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# *In Vitro* Multiplication of Aromatic and Medicinal Plants and Fungicide Activity

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## 1. Introduction

Aromatic and medicinal plants, widely used as folk medicine are, beyond fruits, vegetables grains and spices, the principal source of antioxidant compounds. Several studies demonstrated that antioxidants have also antifungal activity (Jayashree & Subramanyam, 2000; Rasooli & Abyaneh, 2004). More and more, humanity try to replace synthetic metabolites by natural metabolites. Therefore, studies in aromatic and medicinal plants with the capacity to produce a different range of secondary metabolites extremely increase in late years. On the other hand, chemical products, like pesticides, fungicides or bactericides are widely used in agriculture. However, they have disadvantages to the environment, due to contamination of the soils, the final consumers or the producers. Still, the indiscriminate and recurrent use of synthetic fungicides has been found to induce resistance in several fungi, the residual toxicity of these compounds result in human health hazards and requires caution in their use for plant disease control (Singh et al., 2009). Thus, some aromatic and medicinal plants, with antifungal capacity (Soliman & Badeaa, 2002; Goun et al. 2003; Sucharita & Padma, 2010), like genus *Thymus*, *Mentha*, *Calendula* and *Catharanthus* were micropropagated for antifungal activity evaluation.

Medicinal and aromatic plants are important sources for plant secondary metabolites that are involved in many other aspects of a plant's interaction with its immediate environment. The genetic manipulation of plants together with the establishment of *in vitro* plant regeneration systems facilitates efforts to engineer secondary product metabolic pathways (Kumar & Gupta, 2007). Improvement of the yield and quality of these natural plant products through conventional breeding is still a challenge. However, recent advances in plant genomics research has generated knowledge leading to a better understanding of the complex genetics and biochemistry involved in biosynthesis of these plant secondary metabolites (Gómez-Galer et al., 2008). Advances in the cloning of genes involved in relevant pathways, the development of high throughput screening systems for chemical and biological activity, genomics tools and resources, and the recognition of a higher order of regulation of secondary plant metabolism operating at the whole plant

level facilitate strategies for the effective manipulation of secondary products in plants (Kumar & Gupta, 2007).

To overcome the problem of antifungal resistance in human pathogens, plants with antimicrobial properties have been extensively studied for a possible application in food microbiology and as alternative treatments for diseases or to prevent bacterial and fungal growth. Many studies have proven very good fungicide effect of plants (Zabka et al., 2011).

The details of plants screened, their families, vernacular names and their therapeutic uses are given in Table 1.

| Plant species | Family | Common name | Therapeutic use |
|---|---|---|---|
| *Thymus mastichina* and *Thymus zygis* | Lamiaceae | Thyme | Antiseptic, antispasmodic, antitussive (Pina-Vaz et al., 2004) |
| *Mentha rotundifolia* | Lamiaceae | Applemint | Antiseptic, antispasmodic, expectorant, vasoconstrictor (Edris et al., 2003) |
| *Calendula sp.* | Asteraceae | Marigold | Skin problems, fevers, anti-inflammatory, anti-viral, anti-bacterial and fungicide (Hänsel et al., 1992) |
| *Catharanthus roseus* | Apocynaceae | Madagascar periwinkle | Anticancer, antidiabetic, laryngitis, rheumatism, dysmenorrhea (Jaleel et al., 2009) |

Table 1. Ethnomedical information of the studied species.

## 2. *In vitro* multiplication of plants from genus *Thymus*, *Mentha*, *Calendula* and *Catharanthus*

The multiplication by *in vitro* culture, means micropropagation, is a very important methodology to obtain a great number of homogeneous plants in a short period of time. *In vitro* culture is an important system in order to optimize and increase the secondary metabolites production. With this technique, *explants* from different species could be micropropagated under optimized condition of culture media, temperature and photoperiod. In this study, different species of plants with antifungal activity were micropropagated, and the fungicide activity of *in vitro* plants compared with the field plants.

In all the studies presented, the culture media were solidified with 0.7% of agar, and pH was adjusted to 5.6-5.8. Culture media were autoclaved at 121ºC for 15 min. The cultures were maintained in a growth chamber at 24 ± 1 ºC on a 16/8-h photoperiod (73 μmol $m^{-2}$ $s^{-1}$).

Data were subjected to analysis of variance (ANOVA) using STATVIEW 5.0 program, treatment means separated using Fisher´s Least Significant Difference (LSD) test at P = 0.05.

### 2.1 Micropropagation of *Thymus*

Thyme is a perennial herb, a 20 to 50 cm shrub, of the Lamiaceae family, an aromatic plant native to the Mediterranean region (Miguel et al., 1999). The genus *Thymus* is exceptionally rich in species, and due to the diversity and plasticity of these plants, their geographical range is very wide. In Portugal are known, at least, 11 *Thymus* species (Afonso & McMurtrie,

1991). *Thymus* plants are of much interest owing to their use in different applications, in medicine because of their antiseptic properties, in the cosmetic industry or as a food additive for their organoleptic properties (Duke et al., 2002; Torras et al., 2007). *Thymus* species differ with regard to their morphological features and metabolism, which influences their chemical constitution. Within individual species there are chemical variations that are characterized by different plant oil compositions, usually without any morphological differences (Smolik et al., 2009). Increasingly, plant breeding has taken advantage of developments in molecular biology in order to genotype the species of interest in a way that considerably accelerates their selection. These types of approaches consist of choosing desired genotypes on the basis of molecular markers, or having prior knowledge of the genes that determine the formation of a particular trait in a plant (Pradeep-Reedy et al., 2002). There are many publications related to the antibacterial and antifungal activities of thyme essential oil (Urbanczyk et al., 2002; Priestley et al., 2003; Rasooli & Mirmostafa., 2003). It has been used as weed germination inhibitor (Angelini et al., 2003), and the different extracts from thyme leaves have shown the presence of a large number of flavonoids and vitamin E, compounds of great interest in the food industry due to their antioxidant activities (Sotomayor et al., 2004). There is also interest in using thyme essential oil for delaying the autoxidation of food lipids (Youdim et al., 2002).

