants were grown in a commercial orchard at Mundijong (50 km south of Perth, Western Australia) with organic mulch under fertigation which was supplied several times during the day.

Pollination. Pollination was carried out in the field during flowering seasons on 21 month-old plants. Nine pollination combinations were attempted; each self-pollination and all reciprocal crosses were performed on different plants. Flowers opened from the base to the top of stems. Flowers which had just opened were selected

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and each shoot was thinned to 20 of these flowers. At this stage, petals overlapped by about one third, anthesis had just occurred and stigmas were green and receptive, and about 3 mm in length. Sticky, dehiscent anthers were carefully removed with forceps and placed in vials. No contact was made between anthers and stigma at this stage. Within an hour appropriate anthers were rubbed against the stigmatic surface of the designated female parent flowers. To avoid pollen contamination, forceps were dipped in ethanol (70%) between pollinations. It was not necessary to emasculate the flowers, since anthers are located below the stigma and self-pollination does not occur. *Boronia* requires moth pollination (Bussel *et al.* 1995), but no moths or other potential insect vectors were observed in the cultivation area.

Embryo Rescue (Decoated Seed Culture). Five fruits from each shoot of each cross were collected randomly at 4, 5, and 6 weeks after pollination (WAP) and when the seed shed (6.5 WAP). Ovaries were surface sterilized by washing in sodium hypochlorite (2.5%, v/v) for 15 minutes and were rinsed (3x) with sterile distilled water before being placed in sterile petri dishes. Seed were excised from ovaries under a stereo-microscope with forceps and seed coats were removed using a sharp blade before transfer to culture tubes. Decoated seed containing the embryo with endosperm were cultured in screw-cap, 30 ml polycarbonate tubes containing 8 ml nutrient media. Half strength Murashige and Skoog (MS) (Murashige & Skoog 1962) basal media was used for germination. The medium was solidified with 7% agar and pH was adjusted to 6.3 before autoclaving at 121 °C for 20 minutes. Media were solidified with 45 °C sloped surfaces.

Decoated seed were incubated in a growth room at 20-25 °C and exposed to a 16 hour photoperiod using cool white fluorescent tubes (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation). After 4 weeks in germination media, seedlings with shoot and roots were removed from culture and were transferred to pots. Seedlings which only had shoots or roots were transferred to multiplication media containing MS salts supplemented with naphthalene acetic acid (NAA, 0.1 mg/l) + benzyl adenine (BA, 0.4 mg/l). Cultures were transferred to fresh media at 4 weekly intervals. Cultures were monitored and those which produced multiple shoots were divided at transfer.

On the fourth subculture, single shoots were transferred to a rooting medium containing half strength MS salts supplemented with NAA (4 mg/l). Seedlings from either germination or rooting media with roots and shoots were potted in a sterile potting mix (peat: perlite: sand::5:

4:1) and placed in a fogging chamber within a glasshouse for 4 weeks before transfer to an open mist bench for another 4 weeks. Fungicide was applied once a week using a rotation of Benlate (DuPont), Previcur® (Bayer), and Fungarid® (Agnova). Surviving plants were grown in a shade house.

The total number of fruit set, carpels developed and seed produced for each cross was recorded. The proportion of decoated seed that germinated was recorded 4 weeks after culture. Germination was defined as embryo greening accompanied by radical and hypocotyl elongation. Multiplication rate, proportion of plantlets that produced roots, and number of roots formed were recorded after 4 weeks in culture.

RESULTS

Fruit and Seed Development. The phenology of fruit development was described for Mundijong, Perth, Western Australia (Figure 1). Petals had re-closed and carpels had swollen by 2 weeks after pollination (WAP). Swollen carpels were interpreted as a response to fertilization and were an indicator of fruit set. At 2 WAP petals of pollinated flowers were closed, while petals of unpollinated flowers did not and this was used to distinguish pollinated from unpollinated flowers.

Petals wilted by four weeks after pollination (WAP). Fruit harvested at this time were swollen and the number of developing carpels for each fruit varied from 1-4. Most carpels (70%) contained a single seed. A few carpels (5%) contained two seeds and a quarter was empty. At this stage, seed had thin, shiny black seed coats and upon dissection had white soft, gelatinous endosperm, and visible embryos. The gelatinous endosperm made it difficult to remove the seed coat without damaging the endosperm and so the whole seed was cultured.

