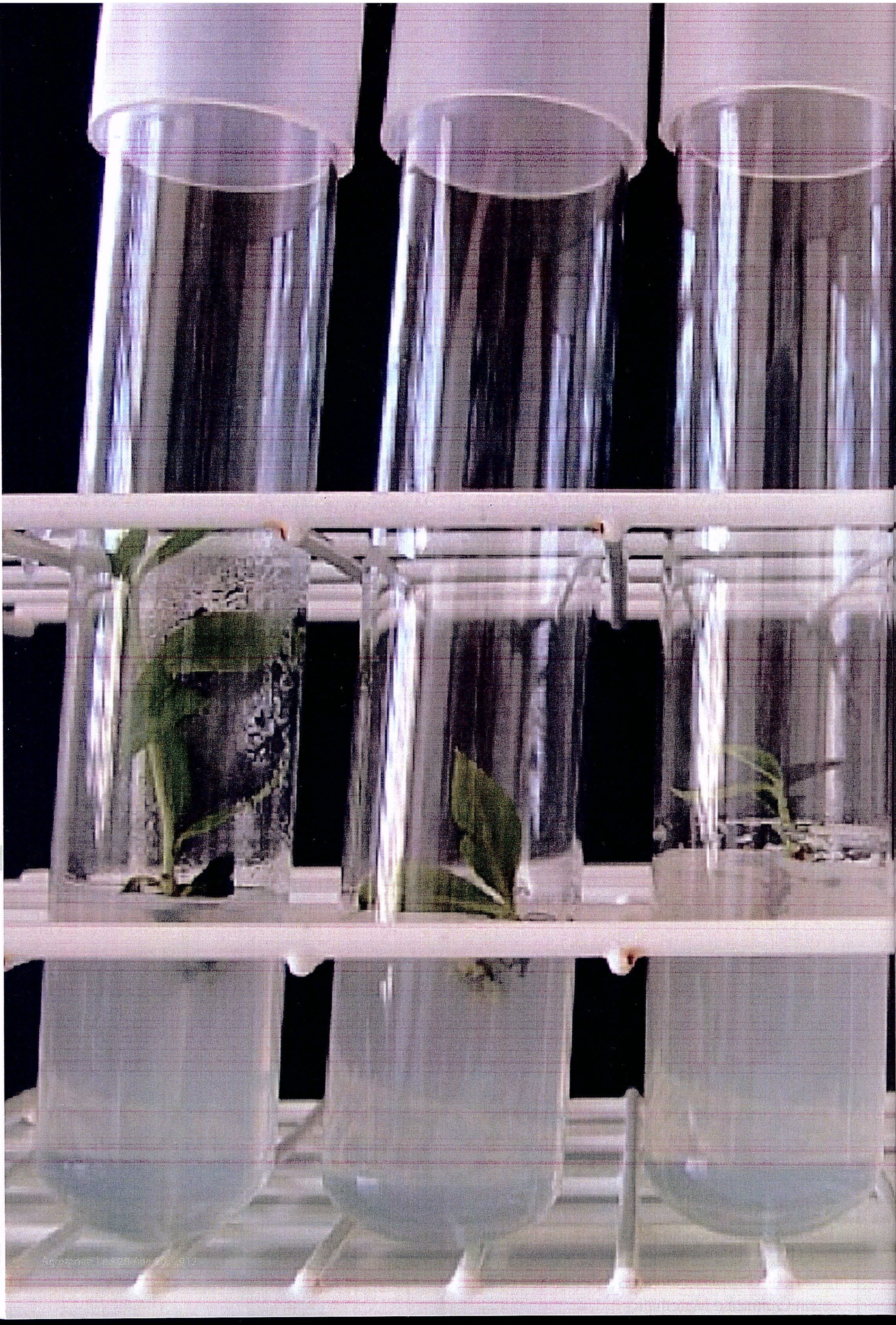

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Common Myrtle *in vitro* Propagation - Establishment and Multiplication Stages

