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Conference presentation

Plant Tissue culture and Ultra High Diluted studies: suggesting a novel model using *in vitro* techniques.

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INTRODUCTION

Plant tissue culture may be defined as the aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions (Pierik, 1987; Torres et al., 1998)

The first reports regarding plant tissue culture date back to 1902 when Gotlieb Harberlandt developed for the first time experiments to maintain mesophyll cells in culture. Since then, *in vitro* techniques have been used in a wide range of applications providing information of great value about problems related to basic and applied aspects of plant science, evaluating the effects of many different substances and/ or conditions in plant growth and development as well as contributing to understanding of factors responsible for growth, metabolism, synthesis of secondary compounds and stress response, for instance.

In vitro culture requires some essential conditions: all plant tissue culture techniques are undergone under axenic and controlled conditions (culture medium composition, light and temperature, for instance). Once more, genetically identical plants commonly used in many studies can be used in order to avoid intraspecific variations.

Many researches on Ultra High Dilutions have been suggesting that standardized laboratory conditions and homogeneous quality of plants may decrease variables that could, in some way, distress that experiments (Majewsky et al.,2009, Jäger et al., 2015).

In vitro culture attempt to these statements providing both controlled conditions and homogeneous cultures by using clonal plants. Considering that, plant tissue culture seems to be a value model for Ultra High Diluted (UHD) but, since now, none studies involving these techniques and UDH research seems to be undergone, once no scientific paper were find.

In order to evaluate a possible use of plant tissue culture to study UHD responses it was chosen *Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson. This specie is a small shrub native of South America that belongs to Verbenaceae family. This plant produces essential oils with analgesic, anti-inflammatory, anticonvulsant, antifungal, and myorelaxant properties (Aguiar et al. 2008; Carmona et al. 2013; Oliveira et al. 2014).

Lippia alba tissue cultures and *in vitro* essential oil production have already been described in scientific literature (Gupta et al., 2001; Tavares et al., 2004; JibinaBai et al., 2014). Other *Lippia* species were still investigated providing many comparable results about the effect of plant growth regulator on *in vitro* development and essential oil profile (Julianni et al., 1999; Peixoto et al, 2006).

Benzilaminopurine is larger used in *Lippia* species cultures and well know as a shoot improver in vitro.

None of all scientific papers evaluated the effects of UHD substances on *in vitro* development or secondary metabolic production.

AIMS

The main goal of this research was to evaluate the use of plant tissue culture to investigate the effects of UHD 6 benzylaminopurine (BA) on *Lippia alba* shoot culture.

METHODS

Whole experiment was performed keeping the cultures at 25°C in controlled environment growth room under 16 day light hours provided by fluorescent day light tubes.

The experiment was conducted in two different laboratories, one of them was in account of preparing test substances and the other responsible for maintain the *in vitro* culture. The experiment was blinded all the time.

Preparing test substances

The experiment was conducted using 6 benzylaminopurine 12CH (10^{-24}), water 12CH, a 3µmol BA solution as positive control and no potentized water as negative control.

All test solutions were prepared freshly on the day of the experiment from the same batch of distilled water.

BA (SIGMA -Aldrich) was dissolved in distilled water in order to obtain the 3µmol stock solution that was used for further potentization process.

According to Brazilian Homeopathic Pharmacopoeia (Brazil, 2011), the whole process consists in successive centesimal (1:99) dilutions steps followed by succussion that, in this case, was carried out through a "mechanic-arm" device model knows as "DENISE". By the same method, 12CH water was prepared. All test solutions were submitted to sterile filtration using membranes with 0,24 µ size pores.

After preparation, all solutions were randomized and coded by a person not involved in the next experimental step.

Plant tissue culture

Nodal segments were obtained from *in vitro Lippia alba* plantlets previously growth on Murashige and Skoog, (1962) basal medium (MS). The nodal segments were subcultured to a double- phase medium that consist in a semi-solid medium with a layer of liquid medium on the top. The semi-solid media was the MS basal medium purchased from Sigma Aldrich solidified with 2.5 g.L-1 Phytagel The pH of all media was adjusted to 5.8 before autoclaving for 15 min at 121°C and 1.1 kg/cm2 pressure. The liquid on the top consisted of 2 ml the sterile test substances, added after the MS medium was sterilized.

Culture vessels were opened in laminar flow chamber and 1 ml of solutions were added to cultures weekly.

Statistical analysis

After 30 days, plantlets were kept out from vessels and evaluated for number of shoots, shoot length, rooted plants (%), callus development (%) and fresh biomass.

The experiment was repeated twice and each one consisted in 3 culture vessel with 5 nodal segments per treatment. The final sample size was 30 plants per treatment.

The data were analysed by one-way analysis of variance (ANOVA) following by Duncan's and t-test ($p<0.05$)

RESULTS

During the 30 days, all treatments promoted morphological responses in *Lippia alba* nodal segments (Figure 1).

