



## “IN VITRO” CONSERVATION OF SOME ALBANIAN POPULATIONS OF *MYRTUS COMMUNIS* L.

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### SYNOPSIS

**Key words:**

*Myrtus communis*,  
light regime,  
micropropagation,  
phytohormones,  
short term  
conservation.

The myrtle (*Myrtus communis* L.) is a shrubby species, typical of the Mediterranean area and very common in Albanian Flora. The goal of this study is to find the optimal micropropagation and “in vitro” conservation protocols. Regarding the two myrtle populations, different media and light regimes were compared. These protocols will serve as a base to establish an “in vitro” genetic collection.

### INTRODUCTION

The myrtle (*Myrtus communis* L.) is a typical species of Mediterranean Flora, shrub or small tree, very common in regions with warm climate, especially in the Southern and Central Albania to 500 m above sea level (Mitrushi, 1955). As a rustic plant, it can grow on poor and dry soils.

Interest for this species is related to landscape and ecological value and the uses of myrtle as aromatic plant in medicinal, food, industrial, handicrafts and cosmetic fields. The liqueur produced from the berries shows antioxidant capacity values, comparable to those of red wine (Vacca et al., 2003). The myrtle is a sacred plant tied to the Goddess Aphrodite and in ancient times it symbolized fertility. The tradition about the use of plants was conserved until the 50's, but many uses were progressively abandoned, because of the change of life style. However, recently, the tradition value has been rediscovered, it represents a fundamental aspect of the Mediterranean area. Recent developments have found success in the field of the decorations. Last years, the myrtle is used in Albania as ornamental plant for indoor decoration and gardening and as aromatic – medicinal plant.

There is a great variability in the natural Albanian populations regarding the leaf and fruit size, type of fruit etc. To develop the cultivation of myrtle as decorative and aromatic plants and to select optimal clones, evaluation of variability and

conservation of different populations are necessary to carry out (Mulas et al., 1999; Ruffoni et al., 2003). Genetic variation is the main problem with seed propagation (Khosh-Kui & Bassiri, 1976).

Micropropagation is a suitable method for obtaining a large quantity of genetically homogeneous and healthy plant material which can be used for planting (Pierik, 1997; Kongjika et al., 2002; Nobre, 1997; Grigoriadou & Leventakis, 2000; Scarpa et al., 2000; Damiano et al., 2008). Clonal propagation of myrtle can be successfully achieved by micropropagation and it would be of interest to make a method available for short-term conservation of "in vitro" cultures (Capuana & Ponti, 2008).

The aim of this study is to determine the optimal method for the micropropagation in order to produce a great number of plants in a short period, "identical" to mother-plants. This protocol will serve as a base to establish an "in vitro" genetic collection of natural population of myrtle by short - term conservation method of minimal growth.

## MATERIALS AND METHODS

**PLANT MATERIAL AND DISINFECTION:** The objects of our study were two different populations of species *Myrtus communis* L., grown in Dajti and Divjaka areas. The myrtle flowers are usually white of extreme flavour (Fig. 1a). The abundant bloom takes place from June to July. The fruits are berries: they can be blue/black (Fig. 2) and mature during the winter.

Axillary buds isolated in spring of 2009 as initial explants were collected from one year old twigs of adult myrtle plants. The buds were treated with 70% ethanol solution and rinsed 3 times in sterile water, after this with HgCl<sub>2</sub> 0.01% solution for 20 min and rinsed again 3 times in sterile water.



Figure 1: Myrtle plants of Divjaka population: a – white flowers; b – blue berries.

PROLIFERATION AND ELONGATION MEDIUM: Basal nutrient medium MS (Murashige & Skoog, 1962) with thiamine-HCl  $0.4 \text{ mg l}^{-1}$ , nicotinic acid  $0.5 \text{ mg l}^{-1}$ , pyridoxine  $0.5 \text{ mg l}^{-1}$ , glicine  $2 \text{ mg l}^{-1}$ , myo-inositol  $100 \text{ mg l}^{-1}$  was used. The buds were inoculated in two variants of proliferation medium:

1. MS medium supplemented wi

One week after explant inoculation in the two media, the organogenesis under the effect of different phytohormones (cytokinins, auxins, gibberellins) were observed. The shoot proliferation and elongation require the combination of the cytokinin BAP with an auxin (Rodriguez et al., 1993). In our case, the optimal combination was shown with the auxin 1-naphtaleneacetic acid (NAA). The presence of the gibberellin GA<sub>3</sub> in the proliferation medium improved the shoot elongation (Fig. 2a).

