Micropropagation of *Agave warelliana* (Baker, 1877): sucrose and light/darkness tests in solid/liquid medium

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Resumen

En este estudio se aplicó la técnica de cultivo *in vitro*, con el objetivo de realizar ensayos preliminares para la micropropagación de *Agave warelliana* a partir de semillas. Para ello se planteó: i) la determinación y constitución de un medio de cultivo (sólido o líquido) para la germinación de semillas, ii) probar concentraciones de sacarosa (0.0, 20 y 30g/L) como fuente de carbono, iii) determinar el efecto de la exposición de los cultivos al factor luz/oscuridad en la proporción de la germinación, iv) ensayo de enraizamiento de brotes con la aplicación de ácido indol butírico (IBA) (2.0 mg/L) y un enraizador comercial (5.0 mg/L), y v) ensayo de aclimatación de plántulas y pase a suelo lográndose un 100% de plantas propagadas. Permitiéndonos aseverar que la producción masiva de *Agave warelliana* es factible mediante el protocolo desarrollado para este estudio.

***Palabras clave**: Agave warelliana, Micropropagación, Plántulas, Citocininas*

Abstrac

In the present research work the in vitro culture technique is applied, with the aim of obtaining the first studies on micropropagation of Agave Warelliana, from seeds, what I imply; i) determination and constitution of a culture medium (solid or liquid) for seeds germination, ii) testing sucrose concentrations (0.0, 20 and 30 g/L) as carbon source, iii) determining the effect of light/dark factor in the germination proportion, iv) shoot rooting test with the application of indole butyric acid (IBA) (2.0 mg/L) and commercial roaster (5.0 mg/L), and v) seedling acclimation trial. What allows us to assert that the mass production of Agave warelliana is feasible through the protocol developed for this study.

***Key words**: Agave warelliana, Micropropagation, Seedlings, Citokininas*

Introduction

In vitro culture of plant cells and tissues is a fundamental alternative in the rescue and conservation of endangered species and also for large-scale multiplication of superior genotypes, and to obtain big clonal populations from selected plants. Modifications can be made in each of the steps of a procedure for *in vitro* propagation to increase the efficiency of this and the quality of the plants. So it is advisable to evaluate the effect of varying the concentration of the components of a culture medium such as mineral salts of Murashige and Skoog (1962), exogenous application of growth regulators (RC) and sucrosa concentration. Little is known about *Agave warelliana* in its natural habitat and in our search bibliography no jobs were found that contributed as alternative solution to the risk faced by this species with respect to his stay in the wild world. Studies with other agave species include Dominguez Rosales (2008) which found that five agave species responded differentially to BA concentration i*n vitro* cultures: the highest shoot production for *Agave cupreata* (Trel. & Berger) and *Agave karwinskii* (Zucc), were obtained in 1.5 and 1 mg/L BA, whereas for *Agave difformis* (Berger) and *Agave obscura* (Schiede), and *A. potatorum* (Zucc), the best responses were obtained to 0.2 mg/L and 3 mg/L, of BA respectively. Santíz et al (2012) multiplied callus in MS medium supplemented with 0,1 g/L de myoinositol, 0,05 g/L of sodium phosphate, 30 g/L of sucrosa, 0.18 µM de 2,4-D and 2,5 g/L de phytagel® as gelling agent, and

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for bud formation added growth regulators; BA (0, 22 y 44 µM), ANA (0; 1,3; 2,6 y 5,2 µM) and 2,4-D (0; 0,55; 1,1 y 2.2 µM) concluding that somatic embryogenesis it is a suitable biotechnological tool the rescue of *Agave grijalvensis*. For *Agave americana* Miguel-Luna et al (2013), used stem tissue as explant and determined that Indole butyric acid in concentrations of 0.5 or 1 mg/L in culture medium stimulated the highest formation of adventitious roots and plant height, compared to shoots established in medium without IBA; all of this under white fluorescent light at 100 umol m^{-2} $s.^{-1}$ of intensity, photoperiod of 16 h and 8 h darkness and temperature of 22-28 °C. Shoots incubated in greenhouse environment developed more leaves, stems of larger diameter and accumulated more total dry matter, but similar amount of roots than shoots incubated in the laboratory. Similarly, Miguel-Luna et al (2014) using similar experimental conditions, reported the Murashige and Skoog (1962) medium (MS), with 6 mg/L of benciladenina (BA) and 40 g/L of sucrose (SA), were the best for new outbreaks of shoots of *Agave americana* var. oaxacensis, quantifying five times the number of shoots with which it started the multiplication culture of propagules. The information void for *A. warelliana* and the differential response of agave species to the *in vitro* culture technique, suggests the need to perform tests for the optimization of a micropropagation protocol for this species. So, in this work, the *in vitro* cultivation technique is applied to *A. warelliana* with the aim of obtain the first study on micropropagation of seeds of this specie in five stages: i) determination and constitution of a culture medium (solid or liquid) for seeds germination, ii) testing sucrose concentrations (0.0, 20 and 30 g/L) as carbon source, iii) determining the effect of light/dark factor in the germination proportion, iv) shoot rooting test with the application of indole butyric acid (IBA) (2.0 mg/L) and commercial roaster (5.0 mg/L), and v) seedling acclimation trial.

