

## MICROPROPAGATION OF *HELIANTHEMUM INAGUAE*, A RARE AND ENDANGERED SPECIES FROM THE CANARY ISLANDS

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**Key words:** Cistaceae, endangered species, *Helianthemum inaguae*, micropropagation, shoot multiplication, rooting

**Abbreviations:** BA–Benzyladenine, Kin–kinetin, IBA–indolebutiric acid, MS–Murashige and Skoog media, NAA–naphthalenacetic acid.

**Palabras clave:** Cistaceae, especies amenazadas, *Helianthemum inaguae*, micropropagación, multiplicación por segmentos nodales, enraizamiento.

**Abreviaciones:** BA–Benciladenina, Kin–kinetina, IBA– Ácido Indolbutírico, MS– medio Murashige y Skoog, NAA– Ácido naftalenacético.

### SUMMARY

A micropropagation protocol has been developed for *Helianthemum inaguae* Marrero Rodr., González Martín & González Artilles, an endangered species from Gran Canaria (Canary Islands, Spain). Shoot tips and nodal segments isolated from *in vitro* germinated seedlings, aged two months, were used as primary explants. Multiple shoot production was obtained using MS medium supplemented with different concentrations of BA, Kin and NAA, showing BA and Kin the best results. Finally, rooting was achieved also in MS medium by adding IBA or without any plant growth regulator. A 72% survival rate was obtained during the acclimatisation process.

### RESUMEN

El presente trabajo da a conocer un protocolo de micropropagación desarrollado para *Helianthemum inaguae* Marrero Rodr., González Martín & González Artilles, una de las especies más amenazadas de la flora endémica de Gran Canaria (Islas Canarias, España). Como explantos iniciales se utilizaron yemas apicales y segmentos nodales aislados de semillas germinadas *in vitro* de unos dos meses de edad. La multiplicación de yemas se realizó en medio MS al que se añadieron diferentes concentraciones de BA, Kin y NAA, observándose los mejores resultados con BA y Kin. Finalmente, el enraizamiento se llevó a cabo en medio MS sin reguladores o con la adición de diferentes concentraciones de IBA. Durante el proceso de aclimatación a las condiciones *ex vitro* se obtuvo un 72 % de supervivencia.

## INTRODUCTION

The genus *Helianthemum* in the Canary Islands is characterised, firstly, for the local distribution of their species and, secondly, for the small number of individuals in their natural populations (SVENETIUS, 1960; BRAMWELL *et al.*, 1977; MARRERO, 1992; MARRERO *et al.*, 1995) which causes a constant loss of alleles that together with the extinction of their habitats could reduce the ability of *Helianthemum* species to increase in number of individuals, to evolve, and ultimately to adapt to future changing circumstances.

Since *Helianthemum inaguae* was described (MARRERO, 1995, *op. cit.*), only one population has been located in La Montaña de los Hornos (Inagua) in the South West of Gran Canaria and, therefore, the *H. inaguae* is considered "in danger of extinction" and included in legal catalogues of threatened plants (CNEA: Catálogo Nacional de Especies Amenazadas (1998) and CEAC: Catálogo de Especies Amenazadas de Canarias (2001)) for its preservation. Moreover, from its last conservation status review (MARRERO *et al.*, in BAÑARES *et al.* 2003), according with the IUCN criteria (IUCN, 2001), *H. inaguae* is also included in the Critically Endangered (CR) list.

In this paper we present a reliable protocol for the micropropagation of *H. inaguae*, in order to be able to increase the number of individuals in the natural populations. Seeds from the collection of Jardín Botánico Canario "Viera y Clavijo", were used as a starting plant material in the micropropagation protocol, a fact that reduces over-collecting the natural population of *H. inaguae*.

## MATERIAL AND METHODS

### 2.1. Plant Material

Seeds of *Helianthemum inaguae* were obtained from the collection of the Jardín Botánico Canario "Viera y Clavijo", which were conserved in the institution since 2002.

### 2.2. Sterilisation Protocol

Seeds were washed in running water and Tween 20 for several minutes and then immersed in ethanol (70%) for 30 seconds. Following this treatment, the seeds were sterilised in two steps with Tween 20 and commercial bleach at two different concentrations ( $2 \text{ g l}^{-1}$  of active chlorine the first one and  $1 \text{ g l}^{-1}$  the second). Finally, all the seeds were washed three times with distilled sterile water before been used in the germination tests.