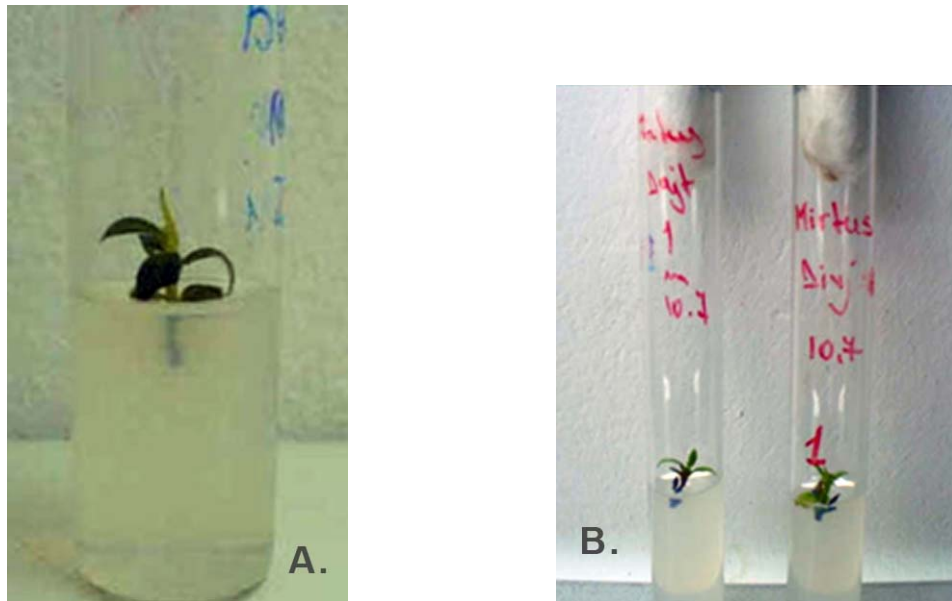
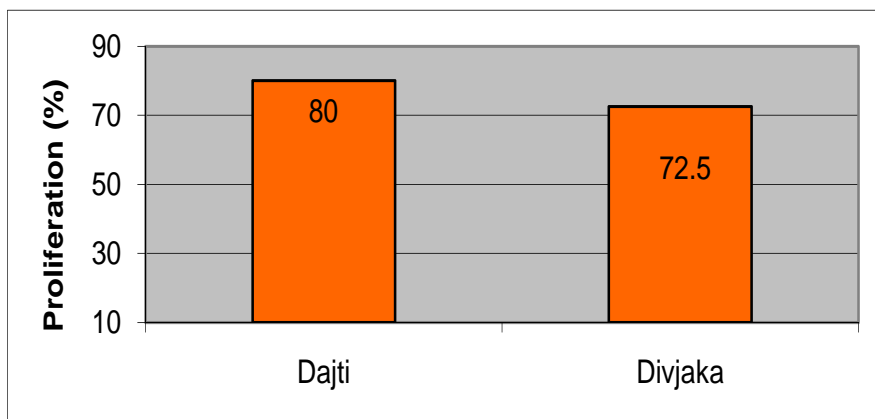


Figure 2: Myrtle explants in culture "in vitro": a - shoot produced in proliferation stage; b - development of explants of two populations.

Graph 1: Proliferation percentage of the explants of two populations in MS medium 1.



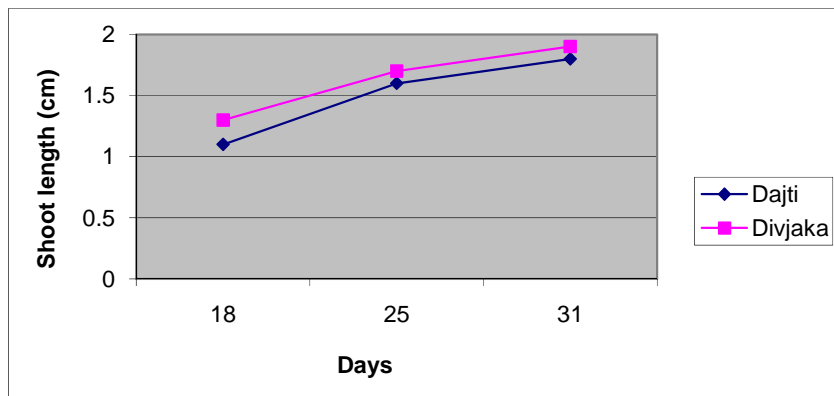
Comparing the behavior of the explants of both populations in the medium 1 (cytokinin/auxin = 65) (Fig. 2b), observed the higher proliferation percentage for Dajt

population (Graph 1). Differences in proliferation of two populations are related with specific reaction of initial explants of different populations inside one species to the conditions of nutrient medium.

