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## Introduction



The Berlengas archipelago is located in the Atlantic Ocean, on the Portuguese continental shelf, on the western side of the Iberian Peninsula, close to Cape Carvoeiro (Peniche). It distances approximately 5.7 miles from mainland. It is a protected area since 1981.



In 2011 Berlengas became part of the list of patrimony of UNESCO, aiming to conserve representative terrestrial and marine ecosystems of the Portuguese coast.



As a result to insular isolation, and to very difficult climatic and edaphic conditions, terrestrial flora evolved differently from the species that grow in mainland, being smaller and more resistant.



There are three endemic taxa, of great conservation value:

- *Armeria berlangensis*,
- *Herniaria lusitanica* subsp. *berlangiana*,
- *Pulicaria microcephala*.

• *P. microcephala*, as well as *A. berlangensis*, are registered in Annex II of the Habitats Directive, due to its conservation relevance, and are considered vulnerable. *Herniaria lusitanica* subsp. *berlangiana* is registered as endangered.

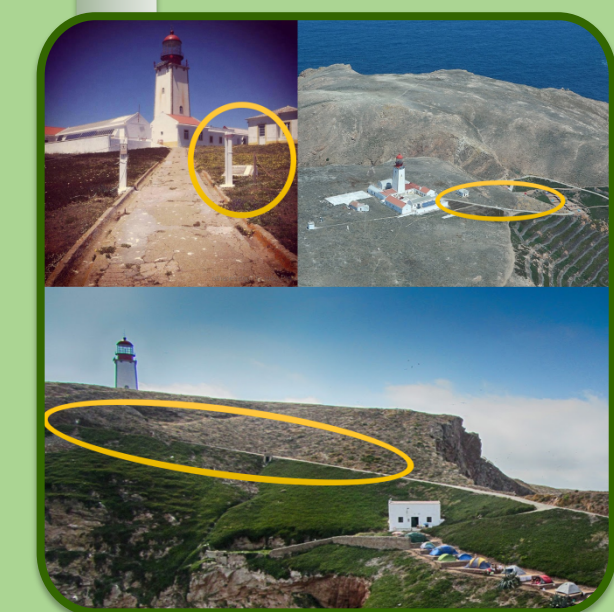


The aim of this project, therefore, was to multiply, *in vitro*, the species *P. microcephala*. Plant shoots were collected in the autumn 2015 from a natural wild population in the Berlengas' Island.

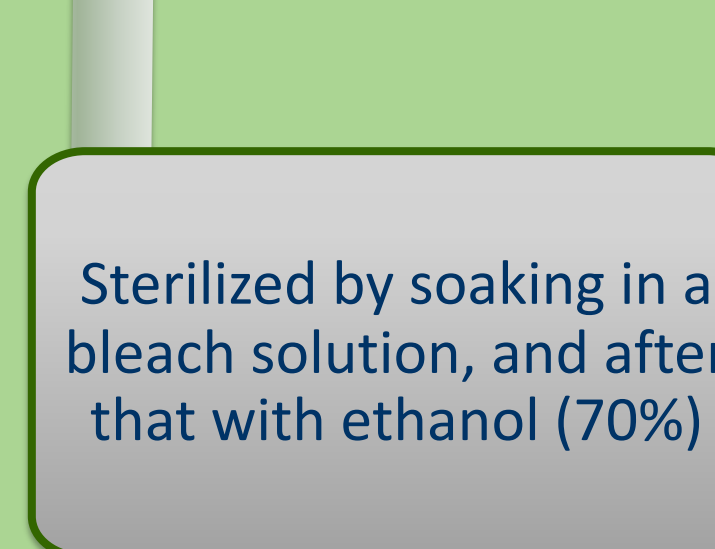
## Materials and methods



Control: Basal medium without the addition of any growth regulators



Multiplication stage: shoots with 0.5-1cm high were placed in MS mineral salts, 3% (w/v) sucrose, 1ml.L<sup>-1</sup> Gamborg vitamin formulation (Sigma), 1.5% (w/v) Phytoagar and different growth regulators

