AN *IN VITRO* INDUCED MUTAGENESIS PROTOCOL FOR THE PRODUCTION OF SUGARCANE TOLERANT TO IMIDAZOLINONE HERBICIDES

By

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Abstract

THE MAIN objective of this study was to establish a protocol for the production of imidazolinone-tolerant sugarcane genotypes through *in vitro* induced mutagenesis. The approach involved using the chemical mutagen ethyl methanesulfonate (EMS) to induce a single target-site mutation in the acetolactate synthase gene, and testing the tolerance of regenerated plants with the herbicide Arsenal (250 g/L imazapyr). The initial work determined the mutagenic (8 mM and 16 mM EMS for 4h) and *in vitro* screening conditions (LD_{50} = 0.042 µM, LD_{90} = 0.08 µM imazapyr in the medium) for 6–10 week-old somatic embryogenic calli of the N12 sugarcane cultivar, and the screening treatment for *ex-vitro* control plants (spraying with Arsenal in the greenhouse). As the culture conditions (which included 2,4-D) alone did not cause a significant production of somaclones, the mutagenic agent was essential to increase the chance of producing herbicide tolerant plantlets. The EMS and imazapyr treatments were then applied in combination and the calli were exposed to increasing levels (0.042–0.16 µM) of herbicide. Putative tolerant plants have been obtained but their regeneration was slower than the control; after acclimatisation they will be sprayed with Arsenal (102 g a.i./ha; 0.39 M). For all treatments, callus mass, number of green, albino and abnormal plantlets, and biomass of acclimatised plants were recorded, and amplified fragment length polymorphism analyses were performed on the regenerated plants.

Introduction

Imidazolinone herbicides (e.g imazapyr) control weeds by inhibiting acetolactate synthase (ALS), which is involved in the biosynthesis of essential branched-chain amino acids. Any of several single target-site (dominant) mutations (Tan *et al.*, 2005), in five conserved regions on the enzyme (Webster and Masson, 2001), reduce target site sensitivity (Yu *et al.*, 2003), thereby conferring resistance to the herbicide. Such mutations occur during *in vitro* morphogenesis caused by culture conditions (e.g. auxins) resulting in somaclonal variant cells, which can then be regenerated into variant plants.

An example in sugarcane is the generation of cell lines, which yielded plants with a five-fold tolerance to glyphosate (Zambrano *et al.*, 2003). This approach can be further enhanced by mutagenic agents (e.g. EMS) (Van Harten, 1998). To-date, efforts in the production of herbicide tolerant sugarcane genotypes have focused on genetic engineering but *in vitro* mutagenesis may offer a potential alternative to recombinant DNA technologies. This study aimed at assessing such an option.

Materials and methods

The somatic embryogenesis protocol of Snyman (2004) was followed using immature leaf rolls of *Saccharum* spp. hybrid var. N12: 1) calli were induced on MS, 20 g sucrose, 8 g agar and 3 mg 2,4-D per L (6–10 weeks); 2) embryo germination was on the same medium devoid of 2,4-D (6–8 weeks); 3) plantlets were established on ½ MS, 5 g sucrose and 8 g agar per L (4 weeks); and 4) plants were acclimatised in a misting chamber (1 min every 6 h) for 3 days, before transfer to a polytunnel. All cultures were subcultured onto fresh media fortnightly.

Calli (0.2 g) were exposed to 10 mL of ethyl methanesulfonate (EMS) (0–96.6 mM) for 4 h, rinsed and placed on callus medium. Imazapyr (Sigma-Aldrich; Pestanal®) was prepared as 5 M in 10 mM potassium phosphate (pH 7.5), and required aliquots were added to the media. Imazapyr concentrations in the media were 0–0.1 µM for callus and 0–20 µM for established plantlets.

