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CONSERVATION VIA IN VITRO PROPAGATION OF ENDANGERED SPECIES FROM GRAZALEMA NATURAL PARK (EARLY RESULTS)

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Abstract

In vitro propagation under controlled culture conditions of the endangered species from Grazalema Natural Park *Atropa baetica* Willk, *Centranthus nevadensis* Boiss, *Echinospartum algibicum* Talavera & Aparicio, *Lavatera maritima* Gouan and *Phlomis margaritae* Aparicio & Silvestre is being investigated. Initially, germination trials were performed with the species *A. baetica, E. algibicum* and *L. maritima* but not with *C. nevadensis* (due to lack of seeds) and *P. margaritae* (a hybrid which does not set seeds). Germination was satisfactory for all the species in the two culture media used (MS and Troncoso in both cases with 3% sucrose) with a higher response and better seedling growth of *A. baetica* and *E. algibicum*. The establishment of micropropagation protocols for all these species has been initiated employing as source of plant material apical and axillary buds, rhizome (only with *P. margaritae*) petioles and leaves. At this early stage of the investigation, *A. baetica* has provided promising and encouraging results in all the micropropagation stages being MS basal salts with 3% sucrose supplemented with different combinations of BAP and NAA a positive nutrient medium. Moreover, adaptation of regenerated *A. baetica* plantlets to greenhouse conditions has been achieved. The response obtained with the other species has been less promising due to high contamination rates and low response to the culture media used. However, this requires further studies which are currently being undertaken (project no. AMB94-1372 funded by CICYT).

Introduction

Nowadays, anthropogenic disturbance of botanical communities in the Mediterranean region have induced reduction by fragmentation of their natural habitats with the subsequent deterioration of the genetic diversity. Therefore, once the endangered biological entity and the cause of the threat have been determined, the third aspect of the integral strategy for the conservation of threatened species is the design of effective methods to solve the specific problem. *In vitro* cultures of endangered species has been conducted as a means to establish the construction of germplasm banks which provide an adequate number of plants, which after their acclimatization in soil, could them be introduced feasibly into their natural habitat, once the threats have been eliminated or diminished (BRAMWELL & al., 1987; BRAMWELL, 1990).

The aim of the present investigation was to conserve these endangered species via *in vitro* propagation. The early results on the germination trials and *in vitro* propagation of some of the threatened species characteristic of Grazalema Natural Park are presented.

Materials and methods

Plant material

The plant material was collected from Grazalema Natural Park, an area which extends to Cádiz and Málaga provinces of southern Spain. The plant species employed in this investigation were as follows: *A. baetica, C. nevadensis, E. algibicum, L. maritima* and *P. margaritae*. Mature seeds only from the species *A. baetica, E. algibicum* and *L. maritima* were used. For *in vitro* culture apical and axillary buds, petioles and leaves and in the case of *P. margaritae* also rhizome fragments were used.

Surface sterilization

The seeds were first washed with water and detergent, followed by a brief immersion in ethanol and sterilization in a 15% solution of NaOCl and finally washed with sterile distilled water 3 times. Surface sterilization of the plant material (buds, leaves, petioles and rhizome fragments) was conducted similarly as described above but with different exposure to ethanol and NaOCl.

Culture medium

For germination trials the seeds were placed onto two different media namely MS (MURASHIGE & SKOOG, 1962) and Troncoso (TRONCOSO & al., 1989) (see Table 1). The number of seeds cultured on the two media was very limited due to the scarcity of seeds available for this investigation.

The composition of the different culture media used for *in vitro* propagation are also listed in Table 1. Rooting was induced for *A. baetica* cultures employing MS/1 medium, supplemented with a higher concentration of NAA (0.04mg l^{-1}) but without BAP. The pH was adjusted to 5.7 before autoclaving and 0.55% of agar was used. Explants were cultured at 25±2°C and a photoperiod of 16h with a light intensity of 30µmol. m^{-2} sec^{-1} photon flux density.