### 2.1.1 Methodology

Branches of *Thymus* plants, species *Thymus mastichina* L. and *Thymus zygis* L., with 10 to 20 cm lenght were collected in University of Trás-os-Montes e Alto Douro (UTAD) Botanical Garden, in Vila Real, Portugal. The *explants* disinfected with commercial bleach 60% (v/v) and washed three times with sterile water, were fragmented into nodal segments and placed in different culture media. Basal culture media MS (Murashige & Skoog, 1962) were evaluated without growth regulators or supplemented with a cytokinin, 1 mg/L BAP (6-benzylaminopurine), alone or combined with an auxin, 0.2 mg/L NAA (α-naphthalene-acetic acid). Two carbon sources, sucrose and glucose, at three concentrations (2, 3 or 4 % w/v) were also tested. Number and length of shoots have been evaluated after four weeks in culture.

### 2.1.2 Results and discussion

The two *Thymus* species studied showed a good response to *in vitro* culture conditions (Fig. 1). After four weeks in culture, *T. mastichina* L. presented higher length of shoots (11.2 mm vs 6.0 mm), while *T. zygis* L. showed bigger shoot number per *explant* (1.9 vs 1.8). The culture media revealed a statistical significant effect ($P<0.05$) for the parameters evaluated, shoot number and length.

In what concern to carbon source, sucrose seems to be the best carbon source to thyme micropropagation. For both species, MS medium containing 3 % sucrose produced the highest shoots length (Fig. 2), similar results were obtained by Bandeira et al. (2007) in *T. vulgaris* L.. With 3 % sucrose concentration the highest shoots length (15.9 mm) was observed in *T. mastichina* L., in MS medium supplied with 1 mg/L BAP. One of the explanations for this is the presence of endogen auxin that releases the addition NAA. The biggest shoot number (9.7) appeared in *T. zygis* L. in MS medium with 2 % sucrose and 1 mg/L BAP. However, considering all the media, the ones with glucose revealed higher values in shoot number when compare with the sucrose ones, 3.6 *vs* 3.3 (though, the difference was not statistically significant $P>0.05$). Cunha and Fernandes-Ferreira (1999) and Harada e Murai (1996) obtain similar results with *Linum usitatissium* L. and *Prunus mume*, respectively.

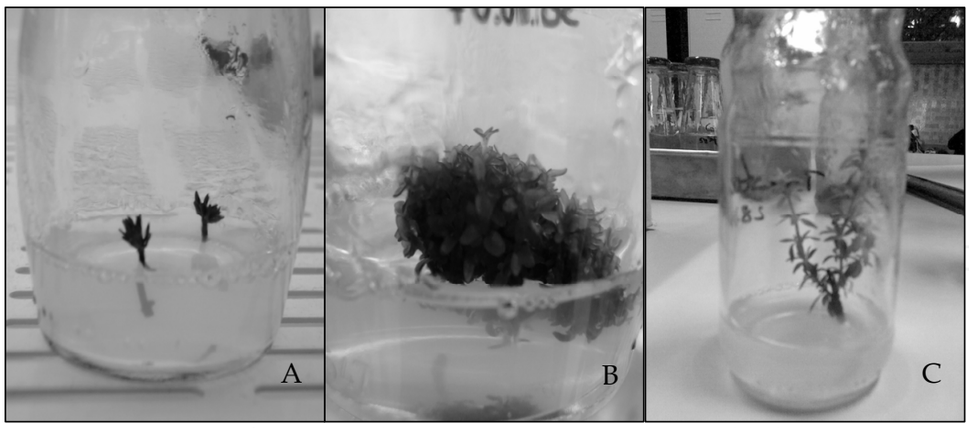


Fig. 1. Micropropagation of *Thymus* plants. A – *Explant* in the establishment medium culture; B – *In vitro* plants of *T. zygis*; C – *In vitro* plants of *T. mastichina*.

