Introgression of yellow flower colour in *Buddleja davidii* by means of polyploidisation and interspecific hybridisation

K. Van Laere, J. Van Huylenbroeck, E. Van Bockstaele

Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit, Applied Genetics and Breeding, Melle, Belgium

Abstract

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To introduce yellow colour in the commercial *Buddleja davidii* ($2n = 4x = 76$) assortment, an interspecific breeding programme with *B. globosa* ($2n = 2x = 38$) was started. The first step was to perform chromosome doubling in *B. globosa*. Two of the obtained tetraploid *B. globosa* plants were subsequently used as male parent in interspecific crosses with the white flowering *B. davidii* cv. Nanhoensis Alba. In total 182 interspecific crosses were made and 18 F1 hybrids were obtained. Genome size measurements, chromosome counts and genomic in situ hybridisation (GISH) analysis proved the hybrid nature of most of the F1 hybrids. Plant morphology also expressed hybrid characteristics. F1 seedlings inherited the yellowish flower colour from *B. globosa*. As for many other woody ornamentals, the creation of hybrids through interspecific hybridisation along with polyploidisation offers new opportunities for breeding in *Buddleja*.

Keywords: chromosome number; genome size; genomic in situ hybridisation (GISH); morphology; ornamental breeding

The genus *Buddleja* L., now classified in the family Scrophulariaceae (Olmstaed et al. 2001), has approximately 125 species naturally found in tropical and subtropical areas of Asia, Africa and North and South America (Leeuwenberg 1979; Norman 2000). The plant grows as an herbaceous perennial and has a dense canopy of foliage and a generous flower display, with scented flowers arranged in long panicles. The genus *Buddleja* contains several flowering shrubs suitable for temperate regions, the most important of which is *Buddleja davidii* L. Other well-known species are *B. alternifolia* Maxim., *B. lindleyana* Fort. Ex Lindl., *B. crispa* Benth. and *B. globosa* Hope. *B. globosa* has particular value due to its orange flowers borne in small spherical clusters. *Buddleja* has come under international scrutiny because of its potential for invasiveness (Ebeling et al. 2008; Tallent-Halsell, Watt 2009).

Few molecular and cytogenetic data for *Buddleja* have been published. In his cyto-taxonomic notes on *Buddleja*, Moore (1960) reported a basic chromosome number of $x = 19$. *Buddleja* species exist with 38 (*B. globosa*), 76 (*B. davidii*) and even up to 228 chromosomes. Great variation in interspecific genome size was also reported (Moore 1960; Hanson et al. 2001; Zonneveld et al. 2005; Van Laere et al. 2009). The high genetic variability in *Buddleja* presents considerable opportunities to develop novel cultivars through interspecific crosses. Controlled interspecific hybridisation in *Buddleja* was first reported by Van De Weyer (1920) when he crossed *B. globosa* with *B. davidii* var. *magnifica* to create *B.* × *weyeriana* Weyer. Ex Rehd. (Van De Weyer 1920), which had the yellow flower colour of *B. globosa*. Cultivars of this hybrid (Golden Glow, Moonlight, Sungold and Honeycomb) are still popular today

(Dirr 1998). Recent genomic in situ hybridisation (GISH) analysis proved the hybrid origin of *B. weyeriana* cv. Sungold (Van Laere et al. 2009). Other interspecific hybrids in *Buddleja* were reported, including the species *B. davidii*, *B. fallowiana* Balf., *B. alternifolia* Maxim., *B. crispa*, *B. madagascariensis* Lam., *B. asiatica*, *B. stenostachya* Reh., *B. salviifolia* Fort., *B. lindleyana* and *B. indica* (Moore 1949; Leeuwenberg 1979; Tobutt 1993; Dirr 1998; Elliott et al. 2004; Lindstrom et al. 2004; Van Laere et al. 2009). Nevertheless, the differences in ploidy level and genome sizes within *Buddleja* species can limit the success rate of interspecific hybridisation. Altering the ploidy level of parent plants can overcome problems with interploidy crosses (Zlesak et al. 2005; Dhooghe et al. 2011). Tetraploidisation was already obtained for *B. globosa* by applying colchicine to nodal sections (Rose et al. 2000). Chromosome doubled plants of *B. davidii* were generated by treating seeds with oryzalin and trifluralin (Eeckhaut 2003) or by applying colchicine to meristem cultures Duron and Morand (1978).