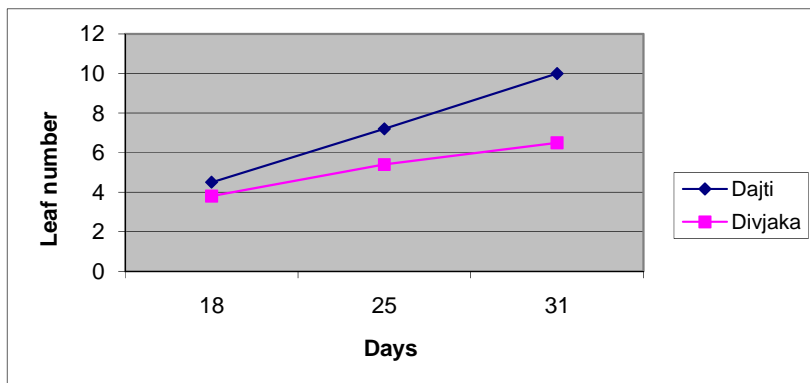
Besides the proliferation percentage are measured biometric parameters (shoot length, leaves number) during the dynamics of development of two populations in tissue culture.

Comparison of biometric parameters of the newly shoots produced in the first stage of proliferation demonstrates that the plantlets originated from Dajti population have little differences in growth from the plantlets derived from Divjaka population. The plantlets of two myrtle populations appeared no remarkable differences regarding to the parameter of plantlet length (Graph 2), while some differences showed on leaves number parameter (Graph 3).

**Graph 2: Comparison of development dynamics of two myrtle populations related to shoot growth.**

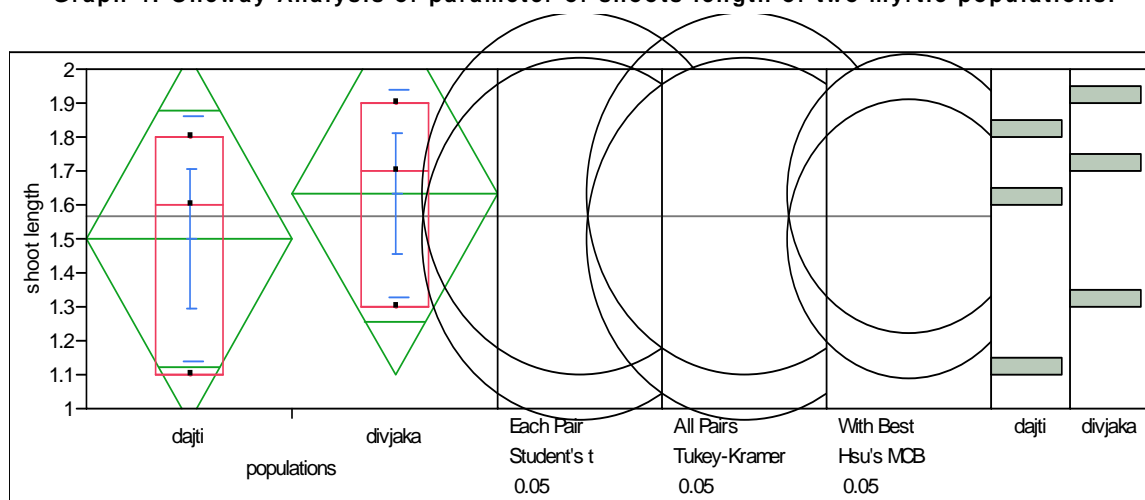


**Graph 3: Comparison of development dynamics of two myrtle populations related to leaves number.**



Anova variance analyze on buds development during the first stage of the proliferation of the explants isolated from two different myrtle populations (Dajt, Divjaka) confirms no remarkable differences among the plantlets of two populations (Graph 4) for the shoot length parameter. On contrary, analyze of variance on the other parameter, leaves number (Graph 5), showed some differences between the plantlets of two populations. One month after the inoculation of explants of Dajti population, the new shoots have the higher main number of leaves (10) in comparing to this parameter of Divjaka population (6.5).