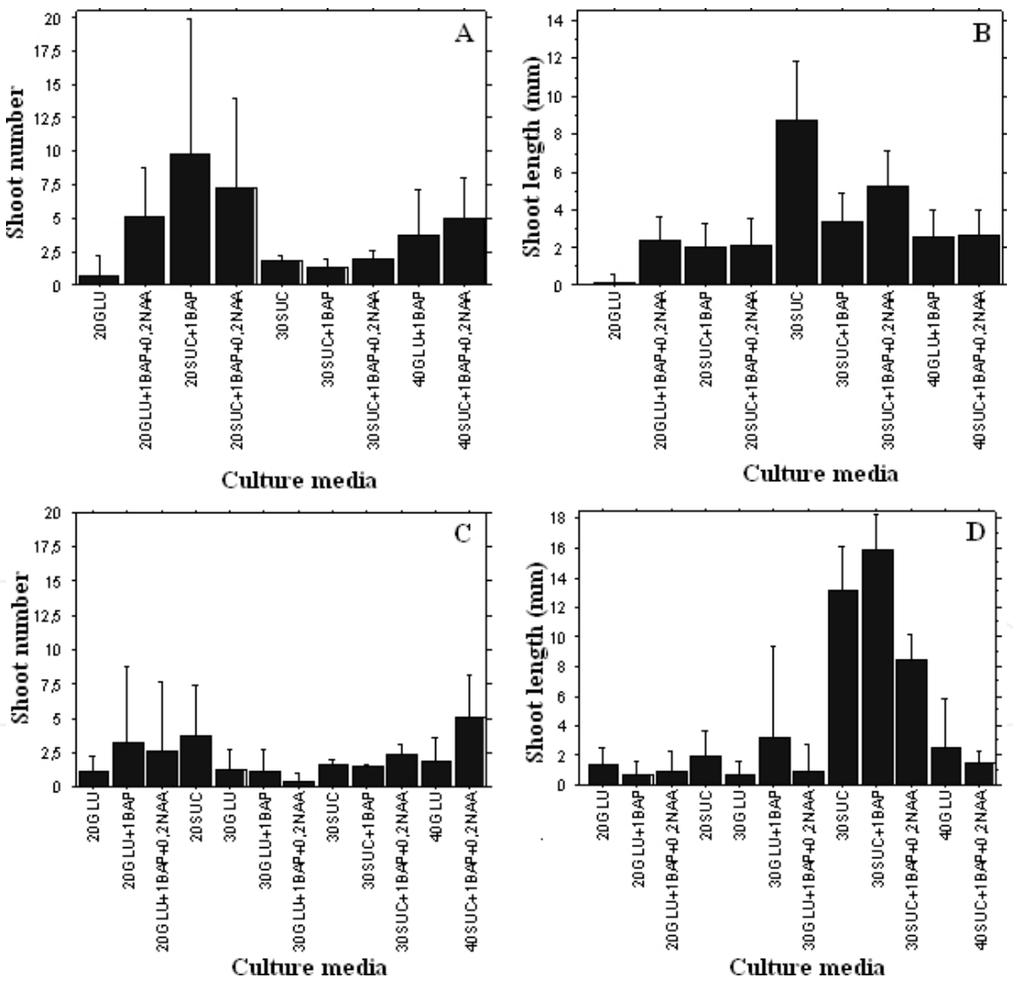


Fig. 2. Effect of culture media (MS) in shoot number and length in *T. zygis* (A and B) and *T. mastichina* (C and D). GLU- glucose, SUC- sucrose, 20- 2%, 30- 3% 40- 4%. Error bars show the 95% confidence interval.

### 2.2 Micropropagation of *Mentha*

The genus *Mentha* belongs to the family Lamiaceae (Labiatae) consisting of about 25 to 30 species mainly found in temperate regions of Eurasia, Australia , South Africa and North America (Shinwari et al., 2011). Mints grow 10 - 120 centimetres tall and can spread over an indeterminate sized area. Mints are used either in the herb form or as an essential oil for flavouring, perfume production and medicinal purposes (Edris et al., 2003). The *Mentha* plant produces secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides. Terpens and quinines are used in food and pharmaceutical, cosmetics and pesticide industries (Khanuja et al., 2000). Some members of this genus are also used as herbal teas and condiments both in fresh and dried form due to their distinct aroma. Morphological markers (such as plant height, leaf shape, colour, etc.) are among the oldest markers used in the evaluation of genetic variability, namely in *Mentha*. However, they are not sufficiently specific and informative because different gene expression in different environments causes wide variability of phenotypic characters in individuals. In some cases congruence between morphology and molecular phylogenetics were reported (Shinwari et al., 2011 as cited in Shinwari, 1995). Genetic diversity refers to the variation at the level of individual gene and provides a mechanism for the plants to adapt in ever changing environment.

The existence of different chemotypes, based on qualitative differences within a taxon, is a common feature in most *Mentha* species and hybrids. As a result, the mint plants produce a number of commercially valuable essential oils, viz. spearmint oil, peppermint oil, pennyroyal oil, etc. (Edris et al., 2003).

#### 2.2.1 Methodology

Nodal segments of *Mentha rotundifolia* plants were collected in the greenhouse of Botanical Garden of UTAD (Vila Real, Portugal). The *explants* were disinfected first with ethanol 70% (v/v) then with commercial bleach 40% (v/v) and washed three times with sterile water, then were fragmented into nodal segments and placed in different culture media. MS culture media with different concentrations of BAP (1.0 or 2.0 mg/L) and NAA (0.1 or 0.2 mg/L) were used.

The number and length of shoots and roots, and the presence and diameter of *calli* were evaluated during the eight weeks of *in vitro* culture. After that acclimatization was done in a mixture of peat and perlite (1:1).

#### 2.2.2 Results and discussion

The micropropagation of Menthe had, among others, the purpose to evaluate the effect of cytokinin and auxin concentrations in the plant development. After four weeks of *in vitro* culture there was the development of plants in all culture media (Fig. 3). The results obtained in MS + 1 mg/L BAP medium with different concentrations of auxin (0.1 or 0.2 mg/L) revealed that the lowest concentration of auxin induces higher values of shoots length (27.0 mm), and the highest concentration causes higher values of the roots length (16.9 mm)(Table 2). These results are due to the fact that a low ratio of auxin / cytokinin stimulates the development of stem sprouts, and a higher ratio to promote root development (Torres et al., 1998). Pascal et al. (1991) found that the addition of a synthetic

auxin (IBA) induces an increase in mulberry shoots. Analyzing the effect of BAP concentration, culture media MS + 1 mg/L BAP + 0.2 mg/L NAA and MS + 2 mg/L BAP + 0.2 mg/L NAA, it is clear that the medium with the lowest BAP concentration led to higher values for all parameters, except for *calli* induction that are similar in both medium (0.7 mm diameter). Similar results were obtained by Erig et al. (2002) with black mulberry tree by checking that an increase in BAP concentration resulted in a decrease in length of shoots.

The results showed that the composition of the culture medium influences the response of Menthe to micropropagation. Overall, MS medium without growth regulators showed better results in terms of the number of shoots (2.0 / *explant*), although shoots length was higher in MS medium with 1 mg/L BAP and 0.1 mg/L NAA (27.0 mm). The plants were successfully acclimatized with a rate of 100%.

