Influence of light on micropropagation of *Pterospartum tridentatum* ecotypes: Malcata, Gardunha and Orvalho

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Abstract

Pterospartum tridentatum is an authoctnous shrub abundantly present in Beira Interior, Portugal. This plant was widely used in traditional medicine due to their bioactive compounds. In this region there are various ecotypes of *P. tridentatum* as Malcata, Gardunha and Orvalho. The objective of this work was to evaluate *in vitro* development capacity of the three ecotypes under three different types of light: dark, fluorescent light (cool-white fluorescent lamps) and LED (DR/B/FR 150). Explants previously established *in vitro* conditions, were introduced in MS medium, supplemented with 4,44 µM of BAP and 2,46 µM of IBA to grow under influence of each type of light. The assay was carried out in cycle of 45 days. At the end of multiplication cycle different biometric and biochemical parameters were recorded. In dark treatment all the explants of all ecotypes showed absent of color in stalks, as well as, the low content of chlorophylls. Another characteristic of this treatment was the highest elongation of the explants. The fluorescent light treatment promoted the higher number of new shoots and fresh weight, with Gardunha had an average of 8 new shoots and bigger fresh weight (0,5049 g). LED treatment produced explants with dark green coloration, however with low fresh weight, in the Orvalho ecotype. As expected the LED treatment showed the highest chlorophylls content. All ecotypes showed capacity of propagation and Gardunha ecotype was the best adapted to the different treatments. However, this experiment permitted to know the behavior of these three ecotypes in different types of illumination and also allowed understanding the best and more efficient *in vitro* development conditions for these ecotypes of *Pterospartum tridentatum.*

Keywords: *in vitro* development, artificial light, dark, LED, prickled broom.

Resumo

Influência da luz na micropropagação de ecótipos de *Pterospartum tridentatum*: Malcata, Gardunha e Orvalho

Pterospartum tridentatum conhecida por carqueja cresce espontaneamente com relativa abundância na Beira Interior. É uma planta bastante utilizada na medicina tradicional para o tratamento de diversas doenças devido à produção de compostos bioativos. Nesta região podemos encontrar diversos ecótipos: Malcata, Orvalho e Gardunha. O objetivo deste trabalho foi avaliar a capacidade de propagação *in vitro* dos três ecótipos sob diferentes tipos de luminosidade: escuro, luz fluorescente e LED

(DR/B/FR 150). Os explantes dos ecótipos, previamente estabelecidos *in vitro*, foram introduzidos em meio MS, suplementado com 4,44 µM of BAP and 2,46 µM of IBA. O ensaio decorreu durante um ciclo de 45 dias, no final do ciclo, foram avaliados parâmetros biométricos e o teor de clorofilas e carotenóides. No tratamento do escuro, as maiores diferenças surgiram na coloração dos explantes, apresentando explantes com ausência de coloração verde no caule, e consequentemente menor teor de clorofilas. Outra característica deste tratamento foi a produção de explantes com maior comprimento (9,5 cm). O tratamento sob luz fluorescente caracterizou-se por apresentar explantes com maior número de novos rebentos e maior peso fresco, no ecótipo Gardunha obteve-se uma média de 8 novos rebentos e um peso fresco médio de 0,5049 g. Como seria de esperar, o tratamento LED produziu explantes com maior teor de clorofilas. Todos os ecótipos revelaram capacidade de propagação nas condições estudadas, verificando-se que o ecótipo Gardunha revelou melhor adaptação aos diferentes tratamentos. Este estudo contribuiu para futuras adaptações de condições mais eficientes no desenvolvimento *in vitro* dos ecótipos de *Pterospartum tridentatum*.

Palavras-chave: propagação *in vitro*, luz fluorescente, LED, escuro, carqueja.