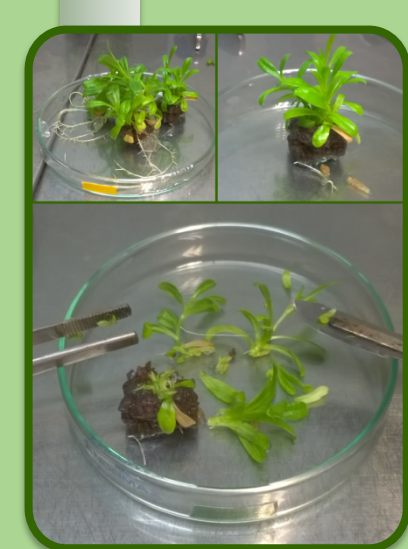


Sterilized by soaking in a bleach solution, and after that with ethanol (70%)

Culture tubes with Murashige and Skoog (MS) mineral formulation (Sigma) supplemented with 0.3% (w/v) sucrose and 0.2% (w/v) gelrite (Sigma, Germany).

Table 1. Concentration of the growth regulators of each medium, with the letter attributed to each combination.

Regulator concentration (mL/L)	NAA			IAA			IBA		
	0,5	1	2	0,5	1	2	0,5	1	2
0,5	A	B	C	J	K	L	S	T	U
Kin 1	D	E	F	M	N	O	V	W	X
2	G	H	I	P	Q	R	Y	Z	ZZ