Various mutagen and herbicide concentrations were tested in combination, and with step-wise increases of imazapyr. *Ex-vitro* plants were sprayed with 9.25–262.5 g a.i./ha; 0.036–1 M) Arsenal (BASF; 250 g/L imazapyr) after acclimatisation. Measurements included callus and plantlet biomass, % plant regeneration and survival, and Amplified Fragment Length Polymorphism (AFLP) analyses.

Results and discussion

The strategy employed in this study was to induce somaclonal variation in embryogenic callus cells (with and without mutagen exposure), followed by *in vitro* selection of imazapyr tolerant cells and then further screening of the putative tolerant plants regenerated from them.

The indirect somatic embryogenesis protocol used here resulted in a relatively low frequency of somaclonal variants, as previously reported for a direct method (Watt *et al.*, 2009).

The results (including AFLPs) obtained from the control (0 EMS, 0 imazapyr) (±7% abnormal plants) and herbicide-only selection indicated that 2,4-D did not induce much somaclonal variation.

This confirmed the premise that a mutagen was essential to increase the frequency of new cell lines in culture. The chemical mutagen EMS was therefore employed as it causes high frequency of gene mutations and low frequency of chromosomal aberrations (Van Harten, 1998).

Each step of the protocol was first established, with conclusions based on biomass, plantlet regeneration and AFLP analyses. From the EMS-only treatment (i.e. no imazapyr in the medium), 8 mM and 16 mM EMS were selected for further studies.

The LD_{50} and LD_{90} for % plantlet inhibition by imazapyr were 0.042 µM and 0.08 µM, respectively. The spraying treatment deemed suitable for screening *ex-vitro* plants in the greenhouse was 102 g a.i./ ha (0.39 M) Arsenal.

Table 1 shows an example of the results obtained for some of the tested combinations of the mutagenic (16 mM EMS) and *in vitro* selection treatments. The indirect somatic embryogenesis protocol (control) routinely yields approximately 400–500 plantlets/g fresh mass; predictably, plantlet yield decreased and regeneration time increased with increasing stringency of EMS and imazapyr regimes.

All of the potentially imazapyr-tolerant subclones, including those treated with 8 mM EMS (results not shown) are being further screened and assessed. The tolerant genotypes will also to be evaluated in the field to determine phenotypic effects and the level of whole plant tolerance to the herbicide.

In sugarcane, glyphosate- (Zambrano *et al.*, 2003) and red rot- tolerant plants (Singh *et al.*, 2008) have been obtained by selection of mutant lines, which arose randomly in response to culture media pressure.

Table 1–Plantlet regeneration after mutagenic treatment with 16 mM EMS and selection on various imazapyr regimes. The standard protocol (no EMS, no imazapyr) yielded 488±99.3 and 35±9.4 plants/g callus fresh mass normal and abnormal (albino and visually chimaeric) plants, respectively. n = 5, mean ± SE.

Culture stages and treatments							No. plants/g callus f. mass	
Culture stage	Callus induction		Embryo germination and lantlet establishment				Normal plants	Abnormal plants
Weeks in culture	2	4	6	8	10	12		
Imazapyr (µM)	0		0.042	0.042	0.063		201±67.3	33± 13.9
				0.063	0.08		204±15.4	14±14.5
				0.08	0.12		26±21.9	4±3.6
	0.042		0.08	0.08			161± 32.7	89±78.6
				0.12			2±2.2	5±4.1
				0.16			2±1.6	0
	0.08			0.08			19±2.4	40±30.3
				0.12			3±1.7	2±1.7
				0.16			9 ±12.9	17±12.9

By increasing the mutation frequency, the protocol reported here has the potential to be more efficient in generating and selecting variability for these and other important traits.

