

## Scaling-up the biomass production of *Cymbopogon citratus* L. in temporary immersion system

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### ABSTRACT

Shoot-tips, collected from greenhouse-grown plants of *Cymbopogon citratus* L. (lemmon grass), were incubated on a semi-solid Murashige and Skoog (MS) medium with 30% (w/v) sucrose, and supplemented with 0.89  $\mu\text{M}$  6-benzyladenine (BA). After three weeks of culture shoots were individualized and then inoculated in 10 litres temporary immersion system (TIS) containing 3 litres of the same basal MS liquid medium. The effects of three immersion frequency (immersion every 12, 6 and 4 hours) on the production of biomass were studied. Three inoculum densities (forty, fifty and sixty shoots/TIS) were also tested. The biomass growth was influenced by the immersion frequency. The highest proliferation rate (17.3 shoots/explants) and the plant length (45.2 cm) were obtained in plants immersed every 4 h. Also, the fresh and dry biomass weight (153.4 gFW and 24.8 gDW, respectively) were higher in this treatment. The maximum biomass accumulation (185.2 gFW and 35.2 gDW) was achieved after 30 days of culture when an inoculum density of 60 explants per TIS was used. For the first time, biomass of *C. citratus* has been produced in 10 litres TIS. These results represent the first step in the scaling-up the biomass production of this medicinal plant in large temporary immersion bioreactors.

Key words: automation, biomass growth, lemmon grass medicinal plant, tissue culture

## Escalado de la producción de biomasa de *Cymbopogon citratus* L. en biorreactores de inmersión temporal

### RESUMEN

Se colectaron ápices meristemáticos de *Cymbopogon citratus* L. (c) (caña santa) a partir de plantas cultivadas en invernaderos. Los ápices fueron incubados en un medio de cultivo semisólido Murashige y Skoog (MS) con 3% (m/v) de sacarosa y 0.89  $\mu\text{M}$  de 6-benciladenina (BA). Los brotes fueron individualizados después de tres semanas de cultivo e inoculados en sistemas de inmersión temporal de 10 litros de capacidad, los cuales contenían 3 litros de medio de cultivo líquido de igual composición. Se estudió el efecto de tres frecuencias de inmersión (cada 12, 6 y 4 horas) en la producción de biomasa. También, se determinó el efecto de la densidad de inóculo. El crecimiento de la biomasa estuvo influenciado por la frecuencia de inmersión. La mayor proliferación de brotes (17.3 brotes/explantes), así como la mayor longitud de la planta (45.2 cm) se obtuvo en el tratamiento con una inmersión cada 4 h. La masa fresca y seca fueron también superiores en este tratamiento (153.4 gMF y 24.8 gMS, respectivamente). Después de 30 días de cultivo la máxima acumulación de biomasa se obtuvo cuando se utilizó una densidad de inóculo de 60 explantes por SIT (185.2 gMF y 35.2 gMS). Por primera vez, se logró producir biomasa de *C. citratus* en sistemas de inmersión temporal de 10 litros. Estos resultados representan el primer paso hacia el escalado de la producción de biomasa de esta planta medicinal en sistemas de inmersión temporal de gran capacidad.

Palabras clave: automatización, caña santa, crecimiento de biomasa, cultivo de tejidos, planta medicinal

Abbreviations: BA, 6-benzyladenine; MS, Murashige and Skoog basal medium; DW, dry weight; FW, fresh weight; SEM, scanning electron microscope; TIS, temporary immersion systems

### INTRODUCTION

Temporary immersion system (TIS) is a cheap technology for automation of *in vitro* plant tissue culture and has been successful used for *in vitro* propagation of medicinal plants (Preil, 2005). From an economical view point, RITA<sup>®</sup> and TIS of 1

litre capacity has been proven to be suitable for research at laboratory scale; but for commercial application, large vessels are frequently used. Moreover, the most important tropical species are commercially propagated in TIS using twin flasks with a capacity ranging from 5 to 10 litres (Jiménez, 2005).