## Results

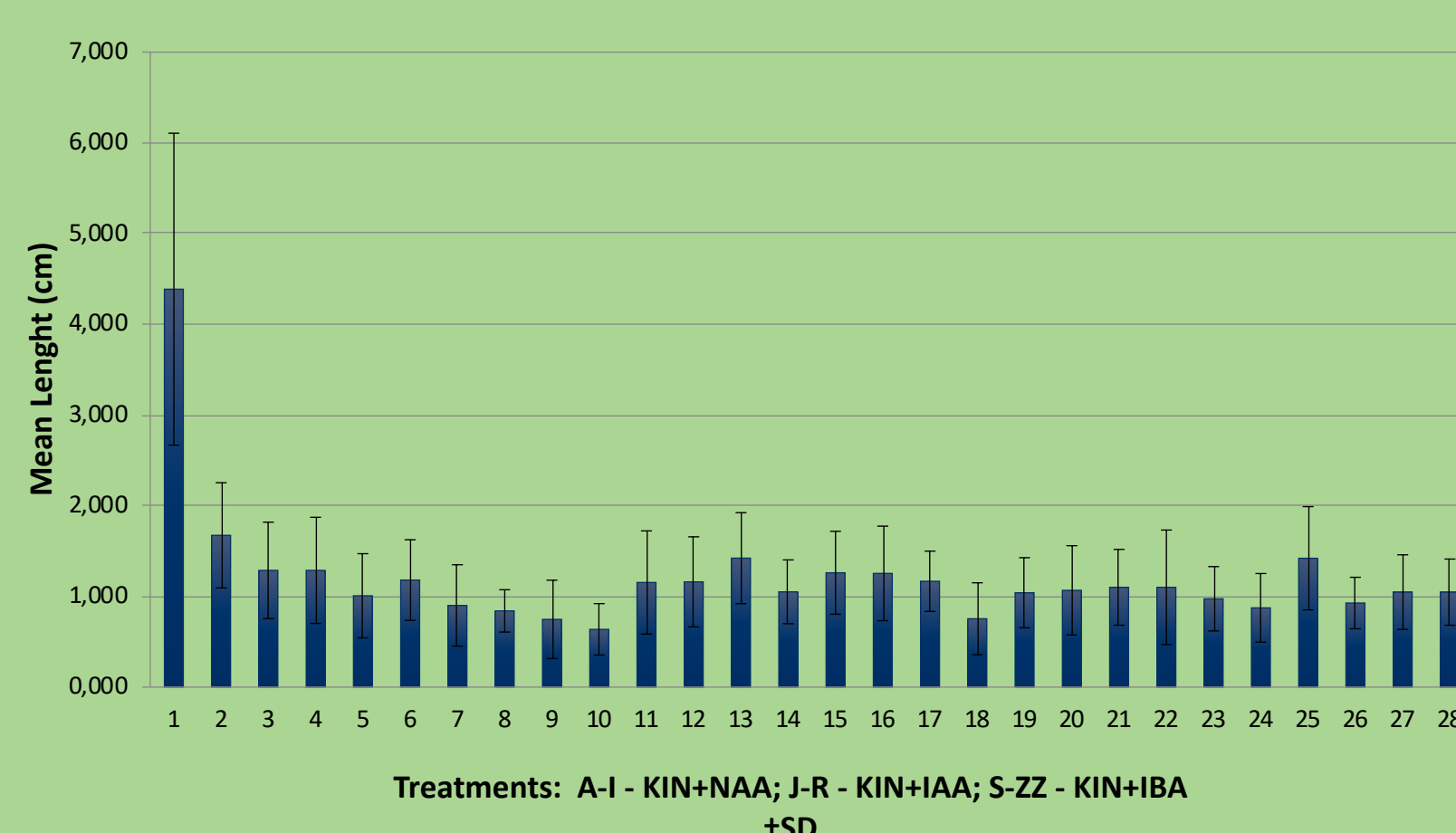
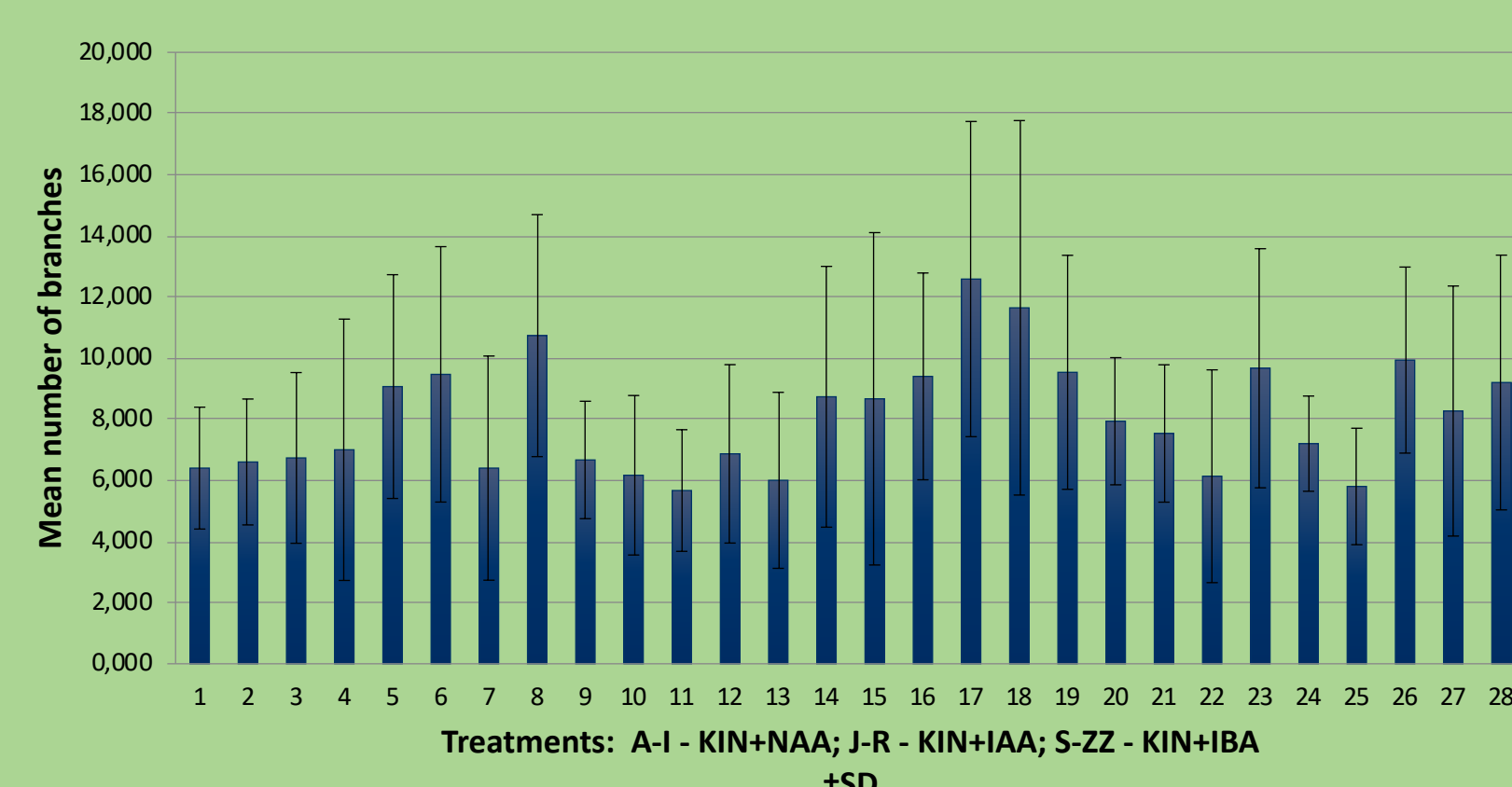