The aim of this study was to obtain a yellow flowered *Buddleja* hybrid with a *B. davidii*-like morphology. First chromosome doubling in *B. globosa* was performed by applying oryzalin and trifluralin to seedlings, seeds and shoots *in vitro*. Induced polyploid *B. globosa* plants were then used as male parent in crosses with *B. davidii*. The hybrid character of the F1 progeny was analysed using morphological parameters, genome size measurements and GISH.

MATERIALS AND METHODS

In vitro polyploidisation of *B. globosa*

In the first experiment, *B. globosa* seeds were sterilised in a 10% NaOCl solution with a drop of Teepol and rinsed three times in sterile water. The seeds were then sown on germination medium containing half-strength MS medium (Murashige, Skoog 1962), 30 g/l sucrose and 7 g/l agar (pH 5.8). When the cotyledons emerged, the resulting seedlings were treated with 2 µl of 0.3 or 3mM trifluralin or 0.3 or 3mM oryzalin during 3 subsequent days (= droplet). After treatment, the seedlings were transferred to fresh germination medium. In the second experiment, *B. globosa* seeds were sterilised and sown on germination medium enriched with 3, 10, 100, 1,000µM trifluralin or oryzalin (= seed sowing). Six weeks after the treatment, the surviving seedlings were transferred to fresh germination medium without mitosis inhibitors. In the third experiment, shoots of *B. globosa* were sterilised and initiated *in vitro* on a multiplication medium containing WPM (Lloyd, McCown 1980), 30 g/l sucrose, 25µM 2-Ip and 7 g/l agar, pH 5.8. After one multiplication cycle, shoots were transferred to fresh multiplication medium enriched with 10, 100 or 1,000µM oryzalin or trifluralin (= solid shoots). After 6 weeks, the surviving shoots were transferred to fresh multiplication medium without mitosis inhibitors. The fourth experiment was the same as the third except for the use of liquid multiplication medium instead of solid medium (= liquid shoots). After one multiplication cycle, the shoots were transferred to liquid multiplication medium and incubated for 3 days on a rotor at 250 rpm. Then 10, 100, or 1,000µM oryzalin or trifluralin was added to the medium and the shoots were further incubated for 3 days on the rotor at 250 rpm. After the treatment, the shoots were transferred to fresh solid multiplication medium.

One hundred seeds or shoots were used for each treatment. As a control, 100 untreated seeds or shoots were grown *in vitro*. The resulting polyploid *B. globosa* plants were acclimatised in the greenhouse at least 4 weeks after growing *in vitro*. The plants were transferred to plastic seedling trays containing a peat mixture (organic matter 20%, dry matter 25%, 1.5 kg/m^3 fertiliser: 12N:14P:24K + trace elements). They were kept in a fog unit (RH > 90%) during 3 weeks. Once the chromosome doubled plants were fully acclimatised, they were planted on the field and evaluated.

Flow cytometry

The ploidy levels of treated seedlings and shoots and genome sizes of parent plants and F1 progeny were measured according to Van Laere et al. (2009). For the measurements of the genome sizes, the reference plant *Pisum sativum* cv. Ctirad was used, which has a genome size of 9.09 pg/2C (Dolezel et al. 1998). At least five repetitions of each sample were measured.

