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Micropropagation of *Aristolochia elegans* (Mast.)

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Abstract

The roots of *Aristolochia elegans* Mast. (Aristolochiaceae) are widely used in Mexican traditional medicine as a remedy for scorpion venom. Current experimental evidence supports its purported antidote properties. However, collection from the wilderness has lead to local extinction of natural populations. In order to contribute to species preservation, cultivation, and standardization of morphological and pharmacological properties, a micropropagation method was developed. This includes *in-vitro* germination of seeds to produce aseptic plantlets, induction of multiple budding, and acclimatization. The treatment with benzylamino purine (10 μM) induced the highest number of buds (3.1 on average) in both types of explants. On the other hand, indolebutyric acid (1.5 μM) caused the highest root index (11.8) per explant. One hundred percent of the micropropagated plantlets developed vigorously after the acclimatization process.

Key words: acclimatization, *Aristolochia elegans*, hydroponic system, *in vitro* propagation, medicinal plant.

Introduction

Aristolochia elegans Mast Aristolochiaceae) is a plant commonly used in Mexican traditional medicine (Avilés and Suárez 1994). In Mexico, it grows in the wild and is locally called "guaco", "pato", or "tlacopalli". The water decoction of the roots is drunk as a remedy against envenomation of scorpion stings, asthma, and rheumatic pain, as well as for inducing uterine contractions during childbirth (Lozoya et al. 1987; Monroy and Castillo 2000).

Pharmacological studies of *A. elegans* root extracts have demonstrated the presence of alkaloids, and other compounds, with a relaxing effect on rat ileum previously contracted with scorpion venom (Jiménez et al. 2005). *A. elegans* roots are obtained exclusively from the wild and uncontrolled collection has lead to a sharp decline of populations in its natural habitat (Jiménez, 2005, personal communication), resembling local extinction suffered by other Mexican medicinal plants such as *Valeriana edulis* (valeriana), *Hippocratea excelsa* (cancerina), and *Mimosa tenuiflora* (tepescohuite), the former two collected for their roots, and the latter for its bark, respectively (Monroy and Castillo 2000; Reyes-Chilpa et al. 2003). In this context, it is important to establish conditions of propagation and cultivation that allow for the production of medicinal plants of homogeneous characteristics in both morphology and pharmacological activity (Rates, 2001). Micro-propagation is a biotechnological tool that allows obtaining healthy and vigorous plants grown at shorter times than required in natural conditions. Additionally, this technique can be helpful to preserve the natural flora of an area (Chawla 2002).

Currently, there are no reports on the propagation of *A. elegans* in the literature. However, the *in vitro* cultivation of other related species such as *A. indica, A. fimbriata*, and *A. manchuriensis*, has been established (Bravo et al. 1999; Manjula et al. 1997; Svensson 2000). The aim of the present study is to establish a methodology for the micropropagation and acclimatization of Mexican medicinal plants *Aristolochia elegans* Mast.

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Materials and Methods

Biological materials

Seeds of *A. elegans* were obtained from the Centro de Investigación Biomédica del Sur (CIBIS, a part of the Instituto Mexicano del Seguro Social IMSS, Xochitepec, Morelos [Mexico]). The plants were previously identified by the personnel

of the herbarium IMSSH and registered with the code HPMIMSS13595.

Medium preparation and culture conditions

A basal culture medium, MS, (Murashige and Skoog 1962) was used, and pH was adjusted to 5.8 with 0.1N HCl before the addition of agar. Other components were added before sterilizing in the autoclave for 20 min. The incubation conditions were: 25 ± 2 °C, photoperiod of 16 h with a light intensity of 310 µmol • m^{-2} • s^{-1}. Each experimental unit (EU) consisted of a baby food-type jar containing 20 mL of the culture medium. These EUs were used for every test.

