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Proteas micropropagation - A review

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Plant tissue culture is one of the key tools of plant biotechnology

- It is define as a collection of techniques used to maintain or grow plant protoplasts, cells, tissues, organs or whole plants under sterile conditions on a nutrient culture medium of known composition and under controlled environmental conditions.

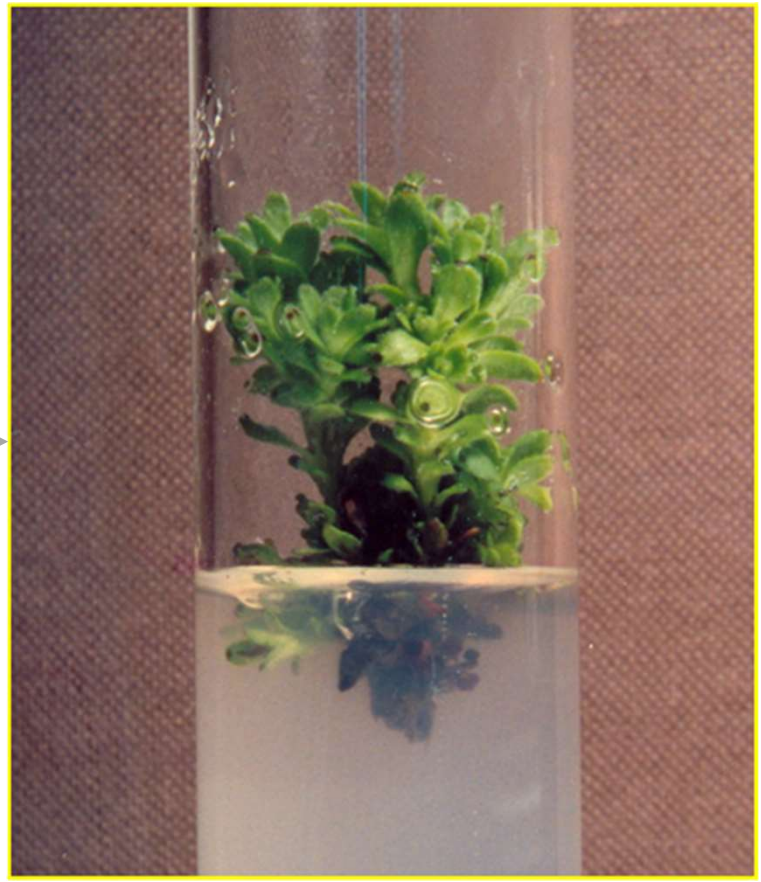


Photo: *Leucadendron* 'Safari Sunset' microshoots growing in a multiplication medium.

Micropropagation

- An important plant tissue culture technique is the vegetative in vitro propagation or **micropropagation**.

- It can be employed for large-scale propagation of disease free clones and gene pool conservation.

- In vitro Rooting Stage of *Leucospermum cordifolium* 'Flame Spike' microshoots
- Photos taken by Suárez, E.

- Generally, micropropagation protocols are divided into four stages that represent points at which the cultural environment needs to be changed:

STAGE I: Establishment of an aseptic culture
STAGE II: Shoot multiplication
STAGE III: Rooting of microshoots
STAGE IV: Acclimatization

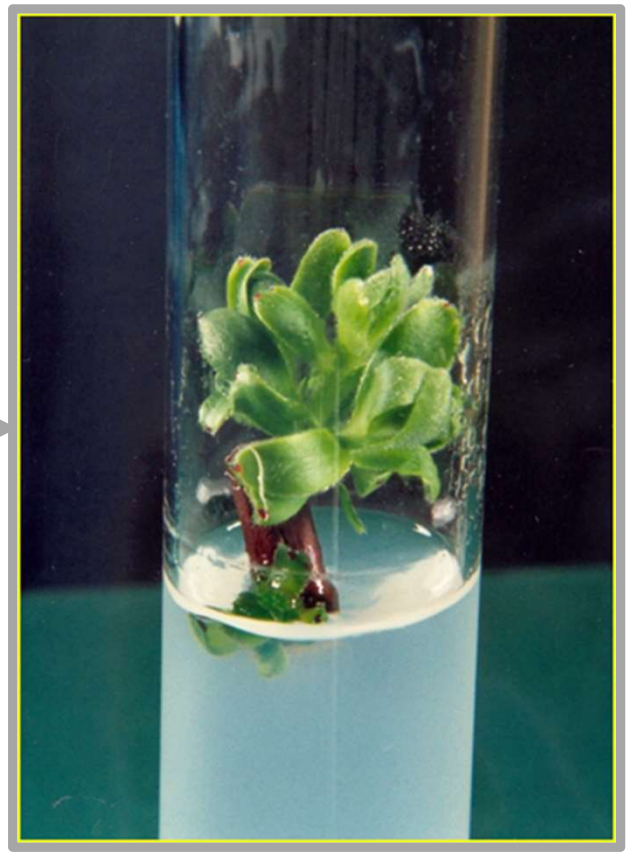
In addition,

A **STAGE 0**, defined as the process of mother plant selection and pre-treatments, is particularly important for micropropagation of proteas.

Three main methods of micropropagation can be distinguished:

1. Propagation by axillary or terminal buds

Axillary bud from a nodal explant of *Leucadendron* 'Safari Sunset'



Three main methods of micropropagation can be distinguished:

1. Propagation by axillary or terminal buds
2. Organogenesis

Clusters of adventitious shoot buds on cotyledons of *Protea cynaroides.*
Selected photo from the PhD thesis of **How-Chiun Wu** (2006) made in the Department of Plant Production and Soil Science, Faculty of Natural and Agricultural Sciences. University of Pretoria.

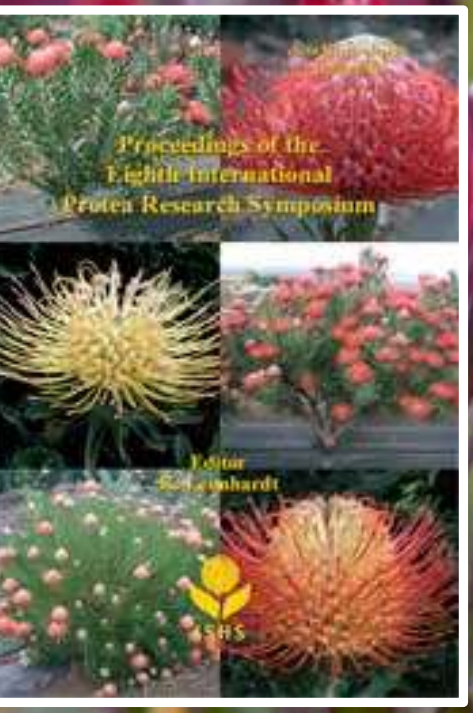
Three main methods of micropropagation can be distinguished:

1. Propagation by axillary or terminal buds
2. Organogenesis
3. Somatic embryogenesis

Germinating somatic embryo of *Protea repens* in vitro.
Photo from RUGGE (1995)

MICROPROPAGATION OF PROTEACEAE

The need to establish protocols for the micropropagation of different types of proteas has been supported by IPA (International Protea Association) at the International Protea Conference held in 2006 in San Diego, California.

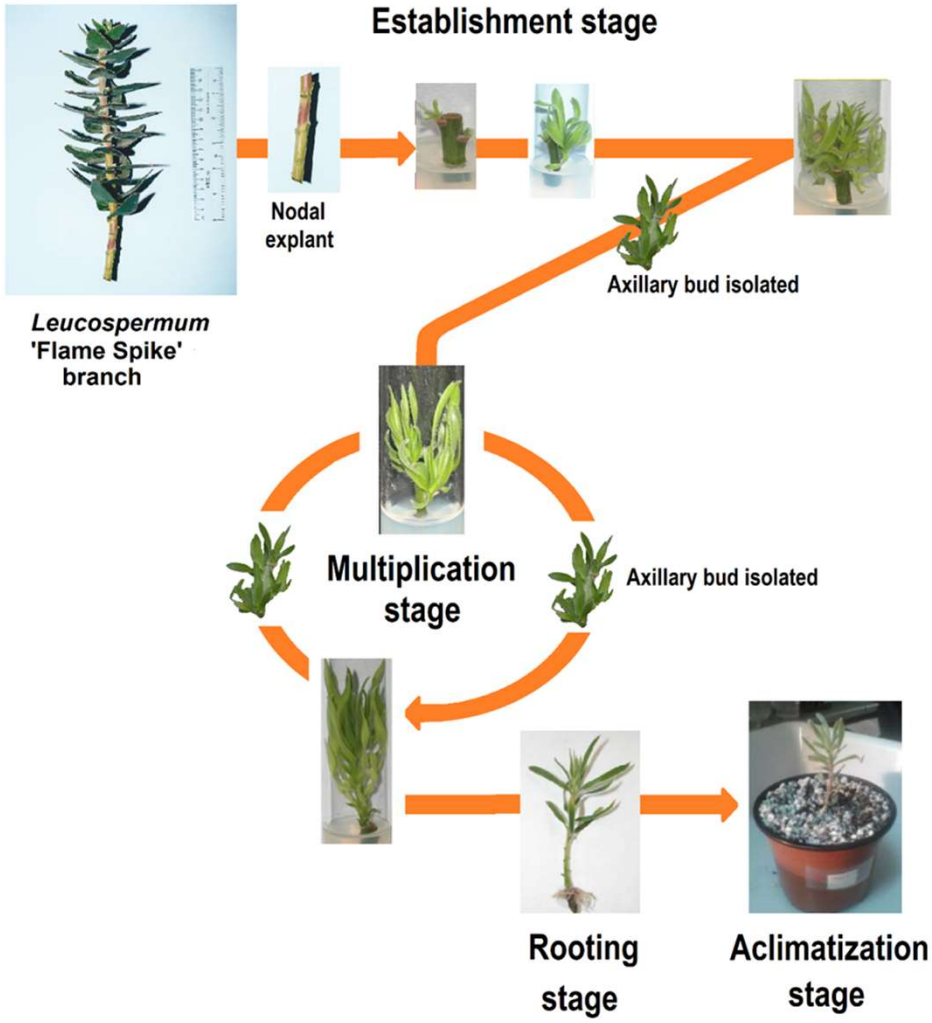


Main objectives of this conference

Due to time constraints, this conference will cover only some aspects of proteas micropropagation through the method of multiplication by axillary buds. For more details, including other methods, see my paper "**Proteas micropropagation - A review**" in Acta Horticulturae.

a. Main research about micropropagation of *Proteaceae* is analysed in this paper, based on the literature obtained from the past four decades.
b. Focused attention is given to axillary or terminal buds proliferation methods.
c. Main problems and new approaches to improve micropropagation protocols and basic and applied research are discussed.

- The most reported technique in *Proteaceae* micropropagation is **axillary or terminal buds proliferation**, in which propagation is based on explants with pre-existing meristems.
- In this method the **genetic stability is usually preserved**.



L. cordifolium 'Flame Spike' micropropagation protocol. (adapted from Suárez et al., 2018, 2019)

- One of the first reports on tissue culture of *Proteaceae* date back to 1970s when Van Staden and Bornman (1976) reported the initiation and growth of *Leucospermum cordifolium* callus.

Van Staden, J. and Bornman, C.H. (1976). Initiation and growth of *Leucospermum cordifolium* callus. J. S. Afr. Bot. 42: 17-23.

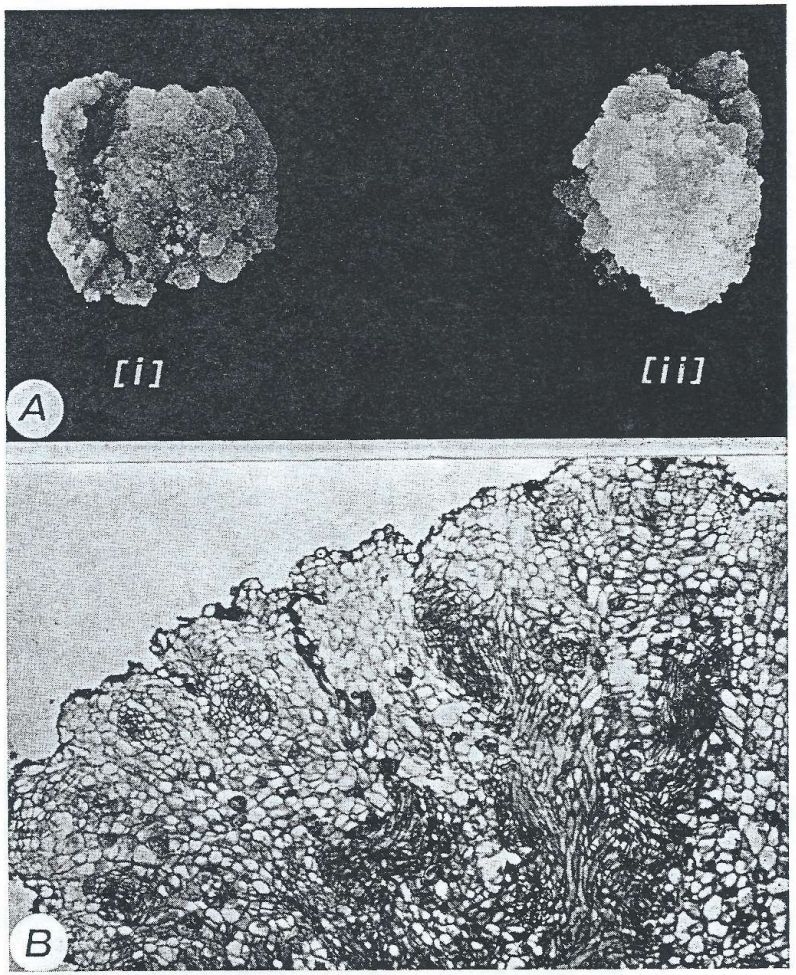


FIG. 1A.
Appearance of *Leucospermum* callus of (i) cotyledonary and (ii) hypocotyledonary origin.
FIG. 1B.
Islets of meristematic cells in callus grown on Miller's (1965) medium in the presence of zeatin.

- Since then, a number of species and cultivars of *Proteaceae* have been successfully culture in vitro, and various authors have reported different studies on proteas micropropagation and other tissue culture techniques.

- The first complete micropropagation protocol was developed for two *Grevillea* hybrids
- Gorst et al. (1978). Tissue culture propagation of two *Grevillea* Hybrids. Comb. Proc. Intl. Plant Propagators Soc. 28:435-446.

Figure 1. An unusual 'Crosbie Morrison' culture showing development of both multiple shoots and an apically dominant shoot, on the same medium. More usually only one type of development occurs.

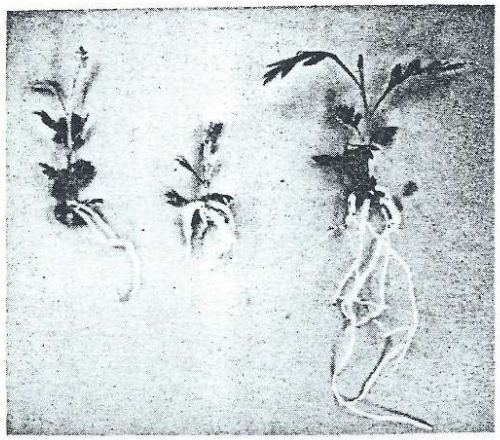


Figure 3. Development of roots in 'Robyn Gordon'. All plants are the same age. The two plants on the left have remained in culture on rooting medium for eight weeks. The plant on the right was transferred to soil after four weeks on rooting medium.

On the right are shown the species and cultivars in which the development of a complete *Proteaceae* micropropagation protocol has been reported (including acclimatization)

- *Alloxylon flammeum*
  - (Donovan et al., 1999)
- Different species and cultivars of *Grevillea*
  - (Bunn and Dixon 1992a; Gorst et al. 1978; Tal et al., 1992b; Watad et al., 1992a; Rajasekaran, 1994; Xiang et al., 2010; Zhou 2014)
- Several *Leucadendron* hybrids
  - (Croxford et al., 2006)
- Different species and cultivars of *Leucospermum*
  - (Kunisaki 1989, 1990; Tal et al., 1992a, b; Suárez et al., 2018, 2019)
- *Protea cynaroides*
  - (Wu and Lin, 2013),
- *Stirlingia latifolia*
  - (Bunn and Dixon, 1992b) and
- *Telopea speciosissima*
  - (Seelye, 1984; Offord et al., 1990, 1992; Offord and Campbell, 1992; Reynoso-Castillo et al., 2001)

MAIN PROBLEMS IN TISSUE CULTURE OF *PROTEACEAE*

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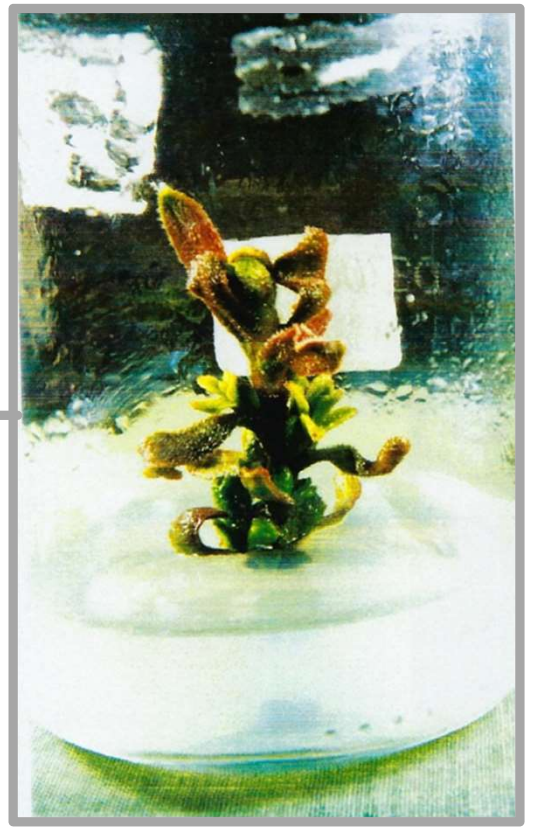
Phenolic oxidation of primary explants is an important factor during the establishment stage (stage I) of several *Proteaceae* species making growth and development too difficult or impossible.



Apical explant of *Leucospermum* 'Sunrise' showing necrosis in the upper leaves

Pérez-Francés, J. F., Raya Ramallo, V. and Rodríguez-Pérez, J.A. (2001a). Micropropagation of *Leucospermum* 'Sunrise' (Proteaceae). Acta Hortic. 545: 161-169.

MAIN PROBLEMS IN TISSUE CULTURE OF *PROTEACEAE*

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A low growth of explants in the stage I and low proliferation rates during multiplication stage (stage II) of some species and cultivars.

Figure 1. Growth and elongation of axillary bud during establishment of *P. cynaroides* nodal shoot segment.

Wu, H.C. and du Toit, E.S. (2012b). In vitro multiplication of *Protea cynaroides* L. microshoots and the effects of high phosphorus concentration on explant growth. Afr. J. Biotechnol. 11(63):12630-12633.

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In vitro rooting (stage III) can be difficult and several species and cultivars have been shown reduced rooting rates. Some species and cultivars can required to include an elongation stage before rooting or during stage I.

Elongation substage prior to rooting of *Leucospermum* 'Tango‘ microshoots
Photo from Suárez, E.

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Acclimatization problems of microcuttings (stage IV) can be extremely important.

