Response Surface Methodology of Glutamine, Asparagine and 2,4-Dichlorophenoxyacetic Acid for *Agave americana* L. Embryo Number and their Optimization in a RITA® Automatic Bioreactor System

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Abstract: Response surface methodology was used to investigate the effect of different concentrations of 2,4-dicholorophenoxyacetic acid (2,4-D), asparagine and glutamine on a number of embryos from callus of *Agave americana* L generated with 0.5mg/L of 2,4-D, treatments obtained according to an experimental design with response surface Box-Behnken with three repetitions at the central point with 0, 1 and 2 mg L^{-1} 2, 4-D; 0, 200 and 500 mg L^{-1} glutamine and 0, 500 and 1000 mg L^{-1} asparagine. The embryo number was optimized using the RITA® automatic bioreactors system using a Programmable Logic Controller (PLC) and a Light Dependent Resistor (LDR) varying the immersion frequencies with similar solid and liquid treatments at the same time for comparative purposes. The results showed that interaction between asparagine and glutamine had a statistically significant effect and the largest embryo number was obtained with the higher concentration of the two amino acids, the coefficient of determination (R2) calculated from the validation data for RSM model was 0.92, The use of the RITA® bioreactor had a positive effect on embryo number at 1 min of immersion time and a frequency of 12 times a day comparing with the liquid system but not at others frequencies, possibly because of the physical conditions inside the reactor. Response surface design was an experimental strategy which led to raise the embryo number using asparagine and glutamine as supplement of MS medium in the callus differentiation *A. americana* L. and using the RITA reactors automatic system was effective to improve the multiplication rate.

Keywords: *Agave americana* L., glutamine, asparagine, somatic embryogenesis, Box-Behnken design, temporal immersion.

1. INTRODUCTION

Agave americana L. is cultivated to obtain textile fiber, used to produce rope, nets and other objects [1] and has been used in traditional medicine as a natural defaunating agent in animals because the leaves contain high concentrations of saponins and fructans used as a prebiotic [2-4]. It is also used in alcoholic beverages like “mezcal” and “comiteco”. Multiplication of *A. americana* occurs through tillers and seed germination, but its propagation rate is low [5] therefore the amount of this a gave is decreasing an accelerating rate as a result of an unsustainable exploitation. *In vitro* methods can be used to propagate and preserve endangered plant species when seed-based methods are inadequate [6]. The methods have been reported with success in the micropropagation of *Agave fourcroydes* Lem [7], *A. tequilana* Weber [8-10], *A. Vera-cruz* Mill [11], *A. angustifolia* Haw [12] and *A. grijalvensis* B. Ullrich [13] in which somatic embryogenesis was an efficient method for the regeneration of plants and has been preferred among other methods, mainly due to increases in the multiplication efficiency of the system, decreases in the costs of production, and partial automation of the process through the use of bioreactors, [14]. Somatic embryogenesis is defined as the process whereby somatic embryogenesis states develop and result in complete plants without fusion of gametes [15]. The main factors affecting the *in vitro* somatic embryogenesis (SE) are the variety, the source of explant and composition of the culture medium, particularly plant growth regulators [16]. However it has also been shown that the addition of some amino acids affects the proliferation of somatic embryos. It has been able to increase the SE of *Triticum aestivum* L. with asparagine (Asn) [17]. Production of somatic embryos and plantlet regeneration in *Medicago sativa* were increased with glutamine, alanine, proline and arginine supplements in regeneration medium [18]. Higher somatic embryos embryogenesis in *Santalum album* L. was obtained after statistical optimisation of medium constituents such as sugar, inorganic nitrogen, and abscisic acid [19].

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Response surface methodology (RSM), a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the relative significance of several independent variables, and determining optimum conditions for desirable responses, [20] has been applied for optimization and process modelling. The development of an optimum procedure to improve the conversion of callus to somatic embryos will reduce the plant production costs and improve the multiplication rate.

In this study, the effect of different concentrations of 2,4-dicholorophenoxyacetic acid (2,4-D), asparagine and glutamine on induction of somatic embryos of *Agave americana* L. were investigated with a surface response design. Also the impact of the immersion frequency of the callus inside the RITA reactors system for optimization of number of embryos was investigated.