Seed disinfection

Two experiments were carried out to establish the best disinfection conditions. 1) The seeds were submerged in NaClO solutions, concentrations, and contact times; conditions are shown in Table 1. The experimental design was random block, with a total of nine treatments by triplicate. Five seeds in MS culture medium (50% w/v) supplemented with 1.5% (w/v) of sucrose were placed in an experimental unit. After two weeks of incubation in the dark, the percentage of disinfected seeds was evaluated. Solutions of NaClO were prepared from a commercial solution of sodium hypochlorite that contained less than 6% of free chloride.

With the aim of increasing the disinfection percentage and germination of the seeds, treatment E (Table 1) was chosen as a starting point to develop the second experiment. 2) The seeds were submerged in ethanol (70% v/v) for 1, 3, and 5 min (Ea, Eb, and Ec respectively) before the immersion in NaClO (Osuna et al. 1999). Then six experimental units were added with five seeds in each one, and kept in the dark until the onset of germination. Afterwards, they were exposed to light (310 µmol • m^{-2} • s^{-1}) to induce the development of the plantlets. In both cases, the evaluated variable was the disinfection percentage.

Table 1. Disinfestations of seeds with solutions of NaClO

Treatment	A	B	C	D	E	F	G	H	I
Solution of NaClO (% free chlorine)	0.5	0.5	0.5	1.0	1.0	1.0	1.5	1.5	1.5
Contact time (min)	5	10	15	5	10	15	5	10	15
Treatment	A	B	C	D	E	F	G	H	I
Solution of NaClO (% free chlorine)	0.5	0.5	0.5	1.0	1.0	1.0	1.5	1.5	1.5
Contact time (min)	5	10	15	5	10	15	5	10	15

Induction of multiple budding

Eight-week-old plantlets obtained from disinfected seeds were used as sources for the explants. These were stem segments 0.5 cm long bearing the apical bud, and 1 cm segments of stem which included axillary buds (knots). Different combinations of BAP and NAA in a MS medium supplemented with sucrose (3% w/v) and agar (0.8% w/v) were tested in order to find the best combination to induce the formation of adventitious buds. For this purpose, an experimental two-factor design was carried out with the following variables: explant type (apical or axillary) and combinations of plant growth regulators (BAP and NAA) in different concentrations, as shown in Table 2.

Table 2. Treatments with BAP and NAA for the induction of multiple budding in two types of explants (apical and axillary buds)

Treatment	NAA	BAP
	Concentration (µM)	
1 (Blank)	0.0	0.0
2	0.0	2.5
3	0.0	5.0
4	0.0	10.0
5	0.5	0.0
6	0.5	2.5
7	0.5	5.0
8	0.5	10.0

Three explants were planted into each experimental unit and every treatment was performed by triplicate. Results were evaluated after an incubation period of six weeks (6 h photoperiod, 310 µmol • m^{-2} • s^{-1} light intensity, 25 ± 2 °C). The amount of buds/EU was counted and the average bud number per explant was calculated. The obtained buds were sub-cultured in MS without growth regulators for three additional weeks to achieve elongation.

Root induction

Stem explants (1 cm) were used to induce the roots. The explants came from aseptic platelets of six weeks of age with an axillary bud. Two explants were placed into an EU with MS medium supplemented with different concentrations of IBA and NAA (Table 3). The EU was incubated under the same conditions chosen for multiple budding. After six weeks, the total number of roots was counted and the root per explant index was calculated. This index represents the relationship between the total root number per UE and the number of transferred explants.

Table 3. Treatments with IBA and NAA for the induction of roots.

Treatment	IBA	NAA
	Concentration (µM)	
1 (Blank)	0.0	0.0
2	0.5	0.0
3	1.5	0.0
4	2.5	0.0
5	5.0	0.0
6	0.0	0.5
7	0.0	1.5
8	0.0	2.5
9	0.0	5.0

Acclimatization

The acclimatization was carried out according to the methodology reported by Ventura et al. (2003), which consists of a hydroponics system with a Hoagland nutritive solution (Salisbury and Ross 1994), where the aerial portion of the plants was covered with a plastic bag which was gradually perforated in such a way that the plants were completely exposed to the moisture of the incubation chamber at the end of the first month. Thereafter, the plants were placed at a photoperiod of 16 h, light intensity of 310 µmol • m^{-2} • s^{-1} and a temperature of 25 ± 2 °C. Then the mineral solution contained in the jars was replaced

with fresh solution and the plants were maintained under the same conditions for another month.