### 2.3. Germination Tests

Germination was carried out in Petri dishes with 20 ml of MS medium supplemented with  $30 \text{ g l}^{-1}$  of sucrose and  $4.5 \text{ g l}^{-1}$  of agar. Incubation was carried out at 20 °C under photoperiod conditions (16 hours light / 8 hours dark). Seeds were soaked in distilled water during 24 hours or scarified with sulphuric acid (98%) during 2 minutes to, later, combine both treatments in order to test the germination ability of *H. inaguae*. Radicle emergence was the criterion for deciding

whether germination had taken place. The amount of germinated seeds was registered every seven days for a period of 4 weeks.

#### 2.4. Micropropagation, Rooting and Acclimatisation

After 30 days, plants from germinated seeds were placed in tubes with 20 ml of basal MS medium. Shoot tips and nodal segments were propagated on MS medium with several concentrations of BA, Kin and NAA (Table 1). One centimetre or longer healthy shoots were rooted by using MS medium with different concentrations of IBA (1, 2, 3, 4 mg l<sup>-1</sup>) or without plant growth regulator. All plant material were maintained at 20 °C with 16/8 hours of photoperiod and subcultured after 30 ± 5 days.

Only healthy well-developed and rooted plants were used for the acclimatisation in the greenhouse. They were previously washed in sterile water to eliminate the excess of agar and then treated with a fungicide solution (Carbendacima, 1 g l<sup>-1</sup>), to be potted into 7.5 cm diameter pots filled with a sterilised soil: peat: vermiculite mix (2:1:1). Finally, they were introduced in trays and covered with transparent plastic. During acclimatisation, aeration was progressively increased by the partial removal of the plastic cover.

All the experiments were repeated at least three times always obtaining similar results. The values represent the mean plus the standard deviation. During the micropropagation, differences between treatments were analysed comparing the means by using the Mann-Whitney test. Significant results were obtained for  $p < 0.05$ .

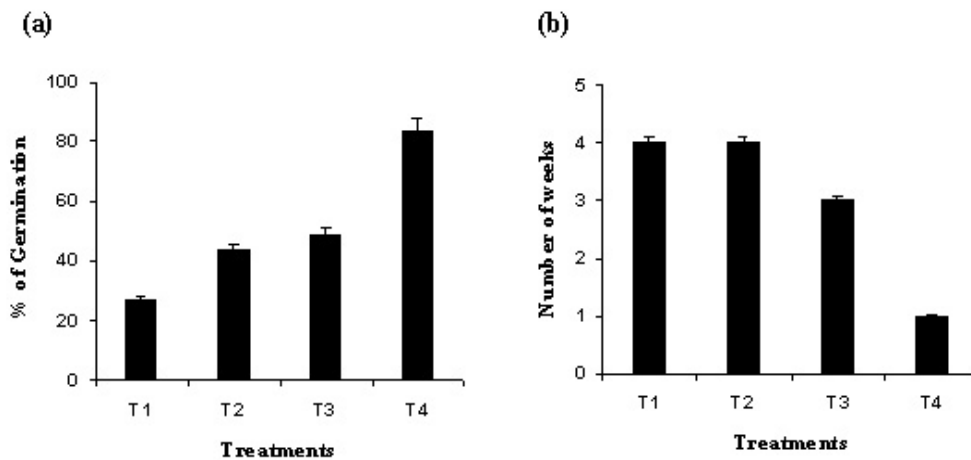
MEDIUM	PLANT GROWTH REGULATOR		
	BA	Kin	NAA
0	-	-	-
1	0	-	-
2	0.5	-	-
3	1	-	-
4	1.5	-	-
5	2	-	-
6	-	0	-
7	-	0.5	-
8	-	1	-
9	-	1.5	-
10	-	2	-
11	1	-	0.1
12	1	-	0.1
13	-	1	0.1
14	-	1	0.1

**Table 1.** Plant growth regulators and concentrations used in mg l<sup>-1</sup> for the micropropagation of *H. inaguae*.

## RESULTS AND DISCUSSION

### 3.1. *In Vitro* Germination of *Helianthemum inaguae* Seeds

The germination of *Helianthemum inaguae* seeds was successfully observed in all the treatments used, although significant differences were obtained. Soaking the seeds in distilled water during 24 hours combined with a scarification in  $H_2SO_4$  (98%) during 2 minutes significantly stimulated seeds radicle emergence, reaching an 80% of germination in one week. All other treatments showed a lower germination rate (below 50%) needing much longer time, two and four weeks (Figure 1).

