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SHORT COMMUNICATION

Transfer of 2,4-D-resistance from *Raphanus raphanistrum* into *Brassica napus*: production of F_1 hybrids through embryo rescue

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Abstract: Development of 2,4-D-resistant *Brassica napus* varieties is valuable for conservation tillage and post emergence control of broadleaf weeds. This research documents successful production and transfer of 2,4-D resistance from *Raphanus raphanistrum* (wild radish) into, *Brassica napus* via embryo rescue.

Key words: 2,4-D, *Brassica napus*, gene transfer, embryo rescue, flow cytometry, triploid.

Résumé : Le développement de variétés de *Brassica napus* résistantes au 2,4-D revêt de l'importance pour le travail de conservation du sol et la lutte contre les dicotylédones après la levée. Cette étude illustre la production d'une résistance au 2,4-D et son transfert de *Raphanus raphanistrum* (radis sauvage) à *Brassica napus* par la technique de la culture d'embryons. [Traduit par la Rédaction]

Mots-clés : 2,4-D, *Brassica napus*, transfert de gènes, culture d'embryons, cytométrie de flux, triploïde.

Brassica crops are commercially important and are grown widely across the globe. *Brassica napus* (rapeseed) is extensively grown for oil seed production; but broadleaf weed competition can significantly reduce yield in this crop. Glyphosate-resistant *B. napus* varieties are commercially available and are widely cultivated. However, increasing evolution of glyphosate-resistant weeds limits the long-term viability of this technology, and warrants need for the development of new herbicide tolerance technology. Auxinic herbicides such as 2,4-D (2,4-dichlorophenoxy acetic acid) are cost-effective and are selective in controlling broadleaf weeds. Development of 2,4-D-resistant *B. napus* will be valuable for post-emergence control of broadleaf weeds in this crop and also facilitate herbicide rotation options for improved weed management.

Raphanus raphanistrum, (wild radish), is an economically important weed in the Brassicaceae family. Many herbicides, including 2,4-D, are used to manage this weed. As a result of extensive and continuous use of 2,4-D in wheat (*Triticum aestivum*) and lupin (*Lupin angustifolius*) cultivation in western Australia, some biotypes of *R. raphanistrum* have evolved resistance to this herbicide (Walsh et al. 2004). 2,4-D-resistant *R. raphanistrum* biotypes are approximately 10 times more resistant than the susceptible biotypes and the resistance is controlled by a single dominant gene (Walsh et al., 2004; Mithila et al., 2013).

Previous research reported successful gene transfer among members of the Brassicaceae family (Bing et al. 1995; Mithila and Hall, 2013; Mithila et al. 2014). Gene transfer may be complicated due to the variation in chromosome numbers among Brassica members, which may result in non-fertile hybrids. However, *in-vitro* techniques (e.g., ovule/embryo rescue) have been used for successful production of interspecific hybrids among *Brassica* members (Momotaz et al. 1998; Mithila et al. 2014). The overall objectives of this research were to: (1) generate hybrids between *B. napus* (2n:38) and 2,4-D-resistant *R. raphanistrum* (2n:18) via embryo culture and regeneration; (2) determine the DNA ploidy to identify true hybrid plants; and (3) confirm the transfer of 2,4-D resistance from *R. raphanistrum* into hybrids.

2,4-D-resistant *R. raphanistrum* and -susceptible *B. napus* were grown from seed in a greenhouse. The seeds were sown in 15 cm plastic pots containing commercial

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potting mixture (Miracle Gro, Marysville, OH, USA) and were grown under a 15/9 h day/night photoperiod, supplemented with 250 μmol m^{-2} s^{-1} illumination provided with sodium vapor lamps and maintained at 25/20 °C day/night. Each pot contained one plant. When plants were flowering, crosses were performed between *B. napus* and 2,4-D-resistant *R. raphanistrum* following the procedure described by Jugulam et al. (2005). Embryo rescue was required for hybrid plant production *in-vitro*. Silique (immature seed pod), ovule culture, and putative hybrid plant regeneration was established according to the procedure described by Mithila and Hall (2013). Plantlets produced *in-vitro* were transferred to soil and were grown in a greenhouse. Upon putative hybrid plant establishment, clonal propagation was achieved by single nodal cuttings.