Each time the plastic cover was perforated, readings of relative humidity (RH) were taken inside the acclimatization system and the incubation chamber. Using these values, the atmospheric hydric potential (Ψ) was calculated for both the chamber and the acclimatization system with the following equation: Ψ (MPa) = -1.06 T log (100 ÷ RH) (Salisbury and Ross 1994), where Ψ is given in MPa and the temperature (T) in °K. In a similar manner, along the acclimatization process the following growth parameters were evaluated: number of knots number of secondary roots, length of the root, and length of the aerial portion (from the interphase root-stem to the apical bud) after 0, 1, and 2 months of growth.

Finally, the plants were transferred to pots of 0.2 L, which contained a sterile mix of peat moss, agrolite, and vermiculite in a proportion of 60:20:20 and a pH between 6.0-6.5. Fifty mL of nutritive solution were added to each pot at the time the plants were being transferred. The plants were kept inside the chamber for three additional weeks under the light, temperature and moisture conditions previously described. Then they were placed in a greenhouse in 2 L pots with the same substrate, where the average conditions were 23 °C, 55% RH, and 1554 µmol • m^{-2} • s^{-1}. A wooden stick of 1.20 m was placed in every pot to support the plant along its growth.

Table 4. Values of relative humidity (RH)1 and hydric potential (») inside the hydroponic system and incubation chamber during five weeks of acclimatization.

Week	RH hydroponic system (%)	RH incubation chamber(%)	Ψ hydroponic system (Mpa)	Ψ incubation chamber (MPa)
1	90.2 ± 1.2	47.2	-14.1	-103.0
2	81.0 ± 1.8	47.3	-28.9	-102.7
3	80.1 ± 1.6	47.8	-30.4	-101.2
4	75.3 ± 1.1	48.7	-38.9	-98.7
5	55.7 ± 1.3	45.0	-80.3	-109.5

[1]Values of RH of the hydroponic system represent the average of ten experimental units ± SD.

Statistical analysis

The software Sigma Stat version 3.0 (SPSS, USA) was used to calculate the statistical parameters. A one-way analysis of variance (ANOVA) was carried out for the disinfection and root induction experiments, while a two-way ANOVA of two factors (explant type and growth regulator treatment) was performed for the experiment of multiple budding. When the ANOVA pointed out a significant difference, the Tukey mean comparison method was used to find different treatments. AP<0.05 significance level was used for all the analyses.

Results

Induction of multiple budding

The results for both tests are shown in Figure 1 as seeds percentage. Test 1 resulted in disinfection values of 6.7 to 60% (Fig. 1A). Disinfection values in the interval of 46.7 to 60% for

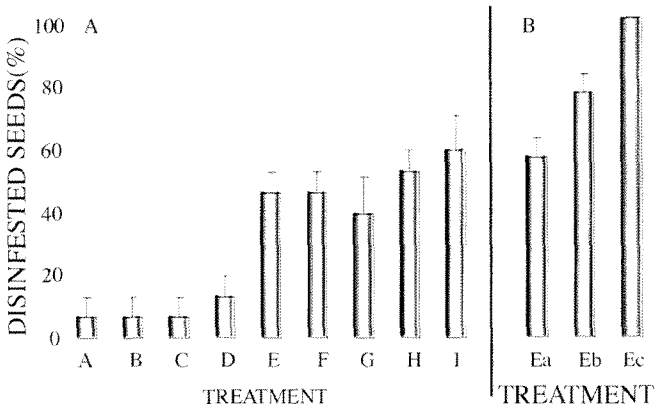

Fig. 1. Results of the disinfection of A. *elegans* seeds: A) Response to exposure time and concentration of HClO as indicated for T1; and B) Response to the treatment with HClO and EtOH (70 % v/v) after 1, 3, and 5 min of contact time (T2). Values represent mean + SD.