Propagação da Murta in vitro - Fases de Estabelecimento e Multiplicação

ABSTRACT

The use of medicinal plants for the extraction of active pharmacological agents and precursors for chemical-pharmaceutical chemosynthesis has recently increased. The aim of this work was to study the establishment and multiplication phases of the in vitro propagation of common myrtle (*Myrtus communis* L.). In the establishment phase, the most suitable disinfection method was found by using increasing concentrations of commercial bleach (10%, 15% and 20%). It was discovered that commercial bleach at 15% allowed better results in the shoots survival rate (87%). The concentrations used to establish the explants from adult plant material did not allow however that all of them survived. This was possibly due to the some release of phenolic substances, and, also, to a certain toxicity of the disinfectant used, especially at the highest concentration.

M. T. Coelho
Department of Life and Food Science. Escola Superior Agrária. Instituto Politécnico de Castelo Branco. Portugal.
mteresacoelho@ipcb.pt

G. Diogo
Department of Life and Food Science. Escola Superior Agrária. Instituto Politécnico de Castelo Branco. Portugal.

R. Reis
Department of Natural Resources and Sustainable Development. Escola Superior Agrária. Instituto Politécnico de Castelo Branco. Portugal.

M. M. Ribeiro
Department of Natural Resources and Sustainable Development. Escola Superior Agrária. Instituto Politécnico de Castelo Branco. Portugal.

During the multiplication phase it was intended to assess the influence of light, through coloured bottle caps, on the shoots growth rate parameters, but we observed that it had no influence on the average of the longest shoot, on the average of the smaller shoot, and also, on the parameters related to the multiplication rate, the number of shoots and the number of shoot segments.

Key words: disinfection method, light factor, micropropagation, *Myrtus communis* L., tissue culture.

RESUMO

O uso de plantas medicinais para extração de agentes farmacológicos e de precursores da síntese de substâncias quimio-farmacêuticas tem vindo a aumentar recentemente. O objetivo deste trabalho foi estudar as fases de estabelecimento e de multiplicação *in vitro* da murta (*Myrtus communis* L.). Na fase de estabelecimento, o método de desinfeção mais adequado foi encontrado usando doses crescentes de lixívia comercial (10%, 15% e 20%). A concentração de lixívia a 15% promoveu a melhor taxa de sobrevivência (87%). Os métodos de desinfeção usados para estabelecer os explantes com base em material adulto não permitiram que todos os explantes estabelecidos sobrevivessem. Isso foi devido, possivelmente, à libertação de algumas substâncias fenólicas e, também, a alguma toxicidade do desinfetante usado, especialmente nas concentrações mais elevadas.

Durante a fase de multiplicação pretendeu verificar-se a influência da luz através da opacidade das tampas dos tubos nos parâmetros de crescimento, mas observámos que esta não influenciou o comprimento médio do maior rebento e do menor rebento e no número de segmentos por rebento.

Palavras chave: Cultura de tecidos; luz; Método de desinfeção; micropropagação; *Myrtus communis* L.

1. INTRODUCTION

Myrtus communis L. (common myrtle), a species from the *Myrtaceae* family, is an evergreen shrub up to 5 m, typical from the Mediterranean area. In Portugal it grows spontaneously all over the country, but not in the Center and Northern inland regions (Bingre et al., 2007). Myrtle, an outcrossed insect-pollinated species, has a flowering period from May to June, and the fruits are multi-seeded berries (Lopez González, 2004).