Medium	Basal salts	Inositol mgl–1	Thiamine mgl–1	NAA mgl–1	BAP mgl–1	IBA mgl–1
MS	MS *	100	1.0	–	–	–
MS/1	MS *	100	1.0	0.025	0.07	–
Troncoso	Troncoso	100	1.0	0.025	0.07	–
MS/2	MS *	100	1.0	0.01	0.50	–
MS/3	MS *	100	1.0	0.02	1.00	–

Table 1. Composition of culture media used.* Murashige & Skoog (1962) without nicotinic acid and pyridoxine HCl.

Results and discussion

The percentage of germination of *A. baetica, E. algibicum* and *L. maritima* seeds is presented in Fig. 1. It can be seen that 100% germination has occurred for *E. algibicum* seeds on both media. Similarly, *A. baetica* seeds showed 75% germination on MS medium and 100% on Troncoso medium (no statistically different t=1.38). Furthermore, the lowest germination rates were observed in *L. maritima* seeds which achieved 50% and 9.1% germination on MS and Troncoso medium respectively (no statistically different t=1.47). These results may indicate the presence of germination inhibitor(s) within the seed covers (SOZZI & CHIESA, 1995) or that the culture media used appear to be unsuitable.

The results obtained for the *in vitro* establishment of *A. baetica*, where apical and axillary buds were employed, are depicted in Fig. 2. It is observed that the three culture media employed provided a positive and satisfactory induction of shoot growth, with the lowest value obtained on MS medium. A slight endogenous bacterial contamination was also noticed but did not appear to affect growth and tended to disappear or decline during culture. After 20d in culture, the new plantlets become branched exhibiting an average shoot length of 5.7cm and a number of 11.9 buds per shoot, thus giving a

