Morphometric and Biochemical Changes in *Agave americana* L. Plantlets Induced By Ethyl Methanesulfonate

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Abstract: *A. americana* L. is a crop with very little genetic variability. In order to evaluate the effect of ethyl methanesulfonate (EMS) to induce variability in *in vitro* plantlets of *A. americana*, different explants (meristems, leaves and roots) were evaluated for the production of callus. MS medium supplemented with ANA (2.68 μM) and BAP (2.68 μM) was used. Callus obtained from apical meristem were treated with 15 mM EMS for two hours after which shoot formation was induced using 2,4-D (0.11 μM) and BAP (44 μM). The EMS induced variations in the morphometric and morphological parameters of the plantlets obtained, with 60% of the plantlets presenting differences such as dwarfism and different leaf forms, without the presence of spines, as well as an increase in fructan content of 30% with respect to the control plantlets. PAL was increased and this activity is related with higher anthocyanins concentration in *A. americana* L. plantlets.

Keywords: Fructans; callus; mutagenesis; flavonoids; anthocyanins; phenylalanine ammonia lyase activity

1 Introduction

A. americana L. is native to Mexico and other parts of tropical America. The plant was taken from its native habitat to Africa, Europe and Asia by the Portuguese and the Spaniards where it quickly naturalized [1]. It is characterized by fleshy, rigid and hard surfaced leaves growing directly out from the central stock to form a dense rosette. The leaves range in length from 1 to 2 m, and the edges of the leaves contain sharp spines [2]. This specie has been used in many ways including its use as a source of fiber, as well as its medicinal and ornamental properties and as a source of fructans [3]. Fructans are the most important sugars in Agaves and inulin is the fructan with the greatest industrial use [4]. The Agave takes about 8 years to mature and flower, using the stored sugar to grow and shortly after flowering, the plant dies. For this reason, the producers stop sexual reproduction by cutting off the inflorescence immediately. Sexual reproduction in Agave is therefore very rare and thus, propagation is achieved by asexual reproduction using rhizomes that emerge at a distance from the parent plants, giving rise to new individuals [5,6].

Genetic improvement through *in vitro* culture using induced mutations has played a fundamental role in the improvement of diverse crops [7-10]. The mutations have allowed the improvement of crops with

economic characteristics, such as high yield, resistance to biotic and abiotic stress and early maturity [11]. Ethyl methanesulfonate (EMS) is the most commonly used chemical mutagen in plantlets [12]. EMS and can cause biological effects on the function and metabolism of plant cells, which produces higher amounts of commercially useful metabolites and can therefore lead to the development of new varieties [13]. EMS, a chemical mutagen, is an alkylating agent, which has been successfully used to introduce point mutations, inducing C-to-T changes resulting in C/G to T/A substitutions [14,15]. EMS is most effective when applied to dividing cells, such as rapidly proliferating callus, given that the probability of incorrect repair of the mutation is the highest when the cells are engaged in DNA replication [16,17]. In the M1 generation, only mutations of dominant characters can be identified, and it is not possible to identify mutations in a recessive character. Moreover, in the M1 generation, there are some signals for mutagen efficiency, for example: pollen sterility, reduction in plant height, late or early flowering, or curled leaves [18].

The objective was to evaluate the changes in morphometric and morphological parameters, fructans, chlorophyll, flavonoids, anthocyanins and phenolic compounds as well as phenylalanine ammonia-lyase activity in *A. americana* L. plantlets obtained from callus treated with EMS.