Common myrtle is widely exploited for essential oils used in the pharmaceutical, cosmetic and food industries, and used, since ancient times, for medicinal, food and spice purposes (Messaoud et al., 2005). Myrtle has been reported to possess hypoglycemic, antimicrobial, and anti-hemorrhagic proprieties (Sepici et al., 2004). Recently, the antimicrobial properties and assessed anticancer activity of some myrtle compounds were confirmed (Fadda and Mulas, 2010, and references therein). Consequently, the myrtle industry is increasing and needs to be supported by sounding crop yields. Vegetative propagation is important in horticulture, because the genotype of valuable cultivar and varieties is usually highly heterozygote in outcrossed species, and requires plant characteristics which are often lost by seed propagation (Hartmann et al., 2010). This species can be propagated by rooting softwood cuttings, but rooting rate depends strongly on seasonality, ranging from 70 to 20%, especially when adult plants are used (Klein et al., 2000). Micropropagation is a suitable method for obtaining a large quantity of genetically homogeneous and healthy plants which can be used for large scale production. Additionally, in vitro propagation will help to overcome seasonality and to establish protocols for rapid multiplication (Canhoto et al., 1999). Some examples in the literature on myrtle micropropagation have reported axillary shoot development (e.g., Scarpa et al., 2000; Parra and Amo-Marco, 1996) and somatic embryogenesis (Parra and Amo-Marco, 1998; Canhoto et al., 1999), but no study has tried to establish a simple disinfection protocol to increase explants survival or studied the effect of light in both the establishment and multiplication phases.

The disinfection method is a key step when plant material from field adult trees is used. When weak disinfection concentration is used contamination by microorganisms might appear, and higher concentrations might be toxic. Thus, establishing a simple and fast disinfection protocol to increases survival of explants is very important for commercial production (Ko et al., 2009).

A pre-incubation period of about a week was required for myrtle shoot establishment to prevent the oxidation of phenolic compounds released at the beginning of the culture, resulting in shoot browning and death (Parra and Amo-Marco, 1996). To avoid the pre-incubation period, we decided to verify if the light decrease, due to the use of a metal cap to seal the culture tubes, could prevent the same phenomenon and increase shoot survival. With the strawberry tree (*Arbutus unedo* L.) the establishment and multiplication phases were more successful when metal caps were used to seal the tubes. Under transparent caps the shoots oxidized, as a result of phenols release oxidation, and shoot growth was significantly lower. Higher mortality was verified when compared with significantly lower mortality and higher shoot growth using metal caps (J.C. Gonçalves, unpublished results). In Mediterranean species, phenolic contents are usually high, and helpful against harsh environmental conditions and predators, thus we have studied the influence of transparent (plastic) versus metal (opaque) caps use in shoot growth during the multiplication phase of myrtle.

The aim of this work was to study the in vitro propagation of common myrtle. In the establishment phase we wanted to know the best sodium hypochlorite concentration to be used together with the light influence, and in the multiplication phase we intended to assess the influence of the light factor through the coloured bottle caps in the shoots growth parameters.

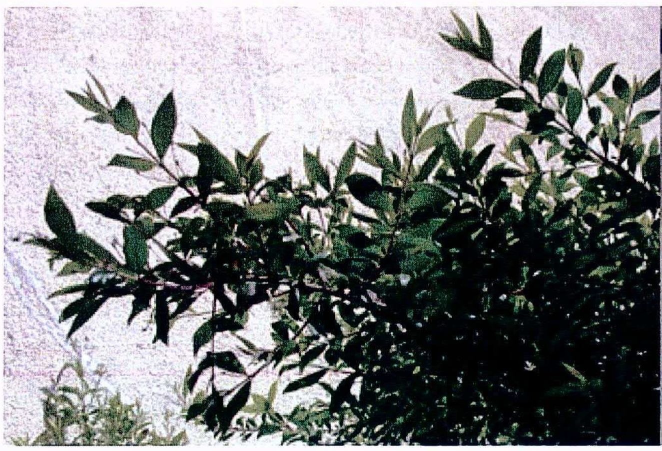
2. MATERIAL AND METHODS

2.1. Plant material, shoot preparation and disinfection

Young softwood shoots (180), with 10 to 15 cm, were collected from a common myrtle plant growing on the campus of the Escola Superior Agrária of Castelo Branco, in early June (Fig. 1a). Afterwards, the shoots were washed with tap water for 5 min., immersed in a fungicide solution (6% Benlate, 50% p/p benomil) for 20 min., and dipped in 70% (v/v) ethyl alcohol for 30 s. Subsequently, the shoots were disinfected using three different commercial bleach concentrations (Domestos containing 5% of sodium hypochloride) 10%, 15% and 20% v/v water (final concentration of 0.5, 0.75 and 1% of sodium hypochlorite, respectively), with one drop of Tween 20 added to the solution, for 10 min., and 60 shoots were used per treatment. Finally, the disinfected shoots were washed three times in sterile water.