Acclimatized plants of *Leucospermum* 'Tango'

Suárez, E., Alfayate, C., Pérez-Francés, J.F. and Rodríguez-Pérez, J.A. (2018Sci. Hortic. 239: 300-307.
Suárez, E., Alfayate, C., Pérez-Francés, J.F. and Rodríguez-Pérez, J.A. (2019). Plant Cell Tissue Organ Cult. 136: 15-27.

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Acclimatization problems of microcuttings (stage IV) can be extremely important.

Poor performance in tissue culture of some rare and threatened species in which young plant material from mature plants are extremely difficult or currently impossible to initiate into culture.

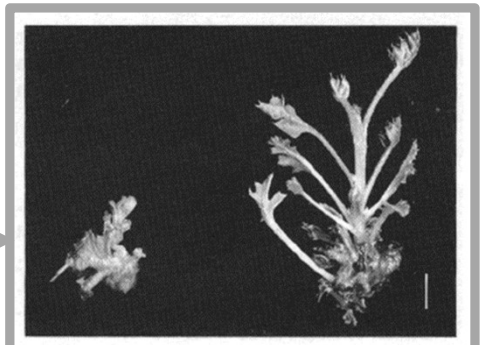


Fig. 2. Adventitious shoot production from excised leaves of in vitro-grown *Grevillea scapigera* 3 weeks (**left**) and 5 weeks (**right**) after initial culture. Bar = 0.3 cm.

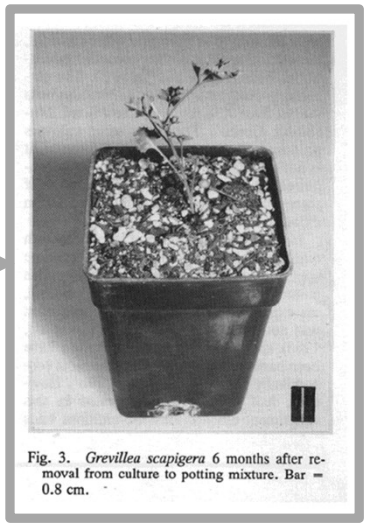
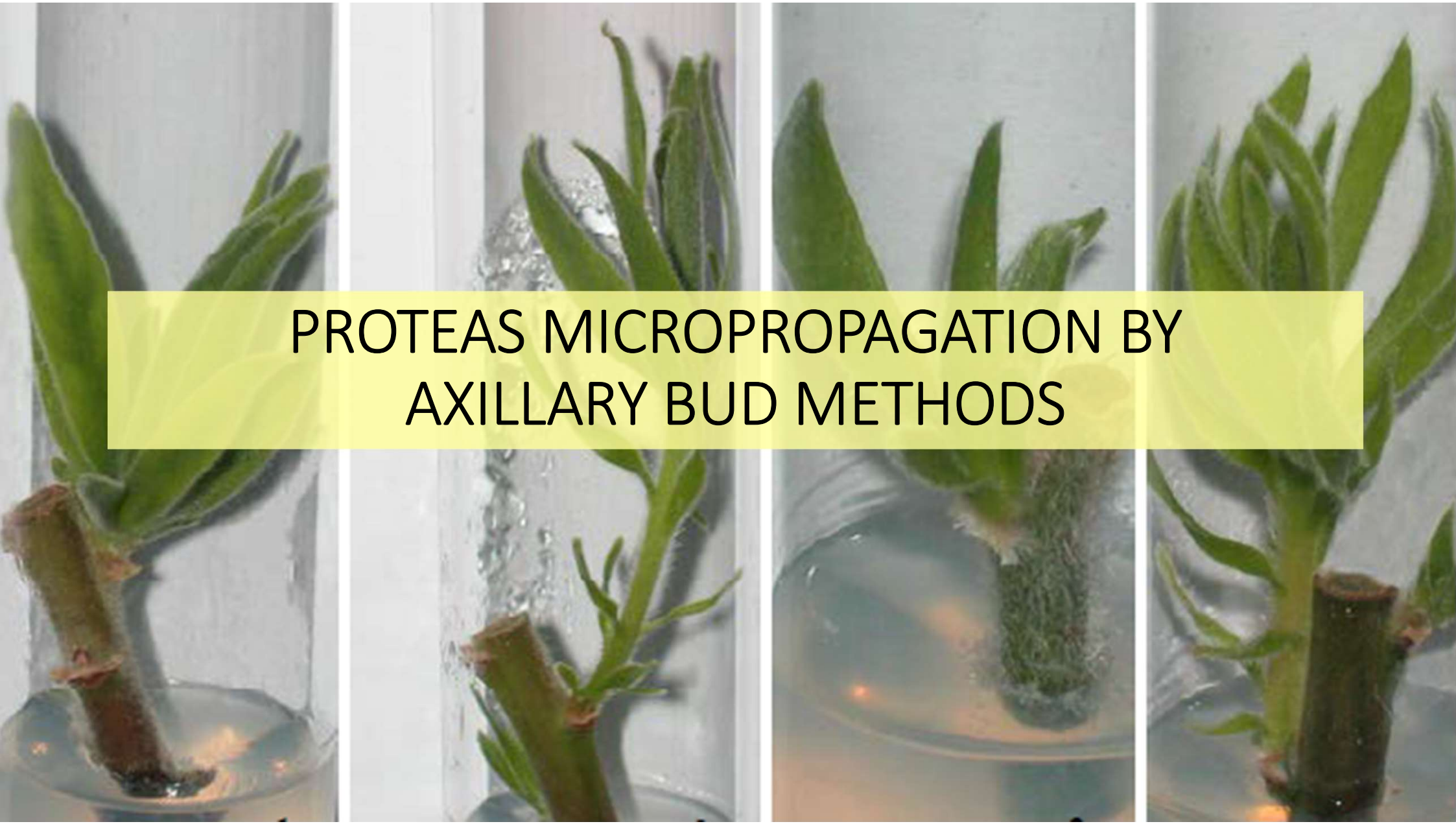


Fig. 3. *Grevillea scapigera* 6 months after removal from culture to potting mixture. Bar = 0.8 cm.

Bunn, E. and Dixon, K.W. (1992a). In vitro propagation of a rare and endangered *Grevillea scapigera*. HortScience. 27(3):261-262.



PROTEAS MICROPROPAGATION BY AXILLARY BUD METHODS

Stage 0: Mother plant selection and pre-treatments

- Pre-treatments of mother plants have proved to be beneficial in reducing the contamination of in vitro cultures and promote physiological changes that increased the success of micropropagation.

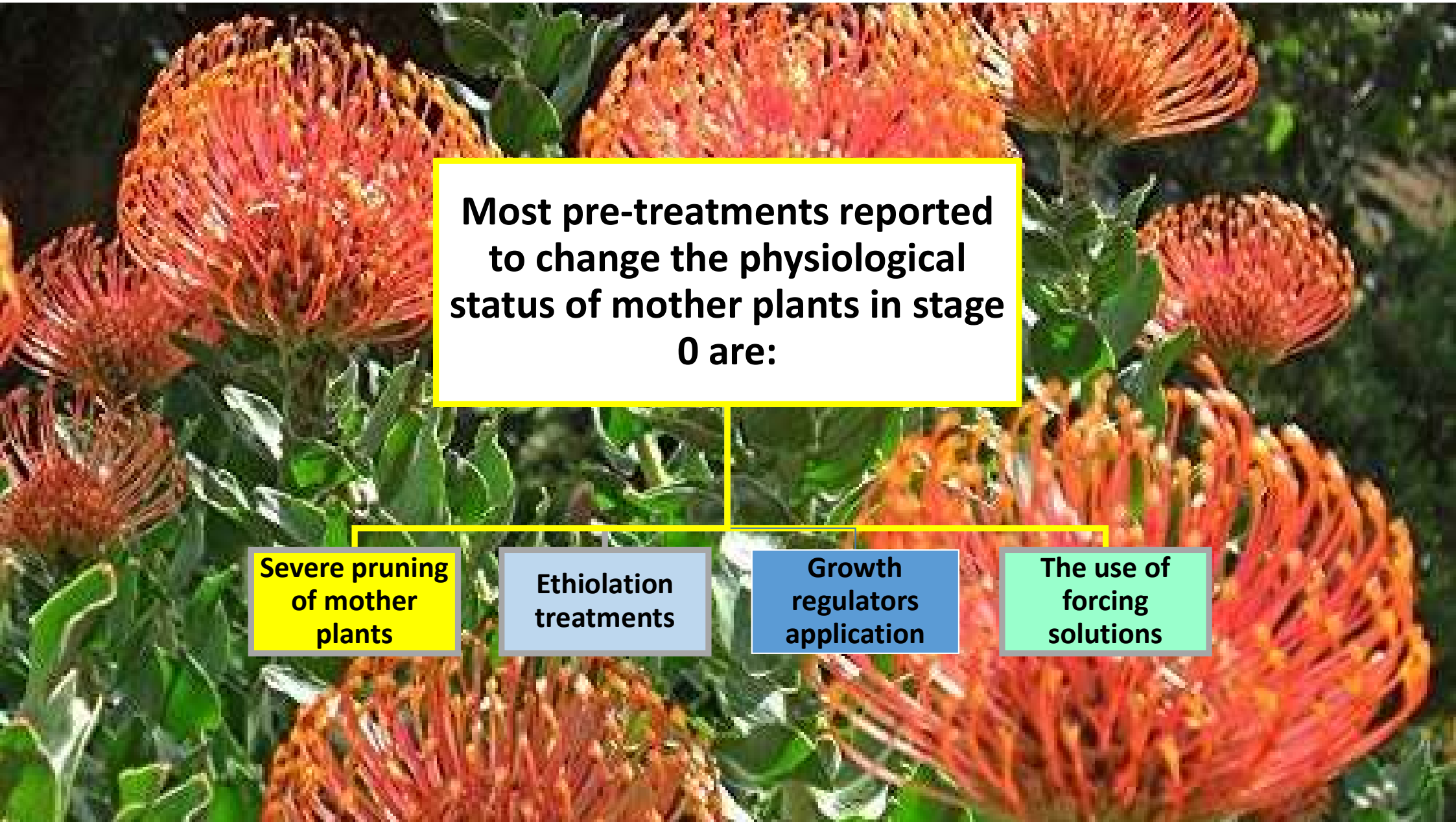
Stage 0: Mother plant selection and pre-treatments

- In many *Proteaceae* species and cultivars this is a very important factor to take in mind.

- Two main objetives has to be considered:
  - Growth of mother plants under more hygienic conditions
  - Modification of the physiological status of mother plants to make explants more suitable as starting material

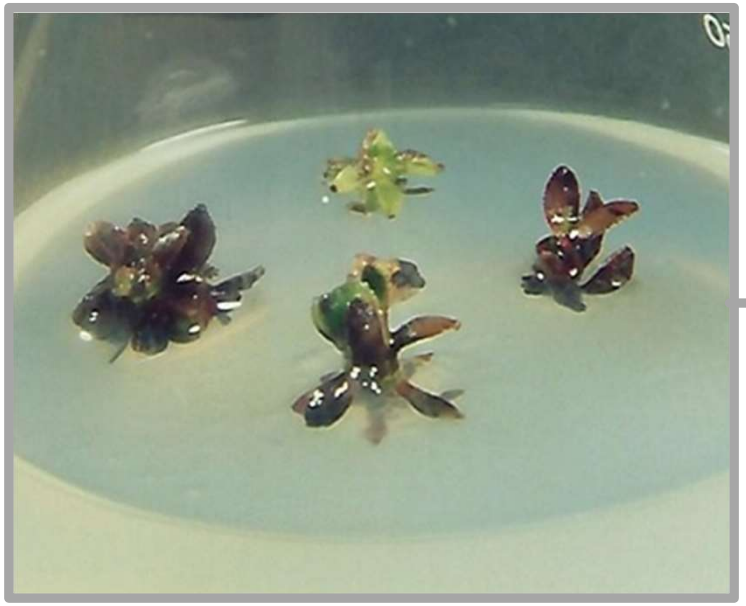
- For critically rare and endangered species, often the only solution is to collect material from the field.
- Low quality of such material and high contamination rates can be important problems for the success of micropropagation of these plants (see e.g., Bunn et al, 2011).

https://florabase.dpaw.wa.gov.au/browse/profile/2091


Most pre-treatments reported to change the physiological status of mother plants in stage 0 are:
Severe pruning of mother plants
Ethiolation treatments
Growth regulators application
The use of forcing solutions

Stage 0: Pre-treatments of mother plants

- As many *Proteaceae* have high phenol contents that can cause explant browning, some authors have used several strategies to decrease this reaction.



Leucadendron 'Safari Sunset' axillary buds with phenol browning

Examples of pretreatments

- **Ethiolation** (e.g. Watad et al., 1992b; Olate et al., 2010).
- **Cytokinin treatment of mother plants**
- **Pretreatments with gibberellins (e.g., Donovan et al., 1999)**

Stage 0: Pre-treatments of mother plants

- A cytokinin treatment of mother plants can reduced browning and even promoted bud sprouting in vitro (Rugge, 1995).

Modified photo from figure 1: Enhanced axillary budbreak of *Protea repens* in vitro. Rugge, B.A. (1995). Micropropagation of Protea repens. Acta Hortic. 387: 121-128.

Stage 0: Pre-treatments of mother plants

- Some authors have reported only an increase in the lateral buds of the mother plants with these cytokinin treatments but it has been important to rejuvenate the material and make it more suitable for in vitro establishment. (Pérez-Francés et al., 2001a; Olate et al., 2010).

Pretreatments with BA to *Leucospermum* 'Sunrise' mother plants to increase the amount of material suitable for in vitro culture initiation. (PÉREZ FRANCÉS et al. 2001)

Stage 0: Pre-treatments of mother plants

- A very interesting approach is to put the source of explants in a forcing solution.

Stage 0: Pre-treatments of mother plants

- A very interesting approach is to put the source of explants in a forcing solution.

In our laboratory, different pre-treatments at stage 0 prior the micropropagation of some *Leucospermum* cultivars are being investigated.
(**Suárez et al. 2021**)

***Leucospermum cordifolium* 'Flame Spike'**

***Leucospermum* 'Tango'**

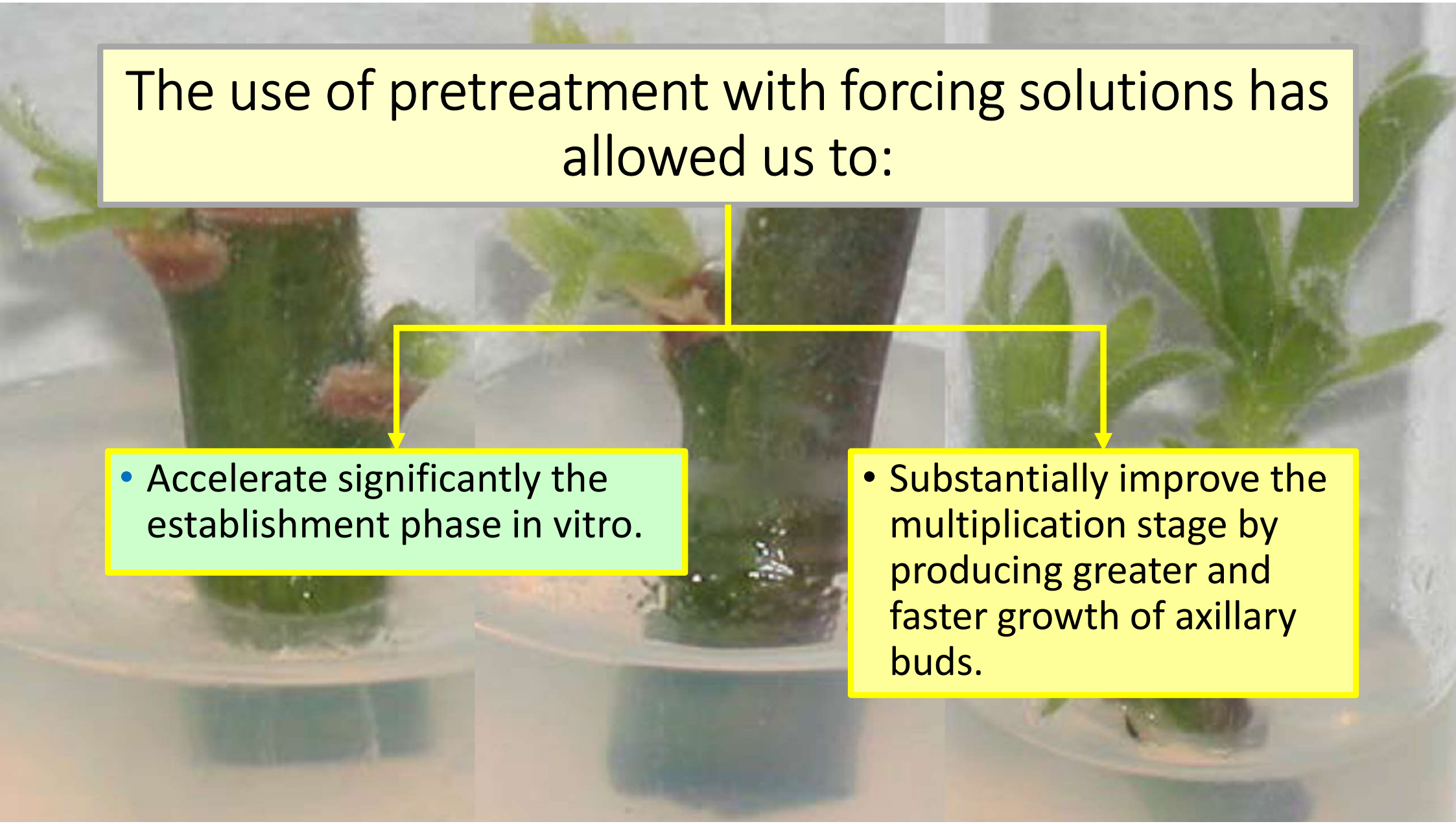
Pretreatments with forcing solutions to branches of *Leucospermum* ‘Flame Spike’ (2 weeks after treatment)

(E. Suárez, pers. Commun., 2020)

Suárez et al. (2021) Effect of forcing solutions used to break the seasonal infuence on in vitro axillary bud sprouting of two *Leucospermum* (R. Br.) cultivars. Plant Cell, Tissue and Organ Culture (PCTOC). https://doi.org/10.1007/s11240-021-02099-y

Composition of the forcing solutions:

- Sucrose
- MS medium diluted to ¼
- Growth Regulators: BA y GA_3



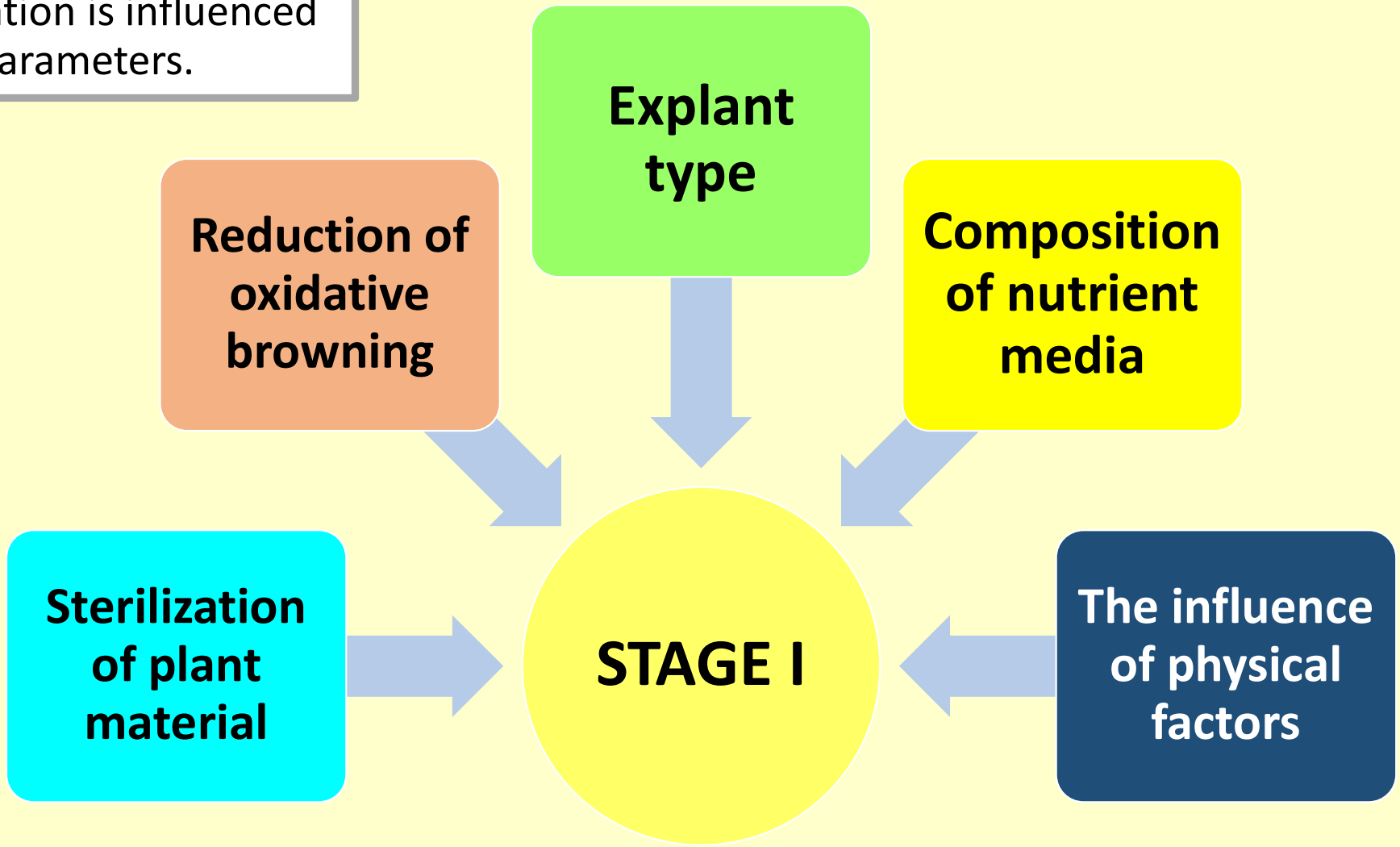
The use of pretreatment with forcing solutions has allowed us to:

- Accelerate significantly the establishment phase in vitro.