Introduction

Pterospartum tridentatum (L.) Willk. is an European endemic shrub, belonging to Fabaceae family (Talavera, 1999) that is very common in the Portugal mountains namely in Beira Interior region. In this region, *P. tridentatum* grows spontaneously in acidic soils and can be found in different mountains as Malcata, Gardunha and Orvalho. This shrub can grow up to 100 cm, presenting yellow flowers, alternates branches and coriaceous winged stems (Teixeira & Pereira, 2004; Ferreira et al., 2012). Rural populations harvest flowers and leaves for use in traditional medicine. After drying it is used for hot infusions for treatment of many health disorders as intestinal problems, rheumatism, colds, stomach ache and diabetes, due to the presence of bioactive compounds (Vitor et al., 2004; Grosso et al., 2007; Neves et al., 2009). It is notable the value that this plant has for local populations and for treatment of their health problems and therefore it is important to establish it in *in vitro* conditions in order to preserve the natural populations. Not only for possible establishment of cultivation fields but also for establishment of efficient protocols of propagation with objective of obtaining bioactive compounds. The micropropagation has been the great tool for propagation of plants at risk or low in abundance and which have some phytochemical interest. Through micropropagation techniques it is possible to obtain a larger production and more uniform plants, avoiding a massive harvest of plants in their natural habitat. Naturally, the growth and development of plants in field is affected by biotics and abiotic factors, namely, temperature, humidity, water and nutrients availability, altitude, herbivore attacks, among others. In *in vitro* conditions, the majority of these factors also may be considered for a suitable development of plants, the quality and physiological condition for micropropagated explants are of great importance both in the subsequent multiplication and acclimatization for *ex vitro* conditions. Light is a crucial factor for development and multiplication of plants, in *in vitro* conditions is used artificial light, namely fluorescent lamps (Gupta & Jatothu, 2013). The power consumption of these lamps is expensive and produces a wide range of wavelengths (350-750 nm) needless for plant growth (Bello-Bello et al., 2017). Light quality plays an important role for photomorphogenesis, phototropism and photosynthesis, influencing the way in which light is absorbed by the chlorophyll (Tripathy & Brown, 1995). Light quality is the most

important propriety of lamps and can be evaluated by spectral distribution and the radiation output (Tantau, 1997). Currently, light emitting diodes (LEDs) have been developed and applied for *in vitro* plant propagation in growth rooms. LEDs show some advantages over existing systems such as wavelength specificity and narrow bandwidth, small size, long lifetime and low power consumption (Massa et al., 2008; Gupta & Jatothu, 2013). Several studies have been realized using LEDs lights on microprogation of different plants, Tripathy & Brown (1995) studied the effect of LEDs on chlorophyll biosynthesis in wheat, another studies confirm the better growth and development of explants using LEDs light compared with conventional systems (Lian et al., 2002; Kim et al., 2004; Nhut et al., 2005). Although light may be essential, some studies were performed in darkness (Leshem et al.,1982; Bach, 1987) where they emphasized the beneficial morphologic effects of darkness on plant growth. Initial studies for *P. tridentatum* micropropagation were established by Diogo et al. (2013) and Coelho (2015) where they was noted that the best composition medium for multiplication of this specie was MS (Murashige & Skoog, 1962) supplemented with 1 mg L^{-1} BAP and 0,5 mg L^{-1} for three ecotypes, using fluorescent light. In this work, we evaluated the growth and development of three *P. tridentatum* ecotypes in different treatments of light, dark, fluorescent light and LEDs and their chlorophylls content.

Materials and methods

Plant material and culture conditions

Nodal segments of *P. tridentatum* ecotypes, Malcata, Gardunha and Orvalho were micropropagated in a gelled medium containing MS nutrients (Murashige & Skoog, 1962), 3% (w/v) sucrose, supplemented with 4,44 µM 6-benzylaminopurine (BAP) and 2,46 µM indole-3-butyric acid (IBA), added 7 g L^{-1} agar and pH adjusted to 5,5 before autoclaving (*Steri 21 Lab*) at 216,9 kPa for 20 minutes at 121 °C. These explants were placed under dark, fluorescent lamps (OSRAM, L58W/840) with 50 µmol m^{-2} s^{-1} PPFD (light intensity) and LED lamps DR/B/FR 150 (PHILIPS, GreenPower LED) with 62,5 µmol m^{-2} s^{-1} PPFD. After 45 days in these conditions, ten nodal segments with 1,5 cm length were placed in glass flasks during a new cycle of 45 days. At the end of cycle the biometrics parameters and chlorophylls and carotenoids contents were evaluated.

Evaluation of culture growth

For evaluation of culture growth, the length of explants, new shoot number, multiplication factor (nodal segments number with 1,5 cm obtained from each explant), callus and roots presence, color of leaves, using a reference grade (figure 1) were evaluated. Fresh and dry weights were also recorded at the end of cycle. Dry weight was determined after drying the material at 105 °C after 24 hours. For growth parameters analysis 20 random explants were selected.