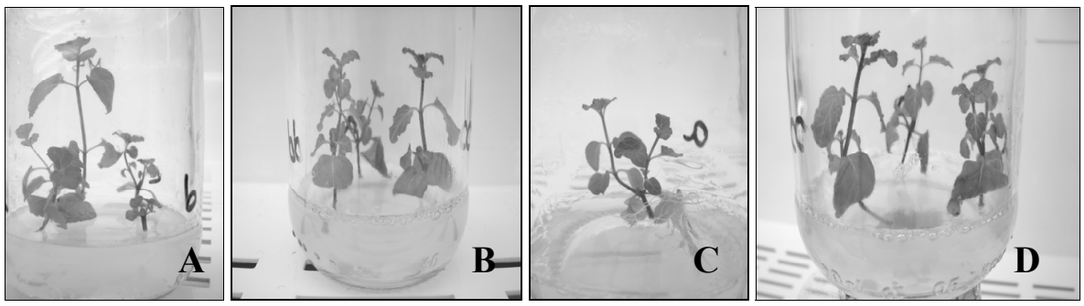


Fig. 3. Micropropagation of *M. rotundifolia*, plants after four weeks in different culture media. A – MS; B – MS + 1 mg/L BAP + 0.1 mg/L NAA; C – MS + 1 mg/L BAP + 0.2 mg/L NAA; D – MS + 2 mg/L BAP + 0.2 mg/L NAA.

| **Culture media** | **MS** | **MS + 1BAP+0.1NAA** | **MS + 1BAP+0.2NAA** | **MS + 2BAP+0.2NAA** |
|---|---|---|---|---|
| Shoot number | 2.0 | 1.9 | 1.8 | 1.5 |
| Shoot length (mm) | 18.0 | 27.0 | 22.2 | 11.8 |
| Root number | 3.2 | 2.6 | 2.5 | 0.4 |
| Root length (mm) | 18.9 | 14.5 | 16.9 | 4.3 |
| *Explants* with *calli* (%) | 0.0 | 15 | 15 | 6 |
| *Calli* diameter (mm) | 0.0 | 0.7 | 0.8 | 0.7 |

Table 2. Number and length of shoots and roots and percentage and diameter of *calli* per explant, during eight weeks of *in vitro* culture.

### 2.3 Micropropagation of *Calendula*

The genus *Calendula* belongs to Asteraceae family, also known as Compositae, and includes several species, namely *Calendula arvensis* and *Calendula officinalis*, which are commonly used

as ornamental and medicinal plants. These plants are known to contain saponins, triterpenic alcohols and their fatty acid esters, carotenoids, flavonoids, coumarins, essential oils, hydrocarbons and fatty acids (Hänsel et al., 1992). *Calendula* is known by its medicinal properties - mainly anti-inflammatory, anti-viral, anti-bacterian and fungicide. Considering secondary metabolites, *C. arvensis* is very similar to *C. officinalis* and therefore, the majority of the traditional or folk medicinal uses are similar for both species, including for the cure of skin problems, fevers, chronic infections, wounds, bites and stings (Grieve, 1984; Chevallier, 1996). *Calendula* sp. micropropagation was achieved for the first time using several types of *explants* (Çoçu et al., 2004). Callogenesis can be useful to obtain suspension cell cultures and regeneration of plants *via* indirect organogenesis. *Calli* induction can be obtained with the addition of growth regulators, namely auxins. However, some genotypes are able to induce callus formation even in its absence. In *C. officinalis*, 2,4-D and IAA associated with cytokinins promoted an efficient callus development (Grzelak & Janiszowska, 2002). As secondary metabolites are accumulated in cell cultures from numerous species, much work is focused on their production in shoot or root cultures formed by dedifferentiation of callus (Banthorpe, 1994). Metabolites extracts can be obtained from *in vitro* material, namely *calli* (Grzelak & Janiszowska, 2002), plantlets (Schmeda-Hirschmann, 2005) and flowers (Hamburger et al., 2003).

### 2.3.1 Methodology

Nodal segments obtained from plants growing in greenhouse conditions were washed in running water, surface-sterilized with a 25% (v/v) sodium hypochlorite solution for 15 min. and finally rinsed 3 times with sterile distilled water (10 min./wash). The *explants* were inoculated in MS media supplemented with BAP (1 or 2 mg/L) alone or combined with 0.1 mg/L of NAA. All the media were supplemented with 30 g/L sucrose. After 4 weeks, the results were analyzed by several parameters like the number and length of shoots and induction and development of *calli*. The *calli* obtained were transferred to MS culture medium added with 3 mg/L 2,4-D, for organogenic development.

### 2.3.2 Results and discussion

In general *calli* induction was detected in all the media. Callogenesis occurred simultaneously with organogenesis and, after 4 weeks of *in vitro* culture, flower induction was obtained (Fig. 4). It was not observed a significant effect of the culture medium on the number and length of shoots. The highest number of shoots was obtained in the medium with 2 mg/L BAP and the larger length was obtained in the medium with 1 mg/L BAP.

Considering all the media tested, the average rate of multiplication of *C. arvensis* was 4.21 shoots/*explant*. ÇoÇu et al. (2004), using different culture media to micropropagate *C. officinalis* obtained similar values. The best results were obtained in the culture medium with 2.0 mg/L of BAP (7.8 shoots/*explant*) (Fig. 5). The presence of the auxin NAA (in combination with the cytokinin BAP), did not induce a positive effect in the number of shoots (Fig. 5). The media with 2.0 mg/L BAP and 0.1 mg/L NAA registered 4.75 shoots/*explant*. The medium with 1 mg/L BAP produced a higher number of shoots/*explant* in the presence of NAA, but not statistically different ($P>0.05$) of the medium without it.