By 5 WAP, petals had started to abscise and fruits were hard, desiccated and brown. By 6.5 WAP (45 days after pollination) some fruits had started to dehisce, and all seeds were harvested to avoid seed loss through the explosive dehiscence of fruits left in the field. Fruit were kept in sealed petri dishes to dry out and fruit dehisced within 2 days. Seed had firm endosperm and fully developed embryos. At this stage, differences in fruit color were observed between genotypes. Fruits born on 'Red' had red fruit color, 'Cameo' fruit were pale pink, whilst 'Moonglow' fruits were pale green.

All crosses within *B. heterophylla* cultivars set fruit and seed but the proportions varied with genotypes

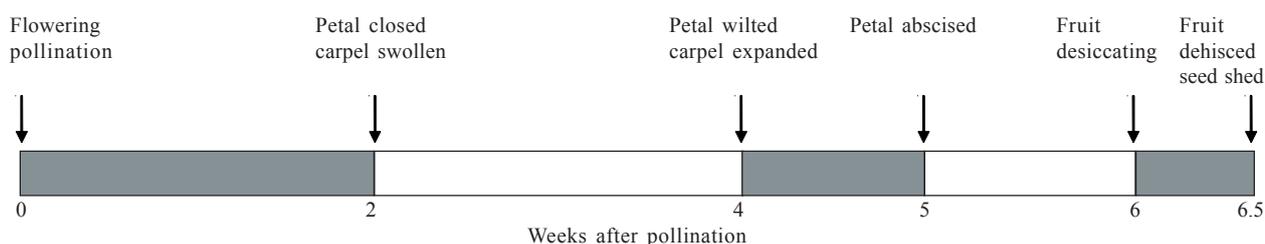


Figure 1. Phenology of *Boronia heterophylla* fruit development.

(Table 1). ‘Moonglow’ and ‘Cameo’ were very successful as female parents and they had a very high proportion of fruit set (>95%). ‘Red’ was less successful with 60-70% fruit set. Fewer fruit were produced on self pollinated than cross pollinated plants. Selfed ‘Cameo’ and ‘Red’ had about 20% fruit set, while selfed ‘Moonglow’ produced more than 70% fruit set, most of the fruit set on ‘Moonglow’ however, produced little or no seed.

The number of swollen carpels per fruit varied between crosses (Table 1). Crosses with ‘Moonglow’ as female parents had the highest number of swollen carpels per fruit, followed by ‘Cameo’ and then ‘Red’. Among selfed plants, ‘Moonglow’ had the highest number of carpels developed (2-3 carpels per fruit), but a quarter of these were empty. Selfed ‘Cameo’ and ‘Red’ flowers produced fruit with a very low number of developed carpels, and generally carpels did not form properly.

The percentage of seed set varied among crosses. Reciprocal crosses between ‘Cameo’ and ‘Moonglow’ were the most successful (Table 1). Both ‘Moonglow’ and ‘Cameo’ produced about 3 seeds per fruit, while ‘Red’ had less than 2 seeds per fruit. All selfed plants produced less than one seed per fruit on average.

In three crosses, M x C, C x R and C x M (R=‘Red’, C=‘Cameo’, and M=‘Moonglow’) carpels on average produced more than one seed. All selfed plants and R x M produced less seed than developed carpels as there were many empty carpels.

Germination in Culture. Germinability varied among crosses (Figure 2). Seed from pollinations with ‘Moonglow’ as the female parent, reached about 60% germination, while seed from pollinations with ‘Cameo’ as the female parent had slightly higher germinability of 60-70%. With ‘Red’ as the female parent seed had 30-60% germinability. C x M crosses had the highest germination

rate, while R x M was the lowest among all seed. Selfed ‘Cameo’ and ‘Red’ plants produced seed which reached about 50% germination, while seed from selfed ‘Moonglow’ had only 30% germination.

