An improved mineral nutrient solution for the *in vitro* propagation of *Pinguicula* species

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Keywords: *in vitro* cultivation, *Pinguicula*, tissue culture.

Introduction

Propagating plants *in vitro* (tissue culture) is widely used to rapidly generate large quantities of disease-free plants. It is applied to hundreds of carnivorous plant species including the genus *Pinguicula* whose species are very sensitive to pathogenic microorganisms and, in some cases, may develop poorly under current cultivation practices (Legendre & Kibellis 2005).

Adams *et al*. (1979) and Gonçalves *et al*. (2008) have respectively reported the successful micropropagation of *P. moranensis* and *P. lusitanica* on a diluted version of the mineral nutrient solution originally published by Linsmaier & Skoog (1965). This was based on the nutrient composition described by Murashige & Skoog (1962) with modifications of the vitamin content. Nevertheless, most *Pinguicula* species cannot be grown on this nutrient solution because they will develop leaf tip burns (*P. moctezumae*), reduce in size after each transplanting (*P. filifolia*), quickly form dormant hibernaculae (all temperate European species), or simply do not grow at all (*P. ramosa*, *P. parvifolia*).

This article aims to share some pieces of personal experience and the composition of an improved mineral nutrient solution that alleviates the above-described symptoms and which can be used to successfully propagate most *Pinguicula* species.

Materials and Methods

Plant material

All species used in this study were obtained from a horticultural company (Nature et Paysages, France) except *P. vulgaris*, *P. gigantea*, and *P. macroceras* seeds which had been collected from the Champagne state of France, Ayautla, Mexico, and Douglas Park, California, USA, respectively. *P.* sp. "huahuapan" had affinity with *P. rectifolia* and *P.* sp. "la vuelta", *P*. sp. "santa maria yukuhiti" had affinity with *P. moranensis*. The hybrid *P. moctezumae* × *P. gigantea* was prepared by the author.

Explant sterilisation

Seeds of all species were surface-sterilized before being introduced to tissue culture vessels. For this, they were soaked for 6 min (with regular vigorous shaking) in a 4% calcium hypochlorite solution supplemented with 0.1% Tween-20 (this solution was filtered immediately before use to discard the detergent-induced bleach precipitates) followed by 3 rinses of 10 min each in sterile distilled water. Dead seeds and plant debris floated in the surface foam while live seeds sank to the bottom of the tubes. This allowed their easy separation either by specifically sucking away the live seeds with

a sterile glass Pasteur pipette or by pouring away the sterilizing and rinsing solutions containing the non-seed material.

Growing conditions

Plants were grown in 5 cm diameter glass vessels (3-6 plants per pot) in a growth chamber maintained at constant temperature (23°C) with an air conditioning unit. They were lit with fluorescent tubes (alternating industrial white and warm, reddish, light tubes, each 10 cm apart) placed 40 cm above the plants. Lights were on 16 h per day.

Liquid media were sterilised by autoclaving at 121°C for 20 min. Plant transfers were made in sterile air in laminar flow hoods.

Plants (0.5 cm diameter) were subcultured 3 times on Mix A (see below) before being transferred to Mix A or Mix B for the comparative study. Their diameter was then measured 8 weeks later.

Mineral nutrient solutions

Distilled water was used to prepare all stock and final solutions.

Mix A: Macronutrients 10X stock (stored at 4ºC for up to 3 months): NH_4NO_3 (16.5 g/l), KNO_3 (19.0 g/l), $CaCl_2$ (3.322 g/l), $MgSO_4$ (1.807 g/l), KH_2PO_4 (1.70 g/l) (Murashige & Skoog 1962).

Micronutrients 1000X stock (stored at –20ºC for several years): KI (0.83 g/l), H_3BO_3 (6.2 g/l), $MnSO_4.H_2O$ (16.9 g/l), $ZnSO_4.7H_2O$ (8.6 g/l), $CuSO_4.5H_2O$ (0.025 g/l), $CoCl_2.6H_2O$ (0.025 g/l), $Na_2MoO_4.2H_2O$ (0.25 g/l). This later substance was dissolved separately in 100 ml water before being added to the final micronutrient mix (Murashige & Skoog 1962).