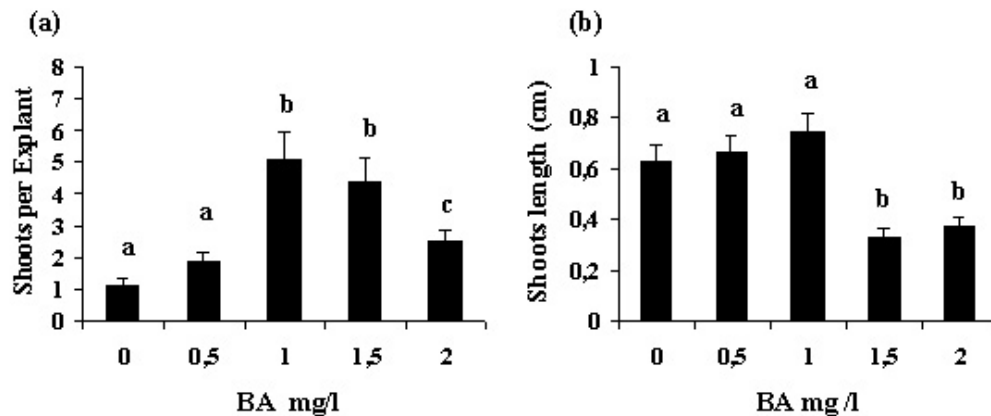


**Figure 1.** Percentage of germinated seeds (a) and the number of weeks needed to reach the percentage of germination (b) for the four different treatments used. T1: no treatment; T2: soaking in distilled water during 24 hours; T3: scarification in  $H_2SO_4$  during 2 minutes; T4: soaking in distilled water during 24 hours and scarification in  $H_2SO_4$  during 2 minutes.

These germination results corroborate previous studies with other species of *Helianthemum* (SANTANA-LÓPEZ *et al.*, 2004; IRIONDO *et al.*, 1995, CORRAL *et al.*, 1990), showing the scarification as a necessary pre-treatment to improve seeds germination. However, in this study the scarification process followed by the sterilisation with commercial bleach inhibited, in 15% of the cases, the development of the germinated seeds, producing necrotic areas in their primary leaves. Nevertheless, despite this problem, combining scarification and soaking during 24 hours was the best treatment to stimulate the germination of *H. inaguae* seeds.

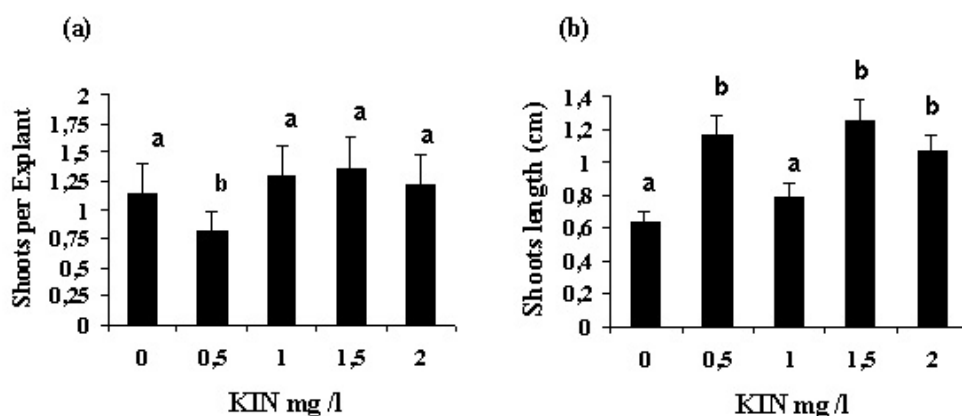
### 3.2. Micropropagation of *Helianthemum inaguae*.

*In vitro* micropropagation of *H. inaguae* was studied by using MS medium with different concentrations of BA, Kin and NAA. Best results were obtained with BA at 1 and 1.5 mg l<sup>-1</sup>, observing higher shoots length only when 1 mg l<sup>-1</sup> was used. Both, lower or higher concentrations of BA, such as 0.5 mg l<sup>-1</sup> or 2 mg l<sup>-1</sup>, showed lower production of new shoots per explant; however, higher shoot length was observed with 0.5 mg l<sup>-1</sup> of BA (Figure 2).



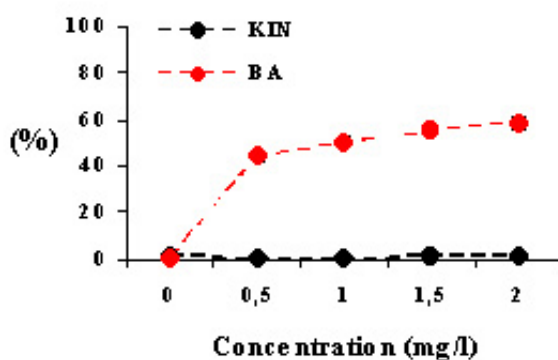
**Figure 2.** Number of shoots per explant (a) and length of the shoots in cm (b) when different concentrations of BA were used. The values represent the mean plus the standard deviation. Significant results were obtained for  $p < 0.05$  and represented with different letters over the bars.