treatments E, F, G, H, and I, were higher and statistically significant compared to treatments A, B, C, and D, which had values of 6.7 to 13.3%. Treatment Ec of the second test (Fig. 1B) achieved a 100% disinfection of the seeds and 93% of germination. Fig. 2 shows the results of the induction of buds in the explants as a result of the treatments previously described. The number of buds for both types of explants increased as the concentration of BAP rises (treatments 2 and 3), reaching a maximum value (3.1 buds per explant) at 10 µM of BAP (treatment 4), which was significantly different from other treatments. Regarding the type of explant, no significant difference was found in the number of buds induced by treatment 4. However, using axillary explants, buds of 1.0-2.0 cm high were obtained, while apical explants yielded buds of 0.5-1.0 cm (Fig. 3).

The combination of NAA and BAP (treatments 6, 7, and 8) did not have a significant effect on the induction of buds of both types of explant. However, it induced a decrease of the number of buds per explant. There was no significant difference between pairs of treatments 2-6 and 3-7 in the case of axillary buds; however, such a difference was found for treatments 4 and 8 which yielded 3.1 and 1.4 buds per explant, respectively. This effect was more evident for apical buds, since they presented the same

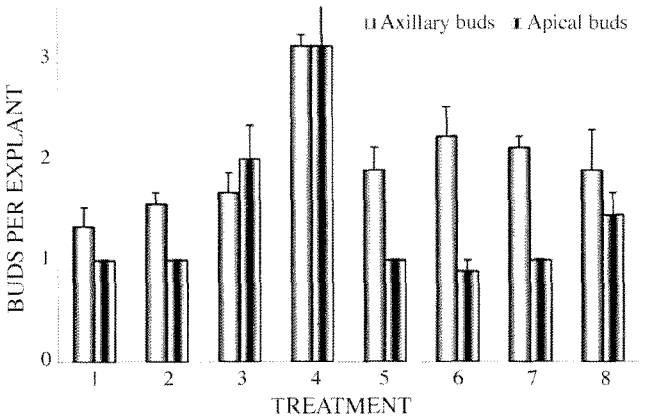

Fig. 2. Effect of different phyto-regulator treatments on the formation of apical and axillary buds. Values represent the mean of three experimental units + SD.

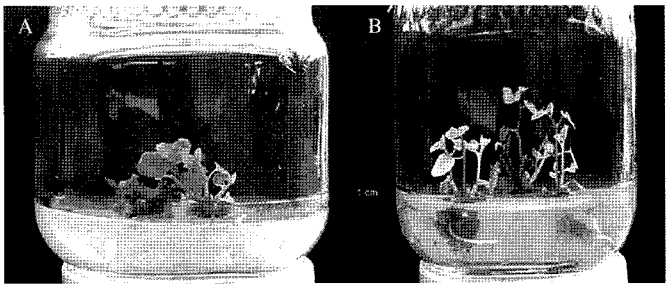

Fig. 3. Images showing multiple budding induced in *A. elegans* plants (8 weeks old) for both types of explants: (A) Apical buds, (B) Axillary buds (MS + BAP 10 µM).

differences observed for treatments 4 and 8, as well as a significant difference between treatments 3 and 7. The addition of NAA 0.5 µM (treatment 5) produced no significant difference as compared to the blank of the two types of explant.

Root induction

The highest index of roots per explant (11.8) after six weeks of culturing (Fig. 4) was achieved with the use of 1.5 µM indolebutyric acid (IBA), while a concentration of 0.5 µM only induced an index of 8.9 roots per explant. On the other hand, there was a negative correlation between concentrations of IBA higher than 1.5 µM and the induction of roots. For the species under study, the addition of NAA had a negligible effect on the induction of roots.