- Substantially improve the multiplication stage by producing greater and faster growth of axillary buds.

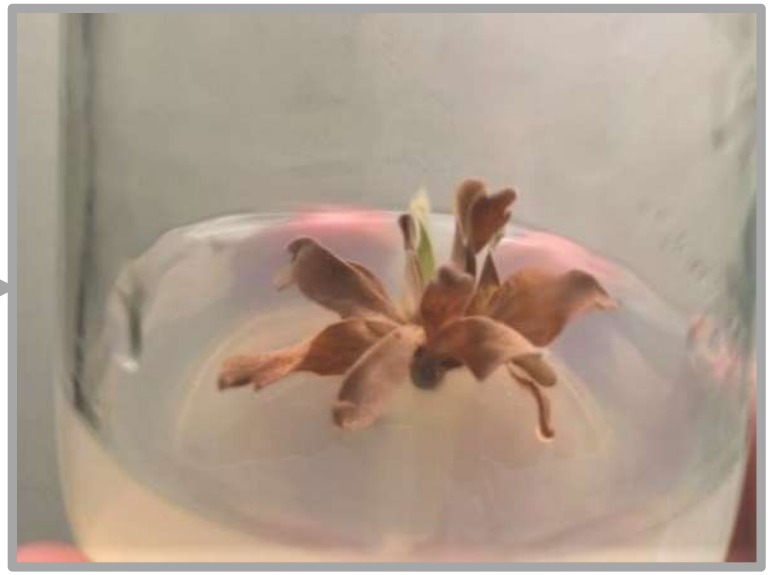
STAGE I: Establishment of an aseptic culture

- The success of the stage I of micropropagation is influenced by different parameters.

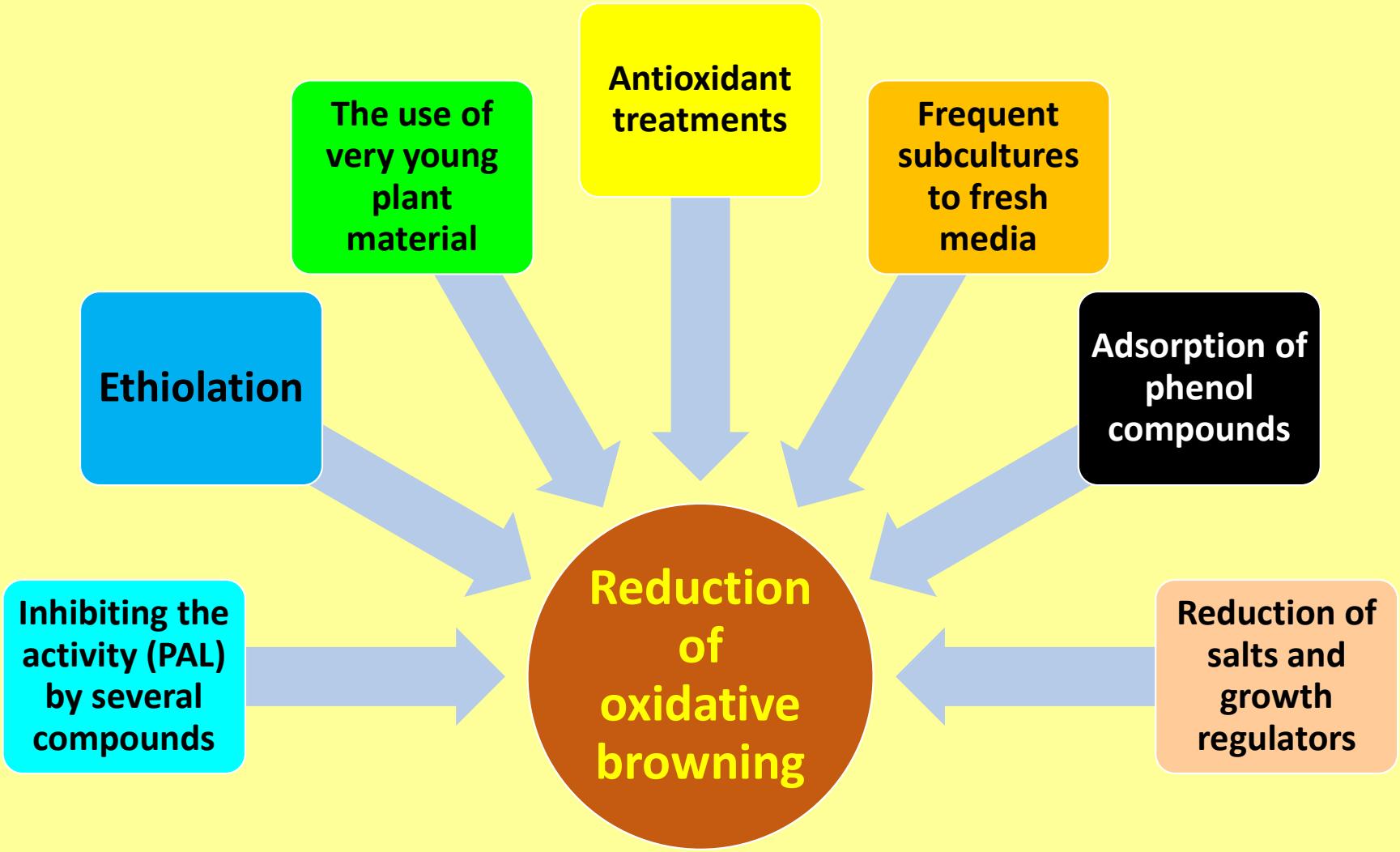


Reduction of oxidative browning

- Explant browning during the stage I is a frequent problem for proteas micropropagation.
- It occurs through the action of oxidized polyphenol-like compounds by triggering defence reactions induced by wouding.



Bud of *Leucospermum patersonii* grown in an establishment medium showing signs of necrosis
Photo taken by González Hernández, S. (2020)


Antioxidant treatments
The use of very young plant material
Frequent subcultures to fresh media
Ethiolation
Adsorption of phenol compounds
Inhibiting the activity (PAL) by several compounds
Reduction of oxidative browning
Reduction of salts and growth regulators

Reduction of oxidative browning

- Frequent subcultures to a fresh medium can sometimes stop browning if the phenolic compounds diffuse into the culture medium.
- (e.g., Rajasekaran, 1994).

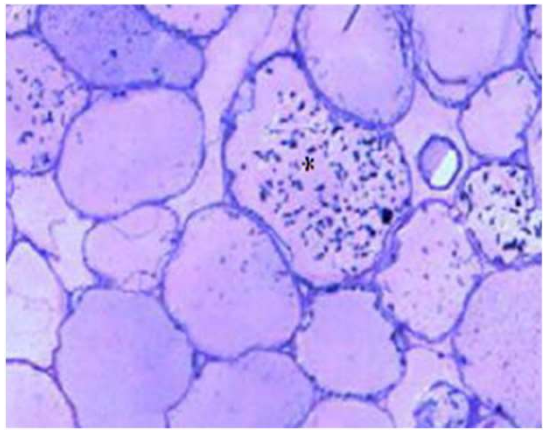
Grevillea robusta axillary buds cultured on a Woody Plant Medium plus 4.4 µM benzyladenine and 0.27 µM naphthaleneactic acid (NAA)

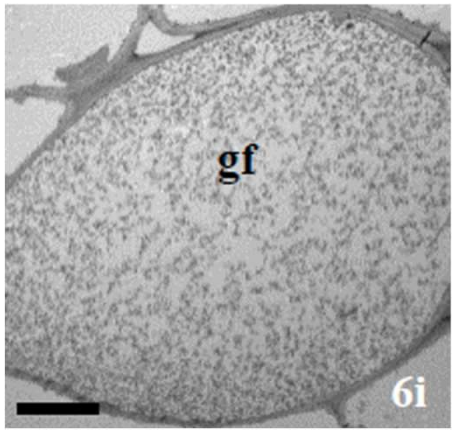
Rajasekaran, P., (1994). Production of clonal plantlets of *Grevillea robusta* in in vitro via axillary bud activation. Plant Cell Tissue Organ Cult. 39: 277-279.

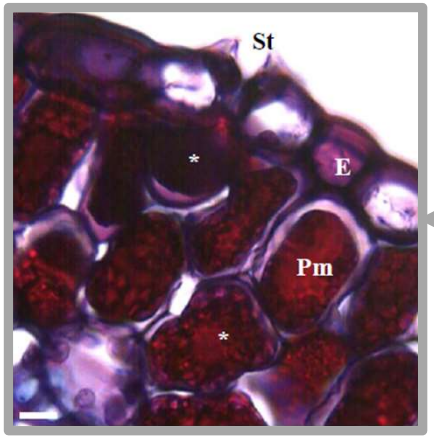
Reduction of oxidative browning

However, in many proteas there are very little diffusion of phenols into de medium.

This is due to the water insolubility of the main polyphenolic compounds in most of the *Proteaceae.*







L. 'Tango' : Phenolic deposits as fine granular

L. 'Flame Spike: Phenolic deposits as scattered drops

Reduction of oxidative browning

- The redox potential of the nutrient medium can be lowered by including chemicals

- into the medium
- sometimes as a preatreament of explants before culture

- **Ascorbic acid**
- **Citric acid**
- An **ascorbic acid and citric acid solution mixture** has been used by several authors (Bunn, 1991; Pérez-Francés et al., 1995; Wu and du Toit, 2004)

Reduction of oxidative browning

- In our laboratory we have observed that the addition of **150 mg L^{-1} ascorbic acid** to culture medium during establishment of explants of two cultivars of *Leucospermum* (*L. cordifolium* ‘Flame Spike’ and *L.* ‘Tango’) was enough to prevent browning
- (E. Suárez, pers. commun., 2019).

L. ‘Tango’

L. cordifolium ‘Flame Spike’

Reduction of oxidative browning

- Another possibility is the use of active charcoal into the culture medium.
- The use of active charcoal it is not always effective.
- Pérez-Francés et al. (1995) reported that the presence of activated charcoal in the multiplication medium clearly inhibited bud proliferation during micropropagation of *Leucandendron* ‘Safari Sunset’.

Reduction of oxidative browning

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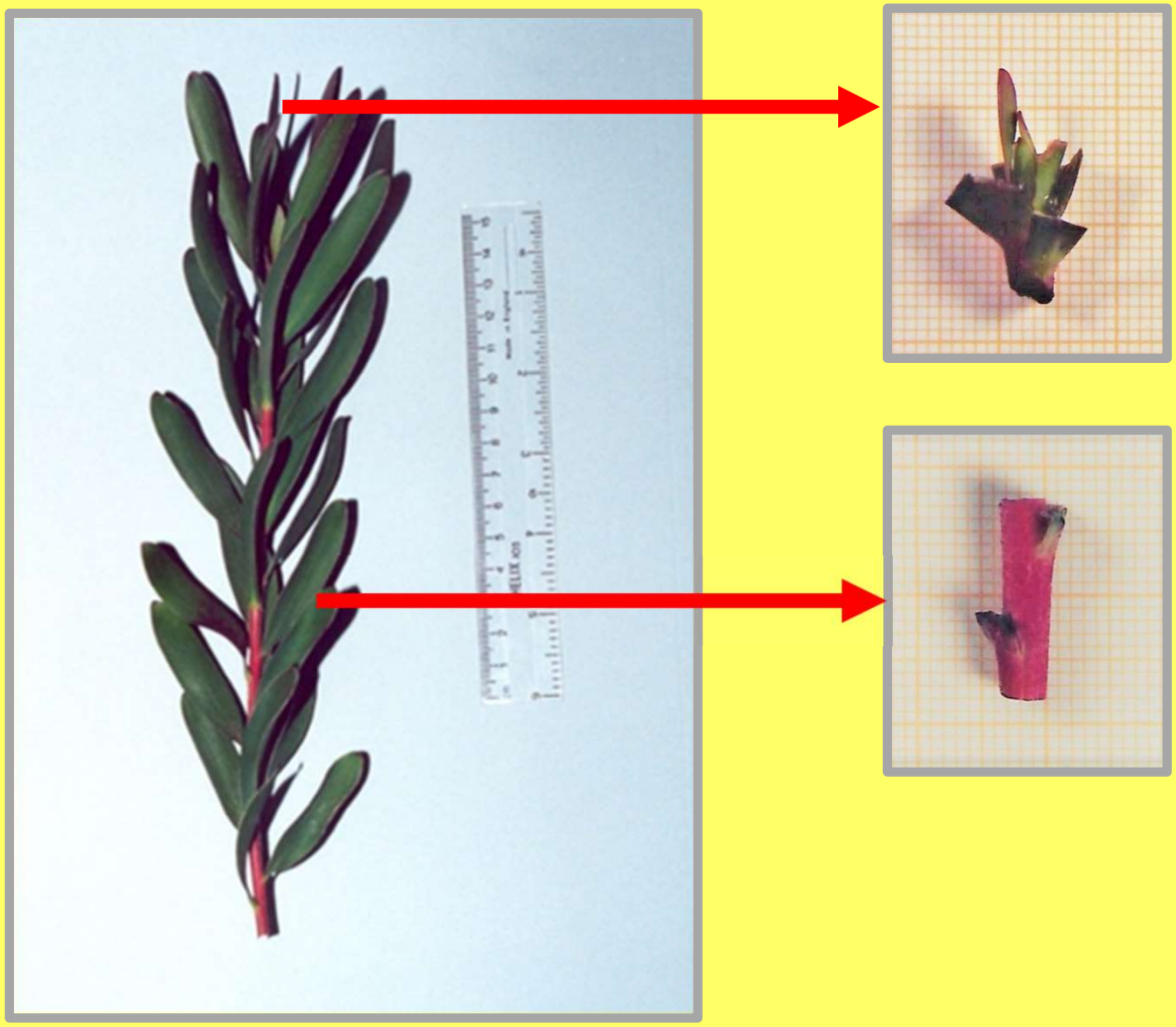
Similar results were obtained by Dias Ferreira et al. (2003) with the same cultivar.

Explant type

- Micropropagation of *Proteaceae* has been reported mainly from axillary or terminal buds, in which propagation is based on pre-existing meristems because it can ensure the highest genetic stability.



Two types of explants from a branch of *L*. 'Safari Sunset'

Explant type

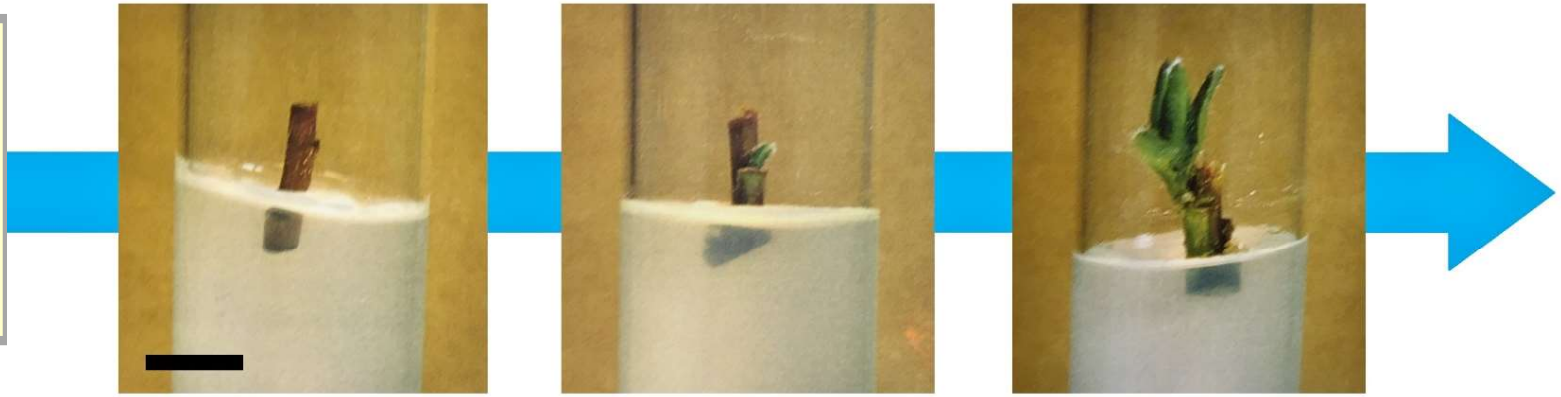
- Thillerot et al. (2006) reported that the use of apical tips as primary explants was better than nodal segments for the initiation stage of *L.* 'High Gold' and three cultivars of genus *Protea*.

Some other authors have also reported the use of apical explants (e.g. Bunn and Dixon, 1992b; Newell et al., 2003; Donovan et al. 1999).

Establishment Stage of apical explant of *Protea* 'Venus'.
Photo from Thillerot, M., Choix, F., Poupet, A. and Montarone, M. (2006). Micropropagation of *Leucospermum* 'High Gold' and Three Cultivars of *Protea*. Acta Hortic. 716:17-24.