Methodology

The present research project was carried out in the plant tissue culture laboratory of the School of Biology Xalapa, University Veracruzana, México, inside of the draft "In vitro culture of plant species of importance ornamental, medicinal, in danger of extinction and its connection to productive processes" under the supervision of the Dra. Blanca Lilia Náder García.

Stage 0

Obtaining seeds.The seeds used were donated by the Biologist David Jimeno Sevilla, collected in the vicinity of Pico de Orizaba, in september of 2006. The systematic identification he made it (Cházaro et al, 2008).

Stage I

Deisinfestation of seeds. Seeds of *A. warelliana*, were washed with running water and soap, then treated with 3 gr^{-1} of agrimicin fungicide for two hours in constant agitation, subsequently and inside laminar flow hood, were included in 25% chlorine solution (Cloralex ®, Elen), plus three drops of bactericide (Bacterin, H24 ®) and a drop of Tween 20 for each 50 ml., chlorine solution during 20 min, in constant agitation. Finally, were washed four times with deionized and sterilized water for four minutes each time and constant agitation. At the end of this treatment, the seeds are kept in sterilized deionized water prior to and during planting.

Stage II

Tests in culture medium Gamborg 1968 (B5), with different concentrations of sucrose and lighting conditions. To the B5 medium, it was added 8 gr/L of bacteriological agar (Bioxon ®) as solidifying agent, supplemented with different concentrations of sucrose (0.0, 20.0, 30.0 gr/L), the pH adjusted to 5.7 (± 0.2), adding HCl (10%) or NaOH (10%). The sowing bottles (20) were sterilized to 121 °C and 1.2 Kg/cm^2 pressure during 25 min. Twenty culture flasks with 15 ml of B5 medium were sterilized under pressure already mentioned and were used for test sucrose concentrations. From three to four seeds per jar were placed and, ten jars for each concentration were incubated in constant lighting conditions under fluorescent white light lamps to 2000 lux for 24 hours and humidity relative (RH) 80-90%, constant temperature of 26 ± 2 °C; the rest of jars, were placed inside a box of polystyrene thermal insulation to keep them in darkness at a constant temperature of 20 ± 2 °C.

Stage III.

Sowing seeds in culture medium B5 with sucrose concentration and experienced luminosity condition. Known such requirements, we proceeded to plant a lot of 105 seeds.

Stage IV

Subculture of plants. To avoid losses of seeds sprouted for phenolization, exhaustion of the medium of cultive, lack of space inside the culture bottle, a subculture was performed on medium B5 new with sucrose concentration correspondent.

Stage V

Rooting. With seedlings that when germinating did not develop root, were subcultured in fresh B5 medium added with 2 mg/L of indolbutyric acid (IBA) and another batch was planted in B5 added with 5 mg/L of commercial rover (CytoRaizan ® 600).

Stage VI

Acclimatization.The plants that had more than two leaves, 5 cm long and had a root, were removed from the medium and transferred to ground. For the above, the roots were washed with deionized and sterilized water. Later they were included in a bactericidal solution (three drops per each 50 ml of Bacterin, H24 ®), during 10 min. Finally went to fungicide solution (Agrimicin, 3 gr/L) by others 10 min. For the sowing were used polyethylene cups No. 4 with a mixture of 60% vermicompost and 40% tepezil (v/v) previously sterilized, were covered with polyethylene bags of 10 X 15 cm. Three waterings were made every three days with B5 medium modified (free of agar, vitamins and sucrose). Subsequently, three more waterings were applied every 6 days with B5 medium modified diluted to 25%. One week after the last irrigation the bags that covered the plants were perforated to allow moderate air flow. 15 days later the perforated bags are removed and continued the irrigation with running water, thus concluding the acclimatization.

Results and discussion

Desinfestation

The process of micropropagation of *Agave warelliana* started with seeds who underwent the process of disinfestation already described and reported excellent results without any contamination coincident with *Agave angustifolia* HAW. (Arzate-Fernández, 2016), *Agave macroacantha* Zucc., (Arizaga y Ezcurra, 2002), and *Yucca aloifolia* L. (Karpov, 2004), which indicated that the seeds are viable and have the potential to germinate. The use of seeds as explants may present some disadvantages, like the ones that quote Wyka et al., (2006) as to what may be unviable, difficult to germinate; however, as they themselves raise, with its use the original plant is not damaged, when you have a copy for explants. In addition to being the ideal material for the conservation of the natural genetic diversity of plants (Ecker (1989), in Martínez-Palacios et al., 2003), Jordan y Nobel (1979) mentioned that in the wild only one seed of between 1.2 million achieves develops into a mature plant.