2. MATERIAL AND METHODS

2.1. Plant Material

The plant material used in this study was obtained from a collection of *A. americana* plants at the experimental site of Tuleaito located in Comitan (Chiapas, México) located at 16 ° 15 '04" north latitude and 92 ° 08' 03" W and at an altitude of 1,622 meters above sea level.

The seeds were germinated by placing them between two layers of wet cotton in an aluminium container at room temperature, keeping constant moisture. Hypocotyls were obtained from seeds germinated for 30 days and washed with liquid soap and rinsed with tap water. Disinfection of the hypocotyls was carried out with an Agrimicin ® Captan ® 3% solution for 20 minutes. They were subsequently rinsed three times with sterile distilled water and then immersed in a solution of 0.01% Rifampicin for 10 minutes. They were then rinsed three times with sterile distilled water and finally were immersed in a solution of sodium hypochlorite at 10% for 5 minutes.

2.2. Induction of Somatic Embryogenesis

For callus induction, disinfected hypocotyls were placed in an MS medium supplemented with 0.5 mg/L of 2,4-D, after 35 days the callus were isolated from the explants and transfer to a liquid MS medium for embryo induction with different concentrations of 2,4-D (0, 1 and 2 mg L^{-1}), asparagine (0, 500 and 1000 mg L^{-1}), glutamine (0, 200, and 500 mg L^{-1}), sucrose 30 g L^{-1}, myo-inositol 100 mg L^{-1} sodium phosphate 50 mg L^{-1} and 0.25% phytagel according to the experimental response surface design, Box-Behnken.

2.3. Experimental Design

A 3-level-3-factor Box-Behnken design was employed with three repetitions at the central point (Table **1**). The variables and their levels selected were asparagine concentration (0-1000 mg L^{-1}), glutamine concentration (0-500 mg L^{-1}), and 2,4-D concentration (0-2 mg L^{-1}). All experimental units were stirred at 100 rpm and incubated at 25±2 ºC with a 16-h photoperiod under fluorescent light (16 µmol s^{-1} m^{-2}). The design was repeated four times for a total of 60 experimental units. Somatic embryos were counted after 20 days of starting the induction stage. After finding the best treatment for embryo numbers, the concentrations of 500 mg L^{-1} Asn, 200 mg L^{-1} Gln and 1 mg L^{-1} 2,4-D was employed to measure the effect of different immersion frequencies 2 to 12 times per day (Figure **3**) at 1 min per immersion of the callus inside the RITA® [(Vitropic S.A, San Mathieu de Tréviers; France). The components are an autoclavable polysulfonate reactor equipped with 2 air vents and 1 netting. Dimensions: height 150 mm, diameter 130 mm, volume 1 litre, weight 350 g, using a automatic control system with a programmable Logic Controller (PLC, altechcorp®, Puebla, México.) with a immersion time of 1 min every 2 hrs using compressed air sterilized with 2 polycarbonate filter of 5µm and 0.01 µm applied directly by an air pump,] in order to obtain the maximum embryo number possible.

Table 1: Range of Variables in the Box-Behnken Design

	Low level	Central level	High level
Variable (mg L^{-1})	(-1)	(0)	(+1)
2,4-D	0	1	2
Glutamine	0	0.2	0.5
Asparagine	0	0.005	0.01

2.4. Statistical Analyses

Embryo number was subjected to a one-way analysis of variance to test for significant difference between the treatments. The StatgraphicPlus Software was used for the regression analysis of the experimental data obtained. The quality of the fit of the model was expressed by the coefficient of determination R^2 and its statistical significance checked by an F-test. The significance of the regression coefficient was tested by a t-test. The level of significance was $P < 0.05$. A differential calculation method was then used to predict the optimum result.