Explant type

- Nodal segments (1-2 nodes) has been used in most of reports



Establishment Stage of *Leucadendron discolor*. Bar = 1 cm

Example

- Pérez-Francés, J. F., Ravelo, B. J.. and Rodríguez-Pérez, J.A. (2001b). In vitro establishment and proliferation of axillary bud cultures of *Leucadendron discolor* (*Proteaceae*). Acta Hortic. 545: 179-185.

Explant type

A feeder leaf sistem seems to be important for axillary bud development in some reports
(e.g., Ben-Jaacov and Dax, 1981; Rugge et al., 1989a; Watad et al., 1992b).

Agricultural Engineering Section, Higher Polytechnic School of Engineering, University of La Laguna

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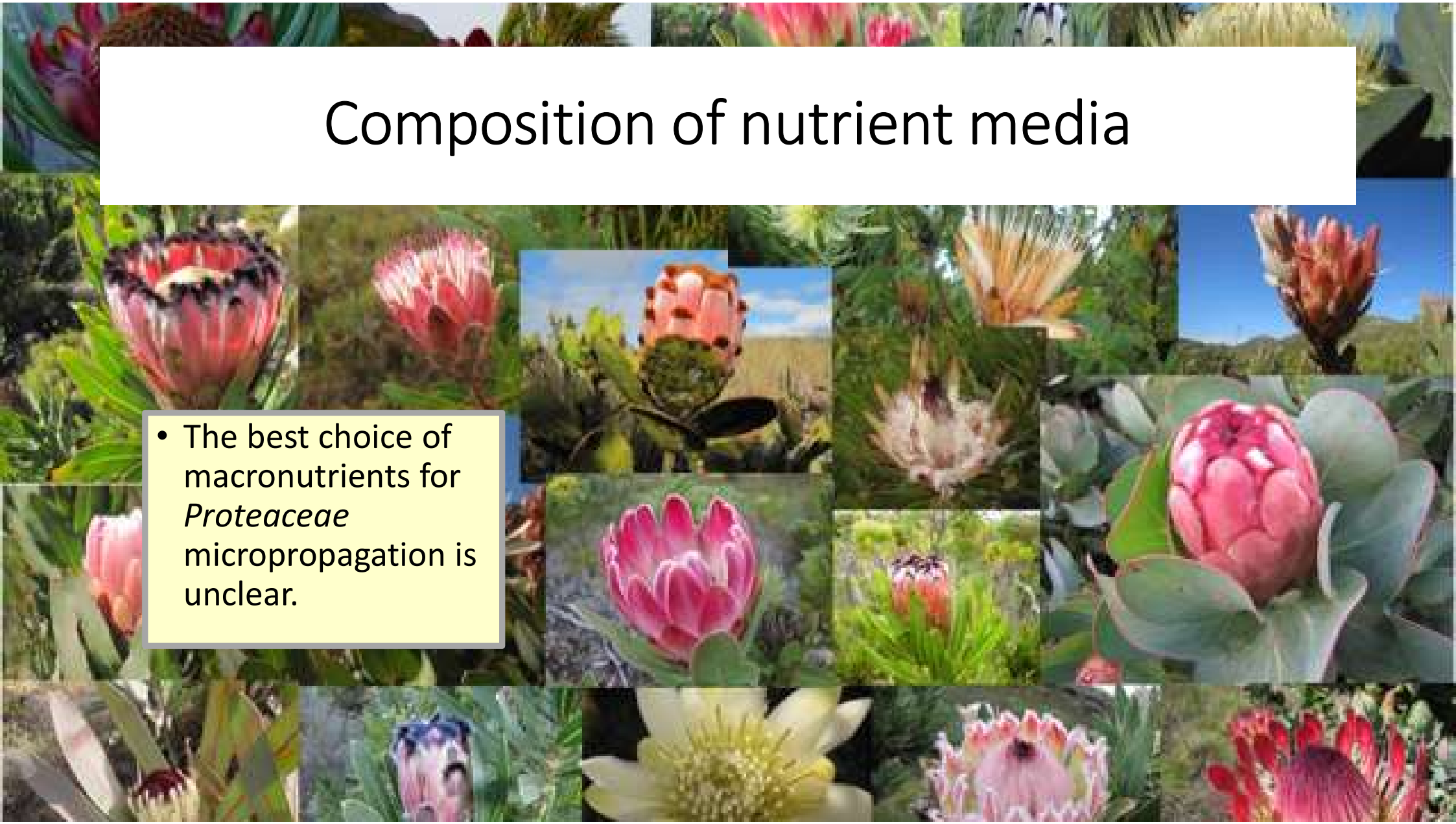
(e.g., Ben-Jaacov and Dax, 1981; Rugge et al., 1989a; Watad et al., 1992b).

- In our laboratory we have carried out studies on the in vitro establishment of *Leucospermum patersonii*.
- The use of nodal segments containing a feeder leaf in contact with the culture medium produced rapid axillary bud formation (unpublished results).

Agricultural Engineering Section, Higher Polytechnic School of Engineering, University of La Laguna

Experiments and photos of Sara González Hernández

Composition of nutrient media



- The best choice of macronutrients for *Proteaceae* micropropagation is unclear.

Composition of nutrient media

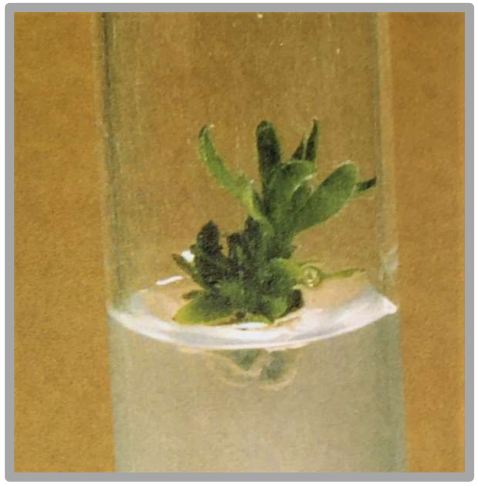
- In my laboratory we have observed that the **MS medium at half strength** has given the best results for the in vitro culture of several protea cultivars.

Leucadendron 'Safari Sunset'
Pérez-Francés et al., 1995
Suárez et al., 2010

Leucadendron discolor
Pérez-Francés et al., 2001

For two cultivars of *Leucospermum*
Pérez-Francés et al., 2001a
Suárez et al., 2019

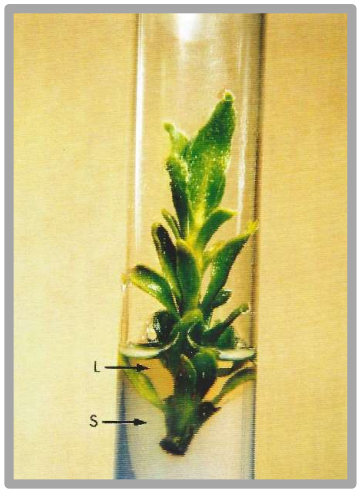
Leucadendron 'Safari Sunset'



Leucadendron discolor

Leucospermum cordifolium 'Flame Spike'

L. 'Tango'



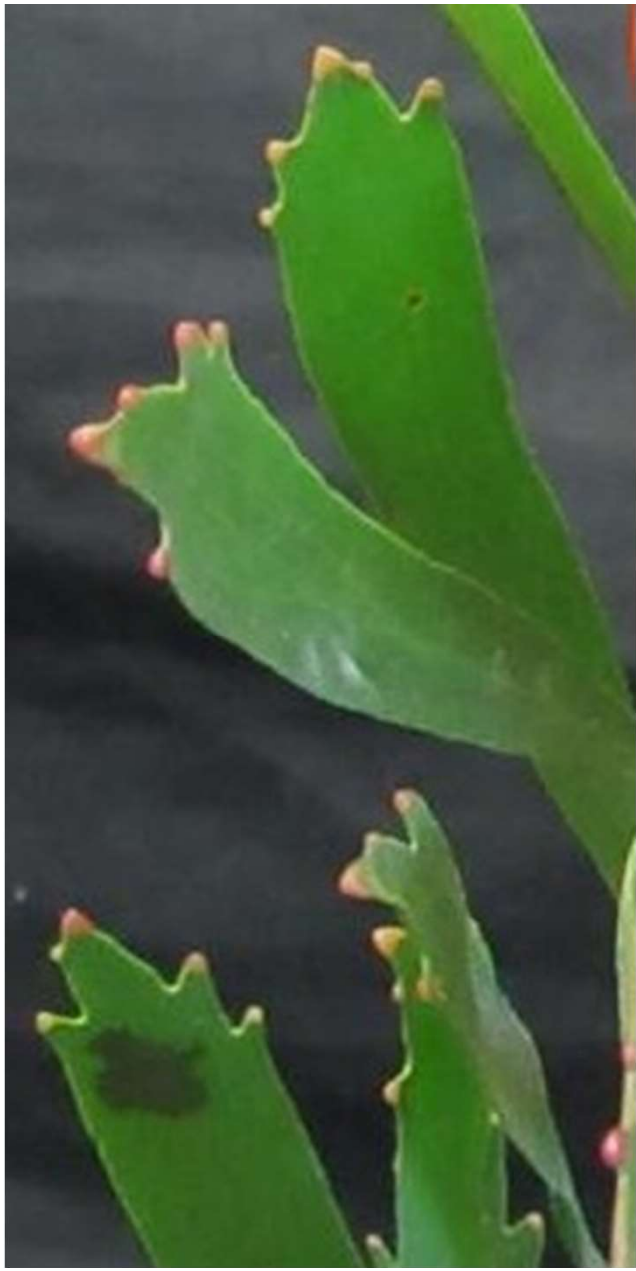
L. 'Sunrise'

Composition of nutrient media

- However, we decided to look for other alternatives for its low rate of buds developed obtained using this medium.

- We develop a method based on the studies of Cos Terrer and Frutos Tomás (2001).

- This method consists of adding macronutrients containing the leaves in a period of maximum growth to the culture medium, since this should provide the best nutritional conditions.



Composition of nutrient media

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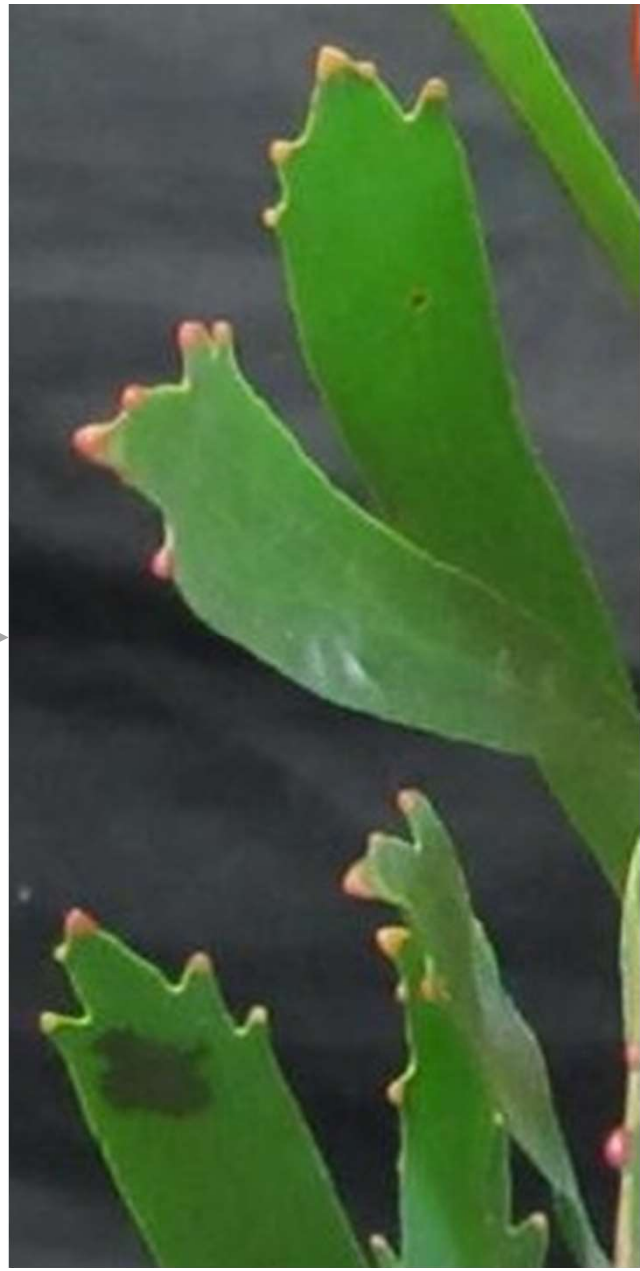

Composition of nutrient media

Table 1. Composition of macronutrients of the Murashige and Skoog (1962) medium at half strength (1/2 MS) and of the specific media for *L*. 'Flame Spike' (MMF) and *L*. 'Tango' (MMT) obtained according to the concentrations of the leaf in the period of maximum growth (Unpublished data taken from E. Suárez).

MACRONUTRIENTES	**½ MS (mg L^{-1})**	**MMF (mg L^{-1})**	**MMT (mg L^{-1})**
KNO_3	950	520	1006
NH_4NO_3	825	2260	1752
$MgSO_4.7H_2O$	185	750	729
KH_2PO_4	85	140	147
$CaCl_2.2H_2O$	220	1519	1498

Unfortunately, no significant differences were observed in the behavior of in vitro plant material between the new media and the medium 1/2 MS.

- Plant growth regulators (PGR) play an important role in all stages of proteas micropropagation protocols.

Cytokinins are particularly important in stage I

Benzyladenine (BA) has been the most used cytokinin

In several studies a **combination of cytokinins and auxins** has been used, with IBA being the most commonly used auxin

- Plant growth regulators (PGR) play an important role in all stages of proteas micropropagation protocols.

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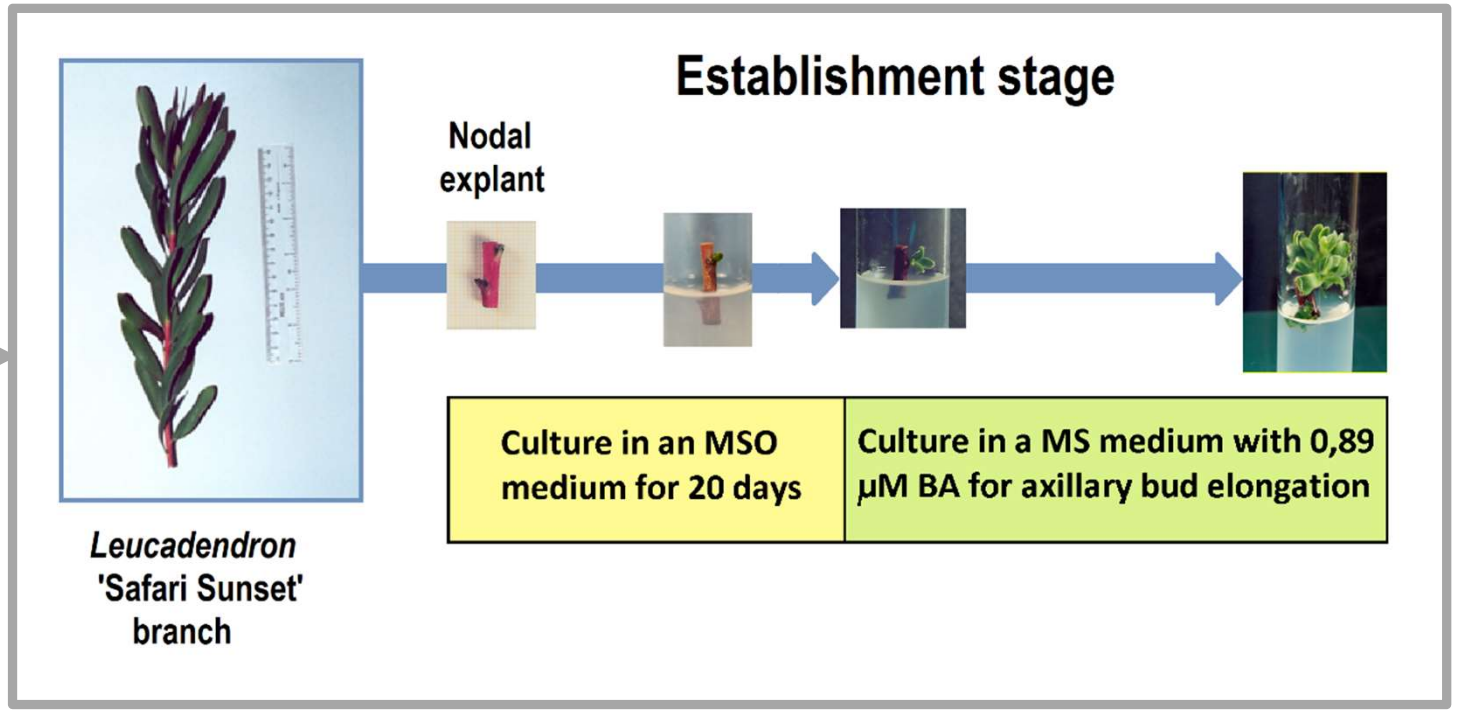
In several studies a **combination of cytokinins and auxins** has been used, with IBA being the most commonly used auxin

Several reports have showed the importance of the use of **gibberellins** (GAs) in stages I and II of some *Proteaceae* species

(Seelye, 1984; Ben-Jaacov, 1986; Tal et al., 1992a; Watad et al., 1992a, b; Rugge, 1995; Mulwa and Bhalla, 2000; Dias Ferreira et al., 2003; Thillerot et al., 2006).

However, some clones can not respond favourably to the addition of GAs to the culture medium.

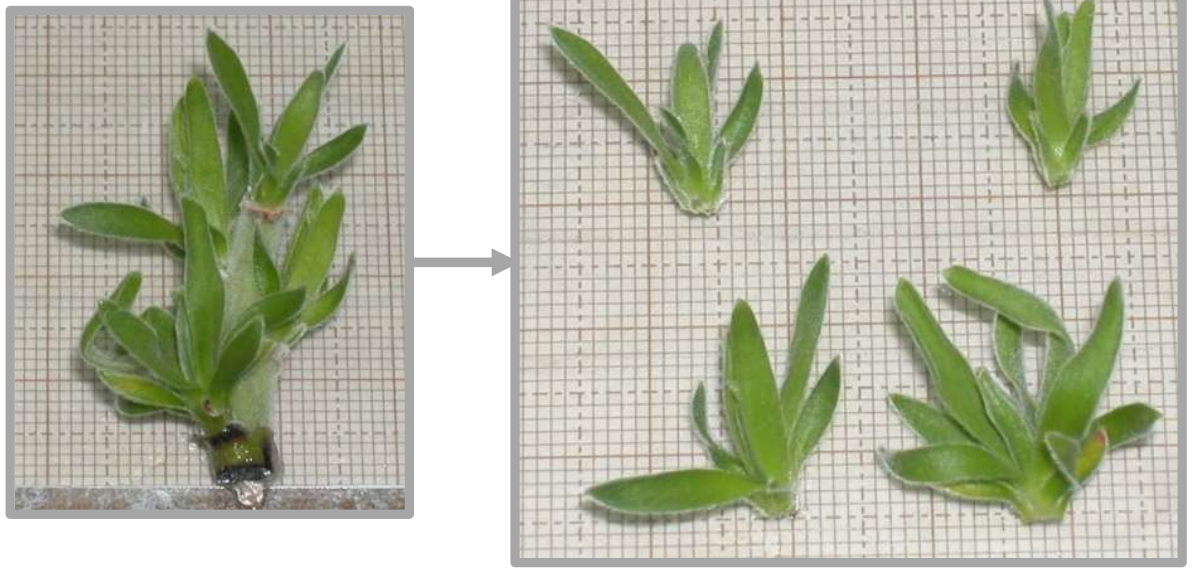
In some reports, the establishment medium does not have PGR in a first phase of the stage I. In these cases, after bud burst, explants are transferred to a medium with the addition of a cytokinin .
(Seelye, 1984, Offord et al., 1990; Pérez-Francés et al., 1995)



STAGE II: Shoot multiplication

Stage II: Multiplication

- The main objective of this stage is to obtain the propagation of plant material without loss of genetic stability.