Fe-EDTA 200X stock (stored at 4ºC away from light for up to 3 months): Na_2EDTA (7.45 g/l dissolved in 900 ml of nearly boiling water), $FeSO_4.7H_2O$ (1.86 g/l added with small increments to boiling hot EDTA). The volume was subsequently adjusted to 1 liter with water. A couple of drops of concentrated HCl were added if iron salts did not dissolve. Crystals formed in the refrigerator during storage but could be re-dissolved by a quick boil in a microwave oven (Dalton *et al*. 1983).

Vitamins 1000X stock (stored at –20ºC for several years): myo-inositol (100 g/l), Thiamine.HCl (0.4 g/l), glycine (2 g/l) (Linsmaier & Skoog 1965).

Final mix: 25 ml/l 10X macronutrient stock, 1 ml/l 1000X micronutrient stock, 3.78 ml/l 200X iron-EDTA stock, 1 ml/l 1000X vitamin stock, 20 g/l sucrose (food grade), pH 5.9 (adjusted with 1 N KOH), 5 g/l Agargel (Sigma A-3301). This specific grade of Agar gave the best results.

Mix B: Macronutrients 10X stock (stored at 4ºC for up to 3 months): NH_4NO_3 (4.125 g/l), KNO_3 (19.0 g/l), $CaCl_2$ (3.322g/l), $MgSO_4$ (1.807 g/l), KH_2PO_4 (1.70g/l).

Micronutrients, Fe-EDTA, and Vitamins stocks were the same as in Mix A.

Final mix: 50 ml/l 10X macronutrient stock, 1 ml/l 1000X micronutrient stock, 3.78 ml/l 200X iron-EDTA stock, 1 ml/l 1000X vitamin stock, 20 g/l sucrose (food grade), pH 5.9 (adjusted with 1 N KOH), 5 g/l Agargel (Sigma A-3301).

Results and Discussion

In a first series of experiments, the growth of *P. moranensis* and *P. agnata* were compared when grown on serial dilutions of Linsmaier & Skoog's (1965) nutrient solution (undiluted and 2-, 4-,

8-, and 16-fold dilutions). The fastest growth was obtained with 4- to 8-fold diluted nutrient solutions in agreement with the formulation used by Adams *et al.* (1979) and Gonçalves *et al*. (2008). A comparison of the growth of the same two species on 4-fold diluted nutrient solutions, the pH of which were either adjusted with NaOH or with KOH, revealed that sodium ions (Na^+) were toxic to these species and the use of KOH yielded faster growth (data not shown). Similarly, the use of the iron-EDTA formulation developed by Dalton *et al.* (1983) was found to significantly improve the performance of these two species (prevents iron, and other metal ions, from precipitating during autoclaving). Because repeated transplanting led to regularly more stunted specimens, the nutrient solution was modified to dilute the macronutrients 4 times while maintaining a constant concentration of the other nutrients (micronutrients, iron-EDTA, and vitamins). Several studies have indeed demonstrated that micronutrients can be growth-limiting if their concentrations are half of optimum values that are, more or less, common to most land plants (Jones 1997). All of these basic adjustments of composition of the nutrient solution have led to the design of Mix A of the present study.

Nevertheless, other species (most noticeably *P. filifolia*, for example) did not grow optimally on Mix A and displayed reduced growth after each subculture. For this reason, a new formulation (called Mix B) was designed and assayed in a comparative study after an initial round of propagation on Mix A. Mix B differed from Mix A by having half the ammonium nitrate and twice the other macronutrients (1/8 the ammonium nitrate and half of the other macronutrients than in Murashige & Skoog, 1962). As shown in Table 1, Mix B allowed most of the tested *Pinguicula* species to develop to larger sizes. Species, like *P. filifolia* and all European temperate species, responded very positively to the change in nutrient solution, while others (from Mexico) were unaffected. Typically, those species that exhibited size reduction during subculturing on Mix A were the ones that fared better on Mix B. All of the species listed in Table 1 were subsequently subcultured 12 times on Mix B with no significant reduction in growth rate or size.

Table 1. Comparative growth of some *Pinguicula* species on tissue culture mineral nutrient solutions Mix A and Mix B.