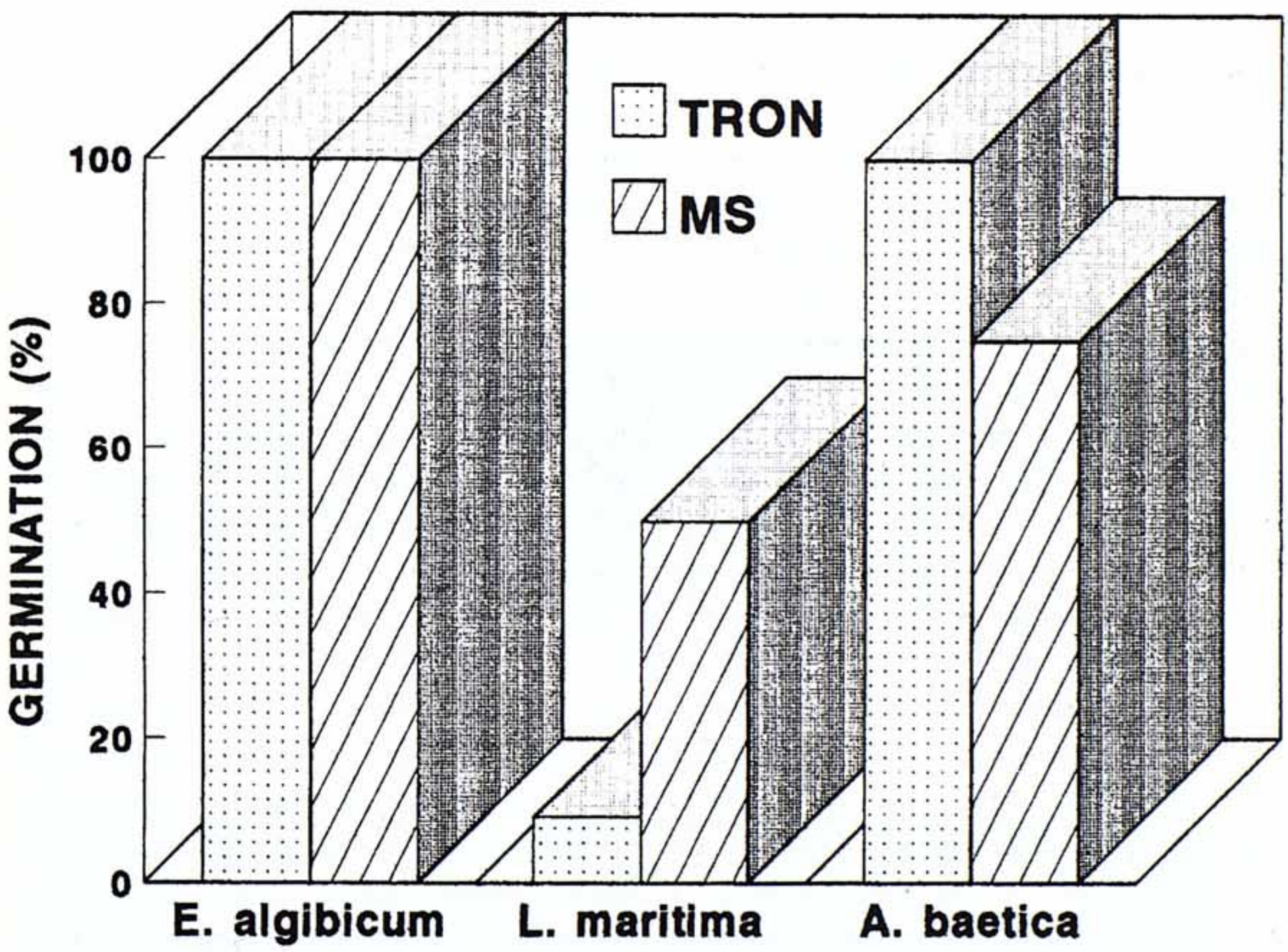


Fig. 1. Influence of nutrient medium on seed germination (after 30d). Seeds number: *Echinospartum algibicum* MS 4, TRON. 7; *Lavatera maritima* MS 2 TRON. 6; *Atropa baetica* MS 2 TRON. 11.