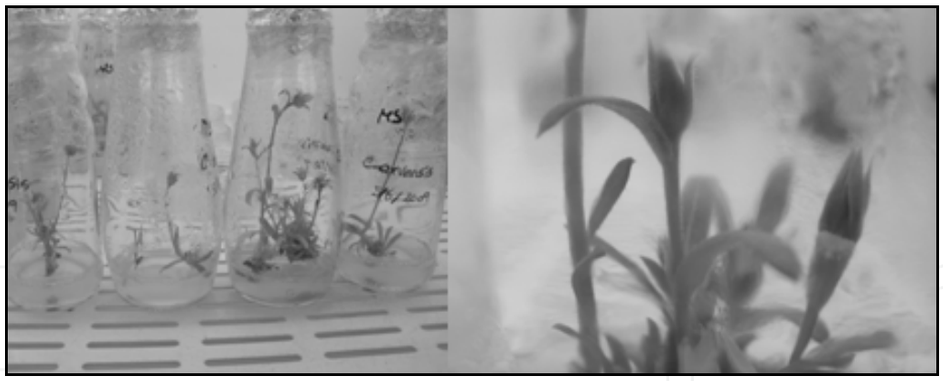

Fig. 4. *C. arvensis* plants after four weeks of *in vitro* culture showing flower development.

The weak response of NAA was also observed by ÇoÇu et al. (2004) with hypocotyl and cotyledon *explants* of C. *officinalis* concluding that the addition of KIN and NAA to culture media reduced the frequency of shoot organogenesis. Considering the length of the shoots, they reached an average of 23.44 mm at the 4th week of *in vitro* culture.

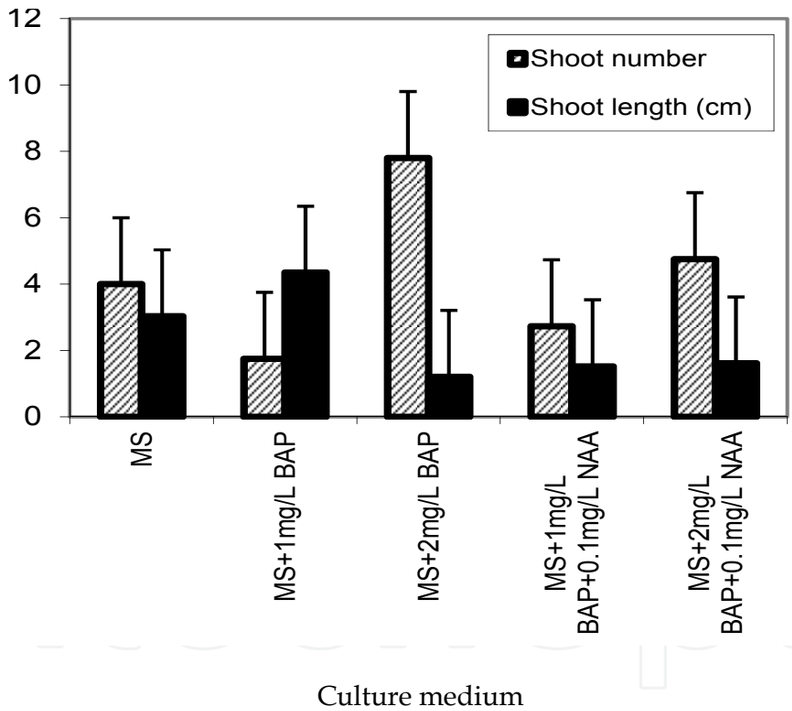


Fig. 5. Effect of different culture media on the number and length of shoots of *C. arvensis*.

### 2.4 Micropropagation of *Catharanthus*

*Catharanthus roseus*, an important medicinal plant of Apocynaceae family, is an herbaceous sub-shrub, also known as Madagascar periwinkle and *Vinca rosea* (synonym). Worldwide has been extensively studied by different groups and has been identified to be a source of numerous active principles of therapeutic importance. It is cultivated mainly for its alkaloids (Taylor et al., 1975), with anticancer activities (Jaleel et al., 2009). *Catharanthus*

*roseus* (L.) G. Don is one of the most important medicinal plants due the accumulation in the leaves of two indolalcaloids vinblastine and vincristine, which were the first natural anticancer agents to be clinically used. The high pharmaceutical value of these secondary metabolites made of *C. roseus* one of the most studied medicinal species. A few protocols for micropropagation of *C. roseus* were reported in the last years (Junaid et al., 2007; Dhandapani et al., 2008; Ilah et al., 2009).

### 2.4.1 Methodology

Nodal segments of *Catharanthus roseus* were collected from plants that were potted. *Explants* were disinfected with a 40% (v/v) sodium hypochlorite solution for 15 min. and finally rinsed 3 times with sterile distilled water (10 min./wash). After that, *explants* were inoculated in MS media alone or supplemented with BAP (1 or 1.5 mg/L) alone or combined with 0.2 mg/L of IBA (indole butyric acid) or NAA. All the media were supplemented with 30 g/L sucrose. To evaluate the effect of culture medium in the development of *explant*s was recorded, over eight weeks, the number and length of their shoots and roots, the number of internodes and the presence and diameter of *calli*.

### 2.4.2 Results and discussion

In this study a protocol was established for the micropropagation of *Catharantus roseus* (Fig. 6), MS culture medium was used because Pietrosiuk *et al* (2007) and Dhandapani *et al* (2008) described good results with this media.


Fig. 6. Different phases of the micropropagation of *C. roseus*. A – *Explants* after two weeks of in vitro culture; B - Plantlet of *C. roseus*, at six weeks of *in vitro* culture; C – Plantlet of *C. roseus* with flower, at eight weeks of *in vitro* culture.