Because TIS allows large scale culturing of plant organs at low cost, it became an attractive alternative for the production of plant secondary metabolites. In this sense, TIS has been described for *in vitro* culture of a wide range of medicinal plant species such as *Lavandula officinalis* Chaix and *Hypericum perforatum* L. (Wilken *et al.*, 2005), *Mentha spicata* L. (Tisserat and Vaughn, 2008), *Saccharum officinarum* L. (Yang *et al.*, 2010), *Camptotheca acuminata* Decne (Sankar-Thomas and Lieberei, 2011), *Panax quinquefolius* L. (Uchendu *et al.*, 2011), *Astragalus membranaceus* (Wuet *et al.*, 2011), *Digitalis lanata* L. (Pérez-Alonso *et al.*, 2012), *Leocojum aestivum* L. (Schumann *et al.*, 2012).

*Cymbopogon citratus* (D.C.) Stapf. is a perennial and medicinal herb belonging to the *Poaceae* family. It has been widely cultivated in tropical and subtropical countries to produce *C. citratus* oil, which fundamentally contains citral, farnesol, nerol, citronellal and myrcene. Citral is an essential oil component, mainly located in the leaves and primarily used to flavour food (Paranagama *et al.*, 2003). Although the biomass production of *C. citratus* has also been described by conventionally suckering (Licea *et al.*, 1999), from callus and cell suspension (Quiala *et al.*, 2002), only in shoots  $\alpha$  and  $\beta$  citrals and free radical scavengers (Wilken *et al.*, 2005; Tapia *et al.*, 2007) have been detected. Moreover in previous research we reported the *in vitro* propagation of *C. citratus* in 1 liter TIS (Quiala *et al.*, 2006). However, there are no experiences on biomass production in large culture vessels. Therefore, this research was conducted to optimize the immersion frequency and the inoculum density in 10 liters temporary immersion bioreactors as the first step in the scaling-up the biomass production of this medicinal plant in large temporary immersion bioreactors.

## MATERIALS AND METHODS

### *Plant material and TIS culture*

Shoot-tips, collected from greenhouse-grown plants of *C. citratus* were surface sterilized in a commercial solution of sodium hypochlorite (3% NaOCl) for 15 min. Under aseptic conditions and with the help of a stereo-microscope, shoot tips of about 1.0 mm size were excised and immediately placed onto a semi-solid Murashige and

Skoog (1962) medium (MS) with 30% (w/v) sucrose, and supplemented with 0.89  $\mu\text{M}$  6-benzyladenine (BA). After three weeks of culture shoots were individualized and used for TIS culture. Single shoots were used in all experiments; they were inoculated in 10 litres TIS containing 3 litres of basal MS medium supplemented with 6-benzylaminopurine (0.89  $\mu\text{M}$ ) and sucrose (30 g l<sup>-1</sup>).

The concept and operation of the TIS used in the experiments were based on the two-vessel system of 10 liters capacity (Nalgene®) as described by (Escalona *et al.*, 1999). The immersion rhythm was regulated by computer software (Byosis version 1.0).

### *Experimental design*

In the first experiment, the effect of three immersion frequencies (every 12, 6 and 4 hours each with 2 min duration) on shoot biomass growth was studied. An inoculum density of forty single shoots (8.3 gMF) per TIS was used.

In the second experiment three inoculum densities (40, 50 and 60 single shoot/TIS) corresponding to 8.8, 10.6 and 12.5 gMF respectively. Immersion frequency was selected from the results in the first experiment.

The systems were incubated at 28 $\pm$ 2°C under a 16/8h (day/night) photoperiod with light supplied by white fluorescent tubes (25  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ). For both experiment, the number of shoots per explant, shoot length (cm), fresh weight (FW) and dry weight (DW) (g) were determined after four weeks of culture.

### *Statistical analysis*

Data were processed using the SPSS software package for Windows ver. 15. Results were analyzed by the Non-parametric test: Kruskal–Wallis. Differences were considered significant at  $p < 0.05$ .

## RESULTS AND DISCUSSION

A fast increase of *C. citratus* biomass production in TIS was obtained in all treatments. Biomass accumulation was influenced by the immersion frequency. Significantly more biomass was accumulated in the shoots immersed every 4h, corresponding to six immersions per day

(Table 1). Also, the highest values of dry weight, proliferation rate, as well as the plants length were obtained with this treatment.

The maximum biomass and growth ratio were observed with an inoculum density of 60 explants (12.5 g of FW) (Table 2). Shoot proliferation was stimulated in the first two weeks (Figure 1a), but at the end of the experiment the shoots elongated and grew up to the top of the vessel (Figure 1b). A high amount of biomass was harvested (185.2 gFW and 35.2 gDW) after 4wks of culture (Table 2) (Figure 1c).