Figure 1. Preliminary results of *in vitro* shoot multiplication rates from *P. microcephala*: length of the highest shoot in response to various media. The shoots were placed in medium with Kin in association with NAA (A-I), IAA (J-R) or IBA (S-ZZ). Letter code according to the table 1. Data represents mean ± Standard Deviation of 15 replicates/treatment and were recorded after 4 weeks of culture.

Figure 2. Preliminary results of *in vitro* shoot multiplication from *P. microcephala*: number of shoots that formed during the assay in response to the presence of various growth regulators in the media. All of them with Kin in association with NAA (A-I), IAA (J-R) or IBA (S-ZZ). Variance also on the concentration according to table 1. Data represents mean ± Standard Deviation of 15 replicates/treatment and was recorded after 8 weeks of culture.



## References

- [1] Gomes, C., Draper, D., Marques, I., Roselló-Graell, A., 2004. Componente Vegetal do Plano de Ordenamento da Reserva Natural das Berlengas. Jardim Botânico.
- [2] Fay, M., 1992. Conservation of rare and endangered plants using *in vitro* methods. *In Vitro Cell Dev Biol*, 29P, 1-4.
- [3] Iriondo, J., Pérez, C., 1990. Application of *in vitro* culture techniques to the conservation of Iberian endemic, endangered plant species. *Botanic Gardens Micropropagation News*, 1, 4-6.
- [4] Queiroga H., Leão F., Coutinho M. (coord), 2010. Nomination of the Berlengas Islands as a UNESCO Biosphere Reserve. Relatório para a Câmara Municipal de Peniche (Portugal).

## Results



Figure 3. a) Expected look after four weeks in *in vitro* culture; b) Expected look after two months in *in vitro* culture; c) Typical result after one month in treatments with hormone regulators; d) Typical result after two months in treatments with hormone regulators; e) Examples of malformations, vitrified plants (1) and plants in rosettes (2); f) *Callus* formation; g) *In vitro* flowering.

