The characterization of herbicide tolerant plants in *Brassica napus* L. after in vitro selection of microspores and protoplasts

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**ABSTRACT**

*Brassica napus* (cv Topas) plants tolerant to chlorsulfuron (CS) were isolated after selection experiments utilizing microspores and haploid protoplasts. The first microspore-derived plant (M-37,) was CS tolerant, haploid and sterile. Normal plant morphology and fertility was restored after colchicine doubling. A CS tolerant plant was also selected from protoplasts (P-26) isolated from microspore-derived embryo tissue and grown on medium containing CS. P-26 was aneuploid, CS tolerant and had very low fertility. The two selected lines produced selfed progeny which were tolerant to from 10-100 times the CS levels of the corresponding Topas plants. Microspores and protoplasts derived from the selfed plants were also CS tolerant. The segregation pattern for CS tolerance from reciprocally crossed progeny of M-37 and Topas was consistent with a semi-dominant nuclear mode of inheritance. Biochemical analysis of the two mutants indicated that the microspore-derived mutant and F1 crosses contained an altered acetohydroxyacid synthase (AHAS) enzyme, while the AHAS activity of the protoplast mutant was similar to Topas. Selfed seed from the M-37 plants have provided tolerance to CS in both greenhouse and field tests. S1 plants from a second microspore selected mutant (M-42) have tolerated 30 g/ha of CS in greenhouse tests. The two single-celled selection systems are fundamentally different both in tissue origin and in genetic variability. The variation from the protoplast system is essentially somaclonal in nature i.e. the variation is the result of spontaneous genetic changes that occur in somatic cells grown under particular nutrient and environmental conditions. The particular mechanisms which underlie somaclonal variation have included individual changes at the nucleotide level, the movement of transposable elements within a chromosome, large chromosomal anomalies including duplications, deficiencies, inversions and translocations and chromosomal changes including the production of aneuploids and polyploids (D'Amato, 1984; Meins, 1983). While the mechanisms are not yet well understood their potential use for crop improvement is clear (Timmerman and Scowcroft, 1981). In contrast, microspore systems in *Brassica* are relatively stable and are increasingly being used in breeding programs for the production of superior clones (Lichter, 1985; Cardy, 1986). It is not clear whether a random segregation of gametes develop into the embryos (Orton and Browers, 1985). In our hands few phenotypic variants are evident when plants are regenerated directly from microspore-derived embryos in *Brassica* (Swanson et al., 1987).

Chlorsulfuron (CS) is a product of E.I. du Pont de Nemours and Co (Wilmington, Delaware, U.S.A.) and is sold under the trade name Glean®. CS tolerance has been isolated in yeast (Falco and Dumas, 1985), bacteria (Yadav et al., 1986), and plants (Chaleff, 1984; Haughn and Somerville, 1986; Sebastian and Chaleff, 1987). The primary enzyme affected by CS in these organisms is acetohydroxyacid synthase (AHAS) which catalyzes the first step in the biosynthesis of the branched chained amino acids. Different tolerance mechanisms involving dominant, semidominant, and recessive mutations were reported in the resistant plants. Glean tolerance could be a valuable attribute for weed control in Brassica and therefore an excellent candidate to establish microspore and protoplast selection methodology.

This paper reports on the first direct use of isolated mutagenized microspores for selection, the first report in *Brassica* of the use of haploid protoplasts for selection and the first report of CS tolerance in the species.
Herbicide tolerant plants of *Brassica napus* L. were derived from mutagenized microspores (M-37 and M-42) and from in vitro selection of protoplasts (P-26) as outlined below. Plants of cv Topas were regenerated from microspores isolated as previously outlined (Swanson et al., 1987) and mutagenized with either 20 mM ethyl nitrosourea (ENU) (M-37) or with 0.5 Krads gamma irradiation (M-42). The mutagenized microspores and non-mutagenized controls were cultured in microspore medium containing 3 ppB (approximately 8.4 x 10^-9 M) chlorosulfuron (CS) (2-chloro-N-[[4-methoxy-6-methyl-1-3-5-triazin-2-yl] aminocarbonyl] benzenesulfonamide). Surviving embryos were subcultured to B5 medium (Gamborg et al., 1968) with 2% sucrose and 0.45% agarose for five days and then transferred to similar medium with 5 ppB CS and plants regenerated.