Germination test

During the seeds germination in medium B5 with 0.0 y 2.0% of sucrose and exposed to light, *A. warelliana* showed at day 8, 20% germination and at 11 days this stopped (fig 1). The germination in the same period with 3.0% sucrose reported only 2.0%, nevertheless, at day 20, it reached 38%. Similarly, seeds exposure to darkness under the same culture medium that those exposed to light, the germination started after 5 days, and it rises to 36-38 % to day 11, but at day 15-18, germination shoots up it rises to 40% in the middle without and with 2.0 of sucrose respectively. For day 11, no seed were germinated in medium B5 with 3% of sucrose, however, at day 13 the germination rose 58% and for day 29, it reached 88% .The factor of luminosity/darkness to which the seeds were subjected during germination originated a process of negative and/or positives phototropism. In seedlings growing in dark conditions a positive phototropism was observed with seedling growing vertically and with pale green coloration; while the most seedlings growing in light conditions, showed negative phototropism, lying horizontally on the culture medium and acquiring an intense green tonality. These observations allowed us to take measures to optimize the micropropagation protocol such as sub cultivate the seedlings to position them correctly, promote root development through exposure to growth regulators, as well as move them from darkness to light so that they begin their photoperiod.

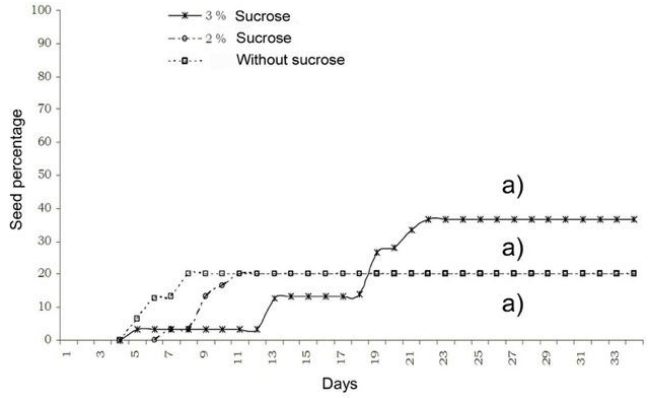

Figure 1. Percentage of *A. warelliana* seed germination in medium B5 with different sucrose concentrations under light conditions. Equal letters indicate non-significant differences (P = 0.2097)

Micropropagation in medium B5 with sucrosa 3% and dark conditions.

Seed germination at days 21 and 28, in medium B5 with 3% of sucrose, resulted in 76% of germination (fig 2). The seedlings coloration was pale green although it changed to dark green when he crops were transferred to illuminated incubation chambers. From germinated seeds, 42 of them developed root, and one or more leaves before being subjected to the process of acclimatization. 18 germinated seed did not develop root and only the first leaf was observed, therefore, they were subjected to the process of rooting. 19 seedlings, were oxidized before they got to measure 1 cm in length, and although they were subcultured in B5 medium for recovery, this was not achieved.

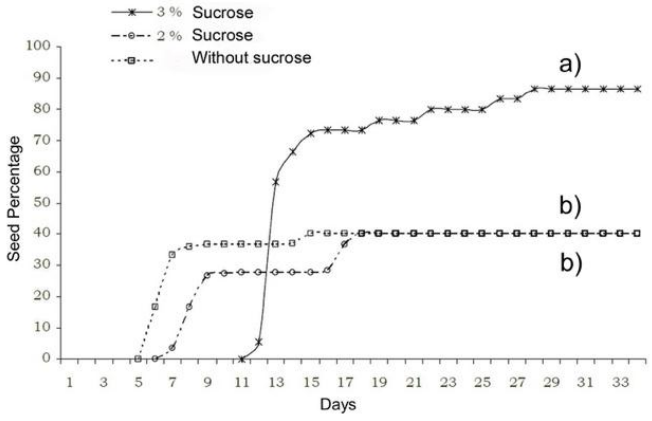

Figure 2 Percentage of *A. warelliana* seed germination in medium B5 with different sucrose concentrations under dark conditions. Different letters indicate significant differences (P = 0.0016)

In the Miguel-Luna (2014) study, the conditions and the culture medium for the stimulation of shoots and roots of *Agave*, coincide with what was tested for *Agave warelliana* in our study. However, the best regulator concentration used and the nutritional composition of the culture medium were not coincident. Therefore, it is shown that micropropagation protocols are specific and must be developed for each specie in particular.