When Kin was present in the medium, almost all concentrations exhibited similar shoots production per explant but significantly lower if compared with BA. However, in contrast with BA, the length of the shoots was well over 1 cm in three of the Kin concentrations tested (Figure 3). These results corroborate previous studies where BA stimulates multiple shoot formation while Kin is more efficient in the elongation process (SANTANA-LÓPEZ *et al.*, 2004; IRIONDO *et al.*, 1995, MORTE and HONRUBIA, 1992). Furthermore, we used low concentrations of Kin (0.2 mg l<sup>-1</sup>) to stimulate shoots elongation after a BA treatment, always obtaining good results (data not shown). Finally, the presence of NAA in the propagation medium, either with BA or Kin, induced callus formation with no morphogenetic structures in a high proportion of the explants.

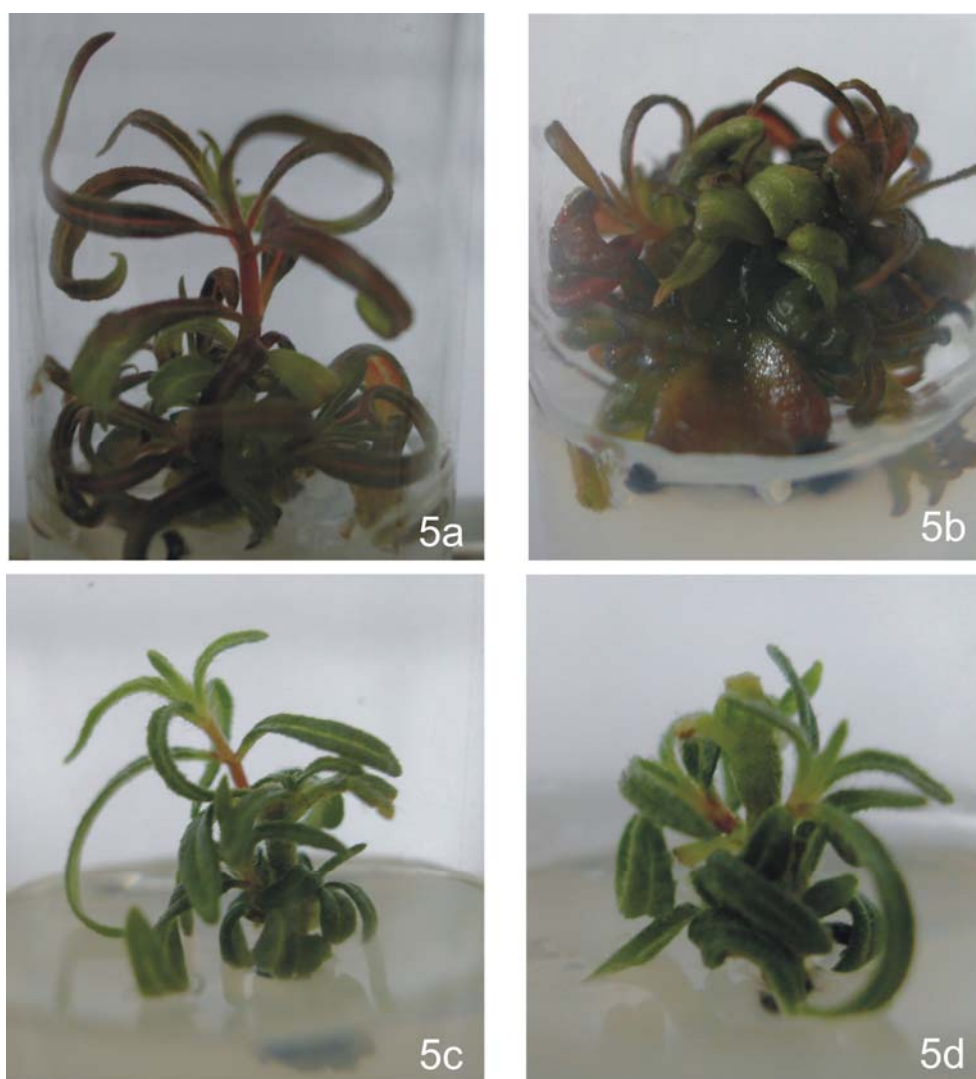


**Figure 3.** Number of shoots per explant (a) and length of the shoots in cm (b) when different concentrations of Kin were used. The values represent the mean plus their standard deviation. Significant results were obtained for  $p < 0.05$  and represented with different letters over the bars.