Regarding the addition of growth regulators and their effect on the development of the *explant*s, it was found that, in general, the number of shoots was favored in medium supplemented with BAP (MS medium + 1 mg/L BAP) and without auxins (2.23 shoots/*explant*) (Table 3). The length of shoots was higher in the media with the highest concentration of BAP but with the addition of the auxin NAA, MS + 1.5 mg/L BAP + 0.2 mg/L NAA (16.07 mm). It is explained, among other things, by the presence of auxin which promotes cell elongation.

| **Culture media** | **MS** | **MS + 1BAP** | **MS + 1BAP+0.2 IBA** | **MS + 1BAP+0.2 NAA** | **MS + 1.5BAP+0.2 NAA** |
|---|---|---|---|---|---|
| Shoot number | 1.33 | 2.23 | 2.08 | 2.16 | 2.15 |
| Shoot length (mm) | 9.45 | 13.58 | 15.16 | 11.62 | 16.07 |
| Root number | 3.13 | 0 | 5.94 | 5.79 | 11.26 |
| Root length (mm) | 3.50 | 0 | 8.86 | 12.88 | 35.40 |
| Number of internodes | 0.78 | 0.78 | 1.0 | 0.89 | 0.91 |
| *Explants* with *calli* (%) | 0 | 0 | 0.27 | 0.44 | 0.45 |
| *Calli* diameter (mm) | 0 | 0 | 1.23 | 3.35 | 3.41 |

Table 3. Number and length of shoots and roots, number of internodes and percentage and diameter of *calli per explant,* during eight weeks of *in vitro* culture.

In rooting, the results were not as expected because, as has been said, the auxin IBA, is related to the root development, so was expect that MS medium + BAP + 0.2 IBA had had a higher rate of rooting, as results obtained by Dhandapani et al. (2008). However, in our study, MS medium supplied with 1.5 BAP + 0.2 NAA revealed the largest number and length of roots, 11.3 roots/*explant* with 35.4 mm. Similar results were verified by Echeverrigaray and colleagues (2005) for thyme cultivars. With regard to the number of internodes, the medium with the addition of IBA (MS + 1 mg/L BAP + 0.2 mg/L IBA) originated the highest values.

This result was not expected since the auxin IBA is more related with rooting, as reported by Dhandapani et al. (2008) also in *Catharanthus roseus*. The culture media with the lowest values for the internodes was MS medium supplemented with NAA (MS + 1 mg/L BAP + 0.2 mg/L NAA). The culture medium MS + 1.5 mg/L BAP + 0.2 mg/L NAA proved to be the best in the number and diameter of *calli* (3.41 mm), this result was expected, since Paramageetham et al. (2007) in *Centella asiática* L. had found that the formation of *calli* occurred in response to an interaction between auxin, its concentration and type of *explant*.

## 3. Fungicide activity

### 3.1 *Aspergillus fumigatus*

*Aspergillus* genus is a famous taxonomic group of molds. Some of its species are very important animal and human pathogens. The disease in humans is caused mainly by *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. Other species, for example,

*Aspergillus terreus* or *Aspergillus nidulans* are quantitatively less prevalent (Karthaus, 2010). *A. fumigatus* is one of the most ubiquitous of the airborne saprophytic fungi that is pathogenic to plants, animals and humans. Inhalation of conidia by immunocompetent individuals rarely has any adverse effect (Latgé, 1999). However, apart from the production of mycotoxins, *A. fumigatus* is a dangerous human pathogenic, which is able to cause very serious human and animal mycoses with a high frequency of resistance to chemical antifungal drugs (Verweij et al., 2009; Lass-Florl et al., 2010; Xu et al., 2010; Zabka et al., 2011). Dramatic increases in the incidence of aspergillosis caused primarily by *A. fumigatus* have occurred in recent years. A high infection-associated death rate of up to and over 50% is attributed even today to these fungi (Karthaus, 2010). *A. fumigatus* has become the most important airborne pathogen in developed countries, causing a significant mortality in invasive aspergillosis (Latgé, 1999; Chamilos & Kontoyiannis, 2005). Patients who have been treated with steroid therapy or those with chronic obstructive pulmonary disease or severe hepatic failure are at high risk for developing pulmonary aspergillosis (Meersseman et al., 2007; Morace & Bhorgi, 2010).

The treatment of aspergillosis is most problematic and questionable owing to toxicity and side effects of the used medicines on the base of synthetic fungicides (Karthaus, 2010). Fungal infections remain a significant cause of morbidity and mortality despite advances in medicine and the emergence of new antifungal agents. Drug resistance in fungi is increasing and is becoming a serious concern and the high use and misuse of antifungal as probably the main cause of this situation. Therefore, there is an urgent need to search for effective new antifungal agents in treatment of infectious diseases at present (Xing et al., 2011).

Currently, there are four classes of antifungal agents with activity against *Aspergillus*: the polyenes, such as amphotericin B its lipid formulations, and nystatin (including liposomal nystatin); the triazoles, including itraconazole, the voriconazole and the investigational posaconazole, the echinocandins, such as caspofungin, the micafungin, and the anidulafungin; and the allylamines such as terbinafine (Groll & Kolve, 2004; Chamilos & Kontoyiannis, 2005; Meersseman et al., 2007; Shi et al., 2010). Clinical resistance of invasive aspergillosis to amphotericin B based therapy is observed frequently in clinical practice (Chamilos & Kontoyiannis, 2005). However, they are intrinsically resistant to fluconazole (Moghaddam et al., 2010).

In other way, pathogenic and toxinogenic fungi are one of the major economic problems of crop and food production (Zabka et al., 2011). In terms of food safety, species of *Fusarium, Aspergillus* and *Penicillium* genera are considered the most significant because they produce the great majority of known mycotoxins (Palumbo et al., 2008; Zabka et al., 2011). There has been increasing concern of the consumers about foods free or with lower level of chemical preservatives because these could be toxic for humans (Bedin et al., 1999; Souza et al., 2005). This resulted in increasing search for new technologies for use in food conservation systems which include alternatives antimicrobials (Brull & Coote, 1999; Souza et al., 2005).