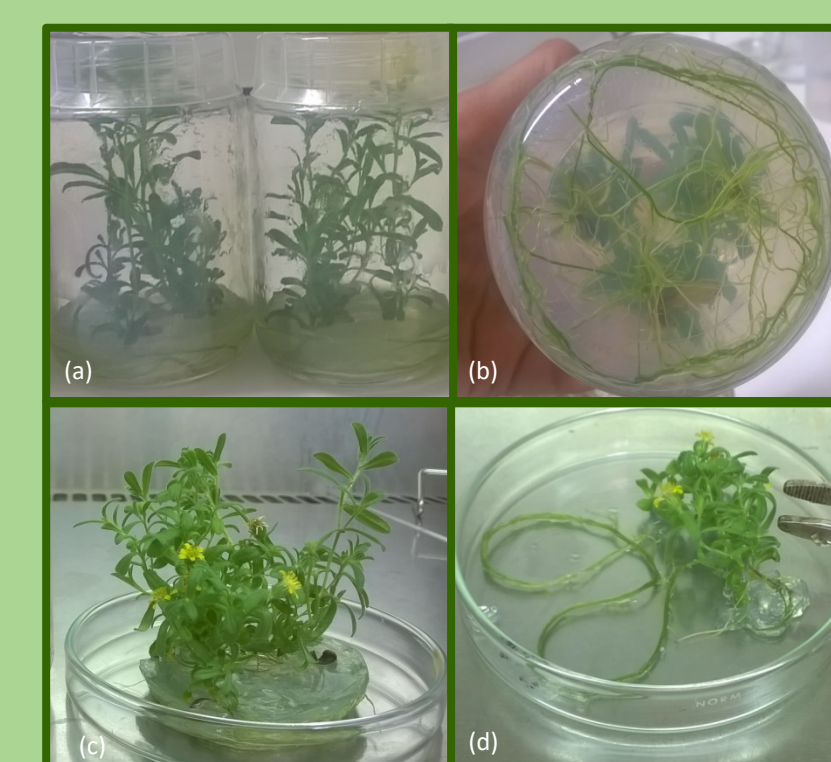


Figure 4. Examples of the look of the shoots under control treatment after a month (a) or two (c) in *in vitro* culture; qualitative and comparative analysis of the plants' roots subjected to this treatment for one (b) or two (d) months respectively showed that plants almost reached the size of the culture flask in both times (a and c) but the roots did not showed differences between b) and d).

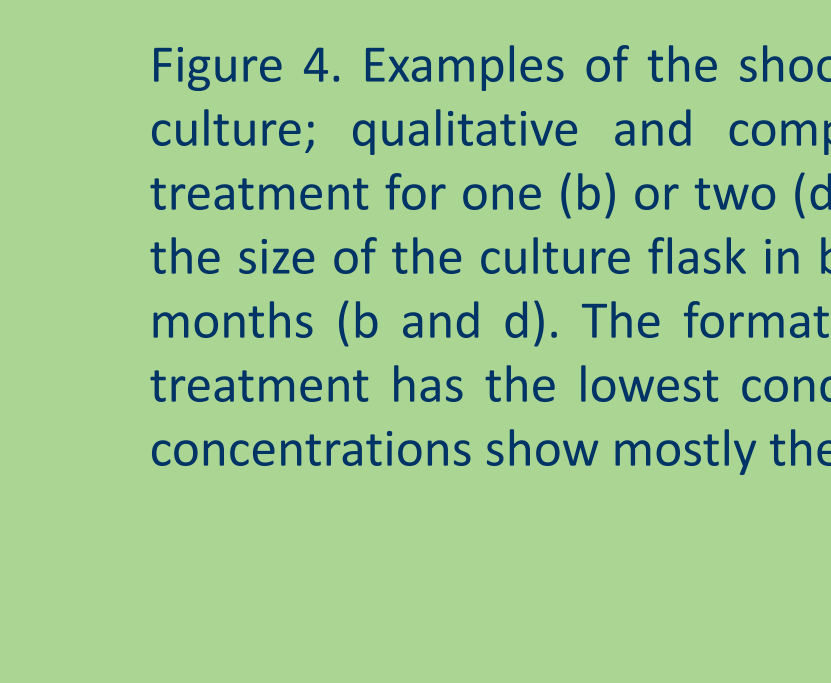


Figure 4. Examples of the shoots under treatment A after a month (a) or two (c) in *in vitro* culture; qualitative and comparative analysis of the plants' roots subjected to this treatment for one (b) or two (d) months respectively showed that plants are almost half of the size of the culture flask in both times (a and c) and the roots have elongated after two months (b and d). The formation of *calli* is evident in this photographs (b and d); This treatment has the lowest concentration of Kin and NAA growth hormones; The others concentrations show mostly the same results.