Species name	Final plant diameter (cm; mean ± SD)*	
	Mix A	Mix B
P. filifolia #	4.0 ± 1.0	8.0 ± 1.0
P. grandiflora #	1.5 ± 0.6	3.7 ± 0.6
P. longifolia subsp *longifolia* #	2.3 ± 0.6	4.7 ± 0.6
P. moctezumae #	3.0 ± 0.9	5.5 ± 0.6
P. vulgaris #	1.2 ± 0.6	3.0 ± 0.1
P. macroceras #	1.7 ± 0.5	2.7 ± 0.6
P. sp. "huahuapan" ns	4.0 ± 1.0	4.3 ± 0.6
P. sp. "la vuelta" #	2.7 ± 0.6	3.7 ± 1.5
P. sp. "santa maria yukuhiti" #	1.5 ± 0.6	2.5 ± 0.6
P. moctezumae × *P. gigantea* ns	2.7 ± 0.9	3.0 ± 0.8

* 8-week old plants – starting plant diameter: 0.5cm; # Significant difference in growth between the two lots of plants (Student t-test, $p<0.05$). ns: non-significant ($p>0.05$). n=3-6.

A set of species that did not develop (or grew very slowly) on Mix A (*P. parvifolia*, *P. heterophylla*, *P. oblongiloba*, and *P. ramosa*) was directly cultured on Mix B. All species developed surprisingly well on Mix B.

Two interesting observations were made during the comparative study of the nutrient solutions, Mix A and B. First of all, long-leaf species such as *P. moctezumae* and its hybrids developed dead leaf tips on Mix A, a phenomenon that also occurs occasionally when plants are grown *ex vitro* (on soil). This disorder was absent from plants grown on Mix B. Additionally, temperate European species did not form winter buds (and entered dormancy) as quickly on Mix B as on Mix A. So, not only did they develop faster on Mix B, but they remained longer in active growth on this mix, growing larger plants in a shorter time. This suggests that both phenomena may be caused by a deficiency of some minerals.

Unlike in Adams *et al.* (1979), the exogenous addition of hormones to the tissue culture medium was not an absolute requirement to induce the multiplication of *Pinguicula* species *in vitro* (also observed by Gonçalves *et al.*, 2008). All species multiplied spontaneously when left long enough on their growth media.

One drawback of *in vitro* technology is that it leads to an inevitable drift in the plants genetic make-up. Though tissue cultured plants are multiplied vegetatively, and are therefore, supposed to be genetically equal, somaclonal variants spontaneously arise (even when no hormones are added exogenously). These variants can be unintentionally selected because of the massive number of plants that are generated in comparison to the small number of specimens that are selected when subculturing fresh media for the next round of propagation, especially in the case of species that grow slowly. I have indeed noticed that I was systematically transferring the best-looking (best-growing) specimens. This led, for example, to the selection, after two years of micropropagation, of a clone of *P. ramosa* that was growing much faster and was making plants twice as large as the mother plant from which it originated.

Acknowledgement: The author wishes to thank Lubomir Adamec for his careful review and suggestions about this manuscript.

References

Adams, R.M., Koenigsberg, S.S., and Langhans, R.W. 1979. *In vitro* propagation of the butterwort *Pinguicula moranensis* H.B.K. HortSci. 14: 701-702.

Dalton, C.C., Iqbal, K., and Turner, D.A. 1983. Iron precipitation in Murashige and Skoog media. Physiol. Plant. 57: 472-476.

Gonçalves, S., Escapa, A.L., Grevenstuk, T., and Romano, A. 2008. An efficient *in vitro* propagation for *Pinguicula lusitanica*, a rare insectivorous plant. Plant Cell Tiss. Organ Cult. 95: 239-243.

Jones, J.B., Jr. 1997. Hydroponics: A practical guide for the soilless grower. St. Lucie Press. Boca Raton, Florida, USA.

Legendre, L., and Kibellis, H. 2005. Disease symptoms in *Pinguicula*: Some causes and remedies. Carniv. Pl. Newslett. 34: 16-23.

Linsmaier, E.M., and Skoog, F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100-127.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.