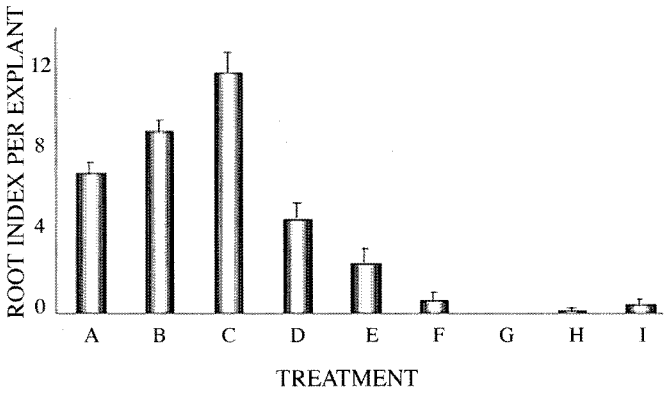

Fig. 4. Effect of different concentrations of auxins IBA and NAA on the formation of roots in 6-week old explants from axillary buds of A. *elegans*. A) Control; B) IBA 0.5 µM; C) IBA 1.5 µM; D) IBA 2.5 µM; E) IBA 5.0 µM; F) NAA 0.5 µM; G) NAA 1.5 µM; H) NAA 2.5 µM; I) NAA 5.0 µM. Values represent the mean of four repetitions + SD.

Acclimatization

Table 4 shows the results of relative humidity (RH) and hydric potential (Ψ) for both the acclimatization system and incubation chamber. A decrease of 66.2% in the values of Ψ between the first and the fifth week of acclimatization was observed in the hydroponic system. Given these conditions, 100% (n = 72) of the micro-propagated plants developed vigorously in the greenhouse after the acclimatization process. Fig. 5 represents the root and aerial parts growth, as well as the increase in the number of secondary roots and knots of micro-propagated plants along the acclimatization process. All the plantlets acclimatized to the conditions of the incubation chamber developed vigorously in the greenhouse (Fig. 6).

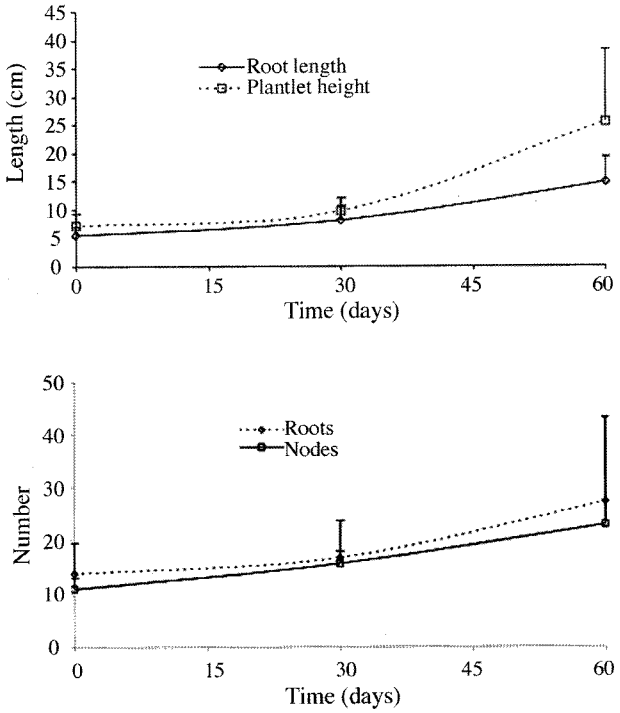

Fig. 5. Development of micro-propagated plants through acclimatization: (A) growth of the largest root and aerial part (the interphase between root and stem was taken as reference), and (B) increase in the number of secondary roots from the root-stem interphase and number of knots. Values represent the mean of 72 plants ± SD.

Discussion

Only three species of the *Aristolochia* genus have been previously propagated *in vitro*. This report is the first dealing with *A. elegans* micropropagation. In this study, the formation of adventitious buds was induced mainly with BAP 10 µM. The maximum number of buds per explant (3.1) observed for *A. elegans* is similar to values reported for other species of the same genus, such as *A. manchuriensis* with 4.2 buds per explant at BAP 2.0 µM, (Svensson 2000), and *A. indica* with 2-3 buds per explant at BAP 13.3 µM + ANA 0.5 µM (Manjula et al. 1997). On the other hand, only one bud per explant was reported for *A. fimbriata* after a treatment with 5.0 µM of BAP (Bravo et al. 1999).