Protoplasts were isolated from microspore-derived embryos which had been subcultured onto B5 medium and were enlarging and forming secondary embryos along the stem axis. These embryos were chopped into 0.5 cm transverse sections and protoplasts isolated following the method of Barsby et al. (1986). Modifications to the protoplast isolation method included the removal of casein hydrolysate from the protoplast medium and the addition of 0.75 ppB CS to the protoplast medium. Surviving colonies were transferred to dilution medium with 2.5 ppB CS. Plant regeneration was accomplished by transferring the surviving colony (P-26) to MS (Murashige and Skoog, 1962) medium containing 1% sucrose and 100 mg/l casein hydrolysate with 2 mg/l kinetin, 2 mg/l zeatin and 0.1 mg/l indoleacetic acid (IAA).

Small plantlets with active root growth were removed from each line, rinsed with water and placed in a beaker with 0.2% colchicine for approximately 6 hours and the roots rinsed with water and repotted. Selfed seed and reciprocally crossed seed to Topas were obtained from the colchicine-doubled microspore-derived, selected plant (M-37). Three additional generations of selfed seed of M-37 were subsequently obtained. Selfed seed was also obtained from the protoplast-derived plant (P-26) and from a second microspore-derived mutant (M-42).

Plants from selfed seed and cuttings of M-37, P-26 and Topas and seed from reciprocally crossed progeny of Topas and M-37 (Topas x M-37 and M-37 x Topas) were sprayed with CS at the 3-5 leaf stage (post emergent (PE)) in a spray chamber (Research Instruments, Guelph, Ontario, Canada). The chamber contained a boom sprayer with a flat fan nozzle (TeeJet TS802) calibrated to deliver 0, 0.5, 1, 3, 6, 12, 20, 25, 30, 40 and 50 g/ha of CS with 0.15% Agral 90 as a surfactant. Four plants of each line were used per treatment. Similarly these lines and seeds from S1 plants of M-42 were tested by germinating ten seeds of each line in Metrox which had been sprayed with 3 and 6 g/ha CS five days prior to sowing (pre-plant incorporated (PPI)).

Aseptic cuttings were produced from young non-flowering nodal explants of M-37, P-26 and Topas and from plants from selfed seed of these plants. The cuttings were placed in petri dishes (100 x 25 mm) containing B5 medium with 0 and 5 ppB CS. One explant from each of the selections was placed in a petri dish with a cutting from Topas as a control. The dishes were placed in a growth cabinet at 25 C and a 16 hour photoperiod of 200 uE m^-2 s^-1. Explant growth and survival was recorded from 20 replicates after 2 weeks.

Inheritance of the CS tolerance from M-37 was examined through an analysis of parents and reciprocal crossed progeny. The seeds were planted in soil treated with 0, 3 or 6 g/ha of CS as previously outlined. Observation were taken on the tolerance of seeds produced from all possible combinations of M-37 parents and the F1 plants (16 combinations). Plants from selfed seed of M-37, P-26, Topas, and the reciprocally crossed progeny of M-37 and Topas were grown under conditions defined for microspore donor plants and the microspores isolated as previously outlined (Swanson et al., 1987). The microspores were cultured with CS concentrations of 0, 2.5, 5, 10, 20, 30, 50, and 100 ppB added to the microspore medium. Ten replicates per treatment were used.

Biochemical analysis of acetohydroxyacid synthase (AHAS) activity from leaf extracts were performed essentially as described by Haughn and Sommerville (1986) using three replicates. Briefly, 1-2 g of young leaf tissue was ground in 3-4 ml ice cold extract buffer and passed through a 10 ml Sephadex G-25 column. One hundred fifty ul of the pooled protein containing fractions were used in each enzyme assay. Background acetalactate/acetoin levels were eliminated from activity determinations by letting the enzyme extract, added after the addition of H2SO4, represent zero enzyme activity. Protein concentrations were determined with the Bio-Rad protein assay dye reagent (#500-0006) using bovine serum albumin to construct the standard curve.

Seed from selfed plants of M-37 and from certified Topas was planted in the field in a randomized complete block-split plot design with four replications. CS was added to plots at 0, 3, and 6 g/ha with 0.15% Agral 90. The seed lots were planted in standard 1x6 meter 6 row plots with approximately 900-1000 seeds/plot using a Wintersteiger (International Precision Inc, Lincoln, Nebraska) plotspider. The time required for each plot to reach 60% flowering, the number of plants per plot at maturity, and the amount of seed produced per plot was recorded.

RESULTS

The M-37 plant which was recovered after microspore selection exhibited the typical haploid characteristics of fine stems, small leaves and petioles, and had small sterile flowers. Normal morphology and fertility was restored to M-37 after colchicine doubling. These results indicate that the selection of this mutant took place at a haploid level and the resultant plant remained haploid until doubled. The M-42 microspore-derived