Interspecific crosses

Interspecific crosses were performed as described in Van Laere et al. (2009). Chromosome doubled

Table 1. Effect of the different trifluralin and oryzalin *in vitro* treatments of *B. globosa* seeds on the doubling of the chromosome number

Method	Mitotic inhibitor	Concentration (µM)	Survivingseedlings (%)	# shoots tested (3 months after treatment)	2*x* plantlets (3 months after treatment)	4*x* plantlets (3 months after treatment)	2*x* + 4*x* plantlets (3 months after treatment)	4*x* plantson the field (1 year after treatment)
Droplet	control	0	98	588	588	–	–	
	oryzalin	300	25	93	46	4	43	–
		3,000	2	2	1	1	–	–
	trifluralin	300	58	183	94	76	13	18
		3,000	29	102	96	3	3	–
Seed sowing	control	0	80	80	80	–	–	
	oryzalin	3	56	56	56	–	–	
		10	39	39	7	–	32	
		100	40	40	23	10	7	5
		1,000	30	30	5	25	–	18
	trifluralin	3	47	47	47	–	–	
		10	79	79	15	–	64	
		100	66	66	61	–	5	
		1,000	0	–	–		–	

B. globosa GLO9 (obtained via droplet method with 0.3mM TRI) and GLO34 (obtained via seed sowing method with 1mM ORY) were used as male parent. The white flowering *B. davidii* cv. Nanhoensis Alba was used as female parent. Since *B. globosa* flowered earlier than *B. davidii*, stored pollen of *B. globosa* had to be used. For storage, pollen was harvested in the morning and dried under a lamp (Osram 40W) (OSRAM, Capelle a/d IJssel, The Netherlands). Dried pollen was then stored at –20°C.

Seeds resulting from the crosses were harvested when fully matured. They were sown directly in the greenhouse in 77-well trays filled with a peat mixture (organic matter 20%, dry matter 25%, 1.5 kg/m fertiliser: 12N:14P:24K + trace elements).

Morphological parameters

Leaf morphology was described on 10 fully developed leaves of a genotype by measuring leaf length (L) and leaf width (W) and calculating the L/W ratio. Flower colours were determined according to the Royal Horticultural Society Color Chart (RHS colours). On 10 fully developed inflorescences of a genotype (parental species and F1 hybrids) the length and/or diameter of the inflorescence was measured. Fertility of a genotype was evaluated by analysing seed set and pollen germination capacity according to the protocol described in Van Laere et al. (2009).

Genomic in situ hybridisation (GISH)

Chromosome preparation for chromosome counting was performed as described in detail in Van Laere et al. (2009). GISH was performed as described by Van Laere et al. (2010), using 25 ng *B. globosa* DNA probe (labeled with biotin or digoxigenin) and 2 µg of *B. davidii* block DNA.

RESULTS AND DISCUSSION

In vitro polyploidisation of *B. globosa*

The favoured method for polyploidisation of woody ornamentals is to apply the mitotic inhibi-

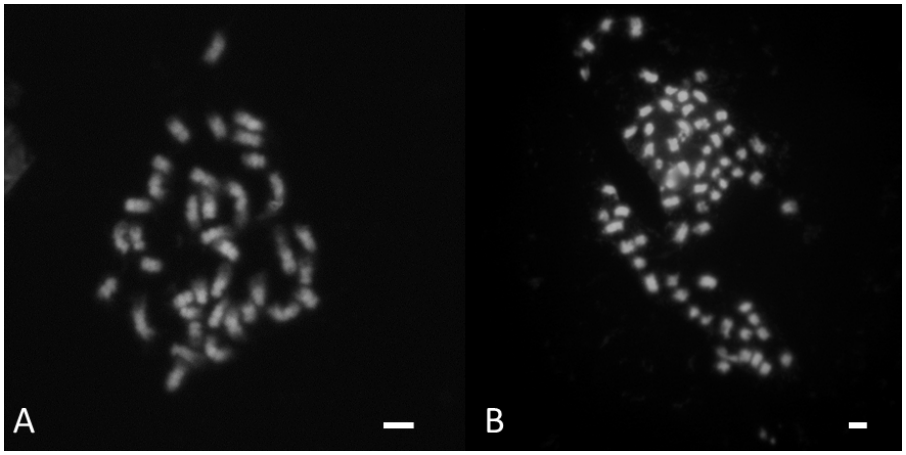

Fig. 1. Metaphase spreads for diploid *B. globosa* having 38 chromosomes (A) and tetraploid *B. globosa* (GLO34) having 76 chromosomes (B) (bar = 5 µm)