3. RESULTS

3.1. RSM Model

The obtained responses, according to the Box-Behnken design varied from 4 to 21 in the results (Table **2**). Among the treatments the highest embryo number was obtained from treatment no. 3 and 8 with an identical number of embryos, however the composition of medium was different-- treatment 3 had 2 mg L^{-1} 2,4-D, 0 mg L^{-1} Gln and 500 mg L^{-1} Asn, and treatment 8 had 1 mg L^{-1} 2,4-D, 200 mg L^{-1} Gln and 500 mg L^{-1} Asn (Table **1**). The lowest embryo number, 4, was obtained from treatment 6 which had a composition of 0 mg L^{-1} 2,4-D, 200 mg L^{-1} Gln and 0 mg L^{-1} Asn.

The model for embryo number was:

$$EN = 18.83+2.19\ (D) - 0.88\ (Gln) + 2.25\ (Asn) - 4.98\ (D)^2 - 1.38\ (D)(Asn) + 3.52\ (D)^2 + 5.88\ (Gln)(Asn) - 3.98\ (Asn)^2$$

$R^2 = 92$ %.

Where EN=Embryo number; D=2,4-dicholoro-phenoxyacetic acid; Gln=Glutamine; Asn=Asparagine.

Maximum value of embryo number (21) could be obtained with 1 mg L^{-1} 2,4-D, 200 mg L^{-1} Gln and 500 mg L^{-1} Asn

3.2. Effect of the 2,4-D, Gln and Asp Concentrations

Embryo number was affected by 2,4-D, Gln and Asn concentrations (Table **2**). Addition of Gln and Asn increased the number of embryos from 19 to 21 compared to the control without Gln and Asn with 1 mg L^{-1} 2,4-D. Figure **1a** shows that only the interaction of Gln and Asn had a significant statistical effect on embryo number. Asn and 2,4-D had a positive effect,

Table 2: Response surface experimental design Box-Behnken with three repetitions in central point for investigate the effect of different concentrations of 2,4-dicholorophenoxyacetic acid (2,4-D) Asp and Gln on embryo number in *Agave americana* L.

	2,4-D	glutamine	asparagine	embryo[a]
Treatment	— (mg L^{-1}) —			— number —
1	1	0. 2	0.005	15.8 ± 4.5
2	0	0	0.005	19.5 ± 9.7
3	2	0	0.005	21.5 ± 8.5
4	0	0.5	0.005	13.3 ± 5.5
5	2	0.5	0.005	15.3 ± 5.0
6	0	0.2	0	4.3 ± 1.2
7	2	0.2	0	13.8 ± 4.5
8	1	0.2	0.005	21.5 ± 6.3
9	0	0.2	0.01	8.8 ± 1.0
10	2	0.2	0.01	12.8 ± 4.3
11	1	0	0	19.3 ± 2,5
12	1	0.5	0	10.3 ± 3.4
13	1	0	0.01	14.8 ± 3.5
14	1	0.5	0.01	20.3 ± 2.2
15	1	0.2	0.005	19.3 ± 3.5

[a]values are the average of four repetitions.