Chlorophylls and carotenoids contents

Chlorophyll a, chlorophyll b and carotenoids contents were estimated based on the procedure described by Sumanta et al. (2014) with some modifications. 30 mg of fresh leaves were weighted and macerated in a mortar with 1 mL of 96 % ethanol, collecting this solution in a microtube. The mortar was cleaned with more 1 mL of 96 % ethanol and this last solution was mixed at the first solution in a same microtube. After, this mixture was centrifuged at 12000 rpm for 10 min at 4 °C. The supernatants were collected and the absorbance of solution was measured at 470, 649 and 665 nm in

spectrophotometer (*Specord, 200 PLUS, Germany*), the same solvent was used as blank. For determination of chlorophyll-a, chlorophyll-b and carotenoids contents were using the following formulas:

$$Chl\ a = \left(13{,}95 \times A_{665} - 6{,}88 \times A_{649}\right)$$

$$Chl\ b = \left(24{,}96 \times A_{649} - 7{,}32 \times A_{665}\right)$$

$$Car = \left(1000 \times A_{470} - \left(2{,}05 \times Chl\ a - 114{,}8 \times Chl\ b\right)\right)/245$$

The results were expressed on μg mL^{-1} of chlorophyll and carotenoids per 100 g fresh weight.

Statistic analysis

To evaluate differences in morphological parameters we used Wilcoxon tests between variables because data did not have a normal distribution, p-value < 0,05 were considered to be significant. The statistical analysis was performed on SPSS software, version 23.

Results and discussion

Effect of lightening in morphologic characteristics

The results of morphologic parameters are presented in Table 1. In dark treatment is observable that the ecotype Malcata had a bigger length comparatively to fluorescent and LED treatments. For Malcata and Orvalho ecotypes the smaller length was obtained under fluorescent light. The highest of new shoot number was verified in Gardunha under dark and fluorescent light with values 6 and 8, respectively. The highest multiplication factor were verified in Gardunha under fluorescent light, even as the highest fresh and dry weights (figure 2) with values 0,5049 g and 0,0762 g, respectively. In agreement with our results, this ecotype was more suitable at different treatments. According to other studies, the use of LED on micropropagation resulted in identical or higher fresh weight than when it was used fluorescent light, as it happenned in Malcata under fluorescent and LED light (Kim et al., 2004; Lian et al., 2002; Shin et al., 2008). The evaluation of fresh weight is important to verify the normal development of culture, however dry weight is also significant because shows compounds content of explants biological structures. For Orvalho ecotype there were no differences in multiplication factor for all treatments. According to scale established previously for color of leaves (figure 1), the dark treatment showed a translucent green, attributed value 4, in fluorescent light the explants shown the level 1-2, as dark green and green, respectively, and LED treatment was characterized by level 1 of color, as dark green. In all treatments it was confirmed the absence of roots. Callus was present in all treatments, namely in dark treatments where callus size increased, which may be due to callus photosensitive to auxin used (IBA) and its accumulation promoted callus formation (Koshiba et al., 1993; Yokawa et al., 2014). The importance of the dark treatments focuses to evaluate the growth capacity of cultures in absence of light, contributing to the reduction of production costs and micropropagation facility. These results support the hypothesis that the explants cultivated in the darkness can be submitted to the next micropropagation stage, cultivation under regular light intensities, and have its phytochrome system reactivated and the tissue re-pigmented. Also the action of plant growth regulators (as auxins and cytokinins) is light-dependent and they show remarkable degradation when exposed to the light, particularly auxins (Neumann et al., 2009). This fact could the response to dark treatment, which is more dependent on media components, for example the growth regulators and their degradation. According

with results it is possible to verify that for the same species, each variety had different responses in their growth according with different light conditions, these aspects are important for to adapt the best *in vitro* conditions for each culture.

