



Asian Journal of Plant Sciences

ISSN 1682-3974

science
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Seed Germination and *in vitro* Propagation of *Agave grijalvensis* an Endemic Endangered Mexican Species

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Abstract: In this study, seed germination and micropropagation protocols for *Agave grijalvensis* were optimized. A 24% *in vitro* seed germination occurred after surface sterilization of capsules in three steps in 70% ethanol, 0.1% HgCl₂ and 3.0% calcium hypochlorite solution and culturing on solid nutrient medium without any growth regulators. Maximum shoot induction was obtained with 38.2 µM 6-benzyl adenine (BA) and 0 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The concentration of 2,4-D increased the callus induction whereas BA and the interaction among BA and 2,4-D had no significant effect. Root induction was best on a ½-MS medium containing 22.7 µM indole butyric acid (IBA) and a 16/8 light regime. This protocol was efficient for obtained vigorous plants for transferred to greenhouse and further planted in field conditions.

Key words: 6-benzyl adenine, indole butyric acid, micropropagation, *Agave grijalvensis*

INTRODUCTION

Agave grijalvensis, an endemic plant from Chiapas, México, is a threatened specie (Mexican norm NOM-059-ECOL-2001). Its population has declined as its natural habitat is decreasing at an accelerating rate due to deforestation and its use for ornamental and food purposes. It multiplies through seeds, but its seed germination is low. Micropropagation is viable alternative, but efficient protocols to obtain a maximum of plantlets in the least possible time have to be designed. Seed germination is a first step to obtain explants for micropropagation purposes (Gutiérrez-Nicolás *et al.*, 2008).

Response Surface Methodology (RSM) is a collection of statistical techniques to design experiments, to build models, to evaluate the effects of factors and to search for optimum conditions for desirable responses (Myers and Montgomery, 2002). This method has been successfully applied in many areas of biotechnology, such as the optimization of medium components for the production of biosurfactants by probiotic bacteria (Rodrigues *et al.*, 2006), protease production by *Microbacterium* sp. (Thys *et al.*, 2006), optimization of a physical concentration process for inulin (Tófano *et al.*, 2007) and for the analysis of solid state growth of *Pleurotus ostreatus* mycelia (Bhak *et al.*, 2005).

The present study was done to obtain seed germination and micropropagation protocols for *A. grijalvensis*. Three disinfection methods for capsules

to obtain aseptic seeds and maximum seed germination were compared, concentrations of 6-benzyl adenine (BA) and 2,4-D were optimized for shoot and callus induction, while amounts of Indole Butyric Acid (IBA) and Murashige and Skoog medium (MS) and changing light regime were determined to maximize the number of roots.

MATERIALS AND METHODS

Seed disinfection methods: *Agave grijalvensis* capsules were collected from the 'Cañon del sumidero' national park 16 47' 56.5 north latitude 93 05' 28.5 west latitude at 966 msnm in Chiapas (Mexico). The capsules were disinfected in three ways.

Method 1: *Agave grijalvensis* capsules were treated with 70% ethanol for 30 s prior to an exposure to a 50% Clorox® solution (5.25% sodium hypochlorite (NaOCl)) for 10 min and rinsed three times with sterile distilled water. Seeds were removed from the capsules under aseptically conditions and placed on seed germination medium (Santacruz *et al.*, 1999).

Method 2: *Agave grijalvensis* capsules were treated with a 20% H₂O₂ solution for 2 h and rinsed at least three times with sterile distilled water. The seeds were placed on a rotor shaker at 100 rpm overnight. Seeds were removed from the capsules under aseptically conditions and the seeds were then placed on seed germination medium (Baogong, 2004).

Table 1: The actual design of experiments for BA and 2,4-D evaluation for callus and shoot induction in *Agave grijalvensis* micropropagation

Trial No.	BA (μM)	2,4-D (μM)
1	0.00	0.00
2	13.30	0.00
3	26.60	0.00
4	39.90	0.00
5	53.20	0.00
6	0.00	0.06
7	13.30	0.06
8	26.60	0.06
9	39.90	0.06
10	53.20	0.06
11	0.00	0.12
12	13.30	0.12
13	26.60	0.12
14	39.90	0.12
15	53.20	0.12
16	0.00	0.18
17	13.30	0.18
18	26.60	0.18
19	39.90	0.18
20	53.20	0.18

Method 3: *Agave grijalvensis* capsules were treated with 0.5% agrimycin-captan® solution. The capsules were then placed on a rotor shaker at 100 rpm for 10 min, rinsed three times with sterile distilled water, submerged in 70% ethanol for 30 s, submerged in 0.1% HgCl_2 for 10 min and rinsed four times with sterile distilled water. The seeds were submerged in 3.0% calcium hypochlorite solution and rinsed four times with sterile distilled water. Seeds were removed from capsules under aseptically conditions and the seeds were then placed on seed germination medium.