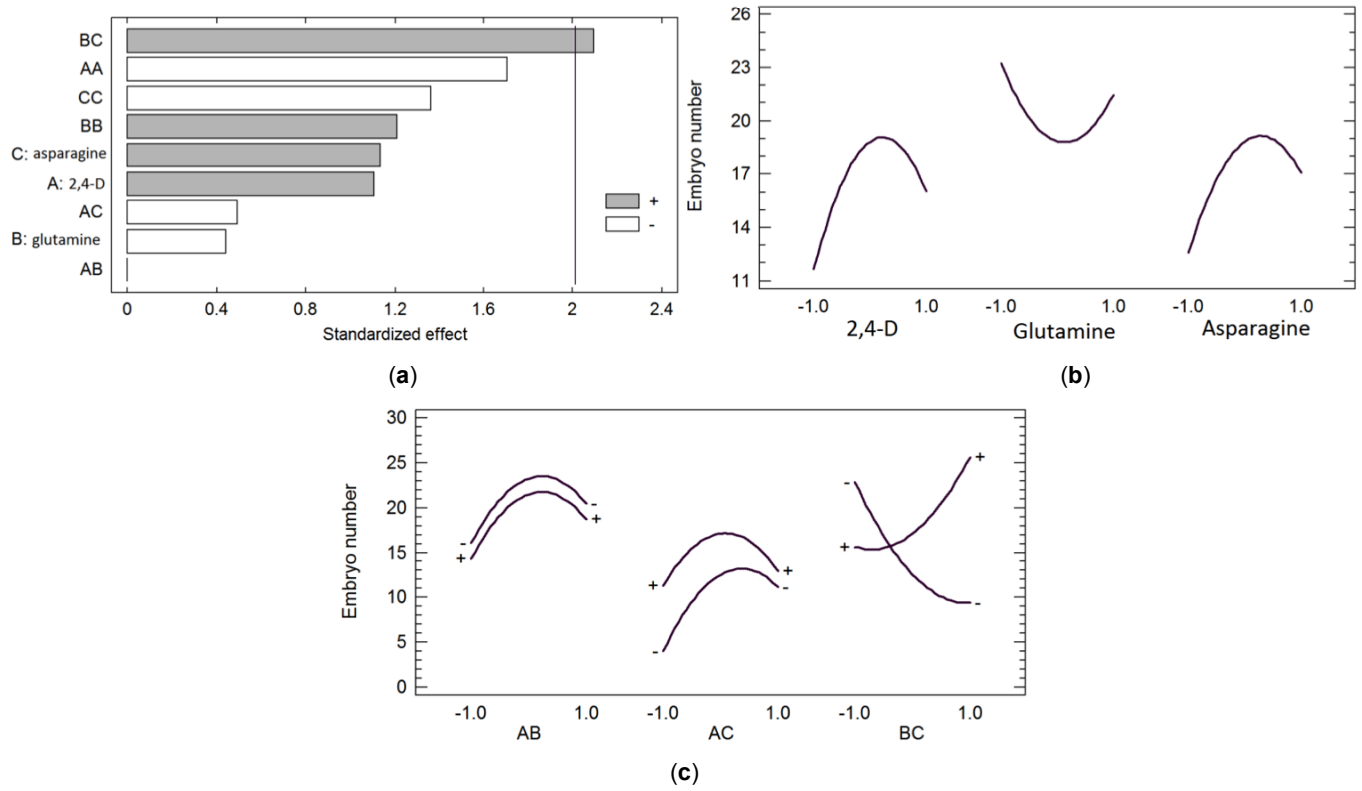

Figure 1: (**a**) Standardized Pareto chart to investigate the effect of glutamine, asparagine and 2,4-D. A: 2,4-D Dichlorophenoxyacetic acid; B: glutamine; C: asparagine; BC is the interaction between glutamine and asparagine; AA is the quadratic term for 2,4-D; BB is the quadratic term for glutamine; CC is the quadratic term for asparagine; AC is the interaction between 2,4-D and asparagine; AB is the interaction between 2,4-D and glutamine. "+" and "-" denoted a positive and negative effect respectively on embryo number. (**b**) main effect of the three factors and (**c**) interaction effects on embryo number in *A. americana* L.

whereas Gln had a negative effect. Figure **1b** shows the principal effect of the 3 factors on embryo number; with low concentrations of 2,4-D the embryo number rose, and with a higher amount of 2,4-D there was a decrease in embryo number. A similar effect was observed with Asn. Gln had an inverse effect. With respect to interactions of 2,4-D, Gln and Asn, only Gln and Asn showed a significant interaction with respect to embryo number (Figure **1c**).

3.2.1. Surface Response Graphics

In the surface response graphic between 2,4-D and Gln it was observed that only 1 mgL^{-1} 2,4-D was enough to produce 17 to 20 embryos regardless of the amount of Gln, and a higher or lower 2,4-D concentration decreased the number to 14-17 (Figure **2a**). The 2,4-D and Asn surface response graphic (Figure **2b**) shows a higher embryo number with 500 mgL^{-1}Asn and 1 mgL^{-1} of 2,4-D and a decreased number when the Asn concentration was largest. In Figure **2c** it was observed that the interaction of a higher concentration of the two amino acids Asn and Gln induced the greatest embryo number.

3.3. Optimization Inside the RITA Reactors

The results of the multiplication phase in RITA bioreactors were observed (Figure **3**), with a maximum of 53 embryos with an immersion frequency of 12 times per day and a minimum of 36 at 2 times per day with 1 min of immersion time. Meanwhile in the liquid system in Erlenmeyer flasks, maximum value was reached at 43 embryos at 100 rpm. Multiple test ranges were employed to observe the effect of different immersion frequency using 95.0 percent of the least significant difference (LSD).