Effect of lightening in chlorophylls and carotenoids content

Chlorophyll a, chlorophyll b and carotenoids are crucial for photosynthetic process in plants, the content of foliar pigments depends on the species and their content varies with internal factors and environmental conditions, namely with light. Several studies proved that blue and red LED are greatly efficient for plant photosynthesis. Blue light is important for chlorophyll biosynthesis, stomatal opening, maturation of chloroplast and photosynthesis (Tibbitts et al. 1983), while red LED exerted beneficial effects on the explants length under *in vitro* conditions (Gupta & Jatothu, 2013). Chlorophyll a (Chl a) is an ubiquitous pigment and can be used as a global biomass indicator (Denant et al., 1991). According Saebo et al. (1995), an important aspect of chlorophyll a is the plantlets with the smallest chlorophyll content thus seem to use the chlorophyll more efficiently than plantlets with excessive chlorophyll. As expected, our results (figure 3) in dark treatments were obtained the smallest chlorophylls content, that confirm the importance of light in development of these biological substances (Tripathy & Brown, 1995). Bello-Bello et al. (2017) also shown lows values of chlorophylls in dark treatments. The highest chlorophyll a content was obtained in Gardunha LED at corresponding with a color level 1 (dark green). For all ecotypes we obtained the highest value of chlorophyll a in LED treatment, that agree with others authors, they also obtained the best values of Chl a in LED treatments comparing with fluorescent light (Shin et al., 2008; Hung et al., 2016).

Conclusions

The quality and development of *in vitro* cultures are very important for their suitable growth. Environmental concerns and research of more efficient *in vitro* methodologies are also an apprehension for industries and research centers. With our results it is possible to verify that the dark treatment is a good way for *P. tridentatum* micropropagation, however further studies are required to confirm their suitable *ex vitro* acclimatization. Also it was verified that for same species but different ecotypes, the lightening effect had an influence in growth *P. tridentatum*, suggesting that there are ecotypes more adapted at light changes than others.

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Figure 1 - Reference grade established for color of leaves of *P. tridentatum* (1- dark green; 2- green; 3- light green and 4- translucent green).

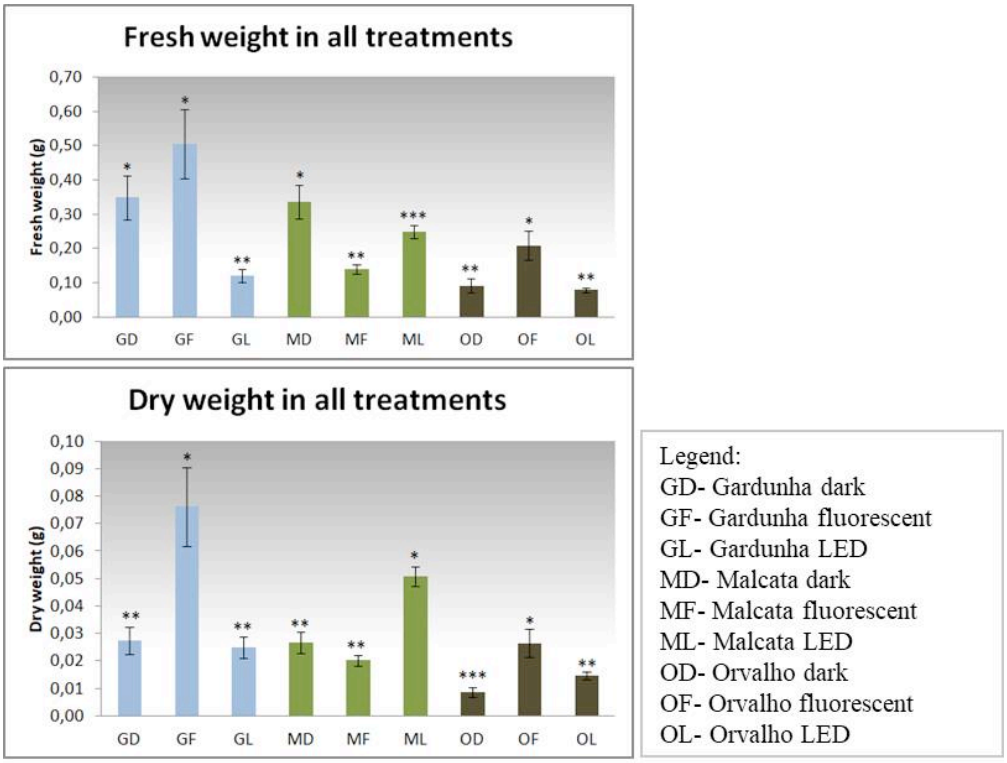

Figure 2 - Results of average fresh and dry weights in dark, fluorescent light and LED treaments for *P. tridentatum* Gardunha, Malcata and Orvalho ecotypes. Different signal (*) means significant differences between same ecotype (p<0,05).