In vitro multiplication of axillary buds of *L.* 'Flame Spike'

Stage II: Multiplication

- In many micropropagation protocols, the medium of establishment coincides with that of multiplication while in others there are some modifications, mainly in the PGR.

As in stage I, **cytokinins** play an important role in stage II.

As a general rule, the increase in the concentration of cytokinins increases the multiplication rate but decreases the length of the shoots produced.

Stage II: Multiplication

- A promising technique is the use of **photoautotrophic cultures** with carbon dioxide enrichment (Wu and Lin, 2013).

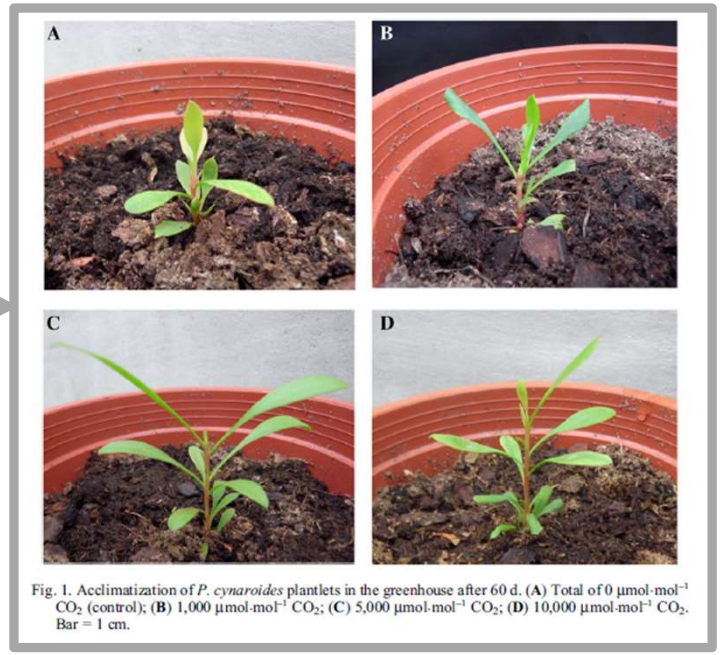


Fig. 1. Acclimatization of *P. cynaroides* plantlets in the greenhouse after 60 d. **(A)** Total of 0 µmol·mol^{-1} CO_2 (control); **(B)** 1,000 µmol·mol^{-1} CO_2; **(C)** 5,000 µmol·mol^{-1} CO_2; **(D)** 10,000 µmol·mol^{-1} CO_2. Bar = 1 cm.

- Also, Wu et al. (2018) have reported an improvement in the growth of microshoots of this species due to the use of a **forced ventilation system** based on modified temporary immersion culture vessels.

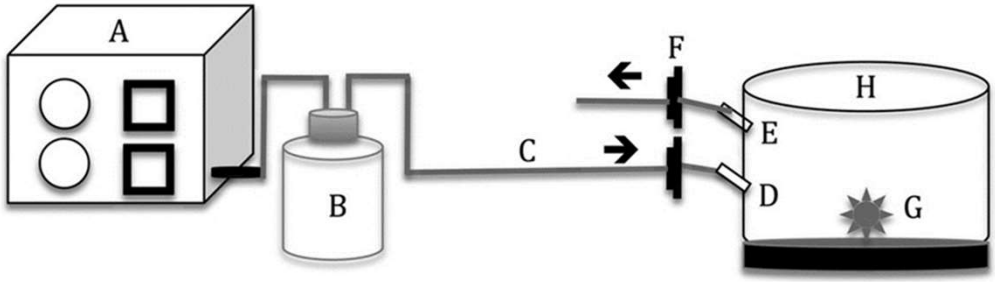
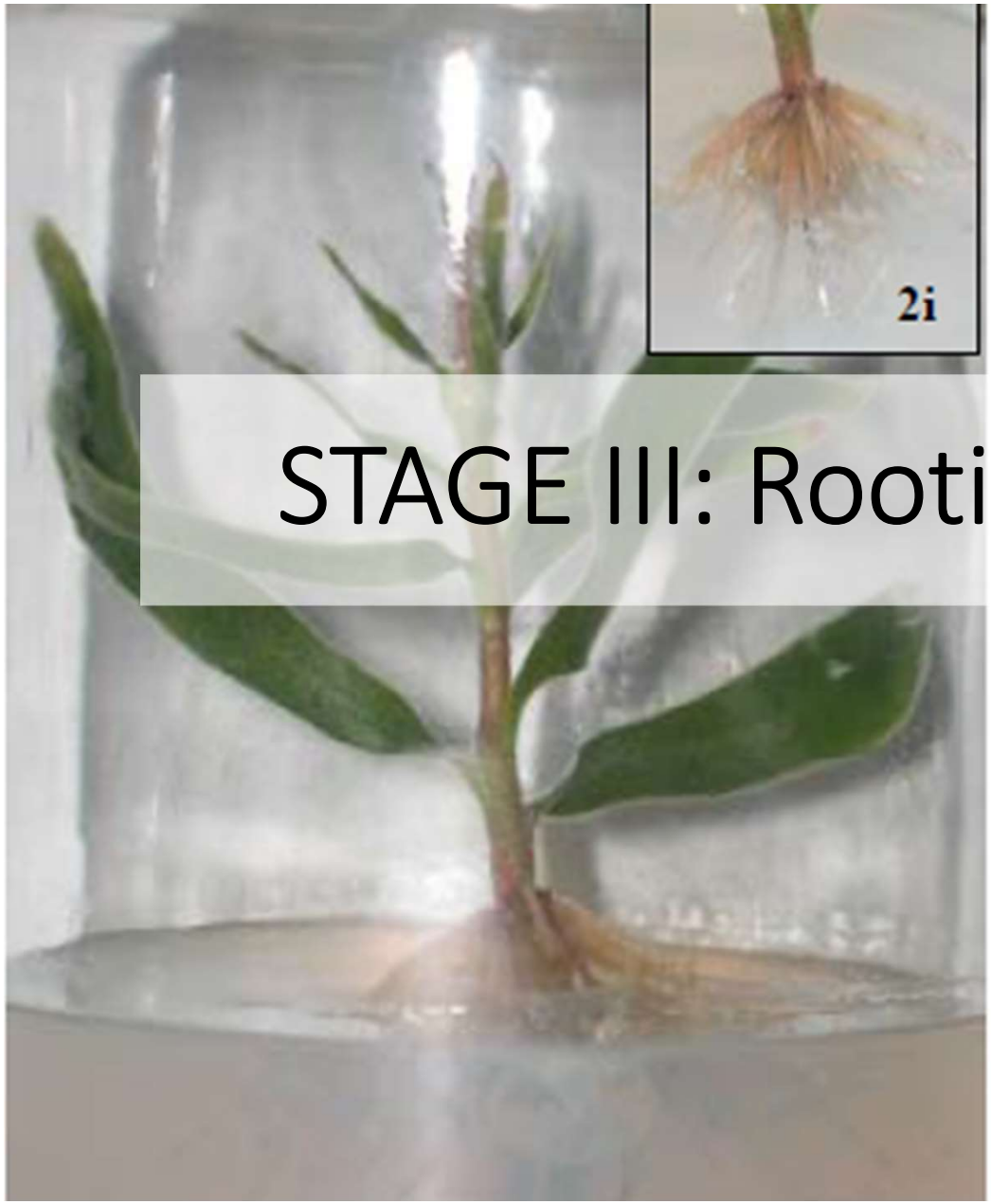


Fig. 1. Schematic drawing of the forced ventilation system using modified temporary immersion culture vessels.



STAGE III: Rooting of microshoots

Stage III: Rooting

- According to Debergh and Maene (1981) this stage can be divided in two substages:

Substage IIIa: the elongation of the buds to shoots and the preparation of uniform shoots.

L. 'Tango'

Substage IIIb: rooting of microshoots

L. 'Flame Spike'

Stage III: Rooting

- This stage and the next stage IV frequently limits the overall success of micropropagation protocols.

- Many *Proteaceae* can be grown as shoot cultures, but have poor rooting and many problems on transfer to soil.

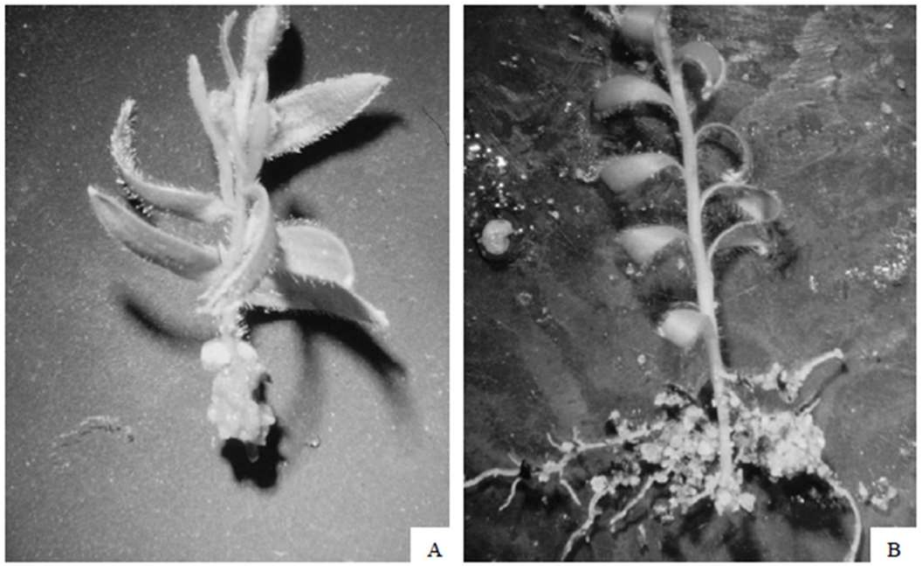


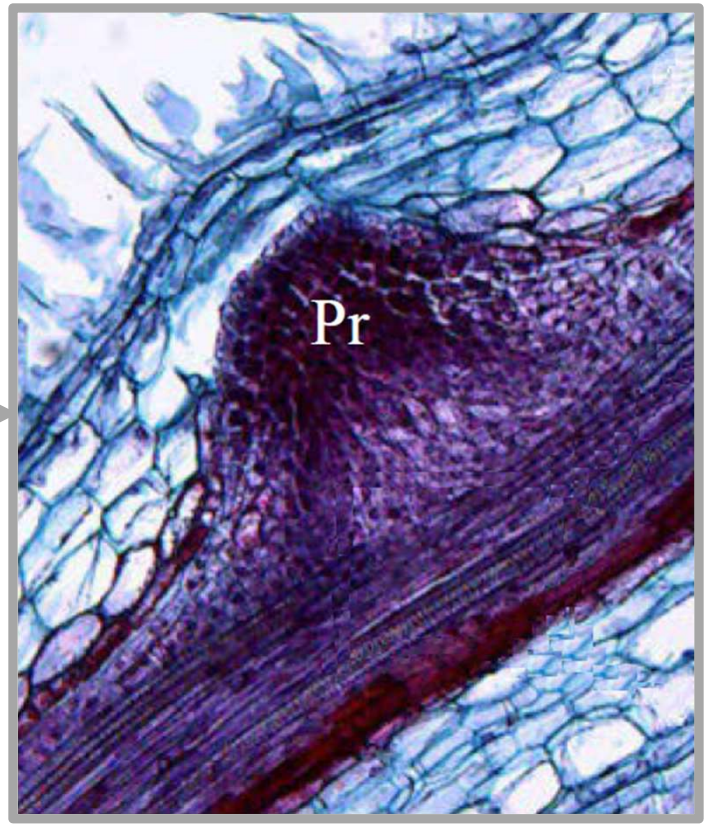
Fig. 3. Rootstrike and growth of tissue cultured cuttings under different conditions – genotype 008. A. Excessive growth of callus and abnormal root formation in agar solidified medium; B. Healthy root formation and growth in sand : peat : perlite medium.

Croxford, B., Yan, G. and Sedgley, R. (2006). Micropropagation of Leucadendron. Acta Hortic. 716:25-34.

Stage III: Rooting

- Rooting stage can be divided into two phases:

Root initiation



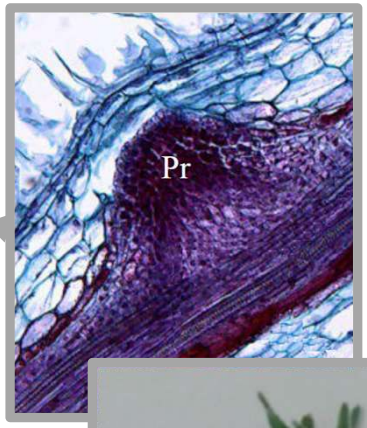
Root primordium from a *Leucospermum* 'Tango' microshoot at 6 days of treatment

Stage III: Rooting

- Rooting stage can be divided into two phases:

Root initiation

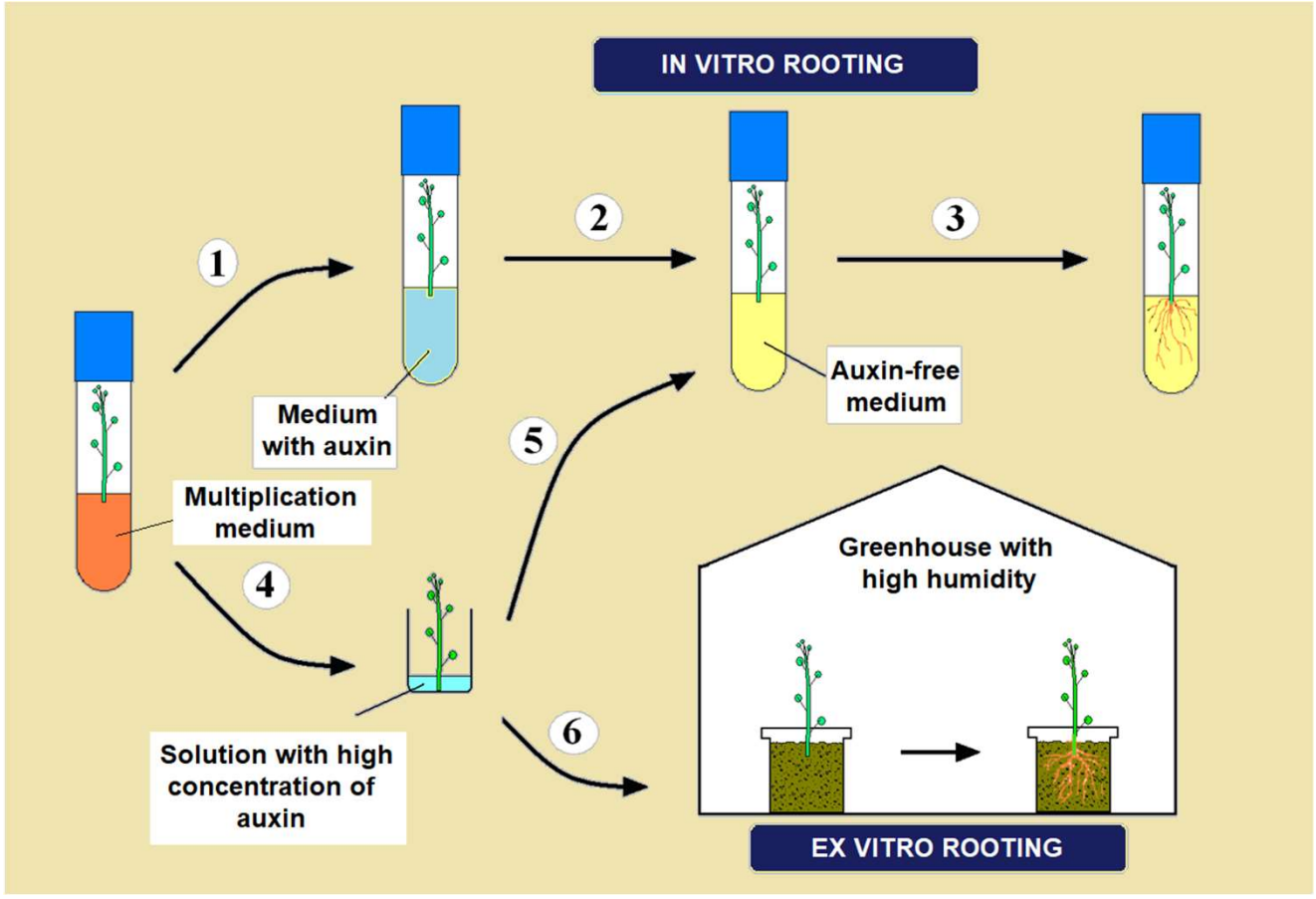
Root elongation

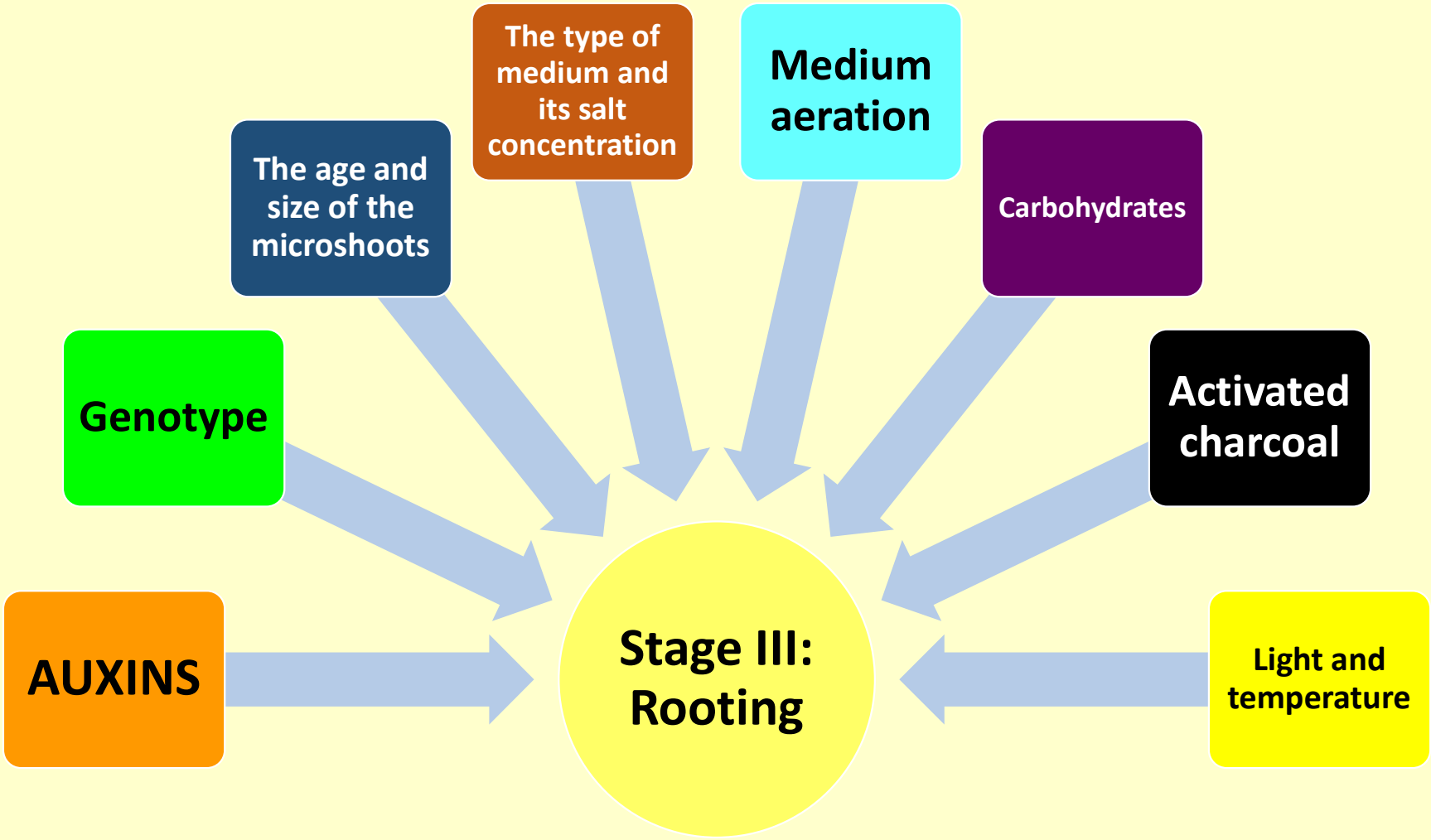


Both phases have different requirements of auxin.