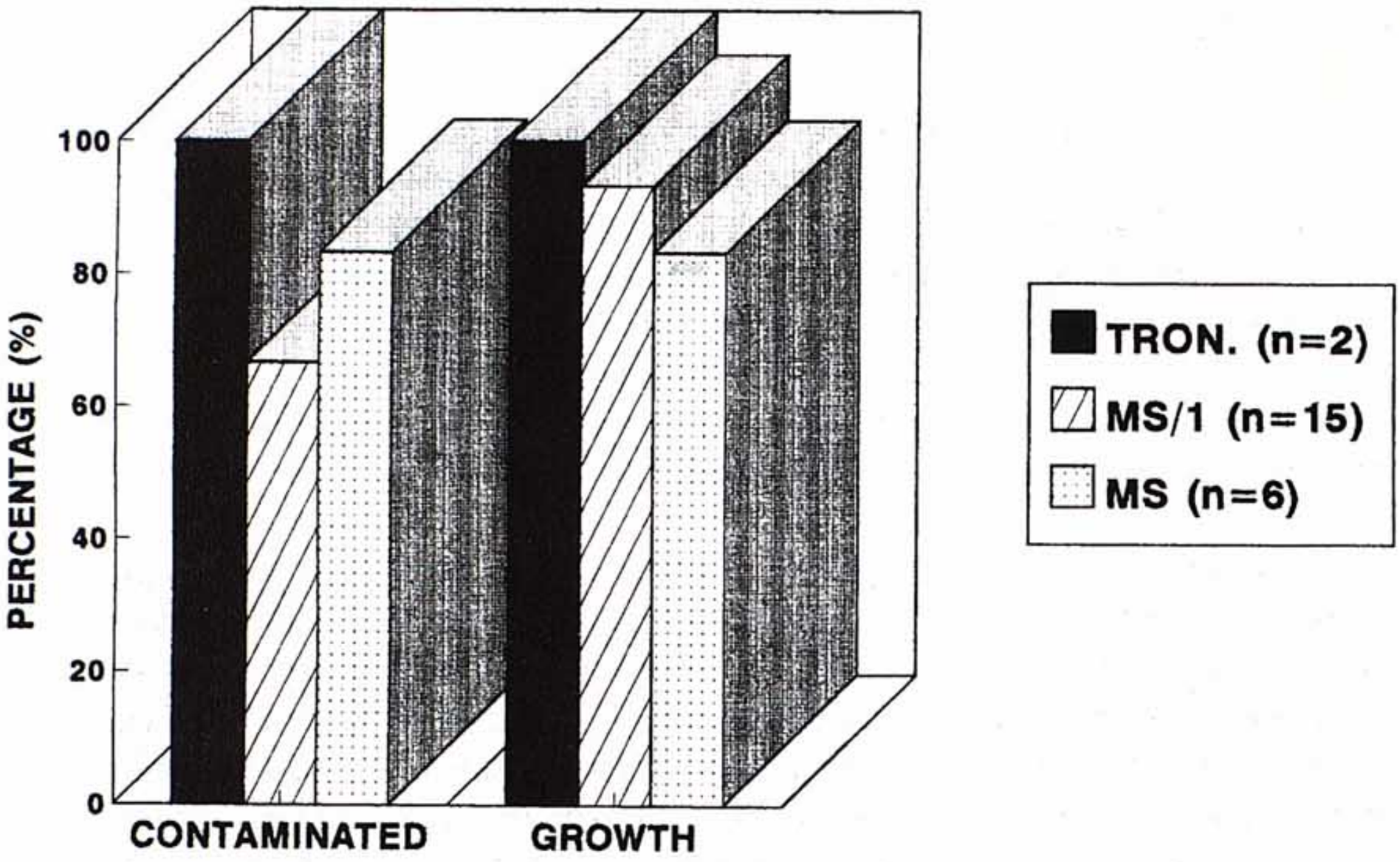
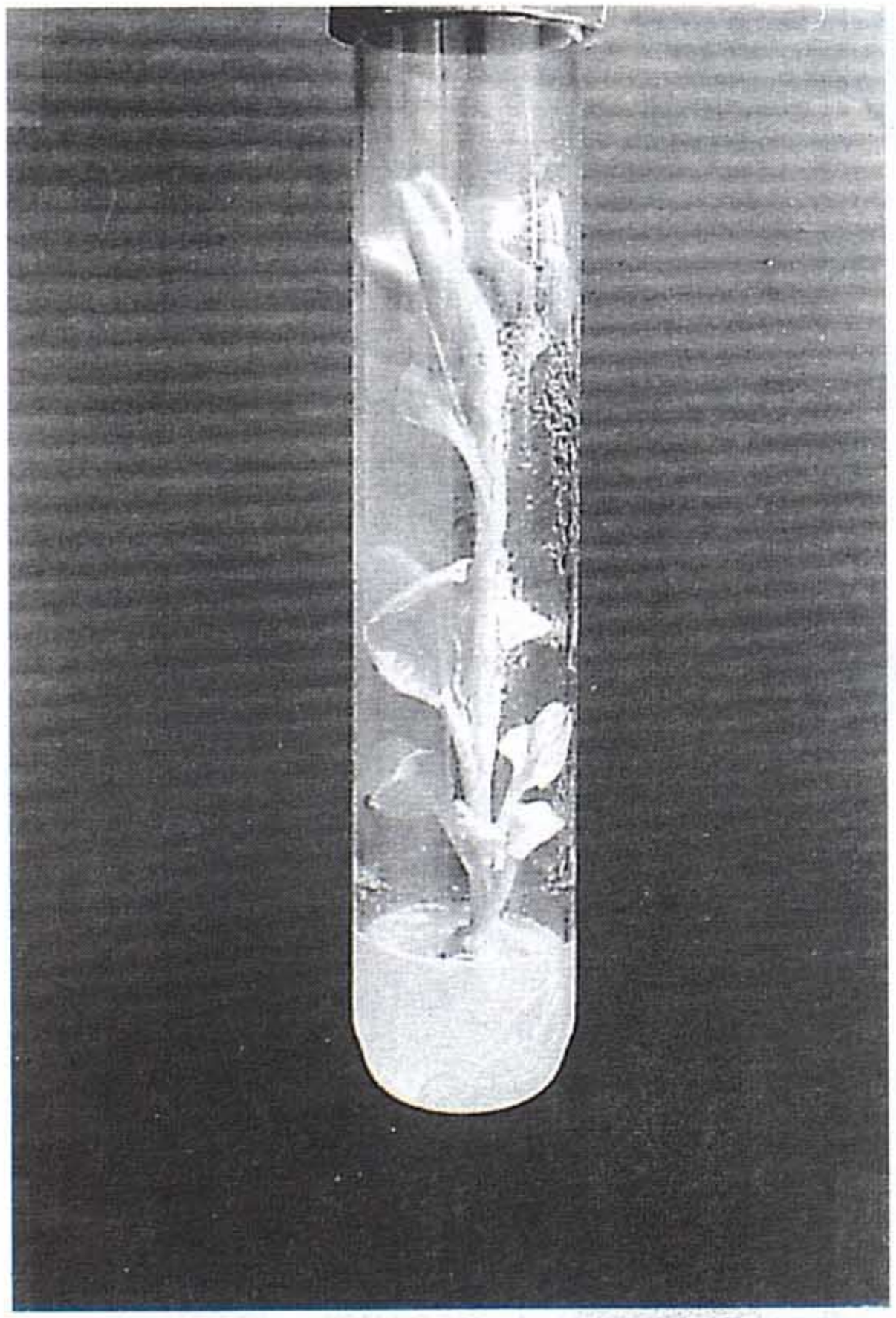