DNA ploidy of putative hybrids and parental plants was assessed by flow cytometry using a BD FACS Calibur flow cytometer (BD Biosciences, San José, USA). Fresh leaf tissue was chopped in a LB01 buffer containing 50 μg mL^{-1} propidium iodide and 50 μg mL^{-1} RNAse, fluorescence area (585/42 nm) was measured, and 2C DNA content was determined relative to an internal standard of *Zea mays* 'CE-777' (5.43 pg/2C; Doležel, et al. 1989). Because the DNA content of the standard exceeded that of 2C *R. raphanistrum* by more than three times, *R. raphanistrum* 4C peaks were used to measure genome size in order to maintain linearity. A minimum of 1000 nuclei per peak was obtained and all CV's < 4.2%. Two replicates were tested per plant. DNA ploidy of putative hybrids was determined by comparison of 2C DNA content to known *R. raphanistrum* diploid and *B. napus* tetraploid.

To determine if resistance to 2,4-D from *R. raphanistrum* was successfully transferred into putative hybrids, 2,4-D dose-response experiment was conducted using putative hybrid clones. Parental plants (*B. napus* and *R. raphanistrum*) as well as putative hybrid clones were grown in a greenhouse (previously described). The putative hybrid clones and parental seedlings (at the four leaf stage) were treated with 2,4-D [100, 250, and 500 g acid equivalent per hectare (ae ha^{-1})] using a bench-type sprayer as described by Mithila et al. (2013). Following 2,4-D treatment, plants were returned to the greenhouse. The putative hybrid plants were classified as 2,4-D-resistant or -susceptible by comparing their injury responses with those of *R. raphanistrum* or *B. napus* plants.

Ovule/embryos rescued from cultured immature siliques facilitated putative hybrid plant production and establishment (Fig. 1). A total of ~1500 *B. napus* buds were pollinated with *R. raphanistrum* pollen. Two to three hundred siliques from the crosses were cultured and ~150 embryos were excised *in-vitro*. Ten putative hybrid plants were produced *in-vitro*. Hybrid plant regeneration via embryo rescue occurred when the experiments were repeated. We determined DNA ploidy of six of these hybrids (as the other four did not establish in soil), along with the parents (*B. napus* and *R. raphanistrum*). Only three hybrid plants were found to be DNA triploids, with DNA contents of 1.71–1.74 pg/2C, intermediate to two parental DNA content (Table 1 and Fig. 2). The other three putative hybrids were found to be DNA tetraploids, with estimated DNA content close to *B. napus* (2.41–2.44 pg/2C; Table 1). These DNA tetraploids may have been true hybrids resulting from the union of an unreduced *R. raphanistrum* gamete and a reduced *B. napus* gamete, or they may have been derived from somatic tissue of the immature ovule. One of the 3× hybrids (No. 1) also survived up to 250 g ae ha^{-1} of 2,4-D application (Fig. 3). After treatment with 2,4-D, the hybrid No. 1 exhibited little or no epinasty (downward curling of plant parts; a typical symptom of auxinic herbicides) and this response was similar to 2,4-D-resistant *R. raphanistrum* plants.

Although, rarely, Warwick et al. (2003) reported production of hybrid plants naturally (a single hybrid plant in 32821 seedlings) between *R. raphanistrum* and *B. napus*, by fusion of unreduced gametes of *R. raphanistrum* and a reduced gamete of *B. napus*. Here, we report for the first time successful production of hybrid plants between

Fig. 1. Production of hybrids between *Brassica napus* and *Raphanus raphanistrum in vitro*, via embryo rescue. Figure appears in colour on the Web.

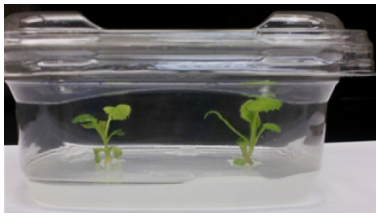


Table 1. 2C DNA content and DNA ploidy of parents and hybrids.