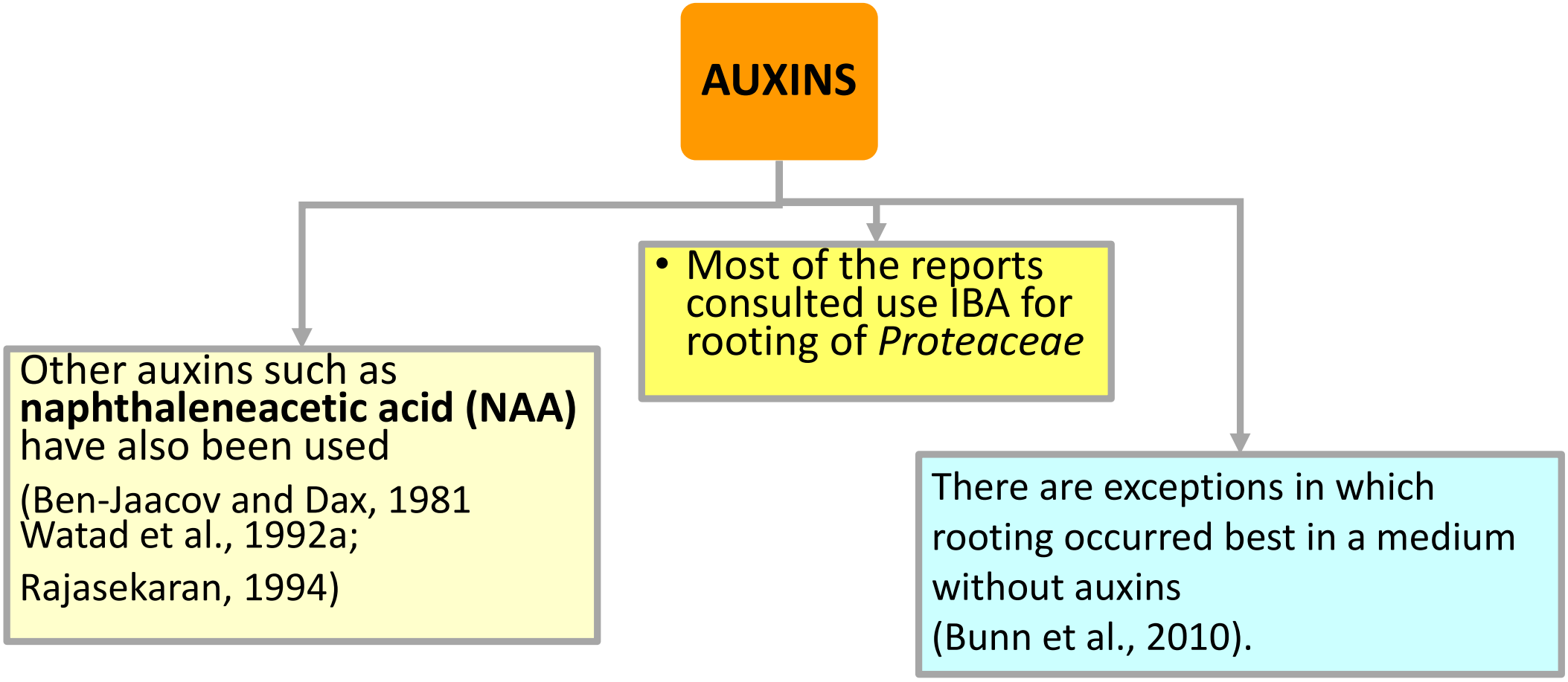
Normally a first treatment of the microshoots with an auxin is used and then they are passed to an auxin-free medium.

In addition, rooting of microcutting can be obtained under in vitro conditions or ex vitro, although this last technique is not always possible.



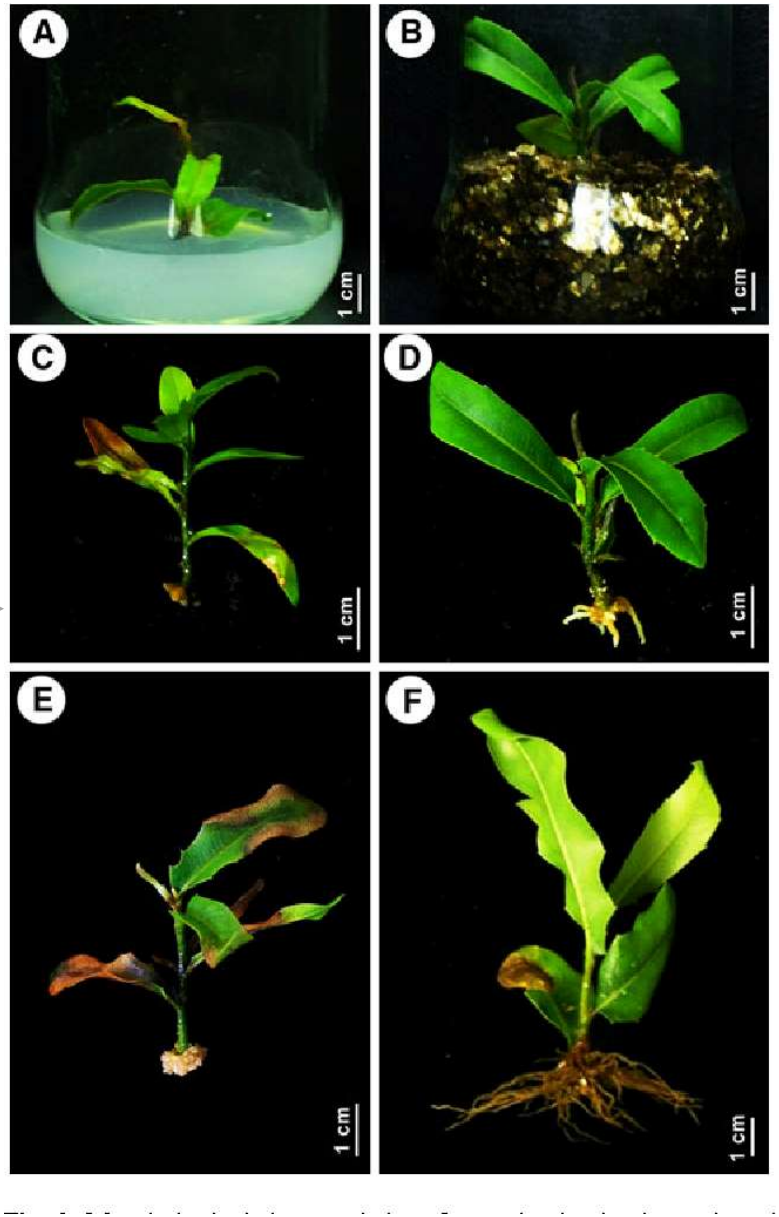

The type of medium and its salt concentration
Medium aeration
The age and size of the microshoots
Carbohydrates
Genotype
Activated charcoal
AUXINS
Stage III: Rooting
Light and temperature

Stage III: Rooting

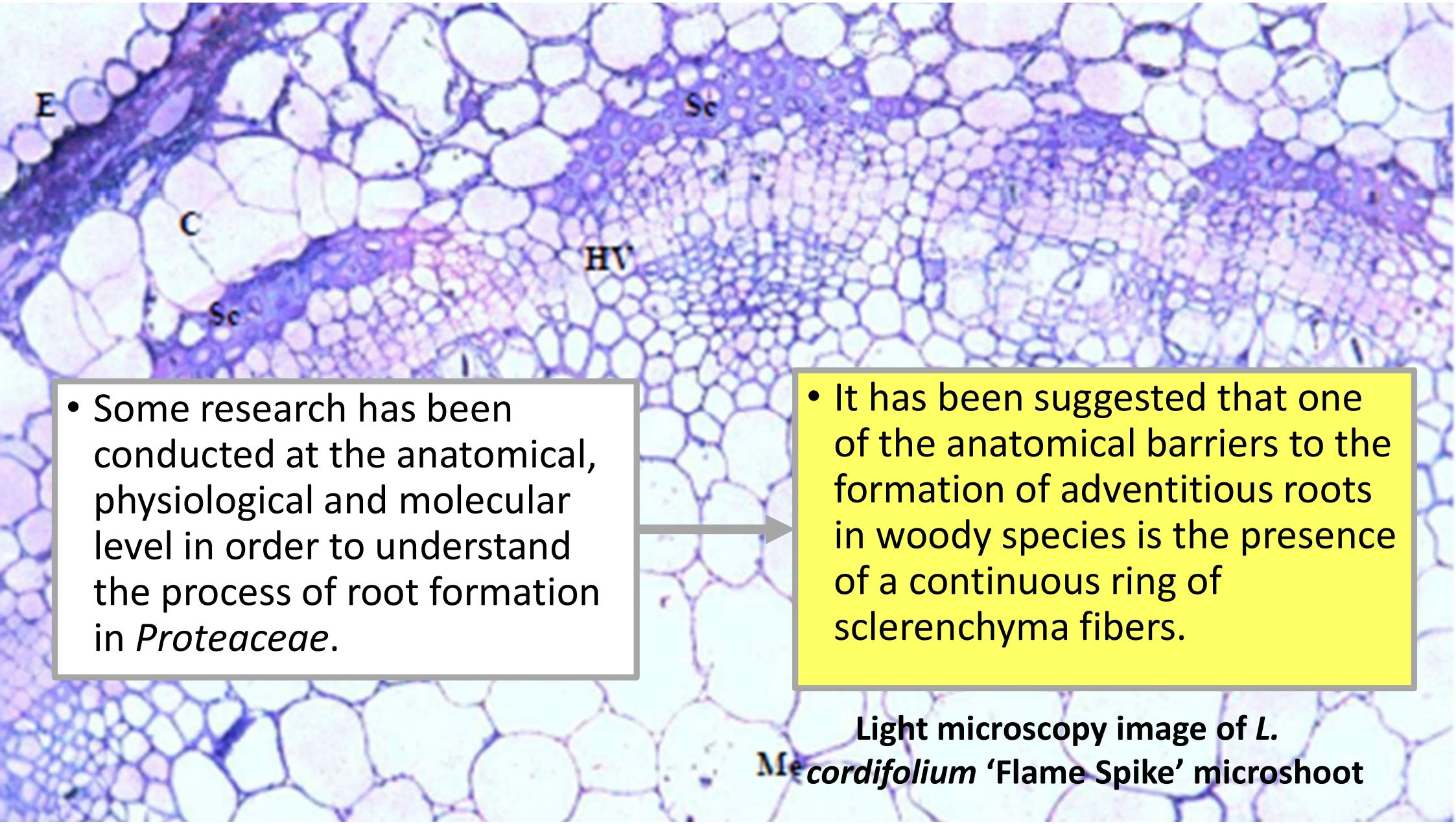


Stage III: Rooting

- Chan-um et al. (2011) reported a successful protocol for root induction and growth of in vitro *Macadamia tetraphylla* plantlets cultured under CO_2-enriched photoautotrophic condictions.



Cha-um et al. (2011). Promoting root induction and growth of in vitro macadamia (Macadamia tetraphylla L. 'Keaau') plantlets using CO2-enriched photoautotrophic conditions. Plant Cell Tissue Organ Cult. 106(3):435-444.



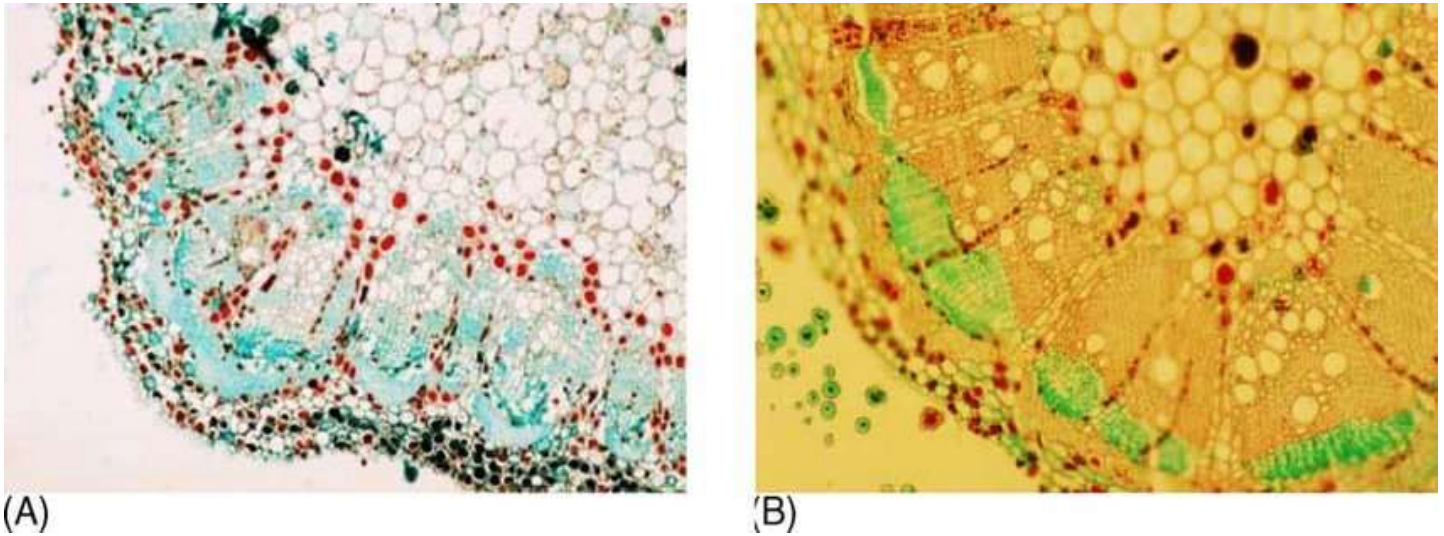
- Some research has been conducted at the anatomical, physiological and molecular level in order to understand the process of root formation in *Proteaceae*.

- It has been suggested that one of the anatomical barriers to the formation of adventitious roots in woody species is the presence of a continuous ring of sclerenchyma fibers.

Light microscopy image of *L. cordifolium* 'Flame Spike' microshoot

Stage III: Rooting

- However, Krisantini et al. (2006) observed that two *Grevillea* cultivars with different ability to root had a similar anatomy with a continuous ring of sclerenchyma fibers.



Cross sections of the easy-to-root *G.*'Royal Mantle' (A) and the difficult-to-root *G.*'Coastal Dawn' (B) taken in winter. Stem anatomy was similar in both cultivars. Magnification 100Â.

Krisantini et al (2006). Adventitious root formation in Grevillea (Proteaceae), an Australian native species. Sci. Hortic. 107: 171-175.

Stage III: Rooting

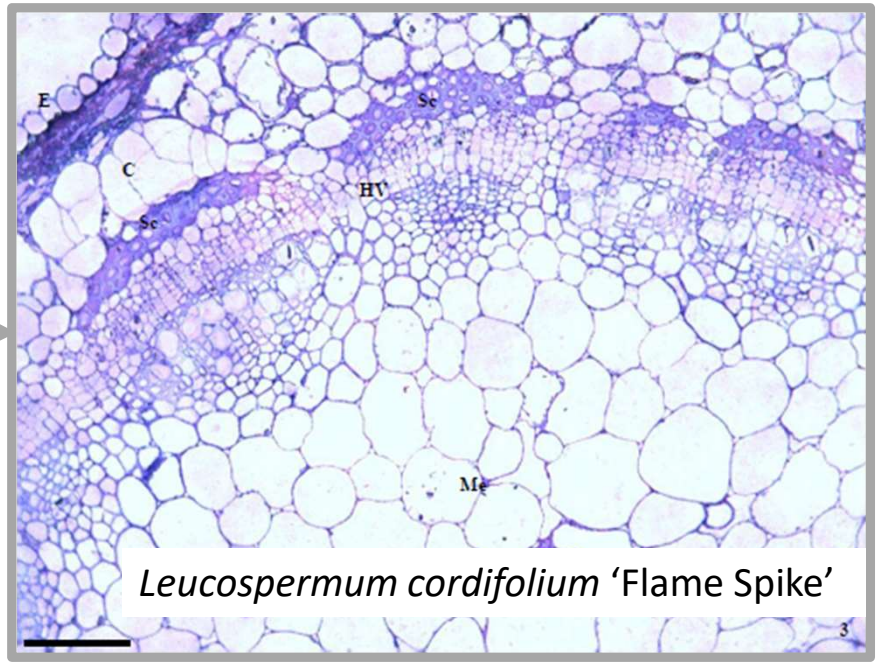
- In our laboratory we have carried out several microscopic studies of rooting, both in vitro and ex vitro, from microcuttings of two micropropagated cultivars of *Leucospermum* (Suárez et al., 2018).