2.2. Establishment phase conditions

Each shoot was cut in 3-4 nodal segments, about 1.5 cm long and leaves were removed. Explants were transplanted into an establishment medium containing MS (Murashige and Skoog, 1962) macronutrients half strength, supplemented with thiamine, pyridoxine, nicotinic acid, ascorbic acid, all at 1 mg/L, 100 mg/L inositol and the following growth regulators 0.2 mg/L 6-benzylaminopurine (BAP) and 0.01 mg/L α-naphthaleneacetic acid (NAA). The media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar and the pH was adjusted to 5.5-5.6 before autoclaving at 121 °C for 20 min.. The nodal segments were inoculated in test tubes containing 15 mL of medium. Agar and growth regulators were supplied from Sigma Chemical Co. Each tube was sealed with a transparent cover or a metal cap. The cultures were kept under controlled environmental conditions in culture ARALABTM room with a 16 h photoperiod, under a photosynthetic photon flux density (PPFD) of 50±5 μmol m-2 s-1 provided by fluorescent light type TLD 18/40 and temperature at 25 °C day and 22 °C at night. After four weeks the shoot survival (S) and mortality (M) per bleach concentration treatment and coloured metal cap was measured.



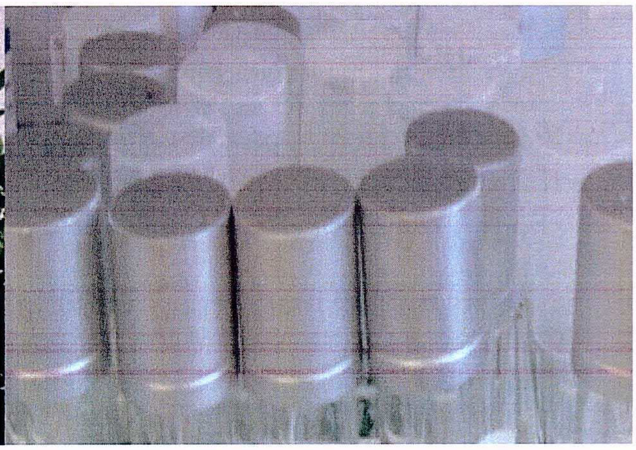


Fig. 1. Mother-plant branch (a). Metal (opaque) and plastic (translucent) stopgaps used in the experiment (b).

2.3. *In vitro* multiplication phase

Afterwards, nodal segments (10 mm) were sub-cultured in test tubes containing 15 mL of the same establishment medium. Each tube was sealed with a transparent cover or a metal cap (Fig. 1b). The growth conditions were the same as described for the establishment phase. The treatments studied were the two types of cap tubes, metal and plastic. After six weeks the following parameters were measured per treatment: the longest shoot length (LSL), the smallest shoot length (SSL), the number of shoots (NS), and the number of segments (NSG).

2.4. Data analysis

The results were analyzed using the SPSS version 17.0. The data gathered in the disinfection procedure was discrete, thus not Gaussian, and we used the contingency tables approach and the G-test (likelihood) to verify an association among the treatments (bleach concentration and cap colour) and the shoot survival/mortality. The null hypothesis was rejected when the probability of type I error was lower than 5%, and the results were factor dependent. The continuous data obtained during the multiplication phase (LSL and SSL, 60 shoots per treatment) was analyzed by one-way analysis of variance. A mixed linear model was used (Steel and Torrie, 1981): $X_{ij} = \mu + P_i + \varepsilon_{ij}$, the number of shoots i=1,...,k, the μ is the general mean, the measured parameter P_i is considered the fixed effect, and ε_{ij} is the experimental error. The discrete data (NS and NSG) obtained during the multiplication phase was analyzed using descriptive statistics.