2 Materials and Methods

2.1 Callus Induction and Proliferation

Three types of explants from 8-month old plantlets of *A. americana* L. cultivated *in vitro* (shoot apical meristem, leaves and roots) were evaluated. In order to obtain the shoot apical meristem, the leaves, stems and roots were removed, explants were placed on MS (Murashige & Skoog) medium [19] supplemented with vitamins, sucrose (30 g/l), myo-inositol (0.228 mM), sodium phosphate (0.362 mM), phytagel (2.5 g/l), supplemented with 2,4-D (0.11 μM) and BAP (44 μM). Explants were incubated at 22°C ± 2 in continuous light [20]. The response was evaluated at eight weeks post culture and the percentage of callus induction was determined in each explant, and calculated with the following formula [21].

$$\text{Callus induction (\%)} = \frac{\text{Explants with callus}}{\text{Explant number}} \text{x100}$$

The explant that gave the best response to callus induction was transferred into solid MS medium with sucrose (30 g/l), KNO_3 (1.90 g/l), NH_4NO_3 (1.65 g/l), phytagel (2.5 g/l), with growth regulators ANA (2.68 μM) and BAP (2.68 μM) were incubated for four weeks at 22°C ± 2 in continuous light for callus proliferation.

2.2 Ethyl Methanesulfonate Treatment

Callus obtained from apical meristem, were used for ethyl methanesulfonate (EMS) treatment, subsequently the callus was immersed in a solution with a 15 mM EMS and left in contact for two hours, after this time, the callus was rinsed three times with sterile water. The controls were not exposed to EMS [10].

2.3 Shoot Regeneration

The EMS-treated and control callus was subcultured in MS medium with 30 g/l sucrose, 1.90 g/l KNO_3 and 1.65 g/l NH_4NO_3 supplemented with 0.11 μM 2,4-D and 44 μM BA, and incubated at 22°C ± 2 in continuous light. Shoot number was evaluated at eight weeks. Shoots with approximately 1 cm long were separated from the calli and were placed in MS medium without growth regulators. The survival of shoots obtained from callus without and with EMS was recorded after two months and the survival rate was calculated [13]. Shoots were placed in MS medium supplemented with indole butyric acid (IBA) for root induction.

2.4 Determination of Morphometric and Morphological Parameters

The morphometric and morphological parameters were determined based on the "Technical Guide for Agave Varieties Description" Which is based on the standard Mexican norm (NOM-001-SAG/FITO, 2013). Five month old plants, regenerated from callus with EMS, were chosen at random to determine the height of the plants, the number of leaves and the percentage of plant survival. These were subsequently compared with the control plants. The Mann-Whitney test, a non-parametric test applied to two independent samples, was used for morphological parameters such as leaf morphology, leaf color, presence of spines, root formation for comparisons among control (plantlets from callus without EMS treatment) and regenerated plantlets from callus with EMS treatment.

2.5 Extraction and Quantification of Carbohydrates

Twenty mg of dry leaf of plantlets both from control and treated with MS were weighed, then milled with a mortar and placed in eppendorf tubes. Ultrapure water was added in a 1:1 (w:v) ratio and the tubes were allowed to stand at room temperature for 30 min. Extract was centrifuged at 12,000 rpm for 20 min at room temperature, the supernatant was transferred to eppendorf tubes and diluted with ultra-pure water (HPLC grade) 1:4 (v:v) having a total volume of 800 µL, which was adjusted to pH 7. High Performance Liquid Chromatography (HPLC) was used for the quantification of the carbohydrates where 10 µL of the extract was injected in a RezexTM RCM-Monosaccharide Column Ca^{+2} and reading was given to the index of refraction. UP water was used as mobile phase, the run conditions were: temperature of 80°C with flow of 0.3 ml/min for 62 min. Carbohydrates were identified based on the comparison of the retention times with the corresponding reference standards: inulin, sucrose, glucose, fructose (Sigma-Aldrich Química, S.L. Toluca, Mexico). It was possible to separate all carbohydrates in a single run. The resulting chromatograph indicated the retention time with which we elucidated the compounds and the concentration was calculated with the area under the peak curve, this area is extrapolated with the calibration curve to obtain the values of each compound.