Fig. 2. Results of *in vitro* establishment of *Atropa baetica* Buds (after 20 d).

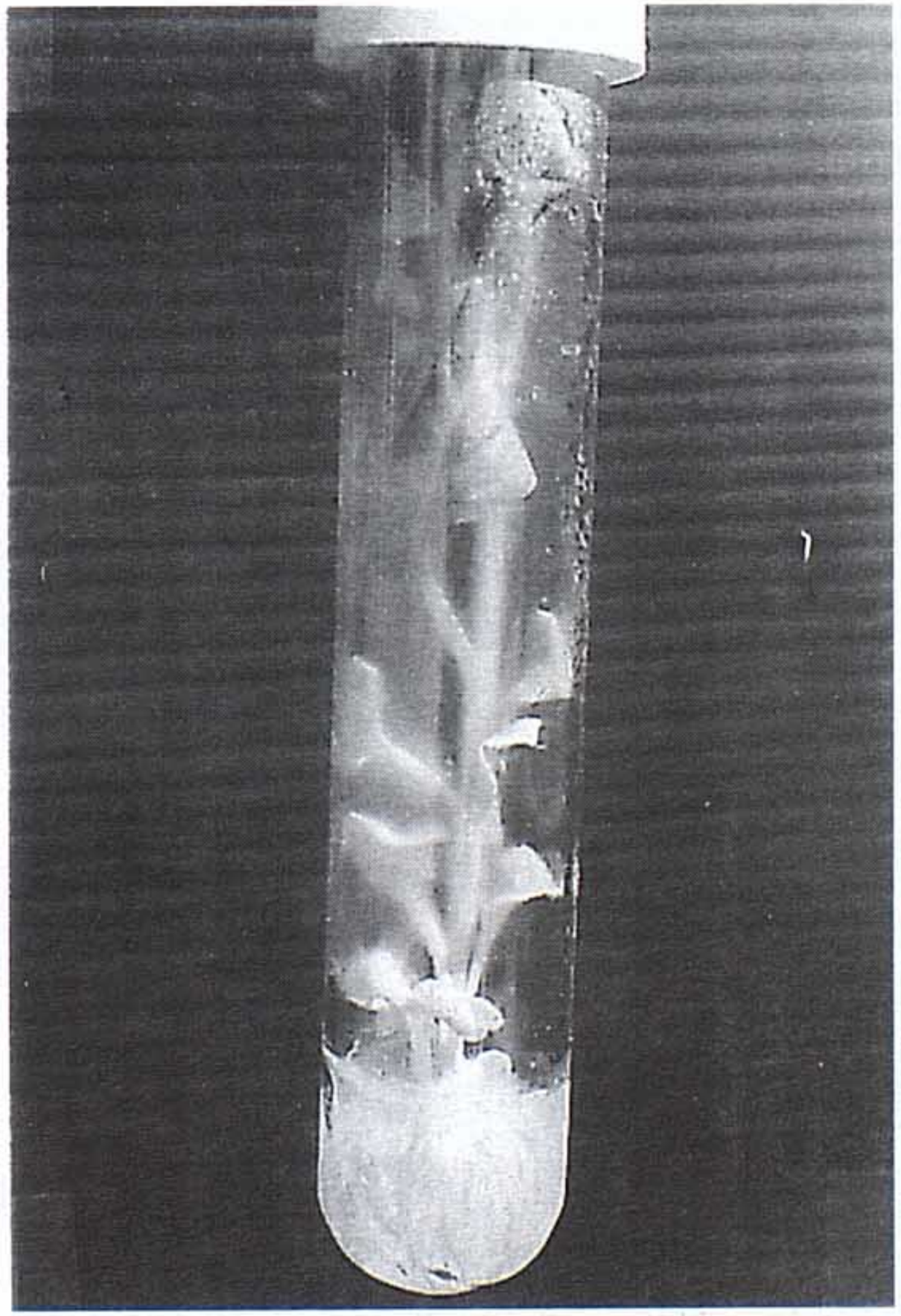
high multiplication rate. It was also noticed that after 40-50d spontaneous rooting of the shoots (*ca.* 4.25%) occurred (see Fig. 3A). However, a much higher root induction (85%) was gained when the culture medium was supplemented with 0.04mg l^{-1} of NAA. This response occurred after 10d and the roots formed were higher in number and more vigorous (see Fig 3B). The acclimatization of rooted plantlets of *A. baetica* in soil was conducted following the method of CANTOS & al. (1993). A percentage of 92.6 of rooted plants survived and adapted readily to *ex vitro* conditions and some of them have already flowered (see Fig. 4).