3. RESULTS

3.1. The effects of bleach concentration and stopgap translucence on shoot survival and mortality

The results showed that the explants survival depended on the bleach concentration used to sterilize the plant material. With the 15% treatment 52 explants survived, but only 40, a significantly lower number, with the 20% concentration. Nevertheless, the G-test revealed that no significant differences existed between the 10 and the 15% of disinfectant concentration. Conversely, the colour of the cap had no influence on the explants survival rate in any bleach concentration treatment (Fig. 2).

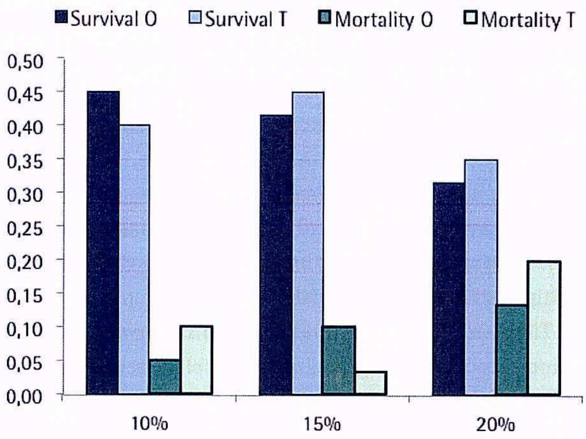


Fig. 2. Percentage of shoot survival and mortality per disinfection treatment and stopgap colour (O=opaque and T=translucent).

3.2. The effects of cap translucence on growth induction

No significant differences were found between the mean of the longest shoot length (LSL) obtained in the tubes sealed with metal (1.3 ± 0.8 cm) or plastic cap (1.2 ± 0.7 cm), clearly perceived through the standard deviation (SD) overlapping (Fig. 3).

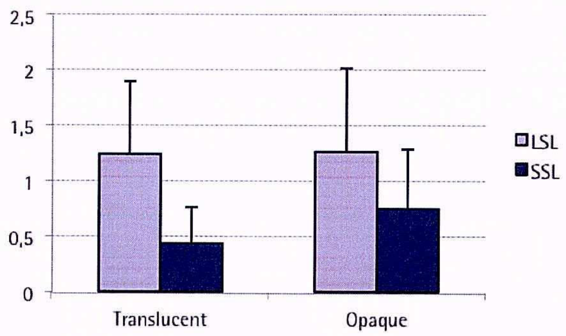


Fig. 3. The mean longest shoot length (LSL) and mean smallest shoot length (SSL) recorded per stopgap translucence treatment.

A similar result was obtained with the mean of the smallest shoot length (SSL) recorded per stopgap translucence treatment. The type I error was low (P = 0.065) and near the 5% threshold, thus the mean SSL value for the shoots in tubes with opaque cap was higher (0.8 cm) than the value for translucent caps (0.4 cm), yet not significant (Fig. 4). Additionally, among shoots length the variability was very high, regardless of the parameter (LSL or SSL). The coefficients of variation (CV) also achieved high values, particularly for SSL (data not shown). In agreement with the previous results, the discrete variables, the mean number of shoots (NS) and the mean number of segments (NSG), were not influenced by the opaque vs. translucent caps, and the SD overlapping between treatments are very clear in Figure 4, in both treatments.