Plants tested	DNA content (pg/2C) mean and 95% CI	DNA Ploidy level
Parents:		
B. napus (plant 1)	2.37 (2.37, 2.37)	4×
B. napus (plant 2)	2.43 (2.22, 2.64)	4×
R. raphanistrum (plant 1)	1.06 (1.04, 1.08)	2×
R. raphanistrum (plant 2)	1.07 (1.03, 1.11)	2×
Predicted 3X hybrid[a]	1.73	3×
Putative hybrids:		
B. napus × *R. raphanistrum* No. 1	1.73 (1.72, 1.73)	3×
B. napus × *R. raphanistrum* No. 2	1.71 (1.64, 1.77)	3×
B. napus × *R. raphanistrum* No. 3	1.74 (1.73, 1.74)	3×
B. napus × *R. raphanistrum* No. 4	2.41 (2.38, 2.44)	4×
B. napus × *R. raphanistrum* No. 5	2.44 (2.15, 2.72)	4×
B. napus × *R. raphanistrum* No. 6	2.42 (2.35, 2.50)	4×

[a]Expected 2C DNA content of a triploid hybrids calculated as the average of the two *B. napus* and two *R. raphanistrum* means.

Fig. 2. Flow cytometry relative fluorescence (fluorescence area) histograms of nuclei extracted from (A) diploid *Raphanus raphanistrum*, (B) tetraploid *Brassica napus*, (C) a triploid hybrid with 2C DNA content intermediate to (A) and (B), and (D) a putative hybrid with 2C DNA content matching *B. napus* (B). Z is the 2C peak for *Zea mays*, included as an internal DNA content standard; R, B, and H indicate *R. raphanistrum*, *B. napus*, and putative hybrid nuclei peaks; 2C, 4C, and 8C peaks are indicated for R, B, and H.

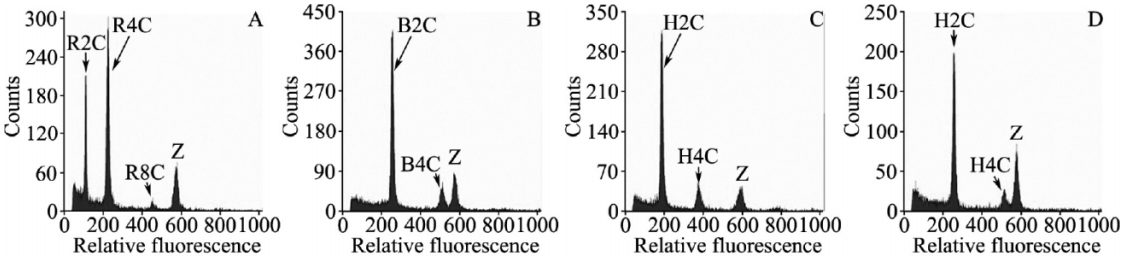
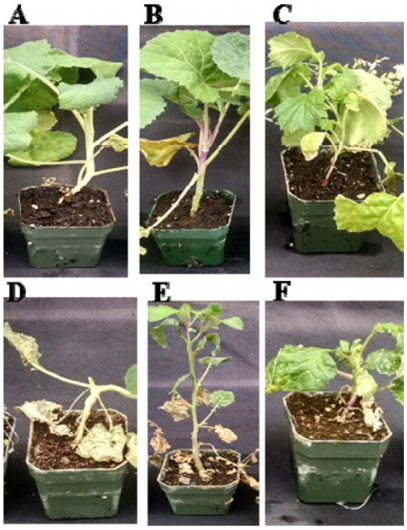


Fig. 3. Plant response of untreated or treated with 250 g ae ha^{-1} of 2,4-D. *a–c* represent untreated *B. napus*, triploid hybrid No. 1, and *R. raphanistrum*, respectively. *d–f* show the response of *B. napus*, triploid hybrid No. 1, and *R. raphanistrum* 3 weeks after treatment with 2,4-D (250 g ae ha^{-1}), respectively. Figure appears in colour on the Web.



B. napus and *R. raphanistrum* and possible transfer of 2,4-D resistance into one of the hybrids generated via embryo rescue. This is the first step towards development of 2,4-D-resistant *B. napus* varieties following this approach. The outcome of this research is encouraging for future development of 2,4-D-resistant *B. napus* cultivars, which may allow farmers to use this herbicide both as pre-emergence as well as post-emergence. Furthermore, such technology will also provide herbicide rotation options to growers and can facilitate effective weed control, less tillage, and possibly minimize evolution of herbicide resistant weeds.

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