**Graph 4: Oneway Analysis of parameter of shoots length of two myrtle populations.**

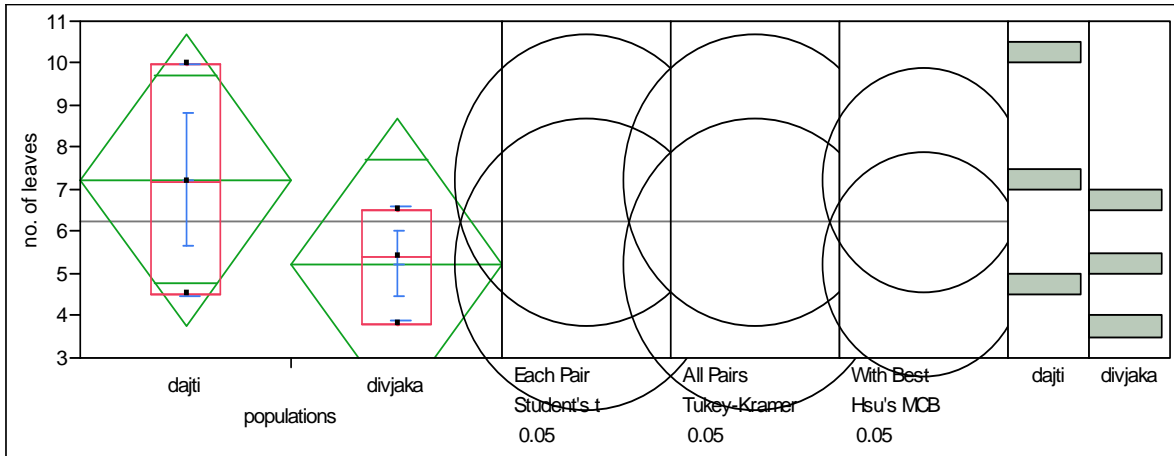


**ANALYSIS OF VARIANCE**

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
populations	1	0.02666667	0.026667	0.2388	0.6507
Error	4	0.44666667	0.111667		
C. Total	5	0.47333333			

The F ratio and its p-value (Prob > F) tell if, overall, there is a statistical difference between any pair of means. The probability of obtaining a F ratio of 0.2388 or more is larger than 0.06507. According to the Analysis of Variance table, the model as a whole is significant (P < 0.05), which implies that: ==> THERE IS NO ANY SIGNIFICANTLY DIFFERENT PAIR OF MEANS.

**Graph 5: Oneway Analysis of parameter of leaves number of two myrtle populations.**

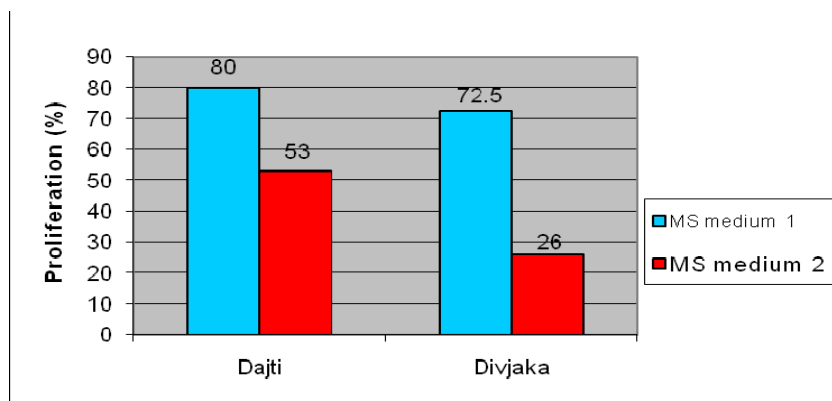


**ANALYSIS OF VARIANCE**

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
populations	1	6.000000	6.00000	1.2757	0.3218
Error	4	18.813333	4.70333		
C. Total	5	24.813333			

The probability of obtaining a F ratio of 1.2757 or more is less than 0.3218. According to the Analysis of Variance table, the model as a whole is significant ( $P < 0.05$ ), which implies that:  $\Rightarrow$  THERE IS AT LEAST ONE SIGNIFICANTLY DIFFERENT PAIR OF MEANS.