Seed had the highest capacity to germinate when they were cultured at 5-6 WAP (Figure 3), and they reached up to 75% overall germination. Seed cultured at 6.5 WAP, that is when the seed dehisced, had half of the germination capacity (40%), and seed cultured at 4 WAP had a very low germinability of less than 20%. Decoated seed cultured at 5 WAP and 6 WAP readily germinated within one week of culture, while germination of decoated seeds cultured at 6.5 WAP took 2 weeks or longer. Germinability was generally less at 4 or 6.5 WAP. Dissection of ungerminated

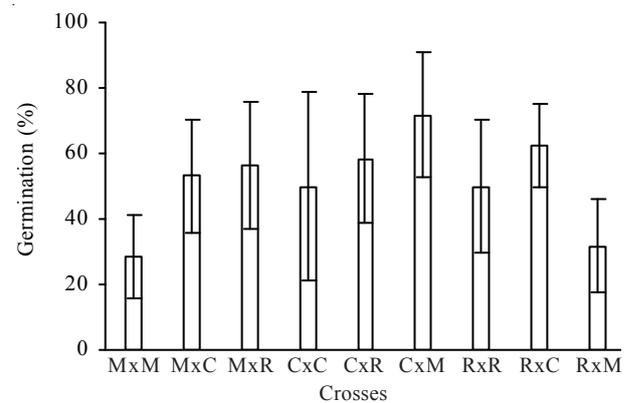


Figure 2. Germination *in vitro* of decoated seed from each intraspecific cross of *B. heterophylla*. M = ‘Moonglow’, C = ‘Cameo’, R = ‘Red’.

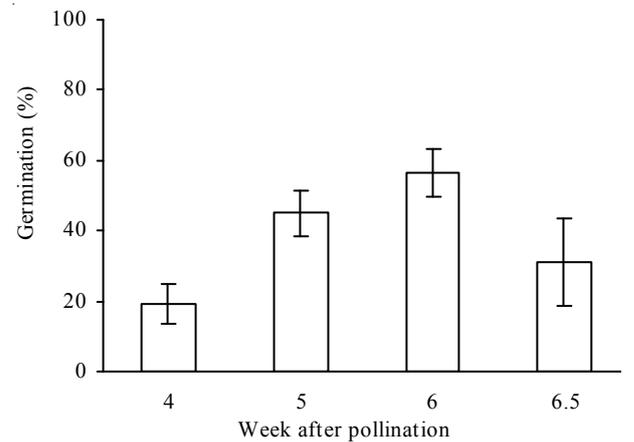


Figure 3. Germination *in vitro* of decoated intraspecific hybrid seed of *B. heterophylla* harvested at different numbers of week after pollination.

Table 1. Proportion of fruit set (n=60), carpels developed per fruit and proportion of seed produced per total potential seed set (x=8, reproductive success) on each cross within *Boronia heterophylla*

Crosses (♀ x ♂)	Fruit set (%)	Carpel	Seed (%)
C x M	100 ± 0	2.9 ± 0.1	82 ± 3
M x C	99 ± 1	3.5 ± 0.1	95 ± 6
M x R	97 ± 2	3.2 ± 0.2	71 ± 7
C x R	96 ± 3	2.5 ± 0.2	71 ± 6
R x C	70 ± 9	1.7 ± 0.2	28 ± 7
R x M	62 ± 11	2.0 ± 0.4	17 ± 5
M x M	73 ± 9	2.4 ± 0.3	19 ± 5
C x C	23 ± 8	1.1 ± 0.3	6 ± 2
R x R	18 ± 6	0.6 ± 0.1	3 ± 1

M= ‘Moonglow’, C= ‘Cameo’, and R= ‘Red’. Mean ± s.e.

Table 2. Proportion of seed germinated from intraspecific *Boronia heterophylla* hybrids harvested at 4-6.5 weeks after pollination (WAP)

Harvest at (WAP)	Germination (%)									
	M x M	M x C	M x R	C x C	C x R	C x M	R x R	R x C	R x M	
4	0	3	0	0	20	15	50	31	17	
5	50	61	74	100	96	88	50	60	60	
6	50	80	66	100	88	98	100	92	50	
6.5	14	69	86	0	29	86	0	67	0	

M= ‘Moonglow’, C= ‘Cameo’, and R= ‘Red’.