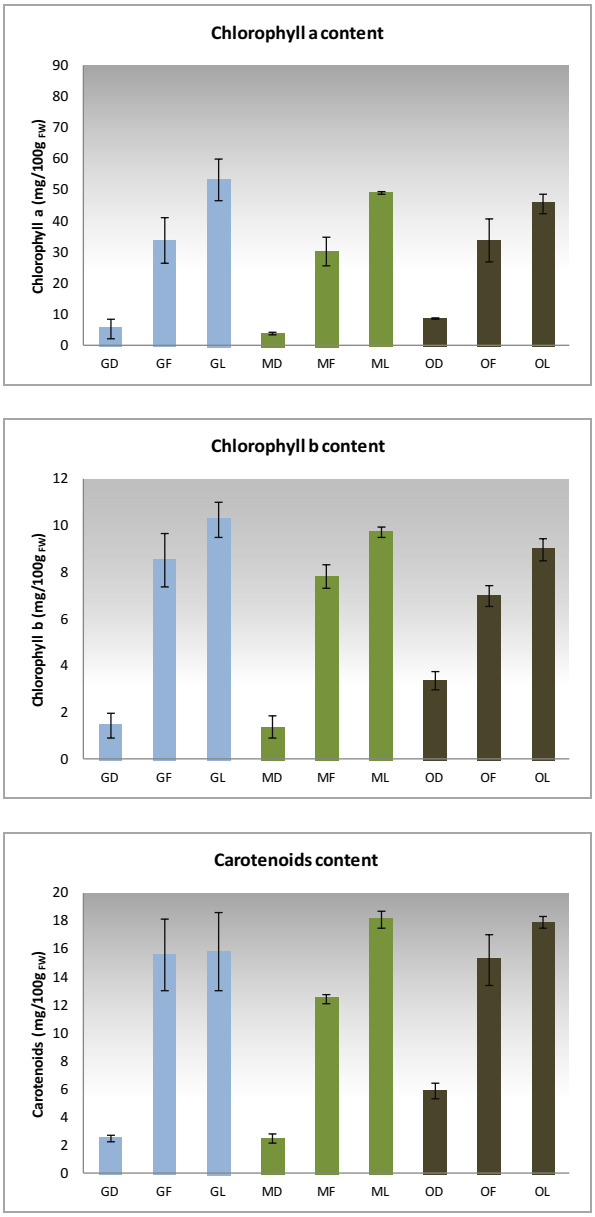

Figure 3 - Results of average chlorophyll a, b and carotenoids content in treatments dark, fluorescent light and LED for *P. tridentatum* Gardunha, Malcata and Orvalho ecotypes. See legend of Figure 2. Results in terms of average and standard deviation of triplicates.

Table 1 - Results of length, new shoot number and multiplication factor of three *Pterospartum tridentatum* ecotypes (M- Malcata, G- Gardunha and O- Orvalho).

Treatment	Length (cm)			New shoot number			Multiplication factor		
	M	G	O	M	G	O	M	G	O
Dark	$9,5\pm1,1^{a;A}$	$5,9\pm1,3^{a;B}$	$3,4\pm1,6^{a,b;C}$	$4\pm1,7^{a;A}$	$6\pm3,1^{a,b;A}$	$3\pm1,6^{b;B}$	$14\pm5,2^{a;A}$	$13\pm7,1^{a;A}$	$4\pm2,8^{a;B}$
Fluorescent	$3,6\pm0,6^{c;B}$	$5,1\pm1,7^{a,b;A}$	$3,2\pm0,7^{b;C}$	$2\pm1,2^{c;C}$	$8\pm3,5^{a;A}$	$5\pm2,3^{a;B}$	$2\pm1,8^{c;C}$	$15\pm8,5^{a;A}$	$5\pm3,7^{a;B}$
LED	$4,1\pm0,7^{b;A}$	$4,3\pm0,7^{b;A}$	$4,2\pm0,9^{a;A}$	$3\pm1,2^{b;B}$	$5\pm2,5^{b;A}$	$3\pm0,8^{b;B}$	$4\pm2,1^{b;B}$	$8\pm3,9^{b;A}$	$4\pm1,0^{a;B}$

Data are presented as average of 20 explants per treatment ± standard deviation. Different lower case letters indicate significant differences between treatments for same ecotype and different capital letters indicate significant differences between ecotypes for same treatment (p<0,05).