Plants from water 12CH and BA 12CH increased the number of new shoots and promoted the highest shoot length (Table 1). The plant growth regulator kinetin, which belongs to the same class of BA, enhanced pea shoot growth (Baumgartner et al., 2004)

Table 1: *In vitro Lippia alba* nodal segments development under different test substances

	Evaluated parameters				
Treatments	New shoots average number	Average shoot lenght (cm)	Fresh biomass (mg)	% Rooted plants (n)	% callus development (n)
Water (no dilution and succession)	0.90 [b]	2.09 [a]	132.7 [a]	60 [a] (18)	20 [b] (6)
BA 3µmol	0.0 [c*]	0.0 [b**]	144.0 [a]	0 [b] (25)	87 [c] (26)
BA 12 CH	1.33 [a]	2.46 [a]	116.4 [a]	70 [a] (21)	10 [ab] (3)
H_2O 12 CH	1.10 [a]	2.44 [a]	130.9 [a]	76.7 [a] (23)	0 [a] (30)

*axilary bud developed but shoot not completely elongated. **shoot smaller than 0.3 cm thus making accurate measure impossible. Mean values followed for same letter, in a column, do not differ significantly in Duncan's test or Student-test (for percentages analyses) at 5% level of statistical significance

We cannot exclude any unspecific physicochemical effects due to succussion as reason for the observed effect of BA 12 CH, since the potencies were analogously prepared as the succussed water controls (Baumgartner et al., 2004)

By adding BA 3µmol the organogenetic response was inhibited since neither shoot nor root were developed. However, it was observed a significant basal callus development. Adding BA to *Lippia alba* leaf explants also induced callus formation in other experiments (JibinaBai et al., 2014) while reduced shoot length developed from nodal segments in other *Lippia* species (Peixoto et al, 2006; Julianni et al., 1999).

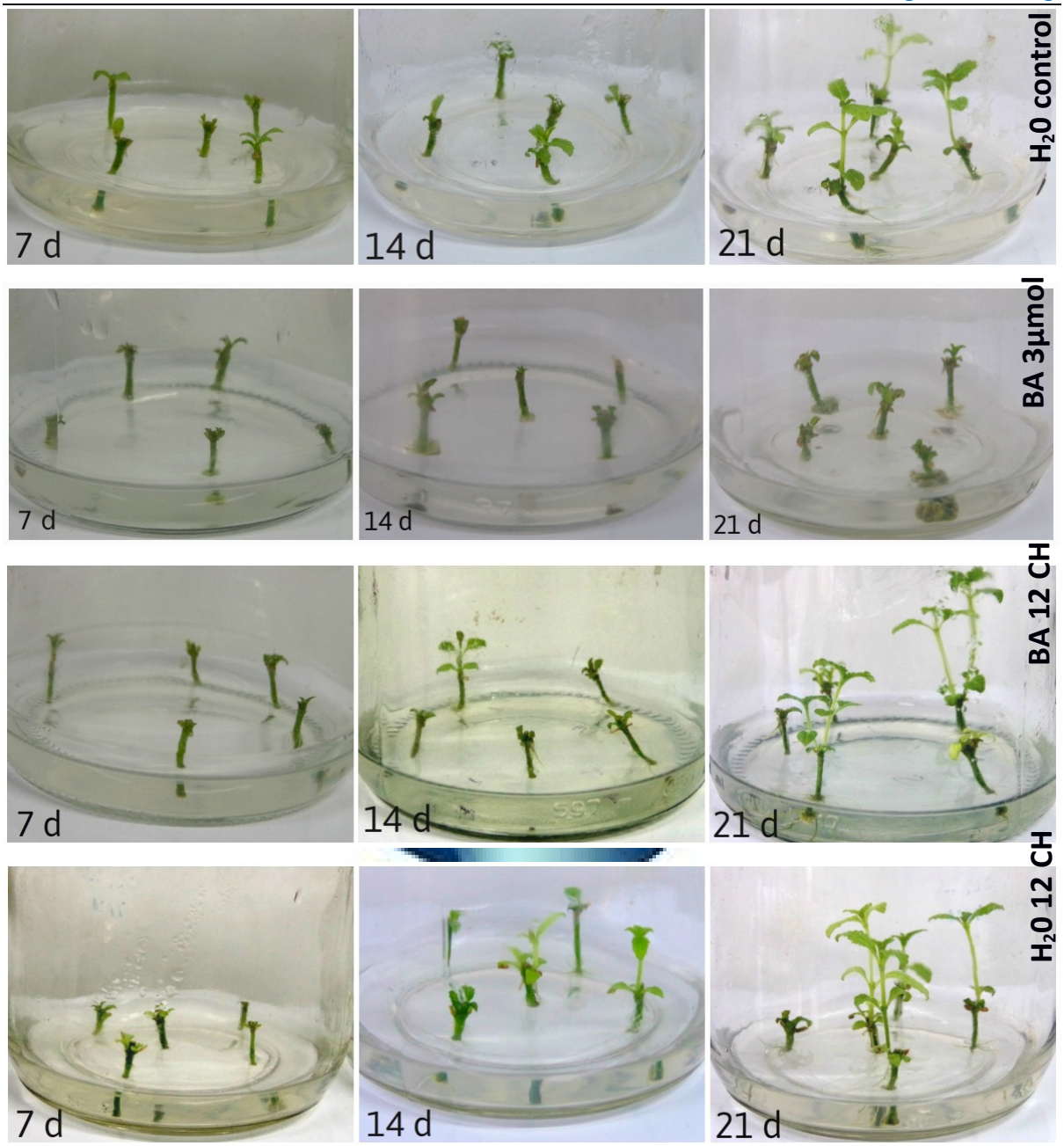

Figure 1: *Lippia alba* nodal segments development under different treatments. 7d – 7days *in vitro*; 14d – 14 days *in vitro*; 21d – 21 days *in vitro*

CONCLUSION

So, these preliminary results suggest that 6 benzilaminopurine modified growth in *Lippia alba in vitro* plants, promoting callus development when used in molar concentration and promoting shoot growth when potentized. Dilution and succussion have significantly different results altering *in vitro* plant development. It is of paramount importance that more experiments be undertaken in order to verify if the test system is stable and reliable. In such way, more studies

are being conducted to analyse other experimental conditions and biochemical and phytochemical parameters especially essential oil profile.

Plant tissue culture could be adapted for UHD studies as it has been extensively used for other purposes providing an opportunity to study UHD on more complex organisms.

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