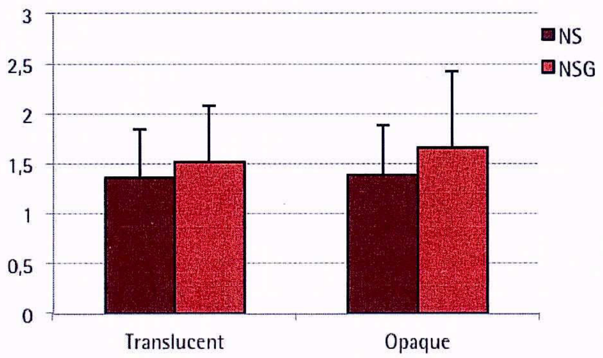


Fig. 4. Mean number of shoots (NS) and mean number of segments (NSG) recorded per stopgap translucence treatment (b)

4. DISCUSSION

The disinfection concentration (for 10 min.) that promoted the highest explant survival (52 out of 60) was 15% of commercial bleach concentration, yet not significantly different from the 10% concentration (0.5% sodium hypochlorite). The latter concentration might be used for economic reasons, since a similar number of explants were established. With the highest bleach concentration (20%) 1/3 of the shoots died, thus a toxic level was probably achieved. We suppose that the antimicrobial properties of the species might help in preventing microbiologic contamination from field collected material and disinfectant at low doses might be enough. In strawberry (*Fragaria vesca* L.) three genotypes survival rates ranged from 89.2 to 100%, in a study by Ko et al. (2009), with a suitable disinfection method: sodium hypochlorite at 0.25%.

Parra and Amo-Marco (1996) used a preincubation period of about a week to establish myrtle from field plants to prevent the oxidation of phenolic compounds and shoots browning. In order to avoid the preincubation period we decided to verify if the decrease in light, with the use of metal cap vs. transparent, could prevent that phenomenon and help increasing shoot survival. Still, the tube cap opacity factor seemed to have no influence on the explants survival. The establishment stage in these species was not influenced by this factor, contrariwise with the results obtained with the strawberry tree (*Arbutus unedo* L.) (J.C. Gonçalves, unpublished results), where metal caps significantly improved explants survival. Ko et al., (2009) with the strawberry, it also showed that light intensity significantly affected explants' survival rate through phenolic production induction, increasing browning at the initial stage and thus causing death. Possibly, the fact that we had collected young softwood shoots, during early spring from the adult mother-plant prevented an excess of phenols being released in the culture medium, avoiding browning and explant survival problems. Another factor that might influence explants establishment is the sampling period. In early June the mother-plant, where shoot samples were collected, was starting the reproductive period and, according to Scarpa et al. (2000), the sampling timing influenced myrtle in vitro success, particularly when adult plants are used. An earlier period (beginning of May) may increase shoots establishment and proliferation, which is due, possibly, to a more suitable endogenous hormonal balance.

During the multiplication phase, the average length of the longest and the shortest shoot were also not influenced by the type of cap used to seal the tubes, which was not the case in the aforementioned strawberry tree experiment, where the use of metal caps provoked lower shoot growth and mortality. In our study, the mean number of shoots (NS) and the mean number of segments (NSG) were not influenced by the caps opacity. The obtained values in both cases were very low (NS = 1.4±0.5 and NSG = 1.5 to 1.7± 0.6 to 0.8). Firstly, the species might be strongly seasonal-dependent to endogenous hormonal balance and, secondly, it is possible that the species' suitable endogenous compounds (antimicrobial properties and possible anti-cancer activity) are released in the medium decreasing shoot proliferation and growth. During the multiplication stage of myrtle, Parra and Amo-Marco (1996) verified that the time between subcultures was longer (eight weeks) than the time required for other woody species (usually one month). Indeed the number of shoots we obtained (1.4 on average) was low and very similar to the value obtained by Scarpa et al. (2000) using explants from the same period and similar multiplication medium (except BAP 10-fold the concentration we used) valuing 1.3, on average. According to these authors, there is an interaction between medium composition and sampling date on the in vitro multiplication. The best result they obtained was NS = 34.8 when the sampling period was early May and the medium composition similar to the one we used, but with 2 mg/L BAP.