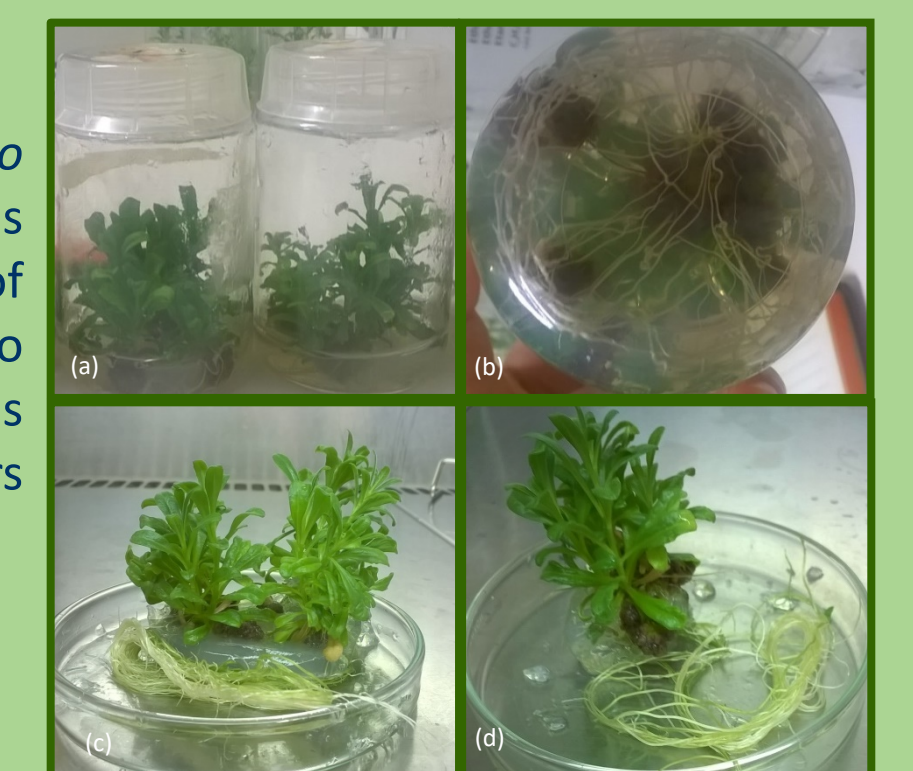


Figure 5. Examples of the behavior of the shoots under treatment J after a month (a) or two (c) in *in vitro* culture; qualitative and comparative analysis of the plants' roots subjected to this treatment for one (b) or two (d) months respectively, showed that: the plants are almost half to a third of the size of the culture flask in both times (a and c) and the roots have elongated after two months (b and d). The formation of *calli* is evident in these photographs (b and d); this treatment is the one with the lowest concentration of Kin and IAA growth hormones; the others concentrations show almost the same results, for the roots; IAA causes the plant to present a lower number of roots, comparing to the control, A or S treatments.