**Graph 6: Proliferation percentage of the explants of two populations under treatment with two different nutrient media.**



*VARIATION OF TWO POPULATIONS UNDER DIFFERENT NUTRIENT MEDIA*

By comparing the results of the reaction of buds organogenesis of two myrtle populations (Graph 6) could distinguish the nutrient medium 1 with greatest impact

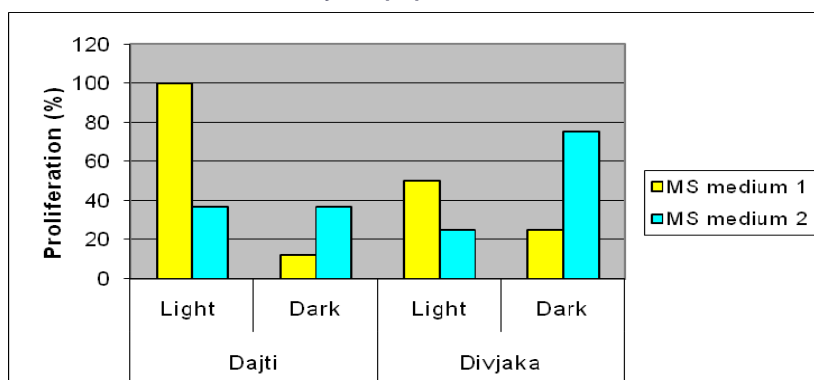
on the proliferation (80 and 72.5%). The survival percentage of the buds in proliferation medium 2 varies 53% for Dajti population and only 26% for Divjaka population. Although the absolute amounts of cytokinin BAP and auxin NAA are higher in the medium 2 comparing to medium 1, the ratio of these different phytohormones is in favour of the cytokinin BAP in the medium 1. This factor positively influences in the first stage of proliferation, when greater amount of cytokinin BAP required for buds development.

#### VARIATION OF PROLIFERATION STAGE IN DIFFERENT LIGHT REGIME

Establishment of "in vitro" cultures of woody plants has shown regeneration problems, because of heavy phenol exudation, which can result in explant necrosis (Singh & Khawale, 2006). Phenols secreted from the cut ends of explants lead to medium browning the 2 days after the culture establishment. This phenomenon can lead to the other problems related to explant growth and organogenesis. For this reason, a part of test-tubes with inoculated myrtle explants was kept in the normal light conditions of "in vitro" chamber, while the other part was maintained in the darkness during a month in the initial period of "in vitro" culture.

According to some authors (Arezki et al., 2004), light favours an increase in phenolic compounds and a reverse variation of peroxidase activity during the culture cycles. These aspects are discussed in terms of a possible regulation of the endogenous auxin level through a light control of peroxidase activity and the level of phenolic compounds.

**Graph 7: Influence of different light conditions in the proliferation stage of buds of two myrtle populations.**



The data of our experiment (Graph 7) on the influence of different light conditions on buds proliferation in two nutrient media showed that the combination of the medium with light regime has a similar effect for two myrtle populations. Explants of two populations, inoculated in the medium 1 (cytokinin BAP  $0.65 \text{ mg l}^{-1}$ , auxin NAA  $0.01 \text{ mg l}^{-1}$ ) grow better in the presence of lighting, whereas the maintenance of the buds in the medium 2 (cytokinin BAP  $2 \text{ mg l}^{-1}$ , auxin NAA  $0.05$

mg l<sup>-1</sup>) in the darkness favors the development of plantlets “in vitro”. Only in the last case of the cultivation in the second medium, the positive effect associated with the influence of the darkness in the inhibition of polyphenolic compounds production, which in its turn, affects buds development in proliferation stage. It is assumed that this effect is related to the increase amount of auxin (5 times higher in medium 2 than in medium 1).

**SUBCULTURE STAGE**

As myrtle shoots grow during elongation stage to several cm with some leaves (Fig. 3), they are ready to pass to the first subculture. During subculture observed not only the production of a considerable number of myrtle plantlets, but even the growth in length of secondary and tertiary adventitious shoots in the presence of cytokinin BAP in the nutrient medium (Fig. 4, Graph8).