The germination medium MS (Murashige and Skoog, 1962) was supplemented with vitamins, 30 g L^{-1} sucrose, 2.5 g L^{-1} phytigel. The culture media was adjusted to pH 5.8 with 0.1 M NaOH before autoclaving at 121°C and 1.2 kg cm^{-2} for 15 min.

The seeds were incubated with a 16/8 light/dark photoperiod at 22°C for 5 days. Contamination was determined by visual inspection for fungal and/or bacterial growth and the number of germinated seeds (shoot elongation) were recorded after 8 weeks.

Experimental designs: A factorial response surface experimental design was used to determine the optimal concentrations of 2,4-D and BA for shoot and callus induction (Table 1). A Box Behnen response surface experimental design was used to determine optimum concentrations of IBA, MS strength and light regime for root number (Table 2). A total of 20 treatments with 6 repetitions were done.

Plantlet acclimatization: The plantlets with 3-5 roots and with well-developed shoots were rinsed with distilled water until agar rest elimination, submerged in 2 g L^{-1}

Table 2: The actual design of experiments for AIB, MS strength and light regime evaluation for root induction of shoot in *Agave grijalvensis* micropropagation

Trial No.	AIB (μM)	MS strength (μM)	Light regime (Light/dark1) (μM)
1	0.00	50	16/8
2	9.80	50	16/8
3	19.70	50	16/8
4	29.50	50	16/8
5	39.30	50	16/8
6	0.00	100	16/8
7	9.80	100	16/8
8	19.70	100	16/8
9	29.50	100	16/8
10	39.30	100	16/8
11	0.00	50	0/24
12	9.80	50	0/24
13	19.70	50	0/24
14	29.50	50	0/24
15	39.30	50	0/24
16	0.00	100	0/24
17	9.80	100	0/24
18	19.70	100	0/24
19	29.50	100	0/24
20	39.30	100	0/24

captan® solution for 5 min and transferred to pots containing a sterilized mixture of peat moss and agrolite (3:2) for hardening at 22±2°C under diffuse light (16/8 h photoperiod). Potted plantlets were covered with polyethylene membranes to ensure high humidity and watered daily with liquid ½-MS medium free of sucrose. After 2 week, the membranes were removed and the plantlets were irrigated with tap water. The plantlets were acclimatized for 1 week in the laboratory conditions and then transferred to a greenhouse for 3 month.

Statistical analysis: Characteristics of the plantlets were subjected to a one-way analysis of variance (ANOVA) to test for significant differences over time (SAS, 1989).

RESULTS AND DISCUSSION

Disinfection and germination: The surface of the explants must be fully sterilized for any tissue culture study. Three methods were compared in this research. The results fluctuated between 65.0 and 98.8%. Method 3 gave the best surface disinfection because the percent of contaminated seed was 1.3 while it was 13.8% method 1 and 35.0% in method. As a result germination were also better with method 3 (Table 3). Germination of seeds sterilized with Clorox was low as chlorine might have suppressed it (Baogong, 2004).

Shoot induction: The concentration of BA affected the shoot induction with p-value of 0.048. 2,4-D and the interaction between BA and 2,4-D did not significantly affect shoot induction (Fig. 1a). Main effects indicated

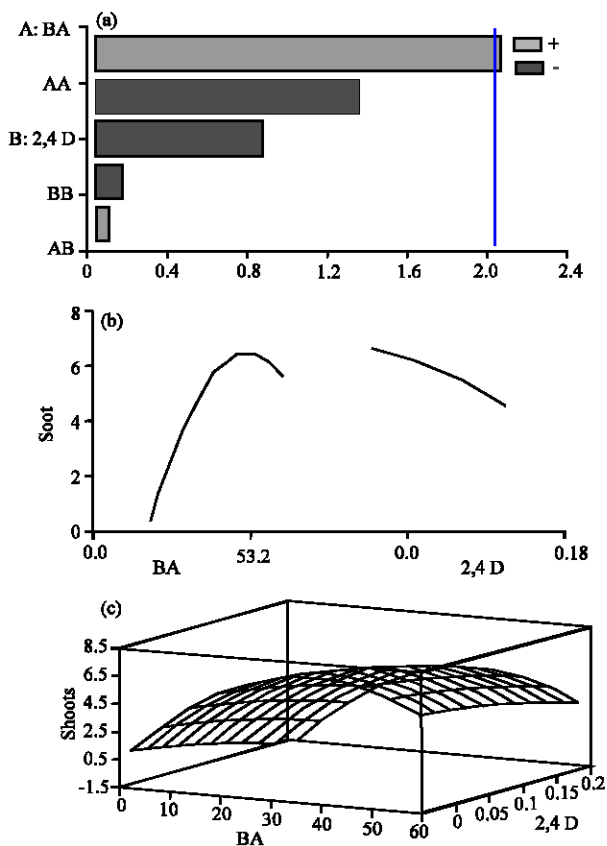


Fig. 1: Standardized Pareto chart (a): Main effects, (b) response surface plot and (c) Investigate the effect of 6-benzyl adenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) on shoot number of *A. grijalvensis*