### 3.2 Aromatic plants and antifungal activity

The spread of multidrug-resistant strains of fungus and the reduced number of drugs available led to a search for therapeutic alternatives, namely among medicinal plants and compounds isolated from them used for their antifungal properties. In these natural sources, a series of molecules with antifungal activity have been found, which are of great importance to

humans and plants. Several molecules obtained from the natural environment are studied and described in bibliography with antimycotic activity. Several extracts are tested for antifungal activities like crude extracts or isolated constituents like, essential oils, terpenoids, saponins, phenolic compounds, alkaloids, peptides and proteins (Abad et al., 2007).

Aromatic plants have been widely used in folk medicine. About three quarter of the world's population relies on plants and plant extracts for healthcare (Parekh & Chanda, 2007). Several plants have been used in folklore medicine in Portugal (Pina-Vaz et al., 2004; Figueiredo et al., 2008). Spices have been used with primary purpose of enhancing the flavor of foods rather than their medicinal and antioxidant properties (Souza et al., 2005).

Plants generally produce many secondary metabolites with antifungal and microbicide activity (Bobbarala et al., 2009). According to the WHO (World Health Organization), plants would be the best source for obtaining a variety of drugs and a possible way to treat diseases caused by multidrug resistant microorganisms (Bhattacharjee et al., 2006). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds (Edeoga et al., 2005; Panghal et al., 2011). Some medicinal plants exert strong antifungal properties and could be conveniently used as a promising alternative source for presently problematic antifungal treatment in many areas with respect to their natural origin (Zabka et al., 2011).

Many commercially drugs used in modern medicine was initially used in crude form in traditional or folk healing practices. Benefits of using plant extracts are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (Panghal et al., 2011).

The genus *Thymus* (Lamiaceae), widely distributed on the Iberian Peninsula, is a taxonomically complex group of aromatic plants traditionally used for medicinal purposes because of their antiseptic, antispasmodic and antitussive properties (Pina-Vaz et al., 2004). Previous studies on the antimicrobial activity of the essential oils of some *Thymus* spp., most of them possessing a large amount of phenolic monoterpenes, showed activity against fungi (Pina-Vaz et al., 2004).

Screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic use (Duraipandiyan et al., 2006).

The main objective of this study was to investigate the inhibitory effects of *Thymus* and *Mentha* extracts against *Aspergillus fumigatus*.

### 3.3 Methodology

Fungal strain was obtained from the collection of pathogenic fungi maintained in the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. Subcultivations on Petri dishes and other manipulations with the strain were carried out in the Bio Security Level 2 (BSL 2) laboratory. The evaluation of antifungal capacity was done by the method of mycelial growth (Zhang et al., 2006). The fungus used in the assays was the fungi *Aspergillus fumigatus*. The mold was grown on potato dextrose agar (PDA). The solution was mixed with PDA culture media respectively to give a series of 5, 10, 20, and 25 mg/mL concentrations of culture media containing the compounds described above. The media

were dispensed into sterilized Petri dishes (9 cm). After solidification, a mycelial disk of 4 mm diameter of the test *Aspergillus fumigatus* taken from 4 days -old fungi culture, was placed at the center of the medium.

The mycelial disks on PDA without any test constituents were performed in the same way and used as control. Radial growth of colonies was measured at two points along the diameter of the plate and the mean of these two readings was taken as the diameter of the fungal colony. After incubation at 25°C in darkness, growth zones were measured at the third, fifth and the seventh day. The growth of the colonies in control sets was compared with that of various treatments and the difference was converted into percent inhibition [(*C* - *T*) x 100/*C*] where *C* and *T* are the radial diameters of the colony in control and treatment, respectively. The percentage of *A. fumigatus* growth inhibition is expressed as a mean of three replicate tests for each treatment. The complete antifungal analysis was carried out under strict aseptic conditions (Zhang et al., 2006).

The analyses were performed using SPSS® (Statistical Package for the Social Sciences) version 19.0. The one-way analysis of variance (ANOVA) followed by Tukey's Test with P = 0.05 were used to detect significant differences in inhibition fungi.

### 3.4 Results

Effect of four different concentrations (5 mg/mL, 10 mg/mL, 20 mg/mL and 25 mg/mL) of *Thymus* and *Mentha* extract plants was tested against *Aspergillus fumigatus*. Antifungal activity was assayed and data on effect of plant extracts on the growth of *Aspergillus fumigatus* in the third, fifth and seventh day is presented in Table 4. The data revealed that reduction in growth of *Aspergillus fumigatus* was observed with extracts of *Thymus* and *Mentha*.

| Plant species | % **Inhibition of *Aspergillus fumigatus*** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Third day | | | | Fifth day | | | | Seventh day | | | |
| | Concentrations of aqueous plant extracts in PDA (mg/mL) | | | | | | | | | | | |
| | 5 | 10 | 20 | 25 | 5 | 10 | 20 | 25 | 5 | 10 | 20 | 25 |
| *Thymus mastichina* | — | — | — | 19.1 | — | — | 4.6 | 16.7 | — | 0.9 | 7.2 | 18.9 |
| *Mentha rotundifolia* | 7.0[a] | 3.9 | — | — | 1.2 | 7.4 | — | — | 3.9 | 9.9 | — | — |

[a]All Values are mean of three replicates.

Table 4. Inhibition effect of plant extracts on *Aspergillus fumigatus* in four different concentrations.

The results indicated that *Thymus mastichina* exhibited antifungal activity against the tested *Aspergillus fumigatus* at two different concentrations of 20 mg/mL and 25 mg/mL. The highest antifungal activity was exhibited at 25 mg/mL in *Thymus*. The percent of inhibition were statistically significant with different concentrations in *Thymus*. The lowest concentration of *Thymus mastichina* did not show any activity against *A. fumigates* in the 3 days, while the other two higher concentrations showed good antifungal activity.