2.6 Quantifications of Chlorophyll and Phenolic Compounds

Physiological parameters were measured in leaves of acclimatized plantlets, EMS-treated and control, which included chlorophyll (Chl), polyphenol contents (Phen), anthocyanin (Anth), flavonoids (Flav) and nitrogen balance index (NBI) using the Dualex sensor (FORCE-A, Orsay, France) according to [22], the content of polyphenols was expressed as Dualex units.

In addition, chlorophyll fluorescence was measured by a Chlorophyll Fluorometer (OS-30p, OPTI-SCIENCE, USA) according to Sanchez-Viveros [23].

2.7 Determination of Phenylalanine Ammonia-Lyase (PAL) Aactivity

Leaf samples (300 mg fresh weight) were extracted in 4 mL of buffer (50 mM Tris pH 8.5, 14.4 mM 2-mercaptoethanol, 1% (w/v) insoluble polyvinylpolypyrrorolidone) and centrifuged at 6000 × g for 10 min at 4°C. The total protein concentrations in soluble enzyme extracts were determined using the Bradford assay [24]. The method of Beaudoin-Eagan & Thorpe [25] was used to estimate PAL activity. The reaction mixture, at a final volume of 3 mL, consisted of 1.9 mL of 50 mM Tris-HCl buffer (pH 8.0), 100 µL of enzyme preparation and 1.0 mL of 15 mM L-phenylalanine for PAL. The assay was started by the addition of enzyme extract after an initial incubation for 60 min at 40°C.

The reactions were stopped by the addition of 200 µL of 6 N HCl. The amounts of formed trans-cinnamic acid were determined by measuring absorbance at 290 nm, against an identical mixture in which D-phenylalanine was substituted for L-phenylalanine. The enzyme activity was expressed in nmoles (trans-cinnamic acid)/mg protein/min, where 1 unit is defined as 1 nmole transcinnamic/mg protein/min.

2.8 Data Analysis

The SAS statistical software [26] SAS was used to analyze data using a confidence limit of 5%. The linear and quadratic values of all factors and the interactions between them were tested. Mann-Whitney test was applied to analyze data obtained from morphological parameters.

3 Results

The highest callus formation index (100%) was found with the apical meristem, followed by the leaves (70%) and the roots presented a low response (30%). Callus induction was obtained with MS medium supplemented with 0.11 μM 2, 4-D and 44 μM BAP (Fig. 1(A)). Callus treated with EMS at 15 mM for two hours did not show changes in colour, consistency or morphology as can be seen in Figure 1B. With respect to shoot induction, there was a statistically significant difference ($p < 0.05$) between the number of shoots generated, in callus control a total of 200 shoots were obtained, while in the EMS treated callus, a total of 71 shoots after eight weeks.

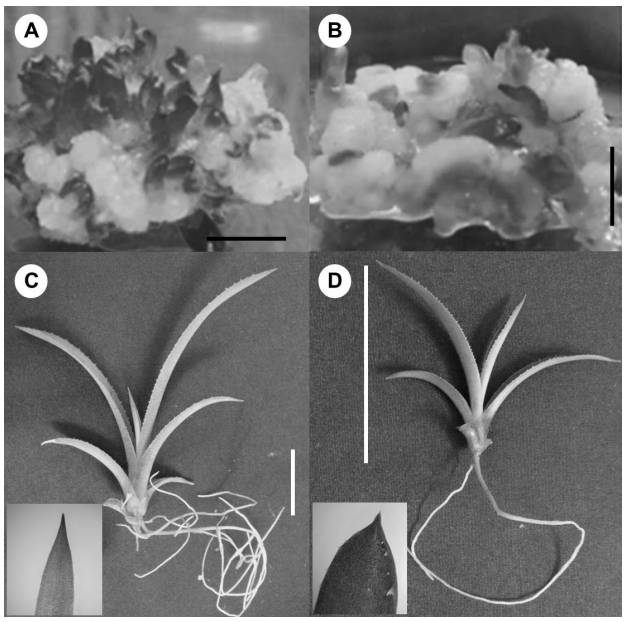

Figure 1: Effect of EMS in the induction of callus and shoots of *A. americana* L. A) Callus obtained with MS medium supplemented with 0.11 μM 2, 4-D and 44 μM BAP (Control). B) Callus treated with EMS at 15 mM for two hours. C) Plantlets of five months (Control), leaf in a lineal form. D) Plantlets of five months derived callus treated with EMS, leaf in an oblong form. Black bars correspond to 1.5 cm; white bars correspond to 4.0 cm