4. DISCUSSION

The results show that interaction between asparagine and glutamine has a significant statistical effect, and the largest embryo number was obtained with the higher concentration of the two aminoacids; this could be because nitrogen originating from amino acids is assimilated quickly into the carbonic skeletons during the metabolism and synthesis of the proteins, when compared to other inorganic N sources [21]. Also, it has been postulated that some amino

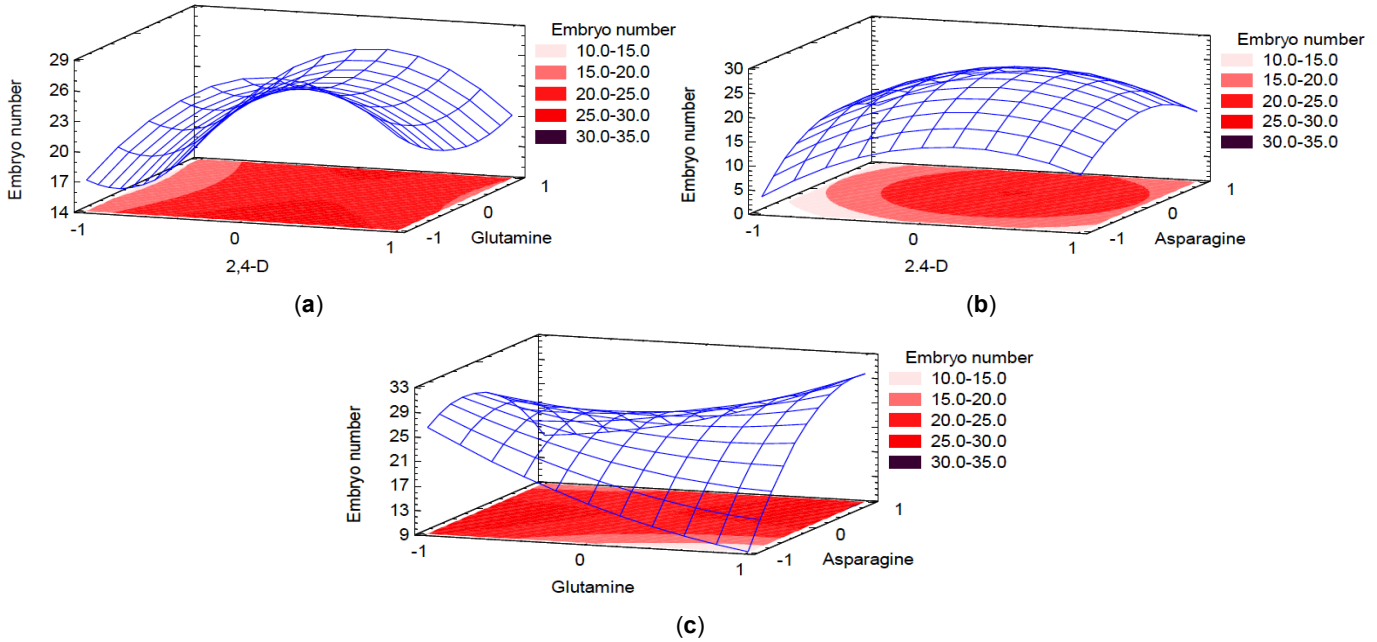

Figure 2: Surface response plot showing the relative effect of (**a**) Glutamine and 2,4-D. (**b**) asparagine and 2,4-D (**c**) Asparagine and glutamine on embryo number in *A. americana* L.