tor on *in vitro* cultured explants (see review by Dhooghe et al. 2011). In our study, after polyploidisation treatment of shoots on solid or liquid medium none of the tested shoots had an altered ploidy level. Nevertheless, chromosome doubling of *B. globosa* seeds or seedlings was successful (Table 1; Fig. 1). The droplet method was most successful using 0.3mM trifluralin; 76 tetraploid plants were obtained *in vitro*, of which 18 tetraploid *B. globosa* plants could be acclimatised on the field. With the seed sowing method, tetraploids were obtained using 100µM oryzalin (10 tetraploid *B. globosa* plants *in vitro*; 5 acclimatised on the field) and 1mM oryzalin (25 tetraploid *B. globosa* plants *in vitro*; 18 acclimatised on the field). In both methods (droplet and seed sowing) mixoploids were also obtained (Table 1). When the mixoploids were retested after 3 months, they all appeared to have reverted to diploids. Eeckhaut (2003) also only regenerated chromosome doubled plants when *B. davidii* seeds were treated with oryzalin and trifluralin. This concurs with results in other genera, such as *Platanus,* where treatment of (ungerminated) diploid seeds with colchicine was the most efficient method to obtain tetraploid seedlings (Liu et al. 2007). The use of nodal sections has some advantages over the treatment of seed(lings); only one selected genotype is treated when using nodal sec-

Fig. 2. Flower morphology of *B. globosa* GLO34 (A), *B. davidii* (B) and the F1 seedlings resulting from *B. davidii* × *B. globosa* GLO34 (C)

Table 2. Overview of the different interspecific crosses within *Buddleja* species after polyploidisation of *B. globosa*

Cross		Pollinations	Fruits	F1 plants on the field
♀	♂			
B. davidii cv. Nanhoensis Alba (2*n* = 4*x*)	*B. globosa* GLO34 (2*n* = 4*x*)	56	4	18
B. davidii cv. Nanhoensis Alba (2*n* = 4*x*)	*B. globosa* GLO9 (2*n* = 4*x*)	126	0	
B. davidii cv. Nanhoensis Alba (2*n* = 4*x*)	*B. globosa* (2*n* = 2*x*)	195	0	

tions. In contrast, our breeding goal – introgression of the yellow colour of *B. globosa* – relied less on the genotype itself; only a chromosome doubled crossing parent was required.

In an early stage, the tetraploid seedlings of *B. globosa* grew faster than the control seedlings. But after 6 to 8 months the growth stagnated, and many of the tetraploid *B. globosa* plants died in the first year. Finally, two of the tetraploid *B. globosa* plants flowered (GLO9 and GLO34) and could be used in interspecific crosses. Pollen germination capacity of *B. globosa* was enhanced by polyploidisation (2% for diploid *B. globosa* versus 8% for GLO9 and 11% for GLO34).

Interspecific breeding

The cross *B. globosa* (2*n* = 2*x*) × *B. davidii* had been reported by Van De Weyer (1920) but the reciprocal cross *B. davidii* × *B. globosa* (2*n* = 2*x*) performed by Van Laere et al. (2009) was not successful. Differences in ploidy level can be an incongruity barrier in interspecific hybridisation resulting in malformation of endosperm, the inhibition of germination (Badger 1988), or spontaneous abortion of the fruits (Van Tuyl et al. 1991; Pickersgill et al. 1993, Sharma 1995). In this study, 182 crosses were made between *B. davidii* cv. Nanhoensis Alba (2*n* = 4*x*) and chromosome doubled *B. globosa* GLO9 and GLO34 (2*n* = 4*x*). These crosses resulted in four fruits and eventually 18 F1 plants on the field (Table 2). As a control, 195 crosses were also performed between *B. davidii* cv. Nanhoensis Alba and *B. globosa* (2*n* = 2*x*). None of these control pollinations yielded fruits (Table 2).