Table 3: Disinfection and germination percents of *Agave grijalvensis* seeds obtained after three methods

Method	Disinfection (%)	Germination (%)
1	86.3b	12.5b
2	65.0c	15.0a
3	98.8a	24.0a

Values with different letters are significantly different at $p < 0.05$

that shoot induction was increased with BA concentration and decreased with 2,4-D concentration (Fig. 1b). The response surface curve indicated that shoot induction increased with 40-50 μM BA combined with 0-0.05 μM 2,4-D (Fig. 1c). Maximum shoot induction was obtained with 38.2 μM of BA and 0 μM 2,4-D.

The model for shoot induction explained 34.9% of the variation found (Table 4). Since the p-value is greater than 0.05, there is no indication of serial autocorrelation in the residuals. Santacruz-Ruvalcaba *et al.* (1999) found that BA, 2,4-D and the interaction between them showed a significant effect on shoot proliferation in *A. parrasana* Berger. They obtained more shoot production when the BA concentration was in the range of 13.3 to 53.2 μM . The

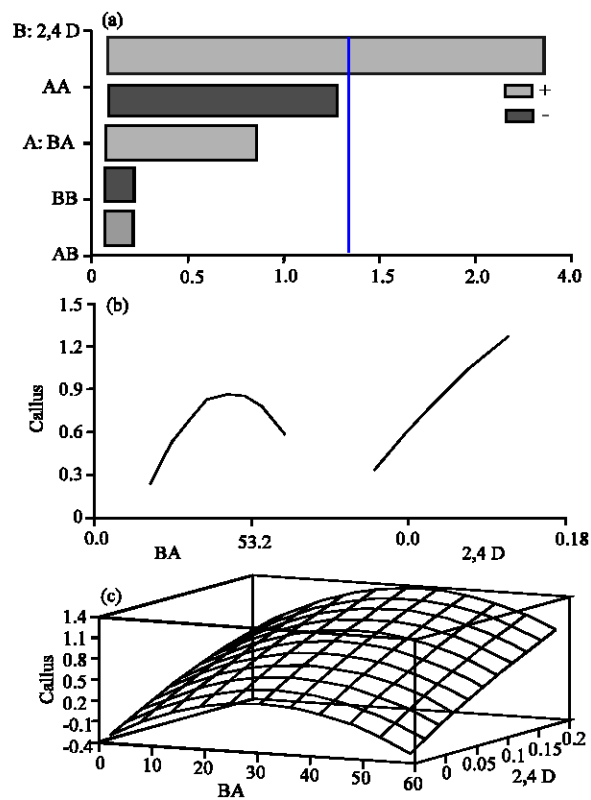


Fig. 2: Standardized Pareto chart (a): Main effects (b) response surface plot and (c) Investigate the effect of 6-benzyl adenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction of *A. grijalvensis*

results obtained in the present study are similar to those with *A. parrasana* indicating that shoot proliferation in *A. grijalvensis* was optimized with 38.2 μM BA while 2,4-D reduced the number of shoots per explant.

Callus induction: The concentration of 2,4-D affected positively the percent of callus induction ($p = 0.02$). BA and the interaction between BA and 2,4-D did not significantly affect callus induction (Fig. 2a). Main effects of BA and 2,4-D indicated that callus induction increased with increased 2,4-D concentration and reached a maximum at 25 μM BA (Fig. 2b). The response surface curve indicated maximum callus induction was found at 30-40 μM BA combined with 0.15-0.2 μM 2,4-D (Fig. 2c). Maximum callus induction was obtained with 32.9 μM BA and 0.18 μM 2,4-D.