Fig. 1(C) shows a control seedling of five months which has a well-developed root system with a height of 8-9 cm; Plantlets deriving from callus treated with EMS presented the same appearance as the control and root development plantlets; however, their height oscillated between 2-3 cm (Fig. 1(D)) (Tab. 1). The number of leaves of EMS-treated plantlets with respect to the control had no statistically significant difference ($p < 0.05$). Percentage of plantlet survival was higher in control in comparison with EMS-derived plantlets (Tab. 1). Different leaf form morphology (oblate, oblong, lanceolate) were observed in plantlets treated with EMS, whereas control plantlets only presented leaves in a lineal form. Variations were also observed with respect to the presence of spines and root formation. This was corroborated by performing the hypothesis test based on the significance of the Mann-Whitney statistic (Tab. 1).

Table 1: Effect of ethyl methanesulfonate on morphometric and morphological parameters of *A. americana* L. plantlets

Treatment	Morphometric parameters			Morphological parameters			
	Plant height	Leaves number	Plantlet survival	LM[4]	LC[5]	PS[6]	RF[7]
	-cm-		-%-	Mann-Whintey test (0.05)			
Control[1]	5.0 a[8]	4.6 a	85 a	72.3 a	61.8 a	82.3 a	80.3 a
EMS[2]	2.8 b	5.2 a	57 b	46.2 b	60.5 a	50.0 b	51.0 b
LSD[3]	0.8	0.6	15	14.2	5.6	12.4	16.4

[1]Regenerated plantlets from callus without EMS treatment; [2]Regenerated plantlets from callus with EMS treatment; [3]Least Significant Difference (0.05); [4]leaf morphology; [5]Leaf color; [6]Presence of spines; [7]Root formation; [8]Values with the same letter are not significantly different between the treatments.

Fructans and fructose concentrations were higher in plantlets from callus exposed to EMS in comparison with control plantlets ($p < 0.05$), whereas sucrose and glucose concentration were not significantly different (Tab. 2). EMS treatment clearly induced an increase in the fructans and fructose content.

Table 2: Effect of ethyl methanesulfonate on sugar concentration in leaves of *A. americana* L. plantlets

Treatment	Fructans	Sucrose	Glucose	Fructose
	mg/mL PS			
Control[1]	1.3 ± 0.4 b[a]	0.15 ± 0.08 a	0.25 ± 0.07 a	0.19 ± 0.10 b
EMS[2]	1.7 ± 0.2 a	0.1 ± 0.03 a	0.25 ± 0.1 a	0.45 ± 0.30 a
LSD[3] (0.05)	0.3	0.007	0.1	0.2

[1]Regenerated plantlets from callus without EMS treatment; [2]Regenerated plantlets from callus with EMS treatment; [3]Least Significant Difference (0.05).

Table 3: Effect of ethyl methanesulfonate on photosynthetic efficiency (Pe), nitrogen content, chlorophyll, flavonoids, anthocyanins and phenylalanine ammonia lyase (PAL) activity in *in vitro* plantlets of *A. americana* L

Treatment	Pe	Nitrogen	Chlorophyll	Flavonoids	Anthocyanins	[3]PAL
	%					-mmol/min x g dw-
Control	[2] 0.71 ± 0.04 a	160.8 ± 39.3 a	40.9 ± 7.3 a	0.26 ± 0.03 a	0.0 ± 0.00 b	0.16 ± 0.0 b
EMS	0.69 ± 0.05 a	127.2 ± 40.9 b	33.9 ± 5.9 b	0.29 ± 0.08 a	0.03 ± 0.04 a	0.29 ± 0.0 a
LSD[1] (0.05)	0.032	25.7	4.2	0.04	0.01	0.002

[1]Least Significant Difference (0.05).