Leucospermum cordifolium 'Flame Spike'

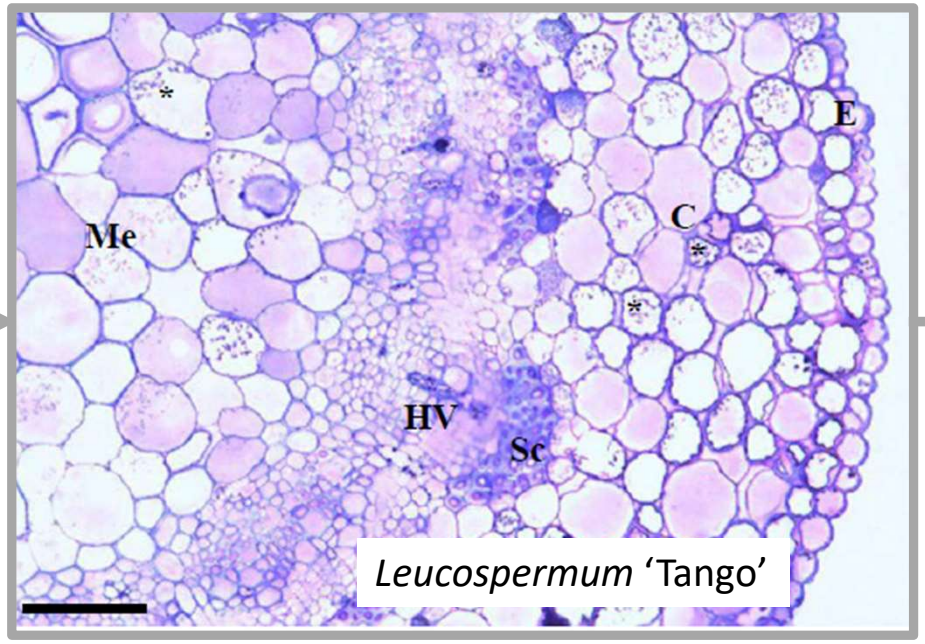
Leucospermum 'Tango'

Stage III: Rooting

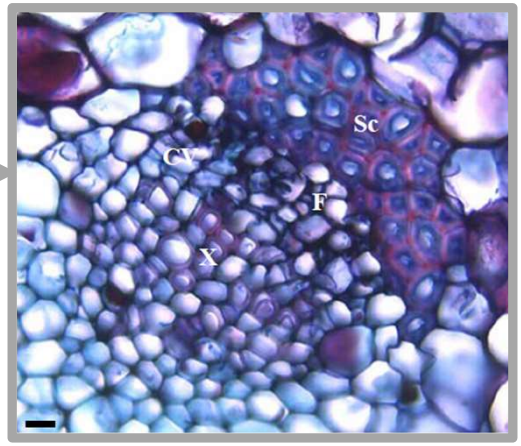
- The in vitro shoots of both cultivars lacked a continuous sclerenchyma ring and only had small groups of sclerenchyma fibers with thickening walls.



Leucospermum cordifolium ‘Flame Spike’

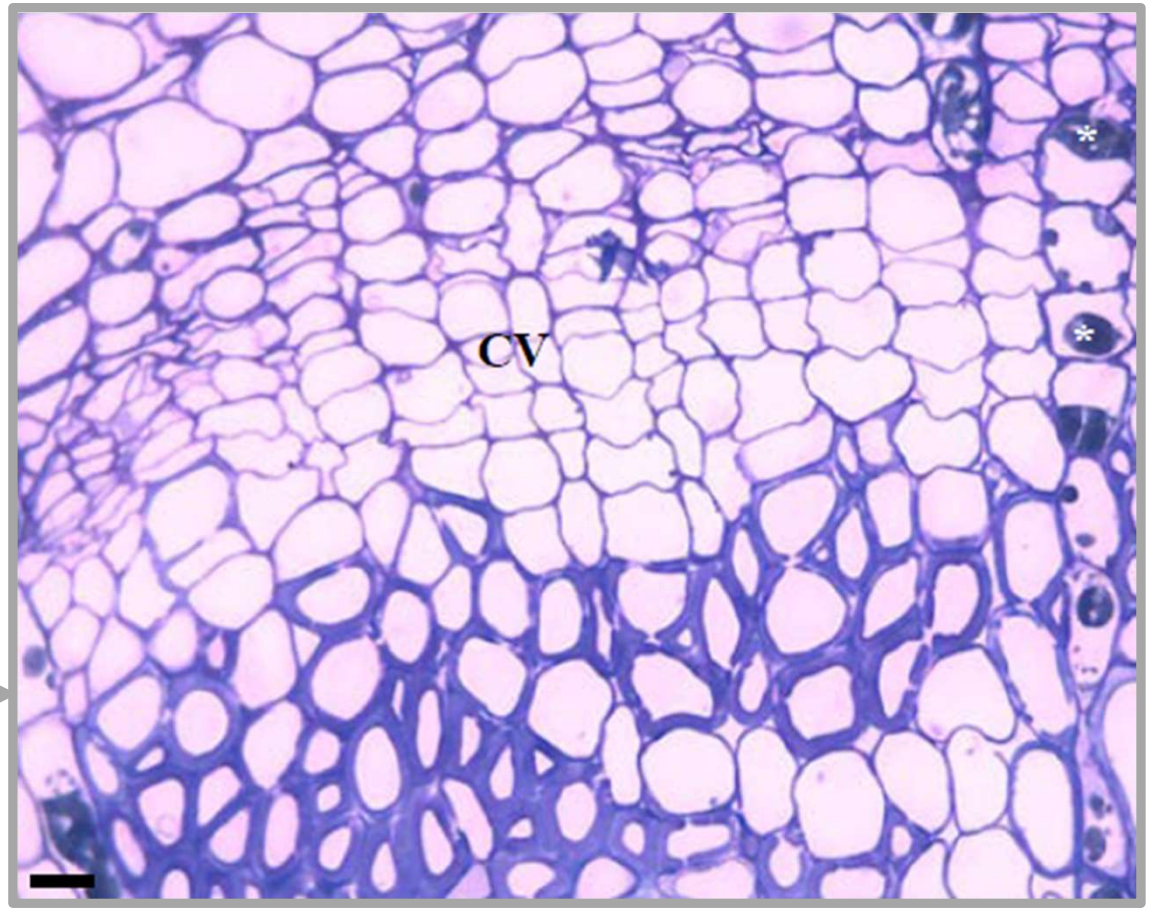


Leucospermum ‘Tango’



Stage III: Rooting

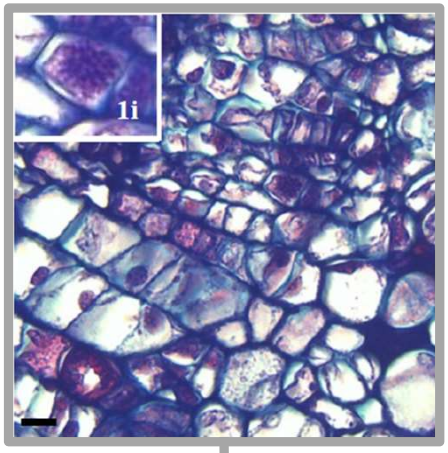
- The sequence of anatomical changes during rooting was similar in vitro and ex vitro and the origin of the adventitious root was from the vascular cambium.



Leucospermum cordifolium ‘Flame Spike’

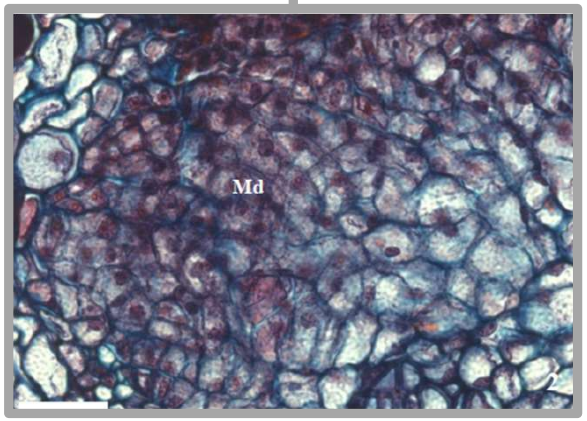
In vitro rooting of *L.* ‘Flame Spike’

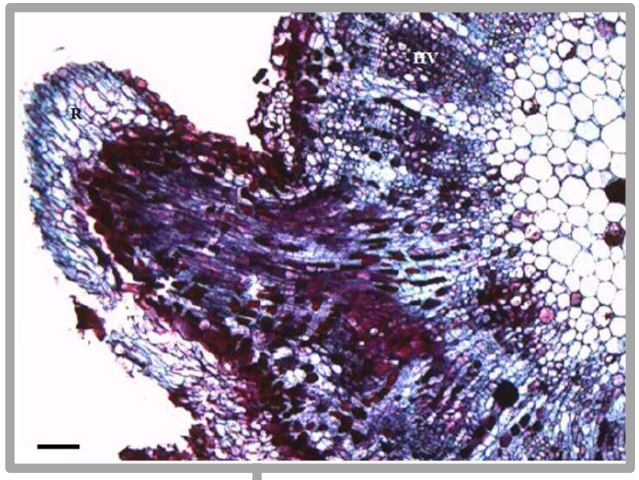
Day 0: treatment with IBA



Days 2-3: a high mitotic activity was observed in the cambial zone at the base of the microcuttings.

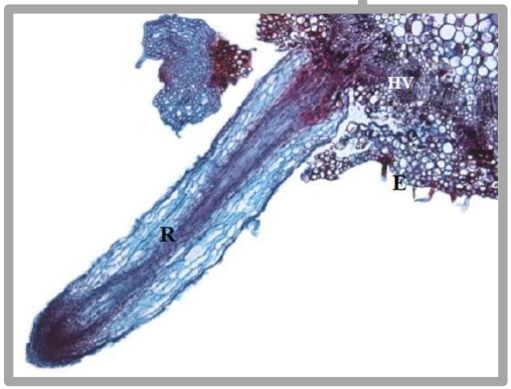
Day 6: meristemoids could be observed that began to produce polarized cell divisions forming the radical primordia.





Day 12: actively growing root primordium

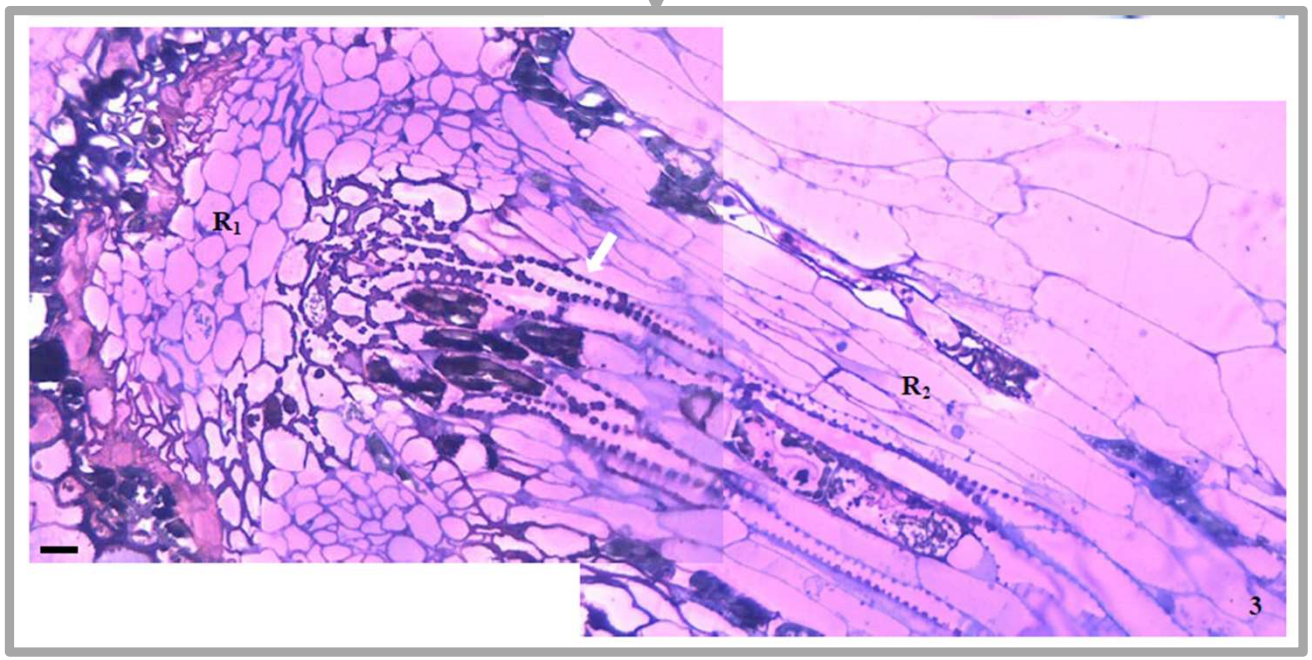
Day 17



Adapted from Suárez et al. 2018

Stage III: Rooting

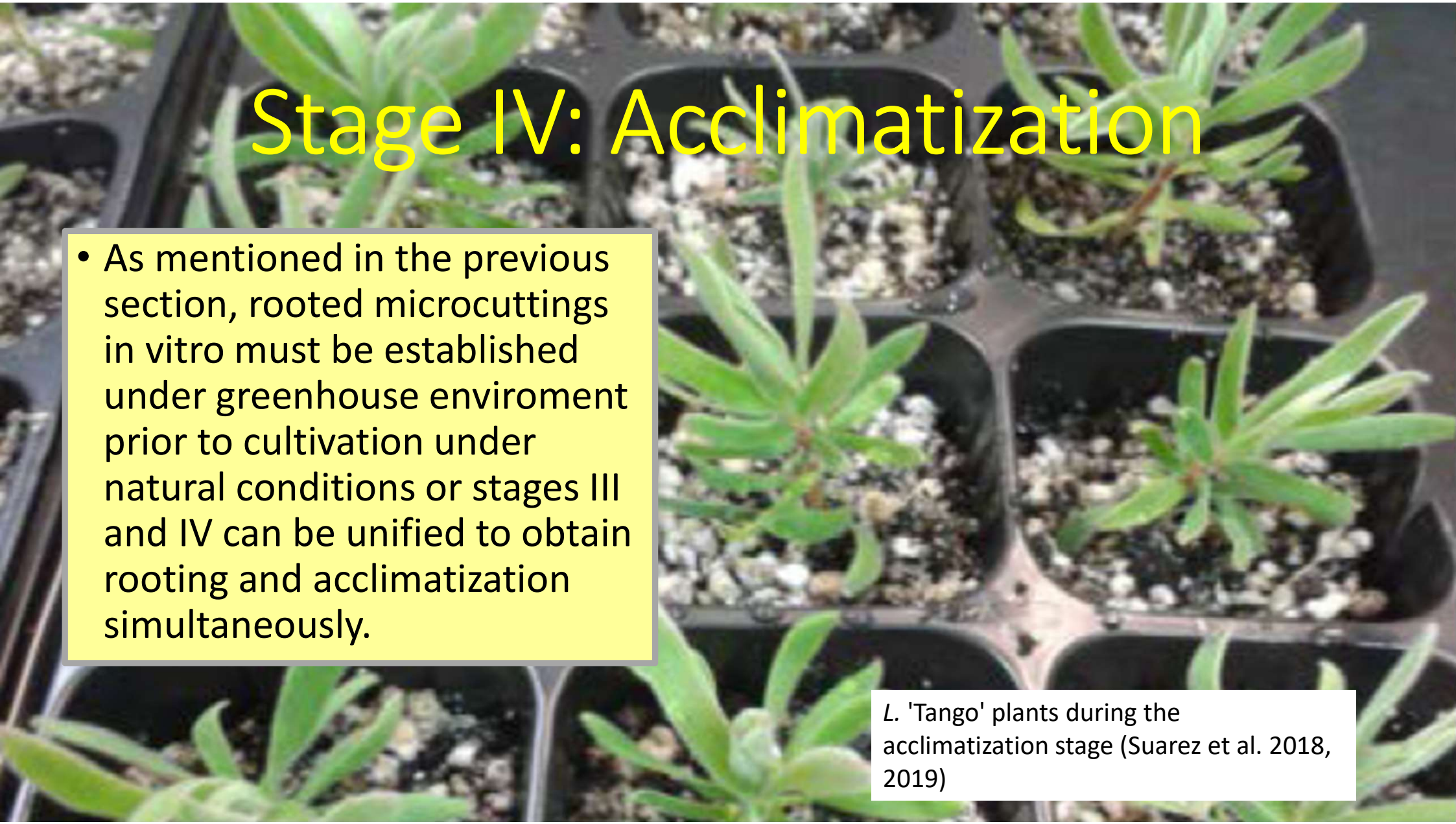
- The acclimatized plantlets showed an adequate radical system with established connections between the stem vascular bundles and the roots, allowing root elongation.



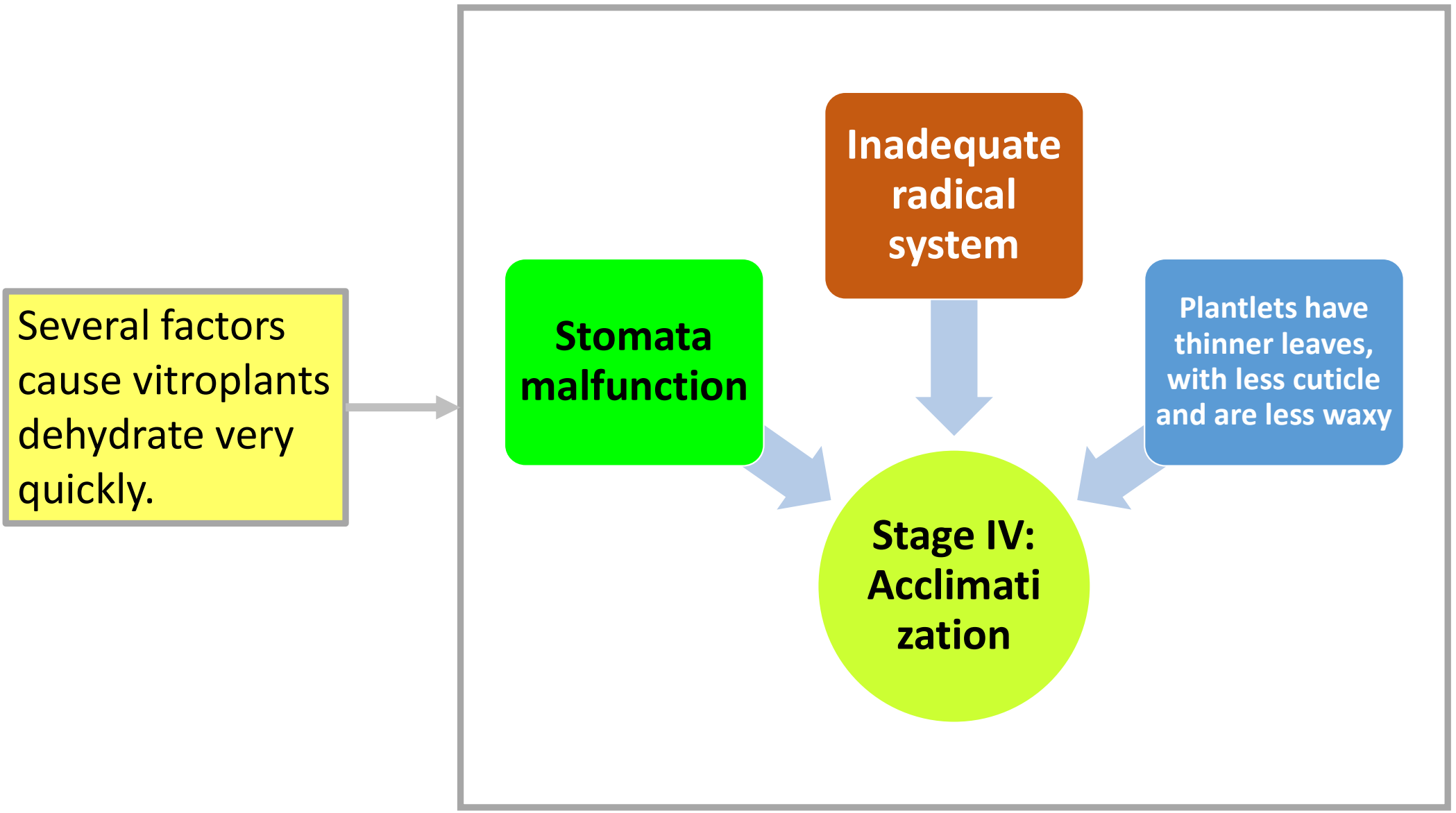
Light microscope photomicrograph of a root of *L. cordifolium* 'Flame Spike' developed ex vitro.