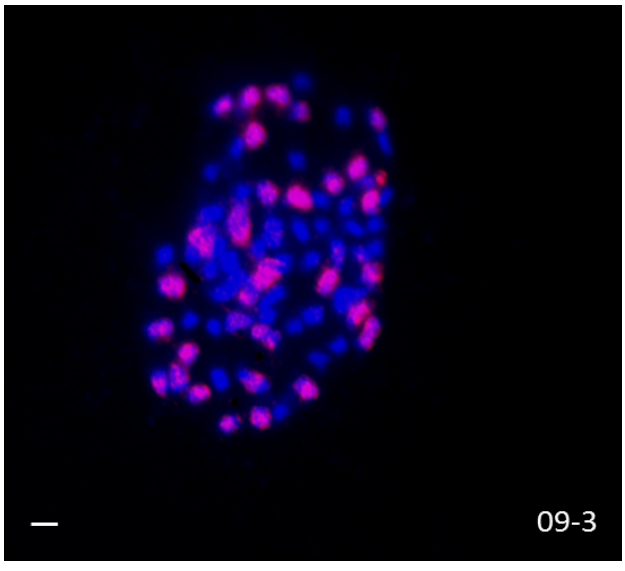

Fig. 3. Discrimination of chromosomes originating from *B. globosa* (red fluorescence) and *B. davidii* (blue fluorescence) in the genome of an F1 seedling obtained after crosses between *B. davidii* × *B. globosa* (tetraploidised) by use of GISH (bar = 5μm)

Plant morphology of seven F1 seedlings was studied in detail (Table 3). The leaves of the F1 seedlings were elliptical with a leaf L/W ratio between 3.24 and 4.5, comparable to the parental species *B. davidii* cv. Nanhoensis Alba (L/W ratio of 4.0) but higher than *B. globosa* GLO34 (L/W ratio of 2.4). The flower panicle had a shape intermediate to *B. davidii* and *B. globosa* and the length of the flower panicles of the F1 seedlings (between 13.0 and 19.46 cm) was longer than *B. davidii* (about 11.6 cm). Most F1 seedlings inherited a yellowish flower colour from *B. globosa* GLO34 (Table 3; Fig. 2) and two seedlings had a purple flower colour with a yellowish centre. Study of the male fertility of the hybrids revealed very low pollen production and no germination of the pollen could be observed in any of the samples analysed. Rose et al. (2001) also successfully crossed doubled *B. globosa* and *B. davidii*. The obtained F1 seedling had yellow flowers tinted with mauve. Our results contrast with Van De Weyer (1920) who wrote that the yellow colour is a recessive trait, occurring only in the second generation of a *B. globosa* × *B. davidii* cross. This indicates that an F2 would need to be created to obtain yellow flowering hybrids.

Morphological analysis, genome size, and GISH proved the hybrid nature of the F1 seedlings. Based on the parents' genome sizes and chromosome

Table 3. Overview of the leaf and flower morphology of 7 F1 seedlings obtained after crossing *B. davidii* cv. Nanhoensis Alba × *B. globosa* (No. 34; tetraploid)

Genotype	Leaf length/ leaf width	Length flower panicle (cm)	Main flower colour (RHS colour chart, yellow group)
B. davidii cv. Nanhoensis Alba	4.0 ± 0.5	11.6 ± 2.4	white group (155B)
B. globosa (2*n* = 2*x*)	3.0 ± 0.3	na[z]	yellow group (23A)
B. globosa GLO34 (2*n* = 4*x*)	2.4 ± 0.1	na	yellow group (23A)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-1)	4.5 ± 0.6	19.5 ± 4.1	yellow group (5D)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-2)	4.2 ± 0.7	16.2 ± 5.4	purple group (76B)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-3)	3.2 ± 0.5	13.0 ± 2.1	yellow group (11D)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-4)	4.2 ± 1.3	16.2 ± 4.4	purple group (76C)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-6)	4.4 ± 0.5	18.1 ± 4.1	yellow group (9D)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-7)	4.5 ± 0.9	13.7 ± 2.8	yellow group (4D)
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-8)	4.3 ± 0.6	17.6 ± 4.2	yellow group (8C)