Among the species tested, *Mentha* was less active. No enhancing effect was observed for *Mentha* extract against *Aspergillus fumigatus* at higher concentrations (20 mg/mL and 25 mg/mL) while the lowest concentrations i.e. 5 mg/mL, 10 mg/mL showed some inhibition activity against the mold strain. The percent of inhibition were statistically significant with different concentrations in *Mentha*.

None of the above concentrations completely inhibited the test fungus. The percent of inhibition ranged from 0.9 to 19.1%.

### 3.5 Discussion

Multi-drug resistance is a medical problem in world-wide and has therefore led researchers in the search for new antimicrobial drugs or resistance, particularly from natural resources (Sharma et al., 2005; Moghaddam et al., 2010). Recently, various natural products or synthetic compounds have been reported to increase the antifungal activity (Duraipandiyan et al., 2006; Bobbarala et al., 2009; Moghaddam et al., 2010; Pai et al., 2010).

Antifungal activity was exhibited by different concentrations extracts. The chronological age of the plant, percentage humidity of the harvested material, the method of extraction were possible sources of variation for the bioactivity of the extracts (Panghal et al., 2011).

The results presented indicate different spectrum of antifungal activity of the two extracts.

The antifungal activity of *Thymus mastichina* extract against the mentioned fungi was dose-dependent and increased with the increase in the plant extract concentrations. It also supports the earlier investigations of other authors (Bobbarala et al., 2009; Moghaddam et al., 2010). Previous studies have shown that *Thymus* possess antimicrobial activity (Pinto et al., 2006; Figueiredo et al., 2008).

In the other way, it was revealed in this study, that the antifungal activity of *Mentha* was enhanced in low concentrations of the extracts.

Therefore, this study suggests that plant extracts of screened plants could be helpful in treating diseases in plants caused by *Aspergillus fumigatus*.

However, there is little information about *Thymus* and *Mentha* and their derivatives in the fungal cell in order to promote fungistatic or fungicide effect (Pina-Vaz et al., 2004; Figueiredo et al., 2008). They have been empirically used as antimicrobial agents, but the mechanisms of action are still unknown (Pinto et al., 2006). Generally, inhibitory action of natural products on fungi involves cytoplasm granulation, cytoplasmic membrane lesion, and inactivation and/or inhibition of intercellular and extracellular enzymes (Cowan, 1999; Pinto et al., 2006) and might be due to various compounds, including terpenoids, phenolics and alkaloids. These compounds jointly or independently, exert different levels of antifungal effect culminating with mycelium germination inhibition (Cowan, 1999). Also, it is reported that plant lytic enzymes act in the fungal cell wall causing breakage of β-1,3 glycan, β-1,6 glycan and chitin polymers (Brull & Coote, 1999). The antimicrobial action of the aqueous extracts could be attributed to the anionic components such as thiocyanate, nitrate, chlorides and sulphates besides other water soluble components which are naturally occurring in the plant material (Darout et al., 2000).

Use of aromatic plants as microbial growth inhibitor in foods is often limited because of flavor considerations as effective antimicrobial dose may exceed the organoleptically

accepted level. Nonetheless, combinations of spices and other antimicrobial barriers could enhance the food shelf stability and microbial safety even in moderated levels (Pandit & Shelef 1994; Brull & Coote, 1999; Souza et al., 2005). In the other way, the use of aromatic plants as remedies in folk medicine, provide a good reason to investigate them scientifically as potential sources of new plant drugs. It is important to prove which plant extracts have a biological activity on some specific medical conditions, *e.g.* antimicrobial and antifungal properties (Tomczykowa et al., 2008).

## 4. Conclusion

It was possible the establishment of a micropropagation protocol in order to multiplicate and maintain *in vitro* the aromatic and medicinal plants, to have enough material to use in future studies of antifungal activity and of genetic variability.

Considering the fact that *in vitro* cannot be directly extrapolated to *ex vitro* effects the results suggests that, the use of plant extracts such as *Thymus* and *Mentha* against *Aspergillus sp.* has potential as a topical antifungal agent as they offer a cheap and effective module for therapeutic and/or preventive purposes.

Our results showed that extracts from *Thymus and Mentha* may be particularly useful against *Aspergillus fumigatus*. These results may justify the popular use of these aromatic plants.

Compound-activity relationship for oils components against fungus organisms must be elucidated to explain its antifungal activity (Tomczykowa et al., 2008).

However, in order to evaluate possible clinical application in food microbiology and therapy of aspergillosis, further studies needed to be made.

Further phytochemical studies are required to determine the types of compounds responsible for the antifungal effects of these species.

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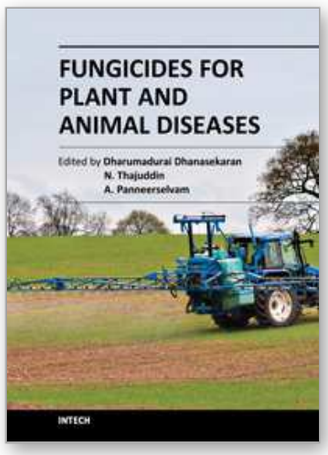


**Fungicides for Plant and Animal Diseases**

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A fungicide is a chemical pesticide compound that kills or inhibits the growth of fungi. In agriculture, fungicide is used to control fungi that threaten to destroy or compromise crops. Fungicides for Plant and Animal Diseases is a book that has been written to present the most significant advances in disciplines related to fungicides. This book comprises of 14 chapters considering the application of fungicides in the control and management of fungal diseases, which will be very helpful to the undergraduate and postgraduate students, researchers, teachers of microbiology, biotechnology, agriculture and horticulture.

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