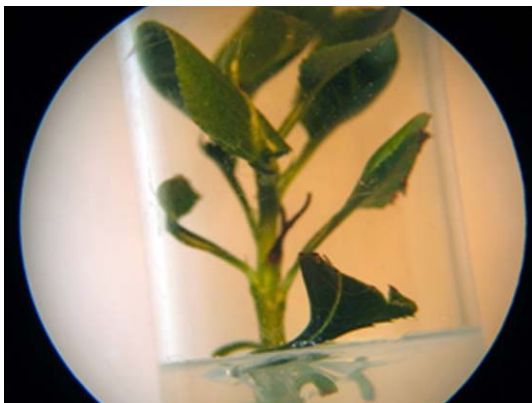
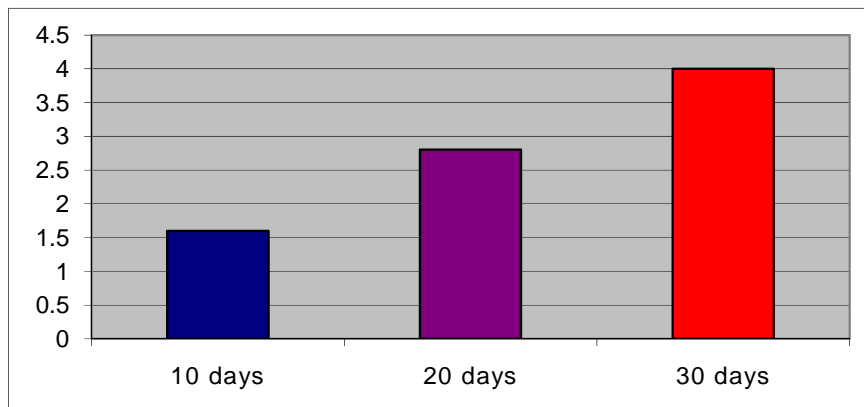


Figure 3: Myrtle shoots ready to transfer to the subculture stage.



Figure 4: Myrtle plantlets with secondary and tertiary adventitious buds.

Graph 8: Increase of adventitious shoots during first subculture of myrtle plantlets.



Increase in length, formation of large numbers of leaves, as well as production of adventitious shoots have a positive impact in micropropagation coefficient (5.2) after the first subculture.



**Figure 5: New myrtle plantlets produced "in vitro" during first subculture.**

#### ROOTING AND PLANTLETS ACCLIMATION

Rooting of myrtle plantlets (Fig. 6 a) resulted very effective (with a percentage of 87%) by adding  $0.1 \text{ mg l}^{-1}$  IBA in nutrient medium. "In vitro" produced plants of *Myrtus communis* kept in little containers and covered with beakers during two weeks are acclimated in specific conditions with high relative humidity of atmosphere, in the low light intensity and in the sterile mixture of soil, peat and perlite (2:1:1). Acclimated plantlets (Fig. 6 b) are able to pass in the permanent place in the green-house.



**Figure 6: Plants of *Myrtus communis* L.  
a - 'in vitro' rooted plant; b - acclimated plants.**

“IN VITRO” CONSERVATION BY MINIMAL GROWTH METHOD

The experimental data obtained from the use of the methods of minimal growth (short term conservation – from some months to a year) at the low temperature (4°C) (Fig. 7) shows that the plantlets conserved for a maximum period of six months in state near the initial size. Extension beyond this period results in a plantlet growth (Fig. 8) and the appearance of necrosis. In this case is necessary to pass the culture in regeneration under normal temperature condition in “in vitro” chamber. The subculture of the plantlets helps to transfer again the plant materials in the short term storage up to six months. These data coincide with results obtained by other authors (Capuana & Ponti, 2008).



Figure 7: “In vitro” collection of myrtle plantlets at low temperature (4°C).



Figure 8: Growth of myrtle plantlets after six months conserved at low temperature.

**CONCLUSIONS**

- The most optimal nutrient medium is considered Murashige & Skoog medium (MS) 1 (cytokinin BAP 0.65 mg l<sup>-1</sup>, auxin NAA 0.01 mg l<sup>-1</sup>) with higher ratio BAP/NAA, which favours the buds development in the first stage of “in vitro” culture;
- Comparing the data of the response of the explants of two populations to “in vitro” culture is observed the difference between two populations. The explants isolated from Dajti population plants present higher percentage of proliferation;
- Comparison of reaction of culture to different lighting regime shows that the medium 1 is favourable in the light, while the medium 2 is most optimal in the darkness;
- During subcultures observed not only the production of a considerable number of plantlets, but even increase in length of secondary and tertiary adventitious shoots in the presence of cytokinin BAP in the nutrient medium MS;

- Method of minimal growth in low temperature gives positive results in the "in vitro" short-term storage of plant material of *Myrtus communis* L. species up to six months

### RECOMMENDATIONS

- Micropropagation as the optimal methods for the production of a great number of homogeneous plants within a short period of plants can be used for myrtle cultivation.

- Experimentation of the other methods of "in vitro" conservation such modification of nutrient medium etc. for short-term storage and cryoconservation for long-term preservation of germplasm of *Myrtus communis* L. species

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