The model used explained 37.6% of the variability in the data (Table 4). The p-value was higher than 0.05 so there was no indication of possible

Table 4: Regression model of response and their significance for shoot, callus and root in *Agave grijalvensis* micropropagation

Induction	Model	R ²	STD	MAE	DW	p-value
Shoot	$1.029+0.33*BA-7.25*2,4-D-0.0043*BA^2+0.05*BA*2,4-D-34.72*2,4-D^2$	0.349	4.07	2.80	1.91	0.1432
Callus	$-0.31+0.039*BA+6.25*2,4-D-0.00066*BA^2+0.019*BA*2,4-D-6.94*2,4-D^2$	0.376	0.67	0.48	2.16	0.1098
Root	$7.08+0.014*IBA-0.004*MS-3.1*LR-0.0016*IBA^2-0.0005*IBA*MS+0.086*IBA*LR-0.008*MS*LR$	0.637	1.45	0.88	2.30	0.0061

R²: Root square, STD: Standard deviation, MAE: Mean absolute error, DW: Durbin-Watson statistics, P: Probability, BA: 6-benzyl adenine, 2,4-D: 2,4-dichlorophenoxyacetic acid, IBA: Indole Butyric Acid

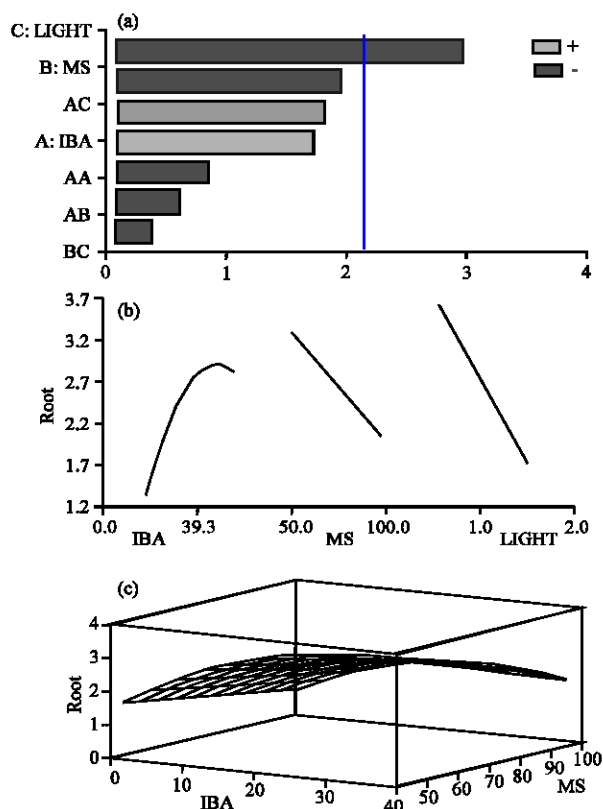


Fig. 3: Standardized Pareto chart (a), Main effects (b) and response surface plot (c) to investigate the effect of strength MS medium, indole butyric acid (IBA) and light regime on root induction of *A. grijalvensis*

serial correlation. Embryogenic calli of *A. vera-cruz* Mill explants were obtained with MS medium supplemented with vitamins, 4.52 μ M 2,4-D or 5.37 μ M α -naphthalene acetic acid (NAA) (Tejavathi *et al.*, 2007). The induction of *A. grijalvensis* callus was obtained with only 0.18 μ M 2,4-D.

Root induction: The light regime affected the root induction, but IBA and MS and the interactions between them did not affect significantly the number of roots (Fig. 3a). The number of roots increased with the concentration of IBA with 50% MS when plantlets were exposed to light regime 1 (16/8) (Fig. 3b). The response

surface curve indicated a maximum number of roots was obtained with 20-30 μ M IBA combined with 1/2-MS and an illumination regime 16/8 (Fig. 3c). Enríquez del Valle *et al.* (2005) found similar results for root induction in *A. angustifolia* shoots. They found that the number of roots per shoot was higher as the IBA concentration increased and the salt concentration in the medium decreased.

The second order model used explained 63.7% of the variability in the data (Table 4). Since the p-value was lesser than 0.05, there was indication of serial autocorrelation in the residuals. For plantlet survival in *ex vitro* conditions, rooting is still a limiting factor (Mata Rosas *et al.*, 2001) and therefore present results are important for micropropagation purposes.

In conclusion, it was found that a method for disinfection of capsules for obtained aseptic seeds and for promotion higher rate of seed germination of *A. grijalvensis*. The Response Surface Methodology (RSM) was employed for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of 6-benzyl adenine (BA) and 2,4-D for shoot and callus induction and also for obtained the optimal levels of IBA, MS and light regime for increased the root number. Maximum shoot induction was obtained with 38.2 μ M BA without 2,4-D. The concentration of 2,4-D increased the callus induction. Root induction was best on a 1/2-MS medium containing 22.7 μ M IBA and a 16/8 light regime. This protocol was efficient for obtained vigorous plants for transferred to greenhouse and further planted in field conditions.

ACKNOWLEDGMENTS

The research was funded by the Dirección General de Educación Superior Tecnológica, Project UR TGZ-07. Micropropagación de plantas endémicas de Chiapas y en peligro de extinción o amenazadas evaluando su variabilidad genética.

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