Stage IV: Acclimatization

- As mentioned in the previous section, rooted microcuttings in vitro must be established under greenhouse enviroment prior to cultivation under natural conditions or stages III and IV can be unified to obtain rooting and acclimatization simultaneously.

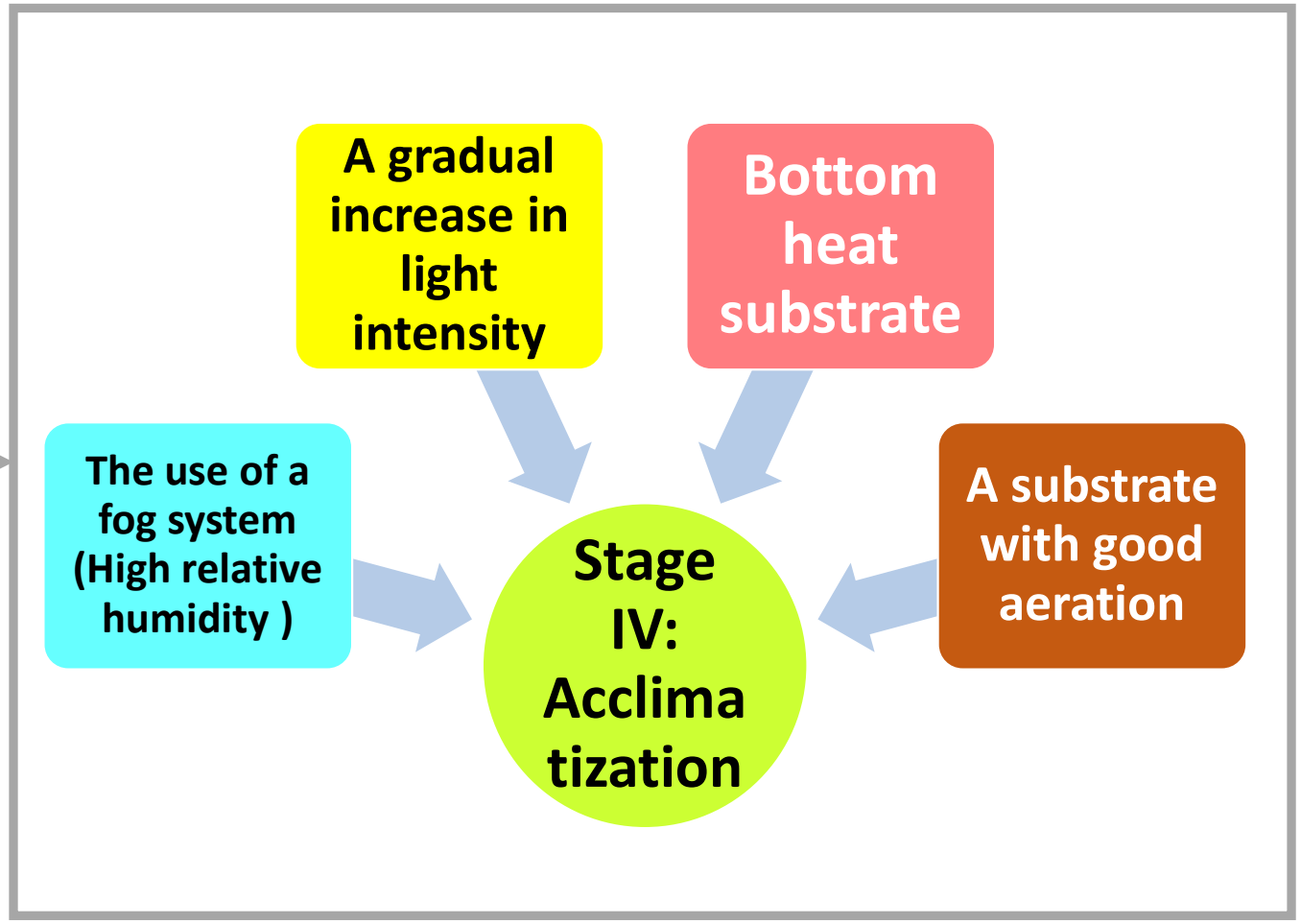


L. 'Tango' plants during the acclimatization stage (Suarez et al. 2018, 2019)


Several factors cause vitroplants dehydrate very quickly.
Stomata malfunction
Inadequate radical system
Plantlets have thinner leaves, with less cuticle and are less waxy
Stage IV: Acclimatization

Stage IV: Acclimatization

- Therefore, they need a period of hardening



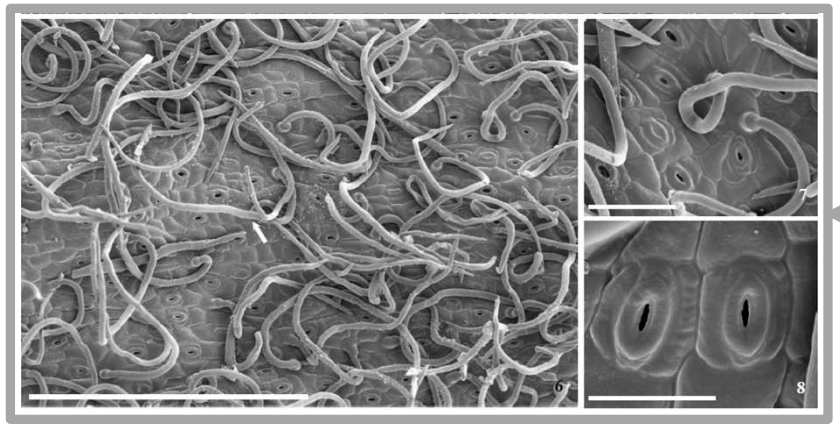
Stage IV: Acclimatization

- In our laboratory we have compared the anatomy of plants grown in the field with plants obtained in vitro and acclimatized plants from two *Leucospermum* cultivars using light, scanning and transmission electron microscopy.

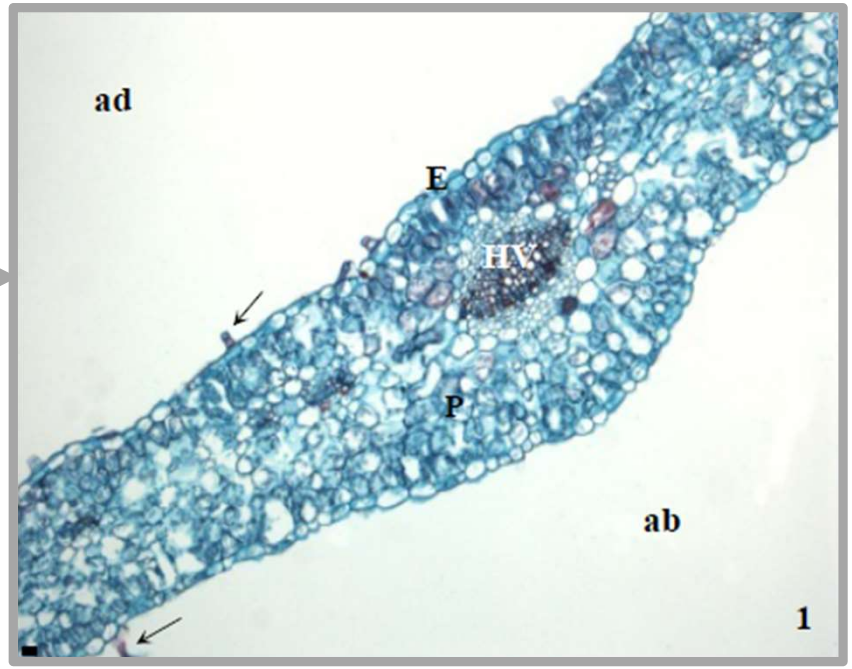
- Stomata of a new leaf of an acclimatized plant of *L. cordifolium* 'Flame Spike' (Suárez et al 2019)

Stage IV: Acclimatization

- The structures developed *in vitro* are not suitable for survival under *ex vitro* conditions.



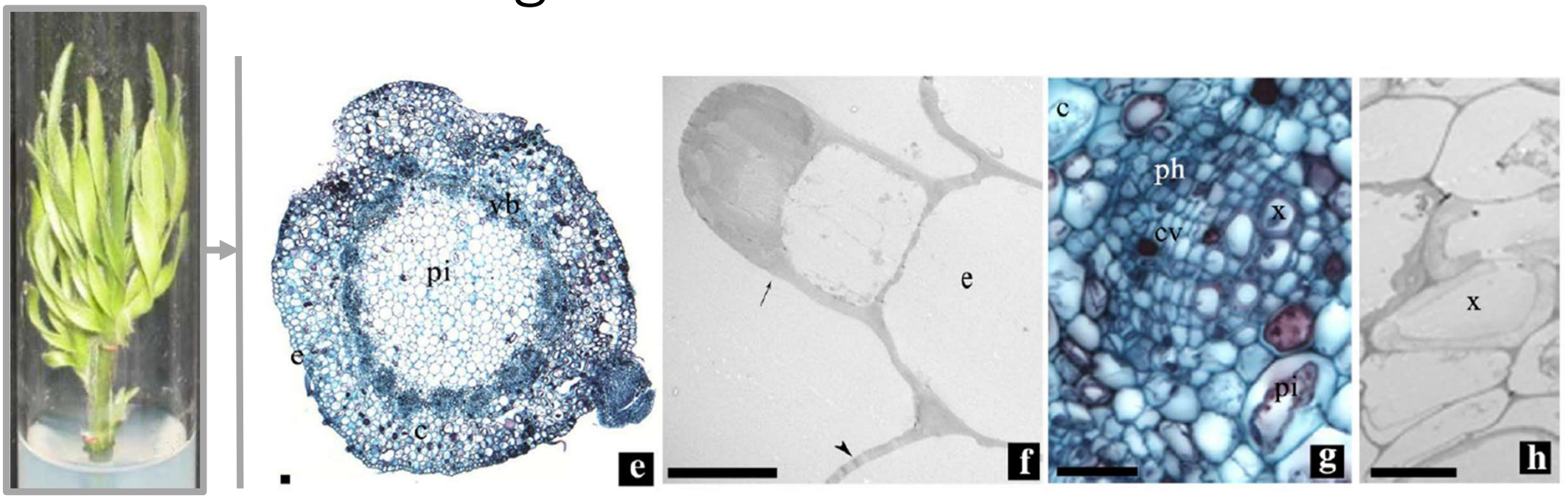
Epidermal cells with frontal view, adaxial surface with stomata and trichomes and a stomatal cluster of two stomata



Section of a leaf of *L. cordifolium* 'Flame Spike' from in vitro culture.
Unilayered epidermis with a thin cuticle and stomata on adaxial and abaxial sides, poor mesophyll.

(Suárez et al., 2019)

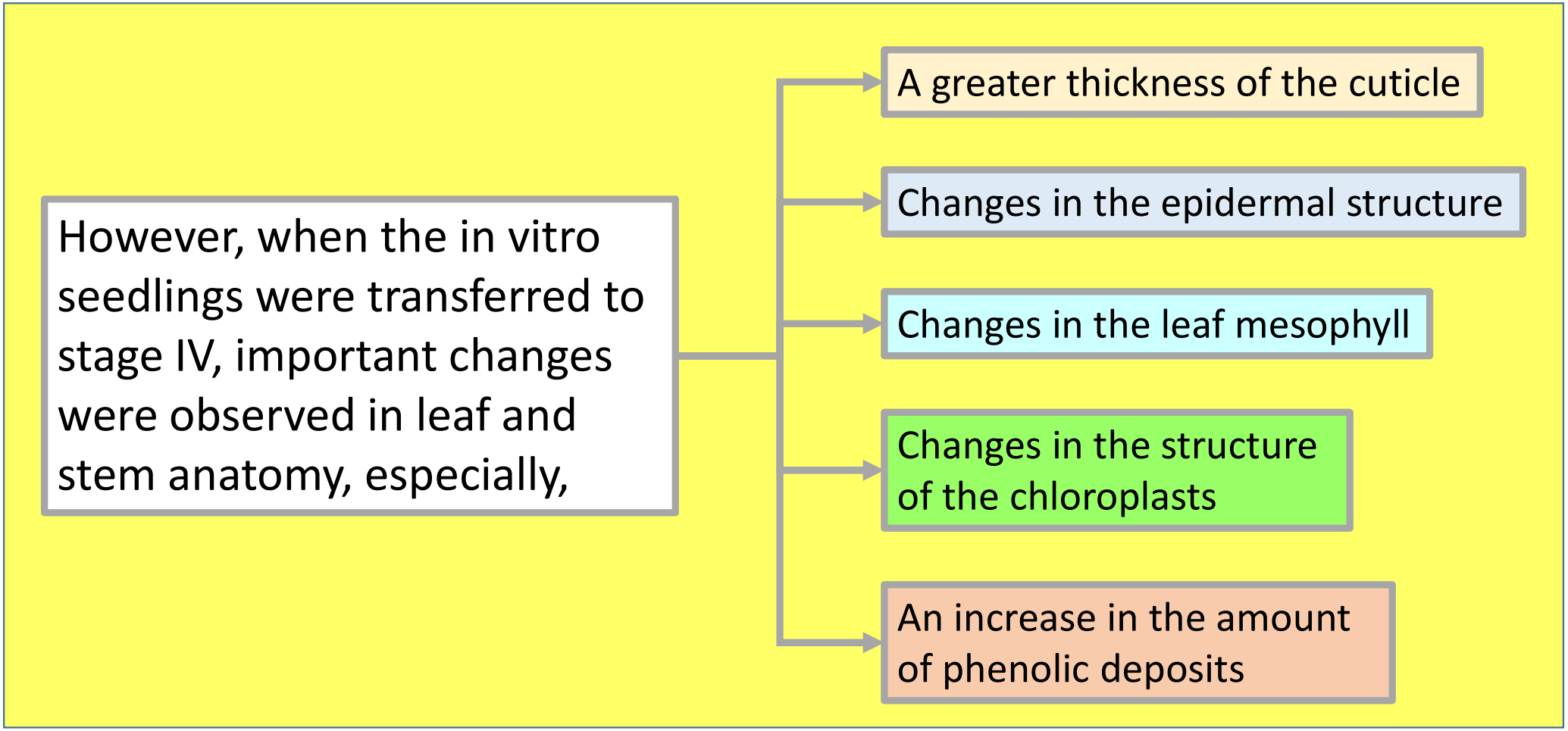
Stage IV: Acclimatization



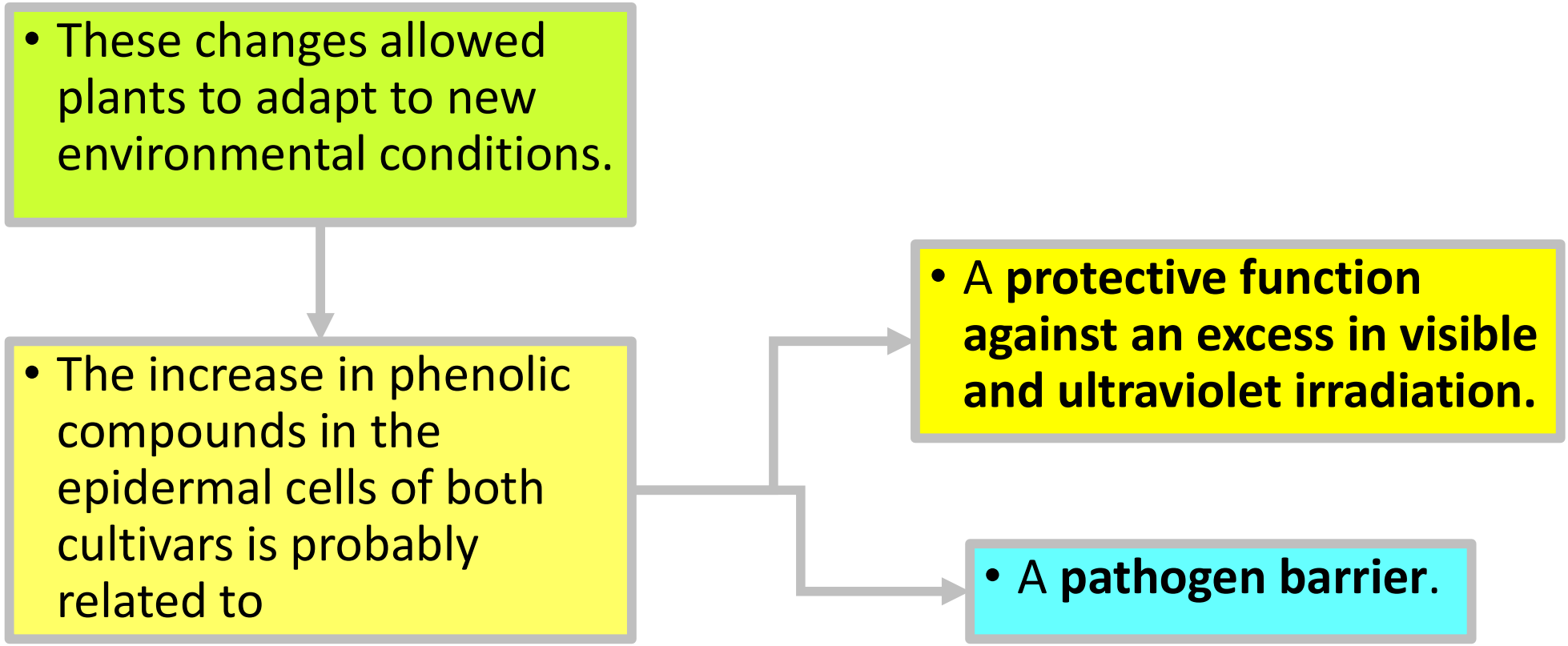
Light and transmission electron microscopy images of *Leucospermum* 'Tango' stem from in vitro culture.
e) Concentric tissue organization. Unilayered epidermis covered by a thin cuticle.
f) Cuticle, epidermis and simple trichome.
g) Collateral vascular bundle.
h) Xylem with thickened wall.

(Suárez et al., 2019)

Stage IV: Acclimatization



Stage IV: Acclimatization



CONCLUSIONS AND FUTURE PROSPECTS

- **The analysis of the literature shows that the micropropagation of proteas is feasible**
  - **for the rapid propagation of select cultivars free of diseases,**
  - **for the introduction of new cultivars and**
  - **for use in conservation programs for endangered species.**

However, more studies are needed for large-scale use for commercial purposes.

CONCLUSIONS AND FUTURE PROSPECTS

- We must focus efforts on reducing costs, incorporating automated processes and controlling and optimizing the microenvironment of culture.

To do this, the improvement of existing protocols must include:

- The shifting of solidified media with **liquid media**, especially for large-scale cultivation in bioreactors and in temporary immersion systems.
- The development of micropropagation protocols for **somatic embryogenesis** and **organogenesis** for large-scale use.
- The regeneration by organogenesis or somatic embryogenesis are a prerequisite for the **regeneration of genetically transformed cells and tissues** that can rapidly produce **new varieties**.
- The development of **photoautotrophic culture systems** is an interesting option that requires more research.

CONCLUSIONS AND FUTURE PROSPECTS

- Finally, it is very important to control **somaclonal variation**.
- There are no studies of this type in proteas and they are necessary even if the protocols are based on multiplication by pre-existing meristems.

- Also, **epigenetic variation**, especially **hyperhydricity**, must always be evaluated in any commercial micropropagation protocol.


END OF THE PRESENTATION
THANKS FOR YOUR ATTENTION
I WISH YOU ENJOY A NICE STAY IN TENERIFE
Pedro Hernández Rodríguez, 2006