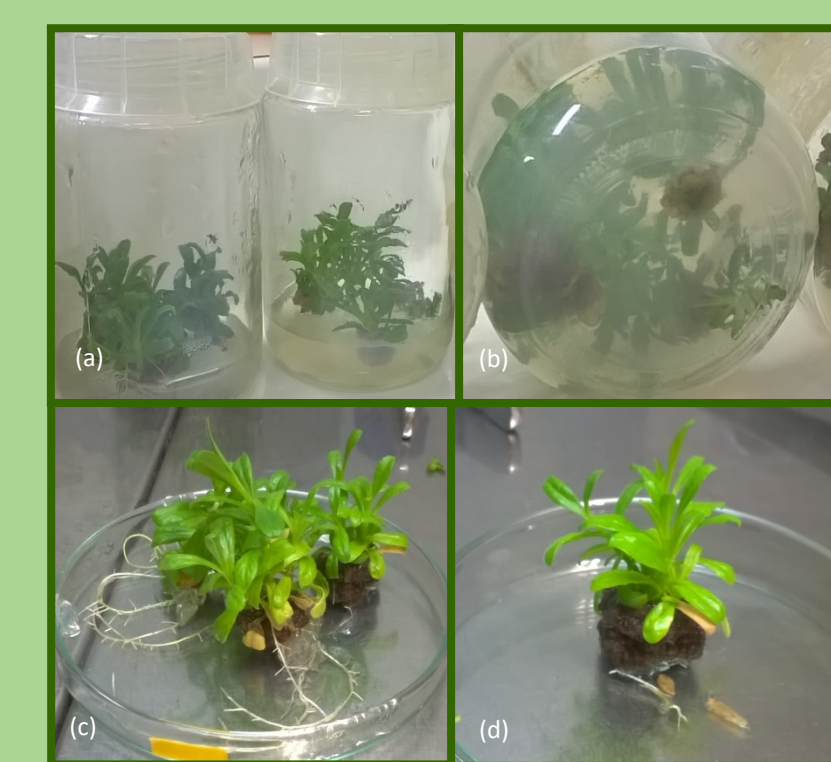
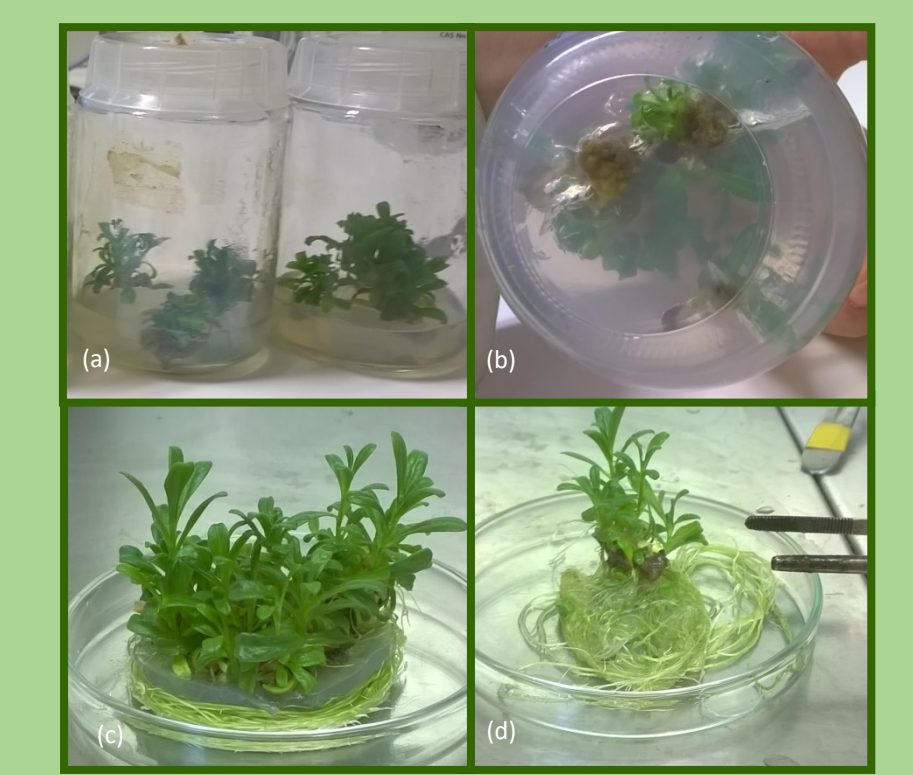


Figure 6. Examples of the behavior of the shoots under treatment S after a month (a) or two (c) in *in vitro* culture; qualitative and comparative analysis of the plants' roots subjected to this treatment for one (b) or two (d) months respectively, showed that the plants are almost half to a third of the size of the culture flask in both times (a and c) and the roots have elongated after two months (b and d). The formation of *calli* is also evident in these photographs (b and d); this treatment is the one with the lowest concentration of Kin and IBA growth hormones; IBA causes the plant to present a higher number of root, when compared to the control, A or J treatments.



## Discussions and Conclusions

After two months of growth the plants demonstrate the impact of the presence of growth regulators in the media.

- Even in the presence of low concentrations of NAA and IBA (0.5mL/L, treatments A and S respectively) the number of roots is high.

The control was the treatment that presents better results in terms of absence of vitrification and abnormal aspects.

- *Pulicaria microcephala*, adapted to the Berlengas island' conditions, didn't performed well in *in vitro* conditions.

The response to the growth hormones present in the media, excluding the control situation, aren't relevant.

- The length of the shoots treated with Kin/NAA and KIN/IAA decreased with the increase of the hormones concentration. The higher the concentration of these hormones the higher the multiplication rates.
- In the treatment with IBA, the impact of this hormone is more evident, as the number of shoots is higher.

It is possible to multiply *in vitro* *P. microcephala* plantlets aiming to increase the number of specimens on the Berlengas island.

- More tests have to be performed in order to assess the success of the acclimatization and rooting stages.

## Acknowledgements

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