In conclusion, if young softwood shoots are sampled from adult trees, the preincubation period may be avoided in myrtle in vitro propagation. The sampling period should be done in early May from adult plants in order to achieve a good establishment and proliferation rate. A 20% commercial bleach solution results in toxic effects upon initiation of softwood cuttings from an adult tree from our test genotype compared to 10-15%, and stopgap translucence had no significant effect on explant survival or a number of multiplication parameters tested. Additionally, in this species, an elongation period is needed, due to strong seasonal-dependent hormonal balance and/or putative endogenous anticancer compounds (Tretiakova, et al., 2008), thus an elongation period is needed without BAP or low BAP concentrations or by adding gibberellins or charcoal or by lower medium macronutrient concentration.

5. LITERATURE CITED

Bingre, P., Aguiar, C., Espírito-Santo, D., Arsénio, P., Monteiro-Henriques, T. (eds.) 2007. Guia de árvores e arbustos de Portugal Continental. Árvores e Florestas de Portugal. Vol. 9. Público, FLAD, LPN. Lisboa.

Canhoto, J., Lopes, M. and Cruz, G. 1999. Somatic embryogenesis and plant regeneration in myrtle (Myrtaceae). Plant. Cell. Tiss. Org. 57:13-21.

Fadda, A. and Mulas, M. 2010. Chemical changes during myrtle (*Myrtus communis* L.) fruit development and ripening. Sci. Horticult. 125: 477-485.

Hartmann, H.T., Kester, D.E., Davies, F. and Geneve Y.R. 2010. Plant propagation: principles and practices.8th ed. Prentice-Hall, Upper Saddle River. New Jersey.

Lopez González, G. 2004. Guía de los árboles y arbustos de la Península Ibérica y Baleares. 2nd ed. Ed. Mundi-Prensa. Barcelona.

Klein, J.D., Cohen, S. Hebbe, Y. 2000. Seasonal variation in rooting ability of myrtle (Myrtus communis L.) cuttings. Sci. Horticult. 83:71-76.

Ko, C-Y., Al-Abdulkarim, A. M., Al-Jowid, S. M. and Al-Baiz, A. 2009 An effective disinfection protocol for plant regeneration from shoot tip cultures of strawberry. Afr. J. Biotechnol. 8:2611-2615

Lopez-González, G. 2004. Guía de los árboles y arbustos de la Península Ibérica y Baleares. 2nd ed. Ed. Mundi-Prensa. Barcelona.

Messaoud, C., Zaouali, Y., Salah, A.B., Khoudja, M.L. and Boussaid, M. 2005. Myrtus communis in Tunisia: variability of the essential oil composition in natural populations. Flavour Frag. J. 20:577-582.

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

Parra, R. and Amo-Marco, J.B. 1996. Effect of plant growth regulators and basal media on in vitro shoot proliferation and rooting of Myrtus communis L. Bio. Plantarum 38:161-168.

Parra R and Amo-Marco J.B. 1998. Secondary somatic embryogenesis and plant regeneration in myrtle (Myrtus communis L.). Plant Cell. Rep. 18:325-330.

Scarpa, G., Milia, M. and Satta, M. 2000. The influence of growth regulators on proliferation and rooting of in vitro propagated myrtle. Plant. Cell. Tiss. Org. 62:175-179.

Sepici A., Gürbüz, I., Çevik, C. and Yesilada, E. 2004. Hypoglycaemic effects of myrtle oil in normal and alloxan-diabetic rabbits. J. Ethnopharmacol. 93: 311-318.

Steel, R.G. and Torrie, J.H. 1981. Principles and procedures of statistics a biometrical approach. Mcgraw-Hill, Singapore.

Tretiakova, I., Blaesius, D., Maxia, L., Wesselborg, S., Schulze-Osthoff, K., Cinalt Jr., J., Michaelis, M. and Werz, O. 2008. Myrtucommulone from Myrtus communis L. indices apoptosis in cancer cells via the mitochondrial pathway involving caspase-9. Apoptosis 13:119-131.

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