[z]the inflorescence of *B. globosa* is not a panicle and could thus not be measured as *B. davidii* inflorescences; results of leaf length, leaf width and length of flower panicle are averages ± SD, *n* = 10

number, a genome size of 1.75 pg/1C and 76 chromosomes are expected in F1 seedlings. This was observed for five of the seven F1 seedlings (Table 4). Two F1 genotypes, 09-4 and 09-8, had a significantly larger genome size of 2.16 pg/1C and 2.04 pg/1C, respectively, while their chromosome number was equal (2*n* = 4*x* = 76).

Three F1 seedlings (09-3, 09-4 and 09-8), each with a different genome size (Table 4), were analysed using GISH. Parental genomes could be clearly distinguished using both biotin and digoxigenin (Fig. 3). As expected, F1 seedlings inherited 38 chromosomes from *B. globosa* and 38 chromosomes from *B. davidii* (Fig. 3), clearly proving the hybrid nature of the F1 seedlings. Some *B. globosa* chromosomes seemed to be partly blocked by *B. davidii* DNA. This is likely due to the considerable homology between the genomes of *B. davidii* and *B. globosa*. On the other hand, the demonstrated possibility of distinguishing between the genomes of *B. globosa* and *B. davidii* indicates a considerable divergence between the middle and highly repetitive DNA sequences of the species. This variation in the repetitive DNA is generally considered to

Table 4. Genome sizes and chromosome numbers of parent plants and F1 hybrids from crosses between *B. davidii* cv. Nanhoensis Alba × *B. globosa* (No. 34; tetraploid)

Genotype	Genome size (1C/pg)	Chromosome number
B. globosa (2*n* = 2*x*)	1.08 ± 0.01	2*n* = 2*x* = 38
B. globosa GLO34 (2*n* = 4*x*)	2.13 ± 0.02	2*n* = 4*x* = 76
B. davidii cv. Nanhoensis Alba'[z]	1.37 ± 0.01	2*n* = 4*x* = 76
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-1)	1.74 ± 0.02	nc[z]
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-2)	1.70 ± 0.03	nc
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-3)	1.76 ± 0.02	2*n* = 4*x* = 76
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-4)	2.16 ± 0.04	2*n* = 4*x* = 76
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-6)	1.81 ± 0.01	nc
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-7)	1.73 ± 0.02	nc
B. davidii cv. Nanhoensis Alba × *B. globosa* (09-8)	2.04 ± 0.05	2*n* = 4*x* = 76

[z]chromosomes not counted; results of genome sizes are averages ± SD, *n* = 5 to 10

be the source of differentiation among species and genera (Dean, Schmidt 1995). It also enables discrimination between the chromosomes of the parental genomes in interspecific hybrids using GISH (Schwarzacher et al. 1992).

CONCLUSION

In our study, interspecific crosses between chromosome doubled *B. globosa* genotypes and *B. davidii* resulted in *B. davidii*-like progeny with a yellow flower colour. Chromosome doubling of *B. globosa* increased both the pollen germination capacity of *B. globosa* and the success rate of the interspecific crosses. The creation of interspecific hybrids, together with the chromosome doubling technology, offers new opportunities for *Buddleja* breeding programmes.

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Corresponding author:

Katrijn Van Laere, Ph.D., Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit, Applied Genetics and Breeding, Caritasstraat 21, 9090 Melle, Belgium
phone: + 329 272 2884, e-mail: katrijn.vanlaere@ilvo.vlaanderen.be