Table 3. Multiplication rate and rooting ability of cultures of intraspecific hybrid seedlings of *Boronia heterophylla*

Hybrid parents	Multiplication rate			Number of roots	Potted plants produced
	Subculture 1	Subculture 2	Subculture 3		
C x M	3.2 ± 0.6	4.0 ± 0.9	7.4 ± 3.5	2.7 ± 0.7	19
C x R	2.8 ± 0.5	4.8 ± 1.3	6.8 ± 2.8	3.3 ± 1.5	15
R x R	2.0 ± 0.0	3.0 ± 0.6	14.3 ± 2.2	2.9 ± 1.0	14
C x C	2.0 ± 0.0	2.0 ± 0.0	19.0 ± 2.3	3.0 ± 0.2	13
R x M	4.7 ± 1.3	9.0 ± 0.6	17.3 ± 3.3	3.5 ± 0.5	12
M x R	2.3 ± 0.3	3.7 ± 0.3	10.0 ± 2.4	1.8 ± 0.4	3
R x C	7.0 ± 1.6	12.0 ± 1.7	36.0 ± 3.4	1.5 ± 0.5	3
M x C	2.5 ± 0.4	3.0 ± 0.2	3.0 ± 0.3	5.1 ± 1.3	2
M x M	-	-	-	-	-

Multiplication rate: number of new shoots per shoot after 4 weeks. M= 'Moonglow', C= 'Cameo', and R= 'Red'. Mean ± s.e.

seed after 6 weeks in culture revealed that half contained dead embryos and brown endosperm and the other half were empty.

Multiplication, Rooting and Transfer to Glasshouse.

On the first subculture into multiplication media, explants did not multiply very well, with a maximum of 5 shoots after 4 weeks in culture. In the second subculture, multiplication rate improved. Roots initiated after 3 weeks in rooting culture media, and after 5 weeks, they were ready to transfer to pots in the glasshouse. All intraspecific hybrids which produced shoots also produced roots (Table 3). Transfer to pots was quite successful with 90% success. Plants were obtained for each intraspecific cross except for selfed 'Moonglow' (Table 3).

DISCUSSION

Boronia heterophylla cultivars were successfully hybridized using hand pollination. The level of fruit and seed set was high. Formation of endosperm and embryos were observed, suggesting that there were no pollination or fertilization barriers between genotypes. Sufficient viable pollen was produced in each set of anthers in flowers from all plants to effect pollination in all crosses. Whilst cross-pollination among genotypes with overlapping flowering periods was possible with fresh pollen, *Boronia* pollen can also be stored (Astarini *et al.* 1999) which would allow for crosses that could further extend the flowering season.

Lethal genes may operate in the pistil, causing sterility within the embryo sac. Crosses using 'Red' as the female parents set only 70% of fruit and 30% of ovules produced seed. This was much lower than other crosses (Table 1), even though the environmental conditions for growth of all plants were held constant. These differences indicated that deleterious or lethal genes may operate in *B. heterophylla* 'Red' egg or polar nuclei. In contrast, reciprocal crosses between 'Cameo' and 'Moonglow' produced almost 100% fruit and seed set suggesting these genes were absent. The high fruit set contrasts with the other genera of Rutaceae, such as *Citrus* which has indehiscent fleshy fruit, where only 5% of flowers set fruit (Plummer *et al.* 1991).

Lack of stigma receptivity may also be a problem in hybridization. Flowers along a stem of *B. heterophylla* did not open at the same time. In *Boronia megastigma*,

flower opening commences in the middle of the stem and advances towards both the apex and base (Robert & Menary 1989). In contracts in *B. heterophylla* anthesis of flowers began at the bottom and advanced to the top over several days. Thus there were differences in stigma receptivity along a branch at any given time. Receptive stigmas in *B. heterophylla* had fully elongated styles (3-4 mm length) and the stigmatic surface was green. Pollen applied at this stage successfully germinated and tubes grew to the ovules. Senesced stigmas were yellow to brown and shriveled. Stigma receptivity in this pollination study was standardized by using flowers of a similar stage. Therefore stigma receptivity was unlikely to be a limiting factor in fruit set when comparing different crosses.