Rooting of seedlings in vitro that did not develop root

To stimulate root development in 18 seedlings that had not done it, are made two tests: eight seedlings were subcultured in B5 medium supplemented with 2 mg/L of IBA and ten were subcultured in the same medium supplemented with 5 mg/L of commercial rooting. The stimulation for root development did not give result; however, seedlings oxidation started by the apical part and eight weeks later, this process was mortal. The same happened in the IBA treatment.

Sowing in soil and acclimatization

After one month the 42 seedlings of *A. warelliana* which were product of the germination, were subcultured individually, transferred to incubation chambers under fluorescent light for allow leaf and root growth. Then, after 4 month, they were removed from incubation chamber, and each seedling was protected with a plastic bag for acclimatization. One hundred percent of micro-propagated plants were obtained. Santíz et al (2012), established a protocol to induce somatic embryogenesis in *Agave grijalvensis* from stem as explant in MS medium, what forced them to redifferentiate with the addition of 22 µM de BA for obtaining shoots. Nevertheless, for microrpogation of *A. warelliana* in this study, we used seed as explant, medium Gamborg (1968) (B5), in a solid and liquid way and applying different concentrations of sucrose as well as light/dark exposure getting a high percentage of germination and subsequent shoots formation. For its part, Dominguez-Rosales (2008) reported for four agave species (*Agave difformis*, Berger, *Agave karwinskii* Zucc, *Agave obscura* Schiede, and *Agave potatorum* Zucc), a 50% of success for seedling acclimatization similar to *Agave wendtti* (Lara, 2018). However, we propose more trials varying the volume of sand for increase the results.

The *in vitro* culture technique as fundamental tool in alternatives for extinction problems, threat, that at present have increased for the vastness of plant populations as well offering results that become a determining background for biochemical, genetics, studies etc, we consider is based on the great diversity of options offered by the modifications in: type of culture medium (composition, state solid/liquid), type explant; seed, stem, leaf, root. Disinfestation Forms, Somatic embryogenesis (direct/indirect). Physical factors: light/darkness, addition or not of chemical components (growth regulators), among others in the technique of *in vitro* culture allows us to achieve satisfactory results regardless of the species that is subjected to this technique, as they show with the research on *Agave angustifolia* (Ríos-Ramírez et al., 2017) evaluated dose of benzylaminopurine (BAP) and indol-3-acetic acid (IAA) in a culture MS medium and their effect on the formation of new adventitious shoots (organogenesis). These same authors, report that the greater proliferation of shoots was in positive relation with the concentration of growth regulators with explants in culture medium added with 4 mg/L BA + 1 mg/L AIA and explant in MS with 0.5 mg/L BA + 0.1 mg/L AIA were formed on average 32.8 and 7.8 shoots with 45 and 24 leaves, respectively. Caraballo et al. (2010), working with *Agave fourcroydes* Lem., grown in MS medium and in combination with 6-benzylamynopurine (BAP) (0.75 mg/L) and indolebutyric acid (IBA) (1.0 mg/L) signicantly improved explant survival and shooting during the *in vitro* establishment of young shoots; combining thidiazuron (TDZ) (0.5 or 0.75 mg/L) with BAP (1.0 mg/L) and IBA (1.0 mg/L) in the basal medium increased the multiplication rate of henequen and signicantly speeded out bud dormancy breaking. The best rooting efficiency was obtained when the basal medium was supplemented with 0.5 or 0.75 mg/L of NAA, giving 100% of rooted explants and an average of 9.40 and 11.55 roots per explants over 94% of micropropagated plants which survived the *ex vitro* weaning step and no morphological disorders were observed in any of the plants. The modification of growth regulators composition in the medium was a key factor to improve the efficiency for the henequen technology micropropagation.

The ecological, economic, medicinal and cultural importance of agaves, like other big groups of plants, is not exempt from overexploitation, alteration of their natural habitats or attack by pathogens. All of this drives to a decrease in their natural populations or even their local extinction. Plant biotechnology, especially plant tissue culture techniques, represents an alternative viable for the massive propagation of species in short time and low costs. This study presents for the first time, a successful protocol for massive micropropagation, regeneration, rooting *in vitro*, acclimatization and conservation *ex situ* of *Agave warelliana*. The micropropagated plants, can be used as source for the chemical characterization of active compounds of importance, industrial and medicinal, as well as for studies at the molecular level, which are used in evolutionary and taxonomics studies. By using *in vitro* culture in this work, it can be specified that seeds of Agave warelliana showed a greater response of germination in B5 medium added with 3% of sucrosa. The use of IBA and commercial rooting did not offerd a solution to the absence of roots in seedlings obtained in germination process. Vermicompost use, of

in combination with tepezil as substrate, were effective to achieve a 100% of acclimatization for plants of *Agave warelliana*.

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