On the other hand, the attempts conducted to establish *in vitro* cultures of the remaining four species (*C. nevadensis, E. algibicum, L. maritima* and *P. margaritae*) have not been successful. The plant material used was also axillary and apical buds and for *P. margaritae* also petioles, leaves and rhizomes.

The main obstacles encountered were the very high contamination rates, which in the case of *C. nevadensis* was reduced with treatment C ($t=2.3$, $P\leq0.05$) (see Table 2) and for *P. margaritae,* when rhizomes were used, this was dramatically high. This may be due to the presence of many contaminants on this underground organ, as reported by some authors who found similar obstacle when rhizome were used (*e.g.* ZARATE, 1994). The other major barrier was the low or lack of response of explants to the culture media used. In the case of *L. maritima* explants which initially seemed to react favourably, began showing symptoms of vitrification (see Fig. 5), which resulted on the death of explants. According to KEVERS (1984) this may have been due to the low amount of agar or the high nitrogen levels in the nutrient medium. Finally, *E. algibicum* and *P. margaritae* have failed to induce *in vitro* cultures despite an initial response (data not presented).



A



B

Fig. 3A. Spontaneous root induction of 45-50 day old *Atropa baetica* plantlet. Fig. 3B. Root induction of *Atropa baetica* plantlet after teatment with NAA. Observe a more prominent root inductio and higher number of roots.

Fig. 4. *In vitro* propagated *Atropa baetica* adult plant showing flowers.