Selfed plants of *B. heterophylla* produced the least seed. Fruit set appeared normal after self pollination, but when the fruits were dissected at 4-6.5 weeks after pollination (WAP), most carpels contained empty seed coats. Empty seed usually indicates embryo abortion (Marchant *et al.* 1994; Grauda & Balode 2004). A reduction in seed set after self pollination compared to cross pollination is widespread among hermaphroditic flowering plants. Gametes can carry either incompatibility factors that react at fertilization, or recessive lethal factors that act post zygotically (James 1992). Post-zygotic control of genetic homozygotes with deleterious or lethal recessive genes most likely operated. Seed set was less than 20% in selfed 'Red', 'Cameo', and 'Moonglow', indicating that 80% of zygotes may have contained deleterious or recessive pairs of genes. Seed abortion systems permit discrimination between selfed and crossed seed so that most of the seed reaching maturity in nature is derived from cross pollination. The selfed seed would be selectively removed by the seed abortion system. Post-zygotic abortion is common in other Western Australian taxa including *Isotoma petraea*, *Stylidium*, *Laxmannia*, and *Eucalyptus* (James 1996). In these groups, seed abortion is an evolved component of the genetic system. Post-zygotic seed abortion system causes self-pollinating species to maintain a genetic architecture that is more characteristics of outbreeding species (James 1996). In *B. heterophylla* post zygotic abortion may also reduce the genetic load following self pollination events.

Lethal genes may also operate on post seed maturation. In the present study, low germination was observed on selfed *B. heterophylla* seeds (Table 2),

suggesting that lethal genes may affect germination, as in *Styloidium* (James 1992). Moreover, in selfed 'Moonglow' all germinated seed died at the seedling stage, and selfed 'Red' and 'Cameo' had low vigor indicated by a low multiplication rate and limited root formation. Also few of these plants survived transfer from *in vitro* to pot culture. *Boronia* may have many deleterious recessive genes which either decrease vigor or are lethal and these may affect any stage of the plant's life cycle (James 1996). Some may operate during seed development and be observed as post-zygotic seed abortion prior to seed shed, others may affect seed longevity, or seeds may germinate but then die as seedlings or juvenile plants before flowering.

Germinability of hybrid seed varied among crosses. This may be due to viability and dormancy factors. Seed viability in *Boronia* species is generally very low, ranging from 0% in *B. molloyae* to 40% in *B. serrulata* (Bell *et al.* 1987). Unless mechanisms can be identified to overcome dormancy it will reduce germinability (Bell 1999). At least two types of dormancy occurred in *Boronia* seeds, physical dormancy imposed by the seed coat and physiological dormancy imposed by the embryo (Baskin & Baskin 1998, 2004). The shiny black, hard seed coat of *Boronia* probably prevented embryo germination through inhibition of water uptake, but it may also inhibit gas exchange, contain chemical inhibitors, act as a barrier against the escape of inhibitors from the embryo, or it could mechanically restrains embryo growth. Germination is prevented or delayed by coat impermeability in *Boronia megastigma* (Sisteberio 1993) and other Rutaceae, such as *Citrus* (Bewley & Black 1982). Seed coat impermeability is due to the structure and chemical composition of the seed coat, for example, tightly packed cells that are impervious to water or intense deposition of callose (Bhalla & Slattery 1984). Variation in structure or chemical deposition may have contributed to the range of germinability observed among intraspecific hybrid seed of *Boronia heterophylla*. Physical dormancy is at least easily overcome by removal of the seed coat (Bell *et al.* 1993; Marchant *et al.* 1994).

Decoated seed culture was preferred to embryo culture for *B. heterophylla* because the young embryos were difficult to excise from the endosperm for culture *in vitro*. Also, high germinability was reached (70%) and thus there was no need to further excise the embryo. Embryo and decoated seed culture also improves germination in *B. megastigma* (Sisteberio 1993; Plummer & Considine 1995). Similar techniques have been successfully applied to several Australian plants such as *Anigozanthos* (Motum *et al.* 1985) and *Chamelaucium* (Anonymous 1993) and many exotic ornamentals such as *Zantheschia* (Yao *et al.* 1995), *Alstromeria* (Buitendijk *et al.* 1995), *Lilium* (Ahn *et al.* 2003) and *Rhododendron* (Eeckhaut *et al.* 2007).

In conclusion, intraspecific hybridization of *B. heterophylla* was successful when pollen was transferred to stigmas when flowers had just opened, petals overlapped by one third and stigmas were green and receptive. Developing hybrid embryos could be excised

5-6 weeks after pollination to avoid dormancy and these readily multiplied and produced roots in culture. This intraspecific hybridization system could provide plants with a range of characteristics, such as improved vigour, different colours and extended flowering time for varietal selection.

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