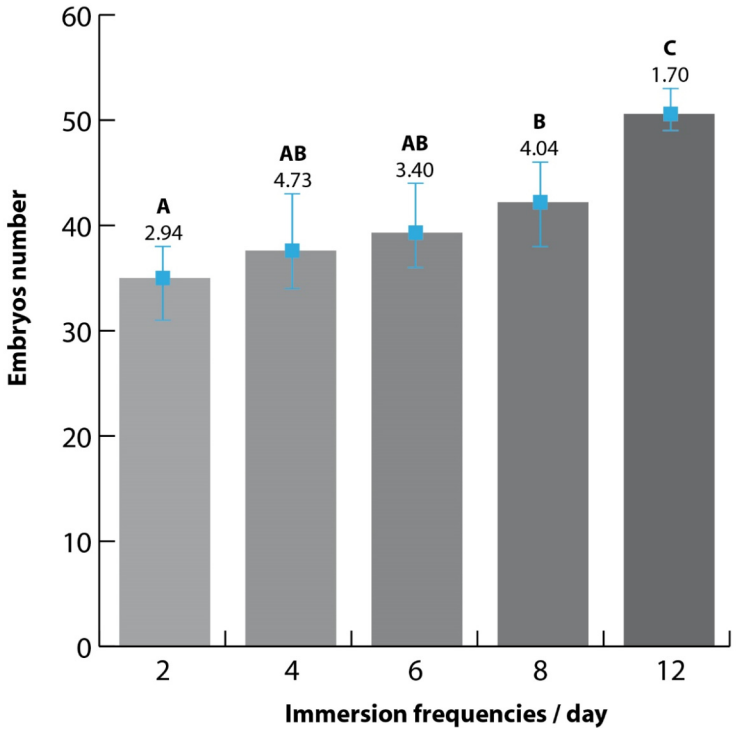

Figure 3: Influence of the immersion frequency in embryo number of *A. americana* L. using the RITA bioreactors system, each column represents the embryo number average at each frequency (P <0.05), Values with different letter are significantly different from each other by DMS test at α = 0.05 (n = 4).

acids allow adaptation to environmental change, especially to those causing cell stress [22] as might be the case *in vitro* morphogenesis. The largest amount of both can contribute to enhance the development of more embryos because the two aminoacids are important compounds in cellular division and are precursors of others aminoacids and energy.

Supplementation with the amino acids, asparagine, aspartic acid and glutamine, increased seedling vigor,

but decreased the starch content of embryos; however the quality of mature somatic embryos correlated with embryo size and was affected by the amount of storage protein and free amino acid, but not by starch [23]. The combination of pyroglutamic acid (a derivative of glutamine), ammonium, and sulphate in the maturation phase 1 medium dramatically increased accumulation of the 2S proteins and all subunits of the 11S medicagin. Application of ammonium nitrate as the major inorganic nitrogen source did not replace the requirement for pyroglutamic acid. Ammonium sulphate and pyroglutamic acid had nutritive and regulatory roles in storage protein accumulation [24]. In the presence of 50 mM glutamine the quantity of high salt soluble S-2 proteins, consisting of mainly medicagin (11S) along with other polypeptides of the storage proteins, increased 2-fold in somatic embryos [18]. A failure to synthesize the full complement of storage proteins (7S, 2S, 11S) was a serious limitation in producing quality embryos in *Medicago sativa* L [25]. In *Musa acuminata* glutamine also had a positive effect on embryo number [26]. The use of the two amino acids increased the embryo number because they probably provided a quick way to assimilate nitrogen into the embryo cells and increase the protein storage linked to production of somatic embryos.

In Figure **3**, the results of the multiplication phase in RITA bioreactors were observed, the maximum of 53 embryosat 12 immersion times per day was better than the other treatments, probably due to the physicochemical conditions generated in the bioreactors during the immersion time that provided the required nutrients, as well as exposure of the explants to oxygen and CO_2levels above those normally present in the liquid medium. Because the atmosphere is completely renewable within the vessel at regular intervals, there was no long accumulation of harmful gases, such as ethylene, which seems to be one of the main reasons for the success of this system [27].

5. CONCLUSION

Surface response design was an experimental strategy that provided a way to increase the embryo number in *A. americana* L using a higher concentration of Gln and Asn as supplement to MS medium due to the interaction between the two amino acids and their chemical properties. Furthermore, the multiplication rate of these embryos was positively affected by an increase in the frequency of immersion up to 12 times per day, with a time of 1 minute per immersion compared with the liquid system. This could have been largely due to the flux of O_2 and CO_2 in every air supply to the RITA reactor which could be stimulating cellular metabolism.

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