[2]Values with the same letter are not significantly different between the treatments.

[3]Phenylalanine ammonia lyase activity expressed as mmol of cinnamic acid/min x g dry weight.

It was observed that photosynthetic efficiency and flavonoid content did not present significant differences between control plantlets and those treated with EMS. Nitrogen and chlorophyll contents were higher in control plantlets, whereas concentrations of anthocyanins and PAL activity were higher in the plantlets treated with EMS (Tab. 3).

4 Discussion

Callus induction was significantly influenced by the type of explant. Apical meristem is a primary tissue that is characterized by being always young. This allows it to be active and it has no reproductive structures, avoiding the biosynthesis of phenolic compounds that may endanger the explant. It also contains stem cells, which allows it to be in constant cell division [27]. When plant cells are in a state of differentiation, the process of the mitotic cell cycle is suppressed, since they have to reacquire the competition of cell proliferation as a central characteristic of callus induction [28]. Berckmans [29] reported that the auxin signal in the cell was translated by ARF transcription factors, especially ARF7 and ARF19 which help to activate the expression of transcription factors of the LBD family, particularly LBD16, LBD17, LBD18, and LBD29 which induce the formation of callus. Callus treated with EMS at 15 mM for two hours did not show morphological changes. Similar results were obtained with rice plantlets to generate callus which were treated with 15 mM EMS, exposed for two hours, the conclusion being that the low dose and less time of the mutagen did not cause apparent lethality in the callus, which is the is one of the main problems associated with chemical mutagenesis [30]. EMS promoted the decrease in the number of shoots. This could be due to the genotoxic effect caused by the EMS on cell totipotency and plasticity [31].

Studies have shown that the use of EMS increases the genetic variability of plantlets thereby overcoming some agronomic and environmental problems [30]. Plantlet-derived seeds of *Capsicum annuum* L. treated with EMS showed significant differences in petal diameter, style length, fruit weight, fruit length, largest and smallest fruit diameter, pedicel length, pericarp thickness, placental length, number of seeds, fruit fresh matter, fruit dry matter, and fruit dry matter content, which indicates that EMS generated variations in plantlets/fruits from seeds [32]. The morphological modifications (leaf shape, absence of spines and roots) observed in plantlets treated with EMS could have been due to some modification in expression patterns of diverse genes [33]. In the same way, it is possible that the plantlets presented somaclonal variation which affects the structural and morphological aspects of the vegetative development of the plantlets. Additionally, the increase in the content of fructans could be due to the fact that the EMS caused a state of stress in the plantlets inducing the signalling response on the enzyme 1-SST, responsible for initiating the synthesis of fructans [34]. On the other hand, when plantlets are under stress they tend to accumulate intracellular osmolytes as a defense mechanism [35]. One of the main solutes is soluble sugars, such as fructose that act as osmoprotectors which allow the maintenance of the turgidity of plantlet tissues in order to continue cell function [36].

The resulting increase of anthocyanins concentration and PAL activity in plantlets treated with EMS could be explained by the fact that anthocyanins are flavonoids formed by phenylpropanoid metabolism from phenylalanine. Phenylalanine ammonia lyase (PAL; E.C.4.3.1.5), which catalyses the biotransformation of L-phenylalanine to trans-cinnamic acid and ammonia is the first and key enzyme of the phenylpropanoid sequence.

5 Conclusion

The results indicated that the EMS caused phenotypical modifications in morphological and morphometric parameters and increased the fructan and fructose content by 30%. PAL was increased and this activity is related with higher anthocyanins concentration in *A. americana* L. plantlets. These results are important given that the increase in PAL activity is related with stress biotic resistance.

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