In an earlier study we found a major production of *C. citratus* biomass with four immersions per day in 1 liter TIS (Quiala *et al.*, 2006). However, in 10 liters TIS the best results were achieved with six immersions per day. Similar results have been described by others authors. The immersion frequency had a significant effect on biomass accumulation and yield of betalains in hairy root cultures (Etienne and Berthouly, 2002). The importance of immersion frequency for the production of biomass and secondary

metabolite was also demonstrated with *Digitalis purpurea* L. (Pérez-Alonso *et al.*, 2009). These authors describe a positive effect of immersion frequency (every four hours) on biomass growth and cardenolides accumulation.

It was demonstrated than 10 liters TIS clearly offered an advantage for the production of a high amount of *C. citratus* biomass. The fast biomass growth in TIS could be the result of the combination of the advantages of both gelled culture (gas exchange) and liquid culture (increased nutrient uptake), which improves the growth of the plantlets (Etienne and Berthouly, 2002). However, renewal of the head space in the TIS with each immersion led to a higher oxygen concentration (Etienne and Berthouly, 2002), probably contributing to the higher biomass of *C. citratus*. In TIS, using larger vessels, large volumes of media can be used. This has a positive effect on the proliferation and growth of the shoots, after immersion a thin layer of culture medium may remain on the surface of the plant, which avoids desiccation and promotes nutrient uptake (Preil, 2005).

Table 1. Biomass accumulation and growth of *C. citratus* shoots after 30 d of culture in 10 liters temporary immersion systems (TIS) under different immersion frequencies.

Immersion frequency (every hour)	Fresh weight/TIS (gFW)	Dry weight/TIS (gDW)	Number of shoot/explant	Shoot Length (cm)
12	41.2±1.45 c	10.3±2.01 c	7.05±1.80 c	10.90± 2.01c
4	116.1±2.03 b	18.1±2.32b	13.50±1.55b	29.17±1.92b
6	153.4±2.71a	24.8±2.28 a	17.3±2.04a	45.20±2.08 a

\*Data are means ± SE from three independent experiments, each with three replicates (n=9). Different letters represent significant differences among treatments according to Kruskal-Wallis and Student-Newman-Keuls ( $p \leq 0.05$ )

Table 2. Effect of inoculum density on biomass accumulation of *C. citratus* after 30 d of culture in a 10 liters temporary immersion system.

Inoculum density (Number of shoots per TIS)	Inoculum		Harvested yield	
	Fresh Weight per TIS (g)	Dry Weight per TIS (mg)	Fresh Weight (gFW per TIS)	Dry Weight (gDW per TIS)
40	8.8	46.1	160.2c	27.1b
50	10.6	55.4	174.6b	30.2b
60	12.5	63.2	185.2a	35.2 a

\*Immersion frequency: every 4h. Data are means ± SE from three independent experiments, each with three replicates (n=9). Different letters represent significant differences among treatments according to Kruskal-Wallis and Student-Newman-Keuls ( $p \leq 0.05$ )



Figure 1. *C. citratus* *in vitro* plants in a 10 litres temporary immersion system after 30 days of culture. a) Shoots cultured in TIS with an immersion frequency of every four hours and inoculum density of sixty shoots/TIS (2 wks culture), b) 4 wks after culture (shoots elongated and grew up to the top of the vessel), c) Harvested biomass at the end of the culture (rule length 15 cm).

For all TIS, the volume of the container, and hence the head space, is much higher than in containers used for conventional procedures. Moreover, containers ranging in size from one to twenty can usually be adapted to the system and it is necessary to adjust some parameters that have been pre-established during the experimental scale in smaller containers (Jiménez, 2005).

In conclusion, the optimization of the immersion frequency and the inoculum density increased the biomass growth of *C. citratus* in large temporary immersion bioreactors. The maximum biomass accumulation was achieved using an immersion frequency every four hours and 60 shoots/TIS per 10 liters TIS (3 liters working volume) as inoculum density. This is the first steps in the scaling-up the biomass production of lemon grass in TIS. The optimization of others parameters such as the frequency of the renovation of culture medium and the optimal volume of them could be a promissory strategy for continuous improving the biomass growth of this medicinal plant in 10 liters temporary immersion bioreactors.

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