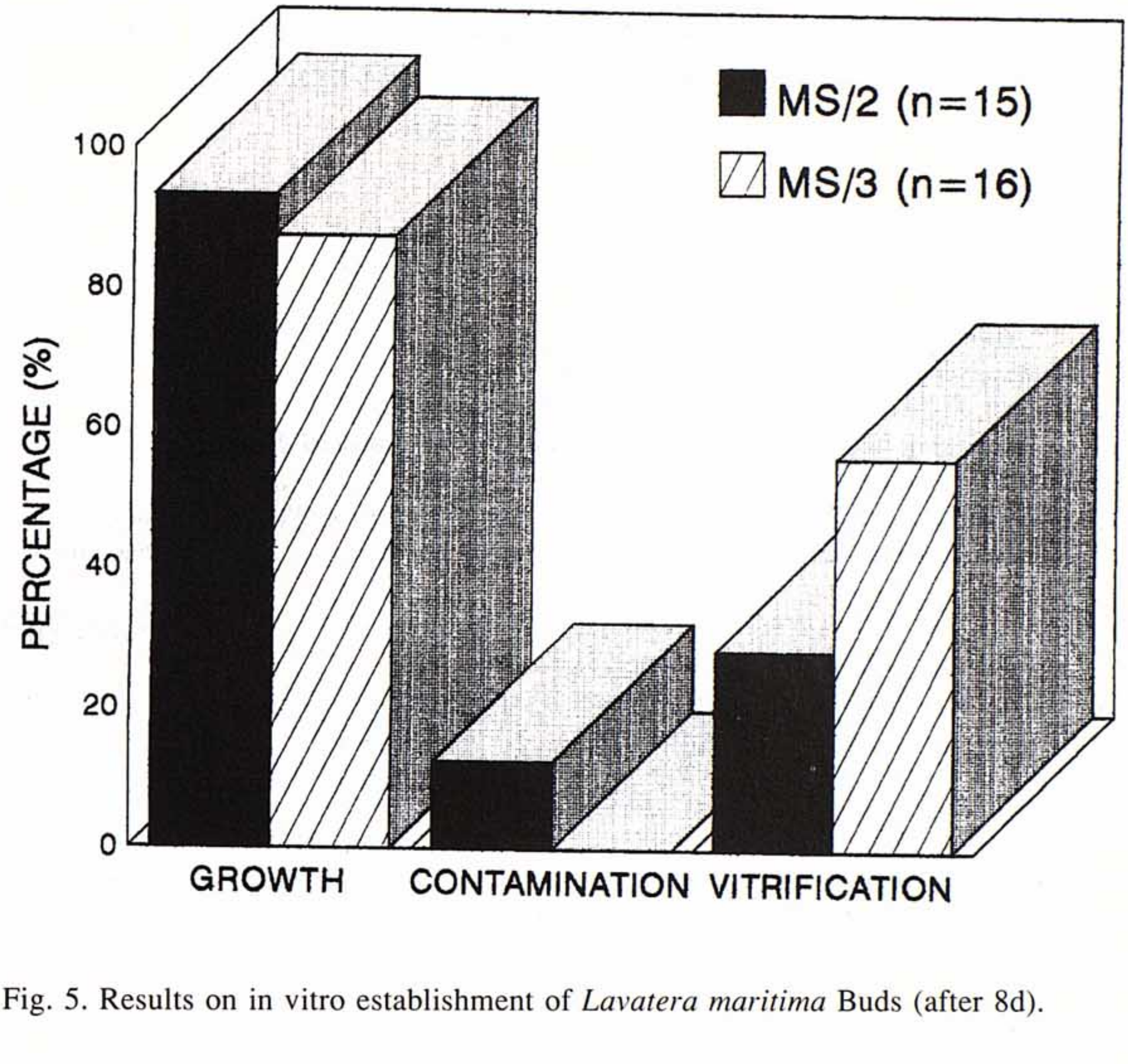


Fig. 5. Results on in vitro establishment of *Lavatera maritima* Buds (after 8d).

Surface steriliz.	Troncoso				MS/1				MS			
	n	C	N	G	n	C	N	G	n	C	N	G
Treatment A	0	–	–	–	9	77.5	100	0	20	15.0	70.0	10.0
Treatment B	3	66.6	33.3	0	8	87.5	62.5	12.5	10	10.0	50.0	10.0
Treatment C	2	0	50.0	0	18	33.3	27.7	16.6	5	0	60.0	20.0
Treatment D	0	–	–	–	10	40.0	70.0	0	12	8.3	58.3	16.6

n = number of explants, C = contamination (%), N = necrosis (%), G = growth (%)
Treatment A EtOH 15s, 15% NaOCl (3.5% active chlorine) 30min
Treatment B EtOH 30s, 15% NaOCl (3.5% active chlorine) 15min
Treatment C EtOH 30s, 15% NaOCl (3.5% active chlorine) 20min
Treatment D EtOH 30s, 15% NaOCl (3.5% active chlorine) 25min

Table 2 Response of in vitro establishment of *C. nevadensis* cultures.

This investigation is still underway and further attempts are being conducted to overcome all these barriers.

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