The response to BAP observed for different species is related mainly to the endogenous concentration of phytohormones. In plants where apical dominance occurs, inhibition of lateral buds development is due to the flow of auxins (such as indole acetic acid) from the apical buds to the lower portions of the plant. In the plant itself, or in the *in vitro* culture, the presence of phytoregulators, such as cytokinins (e.g. BAP) in the culture medium neutralizes (directly or indirectly) the apical dominance effect, resulting in the development of lateral buds. Different species differ in their phytohormone balance and therefore require different concentrations of cytokinins in the culture medium to pre-

Fig. 6. *Aristolochia elegans* acclimatized plants growing in the greenhouse after five months. The plants in the image had an average of 1.30 m of height.

vent apical dominance.

It is known that auxins at low concentrations have the ability to induce the formation of roots, while in high concentrations they induce callus formation. In other reports dealing with different species of *Aristolochia*, the formation of roots was achieved by the use of IBA at high concentrations (2.5 and 5.0 μM); however, this effect is observed in *A. elegans* at a lower concentration of IBA (1.5 μM) with a higher root index (11.8). This can be attributed to the endogenous content of phytoregulators found in different species. It is noteworthy that the formation of roots in explants that received treatment 5 (NAA 0.5 μM) in the bud induction experiment were expected to develop roots, but this did not happen. This behavior may be due to the age of the plantlets from which the explants were taken, since it was different from the others: eight weeks for bud induction and six weeks for root induction. These two weeks of difference in the development of the plantlets could have affected the endogenous content of phytoregulators, which resulted in the development of roots.

Acclimatization is one of the critical stages in micropropagation, since it is during this process that many plants do not survive the environmental conditions. This is due, among other factors, to the 100% RH that exists in the *in vitro* conditions, which damages the closure mechanism of the stomata. When the plants are transferred to the external environment, they face a lower and variable RH (from 30 to 80% depending on the time of the day and season of the year). Under these conditions, the mechanism of the stomata fails causing a fast loss of moisture by transpiration (Pospísilová et al. 1999).

The mass movement of moisture in the substrate-plant-air system takes place as a response to the differences in Ψ soil, Ψ plant, and Ψatmospheric air (-0.5, -1.5, and -95.0 Mpa, respectively) (Salisbury and Ross 1994). Particularly, in the sealed containers that are commonly used for *in vitro* culture, Ψ reaches a value close to zero, and the » in the incubation chamber was close to -103 Mpa which causes a very large difference in this parameter (0 to -103). At these conditions, the plant undergoes a fast loss of water by transpiration. The gradual opening of the cover of the acclimatization system allows the establishment of a progressive equilibrium between the hydric potential in the system and that of the incubation chamber. This may help to regulate moisture loss by transpiration. On the other hand, the nutritive solution contributes to the establishment of equilibrium between the liquid phase and the gaseous phase of the acclimatization system, thus retarding dehydration of the plant tissues.

Regarding growth, it is worth mentioning that during the acclimatization stage (Fig. 5), a very close relationship was observed between the development of the roots and the aerial parts for both length and number. The figure shows a slow growth behavior during the first month, probably due to the beginning of the acclimatization process. However, during the second month, a faster growth was observed as a result of the well-developed roots and aerial parts of the plants.

Another factor that may have contributed to the slow growth of the plants during the first month was the change of the nutritive medium, particularly the carbon source. Sucrose was added to the *in vitro* culture, and therefore the plants were not forced to carry out photosynthesis (mixotrophic culture).

In contrast, the hydroponic system contained no sucrose but only inorganic nutrients. Therefore, the plants were forced to reactivate their photosynthetic system in order to carry out their vital functions of CO_2 fixation and capture of light energy (autotrophic culture). It would be interesting to investigate if *A. elegans* micropropagated plants retain the known chemical and pharmacological properties against scorpion venom.

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