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UN PROTOCOLE POUR LA MUTAGÉNÈSE INDUITE *IN VITRO* POUR LA PRODUCTION DE CANNE À SUCRE TOLÉRANTE AUX HERBICIDES IMIDAZOLINONE

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MOTS CLÉS: Éthylméthane Sulfonate, Imazapyr, Embryogénèse Somatique

Résumé

L'OBJECTIF principal de cette étude était d'établir un protocole pour la production de génotypes de canne à sucre tolérants à l'imidazolinone à travers la mutagénèse induite *in vitro*. L'approche consistait en une utilisation d'un mutagène chimique, l'éthylméthane sulfonate (EMS), pour induire une mutagénèse dirigée dans le gène acétolactate synthase, suivi d'une évaluation de la tolérance des plantes régénérées à l'herbicide Arsenal (250 g/L imazapyr). Une étude initiale a établi la concentration du mutagène (8 mM et 14 mM EMS pendant 4 h), les conditions de sélection *in vitro* (LD50 = 0.042 µM, LD90 = 0.08 µM imazapyr dans le milieu) pour le développement des cals embryogènes somatiques âgés de 6 à 10 semaines de la variété N12 et pour une évaluation des plantes témoins en serre (avec un traitement à l'herbicide Arsenal). Comme les conditions de culture (qui incluaient le 2,4-D) à elles seules n'ont pas produit un nombre significatif de somaclones, l'agent mutagène était essentiel pour augmenter la chance d'obtenir des plantules tolérantes à l'herbicide. Les traitements EMS et imazapyr ont ensuite été appliqués simultanément et les cals exposés à des taux croissants (0.042 – 0.16 µM) d'herbicide. Des plantes tolérantes putatives ont été obtenues mais leur régénération était plus lente que celle du témoin. Après une acclimatation, les plantes seront pulvérisées avec de l'Arsenal (102 g a.i/ha ; 0.39 M). Pour chaque traitement, le poids du cal, le nombre de plantules vertes, albinos et anormales ainsi que la biomasse des plantes acclimatées ont été notés. Des analyses de polymorphisme de longueur des fragments amplifiés (A

PROTOCOLO DE LA INDUCCIÓN *IN VITRO* DE UNA MUTAGÉNESIS PARA LA PRODUCCIÓN DE CAÑA DE AZÚCAR TOLERANTE A LOS HERBICIDAS CON BASE EN IMIDAZOLINONA

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PALABRAS CLAVE: Etil Metanosulfonato, *Imazapyr*, Embriogénesis Somática.

Resumen

EL OBJETIVO principal de este estudio fue establecer un protocolo para la producción de genotipos de caña de azúcar tolerantes a la imidazolinona mediante la inducción *in vitro* de una mutagénesis. El enfoque involucró el uso del mutágeno químico denominado etil metanosulfonato (EMS), el cual induce una mutación en un sitio específico del gen sintasa de acetolactato, y en la evaluación de plantas regeneradas tolerantes al herbicida Arsenal (250 g/L de *imazapyr*). El trabajo inicial permitió definir las condiciones mutagénicas (8 mM y 16 mM EMS por 4h) de criba *in vitro* (LD_{50} = 0.042 µM, LD_{90} = 0.08 µM de *imazapyr* en el medio de cultivo) para callos embriogénicos (de alrededor de 6 a 10 semanas) del cultivar de caña de azúcar N12, y de una metodología de criba *ex vitro* para las plantas control (con aspersión del herbicida Arsenal en el invernadero). Debido a que las condiciones de cultivo (incluyendo el 2,4-D) no ocasionaron por sí mismas una producción significativa de somaclonas, se consideró al agente mutagénico como crucial en el incremento de la producción de plántulas tolerantes al herbicida. Posteriormente, los tratamientos de EMS e *imazapyr* fueron aplicados en conjunto, sometiendo a los callos a diferentes niveles de herbicida (0.042 – 0.16 µM). Si bien se obtuvieron supuestas plantas tolerantes, su regeneración fue más lenta que el control; luego de una aclimatación se les roció con Arsenal (102 g a.i./ha; 0.39 M). En todos los tratamientos, se registraron masas de callo, número de plántulas normales, anormales y albinas, y la biomasa de plantas aclimatizadas. Asimismo, se condujeron análisis del polimorfismo en fragmento amplificado (AFLP, por sus siglas en Inglés) en aquellas plantas regeneradas.