On the other hand, during the multiplication studies with BA hyperhydric tissues were observed in the explants. In fact, when using  $1 \text{ mg l}^{-1}$  or higher concentrations of BA almost 50% of the plant material showed hyperhydric characteristics. However, this trend was not observed when Kin was used (Figure 4). Further studies are needed in order to be able to minimise this effect.



**Figure 4.** Percentage of hyperhydric tissues when different concentrations of BA and Kin were used.



**Figure 5.** Hyperhydric tissues in *Helianthemum inaguae* plants during the multiplication process when BA and KIN were present in the media (a) BA 0,5 mg L<sup>-1</sup> (b) BA 1 mg L<sup>-1</sup>; (c) Kin 0,5 mg L<sup>-1</sup> (d) Kin 1 mg L<sup>-1</sup>. Black arrows indicate plant tissues with hyperhydric symptoms.

### 3.3. Rooting and Acclimatisation

Rooting *in vitro* *Helianthemum inaguae* plants was successful either with different IBA concentrations or without any plant growth regulator. However, significant differences were observed between IBA treatments concerning the number and the length of developed roots (Figure 6). A higher number of roots were observed when using higher concentrations of IBA, however, a higher length was observed when lower concentrations of IBA were used. In addition, higher concentrations of IBA (3 and 4 mg l<sup>-1</sup>) stimulated the formation of callus, which could have a negative effect in their acclimatisation process due to the formation of non-functional roots (Figure 7). Similar results were obtained for other *Helianthemum* species (SANTANA-LÓPEZ, *et al.*, 2004; IRIONDO *et al.* 1995). For their acclimatisation, well-rooted and healthy plants were transferred to the greenhouse and later to an appropriate area in the Jardín Botánico “Viera y Clavijo”, where conditions were similar to their natural habitat. A 72% survival rate was observed after the acclimatisation process.

## CONCLUSIONS

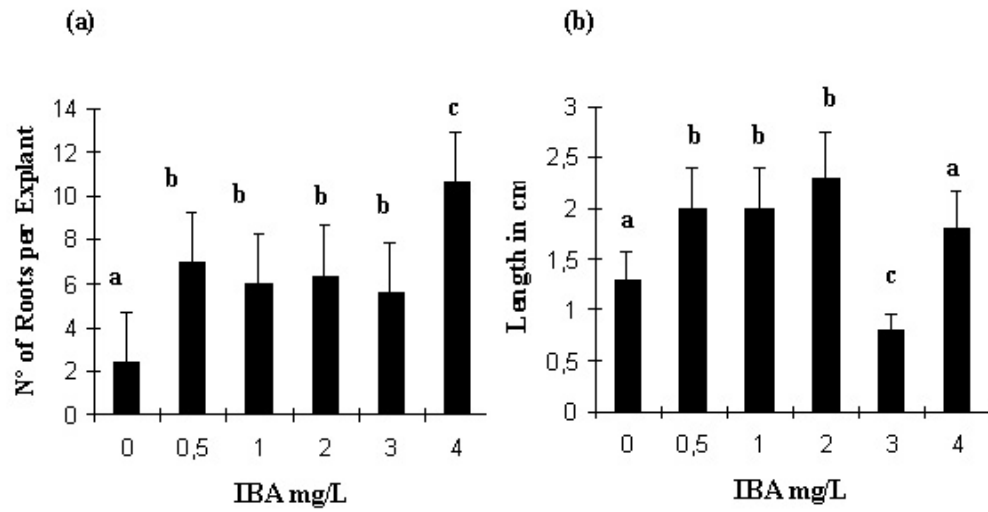
In summary, the results of this study showed that the protocol developed is an efficient method for the micropropagation of *Helianthemum inaguae*. This method allowed us to start with a relative small amount of plant material reaching at the end of the acclimatisation process a 72% plant survival, thus contributing to the species preservation. Despite these positive results some aspects, such as the development of hyperhydric tissues during the multiplication with BA, should be studied in detail in a near future.

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**Figure 6.** Number of roots per explant (a) and length of the roots in cm (b) when different concentrations of IBA were used. The values represent the mean plus the standard deviation. Significant results were obtained for  $p < 0.05$  and represented with different letters over the bars.



**Figure 7.** Callus formation in *H. inaguae* plants with  $3 \text{ mg L}^{-1}$  of IBA (a) and well rooted plants with  $0.5 \text{ g L}^{-1}$  of IBA (b).

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